Individuals under chronic stress suffer anxiety and depression at higher rates than sex- and age-matched controls (O’Donovan et al., 2010; Slavich & Irwin, 2014). In modern society, some of the primary sources of chronic stress are social in nature, relating to relationships and conflicts with other people (McEwen, 2003, 2004; Slavich & Irwin, 2014). As such, the development of appropriate animal models could provide insights into the behavioral, physiological, and neurological consequences associated with chronic social stressors. In addition, using these models to demonstrate how individuals respond to the termination of those same stressors may provide insight into potential therapeutic applications to mitigate symptoms associated with chronic stress with specific implications for anxiety and depressive disorders.
Historically, the most common rodent model that has been utilized to investigate chronic social stress is the social defeat model (a.k.a. “resident-intruder” model; Golden, Covington, Berton, & Russo, 2011; Hollis & Kabbaj, 2014; Iniguez et al., 2014; Kinsey, Bailey, Sheridan, Padgett, & Avitsur, 2007; Watt, Burke, Renner, & Forster, 2009). This model involves a male rodent subject (i.e., the “intruder”) being introduced to a larger and more aggressive male’s cage (i.e., the “resident”). The subject is often attacked repeatedly by the aggressive resident, eventually resulting in the subject expressing behavioral and physiological symptoms that resemble human populations experiencing clinical anxiety and depression (e.g., social withdrawal, anhedonia; Hollis and Kabbaj, 2014; Iniguez et al., 2014; Kinsey et al., 2007; Watt et al., 2009; Zhang, Yuan, Shao, & Wang, 2016).

For at least two reasons, the social defeat paradigm has been insufficient for providing a complete picture of the neuroendocrine correlates of depression and anxiety. First, the social defeat model involves both physical (i.e., attacks by resident male) and psychological (i.e., being placed into an unfamiliar cage) stressors, whereas the most common sources of social stress in modern human populations are psychological. Physical stressors certainly exist, even in Western societies (e.g., malnutrition, infectious diseases), but psychosocial stressors are recognized as frequent precursors to onset of depressive and anxiety disorders (Juster, McEwen, & Lupien, 2010; McEwen, 2004, 2005; Slavich & Irwin, 2014). Second, the social defeat model is typically only effective with male rodents (although, see Harris et al., 2018; Takahashi et al., 2017; Williams et al., 2018) because males tend to be more aggressive than females (Solomon, 2017). However, women report experiencing depressive or anxiety symptoms two times more frequently than men (McLean, Asnaani, Litz, & Hofmann, 2011; Silverstein, 2002). Therefore, a rodent model that involves a primarily psychological stress and is effective with females might provide insights that have been missed when using the social defeat paradigm.

One alternative model has been developed in which male mice either experience or witness social defeat (Warren et al., 2013). Although this testing paradigm allows for the assessment of strictly psychological stress (in the witnesses), it still employs male subjects. Recently, a social instability paradigm was developed and found to be effective at eliciting the predicted behavioral and hypothalamic-pituitary-adrenal (HPA) responses in female subjects (Herzog et al., 2009; Jarcho, Massner, Eggert, & Wichelt, 2016). This paradigm is characterized by frequent and unpredictable changes to the subjects’ social environments including social isolation and social crowding.

The social instability model described above was designed to accurately translate to frequent and substantial changes to one’s social environment in humans. Given that social stressors may last for weeks to months (e.g., family disagreements, feeling excluded from a group), or longer (e.g., end of a marriage, loss of a loved one), assessing glucocorticoid responses over a comparable timeline is ideal. Common methods of sampling glucocorticoids involve the collection of either blood plasma or saliva. Additionally, urinary and fecal samples provide insight to HPA functioning over the preceding hours (Harper & Austad, 2015; Shamim, Yousufuddin, Bakhai, Coats, & Honour, 2000). These methods give “point” values that are highly variable within the same individual, and within a given day. A number of variables are known to affect plasma and salivary samples in particular (e.g., food intake, exercise, time of day/year) and need to be controlled for or taken into account, and repeated samples are required for an accurate understanding of HPA regulation (Davenport, Tiefenbacher, Lutz, Novak, & Meyer, 2006). Sampling glucocorticoids (i.e., corticosterone in rodents, cortisol in primates) in hair, however, is a newer method that allows for the noninvasive assessment of glucocorticoids over a longer period of time with a single sample and has been shown to accurately reflect individual responses to various social stressors in rhesus macaques (Davenport et al., 2006; Dettmer, Novak, Meyer, & Suomi, 2014; Dettmer, Novak, Novak, Meyer, & Suomi, 2009; Dettmer, Novak, Suomi, & Meyer, 2012). Further, because the hair samples reflect HPA activity over the entire period that the hair has been growing (5 weeks in the current study), variables like stage of estrous cycle and time of day are inherently controlled.

Previous work in this lab (Jarcho et al., 2016) has shown that female mice exposed to social instability experience an increase in hair corticosterone, indicating that HPA activity is elevated in these animals throughout the time that they experience social instability. However, we were unable to determine whether our effects were unique to social instability stress itself, or whether they would be common across other social stressors. In addition, previous work by this lab was limited because we did
Comparing Chronic Social Stressors | Jarcho, Avery, Kornacker, Hollingshead, and Lo

not include a measure of neuronal consequences of the stressor, an important measure possibly linking physiological and behavioral patterns. The current study attempted to address both of those questions by incorporating both social instability and social isolation stressors, and by quantifying mRNA concentrations in hippocampal tissue samples in an attempt to elucidate the mechanisms that underlie stress-related alterations in behavior and brain function. Social isolation is characterized by being isolated from social contact (but not, visual, auditory, or olfactory isolation), whereas social instability is characterized by frequent shifts in the social environment including periods of isolation and social crowding.

Although the hippocampus is widely known to be involved in memory and cognition (Squire, 1992), anxiety behavior in rodents requires synchronized activity between the hippocampus and medial prefrontal cortex (Adhikari, Topiwala, & Gordon, 2010) and intrahippocampal kainite injection has been shown to decrease amygdalar volume and increase anxiety-like behavior (O’Loughlin, Pakan, McDermott, & Yilmazer-Hanke, 2014). Thus, we investigated the hippocampus, a region known to respond to social stress (Chang, Hsiao, Chen, Yu, & Gean, 2015; Cheryl M. McCormick et al., 2012) and directly regulate anxiety-like behavior (Mineur et al., 2013), to determine the effect of chronic social stress on mRNA expression. We specifically looked at the involvement of tumor necrosis factor alpha (TNF-α), interleukin 1 receptor beta receptor (IL-1βR), and glial fibrillary acidic protein (GFAP). Expression of proinflammatory cytokines including TNF-α and IL-1βR have been found to increase following chronic stress (Badowska-Szalewska et al., 2013; Liu et al., 2015), and increased levels of these markers have been implicated in the etiology of stress-associated disorders like depression and posttraumatic stress disorder in rat models (Jones, Lebonville, Barrus, & Lysle, 2015; Şahin et al., 2015). Because IL-1β is thought to be a key mediator in a variety of behavioral actions of stress, its receptor has emerged as an attractive target for the treatment of stress-related disorders like depression (Koo & Duman, 2009a, 2009b). Although increased cytokine levels following stress have been demonstrated using chronic variable stress and forced-swim stress models (Badowska-Szalewska et al., 2013; Liu et al., 2015), brain cytokine expression resulting from social instability and social isolation stress has not been investigated. Thus, for the current study, we investigated whether elevated brain TNFα and IL-1βR were also observed in our social stress paradigms.

Our third marker, GFAP, is an intermediate filament component of astrocytes and is often used as an indicator of astrocyte activity and function (Hol & Pekny, 2015). In relation to stress, decreased GFAP expression has been shown to occur in the brains of stressed animals (Araya-Callís, Hiemke, Abumaria, & Flugge, 2012; Imbe, Kimura, Donishi, & Kaneoke, 2013) indicating astrocyte dysfunction. Considering that astrocytes are critical in supporting neuronal functioning, astrocyte dysfunction may lead to neuronal dysfunction, particularly in stress sensitive brain regions, leading to anxiety-like and depressive behaviors (Li, Yang, Ma, & Qu, 2013; Rahati, Nozari, Esrami, Shabani, & Basiri, 2016). Because GFAP expression has been found to be an important stress-related endpoint and indicator of astrocyte function, we wanted to determine how its expression would change in response to our social stress paradigm(s).

Our overall prediction for the current study was that both forms of chronic social stress would induce behavioral, physiological, and neuronal changes when compared to controls, and that social instability would be a more potent chronic social stressor than social isolation. The predicted difference between forms of chronic social stress was based on the fact that social instability is more unpredictable than social isolation, and level of unpredictability is known to enhance the response to the stressor (Thakur, Patel, Gulati, Anand, & Ray, 2015). Behaviorally, based on previous findings (Baranyi, Bakos, & Haller, 2005; Haller, Baranyi, Bakos, & Halász, 2004; Haller, Fuchs, Halász, & Makara, 1999; Herzog et al., 2009), we predicted that females experiencing either social stress would be characterized by increases in expression of anxiety-like and depression-like behaviors when compared to baseline and when compared to control animals not experiencing social stress. We further predicted that animals experiencing social instability would show greater increases in anxiety-like behavioral expression when compared to those experiencing social isolation. However, based on previous results in similar studies, we were cautious with these behavioral predictions (see Jarcho et al., 2016). From the brain tissue samples, we expected to see differences in messenger RNA profiles between those animals that experienced a social stressor and those that did not. Specifically, because neuroinflammation is considered to be a major etiological factor in the development of...
stress-associated depression and anxiety (Lehmann et al., 2016; Weber, Godbout, & Sheridan, 2017), we expected markers of inflammation to be expressed at higher levels in those animals that had experienced chronic social stress (i.e., either isolation or instability), and that animals experiencing instability would express these markers at the highest levels. Given that chronic stress can be accompanied by a proinflammatory profile due to glucocorticoid resistance (Avitsur, Stark, & Sheridan, 2001; Cacioppo, Cacioppo, Capitanio, & Cole, 2015; Hawkley, Cole, Capitanio, Norman, & Cacioppo, 2012), we predicted that subjects showing increased expression of proinflammatory markers would also have elevated hair corticosterone following the stress period. Therefore, we predicted that both groups of animals experiencing social stress would show elevated hair corticosterone levels, and that those experiencing social instability would have the highest hair corticosterone levels.

Method

Subjects and Study Outline

Adult (Age in weeks, \( M = 12.33, SE = 0.45 \) at start of baseline, \( M = 22.33, SE = 0.45 \) at end of Stress period, and \( M = 27.24, SE = 0.78 \) weeks at end of Recovery) female CD-1 mice (\( N = 27 \)) that were bred in our facility were housed in clear plastic cages (10.5” x 19” x 6”, Allentown, Inc., Allentown, NJ) in a temperature- and humidity-controlled animal facility on a 12-hour light-dark cycle with food and water available ad libitum. All behavioral testing occurred within the first 4 hours of the dark (i.e., active) period. Mice were randomly assigned to either the control (\( n = 9 \)), social instability (\( n = 9 \)), or social isolation (\( n = 9 \)) groups, similar to the experimental groups in previous work (Maslova, Bulygina, & Amstislavskaya, 2010). Animals in the control group remained with two familiar females throughout the study, and all animals in the cage were used for the study. Animals in the social instability and social isolation groups spent the first 5 weeks of the study (i.e., “Baseline”) housed with two other females. During the “Stress” period of the experiment, animals in the social instability group experienced an unpredictable and unstable social environment. At varying times of day, these animals were moved every 24–48 hours between social isolation (i.e., housed by themselves) and social crowding (i.e., housed with six female conspecifics in the same cage dimensions) for 5 weeks. This social instability model was based on previous work in rats (Herzog et al., 2009) and mice (Jarcho et al., 2016). For social crowding, animals were returned to the same cage and same cohabitants for each exposure. This housing paradigm is considered stressful because the animals have no control over their social environment, nor are they able to predict exactly how long they will remain in either isolated or crowded social conditions (Baranyi et al., 2005; Haller et al., 1999; Herzog et al., 2009). During this time, animals in the social isolation group were housed continuously in isolation. To control for any handling effects, control and isolated animals were handled on all days that animals in the social instability group were moved. Animals in all experimental groups were returned to their original housing groups (i.e., same subjects housed together as were housed together during baseline) for the final five weeks of the study (i.e., “Recovery”; see Table 1). Adequate measures were taken to minimize pain or discomfort, and all experiments were conducted in accordance with international standards on animal welfare, were compliant with local and national regulations, and were approved by the Institutional Animal Care and Use Committee at Loras College.

Behavioral Testing

All animals were weighed and assessed for behavioral expressions of anxiety and depression once per week throughout the 15-week study in an open field maze and an elevated plus maze (\( n = 9 \) per group during the baseline and stress periods, \( n = 6 \) per group during the recovery period). Mice were tested for 5 minutes on each maze and were video recorded under dim red lighting. Video recordings were scored using Behavior Tracker 1.5 (www.behaviortracker.com) by observers blind to the experimental manipulations. In the open field, the duration of time spent in the center or perimeter of the open field, and frequency of rearing were quantified. In the elevated plus maze, the time spent in the “open” and “closed” arms were quantified.

Brain Tissue Collection

One third of the animals for each group (\( n = 3 \) per group) were randomly selected to be euthanized at the same time for brain tissue collection 24 hours after the end of the stress period at approximately 1300 hours. Animals were euthanized by CO\(_2\) asphyxiation. Brains were extracted and the entire hippocampus, both dorsal and ventral aspects, was collected from each mouse. Tissue was placed in tubes containing RNAlater (ThermoFisher,
Waltham, MA) and stored at 4°C until mRNA analyses were conducted.

**Gene Expression Analyses**
mRNA from mouse hippocampus was isolated using Pure Link spin columns (ThermoFisher, Waltham, MA), and cDNA was synthesized using Verso cDNA synthesis kit (ThermoFisher, Waltham, MA). Real-time polymerase chain reaction (RT-PCR) was performed using PowerUp SYBR Green Master Mix (ThermoFisher, Waltham, MA) and gene specific primers (see Table 2; Integrated DNA Technologies, Coralville, IA). Samples were run in triplicate using a StepOnePlus RT-PCR System (Applied Biosystems, Inc., Foster City, CA). Data were analyzed using the \(2^{-\Delta\Delta C_{t}}\) method (Livak & Schmittgen, 2001), and mRNA expression of target genes was normalized to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are expressed as fold change of levels compared to control mice.

**Hair Sample Collection and Preparation**
To ensure that hair corticosterone samples represented HPA activity during the study, mice were shaved at the start of the baseline period. This hair was not collected or analyzed for corticosterone. Mice were shaved again at the end of the 5-week baseline (\(n = 9\) per group), stress (\(n = 9\) per group), and recovery (\(n = 6\)) periods. Hair was collected from the posterior dorsal portion of the animals (see Figure 1) in order to minimize auto-grooming of the shaved area. Collection was conducted without anesthesia by two trained technicians, one to immobilize the mouse and one to operate the hair clippers (Wahl Clipper Corporation, Sterling, IL). Animals were shaved using the same protocol regardless of experimental group to eliminate any handling effects on hair corticosterone. Hair samples were collected, weighed (mean weight 13.9 ± 0.3 mg), and stored in a -20°C freezer until assay. Samples were prepared following a modified previously published protocol (Davenport et al., 2006). Briefly, samples were washed with isopropanol to remove debris and external corticosterone while minimally affecting corticosterone levels inside the hair (Davenport et al., 2006; Gow, Thomson, Rieder, Van Uum, & Koren, 2010). Washing involved adding 1 ml of isopropanol to each sample for a 5-minute incubation, followed by centrifugation at 13,000 rpm at room temperature prior to removing the solution. Washing was repeated two additional times for all samples. Washed samples were allowed to dry by leaving them in a laminar flow hood for 24 hours. Samples were then chopped into fine pieces with a razor blade to facilitate steroid extraction (Yu et al., 2015). To obtain a holistic measure of HPA activity throughout a given period of the study (e.g., throughout the Baseline period), the entire sample was processed. Steroids were then

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**TABLE 1**

<table>
<thead>
<tr>
<th>Description of Social Instability Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Housing Instability</td>
</tr>
<tr>
<td>Isolation</td>
</tr>
<tr>
<td>Body weight measured</td>
</tr>
<tr>
<td>Behavioral testing</td>
</tr>
<tr>
<td>Shave: sample not assayed</td>
</tr>
<tr>
<td>Week 1–5</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>Week 6 Stress</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>Week 7 Stress</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>1x/wk</td>
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<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>Week 8 Stress</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
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<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>Week 9 Stress</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>1x/wk</td>
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<tr>
<td>1x/wk</td>
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<tr>
<td>Week 10 Stress</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>Single</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>Shave: stress sample</td>
</tr>
<tr>
<td>Brain tissue collection ((n = 3)/(experimental group))</td>
</tr>
<tr>
<td>Week 11–15</td>
</tr>
<tr>
<td>3/cage</td>
</tr>
<tr>
<td>3/cage</td>
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<tr>
<td>3/cage</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>1x/wk</td>
</tr>
<tr>
<td>Shave: recovery sample</td>
</tr>
</tbody>
</table>

Note. All animals were shaved initially to ensure corticosterone concentrations reflected only the study period. The study was comprised of three 5-week phases: (a) baseline, (b) stress, and (c) recovery. During each phase, mice were weighed and assessed behaviorally once per week. At the end of each phase, mice were shaved and hair was collected to assess corticosterone production. All mice were housed three mice per cage for 5 weeks leading up to the study. Controls remained in these groups for the duration of the study. Mice in the isolation group were isolated for 5 weeks, and then returned to the same groups of 3 for 5 weeks. Mice in the instability experienced multiple changes in their housing environment for 5 weeks prior to returning to a stable housing environment of three mice per cage for the final 5 weeks of the study. All mice were weighed and tested behaviorally once per week, and hair samples were collected for corticosterone analyses from all mice at the time points indicated. Three animals from each experimental group were sacrificed at the end of the stress period for collection of brain tissue samples.
extracted from the chopped hair by incubating the samples in methanol for 24 hours. Samples were centrifuged for 5 minutes at 13,000 rpm at room temperature and the steroid-containing methanol solution supernatant was collected. This solution was purified by passing it through Supelco-select HLB SPE tubes (Sigma-Aldrich). Purified extracts were reconstituted with assay buffer (Arbor Assays, Ann Arbor, MI).

Corticosterone Assays
Reconstituted samples were assayed in duplicate(s) for corticosterone via commercially available enzyme immunoassay kits (Arbor Assays, Ann Arbor, MI). The detectable range of corticosterone for these kits was 78.125–10,000 pg/ml, and the intra-assay and inter-assay coefficients of variance were 16.43 and 6.59, respectively. Corticosterone concentrations as detected by enzyme immunoassay were then matched with the original weight of the hair collected in order to account for minor variations in hair quantity collected. Corticosterone concentrations are, therefore, expressed in pg/mg of hair.

Statistical Analyses
Physiological and behavioral patterns were evaluated with a 3 x 3 repeated-measures Analysis of Variance (ANOVA) with group (i.e., social instability vs. social isolation vs. control) and time (i.e., baseline vs. stress vs. recovery) included as main factors, a group by time interaction term, and individual subject identity as a within subject factor. For behavioral trials that were conducted every week (i.e., five trials per mouse per period of the study), averages were calculated for each individual for each period of the study. That is, each subject had three averages for each behavioral measure—one at baseline, one at stress, and one at recovery. Post-hoc t-tests were used to compare social instability values to controls, to compare social isolation values to controls, and to compare baseline to stress to recovery levels within groups. Analyses of mRNA expression levels was assessed with ANOVA. An α of .05 was used in all statistical analyses, and Bonferroni adjustment was used to correct for multiple tests. Effect sizes were calculated as partial eta-squared (η²) for ANOVA and as Cohen’s d for t tests. Post-hoc power analyses were conducted using G*Power software (Faul, Erdfelder, Lang, & Buchner, 2007) with an α level of .05. Power values are reported following estimates of effect size as 1-beta (1-β).

Results
Effect of Social Stress on Body Mass
Animals were weighed once per week throughout the study, and weights were averaged across individuals within experimental groups. Repeated-measures ANOVA with main effects of time and group did not yield significant differences for either main effect, nor was there a Group x Time interaction (all ps > .05).

![FIGURE 1](image_url)

Area of fur collected for corticosterone assays. Following each period of the study (i.e., Baseline, Stress, Recovery) hair samples were collected for corticosterone quantification. Hair was collected from the posterior dorsal surface of the mice, between the tail and hind legs. Shaded area represents target area to be shaved.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Accession</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td>Mouse</td>
<td>NM_010277.2</td>
<td>TGGCCGGGCGCTCAATGCTGGC</td>
<td>GGGCCGACTCCCCGCCATGGCCCTC</td>
</tr>
<tr>
<td>IL-1Beta R</td>
<td>Mouse</td>
<td>NM_010555.2</td>
<td>GGGCCCTAAAGGAAAGAAGATC</td>
<td>TACAGTTGGGAAAGCTTCGC</td>
</tr>
<tr>
<td>TNF alpha</td>
<td>Mouse</td>
<td>NM_013693.2</td>
<td>GAACCTGCGCAAGAGAGGCGACT</td>
<td>AGGCCTTGCCGCAAGAAGACT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Mouse</td>
<td>NM_008884</td>
<td>AACTTTGCGATGTTGAGGAGG</td>
<td>GGGTGGGGGATTGATGTTCT</td>
</tr>
</tbody>
</table>

Note. GFAP = glial fibrillary acidic protein. IL-1Beta R = interleukin 1 receptor beta receptor. TNF alpha = tumor necrosis factor alpha. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.
Comparing Chronic Social Stressors

Effect of Social Stressors on Expression of Anxiety-Like Behaviors

No group differences were seen in the amount of time spent in either the open arms of the elevated plus maze or the center of the open field (all \( p > .1 \)). The only behavior that showed group differences was rearing in the open field maze. This indicator of anxiety remained relatively constant in control mice, but increased during the stress period in the isolation and instability animals (see Figure 2). Rearing frequency was predicted by experimental group, \( F(2, 351) = 6.91, p = .001, \eta^2 = .04, (1-\beta) = .94 \), and post-hoc tests revealed that this effect was primarily driven by differences between rearing patterns of mice in the instability group: compared to controls, \( t(238) = 2.37, p = .018, d = .10, (1-\beta) = .90 \); compared to isolated animals, \( t(238) = 3.87, p < .001, d = .14, (1-\beta) = .92 \), whereas isolated animals did not show different rearing patterns from controls \( (p > .1) \).

Effect of Social Stressors on RNA Expression Patterns in the Brain

In hippocampal samples, IL-1\( \beta \)R mRNA levels differed across experimental groups, \( F(2, 6) = 5.65, p = .045, \eta^2 = .85, (1-\beta) = .99 \) (see Figure 3a), and post-hoc tests revealed significant differences when any two groups were compared, with expression levels being lowest in controls, higher in isolated animals, and highest in instability animals: control vs. isolation, \( t(4) = 5.21, p = .032, d = 2.53, (1-\beta) = .64 \); control vs. instability, \( t(4) = 11.89, p < .01, d = 4.12, (1-\beta) = .96 \); isolation vs. instability, \( t(4) = 5.93, p = .031, d = 2.28, (1-\beta) = .57 \). A similar pattern was observed for TNF\( \alpha \) mRNA with differences in expression across experimental groups, \( F(2, 6) = 8.89, p = .042, \eta^2 = .86, (1-\beta) = .99 \) (see Figure 3b). Post-hoc analyses revealed differences between all groups, again with expression levels being lowest in controls, higher in isolated animals, and highest in instability animals: control vs. isolation, \( t(4) = 8.29, p = .01, d = 3.37, (1-\beta) = .87 \); control vs. instability, \( t(4) = 12.12, p < .01, d = 4.22, (1-\beta) = .96 \); isolation vs. instability, \( t(4) = 5.96, p = .03, d = 2.44, (1-\beta) = .60 \). Hippocampal mRNA levels for GFAP showed a similar pattern of group differences in expression, but in the opposite direction, \( F(2, 6) = 13.37, p = .006, \eta^2 = .82, (1-\beta) = .99 \). Post-hoc analyses revealed lower and lowest expression patterns in isolated and instability animals, respectively: control vs. isolation, \( t(4) = 7.51, p = .02, d = 2.91, (1-\beta) = .76 \); control vs. instability, \( t(4) = 9.48, p = .01, d = 3.58, (1-\beta) = .90 \); isolation vs. instability, \( t(4) = 4.09, p = .05, d = 1.79, (1-\beta) = .39 \) (see Figure 3c).

Effect of Social Stressors on Hair Corticosterone

Hair corticosterone concentrations were assessed by repeated-measures ANOVA, which revealed a significant group by time interaction, \( F(4, 63) = 3.47, p = .013, \eta^2 = .18, (1-\beta) = .89 \) (see Figure 4), indicating different patterns of corticosterone production over time between the three groups. In addition, the phase of the study predicted corticosterone concentrations, \( F(2, 63) = 15.41, p < .001, \eta^2 = .33, (1-\beta) = .99 \). However, the experimental group was not a significant predictor of corticosterone concentrations, \( F(2, 63) = 0.85, p = .42, \eta^2 = .03, (1-\beta) = .17 \). Post-hoc analyses revealed group differences in corticosterone concentrations during the stress period: control vs. isolation, \( t(16) = 2.76, p = .025, d = 1.08, (1-\beta) = .58 \); control vs. instability, \( t(16) = 3.98, p = .004, d = 1.49, (1-\beta) = .84 \), but no differences between groups during either the baseline or recovery periods, and no differences between instability and isolation animals at any period (all \( p > .25 \)).

Discussion

We predicted that both social instability and social isolation would have behavioral, physiological, and neural consequences in adult female mice, and that social instability would have amplified...
consequences. Specifically, we predicted that either social stressor would be associated with increases in hippocampal expression of proinflammatory mRNA, increases in the expression of certain anxiety-like behaviors, and increases in hair corticosterone. We further predicted that social instability would have more potent effects on each of these measures, as a result of being less predictable for the animals experiencing this social stressor. We observed significant increases in markers of neuroinflammation and reduced glial health in animals that experienced both social stressors, and significantly greater changes in animals that experienced social instability. We observed increased rearing behavior only in animals that experienced social instability. Lastly, we observed increased hair corticosterone concentrations in all animals that experienced chronic social stress.

Differences in hippocampal mRNA expression were observed between experimental groups and were greatest between animals that had experienced social instability and controls. Females that experienced social instability were characterized by decreases in a marker of astrocyte structural stability (i.e., GFAP) and increases in markers of neural inflammation (i.e., IL-1βR and TNFα). The decrease in hippocampal GFAP is consistent with other studies showing that chronic stress, and elevated glucocorticoids in particular, leads to a reduction of GFAP within the hippocampus and other areas of the brain (Liu et al., 2011; Tynan et al., 2013; Zhang, Zhao, & Wang, 2015). Glucocorticoids are known to modulate GFAP expression throughout the brain (O’Callaghan, Brinton, & McEwen, 1989), with prolonged corticosterone treatments causing a decrease in GFAP mRNA expression in the hippocampus and cerebral cortex (Nichols et al., 1990). Corticosterone treatment to adult rats also decreases GFAP protein levels in several brain regions whereas adrenalectomy increases the GFAP protein levels (O’Callaghan et al., 1989). Thus, glucocorticoids may be involved in the suppression of GFAP. Although the exact cause of astrocyte atrophy under stressful conditions is poorly understood, there is evidence to suggest that changes in astrocyte morphology and viability is a consequence of immune activation (Lee et al., 2013), specifically attributed to the cytokines TNF-alpha and IL-1beta (van Kralingen, Kho, Costa, Angel, & Graham, 2013), which are likely produced by glucocorticoid-activated microglia.

**FIGURE 3**

Effect of social stress on hippocampal mRNA expression. Quantification of gene expression revealed increased expression of two markers of inflammation. Both IL-1βR (a) and TNFα (b) were up-regulated in animals experiencing social isolation or instability. Further, compared to isolation stress, instability stress resulted in increased expression of both markers of inflammation. GFAP (c) was down-regulated in animals experiencing social stress compared to controls, and was further down-regulated in animals experiencing instability stress compared to those experiencing isolation stress. Data are shown as mean ± SEM of relative delta threshold averaged within sample groups; * indicates significant group differences. For all groups, n = 3.
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Hippocampal inflammation, driven by the cytokines TNF-alpha and IL-1β, has been shown to play a key role in the pathogenesis of depression and anxiety (Abbott et al., 2015; Goshen et al., 2008). In our work, we detected an increase in hippocampal TNF-alpha and IL-1βR mRNA in mice subjected to psychosocial stressors. Consistent with previous reports, social defeat stress has been shown to elevate the expression of cytokines and their receptors in the hippocampus (Joana et al., 2016; McQuaid, Audet, Jacobson-Pick, & Anisman, 2013). Differences in stressor, strain, and sex can all independently vary brain cytokines levels (Deak et al., 2015; Gibb, Hayley, Poulter, & Anisman, 2011; Razzoli, Carboni, Andreoli, Ballottari, & Arban, 2011). Therefore, it is difficult to put the scale of our observations—35-fold increase in hippocampal IL-1βR expression and 62-fold increase in hippocampal TNF-alpha mRNA expression in social instability-stressed mice compared to controls—in the context of other social stressor studies that did not utilize the exact same variables. Although, to the best of our knowledge, there is no direct correlate for our present study, our observed numbers are comparable to the elevation in hippocampal cytokine expression that follows lipopolysaccharide (LPS) administration (Browne, O’Brien, Connor, Dinan, & Cryan, 2012; Czapski, Gajkowska, & Strosznajder, 2010; Henry et al., 2008; Shin et al., 2014).

Given these comparisons, the current study supported previous findings indicating the power of social stressors to promote inflammation in the hippocampus. Our mRNA results indicate that chronic social stress induces an unfavorable neural environment characterized by astrocyte dysfunction and increased neuroinflammation, both of which are implicated in the development of neurological dysfunction and behavioral symptoms associated with stress-related disorders like depression (Bortolato, Carvalho, Soczynska, Perini, & McIntyre, 2015; Cobb et al., 2016).

Importantly, differences in expression patterns were present not just between control and stressed animals, but also between animals experiencing the two types of social stress. This suggests that social instability and social isolation result in distinguishable hippocampal consequences. Coupled with the group differences in rearing behavior, these results indicate that isolation and instability are not experienced in the same way and that instability induces more substantial behavioral and neural consequences than isolation (Maslova, Bulygina, & Amstislavskaya, 2010).

Rearing behavior in the open field, a behavior typically associated with elevated anxiety in mice (Heisler et al., 1998), was exhibited differently between experimental groups. Females subjected to social instability displayed elevated rearing behavior when compared to either isolated animals or controls. A similar pattern was previously observed following social instability stress in this lab, also without other group differences in behavior (Jarcho et al., 2016). It is possible that rearing behavior in the open field is an anxiety-like behavior that is particularly sensitive to the unpredictable nature of social instability stress.

We observed increases in hair corticosterone in animals that experienced either form of social stress, in line with previous work investigating the effects of social stress on the production of glucocorticoids (McCormick, Merrick, Secen, & Helmrreich, 2007; Saavedra-Rodríguez & Feig, 2013). Plasma levels of corticosterone consistently show 3- to 4-fold increases in response to acute social stressors, whereas hair corticosterone increases are less substantial, even in response to repeated social defeat (Yu et al., 2015). However, we did not find a difference in the degree of increase between animals experiencing social instability as...
compared to those experiencing social isolation. We predicted a more substantial increase in hair corticosterone in animals that had experienced social instability than those that had experienced isolation, but observed nearly equal increases in both groups.

The hair corticosterone results combined with the group differences in behavioral and neural markers begs the question of why the experimental groups differed on certain measures of stress and not on the primary measure of HPA activity. One possible explanation might be that there is a ceiling effect of corticosterone deposition in the hair. However, previous work in rats suggest that this is not the case (Scorrano et al., 2015). Another explanation is that, although the HPA response was nearly equivalent in these two groups, other physiological systems are impacted by chronic stress, and may have varying sensitivities to specific chronic stress paradigms (Capitanio & Cole, 2015). That is, chronic social stress, in any form, might increase HPA activity, but the added unpredictability or lack of control associated with social instability (as opposed to isolation, which is unchanging) may more potently increase inflammation in the brain and may be more likely to affect behavior. An additional possibility is that, although the cumulative HPA activity did not differ between the two stress groups, the specific pattern of HPA activity and corticosterone production did. That is, perhaps diurnal patterns were flatter in subjects experiencing social instability than in those experiencing social isolation.

In humans, flattened diurnal cortisol release was observed in individuals who previously experienced anxiety or major depressive disorder (Doane et al., 2013; Jarcho, Slavich, Tylowa-Stein, Wolkowitz, & Burke, 2013), who are battling metastatic breast cancer (Abercrombie et al., 2004), or who endorse higher ratings of loneliness (Doane & Adam, 2010). Similar consequences of diurnal rhythmicity have been observed in rodents. Experimentally flattening the diurnal corticosterone rhythm in mice results in increased expression of anxiety and depression like behaviors in mice (Murray, Smith, & Hutson, 2008), and a similar manipulation in rats resulted in altered hippocampal mRNA expression, demonstrating a possible link between HPA activity, anxiety- and depression-like behaviors, and hippocampal protein expression (Cacioppo et al., 2015; Gartside, Leitch, McQuade, & Swarbrick, 2003; Miller, Maletic, & Raison, 2009). We are unable to assess glucocorticoid reactivity or diurnal patterns in hair samples, but future work will add plasma sampling of corticosterone to address these questions directly.

An alternative explanation for the hair corticosterone patterns that we observed is that the elevations in corticosterone were not a result of the housing paradigms being perceived as stressful, but instead that the two experimental conditions (i.e., isolation and instability) were associated with elevated physical activity patterns. It is certainly true that increased physical activity can increase plasma glucocorticoid concentrations (Few, 1974; Girard & Garland, 2002; Stupnicki & Obminski, 1992), although voluntary exercise has also been shown to mitigate the expected increase in plasma glucocorticoids and downstream health consequences in animals and humans experiencing chronic stress (Adlard & Cotman, 2004; Puterman et al., 2010; Sasse et al., 2008). We cannot rule this possibility out because we did not collect behavioral data on the mice while they were in their home cages. However, it seems highly unlikely that both of these housing paradigms would be associated with increases in physical activity. It should also be noted that, although we did not quantify behavior in the home cages, previous observations in female rats did not detect changes in home-cage behaviors as a result of prolonged isolation stress (McCormick et al., 2007).

Based on previous work investigating the effect of social stressors on behavioral expression of anxiety and depression (Kaushal, Nair, Gozal, & Ramesh, 2012; Kinn Rød et al., 2012; Liu et al., 2013; Reiss, Wolter-Sutter, Krezel, & Ouagazzal, 2007; Treit, 1985; Watt et al., 2009), we expected, but did not observe group differences in the open field and elevated plus maze in the amount of time spent in the center/perimeter or open/closed arms, respectively. Our findings indicate that, although physiological and neural responses were elicited, the primary behavioral measures associated with anxiety (i.e., time in the perimeter of the open field and the closed arms of the elevated plus maze) were not significantly affected by these forms of social stress. It should be noted that previous work in this lab demonstrated a similar lack of group differences on these measures (Jarcho et al., 2016), and other authors did not detect group differences on the forced swim test (Herzog et al., 2009). It is possible that, given the social nature of these stressors, behavioral expressions of anxiety would only have been observable in a more social setting. That is, this lack of observable differences may reflect the nature of the testing apparatuses used,
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not the actual anxiety levels of the mice (Ennaceur & Chazot, 2016). Despite this possibility, group differences on these apparatuses were expected. It is possible that more socially relevant behavioral measures (e.g., social withdrawal) might have revealed significant group differences.

The implications of these findings are limited by certain aspects of the current study. Primarily, the number of animals (n = 9 per experimental group) used in the study was rather small, particularly in our investigation of hippocampal mRNA expression, because only three animals were sacrificed from each group. Future investigations should attempt to increase sample sizes, even if elimination of certain measures that were collected in the present study is necessary for feasibility. A second limitation of the current study is that it focused exclusively on female mice. Females were used in the current study because they show a greater response to psychosocial stress (Haller et al., 1999) and women report higher rates of anxiety disorders than men (Bangasser & Valentino, 2014; McLean et al., 2011). However, to increase the translational value of these findings, future investigations should include both females and males in order to directly observe sex differences that may be relevant to differential rates of stress-induced anxiety disorders in humans. A third limitation is the limited behavioral measures we quantified. Future studies should include additional behavioral tests, particularly those that specifically target indicators of social anxiety (e.g., social withdrawal tests). Lastly, we are unable to determine causality across our dependent variables. For example, it is possible that the changes in hippocampal mRNA were a direct result of social stress. However, it is equally possible that the mRNA effect was mediated by changes in HPA activity. Future studies should attempt to disentangle these variables to determine causality in order to better inform treatment strategies.

These findings support previous work indicating that social stressors are potent enough to elicit behavioral, physiological, and neural responses in adult female mice. In addition, they further support the initial findings that these stressors can induce physiological changes that are detectable in mouse hair, and that the corticosterone concentrations are responsive to the onset and termination of a social stressor. These data also indicate that, although the hair corticosterone responses to both social isolation and instability were similar, the behavioral and neural consequences of these two forms of social stress were quite different. These subtle differences in the form of social stress and the consequences associated with them may be translatable to different sources of social stress in humans and the multitude of mood disorders and other psychological consequences that may result. Additional work is needed to establish a more concrete causal relationship between types of social stress and behavioral, physiological, and neural consequences.

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