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A Novel Adolescent Chronic Social Defeat Model: Reverse-Resident-Intruder Paradigm (rRIP) in Male Rats

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Abstract

Psychosocial stress is linked to the etiology of several neuropsychiatric disorders, including Major Depressive Disorder and Post-Traumatic-Stress-Disorder. Adolescence is a critical neurobehavioral developmental period wherein the maturing nervous system is sensitive to stress-related psychosocial events. The effects of social defeat stress, an animal model of psychosocial stress, on adolescent neurobehavioral phenomena are not well explored. Using the standard Resident-Intruder paradigm, adolescent Long-Evans (LE, residents, $n=100$) and Sprague-Dawley (SD, intruders, $n=100$) rats interacted for five days to invoke chronic social stress. Tests of depressive behavior (forced-swim-test (FST)), fear conditioning and long-term synaptic plasticity are affected in various adult rodent chronic stress models, thus we hypothesized that these phenomena would be similarly affected in adolescent rats. Serendipitously, we observed the Intruders became the dominant rats and the Residents were the defeated/submissive rats. This robust and reliable role-reversal resulted in defeated LE-Residents showing a depressive-like state (increased time spent immobile in the FST), enhanced fear conditioning in both hippocampal-dependent and hippocampal-independent fear paradigms and altered hippocampal long-term synaptic plasticity, measured electrophysiologically in vitro in hippocampal slices. Importantly, SD-Intruders, SD and LE controls did not significantly differ from each other in any of these assessments. This reverse Resident-Intruder-Paradigm (rRIP) represents a novel animal model to study the effects of stress on adolescent neurobehavioral phenomenon.

Keywords

adolescent; depression; fear; hippocampus; LTP; social stress

Introduction

Psychosocial stress (e.g. bullying) across the lifespan is a widespread and global issue with effects persisting many years beyond the bullying episodes. It is linked to the development of several neuropsychiatric disorders such as depression and post-traumatic-stress disorder in children, adolescents and adults (Brunstein et al., 2007; Wang et al., 2011; Hepburn et al.,

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2012; Schneider et al., 2012). Bullying can be classified as conventional: physical (hitting), verbal (name-calling), relational (social isolation) or as non-conventional: cyber-bullying (Wang et al., 2011). In conventional bullying, the strong correlation between bully-associated behaviors and psychological disorders is apparent in the bullies themselves, the bully-victims (individuals that serve in both roles) and the victims (Brunstein et al., 2007; Wang et al., 2011; Hepburn et al., 2012; Schneider et al., 2012). Given the recent national attention on the severity of bullying in the United States (Bullying in the News), understanding the neurobiology of this negative social interaction is imperative in developing novel therapies and interventions for individuals who were previously or are currently involved in a bullying scenario. Social Defeat Stress (SDS), an animal model of psychosocial stress, produces many similar bio-behavioral effects as seen in human victims of bullying stress. Notably, defeated animals develop depressive-like symptoms, exaggerated stress responses, and increased preferences for alcohol and other drugs of abuse (Björkqvist, 2001; Yan et al., 2010; Cruz et al., 2011). These effects make social defeat a putative pre-clinical model to investigate the neurobiological consequences of bullying stress.

While there is a rich animal literature on the effects of adolescent SDS on the adult brain and behavior, few studies have addressed how SDS affects the adolescent animal (Burke & Miczek, 2015; Burke et al., 2016; Buwalda et al., 2011). *Adolescence* is a transitional period, where reliance on parental guidance fades into independence as an adult. In mammalian species, including humans, this transition includes increases in peer influences, sexual competition, novelty-seeking and risk-taking behaviors. Participation in these behaviors is considered to provide the skills necessary to survive without parental caregiving (Spear, 2000; Brenhouse and Andersen, 2011). Underlying these behaviors is a continual neural maturation process in the form of synaptic pruning and sprouting, reorganization of innervating neurotransmitter systems, myelination of nerve fibers and cell proliferation (Spear, 2000; Brenhouse and Andersen, 2011; Lee and Gorzalka, 2012; Eiland and Romeo, 2013). Due to the myriad of neural changes, adolescent brain development is highly vulnerable to insults such as stress. In humans, exposure to stress early in life and adolescence is implicated in the etiology of Major Depressive Disorder (MDD), Post-Traumatic-Stress-Disorder (PTSD) and schizophrenia (Andersen and Teicher, 2008; Eiland and Romeo, 2013; McCormick and Green, 2013). Vulnerability to stress and toxic insults during the adolescent period is also observed in preclinical animal models, suggesting that neural developmental patterns are conserved across species (Burke & Miczek, 2014).

Thus, the purpose of this study was to subject early-to-mid adolescent male Sprague-Dawley (SD) rats to chronic SDS (CSDS) using the standard Resident-Intruder-Paradigm (RIP) and to assess the effects of stress while these rats are still in the window of adolescent development. To model human adolescent social stress, SD-Intruders are exposed to Residents that are adolescent Long-Evans (LE) male rats. Adolescent SD male rats exposed to chronic mild-stress exhibit a depressive phenotype characterized by enhanced hippocampal fear-conditioning and synaptic plasticity (Reich et al., 2013a; Reich et al., 2013b). Thus, we hypothesized that defeated SD rats also would display a depressive phenotype with similar effects on hippocampal-dependent fear learning and synaptic plasticity. Unexpectedly, the rats exhibited a role-reversal in the RIP, whereby the SD-Intruders were the dominant rats and LE-Resident rats were the defeated/submissive rats.

The defeated LE-Residents displayed 1) a depressive-like phenotype, 2) enhanced fear conditioning and 3) both negative and positive modulation of hippocampal synaptic plasticity in adolescent socially-defeated rats. We present this adolescent reverse-Resident-Intruder-Paradigm (rRIP) as a novel animal model to study the effects of stress on neurobehavioral phenomena.

Methods

Animals

Adolescent male Sprague Dawley (SD, 30–33 days old) and Long Evans rats (LE, 38–40 days-old) were single-caged upon arrival (Charles River Laboratories, MA) to the Ramapo College Animal Facility. Rats were acclimated 5–7 days prior to experimental procedures. A total of 200 rats (100 of each strain) were used across the whole study. Rats were housed in white rectangular cages (38.1 × 25.4 cm x h: 21.6 cm) with regular bedding and wire top and placed on a 12-h/12-h light/dark (lights on at 08:00h) cycle in a room temperature of 21°C. Both groups received *ad libitum* access to water throughout the chronic social defeat stress (CSDS) protocol. LE rats were food-restricted for the duration of the experiment, receiving enough daily chow-pellets to ensure rats remained at the 20 percentile of their aggregate body weight. All other groups were given *ab libitum* food access. Rats were acclimated to both the vivarium and food and water schedule for five days prior to experimental procedures. At the start of the experiments, SD rats weighed ~ 100 g and LE rats weighed ~ 200 g. All experimental procedures were carried out in accordance with protocols established and approved by the Institutional Animal Care and Use Committee of Ramapo College.

Chronic Social Defeat Stress

Social defeat stress was induced via the resident-intruder paradigm (RIP). LE rats are generally considered more aggressive than SD rats (Patki et al., 2015) and thus served as the Residents. However, it is important to note that not all male LE rats are equally aggressive and often need to be screened to isolate the more aggressive individuals; however this was not done in the present study (Patki et al., 2015). LE residents are often single-housed, food-restricted, and co-habited with a female to enhance territoriality during the CSDS protocol. Our LE residents were single-housed and food-restricted but did not undergo pre-screening or co-habitation with a female LE rat. LE Residents remained within their home cages throughout the duration of the experiment. Controls included separate single-housed LE and SD rat cohorts that underwent the same food and water conditions as the experimental LE and SD rats, respectively. Resident rats were exposed to a novel Intruder daily for 5 days. Prior to the onset of each encounter, a wire mesh separator cubicle (12.7 × 12.7 cm x 17.8 cm) restricted the SD Intruder and LE Resident rats for 10 min of non-physical sensory contact (i.e., sensory intimidation via visual, auditory and olfactory cues) (Figure 1). Following the 10 min sensory intimidation period, 10 min of physical contact was permitted before the separator cubicle was reintroduced for an additional 8 min. Behavioral displays of dominance and submission were documented according to a behavioral scorecard, with *Dominance* defined as patrolling, pinning and chasing behavior and *Attacks* defined as lunging, injurious and violent behavior. Each day LE Residents encountered a different SD

Intruder to prevent aggressive behavior from becoming artificially reduced as a result of habituation. Each encounter took place mid-day (11:00h-14:00h) in a moderately lit, noise-attenuated room. In the event of injury, laboratory personnel had been instructed to interrupt the interaction and provide the rat with immediate first-aid care. Recommended first-aid was demonstrated by our staff veterinarian and approved by the IACUC. No rats needed first-aid in this study. Twenty-four hours following Day 5 of CSDS, separate cohorts of rats underwent behavioral and neurophysiological assessments.

Forced Swim Analysis

A subset of rats underwent a forced swim test (FST) to assess depressive-like behavior. The FST consists of two testing days, however only the time spent immobile on Day 2 is inferred as a behavioral indicator of *despair*, whereby the urge to escape threatening stimuli is diminished (Yan et al., 2010). Twenty-four hours following CSDS, rats were placed singly inside a plastic cylinder ($r = 30.5$ cm; $h = 46.0$ cm) with 3.0 L of water at room temperature (18–22° C) for 15 min. The procedure was repeated the following day for 5 min. *Immobility*, defined as the time spent motionless at the surface of the water (i.e., absent submerged limb movement), was recorded for each rat via Zeiss (ZSD-808) Stopwatches. Increased immobility is interpreted as a depressive-like phenotype in rats and immobility can be reduced via antidepressant (SSRI) administration (Yan et al., 2010). Mean $\pm$ SEM immobile time during Day 2 (in 5 min) was calculated for each group.

Fear Conditioning

Apparatus—Experiments were performed using an automated, computerized fear-conditioning system (Coulbourn Instruments, White Hall, PA, USA). The system consists of three conditioning chambers (30.5 $\times$ 25.4 $\times$ 30.5 cm) with removable stainless steel grid floors. Footshocks were delivered through the floor grid via a shocker-scrambler unit controlled by custom-designed software (Coulbourn Instruments). Locomotor and freezing activities were monitored through charged-coupled-device (CCD) video cameras mounted at the top of each chamber and subsequently analyzed via FreezeFrame software (Coulbourn Instruments). A house light and speaker (1000 Hz tone, 80 dB) were located on the sidewalls of each chamber. In addition, each chamber was contained in a sound-attenuating cubicle equipped with a ventilation fan (60 dB). Experiments were performed in an isolated testing room to prevent external noise from interfering with the procedure.

Acquisition—To assess hippocampal-dependent and independent fear conditioning, three separate conditioning protocols were used: contextual, trace and delay. The hippocampus processes spatial information and provides a cognitive map of the external world (Clark et al., 2001). Contextual fear conditioning (cFC) involves pairing a footshock (unconditioned stimulus, US) with the foreground environment (conditioned stimulus, CS); thus cFC is hippocampal-dependent. Trace fear conditioning (tFC) selectively recruits the hippocampus by introducing a temporal gap between auditory CS tone- presentation and US foot-shock delivery. Trace conditioning is a model of episodic (autobiographical) memory (Clark et al., 2001). Delay fear conditioning (dFC), however, does not recruit the hippocampus by incorporating overlapping (co-terminating) CS-US presentation. Thus, contrasting results among protocols provide hints as to which neuro-substrates are altered by CSDS.

For trace fear conditioning (tFC), each rat had a 120 sec baseline acclimation period before receiving three presentations of a tone CS (15 sec) followed by a footshock US (2 sec, 0.6 mA), that occurred 30 sec after the CS offset (i.e. trace period). CS-US presentations were separated by 180 sec inter-trial-intervals (ITI). Delay fear conditioning trials were similar to the trace conditioning structure, except the footshock US (2 sec, 0.6 mA) co-terminated with the auditory CS (15 sec). A conventional “foreground” protocol was used for contextual fear conditioning, wherein rats were placed into their corresponding chambers for 10 min and exposed to three footshock (US) presentations (2 sec, 0.6 mA, 30 sec apart) during the last 2 minutes. Rats were returned to the colony room immediately following the conclusion of each session. Each chamber was subsequently cleaned with Formula 409 cleaner. These conditioning protocols are similar to those from recent studies in our laboratory (Reich et al., 2008, 2013a).

Fear Recall—The strength of the CS-US association was assessed 24-h after the acquisition sessions. Trace and delay retention trials consisted of a 120 sec baseline acclimation period followed by three trials of CS-only presentation with 180 sec ITI. Inadvertent contextual conditioning was minimized by altering the visual appearance of each chamber. Plexiglas was used to line the grid floor and bisect the conditioning chamber. For contextual conditioning recall, rats were placed in unaltered chambers identical to those used during acquisition. Once placed in the chamber, freezing was monitored continually for a 10 min period. Freezing is defined as the absence of all movement except for respiration (Fanselow, 1980), lasting a period ≥ 3 sec. For each experiment, time was initially binned into 15 sec intervals and the percentage of time spent freezing was calculated. Then group time-bin means were calculated by averaging across a given 15 sec time-bin for all rats, creating a group run average of an individual experiment (Reich 2008, 2013a). If a rat had baseline freezing scores ≥ 2.5 standard deviations from the group mean, it was removed from further analysis. In this study, no rats were removed from analysis. For the trace and delay recall trials, mean freezing for the first minute following the CS offset was calculated from group run average. In previous studies, we observed that peak freezing to a CS tone occurs during this 60 sec post-CS period (Reich et al., 2008; Reich et al., 2013a). In trace and delay fear recall tests, the first 60 sec post-CS period was used as an index of recall performance. Contextual recall was analyzed using average freezing during the first 120 sec. According to Rescorla-Wagner learning theory, these trials/time periods reflect the strongest potential for memory recall with later trials/time periods serving as extinction trials (Bouton, 2007).

Electrophysiology

Rats were deeply anesthetized with halothane inhalation and decapitated in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee at Ramapo College of New Jersey. The brain was rapidly removed and hippocampi dissected. Transverse hippocampal slices, 400 μ M thick, were cut on a vibratome (Leica-Microsystems). Slices were kept in a holding chamber at room temperature at the interface of artificial cerebrospinal fluid (ACSF) and a humidified 95/5% O₂/CO₂ atmosphere for > 1 hr. The slices were then transferred to a submerged recording chamber and perfused with warm (30° C) ACSF: (mM), NaCl, 120; KCl, 3; MgSO₄, 2; NaH₂PO₄, 1; NaHCO₃, 25;

CaCl₂, 2.5; and glucose, 10 and saturated with 95% O₂-5% CO₂ (pH 7.4). Field excitatory post synaptic potentials (fEPSPs) were recorded from stratum radiatum of CA1 with glass microelectrodes (tip diameter ~ 4–8 μM) filled with ACSF. Stimuli were delivered via a bipolar stimulating electrode located between CA3 and CA1. Signals were recorded with an amplifier (Model 773, WPI Inc. or Model IE-251A, Warner Instruments Inc.), digitized at 10 kHz with an AD interface (Digidata 1440A, Axon Instruments or PCI-6259, National Instruments) and analyzed with either pClamp 10.0 (Axon, Instruments) or WinLTP software. Stable baseline responses (15 min) were recorded prior to experimental manipulation. Synaptic plasticity experiments began with fEPSPs adjusted to ~ 0.5 mV (~50% maximum amplitude) and involved delivering a weak theta-burst stimulation (wTBS; 10 bursts of 5 stimuli, 100 Hz within burst, 200 ms interburst interval) or strong theta-burst stimulation sTBS; 10 bursts of 5 stimuli, 100 Hz within burst, 200 ms interburst interval, *repeated 4 times*) following the baseline period. The initial slope of the fEPSP (mV/ms) was measured within the first two milliseconds of the response immediately after the negative peak of the fiber volley. To standardize responses across experimental conditions, all slope measurements within a given experiment were divided by the average baseline response (15 min). Then, individual experiments within each condition were averaged across each time point. For statistical analyses, the mean baseline value (last 5 min; fifteen consecutive responses) and the mean experimental value (last 5 min; fifteen consecutive responses) were computed. Due to variable effects of stress, only one recording per rat (one hippocampal slice) was performed to minimize any bias from any one rat; thus n is number of rats (only 1 slice per rat).

Statistical Analysis

Data analysis (SPSS, 12.0) for the CSDS, FST and Fear Recall data was performed by repeated measures, one-way and two-way ANOVAs of group means with planned difference pairwise contrasts ($p \leq 0.05$) as indicated. One-way ANOVAs ($p \leq 0.05$) were used to analyze the electrophysiology data.

Results

Role Reversal in Adolescent Social Defeat Stress

Contrary to existing CSDS models, we found LE-Residents consistently are socially defeated. SD-Intruders exhibited significantly more attacking behavior than the LE-Resident across all five testing days ($F(4,136) = 3.51, p \leq 0.001$, RMANOVA (days x condition (resident vs. intruder), see Fig. 2A). This unexpected aggressive Intruder behavior is concomitant with significantly greater dominance over the Residents ($F(4,184) = 12.63, p \leq 0.001$, RMANOVA (days x condition (resident vs. intruder), see Fig. 2B). Note that even though these differences in aggressive behavior were observed on Day 1, the Intruder behavior steadily increased from Day 1 to Day 3 while the Residents' behavior remained consistent. Although the data in Figure 2 are a sample of our total dataset, we reliably observed the same phenomenon in multiple rounds of our CSDS protocol executed over a two year period. Due to the role reversal in Resident and Intruder behavior compared to the conventional Resident-Intruder Paradigm, our protocol is referred to as *Reverse Resident-Intruder Protocol (rRIP)*.

rRIP-CSDS Induces a Depressive-Like Phenotype in Long Evans Residents

A sub-sample of LE and SD rats ($n=6$ each) from Figure 2 subsequently were subjected to a Forced Swim Test (FST). A two-way ANOVA (CSDS (stress vs control) X strain (LE vs SD)) revealed a main effect of CSDS (Fig 3): LE-Residents spent significantly more time (1.9-fold) immobile than LE-controls ($F(1, 19) = 11.96, p = 0.03$). No significant difference was observed between SD-Intruders and SD-Controls ($p = 0.05$, difference pairwise contrast). Significant differences in immobility were also found between LE and SD rats ($F(1, 19) = 4.56, p = 0.046$, Fig 3), however an interaction effect was not observed, $p = 0.05$. Thus, LE-residents spent more time immobile than any of the other groups. This indicates that rRIP induced a depressive-like phenotype in the defeated Residents.

rRIP-CSDS enhances fear conditioning

Initially, effects of CSDS on fear learning were assessed on hippocampal-dependent trace fear conditioning. As indicated by a two-way RMANOVA (CSDS (stress vs control) X strain (LE vs SD)), all groups displayed significantly greater freezing during the cued (60 sec post-CS) period than during the last 60 sec of baseline (pre-CS) period ($F(1, 16) = 154.20, p = 0.000$, Fig. 4A). Importantly, LE-Residents froze significantly more than all other groups (CSDS x strain) following the first CS presentation (60 sec) in tFC memory recall ($F(1, 16) = 3.53, p = 0.035$, Fig. 4A, B). Significant differences were found between LE Residents and LE Controls ($p = 0.05$, difference pairwise comparison); although SD-Intruders and SD-Controls froze at comparable levels ($p = 0.05$, pairwise comparison, Fig. 4B). Baseline (pre-CS) freezing rates were less than 20% with no differences observed among the groups ($p = 0.05$, difference pairwise comparisons, Fig 4A). This indicates generalized fear conditioning was minimal compared to cued conditioning.

As illustrated in Figure 5, CSDS also enhanced freezing during contextual fear conditioning. A two-way ANOVA (CSDS (stress vs control) X strain (LE vs SD)) revealed overall that LE-Residents froze significantly more than SD-Intruders (2.1-fold), LE-controls (2.4-fold) and SD-Controls (1.5-fold) ($F(3, 28) = 3.51, p = 0.03$; Fig. 5). An interaction effect confirmed that LE-residents displayed the highest freezing levels and also indicated that SD-Controls froze more than SD-Intruders ($F(1, 28) = 7.68, p = 0.01$). These observations from both cFC and tFC provide evidence that rRIP-CSDS enhances hippocampal-dependent aversive conditioning in defeated Residents.

However, the enhanced freezing could reflect a general stress-induced effect on fear learning. We therefore tested a separate group of CSDS rats and controls in the hippocampal-independent delay fear conditioning paradigm. Similar to tFC, a two-way RMANOVA (CSDS (stress vs control) X strain (LE vs SD)), revealed significant greater freezing during the cued (post-CS) period than during the baseline (pre-CS) period across all groups ($F(1, 16) = 72.94, p = 0.000$, Fig. 6A). LE-Residents again displayed significantly higher freezing compared to the other groups (CSDS x strain) during memory recall ($F(1, 16) = 26.53, p = 0.000$, Fig. 6A, B). LE Residents and LE Controls differed significantly ($p = 0.05$, difference pairwise comparison), whereas SD-Intruders and SD-Controls did not show significant differences ($p = 0.05$, difference pairwise comparison, Fig. 6B). Baseline (pre-CS) freezing rates were less than 20% with the only significant difference being the LE-

controls (0% freezing, $p = 0.05$, difference pairwise comparisons, Fig 6A). These findings demonstrate that rRIP-CSDS produces enhancement in both hippocampal-dependent and independent fear conditioning. Moreover, the general finding that freezing levels were similar for the SD and LE controls indicates that freezing responses in the two strains are comparable.

rRIP-CSDS modulates hippocampal synaptic plasticity

Long-term-potential (LTP) in the hippocampus is impaired in rats exposed to various forms of chronic stress (Kim and Diamond, 2002; Pavlides et al., 2002; Alfarez et al., 2003; Artola et al., 2006; Holderbach et al., 2007; Reich et al., 2013b). However, we recently reported an increase in LTP in the hippocampi of adolescent rats exposed to a three-week chronic mild stress protocol. This increased LTP was only observed using a weak LTP induction protocol, whereas a strong induction protocol impaired LTP (Reich et al., 2013b). Effects of rRIP-CSDS on in vitro hippocampal LTP were thus assessed using both weak Theta-Burst-Stimulation (wTBS) and strong Theta-Burst-Stimulation (sTBS) protocols.

As previously observed in chronically-stressed rats, a strong LTP-inducing stimulation (sTBS), resulted in impaired LTP in the defeated Residents, control rats (1.7-fold greater; ($F(1,28) = 477.83$, $p = 0.000$); Figure 7A, right). Average baseline responses (5 min prior to sTBS) did not differ between stress (LE residents) and control (LE naive) rats ($p = 0.05$, Fig 7A, right). Conversely, the wTBS produced an increase in LTP in slices from stress rats (1.5-fold greater) compared to control LE rats ($F(1,28) = 26.742$, $p = 0.000$, Fig 7B, right). There were no differences between the average baseline responses. Despite the difference in magnitude, significant LTP was observed for both the Residents and controls ($p = 0.01$, Fig. 7B). In summary, sTBS produced impaired LTP in the defeated Residents, whereas the wTBS yielded enhanced LTP compared to LE control rats.

Discussion

The main finding was that CSDS using adolescent LE-residents and SD-intruders revealed a serendipitous role reversal between LE-Residents and SD-Intruders. Our observations demonstrate that rRIP-CSDS: 1) yields a depressive-like state, 2) enhances fear conditioning and 3) alters hippocampal long-term synaptic plasticity in defeated LE-Residents. Behavioral catalysts, such as food-deprivation and social isolation, conventionally used to enhance the aggressive disposition and territoriality of LE-Residents failed to facilitate these behaviors in the present study. While the exact mechanism for the role reversal was not investigated, previous studies with adolescent and adult LE rats highlight the use of screening methodology to select candidate animals exhibiting heightened levels of aggression (Burke & Miczek, 2015; Buwalda et al., 2011; Patki et al., 2015). In addition, our LE-Residents were not co-housed or pre-exposed to dominant-subordinate social interaction prior to CSDS. Given the age of these rats, a plausible hypothesis is that social isolation for adolescent SD-Intruders promotes enhanced aggression in an attempt to establish dominance in an unfamiliar and preoccupied environment. This enhanced aggression may develop from increases in social exploration and reductions in fear/anxiety behavior following single-housing during adolescence. For example, Burke and Miczek (2015) reported single-housed

SD adolescent rats spent more time “approaching, following and crawling under an adult LE-Resident”. This led to more attacks and bites from the resident, but surprisingly less freezing behavior from the SD-Intruder (Burke & Miczek, 2015). These observations and ours are consistent with increases in novelty-seeking and risk-taking behaviors (i.e. less fear) during adolescence compared to pre-pubertal and adult developmental periods in rodents and humans (Spear, 2000; Brenhouse and Andersen, 2011). Social isolation also deprives juvenile and adolescent rodents of social play needed for proper adult social interactions along with decreasing synaptic densities in several cortical structures (Buwalda et al., 2011). This may explain the more timid behavior of our defeated LE-residents. In standard RIP adolescent studies, social-isolated housing exacerbates the adverse effects on defeated intruders, although pair or group housing of intruders attenuates these effects (Buwalda et al., 2011). Thus the contribution of housing conditions represents a confounding factor that needs to be carefully considered and observed in social stress investigations. Lastly, food restriction may have contributed to the LE-residents less aggressive behavior. Perhaps, food restriction differently affects behavior between adolescence and adults. Future studies will be needed to a) further elucidate the nature of the unexpected social interactions observed in this study and b) explore if the effects of adolescent rRIP-CSDS extend into adulthood. Nevertheless, our results show clear and reliable CSDS with a novel, more naturalistic approach. To our knowledge, it represents the first reliable variant of RIP to induce social defeat in animals designated as Residents.

History of stress is a risk factor in the etiology of MDD and PTSD and various animal models of chronic stress also precipitate depressive-like symptomatology (Andersen and Teicher, 2008; Eiland and Romeo, 2013; McCormick and Green, 2013). Thus, the FST was used to assess if rRIP-CSDS resulted in a depressive phenotype. Indeed, defeated LE-Residents spent significantly more time immobile compared to the LE-Controls, SD-Intruders and SD-controls. The observation that LE-Controls did not differ from both sets of the SD rats indicates that the increase in LE-Resident immobility was not due to strain differences in swimming behavior or anxiety. This demonstrates that the rRIP-CSDS is capable of inducing a *depressive-like state* in defeated LE Residents.

PTSD and MDD are accompanied by dysfunction in fear controllability, thus making Pavlovian fear conditioning a valid model to elucidate the neurobehavioral underpinnings of these disorders (Rau et al., 2005; Jovanovic and Ressler, 2010). In adult male rats, pre-exposure to stress (acute and chronic), footshocks or exogenous administration of corticosterone facilitates fear conditioning (Cordero et al., 2003; Rau et al., 2005). In particular, 21-day exposure to chronic mild stress (CMS) in adolescent SD rats selectively enhanced hippocampal-dependent trace fear conditioning with no effect on contextual or delay fear conditioning (Reich et al., 2013a). Thus, we initially hypothesized that rRIP-CSDS will increase the hippocampal-dependent fear learning in defeated LE-Residents. However, defeated Residents exhibited enhanced fear memory recall in trace, contextual and delay fear conditioning protocols compared to LE-Controls, SD-Controls and SD-Intruders. Since the amygdala gates the expression of fear behavior, these findings indicate that rRIP-CSDS may more profoundly affect the amygdala compared to the hippocampus. However, it should be noted that both the hippocampus and the ventromedial prefrontal cortex are both intricately involved in fear processing and responding (Maren et al., 2013). Thus, further

investigations are required to dissect the effects of rRIP-CSDS on various neuroanatomic loci. The observed general enhancement in fear conditioning further evidence that a history of chronic stress and depressive-like symptomatologies alters neurobehavioral processing of fear.

To begin exploring the neurophysiological effects of rRIP-CSDS, we assessed induction of long-term potentiation (LTP) in the hippocampus of defeated LE-Residents. Our results show that socially defeated, LE-Residents exhibited impaired LTP when using a strong theta-burst stimulation (TBS x 4). Conversely, LTP was *enhanced* compared to LE-Controls using weak theta-burst stimulation (TBS x 1). Similar findings were observed in previous studies from our laboratory using a chronic, mild unpredictable stress (CMUS) protocol in adolescent SD rats (Reich et al., 2013b). This highlights the notion that varying levels and types of synaptic plasticity may subserve different physiologies and behaviors. For example, in a recent study, we observed that this same CMS protocol selectively enhances trace (hippocampal-dependent) fear conditioning, but does not affect contextual fear conditioning, the prototypical hippocampal-dependent fear task, or non-hippocampal fear conditioning (Reich et al., 2013a). However, 21-day chronic restraint stress protocols lead to increases in both hippocampal and non-hippocampal dependent forms of fear conditioning (Conrad et al., 1999; Cordero et al., 2003; Pêgo et al., 2008).

Impairments in LTP via strong induction protocols following chronic stress exposure is well documented (Alfarez et al., 2003; Artola et al., 2006; Holderbach et al., 2007; Pavlides et al., 2002; Reich et al., 2013b), however stress exposure also enhances fear conditioning (Conrad et al., 1999; Cordero et al., 2003; Pêgo et al., 2008). One hypothesis is that chronic stress alters the range of LTP induction, such that LTP becomes saturated at weaker induction stimulation. According to the Bienenstock-Cooper Munro (BCM) theory of synaptic plasticity, induction of LTP or long-term depression (LTD) depends on the current and previous activity of the active synapses (Bienenstock et al., 1982). BCM suggests increases in synaptic activities that initially promote LTP saturate and increase the likelihood of LTD, whereas low levels of synaptic activity promote LTP. Experimental evidence shows that this sliding threshold of synaptic plasticity depends on NMDA-receptor Ca^{2+} influx into the post-synaptic cell (Dudek and Bear, 1993; Kirkwood et al., 1996). Fast rises in intracellular $[\text{Ca}^{2+}]$ result in phosphorylation and insertion of AMPA receptors into the post-synaptic membrane, promoting LTP. Conversely, slower, sustained rises in intracellular $[\text{Ca}^{2+}]$ result in dephosphorylation and removal of AMPA receptors from the post-synaptic membrane promoting LTD (Kirkwood et al., 1996). Both stress-induced impairments of LTP (via strong (standard) induction protocols) and enhanced LTD induction are attributed to shifts in these mechanisms (Artola et al., 2006). However, following exposure to a chronic mild stress protocol, LTP (via a weaker induction protocol (weak-TBS)) is enhanced in the hippocampus of adolescent SD rats. This enhancement is dependent on an increase in cannabinoid-receptor-1 activity, which increases GABA release suppression from cholecystokinin (CCK) interneurons (Reich et al., 2013b). Since decreases in GABA neurotransmission facilitates LTP induction (Wigström and Gustafsson, 1986), chronic stress exposure may modify various pre- and post-synaptic mechanisms to shift LTP/LTD thresholds depending on the type and intensity of the induction stimulation. Increased LTP following weak TBS may contribute to the observed enhanced fear learning in defeated rats

(Reich et al., 2013a; Reich et al., 2013b). It is plausible, for example, that prolonged periods of social stress potentiate the CS-US association by lowering the threshold required for LTP induction. Thus, rRIP-CSDS, like other forms of chronic stress, may lead to dysfunction in fear processing, a condition that increases vulnerability to developing PTSD (Rau et al., 2005; Jovanovic and Ressler, 2010).

In conclusion, our data present a novel variant of adolescent CSDS in which a role-reversal in the conventional resident (dominant)-intruder (subordinate) social interaction is observed. This rRIP-CSDS model produces consistent behavior across several different groups of rats (i.e. the SD-Intruders reliably attacked and the LE-Residents defeated). The lack of pre-screening, female cage-mates, and aggression pre-training makes this a relatively easy paradigm to set-up and execute. Collectively, rRIP-CSDS offers a reliable and less labor intensive alternative to conventional CSDS models to study stress effects on adolescent neurobehavioral phenomena.

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Lay Summary

Adolescent psychosocial stress is implicated in the development of mood and anxiety disorders. Exposing adolescent male rats to a variant model of social stress resulted in stressed rats displaying increased levels of anxiety, depression and fear behaviors as well as impaired synaptic plasticity compared to non-stressed rats. Further investigations with this current model of animal social stress may allow further insights into the neurobehavioral mechanisms underlying mood and anxiety disorders.

Figure 1. Schematic of Chronic Social Defeat Stress (CSDS) design. Male adolescent Long Evans (LE) rats served as the Residents and male adolescent Sprague-Dawley (SD) rats served as the Intruders. Note that Resident-Intruder pairings were unique each day.

Figure 2. Sprague-Dawley (SD)-Intruders exhibit more A) Attacks and B) Dominance over Long-Evans (LE)-Intruders across five days of interactions. Mean $\pm$ S.E.M. Attacks and Dominance are displayed, respectively (as defined in text). A repeated-measure ANOVA with difference pairwise comparisons revealed significant differences between groups across days. Asterisks indicate significant differences, $p < 0.05$. *Note, the differences in number of rats (n) from panels A and B reflects lost Attacks data.*

Figure 3.

Defeated Long Evans (LE)-Residents display enhanced immobility (Mean time spent immobile $\pm$ S.E.M. (seconds)) in the Forced-Swim-Test compared to LE-Controls, Sprague-Dawley (SD)-Controls and SD-Intruders. A two-way ANOVA with difference pairwise comparisons indicated significant differences among the groups and LE Residents. Asterisks = $p < 0.05$.

Figure 4.

Reverse-Resident-Intruder paradigm (rRIP) results in enhanced trace-fear memory recall in Long Evans (LE) residents. A) Box shows that all groups froze significantly more following the first CS (60 sec post-CS trial) than their respective baseline (pre-CS) freezing rates (mean freezing (60 sec) $\pm$ S.E.M.). No differences among baseline freezing rates were observed. B) During the first CS trial (60 sec) LE residents display significant more freezing than any other group. Asterisks indicate significant differences among groups, $p < 0.05$ (two-way repeated-measures ANOVA with difference pairwise comparisons).

Figure 5. Reverse-Resident-Intruder paradigm (rRIP) results in enhanced contextual fear memory recall in LE residents. A two-way ANOVA with difference pairwise comparisons revealed that LE Residents froze significantly (mean freezing (120 sec) $\pm$ S.E.M.) more than all other groups. Asterisks indicate significant differences among groups, $p < 0.05$.

Figure 6.

Reverse-Resident-Intruder paradigm (rRIP) results in enhanced delay-fear memory recall in LE residents. A) Box shows that all groups froze significantly (mean freezing (60 sec) $\pm$ S.E.M.) more following the first CS (post-CS) trial than their respective baseline freezing rates. Oval indicates that LE-controls froze significantly less than other groups during baseline (pre-CS) freezing. B) During the first CS trial (60 sec), LE residents displayed significant more freezing than any other group. Asterisks indicate significant differences

among groups, $p = 0.05$ (two-way repeated-measures ANOVA with difference pairwise comparisons).

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Figure 7.

Reverse-Resident-Intruder paradigm (rRIP) results in altered hippocampal excitatory synaptic plasticity. A) Long-term-potential (LTP) induced by strong (s) Theta-Burst-Stimulation (TBS) is impaired in Long-Evans (LE) Residents compared to LE-controls. B) However, LTP induced by a weak TBS (wTBS) is enhanced in LE Residents compared to LE-Controls. n = number of slices and rats (one slice per rat). Mean $\pm$ S.E.M. field excitatory post synaptic potential (fEPSP) slopes expressed as percent baseline are indicated

and analyzed with one-way ANOVAs. Asterisks indicate significant differences between groups and time course, $p < 0.05$.