THE IMPACT OF STRESS ON COGNITION AND MOTIVATION

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THE IMPACT OF STRESS ON COGNITION AND MOTIVATION

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Stress has a broad impact on animals' behavior, profoundly affects brain regions involved on cognition and motivation and, when maladaptive, is also a trigger for neuropsychiatric disorders. This book focuses on advances in understanding how stressful events impact cognition and motivation, and the neural mechanisms that mediate their effects. Additionally, this book seeks to highlight the most recent efforts to identify individual factors that can alter an organism's response to stressful stimuli, and to describe pharmacological and non-pharmacological interventions that can mitigate the deleterious effects of stress on cognition and motivation.

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EDITORIAL
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Editorial: The Impact of Stress on Cognition and Motivation

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Keywords: cognition, stress, motivation, behavior, decision-making

Editorial on the Research Topic

The Impact of Stress on Cognition and Motivation

Stress is usually defined as an actual or anticipated threat or disruption of organism homeostasis, which usually leads to an acute stress response allowing for adaptation to the new condition. Conversely, chronic stress usually leads to maladaptive responses in various organs and systems, activating pathophysiological mechanisms such as psychiatric disturbances, neurologic disorders, cardiovascular diseases and metabolic dysregulation (Sousa, 2016). The impact of chronic stress in cognition and motivation has been extensively described in the literature. In this Research Topic of Frontiers in Behavioral Neuroscience, a host of new empirical findings on the impact of stress on cognition and motivation was presented in a translational perspective from rodents to humans.

The detrimental impact of stress on behavior can be established early in development, including from exposure before birth. Usually described as programming effects, these are well described after strongly stressful experiences during pregnancy. In one of the papers of this series, Soares-Cunha and co-workers extend our understanding of programming effects by showing that even mild stressful events during this critical period can lead to long-lasting structural and neurochemical alterations and anxious- and depressive-like behaviors in the offspring (Soares-Cunha et al.).

Social stress is also known to induce depressive-like behaviors. Using tests based on association learning, Kúkel'ová et al. show that chronic social stress (CSS) can lead to reductions in reward salience and effort valuation in mice, decision-making deficits that can also be observed in patients depression and schizophrenia (Kúkel'ová et al.), adding to the external validity of the model. Moreover, in another paper of the series, by Yang and collaborators, CSS induced spatial-working and contextual-fear memory deficits were related with decreased levels of N-methyl-D-aspartate receptor subunit 2B (NR2B), impaired long term potentiation (LTP) and NMDA receptor-mediated excitatory postsynaptic currents in the hippocampus (Yang et al.). Importantly, both memory impairments and electrophysiological alterations were attenuated by antidepressant treatment with the NMDA-antagonist ketamine (Yang et al.), adding further evidence to support a role for glutamate excitotoxicity in such stress-related deficits.

Although post-traumatic stress disorder (PTSD) is a widely recognized and often devastating consequence of exposure to intense stress, the biochemical mechanisms underlying the creation of the fear memory remain poorly understood. In a very interesting paper in this regard, Han and co-workers show that the behavioral consequences of exposure to a stress protocol designed to mimic a PTSD like-condition [single prolonged stress (SPS) protocol and immobilization-stress (IM)] are associated with decreased LTP as well as decreased stathmin and increased Rin1 expression in the hippocampus and the amygdala (Han et al.). Of note, both regions are implicated in the regulation of fear memory, further stressing the significance of these findings.

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The serotoninergic system has been involved in both anxiety state and trait, particularly through the activity of 5-HT2A receptors. To assess how anxiety trait can modulate the role of 5-HT2A in stress-induced anxiety, León and collaborators treated animals with high and low freezing responses to contextual cues previously associated with footshock with ketanserin, a preferential 5-HT2A receptor blocker. Their finding of an opposite effect of the drug on the two lines highlights and adds insight into the relevance of genetic variability in the establishment of stress responses (León et al.).

Like serotonin, glial-derived neurotrophic factor (GDNF) has been implicated in the regulation of stress responses. Specifically, it was previously recognized that up-regulation of GDNF expression is associated with increased stress resilience. In a paper in the present Research Topic, Buhusi and co-authors extend this findings by showing that the opposite is also true: GDNF-deficient mice are more vulnerable to stress, failing to express latent inhibition (LI) which results in slower learning of new conditioning associations (Buhusi et al.). Moreover, this LI impairment was associated with a decreased neuronal activation in the nucleus accumbens shell and increased activation in the nucleus accumbens core.

Decision-making processes are among the cognitive-related dimensions widely affected by stress. Work from ours and other groups has shown that chronic stress promotes a switch from a flexible and contextualized goal directed system of responses to a more rigid habit based system (Dias-Ferreira et al., 2009). In line with this, a paper by Maran and colleagues in the present series shows that high arousal states disturb spatial and sequence learning, discrimination of spatial positions and learning of associative sequences, which could all reflect a reduced involvement of the flexible cognitive systems responsible for the sensitivity for contextual details (Maran et al.).

We have previously shown that risk-based decision making is also impacted by chronic stress exposure (Morgado et al., 2015). In the present Research Topic, Simonovic and colleagues translate our findings to humans, showing that stress exposure impairs performance in the Iowa Gambling Task (IGT), delaying the avoidance of the disadvantageous decks (Simonovic et al.). In the same vein, Starcke and colleagues show that the exposure to unsolvable anagrams also induces more disadvantageous decisions on the IGT (Starcke et al.), an effect particularly observed in male participants.

Given the vast consequences of stress exposure and its ubiquitous presence in our everyday lives, research on attenuating measures is of high clinical and even societal relevance. In a very interesting paper of this series, DiMenichi and co-authors show that writing about past failures before experiencing a stressor attenuated subsequent stress responses and reduced their physiological and behavioral effects (DiMenichi et al.), a finding that can be easily transposed to practice.

Altogether, the findings put forward in this research topic contribute to a better understanding of how stress impacts on cognition and motivation, providing a broad range of insights from the molecular and cellular processes that underlie behavioral alterations to new interventions that can ameliorate stress-induced impairments.

AUTHOR CONTRIBUTIONS

PM and JJC wrote this article and approved it for publication.

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ORIGINAL RESEARCH
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Mild Prenatal Stress Causes Emotional and Brain Structural Modifications in Rats of Both Sexes

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Stress or high levels of glucocorticoids (GCs) during developmental periods is known to induce persistent effects in the neuroendocrine circuits that control stress response, which may underlie individuals' increased risk for developing neuropsychiatric conditions later in life, such as anxiety or depression. We developed a rat model (Wistar han) of mild exposure to unpredictable prenatal stress (PS), which consists in a 4-h stressor administered three times per week on a random basis; stressors include strobe lights, noise and restrain. Pregnant dams subjected to this protocol present disrupted circadian corticosterone secretion and increased corticosterone secretion upon acute stress exposure. Regarding progeny, both young adult (2 months old) male and female rats present increased levels of circulating corticosterone and hyperactivity of the hypothalamus-pituitary-adrenal axis to acute stress exposure. Both sexes present anxious- and depressive-like behaviors, shown by the decreased time spent in the open arms of the elevated plus maze (EPM) and in the light side of the light-dark box (LDB), and by increased immobility time in the forced swim test, respectively. Interestingly, these results were accompanied by structural modifications of the bed nucleus of stria terminalis (BNST) and hippocampus, as well as decreased norepinephrine and dopamine levels in the BNST, and serotonin levels in the hippocampus. In summary, we characterize a new model of mild PS, and show that stressful events during pregnancy can lead to long-lasting structural and neurochemical effects in the offspring, which affect behavior in adulthood.

Keywords: prenatal stress, anxiety, depression, BNST, hippocampus

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INTRODUCTION

Early life adversity, including physical and emotional neglect and traumatic experiences, can induce persistent effects on physical and mental health in both animal models and humans (Heim and Nemeroff, 2001; Teicher et al., 2003). Specifically, there is now well-documented evidence that adversity in childhood increases the risk for development of personality disorders, major depression, posttraumatic stress disorder, anxiety and addictive disorders (Agid et al., 1999; Heim and Nemeroff, 2001; Dube et al., 2003; Chapman et al., 2004). The clinical importance of these findings is more evident if one considers that 80% of adults who experienced abuse or neglect early

in life are predicted to suffer at least one episode of a psychiatric disorder such as depression and anxiety or a behavioral disorder such as addiction (Heim and Nemeroff, 2001; Edwards et al., 2003; Gutman and Nemeroff, 2003; McFarland et al., 2003; Espejo et al., 2007). In contrast, the predicted incidence of such disturbances is much lower in individuals abused as adults (Brown and Moran, 1994; McCauley et al., 1997), a finding that points to the existence of critical time windows during which the organism is particularly sensitive to stress-induced pathology later in life.

The stress response works through the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, that induces glucocorticoid (GC) release by the adrenals, which generate a negative feedback that requires mineralocorticoid (MR) and GC receptors (GR) in the brain (Szuran et al., 2000; Herman et al., 2016). The MR are important in the recognition of the stress and the onset of the stress response whereas GRs are activated by the presence of abnormally high levels of corticosterone and are important in the cessation of the stress reaction (de Kloet et al., 2005). Interestingly, much attention has been focused on the ability of early life adversity to program HPA activity (Tarullo and Gunnar, 2006; Heim et al., 2008). Importantly, *in utero* exposure to GC/stress has been found to be associated with long-lasting deficits in mood and affective, as well as addictive behaviors in humans (Heim and Nemeroff, 2001; Sinha, 2001; Malaspina et al., 2008) and in animal models (Oliveira et al., 2006; Mabandla et al., 2008; Markham et al., 2010; Rodrigues et al., 2012; Borges et al., 2013a,b; Soares-Cunha et al., 2014). One of the mechanisms underlying these behavioral changes may be the impact that excessive GCs reaching the fetus have on the development of brain structures involved in mood disorders. In fact, prenatal exposure to high GCs in monkeys causes a dose-dependent degeneration of hippocampal neurons, leading to reduced hippocampal volume (Uno et al., 1990). Similarly, other brain regions such as the prefrontal cortex, amygdala, bed nucleus of stria terminalis (BNST) and nucleus accumbens (NAc) are also affected in rats (Piazza and Le Moal, 1996; Murmu et al., 2006; Oliveira et al., 2006; Lupien et al., 2009; Rodrigues et al., 2012). A second mechanism that contributes to these alterations may be the induction of epigenetic alterations in specific genes. Indeed, pioneering work by Meaney and colleagues showed that the GR can be epigenetically programmed by early life adverse events in both rodents and humans (Weaver et al., 2004; Meaney et al., 2007; McGowan et al., 2009). Moreover, we have shown that prenatal GCs exposure induces long lasting epigenetic changes in dopamine receptor D2 (Rodrigues et al., 2012).

Interestingly, animal studies have also shown that male and female brain exposed to prenatal stress/GCs may undergo different reprogramming. While some argue that prenatal stress causes alterations in the HPA axis response of both male and female rats (Koehl et al., 1999), some studies point to the possibility of sex-specific alterations (Weinstock et al., 1992), thus raising doubt on the existence (or not) of a correlation between the prolonged effects of prenatal stress exposure and sex. Moreover, other studies have shown a differential impact of prenatal stress in mood according to sex (Weinstock et al., 1992; Verma et al., 2011; Weinstock, 2011).

The aim of this study was to develop a novel mild prenatal stress (PS) protocol, which consists in exposure of pregnant dams to unpredicted stress, three times per week, during the gestational days 3–20. Progeny of both sexes was characterized at adulthood (2 months old) for anxious, depressive, impulsive and reward-related behaviors. Moreover, we evaluated structural and neurochemical changes in key brain regions involved in these behavioral dimensions, namely the hippocampus, amygdala, BNST and NAc.

MATERIALS AND METHODS

Animals

Twelve double housed virgin female Wistar han rats (8–10 weeks of age, weighing ∼250 g) were housed overnight with six experienced male Wistar han rats (6 months of age, weighing ∼500 g) and were maintained under standard laboratory conditions with an artificial 12-h light/dark cycle (lights on from 08:00 to 20:00), with an ambient temperature of 21 ± 1°C and a relative humidity of 50%–60%. Standard mating/pregnancy diet (4RF25, Mucedola SRL) and water were given *ad libitum*, except when stated otherwise. Males were removed from the box and females were kept individually housed from pregnancy day 0—which was considered the day when sperm was observed in the vaginal smear—forward.

Progeny from each individual litter were weaned on post-natal day 21 and pair-housed according to sex. The number of animals per litter (male and female) is indicated in **Supplementary Table S1**. Animals were maintained in the same housing conditions as mothers. Male and female progeny derived from at least four different litters were used for all the behavioral, physiological and neurochemical tests, to control for potential litter effects. All behavioral tests were performed when animals were 2–4 months old (61–115 days of age). All animals performed all behavioral tests.

The following behavioral tests were performed from 9:00 to 13:00: Elevated Plus Maze (EPM), Open field (OF), Light/Dark Box test (LDB) and Forced Swimming Test (FST).

The training sessions of the Variable Delay-to-Signal (VDS) and Progressive Ratio (PR) were performed from 9:00 to 19:00; one session was performed in the morning and one session was performed in the afternoon (with an interval of at least 4 h between training sessions). The test sessions of the VDS and PR were performed from 9:00 to 13:00.

The SPT was performed during the dark period, from 21:00 to 22:00.

Health monitoring was performed according to FELASA guidelines. All procedures were conducted in accordance with European Regulations (European Union Directive 2010/63/EU). Animal facilities and the people directly involved in animal experiments were certified by the Portuguese regulatory entity—Direcão-Geral de Alimentação e Veterinária (DGAV—project 023432). All protocols were approved by the Ethics Committee of ICVS.

Prenatal Stress Protocol

From pregnancy day 3 to day 20, six randomly-selected pregnant female rats were subjected to an unpredictable stress protocol (PS, prenatal stress group) that consisted of 4 h of restraint in a cylindric box with 12 cm diameter, stroboscopic lights (Strobeyellow, Disco Pro Light) or exposure to noise (80 dB), given three times per week in a random basis. Control female rats (CTR group, $n = 6$) were left undisturbed in their home cages. At weaning day (post-natal day 21), male and female offspring were house-paired randomly, according with PS treatment (PS or CTR animals), under standard laboratory conditions: artificial 12 h light/dark cycle (lights on from 08:00 a.m. to 08:00 p.m.); room temperature 22°C; food (4RF21, Mucedola SRL) and water were provided *ad libitum*, unless stated otherwise. Animals derived from all litters were used for the experimental procedures.

Body Weight Monitoring

Male and female offspring body weight was monitored from birth until adulthood (from days 1 to 80 post-birth). Body weight was assessed once a week; data is presented as total body weight in grams.

Behavioral Tests

The behavioral timeline is shown in **Figure 3A**.

Anxiety Evaluation

Open Field (OF)

The OF test was conducted in an arena (43.2 cm × 43.2 cm) with transparent acrylic walls and white floor (Med Associates Inc., St. Albans, VT, USA). Rats were placed in the center of the arena and movement was monitored over a period of 10 min with the aid of two 16-beam infrared arrays. Total distance traveled was used as an indicator of locomotor activity.

Elevated Plus Maze (EPM)

The EPM test was carried out under bright white light. Animals were placed individually for 5 min in the center of a black polypropylene plus-shaped platform elevated 72.4 cm above the floor. The apparatus consisted in two open arms (50.8 cm × 10.2 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm; MedAssociates Inc., St. Albans, VT, USA). The number of entries into each arm and the time spent therein were recorded.

Light/Dark Box (LDB)

The LDB test was performed inside the OF arena (43.2 cm × 43.2 cm; MedAssociates Inc., St. Albans, VT, USA). A dark compartment was attached to one side with an opening facing the center of the OF. Animals were individually placed in the center of the illuminated part. The distance traveled and time spent in each compartment was recorded in a single trial of 10 min.

Depressive-Like Behavior Evaluation

Forced Swimming Test (FST)

Rats were placed in cylinders filled with warmed water. After a 5-min pre-test session, animals were retested 24 h later (5 min test). At the end of each session, animals were placed on a heating pad before returned to their home cage. A video camera placed in front of the cylinder was used to record sessions, and later scored by an investigator blind to the experimental details. Time of immobility (passiveness; defined as time spent either immobile or making righting movements to stay afloat), latency to immobility and number of climbing attempts were scored.

Sucrose Preference Test (SPT)

Anhedonia was assessed by the SPT, at two time points, with a 2-day interval. Before each trial, rats were food and water deprived for 12 h. For testing, two pre-weighed bottles containing water or a 2% (m/v) sucrose solution were presented to individually housed animals for 1 h. Sucrose preference (SP) was calculated according to the formula: SP = (sucrose intake/(sucrose intake + water intake)) × 100. Anhedonia was defined as a reduction in SP relative to baseline levels.

Impulsivity Evaluation

Variable Delay-to-Signal (VDS)

The VDS provides rapid and simultaneous assessment of response and decision impulsivity in rodents. This test consists of a series of trials, in a single 30 min session, in which the time period where an action (nose poke) triggers the delivery of a sugared reward is signaled by a light, presented after a variable delay (Leite-Almeida et al., 2013). Animals were kept food-deprived during the whole protocol, which included habituation, training and testing phases. The protocol was performed in a nearly square shaped (25 cm × 25 cm) 5-hole operant chamber (OC; TSE Systems GmbH, Germany). Five square apertures (2.5 × 2.5 cm; #1–#5) with a 3W light bulb and infrared photo beams to detect movements were distributed in a slightly curved wall. On the opposite wall there was another aperture (#6), also equipped with light and photo beams, connected to a food dispenser. Above aperture #6 a house light illuminating the operant chamber. Four chambers were simultaneously used each placed inside sound attenuating chambers, with electrical fans providing ventilation and white noise.

In short, animals were habituated for 2 days. Afterwards, animals were exposed to 10 training sessions that occurred twice daily, with a 5 h interval in between, for five consecutive days. Each *training* session was initiated by turning on the house light and delivering one sugared pellet in the food magazine, the collection of which started an intertrial interval (ITI) of 3 s. Trials then started, consisting of a 3 s period with only the house light on (delay period), followed by lightning of the response aperture for 60 s (response period). Nose pokes in this aperture were either punished with a timeout period in complete darkness, if performed during the delay period (premature responses), or rewarded with the delivery of a pellet if performed during the response period. Collection of a food reward always triggered a 3 s ITI, before a new trial begun. Training sessions were carried until 100 trials were completed or until 30 min had elapsed, on several consecutive days until average premature responding stabilized. Animals failing to complete 100 trials in the 30 min

limit at the end of the training period were removed from the task.

At the end of the training period animals were exposed to the single VDS testing day. The VDS testing session occurred on a single day and consisted of 120 trials, similar to those previously described, with the exception of the delay, which was 3 s in the first and the last 25 trials and randomly either 6 or 12 s in the middle 70 trials (leading to a 3 s—6/12 s—3 s configuration).

Motivation Test

Progressive Ratio (PR) Schedule of Reinforcement

The PR test is a direct reflection of motivation, since it determines the amount of work (breakpoint) that rats are willing to exert to obtain food rewards in an operant task. The protocol was performed according to previous descriptions (Wanat et al., 2010; Soares-Cunha et al., 2016). Rats were placed and maintained on food restriction (∼7 g per day of standard lab chow) to maintain 90% free-feeding weight. Forty-five milligram food pellets (F0021; BioServ), used in the behavioral protocol, were placed in their home cages on the day before the first training session to familiarize the rats with the food pellets. Behavioral sessions were performed in operant chambers (Med Associates).

In short, animals were exposed to 6 days of continuous reinforcement (CRF) training, in which each lever pressing resulted in the delivery of one food pellet, followed by one session of fixed-ratio (FR) 1 (in which one lever pressing is required to obtain one food pellet), four sessions of FR 4 (in which four lever presses are required to obtain one food pellet) and one session of FR8 (in which eight lever presses are required to obtain one food pellet). On the test day, rats were exposed to PR test. PR sessions were identical to FR4 sessions except that the operant requirement on each trial (T) was the integer (rounded down) of $1.4^{(T-1)}$ lever presses, starting at one lever press. PR sessions ended after 15 min elapsed without completion of the response requirement in a trial.

Corticosterone Measurement

Mothers' corticosterone levels were determined in blood samples (250 μl) withdrawn from the tail vein before the beginning of the stress protocol, at two distinct time-points (at the beginning of the light cycle (8:00) and at the beginning of the dark cycle (20:00)), and after the last day of stress protocol (at the beginning of the light cycle (8:00) and at the beginning of the dark cycle (20:00)). Adult progeny's (male and female CTR and PS animals derived from at least four different litters) corticosterone levels were also determined in blood samples (250 μl) withdrawn from the tail vein before a 30 min restraint stress protocol, and 120 min afterward. Corticosterone concentration was determined using an ELISA corticosterone kit (ENZO Life Sciences) according to the manufacturers guidelines. Briefly, corticosterone from 10 μl of plasma was extracted and ran in the corticosterone assay that relies on the recognition of corticosterone from a specific ELISA antibody. Quantification of corticosterone from samples was calculated as the average net optical density bound for each standard and sample.

Corticosterone Levels in Response to Acute Stress

To assess the response of the HPA axis to acute stress, blood from the tail (tail pinching) of the animals was collected at 14:00 (for assessment of corticosterone basal levels). Immediately after this period animals were exposed to an acute stress, consisting of a 30 min restraint protocol. Rats were individually placed in a plastic cylindrical box with holes. Two hours after stress blood was again collected by tail pinching.

Pregnant stress-exposed and control dams were exposed to the corticosterone response to stress assay on gestation day 20.

Progeny (male and female CTR and PS) corticosterone response to stress was assessed on post-natal day 60.

Corticosterone levels were measured in the plasma of rats as described above.

Macrodissection

Male and female CTR and PS animals (n = 6/group, derived from at least four different litters) were deeply anesthetized with lethal dose of pentobarbital, decapitated and heads were immediately snap-frozen in liquid nitrogen. Brain areas of interest were rapidly dissected on ice under a stereomicroscope, observing anatomical landmarks (according to Paxinos and Watson, 2005). Samples were snap-frozen (dry ice) and stored at −80°C until use.

Neurochemical Evaluation

Levels of catecholamines were assayed by high-performance liquid chromatography, combined with electrochemical detection (HPLC/EC) using a Gilson instrument (Gilson, Middleton, WI, USA), fitted with an analytical column (Supleco Supelcosil LC-18 3 mM, Bellefonte, PA, USA; flow rate: 1.0 ml min^{-1}). Samples were stored overnight in 0.2 N perchloric acid at −20°C, sonicated (5 min on ice) and centrifuged at 5000 g. The resulting supernatant was filtered through a Spin-X HPLC column (Costar, Lowell, MA, USA) to remove debris and 150 ml aliquots were injected into the HPLC system, using a mobile phase of 0.7 M aqueous potassium phosphate (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg l^{-1}) and Na-EDTA (40 mg l^{-1}). A standard curve using known concentrations of all catecholamines was run each day.

Histological Procedures

Male and female CTR and PS animals (n = 4/group, derived from at least four different litters) were anesthetized with pentobarbital and were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were removed and placed in 4% PFA. After 4 weeks in PFA, brains were split into two hemispheres by a midsagittal section and processed for stereology, according to the procedure previously described (Keuker et al., 2001). Briefly, they were included in glycolmethacrylate (Tecnovit 7100) and every other microtome-cut section (30 μm) was then collected on a gelatinized slide, stained with Giemsa, and mounted with Entellan New (Merck). The shrinkage factor was calculated according to Madeira et al. (1990).

Stereological Procedures

Volume estimations was obtained using StereoInvestigator® software (MicroBrightField, Williston, VT, USA) and a motorized microscope (Axioplan 2, Carl Zeiss, Hamburg, Germany) attached to a camera (DXC-390, Sony Corporation, Tokyo, Japan). The Cavalieri's principle (Gundersen et al., 1999) was applied to evaluate the volume of each region. For BNST and amygdala sub-regions, central amygdala (CeA) and basolateral amygdala (BLA; brain regions identified using a brain atlas (Paxinos and Watson, 2005)), every 4th section was used. For NAc sub-regions, NAc core and NAc shell (brain regions identified using a brain atlas (Paxinos and Watson, 2005)), every eighth section was used. For hippocampal sub-regions, *cornus ammonis* (CA) 1, CA3 and dentate gyrus (DG; brain regions identified using a brain atlas (Paxinos and Watson, 2005)), every 12th section was used. The volume of the region of interest was calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Total cell numbers were estimated using the optical fractionator method (West et al., 1991). Briefly, a grid of virtual 3D-boxes (30 μm × 30 μm × 15 μm) equally spaced (using the same grid spacing as for volume estimations) was superimposed on every section of the lamina of interest and cells within boxes were counted. Coefficients of error were automatically computed, according to the formulas of Gundersen et al. (1999) for cell numbers and Gundersen and Jensen (1987) for volume estimations. Glial cells were not included in the estimations, and the discrimination between neuronal and glial cell body profiles was based on the criteria described before (Ling et al., 1973; Peinado et al., 1997).

Statistical Analysis

Normality tests were performed for all data (Shapiro-Wilk test). Outliers were identified using Tukey's fences; outliers were removed before proceeding with statistical analysis.

Student's *t*-test was used for analysis of corticosterone levels; stereology data (volume and number of cells); catecholamine levels and the behavioral tests, with the exception of VDS (experimental group × delay) and CRF and FR trainings of the PR test (experimental group × day of training), in which analysis of variance (ANOVA) and Bonferroni *post hoc* test were used.

Since catecholamine levels within the BNST distribution was not normal, non-parametric Mann-Whitney test was applied.

Results are presented as mean ± SEM. All statistical analysis was performed using Prism GraphPad (v7) and results were considered significant for $P \leq 0.05$.

RESULTS

Establishment of a Maternal Chronic Unpredictable Mild Stress Protocol

We developed a model of maternal exposure to chronic unpredictable mild stress, which consisted in exposure to three distinct stressors—loud noise, strobe lights and restraint—with the duration of 4 h per day, three times per week, on a random order. The protocol began on pregnancy day 3 and extended until day 20 of pregnancy (**Figure 1A**).

We were able to observe significant changes in circulating corticosterone levels measured in the plasma of mothers exposed to gestational stress (PS). Before the beginning of the stress protocol, pregnant dams showed normal levels of corticosterone, with a low peak in the morning blood collection and a high peak in the night collection, as expected (**Figure 1B**; CTR: $t_{(6)} = 3.81$, $p = 0.009$; PS: $t_{(5)} = 4.1$, $p = 0.009$). At the end of stress protocol, the circadian rhythm of corticosterone secretion was disrupted (**Figure 1C**; CTR: $t_{(8)} = 4.1$, $p = 0.003$; PS: $t_{(6)} = 0.6$, $p = 0.593$; CTR day vs. PS day: $t_{(7)} = 3.4$, $p = 0.0109$). In addition, we also found that PS-exposed mothers presented increased release of corticosterone upon exposure to an acute stress (**Figure 1D**; $t_{(6)} = 8.5$, $p < 0.001$), suggesting impairment in the negative feedback of the HPA axis.

Prenatal Exposure to Unpredictable Mild Stress Enhances Corticosterone Release in the Progeny

We evaluated the body weight gain of both male and female control (CTR) rats and those prenatally exposed to PS. There were no differences in the weight gain of the progeny since birth until 10 weeks of age (**Figures 2A,B**). We found a trend for increased corticosterone in both sexes of PS group, though it was highly variable between animals (**Figure 2C**; males: $t_{(9)} = 1.8$, $p = 0.103$; females: $t_{(8)} = 1.8$, $p = 0.1143$). Interestingly, both PS males and PS females secreted higher levels of corticosterone in response to an acute stress (**Figure 2D**; males: $t_{(10)} = 3.1$, $p = 0.01$: females: $t_{(10)} = 3.1$, $p = 0.01$), suggesting impairment in the HPA axis activity caused by PS exposure.

Prenatal Exposure to Mild Stress Causes Anxious-Like Behavior in Both Sexes

In order to assess if PS exposure could have an impact in the behavior of adult offspring, we exposed 2-month old rats to the EPM, the OF and the LDB test to assess anxious behavior. In the EPM, both male and female PS animals spent significantly less time in the open arms (**Figure 3B**; males: $t_{(15)} = 2.2$, $p = 0.04$; females: $t_{(20)} = 3.9$, $p = 0.0008$), suggesting increased anxious behavior. In agreement, in the LDB test, PS exposure caused a significant decrease in time spent in the light side of the box (anxiogenic) in comparison with CTR rats in both sexes (**Figure 3C**; males: $t_{(13)} = 2.1$, $p = 0.05$; females: $t_{(13)} = 2.17$, $p = 0.049$). Regarding the OF test, no significant differences were found in the distance traveled (**Supplementary Figure S1A**) or time spent (**Supplementary Figure S1B**) in the center and the periphery of the arena between PS and CTR rats of both sexes.

Prenatal Exposure to Mild Stress Causes Depressive-Like Behaviors in Both Sexes

Next, we also evaluated depressive-like behavior using the FST. PS exposure causes a significant increase in the time rats spend

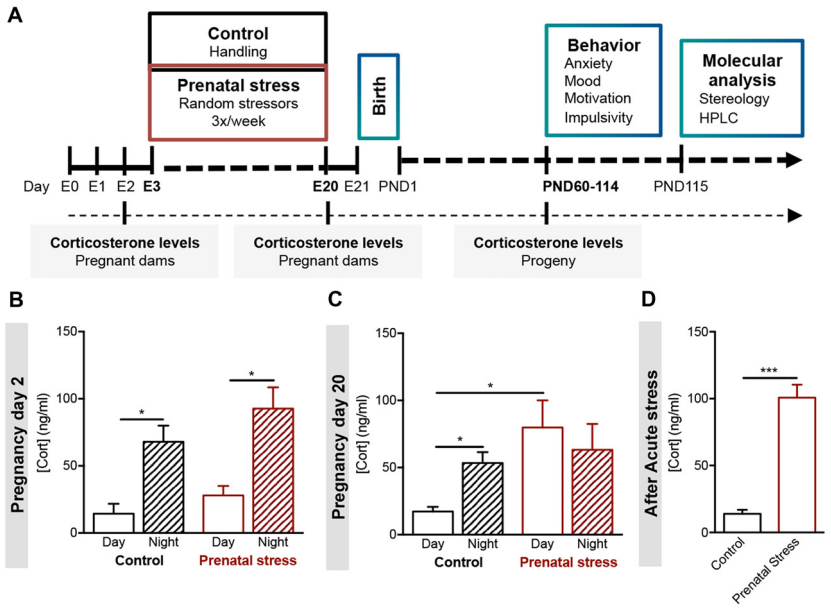

FIGURE 1 | Development of a model of prenatal exposure to unpredictable mild stress. **(A)** Experimental timeline. Pregnant females Wistar han rats (age of 8 weeks, $n = 6$) were exposed to an unpredictable stress protocol (consisting of strobe lights, restraint or noise), applied three times per week in a random fashion from day 3 to day 20 of pregnancy (progeny—prenatal stress group). Progeny of control group derived from age-matched female rats that were handled daily throughout pregnancy. Blood samples were collected from all pregnant dams on day 2 and day 20, and from progeny in adulthood. Different behavioral tests were conducted in young adult male and female progeny. Stereological measurements and catecholamine levels of specific brain regions were evaluated in the progeny. **(B)** Morning (8 am) and night (8 pm) serum corticosterone levels of control and stress-exposed prenatal stress (PS) pregnant dams were measured at the beginning of pregnancy (day 2, $n = 5$) and **(C)** at the end of pregnancy (day 20, $n = 4$). PS pregnant dams present a disruption in corticosterone circadian secretion at later stages of gestation. **(D)** Corticosterone levels of control and PS mothers were also measured after exposure to an acute stressor (restraint for 30 min; $n = 4$). PS mothers present significantly higher corticosterone secretion after acute stress in comparison to controls group. Error bars denote SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

immobile in both sexes (**Figure 3D**; males: $t_{(14)} = 3.1$, $p = 0.0087$; females: $t_{(22)} = 2.56$, $p = 0.0178$), consistent with increased depressive-like behavior. In addition, we examined the hedonic status of PS-exposed rats by performing the SPT. Interestingly, no major differences were observed in the SPT test between groups (**Figure 3E**).

Prenatal Exposure to Mild Stress Does Not Induce Motivational Deficits Nor Causes Impulsivity Traits

Next, we evaluated the motivational status of animals using the PR schedule of reinforcement (PR test), which measures the willingness of the animals to work to obtain a food pellet (reward). The breakpoint, i.e., when animals give up, represents a direct measure of motivation. First, we observed that both male and female animals acquired instrumental conditioning in the CRF training, shown by the increase in lever press over the days of training (**Supplementary Figure S2A**; CONT males vs. PS males: $F_{(4,28)} = 109$, $p < 0.001$; CONT females vs. PS females: $F_{(4,40)} = 91.4$, $p < 0.001$). Similarly, both sexes increased lever pressing over fixed ratio (FR) days of training (**Supplementary Figure S2B**; CONT males vs. PS males: $F_{(4,120)} = 50.7$, $p < 0.001$; CONT females vs. PS females: $F_{(4,160)} = 45.1$, $p < 0.001$), and show significantly more lever pressing in the active lever than in the inactive lever (**Supplementary Figure S2B**; CONT males vs. PS males: $F_{(3,30)} = 136.4$, $p < 0.001$; CONT females vs. PS females: $F_{(3,40)} = 120.8$, $p < 0.001$). These results indicate that PS rats of both sexes are able to learn the task in a similar manner. No significant differences were observed in the breakpoint of PS animals in comparison with CTR rats in both sexes, suggesting that PS did not influence motivation (**Supplementary Figure S2C**; males: $t_{(15)} = 0.6$, $p = 0.531$; females: $t_{(20)} = 1.0$, $p = 0.318$). Similarly, no differences in the number of food pellets consumed during the PR session were observed (**Supplementary Figure S2D**).

We also tested animals in the VDS test, a paradigm designed to assess impulsivity. Interestingly, no significant differences were found in the VDS test (**Supplementary Figure S2E**).

Structural Changes in the Limbic System Caused by Prenatal Stress Exposure

In order to better understand the cause of the behavioral deficits observed in PS rats, we measured the volume and the number of cells, and evaluated catecholamine content of different regions of the limbic system, namely the BNST, hippocampus, amygdala and NAc.

PS causes a significant decrease in the volume of the BSNT in both males and females (**Figures 4A,B**; males: $t_{(9)} = 4.3$, $p = 0.0018$; females: $t_{(6)} = 2.8$, $p = 0.0322$). Females also presented a significant reduction in total number of cells in the same brain region (**Figure 4C**; $t_{(5)} = 4.4$, $p = 0.0071$). Additionally, PS

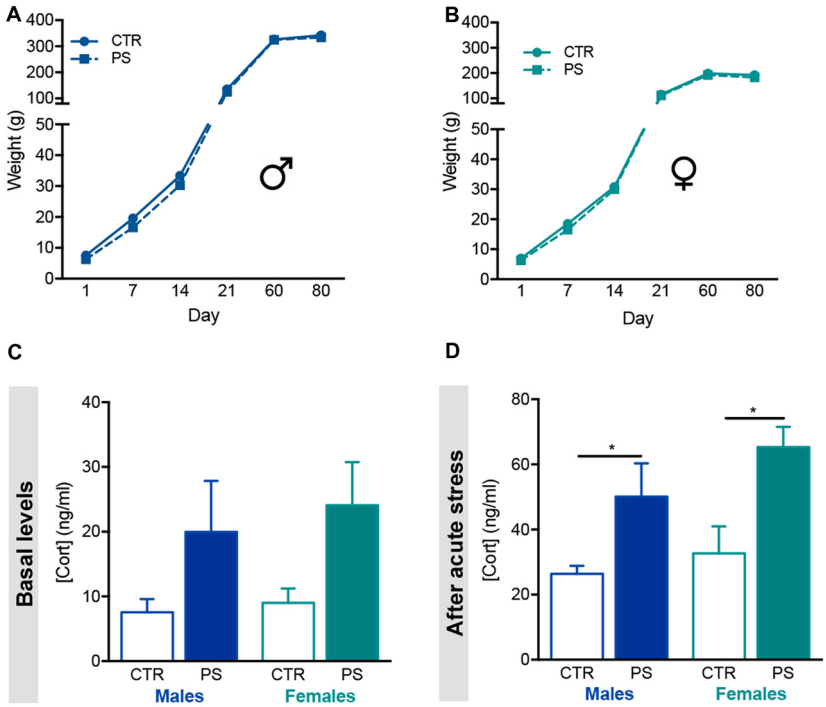

FIGURE 2 | Prenatal mild stress disrupts corticosterone levels in offspring. **(A)** Body weight gain of male rats prenatally exposed to stress (PS, $n = 28$) and control group (CTR, $n = 8$), showing no differences in body weight gain as animals aged. **(B)** Body weight gain of female rats prenatally exposed to stress (PS, $n = 20$) and control group (CTR, $n = 9$), showing no differences between groups. **(C)** Basal serum corticosterone levels of male and female PS is increased in comparison with CTR rats, although not statistically significant ($n_{PS\ males} = 6$, $n_{CTR\ males} = 6$, $n_{PS\ females} = 6$, $n_{CTR\ females} = 4$). **(D)** Upon acute stress exposure (restraint for 30 min), male and female PS rats presented significantly increased serum levels of corticosterone ($n_{PS\ males} = 8$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 6$, $n_{CTR\ females} = 6$). Error bars denote SEM. $^*p < 0.05$.

exposure caused a trend for decreased levels of norepinephrine and dopamine of male PS rats (**Figure 4D**; norepinephrine: $U_6 = 2.0$, $p = 0.114$; dopamine: $U_6 = 2.0$, $p = 0.057$), and a significant decrease in PS female rats in comparison to CTR female rats (**Figure 4D**; norepinephrine: $U_6 = 0.0$, $p = 0.0286$; dopamine: $t_{(6)} = 2.6$, $p = 0.039$).

A significant decrease in the volume and total number of cells of the CA3 layer of the ventral hippocampus (**Figures 5A,B**, volume: $t_{(7)} = 3.4$, $p = 0.0111$; **Figure 5C**, number of cells: $t_{(6)} = 3.4$, $p = 0.0141$) and in the total number of cells in the CA1 sub-region (**Figure 5C**; $t_{(6)} = 5.9$, $p = 0.0011$) in PS males, but not in females. On the other hand, CA3 and CA1 sub-regions of the dorsal hippocampus were significantly enlarged in male PS rats in comparison with male CTR rats (**Figure 5D**; dorsal CA1: $t_{(8)} = 2.6$, $p = 0.031$; CA3: $t_{(6)} = 2.6$, $p = 0.030$), with no differences in the total number of cells (**Figure 5E**). No differences were found in females. Both male and female PS animals presented a significant decrease in the levels of serotonin in the hippocampus, when compared with same-sex controls (**Figure 5F**; males: $t_{(6)} = 5.7$, $p = 0.001$; females: $t_{(6)} = 3.2$, $p = 0.019$).

No differences were found in the volume of the amygdala sub-regions (**Figure 6A**)—CeA (**Figure 6B**) and BLA (**Figure 6C**)—and the NAc (**Figure 6D**)—core (**Figure 6E**) and shell (**Figure 6F**) sub-regions.

DISCUSSION

Since early studies by David J. Barker focusing on the *fetal and developmental origins of adult disease*, a large effort has been made to identify how early life adversity can program adult life. In humans, the large proportion of information about the long-term effects of stress exposure during pregnancy on the offspring of human subjects has been obtained from retrospective studies (Brouwers et al., 2001; Weinstock, 2001; Kofman, 2002; Gutteling et al., 2006). These studies have been helpful to associate maternal stress with impaired metabolic, immune and neuropsychological outcomes in the progeny (Lupien et al., 2009). However, one challenge of human studies is that external variables are not easily controlled, and it is very difficult to evaluate the long-term consequences of PS without interference of daily life stress. In this perspective, the development and study of animal models of PS, in which the timing, duration and intensity of exposure to stress is strictly controlled, poses a great advantage for this type of studies.

In this work, we show that exposure to chronic unpredictable mild stress during pregnancy induces depressive and anxious symptoms in the male and female adult offspring, accompanied by structural and molecular changes in the hippocampus and BNST brain regions. Importantly, these findings corroborate the premise that adverse events occurring during prenatal period can

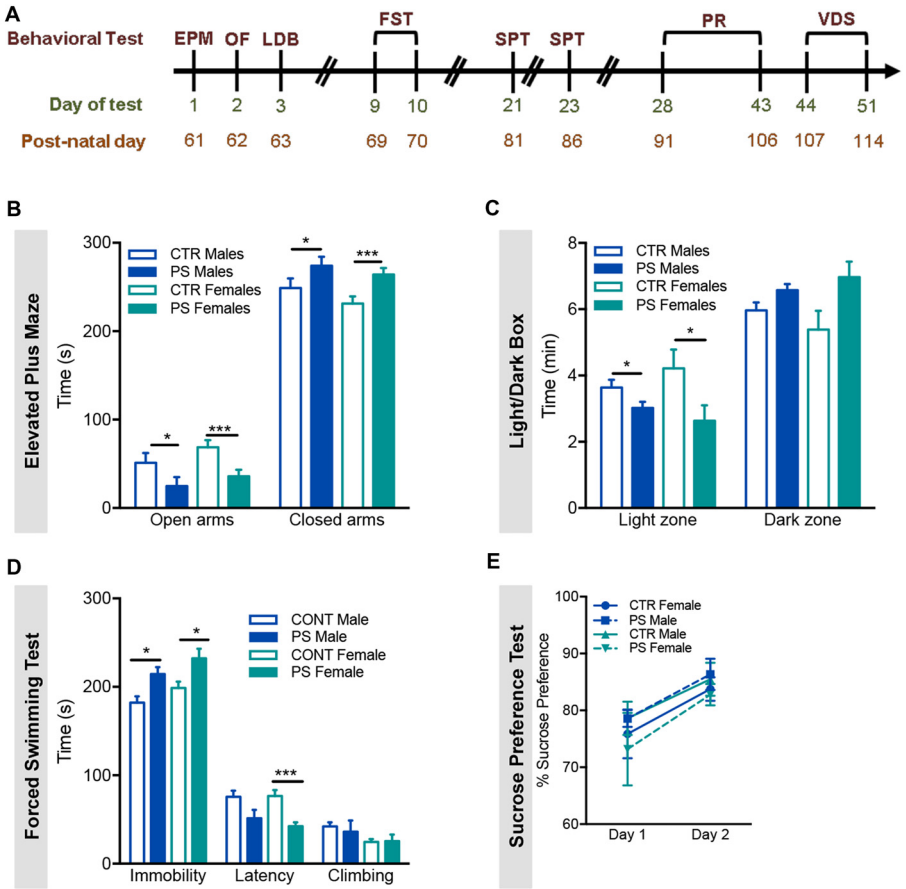

FIGURE 3 | Prenatal mild stress induces anxious- and depressive-like behaviors. **(A)** Timeline of the behavioral tests performed; all rats performed the same behavioral tests in the order shown; EPM, Elevated Plus Maze; OF, Open field; LDB, Light/Dark Box; FST, Forced Swimming Test; SPT, Sucrose Preference Test; PR, Progressive Ratio; VDS, Variable Delay to Signal. **(B)** In the EPM, PS male and female rats exhibited a decrease in time spent in the open arms of the maze, when compared with control group ($n_{PS\ males}$ = 9, $n_{CTR\ males}$ = 9, $n_{PS\ females}$ = 11, $n_{CTR\ females}$ = 12), indicative of anxious behavior. **(C)** In agreement with an anxious phenotype, in the LDB test, PS male and female rats spent significantly less time in the light zone, when compared with same-sex CTR ($n_{PS\ males}$ = 11, $n_{CTR\ males}$ = 9, $n_{PS\ females}$ = 8, $n_{CTR\ females}$ = 7). **(D)** In the FST, PS male and female rats present increased immobility time and decreased latency to immobility and climbing attempts ($n_{PS\ males}$ = 11, $n_{CTR\ males}$ = 9, $n_{PS\ females}$ = 12, $n_{CTR\ females}$ = 12), a measure of behavioral despair, and suggestive of depressive-like behavior. **(E)** No major differences in SPT were found ($n_{PS\ males}$ = 8, $n_{CTR\ males}$ = 8, $n_{PS\ females}$ = 12, $n_{CTR\ females}$ = 12). This test evaluates anhedonia, another dimension of depressive-like behavior. Error bars denote SEM. $^{*}p < 0.05$, $^{***}p < 0.001$.

enhance vulnerability for anxious and depressive phenotypes in adulthood both in animal models and humans (nicely reviewed by (Lupien et al., 2009) and (Weinstock, 2016)). Indeed, in humans, it is well recognized that the offspring of women exposed to stressors during gestation, like natural disasters or adverse life events have a higher risk of psychopathology (Weinstock, 2008; Charil et al., 2010), including hyperanxiety and depression (Van den Bergh et al., 2008; Van Lieshout and Boylan, 2010). Animal studies, in general, mimic these findings; nonetheless there are a large number of models of PS, due to the possibility of controlling stressor intensity and duration. Some works report the use of severe PS paradigms such as electric shocks daily throughout pregnancy (Estanislau and Morato, 2005; Yang et al., 2006). A large number of studies use a single stressor throughout pregnancy that often consists of restraint or bright lights (McCormick et al., 1995; Fujioka et al., 2001; Zuena et al., 2008; Lui et al., 2011; Laloux et al., 2012; Van den Hove et al., 2013). Akin to our study, others prefer the use of different stressors in a random manner, such as protocols of gestational unpredictable mild stress, to reduce the likelihood of habituation to stress (Hougaard et al., 2005; Bourke et al., 2013; Wilson et al., 2013; Wang et al., 2015a,b). Regardless of the type of PS, one consistent finding is the appearance of anxiety and depressive-like behavior in offspring (Estanislau and Morato, 2005; Zuena et al., 2008; Laloux et al., 2012; Wilson et al., 2013; Wang et al., 2015a,b).

One important consideration is that most of the studies apply stressors from day 10 of pregnancy onwards to prevent abortion, but we decided to start stress protocol at day 3. This is an important point because there are different windows of susceptibility to stress effects. For example, it has been shown that the same stressor applied in different stages of pregnancy induces different behavioral outcomes (Liu et al., 2008; Zuena et al., 2008; Fujita et al., 2010; Jia et al., 2010; Głombik et al., 2015).

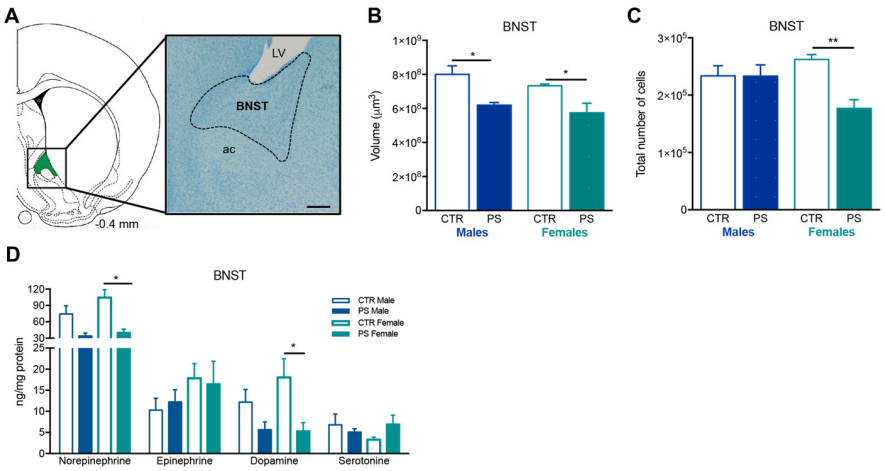

FIGURE 4 | Exposure to prenatal mild stress alters the structure and neurochemical content of the bed nucleus of stria terminalis (BNST). **(A)** Representative image of the BNST anatomy, using a methacrylate coronal section of a rat's brain; numbers represent distance to Bregma; scale bar = 1 mm. **(B)** PS animals of both genders show a significant reduction in the volume of the BNST ($n_{PS\ males} = 7$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 4$, $n_{CTR\ females} = 4$). **(C)** Females also present a decrease in the total number of cells ($n_{PS\ males} = 4$, $n_{CTR\ males} = 3$, $n_{PS\ females} = 3$, $n_{CTR\ females} = 4$), whereas males present a similar number as CTR. **(D)** HPLC measurement of catecholamines revealed that the BNST of female PS animals present a reduction in norepinephrine and dopamine levels in comparison with CTR female rats, and there is a trend for decreased levels of the same amines in male PS rats; no major differences were found in other neurotransmitter levels ($n_{PS\ males} = 4$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 4$, $n_{CTR\ females} = 4$). Error bars denote SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

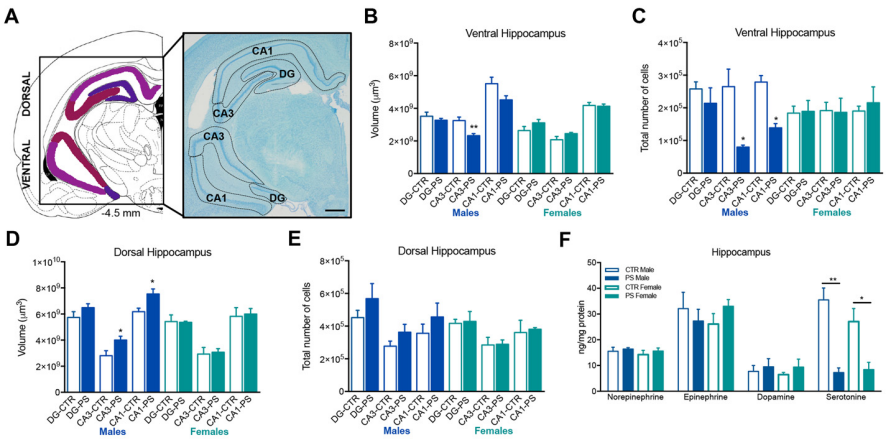

FIGURE 5 | Exposure to prenatal mild stress alters the structure and neurochemical content of the hippocampus. **(A)** Representative image of the ventral and dorsal hippocampus anatomy, using a methacrylate coronal section of a rat's brain; numbers represent distance to Bregma; scale bar = 1 mm. **(B)** Male PS animals showed a significant reduction in the volume of the CA3 sub-region of the ventral hippocampus, with no significant differences in other sub-divisions of this brain region in comparison with male CTR rats; female PS rats did not show any significant difference in comparison with CTR (rats $n_{PS\ males} = 5$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 5$, $n_{CTR\ females} = 4$). **(C)** Male PS rats also presented a reduced number of cells in CA1 and CA3 sub-regions of the ventral hippocampus, but not PS females (rats $n_{PS\ males} = 4$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 4$, $n_{CTR\ females} = 3$). **(D)** Male PS animals showed a significant increase in the volume of the CA1 and CA3 sub-regions of the dorsal hippocampus, with no significant differences in dentate gyrus (DG) in comparison with male CTR rats; female PS rats did not show any significant difference in comparison with CTR female rats ($n_{PS\ males} = 6$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 5$, $n_{CTR\ females} = 4$). **(E)** No differences were observed in the total number of cells between PS and CTR groups in the dorsal hippocampus ($n_{PS\ males} = 6$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 5$, $n_{CTR\ females} = 4$). **(F)** Hippocampal catecholamine profile showing a reduction in serotonin levels in both male and female PS groups in comparison with same-sex CTR rats; no major differences were found in other neurotransmitters (rats $n_{PS\ males} = 4$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 4$, $n_{CTR\ females} = 4$). Error bars denote SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

One important finding is that though we observe anxious and depressive-behaviors, this PS paradigm does not affect other behavioral dimensions such as impulsivity or motivational drive, contrary to other stressors or prenatal GC exposure (Virgolini et al., 2008; Soares-Cunha et al., 2014; Weston et al., 2014). It is important to refer that in this work, animals were tested during the light period of the cycle, which may be a confounding factor in the interpretation of the behavioral data. Though several other studies also evaluate (stress effects in) behavior during the inactive period (Beeler et al., 2006; Roque et al., 2011;

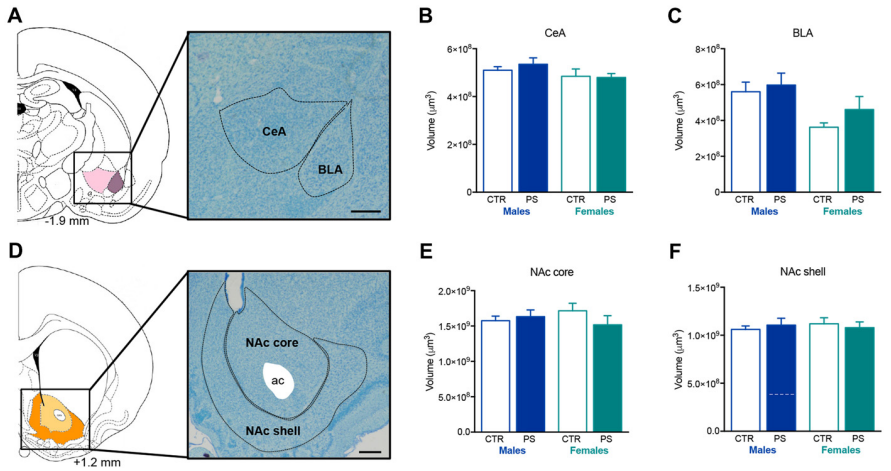

FIGURE 6 | PS exposure does not alter the volume of the amygdala or the nucleus accumbens (NAc). **(A)** Representative image of the amygdala sub-regions, central amygdala (CeA) and basolateral amygdala (BLA), using a methacrylate coronal section of a rat's brain; numbers represent distance to Bregma; scale bar = 1 mm. PS exposure does not alter the volume of **(B)** CeA or **(C)** BLA of both male and female PS rats in comparison with same-sex controls (rats $n_{PS\ males} = 6$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 5$, $n_{CTR\ females} = 3$). **(D)** Representative image of the NAc sub-regions, NAc core and NAc shell, using a methacrylate coronal section of a rat's brain; numbers represent distance to Bregma; scale bar = 1 mm. PS exposure does not alter the volume of **(E)** NAc core or **(F)** NAc shell of both male and female PS rats in comparison with same-sex CTR (rats $n_{PS\ males} = 9$, $n_{CTR\ males} = 4$, $n_{PS\ females} = 5$, $n_{CTR\ females} = 3$). Error bars denote SEM.

Borges et al., 2013b; Alves et al., 2017; Morais et al., 2017), these animals should be tested during their active period as well, which can increase the discriminatory effect of the behavioral tests (Hossain et al., 2004).

Our model of PS caused significant impairment in the production and release of corticosterone in the mothers. Although GCs are necessary in late gestation to promote fetal development (Roberts and Dalziel, 2006; Brownfoot et al., 2013; Moisiadis and Matthews, 2014a,b), inadequate levels of these hormones, such as for example when released early in pregnancy or in high levels, may disrupt the negative feedback of HPA axis, leading to a disturbance in the ability to control the release of these hormones (Roberts and Dalziel, 2006; Brownfoot et al., 2013; Moisiadis and Matthews, 2014a,b). In line with this, we show that, although basal corticosterone levels of PS progeny are similar to control animals, when exposed to an acute stress, these animals present a significant increase in corticosterone levels (in both sexes). This suggests that there is an impairment in the HPA axis, a phenomenon that has been observed in other models of PS (Levine, 1967; Hougaard et al., 2005; Weinstock, 2005; Wilson et al., 2013; Jafari et al., 2017).

Dysregulation of the HPA axis has been associated with different neuropsychiatric conditions such as anxiety and depression in humans (Varghese and Brown, 2001; Wardenaar et al., 2011) and animal models (Varghese and Brown, 2001; Wardenaar et al., 2011). In line with this view, we show that PS animals of both sexes present anxious behavior in two behavioral paradigms. Interestingly, in humans, self-reporting data suggests that PS may also induce a chronic anxiety state in adulthood (Ward, 1991; Van den Bergh and Marcoen, 2004). The anxiogenic phenotype may be caused by the volumetric alterations in the BNST observed in PS progeny, since this brain region has been linked for long to anxiety (Walker et al., 2003; Waddell et al., 2006). Besides the atrophy, the observed catecholaminergic changes may also contribute for the anxious behavior. In fact, other studies have associated an atrophy of the BNST and catecholamine deregulation with anxious behavior (Oliveira et al., 2012). In accordance, several other studies have described a unique role for this brain region in anxiety-like responses in rodents (Walker et al., 2003; Davis et al., 2010).

In addition to the notorious anxious phenotype, PS animals also revealed a significant depressive-like behavior, akin to other animal models of early life stress exposure (Borges et al., 2013a; Palacios-García et al., 2015). One potential explanation for this behavioral trait is the observed decrease in hippocampal serotonin levels of both males and females, given that depression has been strongly linked to impairments in serotonergic neurotransmission (Mahar et al., 2014). In addition to the neurochemical modifications caused by exposure to PS, substantial alterations were also observed in CA1 and CA3 hippocampal structure, being ventral hippocampus hypotrophic and dorsal hippocampus hypertrophic in PS-exposed males. Consistent with our data, other study has reported that PS causes dendritic atrophy of the pyramidal neurons of the CA3 sub-region in offspring (Jia et al., 2010). In addition, our data are in accordance with human studies that show that hippocampal atrophy is associated with recurrent depressive illness (Videbech and Ravnkilde, 2004; McKinnon et al., 2009). Despite this, it is still unclear why PS females present the same behavioral trait without evident changes in hippocampal structure. One cannot rule out that, although structurally intact, the hippocampus may be malfunctioning in the female brain, which is in agreement with the observed serotonergic neurotransmission impairment in these animals.

In addition, one cannot neglect the contribution of other brain regions for depressive behavior such as the amygdala and the NAc. Consistent with the data that we report here showing no volumetric changes in the amygdala of neither PS males nor females, others have also shown the same in depressive human patients (von Gunten et al., 2000; Hickie et al., 2007). However, functional MRI studies show that BLA is hyperactive in several mood disorders, with no apparent volumetric differences (Drevets et al., 1992; Sheline et al., 1996), so one cannot rule out the possibility of this brain region being dysfunctional and (partially) contribute to the observed phenotype. Regarding the NAc, several studies have associated stress-induced changes of this brain region with depressive behavior (Martínez-Téllez et al., 2009; Morales-Medina et al., 2009; Rodrigues et al., 2012; Bessa et al., 2013; Russo and Nestler, 2013; Haim et al., 2014; Francis et al., 2015). However, we found no structural differences in the NAc in this model of PS. Human studies also showed no changes in NAc volume in major depression (Bremner et al., 2000; Hannestad et al., 2006). We also did not find any motivational deficits in PS animals, another core symptom of depressive behavior, nor found any differences in impulsivity which has also been linked to this brain region, suggesting relatively intact NAc (Cardinal et al., 2001; Basar et al., 2010; Soares-Cunha et al., 2016). Yet, functional studies such as *in vivo* electrophysiology or *in vivo* calcium imaging in freely moving animals would be crucial to understand if this brain region (or others) is affected (or not) in this model.

Although we have analyzed the effect of early life exposure to stress in males and females, we could not observe significant differences between sexes. Despite some structural differences of the hippocampus that were observed only in males, both sexes seem to be similarly affected by PS. Curiously, other studies have reported differences between sexes (Roussel et al., 2005; Weinstock, 2007; Boersma and Tamashiro, 2014) and have associated those mainly with hormonal changes (Weinstock et al., 1992; Weinstock, 2007).

In sum, we developed a new model of prenatal mild stress that presents emotional deficits that can now be used to explore in more detail how stress can imprint anatomical, molecular and functional changes in specific brain regions and lead to maladaptive behavior later in life.

AUTHOR CONTRIBUTIONS

AJR, CS-C and NS developed the concept and designed experiments. CS-C and BC performed stress protocol. CS-C, BC and SB performed and analyzed behavior. BC and CS-C performed molecular analysis. CS-C performed statistical analysis on all data. CS-C and AJR wrote the article. All authors discussed and revised the manuscript.

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SUPPLEMENTARY MATERIAL

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FIGURE S1 | Prenatal stress (PS) animals of both genders present similar exploratory activity in the open field (OF). **(A)** Similar time spent in the center and periphery of the open arena between CTR and PS rats (males and females). **(B)** No differences in the distance traveled in the center and the periphery of the arena between groups ($n_{PS\ males}$ = 9, $n_{CTR\ males}$ = 9, $n_{PS\ females}$ = 11, $n_{CTR\ females}$ = 12). Error bars denote SEM.

FIGURE S2 | Prenatal mild stress does not alter motivation for natural rewards or impulsivity. **(A)** Continuous reinforcement (CRF) and **(B)** fixed ratio (FR) trainings of male and female PS rats in the progressive ratio (PR) test, showing no differences in learning curves when compared to same-sex CTR group. **(C)** Breakpoint of PS and control animals, showing that PS exposure does not alter motivation to obtain food ($n_{PS\ males}$ = 9, $n_{CTR\ males}$ = 9, $n_{PS\ females}$ = 12, $n_{CTR\ females}$ = 12). **(D)** The number of food pellets earned during the PR session is similar between groups. **(E)** In the variable delay to signal (VDS), an impulsivity test, the number of premature responses of PS and CTR groups of both males and females is similar within each delay in the VDS test, indicating that PS does not change impulsivity ($n_{PS\ males}$ = 9, $n_{CTR\ males}$ = 9, $n_{PS\ females}$ = 12, $n_{CTR\ females}$ = 12). Error bars denote SEM.

TABLE S1 | Number of pups per sex in each litter of stressed mothers (prenatal stress) and control mothers (control).

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Chronic Social Stress Leads to Reduced Gustatory Reward Salience and Effort Valuation in Mice

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Pathology of reward processing is a major clinical feature of stress-related neuropsychiatric disorders including depression. Several dimensions of reward processing can be impacted, including reward valuation/salience, learning, expectancy and effort valuation. To establish the causal relationships between stress, brain changes, and reward processing pathologies, valid animal models are essential. Here, we present mouse experiments investigating behavioral effects of chronic social stress (CSS) in association learning tests of gustatory reward salience and effort valuation. The reward salience test (RST) comprised Pavlovian pairing of a tone with gustatory reward. The effort valuation test (EVT) comprised operant responding for gustatory reinforcement on a progressive ratio schedule (PRS). All testing was conducted with mice at 100% baseline body weight (BBW). In one experiment, mice underwent 15-day CSS or control handling (CON) and testing was conducted using sucrose pellets. In the RST on days 16–17, CSS mice made fewer feeder responses and had a longer tone response latency, than CON mice. In a shallow EVT on days 19–20, CSS mice attained a lower final ratio than CON mice. In a second CSS experiment, mice underwent CSS or CON and testing was conducted with chocolate pellets and in the presence of standard diet (low effort/low reward). In the RST on days 16–18, CSS mice made fewer feeder responses and had a longer tone response latency, than CON mice. In a steep EVT on days 19–20, CSS and CON mice attained less pellets than in the RST, and CSS mice attained a lower final ratio than CON mice. At day 21, blood levels of glucose and the satiety adipokine leptin were similar in CSS and CON mice. Therefore, CSS leads to consistent reductions in reward salience and effort valuation in tests based on association learning. These reward pathology models are being applied to identify the underlying neurobiology and putative molecular targets for therapeutic pharmacology.

Keywords: social stress, RDoC, reward salience, reward effort, glucose, leptin, mouse model

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INTRODUCTION

Stressful life events are major etiological factors for prevalent neuropsychiatric disorders, including depression. Environmental stressors that precede the onset of depression are often psychosocial and uncontrollable and, therefore, chronic (Kessler, 1997; Kendler et al., 1999; Pryce et al., 2011). Depression is heterogeneous with respect to symptomatology, as indeed are other stress-related neuropsychiatric disorders such as schizophrenia. A core symptom of depression is described as markedly reduced interest or pleasure in (almost) all activities most of the day nearly every day (Dichter et al., 2010). Impaired processing of rewarding events/stimuli also characterizes negative symptoms in schizophrenia (Dichter et al., 2010; Hartmann et al., 2015). The research domain criteria (RDoC) framework places focus on the study and understanding of specific psychological processes, the dysfunction of which is relevant to one or more neuropsychiatric disorders, i.e., transdiagnostic (Cuthbert and Insel, 2013). "Positive valence systems" is the RDoC term for constructs underlying reward-stimulus processing, and examples include: valuation of the salience of a prospective reward (anticipation); learning the association between neutral stimuli and reward (Pavlovian learning), or behavioral actions and reward (operant learning); reward expectancy triggered by stimuli associated with reward; effortful motivation to obtain reward[1].

Automated behavioral tests of such processes are essential for their quantitative study in psychiatric patients compared with healthy control probands, including assessment of the relationship with psychometric measures. Tests of relevance to reward processes that have been applied in depression research to date are based on stimulus discrimination or stimulus choice. For example, Pizzagalli et al. (2005) presented non-clinical subjects with two visual stimuli, one per trial, and correct identification of one stimulus was reinforced-using money-more frequently than correct identification of the other stimulus; such an asymmetric reinforcement ratio leads to a response bias for the more frequently reinforced stimulus. Subjects who scored relatively highly on the self-report psychometric scale, the Beck Depression Inventory, developed a lower reward bias than did subjects with a relatively low depression score. A low response bias for reward is consistent with reduction in one or more of reward valuation, reward learning and reward expectancy, and further tests could be applied to establish which of these is primarily responsible. Treadway et al. (2012) presented depressed and healthy subjects with a choice between high-effort/high-reward and low-effort/low-reward trials, where reward was monetary and, across trials, the size and probability of reward (both low- and high-reward) were variable but predictable. Relative to controls, depressed patients made less high-effort/high-reward choices and were less responsive to size and probability of reward. These effects are clearly consistent with reduced effortful motivation in depression, and also suggest deficient reward valuation and reward learning. In a monetary incentive delay task conducted with functional magnetic resonance imaging (fMRI), one visual stimulus predicts reward (money) and another predicts no reward. In the interval between stimulus response and reward feedback, subjects exhibit increased activity in the ventral striatum/nucleus accumbens. Relative to controls, depressed and schizophrenic patients exhibited lower activation of ventral striatum (Arrondo et al., 2015), and the magnitude of activation was inversely correlated with the Beck Depression Inventory score (Hagele et al., 2015). The nucleus accumbens receives inputs from dopamine neurons in the ventral tegmental area, a major pathway in the mesolimbic dopamine neural circuit of reward processing (Pizzagalli, 2014). Therefore, behavioral tests have identified quantitative deficits in the reward-processing dimensions of approach motivation and possibly also learning, in depressed patients. Interestingly, consummatory pleasure, as measured by the Sweet taste test, is not reduced in depression (Dichter et al., 2010; Treadway, 2016).

Animal models of stress-induced deficits in reward processing analogous to these pathological dimensions in depression are challenging to develop but essential: they enable the causal study of the pathways via which environmental stressors lead to pathophysiological states in reward neurocircuitry and processing, and thereby allow for investigation of novel therapeutic strategies. Given that psychosocial stressors are the major "environmental pathogens" for human emotional disorders (Caspi and Moffitt, 2006), animal models based on manipulations of the social environment would be expected to have particularly high etiological validity (Markou et al., 2009; Pryce and Seifritz, 2011). Examples of social stressors that have been deployed in conjunction with tests of reward include early-life stress in the form of repeated infant-parent separation and chronic adulthood stress in the form of intruder-resident confrontation. In monkeys, for example the common marmoset, early life stress resulted in impaired reversal learning and effort valuation (Pryce et al., 2004), whilst direct neurotoxic depletion of orbitofrontal cortex dopamine also reduced reward sensitivity in a discrimination learning paradigm (Clarke et al., 2014). In rodents, most stress-reward modeling has been conducted in rats to-date. Emphasis has been on the sweet preference test (SPT), in which consumption of sucrose/saccharin relative to water is measured. The initial demonstration (Willner et al., 1987) that chronic unpredictable mild stress (CUMS, a combination of physical and psychosocial stressors repeated over several weeks) led to a reduced sucrose preference that could be restored by antidepressant administration has resulted in a huge number of studies (>1000) with this model (Willner, 2017). However, the SPT is not a test for the dimensions that underlie deficient reward interest in depression, but rather a test for sustained responsiveness during consummatory behavior (Cuthbert and Insel, 2013). Tests of reward bias with analogy to the human test described above (Pizzagalli et al., 2005) have been developed for rats (Der-Avakian et al., 2013, 2016, 2017). In one test version, rats are trained to discriminate between two tone stimuli by pressing the specific lever associated with each tone. In the test phase, they are then presented with ambiguous tones, one of which is more frequently rewarded than the other. Rats exposed to 3 days of social stress had a reduced response bias toward the more frequently rewarded tone (Der-Avakian et al.,

[1]https://www.nimh.nih.gov/research-priorities/rdoc/index.shtml

2017). In another test version, low or high amounts of food reward are each associated with specific substrates (conditioned stimuli); following reward-substrate learning, rats are presented with a substrate choice test with reward now randomized across substrates. Rats treated chronically with the pro-inflammatory cytokine interferon-α developed a low reward bias relative to controls, but retained a normal sucrose preference in the SPT (Stuart et al., 2017). Choice tests for high effort/reward vs. low effort/reward, analogous to the test used to detect effort valuation deficits in depression (Treadway et al., 2012), have also been developed in rats. A major example is concurrent lever pressing for sucrose vs. eating of "free" standard chow; lever pressing is reduced by dopamine antagonists or nucleus accumbens dopamine depletion (Salamone et al., 2007, 2016a,b). Effort valuation of sucrose in the absence of an alternative low reward has been assessed using operant lever pressing on reinforcement schedules, most notably the progressive ratio schedule (PRS; Salomons et al., 2007). Rats exposed to early life stress exhibited, in adulthood, less effort to earn sucrose in a PRS test, a deficit reversed by repeated antidepressant (fluoxetine) treatment (Leventopoulos et al., 2009).

In male mice, we have demonstrated a number of effects on reward processing of a 15-day resident-intruder paradigm that we refer to as chronic social stress (CSS). The paradigm is based on male-male aggression and can therefore not be applied to females; given the relatively high prevalence of depression in women, this is an important caveat. Nonetheless, in male mice, CSS leads, relative to control mice, to reduced operant responding in a time-restricted fixed ratio 1 (FR1) test and a PRS test (Bergamini et al., 2016a, 2018), as well as to fewer spontaneous episodes of approach and FR1 responding to access saccharin or water in a complex home cage environment (Bergamini et al., 2016a). CSS is without effect in a saccharin preference test (Bergamini et al., 2018). Such behavioral effects are consistent with stress-induced deficits in reward salience and effort valuation. Furthermore, in a two-way spatial reversal learning test, CSS mice exhibited less reward-stay responses and made more errors to reversal, consistent with deficits in one or more of reward valuation, learning or expectancy (Bergamini et al., 2016a). Also under two-way spatial learning conditions, CSS mice required more trials to learn that a previously non-rewarded stimulus was now rewarded, consistent with impaired reward learning (Bergamini et al., 2018). Importantly, this latter effect of CSS and that of attenuated PRS responding are also induced by pharmacological nucleus accumbens dopamine depletion (Bergamini et al., 2016b). Furthermore, CSS leads to attenuated mesolimbic dopamine pathway functioning (Bergamini et al., 2018). Building on the findings with this mouse model to-date, the aims of the present study were to investigate CSS effects on gustatory reward valuation and reward learning in a test of Pavlovian conditioning and on reward effort valuation using an operant PRS. We hypothesized that CSS would lead to reduced salience of gustatory reward, particularly in terms of impaired acquisition of the predictive association of a tone with reward availability, and to an additional reduction in reward valuation under conditions of sustained effort to obtain the reward.

MATERIALS AND METHODS

Animals and Housing

The study was conducted with C57BL/6J (BL/6J) male mice bred in-house. Breeding pairs each contributed 1–2 offspring to one or both treatment groups, i.e., CSS, control. Mice were weaned into littermate-pairs at age 3 weeks and remained in these pairs throughout the experiment or until the onset of CSS. Mice in Experiment 1 were transferred from a non-reversed light-dark cycle to a reversed cycle at age 5 weeks, whilst in Experiments 2 and 3 mice were born and remained in reversed cycle conditions. At study onset, mice were aged 12–13, 10–11 and 10–11 weeks in Experiments 1, 2 and 3, respectively (**Figure 1**). Mice were maintained in individually-ventilated type 2L cages containing wood chips, tissue bedding and a sleeping igloo. The temperature was set at 20–22°C and humidity at 50%–60%, and illumination was on a reversed 12:12 h light-dark cycle (white lights off at 07:00–19:00 h). Experimental procedures were conducted during the dark phase, at 08:00–16:00 h. In the home cage, standard chow diet (Complete pellet, Provimi, Kliba AG, Kaiseraugst, Switzerland) was available as detailed below, and water was available continuously. For CSS, the resident mice were ex-breeder males of the CD-1 strain (Janvier Labs, Saint-Berhevin, France) aged 8 months, weighed 37–56 g, and caged singly. This study was carried out in accordance with the recommendations of the Animal Protection Act, Switzerland. The protocol was approved and permit issued (ZH 149/2015) by the Cantonal Veterinary Office, Zurich, Switzerland. All efforts were made to minimize the number of mice studied and any unnecessary stress to those mice that were studied.

Experimental Design

Each of three experiments was conducted with a different, naive mouse cohort (**Figure 1**). Experiments began with mouse handling on each of 5 days. During the next week, baseline body weight (BBW) and food consumption were measured daily. Mice were then food deprived to reduce them to 90%–95% BBW and underwent training with sucrose or chocolate pellet reinforcement during 2 weeks. Post-training, they were then provided with sufficient daily chow to maintain them at 100% BBW for the remainder of the experiment. In Experiment 1, CSS ($N = 19$) and control handling (CON, $N = 18$) were conducted for 15 days, followed by behavioral testing in the reward salience test (RST) and the shallow effort valuation test (EVT) with sucrose pellets. In Experiment 2, mice ($N = 23$) were tested in the RST with sucrose pellets, with or without normal chow in the test cage, the steep EVT with sucrose pellets with or without normal chow, and the steep EVT with chocolate pellets with or without normal chow. In Experiment 3, CSS ($N = 10$) and CON ($N = 10$) were conducted for 15 days, followed by behavioral testing in the RST and the steep EVT, with chocolate pellets. On the day after the end of behavioral testing, blood collection was conducted from these mice for plasma glucose and leptin determination.

Chronic Social Stress

Mice were allocated to CSS and CON groups by counterbalancing for BBW and number of pellets obtained

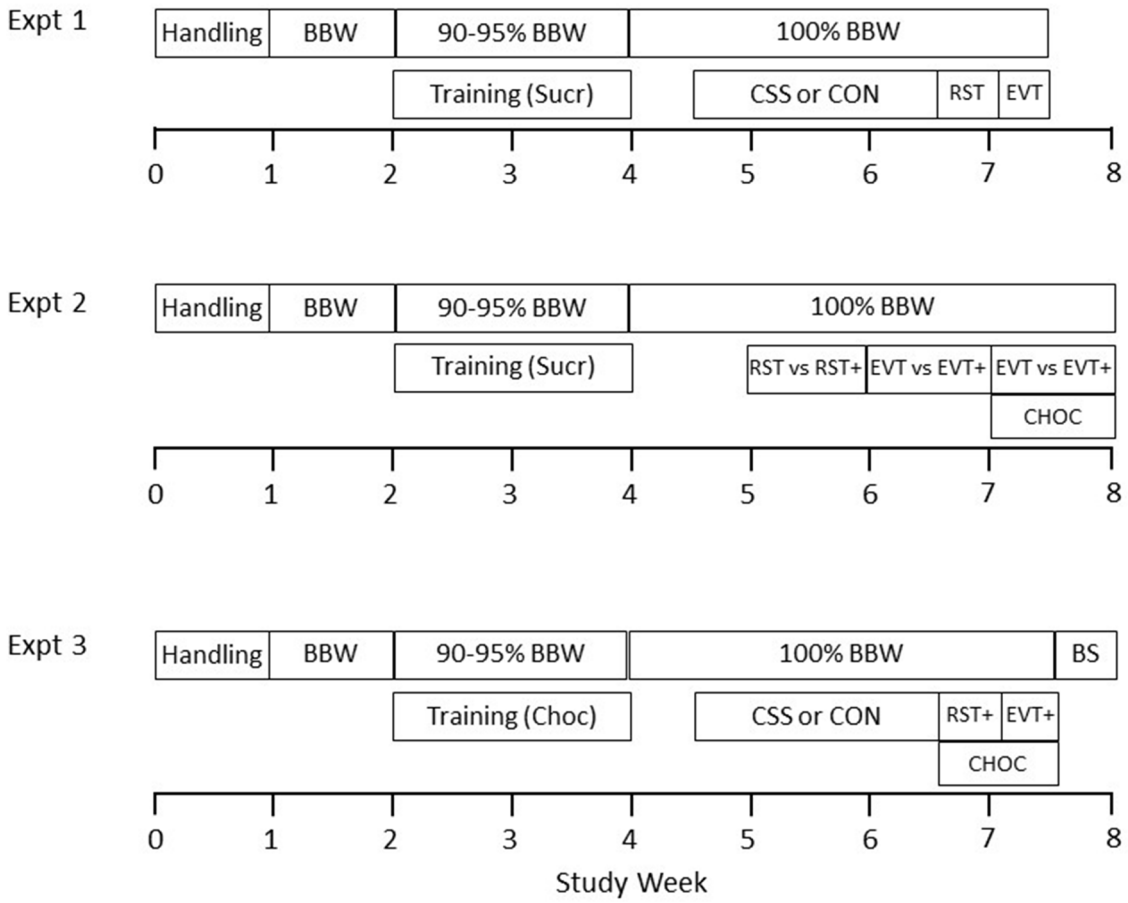

FIGURE 1 | Designs of the three experiments conducted in this study. First, in each experiment, naive mice were handled and then body weight (BW) and chow intake were measured on each day for 7 days, to calculate baselines for body weight (BBW) and chow intake. Second, mice were chow-deprived to reduce BW to 90%–95% BBW, and then placed each day into the behavioral apparatus for training of collection of sweet pellets from the feeder and operant nose poking to initiate pellet delivery. Pellets were either sucrose (Sucr) or chocolate (Choc). Experiment 1: mice were exposed to chronic social stress (CSS) or control handling (CON) handling for 15 days, followed by two daily sessions in the reward salience test (RST) and two daily sessions in the effort valuation test (EVT), with Sucr pellet reward. Throughout this period, mice were fed sufficient daily chow to maintain them at 100% BBW. Experiment 2: mice were given three daily sessions in the RST either with (RST+) or without a chow pellet in the test chamber and with Sucr pellet reward. They were then given two sessions at a 2–3-day interval in the EVT with Sucr pellet reward, one with (EVT+) and one without a chow pellet (order counterbalanced), and finally two sessions at a 2–3-day interval in the EVT with Choc pellet reward, one with and one without a chow pellet (order counterbalanced). Throughout this period, mice were fed sufficient daily chow to maintain them at 100% BBW. Experiment 3: mice were exposed to CSS or CON handling for 15 days, followed by three daily sessions in the RST with a chow pellet and two daily sessions in the EVT with a chow pellet, and Choc pellet reward. Throughout this period, mice were fed sufficient daily chow to maintain them at 100% BBW. On the day following completion of behavioral testing, trunk blood sampling (BS) was conducted for plasma glucose and leptin determinations.

in the final training sessions (see below). CSS was conducted according to the resident-intruder protocol described in detail elsewhere (Azzinnari et al., 2014). Briefly, unfamiliar, aggressive, ex-breeder CD-1 mice were caged singly and with a transparent, perforated plastic divider separating the cage into two equal compartments. On each of 15 days the BL/6J CSS mouse was placed in the same compartment as the CD-1 mouse for a total of 60 s physical attack or 10 min maximum. After the physical attack, the CSS mouse remained in the same compartment and the CD-1 mouse was transferred to the opposite compartment for 24 h, during which the two mice had visual, olfactory, auditory and limited tactile contact. The CSS × CD-1 mouse pairings were rotated so that each CSS mouse was confronted with a novel CD-1 mouse each day. To prevent bite wounds the lower incisors of CD-1 mice were trimmed every 3rd day. From day 15 and throughout behavioral testing, each CSS mouse remained in the same divided cage with the same CD-1 mouse without further attacks. As expected, all CSS mice displayed submissive behavior and vocalization during the 10 min periods of proximate contact, and all CD-1 mice attacked. The mean duration of daily attack received by CSS mice was 45–50 s (Azzinnari et al., 2014). Control (CON) mice remained in their littermate pairs during the dark period and the littermates were separated by a divider during the light period; this was done to increase the similarity of the thermal and metabolic demands on CSS and CON mice during this primarily inactive period (Gordon et al., 1998).

Behavioral Tests

Controlled Feeding and Body Weight

Body weight (BW) and free-feeding chow intake per littermate pair were measured every day for 1 week and mean values provided baselines for each mouse; for food intake, weight eaten per mouse was calculated by assuming chow consumption was proportional to BW. Beginning the following week, mice were chow restricted to 90%–95% of baseline BW (BBW) to ensure adequate motivation during operant training with sweet pellet reinforcement. After completion of training, for 3 days prior to and throughout CSS/CON and behavioral testing in Experiments 1 and 3, and for 1 week prior to and throughout testing in Experiment 2, mice were provided with sufficient standard diet to return to and maintain 100% BBW. As described previously (Bergamini et al., 2016a, 2018), CSS mice require more standard diet per day to maintain 100% BBW than do CON mice. During the behavioral testing period, the daily chow was provided in the home cage 2–3 h after operant training/testing, and all chow had been consumed prior to the time of testing on each day.

Test Apparatus

Behavioral testing was conducted in an infra-red illuminated room adjacent to the mouse holding room, between 09:00 h and 13:00 h. Modular operant chambers (TSE Systems) were used with inner dimensions of 20 × 17 × 18 cm and a house light provided 10 lux illumination. In the middle of one side wall, a feeder port (Ø = 20 mm × depth = 35 mm) was located into which food pellets were delivered from a pellet dispenser. Each nose poke by the mouse into the port was detected via infra-red beam. A tone stimulus could be presented via a speaker located above the feeder port. An operant nose-poke port could be inserted to the side of the feeder port (Ø = 20 mm × depth = 30 mm); a white lamp set into the recess of this port was illuminated to indicate it was active, and nose pokes were detected via an infra-red beam. The center-to-center distance between the nose-poke port and the central feeder was 55 mm. Four such chambers, each placed within an attenuation chamber, were run in parallel by the control PC and interface. The chamber floor and walls were wiped with 70% ethanol between each run. Further details are provided in Ineichen et al. (2012).

Training

Training sessions were conducted on consecutive days and had a maximum duration of 30 min. First, without the operant nose-poke port, mice were trained that sucrose pellets were available in the feeder port. In the first session 15 pellets were placed in the feeder port and one further pellet was delivered automatically each 45 s; in subsequent sessions only one pellet was placed in the feeder port at session onset and mice were required to eat ≥30 pellets in one session; 3–7 sessions were required. At the next stage, mice were required to nose poke once into the feeder to trigger each pellet delivery and learning criterion was two consecutive sessions with ≥30 pellets retrieved and eaten; mice required 2–7 sessions. Then the nose-poke port was introduced and one nose-poke (FR1) into the illuminated port was required to extinguish the light and trigger pellet delivery; pellet retrieval was followed by a 5 s time out and the nose-poke port was then illuminated again. In the first three sessions, 5, 3 and 1 pellets, respectively, were placed in the nose-poke port. Mice were required to earn and eat ≥30 pellets in two consecutive sessions; 3–6 sessions were needed. In Experiments 1 and 2 mice were trained with sucrose pellets (14 mg Dustless Precision Pellets, Bio-Serv), and in Experiment 3 with chocolate-flavor sucrose pellets (20 mg Dustless Precision Pellets, Bio-Serv).

Mice required 8–15 days to complete the three training stages. They were then given sufficient standard diet each day to restore 100% BBW. In Experiments 1 and 3, after 3 days the CSS/CON procedure was then started followed by behavioral testing, and in Experiment 2 behavioral testing was started 7 days after training completion. The number of pellets earned in the final training session and the BBW were used to counterbalance allocation of mice to CSS and CON groups.

Reward Salience Test (RST)

The chamber contained the feeder port and no operant nose-poke port. The session was initiated by presenting a novel tone at 6.5 kHz and 80 dB; the tone had a maximum duration of 30 s and one response into the feeder triggered immediate pellet delivery and tone termination after 1 s. The intervals between consecutive CSs were variable and 50 ± 30 s (inter-trial interval, ITI). Feeder responses during the ITI were counted but were without consequence. Therefore, the tone was a conditioned stimulus (CS) that predicted pellet availability in the feeder. The maximum number of trials per session was 50 and the maximum session duration was 60 min. Two RST sessions were run on consecutive days in Experiment 1 and three such sessions in Experiments 2 and 3. Outputs of interest were number of pellets obtained, latency to respond during CS, and number of responses during ITI. For analysis, the following measures were calculated for the first 30 CS trials per session: pellets obtained; mean CS response latency; mean ITI response latency (ITI duration/ITI responses); CS/ITI reciprocal ratio (1/(CS response latency/ITI response latency)) i.e., if CS response latency < mean ITI response latency, then ratio > 1. Sucrose pellets were used in Experiments 1 and 2 and chocolate pellets in Experiment 3. In Experiment 2, half of the mice were tested without and the other half with a normal chow pellet (3 g) placed on the floor of the chamber. In Experiment 3, all mice were tested with a chow pellet available. The weight of chow pellet eaten was calculated.

Effort Valuation Test (EVT)

The EVT began on the day following completion of the RST. The chamber now contained the operant nose-poke port and feeder port. The session was initiated with illumination of the nose-poke port, and one nose poke resulted in a 1 s tone (6.5 kHz, 80 dB) and delivery of a pellet into the feeder; pellet retrieval was followed by a 5 s time out and then the nose-poke port was illuminated again. In Experiment 1, the following, shallow PRS of reinforcement was used: trials 1–8 = 1 response/trial, 9–16 = 3 responses/trial, 17–24 = 5, 25–32 = 7, 33–40 = 9, and so on. In Experiments 2 and 3, the following, steep PRS was used: trials 1–5 = 1, 6–10 = 5, 11–15 = 9, 16–20 = 13, 21–25 = 17. The

session duration was 45 min. In previous studies (Ineichen et al., 2012; Bergamini et al., 2016a) a break point was used, defined as no response at either one or both of the nose-poke and feeder ports for 600 s; however, since this occurred only rarely we did not use a break point in this study. Outputs/measures of interest were: number of operant responses, number of pellets earned, final ratio attained, total number of feeder responses and feeder response latency, and weight of chow eaten. In Experiment 1, the EVT was run on two consecutive days and sucrose pellets were used. In Experiment 2, all mice were tested in four tests with 2–3-day intervals between consecutive tests: the first two tests were with sucrose pellets and mice were counterbalanced with respect to being tested with a normal chow pellet in the chamber for either the first or second test; these mice were then introduced to chocolate pellets in their home cage on 2 days, and in the following days the EVT ± chow pellet procedure was repeated with chocolate pellets. In Experiment 3, the EVT was run on two consecutive days with chocolate pellets and with a chow pellet in the chamber.

Plasma Glucose and Leptin Determination

In Experiment 3, on the day after completion of behavioral testing, mice were decapitated and trunk blood was collected into EDTA-coated tubes (Microvette 500 K3E, Sarstedt) and placed on ice. Blood samples were centrifuged at 3000 rpm for 15 min at 4°C and plasma aliquots were transferred to Protein Lobind tubes (Eppendorf) and stored at −80°C. Plasma glucose was determined using a hand-held glucose meter (Accu-Chek). Plasma leptin was determined using an ELISA kit (Mouse Leptin EZML-82K, Merck Millipore) according to the manufacturer's protocol: standards (0.23–30 ng/mL) were run in triplicate and samples in duplicate, and leptin concentrations of low and high quality controls provided with the kit were within the expected range.

Data Analysis

For the reward valuation test, each daily session comprising CS 50 trials, data analysis focused on the first 30 CS trials per session i.e., trials 1–30 in session 1, trials 51–80 in session 2 and trials 101–130 in session 3. Mixed factorial analysis of variance (ANOVA) was used: in Experiments 1 and 3, for each output measure there was a between-subject factor of group (CSS, CON) and within-subject factor of trials; in Experiment 2, there was a between-subject factor of chow pellet (with, without) and within-subject factor of trials. For the EVT, in Experiments 1 and 3 the effects of CSS on behavior were analyzed separately for the two daily sessions, using unpaired *t*-tests. For Experiment 2, a 2 × 2 repeated measures ANOVA was used with within-subject factors of reward (sucrose, chocolate) and chow pellet (with, without). In each experiment, findings in the EVT were similar for both daily sessions, and data are reported for the second session specifically. Significant interaction effects were analyzed using *post hoc* pairwise comparisons with Bonferroni correction. Statistical significance was accepted at p-value ≤ 0.05. Effect size values were calculated as partial eta squared (η^2) for ANOVA and as Cohen's d for t-test. Data are presented as means and where an estimate of variance is given this is 1 standard deviation (SD).

RESULTS

Experiment 1

During the 15-day period of environmental manipulation, CON and CSS mice were maintained at 101.4% ± 2.0% and 101.2% ± 2.2% BBW, respectively. This required providing the baseline amount of daily chow to CON mice and 123% of baseline daily chow to CSS mice (**Table 1**). During the subsequent period of behavioral testing, both groups were again maintained at BBW, and this required 95% of baseline daily chow in CON mice and 115% thereof in CSS mice (**Table 1**). In the RST, CSS mice obtained fewer pellets (**Figure 2A**) than CON mice across the first 30 trials of each of the two sessions (Group main effect: $F_{(1,35)} = 23.17$, $p < 0.0001$, $\eta^2 = 0.40$). Both CON and CSS mice increased the number of sucrose pellets obtained from session 1 (trials 1–30) to session 2 (trials 51–80; Trials main effect: $F_{(1,35)} = 22.19$, $p < 0.0001$, $\eta^2 = 0.39$). The CS response latency (**Figure 2B**) was longer in CSS than CON mice ($F_{(1,35)} = 23.83$, $p < 0.0001$, $\eta^2 = 0.41$), whilst in both CON and CSS mice it decreased similarly across sessions ($F_{(1,35)} = 20.94$, $p < 0.0001$, $\eta^2 = 0.37$). For ITI response latency (**Figure 2C**) there was a Group × Trials interaction effect ($F_{(1,35)} = 6.18$, $p < 0.02$, $\eta^2 = 0.15$) and a Group main effect ($F_{(1,35)} = 21.55$, $p < 0.0001$, $\eta^2 = 0.38$): the latency was longer in CSS than CON mice; whilst it decreased in CSS mice from trials 1–30–51–80, it remained low and constant in CON mice. These group differences were reflected in the CS/ITI reciprocal ratio (**Figure 2D**), which provided the measure of CS-reward learning. There was a Group × Trials interaction effect ($F_{(1,35)} = 12.06$, $p < 0.001$, $\eta^2 = 0.26$): both groups had a ratio value of close to 1 at trials 1–30, with the ratio slightly higher in CSS than

TABLE 1 | Details of mouse body weight and home-cage feeding in the three experiments.

Experiment	Group (*N*)	Baseline BW g (WK)[1]	Daily chow during CSS (g)	% Baseline chow during CSS	Age (Wk)[2]	% BBW during testing	Daily chow during testing (g)	% Baseline chow during testing
Experiment 1	CON (18)	28.4 ± 1.9 (14)	3.5 ± 0.3	99.7% ± 8.0%	18	100.4% ± 1.8%	3.2 ± 0.3	95.1% ± 7.6%
	CSS (19)	27.5 ± 2.5 (14)	4.6 ± 0.6***	122.5% ± 14.3%***	18	101.7% ± 2.3%*	4.0 ± 0.4***	115.1% ± 10.8%***
Experiment 2	Naive (21)	28.5 ± 2.2 (12)			15	97.6% ± 2.1%	3.3 ± 0.3	96.7% ± 7.0%
Experiment 3	CON (10)	27.7 ± 2.0 (12)	3.7 ± 0.3	102.4% ± 7.5%	16	100.6% ± 1.3%	3.4 ± 0.3	96.8% ± 6.1%
	CSS (10)	28.6 ± 1.4 (12)	4.5 ± 0.5***	135.0% ± 8.4%***	16	101.4% ± 1.2%	4.2 ± 0.6***	125.0% ± 12.1%***

*[1]Age at assessment of baseline body weight (BBW). [2]Age at onset of behavioral testing. *$p < 0.05$, ***$p < 0.001$, for CSS versus CON in unpaired t-test.*

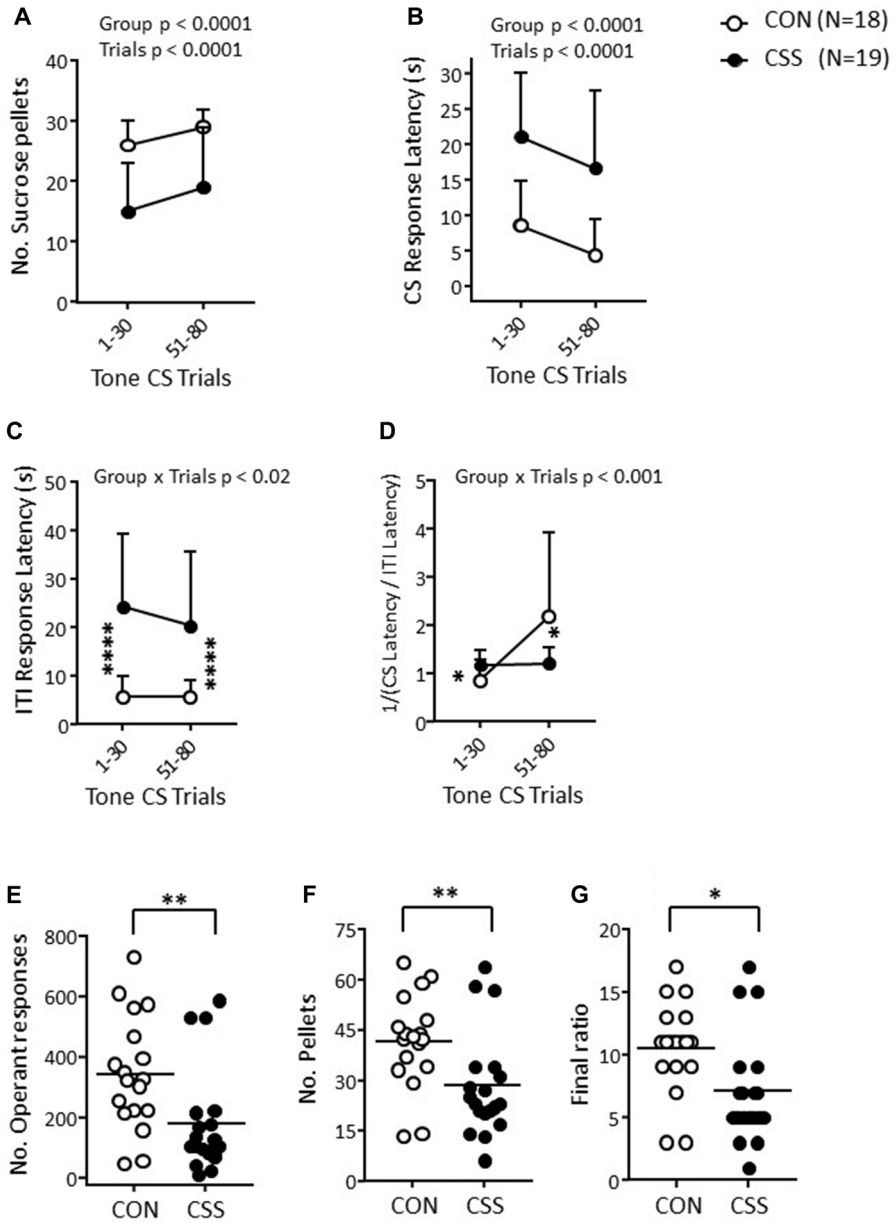

FIGURE 2 | Effects of CSS on days 1–15 on reward-directed behavior in Experiment 1. **(A–D)** Scores for the first 30 trials per session in the RST on days 16–17: **(A)** Number of sucrose pellets obtained. **(B)** Mean response latency to a tone conditioned stimulus (CS; maximum duration 30 s) associated with pellet availability. **(C)** Mean response latency in the inter-trial intervals (ITI, 50 ± 30 s) between successive CS. **(D)** Mean CS/ITI reciprocal ratio. Values are overall mean ± SD. Significant statistical effects indicated were obtained with mixed factorial analysis of variance (ANOVA); $*p < 0.05$, $****p < 0.0001$, in *post hoc* tests of single sessions following Group η Trial interaction. **(E–F)** Scores for the EVT on day 19, using a shallow progressive ratio schedule (PRS). **(E)** Number of operant responses. **(F)** Number of sucrose pellets earned. **(G)** Final ratio attained. Graphs are scatter plots and mean values per group. Significant statistical effects indicated were obtained with unpaired *t*-tests; $*p < 0.05$, $**p < 0.01$.

CON mice ($p < 0.02$, $\eta^2 = 0.16$), and then the ratio increased in CON mice specifically, such that CSS resulted in a relatively low ratio, at trials 51–80 ($p < 0.03$, $\eta^2 = 0.14$). In session 2, across all 50 trials CON mice obtained 46 ± 6 pellets and CSS mice obtained 30 ± 16 pellets ($t_{(35)} = -4.00$, $p < 0.0003$).

In the EVT with a shallow PRS, effortful responding was reduced in CSS mice, as indicated by the lower numbers of operant responses (**Figure 2E**; $t_{(35)} = -2.72$, $p < 0.01$, $d = 0.90$), and, therefore, pellets obtained (**Figure 2F**; $p < 0.01$, $d = 0.90$) and lower final ratio attained (**Figure 2G**; $p < 0.02$, $d = 0.87$).

Experiment 2

Details of BW and home cage feeding are given in **Table 1**. In the RST, mice in the +chow group ate 0.1 ± 0.05 g of chow (**Figure 3E**). There was no effect of the chow

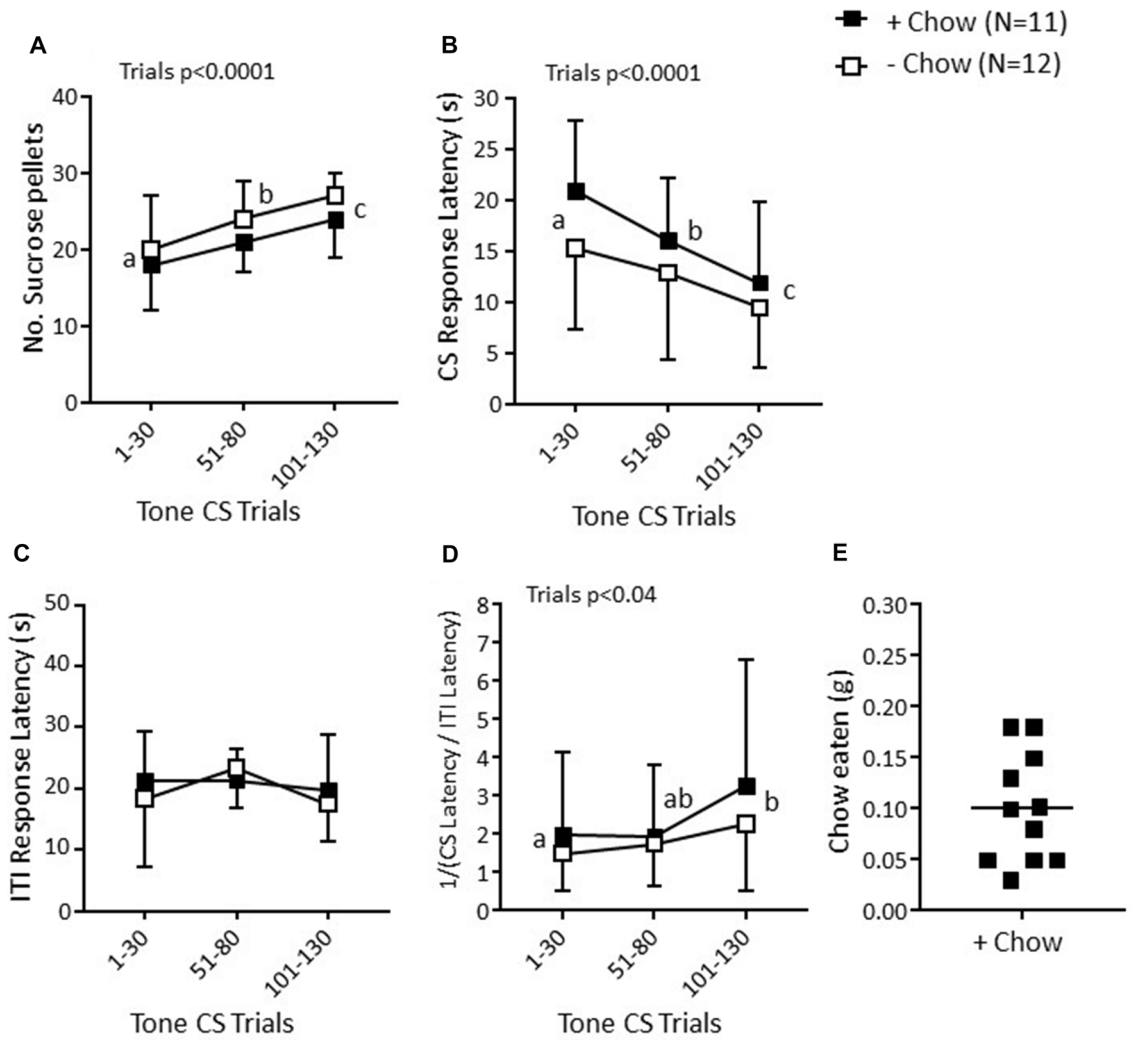

FIGURE 3 | Effects of provision of a chow pellet on behavior in the RST in non-manipulated mice in Experiment 2. Scores are for the first 30 trials per session on three consecutive days. **(A)** Number of sucrose pellets obtained. **(B)** Mean response latency to a tone CS (maximum duration 30 s) associated with pellet availability. **(C)** Mean response latency in the inter-trial intervals (ITI, 50 ± 30 s) between successive CS. **(D)** Mean CS/ITI reciprocal ratio. Values are overall mean ± SD. Significant statistical effects indicated were obtained with mixed factorial ANOVA; trials denoted with different letters were significantly different in *post hoc* Bonferroni tests. **(E)** In the +Chow group, scatter plot and overall mean for the mean weight of chow eaten during the three test sessions.

pellet availability in the test chamber on any RST measure ($p \geq 0.16$, **Figures 3A–D**). The number of sucrose pellets obtained (**Figure 3A**) increased consistently across sessions ($F_{(2,42)} = 22.85$, $p < 0.0001$, $\eta^2 = 0.55$). CS response latency (**Figure 3B**) decreased consistently across sessions ($F_{(2,42)} = 19.04$, $p < 0.0001$, $\eta^2 = 0.50$), whereas the ITI response latency (**Figure 3C**) remained constant across sessions ($p = 0.39$). These measures were reflected in the CS/ITI reciprocal ratio (**Figure 3D**), which was close to 1 at trials 1–30 and then increased significantly ($F_{(2,42)} = 3.71$, $p < 0.04$, $\eta^2 = 0.18$) to being higher at trials 101–130 than 1–30 ($p < 0.03$, $\eta^2 = 0.34$).

In the EVT, mice in the +chow groups ate 0.18 ± 0.11 g chow with sucrose pellets and 0.18 ± 0.12 g chow with chocolate pellets (**Figure 4E**). Despite chocolate pellets (20 mg) being heavier than sucrose pellets (14 mg), the number of operant responses (**Figure 4A**) was higher with chocolate than with sucrose pellets (Pellet main effect: $F_{(1,22)} = 12.89$, $p < 0.002$, $\eta^2 = 0.37$), whilst the presence of chow decreased the number of operant responses (Chow main effect: $F_{(1,22)} = 9.57$, $p < 0.005$, $\eta^2 = 0.30$). Accordingly, mice obtained more chocolate than sucrose pellets (**Figure 4B**) ($F_{(1,22)} = 23.06$, $p < 0.0001$, $\eta^2 = 0.51$) and obtained more pellets with −chow than +chow ($F_{(1,22)} = 12.29$, $p < 0.002$, $\eta^2 = 0.36$). Mice also attained a higher final ratio (**Figure 4C**) with chocolate than sucrose pellets ($F_{(1,22)} = 22.86$, $p < 0.0001$, $\eta^2 = 0.51$) and with −chow than +chow ($F_{(1,22)} = 14.29$, $p < 0.001$, $\eta^2 = 0.39$). There was no significant effect of pellet type ($p = 0.08$) or chow availability ($p = 0.44$) on the latency to retrieve the pellet from the feeder (**Figure 4D**).

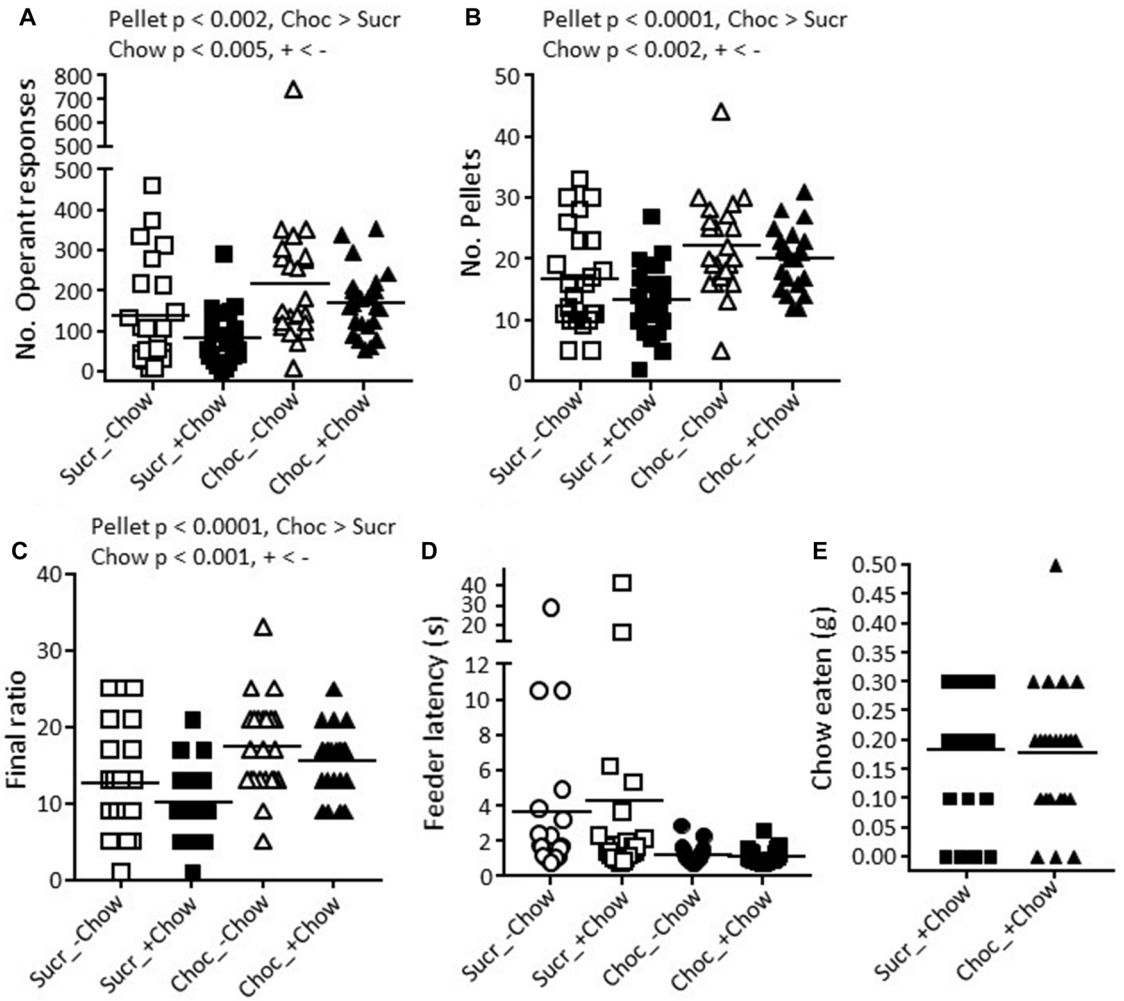

FIGURE 4 | Effects of provision of a chow pellet and of sweet pellet type on behavior in the EVT in non-manipulated mice in Experiment 2. Each mouse was tested under each condition, first with sucrose (Sucr) and then with chocolate (Choc) pellets, and by counterbalancing with respect to whether they were tested first with (+) or without (–) a chow pellet. **(A)** Number of operant responses. **(B)** Number of sucrose pellets earned. **(C)** Final ratio attained. **(D)** Mean latency to collect the pellet from the feeder. Graphs are scatter plots and mean values per group. Significant statistical effects indicated were obtained with 2 × 2 repeated measures ANOVA with within-subject factors of reward and chow pellet. **(E)** In the +Chow conditions, scatter plots and overall means for the weight of chow eaten during the test session.

Experiment 3

During the 15-day environmental manipulation period, CON and CSS mice were maintained at 99.8% ± 0.9% and 98.5% ± 1.1% BBW, respectively. This required providing 102% of baseline daily chow in CON mice and 135% thereof in CSS mice (**Table 1**). During the period of behavioral testing, both groups were again maintained at BBW, and again this required close to the baseline amount of chow in CON mice and 125% thereof in CSS mice (**Table 1**). In the RST, CSS mice obtained less chocolate pellets (**Figure 5A**) than CON mice across the three sessions ($F_{(1,18)}$ = 37.95, $p < 0.0001$, $\eta^2 = 0.68$). Both CON and CSS mice increased the number of pellets obtained across sessions ($F_{(2,36)}$ = 5.37, $p < 0.009$, $\eta^2 = 0.23$), obtaining more in trials 101–130 than trials 1–30. The CS response latency (**Figure 5B**) was higher in CSS than CON mice (F(1, 18 = 28.68, $p < 0.0001$, $\eta^2 = 0.61$). In CON and CSS mice it decreased consistently across sessions ($F_{(2,36)}$ = 7.59, $p < 0.002$, $\eta^2 = 0.30$), being lower in trials 101–130 than trials 1–30 ($p < 0.005$, $\eta^2 = 0.47$). For ITI response latency (**Figure 5C**) there was a Group × Trials interaction effect ($F_{(2,36)}$ = 8.51, $p < 0.001$, $\eta^2 = 0.32$): whereas latency started low and increased in CON mice it started high and decreased in CSS mice; it was higher in CSS than CON mice at trials 1–30 ($p < 0.0001$) and 51–80 ($p < 0.02$) and similar in the two groups in trials 101–130. These group differences were reflected in the CS/ITI reciprocal ratio (**Figure 5D**), where there was also a Group × Trials interaction effect ($F_{(2,36)}$ = 9.92, $p < 0.0001$, $\eta^2 = 0.37$): both groups had a ratio close to 1 at trials 1–30, and then the ratio increased in CON mice specifically, so that CSS resulted in a relatively low ratio at trials 101–130 ($p < 0.003$, $\eta^2 = 0.41$). Both CON

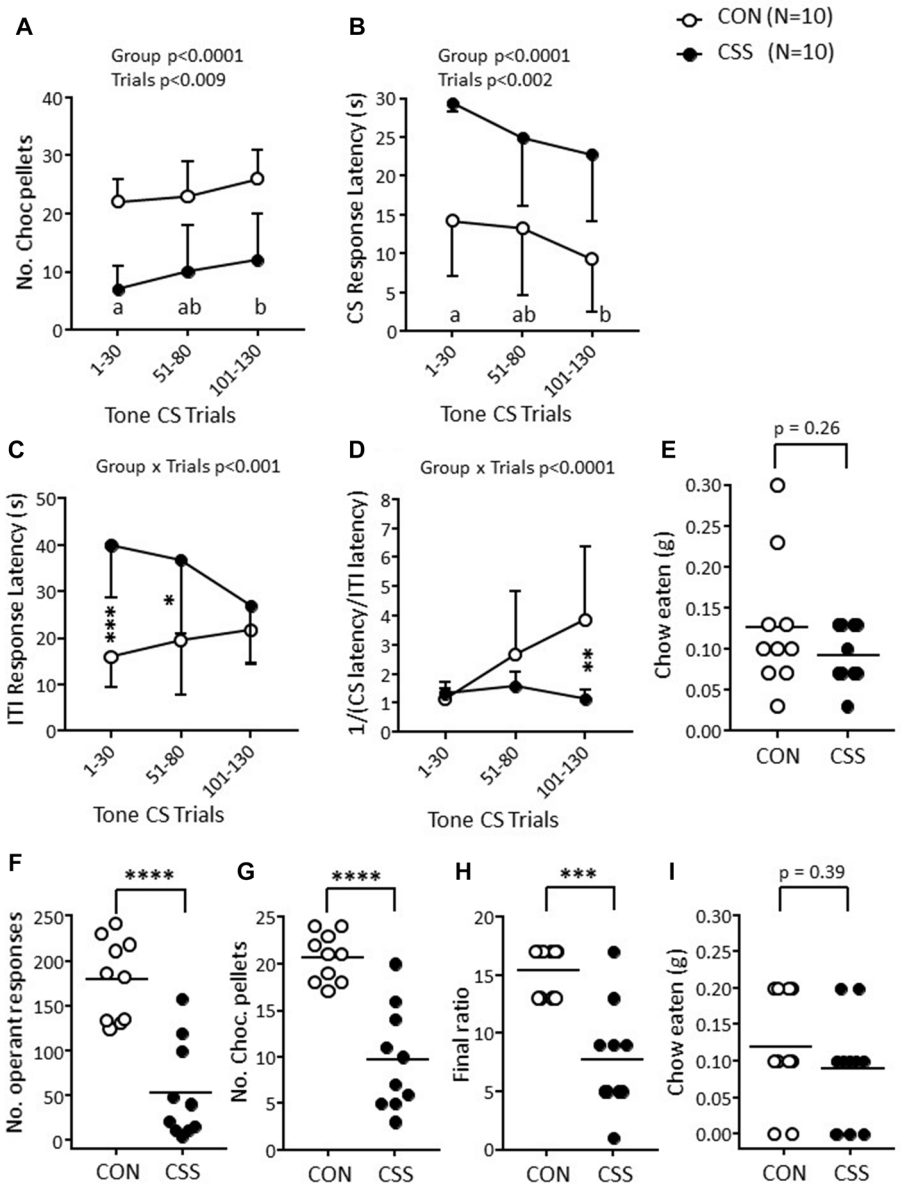

FIGURE 5 | Effects of CSS on days 1–15 on reward-directed behavior in Experiment 3. **(A–D)** Scores for the first 30 trials per session in the RST on days 16–18: **(A)** Number of sucrose pellets obtained. **(B)** Mean response latency to a tone CS (maximum duration 30 s) associated with pellet availability. **(C)** Mean response latency in the inter-trial intervals (ITI, 50 ± 30 s) between successive CS. **(D)** Mean CS/ITI reciprocal ratio. Values are overall mean ± SD. Significant statistical effects indicated were obtained with mixed factorial ANOVA; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, in *post hoc* tests of single sessions following Group × Trial interaction. **(E)** Scatter plot and overall mean for the mean weight of chow eaten during the three test session; *p* value is for the unpaired *t*-test. **(F–I)** Scores for the EVT on day 20, using a steep PRS. **(F)** Number of operant responses. **(G)** Number of chocolate pellets earned. **(H)** Final ratio attained. **(I)** Weight of chow eaten during the session. Graphs are scatter plots and mean values per group. Significant statistical effects indicated were obtained with unpaired *t*-tests; $^{***}p < 0.001$, $^{****}p < 0.0001$.

and CSS mice ate an average of 0.1 g chow during each RST test (**Figure 5E**). In session 3, across all 50 trials, CON mice obtained 39 ± 8 pellets and CSS mice obtained 17 ± 11 pellets ($t_{(18)} = -5.14$, $p < 0.0001$).

In the EVT with a steep PRS, effortful responding was reduced in CSS relative to CON mice, as indicated by the lower numbers of operant responses (**Figure 5F**; $t_{(18)} = -5.69$, $p < 0.0001$, $d = 2.54$) and, therefore, pellets obtained (**Figure 5G**; $p < 0.0001$, $d = 2.56$) and lower final ratio attained (**Figure 5H**; $p < 0.0002$, $d = 2.13$). Both CON and CSS mice ate an average of 0.1 g chow during each EVT test (**Figure 5I**). In contrast to Experiment 1 where a shallow PRS was used and CON and CSS mice obtained a similar number of pellets per session in the RST and EVT (**Figure 2**), in this experiment both CON and CSS mice obtained

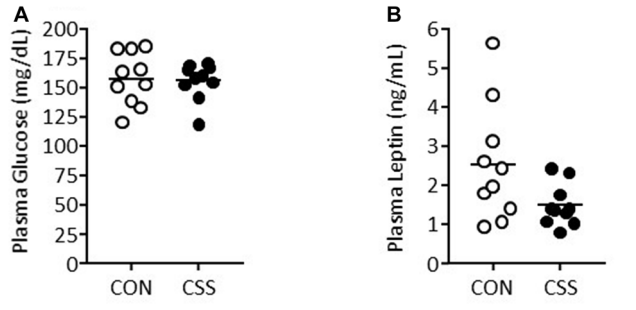

FIGURE 6 | Effect of CSS on days 1–15 on plasma values in Experiment 3: **(A)** Glucose, **(B)** Leptin.

less pellets per session in the steep EVT than in the RST (**Figure 5**).

On the day after completion of behavioral testing (day 21), trunk blood samples were collected. There was no effect of CSS on plasma glucose levels (**Figure 6A**): CON mice had 157 ± 25 and CSS mice 156 ± 16 mg/dL ($p = 0.84$). There was no effect of CSS on plasma levels of the appetite-suppressant adipokine leptin (**Figure 6B**): CON mice had 2.54 ± 1.69 and CSS mice 1.50 ± 0.54 ng/mL ($t_{(18)} = -2.07$, $p = 0.053$, $d = 0.83$).

DISCUSSION

Animal models of stress-induced deficits in reward processing that have analogy with human reward psychopathologies are essential for detailed study of the underlying pathophysiology. Here, we report in mice that CSS leads to deficits in behavioral tests for the dimensions of reward salience and effort valuation relative to gustatory stimuli, and identify some test variables that are important in determining the robustness of these CSS effects.

The RST was used to determine whether CSS would lead to a deficit in one or both of appetitive responding for sweet-tasting reward under minimally effortful conditions and learning the association between a CS and reward availability. In Experiments 1 and 3, CSS mice obtained less sweet pellets than CON mice, due to a longer response latency during the CS. This deficit was consistent across sessions, although both CON and CSS mice obtained more rewards as they progressed through trials/sessions. In contrast to CON mice, there was no evidence for acquisition of the tone-reward association in CSS mice, as demonstrated by their CS/ITI reciprocal ratio remaining at a value close to 1. Of course, these two effects of CSS are inter-dependent, with reduced interest in reward leading to reduced appetitive responding and reduced exposure to the predictive associations of tone-reward and ITI no tone-no reward. Nonetheless, the combination of increased number of pellets obtained across sessions and absence of Pavlovian learning suggests that a deficit in reward learning does contribute to the CSS effect in this test. Reductions in reward salience, learning and expectancy are also implicated in the deficits in development of response bias in a signal-reward detection test as observed in human subjects scoring high on the Beck Depression Inventory (Pizzagalli et al., 2005) and in rats that had experienced 3 days of social stress (Der-Avakian et al., 2017). With regards to the neurobiology underlying the deficits of CSS mice in the RST, the evidence suggests involvement of the basolateral nucleus of the amygdala. In mice exposed to a similar tone-sucrose test, glutamatergic neurons in the basolateral amygdala that projected to the nucleus accumbens displayed an increase in synaptic strength, based on the AMPA receptor/NMDA receptor ratio. This was determined by measuring receptor-specific excitatory post-synaptic current amplitude using whole-cell patch-clamp recording in neurons obtained from tone-sucrose conditioned mice and unconditioned control mice (Namburi et al., 2015). Therefore, it will be important to investigate the effects of CSS on these "reward neurons" projecting from basolateral nucleus to nucleus accumbens (Pryce, 2018).

Comparing the two CSS-RST experiments, the number of pellets obtained was higher and the CS and ITI response latencies shorter in Experiment 1 compared with 3. This was despite Experiment 3 being conducted with chocolate pellets, which were more of an incentive than sucrose pellets, at least according to the direct comparison in the EVT in Experiment 2. Experiment 2 also demonstrated that the presence of normal chow did not impact significantly on sucrose pellets earned in the RST, so this cannot account for the overall reduction in reward salience in Experiment 3 vs. 1. Otherwise, the most notable difference between the two experiments was that the mice in Experiment 1 underwent a shift in the light-dark cycle at age 5 weeks (see "Animals and Housing" section). Given the sensitive inter-relationships between circadian rhythmicity, its disruption, levels of appetite hormones and glucose metabolism (Kim et al., 2015), the circadian shift might have caused a chronic increase in sucrose sensitivity. Whilst this remains to be clarified, it is encouraging that the CSS-RST model is sufficiently robust to accommodate baseline differences in reward salience.

The EVT was used to determine whether CSS would lead to a deficit in appetitive responding for sweet-tasting reward under increasingly effortful conditions. In Experiments 1 and 3, CSS mice obtained less sweet pellets than CON mice due to reduced operant responding. The session duration was always maintained at 45 min, rather than using a break point if there was no operant or feeder response within a certain period; therefore, the overall rate of responding was reduced in CSS mice. Compared with controls, depressed patients made less high-effort/high-reward choices relative to low-effort/low-reward choices (Treadway et al., 2012). Behavior in high-effort/high-reward tests is clearly dependent on reward salience, which the RST had already identified as being reduced in CSS mice. Of particular interest in the EVT is whether there is an additional CSS effect on effort valuation. In Experiment 1, which used a shallow PRS in the absence of a low-effort/low-reward alternative, CON and CSS mice obtained as many sucrose pellets in the EVT as they had in the RST. This is consistent with the EVT not being sufficiently challenging to impact on effort reward valuation and, perhaps related to this, not identifying an increase in effort discounting in CSS mice. Experiment 3 used a steep PRS

for responding for chocolate pellets and also provided a low-effort/low-reward alternative. Under these conditions, both CON and CSS mice obtained fewer chocolate pellets in the EVT than they had in the RST. These data are consistent with this EVT being sufficiently challenging to impact on effort valuation of reward. Furthermore, the decrease in operant responses was particularly marked in CSS mice relative to CON mice (vis effect size >2), consistent with CSS increasing effort discounting in addition to the decrease in reward salience under low-effort conditions.

In the EVT and other effort-based tests, the interval or delay between onset of responding and obtaining of reinforcement requires a neurobiological system that maintains motivation. In humans, using a monetary incentive delay task conducted with fMRI, in the interval between stimulus response and reward feedback subjects exhibit increased activity in the ventral striatum/nucleus accumbens and, relative to controls, depressed and schizophrenic patients exhibit lower activation (Arrondo et al., 2015), with the magnitude of activation being inversely correlated with the Beck Depression Inventory score (Hagele et al., 2015). Accordingly, the nucleus accumbens and the dopamine inputs it receives from neurons in the ventral tegmental area could be a major pathway in the regulation of effortful reward-directed behavior. CSS leads to reduced dopamine turnover in the nucleus accumbens and reduces its sensitivity to dopamine release (Bergamini et al., 2018). Furthermore, the effects of CSS in the EVT are analogous to those obtained with pharmacological (6-hydroxydopamine) reduction of dopamine levels in the nucleus accumbens (Bergamini et al., 2016b). Therefore, the reward-sensitive basolateral amygdala glutamate neurons could be a major regulatory region for reward salience, and the nucleus accumbens, integrating inputs from these amygdala glutamate neurons with those from dopamine neurons projecting from the ventral tegmental area, could be a major regulatory region for effort valuation of reward stimuli. Given that the mice had received multiple exposures to the test environment during training, it is unlikely that increased aversive responding by CSS mice to this environment contributed to the effects observed in the EVT or RST.

The observed effects of CSS on reward-directed behavior co-occur with peripheral changes relating to feeding, energy metabolism and appetite regulation. These include increased food intake to maintain BW and a decrease in the appetite-supressing adipokine leptin (Bergamini et al., 2016a). As in previous studies of CSS effects on reward-directed behavior (Bergamini et al., 2016a, 2018), we provided CSS and CON mice with sufficient daily food to maintain them at BBW during CSS and subsequent behavioral testing. This required provision of CSS mice with 20%–30% more food than CON mice. On days of behavioral testing, food was provided 2–3 h after testing and was consumed, by all mice, several hours prior to behavioral testing on the following day. With regards to blood parameters likely to impact on test behavior, in Experiment 3 on the day after completion of behavioral testing, blood samples were collected for determination of plasma glucose and leptin values. Plasma glucose was at typical levels (Togashi et al., 2016) and similar in both CON and CSS mice, indicating that the additional food provision to CSS mice did not elevate circulating glucose. Despite the additional food received, plasma leptin levels still tended to be reduced in CSS relative to CON mice. In free-feeding mice, plasma leptin is markedly reduced in CSS compared with CON mice (Bergamini et al., 2016a), and the major difference between the present study and this previous finding was the lower leptin levels in CON mice, in line with the food restriction used here to maintain 100% BBW. Nonetheless, the lower average plasma leptin in CSS mice suggests that its appetite-suppressant effects would also be lower in CSS than CON mice. Therefore, with respect to both glucose and leptin levels, there was no evidence that the CSS effects of reduced gustatory-reward salience and effort valuation were attributable to changes in chow intake, energy metabolism and appetite regulation, but rather reflected reduced reward sensitivity. The CSS effects on energy metabolism are nonetheless interesting given that: a common symptom of melancholic depression is weight loss, with reduced interest extending to food, much of which will be sweet tasting (DSM-5, 2013); depression and energy metabolism disorders such as type-2 diabetes are highly co-morbid (Knol et al., 2006); and altered energy metabolism, at least in the brain, is well-described for depression, including increased energy uptake by the amygdala, anterior cingulate cortex and frontal cortex (Price and Drevets, 2010; Harper et al., 2017; Scifo et al., 2018). Furthermore, with respect to leptin specifically, depression is associated with low levels of this adipokine hormone and pharmacological studies indicate that leptin has antidepressant-like efficacy (Lu, 2007; Caron et al., 2018).

This study has demonstrated that CSS leads to reduced gustatory reward salience, learning and expectancy under low-effort conditions and an additional reduction in reward valuation under high effort conditions, in mice. Evidence has also been obtained for the behavioral test conditions that yield the most robust CSS effects, including the use of highly flavored food stimuli, provision of low-effort/low-reward normal diet, and a steep PRS. Importantly, the reward dimensions for which stress-induced deficits have been demonstrated in this study are back-translated from deficits in reward processing described for stress-related neuropsychiatric disorders, most notably depression but also negative symptoms in schizophrenia. It would represent an over-interpretation to attempt to equate these effects to mild, moderate or severe depression. What can be stated is that robust and reproducible effects are obtained, such that the model can be applied for the study of pharmacological reversal of CSS effects. Accordingly, the CSS-RST and CSS-EVT pathology models can now be utilized to increase understanding of the pathophysiologies underlying these reward pathologies and their treatment.

AUTHOR CONTRIBUTIONS

DK and GB conducted the experiments and data analysis and wrote the manuscript. HS conducted the experiments and data analysis. ES wrote the manuscript. BH and CP designed the study and wrote the manuscript.

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
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Effect of Ketamine on LTP and NMDAR EPSC in Hippocampus of the Chronic Social Defeat Stress Mice Model of Depression

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Depression is a common mental disorder that is associated with memory dysfunction. Ketamine has recently been demonstrated to be a rapid antidepressant. The mechanisms underlying how depression induces memory dysfunction and how ketamine relieves depressive symptoms remain poorly understood. This work compared three groups of male C57BL/6J mice: mice exposed to chronic social defeat stress (CSDS) to induce a depression-like phenotype, depression-like mice treated with ketamine, and control mice that were not exposed to CSDS or treated with ketamine. Spatial working memory and long term memory were assessed by spontaneous alternation Y-maze and fear conditioning tests, respectively. We used western blot to analyze the density of *N*-methyl-D-aspartate receptor (NMDAR) subunits in the hippocampus. We recorded long term potentiation (LTP) and NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) in hippocampal slices. We observed that compared with control mice, depression-like mice had significant reductions in spatial working memory and contextual fear memory. The level of NR2B, LTP and NMDA receptor-mediated EPSCs of depression-like mice were decreased. Ketamine treatment attenuated the memory impairment, and increased the density of NR2B and the amplitude of LTP and NMDA receptor-mediated EPSCs in the hippocampus of depression-like mice. In conclusion, depression-like mice have deficits in working memory and contextual fear memory. The decrease of NR2B, LTP induction and NMDA receptor-mediated EPSCs in the hippocampus may be involved in this process. Ketamine can improve expression of NR2B, LTP induction and NMDA receptor-mediated EPSCs in the hippocampus of depression-like mice, which might be part of the reason why ketamine can alleviate the memory dysfunction induced by depression.

Keywords: ketamine, depression, LTP, NMDAR, EPSC

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INTRODUCTION

Depression is one of the most common mental disorders which are associated with high morbidity and mortality (Ménard et al., 2016). People suffering from depression are always seen in disrupted mood, altered sleep and memory dysfunction (Benson et al., 2015). In recent years, ketamine has been demonstrated to relieve depressive symptoms quickly (Blier et al., 2012; Murrough, 2012). Despite extensive research, the mechanisms underlying how depression induces memory dysfunction and how ketamine relieves depressive symptoms remain poorly understood.

Synaptic plasticity is the biological process by which specific patterns of synaptic activity result in changes in synaptic strength, and is thought to contribute to learning and memory (Citri and Malenka, 2008). The effects of depression on synaptic activity involve multiple brain regions, such as the hippocampus, prefrontal cortex (PFC) and amygdale (Liu et al., 2017). The hippocampus is one of most commonly studied brain regions in depression research. First, the hippocampus is part of the limbic system and develops nerve fiber connectivity with emotion-related brain regions, such as amygdala. Second, the hippocampus regulates the hypothalamus-pituitary-adrenal (HPA) axis, which makes it more susceptible to stress and depression (Anacker et al., 2013). Third, it is widely believed that the hippocampus plays a key role in memory encoding and retrieving in central nervous system, and synaptic plasticity in the hippocampus is important for memory (Neves et al., 2008). Preclinical and clinical studies show that depression reduces the size of the hippocampus, and decreases the number of neuronal synapses in hippocampus (Sousa et al., 2000). The changes of synaptic plasticity in the hippocampus may be a possible reason why depression induces memory dysfunction (Duman and Aghajanian, 2012). Long-term potentiation (LTP) and long-term depression (LTD), which indicate the functional indices of synaptic plasticity, are important for memory consolidation and recall (Quan et al., 2010). So the alternations of LTP and LTD in the hippocampus may elucidate the possible mechanism underlying how depression induces memory dysfunction.

It is now well established that dysfunction of the glutamatergic system contributes to the pathophysiology of depression (Sanacora et al., 2008). On the other hand, several glutamate modulating reagents have been used to attenuate the behavioral and molecular alterations presented of depression (Calabrese et al., 2012). N-methyl-D-aspartate receptor (NMDAR) is essential in the fast synaptic glutamate neurotransmission, and ketamine, which is a noncompetitive NMDAR antagonist, shows promise as a novel treatment for depression (Cull-Candy et al., 2001). Tizabi et al. (2012) showed that although ketamine treatment did not result in significant decrease in NMDAR density in the hippocampus of depression-like rats, it induced a significant increase in AMPA/NMDA receptor density ratio in the hippocampus of depression-like rats. NMDAR consists of two obligatory NR1 subunits and two regulatory subunits, usually a combination of NR2A and NR2B (Yashiro and Philpot, 2008). Based on the above results, we guess that function of NMDAR and levels of protein expression of NMDAR subunits in hippocampus may play a role in memory dysfunction induced by depression and in antidepressant effect of ketamine.

In this study, we aimed to investigate the possible mechanism underlying how depression induces memory dysfunction and how ketamine relieves depressive symptoms. To address the issue, we compared three groups of male C57BL/6J mice: mice exposed to chronic social defeat stress (CSDS) to induce a depression-like phenotype (depression-like mice), depression-like mice treated with ketamine (depression-like mice with ketamine), and control mice that were not exposed to CSDS or treated with ketamine. Spatial working memory and long term memory were assessed by spontaneous alternation Y-maze and fear conditioning tests, respectively. We used western blot to analyze the density of NMDAR subunits in the hippocampus. We recorded LTP and NMDA receptor-mediated excitatory postsynaptic currents (EPSCs) in hippocampal slices.

MATERIALS AND METHODS

Experiment Animals

All experimental procedures were conducted in accordance with the institutional ethical guidelines for animal experiments of China, and were approved by Animal Care and Use Committee of Jilin University. The male C57BL/6J mice (8 weeks) and CD-1 mice (18–20 weeks) were housed in a humidity- and temperature-controlled room on a 12-h and 12-h light/dark cycle. Mice accessed to water and food easily.

Chronic Social Defeat Stress Paradigm

An extensive body of experimental and clinical evidence shows that there is a relationship between stress and depression (Moriam and Sobhani, 2013; Warren et al., 2014). The CSDS is considered to be a suitable model for production of depression-like conditions in laboratory rodents (Krishnan et al., 2007; Golden et al., 2011; Yu et al., 2011). In the CSDS model, 8-week-old C57BL/6 male mice, which were regarded as "intruder" mice, were defeated by resident CD-1 mice. The protocol was previously described (Golden et al., 2011). Briefly, C57BL/6J mouse was defeated by a larger CD-1 mouse. Although each individual defeat lasted 5 min, the defeated mouse was subjected to continuous psychological stress from CD-1 mouse through a clear perforated divider in a shared home cage for the remaining 24 h. The C57BL/6 mice were rotated daily while the CD-1 mice were not rotated during the 10-day defeat procedure. In order to select the depression-like mice, 5 min social interaction was recorded on the next day after the last attack of defeat stress. The test was composed of two parts: with and without the target CD-1 mouse in the perforated plastic box. The movements of C57BL/6 mice were recorded with a video recorder. The social interaction ratio is a ratio calculated when CD-1 is present and absent in the interaction zone. In general, the mouse, whose social interaction ratio was <1, had been regarded as depression-like mice (Krishnan et al., 2007). The mouse, whose social interaction ratio was >1, was classified as resilient. In our experiment, approximately 25% of mice showed a resilient phenotype. The mice, which were regarded as "resilient phenotype," were not used for any further tests.

The selected depression-like mice were divided into two groups at random: depression-like mice and depression-like mice with ketamine. We selected to test a dose of ketamine of 5 mg/kg, which emerged as an average dose of ketamine displaying antidepressant-like properties in rodent behavioral paradigms in different studies (Maeng et al., 2008; Franceschelli et al., 2015). Depression-like mice with ketamine received a one-time intraperitoneal (i.p.) injection of ketamine (5 mg/kg), and depression-like mice received an injection of same amount

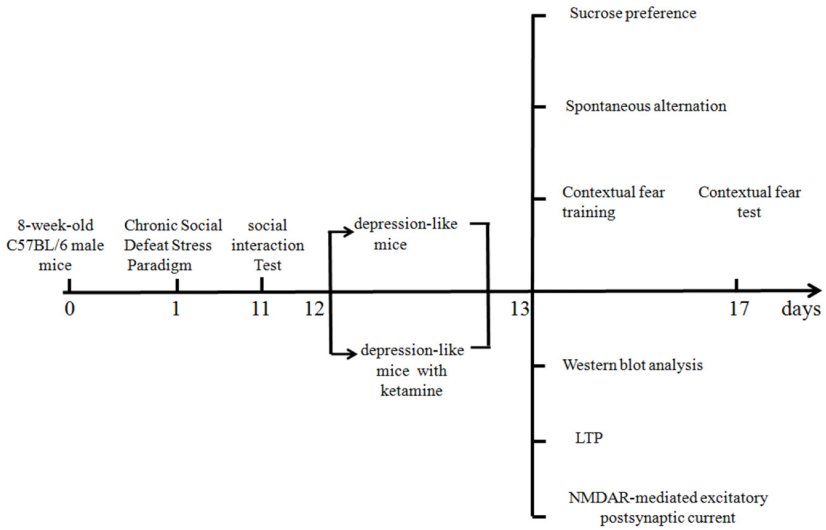

FIGURE 1 | Timetable of the experimental design.

of saline. The control C57BL/6J mice were housed in the same defeat boxes with one mouse per side of the perforated divider. The control mice were rotated every day similar to the mice undergoing defeat, but they were not permitted to contact with CD-1 mouse. Control mice were received a single i.p. injection of saline on the same day when depression-like mice were treated with saline.

A timetable of different experiments is shown in **Figure 1**. Different mice were used for behavioral and electrophysiological experiments. All the experiments were tested 1 day after ketamine or saline treatments.

Behavioral Studies

Sucrose Preference

Thirty-six mice were used to test sucrose preference (control n = 12, depression-like mice n = 12, and depression-like mice with ketamine n = 12). The mice were euthanized after test. Sucrose preference was performed as previously described (Torres-Berrío et al., 2017). Mice were accustomed to drinking water from two bottles with sipper tops for two consecutive days. From the third day, mice were permitted to have a free choice between sucrose water (1% concentration) and regular water for four consecutive days. And the position of the bottles was interchanged after each daily experiment. Sucrose preference was estimated by dividing the volume of consumed sucrose by the volume of consumed sucrose + consumed water. The average of sucrose preference of the four test days was calculated to estimate the total preference for sucrose.

Spontaneous Alternation in the Y-Maze Test

Thirty-six mice were used to test spontaneous alternation performance (control n = 12, depression-like mice n = 12 and depression-like mice with ketamine n = 12). The mice were euthanized after test. Spontaneous alternation in the Y-maze test, which was used to assess spatial working memory of mice, was performed as previously described (Satoh et al., 2007). The symmetrical Y maze consisted of three arms which were 30 cm long, 15 cm high and 8 cm wide. At the beginning of the experiment, each mouse, which was placed in the center of the Y-maze, was permitted to explore freely through the maze for 8 min. The total number of arms mice entered and the sequence were recorded. The percentage of alternation, which was the number of triads containing entries into all three arms divided by the maximum possible number of alternations, was calculated.

Contextual Fear Conditioning

Thirty-six mice were used to test contextual fear conditioning performance (control n = 12, depression-like mice n = 12 and depression-like mice with ketamine n = 12). The mice were euthanized after test. The protocol was previously described by Frankland et al. (2001). Contextual fear conditioning consisted of one training session and a test session. During training section, mice were placed in the conditioning chamber for 7 min. After the first 2 min, mice were presented with five unsignaled foot-shocks (2 s duration, 0.75 mA, 1 min apart). After training, the mice were sent back to their home cage. During testing section, the mice were placed back in the conditioning chamber for 120 s. Freezing time and activity time were recorded by video camera. The percent of freezing was used to assess contextual fear conditioning memory.

Western Blot Analysis

Thirty-six mice were used for western blot analysis (control n = 12, depression-like mice n = 12, and depression-like mice with ketamine n = 12). The mice were euthanized after test. Western blotting was used to evaluate the density of NR1,

NR2A and NR2B subunits on the membrane of hippocampus neurons. Western blot analysis was performed as previously described (Ma et al., 2014). Briefly, the hippocampus was homogenized in lysis buffer A (contained 250 mM sucrose, 50 mM KCl, 20 mM HEPES, 2 mM EGTA and protease inhibitors cocktails). After the lysates were centrifuged at 800 *g* for 10 min, supernatants were collected. The supernatants were centrifuged at 100,000 *g* for 60 min, and the membrane pellets were collected. The membrane pellets were dissolved in lysis buffer B (contained 150 mM KCl, 20 mM HEPES (pH 7.0), 2 mM EGTA, 1% (w/v) CHAPSO and protease inhibitors cocktails), and were incubated at 4°C for 60 min. After the soluble membranes were centrifuged at 100,000 *g* for 60 min, the supernatants were collected. The proteins, which were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), were transferred to polyvinylidene fluoride membranes. After washing, the membranes were blocked with non-fat skim milk, and then were incubated with appropriate primary antibody (NR2A 1:1,000, NR2B 1:1,000, and NMDAR 1:1,000, Cell Signaling Technology, Danvers, MA, USA Inc. β-actin 1:5,000, Sigma). After three times washing, membranes were incubated with the secondary antibody. Visualized by an ECL system, the membranes were developed on Hyperfilm (Amersham). The relative expression levels of all proteins were determined by densitometry and normalized to β-actin.

Brain Slice Preparation

Thirty-six mice were used for LTP and NMDAR-EPSC (control n = 12, depression-like mice n = 12, and depression-like mice with ketamine n = 12). The hippocampal slices of mice were prepared as previously described (Shen et al., 2010). Briefly, mice were anesthetized with isoflurane and decapitated. The brains were rapidly removed from the cranial cavity and submerged in ice-cold artificial cerebrospinal fluid (aCSF) saturated with a mixture gas of 95%O_2+5%CO_2. The aCSF contained (in mM): NaCl 124, $NaHCO_3$ 26, glucose 10, KCl 5, $CaCl_2$ 2, $MgSO_4$ 2 and NaH_2PO_4 1.25, pH = 7.4. The hippocampi were dissected free, and dorsal hippocampal slices (400 μm) were sectioned by a vibrating tissue slicer (Vibratome 3000, Vibratome, USA). The slices were incubated in oxygenated aCSF at 32°C for at least 60 min before the electrophysiological recording.

Long Term Potentiation (LTP)

The strength of synaptic transmission was quantitatively analyzed by the initial slope of the rising phase of the field excitatory postsynaptic potentials (fEPSPs). The procedure of recording fEPSP was previously described by Sarabdjitsingh et al. (2016). Briefly, bipolar stimulation electrodes (60-μm stainless steel wires insulated except for the tip) were placed on the Schaffer collaterals, and glass recording electrodes (filled with buffer, 2–5 MΩ) were placed in the CA1 stratum radiatum. At the start of each experiment, an input-output curve was established to record the slope of the fEPSP, from which maximal and half-maximal slope as well as the corresponding maximal and half-maximal stimulus intensity were determined. The half maximal stimulus intensity that was thus calculated was used throughout the remainder of the recording session. The stable baseline was maintained for at least 30 min. Then, a high frequency stimulus (HFS; 100 pulses delivered at 100 Hz) was applied to the Schaffer collateral pathway. Subsequent data were recorded for further 40 min keeping the same rate or intensity of the stimulus. Based on the records during the last 5 min before HFS, fEPSP slopes were normalized.

NMDA Receptor-Mediated Excitatory Postsynaptic Current (NMDAR-EPSC)

NMDA receptor EPSCs were recorded as previously described (Shen et al., 2010; Xu et al., 2017). The recording aCSF was the same as above except that the aCSF contained 0.5 mM $MgCl_2$ (instead of 2 mM). NMDA receptor EPSCs were recorded from CA1 region using a single glass pipette (3–5 MΩ) in whole cell recording. The intracellular pipette solution contained the following (in mM): KCl 5, Na_2-phosphocreatine 5, lidocaine N-ethyl chloride 5, HEPES 10 and K-gluconate 130, pH = 7.4. The stimulating electrode was placed in the stratum radiatum. CA1 pyramidal cells were voltage-clamed at −70 mV. Evoked by low frequency stimulation (0.05Hz), evoked EPSCs were recorded at −70 mV holding potential in aCSF perfusion containing bicuculline (10 μmol/L) to block $GABA_A$ receptor mediated inhibitory synaptic currents. NMDA receptor EPSCs were isolated at holding potential of −60 mV with NBQX (5 μM). At last, APV (50 μM) was used to show that the current was generated by NMDA receptor.

Statistics

All data were expressed as mean ± SEM. Comparisons among data were carried out by one-way ANOVA analysis followed by Newman-Keuls *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Sucrose Preference

We tested sucrose preference in control mice (n = 12), depression-like mice (n = 12), and depression-like mice with ketamine (n = 12). Control, depression-like mice and depression-like mice with ketamine displayed a sucrose preference over water of 75.6 ± 8.4%, 44.5 ± 10.1% and 61.8 ± 11.3%, respectively (**Figure 2A**). One-way ANOVA revealed significant differences among the three groups ($F_{(2,33)}$ = 29.11, $P < 0.01$). *Post hoc* analysis showed that sucrose preference of depression-like mice was decreased, but ketamine ameliorated the decrease of sucrose preference in depression-like mice ($P < 0.05$: control mice vs. depression-like mice; $P < 0.05$: control mice vs. depression-like mice with ketamine; $P < 0.05$: depression-like mice with ketamine vs. depression-like mice).

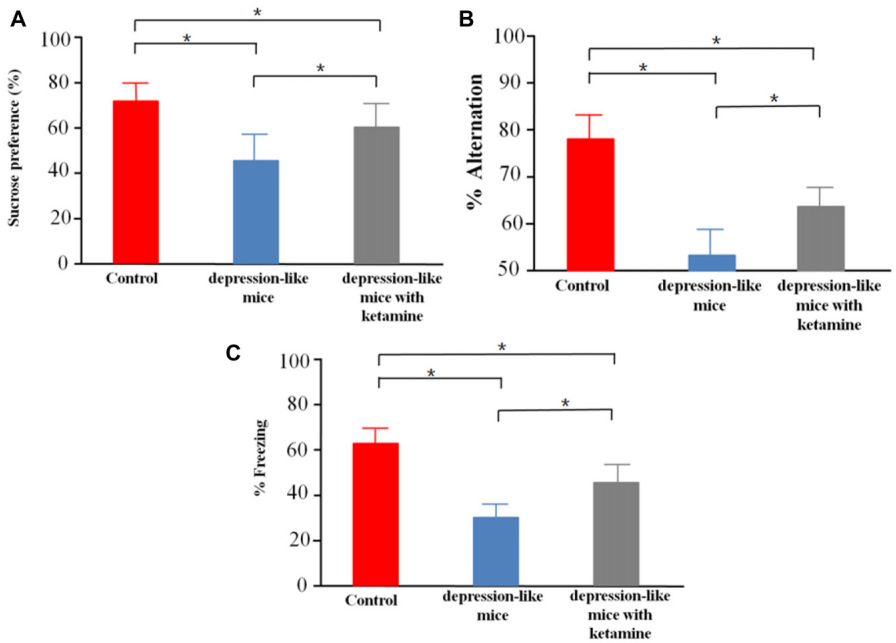

FIGURE 2 | Behavioral studies of mice. **(A)** Depression-like mice had least sucrose preference among the three groups, and ketamine improved sucrose preference performance of depression-like mice. **(B)** Working memory of depression-like mice was impaired, and ketamine treatment attenuated working memory impairment of depression-like mice. **(C)** Contextual fear conditioning memory of depression-like mice was impaired, and ketamine attenuated the memory impairment of depression-like mice. Means were showed, and SEM were represented by error bars, $n = 12$, per group. *$P < 0.05$, one-way ANOVA, *post hoc* Tukey-Kramer.

Spatial Working Memory Test in Y-Maze

Control mice, depression-like mice and depression-like mice with ketamine performed spatial working memory test with 78.4 ± 7.1%, 53.3 ± 6.7%, 65.8 ± 5.3% correct choices, respectively (**Figure 2B**). One-way ANOVA revealed differences among the three groups ($F_{(2,33)} = 45.95$, $P < 0.01$). *Post hoc* analysis showed that spatial working memory of depression-like mice was reduced, but ketamine ameliorated the decrease of spatial working memory in depression-like mice ($P < 0.05$: control mice vs. depression-like mice; $P < 0.05$: control mice vs. depression-like mice with ketamine; $P < 0.05$: depression-like mice with ketamine vs. depression-like mice).

Contextual Fear Conditioning

We tested contextual fear conditioning performance in depression-like mice ($n = 12$), depression-like mice with ketamine ($n = 12$) and control mice ($n = 12$). Control, depression-like mice and depression-like mice with ketamine performed contextual fear memory test with 62.5 ± 7.7%, 35.1 ± 6.1% and 48.7 ± 8.3% of freezing, respectively (**Figure 2C**). One-way ANOVA revealed significant differences among the three groups ($F_{(2,33)} = 40.85$, $P < 0.01$). *Post hoc* analysis showed that contextual fear conditioning of depression-like mice was reduced, but ketamine ameliorated the decrease of Contextual fear conditioning in depression-like mice ($P < 0.05$: control mice vs. depression-like mice; $P < 0.05$: control mice vs. depression-like mice with ketamine; $P < 0.05$: depression-like mice with ketamine vs. depression-like mice).

Membrane Protein Expression of NMDAR in Mouse Hippocampus

Significant differences were encountered with protein expression of NR2B on total hippocampal membranes among the three groups ($p < 0.01$). An additional *post hoc* analysis showed that density of NMDA receptor 2B subunit in depression-like mice was reduced compared with that of control mice ($p < 0.01$). The density of NMDA receptor 2B subunit displayed by depression-like mice treated with ketamine was lower compared with that of control mice ($p < 0.01$), but increased compared with that of depression-like mice ($p < 0.01$; **Figures 3A,D**). Additionally, there was no difference detected among the three groups for the densities of NR1, NR2A subunits in the hippocampus ($P > 0.05$; **Figures 3B,C**).

Long Term Potentiation (LTP)

We recorded evoked fEPSP in the stratum radiatum of the CA1 region in depression-like mice (12 slices from 12 mice), depression-like mice with ketamine (12 slices from 12 mice) and control mice (12 slices from 12 mice). High-frequency stimulation induced stable LTP in control mice and depression-like mice with ketamine. LTP was significantly blocked in depression-like mice (**Figures 4A–C**). There was no difference between control mice and depression-like mice with

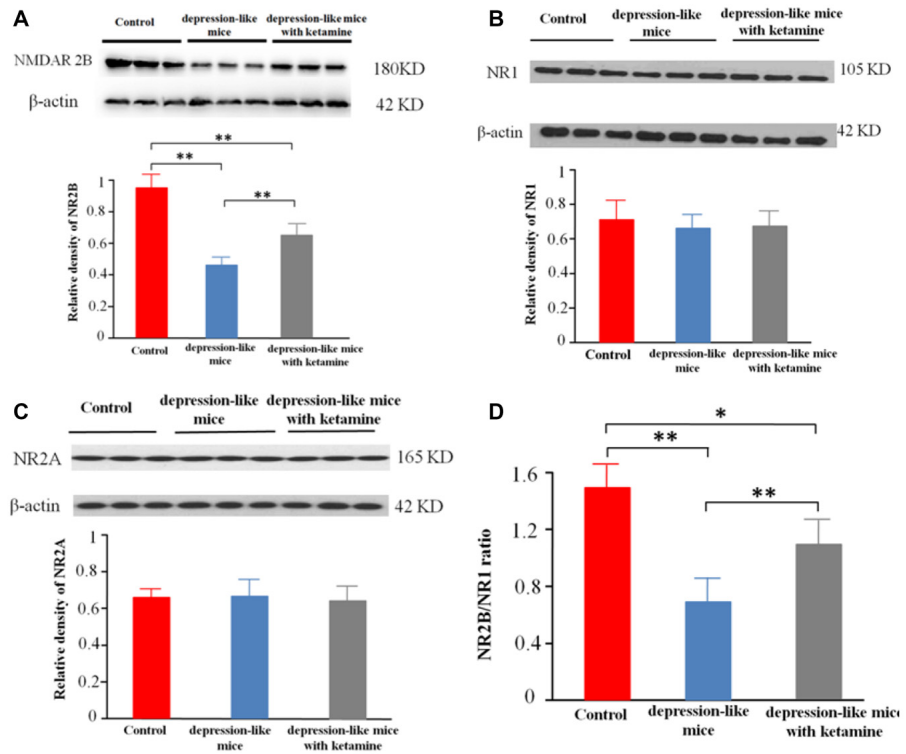

FIGURE 3 | (A) Expression of NR2B subunit on the membrane of hippocampus neurons was tested by western blot. β-actin was selected as an internal standard and control for protein loading. **(B,C)** Expression of NR1 and NR2A on the membrane of hippocampus neurons was shown by western blot. β-actin was selected as an internal standard and control for protein loading. **(D)** NR2B/NR1 ratio. Means were showed, and SEM were shown by error bars, $n = 12$, per group. $^{**}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA, *post hoc* Tukey-Kramer.

ketamine ($P > 0.05$). **Figure 4D** summarized the change of fEPSP slope for each group.

NMDAR-Mediated Excitatory Postsynaptic Current (NMDAR-EPSC)

After blocking GABARs and AMPARs, whole-cell voltage clamp techniques were used to record evoked NMDA receptor-mediated EPSCs from CA1 pyramidal cells in depression-like mice (12 slices from 12 mice), depression-like mice with ketamine (12 slices from 12 mice) and control mice (12 slices from 12 mice). One-way ANOVA revealed differences of amplitudes of NMDA current among the three groups ($F_{(2,33)} = 14.7$, $P < 0.01$). *Post hoc* analysis showed that NMDAR EPSC amplitude of depression-like mice was decreased, but ketamine ameliorated the decrease of NMDAR EPSC amplitude of depression-like mice ($P < 0.01$: control mice vs. depression-like mice; $P < 0.05$: control mice vs. depression-like mice with ketamine; $P < 0.05$: depression-like mice with ketamine vs. depression-like mice). After blockade of GABAR, the ratio of NMDA/AMPA receptor-mediated currents was reduced in depression-like mice compared with those of the other two groups ($F_{(2,33)} = 13.9$, $P < 0.01$; Newman-Keuls *post hoc* test, $P < 0.001$: control mice vs. depression-like mice; $P < 0.05$: control mice vs. depression-like mice with ketamine; $P < 0.05$: depression-like mice with ketamine vs. depression-like mice; **Figure 5**).

DISCUSSION

In this study, we observed that depression-like mice had significant reductions in spatial working memory and contextual fear conditioning memory. The density of NR2B subunit in depression-like mice hippocampus was significantly decreased. Meanwhile, LTP and NMDA receptor-mediated EPSCs in hippocampal slices of depression-like mice were also reduced. Ketamine treatment mitigated the memory impairment of depression-like mice, and ketamine partially restored the levels of NR2B, LTP induction, and NMDAR receptor-mediated EPSCs in hippocampal slices of depression-like mice.

Our work showed that ketamine afforded an antidepressant-like effect in mice in sucrose preference test. Apart from our findings, many preclinical experiments have indicated that ketamine has antidepressant effects in several species and on different behavioral tests. Sałat et al. (2015) and Kara et al. (2017) demonstrated that ketamine reduced immobility of depression-like mice in the forced swim test). Garcia et al. (2008) also reported that ketamine induced

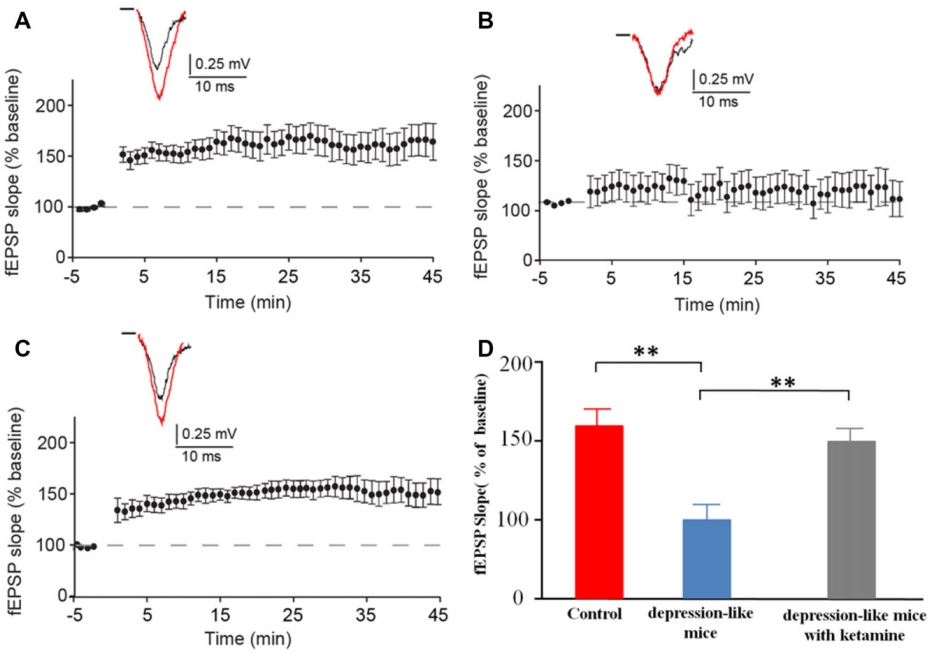

FIGURE 4 | The long-term potentiation (LTP) in the hippocampal Schaffer collateral-CA1 in control mice, depression-like mice, and depression-like mice with ketamine. LTP of depression-like mice was blocked, and ketamine treatment attenuated the blockage of LTP in depression-like mice. **(A)** Control mice. **(B)** Depression-like mice. **(C)** Depression-like mice with ketamine. **(D)** Summarized data of average slope change (normalized to baseline value) from three groups of mice. Means were showed, and SEM were shown by error bars, $n = 12$, per group. $^{**}P < 0.01$, one-way ANOVA, *post hoc* Tukey-Kramer.

antidepressant-like effect in the rat forced swimming test. Maeng et al. (2008) showed that ketamine caused antidepressant-like effects on mice in passive avoidance test. Li et al. (2011) reported that ketamine rapidly ameliorated depression-induced behavioral deficits in novelty-suppressed feeding test. Although ketamine has been reported to have an antidepressant-like effect, there are some reports of inducing depression. Ram et al. (2013) found that the combination of prepubertal onset of chronic stress and ketamine may serve as a valid novel animal model for schizophrenia-like symptoms. The antidepressant effect of ketamine was demonstrated not only in depression-like animals but also in patients. Phelps et al. (2009) and Zarate et al. (2006) demonstrated a rapid antidepressant effect could be achieved with ketamine in patients with major depression. What's more, the antidepressant effects can persist for several days (Liebrenz et al., 2007) to even weeks (Irwin and Iglewicz, 2010; Mathew et al., 2010). In our article, we also showed that ketamine mitigated spatial working memory deficits and contextual fear conditioning memory deficits in depression-like mice. Consistent with our study, Zhu et al. (2015) showed that ketamine effectively attenuated memory impairment of depression-like rats. Besides, the effect of ketamine on memory in depressive patients was reported. Chen et al. (2017) investigated the effects of low doses of ketamine on learning and memory in patients, and found that ketamine attenuated learning and memory impairment.

The hippocampus is one of brain regions susceptible to depression. Many previous studies have shown that the hippocampus is indispensable to proper working memory (Eichenbaum, 2004; Chudasama and Robbins, 2006) and contextual fear memory (Han et al., 2016). Schaffer collaterals are axon collaterals given off by CA3 pyramidal cells in the hippocampus, and transform information from CA3 region to CA1 region. In our article, LTP in the CA1 region of depression-like mice was impaired. Consistent with our study, She et al. (2015) reported that tetanic stimulation failed to induce LTP in Schaffer collateral-CA1 synapses in depression-like rats. Besides, accumulated evidence demonstrated the alterations of LTP in Schaffer fibers-CA1 pyramid synapses in different models of depressive-like rodents. Kaster et al. (2015) reported that the amplitude of LTP in hippocampal slice was reduced in depression-like mice induced by chronic unpredictable stress. Hopeless mice, a bred-based model of depression, displayed an aberrant LTP amplitude in the hippocampus (Machado et al., 2017). Maternal separation, which was an early life stress model, impaired hippocampal LTP (Batalha et al., 2013). However, depending upon the brain area, stress influences LTP differently. For example, chronic psychological stress impairs LTP induction in hippocampal area CA1, while it has no effect on dentate gyrus LTP (Gerges et al., 2001). Our results demonstrate that ketamine attenuates the LTP impairment in CA1 region of depression-like mice. Ketamine enhances hippocampal synaptic plasticity not only

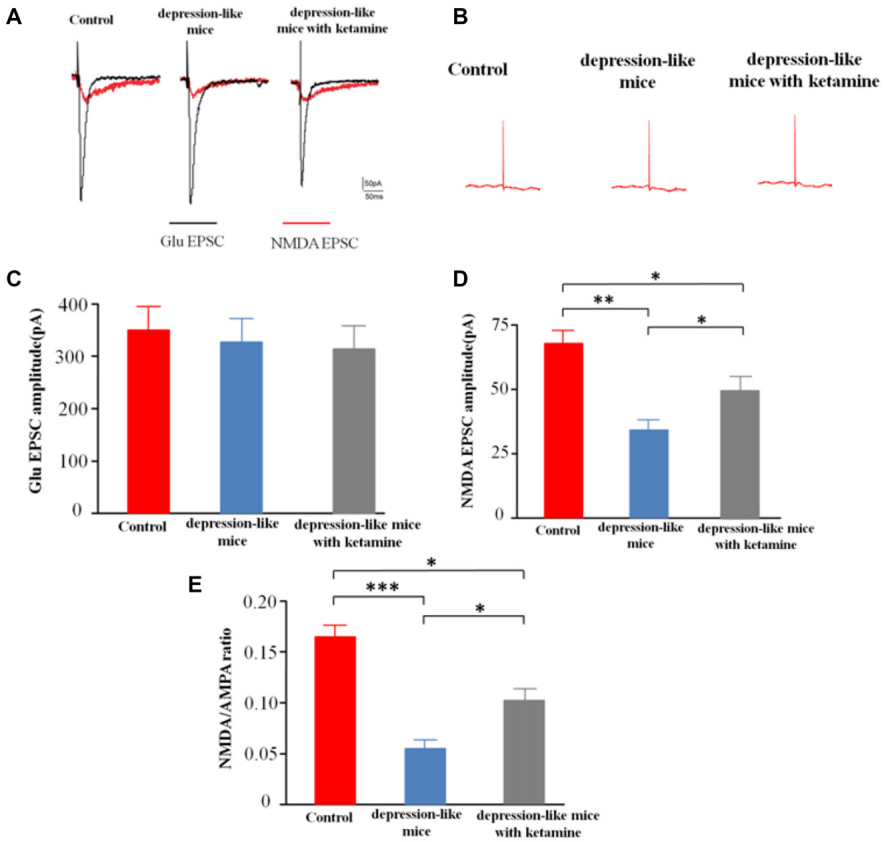

FIGURE 5 | Evoked N-methyl-D-aspartate receptor (NMDAR)-mediated excitatory postsynaptic currents (EPSCs) recorded from CA1 pyramidal cells in mice brain slices. **(A)** Evoke NMDA receptor-mediated EPSC of one mouse from each group. **(B)** EPSC after treatment with APV+NBQX. **(C)** Summarized amplitude of glutamate EPSC of three groups. **(D)** Summarized amplitude of NMDA EPSCs of three groups. **(E)** Summarized NMDA/AMPA ratio of three groups. Means were showed, and SEM were shown by error bars, n = 12 slices from 12 mice, per group. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, one-way ANOVA, *post hoc* Tukey-Kramer.

in depression-like animals but also in nromal animals. Graef et al. (2015) showed that Ketamine enhanced LTP in rat hippocampus 24 h after a single i.p. treatment. Taken together, these studies support a hypothesis that altered hippocampal synaptic plasticity underlie the pathophysiology and treatment of depression.

NMDA receptor is essential for excitatory and inhibitory synaptic transmission (Cull-Candy et al., 2001; Hansen et al., 2017). Moreover, NMDA receptor-dependent synaptic plasticity in the hippocampus has been supposed to be the key mechanism of memory and learning (Zhuo, 2009). We found out that expression of NMDA receptor 2B subunit in the hippocampus of depression-like mice was decreased. On the contrary, Calabrese et al. (2012) demonstrated that chronic stress increases the expression of NR1 and NR2B in rat hippocampus. The following factors might contribute to the differences. Both the experimental animals and depression-like models were different. In our study, the NMDA receptor-mediated EPSCs in hippocampus CA1 region from depression-like mice were decreased. Yuen did the same kind of research in PFC. Yuen et al. (2012) demonstrated that NMDA EPSCs in PFC pyramidal neurons from repeatedly stressed rats were markedly reduced, which caused the detrimental effect on PFC-dependent memory impairment. So the decrease of NMDAR EPSC and the density of NR2B in the hippocampus play a role in memory dysfunction induced by depression. Ketamine attenuates the decrease of NMDAR EPSC and the density of NR2B in the hippocampus, which might underlie the ability why ketamine can alleviate the memory dysfunction induced by depression. Duman and Li (2012) demonstrated another possible mechanism by which ketamine relieves depressive symptoms. Ketamine increases brain-derived neurotrophic factor (BDNF) release and activates mammalian target of rapamycin (mTOR) signaling, which then increases synthesis of synaptic proteins. These effects of ketamine reverse the atrophy of neurons caused by depression.

There are a number of limitations associated with our study. The major limitation of the experimental design is the lack of a control group treated with ketamine. However, recent studies, which have shown the impact of ketamine on hippocampal synaptic transmission and plasticity in the hippocampus of normal rodents, may help justify this limitation.

Ribeiro et al. (2014b) reported that the direct application of ketamine to mouse hippocampal slices reduced LTP. But the decrease of LTP was not persistent. Ribeiro et al. (2014a) also showed that a single i.p. injection of ketamine did not affect LTP 24 h later in adult male C57BL/6 mice. The second limitation of this study was that we only tested LTP in Schaffer collaterals→CA1 pathway of the hippocampus. There are several important pathways which have been shown to sustain LTP in the hippocampus (such as Entorhinal cortex→dentate gyrus, Mossy fibers→CA3, Schaffer collaterals→CA1 and CA1→subiculum; Lynch, 2004). In our article, we showed the alternations of LTP in Schaffer collaterals→CA1 pathway, but we did not test synaptic plasticity in other pathways in the hippocampus. The third limitation was that we didn't test the LTP in PFC of depression-like mice. Depression results in structural alterations (including regulation of neurogenesis, dendrite length and spine density) in hippocampus and PFC (Duman and Aghajanian, 2012). Many previous articles showed that the PFC plays a role in the ketamine's antidepressant actions. For example, Duman and Li (2012) showed that ketamine causes a rapid induction of synaptogenesis and spine formation in the PFC. In our future work, we will test the LTP and NMDA receptor-mediated EPSC in PFC of depression-like animals.

In conclusion, depression-like mice have deficits in working memory and contextual fear memory. The decrease of NR2B, LTP induction, and NMDA receptor-mediated EPSCs in the hippocampus may be involved in this process. Ketamine can improve the density of NR2B, LTP induction, and NMDA receptor-mediated EPSCs in the hippocampus of depression-like mice, which is part of the reason why ketamine can alleviate the memory dysfunction induced by depression.

AUTHOR CONTRIBUTIONS

LS designed the study. YY, WJ and HZ performed the experiments and developed the data analysis. All of the authors discussed the data and co-wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material **(Supplementary Data Sheet 1)** for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00229/full#supplementary-material

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frontiers in Behavioral Neuroscience

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Change of Rin1 and Stathmin in the Animal Model of Traumatic Stresses

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The molecular mechanism of fear memory is poorly understood. Therefore, the pathogenesis of post-traumatic stress disorder (PTSD), whose symptom presentation can enhance fear memory, remains largely unclear. Recent studies with knockout animals have reported that Rin1 and stathmin regulate fear memory. Rin1 inhibits acquisition and promotes memory extinction, whereas stathmin regulates innate and basal fear. The aim of our study was to examine changes in the expression of Rin1 and stathmin in different animal models of stress, particluarly traumatic stress. We used three animal traumatic stresses: single prolonged stress (SPS, which is a rodent model of PTSD), an immobilization-stress (IM) and a Loud sound stress (LSS), to examine the change and uniqueness in Rin1/stathmin expression. Behavioral tests of SPS rats demonstrated increased anxiety and contextual fear-conditioning. They showed decreased long-term potentiation (LTP), as well as decreased stathmin and increased Rin1 expression in the hippocampus and the amygdala. Expression of the stathmin effector, tubulin, and downstream molecules Rin1, Rab5, and Abl, appeared to increase. Rin1 and EphA4 were endogenously coexpressed in primary neurons after SPS stimulation. IM rats exhibited increased anxiety behavior and enhanced fear-conditioning to contextual and auditory stimuli. Similar changes in expression of Rin1/stathmin were observed in IM rats whereas no changes were observed in rats exposed to a loud sound. These data suggest that changes in expression of the Rin1 and stathmin genes may be involved in rodents with SPS and IM stresses, which provide valuable insight into fear memories under abnormal conditions, particularly in PTSD.

Keywords: Rin1, stathmin, fear memory, single prolonged stress, post-traumatic disorder, traumatic stress

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INTRODUCTION

Stathmin, which is also called oncoprotein 18, is a neuronal growth-associated protein that is highly expressed in the lateral nucleus of the amygdala and related thalamic and cortical structures (Shumyatsky et al., 2002). Furthermore, it is reported to stathmin plays an important role in regulating formation/disassembly of cellular microtubules (MTs) and synaptic plasticity (Belmont and Mitchison, 1996). Rin1 is a Ras effector protein that is strongly expressed in telencephalic regions, including the cortex, hippocampus, amygdala, and striatum. Rin1 protein is normally localized in neuronal cell bodies and dendrites (Deininger et al., 2008). Rin1 showed low expression at the postnatal mouse brain, suggesting Rin1 may be dispensable for early brain but implicated Rin1 in mature neurons (Dhaka et al., 2003).

A number of studies have reported high expression of both genes in cancers such as cervical, prostate, and lung cancers (Shan et al., 2012; Biaoxue et al., 2016).

An increasing number of studies have reported that stathmin plays a key role in learning and innate fear, particularly basal fear (Peschanski et al., 1993; Shumyatsky et al., 2002; Brocke et al., 2009). Stathmin knockout mice show decreased memory during amygdala-dependent fear conditioning, fail to recognize the danger in innate fear-aversive environments, and have deficits in long-term potentiation (LTP; Shumyatsky et al., 2002). Stathmin interacts with tubulin and forms heterodimers, which prevent the formation of MTs (Curmi et al., 1997). Tubulin released from the heterodimer enhances formation of MTs after phosphorylation. As a regulator of synaptic plasticity, stathmin is involved in the formation and disassembly of cellular MTs and functions (Belmont and Mitchison, 1996).

Behavioral studies of Rin1−/− mice have demonstrated enhanced learning of conditioned fear, enhanced acquisition of aversive memories, and elevated amygdalar LTP (Deininger et al., 2008; Bliss et al., 2010; Dzudzor et al., 2010), suggesting a critical role for Rin1 in acquisition and persistence of fear conditioning. Rin1−/− and stathmin −/− knockout mice both develop normally and have no alterations in spatial-dependent memory. Two downstream effectors of Rin1 signaling, Abl (Hu et al., 2005) and Rab5 (Tall et al., 2001), regulate cytoskeletal remodeling and endocytosis, respectively. Abl is activated by Rin1 and may contribute to cytoskeletal remodeling of postsynaptic dendritic spines and modulate short-term synaptic plasticity in the hippocampus (Koleske et al., 1998; Brown et al., 2005). Activated Rab5 participates in regulating endocytosis of cell surface receptors in multiple forms of Long-term depression (LTD). Rin1 also contributes to endocytosis of EphA4 in amygdalar neurons (Deininger et al., 2008).

Therefore, both genes are essential in regulating fear memory but not spatial memory. Most genes, such as the N-methyl-D-aspartate receptor, protein kinase C and calmodulin-dependent protein kinase II, are now considered to be involved in regulating multiple learning-memory pathways, including fear- and spatial-dependent memory (Goosens et al., 2000; Humeau et al., 2003; Rodrigues et al., 2004; Fourcaudot et al., 2009). However, only a few genes, including stathmin and Rin1, have been reported to be responsible for fear memory. According to the different expression in behavioral test and LTP in the knockout animals, it is possible that stathmin and Rin1 have different effects or opposite effects on the fear memory.

Post-traumatic stress disorder (PTSD) is a cognitive and emotional disorder that develops after an individual is exposed to a stressful or traumatic event such as violence or an earthquake. PTSD is characterized by re-experiencing the trauma, avoidance, negative changes in cognition/mood, and altered arousal (Adami et al., 2006; Liberzon and Martis, 2006; American Psychiatric Association, 2013). Patients with PTSD may show abnormal consolidation and retrieval of traumatic memories, causing traumatic flashbacks. Normally, traumatic memories are more robust as compared to non-traumatic memories. The hippocampus and amygdala are involved in the fear memory circuit and playing key roles in regulating fear-related emotions and schemas (Rauch and Shin, 1997; Hull, 2002; Shin et al., 2005; Hughes and Shin, 2011). Magnetic resonance imaging (MRI) studies have revealed significant reductions in hippocampal and amygdala volume in adult patients with PTSD. Functional MRI studies have determined that the amygdala is highly activated in patients with PTSD, which is not the case in the hippocampus decreases (Shin et al., 2005; Etkin and Wager, 2007; Morey et al., 2009; Murrough et al., 2011; Xiong et al., 2013). In the present study, we used the single prolonged stress (SPS), which has been widely used as PTSD animal model (Liberzon et al., 1999). During SPS, animals are restrained for 2 h, forced swim for 20-min in 20–24°C water, and finally exposed to ether anesthesia. Neuroendocrinological and behavioral evidences support that SPS rats may be more appropriate and more practical as models of fear-related human condition labeled clinically PTSD. Our previous studies with SPS model found high apoptosis ratios in neurons of both structures (Ding et al., 2010; Li et al., 2010; Han et al., 2013, 2014). Abnormal structure and function of the amygdala and hippocampus may cause abnormal memory-related symptoms.

In the present study, we used the SPS (an animal model that mimics the features of the human condition known as PTSD), an immobilization-stress (IM; a traumatic-like stress) and a Loud sound stress (LSS) to examine the change of Rin1 and stathmin in three different stresses. The changes in expression of the Rin1 and stathmin genes may be involved in traumatic-like stress model, which may be useful in traumatized human populations as well and may provide valuable insight into fear memories that are processed under abnormal conditions.

MATERIALS AND METHODS

Animal Model Preparation and Grouping

Single Prolonged Stress (SPS) Experiment

A total of 80 male Wistar rats (220–250 g) were randomly divided into two groups (40 rats per group): a control group and SPS groups examined on day 7. The control rats remained in their home cages with no handling for 7 days and were killed at the same time as the SPS groups. The SPS rats underwent the SPS procedure on the first day. The SPS procedure was carried out according to the following protocol (Liberzon et al., 1999): a 2 h immobilization (compression with plastic bags), a 20 min forced swim (25°C), a 15 min rest, followed by ether anesthesia (until loss of consciousness). After SPS, the rats were *ad libitum*.

Immobilization-Stressed (IM) Experiment

A total of 45 male Wistar rats (220–250 g) were randomly divided into three groups (15 rats/group): a control group, a restrict stress group (IM) examined on day 1 and a restrained group examined on day 7. The rats underwent the immobilization stress procedure on the first day and underwent 1 h immobilization (compression with plastic bag). The control rats remained in their home cages with no handling.

Loud Sound Stress (LSS) Experiment

A total of 10 male Wistar rats (220–250 g) were randomly divided into two groups (five rats per group): a control group, LSS group

(LSS) examined on day 7. The rats were placed in the chamber (23 × 23 × 35 cm) for 5 min before exposure to the loudspeaker stimulus. A sonic wave (1 min, 1000 Hz, 75 Db) was delivered through the loudspeaker. The control group was placed in the same chamber for 5 min, but without the loudspeaker stimulus.

All experimental animals were maintained as a group on a 12:12 h light/dark cycle, 19–21°C room temperature. The animals had access to food and water. All experimental procedures were approved by the ethics committee of China Medical University and conducted in accordance with the Guidelines Principles on Animal Experimentations for Laboratory Animal Science, China Medical University.

Behavioral Test

For the freezing behavior test, 40 rats per test (20 rats per group) from SPS experiment were included; for open field (OF) test and elevated plus maze (EPM) test, the remaining 20 rats were used. The EPM test was occurred 1 day after OF test. Fifteen rats (five rats per group) from IM experiment underwent the OF test and elevated plus maze (EPM) test. The remaining 20 rats from IM model were used in the fear conditioning test.

Open Field (OF) Test

The open-field test was used to study anxiety-related behavior. The procedure was done as described in Han et al. (2014). The apparatus was surrounded by black walls 40 cm in height, and the floor (100 cm × 100 cm) was divided into 25 squares (20 cm × 20 cm each). During the experiment, each rat was put in the center of the OF (50 cm × 50 cm), and behavior was recorded for 5 min by an automatic analyzing system (Smart 3.0, Panlab, Barcelona, Spain). Time of center cross, the distance of center cross and total cross, and the number of rearing were recorded. The apparatus was cleaned with 70% ethanol using a wet sponge and a paper towel before the introduction of each rat. The percentage of border/center distance (distance into the border (center)/total distance), and the percentage of time in the border/center (time in the border (center)/total time) were calculated.

Elevated Plus Maze (EPM) Test

The procedure was done in Han et al. (2014). The EPM apparatus consists of a plus-shaped maze elevated above the floor with two oppositely positioned closed arms (50 × 10 cm), two oppositely positioned open arms (50 × 10 cm), and a center area (10 × 10 cm). At the beginning, rats were placed in the central area of the maze, facing an enclosed arm. Behavior was recorded with a video camera during the initial 5 min (Smart 3.0, Panlab, Barcelona, Spain). The apparatus was cleaned with 70% ethanol using a wet sponge before the next observation. The number of entries, the time spent and distance into open arms and into closed arms were measured. The percentage of open /closed arm time (time in the open arms (closed arms)/the time in both arms), the percentage of open /closed arm distance (distance in the open arms (closed arms)/the distance in both arms) and open arm entries (number of entries into the open arm/total number of entries in both arms) were calculated. The measures of anxiety are the percentage (%) of open arm entries and the percentage (%) of time spent on the open arms.

Fear Conditioning

In the fear conditioning test, 40 rats (20 rats per group) from the SPS experiment and 20 rats from IM experiment were trained, after training, rats from each experiment were separated into two groups, one for the contextual fear test and the other group for the auditory cued fear test. The procedure was referenced in Bliss et al. (2010). Meanwhile, sensibility test to the foot-shock was done.

Sensibility Test to the Foot-Shock

The rats were placed in the conditioning charmber (23 × 23 × 35 cm) for 3 min. After 3 min, an electrical foot shock stimulation were delivered. Current strength started from 0.05 mA and progressive increase with 0.05 mA. The minimum current strength was recorded when rats appeared the following three behavioral responses: Notice (head toward the reaction), Flinch (hint foot lift from electric shock rod) and Vocalize.

Contextual Fear Conditioning

The rats were placed in the conditioning chamber and the rats allowed to freely explore for 5 min. The degree of freezing during 5 min was considered as baseline freezing. After 5 min of exploration, an auditory cue (1000 Hz, 75 dB, conditioned stimulus (CS)) was presented for 30 s and an electrical foot shock (2 s 1.5 mA, unconditioned stimulus (US)) stimulation were delivered continuously during the last 2 s of the auditory cue. The presentation of CS-US repeats three times per session with 90 s interval during each repeat. Following the final footshock, the rats were returned to home-cage. Forty-eight hours after training, the rats were placed in the chamber which rats were trained and tested for freezing to the contextual fear conditioning. After 5 min, the rat was returned to home cage, the chamber was cleaned and the next phase of the experiments was started.

Auditory Cued Fear Conditioning

Forty-eight hours after training (the training was described in "contextual fear conditioning"), rats from a separate group were placed in a novel chamber and tested for freezing to the tone. After 2 min habituation period (pre-CS), the freezing time was measured immediately after the tone stimulation (post-CS, without foot shock) within 120 s.

For the fear conditioning test, the freezing activity was recorded and measured using Packwin 2.0 software (Panlab, Barcelona, Spain). Freezing time were used as an index of fear conditioning. Freezing was defined as immobility, excluding respiratory movements with a freezing posture. Rats remained still, sluggish, curled or crouched whilst breathing, and had a slight rocking motion.

Long-Term Potentiation (LTP)

The rats (10 rats per group) were deeply anaesthetised by 20% urethane administered i.p. (6.5 ml/kg). The rats were positioned in a stereotaxic instrument (Harvard apparatus,

Holliston, MA, USA), and the scalp was cut and retracted to expose the skull. According to the brain stereotaxic atlas of Paxinos and Watson (1998), insert the stimulating electrode with interelectrode distance of 0.4 mm into the hippocampal CA3 area (coordinates: AP 3.8 mm, ML 3.8 mm, Depth 3.8 mm) and fixed with dental cement. Then insert glass microelectrode (tip 1∼2 microns in diameter, impedance 5∼20 mΩ, filled with 3 mol/L KCl) into the CA1 area (coordinates: AP 3.8 mm, ML 1.8 mm, Depth 2 mm). For the amygdala, insert the stimulating electrode with interelectrode distance of 0.4 mm from the entorhinal cortex area (coordinates: AP 4.8 mm, ML 6.5 mm, Depth 9 mm) and insert glass microelectrode (tip 1∼2 microns in diameter, impedance 5∼20 mΩ, filled with 3 mol/L KCl) into basolateral nucleus of the amygdala (coordinates: AP 1.8 mm, ML 4.5 mm, Depth 8–8.5 mm; Yaniv et al., 2003).

First rats were given a single wave pulse stimulation (7.5 V, 0.1 ms). Each response amplitude were recorded after evoked population spike (PS). The average amplitude of six times evoked PS (1 time each 5 min) were considered as the baseline value (100%). Then single plus stimulation-induced change in PS amplitude and lasting time were recorded after giving the high frequency stimulation with 100 Hz for 5 s (high frequency stimulant, HFS). The change in more than 30% in average amplitude and maintain more than 30 min were defined as LTP and LTD. Higher were LTP; Lower were LTD.

Western Blotting Analysis

Rats (n = 4 per group) without fear conditioning training were decapitated, and the brains were immediately removed and quick frozen in liquid nitrogen and stored at −80°C. The amygdala and the whole hippocampus were then dissected from brain tissue according to the atlas using a stereomicroscope. The amygdala and the hippocampus of each rat was homogenized with a buffer containing 200 mM TBS, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol, and were denatured by boiling for 5 min. Samples (50 μg/lane) were loaded on a 7.5% SDS-polyacrylamide gel, and electro-blotted onto a PVDF membrane (Millipore Corp., Bedford, MA, USA) from the gel by a semi-dry blotting apparatus (Bio-Rad Laboratories, Inc, Hercules, CA, USA). The PVDF membrane was treated with 1.5% skim milk, 0.05% Tween-20 in TBS (TBST) at 4°C overnight, and then incubated with primary antibodies (primary antibodies list were shown in **Table 1**) at 4°C for 24 h. After being washed three times with TBST, the blots were incubated with a second antibody (anti-mouse or anti-rabbit or anti-goat IgG-HRP from Santa Cruz; 1:1000) for 2 h at room temperature. After incubation, blots were washed three times with TBST, and then were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, NJ, USA). The same blots were incubated with antibodies against GAPDH as positive control. The protein levels were evaluated by calculating the OD ratio. The OD of proteins and GAPDH were analyzed on the Gel Image Analysis System (Tanon 2500R, Shanghai, China). The procedures were repeated four times per rat and then calculation of four rats per group to obtain the average value of each group.

The Immunofluorescence Experiment

Rats (four rats per group) were anesthetized with 50 mg/kg body weight sodium pentobarbital and were perfused through the heart with 4% paraformaldehyde in phosphate buffer. The brains were removed from the skull and fixed in the same fixative solution for 24 h. The brains were immersed in 30% sucrose in 0.1 M PB for 3 days for cryosections. The brains were then quickly frozen using powdered dry ice and cut into 25 μm thick frontal sections on a cryostat (Leica CM 3050, Germany). The sections were stored at 4°C before immunofluorescence. The sections were treated with 2% BSA in 0.3% Triton X-100 in PBS for 2 h at RT to block nonspecific reaction. The sections were incubated with primary antibodies (see **Table 1**) overnight at 4°C. For single labeling immunofluorescence, sections were incubated with a primary antibody (Stathmin, Rin1). For double labeling immunofluorescence, sections were incubated with a mixture of two antibodies (Stathmin and NeuN; Stathmin and GFAP; Rin1 and NeuN; Rin1 and GFAP; Rin1 and EphA4). After three washes with phosphate-buffered saline (PBS), sections were incubated at 37°C for 30 min with secondary antibodies. Sections were incubated with DAPI and then washed four more times with PBS and CA1 subregion was observed and the amygdala under a confocal laser scanning microscope (TI-PS100W, Nikon, Japan).

Statistical Analyses

The results were expressed as mean ± SEM. The differences between control group and SPS groups were analyzed by student's T-test after a normality test ($P > 0.05$) using SPSS 13.0 software (Armonk, NY, USA). For the IM experiment, the differences among three experimental groups were analyzed by one-way ANOVA. A level of $P < 0.05$ was considered to be statistically significant.

RESULTS

Behavioral Changes in Single Prolonged Stress (SPS) Rats

SPS Rats Displayed Enhanced Anxiety Behavior

OF test and elevated plus maze (EPM) were utilized to measure anxiety level, exploratory activity, and aversion. In the OF test, the rats were placed in a novel environment and they naturally avoided the open space in the center. The results of the OF test showed a significant decrease in time in the center of rats after exposure to SPS compared with control

TABLE 1 | The following antibodies were used for western blotting and immunofluorescence.

Antibody name	Company	Concentration
Goat polyclonal antibody against stathmin	Santa Cruz, USA	1:500
Mouse monoclonal antibody against tubulin	Boster, China	1:200
Mouse monoclonal antibody against Rab 5	Boster, China	1:1000
Rabbit polyclonal antibody against Rin1	Santa Cruz, USA	1:1000
Mouse monoclonal antibody against EphA4	Boster, China	1:200
Rabbit monoclonal antibody against Abl	Boster, China	1:200
Mouse monoclonal antibody against NeuN	Abcam, USA	1:500
Mouse monoclonal antibody against GAPDH	Boster, China	1:500
Mouse monoclonal antibody against GFAP	Santa Cruz, USA	1:1000

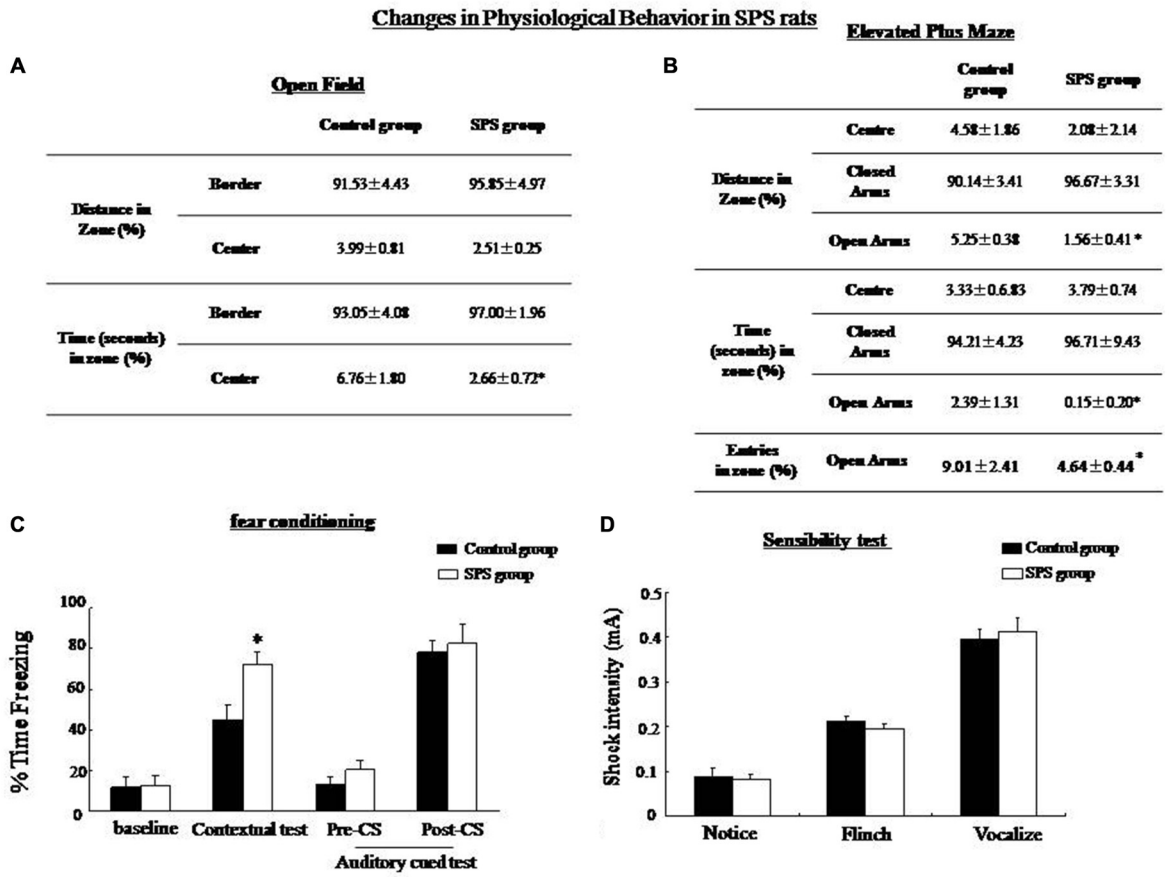


FIGURE 1 | Single prolonged stress (SPS) rats showed decreased exploratory behavior in an open field (OF) test and an elevated plus maze. (A) OF test: SPS rats (n = 10) spent less time in the center zone and showed less rearing compared with the control group (n = 10). **(B)** Elevated plus maze: SPS rats spend shorter distances and spent less number/time in the open arms compared with control rats. **(C)** Conditioning test: the percentage of time spent freezing in contextual fear conditioning was significantly higher in the SPS rats than in the control rats (*$P < 0.05$ vs. the control group), but no difference in auditory cue memory between control and SPS groups. **(D)** Sensibility test to the foot-shock, no significant difference in minimum current strength which induced notice, flinch and vocalize was found between control and SPS rats.

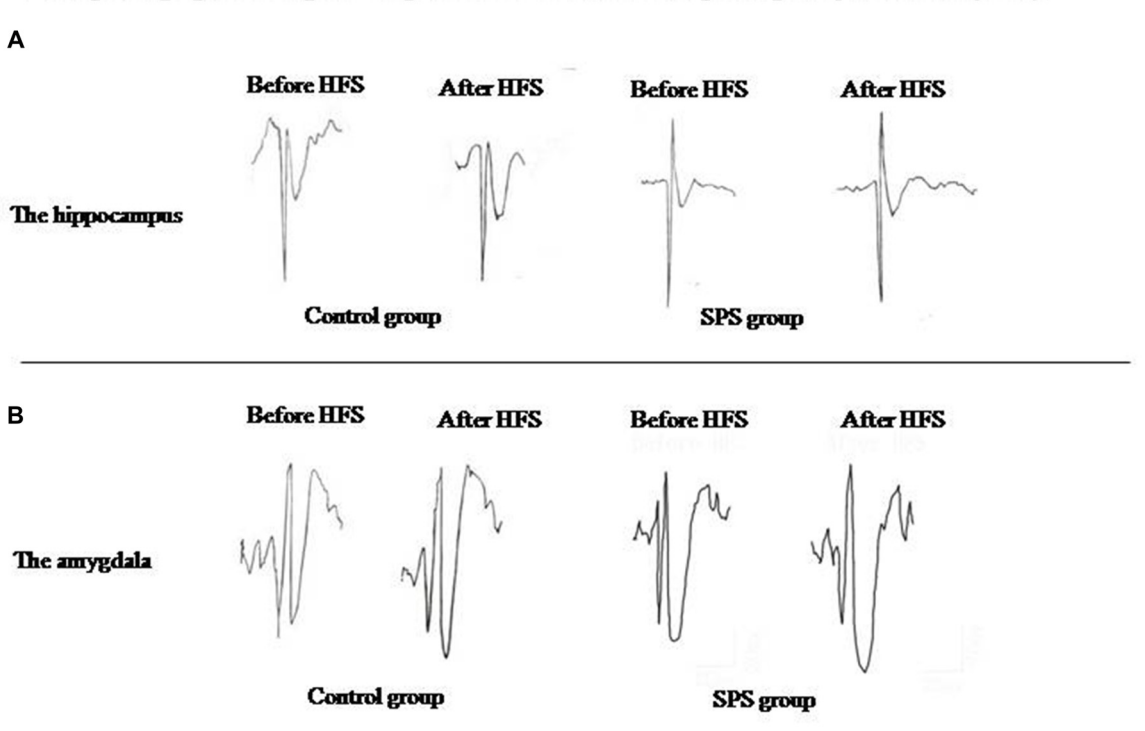
rats (student's T-test, n = 10, $P < 0.05$), which is related to increased anxiety (**Figure 1A**). The EPM was a conflict test between avoiding the open arms of a maze and exploring a new area. The results showed no significant differences in time or distance (student's T-test, n = 10, P = 0.053) in the closed arms. Significantly decreased distance, time and numbers of entries in open arms (student's T-test, n = 10, $P < 0.05$) were observed, suggesting a decrease in exploratory activity, as well as enhanced levels of anxiety and aversion in SPS rats under this environment (**Figure 1B**). Reduced activity of SPS rats in the aversive zones indicated increased anxiety behavior rather than reduced exploration behavior.

Fear Conditioning in SPS Rats

There was no significant difference in the baseline level of freezing between the control and SPS rats. The SPS rats showed increased freezing in comparison with the control group in the contextural test (student's T-test, n = 10, $p < 0.05$). However, no significant difference in the freezing level was seen between the SPS and the control rats in the auditory cued fear test (**Figure 1C**). In the sensibility test to the foot-shock, there was no significant difference in the minimum current which induced notice, flinch and vocalize between control and SPS groups (**Figure 1D**).

Decreased LTP

An increase of 143.53% ± 12.50% in the PS amplitude could be observed in the hippocampus of control rats, whereas that in SPS rats had increased by 121.43% ± 14.87% (**Figure 2A**). Amplitude of evoked PS in the amygdala, had increased by 128.79% ± 10.56% in the control group, while that in SPS rats had increased by only 101.12% ± 13.04% (**Figure 2B**). Thus, differences in enhancement of PS amplitude in both brain regions between two groups were statistically significant (**Figure 2C**). Decreased in LTP after SPS indicated altered plasticity in the amygdala and the hippocampus, which could be associated with formation of altered fear memory.



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	Group	Enhancement rate of PS amplitude
The hippocampus	Control group	143.53% ± 12.50%
	SPS 7 days	121.43% ± 14.87%*
The amygdala	Control group	128.79% ± 10.56%
	SPS 7 days	101.12% ± 13.04%*

FIGURE 2 | (A) Evoked population spike (PS) wave formed before and after high frequency stimulation (HFS) in hippocampus; **(B)** Evoked PS wave formed before and after high frequency stimulation (HFS) in amygdala. **(C)** SPS rats showed significantly decreased enhancement of evoked PS amplitude in hippocampus and amygdala compared with control rats (*$P < 0.05$ vs. the control group).

Changes in Stathmin and Tubulin Expression

Western blot analysis of amygdalar and hippocampal tissues showed a significant decrease in stathmin expression in SPS rats compared with the control group. Stathmin is involved in MT dynamics by regulating both the formation and disassembly of MTs. We found that tubulin expression increased significantly in both brain regions after SPS (**Figure 3**).

To determine the types of cells that express stathmin in the amygdala and hippocampus, we compared the localization of stathmin-immunoreactivity (ir) with the localization of markers of different cell types. We first examined stathmin-ir expression in glial cells by determining its colocalization with glial fibrillary acid protein (GFAP). Dual-immunofluorescence experiments showed that stathmin-ir was expressed in hippocampal glial cells in the control group (**Figures 4A,B**). Next, we found that stathmin-ir was present primarily in neuronal nuclear antigen (NeuN)-positive cells; NeuN is a marker of mature neurons and is expressed in principal cells and interneurons. Cells that coexpressed stathmin-ir and NeuN-ir were observed in the hippocampus (data not shown), the amygdala (**Figures 4C,E**), and the cingulate cortex (**Figure 4D**). However, no coexpression was found in the SPS animals because of a lack of stathmin -ir (**Figure 4F**). The intensity of Tubulin –ir increased significantly in SPS rats (**Figure 4G**) compared with the control group (**Figure 4H**), which was consistent with the western blot results. Finally, we performed dual-immunofluorescence experiments to show the colocalization of stathmin and tubulin. Stathmin- and tubulin-ir colocalized in number of cells of the hippocampal CA1 region

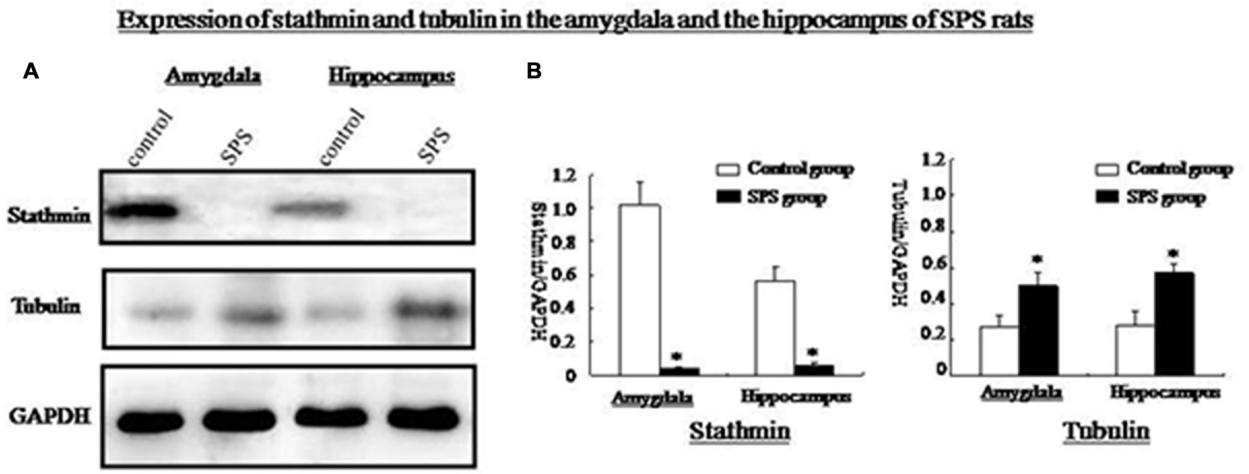

FIGURE 3 | (A) Western blot analysis of stathmin and tubulin in amygdala and hippocampus from control and SPS groups. **(B)** Quantification of western blots showed that, stathmin was significantly decreased while tubulin was remarkably increased in both brain regions of SPS rats compared with control rats (*$P < 0.05$ vs. the control group).

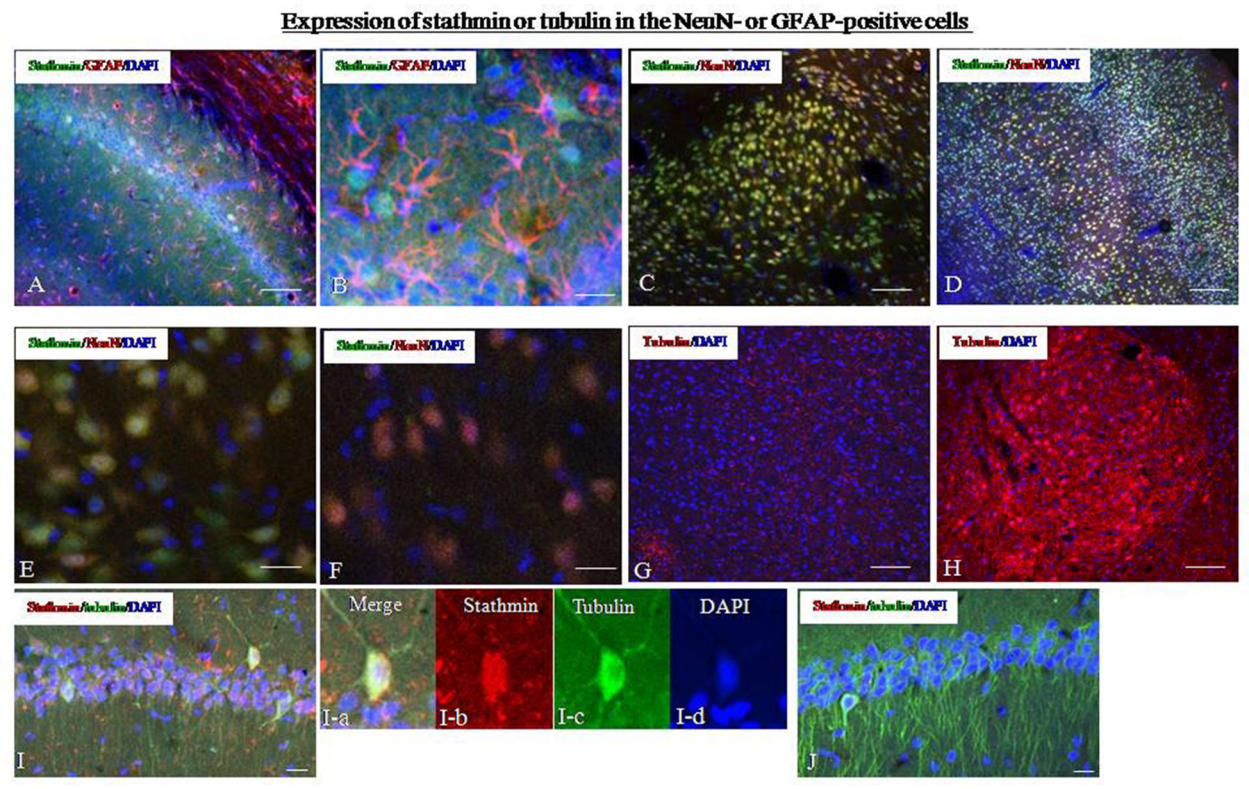

FIGURE 4 | Expression of stathmin and tubulin in the hippocampus. (A) Dual-immunofluorescence image showing stathmin-ir and glial fibrillary acid protein (GFAP)-ir in the hippocampus of the control group. **(B)** A higher magnification image showing colocalization of stathmin-ir and GFAP-ir in the hippocampus of the control group. **(C)** Dual-immunofluorescence image showing stathmin-ir and NeuN-ir in the amygdala of the control group. **(D)** Dual-immunofluorescence image showing stathmin-ir and NeuN-ir in the cingulate cortex of the control group. **(E)** A higher magnification image showing colocalization of stathmin-ir and NeuN-ir in the amygdala of the control group. **(F)** A higher magnification image showing decreased stathmin in the amygdala of the SPS group. **(G,H)** Expression of tubulin in the amygdala of the control group **(G)** and the SPS group **(H)**. **(I,J)** Colocalization of stathmin- and tubulin-ir in the hippocampal CA1 region of control **(I)** and SPS group **(J)**. The magnification image of colocalzation of stahtmin- and tubulin-ir were showed in the **I-a** (merge), **I-b** (stathmin), **I-c** (tubulin0 and **I-d** (DAPI; *$P < 0.05$ vs. the control group; Bar in (**B,D–F,I,J**: 100 μm; Bar in **A,C,G,H**: 20 μm).

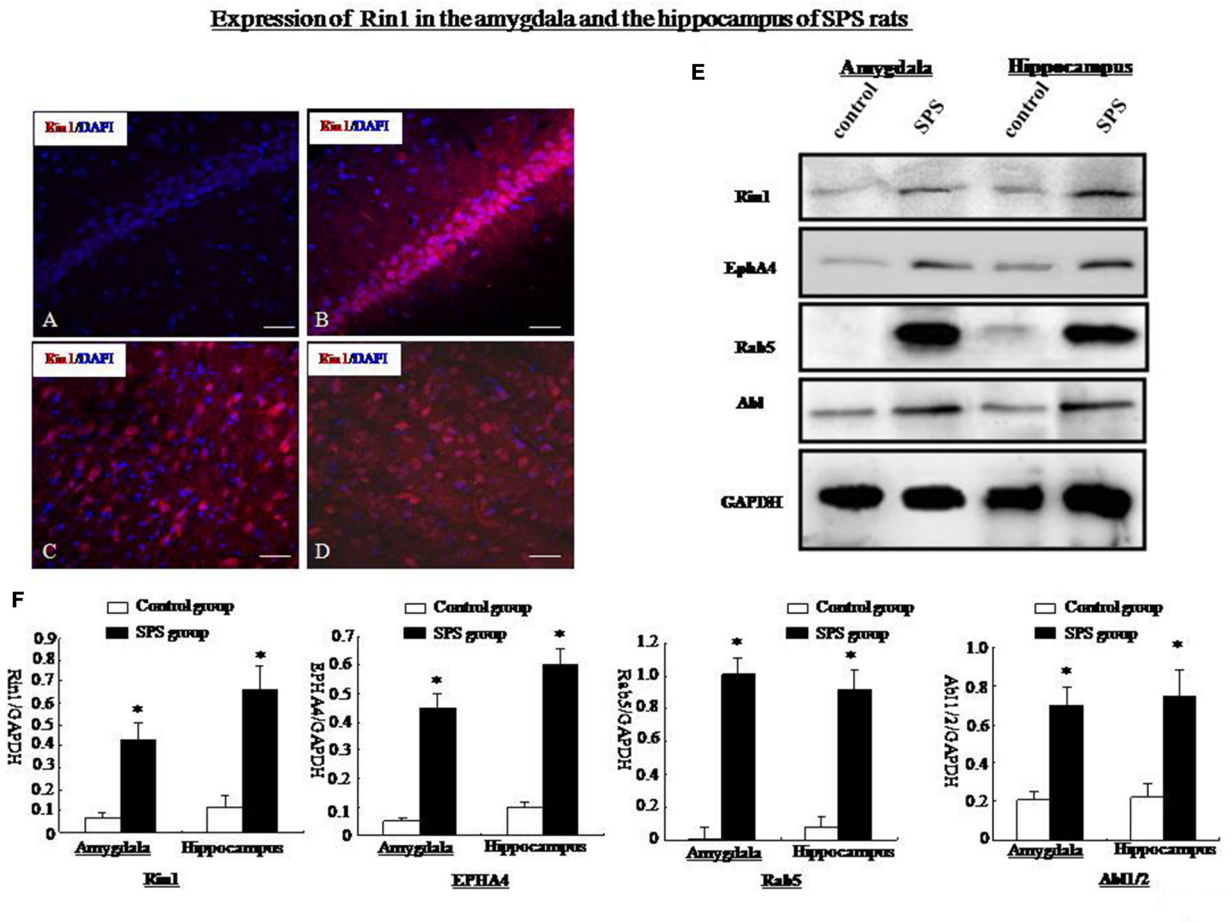

FIGURE 5 | (A) Rin1-ir in the hippocampus of the control group. **(B)** Rin1-ir in the hippocampus of the SPS group. **(C)** Rin1-ir in the amygdala of the SPS group. **(D)** Rin1-ir in the cingulate cortex of the SPS group. **(E)** Western blots showing expression of Rin1, EphA4, Rab5, and Abl in the amygdala and hippocampus of both groups. **(F)** Quantification of western blots showing higher expression of Rin1, EphA4, Rab5, and Abl in the amygdala and hippocampus of the SPS group compared with the control group (*$P < 0.05$ vs. the control group; Bar: 20 μm).

in the control group (**Figure 4I**), but no coexpression cells were found after SPS stimulation because of decreased stathmin-ir (**Figure 4J**).

Changes in Rin1 Expression

The Rin1 gene encodes a Ras effector protein that signals through downstream Rab5 and Abl to positively regulate endocytosis and cytoskeletal remodeling. Therefore, we examined expression of Rin1 and its downstream effectors, EphA4, Rab5, and Abl. Rin1 levels were extremely low in control rats. Therefore, we were unable to obtain precise localization data for Rin1 protein (**Figure 5A**). Other studies have suggested that low Rin1-ir expression is due to a lack of antibody detection of endogenous Rin1 in brain tissue. We found high Rin1-ir expression in the hippocampus, (**Figure 5B**), amygdala (**Figure 5C**), cingulate cortex (**Figure 5D**), and thalamus of SPS rats (data not shown). Western blot analysis showed a significant increase in Rin1 protein in the amygdala and hippocampus of SPS animals compared with control rats (student's *T*-test, $n = 4$, $p < 0.05$). The downstream effectors EphA4, Rab5, and Abl were also expressed at higher levels in both regions of the SPS group than in control rats (student's *T*-test, $n = 4$, $p < 0.05$; **Figures 5E,F**).

Next, we studied the localization of Rin1-ir using markers for different cell types. In SPS animals, Rin1-ir was mainly expressed in NeuN-positive cells in the amygdala (**Figure 6B**) and hippocampus (**Figure 6D**) and was not expressed in GFAP-positive cells (**Figures 6E,F**). In control rats, Rin1-ir was not expressed in these brain regions (**Figures 6A,C**).

Rin1 and EphA4 were coexpressed in primary neurons after stimulation by SPS. Rin1 interacts with EphA4 in excitatory neurons and mediates endocytosis of EphA4. To explore the relationship between Rin1 and EphA4 under SPS conditions, we performed a dual-immunohistofluorescence assay using EphA4 and Rin1 antibodies. We found extremely low levels of Rin1- and EphA4-ir in the brains of control rats (data not shown). Therefore, we were unable to obtain precise Rin1 and EphA4 protein localization data. We found a

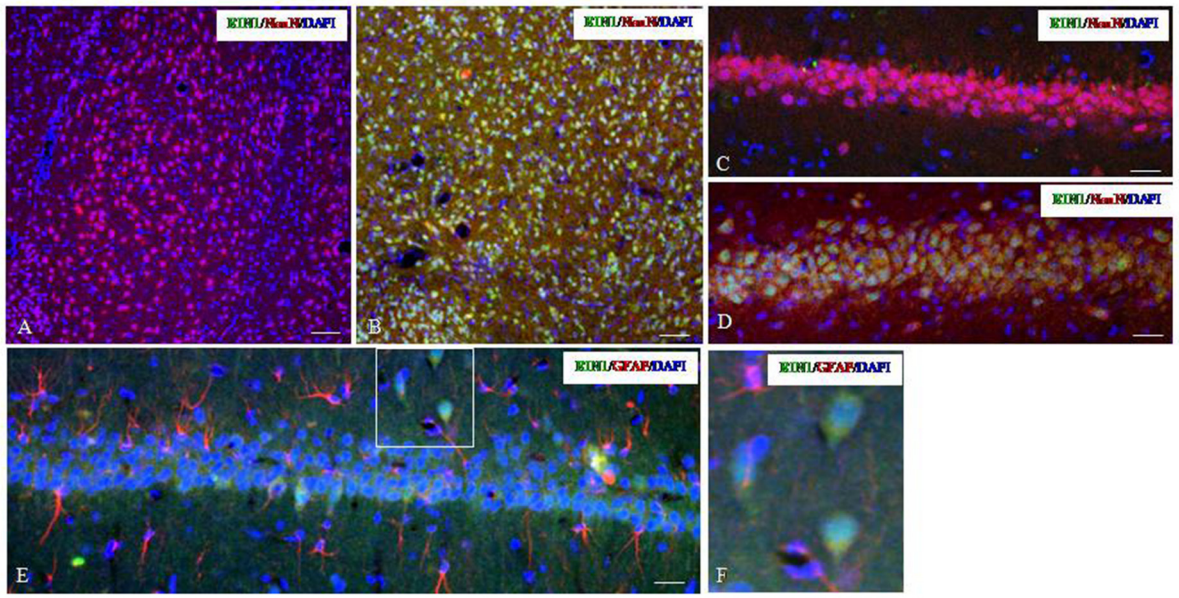

FIGURE 6 | (A,B) Dual-immunofluorescence images for Rin1-ir and NeuN-ir in the amygdala of the control **(A)** and SPS **(B)** groups. **(C,D)** Dual-immunofluorescence images for Rin1-ir and NeuN-ir in the hippocampus of the control **(C)** and SPS **(D)** groups. **(E)** Rin1-ir in GFAP-positive cells in the hippocampus of SPS rats. **(F)** A higher magnification image of the area in the white box in panel **(E)** (Bar in **A,B**: 100 μm; Bar in **C–E**: 50 μm).

high Rin1-/EphA4-ir coexpression ratio in the hippocampus (**Figure 7A**), cingulate cortex (**Figure 7B**), thalamus (**Figure 7C**), and amygdala (**Figure 7D**) of SPS rats. A magnified image of cells in the amygdala revealed colocalization of Rin1-/EphA4-ir (**Figure 7D**, arrow) and Rin1- or EphA4-positive cells (**Figure 7D**, arrowhead). About 88% of EphA4-positive cells were Rin1-positive, and 93% of Rin1-positive cells were EphA4-positive in the amygdala and the hippocampus of the SPS group (**Figure 7E**). Punctate fluorescence was detected in the cytoplasm and axons of cells in which EphA4- and Rin1-ir colocalized (**Figure 7F**). Cluster intensity was analyzed using single-channel pseudo-color mode, and we found that the high intensity clusters were only EphA4. We selected two clusters (a and b) and measured their intensity by positioning the coordinates (intersection of two pink lines/yellow lines). EphA4 showed peak intensity at points a and b (**Figure 7G**, green line), whereas Rin1 did not (red line), indicating that the frequency of the peak between EphA4 and Rin1 was inconsistent and that EphA4 immunoreactive intensity was higher at a/b than that at other points.

Roles of Rin1 and Stathmin in Response to Immobilization (IM) and a Loud Sound

SPS consisted of multiple stresses. An IM stimulus was provided to detect changes in Rin1 and stathmin expression under a non-SPS like stress condition, with an aim to explore whether changes in both genes were specific to SPS. The OF test showed a significant increase in distance from the center zone after 7 days in the IM rats compared with control rats (One-way ANOVA, $n = 5$, $p < 0.05$; **Figure 8A**). The EPM results revealed a significant decrease in distance, time and the number of entries into the open arms 7 days after IM exposure (One-way ANOVA, $n = 5$, $p < 0.05$; **Figure 8B**). Contextual and auditory cue fear conditioning test also revealed the significant increase in level of freezing in IM rats compared with control rats (One-way ANOVA, $n = 10$, $p < 0.05$; **Figure 8C**). In the sensibility test to the foot-shock, there was no significant difference in the minimum current which induced notice, flinch and vocalize between control and IM groups (**Figure 8D**).

Western blotting showed that Rin1, EphA4, Abl, Rab5, and tubulin expression decreased significantly in the amygdala and the hippocampus at 1 day and increased significantly at 7 days after the IM stimulation, whereas stathmin expression decreased at 1 day and 7 days after the IM stimulation (One-way ANOVA, $n = 4$, $p < 0.05$; **Figure 9**). These results were consistent with those observed after SPS, despite the distinct magnitude of changes in expression. Changes in stathmin or Rin1 expression could not be observed after a loud sound stimulus (student's T-test, $n = 4$, $p < 0.05$; **Figure 10**).

DISCUSSION

Behavioral Change in SPS Rats

Three behavioral tests (namely, OF test, elevated plus maze and fear-conditioning test) are tested in the present study to examine the behavioral changes in SPS- and immobilization-stressed rats. OF and EPM were used to be measured anxiety level, exploratory activity, and aversion. EPM results showed that

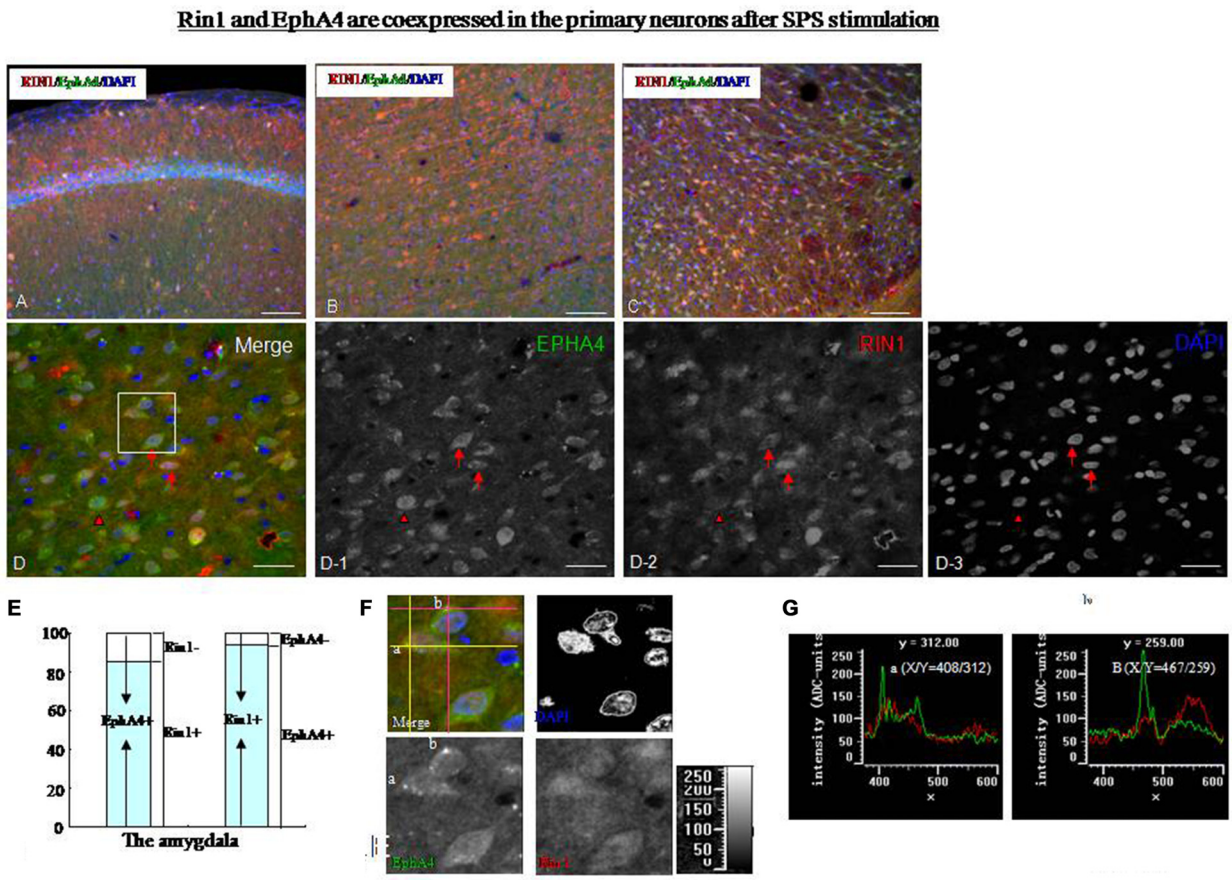

FIGURE 7 | (A–C) Dual-immunofluorescence images showing that Rin1-ir and EphA4-ir were colocalized in the hippocampus **(A)**, cingulate cortex **(B)**, and thalamus **(C)** of SPS rats. **(D)** Higher magnification image of the amygdala shows colocalization of Rin1/EphA4 (arrow) and EphA4- or Rin1- positive cells (arrowhead). EPHA4: **D-1**; RIN1: **D-2**; DAPI: **D-3**. **(E)** Statistical analysis indicated that about 88% of EphA4-positive cells were Rin1-positive, and 93% of Rin1-positive cells were EphA4-positive in the amygdala. **(F)** Higher magnification images of the area in the white box in panel **(D)**. Some bright clusters were detected (merge). Two clusters (a and b) were selected by positioning the coordinates (a: intersection of two yellow lines; b: intersection of two pink lines). **(G)** Intensity of points a and b. The green line shows the intensity of EphA4, and the red line shows the intensity of Rin1. EphA4 was expressed at peak intensity at points a (X/Y = 408/312) and b (X/Y = 467/259; $^*P < 0.05$ vs. the control group; Bar in **A–C**: 100 μm; Bar in **D**: 20 μm).

SPS induced decreased levels in entries number/time/distance of the open arms and increased level in the closed arms. OF test exhibited decreased locomotor activity within the inner regions of the field. These results suggested distinctly enhanced anxiety level, enhanced aversion and decreased exploratory in SPS rats in comparison with control rats, which are consistent with results from other studies on SPS and may be important for understanding the human condition of PTSD. Our fear-conditioning tests showed higher freezing level in SPS rats than in control group in contextual memory, but not for auditory cue memory. It is well known that the amygdala has an important role in auditory-cued and contextual fear conditioning. In auditory-cue conditioning, direct projections from the thalamus and/or from the auditory cortex to the lateral amygdala (LA) are thought to be critical (Romanski and LeDoux, 1992; Li et al., 1996; LeDoux and Muller, 1997). Thus, lesions of the LA, but not of the basolateral amygdala (BLA), accessory basal, or medial nucleus of the amygdala can block auditory-cue conditioning (Nader et al., 2001). Therefore, the different behavioral and electrophysiological outcomes obtained in contextual and auditory-cued fear conditioning may be due to a different expression in Rin1 and Stathmin in subnuclei of the amygdala after SPS stimulation. Studies from Toledano and Gisquet-Verrier (2014) found that SPS rats showed decreased or unchanged level of acoustic startle response, which is consistent with our results. Studies from Imanaka et al. (2006) also reveals markly elevated freezing level in acquisition of fear conditioning in SPS rats compared with that in control rats. But studies from Knox et al. (2012) shows no effect of SPS on the acquisition of fear conditioning. It has been reported that the hippocampus and the amygdala are involved in contextual conditioning and encode memories in multiple cues is associated with the aversive event (Phillips and LeDoux, 1994; Calandreau et al., 2005). During traumatic process, dysfunction in both regions can bias the formation of multiple cues and exaggerate fear responses under multiple environments (or

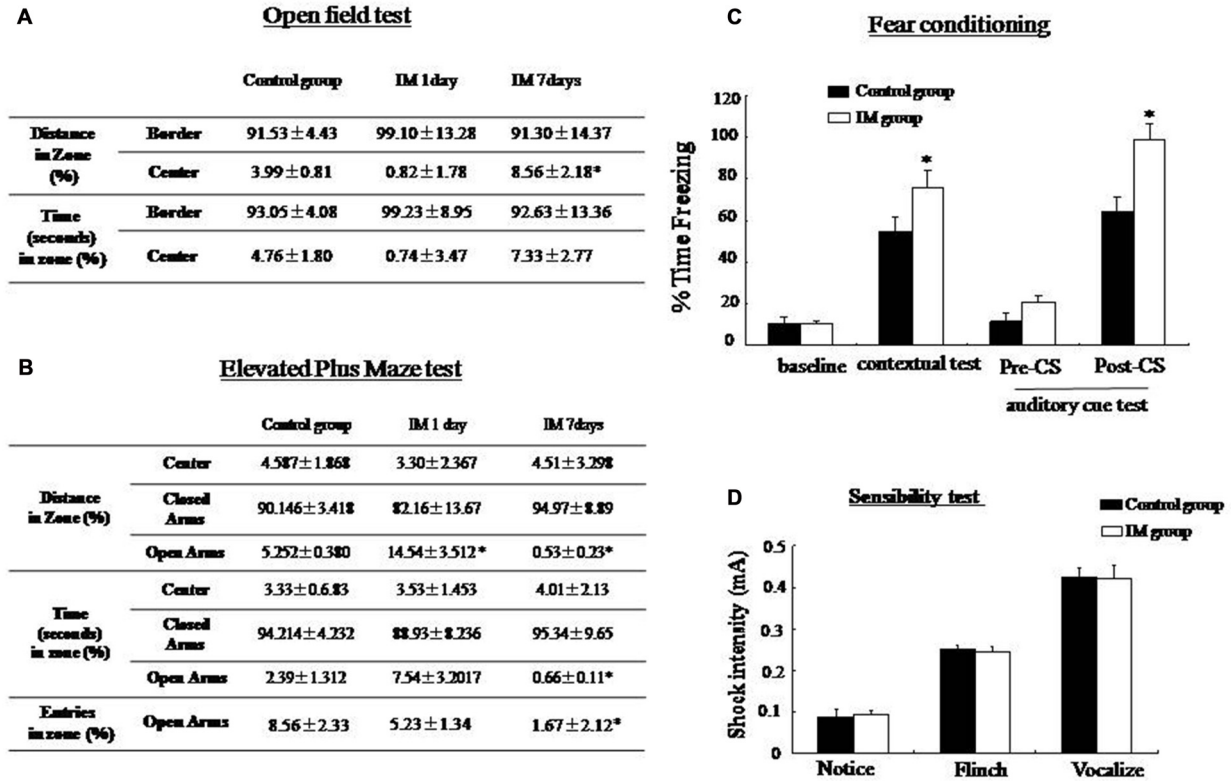

FIGURE 8 | (A) Open-field test: The immobilization (IM)-stressed rats showed a greater percentage of distance in the border zone compared with control rats. **(B)** Elevated plus maze: IM-stressed rats showed shorter distance, less time and less entry number in the open arm compared with control rats. **(C)** Conditioning test: The percentage of time spent freezing in contextual and auditory cue fear conditioning (post-CS) were significantly higher in the IM rats than in the control rats (*$P < 0.05$ vs. the control group). No significant difference was observed in baseline and pre-CS of the auditory cue fear conditioning between control and IM rats. **(D)** sensibility test to the foot-shock, no significant difference in minimum current strength which induced notice, flinch and vocalize was found between control and IM rats.

contexts; Achesona et al., 2012). Beyond that, we also found similar behavioral changes in immobilization-stressed rats. IM rats showed an enhanced contextual memory and auditory cued memory in comparison with control rats, which is different from what was observed in the SPS rats; this suggests that SPS and other traumatic stressors could induce different changes in different cued fear conditioning.

Dysfunction in Stathmin after SPS Exposure

Our results suggest a loss of stathmin expression after SPS exposure. It is first found that stathmin is highly expressed in most brain regions, such as the hippocampus and the amygdala, which are the main regions regulating emotional memory. Stathmin knockout mice exhibited normal neuronal morphology, decreased memory, and recognizeably reduced less danger in innately aversive situations, suggesting that the loss of stathmin expression impacts innate and learning anxiety-related behavior (Shumyatsky et al., 2002). Our OF test results showed that SPS rats spent less time in the center region, which is in contrasts with results from the knockout mice. These opposite results may be attributed to different manifestations of a basic-fear disorder or the comprehensive effects of changes on expression of multiple genes in SPS animals. Stathmin is probably regulated by a basic fear because it is highly expressed under normal conditions. Studies from knockout animals found reduced contextual fear memory and impaired dentate gyrus LTP in stathmin−/− mice, indicating that stathmin was a positive regulator of fear memory (Shumyatsky et al., 2002; Uchida et al., 2014; Zhang et al., 2015). In addition, abilities to properly assess a threat, provide parental care, and interact socially as adults are deficient in stathmin knockout animals (Martel et al., 2008). Stathmin plays a negative role in regulating MT formation. A lack of stathmin and increased tubulin expression may be indicative of greater MT formation and decreased MT dynamics in SPS rats. MTs may be important for synaptic activity and cellular transport in the case of transporting important molecules and organelles to the synapse (Westermann and Weber, 2003). Enhanced

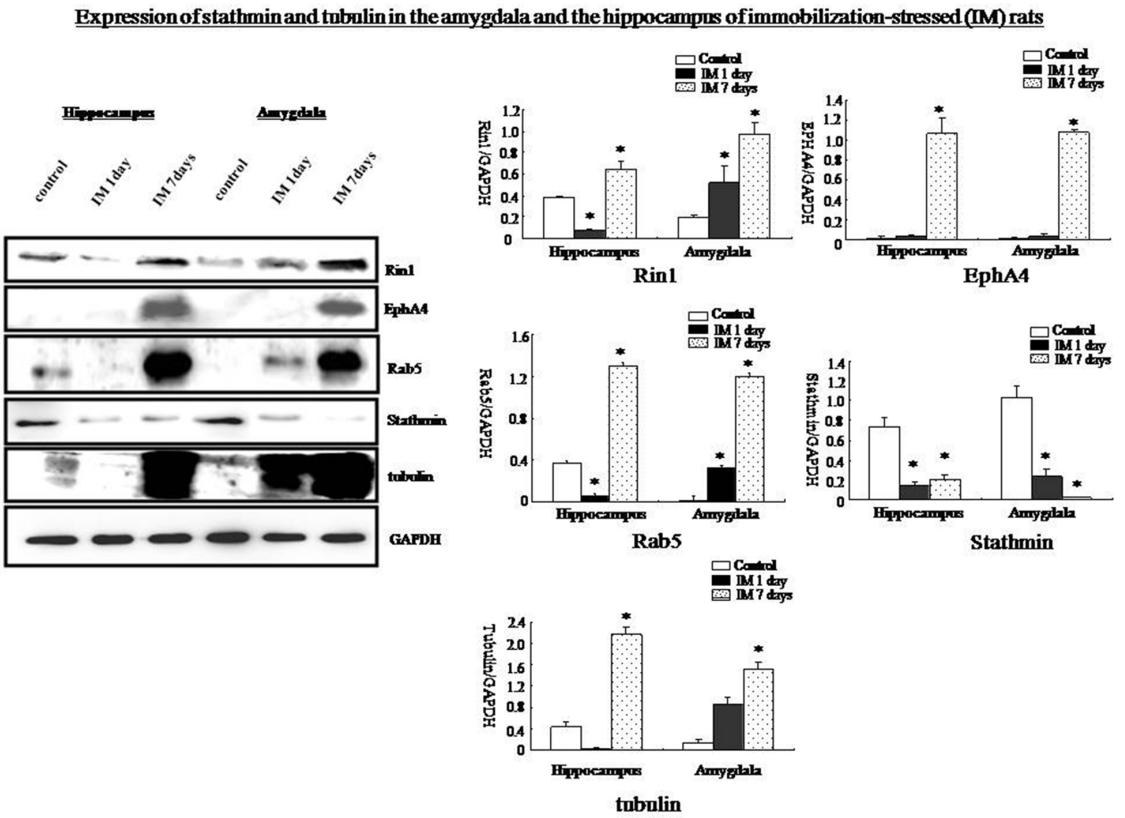

FIGURE 9 | Western blots showing that Rin1, EphA4, Abl, Rab5, and tubulin expression increased significantly in the amygdala and hippocampus 7 days after the IM-stress stimulation compared with the control rats; in contrast, stathmin expression decreased. Quantification of western blots showing lower expression of Rin1, EphA4, Rab5, and tubulin in the amygdala and hippocampus of the IM 1 day group and higher expression in the IM 7 days group compared with the control group except stathmin (*$P < 0.05$ vs. the control group).

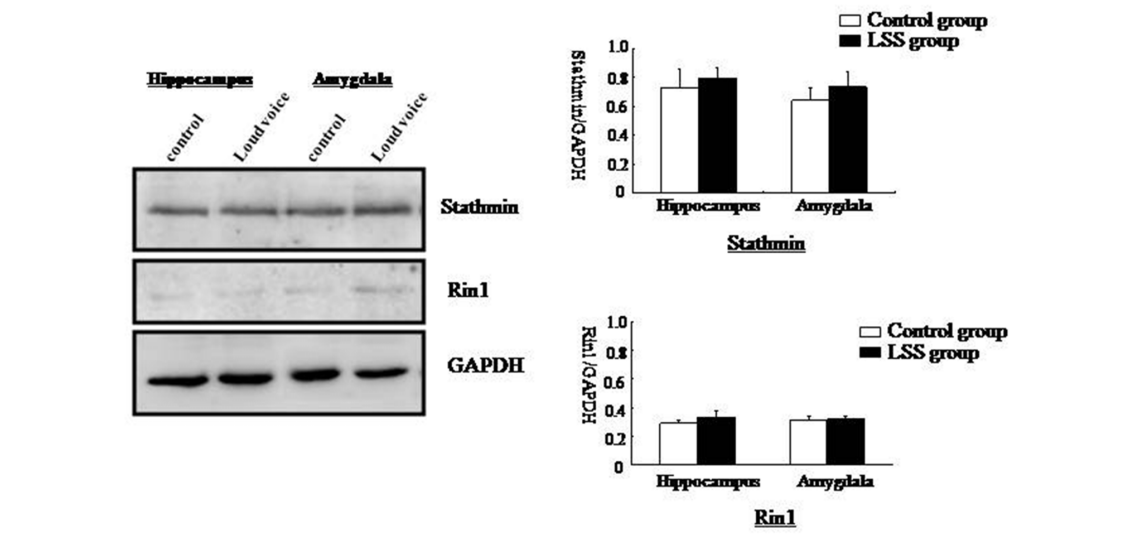

FIGURE 10 | Western blots showing no changes in stathmin or Rin1 expression after the loud sound stimulus.

MT function may be required to maintain basic physiological functions of cells under the condition with SPS-induced high apoptosis ratio condition (Hirokawa and Takemura, 2005). Changes in cytoskeletal proteins integrin, vinculin and connexin 43 are also found in our previous study (Li et al., 2015), which are consistent with increased MTs. Few studies at present have focused directly on stathmin expression in fear-related psychological disorders. A recent study from Uchida showed deficit of stathmin reduced contextual fear memory and impaired dentate gyrus LTP (Uchida et al., 2014). As is reported in one clinical study, stathmin expression is associated with re-experiencing of PTSD symptoms (Cao et al., 2013). Finally, an opposite change in stathmin expression is found in one study; besides, blast-related traumatic brain injury can increase stathmin expression in amygdala as well as anxiety levels (Elder et al., 2012).

Rin1 Functions after Stimulation by SPS

Rin1 is expressed postnatally in the brain, which is dramatically reduced in expression in adult brains (Bliss et al., 2010). Here, we showed increased expression of Rin1 and its downstream effectors Rab5 and Abl in the hippocampus and amygdala of SPS rats. Our behavioral tests showed an enhancement of freezing time in SPS rats compared with the control group in the contextual fear conditioning, not auditory fear. It is discovered in our previous study that spatial-dependent memory in SPS rats is deficient as well. Rin1−/− mice display enhanced auditory fear conditioning through increasing fear acquisition/retention with deficits in extinction (Dhaka et al., 2003; Bliss et al., 2010). As is reported by Bliss, Rin1−/− mice had reduced latent inhibition, indicating that Rin1 has a limited effect on establishing memories (Bliss et al., 2010). Few existing studies that investigated the relationship between Rin1 and fear have been limited to Rin1 knockout animals. Direct evidence showing expression and function of Rin1 in a fear-related psychological disorder is lacking at present. Increased Rin1 level in hippocampus and amygdala of SPS rats is found in this research, suggesting a possibility of altered amygdala-hippocampal interactions. However, according to the present data, no direct evidence is available to conclude that increased Rin1 expression can inhibits fear memory in SPS rats. Instead, it can only be achieved by interventional studies in amygdala and hippocampus. Abl is reported to affect short-term synaptic plasticity (Moresco et al., 2003). Rab5 controls endocytosis of cell surface receptors such as AMPA (Bliss et al., 2010). It has been also reported that stathmin mutations disrupt GluA2 (one subunit of AMPA) localization (Uchida et al., 2014), suggesting AMPA could be a common target of stathmin and Rin1. It would be helpful for understanding functional link between both independent molecules in stress disorder. Rin1 coordinates stimulation of the Abl and Rab5 signaling pathway by integrating actin remodeling, recycling receptors by endocytosis, and trafficking at the postsynaptic membrane. Our dual-immunofluorescence assay revealed that Rin1 was expressed in primary neurons. Rin1 and EphA4 coexpression in primary neurons of SPS rats suggests that Rin1 interacts with EphA4 while regulating endocytosis, which is relevant to neuronal plasticity (Deininger et al., 2008). Interactions between Rin1 and EphA4 have been examined in several cell lines. Increased Rin1 expression inhibited enhancement of LTP in the amygdala by suppressing EphA4 internalization and function in SPS rats. High intensity EphA4-stained clusters were localized on the surface of primary cells, which is an area of internalization.

Taken together, our data strongly suggest that the change in stathmin and RIN1 can be observed in the hippocampus and the amgydala in SPS rats. However, behavioral expression of knockout animals with both genes suggests that increased RIN1 and decreased stathmin should inhibit fear memory formation, which contradicts our behavioral test results (increased contextual freezing and unchanged auditory freezing). Two possibilities that may explain the inconsistencies between the observed behaviors and expression of the genes: first, the interaction of RIN1 with stathmin may induce different expression of the individual knockout gene since they share a common target, which is AMPA in the pathways of stathmin and Rin1. Second, such inconsistency may derive from the comprehensive effect of changes in expression of multiple genes in SPS animals.

Stathmin and Rin1 Expression after Other Traumatic Stressors

SPS represents a very specialized stress, but whether other traumatic stressors can change stathmin and Rin1 expression remains unknown. Two additional stressors, IM stress and LSS, are used in this research. Expression of Rin1, EphA4, Abl Rab5 and tubulin in amygdala and hippocampus is distinctly increased after IM stimulation, whereas stathmin expression is decreased, which is consistent with SPS results. Behavioral tests indicate abnormal innate fear and enhanced fear, demonstrating that not only SPS but also other traumatic stressors can change expression of stathmin and Rin1. Thus, changes in both genes may not be specific to PTSD-like stress; instead, they may be specific to a broader spectrum of traumatic stresses. The lack of changes in stathmin and Rin1 expression after a LSS is consistent with our hypothesis. However, changes in stathmin and Rin1 expression should be further examined in additional trauma-related psychological disorders.

Unexpected Finding and the Limitation

It is shown in the present study that SPS induces increased contextual fear conditioning, increased Rin1 level while decreased Stathmin level in hippocampus and amygdala. However, Rin1 knockout mice demonstrate enhanced fear conditioning, while Stathmin knockout mice display decreased fear memory. The inconsistency between context conditioning and expression of Rin1/Stathmin may be explained by the comprehensive effects of changes on expression of multiple genes in SPS animals. On the other hand, Stathmin is likely regulated by a basic fear, which may be different from Rin1 in regulating fear conditioning. Therefore, further studies will be required to explicitly address the effects of Rin1/Stathmin on fear conditioning in SPS model.

Our study does not illustrate a direct involvement of Stathmin or Rin1 in the enhanced fear conditioning observed in rats subject to SPS or to immobilization. Therefore, interventional studies should be enrolled in future study. Such as it is, our results may provide new insight into the molecular mechanism of abnormal fear memory after exposure to trauma and a better understanding towards individual variations in PTSD susceptibility and therapy.

CONCLUSION

Our data show that changes in stathmin and RIN1 in hippocampus and amgydala can be seen under the conditions of SPS and IM, suggesting that the changes in the expression of Rin1 and stathmin genes may be involved in SPS and immobilization stress. But changes in the expression of both genes may not be specific to PTSD-like stress.

AUTHOR CONTRIBUTIONS

FH and YS contributed to experimental design; FH, JJ, JD and HL contributed to acquisition of data; FH, BX and HL contributed to analysis of experimental data.

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Behavioral Effects of Systemic, Infralimbic and Prelimbic Injections of a Serotonin 5-HT$_{2A}$ Antagonist in Carioca High- and Low-Conditioned Freezing Rats

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The role of serotonin (5-hydroxytryptamine [5-HT]) and 5-HT$_{2A}$ receptors in anxiety has been extensively studied, mostly without considering individual differences in trait anxiety. Our laboratory developed two lines of animals that are bred for high and low freezing responses to contextual cues that are previously associated with footshock (Carioca High-conditioned Freezing [CHF] and Carioca Low-conditioned Freezing [CLF]). The present study investigated whether ketanserin, a preferential 5-HT$_{2A}$ receptor blocker, exerts distinct anxiety-like profiles in these two lines of animals. In the first experiment, the animals received a systemic injection of ketanserin and were exposed to the elevated plus maze (EPM). In the second experiment, these two lines of animals received microinjections of ketanserin in the infralimbic (IL) and prelimbic (PL) cortices and were exposed to either the EPM or a contextual fear conditioning paradigm. The two rat lines exhibited bidirectional effects on anxiety-like behavior in the EPM and opposite responses to ketanserin. Both systemic and intra-IL cortex injections of ketanserin exerted anxiolytic-like effects in CHF rats but anxiogenic-like effects in CLF rats. Microinjections of ketanserin in the PL cortex also exerted anxiolytic-like effects in CHF rats but had no effect in CLF rats. These results suggest that the behavioral effects of 5-HT$_{2A}$ receptor antagonism might depend on genetic variability associated with baseline reactions to threatening situations and 5-HT$_{2A}$ receptor expression in the IL and PL cortices.

Highlights

- CHF and CLF rats are two bidirectional lines that are based on contextual fear conditioning.
- CHF rats have a more “anxious” phenotype than CLF rats in the EPM.
- The 5-HT$_{2A}$ receptor antagonist ketanserin had opposite behavioral effects in CHF and CLF rats.

- Systemic and IL injections either decreased (CHF) or increased (CLF) anxiety-like behavior.
- PL injections either decreased (CHF) anxiety-like behavior or had no effect (CLF).

Keywords: breeding lines, freezing, contextual fear conditioning, elevated plus maze, serotonin, 5-HT_{2A} receptors, medial prefrontal cortex

INTRODUCTION

Several studies indicate that contextual fear conditioning represents one of the simplest animal models of investigating anticipatory anxiety (Brandão et al., 2008). It involves placing an animal (e.g., a rat) in a novel environment and delivering a brief unsignaled footshock several minutes later. The next day, the animal freezes when it is returned to the same chamber in the absence of footshock (Landeira-Fernandez, 1996). Conditioned freezing is a direct function of shock intensity (Sigmundi et al., 1983) and depends on the association between the contextual cues associated with the experimental chamber and footshock (Landeira-Fernandez et al., 2006). Classic anxiolytic benzodiazepines, such as midazolam and diazepam (Fanselow and Helmstetter, 1988), and non-benzodiazepine anxiolytics, such as the serotonin (5-hydroxytryptamine [5-HT])-$_{1A}$ receptor agonist ipsapirone (Inoue et al., 1996) and 5-HT reuptake inhibitors citalopram and fluvoxamine (Hashimoto et al., 1996), reduced conditioned freezing. Anxiogenic substances, such as the benzodiazepine inverse agonist dimethoxy-β-carboline, induced freezing behavior similarly to fear conditioning (Fanselow et al., 1991).

Two lines of Wistar rats, termed Carioca High-conditioned Freezing and Low-conditioned Freezing (CHF and CLF, respectively), were selectively bred for high and low levels of freezing in response to contextual cues that were previously associated with footshock (Castro-Gomes and Landeira-Fernandez, 2008). The results of our ongoing breeding program have shown a clear divergence of the conditioned freezing phenotype after only three generations (Castro-Gomes and Landeira-Fernandez, 2008). The presence of different levels of anxiety-like behavior, that are characteristic of each line, can be assessed using several behavioral tests, including the elevated plus maze (EPM), the social interaction test and defensive responses that are induced by electrical stimulation of the dorsal periaqueductal gray (Dias et al., 2009; Galvão et al., 2010; Castro-Gomes et al., 2011, 2014; Salviano et al., 2014). These two lines represent an important tool for investigating the anxiogenic/anxiolytic pharmacological profiles of various compounds (Castro-Gomes et al., 2013).

5-HT is an indoleamine that is intimately connected to the neurocircuitry that underlies anxiety (for review see Millan, 2003; Graeff, 2004). It exerts its behavioral and physiological effects by acting at different receptor subtypes that are distributed into seven G-protein-coupled receptor families (Hoyer et al., 2002; Hannon and Hoyer, 2008). The 5-HT_2 receptor family (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) has been the focus of much research interest because of its critical role in modulating anxiety-like behavior in animals and humans (Naughton et al., 2000; Graeff, 2002; Wood, 2003; Gordon and Hen, 2004). The predominant effect of 5-HT in this metabotropic receptor family (especially the 5-HT_{2A} subtype) is excitatory and appears to mediate depolarizing effects (Davie et al., 1988; Eison and Mullins, 1996; Hasuo et al., 2002), but it may also have inhibitory activity or even interact with a number of other inhibitory interneurons (Avesar and Gulledge, 2012; Halberstadt, 2015; Wang et al., 2016).

Paradoxically, the effects of 5-HT_2-acting drugs on anxiety have been highly inconsistent in animals and humans. For example, systemic administration of 5-$HT_{2A,2C}$ agonists exerts both anxiogenic-like (Charney et al., 1987; Lowy and Meltzer, 1988; Bastani et al., 1990; Rodgers et al., 1992; Gibson et al., 1994; Setem et al., 1999; Jones et al., 2002; Bull et al., 2003; Durand et al., 2003) and anxiolytic-like (Ripoll et al., 2006; Hughes et al., 2012) effects. Similarly, 5-$HT_{2A,2C}$ antagonists have been reported to exert anxiolytic-like effects (Critchley and Handley, 1987; Motta et al., 1992; Kennett et al., 1994; Nic Dhonnchadha et al., 2003), anxiogenic-like effects (Pellow et al., 1987), or no effects (Chaouloff et al., 1997; Setem et al., 1999). Similar inconsistencies have also been reported following direct infusions of 5-HT_2 agonists and antagonists in anxiety-related postsynaptic brain sites (for review see Menard and Treit, 1999; Graeff, 2002, 2004). For example, 5-$HT_{2A/2C}$ agonist administration in the amygdala (Campbell and Merchant, 2003; de Mello Cruz et al., 2005) and ventral hippocampus (Alves et al., 2004) has been shown to be anxiogenic, whereas microinjections in the dorsal periaqueductal gray have been reported to be anxiolytic (Graeff et al., 1993).

Different experimental procedures, brain sites and selectivity for 5-HT_2 receptors might contribute to these discrepancies. Moreover, the role of the 5-HT_2 receptor family in anxiety might depend on genetic variables that are associated with different trait levels of defensive reactions. One of the purposes of the present study was to investigate the effects of systemic injections of a 5-HT_{2A} receptor antagonist in CHF and CLF animals in the EPM. Ketanserin was chosen in this study because of our 25 years' experience with the use of this drug as a pharmacological tool for the study of the neurobiology of anxiety and fear (Motta et al., 1992; de Luca et al., 2003; Oliveira et al., 2007; Almada et al., 2009). Ketanserin is a 5-$HT_{2A/2C}$ receptor antagonist that has higher affinity for 5-HT_{2A} receptors than 5-HT_{2C} receptors (Kristiansen and Dahl, 1996; López-Giménez et al., 1997; Knight et al., 2004).

EPM is based on rodents' innate fear of open spaces (Treit et al., 1993). This model has been, behaviorally, physiologically and pharmacologically, validated as an animal model of anxiety in rats (Pellow et al., 1985; Pellow and File, 1986; Reibaud and Böhme, 1993). Factor analyses have also indicated that this test reliably dissociates the anxiety-related effects (open arm entries) from locomotor effects (closed arm entries) of several anxiolytic and anxiogenic agents (File, 1992; Cruz et al., 1994).

The role of 5-HT_{2A} receptors in anxiety might also depend on serotonergic activity in different brain regions. The ventromedial prefrontal cortex (vmPFC) is composed of the infralimbic (IL) and prelimbic (PL) subregions. Serotonergic neurons in both the dorsal and medial raphe nuclei send robust projections to the vmPFC (Azmitia and Segal, 1978; Steinbusch, 1981; Blue et al., 1988). Moreover, 5-HT_2 receptors, but mainly the 5-HT_{2A} receptor subtype, are widely and densely distributed in both the PL and IL (Pazos et al., 1985; Pompeiano et al., 1994; Santana et al., 2004).

Behavioral results that have been generated with different animal models of anxiety indicate that the IL and PL play distinct and complex roles in conditioned and innate defensive reactions. The IL appears to inhibit the expression of anxiety-like behavior, whereas the PL facilitates its expression through descending projection to the basolateral complex of the amygdala (Likhtik et al., 2014). For example, stimulation of the IL reduced the expression of auditory fear conditioning (Vidal-Gonzalez et al., 2006) but produced anxiety-like behavior in the EPM (Bi et al., 2013). Moreover, inhibition of the IL impaired the acquisition, consolidation and expression of conditioned fear extinction (Sierra-Mercado et al., 2006, 2011; Corcoran and Quirk, 2007). Inactivation of the IL had an anxiolytic-like effect in the EPM (Bi et al., 2013). Stimulation of the PL increased the occurrence of conditioned fear (Vidal-Gonzalez et al., 2006) and anxiety-like behavior in the EPM (Wang et al., 2015). Inactivation of the PL reduced the expression of conditioned fear (Corcoran and Quirk, 2007) and anxiety-like behavior in the EPM (Wang et al., 2015), but PL blockade did not have any effects on innate fear reactions (Corcoran and Quirk, 2007). Neither stimulation nor inactivation of the PL caused any changes in the extinction of contextual (Laurent and Westbrook, 2009) or auditory (Sierra-Mercado et al., 2011) fear conditioning.

These results suggest that the IL and PL play opposite roles in fear conditioning that might depend on nature (i.e., innate or learned) of the threatening stimulus (Lisboa et al., 2010). Therefore, considering the nature of anxiety as well as the presence of 5-HT_{2A} receptors in the PL and IL cortices, the present study also compared in two other experiments the effects of microinjections of the preferential 5-HT_{2A} receptor antagonist ketanserin in the IL and PL in CHF and CLF rats in both innate (EPM) and learned (contextual fear conditioning) models of anxiety.

MATERIALS AND METHODS

Materials

Animals

The animals that were used in the present study were selectively bred for high (CHF) and low (CLF) contextual fear conditioning according to procedures described in our previous work (Castro-Gomes and Landeira-Fernandez, 2008). Briefly, albino Wistar rats were selectively bred for differences in defensive freezing behavior in a contextual fear-conditioning paradigm. This protocol involved one acquisition session and one test session. During acquisition, each animal was placed in the observation chamber for 8 min. At the end of this period, three unsignaled 0.5 mA, 1 s electric footshocks were delivered with an intershock interval of 20 s. Three minutes after the last footshock, the animal was returned to its home cage. The test session was conducted approximately 24 h after training. This test consisted of placing the animal for 8 min in the same chamber where the three footshocks were delivered the previous day. No footshock or other stimulation occurred during this period. All of the animals were phenotyped before beginning each experiment. The phenotyping procedure consisted of evaluating the amount of conditioned freezing during the test session (Castro-Gomes and Landeira-Fernandez, 2008). The experiments began 2 months after the phenotyping procedure. The first experiment investigated the effects of systemic intraperitoneal ketanserin administration in CHF and CLF animals of the 10th generation. The second experiment investigated the effects of ketanserin injections in the IL in CHF and CLF animals of the 15th generation. The third experiment investigated the effects of ketanserin injections in the PL in CHF and CLF animals of the 20th generation. Male rats from both the CHF and CLF lines were 4–6 months old at the beginning of each of the three experiments in the present study.

All the animals were born and maintained in the colony room of the PUC-Rio Psychology Department at controlled room temperature (24 ± 1°C) and a 12 h/12 h light/dark cycle (lights on 7:00 AM–7:00 PM). They were housed in groups of 3–5 according to their respective lines in polycarbonate cages (18 cm × 31 cm × 38 cm) with food and water available *ad libitum*. All of the behavioral experiments were conducted during the light phase of the light/dark cycle. The animals were handled once daily for 2 min for 5 days before the beginning of each experiment. The experimental procedures were performed in accordance with the guidelines for experimental animal research that were established by the Brazilian Society of Neuroscience and Behavior (SBNeC) and National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Animal handling and the methods of sacrifice were reviewed and approved by the Committee for Animal Care and Use of PUC-Rio (protocol no. 20/2009).

Apparatus

Contextual fear conditioning was performed in four observational chambers (25 cm × 20 cm × 20 cm) inside sound-attenuating boxes. A video camera was mounted on the back of each observational chamber so that the animal's

behavior could be observed on a monitor in an adjacent room. Background noise (78 dB) was supplied by a white-noise generator. The chamber had a grid floor (15 stainless steel rods spaced 1.5 cm apart) connected to a shock generator (0.5 mA, 1 s duration) and scrambler (AVS, SCR04; São Paulo, Brazil). An interface with four channels (Insight, Ribeirão Preto, Brazil) connected the shock generator to a computer, which allowed the experimenter to apply an electric footshock. A digital multimeter was used to calibrate the shock intensities before each experiment. A 5% ammonium hydroxide solution was used to clean the chamber before and after each test.

The EPM was elevated 50 cm above the floor and had two open arms (50 cm × 10 cm, with 1 cm high edges) and two closed arms (50 cm × 10 cm, with 40 cm high walls) arranged so that arms of the same type were opposite each other. All of the arms were connected by an open central area (10 cm × 10 cm). The tests were performed in a room that was illuminated by a 100-W light bulb that was suspended 1.75 m above the central part of the maze. A 20% alcohol solution was used to clean the maze between trials.

Drug

Ketanserin tartrate 97% (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 2% dimethylsulfoxide (DMSO) vehicle solution. Both the systemic (0.5 mg/kg) and central (5 nmol/μl) doses were selected according to our previous work (Motta et al., 1992; de Luca et al., 2003; Oliveira et al., 2007). Rats in the control group received 2% DMSO dissolved in saline (vehicle).

Stereotaxic Surgery and Histology

The rats in Experiments 2 and 3 were implanted with bilateral cannulae in the IL or PL. The rats were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine hydrochloride (ketamine 50®, Holliday-Scott SA) and 10 mg/kg xylazine (Seton®, Calier) and mounted in a stereotaxic instrument with the incisor bar set 3.3 mm below the interaural line. Each rat was bilaterally implanted with stainless steel guide cannulae (outer diameter, 0.7 mm) aimed 0.5 mm above the target area. With bregma as the reference for each plane (Paxinos and Watson, 1986), the coordinates for IL cannula implantation were the following: anterior/posterior, 3.2 mm; lateral, ± 0.4 mm; depth, 4.2 mm. The coordinates for PL cannula implantation were the following: anterior/posterior, 3.2 mm; lateral, ± 0.5 mm; depth, 2.4 mm. The guide cannulae were anchored to the skull by dental acrylic and one stainless steel screw. After implantation, the guide cannulae were sealed with a stainless steel wire to prevent blockage. Immediately after cannula implantation, the animals received 0.1 ml of a combination of antibiotics (benzylpenicillin benzathine, 600,000 UI; benzylpenicillin procaine, 300,000 UI; potassium benzylpenicillin, 300,000 UI; dihydrostreptomycin sulfate, 250 mg; streptomycin sulfate, 250 mg, intramuscular) and an analgesic (flunixin, 50 mg/kg, subcutaneous) to prevent infections and decrease post-surgical pain.

At the end of the experiments, the animals were bilaterally injected with 2 μl of 1% Evan's Blue dye in the IL and PL to verify the accuracy of the injections. The animals were then sacrificed with an overdose of urethane (1.25 g/kg, intraperitoneal; Sigma-Aldrich, St. Louis, MO, USA) and intracardially perfused with 0.9% saline and 4% formalin through the left ventricle. The brains were removed and stored in 10% formalin for at least 2 weeks and then sectioned using a cryostat (CM-1900, Leica, Germany) into 60 μm sections. The injection sites were identified using the rat brain atlas of Paxinos and Watson (1986). Only rats with bilateral cannula sites in the IL or PL were considered for the statistical analysis.

Methods

Experiment 1

The CHF and CLF animals were randomly assigned to the ketanserin (0.5 mg/kg) or vehicle group. Thirty minutes after the intraperitoneal injection, each animal was placed in the center of the EPM facing one of the closed arms. The experimental session lasted 5 min. A highly trained observer who remained blind to the treatment conditions recorded the number of entries into and time spent on the open and closed arms with the aid of computer software. The percentage of open arm entries (100 × open arm entries total arm entries) and percentage of time spent on the open arms (100 × time open/[time open + time closed]) were calculated for each animal as indices of anxiety-like behavior. Based on factor analysis of the rats' behavior in the EPM (File, 1992; Cruz et al., 1994), the absolute number of closed arm entries was interpreted as a reliable index of general locomotor activity.

Experiments 2 and 3

One week after surgery, CHF and CLF animals were randomly assigned to the ketanserin (5 nmol/μl) or vehicle group. Experiment 2 investigated the behavioral effects of ketanserin microinjections in the IL. Experiment 3 investigated the behavioral effects of ketanserin microinjections in the PL. Bilateral infusions were performed through an internal cannula (outer diameter, 0.3 mm) that extended 0.5 mm beyond the guide cannula tip. The cannula was attached to a 10 μl Hamilton syringe via polyethylene-10 tubing. Confirmation of a successful infusion was achieved by monitoring the movement of a small air bubble inside the polyethylene-10 tubing. A volume of 0.5 μl/side was delivered over approximately 2 min through the 10 μl Hamilton syringe, driven by a Harvard syringe pump. Following the infusion, the internal cannula was left in place for an additional 2 min to minimize reflux up the cannula shaft. Previous results from our laboratory demonstrated the effectiveness of these parameters when ketanserin was injected locally into brain structures (Motta et al., 1992; de Luca et al., 2003; Oliveira et al., 2007; Almada et al., 2009). Other results suggest that the drug may diffuse approximately 0.5–1.0 mm from the tip of the infusion cannula (Allen et al., 2008).

Five minutes after the injection, each animal was tested in the EPM. At the end of the EPM test, each animal was returned to the same chamber where contextual fear conditioning occurred to test the duration of freezing as a measure of the conditioned

fear response. The animal stayed there for 8 min with no footshock or other stimulation. A time-sampling procedure was used to assess fear conditioning in response to contextual cues. Every 2 s, a well-trained observer recorded episodes of freezing, which were defined as the total absence of movement of the body or vibrissa, with the exception of movement required for respiration.

Statistical Analysis

The results were statistically analyzed by two-way analysis of variance (ANOVA) to detect overall differences. One independent factor was treatment (ketanserin and vehicle), and the other independent factor was rat line (CLF and CHF). Tukey's Honestly Significant Difference test was used for *post hoc* pairwise comparisons between groups. In all cases, values of $p < 0.05$ were considered statistically significant. All analysis were performed with SPSS software.

RESULTS

Systemic Injection of Ketanserin Induced an Anxiolytic-Like Effect in CHF Animals and Anxiogenic-Like Effect in CLF Animals in the Elevated Plus Maze

The number of animals in each experimental condition in this experiment was the following: CLF animals injected with vehicle ($n = 8$), CLF animals injected with ketanserin ($n = 10$), CHF animals injected with vehicle ($n = 8$), and CHF animals injected with ketanserin ($n = 8$). **Figure 1** shows the behavioral effects in CHF and CLF that received systemic injections of ketanserin or vehicle in the EPM. The two-way ANOVA of the percentage of open arm entries (**Figure 1A**) indicated a main effect of rat line ($F_{(1,30)} = 4.71$, $p < 0.05$) and a rat line × treatment interaction ($F_{(1,30)} = 21.66$, $p < 0.01$). No main effect of treatment was found ($F_{(1,30)} = 0.01$, $p > 0.90$). The *post hoc* comparisons revealed that systemic ketanserin administration significantly increased the percentage of open arm entries in CHF animals compared with the vehicle but significantly decreased this measure in CLF animals (both $p < 0.05$).

A similar pattern was observed for the percentage of time spent in the open arms (**Figure 1B**). The two-way ANOVA revealed a main effect of rat line ($F_{(1,30)} = 7.31$, $p < 0.05$) and a rat line × treatment interaction ($F_{(1,30)} = 15.91$, $p < 0.01$). No main effect of treatment was found ($F_{(1,30)} = 0.01$, $p > 0.90$). The *post hoc* comparisons revealed that systemic ketanserin administration significantly increased the percentage of time spent on the open arms in CHF animals compared with vehicle but significantly decreased this measure in CLF animals (both $p < 0.05$).

Figure 1C shows the effects of ketanserin and vehicle on the absolute number of closed arm entries in CHF and CLF animals. No differences were observed among groups. The two-way ANOVA revealed no main effect of rat line or treatment and no interaction between factors (all $p > 0.05$).

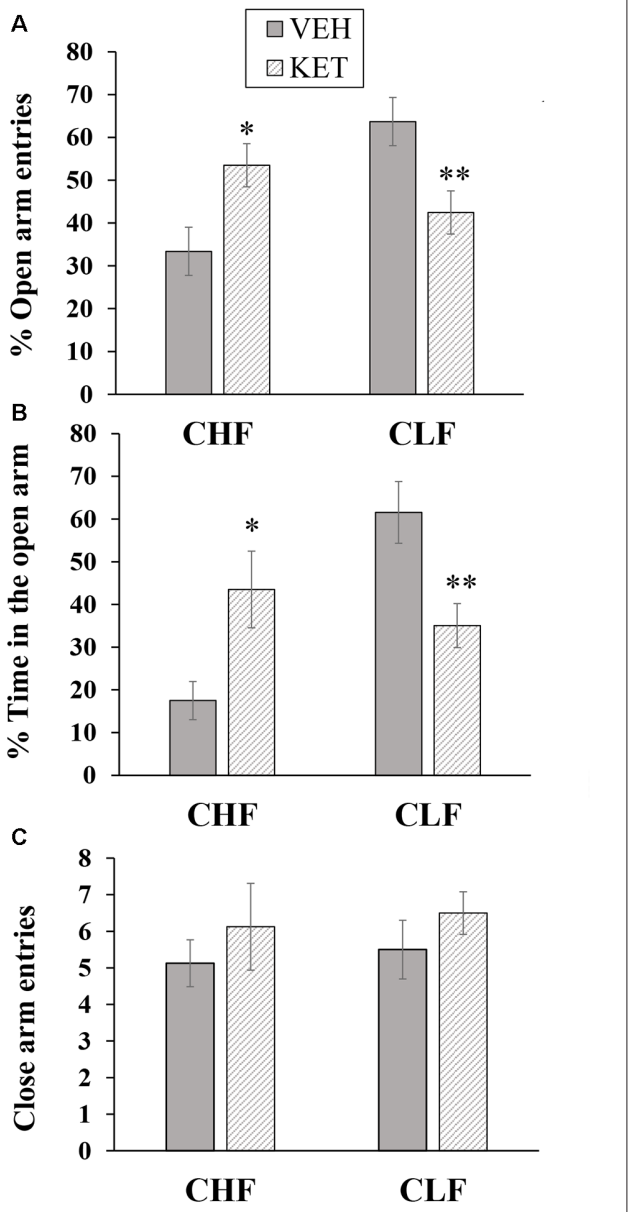

FIGURE 1 | Mean + SEM percentage of open arm entries **(A)**, percent time spent on the open arms **(B)** and closed arm entries **(C)** in the elevated plus maze (EPM) in Carioca High-conditioned Freezing (CHF) and Carioca Low-conditioned Freezing (CLF) animals that received systemic ketanserin (KET) or vehicle (VEH) injections. *$p < 0.05$ KET vs. VEH among CHF animals; **$p < 0.05$ KET vs. VEH among CLF animals.

Intra-IL Injection of Ketanserin Induced an Anxiolytic-Like Effect in CHF Animals and Anxiogenic-Like Effect in CLF Animals in Both the Elevated Plus Maze and Contextual Fear Conditioning Paradigm

Histology

The histological analysis of the cannula placements confirmed that the infusions were made in the IL region in all animals

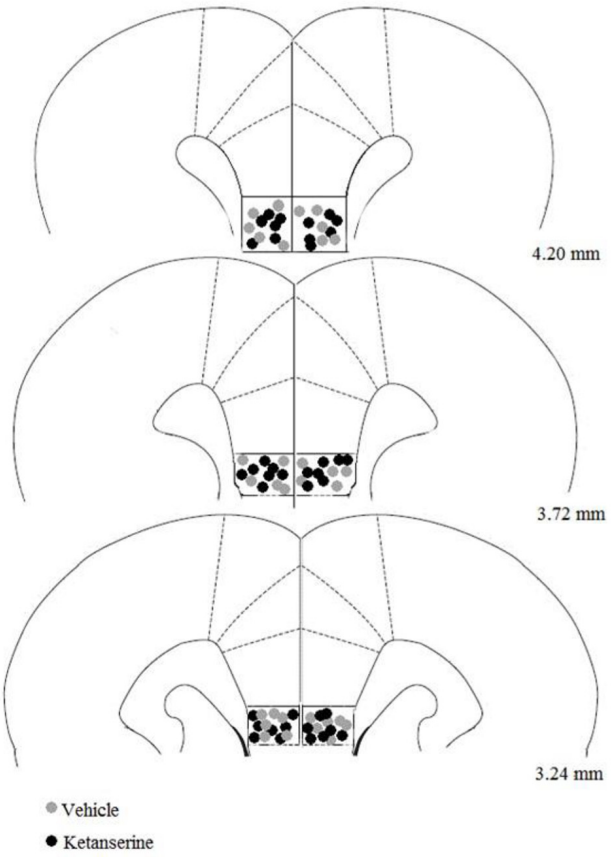

FIGURE 2 | Site of microinjection tips in the infralimbic (IL) cortex. Gray square indicates site of vehicle injection. Black circle indicates site of ketanserin injections. Plates are taken from Paxinos and Watson (1986) and the numbers on the right side of each plate indicate the distance (in millimeters) from bregma.

that were included in the statistical analysis. Four of forty rats in the experiment were excluded because their cannula missed the IL. **Figure 2** shows the bilateral microinjection sites in the IL. The final sample size for each group was the following: CLF animals injected with vehicle ($n = 9$), CLF animals injected with ketanserin ($n = 11$), CHF animals injected with vehicle ($n = 7$), and CHF animals injected with ketanserin ($n = 9$).

Elevated Plus Maze

Figure 3 shows the mean ± SEM percentage of open arm entries, percent time spent on the open arms and closed arm entries in the EPM in CHF and CLF animals that received ketanserin or vehicle microinjections in the IL.

The two-way ANOVA of the percentage of open arm entries (**Figure 3A**) indicated a main effect of rat line ($F_{(1,32)} = 8.81$, $p < 0.05$) and a rat line × treatment interaction ($F_{(1,32)} = 15.61$, $p < 0.01$). No main effect of treatment was found ($F_{(1,32)} = 0.09$, $p > 0.76$). The *post hoc* comparisons revealed that ketanserin microinjections in the IL significantly increased the percentage of open arm entries in CHF animals compared with the vehicle

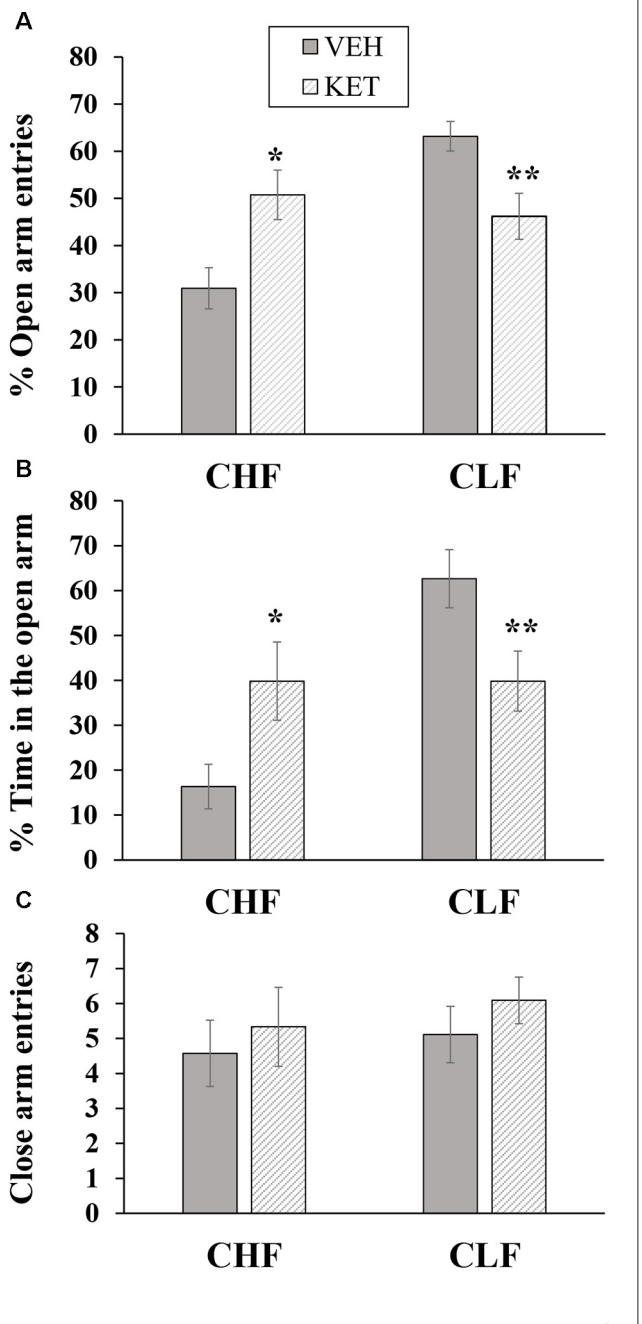

FIGURE 3 | Mean + SEM percentage of open arm entries **(A)**, percent time spent on the open arms **(B)** and closed arm entries **(C)** in the EPM in CHF and CLF animals that received ketanserin (KET) or vehicle (VEH) microinjections in the IL cortex. *$p < 0.05$ KET vs. VEH among CHF animals; **$p < 0.05$ KET vs. VEH among CLF animals.

but significantly decreased this measure in CLF animals (both $p < 0.05$).

The two-way ANOVA of the percentage of time spent on the open arms (**Figure 3B**) revealed a main effect of rat line ($F_{(1,32)} = 10.41$, $p < 0.05$) and a rat line × treatment interaction

($F_{(1,32)} = 10.41$, $p < 0.05$). No main effect of treatment was found ($F_{(1,32)} = 0.01$, $p > 0.90$). The *post hoc* comparisons revealed that ketanserin significantly increased the percentage of time spent on the open arms in CHF animals compared with the vehicle but significantly decreased this measure in CLF animals (both $p < 0.05$).

The two-way ANOVA of the absolute number of closed arm entries in CHF and CLF animals that received microinjections of ketanserin or vehicle (**Figure 3C**) revealed no main effect of rat line or treatment and no interaction between these factors (all $p > 0.05$).

Contextual Fear Conditioning

Figure 4 shows the mean ± SEM percentage of time spent freezing in CHF and CLF animals that received microinjections of ketanserin or vehicle in the IL. The two-way ANOVA indicated main effects of rat line ($F_{(1,32)} = 69.12$, $p < 0.01$), treatment ($F_{(1,32)} = 70.61$, $p < 0.01$) and a significant rat line × treatment interaction ($F_{(1,32)} = 66.15$, $p < 0.01$). The *post hoc* comparisons indicated that ketanserin microinjections in the IL significantly decreased the percentage of time spent freezing in CHF animals compared with the vehicle but significantly increased this measure in CLF animals (both $p < 0.05$).

Intra-PL Injection of Ketanserin Induced an Anxiolytic-Like Effect in CHF Animals but no Effect in CLF Animals in Both the Elevated Plus Maze and Contextual Fear Conditioning Paradigm

Histology

Figure 5 shows coronal sections of the injection sites in the PL. The injections were distributed throughout the entire rostral-caudal extent of the target area within the PL. Three of thirty-nine rats in the experiment were excluded because their cannula missed the PL. The final sample size for each group was the following: CLF animals injected with vehicle ($n = 10$), CLF animals injected with ketanserin ($n = 9$), CHF animals injected with vehicle ($n = 8$) and CHF animals injected with ketanserin ($n = 9$).

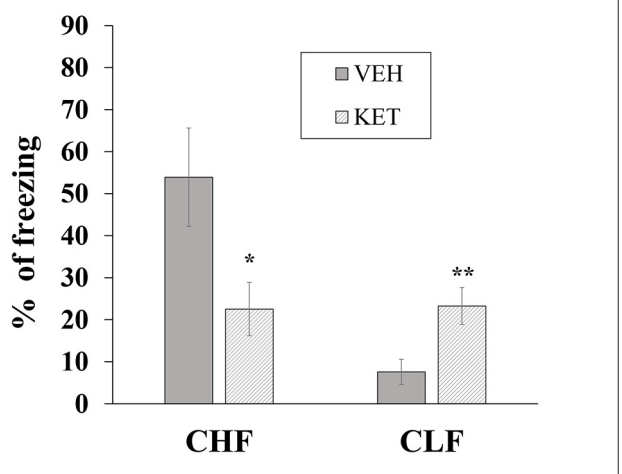

FIGURE 4 | Mean + SEM percentage of time spent freezing in CHF and CLF animals that received ketanserin (KET) or vehicle (VEH) microinjections in the IL cortex. *$p < 0.05$ KET vs. VEH among CHF animals; **$p < 0.05$ KET vs. VEH among CLF animals.

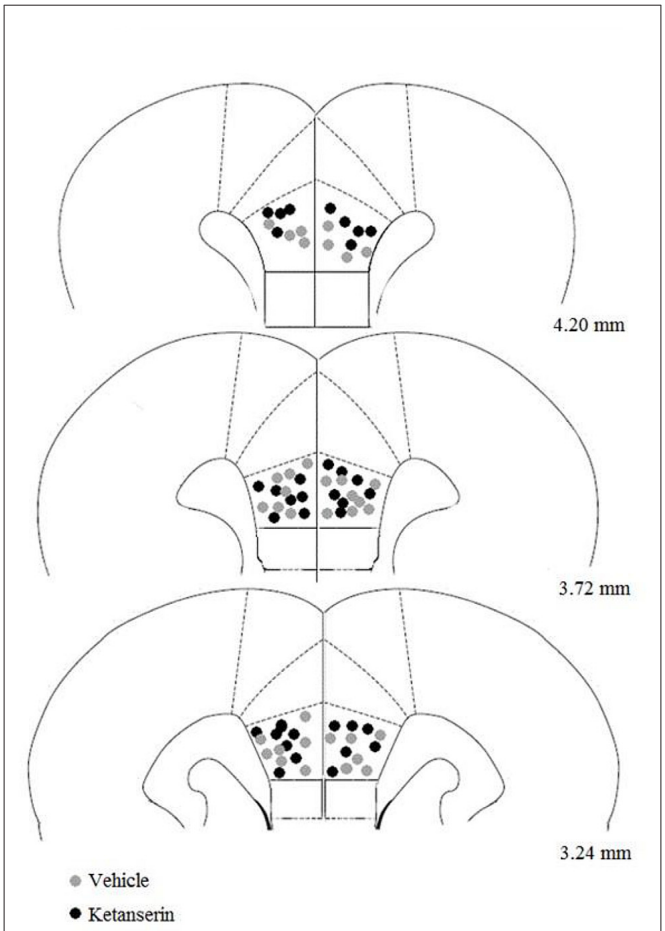

FIGURE 5 | Site of microinjection tips in the prelimbic (PL) cortex. Gray square indicates site of vehicle injection. Black circle indicates site of ketanserin injections. Plates are taken from Paxinos and Watson (1986) and the numbers on the right side of each plate indicate the distance (in millimeters) from bregma.

Elevated Plus Maze

Figure 6 shows the mean ± SEM percentage of open arm entries, percent time spent on the open arms and closed arm entries in the EPM in CHF and CLF animals that received microinjections of ketanserin or vehicle in the PL.

The two-way ANOVA of the percentage of open arm entries (**Figure 6A**) revealed a main effect of treatment ($F_{(1,32)} = 11.01$, $p < 0.05$) and a rat line × treatment interaction ($F_{(1,32)} = 7.32$, $p < 0.01$). No main effect of treatment was found ($F_{(1,32)} = 3.81$, $p > 0.05$). The *post hoc* comparisons revealed that ketanserin microinjections in the PL significantly increased the percentage of open arm entries in CHF animals compared with vehicle

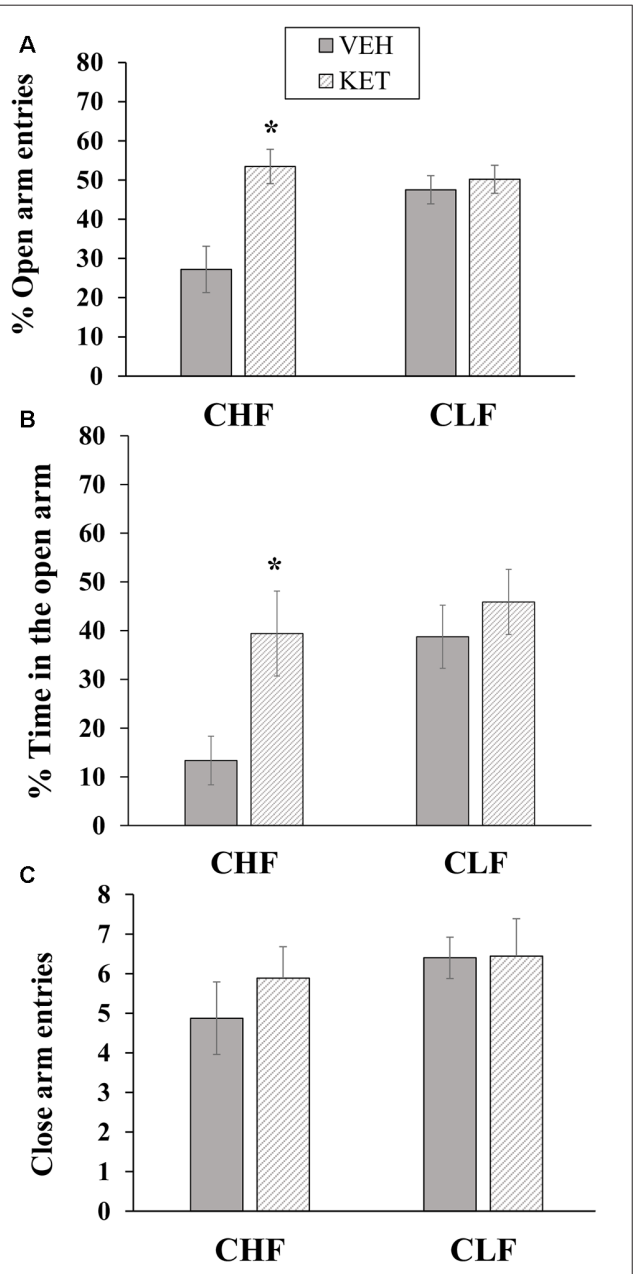

FIGURE 6 | Mean + SEM percentage of open arm entries **(A)**, percent time spent on the open arms **(B)** and closed arm entries **(C)** in the EPM in CHF and CLF animals that received ketanserin (KET) or vehicle (VEH) microinjections in the PL cortex. *$p < 0.05$ KET vs. VEH among CHF animals.

($p < 0.05$) but had no effect on this measure in CLF animals ($p > 0.05$).

The two-way ANOVA of the percentage of time spent on the open arms (**Figure 6B**) revealed main effects of treatment ($F_{(1,32)} = 6.47$, $p < 0.05$) and rat line ($F_{(1,32)} = 5.98$, $p < 0.05$). No interaction between rat line and treatment was found ($F_{(1,32)} = 2.10$, $p > 0.15$).

Figure 6C shows the absolute number of closed arm entries in the EPM maze in CHF and CLF animals that received

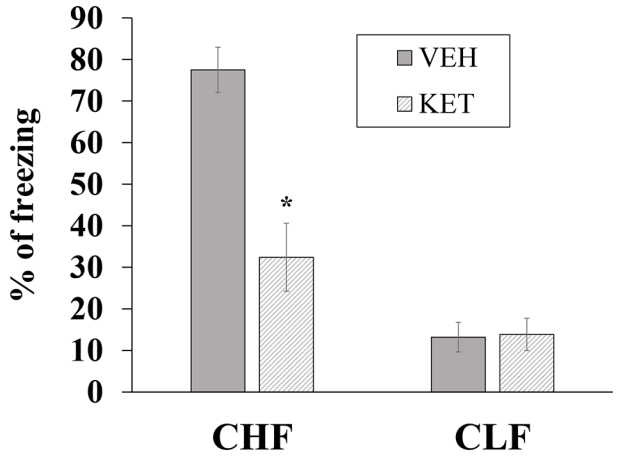

FIGURE 7 | Mean + SEM percentage of time spent freezing in CHF and CLF animals that received ketanserin (KET) or vehicle (VEH) microinjections in the PL cortex. *$p < 0.05$ KET vs. VEH among CHF animals.

microinjections of ketanserin or vehicle. The two-way ANOVA revealed no main effect of rat line or treatment and no interaction between these factors (all $p > 0.05$).

Contextual Fear Conditioning

Figure 7 shows the mean ± SEM percentage of time spent freezing in CHF and CLF animals that received microinjections of ketanserin or vehicle in the PL. The two-way ANOVA indicated main effects of rat line ($F_{(1,32)} = 26.02$, $p < 0.01$), treatment ($F_{(1,32)} = 27.30$, $p < 0.01$) and a significant rat line × treatment interaction ($F_{(1,32)} = 28.42$, $p < 0.01$). The *post hoc* comparisons indicated that ketanserin microinjections in the PL significantly decreased conditioned freezing in CHF animals compared with vehicle ($p < 0.05$) but had no effect in CLF animals ($p > 0.05$).

DISCUSSION

Although there are several animal models of anxiety, very few have highlighted individual differences in the vulnerability to threatening situations as an important variable for studying behavioral responses to pharmacological compounds (Steimer, 2011; Beckers et al., 2013). Selectively bred models of trait anxiety represent an important tool for investigating neural pathophysiological mechanisms associated with anxiety disorders (Castro-Gomes et al., 2013). The present study evaluated the behavioral response to the preferential 5-HT_{2A} receptor antagonist ketanserin in two lines of animals that were selectively bred for high (CHF) and low (CLF) freezing responses to contextual cues that were previously associated with footshock.

The present results from these two lines of animals from the 10th, 15th and 20th generations consistently showed that CHF rats displayed a significantly more "anxious" phenotype, reflected by open arm parameters in the EPM compared with CLF animals. No differences were found between CHF and CLF rats in the number of closed arm entries, suggesting that

the anxiety-like profile of CHF animals was not attributable to locomotor impairment but rather to increases in aversion to the open arms. These behavioral differences between CHF and CLF rats in the EPM are consistent with previous studies from our laboratory and indicate that the conditioned freezing parameter that is used for our breeding program remained stable in a different threatening situation across different generations (Hassan et al., 2013, 2015; Dias et al., 2014; Mousovich-Neto et al., 2015).

Importantly, a biological phenomenon known as genetic drift (Falconer and Mackay, 1996) might represent a confounding factor in our breeding program, in which allele frequencies significantly increase or decrease, possibly leading to differential fixation of the alleles in the CHF and CFL lines as a result of the small size of the two populations. Nevertheless, the present results and past findings from our group strongly indicate that our artificial selection program has indeed resulted in contrasting phenotypes between our two lines that are not attributable to random genetic effects, which cannot be entirely excluded in any evolutionary process that involves small breeding groups.

Systemic injections of ketanserin induced opposite effects in these two lines of rats when the animals were evaluated in the same animal model of anxiety. Ketanserin administration in CHF animals increased both the percentage of entries into and time spent on the open arms of the EPM, without changing the absolute number of closed arm entries. This indicates a selective anxiolytic-like effect without locomotor interference in this test. Conversely, CLF animals that received systemic ketanserin injections exhibited a behavioral pattern suggestive of an anxiogenic-like action in the EPM, reflected by a decrease in open arm exploration and no changes in closed arm entries. These results are consistent with a previous study that reported a bidirectional effect of systemic ketanserin administration in the EPM in female rats with different basal levels of anxiety associated with hormonal states (Díaz-Véliz et al., 1997). Ketanserin produced an anxiogenic-like effect in low-anxiety females that had high estrogen levels (proestrus) but produced an anxiolytic-like effect in high-anxiety females that had low estrogen levels (diestrus). León et al. (2009) showed that methylenedioxymethamphetamine and fluoxetine administration also had opposite effects, depending on whether the subjects were pre-exposed to chronic mild stress, suggesting differential effects of the drug that depended on basal conditions (León et al., 2009). Therefore, differences in baseline levels of anxiety appear to be important for determining the behavioral effects of serotonergic manipulations in the EPM. We previously reported in the rat EPM that the anxiogenic-like effects of the 5-HT_2 receptor agonist TFMPP administered systemically were prevented by intra-amygdala infusions of the mixed 5-$HT_{2A/2C}$-receptor antagonist ritanserin, which does not affect basal levels of anxiety in this animal model (de Mello Cruz et al., 2005).

The behavioral effects of pharmacological interventions are a dynamic process that involves both environmental and genetic factors. The present results highlight the importance of considering the underlying heterogeneity of a given experimental animal population. This is particularly important in basic research that investigates the role of serotonergic activity in anxiety. Conflicting results might be found because most experiments use very heterogeneous populations with considerable variations in anxiolytic-like responses to the same threatening situation. Ignoring the impact of individual differences in behavioral pharmacology research that is performed with heterogeneous populations of animals might mask behavioral effects due to an average result across different levels of defensive responses that these animals might present to cope with a threatening situation (Veenema et al., 2004; Beerling et al., 2011; Castro et al., 2012; Duclot and Kabbaj, 2013). Human and animal studies have shown individual differences in ways of coping with environmental challenges (Blanchard et al., 2001; de Kloet et al., 2005; Bardi et al., 2012; Metna-Laurent et al., 2012; Coppens et al., 2013). Using similar populations, some individuals display higher vulnerability to the development of anxiety-related disorders when faced with threatening situations, whereas other individuals seem to be more resilient to the development these types of pathologies (Bolger and Zuckerman, 1995; Hammen, 2005; Aisa et al., 2008; Uchida et al., 2008; Sandi and Richter-Levin, 2009; Oitzl et al., 2010). Therefore, individual variability needs to be taken into account in animal models of anxiety. Using animal lines that express an anxious-like phenotype is an important methodology for investigating the ways in which the underlying neuropharmacology might contribute to the observed behavioral differences.

The present study also investigated the participation of 5-HT_2 receptors in the IL and the PL subregions of the vmPFC in CHF and CLF animals in the EPM and contextual fear conditioning paradigm. Intra-IL acute infusions of ketanserin induced the same bidirectional behavioral effects in the EPM as systemic injections (i.e., an anxiolytic-like effect in CHF rats but an anxiogenic-like effect in CLF rats), and these effects extended to contextual fear conditioning. Intra-PL acute infusions of ketanserin also had an anxiolytic-like effect in CHF animals but no effect in CLF rats in both models of anxiety. These results suggest that participation of the IL and PL on anxiety-like behavior might not depend solely on the nature (i.e., innate or learned) of the threatening stimulus, as suggested by Corcoran and Quirk (2007) and Sierra-Mercado et al. (2006), but rather on the genetic vulnerability of specific animals to the threatening situation. Moreover, ketanserin injections in either the IL or PL produced the same anxiolytic-like effect in CHF animals. Behavioral differences between ketanserin injections in the IL and PL were only observed in CLF animals, in which an anxiogenic-like effect was only observed when it was injected in the IL and not in the PL.

The IL sends descending projection to the basolateral complex of the amygdala (Likhtik et al., 2014). These projections seem to play an important role in the extinction of conditioned fear (McDonald, 1998; Vertes, 2004). Inactivation of the IL impaired the consolidation and retrieval of extinction of fear conditioning (Quirk et al., 2000; Laurent and Westbrook, 2009). The present study only found an anxiogenic-like effect in CLF rats when the IL region was microinjected with ketanserin. Surprisingly, CHF animals displayed an

anxiolytic-like response to microinjection of the same 5-HT_2 antagonist. This could be related to differences in 5-HT_2 receptor expression in this subregion of the vmPFC in both lines of animals. 5-HT_2 receptor downregulation after chronic treatment with antidepressants (Peroutka and Snyder, 1980) and serotonin receptor agonists has been reported (Conn and Sanders-Bush, 1986; Leysen et al., 1987a,b; Smith et al., 1999).

Neurons in the PL play an excitatory role in conditioned fear behavior. This excitatory effect appears also to be mediated by descending projections to the basolateral complex of the amygdala (Likhtik et al., 2014). Previous results indicated that pharmacological inhibition of the PL reduced the expression of conditioned fear (Corcoran and Quirk, 2007). Furthermore, immunohistochemistry indicated that the PL exhibits greater activation when animals are reexposed to contextual cues that were previously associated with footshock (Lemos et al., 2010). Our results in CHF animals are consistent with these previous studies and highlight the participation of postsynaptic 5-HT_{2A} receptors in the PL in contextual fear conditioning. However, ketanserin did not induce any reduction in conditioned fear among CLF animals, which might be attributable to a floor effect because the animals that received vehicle injections already had very low levels of the freezing response.

Importantly, the present study has several limitations that need to be considered when interpreting the findings. One of the limitations refers to the selectivity of ketanserin. Although this drug has been extensively used as a pharmacological tool to efficiently block 5-HT_{2A} receptors (Kristiansen and Dahl, 1996; López-Giménez et al., 1997; Knight et al., 2004), it also has high binding affinity for both histamine H_1 (Wouters et al., 1985; Ghoneim et al., 2006) and α_1-adrenergic (Hoyer et al., 1987; Israilova et al., 2002) receptors. Thus, the participation of vmPFC 5-HT_{2A} receptors in bidirectional behavioral changes in CHF and CLF rats should not be considered in isolation. Although beyond the scope of the present study, consideration should be given to the possible influence of H_1 and α_1-adrenergic receptors in the present results. Future studies should further investigate the involvement of 5-HT_{2A} receptors in the vmPFC in CHF and CLF animals.

AUTHOR CONTRIBUTIONS

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and drafting the work or revising it critically for important intellectual content; and final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Impaired Latent Inhibition in GDNF-Deficient Mice Exposed to Chronic Stress

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Increased reactivity to stress is maladaptive and linked to abnormal behaviors and psychopathology. Chronic unpredictable stress (CUS) alters catecholaminergic neurotransmission and remodels neuronal circuits involved in learning, attention and decision making. Glial-derived neurotrophic factor (GDNF) is essential for the physiology and survival of dopaminergic neurons in substantia nigra and of noradrenergic neurons in the locus coeruleus. Up-regulation of GDNF expression during stress is linked to resilience; on the other hand, the inability to up-regulate GDNF in response to stress, as a result of either genetic or epigenetic modifications, induces behavioral alterations. For example, GDNF-deficient mice exposed to chronic stress exhibit alterations of executive function, such as increased temporal discounting. Here we investigated the effects of CUS on latent inhibition (LI), a measure of selective attention and learning, in GDNF-heterozygous (HET) mice and their wild-type (WT) littermate controls. No differences in LI were found between GDNF HET and WT mice under baseline experimental conditions. However, following CUS, GDNF-deficient mice failed to express LI. Moreover, stressed GDNF-HET mice, but not their WT controls, showed decreased neuronal activation (number of c-Fos positive neurons) in the nucleus accumbens shell and increased activation in the nucleus accumbens core, both key regions in the expression of LI. Our results add LI to the list of behaviors affected by chronic stress and support a role for GDNF deficits in stress-induced pathological behaviors relevant to schizophrenia and other psychiatric disorders.

Keywords: c-Fos, chronic stress, glial-derived neurotrophic factor, latent inhibition, nucleus accumbens, schizophrenia

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INTRODUCTION

Stress initiates integrated organismal responses, ranging from biochemical, endocrine and immune processes to behavioral alterations, in order to adapt and ensure the survival of the individual. Acute stress usually induces adaptive time-limited responses, i.e., rapid detection of threat through reallocation of resources to a network promoting vigilance, at the cost of the executive network, adequate fight-or-flight responses and restoration of homeostasis when threats are no longer present. On the other hand, persistent changes resulting from long-term chronic stress can have deleterious implications for the health and survival of the organism (De Kloet et al., 2005; Pardon and Marsden, 2008; Herman, 2013).

Cognitive dysfunction is a hallmark of chronic stress in both humans (Lupien et al., 2007; Marin et al., 2011) and experimental animals (Holmes and Wellman, 2009; Moreira et al., 2016). In rodents, chronic stress impairs performance in spatial learning and memory tasks, novel object

recognition (for review see Moreira et al., 2016), behavioral flexibility (Hurtubise and Howland, 2017; Jett et al., 2017) and decision making (Dias-Ferreira et al., 2009; Buhusi et al., 2016).

The biological underpinnings of stress-induced behavioral modifications are related to the effects of stress hormones (CRF, glucocorticoids) and changes in neurotransmission (Linthorst and Reul, 2008; Joels and Baram, 2009; Herman, 2013). Chronic stress is associated with impaired glutamatergic neurotransmission (Jett et al., 2017) and altered inhibitory GABA responses in the prefrontal cortex (McKlveen et al., 2016), as well as changes in *dopamine* (DA; Ahmad et al., 2010; Belujon and Grace, 2015) and norepinephrine release (Arnsten, 2011; Jett and Morilak, 2013).

Two recent studies (Uchida et al., 2011; Bian et al., 2012) identified a major role for the *Glial-derived neurotrophic factor* (GDNF) in response to *chronic unpredictable stress* (CUS): Increased GDNF expression in the *nucleus accumbens* (Acb) and the hippocampus promotes resilience and recovery from CUS. Instead, individuals who cannot up-regulate GDNF during stress exhibit anxiety, anhedonia and avoidance of social interactions, possibly due to the negative consequences of chronic stress on the DA circuits. GDNF, a neurotrophic factor, is particularly important for the physiology of catecholaminergic neurons. GDNF and its receptors are required for neuronal development (Strömberg et al., 1993; Burke, 2006), the expression of Tyrosine Hydroxylase (the enzyme required for catecholamine synthesis; Beck et al., 1996) and the DA Transporter (required for high affinity DA uptake; Lin et al., 1993), the survival of DA neurons in the substantia nigra (Granholm et al., 2000; Boger et al., 2006; Pascual et al., 2008; Zaman et al., 2008), and the survival of noradrenergic neurons in the locus coeruleus (Zaman et al., 2003; Quintero et al., 2004; Pascual et al., 2011). Acb-derived GDNF is a retrograde enhancer of DA tone in the mesocorticolimbic system (Wang et al., 2010). Several lines of genetically engineered mice have been developed to explore the role of GDNF and its receptors in DA neuron development and survival (Pichel et al., 1996; Kramer et al., 2007; Pascual et al., 2008).

A recent study (Knapman et al., 2010) revealed that mice highly reactive to stress exhibit reversal learning and *latent inhibition* (LI) deficits. LI is defined as the loss of future associability by a stimulus that has been repeatedly presented without consequence (Lubow and Moore, 1959). LI results in slower learning of a new *conditioned stimulus* (CS)—*unconditioned stimulus* (US) association, when the *pre-exposed* (PE) stimulus is afterwards presented with consequences.

Given that LI is a process highly dependent on DA (Young et al., 2005; Weiner and Arad, 2009; Arad and Weiner, 2010), which in turn is regulated by GDNF levels, here we tested the hypothesis that stressed *GDNF-deficient* (*heterozygous*, HET) mice would be less able to increase levels of GDNF (due to having a single functional allele, Griffin et al., 2006) than their *wild-type* (WT) littermates, with negative consequences on DA function and deficits in LI. We also comparatively evaluated neuronal activation (c-Fos+ cell counts) in brain regions known to be important for LI expression—Acb and ventral hippocampus (vHipp)—in GDNF HET mice and their WT littermates.

MATERIALS AND METHODS

Subjects

The subjects were 52 3–4 month-old male GDNF-deficient (HET, $n = 26$) mice and their WT ($n = 26$) littermate controls from a GDNF colony (Granholm et al., 1997) maintained on C57BL/6J background for at least 10 generations. Genotypes were confirmed by PCR amplification from tail biopsy samples. The mice were further divided into *Stress* (S, $n = 26$) and *No-Stress* (NS, $n = 26$) groups. Mice were housed in a temperature-controlled room under a 12-h light-dark cycle. Mice were maintained at 85% of their *ad libitum* weights by restricting access to food (Teklad Diet 8604, Envigo, Denver, CO, USA). All experimental procedures were conducted in accordance with the standards for the ethical treatment and approved by Utah State University IACUC Committee.

Chronic Unpredictable Stress (CUS)

Stress mice received 21 days of CUS as in Dias-Ferreira et al. (2009), using the following daily randomly-chosen stressors: 30 min restraint, 10 min forced swim, or 10 min exposure to an aggressive Balb/c male mouse. We have chosen this 3-week CUS protocol since stressed C57Bl/6J mice seem to be resilient to this CUS (e.g., Buhusi et al., 2016), and the aim was to comparatively evaluate GDNF-deficient mice relative to their WT littermates. Please note that when exposed to a longer (8-week), more complex CUS protocol (Monteiro et al., 2015), C57Bl/6J mice do show changes in anxiety, depressive-like and exploratory behaviors.

Apparatus

The apparatus consisted in eight standard mouse operant chambers housed inside sound-attenuating cubicles (Med Associates, St. Albans, VT, USA) equipped with a house light, a fan, two nosepokes on the front wall and one nosepoke on the back wall, a programmable audio generator, a shocker/scrambler module, a lever, and a standard mouse 20-mg pellet feeder. The *pre-exposed* (PE) and *non-pre-exposed* (NPE) conditioned stimuli were a 80-dB tone and a 10-Hz click. The US was a 1-s 0.5 mA footshock.

Latent Inhibition (LI)

LI was assessed using an "on baseline" *conditioned emotional response* (CER) procedure consisting of baseline, pre-exposure, conditioning, rebaseline and test phases (i.e., allowing the mouse to eat during the all stages of the LI paradigm; Buhusi et al., 2017). Mice were assigned either to a PE tone/NPE click or PE click/NPE tone in a counterbalanced manner. Mice were shaped to nosepoke for food pellets on an FR1 schedule throughout the LI task, which consisted of four daily sessions as follows: During the 60-min pre-exposure session mice received 40 30-s presentations of the PE stimulus separated by a 60-s *inter-stimulus interval* (ISI). During the 30-min conditioning session, the PE and NPE stimuli were presented for 30 s three times, separated by a 240-s ISI. The last presentation of the PE and NPE stimuli was paired with a 1-s, 0.5-mA footshock.

On the next day mice were given a 60-min rebaseline session during which mice were reinforced for nosepoking on an FR1 schedule. During a 30-min test session, mice were presented with 3-min PE and NPE stimuli with an 8-min ISI. Mouse behavior was video recorded and the duration of freezing behavior was estimated using FreezeScan software (CleverSys Inc., Reston, VA, USA; Buhusi et al., 2017).

c-Fos Immunostaining

To evaluate neuronal activation, we performed c-Fos immunostaining using standard procedures (Buhusi et al., 2016). Two hours after the start of the test session 5–9 mice in each group were deeply anesthetized and transcardially perfused with a paraformaldehyde solution (4% in 0.1 M phosphate buffer, pH 7.4). Brains were collected and sectioned on a vibrating microtome (VT1200S, Leica, Germany). Free-floating brain sections (50 μm) were incubated with a blocking and permeabilization solution (5% donkey serum, 0.3% Triton X-100 in PBS) for 2 h and then incubated overnight at 4°C with the c-Fos primary antibody (Cell Signaling Technologies, 1:300 dilution). The sections were rinsed in PBS, 0.1% Tween-20 and incubated for 2 h with Alexa488-conjugated donkey anti rabbit secondary antibody and NeuroTrace 530/615 (Fisher Scientific/Invitrogen, Carlsbad, CA, USA). NeuroTrace neuronal labeling was used to identify the neuroanatomical regions of interest. The sections were rinsed in PBS before mounting with Prolong Gold (Fisher Scientific/Invitrogen, Carlsbad, CA, USA).

Neuronal Activation Analysis

Fluorescence images were acquired on a Zeiss LSM710 laser scanning confocal microscope using appropriate filter sets. Analysis of neuronal activation was performed by counting c-Fos-positive nuclei, in same-size areas in two sections/region of interest/mouse in the following areas of interest: *prelimbic cortex* (PrL: Bregma 2.1–1.54), *ventral hippocampus* (vHipp: Bregma −2.92 to −3.40), *nucleus accumbens shell* (Acb-shell: Bregma 1.78–1.1), and *nucleus accumbens core* (Acb-core: Bregma 1.78–1.10; Franklin and Paxinos, 2008), by two independent observers unaware of genotype. Neuronal activation in each region was averaged over observers and subjected to statistical analyses.

Statistical Analyses

The estimated duration of freezing behavior in the first 60 s of the presentation of the PE and NPE stimuli during the conditioning and test sessions was subjected to mixed ANOVAs with between-subjects variables stress (S, NS) and genotype (HET, WT), and within-subjects variable pre-exposure (PE, NPE), followed by *post hoc* analyses. The latency to freeze (to the context) during the conditioning and test sessions was subjected to mixed ANOVAs with between-subjects variables stress (S, NS) and genotype (HET, WT), and within-subjects variable session (conditioning, test), followed by *post hoc* analyses. The difference in freezing between NPE and PE, the number of rewards and nosepokes during the test session, and the neuronal activation (c-Fos+ cell counts in each brain region) were subjected to two-way ANOVAs with factors stress (S, NS) and genotype (HET, WT), followed by LSD *post hoc* analyses. To further explore data, results were collapsed over stress and/or genotype (to yield larger groups), and correlational analyses were conducted between LI (the difference in freezing to the NPE and PE stimuli) and neuronal activation (c-Fos+ cell counts) for Acb-shell and Acb-core. All statistical analyses were conducted at an alpha level 0.05.

RESULTS

Latent Inhibition

The average freezing duration during the PE and NPE stimuli during the test session is shown in **Figure 1**. Analyses

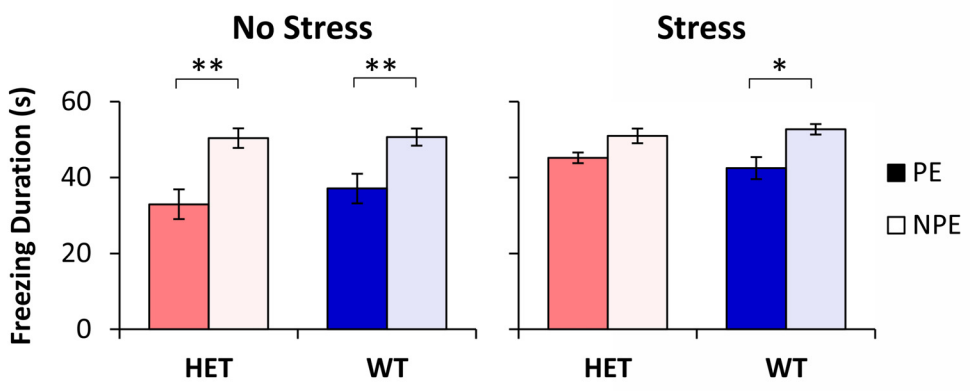

FIGURE 1 | Latent inhibition (LI) by stress and genotype. Average duration of freezing (±SEM) to the pre-exposed (PE) and non-pre-exposed (NPE) stimulus in glial-derived neurotrophic factor (GDNF) heterozygotes (HET) and wild type (WT) littermate controls under no-stress (left) and chronic unpredictable stress (CUS; right). A significant LI (significantly larger freezing to NPE than PE) was observed in all groups except in stressed GDNF HET mice. *$p < 0.05$; **$p < 0.01$.

indicated a main effect of pre-exposure ($F_{(1,48)}$ = 52.96, $p < 0.01$), suggesting that mice froze longer during the NPE stimulus than during the PE stimulus (LI). However, LI was not expressed equally in all groups: Analyses indicated a significant main effect of stress ($F_{(1,48)}$ = 5.51, $p < 0.05$), suggesting that NS mice showed more LI than S mice. Furthermore, analyses indicated a significant pre-exposure × stress interaction ($F_{(1,48)}$ = 5.34, $p < 0.05$), suggesting that stress increased freezing to the PE stimulus, but not to the NPE stimulus. A *post hoc* LSD test indicated a significant difference in freezing between NPE and PE in all NS mice (all ps < 0.01), and in the stressed WT mice ($p < 0.05$), but not in stressed GDNF HET mice ($p > 0.05$), indicating that all mice showed LI except stressed GDNF HET mice.

Analyses of LI, the difference in freezing between NPE to PE, supported the above results: a factorial ANOVA with factors stress and genotype indicated a significant main effect of stress ($F_{(1,48)}$ = 5.34, $p < 0.05$). LI was large in NS mice (13.5 ± 3.9 s in WTs, and 17.4 ± 3.5 s in HETs), but it decreased in stressed mice (10.3 ± 2.9 s in WTs, and 4.8 ± 2.5 s in HETs). A *post hoc* LSD test indicated that LI did not change with stress in WT mice ($p > 0.05$), but was significantly decreased in stressed GDNF HET mice relative to no-stress mice ($p < 0.05$). Taken together, these results provide support for a "two-hit" model under which environmental factors (stress) potentiate the effect of genotype to reveal the disruption of LI in stressed GDNF HET mice but not in the other groups.

Unconditioned Freezing

To evaluate the hypothesis that the difference in freezing to PE and NPE stimuli in **Figure 1** may be due to the intrinsic (unconditioned) differences in freezing to the two stimuli, we performed analyses of freezing behavior to the PE and NPE stimuli in the conditioning session, before these stimuli were paired with footshock. These analyses failed to indicate any main effects of stimulus (PE/NPE; $F_{(1,48)}$ = 2.07, $p > 0.05$), genotype ($F_{(1,48)}$ = 1.49, $p > 0.05$), stress ($F_{(1,48)}$ = 0.15, $p > 0.05$), or any interactions (all Fs < 1.99, $p > 0.05$), suggesting no differences in unconditioned freezing to the PE and NPE stimuli, irrespective of genotype and stress condition. This result indicates that the differences in freezing between groups in **Figure 1** are not due to differences in unconditioned freezing, but reflect differences in conditioned freezing (associability/learning), thus describing true differences in LI.

Reactivity to Shock

Another possibility is that stressed GDNF HET mice became more reactive to shock than the other groups. To evaluate this hypothesis we followed three lines of evidence: first, a *post hoc* LSD test of the duration of freezing during the test session (see "Latent Inhibition" Section) failed to indicate differences between genotypes in duration of freezing to the NPE stimulus (all ps > 0.05; see **Figure 1**); same analyses also failed to indicate differences in duration of freezing to the NPE stimulus between unstressed and stressed mice for each genotype (all ps > 0.05; see **Figure 1**). Taken together, these analyses suggest that all mice learned similarly about the NPE stimuli, thus making it unlikely that they had different reactivity to shock.

Second, analyses of the latency to freeze in the conditioning session (before exposure to shock) and in the test session (after exposure to shock) failed to indicate any effects of session ($F_{(1,48)}$ = 0.74, $p > 0.05$), genotype ($F_{(1,48)}$ = 2.21, $p > 0.05$), stress ($F_{(1,48)}$ = 2.31, $p > 0.05$), or any interactions (all Fs$_{(1,48)} < 3.27$, all ps > 0.05), suggesting that the propensity to freeze in the given context did not change after exposure to shock, and did not vary with stress and genotype, thus making it unlikely that mice differed in their reactivity to shock.

Finally, analyses of the number of rewards earned, and number of nosepoke responses emitted, during the test session failed to indicate any effects of genotype (all Fs$_{(1,48)} < 0.02$, all ps > 0.05), stress (all Fs$_{(1,48)} < 3.61$, all ps > 0.05), or stress × genotype interaction (all Fs$_{(1,48)} < 0.18$, all ps > 0.05), suggesting that mice responded similarly, and earned food similarly, irrespective of stress and genotype. These results indicate that stressed GDNF HET mice nosepoked and were rewarded similarly with the other mice, thus making it unlikely that the absence of LI in stressed GDNF HET mice is due to these mice being more reactive to shock than the other mice.

In summary, the three lines of evidence indicate that all groups were similar in nosepoking, earning food, learning about the context and learning about the NPE stimulus, suggesting that all groups were similarly reacting to and/or learning about the footshock. Nevertheless, groups differed in their freezing to the PE stimulus (see **Figure 1**): Freezing to the PE stimulus was significantly smaller than freezing to the NPE stimulus in all unstressed mice and in the WT stressed mice, but increased (to levels not significantly different than freezing to the NPE stimulus) only in the stressed GDNF HET mice, indicative of impaired LI.

Neuronal Activation

As previously shown in Sotty et al. (1996), we have assessed neuronal activation during LI through analyses of expression of the immediate early gene c-Fos in brain regions known to be relevant to LI through lesion or pharmacological studies (Yee et al., 1995; Pouzet et al., 2004; Schiller and Weiner, 2004; Gal et al., 2005; Schiller et al., 2006; Ouhaz et al., 2014). **Figure 2A** indicates three different patterns of neuronal activation: first, neuronal activation in vHipp was affected only by stress ($F_{(1,22)}$ = 5.39, $p < 0.05$), but not by genotype ($F_{(1,22)}$ = 0.21, $p > 0.05$), or interactions ($F_{(1,22)}$ = 1.11, $p > 0.05$). Second, Acb-shell was independently affected by stress ($F_{(1,22)}$ = 4.55, $p < 0.05$) and genotype ($F_{(1,22)}$ = 5.06, $p < 0.05$), but not by the stress × genotype interaction ($F_{(1,22)}$ = 1.08, $p > 0.05$). Third, Acb-core activation was not affected by neither stress alone ($F_{(1,23)}$ = 1.14, $p > 0.05$) nor genotype alone ($F_{(1,23)}$ = 0.83, $p > 0.05$), but was significantly affected by a stress × genotype interaction ($F_{(1,23)}$ = 4.80,

$p < 0.05$). A *post hoc* LSD test indicated that Acb-core activation was significantly increased in GDNF-HET mice relative to the other groups ($p < 0.05$). Finally, in the present study, PrL neuronal activation was not affected by either stress, genotype, or their interaction (all $Fs_{(1,27)} < 0.31$, all $ps > 0.05$). These results indicate that various brain regions relevant to LI are differentially affected by stress, genotype, and their interaction, thus supporting a complex "two-hit" stress × genotype model.

To further understand the effect of stress and genotype on these brain regions, we evaluated the relationship between LI (the difference in freezing duration to NPE and PE stimuli) and neuronal activation (number of c-Fos+ cells) in the nuclei that control the behavioral output (Schmajuk et al., 1997; Weiner, 2003), Acb-shell and Acb-core, as shown in **Figures 2B–D**. **Figures 2B,C** shows the relationship between LI and neuronal activation in Acb-shell (**Figure 2B**) and Acb-core (**Figure 2C**) in WT and HET mice when data are collapsed over the stress variable. Consistent with previous studies (Sotty et al., 1996), **Figure 2B** indicates that irrespective of the stress condition, LI correlated with Acb-shell activation in WT mice ($R^2_{(10)} = 0.53$, $p < 0.05$), but not in GDNF HET mice ($R^2_{(12)} = 0.01$, $p > 0.05$). Similarly, **Figure 2C** indicates that irrespective of the stress condition, LI correlated with Acb-core activation in WT controls ($R^2_{(10)} = 0.34$, $p < 0.05$), but not in GDNF HET mice ($R^2_{(13)} = 0.07$, $p > 0.05$). Indeed, as shown in **Figure 2A**, stress determined a decrease in Acb-shell activation and an increase in Acb-core activation in stressed GDNF HET mice, such that stressed GDNF HET mice, but not stressed WT mice, showed impaired LI (**Figure 1**).

Moreover, **Figure 2D** shows that the relationship between LI and Acb-core activation differs in no-stress (NS) and stress (S) mice when data are collapsed over genotype: Irrespective of genotype, LI correlated with Acb-core activation in S mice ($R^2_{(10)} = 0.47$, $p < 0.05$), but not in NS mice ($R^2_{(13)} = 0.01$, $p > 0.05$). Indeed, **Figure 2A** indicates that there was no significant difference in Acb-core activation in S and NS WT, which showed LI (**Figure 1**), while stressed GDNF HET mice showed an increase in Acb-core activation (**Figure 2A**), and failed to show LI (**Figure 1**). In summary, all three patterns in **Figures 2B–D** contributed to the disruption of LI in stressed GDNF HET mice, and to the significant LI in the other mice, as shown in **Figure 1**.

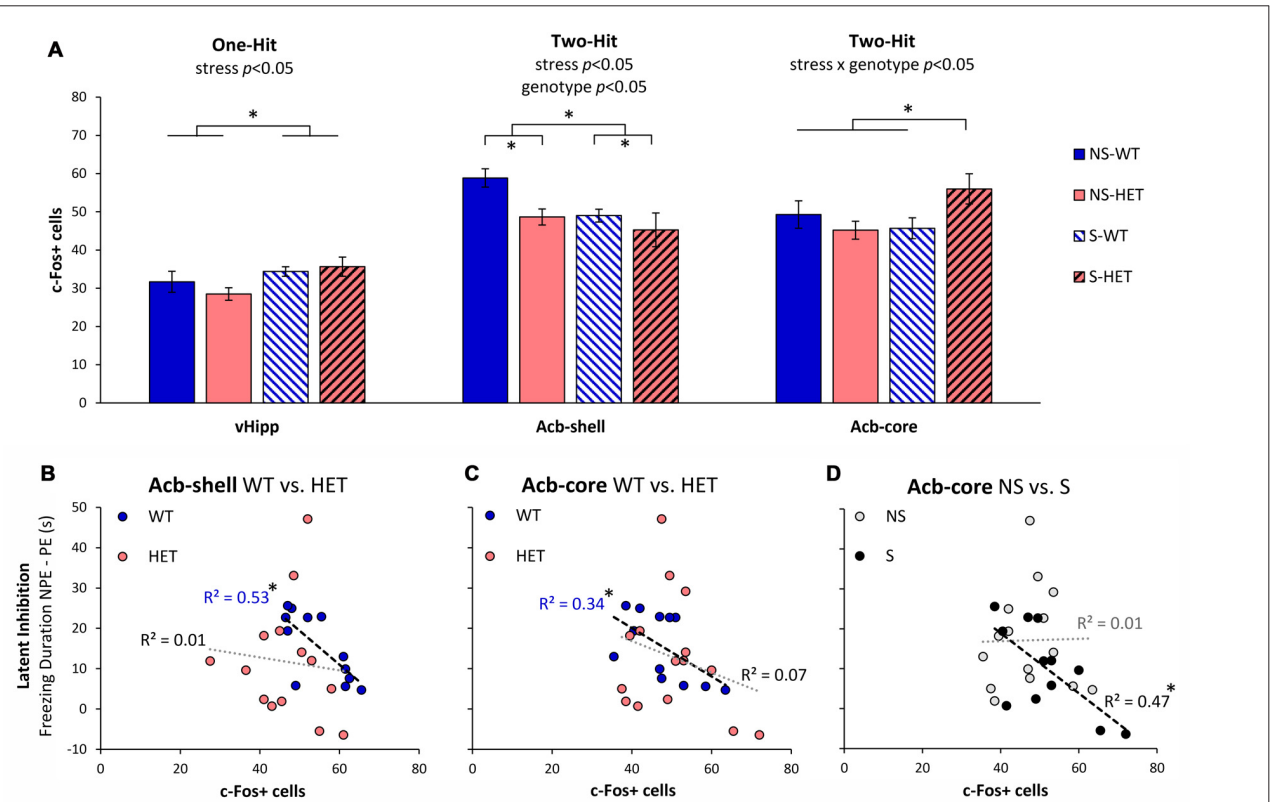

FIGURE 2 | Neuronal activation during LI testing. **(A)** Average c-Fos+ cell counts (±SEM) in ventral hippocampus (vHipp), nucleus accumbens shell (Acb-shell), and nucleus accumbens core (Acb-core) in the stress (S) and no-stress (NS) GDNF-deficient mice (HET) and WT littermate controls. Analyses indicated different patterns of effects of stress and genotype on neuronal activation in these brain regions: vHipp activation was affected only by stress (one-hit), Acb-shell activation was independently affected by stress and genotype (independent two-hit), while Acb-core activation was affected by the interaction stress × genotype (two-hit interaction). **(B–D)** Correlations between LI (difference in freezing duration to the NPE, and PE, stimuli) and neuronal activation (number of c-Fos+ cells) in Acb-shell **(B)** and Acb-core **(C,D)** when data are collapsed across stress **(B,C)** or genotype **(D)**. *$p < 0.05$.

DISCUSSION

Using an "on baseline" within-subject CER LI procedure developed in our lab (Buhusi et al., 2017), the current study found that WT mice showed LI, consistent with previous findings (Gould and Wehner, 1999). Additionally, results indicated that GDNF HET mice in C57BL/6J background showed LI under baseline, no-stress conditions. However, after exposure to a CUS regimen, GDNF HET mice failed to show LI, while WT littermates continued to show LI. These results are unlikely to be due to differences in unconditioned freezing to the two stimuli, or to differences in reactivity to shock, as all mice froze similarly to the two stimuli (before they were paired with shock), learned similarly about the NPE stimulus and context, nosepoked similarly and were rewarded similarly in the FR1 task. Further studies are required to evaluate whether altered LI as a consequence of the stress × GDNF-deficit interaction reflects anomalies in either acquisition or expression of LI.

Neuronal activation analyses (c-Fos+ cell counts) in brain regions involved in LI indicated that in some brain regions activity was affected solely by stress (vHipp), while in others it was affected by both stress and genotype (Acb-shell) or their interaction (Acb-core). Our results revealing the combined effects (including an interaction) of stress and genetic factors on neuronal activation in the Acb support current neurobiological (Weiner, 2003) and neuro-computational models (Schmajuk et al., 1997, 1998; Buhusi et al., 1998) of LI.

Neural Substrates of Latent Inhibition

LI is a phenomenon observed both in humans and other species by which repeated presentation of a neutral stimulus with no consequences reduces its future associability relative to learning about other novel stimuli (Lubow and Moore, 1959; reviewed in Lubow, 1989). Most theories relate LI to selective attention, i.e., during pre-exposure of an inconsequential CS, the animal or participant learns not to attend to it (Pearce and Hall, 1980; Lubow and Gewirtz, 1995; Schmajuk et al., 1997). Weiner and Feldon (1997) suggested a "switching" theory in which the nucleus accumbens plays a major role (see **Figure 3**): during pre-exposure a CS-noUS association is learned (with the involvement of the hippocampus), after which the new CS-US association requires switching controlled by the core of the nucleus accumbens (with the shell having a modulatory role; Weiner, 2003; Gray and Snowden, 2005). According to this theory, LI is disrupted by the change of context between pre-exposure and conditioning (Lubow et al., 1976), suggesting that the hippocampus may be important for detecting the mismatch.

Interestingly, the results of our study support a computational model suggesting that LI is affected by the interaction between environmental stimuli and brain insults (Schmajuk et al., 1997; Buhusi et al., 1998; see **Figure 3**). In this neural network model LI depends on the novelty of the PE and NPE stimuli relative to the context (computed in the VTA and modulating activity in the accumbens), which relies on learned associations between stimuli (which in turn depend on normal hippocampal function). Thus, according to this model, current data could be explained by genetically-induced alterations in brain function combined with environmental factors (e.g., decreased expression of GDNF and inability to up-regulate GDNF expression in the hippocampus during stress), which interact to alter novelty computation and activity in the accumbens, and impair LI in stressed GDNF HET mice.

Although Weiner's "switching model" and Schmajuk's "novelty" model describe activity in the same network (investigated in this article, and shown in **Figure 3**), the interpretation of neuronal activity and its behavioral correlates (**Figure 2** in this article) is very different in the two models: Weiner's model interprets neuronal activity as reflecting changes in switching, while Schmajuk's model interprets neuronal activity as reflecting changes in novelty. Further studies are required to evaluate and differentiate these models, although it is notable that Schmajuk's "novelty" model already incorporates and addresses environment × novelty interactions (Buhusi et al., 1998), thus possibly addressing the data from the current study.

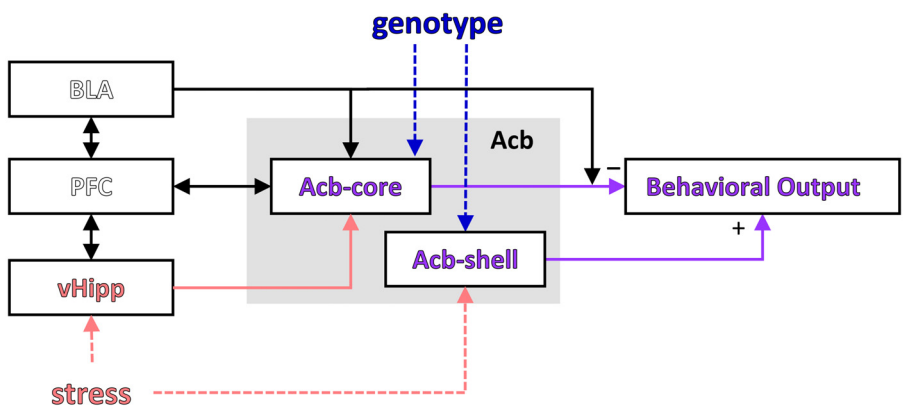

FIGURE 3 | Modulation of a putative LI circuit by stress or the GDNF genotype. A putative circuit for LI (modified after Schmajuk et al., 1997; Weiner, 2003) indicating the brain regions where activity was affected by stress and/or genotype. PFC, prefrontal cortex; vHipp, ventral hippocampus; Acb, nucleus accumbens; Acb-core, nucleus accumbens core; Acb-shell, nucleus accumbens shell.

As suggested by the above theories, multiple studies have shown that the Acb and the hippocampus are indeed key structures in LI acquisition and expression. Lesion studies revealed opposing roles of Acb-shell and core in LI: lesions of the Acb-shell impair LI (Weiner et al., 1999), while lesions of Acb-core or Acb-shell+core are associated with persistent LI (Weiner et al., 1999; Gal et al., 2005). Our results showing that stressed GDNF HET mice which have impaired LI also have decreased c-Fos+ cell counts in the Acb-shell and increased neuronal activation in the Acb-core are consistent with these previous findings.

Hippocampal lesions revealed maintenance of LI, but loss of context specificity of the CR and LI (Good and Honey, 1991; Honey and Good, 1993; Coutureau et al., 1999), however LI is disrupted after ventral hippocampal (vHipp)/ventral subiculum (vSub) NMDA receptor activation (Pouzet et al., 2004; Lodge and Grace, 2008). Our findings that stress increases c-Fos+ cell counts in the ventral hippocampus in the LI procedure also support a role for the increased vHipp activity in the disruption of LI.

The involvement of the prefrontal cortex, which is bi-directionally connected with the hippocampus and amygdala and projects to the Acb (Del Arco and Mora, 2008, 2009; see **Figure 3**), has also been evaluated in relation to LI, with mixed results: excitotoxic lesions of the medial prefrontal cortex did not affect LI (Lacroix et al., 1998), while catecholaminergic depletion within the medial prefrontal cortex enhanced LI (Nelson et al., 2010). In our study, assessment of neuronal activation in the prelimbic cortex (part of the medial prefrontal cortex) during the LI task has revealed no differences between experimental groups, consistent with the Lacroix et al.'s (1998) study. The absence of differences in the prelimbic cortex activation between experimental groups in the LI task further suggests that in our study the changes in neuronal activity were not general, but were rather specific to certain brain areas.

Stress, Latent Inhibition and Schizophrenia

Chronic stress induces changes in gene expression (including an up-regulation of GDNF expression in resilient individuals, see Uchida et al., 2011), and alters neuronal morphology and function in many brain regions, including regions relevant for the acquisition and expression of LI. For example, after stress pyramidal neurons in the cortex and hippocampus exhibit altered dendritic and spine morphology and decreases in spine density (Cook and Wellman, 2004; Maras and Baram, 2012; Leuner and Shors, 2013; McEwen and Morrison, 2013). Interestingly, CUS is associated with increased dendritic complexity in the Acb-core, while decreased dendritic complexity is found in the Acb-shell (Taylor et al., 2014); these results may explain the differences in neuronal activation observed in the two regions of Acb in our study.

Stress also induces alterations in DA neurotransmission, which is particularly important for the acquisition and expression of LI (Young et al., 2005; Weiner and Arad, 2009): Rats exposed to CUS have a decreased DA output in the Acb-shell accompanied by a decrease in the number of DAT binding sites (Scheggi et al., 2002). Indeed, stress was shown to attenuate LI in humans (Braunstein-Bercovitz et al., 2001) or rats (Hellman et al., 1983), although, in some cases, it may potentiate it (Melo et al., 2003). Knapman et al. (2010) reported a reduction in LI in mice highly reactive to stress supporting our own observation that genetic factors are major contributors to vulnerability to stress: in our study only stressed GDNF HET mice, but not stressed WT littermates, failed to show LI.

In vulnerable individuals chronic stress can precipitate psychiatric disorders (Bale, 2006; Deppermann et al., 2014; Nestler et al., 2016), including schizophrenia (Aiello et al., 2012; Holtzman et al., 2012, 2013). SZ is a chronic neuropsychiatric disorder, characterized by delusions, hallucinations, disorganized behavior and speech, and attentional control deficits, symptoms that can lead to severe impairments in adaptive function and social integration (van Os and Kapur, 2009). Interestingly, disrupted LI is an important feature in SZ, particularly in drug-naïve patients or during acute episodes (Baruch et al., 1988; Gray et al., 1995; Williams et al., 1998; Rascle et al., 2001). Abnormal LI in SZ may be explained by patients having selective attention deficits and continuing to attend irrelevant stimuli (Lubow, 1989), having a hyperactive "switching" mechanism (Hemsley, 1993), or a hyperactive novelty system (Schmajuk et al., 1997; Buhusi et al., 1998). Impaired LI is thought to underlie the positive symptoms of SZ (Morris et al., 2013). Drug treatment, using either typical or atypical neuroleptics, leads to normalization of LI (Leumann et al., 2002), possibly by modifying neuronal activation threshold in specific brain areas.

GDNF and Schizophrenia

Several neuropsychiatric diseases are associated with alterations in GDNF expression levels (Rosa et al., 2006; Tseng et al., 2013; Tunca et al., 2015); serum levels of GDNF and other neurotrophic factors are also modified by treatment (Zhang et al., 2008). Interestingly, although serum GDNF levels are decreased in SZ (Tunca et al., 2015), genetic association studies between GDNF and SZ produced mixed results (Lee et al., 2001; Michelato et al., 2004; Williams et al., 2007). However, GDNF-receptor genes GFRA1, GFRA2 and GFRA3 are located in chromosomal regions with suggestive linkage to SZ. A recent study (Souza et al., 2010) found evidence for genetic associations between GFRA1 and 3 and schizophrenia, as well as evidence for GFRA2 variants modulating the therapeutic response to clozapine. Our results support a role for the GDNF signaling pathway and its interaction with stress in the development of abnormal behaviors relevant to SZ and other mental disorders.

CONCLUSION

This study identifies a disruption of LI in stressed GDNF-deficient mice, providing strong evidence for a role of chronic stress in LI alterations in individuals with particular genetic vulnerabilities. The disruption of LI may be the result of small changes in neuronal function or connectivity related to genotype which is potentiated as a result of chronic stress. Our results add LI to the list of behaviors affected by chronic stress and support a role for GDNF deficits in stress-induced

pathological behaviors relevant to schizophrenia and other psychiatric disorders.

AUTHOR CONTRIBUTIONS

MB: experimental design and immunostaining and imaging. CKB and CVB: behavior. MB and CVB: data analysis and wrote the article.

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Lost in Time and Space: States of High Arousal Disrupt Implicit Acquisition of Spatial and Sequential Context Information

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Biased cognition during high arousal states is a relevant phenomenon in a variety of topics: from the development of post-traumatic stress disorders or stress-triggered addictive behaviors to forensic considerations regarding crimes of passion. Recent evidence indicates that arousal modulates the engagement of a hippocampus-based "cognitive" system in favor of a striatum-based "habit" system in learning and memory, promoting a switch from flexible, contextualized to more rigid, reflexive responses. Existing findings appear inconsistent, therefore it is unclear whether and which type of context processing is disrupted by enhanced arousal. In this behavioral study, we investigated such arousal-triggered cognitive-state shifts in human subjects. We validated an arousal induction procedure (three experimental conditions: violent scene, erotic scene, neutral control scene) using pupillometry (Preliminary Experiment, n = 13) and randomly administered this method to healthy young adults to examine whether high arousal states affect performance in two core domains of contextual processing, the acquisition of spatial (spatial discrimination paradigm; Experiment 1, n = 66) and sequence information (learned irrelevance paradigm; Experiment 2, n = 84). In both paradigms, spatial location and sequences were encoded incidentally and both displacements when retrieving spatial position as well as the predictability of the target by a cue in sequence learning changed stepwise. Results showed that both implicit spatial and sequence learning were disrupted during high arousal states, regardless of valence. Compared to the control group, participants in the arousal conditions showed impaired discrimination of spatial positions and abolished learning of associative sequences. Furthermore, Bayesian analyses revealed evidence against the null models. In line with recent models of stress effects on cognition, both experiments provide evidence for decreased engagement of flexible, cognitive systems supporting encoding of context information in active cognition during acute arousal, promoting reduced sensitivity for contextual details. We argue that arousal fosters cognitive

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adaptation towards less demanding, more present-oriented information processing, which prioritizes a current behavioral response set at the cost of contextual cues. This transient state of behavioral perseverance might reduce reliance on context information in unpredictable environments and thus represent an adaptive response in certain situations.

Keywords: arousal, stress, context processing, associative learning, spatial learning, multiple memory systems

INTRODUCTION

Cognitive adaptations during high arousal states play an important role in models of development of psychopathology, e.g., post-traumatic stress disorder or phobias (Acheson et al., 2012; Pitman et al., 2012; de Quervain et al., 2017), shooting decisions during police actions (Nieuwenhuys et al., 2015), implementation of military operations on the battlefield (Lieberman et al., 2016) and forensic considerations in the face of crimes committed in rage (Brookman, 2015). Thus, examining cognitive functioning in humans *during* extreme arousal states has important implications on basic and applied research. Beyond everyday fluctuations of general arousal (Berridge, 2008; Carter et al., 2010; de Lecea et al., 2012; Nielsen and Mather, 2015; Nielsen et al., 2015), extreme increases occur in response to challenging situations (Berridge and Waterhouse, 2003), such as panic or sexual excitement (Calderon et al., 2016), and promote fundamental changes in cognition in response to immediate environmental demands (Shields et al., 2016a). The current study complements existing research on arousal effects on cognition, examining whether states high in arousal alter sensitivity for spatial relations or sequential order, two core aspects of episodic memory formation.

Temporal-spatial context of a past event represents an immanent part of episodic memory formation (Tulving, 1984). Encoding and/or retrieval of both types of context information have been shown to strongly rely on a single hippocampus- and prefrontal cortex-dependent memory system (Rajah et al., 2010a,b, 2011; Kraus et al., 2013; Cabral et al., 2014). This network is supposed to support the construction of a schematic model of a situation based on contextual information (Bar, 2004; Ranganath and Ritchey, 2012), such as spatial and temporal relationships, therefore it is required to form contextualized, detailed representations of events and for allocentric spatial navigation (Squire et al., 2004; Howard and Eichenbaum, 2013; Pezzulo et al., 2014; Davachi and DuBrow, 2015). However, processing of spatial and temporal details is prone to interference by acute stress. At least for long-term memory formation, it has been shown that events encoded immediately after stressful encounters are embedded less in contextual details and lack precision (Schwabe et al., 2009). In addition, encoding during arousal leads to more gist-like memories at the cost of peripheral details (Kensinger et al., 2007). This narrowed focus on the essential core of experiences lacking contextual embeddedness and depth of detail is reflected by an increased rate of false alarms (Payne et al., 2002) and is directly related to autonomic arousal (Qin et al., 2012).

An explanation for these findings is offered by a recent account that builds on evidence showing that acute stress leads to an upregulation of the salience network (Hermans et al., 2011, 2014), which then biases competition of learning and memory between multiple underlying systems (Poldrack and Packard, 2003; Mizumori et al., 2004; Squire and Dede, 2015). Stress produces a shift toward the use of "habit" memory by impairing hippocampus- and probably prefrontal cortex-dependent "cognitive" memory (Arnsten et al., 2012; Packard and Goodman, 2012; Schwabe and Wolf, 2013; Schwabe, 2013, 2016; Gagnon and Wagner, 2016). Existing evidence fits in well with this notion, showing that stressful encounters decrease hippocampal activation (Pruessner et al., 2008; Henckens et al., 2009; Cousijn et al., 2012; Schwabe and Wolf, 2012) and thereby strengthen the dorsal striatum-dependent system (Poldrack and Packard, 2003; Vogel et al., 2015a, 2017), which then supports incremental strengthening of stimulus-response associations (Packard and Knowlton, 2002; Devan et al., 2011).

With a focus on cognitive functioning, stress hijacks active cognition by impairing working memory (Schoofs et al., 2008, 2009; Luethi et al., 2009; Qin et al., 2009), cognitive flexibility (Plessow et al., 2011, 2012; Shields et al., 2016b) and cognitive inhibition (Mahoney et al., 2007; Sänger et al., 2014), but simultaneously enhances behavioral control in terms of response inhibition (Schwabe et al., 2013; Weinbach et al., 2015). At the same time, increases in arousal lead to reduction of the range of cue use by narrowing attention to prioritized cues at the expense of surrounding information (Harmon-Jones et al., 2013; Sakaki et al., 2014; Weinbach and Henik, 2014; Maran et al., 2017). In addition, working memory performance under stress is characterized by a higher rate of false alarms, indicating less specific representations and more liberal responding (Duncko et al., 2009). Schwabe and Wolf (2013) pointedly termed this bias of the relative use of multiple memory systems a shift "from thinking to doing".

Altogether, these findings provide strong evidence for dynamic adjustment of ongoing information processing depending on arousal state, suggesting a switch from contextual "cognitive" to rigid "habit" strategies underlying active cognition, leading to reduced reliance on contextual information. The current study focuses on this suggested consequence of high arousal states on cognitive performance, meaning impaired ability to use contextual cues to inform actual responses. The first aim of this study was to examine how high arousal states affect two core aspects of context processing, the acquisition of spatial (Experiment 1) and sequential (Experiment 2) context information. More specifically, we focused on whether aroused subjects *implicitly* acquire contextual details by assessing how

context information affects performance depending on different arousal states. Although evidence strongly indicates that arousal, regardless of its motivational direction, drives adaptations in cognition (Mather and Sutherland, 2011; Harmon-Jones et al., 2013; Maran et al., 2015), previous work on this topic focused solely on the effect of aversive stressors (e.g., fear). Thus, the second aim of this study was to investigate whether both aversive and appetitive arousal states (i.e., sexual excitement) lead to similar alterations of context acquisition and thereby further support the notion that the tested cognitive adaptations are mainly being driven by variations in arousal rather than emotional valence.

We implemented two experimental designs to measure the implicit acquisition of spatial and sequential context, each with arousal state varying as between-subject condition. Our first prediction proposes that states high in arousal disrupt implicit acquisition of context information, more specifically the use of both spatial and sequential context of ongoing events. Since the moment-to-moment processing of contextual details facilitates task execution in both paradigms described below, impaired performance would support this first hypothesis. Second, we expect that increases in arousal exert their effects regardless of its motivational direction, thus exposure to both aversively and appetitively arousing events should result in the same performance decrements. Evidence showing any differences in arousal-induced performance decrements between the aversive and appetitive states would support this second hypothesis.

Our predictions were tested in two behavioral experiments using established experimental paradigms, assessing implicit acquisition of spatial relations (spatial discrimination paradigm; Marshall et al., 2016; Experiment 1) and predictive sequences (learned irrelevance paradigm; Orosz et al., 2008, 2011; Experiment 2). The elicitation procedure comprised three cinematographic fragments: a social conversation, a violent encounter and sexual intercourse. To ensure the effectiveness of the arousal elicitation method, we conducted a preliminary study to evaluate the procedure by measuring pupillary responses. We implemented the arousal elicitation method between practice and testing, therefore the arousing encounter can be considered as part of the task context (Joëls et al., 2006; Diamond et al., 2007). Since effects of arousal on cognition are strongly time dependent (Joëls and Baram, 2009; Joëls et al., 2011; Henckens et al., 2012), it is noteworthy that we focused on immediate cognitive adaptations after exposure to an arousing event. Thus, both tasks took less than 20 min to perform, well before cortisol secretion peaks in response to an arousing encounter (after about 25 min, Schwabe et al., 2008).

In the following, we first present the Preliminary Experiment which aimed to validate the general arousal elicitation procedure by analyzing tonic pupillary changes and self-report mood states (Preliminary Experiment: Validation of the general arousal elicitation procedure). Second, in Experiment 1 we examined whether states high in arousal affect implicit acquisition of spatial context (Experiment 1: Arousal and spatial context processing) and third, Experiment 2 explored how alterations in arousal impact acquisition of sequence information (Experiment 2: Arousal and sequence acquisition).

PRELIMINARY EXPERIMENT: VALIDATION OF THE GENERAL AROUSAL ELICITATION PROCEDURE

In the following experiment, cinematographic material was validated by analyzing tonic pupillary changes with regard to their ability to induce different states of arousal. Three scenes from existing feature films were chosen, the first scene showing a casual conversation during shopping (control condition), the second scene a highly aversive, violent homicide (aversive arousal) and the last clip romantic, explicitly sexual intercourse (appetitive arousal). Although we investigated effects of arousal referring to these distinct experimental conditions, it should be noted that arousal is a continuous neurobiological function (Pfaff and Banavar, 2007).

The use of emotional clips in order to induce arousal states represents an established and efficient method (e.g., Gabert-Quillen et al., 2015; Samson et al., 2015; Gilman et al., 2017). Since the chosen scenes depict realistic events, they match extremely arousing situations in real life which can be considered as unpredictable and uncontrollable (i.e., involvement in violent encounters or sexual interactions).

Validation of the method used was ensured by comparison of tonic pupil response immediately after stimulus exposure. Pupillary dynamics has been shown to be a reliable, non-invasive indicator of arousal (Bradley et al., 2008), mediating neuromodulatory actions (Hou et al., 2005) and corresponding locus coeruleus recruitment (Rajkowski et al., 1994; Murphy et al., 2014). Furthermore, recent evidence shows that locus coeruleus activity anticipates changes in pupil diameter (Joshi et al., 2016). Thus, since we expect arousal to be the driving force behind the hypothesized disruption of implicit acquisition of context information, capturing differential tonic pupil responses between conditions represents a precise physiological marker to validate the arousal elicitation procedure used for both of the following experimental designs.

To be considered effective to induce a state of enhanced arousal, the scene presenting violent and sexual interactions should induce a larger pupil dilatation compared to the control clip showing an everyday life scene. In addition, since the material should induce arousal states of different valence, i.e., aversive and appetitive, self-reported mood states should reflect specific alterations in negative and positive affect, respectively.

Materials and Methods

Design and Procedure

In a within-subject design, participants were presented with three different cinematographic scenes (control, erotica, violence). The subjects saw an initial fixation cross for the duration of 7 s, followed by a 60 s clip and another fixation cross of 90 s. Each participant watched all three clips between 10–12 am on consecutive days, approximately 24 h apart. We presented one clip a day in a randomized order. Pupil diameter was sampled throughout the procedure by a Tobii TX 300 eye-tracker. Subjects were sat 30 cm away from the eye-tracker.

To check the effects on positive and negative affectivity, we registered participants' affect at the beginning and the end of the experimental procedure by using the "Positive and Negative Affect Schedule" (PANAS; Watson et al., 1988; German translation by Krohne et al., 1996, five-point Likert scale). This scale allows capturing current mood states by an evaluation of a series of words which describe various feelings.

Participants

Thirteen young adult participants (6 females, 7 males; M_{age} = 23.77 years, SD = 2.89; age range: 20–30 years) were healthy volunteers recruited from the University of Innsbruck and received research credits for participation in the experiment. All had normal or corrected-to-normal visual acuity. None of the participants indicated suffering from a diagnosed psychiatric disease or having first-degree relatives who did, being under the influence of psychoactive substances or psychopharmacologic treatment, or having suffered severe head injuries in the course of their lives (self-report). In addition, participants had no history of being exposed to a severe traumatic event, had frequently watched violent movies or played violent video games. This study was carried out in accordance with the recommendations of the guidelines of the Ethics Committee of the Department of Psychology, University of Innsbruck, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Department of Psychology, University of Innsbruck.

Arousal Elicitation Procedure

Three short fragments from existing feature films were used to induce altered states of arousal. In doing so, we intended to experimentally induce three sustained states with the longest possible duration during completion of the tasks described below: (a) a neutral, low arousal state serving as control condition; (b) an aversive, high arousal state; and (c) an appetitive, high arousal state. The selected fragment for induction of valence after highly aversive arousal showed a distressing scene of violence (an aggressive and violent encounter between men during which one gets killed by a fire extinguisher) and that for induction of highly appetitive arousal showed an explicitly sexual scene (a man and woman during sexual intercourse, including close-up images of genitalia), whereas a social interaction (shopping scene featuring two women) was presented as control condition.

All selected fragments had matched audiovisual characteristics. The first and last scenes have successfully been used in previous studies investigating states of stress (e.g., Hermans et al., 2011). Providing both an appetitive and aversive state of high arousal allows for experimentally ruling out specific alterations due to the valence of the scenes and therefore the motivational direction of a corresponding state (Mather and Sutherland, 2011; Harmon-Jones et al., 2013).

The cinematographic material was approved by the Austrian Commission for Media for Youth (JMK) for viewers above 16 years and participants have previously been informed that the scenes they were about to watch might contain offensive or distressing content. Subjects could end their participation in the experiment at any time if desired.

Pupil-Diameter Measurements

Pupil diameter was sampled at 300 Hz and recorded throughout the task using an infrared video eye-tracker (Tobii TX 300). Pupil-diameter measurements were processed using a newly developed open source tool, Cheetah Experimental Platform Web, 2.0 (CEP–Web; Zugal et al., 2017), which allows performing the following evaluation stages. First, we substituted any missing values from one pupil by the values determined for the respective other pupil. Second, all values that differed more than three standard deviations from the mean value were considered outliers and therefore removed. Third, the blink detection filter implemented in CEP–Web which, based on a heuristic of missing values and gaze position, detects and clips out blinks 200 ms section before and after each identified blink, (Pedrotti et al., 2011). Next, a filter for linearly interpolating missing data by linear interpolation of values measured just before and after each blink provided by CEP-Web was applied. Finally, based on the continuous pupil measurements, data were low-pass filtered using a third order low pass Butterworth with a cutoff frequency of 4 Hz. Even though a low pass Butterworth filter can be used for processing measurement artifacts, its application introduces a phase response to the filtered signal towards the past. In order to compensate for this phase response, CEP-Web calculates the expected phase response and automatically reshifts the processed signal so that the phase response is equalized.

Relative pupil dilatation was calculated using the mean pupil dilatation 5000 ms before the scene as baseline. Since we were not interested in the pupillary response during the scenes, we focused on mean relative pupillary dilatation during the duration of the second fixation cross as an index of sustained alteration in tonic arousal. Measurements within the first 2000 ms were removed, since they reflected the initial pupillary adaptation to the low level differences between the cinematographic material and the following fixation cross.

Data Analysis

To examine the effects of different scenes on tonic pupil dilatation as well as self-reported mood and arousal, an ANOVA for independent measures was applied to the preprocessed mean pupil-diameter measurements with scene type (neutral, violence, erotica) as between-subject variable.

Sphericity was tested using Mauchly's test and in case of deviance from sphericity, Type I error was controlled by adjusting the degrees of freedom using the Greenhouse-Geisser correction. All reported p values are two-tailed. Alpha levels were set at 0.05. In addition, we applied Bayesian inferential procedures for each hypothesis testing, which allows quantifying the relative strength of evidence for one hypothesis compared to the other. Data were analyzed using SPSS (Version 24) and JASP (Version 0.8; JASP Team 2016).

Results

Results from all participants were subject to data analysis. Effect sizes are reported by partial eta squared η^2_{Part} (0.01 = small; 0.06 = medium; 0.14 = large) for analyses of variance.

First, we applied repeated measures ANOVAs on pupil dilatations during presentation of the second fixation cross immediately after the arousal-inducing material. Two dependent variables were defined, pupillary response relative to a 5 s baseline before observation of the scenes and absolute pupil dilatation in pixels during the second fixation interval (**Table 1**). Considering the small sample size, we additionally applied non-parametric analyses.

Statistical testing on relative pupil sizes indicated a strong main effect for the arousal group, $F_{(2,24)} = 5.07$, $MSE = 0.001$ $p = 0.015$, $\eta^2_{\text{Part}} = 0.30$, $BF_{10} = 3.98$. Mauchly's test showed that the assumption of sphericity had been fulfilled for the within-subject variable arousal condition, $\chi^2_{(2)} = 0.57$, $p = 0.168$. Whereas pupillary response did not differ between high arousal groups, $\Delta = 0.01$, $SE = 0.02$, Bonferroni-adjusted $p = 1$, compared to the neutral group, relative pupil size was higher for both the aversive group with a difference of 0.04 ($SE = 0.01$), Bonferroni-adjusted $p = 0.017$, and in the appetitive group with a difference of 0.03 ($SE = 0.01$), Bonferroni-adjusted $p = 0.033$. Likewise, absolute pupil response differed across arousal conditions, $F_{(2,24)} = 10.63$, $MSE = 0.012$ $p < 0.001$, $\eta^2_{\text{Part}} = 0.47$, $BF_{10} = 51.29$. Mauchly's test confirmed that the assumption of sphericity had been fulfilled for the within-subject variable arousal condition, $\chi^2_{(2)} = 0.70$, $p = 0.705$. Again, the aversive and appetitive conditions did not differ regarding absolute pupil size during the fixation interval after the arousing material, $\Delta = 0.08$, $SE = 0.04$, Bonferroni-adjusted $p = 0.191$. By contrast, participants' pupil dilatation was larger after the violent clip compared to the control clip with a difference of 0.20 ($SE = 0.05$), Bonferroni-adjusted $p = 0.004$, and even more enlarged after exposure to the appetitive compared to the neutral material with a difference of 0.11 ($SE = 0.04$), Bonferroni-adjusted $p = 0.041$.

In addition, Friedman's tests overall confirmed these effects showing alterations in relative, $\chi^2_{(2)} = 6.00$, $p = 0.050$, and absolute tonic pupil responses, $\chi^2_{(2)} = 12.91$, $p = 0.002$, after application of the arousal induction procedure. These results support the validity of the arousal elicitation method, showing that both cinematographic fragments, the violent and explicitly sexual scenes, induced a tonically enlarged pupil dilatation, which indicates a state of increased arousal.

TABLE 1 | Effects of different conditions of the arousal elicitation method on relative and absolute tonic pupil dilatation as well as self reported mood.

	Arousal state					
	Control		Violence		Erotica	
Lure-Type	***M***	***(SE)***	***M***	***(SE)***	***M***	***(SE)***
Tonic pupil size$_{\text{Rel.}}$	0.92	(0.01)	0.96	(0.01)	0.96	(0.01)
Tonic pupil size$_{\text{Abs.}}$	2.94	(0.03)	3.14	(0.03)	3.05	(0.02)
Negative affectivity	−0.29	(0.16)	−0.28	(0.11)	0.47	(0.14)
Positive affectivity	0.12	(0.11)	0.87	(0.15)	0.17	(0.12)

Standard errors in parentheses (standard errors were corrected for repeated measures).

Second, in order to assess the effects of the arousal induction method on current subjective mood, repeated measures ANOVAs were applied on changes in mood state, calculated by subtracting mood state values before and after participants underwent the procedure. For negative affectivity, Mauchly's test indicated that the assumption of sphericity had been fulfilled for the within-subject arousal condition, $\chi^2_{(2)} = 1.29$, $p = 0.525$. Results revealed a strong effect of arousal conditions on self reported negative affect state, $F_{(2,24)} = 7.22$, $MSE = 0.319$, $p = 0.004$, $\eta^2_{\text{Part}} = 0.38$, $BF_{10} = 25.71$. Participants in the aversive arousal condition reported higher negative mood compared to those in the low arousal control condition with a difference of 0.75 ($SE = 0.25$), Bonferroni-adjusted $p = 0.020$, as well as compared to participants in the appetitive arousal condition with a difference of 0.70 ($SE = 0.23$), Bonferroni-adjusted $p = 0.045$. By contrast, there was no difference between the control group and the appetitive arousal group, $\Delta = 0.05$, $SE = 0.18$, Bonferroni-adjusted $p = 1$.

Regarding positive affectivity, again we found a strong main effect for arousal group, $F_{(2,24)} = 6.43$, $MSE = 0.387$ $p = 0.006$, $\eta^2_{\text{Part}} = 0.35$, $BF_{10} = 16.84$. Mauchly's test indicated that the assumption of sphericity had been fulfilled for the within-subject arousal condition, $\chi^2_{(2)} = 1.99$, $p = 0.371$. Specifically the appetitive arousal group reported higher positive mood compared to both the aversive arousal group with a difference of 0.75 ($SE = 0.20$), Bonferroni-adjusted $p = 0.008$, and the low arousal control group, with a slightly significant difference of 0.76 ($SE = 0.29$), Bonferroni-adjusted $p = 0.062$. On the other hand, the latter two groups did not differ with regard to self-reported positive affect, $\Delta = 0.01$, $SE = 0.24$, Bonferroni-adjusted $p = 1$.

Friedman's tests confirmed these effects, showing alterations in mood state between repeated measures for both negative, $\chi^2_{(2)} = 10.80$, $p = 0.005$, and positive affect, $\chi^2_{(2)} = 12.04$, $p = 0.002$.

Altogether, results of the self-report data confirm successful induction of the targeted differences in valence, showing that arousal induction conditions induce states with opposite motivational direction (aversive and appetitive).

Our results provide strong evidence that both of the selected cinematographic fragments, which depicted violence and erotica, presented an effective measure to elicit a state of increased arousal and different valence. Participants showed increased pupil sizes following exposure to the high arousal scenes compared to the control clip. The uniform tonic change of pupil dilatation in response to the arousal-inducing material suggests that the material used meets the criteria to experimentally create a situation of sufficient strength (Lissek et al., 2006), thus being able to effectively evoke the targeted normative cognitive adaptations as aimed for in the following experiments.

EXPERIMENT 1: AROUSAL AND SPATIAL CONTEXT PROCESSING

Spatial context represents a crucial source of information in everyday life as it informs spatial navigation in terms of way-finding (Wiener et al., 2004). Notably, both working memory for spatial context as well as memory encoding

and retrieval thereof have been shown to be a hallmark of hippocampal function (Rajah et al., 2010a,b; Spellman et al., 2015; Esfahani-Bayerl et al., 2016). Yet, the engagement of hippocampal-centered processing in learning and memory has been shown to be impaired by increased arousal (Packard and Goodman, 2012; Schwabe and Wolf, 2013). In fact, acute stress is associated with an impairment of spatial working memory in rodents and monkeys (Gamo et al., 2015), healthy humans (Moriarty et al., 2014; Olver et al., 2015) and psychiatric populations (Smith and Lenzenweger, 2013), as well as impaired spatial learning in rodents (Akirav et al., 2001, 2004; Herrero et al., 2006) and monkeys (Arnsten and Goldman-Rakic, 1998). In humans, exposure to stress strengthens reliance on egocentric, route-based strategies at the cost of allocentric, cognitive map-based context information (van Gerven et al., 2016; Brunyé et al., 2017). Moreover, further research showed that under acute stress, spatial navigation is supported less by a hippocampus-dependent strategy, which maps flexible spatial relations using multiple cues (Schwabe et al., 2007; Vogel et al., 2017).

To assess whether states high in arousal affect implicit acquisition of spatial context, we applied the spatial mnemonic discrimination paradigm (Reagh et al., 2014). After participants underwent the arousal induction procedure, they had to respond to a sequence of objects, which were presented at different spatial locations. In a subsequent surprise recall, participants viewed the same objects at either the same or stepwise vertically or horizontally displaced locations and had to decide whether the object had remained in the same location or whether it had been moved. Recently, this paradigm has been applied successfully to study early forms of hippocampal impairment in elderly humans (Reagh et al., 2014) and the impact of experienced life-time stress on age-related hippocampal function (Marshall et al., 2016). In addition, spatial discrimination ability in this spatial task was related to performance in the Rey Auditory Verbal Learning Test, a neuropsychological test of hippocampus related, declarative memory (Reagh et al., 2014). Thus, this experimental paradigm can be considered sensitive when assessing hippocampus-based processes.

As formulated in the hypothesis section, we expect a compromised hippocampal-related acquisition of spatial context as measured by spatial discrimination ability during states high in arousal. Impairments in spatial context processing might result from an arousal-induced over-reliance on the introduced stimulus response strategy as provided by the instructed and trained task representations. Evidence supporting this prediction would be impaired performance in the spatial mnemonic discrimination paradigm in both increased aversive and appetitive arousal states as compared to the control condition.

Materials and Methods

Design and Procedure

In a 3 (arousal state) × 5 (lure-type) factorial design, each participant was randomly allocated to one of the three conditions (control, violence, erotica; between-subject variable) and performed the spatial discrimination paradigm consisting of an implicit learning and a surprise recall phase with correct spatial positions and five lure displacements (1-Move, 2-Move, 3-Move, 4-Move, Corner-Move; within-subject variable). All tests took place between 10 am and 12 am.

The experimental task was developed using E-Prime software (Version 2.0; Psychology Software Tools, Pittsburgh, PA, USA; Schneider et al., 2012) and presented on a Dell 22 Monitor P2217H monitor (resolution 1920 pixels × 1080 pixels, refresh rate = 60 Hz).

Participants

All participants were healthy volunteers recruited from the University of Innsbruck und met the same criteria as the sample for validation of the arousal induction method. Sixty-six participants (40 females, 26 males; M_{age} = 22.23 years, SD = 2.61; age range: 18–33 years) were tested and informed consent was obtained according to the guidelines of the Ethics Committee of the Department of Psychology, University of Innsbruck.

Experimental Manipulation of the Arousal State

The arousal induction method was applied to the subjects of whom each was randomly allocated to one of the three arousal conditions (control, violence, erotica; n = 22 per condition).

Spatial Mnemonic Discrimination Task

The Spatial Mnemonic Discrimination Task (Reagh et al., 2014; Marshall et al., 2016) consists of 140 common objects and is structured in two sequences, an encoding and a retrieval phase. At first, participants had to judge whether each presented item is more likely to be used indoors or outdoors by responding with their right or left index finger. The objects were located in a 5 × 7 grid due to the dimensions of common widescreen displays and appeared for 2500 ms, assigned pseudo randomly (Marshall et al., 2016). This first sequence conduces incidental learning (Reagh et al., 2014).

After a 5 min delay, in the second sequence participants judged whether the same objects presented in the first sequence were located in the same or a different location. In this setting, 40 objects were placed in a repeated grid space and 100 objects had been moved. The moved objects can be divided in five different lure-types containing 20 objects each. The objects were categorized in 1-Move, 2-Move, 3-Move or 4-Move dimensions in horizontal and vertical direction and as a fifth category Corner-Move Lures, which are objects relocated to the opposite corner of the grid. Diagonal displacements were excluded in the current setting (Reagh et al., 2014). This differentiation enables one to make parametric comparisons across levels of mnemonic interference (Marshall et al., 2016). Each space of the grid is equally likely to contain an object and direction of displacements also appeared for 2500 ms assigned pseudo randomly for each participant. The task was programmed using E-Prime 2.0 (Schneider et al., 2012).

Data Analysis

Change detection performance was quantified using d-prime (d') as a measure of sensitivity according to signal detection theory

(Macmillan and Creelman, 1991). Based on the z-transformed probability of correct match responses (hits, *H*) and incorrect match responses (false alarms, *F*) for each displacement step and condition, we calculated sensitivity separately: *d'* = z(*H*) – z(*F*). Corrections for extreme values in hit rates or false alarms were applied following the log-linear approach (Snodgrass and Corwin, 1988; Stanislaw and Todorov, 1999) by adding 0.5 to both the number of hits and the number of false alarms and adding 1 to both the number of signal trials and the number of noise trials. The log-linear approach results in less biased estimates of sensitivity *d'* than does the 1/(2N) rule (Hautus, 1995).

To examine the effects of different arousal states on spatial learning performance, a 3 × 5 mixed-measures ANOVA was applied to the estimates of sensitivity *d'* with arousal state (control, erotica, violence) as between-subject variable and lure-types (1-Move, 2-Move, 3-Move, 4-Move, Corner-Move) as within-subject variable. Planned contrasts were used to decompose significant effects of arousal states on spatial discrimination ability for different lure-types. Sphericity was tested using Mauchly's test and in case of deviance from sphericity Type I error was controlled by adjusting the degrees of freedom using the Greenhouse-Geisser correction. An alpha-level of 0.05 was used for all statistical tests. All reported *p* values are two-tailed. We determined Bayes factors for each hypothesis, which allows quantifying the relative strength of evidence for one hypothesis compared to the other. Data were analyzed using SPSS (Version 24) and JASP (Version 0.8; JASP Team 2016), respectively.

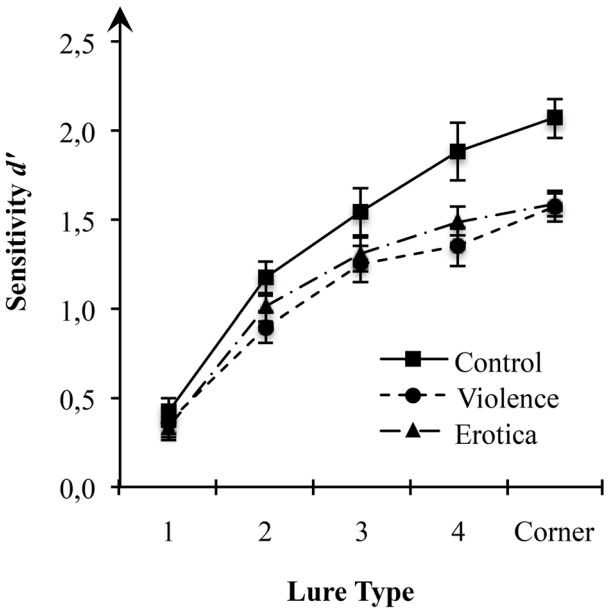

FIGURE 1 | Effects of alterations in arousal on spatial discrimination performance. Compared to the control condition, both high arousal groups showed reduced sensitivity of spatial displacement as measured by d-prime (*d'*) for spatial locations at a moderate to low level of mnemonic interference. Standard errors are represented by the error bars attached to each column in the figure.

Results

Data from all participants were used for statistical analysis. Effect sizes are reported by partial eta squared η^2_{Part} (0.01 = small; 0.06 = medium; 0.14 = large).

Effects of Arousal States on Spatial Discrimination

Considering the effect of arousal states on acquisition of spatial information as predicted in our hypothesis, we first performed a 3 (arousal states) × 5 (lure-types) mixed-measures ANOVA on estimates of sensitivity *d'* for spatial locations with arousal state (control, violence, erotica) as between-subject variable and lure-types (1-Move, 2-Move, 3-Move, 4-Move, Corner-Move) as within-subject variable (**Figure 1**). Results showed a strong main effect for the between-subject variable arousal state, $F_{(2,63)} = 5.13$, $MSE = 0.133$, $p = 0.009$, $\eta^2_{\text{Part}} = 0.14$, $BF_{10} = 2.53$. For all lure-types, planned contrasts revealed better performance in the control group ($M_{\text{Control}} = 1.42$, $SE_{\text{Control}} = 0.10$) as compared to both the aversive arousal group ($M_{\text{Violence}} = 1.09$, $SE_{\text{Violence}} = 0.08$) with a difference of 0.33 ($SE = 0.11$), Bonferroni-adjusted $p = 0.012$, as well as the appetitive arousal group ($M_{\text{Erotica}} = 1.14$, $SE_{\text{Erotica}} = 0.05$) with a difference of 0.27 ($SE = 0.11$), Bonferroni-adjusted $p = 0.048$. By contrast, there was no difference in spatial discrimination scores between the high arousal groups, $\Delta = 0.06$, $SE = 0.11$, Bonferroni-adjusted $p = 1$.

This result pattern indicates strong impairment of spatial context processing due to the experimental arousal elicitation. Interestingly, since the latter result rules out valence specific alterations in estimates of sensitivity *d'* for spatial information, impairments in spatial discrimination are attributable to experimental variations in arousal, regardless of the appetitive or aversive direction.

Interaction of Arousal States and Lure Displacements

Assessing the interaction between arousal states and lure displacement, the 3 × 5 mixed-measures ANOVA revealed a moderate interaction between the two factors arousal states and lure-types on estimates of sensitivity *d'* (see **Table 2**), $F_{(8,252)} = 2.21$, $MSE = 0.111$, $p = 0.028$, $\eta^2_{\text{P}} = 0.07$, main effects model: $BF_{10} = 7.44\text{e+}68$; full effects model: $BF_{10} = 9.23\text{e+}68$, adding the interaction increases the degree of this support by 1.80. Mauchly's test indicated that the assumption of sphericity had been fulfilled for the within-subject variable lure-types $\chi^2_{(9)} = 13.59$, $p = 0.138$. To decompose this statistically significant interaction between arousal states and sensitivity for lure displacement, we applied contrasts defining the lure-type with the highest level of mnemonic interference, i.e., 1-Move lures, as reference level. Planned contrasts comparing the 1-Move lures with the other levels of the factor lure-type revealed strong effects of arousal states for Corner-Move lures, $F_{(2,63)} = 5.52$, $MSE = 0.226$, $p = 0.006$, $\eta^2_{\text{P}} = 0.15$, as well as moderate effects for 4-Move lures, $F_{(2,63)} = 4.84$, $MSE = 0.252$, $p = 0.011$, $\eta^2_{\text{P}} = 0.13$, but not for 3-Move lures, $F_{(2,63)} = 1.04$, $MSE = 0.271$, $p = 0.359$, or for 2-Move lures, $F_{(2,63)} = 1.67$, $MSE = 0.141$, $p = 0.197$. The means reveal contrast effects reflecting altered spatial discrimination

TABLE 2 | Effects of alterations in arousal on spatial discrimination performance: estimates of sensitivity *d*-prime (*d*') for spatial locations in the Spatial Mnemonic Discrimination Paradigm (Reagh et al., 2014; Marshall et al., 2016) for each lure displacement and arousal state.

	Arousal state					
	Control		Violence		Erotica	
Lure-Type	*M*	*(SE)*	*M*	*(SE)*	*M*	*(SE)*
1-Move	0.42	(0.08)	0.36	(0.08)	0.33	(0.07)
2-Move	1.17	(0.10)	0.90	(0.09)	1.01	(0.08)
3-Move	1.54	(0.14)	1.25	(0.10)	1.31	(0.10)
4-Move	1.88	(0.16)	1.35	(0.11)	1.49	(0.08)
Corner-Move	2.07	(0.11)	1.57	(0.08)	1.59	(0.07)

Standard errors in parentheses.

ability for high arousal states (violence, erotica) when compared to the low arousal condition (control). The lack of effects of arousal states on recognition performance in lure-types near the correct spatial position is interpreted as a result of item difficulty being too high, leading to floor effects. Lure displacements with levels of mnemonic interference too high are insensitive to effects of arousal states on spatial discrimination.

In fact, the main effect for lure-type, $F_{(4,252)} = 176.63$, $MSE = 0.111$, $p < 0.001$, $\eta^2_P = 0.74$, $BF_{10} = 1.31e{+}68$, shows that increasing spatial proximity of the lure to the correct original position leads to decreased discrimination performance. This finding is in line with previous results obtained by using this paradigm to assess age-related declines in spatial discrimination ability, showing that differences between young and old participants appear in 3-Move lures and 4-Move lures, thus in the intermediate interference range (Reagh et al., 2014; Marshall et al., 2016). The authors interpreted the absence of differences in 1-Move lures and 2-Move lures, as well as Corner-Move lures, as a result of mnemonic interference being too high in the first two and too low in the latter.

Effects of Arousal State for Each Lure Displacement

To further decompose significant effects of arousal states on spatial discrimination performance, planned contrasts were performed comparing estimates of sensitivity *d*' for spatial locations between all three arousal states.

One-way ANOVA with the between-subject variable arousal state on performance scores for 4-Move lures showed strongly altered spatial discrimination depending on arousal state, $F_{(2,63)} = 5.24$, $MSE = 0.317$, $p = 0.008$, $\eta^2_{Part} = 0.14$, $BF_{10} = 5.93$. Similar to the contrasts decomposing the main effects of arousal states, we found better performance in the control group when compared to the aversive arousal group with a difference of 0.53 ($SE = 0.17$), Bonferroni-adjusted $p = 0.009$. Likewise, the appetitive arousal group showed slightly impaired spatial discrimination performance than the control group with a difference of 0.39 ($SE = 0.17$), Bonferroni-adjusted $p = 0.072$, but their sensitivity *d*' did not differ from the aversive arousal group, with a difference of 0.14 ($SE = 0.17$), Bonferroni-adjusted $p = 1$.

Next, we examined the effect of arousal states on spatial discrimination for Corner-Move lures via one-way variance analysis on estimates of sensitivity *d*'. We found a strong alteration of performance scores between arousal groups, $F_{(2,63)} = 9.69$, $MSE = 0.182$, $p < 0.001$, $\eta^2_{Part} = 0.24$, $BF_{10} = 128.20$. In line with our prediction, both the aversive arousal group and the appetitive arousal group showed impaired spatial discrimination compared to the control group. The former with a difference of −0.50 ($SE = 0.13$), Bonferroni-adjusted $p < 0.001$, and the latter with a difference of −0.48 ($SE = 0.13$), Bonferroni-adjusted $p < 0.001$. Again, the difference in performance scores of 0.02 ($SE = 0.13$) between the high arousal states did not reach significant levels, Bonferroni-adjusted $p = 1$.

By contrast, as indicated by planned contrasts, one-way ANOVAs confirmed that neither spatial discrimination for 1-Move, $F_{(2,63)} = 0.38$, $MSE = 0.140$, $p = 0.685$, $BF_{10} = 0.17$, nor for 2-Move, $F_{(2,63)} = 2.18$, $MSE = 0.182$, $p = 0.122$, $BF_{10} = 0.64$, or for 3-Move lures, $F_{(2,63)} = 0.288$, $MSE = 0.52$, $p = 0.173$, $BF_{10} = 0.48$, were affected by arousal states.

In line with our previous statistical analysis, results further suggest that estimates of sensitivity *d*' for spatial locations of the three groups begins to diverge at a moderate level of mnemonic interference (4-Move lures) and reaches its strongest alteration at a low level of mnemonic interference (Corner-Move lures). Once again, our results uniformly show impaired spatial discrimination in high arousal states relative to a neutral state.

Compared to the control condition, both high arousal groups showed strong impairment of spatial context acquisition. Presumably due to floor effects, alterations in performance reached significance at a moderate to low level of mnemonic interference. Our behavioral data support the idea that states high in arousal disrupt hippocampus-dependent acquisition of spatial context information (Schwabe et al., 2007; Brunyé et al., 2017; Vogel et al., 2017) as indicated by impaired spatial discrimination ability (Reagh et al., 2014; Marshall et al., 2016). Consistent with our second hypothesis, high arousal states did not differ in the direction and magnitude they hamper performance, thus excluding a role of their valence, i.e., appetitive or aversive (Mather and Sutherland, 2009; Harmon-Jones et al., 2013).

EXPERIMENT 2: AROUSAL AND SEQUENCE ACQUISITION

The purpose of Experiment 2 was to explore how arousal states affect acquisition of sequence information. Sequence information refers to a type of context which informs actual behavior about the chronological order of events, thus allowing to predict a relevant event by preceding cues. In contrast to the formation of simple stimulus–response (S–R) associations underlying habit formation (Hull, 1943), prediction learning requires acquisition of stimulus–stimulus (S–S) relationships (Tolman, 1948), therefore the two processes might rely on different memory systems (Bornstein and Daw, 2012; Mattfeld and Stark, 2015). Both neuroimaging (Ross et al., 2009) and neuropsychological evidence (Schapiro et al., 2014) support the role of hippocampus in formation of sequential associations, presumably based on its function in predicting the next event in a sequence (Turk-Browne et al., 2010; Moustafa et al., 2013; Davachi and DuBrow, 2015). The

hippocampus is assumed to promote binding of temporally close events, thus embedding incoming information in a temporal context (Staresina and Davachi, 2009; Hsieh et al., 2014).

Previous findings regarding stress effects on associative learning using eye-blink conditioning paradigms have proved equivocal. Whereas learning performance in a delay and trace eye-blink conditioning paradigm requiring the acquisition of simple relations is enhanced under acute arousal (Duncko et al., 2007), providing additional context using eye-blink conditional discrimination learning conditioning is strongly impaired in aroused humans (Wolf et al., 2012). Instead of simply learning CS-US pairings, the latter paradigm requires acquisition of a contextual cue in terms of a preceding discriminative stimulus, which indicates the reliability of the CS to predict the US, a process related to hippocampal function (Green and Woodruff-Pak, 2000; McDonald et al., 2002; MacDonald, 2008).

Based on the findings that arousal hampers the engagement of memory systems supporting context acquisition (McDonald et al., 2007; Packard and Goodman, 2012; Schwabe and Wolf, 2013), we hypothesize disrupted acquisition of sequential context by increased arousal states. To test our prediction, human subjects underwent the validated arousal induction procedure to experimentally modulate arousal state. Thereafter, participants performed an associative learning paradigm, the learned irrelevance task (Orosz et al., 2008), which requires continuous acquisition of sequences to predict a target cue. The capability to acquire sequential context was inferred from their ability or inability, respectively, to accelerate responses to the behavioral relevant cue using reliable predictor cues. Slower target responses after reliable cues due to increases in arousal would indicate disrupted context acquisition, providing evidence for our main hypothesis. In addition, the absence of differences in performance during both states high in arousal would suggest that alterations in arousal could be considered pivotal, regardless of their motivational direction.

Materials and Methods

Design and Procedure

We designed a 3 (arousal state) × 3 (predictive association) factorial experiment. Participants were randomly allocated to one of the three conditions (control, erotica, violence; between-subject variable) and performed the learned irrelevance paradigm consisting of an implicit learning and a recall phase. Participants were told they would perform a simple response speed testing task, during which they had to respond as fast as possible to a target letter. However, each target letter was predicted by three types of conditioned stimuli (random, pre-exposed (PE), non pre-exposed (NPE); within-subject variable) which differed in their predictive reliability. Like in Experiment 1, all tests took place between 10 am and 12 am.

The experimental task was developed using E-Prime software (Version 2.0; Psychology Software Tools, Pittsburgh, PA, USA; Schneider et al., 2012) and presented on a Dell 22 Monitor P2217H monitor (resolution 1920 pixels × 1080 pixels, refresh rate = 60 Hz).

Participants

Eighty-four healthy volunteers (51 females, 33 males; M_{age} = 22.85 years, SD = 2.36; age range: 19–34 years) recruited from the University of Innsbruck were tested. All participants met the same inclusion criteria as the sample for validation of the arousal induction method. Informed consent was obtained according to the guidelines of the local Ethics Committee.

Experimental Manipulation of the Arousal State

Participants underwent the arousal induction method, each one being randomly allocated to one of the three arousal conditions (control, violence, erotica; n = 28 per group).

Learned Irrelevance Paradigm

The Learned Irrelevance Paradigm was designed as a visual target detection task (Orosz et al., 2008, 2011). Participants saw a series of letters in equal size and white color in the middle of a black screen and were instructed to respond as fast as possible to the target letter X immediately when it occurred by pressing the space bar on the keyboard. There were 450 letters (75 target and 375 non-target), each presented for one second one after another without interstimulus interval, resulting in an overall test duration of 7.5 min.

In addition to the target letter "X", 10 additional letters were presented. These non-target characters were divided into two groups, the PE character group consisting of a selection of five consonants (B, D, T, Y and Z) and the NPE character group consisting of five vowels (A, E, I, O and U). The predictability of the target by the pre-target letter was manipulated in three steps as described in the following sequence type. First, in the random condition (R) each letter from the PE group randomly appeared between the target letters. Hence, the pre-target letter did not predict the target letter. Second, in each block of the NPE condition the target letter was predicted five consecutive times by the same letter from the NPE group, therefore representing a CS for the occurrence of the target letter. Since one NPE character was used as CS only one single time in the whole task and was never presented elsewhere, NPE-CS should have a high predictive value and associability. By contrast, in the third and other PE condition the target was predicted five consecutive times by just one particular PE-letter and similar to the NPE condition, each character was used for one block only. The crucial distinction was that unlike letters from the NPE group, PE letters were presented as filler letters in each preceding block. Since PE-CS occurred both as fillers and pre-target letters, they constituted less reliable predictors of the target letter and it should be harder to learn an association between PE-CS and the target.

Each condition was segmented in five blocks containing 30 characters with five targets, five target predictors and 20 fillers, resulting in 15 blocks overall. The number of filler letters between target and following predictor varied from one to eight with an average of four. Filler characters were letters from the PE group. Blocks were presented in a fixed order, always beginning with R

and subsequently being counterbalanced with either PE or NPE. Two successive blocks never belonged to the same condition.

According to the degree of prediction, the latency measured between the target onset and key pressing was expected to be the lowest in NPE, somewhat higher in PE and highest in R. Faster responses in PE- and NPE-blocks compared to R-blocks would indicate that participants have learned the associative sequences, i.e., the predictive letter-letter associations. In addition, the average reaction time (RT) in PE is expected to be significantly higher than in NPE. This would indicate that participants had learned from previous trials involving PE characters that this group of letters does not predict the target letter. Therefore, differences in RT in the NPE- and the PE-condition represent a measure of the learned irrelevance effect (Orosz et al., 2008).

Data Analysis

Supposing that the first two predictor-target pairings generated a predictive association enabling anticipations of the following target sequences, only the last three target RTs were utilized for the analysis (Orosz et al., 2011). The mean RT was operationalized as dependent variable, whereby differences between trial types around and below zero indicated disrupted associative learning (Orosz et al., 2008).

As opposed to the analysis strategy in Experiment 1, where comparisons between groups within each lure displacement were crucial to test our hypothesis, this time we compared response latencies between sequence types within each arousal state. Since alterations in response times indicate whether stable associations between within predictor-target pairings were built, the absence of accelerated responses after a predictor suggests impaired acquisition of predictive sequences whereas faster responses prove successful learning of context information. First, we applied a 3 × 3 mixed ANOVA with the between-subject factor arousal state (control, violence, erotica) and the within-subject factor sequence type (R, PE, NPE). Second, in order to decompose interaction effects, we calculated planned contrasts in terms of additional repeated measures ANOVAs for each factor level of the between-subject variable arousal state.

All reported p values are two-tailed and alpha levels were set at 0.05. Again, we applied Bayesian inferential procedures for each hypothesis testing. Data were analyzed using SPSS (Version 24) and JASP (Version 0.8; JASP Team 2016).

Results

Response times for correct responses from all participants were used for data analysis. To deal with outliers, we applied the median absolute deviation method (Leys et al., 2013) to response times for each condition of each factor separately. In doing so, a total of 3.62% of all trials were identified as outliers and therefore eliminated. Data from all tested participants were included for data analysis.

Interaction of Arousal States and Sequential Stimulus–Stimulus Pairings

The effects of alterations in arousal on behavioral responses (see **Table 3** and **Figure 2**) was analyzed using a 3 (arousal state) × 3 (sequence type) mixed measures ANOVA with different groups (control, violence, erotica) as between-subject variable and types of *sequences* (random, PE, NPE) as within-subject variable. Mauchly's test indicated that the assumption of sphericity had been violated for the within-subject variable $\chi^2_{(2)} = 9.76$, $p = 0.008$. The degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.90$). We found a strong interaction between both factors, $F_{(3.59,145.31)} = 9.44$, $MSE = 478.641$, $p < 0.001$, $\eta^2_P = 0.19$, main effects model: $BF_{10} = 1.43e+6$; full effects model: $BF_{10} = 2.85e+10$. In line with our prediction, means revealed effects reflecting altered prediction learning for high arousal states in comparison to the low arousal condition. In addition, results revealed a main effect for CS-type, $F_{(1.79,145.31)} = 20.72$, $MSE = 478.641$, $p < 0.001$, $\eta^2_P = 0.20$, $BF_{10} = 77,025.39$. To further decompose both the interaction of arousal states and sequence type as well as the main effect of sequence type, in the next paragraph we applied one-way ANOVAs on sequential stimulus–stimulus learning performance for each arousal group individually.

Considering the effect of alterations in arousal on overall response times, the 3 × 3 ANOVA showed a strong main effect for the factor arousal state, $F_{(2,81)} = 6.81$, $MSE = 2,308.874$, $p = 0.002$, $\eta^2_P = 0.14$, $BF_{10} = 19.30$. The appetitive arousal group ($M_{Erotica} = 437.07$ ms, $SE_{Erotica} = 12.23$) responded more slowly to the target letters compared to the control group ($M_{Control} = 393.95$ ms, $SE_{Control} = 8.34$) with a difference of 43.13 ms ($SE = 12.84$), Bonferroni-adjusted $p = 0.003$. Likewise, compared to the aversive arousal group ($M_{Violence} = 398.48$ ms, $SE_{Violence} = 5.31$), RTs in the appetitive arousal condition slowed down with a difference of 38.59 ms ($SE = 12.84$), Bonferroni-adjusted $p = 0.012$. By contrast, the difference of 4.54 ms ($SE = 12.84$) between the control and the negative arousal conditions was not significant, Bonferroni-adjusted $p = 1$. Although exposure to aversive content had no effect on response times, an increase in appetitive arousal led to adeceleration of behavioral responses.

Effects of Arousal State for Each Sequence Type

To assess whether increases in arousal change the acquisition of stimulus–stimulus sequences, we tested for discrepancies in response times for different predictive associations for all three arousal states separately.

First, we tested learning performance operationalized as alterations in response times between sequence types in the control condition. As Mauchly's test indicated that the

TABLE 3 | Effects of alterations in arousal on associative learning performance: response times in the Learned Irrelevance Paradigm (Orosz et al., 2008) for each trial type and arousal state.

	Arousal state					
	Control		Violence		Erotica	
Sequence type	*M*	*(SE)*	*M*	*(SE)*	*M*	*(SE)*
Random	418.08	(7.77)	404.48	(5.70)	436.60	(11.76)
Pre-exposed	393.08	(8.19)	398.60	(6.05)	440.11	(13.26)
Non pre-exposed	370.68	(10.88)	392.36	(7.11)	434.51	(12.43)

Standard errors in parentheses.

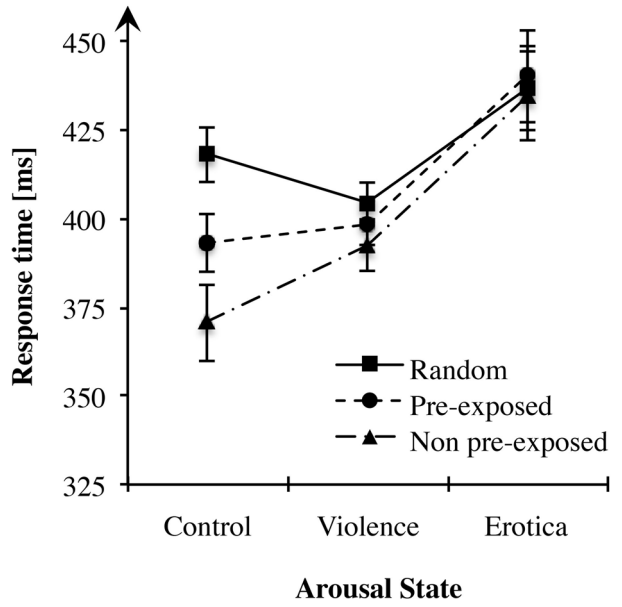

FIGURE 2 | Effects of alterations in arousal on associative learning performance. In both high arousal conditions response times did not differ between sequence types, whereas in the control group participants showed fastest responses in non pre-exposed (NPE)-trials, slowest responses in R-trials with pre-exposed (PE)-trials lying in between, thus learning performance remained unchanged. Standard errors are represented by the error bars attached to each data point in the figure.

assumption of sphericity had been violated $\chi^2_{(2)} = 7.46$, $p = 0.024$, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.80$). Repeated measures ANOVA produced a strong main effect for the within-subject variable sequence type, $F_{(1.60,43.22)} = 30.15$, $MSE = 652.633$, $p < 0.001$, $\eta^2_{\text{Part}} = 0.53$, $BF_{10} = 5.01\text{e+}6$. More precisely, contrasts revealed that participants achieved faster responses after both the PE, $F_{(1,27)} = 23.77$, $MSE = 736.465$, $p < 0.001$, $\eta^2_{\text{Part}} = 0.47$, and the NPE predictors, $F_{(1,27)} = 40.24$, $MSE = 1,563.369$, $p < 0.001$, $\eta^2_{\text{Part}} = 0.60$, than after random stimuli. Likewise, faster reactions were measured for target letters predicted by NPE compared to PE stimuli, $F_{(1,27)} = 16.85$, $MSE = 833.912$, $p < 0.001$, $\eta^2_{\text{Part}} = 0.38$. These results are in accordance with previous studies, clearly replicating the learning effects in the learned irrelevance paradigm within the control *condition* (Orosz et al., 2008).

Second, we applied a repeated measures ANOVA on learning performance of the aversive arousal group. Mauchly's test indicated that the assumption of sphericity had been violated $\chi^2_{(2)} = 6.21$, $p = 0.045$. Again, degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity ($\varepsilon = 0.83$). Results showed no effect of sequence type on RTs, $F_{(1.65,44.54)} = 2.09$, $MSE = 596.042$, $p = 0.143$, $BF_{10} = 0.52$, indicating that participants did not anticipate the target using the prediction stimuli. Hence, acquisition of sequential context was abolished within the aversive arousal condition.

Next, we addressed differences between predictive stimulus-stimulus associations within the appetitive arousal condition. Like for the aversive arousal group, repeated measures ANOVA indicated no alterations in response times depending on the levels of the experimental factor sequence type, $F_{(2,54)} = 0.82$, $MSE = 274.098$, $p = 0.446$, $BF_{10} = 0.20$. Mauchly's test indicated that the assumption of sphericity had been fulfilled for the within-subject variable lure-types $\chi^2_{(2)} = 0.93$, $p = 0.627$. Thus, appetitive arousal abolished acquisition of sequence information and thereby anticipation of the required response.

The general objective of Experiment 2 was to examine effects of high arousal states on sequential context processing. To summarize, in highly aroused subjects response times did not differ between sequence types, indicating abolished learning of simple predictive pairings, whereas in the control group, learning performance was fully intact and adapted to the experimentally manipulated predictive value of the pre-target stimulus. This result pattern supports our prediction stating disrupted acquisition of sequential context by states of increased arousal associated with a hippocampus related, "cognitive" system (Rajah et al., 2010a; Vogel et al., 2015a). Intriguingly, high arousal states modulated context processing regardless of their motivational direction. By contrast, in a control state not manipulated, human subjects learned stimulus-stimulus associations and used this sequence information to predict the behaviorally relevant target as indicated by accelerated response times.

DISCUSSION

We actively represent ongoing events within a spatial-temporal context, which informs behavior to respond adaptively to current demands. In everyday life, binding spatial-temporal contextual details from the incoming flow of information to a current event mainly occurs in an implicit way. The goal of the present experiments was two-fold: first, we aimed to examine how variations in arousal impact two kinds of context processing, the acquisition of spatial (Experiment 1) and sequential (Experiment 2) associations. Second, we intended to test whether arousal states of different motivational direction, i.e., aversive and appetitive valence, promote different effects on context acquisition. Our findings show that regardless of valence, increased arousal interferes with implicit learning of contextual information to support task performance. Both aversively and appetitively aroused human subjects showed impaired ability to acquire spatial relations in a spatial discrimination paradigm (Experiment 1) and failed to detect predictive sequences in an associative learning paradigm (Experiment 2). Thus, under increased arousal, participants failed to bind or use spatial and sequential context to inform behavioral responses and thereby facilitate performance. States high in arousal might favor reflexive action by narrowing the attentional scope on executing implemented stimulus-response bindings (Packard and Goodman, 2012; Schwabe and Wolf, 2013; Gagnon and Wagner, 2016; Schwabe, 2016). As a consequence, sensitivity for spatial-temporal contextual details is reduced, promoting impaired context processing. We conclude that sensitivity for spatial-temporal context in terms of implicit acquisition of

spatial and sequential associations varies as a function of arousal state.

How can this variation in the use of contextual cues altered by arousal be explained? One line of research which offers an explanation showed decreased engagement of hippocampal and presumably prefrontal cortical systems under stress (Schwabe and Wolf, 2013). Neuropsychological evidence supports the role of hippocampus in constructing spatially coherent internal scenes and an inflexible focus on specific fragments in patients with selective bilateral hippocampal damage (McCormick et al., 2017). In addition, at least in young adults, changes in the ability to retrieve both spatial and temporal contextual details are related to inter-individual differences in hippocampal volumes (Rajah et al., 2010a). In concert with other brain sites, the interconnected hippocampus (Ranganath and Ritchey, 2012; Yonelinas, 2013) supports binding of spatial-temporal contextual details with item information while required to integrate actual experience in a contextualized episodic event (Chun and Phelps, 1999; Hannula and Ranganath, 2008; Shimamura and Wickens, 2009; Zeidman and Maguire, 2016). Thus, weakening the engagement of hippocampus-centered learning strategies may hamper the processing of spatial-temporal context. Increased arousal is one influential factor among others (Packard and Goodman, 2013) that bias the competition of active learning strategies towards a dominance of striatum-dependent, habit-like stimulus-response learning and weakens hippocampus-dependent, cognitive learning (Packard and Goodman, 2012; Schwabe and Wolf, 2013; Schwabe, 2016). In the light of our findings, impaired performance by states high in arousal results from a reduced use of spatial-temporal contextual cues, which might reflect weakened engagement of a hippocampus-centered "cognitive strategy" (Vogel et al., 2015a,b, 2017) leading to a measurable behavioral impairment in task performance. Nevertheless, it is noteworthy that both experiments were of behavioral nature, therefore assumptions about the involved brain areas remain hypothetical.

Arousal and underlying locus correuleus-norepinephrine activity orchestrate cognition in several ways (Berridge and Waterhouse, 2003) by promoting exploitation of active behavioral sets (Aston-Jones and Cohen, 2005) as well as reorienting (Bouret and Sara, 2005; Sara and Bouret, 2012; Sara, 2015) and narrowing attention to high-priority information in memory and cognition (Mather and Sutherland, 2011; Harmon-Jones et al., 2013; Mather et al., 2016). These consequences of increased arousal for cognition might be reflected by our findings. In our study, states high in arousal might have reoriented attention to the active stimulus-response set, which has been established by task instructions and completion of practice trials, thus narrowing active cognition to the behaviorally relevant cues (i.e., indoor or outdoor objects in Experiment 1, target letter in Experiment 2) at the cost of implicit sensitivity to their spatial or temporal context.

In the accounts mentioned, arousal-biased cognition is assumed to exert an adaptive value by promoting a less resource-demanding mode as reflected by enhanced reliance on stimulus-response learning and habitual responding, as well as reduced engagement of more complex cognitive strategies (Packard and Goodman, 2012; Schwabe and Wolf, 2013). As demonstrated by our experiments, these changes in information processing lead to impaired implicit acquisition of spatial and sequential cues at the same time, resulting in performance decrements in tasks which require context processing to facilitate task execution. But how can reduced sensitivity for spatial-temporal contextual details be an adaptive response in challenging situations? One reasonable suggestion comes from more recent research in the domain of perceptual decision-making. Krishnamurthy et al. (2017) provided evidence by showing that arousal adaptively adjusts the influence of prior expectations on perceptual judgments. In their study, increased arousal in highly dynamic environments promoted less influence of priors on the perception of a stimulus. Similarly, in our study participants were exposed to highly arousing events which might have signaled an uncontrollable situation with unpredictable outcomes. Thus, reduced use of contextual details to inform behavioral responses might be adaptive in unpredictable environments, since information on spatial positions or sequential orders of events may change quickly and therefore represent unreliable information.

Our results are well in line with previous findings, showing impaired spatial cognition (e.g., Olver et al., 2015) and associative learning (e.g., Wolf et al., 2012) by stress. The present findings, however, extend previous evidence in several important ways. First and foremost, most previous research focused on stress-induced impairments in behavioral tasks directly addressing the instructed and therefore explicit remembering of contextual cues. By contrast, in the current experiments, paradigms required acquisition of spatial-temporal associations in an implicit manner. Second, we considered differences in valence of arousal states, showing that acute arousal *per se*, regardless of the motivational direction, hampers implicit context processing. Third, whereas stress effects on long-term memory are very well documented (Roozendaal et al., 2009; Joëls et al., 2011; Schwabe, 2016), our study addressed how context processing is affected *within* a state of increased arousal, i.e., immediately after a challenging encounter, which occurs in the context of the experimental task. Finally, since the presence of alterations in brain responses as measured by neurophysiological assessments can be associated with normal cognitive functioning, our experimental design allowed us to measure arousal effects on cognition on a behavioral level (Harvey, 2012).

Despite the application of reliable experimental paradigms (Orosz et al., 2008; Marshall et al., 2016) and results providing strong evidence (Lee and Wagenmakers, 2014) for the derived predictions, the present study has some limitations. One clear limitation of our study is that although we refer to neurobiological models of arousal driven modulations of cognitive processes, no neurophysiological techniques were applied. Moreover, arousal induction method was applied immediately after participants were instructed and performed the practice trials and instantly before task execution. Arousing material probably acted as distractor occupying working memory resources and thereby taxing the implicit acquisition of contextual information. Although performance decrements by arousal and distraction in concert are not exclusive, existing

evidence shows that neither implicit spatial learning (Vickery et al., 2010) nor implicit sequence learning (Kaufman et al., 2010) are affected by interference from working memory load. We suggest that increased arousal impairs sensitivity to contextual details by a narrowed focus on the active stimulus-response set, at the cost of information surrounding an event. Putatively, at a neural level this shift in processing mode is reflected by decreased engagement of hippocampal- and prefrontal-centered cognitive systems supporting context processing.

To conclude, we shed light on the online processing of context information as a crucial aspect of active cognition (Davachi and DuBrow, 2015), whereas most other studies focused on the effects of states high in arousal on storing information over the long term. Our findings go beyond the known effects of stress on working memory related functions (Shields et al., 2016b), showing that increased arousal impairs implicit acquisition of spatial and sequential context of an event. Decreased sensitivity for contextual details is attributable to changes in arousal state, regardless of its motivational direction. This finding emphasizes that this bias might occur in aversive as well as appetitive conditions, such as panic and likewise in states of sexual excitement. Our study highlights the ability of alterations in general arousal to promote adjustments of ongoing cognition, thus extending our understanding of information processing during states high in arousal in everyday life and clinical populations with hyper-arousal disorders.

AUTHOR CONTRIBUTIONS

TM initiated and designed the study. TM, MM, MF and PS collected the data and interpreted the results. TM, BW, SZ and JP performed the preprocessing of pupil measurements. TM performed the statistical analyses and drafted the article. TM, MM, MF, BW, JP, SZ and PS read and corrected versions of the manuscript.

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Performance Under Stress: An Eye-Tracking Investigation of the Iowa Gambling Task (IGT)

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Stress pervades everyday life and impedes risky decision making. The following experiment is the first to examine effects of stress on risky decision making in the Iowa Gambling Task (IGT), while measuring inspection time and conscious awareness of deck contingencies. This was original as it allowed a fine grained rigorous analysis of the way that stress impedes awareness of, and attention to maladaptive financial choices. The extended Cognitive Reflection Task (CRT) further afforded examination of the impact of impaired reflective thinking on risky decision making. Stressed participants were slower to avoid the disadvantageous decks and performed worse overall. They inspected disadvantageous decks for longer than the control condition and were slower in developing awareness of their poor deck quality compared to the control condition. Conversely, in the control condition greater inspection times for advantageous decks were observed earlier in the task, and better awareness of the deck contingencies was shown as early as the second block of trials than the stress condition. Path analysis suggested that stress reduced IGT performance by impeding reflective thinking and conscious awareness. Explicit cognitive processes, moreover, were important during the preliminary phase of IGT performance—a finding that has significant implications for the use of the IGT as a clinical diagnostic tool. It was concluded that stress impedes reflective thinking, attentional disengagement from poorer decks, and the development of conscious knowledge about choice quality that interferes with performance on the IGT. These data demonstrate that stress impairs risky decision making performance, by impeding attention to, and awareness of task characteristics in risky decision making.

Keywords: decision making, cognitive reflection test (CRT), stress, Iowa Gambling Task (IGT), somatic marker hypothesis, eye-tracking, conscious knowledge

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INTRODUCTION

Stress is a mental tension that arises in uncontrollable situations and results in a compensatory psychological and physiological response (Lovallo, 2016). Stress moreover, alters cognitive and emotional processes implicated in decision making (Reimann and Bechara, 2010; Simonovic et al., 2017a; Starcke et al., 2017). Traditionally, emotion was characterized as disruptive to cognitive processes in decision making however, the Somatic Marker Hypothesis (SMH) made a compelling case that emotional factors and arousal facilitate effective decision making (Damasio, 1994). The SMH postulates that emotion plays a pivotal role in complex decision making (Bechara and Damasio, 2005) and emotion-based learning (Damasio, 1996; Starcke et al., 2017).

The goals of this article are two fold: first, to examine the effect of stress on cognitive reflection, conscious awareness and attention in predicting risky decision making performance; and second, to test theoretical explanations of the Iowa Gambling Task (IGT) and consider its suitability as a diagnostic clinical tool.

The IGT was developed to test the SMH and emotion-based learning, by mimicking real life decision making with risks, rewards and punishments (Bechara et al., 1994). IGT participants select cards from four decks which have different frequencies of reward and punishment. Advantageous decks offer moderate rewards and small punishments whereas disadvantageous decks offer larger rewards and substantial punishments, resulting in an overall loss. Participants should make as much notional money as possible over the course of the task. It is assumed that participants develop a "gut feeling" (or somatic marker) about the "goodness" or "badness" of decks and progressively acquire conceptual knowledge and awareness about task contingencies. Early evidence indicated that somatic markers help advantageous decision making during IGT performance (e.g., Bechara et al., 1994; Bechara and Damasio, 2005), however subsequent studies challenged this view—showing that analytic thinking and explicit knowledge of the deck contingencies played a more significant role in the IGT than previously thought (e.g., Maia and McClelland, 2004; Bowman et al., 2005; Simonovic et al., 2017a,b). Indeed, Simonovic et al. (2017a,b) argued that cognitive processes and conscious awareness influence the development of somatic markers and suggested that the IGT performance is best understood through the interplay between emotional and analytic processes within a dual process account (see e.g., Kahneman, 2011).

Brevers et al. (2013) use a dual-process framework that contrasts intuitive, effortless, emotional and unconscious processes (Type I) with effortful conscious and controlled processes (Type II). The SMH can account for intuitive processes such as emotional responses or gut feelings that shape IGT performance and are measured through physiological techniques (Glöckner and Witteman, 2010). Brevers et al. (2013) further proposed that "cool" reflective processing is needed for evaluation of "hot," affective choices made early in the IGT. Hence, current evidence suggests a complex interplay between Type I and Type II processes in determining IGT performance. These Type II processes require further investigation by measuring attention and conscious awareness about the task.

Stress

According to the SMH, the connection between somatic markers and decision making can be interrupted by stress (e.g., Reimann and Bechara, 2010). Stress interferes with the learning process in healthy controls, increases risk-taking behavior and leads to disadvantageous card selections on the IGT (Preston et al., 2007; Miu et al., 2008; van den Bos et al., 2009; Robinson et al., 2015; Simonovic et al., 2017a; Starcke et al., 2017). Preston et al. (2007) demonstrated that stressed participants learned more slowly on the IGT than the control group. Thus, incidental anticipatory stress interfered with the development of somatic markers which may have been due to working memory disruption (Hinson et al., 2002). Furthermore, Preston et al. (2007) suggested that anticipatory stress shifts cognitive processes away from deliberative processing towards automatic processing, and thus impairs differentiation between advantageous and disadvantageous choices—a proposal that warranted further investigation.

There is however disagreement about the nature of the impact of stress on learning and performance in the IGT. Starcke et al. (2017) argued that performance on the early trials of the IGT is determined by emotional feedback processing. However, when Simonovic et al. (2017a) replicated and extended Preston et al. (2007) work, demonstrating that reflective thinking was also important early in the task. The correlations reported in Simonovic et al.'s (2017a) study also indicated that reduced reflective thinking in the stress group led to increased disadvantageous deck selection. The effect of stress on IGT performance was predicted by analytic thinking and thus challenged the primacy of emotional learning in early trials (see Bechara et al., 2000). The results from these studies resonate with previous research suggesting that stress reduces cognitive capacity and consequently diminishes learning from negative choices (Lighthall et al., 2009; Petzold et al., 2010). However, it is difficult to unpack whether impaired IGT performance is due to stress inhibiting the development of somatic markers or the reduced capacity for reflective thinking, or both.

Stress disrupts cognitive control (Schwabe and Wolf, 2009), and changes from goal-directed to automatic control of action (Margittai et al., 2015). Margittai et al. (2015) tested participants who received placebo, cortisol and yohimbine, a drug that increases noradrenergic stimulation, before performing the Cognitive Reflection Task (CRT; Frederick, 2005). Their results showed that an increase in cortisol reduced reflective processing and increased intuitive processing. The results showed that cortisol mediates the engagement of cognitive processes and supports the view that stress during the IGT reduced capacity for Type II processes (e.g., Simonovic et al., 2017a). Margittai et al.'s (2015) results also accord with research showing Type II processes to be cortisol dependent (Schwabe and Wolf, 2009, 2013).

Conscious awareness choice quality is important in risky decision making generally, and also the IGT. Maia and McClelland (2004) challenged Bechara et al.'s (1994) view that conscious awareness and performance on the IGT are unrelated by showing that explicit knowledge about decks improved deck selection. Bowman et al. (2005) further demonstrated an explicit evaluation of affective choices could guide future decision making. Fernie and Tunney (2013) however, showed that not all participants attain conscious awareness of deck quality, and that conceptual knowledge was not essential for advantageous selections. Newell and Shanks (2014) further argue that conscious awareness diverts attention to positive choices and recruits Type II goal-directed cognitive processes. Thus, conscious awareness initiates executive attention that further prompts executive functioning and enhances decision making. Thus, risky decision making can be influenced by both emotion-mediated, explicit knowledge and analytic thought, but the precise nature of the

interaction between these processes remains an open question, particularly under stressful conditions.

While the impact of stress on conscious awareness during the IGT has not been investigated there is evidence that stress impedes attentional monitoring of the accuracy of choices (Reyes et al., 2015) and impedes information processing (Hardy et al., 1996). Furthermore, stress may impair preconscious selective attention to avoid negative stimuli (e.g., Roelofs et al., 2007). These findings have implications for explaining the way that stress impairs IGT performance and risky choice *per se*.

The present study extends Simonovic et al. (2017a) using an eye-tracking methodology; an extended, more reliable CRT (Toplak et al., 2014, see Stupple et al., 2017) and Maia and McClelland's (2004) Conscious Awareness Test to provide a more comprehensive analysis of when and where Type II processes are implicated during the IGT. These analyses are informative for testing theories of IGT performance, but also understanding risky decision making under stress.

We propose that stress impairs decision making by reducing capacity for analytic processing which impedes attention to, and awareness of, situational factors. Thus, we propose that when performing IGT under stress, analytic processing would be less predictive of performance; that participants will persist in fixating on disadvantageous decks for more trials and show less awareness of task characteristics.

This leads to a series of hypotheses about: (1) performance; (2) the role of reflection; (3) inspection times; (4) conscious awareness; and (5) predicting IGT performance. Thus: (1) the stress manipulation will inhibit performance on the IGT and delay the elimination of disadvantageous deck selections; (2) there will be more significant correlations between CRT and IGT performance in the control condition than the stress conditions; (3) there will be increased inspection time for disadvantageous decks which will persist across more blocks in the stress condition. There will also be decreased inspection time for advantageous decks which will persist across more blocks in the stress condition; (4) there will be poorer estimates of deck quality in the stress condition; and finally, (5) the relationship between inspection time, conscious knowledge, CRT scores, stress and IGT performance will be tested by path analyses; we predict that inspection time, conscious knowledge, CRT scores and Systolic Blood Pressure (SBP) reactivity will indirectly predict IGT performance.

MATERIALS AND METHODS

Participants

Twenty-three male and 53 female undergraduate students aged 19–56 years, were recruited and randomly allocated to stress and control groups. Participants had normal or corrected to normal vision. This study was carried out in accordance with the recommendations of the British Psychological Society. The protocol was approved by the University of Derby Human Sciences Research Ethics Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki. People under the age of 18 years old and people who reported depression, anxiety, any cardiovascular disease, high blood pressure or a history of neurological illnesses were excluded from participation.

Materials

Stress Manipulation

The study used an anticipatory speech task (Simonovic et al., 2017a), based on a modified version of Preston et al. (2007) anticipatory speech task. A video camera was installed that simulated recording; before the experiment; only participants in the experimental group were told that they would be video-recorded during their performance and they would have to deliver a speech to summarize their experience at the end of the experiment. Control participants were not exposed to any aspect of the stress manipulation while completing the experiment.

Physiological Measurement

SBP and Heart Rate (HR) responses to stress were measured to check whether the manipulation was effective using a continuous, non-invasive cardiovascular Finometer (Finapres Medical System, Amsterdam, Netherlands). Baseline SBP and HR measurements were taken for 5 min before the initiation of IGT followed by SBP and HR measurements taken during the IGT performance. SBP and HR reactivity were calculated by subtracting the average of the performance SBP/HR measurements from the average of resting SBP/HR measurements.

Conscious Awareness Test

Maia and McClelland's (2004) Test of Awareness measures the emergence of conceptual knowledge about deck contingencies. We obtained deck ratings of −10 to +10 to measure awareness of deck quality (Deck Rating).

Eye Tracking Measurements

Eye movements were recorded with the Eye-gaze binocular system Tobii-X2-30 (Inquisit 4 ms plugins), with a remote binocular sampling rate of 30 Hz and an accuracy of approximately 0.45°. The X2 Eye Tracker is a stand-alone eye tracker, and was attached to a laptop (Dell, Precision M6700, 2.70G hz). Participants were seated approximately 0.7 m from the laptop monitor. The Tobii measured 184 mm in length and enabled tracking at close distances (up to 36° gaze angle). Fixations were identified using a fixation radius of 20 pixels and a minimum fixation duration of 100 ms or above. Before starting the experiment, a 9-point calibration routine was executed. Each data point was identified with a timestamp and "X, Y" coordinates, and these coordinates were processed further into fixations and overlaid on a video recording of the IGT. Choices, decision times, and basic eye-tracking parameters such as inspection time and coordinates were recorded. To avoid methodological artifacts, eye tracking metrics were delineated through fixation filters. Eye-tracking parameters such as were recorded for both eyes and then aggregated. To avoid methodological artifacts, eye tracking metrics were delineated through fixation filters. Fixation filters were used to remove blinking points and extrapolate the data correctly into

fixations. Non-overlapping areas of interest (AOI) around each cell in the matrix were defined, each containing different decks. Hence, four AOIs were obtained with the size of 690 × 458 pixels for decks (36° gaze angle). For each participant and each decision, the inspection time within each AOI was calculated.

IGT

Bechara et al.'s (1994) computerized version of IGT and standard instructions were used. Inquisit 4 (Millisecond Software; Seattle, WA, USA) was used to run the IGT script; participants were required to choose individual cards from four decks that provide financial rewards and punishments. Bechara et al.'s (1994) IGT instructions for computerized version were followed. One-hundred and forty trials (seven Blocks of 20) were completed.

CRT

The seven-item CRT (Toplak et al., 2014) was used to measure analytic ability. The score was the total number of correct answers. Higher CRT scores indicated higher reflective ability. The CRT consists of problems where an intuitive answer must be resisted to reach the correct solution. An example question is "If John can drink one barrel of water in 6 days, and Mary can drink one barrel of water in 12 days, how long would it take them to drink one barrel of water together?" The correct answer is 4 days and the intuitive answer is 9 days. The Cronbach alpha for correct CRT responses was $\alpha = 0.77$.

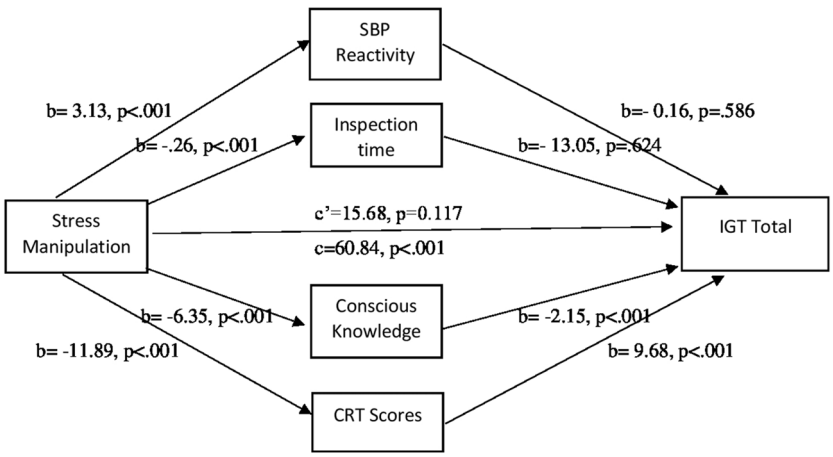
Procedure

Following consent, participants sat for a 5-min resting period, and then baseline SBP/HR measurements were taken. Next, they were randomly allocated to groups; they were no differences in age or gender. The instructions regarding the presentation to the camera were only given to the experimental group, and they were shown the camera which was then switched on. The CRT was administrated followed by the IGT. Eye tracking measures and SBP/HR measurements were taken continuously during the IGT performance. Also, conscious awareness per Block was assessed during the task. After the completion of the IGT task, participants in the experimental group were told that they would not have to give the speech at that point. Finally, participants were debriefed, and post-task SBP/HR measurements were taken to ensure readings had returned to baseline.

Analytic Strategy and Scoring

Initial analyses focused on checking that the stress manipulation was effective: ANOVA was used to determine if SBP and HR reactivity differed by condition. Next, a 2 (condition) × 7 (Block) mixed ANOVAs were used to determine the effect of the manipulation on IGT scores across the seven Blocks. Standard scoring was derived by deducting total disadvantageous card picks (A + B) from total advantageous picks (C + D).

As parametric assumptions were not met, a Mann–Whitney test was used to test for differences in CRT scores between the two



Direct effect, $R^2 = 0.68$, b= 15.68, t(70.00) = 1.58, p = 0.117 c'

Total effect, $R^2 = 0.44$, b= 60.84, t(74.00) = 7,70, p< 0.001 c

Indirect effect CRT, b=30.32 (SE=6.52), 95% CI [19.17, 45.90]

Indirect effect Inspection time, b=3.45 (SE=7.78), 95% CI [-10.21, 20.76]

Indirect effect Conscious knowledge, b=13.64 (SE=4.83), 95% CI [5.70, 24.93]

Indirect effect SBP reactivity, b=-1.86 (SE=3.21), 95% CI [-6.57, 6.79]

FIGURE 1 | Model of stress manipulation as a predictor of Iowa Gambling Task (IGT) scores, mediated by Systolic Blood Pressure (SBP) reactivity, inspection time, Cognitive Reflection Task (CRT) and conscious knowledge. The Confidence Interval (CI) for the indirect effect is a BCa bootstrapped CI based on 10,000 samples.

conditions. Bivariate correlations were examined relationships between CRT scores and disadvantageous deck picks (A + B) during each Block, for each group separately.

Inspection time was also examined across Blocks; 2 (condition) × 7 (Block) mixed ANOVAs were used to determine the differences in inspection time for disadvantageous and advantageous decks across Blocks. Next, a 2 (condition) × 7 (Block) mixed ANOVA was used to determine the effect of manipulation on overall deck ratings (C + D – A + B) across Blocks.

Finally, a bootstrapped mediation model tested the conceptual model outlined in **Figure 1**. All hypotheses were tested simultaneously using the Process macro for SPSS (Hayes, 2012), with 10,000 bootstrapping re-samples and bias-corrected 95% Confidence Intervals (CIs) for each indirect effect. In bootstrapping analyses, bias-corrected CIs that do not contain 0 signify a significant mediational effect (Preacher and Hayes, 2004, 2008). Direct effects estimate how much two cases differing on the independent variable (stress manipulation) also differ on the dependent variable (total IGT score: (C + D) − (A + B)), independent of the effect of the mediator variables (SBP reactivity, inspection time, CRT scores and conscious knowledge) on the dependent variable. Total effects are the sum of the indirect and direct effects of the independent variable (stress manipulation) on the dependent variable (IGT scores; Hayes, 2012). To balance concerns related to Type I and Type II errors the alpha level for all analyses was adjusted to $p < 0.005$. Analysis was conducted using IBM SPSS 24 for Windows. All analyses were repeated with gender as either a covariate or a moderator; no outcomes were affected.

RESULTS

Manipulation Checks

Stress induction—ANOVA revealed a condition (stress vs. control) effect for SBP reactivity, $F_{(1,74)} = 13.63$, $p < 0.001$, $\eta_p^2 = 0.16$; reactivity was larger in the stress condition than in the control condition (**Table 1**). Further, ANOVA revealed a condition (stress vs. control) effect for HR reactivity, $F_{(1,74)} = 4.07$, $p < 0.05$, $\eta_p^2 = 0.05$; reactivity was larger in the stress condition than in the control condition (**Table 1**).

Cognitive reflection task (CRT) performance under stress— A Mann–Whitney test showed differences in CRT scores between the two groups: participants in the stress condition had lower CRT scores (Median = 1, IQR = 5) than participants in the control condition (Median = 4.5, IQR = 7) $U = 128$, $p < 0.001$, $r = 0.72$ demonstrating that reflective thinking was inhibited by stress.

TABLE 1 | Mean (SD) Systolic Blood Pressure (SBP) and Heart Rate (HR) at baseline and during Iowa Gambling Task (IGT) performance.

	SBP		HR	
	Baseline	During	Baseline	During
Stress	122.05 (15.19)	139.16 (13.76)	80.46 (14.44)	85.08 (14.28)
Control	120.22 (8.70)	122.60 (7.19)	77.33 (7.95)	77.50 (8.24)

TABLE 2 | Mean (SD) standard IGT scores per Block for control and stress group.

Blocks	Stress	Control	Total
1	−4.05 (4.48)	−3.16 (8.29)	−3.60 (6.63)
2	−2.32 (6.39)*	4.53 (8.02)	1.10 (7.98)
3	−0.53 (7.56)*	9.40 (6.94)	4.43 (8.77)
4	−0.89 (6.23)*	10.32 (7.22)	4.71 (8.76)
5	2.00 (7.45)*	11.74 (7.70)	6.87 (8.99)
6	1.21 (7.81)*	12.10 (7.88)	6.65 (9.53)
7	1.89 (9.21)*	13.16 (7.70)	7.53 (10.16)
Total	−2.68 (31.15)	58.16 (37.47)	

**Denotes significant $p < 0.005$.*

IGT Performance—Deck Selection Analysis

A Greenhouse-Geisser adjusted ANOVA was used to determine the effect of stress condition on the standard IGT scoring, (C + D) − (A + B) across the seven Blocks of the IGT. There was a main effect of stress condition $F_{(1,74)} = 58.80$, $p < 0.001$, $\eta_p^2 = 0.44$, participants in the stress condition had lower IGT scores than the control group. There was a main effect of Block, $F_{(4.25,314.32)} = 33.29$, $p < 0.001$, $\eta_p^2 = 0.31$. with IGT scores increasing after the first Block. The adjusted *post hoc* pairwise comparisons demonstrated that IGT scores in Block 1 were significantly lower than all other Blocks (all $p < 0.005$). Furthermore, IGT scores in Block 2 were significantly lower than Blocks 4, 5, 6 and 7 (all $p < 0.005$). There were no other significant differences between Blocks. There was a significant Block × Stress manipulation interaction, $F_{(4.25,314.32)} = 7.44$, $p < 0.001$, $\eta_p^2 = 0.09$, independent *t*-tests revealed that participants in the stress conditions had lower IGT scores in all but Block 1 (all significant $p < 0.001$) compared with participants in the control condition (see **Table 2** for descriptive statistics).

Correlations Between CRT and IGT by Block

Correlations between disadvantageous card selections (A + B) per Block revealed medium to large correlations across both conditions. Further correlations between disadvantageous card selection scores for each Block and CRT scores were calculated for control and stress conditions separately. Significant negative correlations between disadvantageous card selection scores and CRT scores were observed in Blocks 1, 6 and 7 in the stress condition, and in Blocks 3, 5, 6 and 7 in the control condition.

TABLE 3 | Correlations between CRT scores and disadvantageous card selection scores (A + B).

Blocks	Stress	Control
1	$r = -0.321$, $p = 0.049$	$r = 0.007$, $p = 0.966$
2	$r = -0.005$, $p = 0.977$	$r = -0.186$, $p = 0.264$
3	$r = -0.175$, $p = 0.294$	$r = -0.442$, $p = 0.005$*
4	$r = -0.124$, $p = 0.458$	$r = -0.201$, $p = 0.227$
5	$r = -0.234$, $p = 0.158$	$r = -0.476$, $p = 0.003$*
6	$r = -0.354$, $p = 0.029$	$r = -0.519$, $p = 0.001$*
7	$r = -0.327$, $p = 0.045$	$r = -0.484$, $p = 0.002$*
Total	$r = -0.354$, $p = 0.029$	$r = -0.462$, $p = 0.003$*

**Denotes significant $p < 0.005$.*

TABLE 4 | Mean (SD) inspection time for disadvantageous decks per Block for control and stress group.

Blocks	Stress	Control	Total
1	0.21 (0.08)	0.23 (0.15)	0.22 (0.12)
2	0.45 (0.14)*	0.15 (0.14)	0.30 (0.20)
3	0.49 (0.18)*	0.13 (0.08)	0.31 (0.23)
4	0.53 (0.20)*	0.17 (0.17)	0.35 (0.26)
5	0.38 (0.23)*	0.12 (0.10)	0.25 (0.22)
6	0.20 (0.16)	0.13 (0.12)	0.17 (0.15)
7	0.19 (0.15)	0.14 (0.14)	0.17 (0.15)
Total	0.35 (0.10)	0.15 (0.07)	

**Denotes significant $p < 0.005$.*

TABLE 5 | Mean (SD) inspection time for advantageous decks per Block for control and stress group.

Blocks	Stress	Control	Total
1	0.22 (0.10)	0.22 (0.14)	0.22 (0.12)
2	0.19 (0.10)*	0.30 (0.14)	0.25 (0.13)
3	0.23 (0.15)*	0.41 (0.23)	0.32 (0.21)
4	0.23 (0.16)*	0.46 (0.30)	0.35 (0.27)
5	0.19 (0.12)	0.22 (0.18)	0.21 (0.15)
6	0.23 (0.15)	0.18 (0.16)	0.21 (0.16)
7	0.23 (0.14)	0.19 (0.18)	0.21 (0.17)
Total	0.22 (0.08)	0.29 (0.10)	

**Denotes significant $p < 0.005$.*

Higher CRT scores were associated with better performance in those Blocks (**Table 3**).

Deck Inspection-Time Analyses

A Greenhouse-Geisser-adjusted ANOVA with log transformed data was used to determine the effect of stress condition on the inspection time for disadvantageous choices (A + B) across the seven Blocks of the IGT. There was: a main effect of stress condition, $F_{(1,74)} = 89.25$, $p < 0.001$, $\eta_p^2 = 0.54$, such that longer inspection-times were observed in the stress group compared to the control group. There was a main effect of Block, $F_{(4.14,306.16)} = 21.81$, $p < 0.001$, $\eta_p^2 = 0.23$, such that there was an increase in inspection time from Block 1 until Block 4 (all $p < 0.001$). The *post hoc* pairwise comparisons (threshold alpha $p < 0.005$) demonstrated that inspection time in Block 1 was lower than inspection time in Blocks 2 and 3 (all $p < 0.005$). Inspection times in Blocks 2 and 3 were significantly higher than inspection times in Blocks 6 and 7. Furthermore, inspection time in Block 4 was significantly higher than inspection times in Blocks 5, 6 and 7 (all $p < 0.005$). There was a significant Block × stress manipulation interaction, $F_{(4.14,306.16)} = 26.16$, $p < 0.001$, $\eta_p^2 = 0.26$. Independent *t*-tests revealed longer inspection time in the stress conditions ($p < 0.005$), in all but Block 1, 6 and 7 compared with participants in the control condition (see **Table 4** for descriptive statistics).

A Greenhouse-Geisser-adjusted ANOVA with log-transformed data was used to determine the effect of stress condition on the inspection time for advantageous choices (C + D) across the seven Blocks of the IGT. There was: a main effect of stress condition $F_{(1,74)} = 7.52$, $p = 0.008$, $\eta_p^2 = 0.09$, such that longer inspection time was observed in the control group compared to the stress group. There was a main effect of Block, $F_{(3.78,280.19)} = 12.12$, $p < 0.001$, $\eta_p^2 = 0.14$, such that there was an increase in inspection time from Block 1 until Block 4 (all $p < 0.05$). The *post hoc* pairwise comparisons ($p < 0.005$), demonstrated that inspection time for advantageous decks in Block 1 was significantly lower than Blocks 3 and 4 (all $p < 0.005$). Furthermore, inspection time for advantageous decks in Blocks 3 was significantly higher than Blocks 5 and 6 (all $p < 0.005$). Additionally, inspection time for advantageous decks in Block 4 was significantly higher than Blocks 5, 6 and 7 (all $p < 0.005$). There was a significant Block × stress manipulation interaction, $F_{(3.78,280.19)} = 10.88$, $p < 0.001$, $\eta_p^2 = 0.13$, on inspection time. Independent *t*-tests revealed longer inspection time for participants in the control condition in Blocks 2, 3 and 4 compared with participants in the stress condition (see **Table 5** for descriptive statistics).

Conscious Awareness of Deck Quality

A Greenhouse-Geisser-adjusted ANOVA was used to determine the effect of stress condition on the overall deck ratings (C + D − A + B) across the seven Blocks of the IGT. There was: a main effect of stress condition $F_{(1,73)} = 12.90$, $p = 0.001$, $\eta_p^2 = 0.15$, such that the stress deck ratings were lower compared to the control group. There was a significant main effect of Block, $F_{(2.75,200.67)} = 26.43$, $p < 0.001$, $\eta_p^2 = 0.27$, such that there was increase in deck ratings across blocks. The adjusted ($p < 0.005$) *post hoc* pairwise comparisons demonstrated that deck ratings in Block 1 was significantly lower than all Blocks (all $p < 0.001$). There were no other significant differences between Blocks. and a non-significant Block × stress manipulation interaction, $F_{(2.75,200.67)} = 1.62$, $p = 0.13$, $\eta_p^2 = 0.02$, on overall deck ratings. This indicated that participants in the control conditions started to develop understanding of the patterns of gains and losses after the first Block (see **Table 6** for descriptive statistics).

Path Analyses of Determinants of IGT Performance

The assumption of normality was met and there were no outliers. For the path analyses, stress manipulation was an independent variable and overall IGT score was the dependent variable. CRT scores, inspection time, conscious knowledge and SBP reactivity were indirect pathways. Initially, it was checked if the stress manipulation predicts chosen mediators. Stress manipulation significantly predicted all the mediators (**Table 7**).

TABLE 6 | Mean (SD) for overall deck ratings (C + D – A + B) per Block for control and stress group.

Blocks	Stress	Control	Total
1	−5.84 (7.48)	1.37 (7.01)	−2.28 (8.07)
2	1.18 (9.36)	4.83 (9.62)	2.99 (9.13)
3	1.60 (8.64)	8.37 (8.81)	4.94 (9.31)
4	3.47 (9.28)	8.51 (11.15)	5.96 (10.49)
5	3.13 (8.08)	9.51 (11.38)	6.28 (10.30)
6	2.39 (8.27)	10.48 (10.65)	6.39 (10.30)
7	2.45 (8.00)	10.16 (10.46)	6.25 (10.01)
Total	1.19 (6.90)	7.61 (8.32)	

TABLE 7 | The overall model and effect of stress manipulation on mediators.

	Overall model	Stress manipulation effect
CRT	$F_{(1,74)} = 80.35$, $p < 0.001$, $R^2 = 0.52$	$b = 3.13$ (SE = 0.35), $t_{(74)} = 8.96$, $p < 0.001$
SBP reactivity	$F_{(1,74)} = 21.54$, $p < 0.001$, $R^2 = 0.22$	$b = -11.89$ (SE = 2.56), $t_{(74)} = -4.64$, $p < 0.001$
Inspection time	$F_{(1,74)} = 90.93$, $p < 0.001$, $R^2 = 0.55$	$b = -0.26$ (SE = 0.03), $t_{(74)} = -9.54$, $p < 0.001$
Conscious knowledge	$F_{(1,74)} = 12.98$, $p < 0.001$, $R^2 = 0.15$	$b = -6.35$ (SE = 1.76), $t_{(74)} = -3.60$, $p < 0.001$

The results were significant for all the indirect pathways. Further path analyses indicated that the direct effect of stress manipulation on IGT was not significant when controlling for indirect pathways, $b = 15.30$ (SE = 12.15), $t = 1.26$, $p = 0.21$. However, there was a significant indirect effect of stress manipulation on IGT scores through CRT, $b = 30.32$ (SE = 7.40), $Z = 4.09$, $p < 0.001$ and conscious knowledge, $b = 13.64$ (SE = 4.67), $Z = 2.92$, $p = 0.003$. Conversely, the indirect effect of stress manipulation through inspection time, $b = 3.45$ (SE = 7.07), $Z = 0.49$, $p = 0.63$, and SBP reactivity, $b = -1.86$ (SE = 3.50), $Z = -0.53$, $p = 0.49$ was not significant. The full model of stress manipulation as a predictor of IGT scores is outlined in **Figure 1**.

DISCUSSION

To summarize our findings: compared to the control condition, stressed participants were slower to avoid the disadvantageous decks and performed worse overall, they inspected disadvantageous decks for longer, and were slower in developing awareness of their poor deck selections compared to the control condition. Conversely, the control condition had longer inspection times for advantageous decks earlier in the task, and earlier accuracy in awareness of the deck contingencies than the stress condition. Path analysis demonstrated that stress reduced performance by impeding reflective thinking and conscious awareness. We now present a more detail review of the findings in relation to our hypotheses.

Inspection time differences between disadvantageous and advantageous decks were observed in the stress condition persisted for longer than in the control group. It was hypothesized that the stress would inhibit the learning of deck contingencies. Participants in the control conditions had more accurate estimates of deck quality in all but Blocks 2 and 4. Finally, path analysis examined direct and indirect effects of the stress manipulation, SBP reactivity, inspection time, CRT and conscious knowledge upon IGT scores. This analysis demonstrated the stress manipulation indirectly affected IGT scores by reducing cognitive reflection and conscious knowledge but did not have a direct effect. These findings are discussed in turn.

The hypotheses related to replication of Simonovic et al.'s (2017a) findings were partially supported. The stress manipulation delayed the optimization of deck selections and reduced reflective ability as indexed by CRT scores. It was hypothesized that CRT scores would correlate in the earlier Blocks for both conditions—this was not consistently observed; however, CRT scores and disadvantageous deck picks were correlated in Block 3, 5, 6 and 7 in the control condition but were not significant in the stress condition.

Manipulation Check and IGT Performance

The stress manipulation successfully increased SBP/HR reactivity. The results showed that stressed participants selected more cards from disadvantageous decks, after the first Block, indicating that their learning was impaired. These findings support Simonovic et al.'s (2017a) findings on a standard extended version of the IGT. These data support previous findings that stress impairs learning and leads to a slower elimination of disadvantageous deck selection (Preston et al., 2007; Starcke et al., 2017; Wemm and Wulfert, 2017). Our findings show that deck selection in the stress condition improved after the fourth trial compared to the control condition where deck selection improved after the first trial.

CRT Results

As with Simonovic et al.'s (2017a), participants in the stress condition had significantly lower CRT scores, indicating that stress reduced reflective ability. These data support the dual process account of IGT where it is assumed that "cool" reflective processes are important in overriding "hot" processes that favor short-term gain (Brevers et al., 2013). According to Brevers et al. (2013) "cool" systems are associated with monitoring options associated with risk and gain. When "hot" systems do not allow risk assessments of the choices, "cool" systems evaluate the risk and benefits of the choices. The overall correlation between the CRT scores and disadvantageous deck selections observed for both conditions indicate that reflective processes are implicated in disambiguation of the disadvantageous deck contingencies. Correlational data further indicate that the reflective processing is significant in both early and late trials (e.g., Simonovic et al., 2017a) rather than being located only when participants have learned the rules of the task (e.g., Starcke et al., 2017). The CRT scores in the control condition correlated with disadvantageous deck selection from early in the task. This indicates that the importance of reflective thinking emerged after the second Block and persisted until the end of the task in the control condition, showing that less reflective participants were more likely to make a disadvantageous choice. However, reflective processing was not a reliable correlate with performance in the stress condition.

This is also in line with Margittai et al.'s (2015) study that demonstrated that higher cortisol levels impaired performance on the original CRT. This indicates that stress disrupts higher order control, mediated by Prefrontal Cortex (PFC; e.g., Arnsten, 2009; Schwabe and Wolf, 2011, 2013). Since

neurochemicals released in response to stress (e.g., dopamine and glucocorticoids) have receptors in the PFC, decision making processes that depend on PFC can be directly affected by stress (e.g., Preston et al., 2007). This also supports Preston et al.'s (2007) argument that under stress, decision making shifts away from deliberative PFC processing towards subcortical areas of the brain and allows automatic, amygdala-mediated processing to dominate. This can explain non-optimal performance and a lack of reflective thinking in the stress condition. Although, cortisol was not measured in this study, the success of the stress induction technique (evidenced by blood pressure and HR responses) means that it is highly likely that cortisol levels affected learning and consequent performance.

Inspection Time

Greater inspection time for disadvantageous decks was observed for participants in the stress condition, particularly from Blocks 2 to 5. One possibility is that there are differences in attentional control between the two conditions due to impaired ability to disengage from the negative choices associated with disadvantageous decks in the stress condition. It may be the case that these findings indicate that participants are merely looking at the decks they select, however the eye-tracking measure is a much finer-grained index of participant behavior and would pick up on subtle differences in attentional focus that a gross deck selection measure may miss. These data are consistent with findings where increased attention towards negative choices are associated with increased negative preferences and poorer learning from punishment (Sapolsky, 2000; Ononaiye et al., 2007; Sposari and Rapee, 2007; Cavanagh et al., 2011). Thus, the findings support the proposal that stress inhibited attentional disengagement from the negative choices. Alternately, the stress condition may have reduced participant learning from feedback by "hijacking" cognitive processes such that somatic markers or deck outcomes were attended to less. Greater inspection time for advantageous decks was observed in Blocks 2, 3 and 4, in the control condition before participants are expected to show awareness of the advantageousness of decks according to traditional SMH accounts (e.g., Bechara et al., 2000; Brand et al., 2007; Starcke et al., 2017). Thus, it is possible that stress impaired both learning from the positive and negative feedback of disadvantageous/advantageous decks, reduced participants' ability for attentional disengagements of disadvantageous choices and increased an awareness of the advantageousness of the good decks in control group.

Conscious Awareness

On average participants in the control condition showed sufficient knowledge of deck quality to guide advantageous long-term choices after the first Block. Deck rating scores in the stress condition suggest that stress interfered with learning as participants failed to develop sufficient knowledge to guide their performance compared to the control group. These data are consistent with previous studies that have shown that the IGT can be performed through access to explicit, conscious knowledge (e.g., Maia and McClelland, 2004, 2005; Fernie and Tunney, 2006, 2013). Thus, while the possibility that somatic markers contribute to IGT performance cannot be ruled out, the results reliably show that stress impaired the conscious processes which are integral to IGT performance. However, it should be noted that the nature of the IGT and the design of Maia and McClelland's (2004) test could promote additional cognitive rather than intuitive processing, because participants are asked to assess deck quality at regular intervals. The present results are, however, in line with the Simonovic et al. (2017a) findings where no measure of conscious awareness was employed. Thus, these data challenge previous research that suggests non-conscious intuitive signals dominate decisional choices in the early stages of the IGT (e.g., Bowman et al., 2005; Maia and McClelland, 2005; Fernie and Tunney, 2006, 2013).

Differences in conceptual knowledge and inspection time for advantageous decks emerged after the second Block. This indicated that the control group had gained sufficient knowledge about the deck contingences and was more focused on the good decks. This also suggested that explicit knowledge runs in parallel with deck selection. Thus, good awareness of deck quality activates cognitive processing about the payoff structure leading to a more optimal decision making strategy. Konstantinidis and Shanks (2014) reported similar findings in a study that used wagering to examine conceptual awareness; participants developed preferences towards the advantageous decks and accurately justified their preferences. This is consistent with Newell and Shanks's (2014) suggestion that conscious awareness diverts attention to positive decisional choices and recruits cognitive processes related to goal-directed behavior. According to this view, conscious awareness initiates executive attention that further initiates executive functioning (e.g., working memory) to reflect on the specific components of the task. Our data indicated that conscious awareness, attentional processing and analytic ability arise early in the task for the control group, however the precise nature of the correlates and causal relationships with learning and performance require further investigation. The results indicated that participants who were more reflective have greater awareness of deck contingences and are more focussed on good decks earlier in the task, implicating Type II processing throughout the task.

Path Analysis

Path analysis revealed that the effect of stress on performance occurs through reduced reflective and conscious awareness rather than different routes. Furthermore, knowledge of deck quality emerged as an additional mediator to reflective ability. The mediators reduced the direct effect of stress manipulation on IGT scores. However, the analysis revealed a weak relationship between the mediators and overall IGT scores. This raises the possibility that the mediators are not strongly related to each other. Preston et al. (2007) and Simonovic et al. (2017a) argued that stress disrupts Type II cognitive processes indicating that performance was not primarily dependent on emotional processing and is more consistent with Brevers et al. (2013) dual

process account of the IGT. However, contrary to Brevers et al.'s (2013) suggestion that "hot," Type I processes guide successful performance, our data indicate that Type II processes guide decision making in the absence of stress. Emotional processing may guide Type II processing early in the task, as it could be argued that a complex interaction between these components give rise to somatic markers. Unpacking this issue is complicated by the evidence that conflict between Type I and Type II processes can be physiologically arousing (e.g., Evans, 2003; De Neys and Glumicic, 2008; De Neys et al., 2010). Thus, it could be argued that rather than emotional processing generating the arousal, it is instead due to the cognitive effort being employed to learn deck contingences.

CONCLUSION

In conclusion, this experiment provides the first examination of conscious awareness under stress on the IGT and the first direct measure of attentional focus during IGT performance. The results of this experiment further clarify the findings from studies that demonstrate a link between stress and IGT performance. Moreover, we demonstrated the importance of reflective cognition, attention and conscious knowledge in later trials but also in the earlier trials traditionally associated with learning rather than performing the task. Induced stress not only impaired decision making performance, but also impeded attention to, and awareness of task characteristics in risky decision making. This was evidenced with the reduced capability for Type II thinking under stress and increased dominance for Type I thinking. These findings are also problematic for the use of the IGT as a diagnostic clinical tool—the importance of reflective processing, early conscious awareness of deck quality and impairment of these through stress all undermine the view that the IGT can diagnose a specific impairment of emotional processes. The results of this experiment support a dual-process account of risky decision making as conscious and effortful processing is impaired in the presence of stress and implicated in its absence.

AUTHOR CONTRIBUTIONS

All authors (BS, ES, MG and DS) made substantial contributions to the conception and design of the study and revised the manuscript for important intellectual content. BS collected the data and drafted the initial manuscript. BS, ES and DS analyzed and interpreted the data.

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Exposure to Unsolvable Anagrams Impairs Performance on the Iowa Gambling Task

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Recent research indicates that external manipulations, such as stress or mood induction, can affect decision-making abilities. In the current study, we investigated whether the exposure to an unsolvable task affected subsequent performance on the Iowa Gambling Task. Participants were randomly assigned to a condition in which they were exposed to unsolvable anagrams (n = 20), or a condition in which they worked on solvable anagrams (n = 22). Afterwards, all participants played the Iowa Gambling Task, a prominent task that measures decision making under uncertain conditions with no explicit rules for gains and losses. In this task, it is essential to process feedback from previous decisions. The results demonstrated that participants who worked on unsolvable anagrams made more disadvantageous decisions on the Iowa Gambling Task than the other participants. In addition, a significant gender effect was observed: Males who worked on unsolvable anagrams made a more disadvantageous decisions than the other male participants. Females who worked on unsolvable anagrams also made more disadvantageous decision than the other female participants, but differences were small and not significant. We conclude that the exposure to unsolvable anagrams induced the experience of uncontrollability which can elicit stress and learned helplessness. Stress and learned helplessness might have reduced the ability to learn from the given feedback, particularly in male participants. We assume that in real life, uncontrollable challenges that last longer than a single experimental manipulation can affect decision making severely, at least in males.

Keywords: decision making, uncontrollability, stress, learned helplessness, cognition, emotion, motivation

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INTRODUCTION

Weber and Johnson (2009) differentiate decision-making situations according to their degree of uncertainty. They can range from complete ignorance (not even the possible outcomes are known) through uncertainty/ambiguity (the outcomes are known but their probabilities are not) to risk (the outcome probabilities are known), and to certainty (only a single outcome is possible). In neuropsychological decision-making research many studies investigated decision making under ambiguity and risk (Brand et al., 2006). In situations of ambiguity the exact contingencies between options and their outcomes are initially unknown. It is not possible to exactly calculate the advantages and disadvantages of an option on the basis of probabilistic calculations. In these situations, learning from feedback is very important. Examples are the choice of a partner or the choice of holiday locations.

Decisions under initial ambiguity are often simulated with the Iowa Gambling Task (IGT; Bechara et al., 1994, 2000). In this task, participants are exposed to four card decks and can choose one card at a time in 100 trials. Each card selection is associated with a financial gain, but in between, money is lost. Card decks differ in net gains which are unknown to the participants at the beginning of the task and must be learned through the given feedback. However, those decks that initially offer high gains are associated with high losses in the long run and participants must learn that the decks with moderate gains are the most advantageous ones in the long run. It has been proposed that emotional and cognitive processes are both involved in task solution (e.g., Guillaume et al., 2009). Early studies with the IGT investigated decision-making abilities in patients with circumscribed brain lesions, for example in patients with prefrontal cortex or amygdala lesions. Patients with lesions or dysfunctions of the ventromedial prefrontal/orbitofrontal cortex or the amygdala often choose the disadvantageous options in the IGT (Bechara et al., 1999). It was concluded that they lack the ability to experience the rewards and punishments or to integrate previous experiences for upcoming decisions. After these early studies, many patient groups with neurological or psychiatric diseases were examined with the IGT (Dunn et al., 2006; Buelow and Suhr, 2009). For example, patients with basal ganglia dysfunction due to Parkinson's disease show disadvantageous performance (Kobayakawa et al., 2008, 2010). Deteriorations in patient groups were interpreted as emotional and cognitive deficits to integrate prior consequences into the current decision-making process. In addition, healthy participants were examined with the IGT and their performance has been related with several personality traits and other trait- and state-variables. For example, it has been demonstrated that IGT performance is negatively related to trait anxiety (Miu et al., 2008) and a negative relationship between IGT performance and neuroticism has been found in older adults (Denburg et al., 2009). The IGT is also sensitive to external manipulations such as stress induction (Preston et al., 2007; van den Bos et al., 2009; Wemm and Wulfert, 2017) and mood induction (de Vries et al., 2008). The results indicate that stress overall deteriorates performance in the IGT. Participants in a positive mood perform better in the early trials of the IGT compared to participants in a negative mood.

The current study investigates how the exposure to unsolvable anagrams affects subsequent IGT performance. Unsolvable tasks elicit a psychological state of uncontrollability. Demands that go beyond the capacities of an individual and that are unpredictable or uncontrollable elicit stress (Dickerson and Kemeny, 2004; Koolhaas et al., 2011). Unsolvable tasks have been used as stress induction procedures in recent studies, for example, the Montreal Imaging Stress Task (Dedovic et al., 2005). Participants are exposed to arithmetic tasks that are unsolvable within the given time limit and a fictitious average and expected performance is presented. Participants believe that the tasks should be solvable, but that they are unable to do so. This procedure leads to increases of the stress hormone cortisol. A recent study indicated that those participants who show a high cortisol response also showed an increase in activation in dorsomedial and dorsolateral prefrontal cortex regions (Dedovic et al., 2009b). When their performance was negatively evaluated, their brain activity was reduced in the medial orbitofrontal cortex and the hippocampus. Acute stress induction is proposed to affect decision making in two ways (Starcke and Brand, 2016): increased reward seeking and risk taking due to alterations in dopamine firing rates (motivational and emotional changes); and reduced executive control due to suboptimal prefrontal cortex functioning (cognitive changes). The stress hormone cortisol leads to increased dopaminergic activity (Ungless et al., 2010) which influences reward prediction and feedback learning (Shohamy et al., 2008). Dopaminergic neurons particularly respond towards stimuli that predict high and immediate rewards (Morris et al., 2006; Kobayashi and Schultz, 2008). Focusing on immediate and initially high rewards is dysfunctional in the long run when performing the IGT. Furthermore, stress is supposed to impair executive functions (Hermans et al., 2014) because the release of stress hormones can impair prefrontal cortex functioning. Executive functions are involved in the latter trials of the IGT (Brand et al., 2007).

The exposure to unsolvable tasks not only induces stress, but also learned helplessness (Peterson et al., 1993). Learned helplessness is a psychological state in which individuals experience that none of their actions affects outcomes and they cannot control the situation. Thus, they experience no contingency between action and outcome no matter which action they undertake. Many people react with motivational, emotional and cognitive distortions, i.e., they become passive, depressed, and are unable to discover an adaptive behavioral reaction although an adaptive reaction exists (Seligman, 1975; Maier and Seligman, 1976). According to the theory of learned helplessness, previous experiences of uncontrollability result in the belief that situations are always uncontrollable. As a consequence, individuals who learned to be helpless have difficulties in finding solutions even in situations in which solutions exist. That means, they perform worse than participants who were not exposed to uncontrollable situations before (Hiroto and Seligman, 1975). On a neural level, experimentally induced learned helplessness affects cerebral blood flow in the amygdala and the hippocampus (Schneider et al., 1996). During exposure to unsolvable anagrams, activity in the amygdala increased, while activity in the hippocampus decreased. Altered amygdala activation could affect the experience of rewards and punishments during IGT performance, and decreased hippocampal activation could decrease learning processes in the IGT.

In the current study, we hypothesize that participants who are exposed to unsolvable anagrams subsequently perform worse in the IGT than control participants. The manipulation should induce the experience of uncontrollability which elicits stress and learned helplessness. To the best of our knowledge this is the first study that examined the effect of unsolvable tasks on the IGT in humans. However, recent studies indicate that stress induction with social evaluative stressors affects performance on

the IGT (Preston et al., 2007; van den Bos et al., 2009; Wemm and Wulfert, 2017). In addition, a recent animal study reported effects of inescapable footshocks on a rat gambling task (Nobrega et al., 2016). Rats that were exposed to inescapable footshocks showed an increase in disadvantageous choices relative to control rats. Inescapable footshocks reliably induce the experience of uncontrollability in animals with stress and learned helplessness as a consequence.

PARTICIPANTS AND METHODS

Participants

Overall, 54 participants took part in the study. Most of them were students and received course credits for their participation and no financial compensation. The other participants did not receive any course credits and were not paid either. Due to ethical reasons they were asked if they had chronic or acute diseases (including psychiatric diseases), or acute psychological problems. If they affirmed one of these questions they were excluded from participation. The study was approved by the local ethics committee (division of Computer Science and Applied Cognitive Sciences at the Faculty of Engineering, University of Duisburg-Essen) and all participants provided written informed consent. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the committee of the division of Computer Science and Applied Cognitive Sciences at the Faculty of Engineering, University of Duisburg-Essen. Half of them were randomly assigned to the experimental group (EG) in which they worked on unsolvable anagrams, and the other half was assigned to the control group (CG) in which they worked on solvable anagrams.

Methods

Solvable and Unsolvable Anagrams

All participants received 20 anagrams with four letters and were required to form a word out of these letters. However, only the anagrams given to the CG were solvable, whereas the anagrams given to the EG were unsolvable. All anagrams were designed in the participants' native language, i.e., German. Participants were instructed to form one new German word out of each anagram. Given names and homonyms were not allowed. Examples of solvable anagrams were given before the task started. In the CG, solvable anagrams were then presented. An example of a solvable anagram is the word EURE (yours) which can be converted into REUE (regret). In the EG, unsolvable anagrams were presented. An example of an unsolvable anagram is the word KIND (child) which cannot be converted into any other German word. Participants worked 15 min on the anagram task. The anagrams were presented as a paper and pencil task and participants were given all anagrams at once with the time limit of 15 min. They were not allowed to ask any questions within task performance. The anagrams were tested in a pre-study in order to ensure that the solvable anagrams are solvable within the given time limit, but were not so easy that they could be completed immediately. The complete list of anagrams can be seen in **Table 1**.

Decision-Making Performance

All participants performed the computerized version of the IGT (Bechara et al., 2000). In the IGT, participants are exposed to four card decks, A, B, C, and D, and can choose one card at a time (for recent research on construct validity and reliability see Buelow and Suhr, 2009; Buelow and Barnhart, 2017). They are required to gain as much fictitious money as possible and to lose as few money as possible. The task has 100 trials and each card is associated with a financial gain, but in between, money is lost. The contingencies are unknown to the participants, but they receive a feedback after each choice. The exact amount of money that is gained or lost is displayed on the screen (you win/you lose) and visual (smiley or frowny) and acoustic signals (pleasant or unpleasant sound) accompany the feedback. Card decks differ in net gains: Decks A and B are disadvantageous in the long run, whereas decks C and D are advantageous in the long run. Decks A and B offer high gains at the beginning of the task, but during task performance high losses occur in between that exceed the gains in the long run. Decks C and D offer medium gains, but only small losses. This must be discovered by the participants through the processing of the given feedback. To analyze the results, the netscore is calculated: the number of disadvantageous choices is subtracted from the number of advantageous choices. A positive netscore indicates that more advantageous than disadvantageous decisions were made and *vice versa*. The course of the IGT can be subdivided into five blocks of 20 trials. A netscore for each block of trials (1–20, 21–40, 41–60, 61–80, 81–100) can be built.

Measurement of Current Affect

To measure current affect prior to and after the experimental manipulation, the Positive and Negative Affect Schedule (Watson et al., 1988) was used. The questionnaire consists of 10 positive and 10 negative adjectives that should be answered on a five point Likert scale from 1 "very few or not at all" to 5 "very much". Scores were averaged for the positive and the negative affect dimension separately. Thus, results for positive and negative affect can each range from 1 to 5.

Measurement of Personality

To measure the big five personality characteristics, the short version of the Big Five Inventory (Rammstedt and John, 2007) was used. The five personality dimensions conscientiousness, neuroticism, extraversion, openness to experiences and agreeableness are assessed with two items each. Items are answered on a five point Likert scale from 1 "not at all" to 5 "completely". After recoding inverted items scores were averaged for each dimension separately. Thus, scores for each personality dimension can range from 1 to 5. Personality has been assessed to demonstrate that EGs show no major differences in their personality traits.

Measurement of General Response Style

To measure the general response style towards dysphoria and depression, the Response Styles Questionnaire (Kühner et al., 2007) was used. The questionnaire consists of 32 items of which 21 measure the response style rumination and 11 measure the

TABLE 1 | Solvable and unsolvable anagrams.

Solvable anagrams (translation)	Possible solution (translation)	Unsolvable anagrams (translation)
LEIB (body)	Beil (axe)	KIND (child)
BRIE (brie)	Rieb (rubbed)	MORD (murder)
ODEM (breath)	Mode (fashion)	ALSO (thus)
BAUT (builds)	Taub (deaf)	AURA (aura)
RIEF (called)	Reif (ripe)	BANN (ban)
AMTS (official)	Mast (pole)	BAUM (tree)
EGAL (whatever)	Lage (position)	HILF (help)
LIEH (borrowed)	Heil (salvation)	BLUT (blood)
FLAU (slack)	Lauf (run)	HOSE (trousers)
ROTE (red)	Tore (gates)	NOTE (note)
ADLE (ennoble)	Lade (lade)	HAND (hand)
KLEE (clover)	Ekel (disgust)	AUTO (car)
EURE (yours)	Reue (regret)	SINN (meaning)
SIEL (tide gate)	Seil (rope)	BALL (ball)
FEIL (for sale)	Fiel (fell)	WACH (awake)
TORS (genitive of gate)	Rost (rust)	REGE (active)
HELM (helmet)	Mehl (flour)	HUND (dog)
REBE (vine)	Eber (boar)	BAHN (train)
HALM (stalk)	Mahl (meal)	HAUS (house)
EDER (a German river)	Rede (speech)	VERB (verb)

Translations and solutions are examples of possible translations and solutions.

response style distraction. Each item can be answered on a four point Likert scale from 1 "nearly never" to 4 "nearly always" and scores are summed up. Scores for rumination can range from 21 to 84, scores for distraction can range from 11 to 44. General response style has been assessed to demonstrate that EGs show no major differences in their general coping styles.

Design and Procedure

After providing written informed consent, participants filled out questionnaires on current baseline affect and personality. Then, they were exposed to the experimental manipulation with either solvable or unsolvable anagrams. Affect was measured again after the manipulation. After that, all participants played the IGT. Then, general response style was measured. Finally, demographic variables were assessed and participants were asked if they knew the IGT. All participants were fully debriefed and thanked for participation after they finished the study.

Statistical Analyses

Data were analyzed with SPSS version 20 (IBM, Armonk, NY, USA). Differences between the EG and the CG concerning age, response style, and personality were calculated with *t*-tests. Group differences concerning the gender distribution were calculated with a chi-square test (X^2). Potential changes in current affect before and after the experimental manipulation were calculated with repeated-measures analysis of variances (ANOVAs) with "group" as between-factor, and "point in time" as within-factor. The decision-making performance was also calculated with a repeated-measures ANOVA with "group" as between-factor, and "IGT-blocks" as within factor. Greenhouse-Geisser correction was applied when appropriate and partial eta squared (η_p^2) was used as effect size. In order to analyze moderating effects of age, affect and openness to experiences moderated regression analyses were used in which the "IGT netscore" was the dependent variable, and "group" was the predictor. "Age", "positive and negative affect" after the experimental manipulation, and "openness to experiences" were included as moderating variable in each of the regressions. An ANOVA with "group" and "gender" as factors and "IGT netscore" as dependent variable was performed to analyze potential gender effects.

RESULTS

Participants

None of the participants quit the study. However, at the end of the study participants were asked if and if yes from where they knew the IGT. Twelve participants admitted to know the task (mainly from other studies, seven in the EG and five in the CG). They were excluded from further analysis because participants usually receive a debriefing after playing the IGT and thus know the contingencies of the decks. In the EG 20 participants and in the CG 22 participants remained. The excluded participants had a significantly higher IGT netscore than the IGT naive included participants ($p = 0.005$). Of the 42 included participants 30 were females and participants' age ranged from 18 to 54. Groups did not differ concerning age (mean EG = 21.50, $SD = 3.25$, mean CG = 22.36, $SD = 7.66$, $t = -0.47$, $df = 40$, $p = 0.64$) and gender (EG = 14 females, CG = 16 females, $X^2 = 0.04$, $df = 1$, $p = 0.85$). Most of the participants studied Applied Cognitive Sciences at the University of Duisburg-Essen (17 in the EG and 16 in the CG) and received course credits for their participation. The other participants were acquaintances of the investigator JDA and studied other fields or worked in a graduate occupation (six), worked in a skilled occupation (two), or went to school (one). Results for response style and personality indicate that groups did not differ, except concerning openness to experiences which is higher in the EG

TABLE 2 | Results for personality and response style in both groups.

	EG mean (*SD*)	CG mean (*SD*)	*T*	*df*	*p*
Neuroticism	3.20 (1.01)	2.95 (0.94)	0.82	40	0.42
Extraversion	3.20 (0.83)	3.32 (1.04)	−0.40	40	0.69
Openness	4.40 (0.60)	3.59 (1.10)	3.00	33.06	0.005
Agreeableness	3.05 (0.89)	2.80 (0.98)	0.88	40	0.39
Conscientiousness	3.35 (1.09)	3.18 (0.84)	0.56	40	0.58
Distraction	27.60 (6.33)	25.14 (5.97)	1.30	40	0.20
Rumination	46.80 (11.36)	49.14 (11.00)	−0.68	40	0.50

EG, experimental group; CG, control group; SD, standard deviation.

(see **Table 2**). Results predominantly demonstrate successful randomization.

Changes in Current Affect

The 2 × 2 repeated measures ANOVA for positive affect demonstrates that there was no significant main effect for "point of measurement", $F_{(1,40)} = 0.76$, $p = 0.39$, $\eta_p^2 = 0.02$, no significant main effect for "group", $F_{(1,40)} = 1.44$, $p = 0.24$, $\eta_p^2 = 0.04$, but a significant interaction of "group" × "point of measurement", $F_{(1,40)} = 4.04$, $p = 0.05$, $\eta_p^2 = 0.09$. Concerning negative affect there was no significant main effect for "point of measurement", $F_{(1,40)} = 1.72$, $p = 0.20$, $\eta_p^2 = 0.04$, a significant main effect for "group", $F_{(1,40)} = 8.16$, $p < 0.01$, $\eta_p^2 = 0.17$, and no significant interaction of "group" × "point of measurement", $F_{(1,40)} = 1.82$, $p = 0.19$, $\eta_p^2 = 0.04$. Results can be seen in **Figures 1**, **2**. In the CG there was a small increase in positive affect, whereas participants in the EG experience a decrease in positive affect after the experimental manipulation. The EG had a higher negative affect than the CG prior to and after the experimental manipulation.

Decision-Making Performance

The 5 × 2 repeated measures ANOVA for IGT performance demonstrates that there was a significant main effect for "block", $F_{(3.13,125.06)} = 6.98$, $p < 0.001$, $\eta_p^2 = 0.15$, a significant main effect for "group", $F_{(1,40)} = 3.99$, $p = 0.05$, $\eta_p^2 = 0.09$, but no significant interaction of "group" × "block", $F_{(3.13,125.06)} = 1.09$, $p = 0.36$, $\eta_p^2 = 0.03$. Results can be seen in **Figure 3**. Results indicate that performance increased during the course of the task in both groups, but that the EG overall performs worse than the CG (mean netscore EG = −2.20, *SD* = 28.76, mean netscore CG = 14.64, *SD* = 25.86). No different learning curves of the EG and the CG were observed as there was no significant interaction between groups and blocks. Age, openness to experiences and post manipulation affect did not moderate the effect of the predictor "group" on "IGT netscore" (*p*s > 0.05). The factor "gender" interacted with the factor "group" ($F_{(1,38)} = 4.78$, $p < 0.05$, $\eta_p^2 = 0.11$): Females of the EG and CG differed from one another, but only on a descriptive level (mean netscore $EG_{females} = 3.14$, *SD* = 31.55, mean netscore $CG_{females} = 8.75$, *SD* = 23.25, $t_{(28)} = -0.56$, $p = 0.58$). Male participants of the EG performed significantly worse than male participants of the CG (mean netscore $EG_{males} = -14.67$, *SD* = 16.95, mean $netscore_{males}$ CG = 30.33, *SD* = 27.93, $t_{(10)} = -3.37$, $p < 0.01$). In the CG, there was a trend towards males outperforming females ($t_{(20)} = -1.84$, $p = 0.08$), while in the EG no differences between males and females were observed ($t_{(18)} = 1.29$, $p = 0.21$).

DISCUSSION

The results of the current study indicate that the exposure to an unsolvable anagram task led to a decrease in decision-making performance in the IGT, which is initially an ambiguous decision-making task. A similar result has been reported for rats in a rat gambling task who were exposed to inescapable footshocks (Nobrega et al., 2016). The exact result pattern of the current study indicates that the individuals exposed to

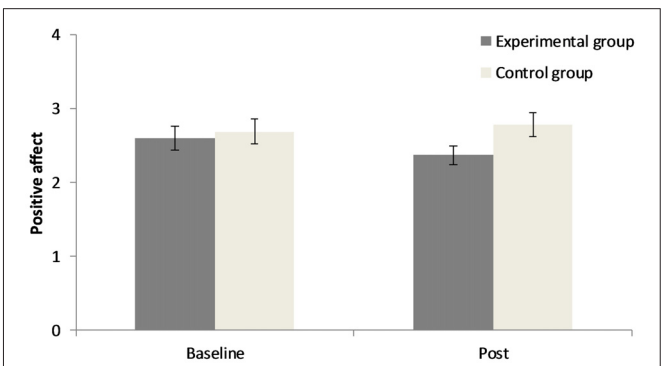

FIGURE 1 | Changes in positive affect before and after the experimental manipulation in both groups. Error bars represent ± one standard error of the mean.

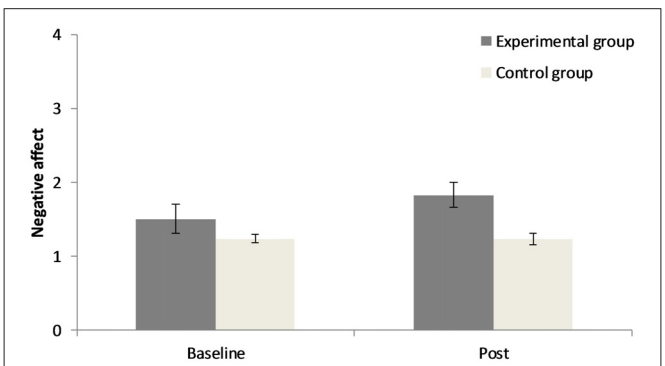

FIGURE 2 | Changes in negative affect before and after the experimental manipulation in both groups. Error bars represent ± one standard error of the mean.

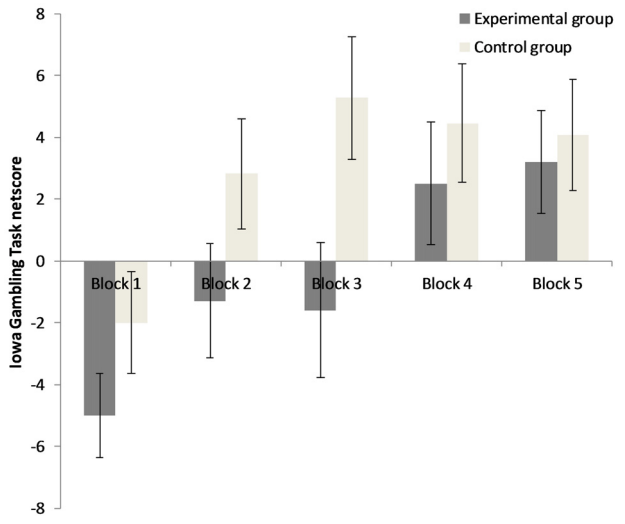

FIGURE 3 | Decision-making performance in both groups for the five blocks of 20 trials. Error bars represent ± one standard error of the mean.

unsolvable anagrams reach a positive IGT netscore during trials 61–80, while control participants already reached a positive netscore during trials 21–40. Brand et al. (2007) postulate, that the early trials of the IGT (1–40) are ambiguous and rely on emotional feedback processing, while the latter trials (41–100) have a more explicit character because contingencies have already been learned by several participants. They demonstrated that only the latter trials are related to executive functions and strategic decision making. However, the shift from ambiguity to explicitness varies from person to person and has no clear cut-point. In the current study, the control participants of our healthy sample showed the typical profile of healthy participants who prefer the advantageous options after a number of trials. In contrast, participants exposed to the unsolvable task show a delayed preference for the advantageous options on a descriptive level. However, the interaction between group and block of trials did not reach significance, so we observed an overall deteriorating effect and no different learning curves. A significant gender effect was observed: Males exposed to the unsolvable anagrams showed worse IGT performance than males in the CG. In females, differences pointed in the same direction, but only on a descriptive level. In the CG, males tended to outperform females. Both findings are in line with recent studies in the field: males are (on a descriptive level) more prone to stress induced deteriorations in the IGT compared with females (Preston et al., 2007) and the relationship between individual stress responses and IGT performance is different for males and females (van den Bos et al., 2009; Wemm and Wulfert, 2017). Under no stress conditions, males make more advantageous decisions than females (van den Bos et al., 2013). We propose that the unsolvable anagrams induced a state of uncontrollability which elicits stress and learned helplessness (Peterson et al., 1993; Dedovic et al., 2005) affecting emotion, cognition and motivation, particularly in our male participants.

Participants who worked on unsolvable anagrams had decreased positive affect after the experimental manipulation and a higher negative affect than control participants from the beginning. This mood state might have interfered with the development of emotional signals that guide the decision process in an advantageous direction. The IGT strongly relies on emotional feedback learning (Bechara et al., 1999). According to the somatic marker hypothesis (Damasio, 1996), somatic signals "mark" the advantageous options even before conscious knowledge about their valence exists. Thus, participants might feel which options are advantageous even before they explicitly know. The reliance on feelings in the IGT appears to be easier when one is currently in a good mood (Suhr and Tsanadis, 2007; de Vries et al., 2008). In the current study, negative affect and reduced positive affect could have reduced emotional feedback learning. However, affect after the experimental manipulation did not moderate the main results.

Participants who were exposed to unsolvable anagrams also might have had reduced cognitive capacities in recognizing response-outcome relations. During the course of the IGT many participants learn which are the advantageous options and know them explicitly at the end of the game (Guillaume et al., 2009). Those participants outperform participants without explicit knowledge. In this case, the task loses its ambiguous character. As mentioned before, during the latter trials of the IGT relationships with other cognitive tasks were observed (Brand et al., 2007). Thus, cognitive abilities play a role in the IGT at the later trials of the task. Classical studies indicate that exposure to uncontrollability leads to disturbances in cognitive tasks (Hiroto and Seligman, 1975; Miller and Seligman, 1975) and a recent study also demonstrated a tendency in that direction (Taylor et al., 2014). Recent research suggests that stress can also lead to reduced performance in cognitive tasks (Starcke et al., 2016). In the current study, cognitive deteriorations might have led to reduced explicit knowledge about the winning and losing probabilities of each deck.

Participants in the unsolvable task condition might also have had motivational deficits. A necessary precondition for successful IGT performance is the motivation to do so. That means, participants must continue their effort in task performance even if they do not recognize the contingencies quickly. Reduced persistence might lead to random deck choices instead of attentive exploration. Motivational deficits are discussed as a feature of learned helplessness (Maier and Seligman, 1976) which means that individuals stop exploring potential solutions too early. Another feature of the IGT is that the options that offer high gains lead to high losses in the long run. Thus, participants have to override the urge to choose options with potential high gains. Under stress, potential high gains have a particularly high salience and potential losses might be ignored (Mather and Lighthall, 2012). The willingness to choose the decks with small, but long-term gains might have been reduced in the EG of the current study.

Participants who worked on unsolvable anagrams might also have been affected by a specific neural pattern. The IGT is thought to rely on numerous brain regions including the ventromedial prefrontal/orbitofrontal cortex, the limbic system

(Bechara et al., 1999), and the basal ganglia (Kobayakawa et al., 2008). Learned helplessness and stress have been found to alter activation in these brain regions: Schneider et al. (1996) measured cerebral blood flow with positron emission tomography in participants at baseline and when exposed to solvable and unsolvable anagrams. In the solvable anagram condition, blood flow increased in the hippocampus and decreased in the mammillary bodies, while in the unsolvable condition, blood flow increased in the mammillary bodies and the amygdala and decreased in the hippocampus. A neural pattern like this might alter normal amygdala activation during IGT performance and decrease hippocampal mediated learning abilities. Acute stress also leads to metabolic changes in the prefrontal cortex, limbic system and basal ganglia, and to the secretion of stress hormones such as cortisol (Dedovic et al., 2009a; Pruessner et al., 2010). Excessive cortisol secretion can lead to reduced prefrontal functioning and impair executive functions (Hermans et al., 2014). Increased dopaminergic activity due to cortisol secretion (Ungless et al., 2010) can influence reward prediction and feedback learning (Shohamy et al., 2008). More precisely, stress increases the salience for potential high gains while potential losses are ignored (Mather and Lighthall, 2012).

The above mentioned mechanisms (with the exception of current affect) have not been investigated directly in the current study which limits the conclusions that can be drawn. Future studies should address in more detail the emotional, cognitive, motivational and brain alterations that potentially mediate the effects of unsolvable tasks on decision making. The manipulation check was restricted to changes in current affect. We did not assess the experience of stress and learned helplessness with questionnaires directly because we did not want to evoke the suspicion that the anagrams were unsolvable in the EG. However, more fine grained manipulation checks would be helpful, for example the assessment of physiological and endocrine stress responses, or a questionnaire that measures acute feelings of helplessness in a subtle way. The manipulation with unsolvable anagrams could induce a lot of psychological states such as stress, learned helplessness, ego-depletion, frustration, insecurity, reduced self-esteem, or mood changes. Therefore, it cannot be truly concluded whether the effects reported primarily depend on cognitive, emotional or motivational factors. A further limitation is that the poor IGT performance after the exposure to the unsolvable anagrams can be attributed to the relative small subgroup of male participants. Thus, conclusions can be drawn for males only so far. However, the effects of our female participants point in the same direction, albeit they are smaller and not significant. Future studies should examine equal sized groups of males and females and explore reasons for the gender differences that were observed in the current study. Furthermore, EGs differed concerning their openness to experiences. However, this personality trait did not moderate the main results of the study and recent research also suggests that openness to experiences is unrelated to IGT performance (Denburg et al., 2009).

Current results indicate that the single exposure to an unsolvable task impairs decision-making abilities in an ambiguous situation, particularly in males. In real life, exposure to uncontrollable situations can be long lasting, such as excessive demand in school or job, or continuing failure in finding a job or partner. This might lead to severe deteriorations in real life decision making in ambiguous situations. It is possible that the risk of developing depressive symptoms increases. It has been demonstrated that participants who were exposed to an uncontrollable situation showed cognitive performance similar to patients who suffer from depression (Miller and Seligman, 1975). In extreme cases, people could give up decision making completely. Indecisiveness is included as a diagnostic characteristic of depression in the current Diagnostic and Statistical Manual for Mental Disorders (American-Psychiatric-Association, 2013).

AUTHOR CONTRIBUTIONS

KS designed the study and wrote the manuscript; JDA designed the study, collected the data, and analyzed the data; MB designed the study and revised the manuscript.

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Writing About Past Failures Attenuates Cortisol Responses and Sustained Attention Deficits Following Psychosocial Stress

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Acute stress can harm performance. Paradoxically, writing about stressful events—such as past failures—has been shown to improve cognitive functioning and performance, especially in tasks that require sustained attention. Yet, there is little physiological evidence for whether writing about past failures or other negative events improves performance by reducing stress. In this experiment, we studied the effects of an acute psychosocial stressor, the Trier Social Stress Test, on attentional performance and salivary cortisol release in humans. Additionally, we investigated whether an expressive writing task could reduce the detrimental effects of stress, both on performance and physiological response. We found that when individuals were asked to write about a past failure before experiencing a stressor, they exhibited attenuated stress responses. Moreover, those who wrote about a past failure before being exposed to stress also exhibited better behavioral performance. Our results suggest that writing about a previous failure may allow an individual to experience a new stressor as less stressful, reducing its physiological and behavioral effects.

Keywords: stress, cortisol reactivity, expressive writing, Trier Social Stress Test (TSST), sustained attention, psychosocial stress, cortisol

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INTRODUCTION

Acute stress can be harmful to performance. In a real world setting, high levels of stress have been known to cause individuals to "choke under pressure," resulting in suboptimal performance (Beilock and Carr, 2005). "Choking under pressure" has been found to occur in both physical settings, such as high-stakes sporting events (Baumeister, 1984), and in classroom settings, such as during important exams (Beilock and Carr, 2005). Acute stress seems particularly detrimental to performance on tasks that require high levels of sustained attention. In the laboratory, acute stress has been shown to lead to higher rates of error on tasks requiring high levels of sustained attention (Qian et al., 2015).

Because acute stress is harmful to performance, there has been a recent interest in developing stress reduction interventions. Expressive writing, particularly about negative events such as current anxieties, has been shown to lead to *improvements* in performance (DiMenichi and Richmond, 2015), even in a high-stress environment (Ramirez and Beilock, 2011). Although this outcome is counterintuitive, it has been proposed that writing about negative life events leads to positive outcomes because it relieves stress that normally occurs as a result of attempting to inhibit

thoughts about these negative life events (Pennebaker, 1997). However, the assertion that stress reduction is the mechanism by which expressive writing about negative events leads to positive outcomes has been understudied. Specifically, writing about failures has been shown to lead to performance improvements on tasks requiring sustained attention (DiMenichi and Richmond, 2015). However, it remains unknown whether writing about failures improves sustained attention because writing about failures reduces stress, or because it allows an individual to perform better *despite* experiencing physiological stress, perhaps by boosting psychological resources (Hemenover, 2003). If writing about past failures prior to an acute stressor reduces stress, then we would observe a reduction in endocrine response to that acute stressor, along with less of an impairment on performance in a sustained attention task following stress.

Acute stress has been shown to activate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of the hormone cortisol in both animals and humans (Hanson et al., 1976). Furthermore, cortisol reliably peaks in the saliva in humans about 20 min after an individual experiences a stressor (Kirschbaum et al., 1993; Dickerson and Kemeny, 2004). However, there is evidence that this response can be buffered with proper stress-reduction interventions (Smyth et al., 2008). Thus, measuring cortisol during and after a laboratory stressor may shed light on whether expressive writing about a negative event prior to stress can act as a stress-reduction intervention.

In the current study, we examined whether expressive writing about a past failure reduces one's cortisol response to a new psychosocial stressor. We hypothesized that experiencing a psychosocial stressor would result in an increase in cortisol, but writing about a failure before experiencing the stressor would attenuate this cortisol response. We also examined whether expressive writing about past failures improves performance on a task requiring persistent, sustained attention directly after experiencing psychosocial stress. We predicted that stress would harm performance, and that writing about a past failure would attenuate this effect.

MATERIALS AND METHODS

Participants

One hundred and two participants were recruited from the surrounding area of Rutgers University, Newark. Our sample size was based on the performance effect of DiMenichi and Richmond (2015) and the cortisol effect of Kirschbaum et al. (1993). We also ran an additional power analysis based on the averaged effect size from two previous studies that utilized stress interventions ($f^2 = 0.28$) on cortisol after the Trier Social Stress Test (TSST) (Gaab et al., 2003; Hammerfald et al., 2006). With stress group, writing group, and gender as factors, as well as an error probability of 0.05, this analysis suggests a total sample size of 86 participants. We are therefore confident that our sample size meets adequate power requirements.

The study was approved by the Institutional Review Board at Rutgers University. Participants (mean age = 24.09, *SD* = 7.36; 54% female; 21% white/Caucasian, 25% black/African American, 34% Asian, 1% Native American, 12% "other") were paid $15 for 1.5 h of participation. All participants completed the study between 1 and 5 p.m., in order to control for circadian fluctuations of cortisol (Dickerson and Kemeny, 2004). Subjects were naive to the purpose of why the saliva samples were being collected. Our saliva testing lab alerted us that two participants produced saliva samples that were contaminated (presumably from food content in the saliva); therefore, their data were not analyzed. Two additional participants' data were removed from analyses after participants failed to follow instructions (i.e., did not write about their assigned writing prompt).

Task

Procedure Timeline

Six cortisol samples were obtained throughout the experiment using salivary cheek swabs. After arriving at the laboratory and following giving written consent, participants provided the first salivary cortisol sample (T0), which served as a baseline measurement. Participants were then pseudo-randomly assigned to complete the "failure" or "control" writing manipulation (see below for a detailed description of the writing manipulation). After completing the 10-min writing manipulation, a second salivary cortisol sample was obtained (T1; 15 min elapsed since T0; 20–25 min since arrival). Participants were then pseudo-randomly assigned to complete the TSST (Kirschbaum et al., 1993) or a control task. After completing the TSST or control task, a third saliva sample was taken (T2; 35 min elapsed since T0). Participants then completed the sustained attention to response task (SART; McVay and Kane, 2009; DiMenichi and Richmond, 2015) immediately after completing the TSST to examine the effect of psychosocial stress on attentional performance. Halfway through the SART, a fourth saliva sample was obtained (T3; 55 min elapsed since T0). Finally, the fifth saliva sample was collected at the conclusion of the SART (T4; 70 min elapsed) and the sixth was collected at the conclusion of the survey battery (T5; 85 min elapsed). See **Figure 1** for experimental groups and cortisol timeline.

Cortisol collection and assay procedures

Participants were asked to refrain from eating or drinking anything (besides water) at least 1 h prior to participating in the study. Salivary cortisol samples were collected using Salimetrics Oral Swabs. Participants were asked to hold swabs in their cheek for approximately 2 min and to saturate each swab as much as possible with saliva. After this time elapsed, participants were asked to spit the swab into a Salivette vial. Vials were stored in a freezer at −20° before being shipped on dry ice to Salimetrics LLC (Carlsbad, CA, United States), where each sample was assayed twice. The intra-assay variability was 4.66% and the inter-assay variability was 4.47%.

Writing manipulation

Participants were pseudo-randomly assigned to complete a writing manipulation adapted from DiMenichi and Richmond (2015). In the "failure" condition, participants saw a prompt on a computer screen that asked them to spend the next 10 min

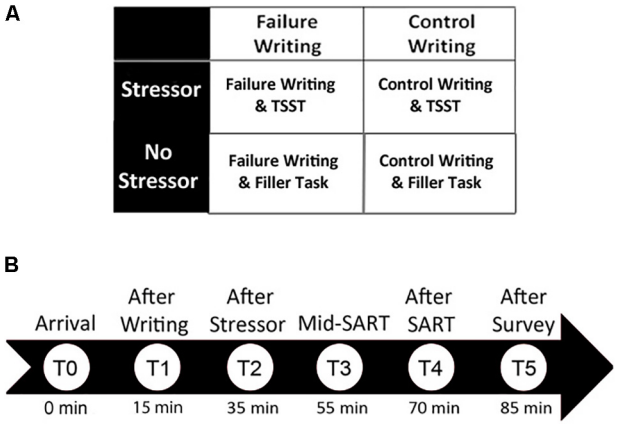

FIGURE 1 | Experimental method. **(A)** Participants were assigned to one of four conditions in which they wrote about a failure or a control topic, and then experienced a stressor or control activity. **(B)** Six salivary cortisol samples were obtained throughout the experiment. Because previous research has found that cortisol peaks about 20 min after a stressor is experienced, all samples represent peak cortisol as a result of the previous event.

writing about a difficult time in which they did not succeed. They typed their response on the computer. Participants pseudo-randomly assigned to the "control" condition were prompted to write about the plot of a movie they had recently viewed. In order to control for the effect of mood, a follow-up study verified that asking participants to write about a sad movie did not have an effect on attentional performance, suggesting that mood alone is not likely to be the mechanism by which failure writing improves performance on the SART (see Supplementary Material).

Since previous research has found that individual differences within each writing sample (e.g., emotional intensity) can lead to individual differences in outcomes (Harber et al., 1992), two research assistants blind to cortisol and behavioral results read each participant's writing sample and coded the writing sample for the following five elements: valence (overall positive and negative tone of writing), emotional arousal (i.e., a rating pertaining to how emotional the sample was), compliance with the prompt, relation to oneself, and relation to persistence). Each category was rated with a single score from 1 to 5.

Trier Social Stress Test (TSST)

Immediately after completing the writing manipulation, participants assigned to the stress condition completed the TSST (Kirschbaum et al., 1993). The TSST proceeded as follows: the experimenter asked participants about their current career or major, and probed them about their "dream job." Then, participants were told they would have 6 min to prepare a 5-min speech about why they possess the qualities for their "dream job." They were also told that they would have to give their 5-min speech in a job-interview format to a "speech expert" while being videotaped and behaviorally analyzed (the "speech expert" was actually a research assistant from the lab). While the participant gave his or her speech, the confederate responded in a cold and unsympathetic manner. If participants did not take the entire 5 min to complete the speech, the speech expert alerted them of the time remaining, and asked them to continue. After 5 min, the speech expert asked the participant to count backwards from 2063 by 13. If the participant made a mistake, he or she was asked to start over from 2063. After 5 min, the speech expert asked the participant to stop.

Participants pseudo-randomly assigned to the control task were probed about their career goals, and then were asked to complete an innocuous personality survey tapping the five OCEAN personality traits for 16 min while alone in a testing room.

Sustained Attention to Response Task (SART)

Immediately following the conclusion of the psychosocial stress manipulation or control task, all participants completed a SART (McVay and Kane, 2009; DiMenichi and Richmond, 2015). In this simple "go/no-go" task, participants were told to press the space bar as soon as a letter appeared on the screen, unless that letter was a vowel. Participants were given 2 s to respond to each trial, and the entire SART lasted about 30 min in order to require persistent attention to complete. There were 600 trials, and 20% of trials were vowels (all letters were included except Y).

Survey battery

After completing the SART, participants provided information about demographics and daily habits, including smoking habits, contraceptive use, and information about menstrual cycles, since these factors may affect cortisol levels. Furthermore, we distributed a survey battery so that we could explore whether individual differences in cortisol response or SART performance were related to personality traits. The battery included the General Causality Orientations Scale, which assesses intrinsic vs. extrinsic motivations, as well as how much an individual believes circumstances are mostly a matter of luck (Deci and Ryan, 1985); the Connor-Davidson Resilience Scale, which measures individual differences in trait resiliency (Connor and Davidson, 2003); and the Achievement Goal Questionnaire, which examines preference for wanting to achieve goals in order to master a new skill, perform well, or avoid failure (Elliot and Church, 1997). Surveys that examined emotional tendencies included the Beck Depression Inventory-II (Beck et al., 1988), and the Perceived Stress Scale (Cohen et al., 1983), which assesses the extent to which stressors have felt uncontrollable in the last month. The Marlow-Crown Social Desirability Scale was also included to measure any bias in responding on the survey battery (Crowne and Marlowe, 1960). Surveys were completed on a computer via the website Qualtrics (2013) (Provo, UT, United States) and presentation order was randomized by the computer to prevent order effects.

Analyses

Cortisol

Preprocessing

Before conducting cortisol analyses, in order to fulfill the requirement for homoscedasticity required for most statistical

tests, we examined the skewness of the cortisol measure at each of our 6 timepoints. Across all subjects, every timepoint had a positive skew, averaging 2.01 across all 6 timepoints. Therefore, we performed a power transformation to normalize our cortisol data. Based on a review by Miller and Plessow (2013) that examined the most effective transformations for cortisol time course data, we selected the power transformation $x' = (x^{0.26} - 1)/0.26$. After transformation, the skew of all timepoints averaged 0.04. Since there are individual differences in baseline cortisol values, we subtracted each transformed T0 value from the remaining five transformed cortisol timepoints (Mehta and Josephs, 2006). All following analyses use these transformed and baseline-adjusted values.

Preliminary manipulation checks

We conducted several preliminary analyses to ensure that our findings were not a result of extraneous variables. First, we conducted a two-way ANOVA examining main effects and an interaction effect of stress group and writing group on the T0 cortisol measurements to ensure that there were no significant differences in baseline cortisol between groups.

We conducted a one-way ANOVA that examined the effect of writing group on cortisol levels at time point T2 (i.e., peak cortisol response since the writing exercise) to examine whether individuals who wrote about a failure showed an increase in cortisol in comparison to individuals who wrote about a control topic. In other words, we sought to ensure that the failure writing exercise did not itself act as an acute stressor.

Since previous studies have shown that gender can influence cortisol levels (Kirschbaum et al., 1995), we also conducted a three-way ANOVA that examined the effect of gender, stress, and writing group on cortisol levels using an area under the curve with respect to increase (AUCi) analysis (Pruessner et al., 2003). We utilized the trapezoidal method, with T0 as our baseline value and points T1–T5 as points in the analysis. Furthermore, since oral contraceptive use has been shown to affect cortisol responsivity (Kirschbaum et al., 1995), among our female participants, we conducted a one-way ANOVA that examined the effect of oral contraceptives on AUCi for female participants. We also tested if smoking habits affected peak cortisol levels by examining whether the number of cigarettes smoked per week significantly correlated with AUCi levels.

Main analysis

We conducted a two-way ANOVA examining the effect of writing group and stress group on the AUCi of participants' cortisol responses. Since we hypothesized that writing about a past failure would attenuate the release of cortisol, we expected to find a significant interaction of stress group and writing group on AUCi levels.

Behavior

To examine if reflecting on failures improved performance on the SART after a stressor, we conducted a two-way MANOVA examining the effects of stress group and writing group on errors of commission on the SART (i.e., pressing when the correct answer should be to omit a response), errors of omission on the SART (i.e., failing to press when the correct answer should be to respond), and reaction time on the SART. To examine whether individual differences in cortisol response predicted performance effects, we added AUCi cortisol values as a continuous predictor.

Writing Sample Content and Survey Battery

To explore how individual differences related to cortisol response or SART performance, we conducted correlations examining the relationships between (1) baseline cortisol, (2) AUCi of participants' cortisol levels, (3) SART errors, (4) SART reaction time, (5) scores from all questionnaires in the survey battery, and (6) writing sample ratings.

RESULTS

Cortisol Results

Results of Manipulation Check Analyses

We conducted several analyses to ensure that our main results were not caused by extraneous variables. To ensure that groups did not differ with respect to cortisol at baseline, we conducted a two-way ANOVA examining the effects of stress group and writing group on baseline cortisol. This analysis did not yield significant main effects [stress: $F(1,95) < 0.01$, $p = 0.962$, $\eta_p^2 < 0.01$; writing: $F(1,95) = 0.38$, $p = 0.540$, $\eta_p^2 < 0.01$)] or an interaction effect, $F(1,95) = 1.95$, $p = 0.166$, $\eta_p^2 = 0.02$. Moreover, the ANOVA that tested whether the two writing groups differed in cortisol level after the writing manipulation did not yield significance, $F(1,95) = 0.16$, $p = 0.686$, $\eta_p^2 < 0.01$, suggesting that writing about past failures itself did not cause a differential increase in cortisol.

Groups also did not differ significantly from each other in gender, $\chi^2 = 0.83$, $p = 0.843$, $W = 0.18$, or age, $F(1,95) = 1.68$, $p = 0.199$, $\eta_p^2 = 0.02$. See **Table 1** illustrating the number of female and male participants in each group. Although we found a significant effect of gender on AUCi values of cortisol, $F(1,95) = 6.32$, $p = 0.014$, $\eta_p^2 = 0.07$, we did not find a significant interaction of stress group and gender on AUCi values, $F(1,95) = 0.04$, $p = 0.843$, $\eta_p^2 < 0.01$, nor did we find a significant interaction of writing × gender, $F(1,95) = 0.20$, $p = 0.656$, $\eta_p^2 < 0.01$. Our results suggest that although males in our sample tended to have higher cortisol than the females in our sample, these results were not a result of our stress and/or writing manipulations.

When examining changes in cortisol within our female participants, we did not find a significant effect of oral

TABLE 1 | Number of male and female participants across conditions.

	Female	Male
Failure Writing × TSST	15	9
Control Writing × TSST	12	12
Failure Writing × Filler Task	13	11
Control Writing × Filler Task	15	11
Total	**55**	**43**

contraceptives on AUCi values of cortisol, $t(53) = -0.73$, $p = 0.467$, $d = 0.46$. However, only 4 of the 55 women in our sample reported taking oral contraceptives, and all 4 of these women were in the non-stress condition (3 failure writing and 1 control writing). Furthermore, removing these women from our data analysis led to qualitatively similar results. Moreover, the number of participants in each week of menstrual cycle did not differ across condition, $\chi^2 = 9.54$, $p = 0.656$, $W = 0.02$, nor did day in menstrual cycle significantly correlate with AUCi values across all female participants, $R = 0.05$, $p = 0.745$, or within female stress subjects, $R = 0.15$, $p = 0.462$. See Supplementary Material for Table detailing number of participants in each week of menstrual cycle across conditions.

Groups did not differ from each other in terms of proportion of smokers (failure writing and TSST, $n = 2$; control writing and TSST, $n = 1$; failure writing and filler task, $n = 1$; control writing and filler task, $n = 1$) and non-smokers (failure writing and TSST, $n = 22$; control writing and TSST, $n = 24$; failure writing and filler task, $n = 23$; control writing and filler task, $n = 24$), $\chi^2 = 0.17$, $p = 0.876$, $W = 0.08$. Furthermore, we did not find a significant correlation between number of cigarettes smoked per week and AUCi levels of cortisol, both across all participants, $r = -0.18$, $p = 0.083$, and within just the stress participants, $r = -0.231$, $p = 0.113$.

Writing About Failures Buffers Physiological Stress Responses to the TSST

When examining the effect of stress group and writing group on AUCi values of cortisol, we did not find a significant main effect of stress on AUCi, $F(1,95) = 2.16$, $p = 0.145$, $\eta_p^2 = 0.02$. Moreover, we did not find a significant effect of writing on AUCi values, $F(1,95) = 0.01$, $p = 0.923$, $\eta_p^2 < 0.01$. In line with our hypothesis, we found a significant interaction effect of stress group × writing group, $F(1,95) = 4.61$, $p = 0.034$, $\eta_p^2 = 0.05$. These results suggest that those who wrote about a past failure before undergoing the TSST exhibited significantly reduced cortisol levels.

We also conducted several least-squared differences *post hoc* analyses that examined group differences in AUCi values of cortisol. Specifically, in the control writing groups, the stress manipulation significantly increased cortisol (mean AUCi difference = 19.01, $p = 0.011$). However, this was not the case among participants who wrote about a past failure (mean AUCi difference = 3.56, $p = 0.636$). Thus, our findings suggest that writing about a past failure before undergoing acute stress significantly attenuated the cortisol response to a psychosocial stressor (**Figure 2**). See **Table 2** for AUCi results across groups, and **Table 3** for complete results of the two-way ANOVA.

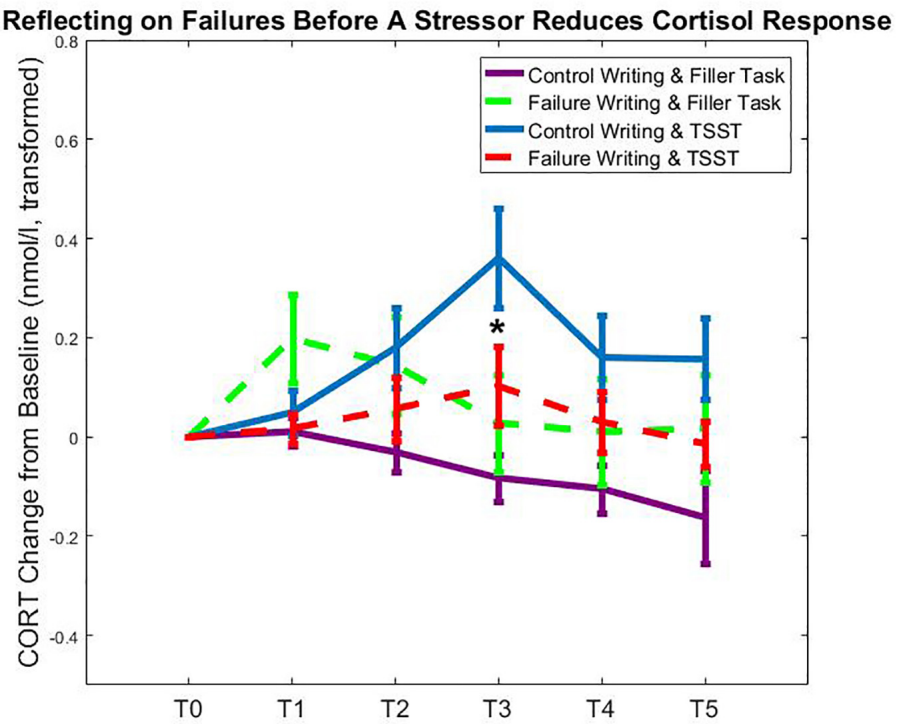

FIGURE 2 | Results. T0 = baseline; T1 = finish writing, 15 min since baseline; T2 = 10 min since stressor onset, 35 min since baseline, expected peak cortisol after writing; T3 = 30 min since stressor onset, 55 min since baseline, expected peak cortisol after stressor; T4 = 45 min since stressor onset, 70 min since baseline; T5 = 60 min since stressor onset, 85 min since baseline. Participants who were subjected to the psychosocial stressor exhibited cortisol increases from baseline (blue line), especially at peak cortisol since stressor conclusion (T3); however, participants who reflected on failures before experiencing the psychosocial stressor exhibited a reduced cortisol response (red line). Participants did not exhibit significant differences at peak cortisol since completing our writing manipulation (T2). Cortisol values represent transformed and baseline-adjusted values (see section "Materials and Methods").

TABLE 2 | Area under the curve from increase (AUCi) values across conditions.

	Mean	*SD*
Failure Writing × TSST	3.57	20.38
Control Writing × TSST	14.35	27.71
Failure Writing × Filler Task	7.13	36.88
Control Writing × Filler Task	−4.66	14.14

TABLE 3 | Effects of stress and writing manipulations on AUCi cortisol levels (full two-way ANOVA results).

	AUCi
Corrected model	$F(1,95) = 2.31$
	$p = 0.081$
Stress group	$F(1,95) = 2.16$
	$B = 0.3.56$
	$p = 0.145$
Writing group	$F(1,95) = 0.01$
	$B = -10.78.$
	$p = 0.923$
Stress and writing interaction	***F(1,95) = 4.61****
	B = 22.57
	p = 0.034

*B, beta weight; *p < 0.05; **p < 0.01.*

Behavioral Results

Writing About a Failure Before Stress Buffers Against Stress's Effect on Performance

We examined the effect of writing group and stress group on errors of commission, errors of omission, and reaction time in the SART task. We found a significant main effect of writing group on reaction time, $F(1,96) = 4.89$, $p = 0.029$, $\eta_p^2 = 0.05$, whereby writing about failures (regardless of whether the participant experienced a stressor) resulted in significantly slower reaction times on the SART ($M = 637.83$ ms, $SD = 104.69$ ms) compared to those who did not write about a past failure ($M = 591.03$ ms, $SD = 104.79$ ms). We also found a significant interaction of stress group and writing group on errors of commission on the task, $F(1,96) = 4.55$, $p = 0.036$, $\eta_p^2 = 0.05$; participants who wrote about past failures before experiencing a stressor made significantly fewer errors of commission ($M = 7.75$, $SD = 7.99$) than those who did not write about a past failure before experiencing a stressor ($M = 13.58$, $SD = 7.99$). Our results suggest that writing about a past failure resulted in slower reaction times on the SART. Furthermore, writing about a past failure before stress resulted in improved performance. This is consistent with a previously documented speed-accuracy tradeoff in this task (DiMenichi and Richmond, 2015); indeed, here we also found a significant negative correlation between RT and error rates on the SART, $r = -0.215$, $p = 0.034$. See **Table 4** for full MANOVA results and **Table 5** for SART performance across condition.

We also conducted the same MANOVA described above, and added AUCi as a continuous variable. While previously significant predictors remain unchanged, AUCi did not significantly predict any aspect of SART performance.

TABLE 4 | Effects of stress and writing manipulations on SART performance (full two-way MANOVA results).

	Commission	Omission	Reaction time
Stress group	$F(1,95) = 0.40$	$F(1,95) = 3.09$	$F(1,95) = 2.37$
	$B = -2.42$	$B = 7.04$	$B = -39.40$
	$p = 0.527$	$p = 0.082$	$p = 0.127$
Writing group	$F(1,95) = 2.20$	$F(1,95) = 0.54$	***F(1,95) = 4.89****
	$B = -5.83$	$B = 4.08$	***B = 39.95***
	$p = 0.142$	$p = 0.464$	***p = 0.029***
Stress and writing interaction	***F(1,95) = 4.55****	$F(1,95) = 0.46$	$F(1,95) = 0.11$
	B = 6.89	$B = -3.92$	$B = 13.68$
	p = 0.036	$p = 0.500$	$p = 0.747$

*$p < 0.05$.

Individual Differences in Writing Sample Content and Survey Battery

After controlling for multiple comparisons, we did not find any significant relationships across conditions regarding our individual differences measures (both survey and writing content ratings) and our physiological and behavioral results. See Supplementary Material for a correlation table of our survey battery results.

DISCUSSION

Previous research has suggested that acute stress is harmful to sustained attentional performance (e.g., Qian et al., 2015). However, previous research has also suggested that reflecting about past traumas or current anxieties can improve well-being (Pennebaker, 1997; Niles et al., 2014) and immediate performance (Ramirez and Beilock, 2011). We examined the effect of writing about past failures on cortisol responses to a new psychosocial stressor and sustained attentional performance after stress. We found that when individuals were subjected to the TSST, they exhibited increased cortisol levels, a typical response to a stressful event (Kirschbaum et al., 1995; Dickerson and Kemeny, 2004). However, when individuals wrote about a past failure before experiencing the psychosocial stressor, their cortisol response was attenuated, suggesting that writing about a past failure before experiencing a new stressor may lead to some reduction in one's physiological experience of stress. Moreover, higher stress responses were associated with poorer performance on a sustained attention task, but writing about failures before a stressor protected against the typical detrimental effect of acute stress on performance. Specifically, while stressed individuals who wrote about a control topic made the most errors of commission, stressed participants who had reflected on failures made the fewest errors of commission.

We did not find evidence that writing about failures alone leads to a significant increase or decrease of cortisol levels, counter to some literature that suggests that writing about past traumas itself affects stress (Pennebaker, 1997). Instead, we propose that writing about failures may make a new stressor seem subjectively less stressful by comparison.

TABLE 5 | SART performance across stress and writing group.

	Failure Writing × TSST	Control Writing × TSST	Failure Writing × Filler Task	Control Writing × Filler Task
Errors of commission	Mean = 7.75	Mean = 13.58	Mean = 10.17	Mean = 9.12
	SD = 6.46	*SD* = 9.12	*SD* = 8.95	*SD* = 7.15
Errors of omission	Mean = 10.21	Mean = 6.13	Mean = 3.17	Mean = 3.00
	SD = 22.28	*SD* = 16.19	*SD* = 6.00	*SD* = 6.23
Reaction time	Mean = 618.13	Mean = 578.18	Mean = 657.53	Mean = 603.89
	SD = 112.50	*SD* = 107.33	*SD* = 118.93	*SD* = 77.12

Longitudinal data provides support for this claim, as past stressful experiences have been shown to allow an individual to adapt better to a new stressor (Homberg, 2012). Specifically, Stress Inoculation Theory suggests that individuals who have experienced some level of lifetime adversity are more likely to exhibit resilience to a new stressor (Lyons et al., 2009). Furthermore, early life adversity can lead to more adaptive cognition (Frankenhuis and de Weerth, 2013). In the same way that past stressful experiences may allow an individual to adapt to new stressors, writing about a past failure may allow an individual to adapt to a new immediate stressor.

Writing about a failure before experiencing psychosocial stress resulted in reduced cortisol reactivity, as well as better performance on the SART. Although some research has suggested that stress may affect performance in a U-shaped manner (Yerkes and Dodson, 1908; Lupien et al., 2009), others have found that increases in stress result in linear decreases in performance (Domes et al., 2004; Van den Bos et al., 2009). Future work could further examine if there is a linear relationship between stress levels and performance in a sustained attention task.

We also found that writing about a failure resulted in increased reaction time on the SART. All participants in our experiment exhibited a speed-accuracy tradeoff: participants who had the slowest reaction times on the SART also exhibited the best performance. Taken together, these findings support previous claims that writing about a past failure may cause an individual to make slower, more deliberate choices in order to avoid another future failure, resulting in better performance (DiMenichi and Richmond, 2015).

One limitation of the current study is that we did not assess self-reported stress levels throughout the experimental session. A behavioral pilot study we conducted suggested that repeatedly asking individuals about their stress levels after writing about a past failure eliminated the behavioral effects of the writing manipulation. Post-event processing literature suggests that asking participants to repeatedly reflect on stressful feelings about an event can increase negative feelings about that event (Mellings and Alden, 2000), and introspecting on an emotional response may actually change the response (Silvia, 2002; Hutcherson et al., 2005).

Although we found a significant interaction of stress and writing prompt on errors of commission, we did not find a significant main effect of writing group on errors of commission, unlike DiMenichi and Richmond (2015) and our follow-up study described in the Supplementary Material. This could have occurred because the task structure of our task varies from the task structure described in the two other studies: in this study, participants took a 2-min break halfway through the SART to provide a cortisol sample. This break could have improved attention on the SART, resulting in improved performance for all groups, and smaller performance differences between writing groups. Furthermore, in the two other studies, participants wrote about a past failure or control topic and immediately completed a sustained attention task. However, in the non-stress condition in this study, participants completed a filler task before completing the sustained attention task. Perhaps adding this filler task somehow affected performance on the sustained attention task, either because of the task itself or because of experimental timing.

While previous research has suggested that journaling may be beneficial to mental health (Barak and Grohol, 2011), the current study suggests that writing about one's past failures might not only improve mental health and well-being, but also change the way an individual reacts to future stressors. Perhaps writing about a past failure increases perceived controllability over challenges. It has been shown that increasing perceived control alters the effect of stress on persistence (Bhanji et al., 2016). Future studies might investigate this possibility by assessing or manipulating perceived controllability during stress. Also, although we assessed various traits and tendencies that could contribute to our observed effects, it is unknown if there are other individual differences (e.g., a tendency to disclose, or previous experience with life stressors) that could moderate how strongly writing about a past failure affects stress and performance.

CONCLUSION

We found that writing about past failures reduced one's physiological stress response to a new psychosocial stressor. Most importantly, we found that writing about a past failure before a stressor buffers against decreases in performance that are associated with high levels of stress. In a real-world setting, this information may be valuable to clinicians, as well as educators hoping to improve attentional performance. Since writing about test anxieties has already been shown to protect against the negative effects of stress on performance on a high-stakes exam in a classroom setting (Ramirez and Beilock, 2011), this writing

manipulation may be especially valuable to populations who exhibit high levels of performance anxiety.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Rutgers University Institutional Review Board with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Rutgers University Institutional Review Board.

AUTHOR CONTRIBUTIONS

BD and ET conceived of the experiments and design. BD and CB performed the experiments. BD and KL analyzed the data. All authors contributed to the written manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00045/full#supplementary-material

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