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ORIGINAL PAPER

The glutamate agonist NMDA blocks gonadal regression and enhances antibody response to an immune challenge in Siberian hamsters (*Phodopus sungorus*)

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Abstract Seasonal variation in behavior and physiology, including changes in immune function, are common. This variability is elicited by changes in photoperiod and often covaries with fluctuations in both energy reserves and reproductive state. It is unclear, however, whether changes in either variable alone drive seasonal changes in immunity. We investigated the relative contributions of reproduction and energy balance to changes in immune function. To accomplish this, we uncoupled seasonal changes in reproduction from those related to energy balance via daily injections of N-methyl-D-aspartate (NMDA) in Siberian hamsters (*Phodopus sungorus*). NMDA is a glutamatergic agonist that blocks short day-induced gonadal regression, while leaving short-day declines in body mass unaffected. In Experiment 1, we examined the effect of differing doses of NMDA on testosterone production as a proxy for NMDA effects on reproduction; a dose-dependent rise in testosterone was observed. In Experiment 2, animals were maintained on long or short days and received daily injections of NMDA. After 8 weeks, all animals underwent a humoral immune challenge. Short-day animals receiving daily injections of NMDA maintained long day-like gonads; however, contrary to our predictions, no trade-off between reproduction or energy balance and immune function was observed. Unexpectedly, NMDA treatment increased immunoglobulin levels in all groups, suggesting that NMDA may provide an immunomodulatory signal, presumably through actions on peripheral glutamate receptors. These results support a previous finding that NMDA blocks reproductive regression. In addition, these findings demonstrate a general immunoenhancing effect of NMDA that appears independent of changes in reproductive or energetic state of the animal.

Keywords Energy trade-off · Photoperiod · Seasonal reproduction

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Introduction

Most animals display marked seasonal variation in physiology, morphology and behavior. In the temperate zone, these changes are triggered in response to changes in the ambient photoperiod (for reviews see Dawson et al. 2001; Goldman 2001). Seasonal responses are considered to be adaptations that allow animals to optimally time breeding cycles during periods of sufficient energetic availability, thus enhancing reproductive success and survival (Baker 1938; Bronson 1989). For example, many temperate breeding rodents encounter reduced resources and high thermoregulatory demands during the winter. In turn, these

animals restrict breeding to the long days of spring and summer, thereby enhancing offspring success and parental survival (Bronson 1989).

Immune function also varies in response to photoperiod in a number of species (reviewed in Martin et al. 2008; Nelson 2004; Nelson and Demas 1996). These responses are complex; specific aspects of immunity are affected differentially in response to changes in photoperiod. For example, in the photoperiodic Siberian hamster (*Phodopus sungorus*), a reduction in antibody response to antigens is observed in animals housed in short winter-like days (Demas 2002; Demas et al. 2002; Drazen et al. 2001; Drazen et al. 2000; Yellon et al. 1999a), whereas measures of cell-mediated immunity are enhanced in short-day housed Siberian hamsters compared with those housed in long days (Bilbo et al. 2002; Prendergast et al. 2008; Yellon et al. 1999a). Although a robust antibody response to a potential pathogen enables individuals to fight off potentially deadly infections, mounting an antibody response is energetically costly (Demas et al. 1997; Martin et al. 2003). When resources are scarce or energy reserves are low, as is the case during temperate zone winters, survival may be compromised if an individual allocates already limited resources to other costly processes, such as reproduction, thereby limiting the resources available for adequate immune responses. Maintenance of reproductive functionality during periods of decreased energy availability should lead to trade-offs with other energetically costly functions, including immunity. In fact, such trade-offs have been observed; in birds, experimentally increasing clutch size leads to a reduced antibody response, and in tree lizards, vitellogenic females display decreased wound-healing capabilities (Cichon et al. 2001; Deerenberg 1997; French et al. 2007).

The goal of the current investigation was to experimentally test whether such a trade-off exists by manipulating the reproductive status of male Siberian hamsters, which display natural decreases in food intake and energy reserves (e.g., fat) and undergo reproductive regression when held under winter-like photoperiods (Bartness and Wade 1985; Goldman 2001; Hoffmann 1973), and performing an immune challenge on these individuals. We sought to take advantage of these natural photoperiod-induced changes in food intake, body mass and fat stores to experimentally dissociate reproductive and energy status by providing daily administration of the glutamate agonist N-methyl-D-aspartate (NMDA) to male Siberian hamsters (*Phodopus sungorus*) housed either on winter-like short days or summer-like long days. Glutamate, acting on the NMDA sub-type of glutamatergic receptors, stimulates gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus; activation of these receptors plays a modulatory role in the activity of the reproductive neuroendocrine axis (reviewed in (Brann and Mahesh 1997). Furthermore, a previous study in Siberian hamsters demonstrated that NMDA blocked gonadal regression, presumably via stimulation of GnRH release, in short-day housed animals without altering other phenotypic changes (e.g., body mass) associated with short days (Ebling et al. 1995). Importantly, NMDA does not block short day-induced reductions in body mass and presumably body fat (Ebling et al. 1995).

In Experiment 1, we demonstrated a dose-dependent rise in testosterone in response to NMDA, complementing a previous report in this species of NMDA-induced elevation of serum LH (Ebling et al. 1995). In Experiment 2, we attempted to uncouple reproductive and energetic states to assess the relative contribution of reproductive versus energetic status in humoral and innate immune responses. We confirmed previous findings that daily injections of NMDA block photoperiod-induced gonadal regression, while leaving the typical reductions in body mass unaffected (Ebling et al. 1995). In addition, we also measured antibody production in response to antigenic challenge and ex vivo bacterial killing ability. We predicted that the resulting energetic bottleneck caused by a photoperiod-induced reduction in energy reserves, combined with energy directed toward maintaining reproductive function, would exacerbate short-day decreases in immune function observed in previous studies (Demas 2002; Demas et al. 2002; Drazen et al. 2001; Drazen et al. 2000; Yellon et al. 1999a). In contrast to our predictions, NMDA enhanced immune responses in both long- and short-day hamsters, suggesting that NMDA, likely acting on glutamate receptors, enhances immunity irrespective of reproductive or energetic state in Siberian hamsters, and likely other species.

Methods

Animals and housing

Adult (>60 days old) male Siberian hamsters (*Phodopus sungorus*) were obtained from our breeding colony at Indiana University. The progenitors of these animals were generously provided by Dr. Randy Nelson (Ohio State University) and Dr. Timothy Bartness (Georgia State University). All animals were initially group housed (2–4 with same sex siblings on weaning at 18–21 days of age) in long-day photoperiods (light:dark, 16:8). Temperature (20 ± 2°C) and humidity (50 ± 10%) were maintained at constant levels. Animals in Experiment 1 ($n = 63$) were maintained on long days (16:8) and individually housed in polypropylene cages (27.8 × 17.5 × 13.0 cm). Animals in Experiment 2 were randomly assigned to one of two photoperiodic conditions: long day (light:dark, 16:8, $n = 19$)

or short day (light:dark, 8:16, $n = 36$), housed individually in polypropylene cages (27.8 × 17.5 × 13.0 cm) and weighed. Siberian hamsters display voluntary reductions in food intake in response to short-day photoperiods, leading to marked reductions (up to 40%) in body mass and available energy (i.e., fat) stores compared to animals held in long-day photoperiods (Bartness et al. 1989; Bartness and Wade 1985; Mercer et al. 2001; Reddy et al. 1999; Steinlechner et al. 1983). Thus, although all animals were given ad libitum access to food (PMI LabDiet 5012, Rat Diet, St. Louis, MO) and tap water throughout the study, animals held in short-day photoperiods displayed a reduction in energy consumption and body mass similar to reductions observed in animals held in long days under a restricted diet (Mercer et al. 2001). All animals were treated in accordance with the Bloomington Institutional Animal Care and Use Committee (BIACUC).

Experiment 1: effect of NMDA on testosterone secretion

Experimental treatments

To determine the optimal dose of NMDA necessary to induce activation of the hypothalamo-pituitary–gonadal (HPG) axis, gonadal sex steroid secretion was measured in long-day housed males that received a single subcutaneous (s.c.) injection of 10 ($n = 17$), 20 ($n = 14$) or 40 mg/kg ($n = 16$) NMDA (#M3262, Sigma Chemical, St. Louis, MO) dissolved in 0.1 M phosphate buffer (PB) or PB vehicle alone ($n = 16$). At 15 min after the injection (Ebling et al. 1995), hamsters were lightly anesthetized under isoflurane vapors (#NDC 100190773-40, Baxter, Deerfield, IL) and a small (~150 µl) blood sample was obtained from the retro-orbital sinus. Samples were allowed to clot for 1 h, the clots were removed, and the samples centrifuged (at 4°C) for 30 min at 5,000*g*. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at −80°C until assayed for testosterone. All samples were collected between 1,000 and 1,200 h. This experiment consisted of two cohorts [cohort 1 ($n = 31$); cohort 2 ($n = 32$)]. Each injection treatment was represented in both cohorts. Cohorts did not differ in post-injection testosterone titers and were combined for all subsequent analyses.

Assessment of circulating testosterone titers

Serum testosterone was assessed using a commercial EIA kit (Correlate-EIA kit #900-065; Assay Designs, Ann Arbor, MI). Samples were diluted to 1:20 and run in duplicate. Samples were run on two plates, with all samples from the same cohort run on the same plate. The sensitivity of the assay was 3.82 pg/ml. The inter-assay coefficient of variation (CV) was less than 15%; individual samples that had CVs greater than 15% were excluded from subsequent analysis. The intra-assay coefficient of variation was 3.5%.

Experiment 2: effects of daily NMDA injections on reproduction, body mass and immune function

Experimental treatments

Animals were housed in either long- (L:D 16:8) or short-day photoperiods (8:16). All animals received daily s.c. injections of either NMDA (20 mg/kg) suspended in 0.1 ml of phosphate buffer, or phosphate buffer (0.1 ml) with no NMDA, yielding four distinct treatment groups: long-day NMDA injected ($n = 9$); long-day vehicle injected ($n = 10$); short-day NMDA injected ($n = 18$); and short-day vehicle-injected ($n = 18$). The dose of NMDA (20 mg/kg) was chosen primarily based on its effectiveness to significantly activate the HPG axis in our animals, as demonstrated by elevated testosterone levels (Experiment 1, see "Results") as well as from previous findings (Ebling et al. (1995), indicating that this dose significantly elevated the levels of luteinizing hormone.

After 8 weeks of daily injections, all hamsters received a single s.c. injection of 100 µg of the antigen keyhole limpet hemocyanin (KLH, #374805, Calbiochem, EMD Biosciences, Gibbstown, NJ) (suspended in 0.1 ml sterile saline solution), to which all animals were previously naive. KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Megathura crenulata*). KLH was used because it generates a robust antigenic response in rodents, but does not make the animals sick (e.g., prolonged inflammation or fever) (Demas 2002; Dixon et al. 1966). Body mass was measured initially upon individual housing, prior to injection and photoperiod treatments, and then weekly throughout the experiment. Body mass was important to assess both photoperiodic condition of the animal (i.e., short-day animals are significantly smaller than long-day animals), and the health and condition of animals within a photoperiod.

Blood sampling

On days 5 and 10 post-KLH injection, a blood sample was taken via the retro-orbital sinus, to measure serum concentrations of KLH-specific antibodies. Days 5 and 10 incorporate the peak rises in immunoglobulins, IgM and IgG, respectively. IgM is the initial immunoglobulin response following an immune challenge, and IgG is the principal circulating immunoglobulin throughout an immune response (Demas et al. 1997; Drazen et al. 2000).

On the day of sampling, animals were brought into the surgery room, lightly anesthetized with isoflurane vapors, and blood samples were drawn from the retro-orbital sinus between 1,000 and 1,200 h. Samples were allowed to clot for 1 h, the clots were removed, and the samples centrifuged (at 4°C) for 30 min at 5,000*g*. Serum aliquots were aspirated and stored in sealable polypropylene microcentrifuge tubes at −80°C until assayed for IgM and IgG.

Necropsies and assessment of adipose tissues

On day 10 after the final bleed, animals were euthanized and necropsies were performed to assess paired testes and fat pad mass. Testes mass was important both to assess the reproductive condition and also the effectiveness of NMDA treatment, where short-day NMDA animals were predicted to have large, non-regressed testes. Fat stores are also an important indicator of reproductive condition where short-day animals are non-reproductive, have smaller gonads and have smaller fat stores than long-day reproductive animals. We removed epididymal white adipose tissue (EWAT), retroperitoneal WAT (RWAT) and inguinal WAT (IWAT) pads from all hamsters. All tissues were cleaned of connective tissue and weighed to the nearest 0.1 mg.

Assessment of humoral immunity

To assess humoral immunity, serum anti-KLH IgM and IgG concentrations were assayed using an enzyme-linked immunosorbent assay (ELISA) as previously described (Demas et al. 2003). Specifically, microtiter plates were coated with antigen by incubating overnight at 4°C with 0.5 mg/ml KLH in sodium bicarbonate buffer (pH 9.6). Plates were then washed with phosphate-buffered saline (PBS; pH 7.4) containing 0.05% Tween 20 (PBS-T; pH 7.4) and blocked with 5% non-fat dry milk (Mix'n Drink, Saco Foods, Middleon, WI, USA) in PBS-T overnight at 4°C to reduce non-specific binding, and then washed again with PBS-T. Serum samples collected from animals 5 and 10 days after KLH injections (see "Blood Sampling") were thawed and diluted to 1:20 in PBS-T. As much as 150 µl aliquots of this serum dilution were added in duplicate to the wells of the antigen-coated plates. Positive control samples (pooled sera from hamsters previously determined to have high levels of anti-KLH antibody, similarly diluted with PBS-T) and negative control samples (pooled sera from KLH-naive hamsters, similarly diluted with PBS-T) were also added in duplicate to each plate. The plates were sealed, incubated at 37°C for 3 h, then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated anti-hamster IgG; #707405-002, Rockland, Gilbertsville, PA, and anti-mouse IgM, #59295 Cappel, Durham, NC, diluted 1:500 with PBS-T) was added to the wells, and the plates were sealed and incubated for 1 h at 37°C. Plates were washed again with PBS-T and 150 µl of the enzyme substrate p-nitrophenyl phosphate (#N9389, Sigma Chemical, St Louis, MO; 1 mg/ml in diethanolamine substrate buffer) was added to each well. The plates were protected from light during the enzyme–substrate reaction. The optical density (OD) of each well was determined using a plate reader (Bio-Rad, Benchmark; Richmond, CA) equipped with a 405-nm wavelength filter and the mean OD for each set of duplicate wells was calculated. To minimize intra-assay variability, the mean OD for each sample was expressed as a percentage of its plate positive control OD for statistical analyses.

Assessment of innate immunity

The ability of the innate immune system to neutralize Gram-negative bacteria was assessed via an ex vivo bactericidal assay. All procedures were modified from (Tieleman et al. 2005) and performed in a sterile laminar flow hood. In short, a bacterial stock solution was prepared by adding one pellet of lyophilized *Escherichia coli* ($E^{powerTM}$ Microorganisms #0483E7, ATCC 8739, MicroBioLogics, St. Cloud, MN) to 40 ml of 1 M sterile PBS. The solution was activated via incubation at 37°C for 30 min. Immediately following incubation, the bacterial working solution was prepared by diluting 2 ml of the stock solution into 8 ml 1 M PBS. Meanwhile, serum samples were diluted to 1:20 in CO_2-independent media (Gibco #18045, Carlsbad, GA) containing 2.34 mg of L-glutamine (Sigma–Aldrich). To each diluted sample, 20 µl of the bacterial working solution was added and the mixture was allowed to incubate at 37°C for 30 min to induce bacterial killing. After incubation, 50 µl of each sample was added to tryptic soy agar plates in duplicate. All plates were covered and left to incubate upside down overnight at 37°C. Colony numbers were counted and bactericidal capacity was calculated as the mean number of colonies for each sample divided by the mean of colonies on control plates (containing only media and bacterial solution) and expressed as percentage of bacteria killed relative to the control.

Statistical analyses

Experiment 1

Differences in the testosterone response to vehicle injection or an injection with 10, 20 or 40 mg/kg NMDA were determined using a one-way analysis of variance (ANOVA). Pairwise comparisons were probed using Fisher's LSD tests. To meet the assumptions of normality

for parametric statistics, all testosterone values were log-transformed prior to analysis.

Experiment 2

A polymorphism in responsiveness to short-day photoperiods (e.g., reproductive condition, body mass, pelage coloration) has been documented in a laboratory population of Siberian hamsters. Specifically, reproductive non-responders fail to respond to photoperiodic information and remain reproductively active despite exposure to short days (Kliman and Lynch 1992; Lynch and Lynch 1986; Prendergast et al. 2001); the remaining animals (i.e., responders), in contrast, display the typical gonadal regression in response to short days. Additionally, animals responsive to short days display a dramatic decline in body mass (typically a decrease of >10% long-day values) (Bartness and Wade 1985; Hoffmann 1973) and molt their breeding season pelage and replace it with a thicker, whiter fur; reproductive non-responders maintain their breeding season body mass and pelage. Pelage coloration and change in body mass was noted throughout the duration of the experiment. Of the short-day animals, ten NMDA treated animals and ten vehicle-treated controls were deemed short-day non-responders because they did not display a reduction in body mass (>10% reduction) or typical changes in pelage coloration and were excluded from subsequent analyses (non-responder NMDA $n = 10$, non-responder vehicle $n = 10$). Resultant sample sizes for each group were: long-day NMDA $n = 9$, long-day vehicle $n = 10$, short-day NMDA $n = 6$, short-day vehicle $n = 8$.

Differences among all dependent measures were determined using separate two-way ANOVAs (photoperiod x injection treatment). Post hoc comparisons between pairwise means were conducted using Fisher's LSD tests when the overall ANOVAs were statistically significant. Paired testis mass data were log-transformed prior to analysis to meet assumptions of parametric statistics. In all cases, differences between group means were considered statistically significant if $P \leq 0.05$. All analyses were performed on JMP 7.0.1 (SAS Institute Inc., Cary, NC, USA) for Windows.

Results

Experiment 1

Serum testosterone

The injection dose of NMDA significantly altered circulating levels of serum testosterone ($F_{3,63} = 3.52$, $P = 0.02$). Specifically, animals injected with 20 and 40 mg/kg NMDA had significantly elevated levels of serum testosterone compared with vehicle-injected animals ($P < 0.05$) (Fig. 1). Animals injected with 10 mg/kg NMDA displayed testosterone levels intermediate to vehicle-injected animals, and testosterone levels in animals injected with either 20 or 40 mg/kg NMDA did not significantly differ from any other treatment ($P > 0.05$) (Fig. 1).

Experiment 2

Body mass

There was a significant effect of photoperiod on final body mass, such that short-day animals were significantly smaller than long-day animals at the end of the study ($F_{1,31} = 7.20$, $P = 0.01$; Fig. 2). There was no effect of NMDA treatment and no interaction between NMDA treatment and photoperiod (all $F < 1.68$, all $P > 0.20$).

Testes and fat mass

Neither photoperiod nor NMDA treatment significantly affected the overall final paired testes mass (Photo $F_{1,29} = 3.87$, $P = 0.06$; NMDA $F_{1,29} = 3.43$, $P = 0.07$; Fig. 2). However, there was a significant interaction between the two treatments ($F_{1,29} = 76.29$, $P < 0.01$). According to post hoc comparisons, short-day control animals treated daily with vehicle have significantly smaller paired testes mass than all other groups: long-day NMDA, long-day vehicle, and short-day NMDA. Therefore, NMDA treatment was effective in maintaining larger

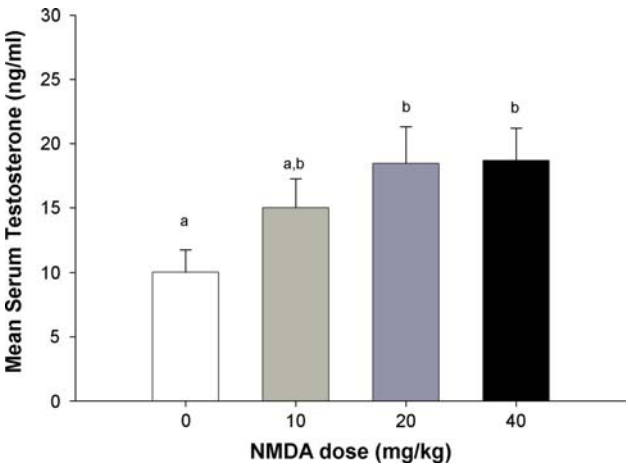

Fig. 1 Effects of NMDA treatment on the reproductive axis: NMDA activates the HPG axis, stimulating elevations in testosterone in a dose-dependent manner. Hamsters receiving either 20 or 40 mg/kg of NMDA subcutaneously had significantly elevated testosterone levels compared with vehicle-injected animals. *Different letters* denote groups that differ significantly ($P < 0.05$)

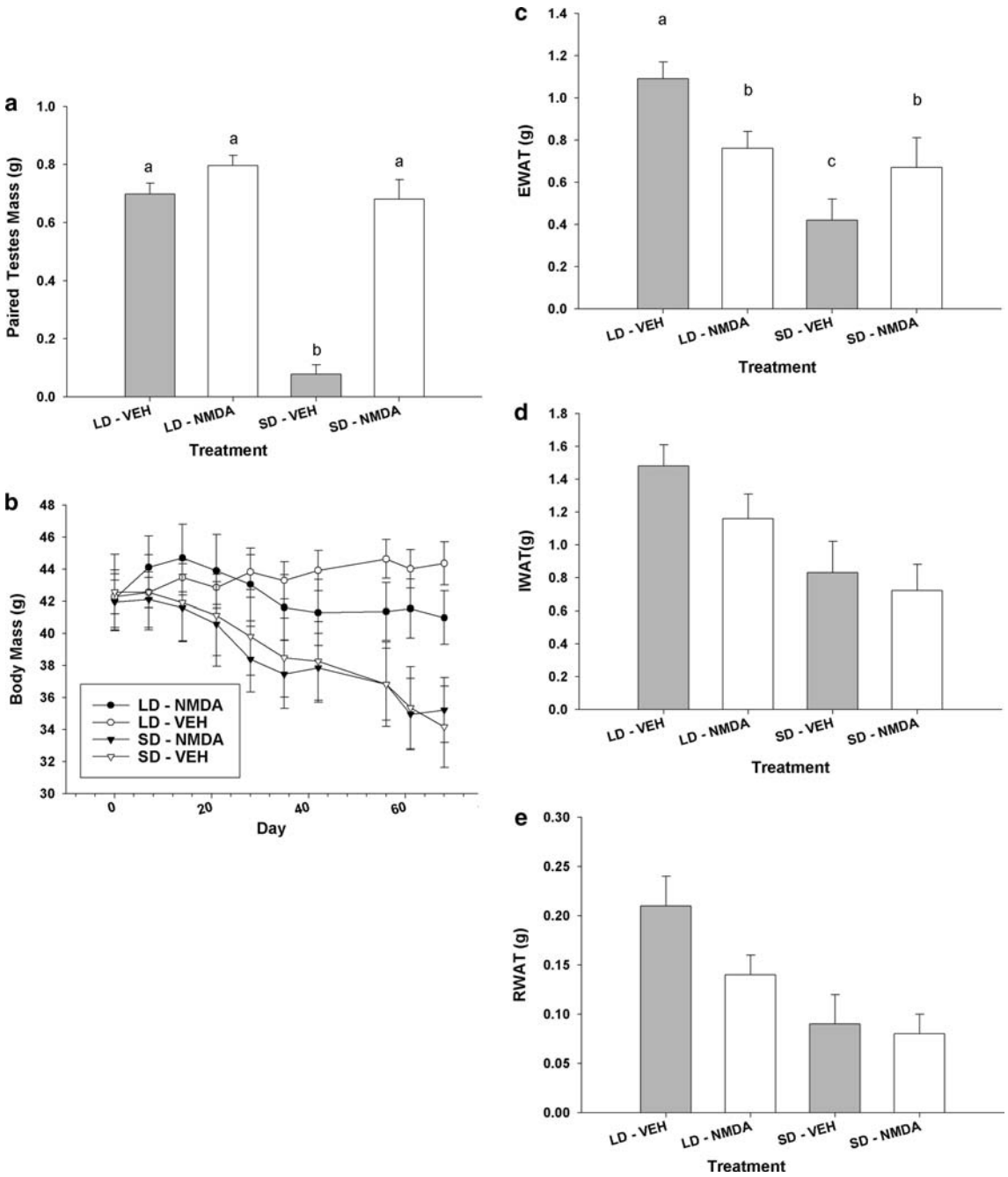

Fig. 2 Effects of NMDA and photoperiod-induced morphology: NMDA led to long day-like paired testes mass in short-day animals (**a**), while NMDA did not alter typical short day-induced reductions in body mass (**b**). Interestingly, the fat pad surrounding the gonads (the epididymal white adipose tissue [EWAT]) of short-day NMDA treated animals were larger than short-day controls, presumably due to the enlarged gonads (**c**). Non-gonadal fat stores were not affected by NMDA treatment; no significant differences were observed in either retroperitoneal (RWAT) (**d**) or inguinal white adipose tissue (IWAT) (**e**). *Different letters* denote groups that differ significantly ($P < 0.05$)

gonads in short-day animals that otherwise responded to photoperiod treatment (i.e., significantly decreased body mass) (Fig. 2).

Final epididymal white adipose tissue (EWAT) pad mass was significantly affected by NMDA treatment (NMDA $F_{1,29} = 6.68$, $P = 0.01$; Fig. 2), and the effects of NMDA treatment differed according to photoperiod (photo × NMDA $F_{1,29} = 8.54$, $P < 0.01$). According to post hoc comparisons, long-day control animals had the largest fat stores, followed by long-day and short-day NMDA-treated animals. Finally, short-day vehicle-treated animals had the smallest fat stores. There was no overall

effect of photoperiod on EWAT (photo $F_{1,29} = 0.42$, $P = 0.52$). Likewise, there was no significant effect of photoperiod, NMDA treatment or interaction for either final retroperitoneal WAT (RWAT) or final inguinal WAT (IWAT) pads (all $F < 3.41$, all $P > 0.08$; Fig. 2).

Humoral immunity

NMDA treatment significantly affected both circulating IgM ($F_{1,29} = 4.58$, $P = 0.04$; Fig. 3) and IgG ($F_{1,29} = 4.12$, $P = 0.05$; Fig. 3) levels. Post hoc comparisons revealed that IgM and IgG levels were both elevated in NMDA-treated animals relative to vehicle-treated controls. There was no significant effect of photoperiod or NMDA treatment by photoperiod interaction for either IgM or IgG (all $F < 0.43$, all $P > 0.52$).

Serum bacterial killing capacity

There was no significant effect of NMDA treatment, photoperiod or an interaction with bacterial killing (all $F < 0.02$, all $P > 0.88$; Fig. 3).

Discussion

The goal of the current study was to examine energetic trade-offs between reproduction and immune function in a seasonally breeding rodent, the Siberian hamster. A previous report indicated that short-day housed hamsters receiving daily injections of NMDA elevate circulating levels of LH and maintain breeding season-like gonads, while displaying a short day-like decrease in body mass (Ebling et al. 1995). Based on these findings, we predicted that NMDA would induce a dose-dependent rise in serum testosterone and that, similar to the previous report, short-day housed hamsters receiving NMDA would maintain long day-like reproductive status while displaying the typical short-day reduction in energy reserves (e.g., reduced body fat and body mass). Indeed, hamsters housed in short-day photoperiods provided with daily NMDA injections displayed long day-like gonadal morphology and short day-like reductions in body mass; short day-like reductions in fat pad mass was also observed in the current study. Further, we predicted short-day hamsters would have a reduced immune response compared with controls, owing to an energetic trade-off between reproduction and immune function. This predicted reduction in immunity, would be driven by short day-induced decreases in energy reserves (e.g., fat stores). Contrary to these predictions, however, NMDA treatment led to greater antibody production in response to KLH, regardless of photoperiodic treatment. These findings suggest that NMDA enhances immune responses and that this immunoenhancement appears to be independent of changes in energetic state.

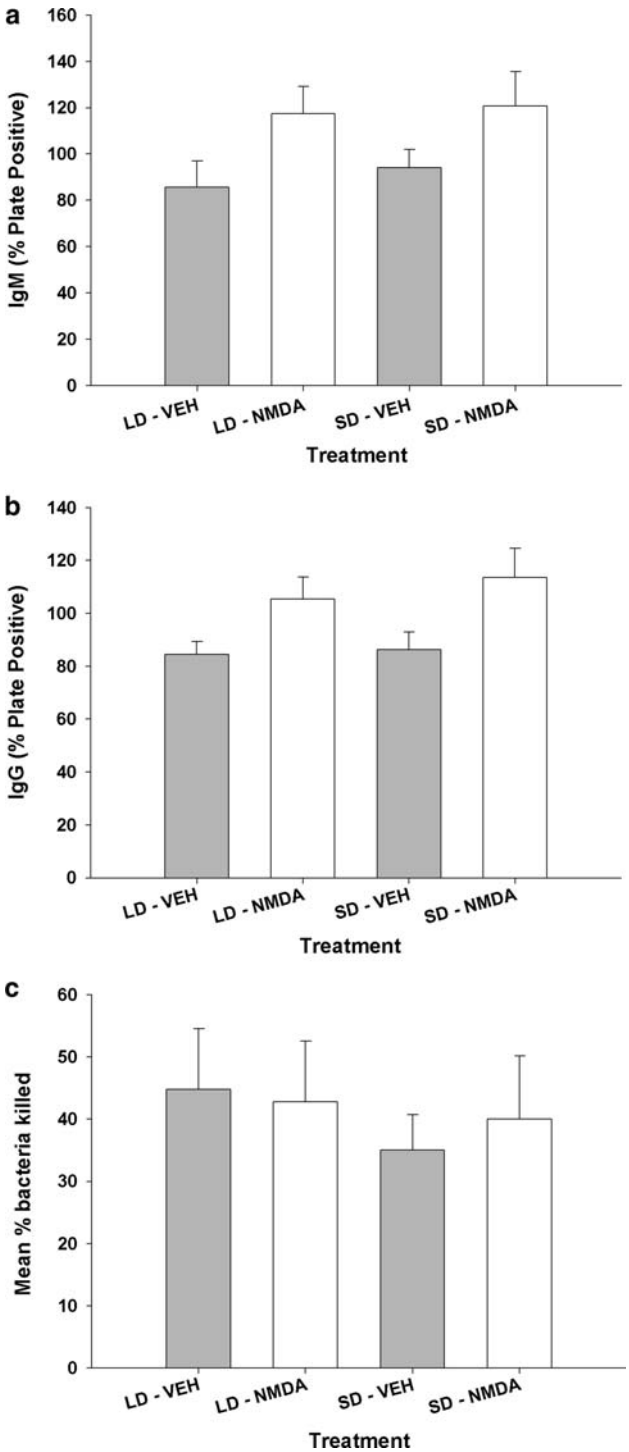

Fig. 3 Effects of NMDA and photoperiod treatment on immune responses: NMDA injections significantly elevated both anti-KLH IgM (**a**) and IgG (**b**) production compared with animals that received vehicle injections. This was observed regardless of photoperiod treatment. No effect of treatment was observed in the capability of serum to kill *E. coli* bacteria in vitro (**c**)

Mature reproductive organs are maintained by activation of the hypothalamo-pituitary–gonadal (HPG) axis. Seasonally breeding rodents typically experience a marked decline in reproductive function, and thus reproductive success, when exposed to winter-like short days (Beery et al. 2007). For example, hamsters transferred from long summer-like to short winter-like days display significant reductions in circulating levels of luteinizing hormone (LH) and testosterone, followed by subsequent involution of the testes, driven primarily by gonadal apoptosis (Maywood and Hastings 1995; Young et al. 1999). Short day-induced reproductive suppression is orchestrated, in part, by changes in glutamatergic influences on hypothalamic GnRH neurons (Ebling et al. 1995).

The current study sought to confirm that NMDA activates the HPG axis and thus stimulates maintenance of a long day-like reproductive state in hamsters transferred to short days. Consistent with this hypothesis, injections of NMDA induced a dose-dependent rise in circulating testosterone titers in adult male Siberian hamsters, with the two highest doses (20 mg/kg and 40 mg/kg) eliciting similarly pronounced elevations in testosterone. This finding supports a previous report in this species, which demonstrated NMDA-induced up-regulation of pituitary LH release (Ebling et al. 1995).

Next, to examine whether prolonged NMDA activation of the HPG axis maintains reproductive status in hamsters exposed to 8 weeks of short-day photoperiods, a period that typically leads to full gonadal regression and significant reductions in body mass and fat stores (Wade and Bartness 1984; Young et al. 1999), hamsters received daily NMDA injections of 20 mg/kg for 8 weeks, which corresponds to the smallest dose from Experiment 1 that elicited a significant elevation in testosterone. Consistent with a previous report (Ebling et al. 1995), the gonads of hamsters housed in short-day photoperiods that received daily NMDA injections were similar in size to those housed in long-day photoperiods. Interestingly, although these animals had long day-like reproductive organs, they exhibited a normal short day-like decrease in body mass (e.g., (Wade and Bartness 1984). Because photoperiodic changes in body mass are due in large part to changes in total body fat in this species (Wade and Bartness 1984), these findings suggest that NMDA did not alter total body fat. Indeed, short-day hamsters receiving NMDA or vehicle had similarly sized fat pads that were considerably smaller than animals held in long days. Thus, as expected, NMDA injections in short day-housed animals induced dissociations between energy reserves (e.g., fat) and reproductive function (e.g., gonads), suggesting that NMDA enables selective manipulation of the reproductive system without significant alterations in energetic state.

It has previously been demonstrated, by our laboratory and others, that short-day hamsters exhibit a depressed antibody response to an immune challenge compared with long-day hamsters (Demas et al. 2002; Drazen et al. 2001; Yellon et al. 1999b; Zysling and Demas 2007). This decreased antibody response occurs concomitantly with short day-induced decreases in energy stores, suggesting that decreased energy availability drives the observed changes in immunity (Demas 2004). Thus, we hypothesized that experimentally increased investment in an energetically expensive physiological function, reproduction (experimentally activated via NMDA injections), coupled with the typical reductions in energy reserves in short-day hamsters would lead to a trade-off in responses, thus eliciting a further suppression of antibody production. This outcome, however, was not observed; antibody responses in NMDA-injected hamsters displayed greater antibody titers in response to an immune challenge. These findings suggest a direct effect of NMDA on immune responses in this species.

Previous investigations have reported a significant decline in antibody responses in hamsters held on short versus long days (Demas 2002; Demas et al. 2002; Drazen et al. 2001; Drazen et al. 2000; Yellon et al. 1999a). However, in this investigation, no differences were observed between the vehicle-injected animals housed in differing photoperiods. The lack of clear photoperiod-induced changes in immune function in vehicle-treated animals may be due to the chronic mild stress imposed by daily injections. A recent report (Zysling and Demas 2007) observed that anti-KLH IgG levels in animals receiving multiple injections of saline every other day over 12 days did not differ compared with controls, consistent with the idea that multiple injections may indeed increase stress levels, reducing the ability to detect naturally occurring photo-induced reductions in immunity.

Within the central nervous system, the excitatory amino acid glutamate acts on distinct classes of receptors, which coordinate distinct physiological responses. NMDA acts on one such class of receptors, the so-called NMDA receptor subtype. NMDA glutamatergic receptors have recently been identified in the immune system on lymphocytes (Boldyrev et al. 2004; Kostanyan et al. 1997). These receptors have been shown to mediate the activation of T-cells and the potentiation of T-cell antibodies (Boldyrev et al. 2004; Lombardi et al. 2001; Miglio et al. 2005). In the current study, increasing antibody production in response to NMDA injections may be possible through direct activation of the immune system through actions on T-cells. KLH, employed in the present study, is a T-cell dependent antigen; thus, antibody production requires coordination of both T and B lymphocytes (e.g., (Julius et al. 1972). This idea of a direct effect of NMDA on antibody production is

supported by our observation of elevated antibody responses in all animals receiving NMDA injections regardless of photoperiod. Both long and short-day NMDA-injected animals displayed significantly greater antibody production compared with animals receiving vehicle. Future studies will be needed to confirm this hypothesis. If true, greater antibody production in all NMDA-treated hamsters regardless of photoperiod likely limited our ability to detect our hypothesized energetic trade-off between the reproductive and immune systems.

In addition to assessment of acquired immunity, the effects of daily NMDA injections on one aspect of the innate immune response were assessed by quantifying the ability of serum to kill *E. coli* bacteria in vitro. No significant differences were observed in serum bactericidal capacity; however, in short-day NMDA-injected animals, the mean percentage of bacteria killed was slightly ($\sim$5%) higher, suggesting that NMDA may not only alter the acquired immune system, but also play a minor mediating role in innate immune defenses. Consistent with this idea, NMDA activation increases intracellular reactive oxygen species (ROS) in lymphocytes (Boldyrev et al. 2004), and ROS can act as key antimicrobial agents of the innate immune system (Bogdan et al. 2000). Further work will be needed to address the potential role of NMDA in modulating innate immune system functioning.

In the present study, we utilized NMDA to dissociate reproductive and energetic responses to photoperiod. We hypothesized that this manipulation would provide a powerful method to investigate the cost of "mistimed" reproductive responses in seasonal mammals. The current study investigated this hypothesized cost in immune investment. The data from the current investigation suggest that this manipulation, while useful for manipulation of reproductive function, should be used with caution when addressing complex physiological interactions. Future studies may, however, be able to utilize this method to investigate other traits of behaviors associated with this uncoupling of physiological (large gonads) and morphological (pelage coloration and body mass) traits. For example, investigations of behavioral interactions between reproductive and non-reproductive individuals (e.g., Carter et al. 1980; Dluzen et al. 1981) housed in the same photoperiod may yield interesting insights into the ability of a reproductive individual to alter the behavior or physiology of a non-manipulated animal. The current study employed peripheral injections of NMDA, which likely led to direct modulation of the immune system. Infusion of NMDA directly into the central nervous system may enable future studies to utilize this neurotransmitter to uncover potential indirect effects on the immune system associated with changes in HPG neuroendocrine activity.

The current results support a previous finding (Ebling et al. 1995) demonstrating that the glutamate agonist NMDA blocks photoperiod-induced gonadal regression, while leaving photoperiod-induced changes in energy intake or stores unaffected. We further documented a stimulatory effect of NMDA on antibody production, regardless of photoperiod. This observation of greater antibody production in animals treated daily with NMDA was unexpected and may demonstrate a potential key role for glutamate signaling, regulating interactions between the central nervous and the immune system. Indeed, evidence of interactions between the central nervous system and the immune system is becoming increasingly well-documented (for a recent review see Levite 2001). Collectively, these findings, combined with the known role of glutamate in the regulation of GnRH neurons (reviewed in Brann and Mahesh 1997), suggest that glutamate, through actions on NMDA receptors, may play a key modulatory role in regulating seasonal cycles of reproduction independent of photoperiodic-induced changes in adipocity, and that NMDA enhances humoral immunity via mechanisms that appear independent of changes in energy balance. Lastly, these data are consistent with the idea that the neurotransmitter glutamate may serve as a potential mechanism for communication between the central nervous and immune systems.

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