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Repeated Social Defeat Causes Increased Anxiety-Like Behavior and Alters Splenocyte Function in C57BL/6 and CD-1 Mice

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Abstract

The experimental model, social disruption (SDR), is a model of social stress in which mice are repeatedly attacked and defeated in their home cage by an aggressive conspecific. In terms of the impact of this stressor on the immune response, SDR has been reported to cause hyperinflammation and glucocorticoid insensitivity. To this point however, the behavioral consequences of SDR have not been thoroughly characterized. Because social defeat has been reported to cause anxiety- and depressive-like behaviors, the current study was designed to assess whether SDR also causes anxiety- and depressive-like behaviors. Using the light/dark preference test and the open field test as tools to measure behaviors characteristic of anxiety, the data showed that C57BL/6 and CD-1 male mice subjected to SDR displayed increased anxiety-like behavior. The increase in anxiety-like behaviors persisted for at least one week after the cessation of the stressor. In contrast, depressive-like behaviors were not elicited by SDR as assessed by the forced swim test or the tail suspension test. These data indicate that social disruption stress causes an increase in anxiety-like behaviors, but not depressive-like behaviors.

Keywords

Social disruption; social stress; anxiety; depression; bullying; strain differences; aggression; social defeat

Introduction

Social defeat stress causes changes in immune function and behavior. In an animal model of social stress termed social disruption (SDR), resident mice are subjected to repeated attack and defeat in their home cage by an aggressive intruder mouse (Stark et al., 2001). Mice that display submissive behaviors during SDR are still attacked by the aggressor (Avitsur et al., 2001). After the two-hour session of SDR, defeated mice show a two-fold increase in plasma

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corticosterone (Avitsur et al., 2001) demonstrating the high magnitude of the ensuing stress response.

Social defeat has been shown to have immunosuppressive effects. For example, socially defeated mice had reduced mitogen-induced splenocyte proliferation, as compared with handled controls (Beitia et al., 2005). Similarly, splenocytes from socially defeated mice immunized with sheep red blood cells formed significantly fewer plaque-forming and rosette-forming cells than controls (Devoine et al., 2004). Defeat in the SDR model, however, causes an increase in the production of the proinflammatory cytokines IL-1 α , IL-6, and TNF- α (Avitsur et al., 2002; Avitsur et al., 2003) and a marked insensitivity to the glucocorticoid hormone corticosterone (Stark et al., 2001). Similar to the observed differences in the immune effects of SDR, the biobehavioral response to SDR may also differ from that of other social stressors.

Social defeat has also been shown to cause lasting behavioral changes in rodents, including the development of anxiety-like behaviors. For example, rats that observed a partner rat as it received inescapable foot shock displayed increased defensive burying, a behavior associated with anxiety (Gutiérrez-García et al., 2006). Similarly in mice, repeated social defeat increased anxiety-like behavior in the light/dark preference test, with defeated mice spending more time in the light area of the apparatus, but had no effects on depressive-like behavior in the Porsolt forced swim test (Keeney and Hogg, 1999). However, mice that received just one eightminute session of social defeat showed increased immobility in the Porsolt forced swim test (Hebert et al., 1998).

The effects of repeated social defeat in the social disruption model on anxiety- and depressive-like behaviors have not been explored. Like the immunological effects of SDR, the behavioral response to social disruption may differ from other social stressors. Thus, this study tested the effects of SDR on anxiety-like and depressive-like behaviors. Changes in cytokine production, splenic cell populations, and glucocorticoid insensitivity were also measured. These effects were measured in both inbred and outbred mouse strains.

Methods

Animals

Male C57BL/6 and CD-1 mice were purchased from Charles River and housed in groups of 3-5 in polycarbonate cages and maintained under 12:12 light cycle in a temperature ($21 \pm 1^{\circ}$ C) and humidity ($50 \pm 5\%$) controlled, Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility at the Ohio State University for the duration of this experiment. Standard lab diet and tap water were available *ad libitum*. All mice were aged 8-10 weeks and were experimentally naïve at the beginning of the experiments. Mice were handled minimally, for the purpose of general husbandry. All procedures were approved by the Institutional Laboratory Animal Care and Use Committee (ILACUC) at the Ohio State University.

Social Disruption (SDR)

The social disruption paradigm was described previously (Avitsur et al., 2001, Stark et al., 2001). Briefly, social disruption consisted of introducing an aggressive intruder mouse into the home cage of the resident mice. The resident mice were the subjects of these experiments. Aggressors were introduced to each cage for six daily cycles lasting two hours each, starting at approximately 1700 EST. The aggressor mouse usually attacked the resident mice within 5 minutes of being introduced into the cage. Aggressors were C57BL/6 or CD-1 males, selected based on having a history of agonistic behavior. Aggressors were single housed for a period

of several weeks to further induce aggressive behavior. Sessions were monitored to ensure that the intruder repeatedly attacked and consistently defeated the resident mice. If the aggressor did not attack the residents, or if the residents defeated the aggressor, then the aggressor was removed and replaced by a new aggressor mouse. During SDR, the resident mice generally displayed submissive behaviors, including upright submissive posture, fleeing, and crouching. A different aggressor was used during each cycle to prevent habituation. Control mice were left undisturbed in their home cages during SDR sessions.

Wound Severity

Cutaneous wounds were assessed at sacrifice, as described previously (Avitsur et al., 2001), with some changes. Wounding was quantified by a trained observer on a 5-point scale, ranging from 0 to 4, with 0 representing no visible wounds, and 4 representing large wounds.

Glucocorticoid Insensitivity Assay

Glucocorticoid (GC) insensitivity can be quantified by measuring the viability of cells cultured with various physiological and pharmacological concentrations of glucocorticoid in the absence or presence of LPS (lipopolysaccharide). High doses of corticosterone typically reduce the viability of LPS-stimulated splenocytes by inducing apoptosis. However, splenocytes harvested from SDR mice remain viable even in the presence of high concentrations of corticosterone (Stark et al., 2001). In the present study, mice were sacrificed by CO₂ asphyxiation on the morning following the last day of behavioral testing (for all groups: 15 days after the first cycle of SDR). Wound severity and individual body weights were assessed at this time. Spleens were harvested, weighed, and homogenized for one minute by a stomacher (Stomacher 80 Biomaster, Seaward, London, England) as per manufacturer's instructions (Bailey et al. 2004; Stark et al. 2001). Red blood cells were lysed with a hypotonic solution (0.16M NH₄Cl, 10mM KHCO₃, and 0.13 mM EDTA). The cell suspension was washed in HBSS/10% FBS and passed through a 70 m nylon filter. Triplicate samples of splenocytes were cultured 100 l/well at 2.5×10^5 cells per well in flat-bottom 96-well tissue culture plates in complete RPMI (containing 10% heat-inactivated fetal bovine serum, 0.075% sodium bicarbonate, 10mM Hepes buffer, 100 U/ml penicillin G, 100 g/ml streptomycin sulfate, 1.5 nM L-glutamine, and 0.0035% 2-mercaptoethanol). Cultures were stimulated with 0.40 g/ml LPS and corticosterone (dose range 0.005 - 0.05 M) for 48 hr at 37° C and 5% CO₂. Cell viability was measured with a tetrazolium substrate solution (Cell Titer 96 non-radioactive proliferation kit, Promega), and read at 490 nm by an ELISA plate reader. Viability of each sample was expressed as the mean optical density (OD) of each LPS-stimulated sample, minus OD of nonstimulated samples treated with the same corticosterone concentration. For comparison purposes, data were converted into a corticosterone resistance index, which represents the viable cells in each corticosterone treatment group, as a percentage of non-corticosterone treated cultures from the same treatment group (Avitsur et al., 2001,2002).

Cytokine ELISAs—Splenocytes were processed as above, suspended 2.5×10^6 cells/ml in RPMI, plated 200 l/well in cell culture treated 96-well plates, and incubated for 18 hr at 37° C in 5% CO₂. Pro-Inflammatory cytokines (IL-6 and TNF- α) from splenocytes culture supernatants were quantified by a standard sandwich ELISA, as described previously (Avitsur et al., 2005; Stark et al., 2001). For IL-6 determination, rat anti-mouse IL-6 antibody was used (BD Pharmingen, San Diego, CA). The ELISA for TNF- α used rat anti-mouse TNF- α antibody (BD Pharmingen, San Diego, CA) and was also performed as per manufacturer's instructions, with the modification that assay diluent was phosphate-buffered saline plus 2% bovine serum albumin.

Flow Cytometry—Cell suspensions of 2.5×10^5 cells in RPMI were incubated for 45 minutes at 4°C with FITC-conjugated Gr-1/Ly-6G (clone RB6-8C5), PE-conjugated anti-mouse Pan-

NK (clone DX5), PerCP-conjugated anti-mouse CD45R/B220 (clone RA3-6B2) and APCconjugated anti-mouse CD11b/Mac-1 (clone M1/70). All monoclonal antibodies were purchased from BD Pharmingen (San Diego, CA). Cells were stained using a standard lyse/wash procedure using PBS (Dulbecco's PBS without Ca and Mg, 2% FBS, and 0.1% NaN₃). A total of 10,000 events from each sample were analyzed on a dual-laser flow cytometer (FACSCalibur, BD Immunocytometry Systems) using Cell Quest Pro and Attractions software. Forward and side scatter characteristics as well as differences in antibody staining were used to identify lymphocytes, neutrophils, and monocytes/macrophages. Matched isotype controls were used to set negative staining criteria.

Tests of anxiety-like behavior and locomotion

Two tests were used to assess anxiety-like behaviors in defeated mice: the light/dark preference test and the open field test. The light/dark preference test, also known as the black-white test, is a commonly used test for anxiety-like behavior (Crawley, 1981; Crawley and Goodwin, 1980; Flint, 2002; Holmes et al., 2001; Ohl, 2005). The apparatus consisted of two Plexiglas boxes, connected by a small passage at floor level. The larger box (30 × 20 cm) was lit by a bright incandescent light bulb (4100 lux) directly overhead, and the smaller box (30 × 10 cm) was made of black Plexiglas and was covered, making it much darker (< 3 lux). Mice that express anxiety-like behavior tend to spend less time in the brightly lit box and take longer to emerge from the dark box after entering. These effects are reversed by anxiolytic compounds (Bourin and Hascoët, 2003; Crawley, 1981; Crawley and Paylor, 1997). The open field apparatus consisted of a 30 × 30 × 25 cm Plexiglas box with a solid floor and was lighted by overhead room lighting (1300 lux). A grid was drawn on the floor that divided the floor into 36 squares. The open field test is designed to take advantage of a rodent's natural tendencies to explore the environment while avoiding open spaces. Mice that express anxiety-like behavior tend to spend less time in the center of the open field and also tend to locomote near the walls of the apparatus (thigmotaxis). These effects are also reversed by anxiolytic compounds (Bhatnagar et al., 2004; Crawley, 1999; Dulawa et al., 2004; Prut and Belzung, 2003; Sullivan et al., 2003). For this experiment, the dependant variables were [a] total number of line crosses, [b] rearing, [c] total time spent in the center/perimeter of the open field, and [d] number of transitions between the center/perimeter of the open field. Each test apparatus was cleaned with water-dampened cloths between subjects. Both tests were recorded for 5 minutes.

Tests of depressive-like behavior

Two tests were used to assess depressive-like behaviors in defeated mice: the Porsolt forced swim test and the tail suspension test. In the forced swim test, individual mice were placed in a glass cylinder (43 cm high, 22 cm diameter) filled with roomtemperature water to a depth of 15 cm. The rim of the cylinder was high enough that the mouse could not climb or jump out, and the water was deep enough that the mouse could not touch the bottom of the cylinder with its tail (Crawley and Paylor, 1997; Hebert et al. 1998; Porsolt, 1977; Porsolt, 1997). This test was recorded for 5 minutes in the dark (< 3 lux), using infrared lighting. Struggling behavior was measured as time spent actively swimming around the apparatus, climbing the walls of the tank (forepaws break the surface of the water), or floating (which included minor movements to keep the head above water). The water was replaced between subjects. Like the Forced Swim test, the tail suspension test models depression in mice by placing the animal in an inescapable, uncomfortable situation and measuring passive behavior (Dalvi and Lucki, 1999; Mayorga and Lucki, 2001; Steru et al., 1985). Individual mice were suspended by their tails with adhesive tape, from a horizontal bar 25cm above the tabletop. This test was recorded for 6 minutes under ambient room lighting. Struggling behavior was measured as time spent actively kicking and struggling vs. hanging motionless.

General Procedure

In the first experiment, anxiety-like behavior was measured on the morning immediately following the sixth or final cycle of social disruption. Mice were randomly assigned to either home cage control (HCC; n=19) or social disruption (SDR; n=22) treatment groups. All behavioral tests began at approximately 1000 h. Mice were transported to the testing area and left undisturbed for 30 minutes before testing began. Behavior was measured in the light/dark preference test. During testing, the investigators left the room and behavior was videotaped for later analysis. The videos were digitized and scored by a trained observer blinded to the treatment groups, using the Observer software (Noldus Information Technologies, the Netherlands). On the following morning, mice were sacrificed by CO₂ asphyxiation at which time spleens were harvested, weighed, and processed as described above for assays of GC insensitivity, cytokine production, and phenotyping by flow cytometry.

The second experiment focused on temporal defeat-induced changes in behavior. Separate groups of mice were tested for anxiety- or depressive-like behaviors in the open field, forced swim test, or tail suspension test, on the mornings following day 1 and day 6 of SDR. A third group received 6 cycles of SDR and was tested after 8 days of rest (day 14 after the first cycle of SDR). A total of 51 C57Bl/6 mice were used in the open field test (mice were randomly assigned to HCC and SDR groups as follows: Day1 (10, 10); Day6 (9, 9); Day14 (3, 10)). A total of 48 C57Bl/6 mice were used in the forced swim test (mice were randomly assigned to HCC and SDR groups as follows: Day1 (10, 10); Day6 (5, 10); Day14 (3, 10)). A total of 60 C57Bl/6 mice were used in the tail suspension test (mice were randomly assigned to HCC and SDR groups as follows: Day1 (10, 10); Day6 (10, 10); Day14 (10, 10)). Because separate groups of mice were tested on each day, there was no habituation to either test. The open field test was scored by two trained observers and inter-observer reliability was > 96%. Mice were sacrificed on the morning following the last day of behavioral testing (for all groups: 15 days after the first cycle of SDR), at which time spleens were harvested, weighed, and processed as described above for assays of GC insensitivity, cytokine production, and phenotyping by flow cytometry.

The third experiment measured SDR-induced changes in anxiety- and depressive-like behaviors in the outbred CD-1 strain. As in the first experiment, mice were randomly assigned to SDR or HCC treatment, and the SDR groups were subjected to 6 cycles of SDR over 6 days. On the morning following the 6th cycle of SDR, a total of 60 CD-1 mice were tested in the open field test (HCC(6); SDR(8)), light/dark preference test (HCC(10); SDR(10)), forced swim test (HCC(10); SDR(16)), or tail suspension test (HCC(10); SDR(10)). Mice were sacrificed 15 days after the first cycle of SDR, at which time spleens were harvested, weighed, and processed as described above for assays of GC insensitivity, cytokine production, and phenotyping by flow cytometry.

Statistics

A repeated measures 2-way (stress vs. corticosterone concentration) ANOVA was used to assess glucocorticoid insensitivity. Between groups 1-way ANOVA was used to compare spleen mass, cytokine concentrations, and cell population phenotypes. A between groups, 2-way (stress vs. ethological measure) ANOVA was used to compare behavioral measures on separate time points. Correlation comparisons were made between behavioral measures and immunological measures using Fisher's *r* to *z* conversion to establish statistical significance. Follow-up comparisons were performed using Fisher's PLSD tests, where appropriate. All statistical comparisons were made using Statview software (SAS Institute, Inc.). Statistical significance was set at $p < 0.05$.

Results

Experiment 1: Effects of social disruption on anxiety-like behavior in C57BL/6

Social disruption stress caused an increase in anxiety-like behaviors in the light/dark preference test. Individual mice were placed in the corner of the brightly lit box and allowed to freely explore the apparatus. Each mouse moved about and explored the apparatus. Defeated mice spent significantly more time in the smaller, dark box of the apparatus than did non-defeated controls ($F(1,39) = 4.13$; $p < 0.05$; Fig. 1A). Latency to enter the dark box did not differ between treatment groups; both defeated and non-defeated mice entered the dark box after approximately 1 minute ($p > 0.96$). However, after entering the dark box, the defeated mice took significantly longer to reemerge than did controls ($F(1,39) = 6.16$; $p < 0.05$; Fig. 1B).

Experiment 2: Temporal effects of social disruption on immune function and behavior in C57BL/6

Immunological measures—Consistent with previous data from our lab and others, SDR caused an increase in spleen mass. Mean spleen mass was 133.0 ± 11.3 mg in SDR mice and 84.8 ± 6.1 mg in HCC mice ($F(1,45) = 10.15$; $p < 0.01$); this effect persisted when corrected for individual body mass ($F(1,45) = 10.68$; $p < 0.01$; Fig. 2A). Also consistent with previous findings, the cellular makeup of the spleen, as measured by flow cytometry, indicated that the enlarged spleens of SDR mice had more macrophages/monocytes than control mice, with SDR mice having a mean number of 4.79×10^6 cells per spleen, compared with 1.58×10^6 ($F(1,44) = 6.07$; $p < 0.05$). Glucocorticoid insensitivity developed in SDR mice but not in controls ($F(1,43) = 5.18$; $p < 0.05$; Fig. 2B). More specifically, follow-up comparisons revealed significantly higher cell viability in the SDR splenocytes stimulated with 0.5M corticosterone and 5.0M corticosterone. In addition, supernatants from cultured splenocytes stimulated with LPS revealed an increased production of the proinflammatory cytokine IL-6 ($F(1,24) = 11.75$; $p < 0.01$; Fig. 2C) and TNF- α in SDR mice compared to controls ($F(1,39) = 7.62$; $p < 0.01$; Fig. 2D). As reported previously, cytokine production and cell proliferation were greatly reduced in non LPS-stimulated samples (Avitsur et al., 2003). Mean wound severity within SDR treatment groups was 1.73 ± 0.22 , and did not differ significantly between behavioral tests.

Open field test—Social disruption (SDR) caused an increase in anxiety-like behaviors in the open field test. As in the light/dark preference test, all mice moved about and explored the open field. However, on all days tested, mice subjected to SDR spent significantly less time in the center of the open field than controls ($F(1,45) = 14.89$; $p < 0.001$; Fig. 3A). Although mice from both treatment groups entered and explored the center of the open field, SDR mice entered the center fewer times than controls ($F(1,45) = 7.50$; $p < 0.01$; Fig. 3B). This difference was not due to changes in locomotion or activity as line crosses did not differ between treatment groups on any of the days tested ($p > 0.62$; Fig. 3C).

Porsolt forced swim test—Social disruption did not have a strong depressive-like effect in the forced swim test. After being placed in the water tank, defeated mice displayed a shortened latency to stop swimming and become immobile after 1 and 6 cycles of SDR compared with controls ($F(1,18) = 4.80$; $p < 0.05$ and $F(1,13) = 7.29$; $p < 0.05$; Fig. 3D). However, the total time spent immobile did not differ between treatment groups ($p > 0.12$; Fig. 3E). Similarly, overall time spent swimming and climbing the walls of the tanks did not differ between groups ($p > 0.33$ and 0.23 , respectively).

Tail suspension test—Like the forced swim test, social disruption did not affect depressive-like behavior in the tail suspension test. Social disruption had no effect on time spent immobile

on all days tested ($p > 0.42$; Fig. 3F). There were no observed cases of tail climbing during testing.

Experiment 3: Effects of SDR on immune function and anxiety-like behavior in outbred mice

Immunological measures—Social disruption also caused an increase in spleen mass in outbred CD-1 mice. Mean spleen mass was 195.4 ± 33.7 mg in SDR mice and 104.1 ± 7.9 mg in HCC mice ($F(1,13) = 6.03$; $p < 0.05$), and this effect persisted when corrected for individual body mass, ($F(1,13) = 6.23$; $p < 0.05$; Fig. 4A). Glucocorticoid insensitivity developed in defeated outbred CD-1 mice but not in controls ($F(1,13) = 9.46$; $p < 0.01$; Fig. 4B). Follow-up comparisons revealed significantly greater cell viability in the SDR mice splenocytes stimulated with 0.005, 0.1, 0.5, and 5.0M corticosterone. Also consistent with previous findings from inbred mouse strains, supernatants from cultured splenocytes stimulated with LPS revealed an increased production of the proinflammatory cytokines IL-6 ($F(1,13) = 12.56$; $p < 0.01$; Fig. 4C) and TNF- α ($F(1,13) = 18.24$; $p < 0.001$; Fig. 4D) in SDR mice compared to controls. Cytokine production and cell proliferation were greatly reduced in non LPS-stimulated samples. Mean wound severity within SDR treatment groups was 2.22 ± 0.15 , and did not differ significantly between behavioral tests.

Open field and light/dark preference tests—Outbred CD-1 mice subjected to six sessions of SDR showed anxiety-like behavior in the open field test, but not in the light/dark box. In the open field test, defeated outbred CD-1 mice spent significantly less time in the center of the open field than controls ($F(1,12) = 13.90$; $p < 0.01$; Fig. 5A). Defeated mice entered the center of the open field about as many times as controls ($p > 0.22$; Fig. 5B). As in C57BL/6 mice, social disruption had no effect on locomotion ($p > 0.85$; Fig. 5C). In the light/dark box test, there was no difference between the SDR and control groups, which both spent a mean 164 ± 27 s and 166 ± 28 s, respectively, in the dark box ($p > 0.83$). Similarly, latency to reemerge from the dark box after entering did not differ ($p > 0.80$). Mean time to reemerge was 26.2 ± 6 s in HCC and 28.3 ± 6 s in SDR mice.

Forced swim and tail suspension tests—As with the inbred mouse, no observable differences were found in depressive-like behaviors in defeated outbred CD-1 mice. In the forced swim test, there was no difference in latency to become immobile ($p > 0.75$; Fig. 5D) or total time spent immobile ($p > 0.93$; Fig. 5E). Overall time spent swimming and climbing the walls of the tanks did not differ between groups ($p > 0.93$). Similarly, the tail suspension test revealed no differences in immobility ($p > 0.88$; Fig. 5F). As with the C57BL/6 strain, no tail climbing behaviors were observed in outbred CD-1 mice.

Discussion

The data confirm and extend previous reports on the effects of repeated social defeat on the immune system (Avitsur et al., 2001; Bailey et al., 2004; Padgett et al., 1998), showing that the noted effects are not limited to inbred mouse strains. Social disruption (SDR) stress resulted in a significant enlargement of the spleen, due to an increase in trafficking of CD11b+ myeloid cells from the bone marrow to the spleen (Engler et al., 2005). In addition, splenocytes from mice exposed to SDR produced higher levels of IL-6 and TNF- α upon *in vitro* stimulation with LPS and remained viable in culture, even in the presence of high pharmacological doses of corticosterone, a phenomenon referred to as glucocorticoid resistance (Stark et al., 2001).

Beyond simply extending the observations concerning SDR from the inbred to the outbred CD-1 mouse strain, the goal of this study was to assess whether the stress of SDR affected behavior. A close look at the data generated from the forced swim test showed that, in C57BL/6 mice, SDR caused a decrease in the latency to become immobile; this was not true in the

CD-1 animal. However, in spite of the decrease in latency to become immobile, overall immobility was not significantly affected by SDR in either strain of mouse. Likewise, no significant differences were found among the treatment groups as assessed by the tail suspension test. Although additional tests to assess depressive-like behaviors (e.g., sucrose consumption test) could be employed, taken together and keeping in mind limitations inherent to the forced swim test and tail-suspension test, the data suggest that SDR does not cause an increase in depressive-like behavior.

Therefore, the data reveal that on the one hand, SDR had little to no effect on depressive-like behavior as assessed by forced-swim or tail-suspension testing. However, on the other hand, by using measures commonly used to assess anxiety-like behaviors in rodents (Bhatnagar et al., 2004; Bourin and Hascoët, 2003; Crawley, 1981, 1999; Crawley and Paylor, 1997; Ohl 2005), the data described herein reveal that SDR of either inbred C57BL/6 or outbred CD-1 male mice resulted in an increase in anxiety-like behavior. The development of this anxiety-like behavior was evident in C57BL/6 mice after a single cycle of SDR and lasted for at least one week following the cessation of the stressor.

The extant literature indicates that forms of social defeat can be associated with decreased locomotion (Avgustinovich et al, 1997; Kudryatseva et al., 2004). If true for SDR, such reduced activity would undoubtedly bias the interpretation of our tests for anxiety. However, data from the open field test indicated that SDR had no effect on locomotion, regardless of strain, thus suggesting that SDR increases anxiety-like behaviors without affecting overall activity levels. This further indicates that the observed reduction in time spent in the center of the open field was not just an artifact of decreased mobility, but was directed by different exploratory patterns of defeated vs. non-defeated mice. The observed differences in mobility indicate that there are qualitative differences between SDR and other social stressors.

The SDR-induced increase in time spent in the dark box of the light/dark preference test seen in C57BL/6 mice did not replicate in outbred CD-1 mice. This is possibly due to strain-specific differences in the baseline expression of anxiety-like behaviors. Compared to other strains, C57BL/6 mice are considered to be a relatively “low anxiety” strain in the L/D box (Bouwknicht and Paylor, 2002; Rodgers et al., 2002). In fact, data from the present study indicate that during the 5 minute test, control C57BL/6 mice spent 125 seconds (41.6%) in the dark box whereas outbred CD-1 controls spent 165 seconds (55%) in the dark. Thus, because of the higher baseline ‘anxiety’ of the CD-1 mouse, which spends comparatively more time in the dark, the effects of SDR may be too subtle to detect in this strain of mouse. This reduced effect can not be explained by strain-specific wound severity, as both C57BL/6 and CD-1 mice were wounded to a similar extent in all experiments.

Although a direct causal relationship between anxiety-like behavior and GC insensitivity has not yet been established in this model, high expression of anxiety-like behaviors was seen only in defeated mice, and not in non-defeated controls. Mice subjected to SDR that expressed high levels of GC insensitivity and splenomegaly also tended to express higher levels of anxiety-like behavior, whereas control mice did not develop GC insensitivity and expressed low levels of anxiety-like behavior. Unfortunately, at an individual level within the SDR-treated group, the data did not reveal statistically significant correlations between the development of anxiety and any immunological measures or wound severity. However, previously published data has shown a connection between submissive behavior during SDR and the development of GC insensitivity (Avitsur et al., 2001). Although submissive behavior was not assessed in the present study, those data raise the question of whether mice that display submissive behavior during SDR are also more likely to develop anxiety-like behaviors as compared to cagemates that were not submissive. To draw any such conclusion, further studies are needed to assess

any such causal links among submissive behaviors, anxiety-like behaviors, and the immunological consequences of SDR.

The stress response is a well-characterized psychophysiological reflex that affects immunological regulation in most animal species, including humans. These stress-induced effects are not inconsequential; high stress reactivity has been linked to increased susceptibility to infection and immune dysfunction (Black, 2002; Padgett and Glaser, 2003; Glaser and Kiecolt-Glaser, 1998; Kiecolt-Glaser et al., 2002; Liu et al, 2002; Rohleder et al., 2001; Cohen and Hamrick, 2003). Some of these effects can be modeled on the laboratory, and as we and others have shown, social defeat activates the core stress responses and can have deleterious immunological effects on defeated mice (Devoino et al., 2003; Merlot et al., 2003; Padgett et al., 1998; Sheridan et al., 2000), including increased proinflammatory cytokine production, splenomegaly, trafficking of lymphocytes to the spleen, and insensitivity to the antiproliferative effects of glucocorticoids in LPS-stimulated splenocytes (Avitsur et al., 2003; Bailey et al., 2006; Engler et al., 2005; Stark et al., 2001). Although slight differences occurred between C57BL/6 mice and CD-1 mice, the present data indicate that the development of anxiety-like behavior can be added to the list of consequences mediated by social disruption. Whether or not the immune and behavioral changes associated with social disruption are covariates or are dependent upon one another remains to be evaluated fully.

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References

- Avgustinovich DF, Gorbach OV, Kudryavtseva NN. Comparative Analysis of anxiety-like behavior in partition and plus-maze tests after agonistic interactions in mice. *Physiol Behav* 1997;61:37–43. [PubMed: 8976531]
- Avitsur R, Kavelaars A, Heijnen C, Sheridan JF. Social stress and the regulation of tumor necrosis factor- α secretion. *Brain Behav Immun* 2005;19(4):311–317. [PubMed: 15944070]
- Avitsur R, Padgett DA, Dhabhar FS, Stark JL, Kramer KA, Engler H, Sheridan JF. Expression of glucocorticoid resistance following social stress requires a second signal. *J Leukoc Biol* 2003;74:507–513. [PubMed: 12960258]
- Avitsur R, Stark JL, Sheridan JF. Social stress induced glucocorticoid resistance in subordinate animals. *Horm Behav* 2001;39:247–257. [PubMed: 11374910]
- Avitsur R, Stark JL, Dhabhar FS, Padgett DA, Sheridan JF. Social disruption-induced glucocorticoid resistance: kinetics and site specificity. *J Neuroimmunol* 2002;124:54–61. [PubMed: 11958822]
- Bailey MT, Engler H, Sheridan JF. Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice. *J Neuroimmunol* 2006;171(12):29–37. [PubMed: 16253348]
- Bailey MT, Avitsur R, Engler H, Padgett DA, Sheridan JF. Physical defeat reduces the sensitivity of murine splenocytes to the suppressive effects of corticosterone. *Brain Behav Immun* 2004;18(5):416–24. [PubMed: 15265534]
- Beitia G, Garmendia L, Azpiroz A, Vegas O, Brain PF, Arregi A. Time-dependent behavioral, neurochemical, and immune consequences of repeated experiences of social defeat stress in male mice and the ameliorative effects of fluoxetine. *Brain Behav Immun* 2005;19(6):530–539. [PubMed: 16214024]
- Bhatnagar S, Sun LM, Raber J, Maren S, Julius D, Dallman MF. Changes in anxiety-related behaviors and hypothalamic-pituitary-adrenal activity in mice lacking the 5-HT-3A receptor. *Physiol Behav* 2004;81:545–555. [PubMed: 15178147]

- Black P. Stress and the inflammatory response: A review of neurogenic inflammation. *Brain Behav Immun* 2002;16:622–652. [PubMed: 12480495]
- Bourin M, Hascoët M. The mouse light/dark box test. *Eur J Pharm* 2003;463:55–65.
- Bouwknicht JA, Paylor R. Behavioral and physiological mouse assays for anxiety: a survey in nine mouse strains. *Behav Brain Res* 2002;136(2):489–501. [PubMed: 12429412]
- Cohen S, Hamrick N. Stable Individual Differences in Physiological Response to Stressors: Implications for Stress-Elicited Changes in Immune Related Health. *Brain Behav Immun* 2003;17:407–414. [PubMed: 14583231]
- Crawley JN. Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. *Pharmacol Biochem Behav* 1981;15:695–699. [PubMed: 6118883]
- Crawley JN. Behavioral phenotyping of transgenic and knockout mice: experimental design and evaluation of general health, sensory functions, motor abilities, and specific behavioral tests. *Brain Res* 1999;835:18–26. [PubMed: 10448192]
- Crawley JN, Goodwin FK. Preliminary report of a simple animal model for the anxiolytic effects of benzodiazepines. *Pharmacol Biochem Behav* 1980;13:167–170. [PubMed: 6106204]
- Crawley JN, Paylor R. A proposed test battery and constellations of specific behavioral paradigms to investigate the behavioral phenotypes of transgenic and knockout mice. *Horm Behav* 1997;31:197–211. [PubMed: 9213134]
- Dalvi A, Lucki I. Murine models of depression. *Psychopharmacology (Berl)* 1999;147(1):14–6. [PubMed: 10591858]
- Devoino L, Alperina E, Pavina T. Immunological consequences of the reversal of social status in C57BL/6J mice. *Brain Behav Immun* 2003;17:28–34. [PubMed: 12615047]
- Devoino L, Alperina E, Podgornaya E, Ilyutchenok R, Idova G, Polyakov O. Regional changes of brain serotonin and its metabolite 5-hydroxyindolacetic acid and development of immunosuppression in submissive mice. *Int J Neurosci* 2004;114(9):1049–62. [PubMed: 15370173]
- Dulawa SC, Holick KA, Gundersen B, Hen R. Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology* 2004;29(7):1321–30. [PubMed: 15085085]
- Engler H, Engler A, Bailey MT, Sheridan JF. Tissue-specific alterations in the glucocorticoid sensitivity of immune cells following repeated social defeat in mice. *J Neuroimmunol* 2005;163(12):110–119. [PubMed: 15885313]
- Flint, J. Animal models of anxiety. In: Plomin, R.; DeFries, JC.; Craig, IW.; McGuffin, P., editors. *Behavioral Genetics in the Postgenomic Era*. American psychological Assn.; Washington, D.C.: 2002. p. 425–441.
- Glaser R, Kiecolt-Glaser JK. Stress-associated immune modulation: relevance to viral infections and chronic fatigue syndrome. *Am J Med* 1998;105(3A):35S–42S. [PubMed: 9790480]
- Gutiérrez-García AG, Contreras CM, Mendoza-Lopez MR, Cruz-Sanchez S, Garcia-Barradas O, Rodriguez-Landa JF, Bernal-Morales B. A single session of emotional stress produces anxiety in Wistar rats. *Behav Brain Res* 2006;167(1):30–5. [PubMed: 16216347]
- Hebert MA, Evenson AR, Lumley LA, Meyerhoff JL. Effects of acute social defeat on activity in the forced swim test: parametric studies in DBA/2 mice using a novel measurement device. *Aggress Behav* 1998;24:257–269.
- Holmes A, Iles JP, Mayell SJ, Rodgers RJ. Prior test experience compromises the anxiolytic efficacy of chlordiazepoxide in the mouse light/dark exploration test. *Behav Brain Res* 2001;122(2):159–67. [PubMed: 11334646]
- Keeney AJ, Hogg S. Behavioural consequences of repeated social defeat in the mouse: preliminary evaluation of a potential animal model of depression. *Behav Pharmacol* 1999;10(8):753–64. [PubMed: 10780291]
- Kiecolt-Glaser JK, McGuire L, Robles TF, Glaser R. Psychoneuroimmunology and psychosomatic medicine: back to the future. *Psychosom Med* 2002;64(1):15–28. [PubMed: 11818582]
- Kudryavtseva NN, Bondar NP, Avgustinovich DF. Effects of repeated experience of aggression on the aggressive motivation and development of anxiety in male mice. *Neurosci Behav Physiol* 2004;34(7):721–30. [PubMed: 15526428]
- Liu LY, Coe CL, Swenson CA, Kelly EA, Busse WW. School examinations enhance airway inflammation to antigen challenge. *Am J Respir Crit Care Med* 2002;165:1062–1067. [PubMed: 11956045]

- Mayorga AJ, Lucki I. Limitations on the use of the C57BL/6 mouse in the tail suspension test. *Psychopharmacology (Berl)* 2001;155(1):110–2. [PubMed: 11374330]
- Merlot E, Moze E, Dantzer R, Neveu PJ. Importance of fighting in the immune effects of social defeat. *Physiol Behav* 2003;80:351–357. [PubMed: 14637235]
- Ohl F. Animal models of anxiety. *Handb Exp Pharmacol* 2005;169:35–69. [PubMed: 16594254]
- Padgett DA, Glaser R. How stress influences the immune response. *Trends Immunol* 2003;24(8):444–448. [PubMed: 12909458]
- Padgett DA, Sheridan JF, Dorne J, Berntson GG, Candelora J, Glaser R. Social stress and the reactivation of latent herpes simplex virus type 1. *Proc Natl Acad Sci USA* 1998;95:7231–7235. [PubMed: 9618568]
- Porsolt RD. Historical perspective on CMS model. *Psychopharmacology* 1997;134(4):363–4. [PubMed: 9452177]
- Porsolt RD, Le Pichon M, Jalfre M. Depression: a new animal model sensitive to antidepressant treatments. *Nature* 1977;266(5604):730–2. [PubMed: 559941]
- Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol* 2003;463(13):3–33. [PubMed: 12600700]
- Rodgers RJ, Boullier E, Chatzimichalaki P, Cooper GD, Shorten A. Contrasting phenotypes of C57BL/6JOLA^{Hsd}, 129S2/SvHsd and 129/SvEv mice in two exploration-based tests of anxiety-related behaviour. *Physiol Behav* 2002;77(23):301–10. [PubMed: 12419406]
- Rohleder N, Schommer NC, Hellhammer DH, Engel R, Kirschbaum C. Sex differences in glucocorticoid sensitivity of proinflammatory cytokine production after psychosocial stress. *Psychosom Med* 2001;63:966–972. [PubMed: 11719636]
- Sheridan JF, Stark JL, Avitsur R, Padgett DA. Social disruption, immunity, and susceptibility to viral infection. *Ann N Y Acad Sci* 2000;917:894–905. [PubMed: 11270350]
- Stark JL, Avitsur R, Padgett DA, Campbell KA, Beck FM, Sheridan JF. Social stress induces glucocorticoid resistance in macrophages. *Am J Physiol Regul Integr Comp Physiol* 2001;280:R1799–R1805. [PubMed: 11353685]
- Steru L, Chermat R, Thierry B, Simon P. The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* 1985;85(3):367–70. [PubMed: 3923523]
- Sullivan GM, Apergis J, Gorman JM, LeDoux JE. Rodent doxapram model of panic: behavioral effects and c-Fos immunoreactivity in the amygdala. *Biol Psychiatry* 2003;53(10):863–70. [PubMed: 12742673]

Figure 1.

Male C57BL/6 mice were subjected to repeated social disruption (SDR) for 6 consecutive days. On the morning following the sixth day of defeat, the mice were individually tested in the light/dark preference test for 5 minutes. (a) Defeated mice (SDR) spent significantly more time in the dark box of the test apparatus than non-defeated home cage controls (HCC). (b) Defeated mice took longer to emerge from the dark box than controls. * $p < 0.05$ vs. controls.

Figure 2.

Social disruption caused changes in immune regulation in C57BL/6 mice. Mice were subjected to six consecutive days of social disruption stress (SDR) then spleens were harvested, weighed, and cultured. (a) Mice subjected to SDR had heavier spleens than controls (HCC) mice, even when corrected for body weight. (b) Splenocytes were cultured for 48 hours, stimulated with LPS and various concentrations of the glucocorticoid hormone corticosterone. Splenocytes from defeated mice were insensitive to the pro-apoptotic effects of corticosterone. (c, d) Splenocytes were cultured for 18 hours, stimulated with LPS. Supernatants from these cells were assayed for IL-6 and TNF via sandwich ELISA. * $p < 0.05$; ** $p < 0.01$ vs. control.

Figure 3.

Behavioral effects of social disruption in C57BL/6 mice. Mice were subjected to 6 consecutive days of social disruption (SDR; filled bars). Separate groups of mice were tested on the day after 1 day of defeat (Day 1), after the 6th day of defeat (Day 6), or after 6 days of defeat, then rested for 8 days with no SDR (Day 14). Non-defeated controls (HCC; open bars) were also tested on each of these days. (a) Mice subjected to SDR spent less time in the center of the open field and (b) entered the center of the open field less often than controls. (c) Locomotion was not affected by SDR. (d, e) In the forced swim test, defeated mice became immobile more quickly than controls on Day 1 and Day 6, although total time spent immobile did not differ

between groups. (f) Immobility in the tail suspension test was not affected by SDR. * $p < 0.05$ vs. controls.

Figure 4.

Immune effects of SDR in CD-1 mice. Mice were subjected to 6 consecutive days of social disruption (SDR). Spleens were harvested, weighed, and cultured. (a) As with inbred strains, spleens from SDR mice were larger than those from control (HCC) mice. (b) Cultured splenocytes from SDR mice were insensitive to the pro-apoptotic effects of corticosterone, as compared with controls, even at high concentrations. (c,d) Supernatants from LPS-stimulated splenocytes from defeated mice contained higher concentrations of IL-6 and TNF than those from control mice. * $p < 0.05$; † $p < 0.001$

Figure 5.

Behavioral effects of SDR in CD-1 mice. Mice were subjected to 6 consecutive days of social disruption (SDR), and behavior was measured on the morning following the 6th day of defeat. (a) In the open field test, defeated mice spent less time in the center of the open field than controls (HCC) and (b) Entered the center of the open field fewer times than controls. (c) Locomotion was not affected by SDR. (d, e) In the forced swim test, CD-1 mice showed no differences in latency to become immobile or in overall time immobile, regardless of defeat treatment. (f) No differences were found in time immobile in the tail suspension test. ** $p < 0.01$