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Trait-specific effects of exogenous triiodothyronine on cytokine and behavioral responses to simulated systemic infection in male Siberian hamsters

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ABSTRACT

Seasonal changes in day length enhance and suppress immune function in a trait-specific manner. In Siberian hamsters (*Phodopus sungorus*) winter-like short days (SDs) increase blood leukocyte concentrations and adaptive T cell dependent immune responses, but attenuate innate inflammatory responses to simulated infections. Thyroid hormone (TH) signaling also changes seasonally and has been implicated in modulation of the reproductive axis by day length. Immunologically, TH administration in long days (LD) enhances adaptive immune responses in male Siberian hamsters, mimicking effects of SDs. This experiment tested the hypothesis that T_3 is also sufficient to mimic the effects of SD on innate immune responses. Adult male hamsters housed in LDs were pretreated with triiodothyronine (T_3; 1 μg, s.c.) or saline (VEH) daily for 6 weeks; additional positive controls were housed in SD and received VEH, after which cytokine, behavioral, and physiological responses to simulated bacterial infection (lipopolysaccharide; LPS) were evaluated. SD pretreatment inhibited proinflammatory cytokine mRNA expression (i.e. interleukin 1β, nuclear factor kappa-light-chain-enhancer of activated B cells). In addition, the magnitude and persistence of anorexic and cachectic responses to LPS were also lower in SD hamsters, and LPS-induced inhibition of nest building behavior was absent in SD. T_3 treatments failed to affect behavioral (food intake, nest building) or somatic (body mass) responses to LPS in LD hamsters, but one CNS cytokine response to LPS (e.g., hypothalamic TNFα) was augmented by T_3. Together these data implicate thyroid hormone signaling in select aspects of innate immune responses to seasonal changes in day length.

1. Introduction

Immune function varies over time: constitutive (Dhabhar et al., 1994), adaptive (Prendergast et al., 2013) and innate (Castanon-Cervantes et al., 2010) assays of immunity are modulated over a circadian timescale. Immune responses are also dynamically regulated over seasonal timescales (Nelson, 2004; Stevenson and Prendergast, 2015). In the laboratory, changes in day length induce striking changes in innate and adaptive immunity. Following adaptation to short winter photoperiods (short days; SDs), multiple aspects of Siberian hamster immune function adopt a winter phenotype: circulating leukocyte subpopulations are augmented or depleted (Bilbo et al., 2002a; Prendergast et al., 2004), delayed-type hypersensitivity (DTH) responses are augmented (Bilbo et al., 2002a; Stevenson et al., 2014), and infection-induced acute-phase proinflammatory cytokine production, fever and sickness behaviors are attenuated (Bilbo et al., 2002b; Prendergast et al., 2003; Navara et al., 2007).

Activity of the immune system engages a complex, reciprocal activation of neuroendocrine systems. In addition to the well-described influences of adrenal hormones on immunity, thyroid hormones are potent regulators of immune function (Dorshkind and Horseman, 2000). Systemic hyper- or hypo-thyroidism alters antibody responses, cytokine production, and lymphocyte proliferation (De Vito et al., 2011); conversely, inflammation dynamically upregulates thyroid hormone-activating enzyme type II deiodinase (DIO2) in a nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) dependent manner (de Vries et al., 2016) in CNS (Fekete et al., 2004) and peripheral tissues (Kwakkel et al., 2014).

Abundant data indicate that thyroid hormones participate in seasonal photoperiodic transitions in the reproductive neuroendocrine system (Nakane and Yoshimura, 2014; Stevenson et al., 2017), and emerging evidence suggests that T_3 also figures prominently in

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photoperiod-driven changes in immune function (Stevenson et al., 2014; Banks et al., 2016). Siberian hamster adaptation to SD reduces expression of iodothyronine deiodinase Type III (*dio3*) in blood leukocytes, which inhibits conversion of T_4 into the biologically-active hormone T_3 in these cells, yielding greater levels of lymphocyte cytoplasmic T_3 (Stevenson et al., 2014). In addition, exogenous T_3 is sufficient to induce the SD phenotype in blood leukocyte concentrations and DTH in hamsters housed in long, summer days (LDs; Stevenson et al., 2014; Banks et al., 2016).

Lipopolysaccharide (LPS) is a TLR4 ligand and a potent activator of innate immune responses (Bryant et al., 2010). Peripheral LPS treatment upregulates expression of proinflammatory signaling cascades in the CNS, inducing NFκB-dependent IL-1β and TNFα expression, which culminates in behavioral symptoms of infection (Hart, 1988; Kent et al., 1992). In male and female Siberian hamsters, proinflammatory cytokine production in the periphery and in the CNS is downregulated in SD (Bilbo et al., 2002b; Prendergast et al., 2003; Pyter et al., 2005), as is behavioral responsiveness to cytokines (Wen and Prendergast, 2007). Consequently, physiological and behavioral sickness responses to LPS are diminished in hamsters following adaptation to SD (Bilbo et al., 2002b; Baillie and Prendergast, 2008; Prendergast et al., 2008). Attenuated innate inflammatory responses in winter may conserve energy at a time of year when hamsters are in negative energy balance (Nelson, 2004). Whether T_3 participates in winter attenuation of innate immune responses has not been examined.

This experiment examined the role of T_3 in seasonal immune biology. Behavioral, physiological, and molecular aspects of the acute phase response to infection were examined in hamsters treated with T_3. If, in common with constitutive and adaptive immune responses, seasonal increases in thyroid hormone signaling participate in the effects of photoperiod on innate immune responses, then we expect that T_3 will attenuate inflammatory responses to LPS. If this outcome is not obtained, then we can exclude the hypothesis that the upregulation of T_3 signaling in immune cells participates in photoperiodic attenuation of inflammatory responses to LPS.

2. Methods

2.1. Animals

Siberian hamsters (*Phodopus sungorus*) were obtained from a breeding colony which provided 15 h of light per day (LD; lights off at 18:00 CST). Adult male hamsters were singly-housed in polypropylene cages, with food (Harlan, Teklad) and filtered tap water provided ad libitum; cotton nesting material was also available in the cage. Ambient temperature and relative humidity were maintained at 19 ± 2 °C and 53 ± 10%, respectively. All procedures were approved by the Animal Care and Use Committee at the University of Chicago.

2.2. Photoperiod and hormone pretreatment

To assess effects of photoperiod and T_3 on innate immune adaptations, hamsters were pretreated for 6 weeks with photoperiod and/or T_3 as follows ("pretreatment" groups): hamsters housed in LD received daily subcutaneous (s.c.) injections of 1 μg T_3; triiodothyronine (T2877, Sigma Aldrich) or sterile 0.9% saline ('VEH', 0.1 mL) for 6 weeks before sickness responses were elicited (see below). Injections were administered 3 h before the onset of darkness. This injection regimen blocks gonadal regression in hamsters exposed to reproductively-inhibitory short photoperiods (Freeman et al., 2007). This dosage of T_3 was chosen because it mimics treatments used in prior studies of T_3-immune relations in this species (Stevenson et al., 2014). Additional control hamsters were housed in SDs (9L:15D; lights off at 18:00 CST) and received saline (0.1 mL) injections only. In both experiments (see below) injections continued on a daily basis up to and including the final day of data collection.

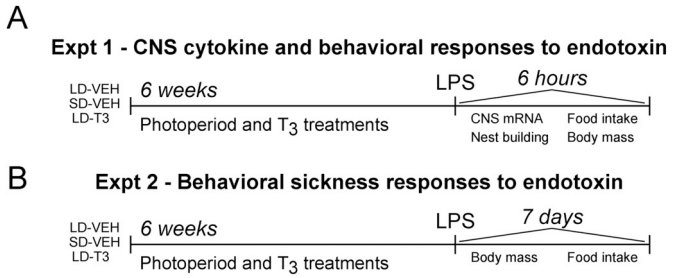

Fig. 1. Experiment timeline (A) Experiment 1: Behavioral effects of six weeks of T_3 pretreatment on hypothalamus and cortex cytokine expression and behavioral responses to LPS. Data collected 6 h after injection. (B) Experiment 2: Effects of six weeks of T_3 pretreatment on behavioral responses to LPS. Data collected every 24 h following injection for 7 days. Not depicted is the second round of injection. Hamsters injected with LPS in the first round were injected with SAL in the second round, and vice versa.

2.3. Experiment 1 – effects of T_3 on CNS cytokine and behavioral responses to endotoxin

2.3.1. Pretreatment

On *week 0*, hamsters were housed in LD (n = 30) or were transferred to SD (n = 16). Beginning on *week 0* and continuing the next 6 weeks, LD hamsters received daily T_3 (n = 14) or saline (n = 16) injections; SD received daily vehicle injections. Timeline shown in Fig. 1A. Reproductive responses to LD and SD treatments were confirmed via testes mass acquired at sacrifice.

2.3.2. LPS treatments

On *week 6*, sickness responses were elicited using a simulated bacterial infection. LPS (*E. coli* 026:B6; lyophilized powder; Sigma) or control injections (0.1 mL of 0.9% sterile saline; SAL) were administered 30 min before the onset of darkness (17:30 h CST) to hamsters in all 3 pretreatment groups. LPS was reconstituted in sterile 0.9% saline and delivered intraperitoneal (i.p.) at a dose of 0.625 mg/kg (volume of ~0.1 mL; cf. Wen and Prendergast, 2007).

2.3.3. Sickness responses

Food intake, nest-building behavior, and body mass changes were measured during the first 6 h after LPS and SAL injection treatments. At the time of injection, each hamster was given a small (mean ± SEM = 2.56 ± 0.02 g) square of pre-weighed compressed cotton batting, in order to assess nesting material use. An equivalent amount of nesting material was provided to all 3 pretreatment groups. The amount of cotton incorporated into the nest was determined by weighing the unused portion at the time of tissue collection. Nest building behavior was operationally defined as the percentage of the cotton used (Wen and Prendergast, 2007; Carlton and Demas, 2015). Body mass and the mass of food pellets in the food hopper were determined (to 0.1 g resolution) at the time of injections and again at the time of brain tissue collection (6 h after injection, see below). Body mass loss was calculated as the difference between initial and terminal body mass and was expressed as a percentage of initial body mass. Mass-specific food intake (MSFI) was calculated as the quotient of 6 h food intake divided by initial body mass (in g/kg units). Extreme sickness responses to LPS resulted in the removal of 3 hamsters from study at the 6 h timepoint.

2.3.4. Tissue collection

Brain tissues were collected 6 h after LPS/SAL injections. Hamsters were anesthetized with 3% isoflurane (in O_2) and euthanized via rapid decapitation. Whole hypothalami and a section of cortex from the lateral aspect of the right frontal lobe were dissected according to procedures described previously (Prendergast et al., 2013; Stevenson and

Prendergast, 2013; Prendergast et al., 2014) and stored at −80 °C until nucleic acid extraction.

2.3.5. RNA isolation

Hypothalamic total RNA was extracted using RNAeasy kits (Qiagen; Germantown, MD) following the manufacturer's instructions. RNA concentration and quality were determined by spectrophotometer (Nanodrop, Thermo Scientific; Wilmington, DE). cDNA was synthesized using Superscript III (Invitrogen; Carlsbad, CA), and cDNA were stored at −20 °C.

2.3.6. Quantitative PCR (qPCR)

qPCR was used to quantify the transcriptional activity in CNS cytokine pathways that have been implicated in the expression of sickness behaviors. Hypothalamus and frontal cortex expression of *il1β*, *tnfα*, and *nfκb* mRNAs were measured using qPCR in triplicate technical replicates. PCR specificity of primers was established by single-bands in 2.5% agarose gel and PCR product sequencing at the University of Chicago Comprehensive Cancer Center. Accession numbers are as follows: *il1β* (KJ996172.1), *tnfα* (KJ996171.1), *nfκb* (MH388471) and *gapdh* (KC153108.1). Furthermore, all sequences completely aligned with our Siberian hamster genome (BioProject Accession PRJNA318271). qPCR reactions were performed using a BIORAD CFX384 system using the following steps: an initial denaturation at 95 °C for 30 s, then 39 cycles of [i] 95 °C for 30 s, [ii] annealing dependent on target mRNA amplicon sequence for 30 s and [iii] an extension at 72 °C for 30 s (see Table S1 for primer sequences and amplicon-specific qPCR reaction conditions). Melting curve analyses were included to confirm the quality and specificity of each reaction. Quantification of mRNA expression levels was accomplished with iQ Sybr Green Supermix (BioRad, Hercules, USA). PCR Miner software (Zhao and Fernald, 2005) was used to calculate reaction efficiencies (E) and cycle thresholds (CTs). Only efficiency values $0.8 > E < 1.2$ were included in statistical analyses. Relative expression of each target gene of interest relative to *gapdh* was determined using $2^{-(\Delta\Delta Ct)}$ (Livak and Schmittgen, 2001). mRNA expression data were $\log_{10}$-transformed in order to perform parametric statistical analyses (see Statistical analyses). Six out of 370 individual mRNA expression values (LD-VEH: n = 3, SD-VEH: n = 2, LD-T_3: n = 1) were identified as outliers using Grubb's test and were omitted from subsequent analyses.

2.4. Experiment 2 - effects of T_3 on behavioral sickness responses to LPS treatment

2.4.1. Pretreatment

As in Experiment 1, on *week 0* adult male hamsters were maintained LD (n = 47) or were transferred to SD (n = 24). For the next 6 weeks, LD hamsters received daily T_3 (n = 23) or VEH (n = 24) injections; SD hamsters received daily VEH injections (n = 24). Timeline shown in Fig. 1B.

2.4.2. LPS treatments

On *week 6*, sickness responses were elicited again using LPS (*E. coli* 026:B6; 0.625 mg/kg; i.p.; as in Experiment 1). LPS and saline injections were delivered 30 min before dark onset to hamsters in all 3 pretreatment groups in a randomized, repeated-measure, counterbalanced design. The final sample sizes were: LD-VEH-LPS (n = 12), LD-VEH-SAL (n = 12), LD-T_3-LPS (n = 11), LD-T_3-SAL (n = 12), SD-VEH-LPS (n = 12), and LD-VEH-SAL (n = 12).

2.4.3. Sickness responses

On week 6 somatic and behavioral sickness responses to injections were evaluated in all hamsters using daily body and food weights obtained 15–45 min before dark onset. After 3 days of baseline measurements, hamsters were injected with either LPS or SAL, in a repeated-measures design, with successive injections separated by 10 days. To assess nest building behavior, at the time of each injection hamsters were given ~3 g squares of pre-weighed cotton batting, which were weighed again 16 h later (cf. 6 h in Experiment 1). Food and body masses were obtained daily 15–45 min before dark onset until 7 days after the 2nd LPS or SAL injection, at which point Experiment 2 was concluded.

2.4.4. Statistical analyses

In Experiment 1, food intake and body mass responses 6 h after injection (LPS or SAL) treatments and $\log_{10}$-transformed mRNA expression levels were compared among treatment groups using a 3 (pretreatment: LD-VEH, SD-VEH, LD-T_3) × 2 (immune challenge treatment: LPS, SAL) factorial ANOVA. In Experiment 2, between- and within-groups repeated-measures ANOVAs were used to evaluate longitudinal changes in food intake and body mass responses to LPS treatment. In both experiments, Kruskal-Wallis *H* and Mann-Whitney *U* tests were used to assess nesting material use among and between groups. Where warranted by significant omnibus *F* statistics, pairwise comparisons were performed using unpaired *t*-tests (Experiment 1) and Fisher's LSD tests (for longitudinal comparisons in Experiment 2). Differences were considered significant if $P \leq 0.05$.

3. Results

3.1. Experiment 1 – effects of T_3 on CNS cytokine and behavioral responses to endotoxin

3.1.1. Response to pretreatments

Photoperiod and hormone pretreatment significantly altered body mass ($F_{2,70} = 11.16$, $P < 0.001$; LD-VEH: 38.4 ± 1.1; SD-VEH: 31.8 ± 1.1; LD-T_3: 37.3 ± 1.0 [mean ± SEM]). SD-VEH hamsters weighed less than LD-VEH and LD-T_3 hamsters ($P < 0.001$, both comparisons, not illustrated), and body weights of LD-T_3 hamsters were comparable to those of LD-VEH hamsters ($P > 0.40$), immediately prior to endotoxin treatments. SD-VEH hamsters showed decreased testes mass ($P < 0.001$) relative to LD-SAL hamsters. SD-VEH hamsters showed decreased testes mass compared to LD-VEH and LD-T_3 groups ($P < 0.001$ for both comparisons). LD-VEH testes mass did not differ from the LD-T_3 group ($P > 0.50$).

3.1.2. Proinflammatory mRNA expression – cortex

LPS treatments increased *nfκb* ($F_{1,62} = 61.294$, $P < 0.001$), *il1β* ($F_{1,65} = 278.112$, $P < 0.0001$), and *tnfα* ($F_{1,60} = 133.882$, $P < 0.0001$) mRNA levels in cortex (Fig. 2). Cortical *nfκb* was greater in LPS- relative to SAL-treated hamsters in all 3 pretreatment groups ($P < 0.01$, all comparisons; Fig. 2A). LPS-induced cortical *nfκb* mRNA was lower in SD-VEH relative to LD-VEH hamsters ($P < 0.05$). LPS-induced cortical *nfκb* mRNA trended toward significance with lower expression in SD-VEH relative to LD-T_3 hamsters ($P = 0.0924$). *il1β* mRNA was also upregulated by LPS in all groups ($P < 0.001$, all comparisons; Fig. 2B). LPS-induced cortical *il1β* mRNA tended to be lower in SD-VEH relative to LD-VEH hamsters ($P = 0.0786$). Lastly, cortical *tnfα* was increased by LPS in all pretreatment groups ($P < 0.001$, all pairwise comparisons; Fig. 2C).

3.1.3. Proinflammatory mRNA expression – hypothalamus

Overall, LPS increased whole hypothalamic *nfκb* ($F_{1,56} = 4.697$, $P < 0.05$), *il1β* ($F_{1,58} = 117.64$, $P < 0.001$), and *tnfα* ($F_{1,57} = 86.13$, $P < 0.001$) mRNA expression 6 h after injection (Fig. 3). Pairwise comparisons did not identify differences in *nfκb* expression between LPS and SAL samples within any of the 3 pretreatment groups (LD-VEH: $P > 0.25$; SD-VEH: $P > 0.25$; LD-T_3: $P > 0.15$; Fig. 3A). LPS elicited significant increases in *il1β* mRNA in all 3 pretreatment groups ($P < 0.01$ vs SAL, all comparisons; Fig. 3B). LPS-induced hypothalamic *il1β* mRNA was lower in SD-VEH relative to LD-VEH and LD-T_3 hamsters ($P < 0.05$, $P < 0.01$, respectively). Lastly, *tnfα* mRNA was

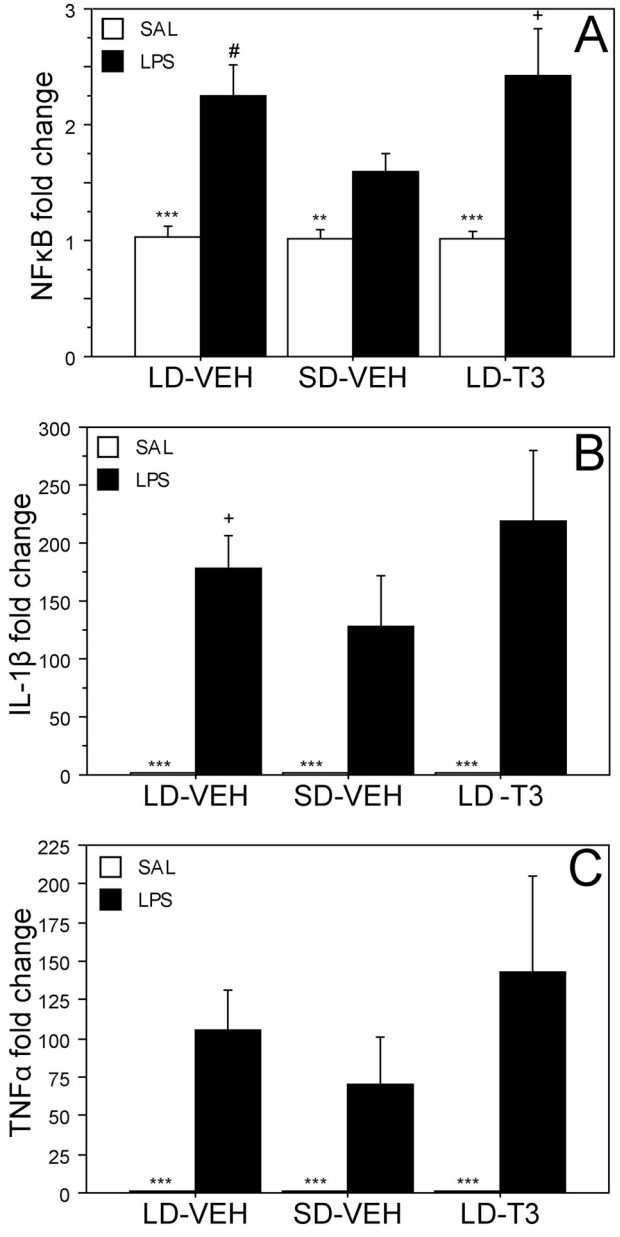

Fig. 2. Mean ± SEM cortical (A) *nfκb*, (B) *il1*β and (C) *tnfα* mRNA expression (relative to the control gene *gapdh*) 6 h after LPS (0.625 mg/kg) or saline (SAL) injection of male Siberian hamsters that were housed in a long day photoperiod and treated with saline (LD-VEH) or 1 μg T_3 (LD-T3) for 6 weeks. An additional control group was housed in a short day photoperiod and was treated with saline daily (SD-VEH). ***P < 0.001 **P < 0.01 vs LPS-treated group. #P < 0.05 vs SD-VEH LPS injected hamsters. Fig. A +P = 0.0924 vs SD-VEH LPS injected hamsters. Fig. B +P = 0.0786 vs SD-VEH LPS injected hamsters.

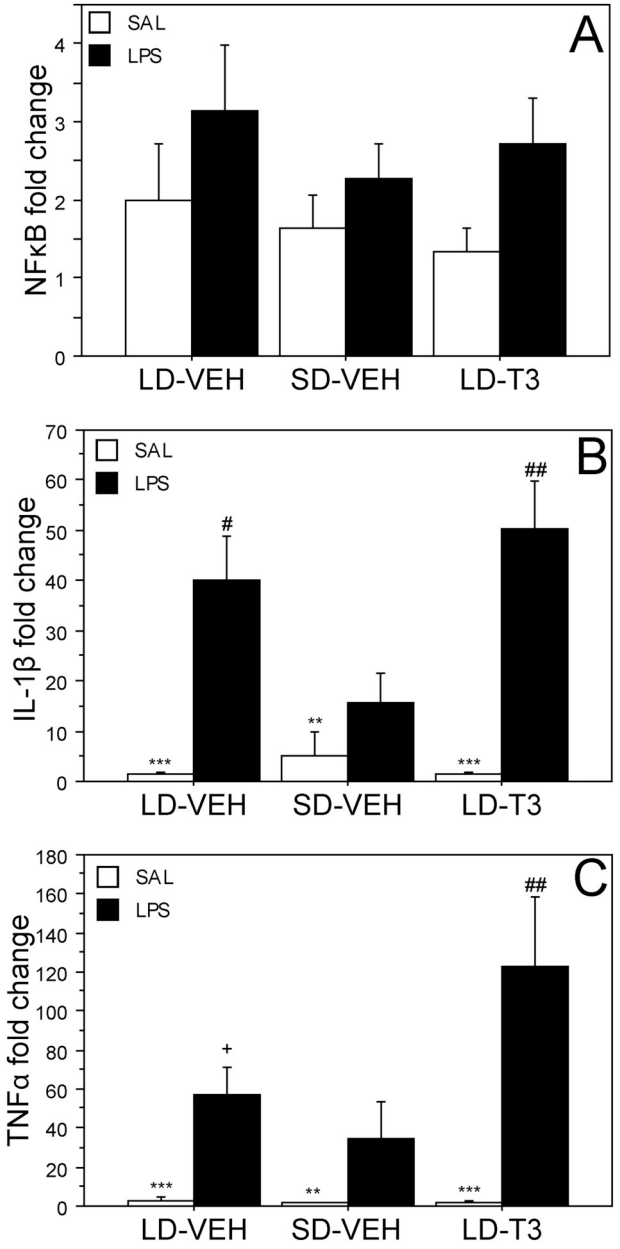

Fig. 3. Mean ± SEM hypothalamic (A) *nfκb*, (B) *il1*β and (C) *tnfα* mRNA expression (relative to the control gene *gapdh*) 6 h after LPS (0.625 mg/kg) or saline (SAL) injection of male Siberian hamsters that were housed in a long day photoperiod and treated with saline (LD-VEH) or 1 μg T_3 (LD-T3) for 6 weeks. An additional control group was housed in a short day photoperiod and was treated with saline daily (SD-VEH). ***P < 0.001 **P < 0.01 vs LPS-treated group. ##P < 0.01 #P < 0.05 vs SD-VEH LPS injected hamsters. +P = 0.059 vs SD-VEH LPS injected hamsters.

upregulated by LPS in all pretreatment groups (LD-VEH and LD-T_3: P < 0.01 vs SAL; SD-VEH: P < 0.05 vs SAL; Fig. 3C). LPS-induced *tnfα* expression was lower in SD-VEH relative LD-T_3 hamsters (P < 0.05).

3.1.4. Nest building

In the first 6 h after treatment, SAL-injected hamsters used nearly all of the nesting material provided to them (LD-VEH: 98.7 + 1.3%; SD-VEH: 97.0 = 3.0%; LD-T_3: 96.1 + 2.7%; H = 0.56, df = 2, P > 0.70; Fig. 4A). LPS suppressed nest building over the same interval, in a manner that varied markedly across pretreatment groups (H = 9.45, df = 2, P < 0.01; Fig. 4A). Relative to SAL-treated controls, LPS suppressed nest building in LD-VEH (P < 0.005) and LD-T_3 hamsters (P < 0.05) but failed to do so in SD-LPS hamsters (P > 0.90). Nesting material use after LPS treatment was greater in SD-VEH compared to LD-VEH hamsters (P < 0.005) and LD-T_3 hamsters (P < 0.01); LPS-induced nesting material use did not differ between LD-VEH and LD-T_3 hamsters (P > 0.50).

3.1.5. Food intake

Among SAL-injected hamsters food intake (during the 6 h between injection and tissue collection) did not differ significantly as a function of pretreatment (LD-VEH: 0.88 + 0.7 g; SD-VEH: 0.78 + 0.6 g; LD-T_3: 1.02 + 0.1 g; $F_{2,33}$ = 2.31, P > 0.10; not illustrated); but consistent with prior work, SD hamsters tended to eat less than LD hamsters

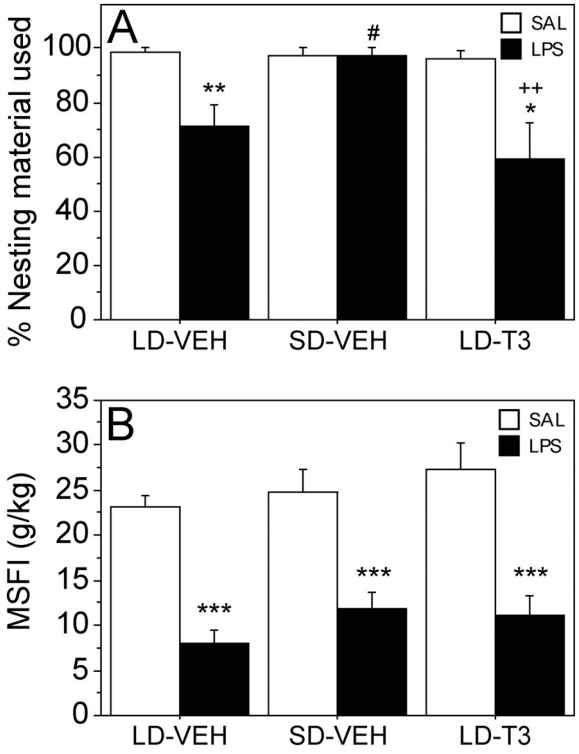

Fig. 4. Mean ± SEM (A) nest building behavior (nesting material usage) and (B) mass specific food intake during the first 6 h after LPS (0.625 mg/kg) or saline (SAL) treatment of male Siberian hamsters that were housed in a long day photoperiod and treated with saline (LD-VEH) or T_3 (LD-T3), or housed in short days and treated with saline daily (SD-VEH). *P < 0.05 **P < 0.01 ***P < 0.001 vs saline-treated group. #P < 0.05 vs LD-VEH LPS injected hamsters. ++P < 0.01 vs SD-VEH LPS injected hamsters.

(P = 0.0516). Food intake values were corrected for differences in body mass to control for week 6 differences in body weight, established photoperiodic differences in hoarding and ingestive behavior (Bartness, 1996) and empirical differences in early-scotophase food intake (present data) and are expressed as mass-specific food intake (MSFI; expressing food intake as MSFI did not affect ANOVA main effects or pairwise comparisons). LPS acutely inhibited MSFI on the night of injection ($F_{1,64}$ = 75.41, P < 0.001; Fig. 4B); this effect was evident in all pretreatment groups (P < 0.001, all comparisons; Fig. 4B); overall MSFI was 2–3 fold higher in SAL-relative to LPS-treated hamsters, but did not differ among LPS groups (P > 0.10, all comparisons).

3.1.6. Body mass

Acute immune activation ($F_{1,64}$ = 4.42, P < 0.05) and photoperiod/T_3 pretreatment ($F_{2,64}$ = 4.21, P < 0.05) independently affected changes in body mass (Fig. 5). Both LPS and SAL treatment groups lost body mass in the first 6 h after injection. The magnitude of LPS-induced body mass loss was comparable in all 3 pretreatment groups (P > 0.05, all comparisons).

3.2. Experiment 2 - effects of T_3 on behavioral responses to LPS treatment

3.2.1. Nest building

In Experiment 2, nest building responses to SAL injections were not affected by pretreatments (H = 1.12, df = 2, P > 0.50; Fig. 6A). However, the effects of LPS on nest building behavior varied across pretreatment groups (H = 8.64, df = 2, P < 0.05). Relative to SAL-treated controls, LPS suppressed nesting material use in LD-VEH (P < 0.001), LD-T_3 hamsters (P < 0.001) and SD-VEH hamsters (P < 0.005). Among LPS-treated hamsters, nest building was greater in SD-VEH compared to LD-VEH hamsters (P < 0.01) and LD-T_3 hamsters (P < 0.05); LPS-induced nesting material use did not differ between LD-VEH and LD-T_3 hamsters (P > 0.70).

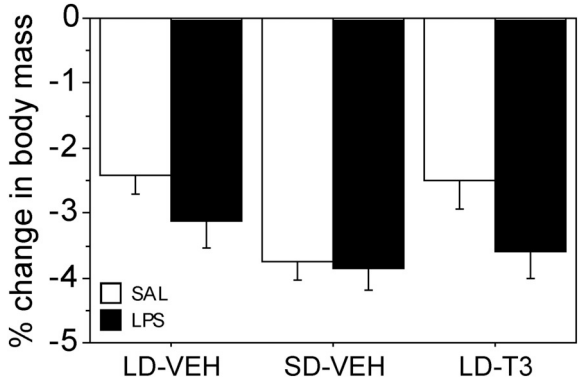

Fig. 5. Mean ± SEM percent body mass during the 6 h immediately after LPS (0.625 mg/kg) or saline (SAL) treatment of male Siberian hamsters that were housed in a long day photoperiod and treated with saline (LD-VEH) or T_3 (LD-T3), or housed in short days and treated with saline daily (SD-VEH).

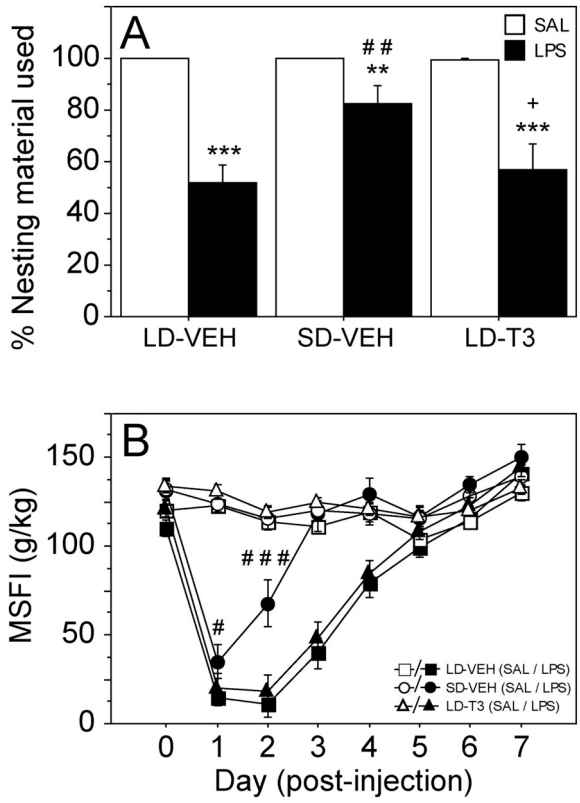

Fig. 6. Mean ± SEM (A) nest building behavior and (B) daily mass specific food intake after LPS (0.625 mg/kg) or saline (SAL) treatment of male Siberian hamsters that were housed in a long day photoperiod and treated with saline (LD-VEH) or T_3 (LD-T3), or housed in short days and treated with saline daily (SD-VEH). **P < 0.01 vs saline-treated group. ***P < 0.001 vs saline-treated group. #P < 0.05 ##P < 0.01 ###P < 0.001 vs LD-VEH LPS injected hamsters. +P < 0.05 vs SD-VEH injected hamsters.

3.2.2. Food intake

Photoperiod/hormone pretreatment altered the anorexic response to LPS treatment (pretreatment × injection: $F_{14,602}$ = 4.81, P < 0.001; Fig. 6B); this interaction was characterized by effects of photoperiod on VEH pretreated groups. LPS caused anorexia in all 3 pretreatment groups during the first 2 days (P < 0.001 vs SAL controls, all comparisons); however, in SD-VEH hamsters anorexia magnitude was reduced

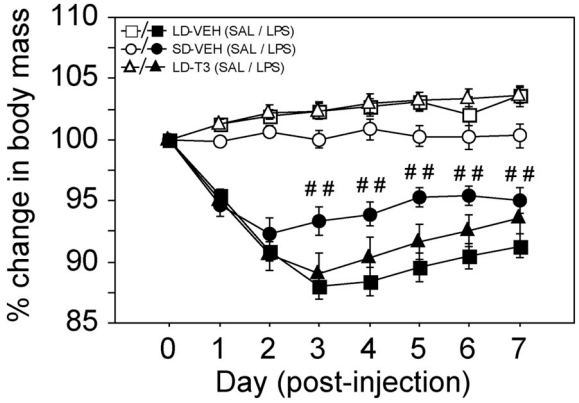

Fig. 7. Mean ± SEM percent body mass loss measured daily after LPS (0.625 mg/kg) or saline (SAL) treatment of male Siberian hamsters that were housed in a long day photoperiod and treated with saline (LD-VEH) or T_3 (LD-T_3) or housed in short days and treated with saline daily (SD-VEH). $^{\#\#}P < 0.01$ in SD-VEH relative to LD-VEH hamsters treated with LPS.

relative to LD-VEH (day +1: $P < 0.05$; day +2: $P < 0.001$) and LD-T_3 (day +2: $P < 0.001$) hamsters. LPS-induced anorexia was fully resolved in SD-VEH hamsters on day +3 ($P > 0.80$ vs SAL controls) but did not resolve in LD-VEH or LD-T_3 hamsters until day +5 ($P > 0.50$, $P > 0.20$, respectively). Anorexic responses of LD-VEH and LD-T_3 hamsters were comparable on all experimental days ($P > 0.20$, all comparisons; Fig. 6B).

3.2.3. Body mass

Pretreatment also significantly affected the pattern of change in body mass following LPS treatments (pretreatment × injection: $F_{14,602} = 5.52$, $P < 0.001$; Fig. 7). All groups lost body mass monotonically during the first 48 h after LPS treatment. Whereas SD-VEH hamsters reached body mass minima of approximately −7.5% on day +2, this nadir was not reached in LD groups until day +3 and exceeded −10%. Body mass loss due to LPS treatment was attenuated in SD-VEH relative to LD-VEH hamsters on day +3 through day +7 ($P < 0.01$, all comparisons). Body mass loss did not differ between LD-VEH and LD-T_3 hamsters at any timepoint post-injection ($P > 0.10$, all comparisons).

4. Discussion

The present data indicate that acute phase inflammatory cytokine and behavioral responses to simulated infection are attenuated by adaptation to short photoperiods in male Siberian hamsters, replicating and extending prior work (Bilbo et al., 2002a; Prendergast et al., 2003; Nelson, 2004; Navarra et al., 2007) demonstrating photoperiod-dependent modulation of sickness responses to LPS. Short days reduced hypothalamic *il1β* and cortex *nfκb* expression, and the magnitude of anorexic and cachectic responses to LPS injections relative to long day hamsters pretreated with vehicle. In the present work, T_3 treatments did not reduce the acute induction of *nfκb*, *il1β* or *tnfα* in the cortex nor hypothalamus and did not mitigate the effects of LPS on thermoregulatory or ingestive behavior. Whereas previous experiments show that T_3 mimicked the effects of SD on blood leukocyte concentration (Stevenson et al., 2014; Banks et al., 2016) and adaptive skin inflammatory responses (Stevenson et al., 2014), the present data reveal a different role for T_3 in mediating photoperiodic changes in the response to innate immune challenges. Prior reports indicate T_3 decreases proinflammatory activities of monocytes and macrophages in mice (De Vito et al., 2011). Additionally, T_3 protects mice against lethal doses of LPS-induced endotoxemia (Perrotta et al., 2014).

Many seasonally-breeding species modulate immune responses over the annual cycle (Nelson, 2004; Stevenson and Prendergast, 2015). Male Siberian hamsters housed in short days for 8–12 weeks increase blood leukocyte concentrations (Bilbo et al., 2002a; Prendergast et al., 2008; 2008; Stevenson et al., 2014). The increase in leukocytes can be attributed to an increase in lymphocytes (T-cells and natural killer cells), as B-cells, neutrophils and monocytes remain constant (Bilbo et al., 2002a). The CNS and the immune system interact to generate seasonal rhythms in immune responses. For example, seasonal changes in sickness behavior, which are of lower magnitude in the winter months, are driven by decreases in cytokine responses to inflammogens (Bilbo et al., 2002b; Prendergast et al., 2003) and by photoperiodic decreases in behavioral responsiveness to cytokines (Wen and Prendergast, 2007). In the present study, the activation of CNS proinflammatory pathways was affected by photoperiod and T_3 in a site- and molecule-specific manner. In cortex *nfκb* responses to LPS were reduced by adaptation to SD, while remaining similar in the hypothalamus. This result prompts the conjecture that photoperiodic modulation of CNS cytokine production may occur downstream of NFκB, dependent on the brain region. However, additional empirical evidence would be required to support such a conjecture. For example, qPCR approaches used in the present work allow for quantification of mRNA, but are obtained from non-homogenous brain dissections (i.e., cortex dissections likely include motor cortex, anterior cingulate area, frontal cortex). Whole hypothalamus dissections likewise contain many nuclei, each with different roles in homeostasis. Additionally, the single, 6 h, sampling interval used here may have missed differences in *nfκb*, induced by pretreatment or LPS. Also, other mechanisms that regulate CNS IL-1β and TNFα expression, e.g., p38 MAPK (Huang et al., 2011) may be a mechanism of photoperiodic modulation. Lastly, we examined only one dose of T_3 in the present work, and other studies in male hamsters have suggested dose-response effects of T_3 on immunity (Stevenson et al., 2014). Previous studies have identified photoperiod effects on constitutive *nfκb* expression in the cells lining the caudal region of the hypothalamic third ventricle of F344 rats (Stoney et al., 2017). Here we identified *nfκb* mRNA expression as greater in LD relative to SD hamsters; however, differences in species, measurement techniques, and/or tissues analyzed, may account for these different observations.

T_3 was considerably less effective than SD in attenuating the innate immune response (CNS cytokine expression; Figs. 2 & 3) compared to previous reports of its effects on adaptive immunity. In light of the complex role of T_3 in immunobiology (see review De Vito et al., 2011), it is possible that innate and adaptive immune processes in Siberian hamsters are influenced differently by T_3. In LD-T_3 hamsters, *nfκb* responses to LPS were similar to those exhibited by LD-VEH hamsters. Cortical and hypothalamic *il1β* responses were likewise similar in VEH and T_3-treated hamsters housed in LD. Where the LD-VEH hamsters showed a trending increase in hypothalamic *tnfα* relative to SD-VEH when treated with LPS, LD-T_3 showed a significant upregulation compared to SD-VEH hamsters (Fig. 3C). In parallel with previous reports of T_3 on spleen *il1β* expression (Banks et al., 2016), the present data support the hypothesis that T_3 has cell- or tissue-specific effects. Despite this difference in cytokine expression, acute thermoregulatory and ingestive behavior (Fig. 4) and longer-term ingestive behavior (Fig. 6) were unaffected by T_3 treatments. Although it remains possible that longer intervals of T_3 treatment, or different dose regimens of T_3, might be sufficient to induce the SD phenotype in these features of the innate immune response. Interestingly, LD-T_3 hamsters showed upregulation of hypothalamic *tnfα* compared to SD hamsters. Previously published data show that T_3 treatments are sufficient to mimic effects of SD on adaptive and constitutive immune responses (Stevenson et al., 2014 & Banks et al., 2016) but are inadequate to elicit SD-like responses in innate inflammatory responses to LPS. Cell-specific regulation of *dio3* expression by photoperiod may allow tissue-specific flexibility in the direction of change in thyroid hormone signaling to allow seasonally-appropriate changes in reproductive and immune function to occur concurrently in response to a common photoperiodic cue (Stevenson et al., 2014). Banks et al. (2016) showed that T_3 treatment in SD

attenuated or exaggerated splenic *il1β* and *tnfα* mRNA levels, respectively. Absent a SD-T_3 group, the present report does permit insights into whether increased T_3 might be sufficient to promote a LD innate immune phenotype in SD Siberian hamsters. Exogenous T_3 appears to influence the immune system in a trait-specific manner.

The mechanisms for photoperiodic immunoenhancement have not been fully elaborated, but may be driven by local thyroid hormone signaling. Seasonal changes in the neuroendocrine control of reproduction involves genomic (Barrett et al., 2007; Yoshimura et al., 2003; Prendergast et al., 2002; Webster et al., 1991; Yasuo et al., 2006; Stevenson et al., 2012) and epigenomic (Stevenson and Prendergast, 2013) switches in the hypothalamus that result in greater T_3 concentration in LD compared to SD hamsters. Peripheral blood leukocytes exhibit photoperiodic changes in *dio3* expression which lead to increased cytoplasmic concentrations of T_3 in SD. Circulating leukocytes are also more numerous in SD as compared to LD. Peripheral injections of 1 μg/day T_3 to LD housed hamsters for 6 weeks mimicked the effects of SD on blood leukocyte concentrations and on adaptive immunological delayed-type hypersensitive responses (Stevenson et al., 2014), pointing to thyroid hormone involvement in seasonal variations in immune function. Here, we show that this 1 μg/day dose of T_3, provided for 6 weeks, fails to significantly attenuate many photoperiod-dependent sequelae of the innate immune challenge with LPS. The artificially-elevated circulating T_3 concentrations in hamsters in the present study may not be sufficient to overcome photoperiod-dependent regulation of acute bacterial sickness responses. The influence of T_3 on innate immune responses in SD hamsters was not examined in this study, as we hypothesized that T_3 administration in innate immune response would mimic its effects in SD. A higher T_3 dose may be required to induce the SD phenotype in the innate responses to LPS.

In summary, exposure to SD attenuated neural cytokine and long-term behavioral sickness responses to a simulated bacterial infection. In contrast to the effects of T_3 on adaptive immunity, exogenous T_3 failed to mimic the effects of SD on cytokine expression with only modest sickness responses following LPS administration. A limitation of the work is that experiments only examined a single dose of T_3. We conclude that T_3 may play only a minor role in imparting the effects of SD onto innate immune responses. However, innate and adaptive immune responses in male hamsters may also differ in responsiveness to T_3.

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