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TITLE: Divergent immune responses in behaviorally-inhibited vs. non-inhibited male rats

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HIGHLIGHTS:

- Different immune responses may explain temperament-specific health outcomes.
- Rats were tested for stable behavioral phenotypes by testing on two different arenas.
- Behaviorally-inhibited (BI) rats had a greater innate response than non-inhibited (NI).
- BI rats had a dampened delayed-type hypersensitivity response compared to NI rats.

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ABSTRACT

Stable behavioral traits (temperament, personality) often predict health outcomes. Temperament-specific differences in immune function could explain temperament-specific health outcomes, however, we have limited information on whether immune function varies by personality. In the present study, we examined the relationship between a basic behavioral trait (behavioral-inhibition vs. non-inhibition) and two immune responses (innate inflammation and delayed-type hypersensitivity, DTH) in a rodent model. In humans, behavioral inhibition (fearful temperament) is associated with altered stress physiology and allergies. In laboratory rats, the trait is associated with elevated glucocorticoid production. We hypothesized that behavioral inhibition is associated with glucocorticoid resistance and dampened T-helper 1 cell responses often associated with chronic stress and allergies. Further, this immune profile would predict poorly-regulated innate inflammation and dampened DTH. In male Sprague-Dawley rats, we quantified consistent behavioral phenotypes by measuring latency to contact two kinds of novelty (object vs. social), then measured lipopolysaccharide(LPS)-induced innate inflammation or keyhole limpet hemocyanin(KLH)-induced DTH. Behaviorally-inhibited rats had heightened glucocorticoid and interleukin-6 responses to a low/moderate dose of LPS and reduced DTH swelling to KLH re-exposure compared to non-inhibited rats. These results suggest that behavioral inhibition is associated with a glucocorticoid resistant state with poorly regulated innate inflammation and dampened cell-mediated immune responses. This immune profile may be associated with exaggerated T-helper 2 responses, which could set the stage for an allergic/asthmatic/atopic predisposition in inhibited individuals. Human and animal models of temperament-specific immune responses represent an area for further exploration of mechanisms involved in individual differences in health.

Keywords: innate immune response, delayed type hypersensitivity, temperament, personality, individual differences, inflammation

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1. Introduction

Specific temperaments (behavioral phenotypes or personalities) have been associated with specific health outcomes. One mechanism by which temperament may moderate health outcomes is through differential temperament-specific immune regulation (Capitanio et al., 2008; Zozulya et al., 2008; Rangassamy et al., 2016). To date, we have relatively limited information on the relationship between temperament and immune function. Many studies have investigated the immune parameters of a variety of animal species and populations in response to environmental demands, such as parasite load (Koprivnikar et al., 2012; Barber & Dingemanse, 2010) and social competition/aggression (Azpiroz et al., 2008; Capitanio et al., 2008, 2011; Chun et al., 2017). This has been researched at the behavioral level (e.g. behaviors that affect exposure to parasites/antigens) and at the population level, but potential physiological mechanisms at the individual level are still unclear.

In the current study, we examined immune responses in laboratory rats that were characterized as behaviorally-inhibited, non-inhibited, or mixed. At the individual level, inhibition and exploration are behavioral traits that can be relatively stable over time and across conditions and may involve stable differences in underlying physiology (Cavigelli & McClintock, 2003, Roberts et al., 2006; Réale et al., 2007; Bell et al., 2009; Cavigelli et al., 2009). In humans, Behavioral Inhibition (BI) is defined as a fearful or avoidant response to a wide array of novel experiences and is evident in approximately 20% of children studied in the US (Garcia-Coll et al., 1984; Kagan et al., 1984). BI humans are prone to develop environmental allergies and asthma, conditions that are broadly considered to reflect a T-helper 2 cell (T_H2) immune polarization (Kagan et al., 1991; Bell, 1992; Kim et al., 2006; Gulec et al., 2010; Heffner et al., 2014). BI individuals also show elevated basal glucocorticoid production (Kagan et al., 1987; Schmidt et al., 1997; Smider et al., 2002; Baugh et al., 2017) associated with negative health and immune outcomes (summarized in Chrousos & Kino, 2009; Miller et al., 2002; Webster Marketon & Glaser, 2008; Picard et al., 2014). In animals, BI/neophobia/exploration (defined by approach latency, locomotion, and/or time interacting with novel objects) are relatively stable traits over time and across contexts (Verbeek et al., 1994; Wilson et al., 1994; Dingemanse et al., 2002; Cavigelli & McClintock, 2003; Mason et al., 2006; Cavigelli et al., 2007; Krajl-Fiser et

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al., 2007; Bell et al., 2009; Caruso et al., 2014). Like in humans, BI male rats, defined as having consistently slower-than-median approach latencies in two novel conditions, have low-grade elevation in glucocorticoid production which is associated with shortened life span (Cavigelli & McClintock, 2003; Cavigelli et al., 2007; Cavigelli et al., 2009). An animal model of temperament-associated differences in immune responses provides a system to experimentally test origins and mechanisms that underlie the relationship between BI/fearful temperament and health risks.

Studies that have linked *individual* behavioral profiles to immune system competence do not converge on a clear pattern. In free-ranging house finches, low-exploratory individuals invested less in constitutive defenses in favor of inducible defenses (i.e. occasionally spending a lot of energy to fight infections rather than investing in a continuous low-level prevention; Zylberberg et al. 2014), but in fairy wrens, low-exploratory individuals had *higher* investment in constitutive defenses (Jacques-Hamilton et al., 2017). In greenfinches, reactive individuals mounted higher innate and adaptive immune responses compared to proactive birds (Sild et al., 2011). In farm pigs, low-active animals had decreased lymphocyte proliferation in response to KLH exposure and lower expression of transcripts associated with innate immunity at baseline, with no T_H1/T_H2 shift (Bolhuis et al., 2003; Oster et al., 2015; cf. Geverink et al. 2004). In lab-reared zebrafish, individuals neophobic to novel objects demonstrated a heightened basal pro-inflammatory profile (i.e. increased brain IL-1β and reduced IL-10 expression), and fish that had less preference for social partners had reduced brain IFN-γ expression, indicating dampened cell-mediated immunity (Kirsten et al., 2018). Similarly, in wild male mice exposed to repeated social confrontations, anxious/neophobic mice showed a comparatively lower T helper/regulatory T cell ratio in the spleen indicating general immunosuppression compared to less fearful mice (Rangassamy et al., 2016). These results suggest that inhibition/exploration behavioral phenotypes are associated with different immune regulation, but that the specific direction of this regulation is unclear.

Population-level studies have shown that inhibited or low-exploration species tend to invest less in innate immune responses and more in adaptive immunity (Lee, 2006; Martin et al., 2006; 2007; c.f. Johnson et al., 2012). But on the individual level, the opposite has been

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proposed: less exploratory individuals invest more in innate immunity (Zuk & Stoehr 2002; Lee, 2006; Adelman & Martin, 2009), perhaps at the cost of immune tolerance (e.g. Medzhitov et al., 2012). Some of the complications in this area are that free-ranging individuals with different temperaments expose themselves to different kinds and amounts of antigens; and in more controlled settings, immune function is quantified in many different ways, including estimates of immune function in the absence of an immune challenge. In addition, there are a variety of ways to define and measure behavioral phenotypes. This is particularly true for exploration-related behaviors, which could be driven by two different underlying motivations - i.e. harm avoidance vs. novelty seeking (Budaev 1998; Ray & Hansen, 2004). To test whether immune responses are functionally different among behavioral phenotypes, we need well-characterized and conceptualized behavioral phenotypes and tests of immune function that use standardized antigens presented in relatively antigen-free environments (i.e. low bacterial, viral, and parasite loads). In the current study, we examined how a basic behavioral trait (Inhibition vs. Non-Inhibition) related to two basic functional immune responses (innate inflammation and delayed-type hypersensitivity, DTH) in a social mammal, the Sprague-Dawley rat (*Rattus norvegicus*).

Briefly, the immune system can be divided into two broad domains: innate and adaptive. Cells involved in the innate system are the first to respond at a site of potential infection and can mount an inflammatory response within hours (Copeland et al., 2005). They are non-specific but recognize a “library” of pathogen-associated molecular patterns (PAMPs), of which lipopolysaccharide (LPS) is a prominent example. LPS is an endotoxin found in the cell walls of Gram-negative bacteria and is detected by a variety of innate immune cells that secrete cytokines that in turn rapidly attract and activate other immune cells; notably macrophage-secreted interleukin-6 (IL-6) which triggers the sickness response (Papanicolaou et al., 1998; Bluthé et al., 2000; Visintin et al., 2001). Adaptive responses are slower than innate responses and are mediated by T-helper cells: intracellular pathogens stimulate dendritic cells to signal naive T-helper cells to mature into T-helper 1 cells (T_H1) that guide cell-mediated adaptive immunity, while extracellular pathogens stimulate T-helper cells to mature into T-helper 2 cells (T_H2) that guide humoral adaptive immunity. Typically, once T_H1/T_H2 differentiation occurs,

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resulting cytokines promote continued similar T-helper cell differentiation. Some have argued that individuals can develop a bias toward a greater T_H1 or T_H2-mediated adaptive response, e.g. an allergic phenotypes are often referred to as T_H2 polarization. In terms of adaptive responses, delayed-type hypersensitivity (DTH) is an easily-observed T_H1 cell-mediated response that can be measured non-invasively. DTH involves a sensitization phase where naive T-helper cells develop into antigen-specific T_H1 cells, and an effector phase where antigen re-exposure causes antigen-specific T_H1 cells to secrete cytokines that recruit large numbers of non-specific inflammatory cells to the antigen which results in localized induration within 24-72 hours (Punt et al., 2018).

Experimentally-induced chronically-elevated glucocorticoid exposure can alter both innate and T_H1-mediated immune function. Chronic stress can induce glucocorticoid resistance in macrophages and contribute to an innate pro-inflammatory profile (Stark et al., 2001). Glucocorticoid drugs can inhibit macrophage-induced T_H1 response with little effect on macrophages, thus stimulating exaggerated and prolonged innate immune activity (Mosser & Edwards, 2008; van de Garde et al., 2014; Kelly et al., 2018). On the flip side, IL-6 stimulates HPA axis activity in the short term , though over time may blunt the ACTH response (Lyson & McCann, 1991; Mastorakos et al., 1993). The DTH immune response is also affected by environmental stressors and glucocorticoid activity. Chronic or inescapable stress in rodents leads to elevated glucocorticoid production and suppressed DTH response, that is partially glucocorticoid-mediated, with a resulting skew toward increased T_H2-associated responses and reduced glucocorticoid sensitivity (Dhabhar & McEwen, 1997; 1999; Jasnow et al., 2001; Bartolomucci et al., 2003; Fleshner et al., 1995; Gazda et al., 2003; Bailey et al., 2009; Li et al., 2013; reviewed in Haczku & Panettieri, 2010). Last, chronic corticosteroid overexposure (*in vitro* and *in vivo*) reduces IL-12 production thereby reducing T_H1 proliferation - which indirectly drives T_H2 polarization (DeKruyff et al., 1998). Failure to suppress T_H2 development may result in the heightened allergic responses seen in behaviorally inhibited people.

In the current study, we examined whether rat behavioral inhibition, which is associated with increased basal glucocorticoid production, is also associated with altered immune function. The working hypothesis is that behavioral inhibition is comparable to chronic stress

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and/or long-term overexposure to glucocorticoids, and thus is associated with immunological profiles associated with chronic glucocorticoid elevations, even in the context of low-stress. We predicted that behaviorally-inhibited individuals will show signs of innate inflammatory immune cell glucocorticoid resistance and T_H2 polarization which will be displayed as elevated systemic innate pro-inflammatory response and attenuated T_H1 response like localized DTH (Stark et al., 2001; Elenkov, 2004). To test this hypothesis, we compared immune responses of behaviorally-inhibited (BI), non-inhibited (NI), and mixed behavior rats in two broad functional immune responses that are known to be affected by chronic stress and/or chronic glucocorticoid overexposure: lipopolysaccharide (LPS)-induced innate pro-inflammatory IL-6 production and keyhole limpet hemocyanin (KLH)-induced DTH swelling.

2. Methods

We conducted four studies to compare immune responses among behaviorally-inhibited, non-inhibited, and mixed behavior male rats. In the first two studies, we assessed the innate immune response by quantifying circulating IL-6 responses to LPS. In the third and fourth study we quantified T_H1-mediated DTH by measuring localized swelling following sensitization with and reintroduction of KLH. The methods for housing rats and for classifying rat temperament were the same for all studies, as described below. For this initial study on temperament and immune function in a controlled laboratory condition, we focused on male rats because behavioral inhibition has been associated with elevated glucocorticoid production and a truncated life span in males (Cavigelli & McClintock, 2003; Cavigelli et al., 2007; 2009) and because sex hormones differentially affect behavior and T_H1 and T_H2 responses (Ray and Hansen, 2004; Roved et al., 2017).

2.1. Animals

Young male Sprague-Dawley rats (55-60 days of age) were purchased from Charles River Laboratories (Raleigh, NC), individually-housed in solid bottom plastic cages (43.5 x 23.5 x 20.5 cm), and maintained on a 12L:12D lighting schedule (lights off at 10:00 h and on at 22:00 h EST). Food and water were available *ad libitum* and cages were cleaned once a week by animal

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facility personnel trained in animal care and handling. The colony room was maintained at 22°C with ~50% humidity. Rats were allowed to acclimate to laboratory housing and daily handling for 1-2 weeks prior to testing. All methods in this study were approved by the Pennsylvania State University Institute for Animal Care and Use Committee and adhered to methods specified in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011). Across the 4 studies we used a total of 113 rats (specific numbers in each study given below).

2.2. Behavioral Response to Novelty

To determine generalized and consistent trait-like inhibited/non-inhibited behavior, we measured approach latency in two different low-threat novel environments: one with rat-sized objects ('novel object arena'; Figure 1a) and one with an unfamiliar rat ('novel social arena', Figure 1b) (Cavigelli et al. 2007; 2009). Behavior in one arena does not predict behavior in the other arena, but individuals that are consistently slow to approach novelty in both arenas maintain this behavioral tendency over time (Cavigelli et al. 2007; 2009). This generalized measure of slow novelty engagement across different contexts is based on early studies of behavioral inhibition in children where this behavioral designation was used to identify those that had reliably slow approaches to a wide array of novel experiences, both social and non-social (Garcia-Coll et al., 1984; Kagan et al., 1984). In prior studies, we have found that inhibition in the social arena is more predictive of basal and reactive circulating glucocorticoid levels, thus, in the current study we also considered whether inhibition in the social vs. object arena was more closely related to immune function (Cavigelli et al., 2007). Behavioral tests were conducted at 70-75 days of age during the active period (13:00-15:00 h) in a room adjacent to the colony room, illuminated with two 25-watt red bulbs that provided ~6 lux of illumination at the center of the test arena. These two behavioral tests were conducted with all rats in all four studies, with 3-4 days between each test and the 'novel object arena' tested before the 'novel social arena'.

For both arenas, latency to approach novelty was coded as the time from placement in the arena to first contact with a novel object or social partner. In the novel social arena, 3 males

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never approached novelty and their latency was coded as 310 seconds (i.e. 10 seconds longer than test duration). Rat temperament was assigned according to latencies in the two novel arenas: *Behavioral-Inhibition* was defined as slower-than-median in both arenas, *Non-Inhibited* as faster-than-median on both arenas, and *Mixed-Behavior* as faster-than-median in one arena and slower-than-median in the other (Cavigelli et al., 2007; 2009). Using these criteria, 20-30% of rats tested were characterized as Inhibited or Non-Inhibited and the remaining 70-80% characterized as Mixed-Behavior. This method of characterizing individuals allows us to identify consistently inhibited vs non-inhibited rats across different contexts, as has been done with children.

2.2.1. Novel Object Arena. The arena was designed to be minimally anxiety-provoking with familiar colony-room odors, low-light conditions, high walls, and a Plexiglas cover (Cavigelli & McClintock, 2003). To provide a complex rat odor, the floor was covered with clean corn cob bedding and sprinkled with bedding (feces removed) from all cages in the colony room. Beyond removal of new feces, the arena floor was not further altered between each rat. The square test arena (120 cm x 120 cm) had white polypropylene walls (46 cm high) and a clear plastic cover. Three of the four corners contained a novel rat-sized object (a plastic tube, an inverted bowl, or a wire tunnel; Figure 1a). During testing, rats were placed into a protected container (clean ceramic bowl with 5 cm sides or red acrylic tube) and lowered into the empty arena corner. Rats were videotaped for 5 min with a camera placed above the arena and returned to their home cage immediately after testing.

2.2.2. Novel Social Arena. The novel social arena was the same size and height as the Novel Object Arena, but instead of rat-sized novel objects, the arena contained two cages – an empty cage and one with an unfamiliar same-sex rat of similar size and age as the test rat (Figure 1b) (Cavigelli et al., 2007; 2009). Rats were introduced to the arena, videotaped, and start bowl cleaned as described for the novel object arena.

2.3 Innate Immune Response – Rapid circulating IL-6 response to lipopolysaccharide (LPS).

To quantify the innate pro-inflammatory response, we injected rats with LPS and measured circulating IL-6 levels at regular intervals during the following 10 hrs. For this initial

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set of studies on rat temperament and immune function, we limited analyses to IL-6. The first of the two studies was used to identify an intermediate LPS dose to mimic a common immune challenge where individual variability in responses can be documented (as opposed to commonly-used high doses used to stimulate strong sickness behavior responses.

2.3.1 Innate Immune Response, Study 1: Identify Appropriate Dose. We used 4 different doses: a commonly-used high dose (125 µg/kg), two intermediate doses (5 and 25 µg/kg), and a control dose (0 µg/kg) (Givalois et al., 1994; Salome et al., 2008). LPS, derived from *Escherichia coli*, serotype 0111:B4, with the same lot number within each study (cat. no. L3012; Sigma-Aldrich, St. Louis, MO) was diluted with sterile endotoxin-free (USP) saline (Cat. No. 2F7124; Baxter, Deerfield, IL) to arrive at an injection volume of 0.5 ml. At ~80 days of age, all rats (*N*=26) were injected intraperitoneally (IP) with one of the four LPS doses 1 hr before the dark phase of the light cycle. Six rats were assigned to each of the 0 and 25 µg/kg conditions and 7 rats to each of the 5 and 125 µg/kg conditions. To measure circulating IL-6, blood was collected by tail-tip amputation immediately before LPS injection and at 2, 4, 6, and 10 hours post-injection. Blood was collected into untreated tubes, stored on ice for approximately an hour, then centrifuged and serum aliquots stored at -80°C until assayed for IL-6.

Serum IL-6 concentrations were measured with a commercial ELISA kit (Rat IL-6 Quantikine, cat no. R6000B; R&D Systems, Minneapolis, MN) with a standard range of 62.5-4000 pg/ml and a sensitivity of 36 pg/ml. Samples were processed according to instructions supplied with the kit. Briefly, 75 µl of frozen rat serum was thawed and diluted 1:2 with diluent supplied with the kit and duplicate samples (50 µl) analyzed for each rat time point. Mean intra-assay and inter-assay coefficients of variation were 7.14 and 14.52.

2.3.2 Innate Immune Response, Study 2: Compare Innate Pro-inflammatory Responses among Behaviorally-Inhibited (BI), Non-Inhibited (NI), and Mixed-Behavior Rats. We used 30 rats that had been characterized as either BI (*n*=6), NI (*n*=7), or Mixed (*n*=17) (according to methods in section 2.2). Methods were identical to LPS Study 1, except only the 25 µg/kg LPS dose was used, and an additional blood sample at 8 hrs post-LPS injection was collected based on results from the prior study. In addition, to test the glucocorticoid resistance hypothesis, we compared post-LPS IL-6 to glucocorticoid secretion; blood samples were analyzed for both IL-6

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and corticosterone. Corticosterone concentration was analyzed with a commercial radioimmunoassay kit (cat. no. 07-120103; MP Biomedicals, Solon, OH) with a standard range of 25-1000 ng/ml and a sensitivity of 25 ng/ml. Samples were processed in duplicate according to instructions supplied with the kit, with some minor alterations. Intra- and inter-assay coefficients of variation were 3.44 and 15.79 for a low control sample and 0.78 and 4.65 for a high control sample across 3 assays.

2.4 Delayed-Type Hypersensitivity – Peripheral swelling response to keyhole limpet hemocyanin (KLH) re-exposure.

KLH is an extremely large heme protein found in the giant keyhole limpet *Megathura crenulata.* It is highly immunogenic, and because KLH is not homologous to any vertebrate proteins, it is unfamiliar to mammals commonly used in research and thus ideal for examining immune responses to a novel antigen. In addition, KLH causes dendritic cells to sensitize T_H1 cells but macrophages do not trigger a large inflammatory response. KLH is therefore an ideal antigen to experimentally-induce a delayed-type hypersensitivity reaction. KLH is introduced systemically, then reintroduced locally several weeks later using a subcutaneous injection, and DTH response estimated from relative swelling at the injection site. This provides a minimally-invasive measure of immune response to antigen re-exposure, which is ideal for longitudinal and lifespan studies.

2.4.1 Delayed-Type Hypersensitivity Response, Study 1: Identify Initial Dose/Injection Site and Re-Exposure Swelling Response Dynamics. In prior studies with KLH-induced DTH responses, several different injection doses and sites have been used for initial KLH exposure, and to estimate DTH swelling, induration measured at different times after re-exposure. To identify the best method to document individual variability in DTH responses, we compared swelling at 1, 2, and 3 days post-re-exposure after initial exposure at one of three locations/doses (Exon et al., 1990; Wood et al., 1993; Fleshner et al., 1995; Gazda et al., 2003). We used 27 male Sprague-Dawley rats classified as Behaviorally-Inhibited (*n*=8), Non-Inhibited (*n*=8), and Mixed-Behavior (*n*=9) with rats in each temperament group evenly distributed across three KLH initial injection sites/doses: (1) 500 µg/kg intraperitoneal (IP; injection volume 0.5 ml;

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n=8), (2) 3mg/kg subcutaneous in caudal tail fold (SC; injection volume 0.2 ml; *n*=9), or (3) both sites (IP+SC; *n*=10; KLH cat. no. 374825, Calbiochem, San Diego CA). Within each injection site, KLH dose/volume was the same for all animals. The initial KLH injection occurred at 95 days of age and rats were re-exposed to heat-aggregated KLH (100 µl injection at 20 mg/ml concentration) injected into the right hind footpad thirty days later (Exon et al., 1990). As a control, an equal volume of saline was injected into the left hind footpad. Right and left footpad swelling was measured 1, 2, or 3 days following re-exposure with a spring-loaded micrometer (Mitutoyo, catalog no. 7301). Analyses were conducted on the difference in swelling between right (KLH-treated) and left (saline-treated) footpads.

2.4.2 Delayed-Type Hypersensitivity Response, Study 2: Compare Delayed-Type Hypersensitivity Swelling among Behaviorally-Inhibited (BI), Non-Inhibited (NI), and Mixed-Behavior Rats. Based on the results of Study 1, for Study 2 we used only the IP method of KLH sensitization (using 500 µg/kg, IP, injection volume 0.5 ml) and measured foot swelling 1 day after KLH re-exposure (*N*=30 male rats). Sensitization occurred at 75 days of age and re-exposure 30 days later with non-heat-aggregated KLH using the same protocol as KLH Study 1. Behavioral and foot-swelling measurements were conducted as in DTH Study 1.

2.5 Statistical Analyses.

In the Innate Immune Response Study 1, a repeated measures ANOVA was used to compare IL-6 responses (2, 4, 6, 10 hr levels) between doses (low, mid, high) as a fixed factor, with IL-6 levels at the time of injection (0 hr) as a covariate. To compare IL-6 responses among temperaments, overall IL-6 production in response to low and mid LPS doses was estimated using area-under-the-curve with respect to ground (AUCg; Pruessner et al., 2003), then AUCg compared across temperaments using ANOVA with temperament (BI, NI, Mixed) and dose (low, mid) as fixed factors, IL-6 AUCg as dependent variable, and IL-6 concentration at the time of injection as a covariate. To further compare IL-6 responses across temperaments a regression analysis was used to compare mean approach latency across the two novel arena tests (or latency on either test) and LPS dose (predictor variables) to IL-6 AUCg (dependent variable). In the Innate Immune Response Study 2, similar analyses were conducted (excluding dose as a

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variable) to compare IL-6 responses to a mid-LPS dose among temperaments. In both studies, several LPS-injected rats had peak IL-6 levels that were very low and comparable to those in control (saline-injected) rats (< 200 pg/ml at any time point as compared to a mean peak of 2925 pg/ml in Study 2). Non-responders were defined as LPS-injected rats that had IL-6 levels less than 200 pg/ml at all time points, and these individuals were excluded from analyses (4 LPS-injected rats in Study 1 – all in the 5μg/kg and 25μg/kg groups – and 3 rats in Study 2, 2 BI, 1 NI). In Study 2, corticosterone responses to LPS injections were compared among temperaments using similar methods described above for IL-6; a one-factor ANOVA with temperament as a factor, corticosterone AUCg as the dependent variable, and concentration at the time of LPS injection as a covariate. We also used a repeated measures ANOVA with temperament as factor, corticosterone concentrations at each time point after LPS injection as the dependent variable and corticosterone levels at the time of injection as a covariate. Total glucocorticoid production was linearly compared to temperament using a correlation analysis with mean approach latency across novel arenas and corticosterone AUCg as variables. To compare the relative relationship between temperament and glucocorticoid and IL-6 production following LPS challenge, we used a multiple regression analysis with mean latency on both novel arenas and glucocorticoid concentration at the time of LPS injection and during the 10 hours after LPS (AUCg) as predictor variables, and IL-6 AUCg as the dependent variable. To determine if a specific form of inhibition (i.e. social vs. non-social) was most closely associated with immune function, these analyses were repeated with latency on each arena separately instead of mean latency across both arenas.

DTH responses were estimated from relative footpad swelling calculated as the difference in cross-section between the saline- and KLH-treated feet. In DTH Study 1, swelling response differences between the IP, SC, and IP+SC initial injection methods were assessed by ANOVA. To compare swelling responses among temperaments in DTH Study 2, an ANOVA was used with temperament as the fixed effect factor and swelling response as dependent variable. Two rats were identified as outliers with footpad swelling difference scores further than two standard deviations from the mean; these two males were excluded from analysis (2 NI), leaving 9 BI, 6 NI, and 13 Mixed rats. In addition, correlational analyses were conducted to

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compare swelling response to mean latency on the two arenas and to latency on each arena separately. For all analyses, dependent variables were inspected for normality and for those variables that showed a right skew, natural-log-transformation values were used in statistical analyses to meet normal distribution requirements. For clarity, means and SEMs of raw values are presented in figures.

3. Results

3.1. Innate Immune Response Results

3.1.1. Study 1: Dose effects of LPS on IL-6 response.

As expected, increasing LPS doses led to increased circulating IL-6 concentrations (Dose: F (3,22) = 8.39, $p < 0.001$; Time: F (4,19) = 305.48, $p < 0.0001$; Dose x Time: F (12,53) = 5.80, $p < 0.0001$; Figure 2). In the control group (0 µg/kg), IL-6 levels did show a mild increase over time, likely a response to the injury of the tail clip required for repeat blood collection (F (4,20) = 24.81, $p < 0.0001$). There was no significant difference in IL-6 responses between the 5 and 25 µg/kg doses (F (1,7)=1.35, p = 0.283), and thus some analyses were conducted merging across doses. The 125 µg/kg dose created the expected ceiling effect in all animals.

3.1.2. Study 1 & 2: Behaviorally-Inhibited (BI) rats have a greater interleukin-6 and glucocorticoid response to moderate LPS stimulation than Non-Inhibited (NI) rats.

In Study 1, at the moderate doses of 5 µg/kg and 25 µg/kg, Behaviorally-Inhibited rats – those with the longest approach latencies in the novel arenas – had significantly higher circulating IL-6 AUCg values (mean latency across arenas: adjusted R^2 = 0.85, n = 9, t = 3.81, p = 0.0089). Latencies within each arena were less predictive of individual rat IL-6 AUCg after LPS, with object approach latencies more closely related to IL-6 responses than social approach latencies (object: adjusted R^2 = 0.73, n = 9, t = 2.36, p = 0.056; social: adjusted R^2 = 0.58, n = 9, t = 1.21, p = 0.275). At the higher LPS dose (125 µg/kg), there was no relationship between approach latencies and IL-6 responses to LPS (R^2 = 0.09, n = 7, t = -0.71, p = 0.509).

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Study 2 confirmed this accentuated IL-6 response in Behaviorally-Inhibited rats. Basal circulating IL-6 concentrations at the time of LPS injection were no different among temperaments (F (2,24) = 0.361, p = .701, η^2 = 0.029; Figure 3a). However, in response to the LPS challenge, BI rats had peak and AUCg circulating IL-6 levels that were 2-3 times greater than NI and Mixed rats (IL-6 AUCg ANOVA: F (2,23) = 5.044, p < 0.05, η^2 = 0.305; repeated measures ANOVA: F (2,23) = 3.802, p < .05, η^2 = 0.248; Figure 3a). Tukey's HSD tests indicate that the BI rats were significantly different from both the NI and Mixed groups (ps = 0.012 and 0.020). There was a significant positive correlation between mean latency on the novelty arenas and IL-6 production (AUCg) in response to the moderate LPS dose (r = 0.453, n = 27, p = 0.009). Latency on the novel social arena was more closely related to IL-6 production than latency on the novel object arena (r = 0.404, n = 27, p = 0.018 vs. r = 0.225, n = 27, p = 0.129).

There was also a trend toward temperament-specific differences in glucocorticoid production in response to the moderate LPS challenge. At the time of LPS challenge, basal corticosterone levels did not differ among temperament groups (F (2,23) = 0.868, p = 0.434, η^2 = 0.070; Figure 3b), but there was a significant temperament difference in corticosterone secretion after LPS injection. BI rats had significantly greater corticosterone AUCg compared to Mixed rats (corticosterone AUCg ANOVA: F (2,22) = 4.755, p = 0.019, η^2 = 0.302; repeated measures ANOVA: F (2,22) = 4.201, p = 0.028, η^2 = 0.276; Figure 3b). In the case of glucocorticoid production, there were no significant linear relationships between approach latency in the novel arenas and total corticosterone production (AUCg) after LPS stimulation (mean latency in both arenas: r = 0.163, n = 27, p = 0.208; novel social: r = 0.049, n = 27, p = 0.403; novel object: r = 0.172, n = 27, p = 0.195). Variance in IL-6 response to LPS was more closely related to behavioral measures of temperament (mean latency to approach) than to basal corticosterone level at the time of LPS challenge or to corticosterone responses (AUCg) to the challenge (regression statistics for mean approach latency, basal corticosterone, and corticosterone AUCg to LPS: $ß$ = 0.436, t = 2.377, p = 0.026; $ß$ = -0.233, t = 1.052, p = 0.304, $ß$ = 0.202, t = 0.903, p = 0.376).

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3.2. Delayed-Type Hypersensitivity Results

3.2.1 Study 1: Temporal dynamic of footpad swelling after KLH re-injection did not differ depending on location of initial injection.

In Study 1, on the day after KLH re-exposure, the KLH-treated footpad was thicker than the saline-treated footpad (paired t (8) = 6.502, $p < 0.0001$; KLH vs. saline mean ± SD: 5.032 ± 0.227 vs. 4.658 ± 0.166 mm). This difference in swelling between the right (KLH-treated) and left (saline-treated) footpads did not differ depending on the location of the initial KLH injection (intraperitoneal, caudal fold, or both; F (2,21) = 0.513, p = 0.606) but did differ according to days following re-exposure (F (2, 22) = 7.453; p = 0.003). Footpad swelling was greatest 1 day after re-exposure, intermediate 2 days after re-exposure, and resolved by day 3 (mean ± SD on Days 1, 2, 3: 0.375 ± 0.172, 0.192 ± 0.221, -0.008 ± 0.222 mm). Based on these results, analyses of temperament-related swelling were conducted with footpad swelling measured on Day 1 after KLH re-exposure.

3.2.2. Study 1 & 2: Behaviorally-Inhibited (BI) rats had a dampened (or slower) DTH response to KLH than Non-Inhibited (NI) rats.

In Study 1, rats that were fast to approach novelty (NI) had more footpad swelling one day after KLH re-exposure than rats that were slower to approach novelty (BI) (r = -0.65, n = 10, p = 0.041). Two days after KLH re-exposure, there was a trend for greater KLH-induced footpad swelling in BI vs. NI males (r = 0.67, n = 8, p = 0.069), suggesting a possible delayed DTH response in BI. Study 2 outcomes were similar; NI males had a heightened swelling response one day after KLH footpad re-exposure compared to Mixed- and BI males (F (2,25) = 3.53, p = 0.045; Figure 4). There was a significant negative correlation between mean approach latencies on the two novelty arenas and footpad swelling responses (r = -0.534, n = 28, p = 0.003). Again, like for the IL-6 response to LPS, latency on the novel social arena was more closely related to footpad swelling than latency on the novel object arena (r = -0.47, n = 28, p = 0.012 vs. r = -0.33, n = 28, p = 0.083).

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4. Discussion

In the current study, we found that behavioral inhibition, a trait associated with moderate, chronic glucocorticoid overproduction, was also associated with a specific immune response profile. Male rats that showed behavior inhibition had an elevated innate inflammatory response with concurrent elevated circulating glucocorticoids, and dampened T_H1-mediated delayed-type hypersensitivity response compared to non-inhibited rats. These results suggest that behavioral inhibition involves innate cell glucocorticoid resistance and a skew toward less T_H1-mediated immune responses. Taken together, immune function was systematically related to a behavioral trait even in the absence of differential antigen exposure among behavioral types.

The results of the current study are similar to those documented in prior studies with mice, primates, and cattle (Azpiroz et al., 2008; Capitanio et al., 2011; Hulbert et al., 2011; Kirsten et al., 2018) and differ from other studies that have shown increased innate immunity in free-ranging avian risk-takers and heightened DTH responses in socially-inhibited human adults (e.g. Cole et al., 1999; Zylberberg et al., 2014). Different findings across studies may result from different behavioral categorization methods, species differences in evolutionary histories, sex differences, disease history, genetic predispositions, and/or different environmental conditions across studies. For example, laboratory-bred, farm-bred, and wild-caught animals all have vastly different breeding histories, life experiences, and pathogen exposures. Innate immune responses are associated with significant caloric cost, and for wild-living animals sickness behavior may lead to lost opportunities to forage, socialize, and mate (Rauw, 2012; McDade, 2005; Lochmiller & Deerenberg, 2000) and may cause tissue damage (i.e. immunopathology; Medzhitov et al., 2012; Peiris et al., 2010; La Gruta et al., 2007). Thus, innate immunity as it relates to temperament may differ between free-ranging and lab/farm-raised animals.

Additionally, variance among studies may be driven by differential behavioral characterization methods. Behavioral response to novelty is likely driven by two distinct motivations: harm avoidance vs. sensation-seeking; specific testing conditions may access one motivation more readily than the other (e.g. Ray & Hansen 2004). Tests to access these two underlying motivations are not strongly-established in rodents. In the current study, we used

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tests that mimic those used to measure behavioral inhibition (fearful temperament) in children: low-threat, moderately-complex testing environments that maximize movement in most animals. Sensation-seeking in humans has been defined as willing to take risks for the sake of varied, novel, and complex sensations and experiences (Zuckerman 1979). Thus, by testing in a low-threat environment (low light, protected start area, covered arena with high walls), we likely did not have a strong metric of sensation-seeking but rather of harm avoidance/fearful temperament/behavioral inhibition. Variance in immune profiles as they relate to exploration-related behaviors may result from a high degree of behavior test variance, both within and between species. Interestingly, studies that used similar behavioral characterization methods as those used in the current study, showed similar glucocorticoid and immune response biases (passive-reactive male mice, Azpiroz et al., 2008; nervous infant rhesus monkeys, Capitanio et al., 2011; temperamental Brahman bulls, Hulbert et al., 2011; high responder zebrafish, Kirsten et al., 2018).

Another source of variance among studies may result from sex of the study animals. Behavioral patterns and basal and stimulated glucocorticoid circulation and immune function is quite different between males and females and this difference must be taken into account when studying the relationship between behavioral and immune phenotypes (Cavigelli et al. 2008; Immonen et al., 2018; Tarka et al., 2018). For example, in female humans social-inhibition was associated with elevated DTH responses (Cole et al., 1999) whereas the current study with male rats showed the opposite result. Further work is required to determine if immune function is systematically associated with behavioral phenotypes, and if so how these systematic immune differences affect health and fitness outcomes, in both free-ranging and domesticated animals, males and females, and harm avoiding vs. sensation-seeking individuals.

Prior work has demonstrated effects of chronic stress and glucocorticoid production on a variety of immune functions, including innate immunity and delayed-type hypersensitivity responses. The present study tested whether a simple behavioral trait associated with elevated glucocorticoid production was also associated with a biased immune response profile. Inhibited rats had heightened IL-6 and glucocorticoid responses to a low/moderate dose of lipopolysaccharide, and a dampened swelling response to keyhole limpet hemocyanin re-

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exposure compared to non-inhibited rats. Behavioral measures of temperament were linearly-related to rapid IL-6 responses to LPS, but glucocorticoid production was not, suggesting that behavior-associated immune alterations are not solely mediated by glucocorticoid profiles (e.g. Sloan et al., 2008; Azpiroz et al., 2008; Capitanio et al., 2011). On the other hand, a heightened IL-6 and corticosterone response to LPS suggests that behavioral inhibition may involve some degree of glucocorticoid resistance. Glucocorticoid administration can inhibit macrophage-triggered T_H1 inflammatory responses, although macrophages quickly develop glucocorticoid resistance, which leads to enhanced innate immune activity and dysregulation of immune balance (Mosser and Edwards, 2008; Stark et al., 2001; van de Garde et al., 2014). This dysregulation occurs in disorders like autoimmune diseases, chronic infections, major depression, and atherosclerosis – many of the health conditions that have been associated with behavioral inhibition (Calcagni & Elenkov, 2006). In addition, long-term exposure to elevated glucocorticoids can produce tolerance in specific immune cells, which trigger runaway inflammation and T_H2 responses (i.e. macrophages, via innate lymphoid cells) while other cell types remain inhibited (Schmidt et al., 2010; Banuelos & Lu, 2016). Chronically-elevated corticosterone levels in inhibited individuals may inhibit T_H1 adaptive immune responses but allow for elevated innate inflammatory and T_H2 responses.

T_H2 polarization may be adaptive for coping with acute stressors - a strong T_H2 response could help moderate an elevated T_H1 response initiated to cope with skin wounding and infection (Calcagni & Elenkov, 2006; Munck & Náray-Fejes-Tóth, 1994; Dhabhar & McEwen, 1997; Gause et al., 2013). However, in the face of chronic stress or chronically-elevated glucocorticoid production, and in the absence of wounds, this regulatory response may lead to long-term T_H2 activation – a risk-factor for allergic and atopic responses (Elenkov & Chrousos, 1999). Additionally, a major function of the immune system is also to defend against damaged self-cells via the DNA damage response and repair (DDR/R) pathway. Immune dysregulation toward T_H2 inhibits this DDR/R cascade, allowing cancerous cells to proliferate unchecked (Pateras et al., 2015). Although not specifically tested in the current study, a dampened T_H1-mediated response in behaviorally-inhibited males may be linked to elevated T_H2-mediated responses – a strong anti-parasite defense in wild-living animals, but a hallmark of

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allergies/atopic disorders/asthma in clean, protected lab-living animals and humans (see Maggi, 1998).

Future mechanisms to explore include the function of regulatory T cells (T_{reg}). T_{reg} cell activity reduces allergic airway inflammation by suppressing T_H2 cell differentiation (Duan et al., 2011) or modulating the activity of T_H1 and T_H2 cells (Akdis et al., 2004). In humans with asthma and in allergic asthma mouse models, the ability of T_{reg} cells to influence monocyte activity and IL-6 production is altered (Lee et al., 2007; Matsumoto et al., 2009; Hamzaoui et al., 2010). Inhibited recruitment of T_{reg} cells in response to allergen can result in excessive T_H2 responses in sensitized asthmatics (Wang et al., 2009). In addition, T_{reg} cells are inhibited by T_H17 cells, which are resistant to glucocorticoid suppression (Schmidt et al., 2010; Banuelos & Lu, 2016). Thus, chronic glucocorticoid production may have minimal impact on these processes. Future work on temperament-associated immune biases might focus on T_{reg} cell function in fearful humans as a possible mechanism underlying risk for allergy-related health outcomes.

The results of the current study provide support that a rodent model of human behavioral inhibition is possible. This and prior studies indicate that male rats that are slow to explore a novel, complex, protected environment have a shortened lifespan, elevated basal and stress-reactive levels of glucocorticoids, a heightened pro-inflammatory innate immune response, and dampened delayed-type hypersensitivity compared to rats that are not inhibited in this setting (Cavigelli & McClintock, 2003; Cavigelli et al. 2007; 2009). Chronic glucocorticoid overexposure, such as that associated with behavioral inhibition, may lead to these specific immune alterations, although this requires further study. An animal model of fearful or harm avoidant or behavioral inhibition temperament with similar underlying physiological biases as those seen in similarly-behaving humans provides a system in which to experimentally-test how genetic, environmental, and developmental conditions affect the relationship between temperament and health.

An important aspect of this model is that latency to approach a novel conspecific (social partner) was more predictive of immune dysregulation than latency to approach a novel object. Social inhibition, rather than inhibition in non-social contexts, may be a more appropriate model of temperament-specific immune dysfunction. Social stress has been shown to enhance

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the T_H2 response to allergen and reduce glucocorticoid sensitivity in many studies (Bailey et al., 2009; Li et al., 2013; reviewed in Haczku & Panettieri, 2010). Zebrafish showing low social exploration had reduced expression of IFN-γ (Kirsten et al., 2018). Mice deficient in adaptive immunity (SCID mice) have been shown to exhibit defects in social behavior that can be reversed by IFN-γ, a cytokine critical for T_H1 development (Filiano et al., 2016). In humans, loneliness and social isolation are associated with negative health outcomes, including mortality (Steptoe et al., 2013; Ellwardt et al., 2015; reviewed in Holt-Lunstad et al., 2015), and social inhibition predicted DTH responses during social engagement (in adults with autoimmune disorders; Cole et al. 1999). Thus, an animal model of social inhibition may provide the best system in which to study fearful temperament-specific physiology and health.

Further work is required to identify mechanisms by which temperament-specific immune alterations occur. Future studies should include measures of central nervous system function, and experimental manipulation of glucocorticoid activity to determine the relative role of these mechanisms in temperament-related immune response biases. An important caveat for this future work is that experimentally-manipulated exogenous glucocorticoid exposure should mimic glucocorticoid profiles seen in distinct temperaments, recognizing that endogenous glucocorticoid profile mimicry can be difficult with exogenous glucocorticoids (Pruett et al. 1999; Fleshner et al., 2001). Specific aspects of central nervous system alterations include serotonergic function (Takano et al., 2007; Donner et al., 2016), hippocampal and prefrontal cortex glucocorticoid signaling (Cerqueira et al., 2005; Patel et al., 2008; Sorrells et al., 2014), the kynurenine pathway (Brooks et al., 2016), and pro-inflammatory cytokine regulation throughout the brain (Capitanio et al., 2008; reviewed in Ménard et al., 2016). Several cytokines, particularly IL-1 and TNFα, can affect central nervous system functioning (reviewed in Rochfort & Cummins, 2015). In turn, central nervous system processes can affect immune function by triggering secretion of immunomodulating hormones or autonomic stimulation of lymph nodes, all of which may be associated with an inhibited temperament (e.g. Sloan et al., 2008). Further research on mechanisms could provide insight into targeted interventions in order to improve both psychological and physical well-being in behaviorally-inhibited people.

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Figure Legends

Figure1. Overhead view of the novel object (a) and novel social (b) arenas. Test rat is placed in a clean ceramic bowl and lowered into the corner of the arena and allowed to explore freely for 5 minutes.

a.



b.

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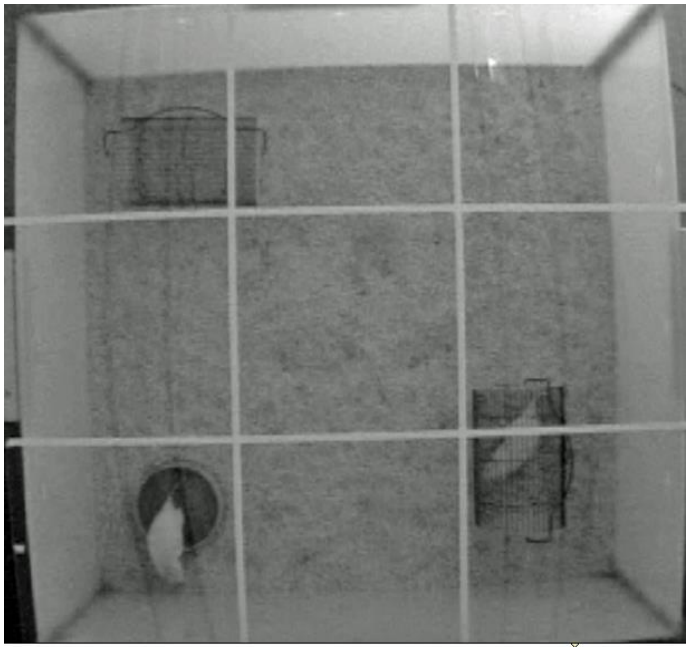


Figure 2. Male Sprague-Dawley rat circulating interleukin-6 (IL-6) concentration at baseline and in response (at 2, 4, 6, 10 hr) to 4 different doses of intraperitoneal lipopolysaccharide (LPS; 0, 5, 25, 125 µg/kg; n = 6-7 rats in each dosage group respectively). Circles indicate means, error bars indicate S.E.M.

Figure 2.

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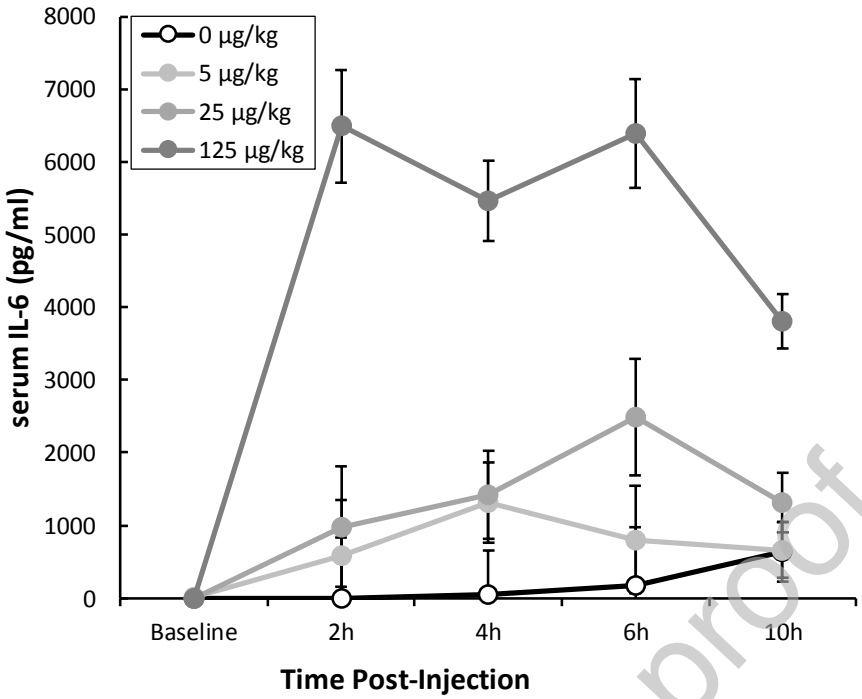
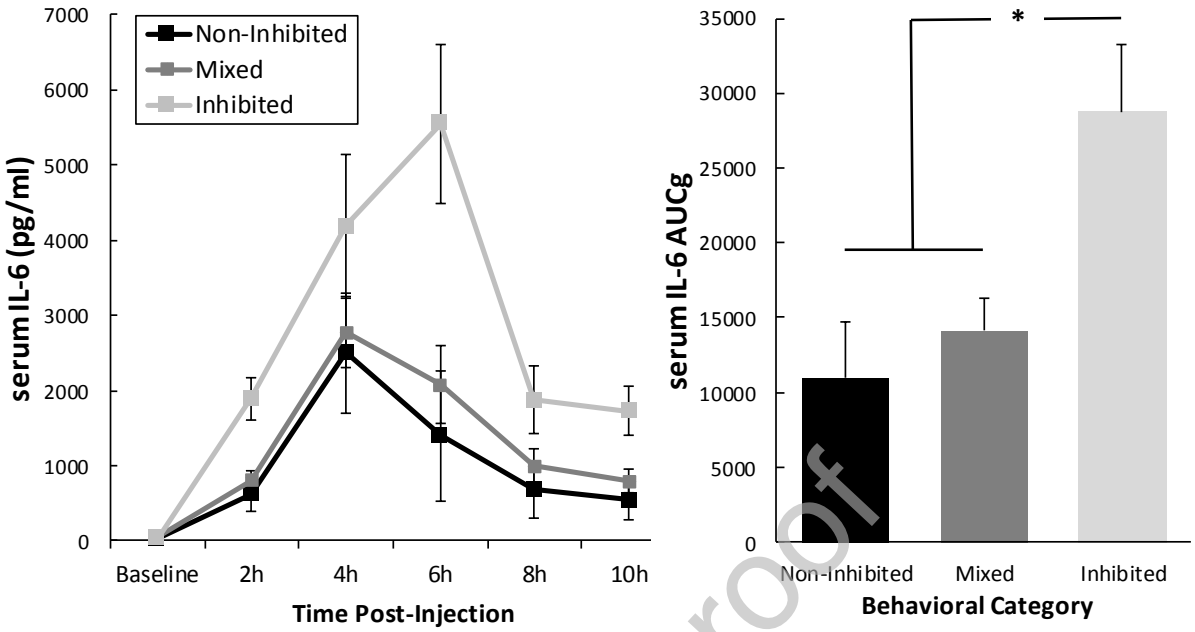
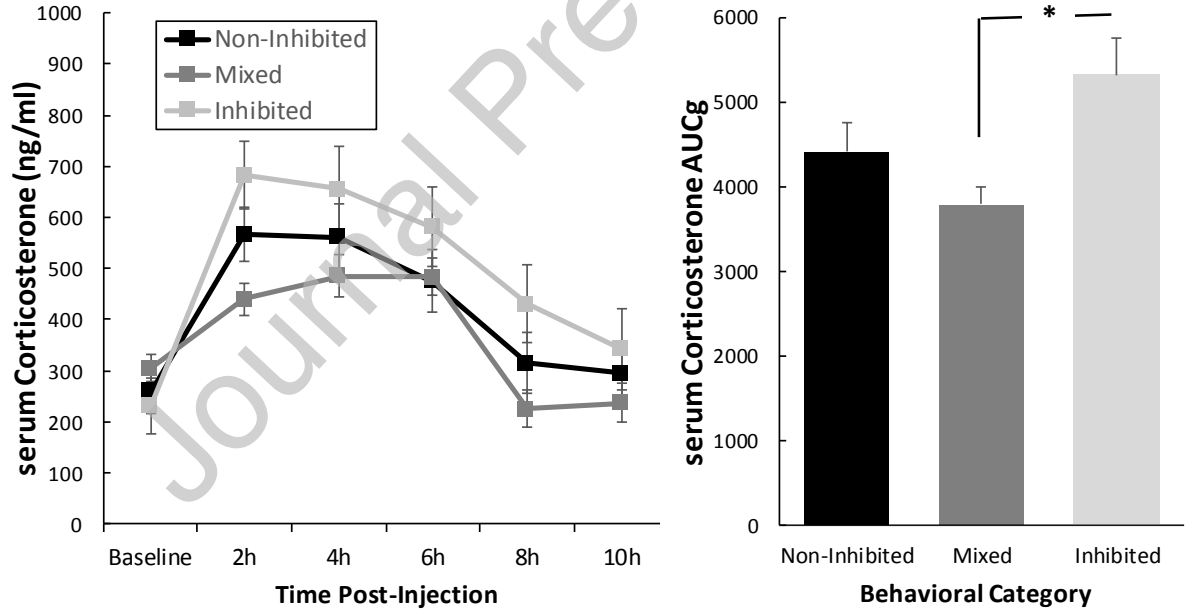


Figure 3a,b. (a) Mean circulating interleukin-6 (IL-6) concentration at baseline and after intraperitoneal lipopolysaccharide (LPS) injection for Behaviorally-Inhibited (BI), Non-Inhibited (NI), and Mixed-Behavior male rats (n = 4, 6, 17 respectively). Left-hand figure depicts mean IL-6 concentration at each sampling time, right-hand figure depicts the total IL-6 response to LPS represented as area-under-the-curve relative to ground (AUCg) during the 10 hrs following LPS injection. Error bars indicate S.E.M. (b) Mean circulating corticosterone concentration at baseline and after intraperitoneal lipopolysaccharide (LPS) injection for BI, NI, and Mixed male rats (n = 4, 6, 17 respectively; same rats as those whose data are depicted in Figure 3a). Left-hand figure depicts mean corticosterone concentration at each sampling time, right-hand figure depicts the total corticosterone response to LPS represented as area-under-the-curve relative to ground (AUCg) during the 10 hrs following LPS injection. Error bars indicate S.E.M. For both AUCg figures, asterisk indicates significant differences ($p<.05$) between groups in post-hoc comparisons.

a.



b.

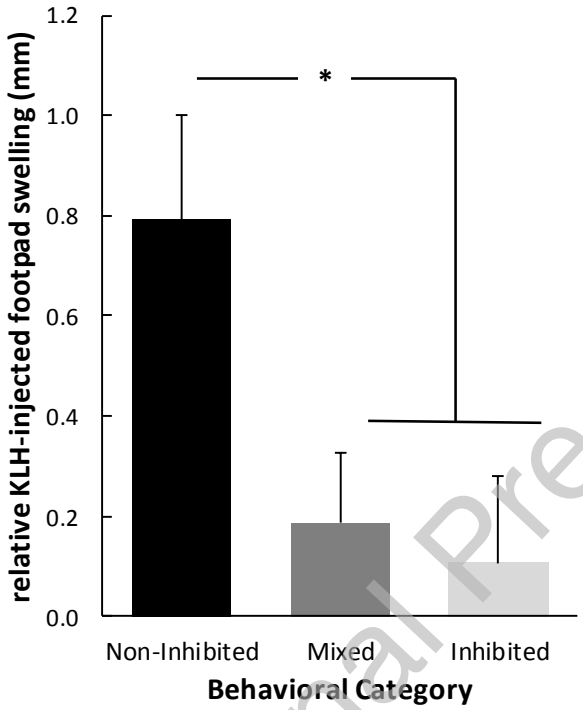


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Figure 4. Delayed-type hypersensitivity response, as measured by relative footpad swelling in response to keyhole limpet hemocyanin (KLH) re-exposure in Behaviorally-Inhibited (BI),

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Non-Inhibited (NI), and Mixed-Behavior male rats (n = 9, 6, 13 respectively). Relative swelling represents the difference in footpad thickness between the saline-exposed footpad and the KLH re-exposed footpad. Error bars indicate S.E.M. Asterisk indicates significant difference (p<.05) between groups in post-hoc comparisons.



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