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Epstein–Barr virus (EBV)-encoded dUTPase and chronic restraint induce impaired learning and memory and sickness responses

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HIGHLIGHTS

- EBV-encoded dUTPase reduces activity and increases body temperature.
- EBV-encoded dUTPase combined with chronic restraint exacerbates sickness behavior.
- Restraint stress impaired learning and memory.

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ABSTRACT

Most adult humans have been infected with Epstein–Barr virus (EBV) and carry the latent virus. The EBV genome codes for several proteins that form an early antigen complex important for viral replication; one of these proteins is deoxyuridine triphosphate nucleotidohydrolase (dUTPase). The EBV-encoded dUTPase can induce sickness responses in mice. Because stress can increase latent virus reactivation, we hypothesized that chronic restraint would exacerbate sickness behaviors elicited by EBV-encoded dUTPase. Male Swiss-Webster mice were injected daily for 15 days with either saline or EBV-encoded dUTPase. Additionally, half of the mice from each condition were either restrained for 3 h daily or left undisturbed. Restraint stress impaired learning and memory in the passive avoidance chamber; impaired learning and memory was due to EBV-encoded dUTPase injected into restrained mice. EBV-encoded dUTPase induced sickness responses and restraint stress interacts with EBV-encoded dUTPase to exacerbate the sickness response. These data support a role for EBV-encoded dUTPase and restraint stress in altering the pathophysiology of EBV independent of viral replication.

1. Introduction

Epstein–Barr virus (EBV) is a member of the herpes virus family. Approximately 95% of adults in the United States between the ages of 35 and 40 are infected with EBV [1]. In common with many viruses, EBV can induce a classical sickness response including fever and lethargy. However, EBV infections are life-long and can alternate between latent and reactivated states. Latent infections with EBV have been linked to the development of cancer; particularly Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin lymphoma, and diffuse large B cell lymphoma [1–3].

The EBV genome encodes several proteins important for lytic viral replication that are part of an early antigen complex [4–9]. One of these EBV-encoded proteins, deoxyuridine triphosphate nucleotidohydrolase (dUTPase), is capable of inducing immune dysregulation *in vitro* by inhibiting cellular replication and altering production of pro-inflammatory cytokines [10–13]. The ability of EBV-encoded dUTPase to dysregulate immune function suggests that it may play a role in the pathophysiology of EBV infection [10–13]. In a previous study, we reported that EBV-encoded dUTPase induces sickness responses in mice that was characterized by increased temperature and reduced activity [14]. Splenic and lymphatic cells taken from EBV-encoded dUTPase-injected mice reduced proliferation of T-cells and production of inflammatory cytokine interferon-γ when stimulated with a T-cell mitogen [14].

Stress can also dysregulate the immune system [15]. Acute stress enhances, whereas chronic stress tends to impair immune function [16]. The stress response leads to the prolonged production of glucocorticoid

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hormones that can then dysregulate the immune system [17]. Cytokines activate the hypothalamic–adrenal–pituitary (HPA) axis, producing glucocorticoids that provide negative feedback to activated immune cells to downregulate the synthesis and release of proinflammatory cytokines [18]. Therefore, short periods of restraint stress, which increase glucocorticoids, can decrease proinflammatory cytokine expression even in response to LPS injections [18]. Acute stress may enhance the immune response, whereas prolonged stressful situations, such as preparation for exams by medical students, increase latent viral reactivation due to shifts in the immune system [19,20]. Additionally, treatment of EBV positive Burkitt's lymphoma cells with glucocorticoids causes reactivation of latent EBV by inducing immediate early genes [21]. Thus, glucocorticoids, which typically increase with stress as well as during an immune response, can reactivate a latent virus not only by altering immune physiology, but also by activating viral immediate early genes.

To assess changes in the sickness response, mice were injected with EBV-encoded dUTPase for 15 days and restrained 3 h each day. Locomotor activity and temperature were continuously monitored. On day 14 mice were also behaviorally tested to assess changes in learning and memory. We hypothesized that chronic restraint stress exacerbates sickness behavior elicited by EBV-encoded dUTPase.

2. Materials and methods

2.1. Subcloning and purification of EBV-encoded dUTPase

The subcloning of the EBV (BLLF3 pET3A was kindly provided by Dr. Peter Sommer (Institut fur Mikrobiologic und Hygiene, Abteilung Virolgie)) was conducted by PCR amplification using the forward (5′-CCGGTTAAGCTTGGATCCATGGAGGCC TGTC-3′) and reverse (5′-GCGA ATTCTCATTGACCCGACGATCC-3′) primer sets (125 pmol of each), DNA (140 ng), high fidelity PCR supermix (Invitrogen, Gary Island, NY, USA) and the following PCR conditions: Denaturation at 94 °C for 3′ (1 cycle) followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1′ and 1 cycle at 72 °C for 20′. The PCR product was purified using the QIAquick gel extraction kit (QIAGEN) and cloned into the protein expression vector pTrcHis Topo (Invitrogen, Gary Island, NY, USA). Twenty individual clones were isolated following transformation of *Escherichia coli* (*E. coli*) Top 10 competent cells, DNA was then purified using the QIAPrep Spin Miniprep kit (QIAGEN, Valencia, CA, USA), screened by PCR for the presence of specific dUTPase genes and the sequence verified by DNA sequencing analysis. The pTrcHisdUT constructs, containing the EBV-encoded dUTPase gene in the correct orientation and in frame, were used to transform *E. coli* BL21(DE3)*plyS* competent cells for purification of recombinant proteins as described below.

The recombinant EBV-encoded dUTPase protein was purified using HisPur™ Spin columns (3 ml resin bed) as described by the manufacturer (Pierce, Rockford, IL, USA). Briefly, BL21(DE3)plyS containing a specific PTrcHisDUT construct was grown in LB medium containing chloramphenicol (25 μg/ml) and ampicillin (100 μg/ml) at 37 °C for 2.5 h. IPTG (1 mM final concentration) was added and the culture was incubated an additional 2 h at 37 °C. Bacteria were collected from 1 to 2 l of medium by low speed centrifugation and the bacterial pellet was resuspended in 50 ml of extraction buffer (50 mM sodium phosphate, 300 mM NaCl and 10 mM imidazole, pH 7.4). Bacteria were lysed by ultrasonication. The resulting homogenate was centrifuged (15,000 ×*g*, 30 min at 4 °C), and the supernatant was applied to a HisPur™ spin column, which was equilibrated in an extraction buffer. The column was washed three times with two-resin bed volumes of extraction buffer and the EBV-encoded dUTPase protein was eluted by washing the column four times with one resin-bed volume of 50 mM sodium phosphate, 300 mM NaCl and 150 mM imidazole, pH 7.4. Fractions were assayed for the EBV-encoded dUTPase activity as described previously [10] and for protein using the Coomassie Brilliant Blue dye-binding assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard. A unit of dUTPase activity was defined as the amount of enzyme required to convert 1 nM of dUTP to dUMP and pyrophosphate per min at 37 °C under the assay conditions. Purity of EBV-encoded dUTPase was determined by SDS-PAGE as described previously [10]. Proteins were visualized using EZBlue™ protein gel stain as described by the manufacturer (Sigma Aldrich, St. Louis, MO). The EBV-encoded dUTPase protein preparations were tested as described previously [10] and were free of detectable levels of LPS, peptidoglycan (SLP-HS), DNA, or RNA.

2.2. Animals

Thirty-eight adult male Swiss-Webster mice (~8 weeks) were obtained from Charles River Labs (Wilmington, MA, USA). Mice were group-housed, five per cage, in polypropylene cages (33 cm × 18 cm ×14 cm) at an ambient temperature of 22 ± 2 °C and relative humidity of 50% ± 10%. Animals were given *ad libitum* access to Harlan Teklad 8640 food (Madison, WI, USA) and filtered tap water. Mice were placed in a 14:10 light dark cycle and allowed to acclimate to the facility for at least one week. A 14:10 light dark cycle was chosen because it gives a clear long day signal to all animals as opposed to the ambiguous 12:12 light cycle. Behavior and sickness responses can be altered by day length so we chose an unambiguous light dark cycle. All experimental procedures were approved by the Ohio State University Institutional Animal Care and Use Committee.

2.3. Mini Mitter implants

One week after arrival, mice had radiotelemetric transmitters (Mini Mitter, Respironics, Bend, OR, USA) implanted in the peritoneal cavity while under isoflurane anesthesia. Mice were single-housed and allowed to recover for one week. Home cages were placed on TR-4000 receiver boards connected to DP-24 DataPorts (Mini Mitter) that continuously collected temperature and activity data in 30 min bins. These 30 min bins of activity and temperature data were extracted from the VitalView (Mini Mitter, Respironics, Bend, OR, USA) program at the conclusion of the experiment. Temperature and activity from individual days were significant. Due to the number of days and that mice were removed from receiver boards during behavioral testing on day 14, data were collapsed by taking an average of the first 4, 7 or 14 days of data to make presentation clearer.

2.4. Treatment with EBV-encoded dUTPase and restraint

Following one week of recovery, mice were injected in the right hindlimb daily between zeitgeber time (ZT) 8–9 with 10 μg EBV-encoded dUTPase in saline or an equal volume of 0.9% saline (Table 1). A 10 μg dose of EBV dUTPase for 14 days has been previously used to induce sickness behavior in mice [14]. ZT is a standard of time based on the period of a zeitgeber, ZT 0 is defined as the time when the lights come on and in this study ZT 14 is when the lights went off. Mice were injected for 15 consecutive days. Gait was not altered following injections; mice were observed to have normal locomotor abilities. Mice that developed bruises or alterations in gait following injections were removed from the study. Beginning the first day of injections, mice in the restraint experimental groups were placed in ventilated 50 ml conical restraint tubes for 3 h/day at random times during the light phase (Table 2). Unrestrained mice were left undisturbed with access to food and water while the other mice were restrained.

Table 1,
The number of mice in each group.

	Unrestrained	Restrained
0.9% saline	10	9
EBV-encoded dUTPase	10	9

Table 2
Restraint times for mice using zeitgeber time (ZT), ZT 0 is lights on. Injections occurred daily between ZT 8 and 9.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15
ZT	8.5	11.5	8.5	9.25	5.25	11	8.5	11.5	10	8.25	10.5	5.25	10	9	10.25

2.5. Daily measurements

Body weight and food intake were measured daily starting two days before injections at ZT 8–9. Additionally, mice were given a 1:1 solution of water and sweetened condensed milk daily for 3 h/day at the onset of the dark phase, ZT 14 [22]. Food and water were removed while mice had access to the sweetened condensed milk. On days that mice were behaviorally tested, mice did not receive sweetened condensed milk. Milk intake was also averaged for the first 4, 7, or 14 days so any general changes could be observed.

2.6. Passive avoidance

The passive avoidance test assesses learning and memory by teaching mice an association between escaping from an aversive stimulus by using light and a foot shock. Retention of this pairing is assessed 24 h after initial trial, and latency to enter the dark chamber is used to assess retention. During the dark phase on day 14, mice were placed in the passive avoidance chamber (Gemini Avoidance System, San Diego Instruments Inc., San Diego, CA) and assessed as previously described [23].

2.7. Statistical analysis

Main effects of restraint (no restraint vs. restraint) and injection type (saline, EBV-encoded dUTPase) and interactions between the two variables were assessed. Body temperature, home cage activity, and percent milk intake across days were analyzed as a 2×2 repeated measures ANOVA. Total food intake, percentage of body mass gained and averaged milk intake on days 4, 7, and 14 were run as 2×2 univariate ANOVAs. Passive avoidance was run as a 2×2 multivariate ANOVA. Statistics were performed using SPSS 19 for Windows (IBM, Armonk, NY, USA). For body temperature and home cage activity, some of the Mini Mitters failed during the experiment, and these mice were excluded from subsequent body temperature and activity analysis. Mean differences were considered statistically significant when p was <0.05. Significant differences were followed up with Bonferroni post hoc tests to correct for multiple comparisons.

3. Results

3.1. Temperature

Body temperature on days 1 and 2 of injections was increased by restraint ($F_{1,1269} = 3.31$, $p < 0.01$; $F_{1,1316} = 5.21$, $p < 0.01$; Fig. 1, A) and was increased by EBV-encoded dUTPase injections compared to saline ($F_{1,1269} = 1.62$, $p < 0.01$; $F_{1,1316} = 1.90$, $p < 0.01$; Fig. 1, A). On day 1 there was an interaction between restraint and dUTPase on body temperature ($F_{1,1269} = 2.07$, $p < 0.01$; Fig. 1, A); however, there was no interaction on day 2 ($p > 0.05$). Average body temperature over the first 4, 7, and 14 days of injections was increased by restraint ($F_{1,1363} = 6.13$, $p < 0.01$; $F_{1,1316} = 8.66$, $p < 0.01$; $F_{1,1316} = 11.72$, $p < 0.01$; Fig. 1, B, C and D respectively). Body temperatures averaged over the first 4 and 7 days were also increased by EBV dUTPase injection compared to saline ($F_{1,1363} = 1.76$, $p < 0.01$; $F_{1,1363} = 1.90$, $p < 0.01$; Fig. 1, B and C); however, body temperature averaged over 14 days was not altered by saline or EBV-encoded dUTPase injections

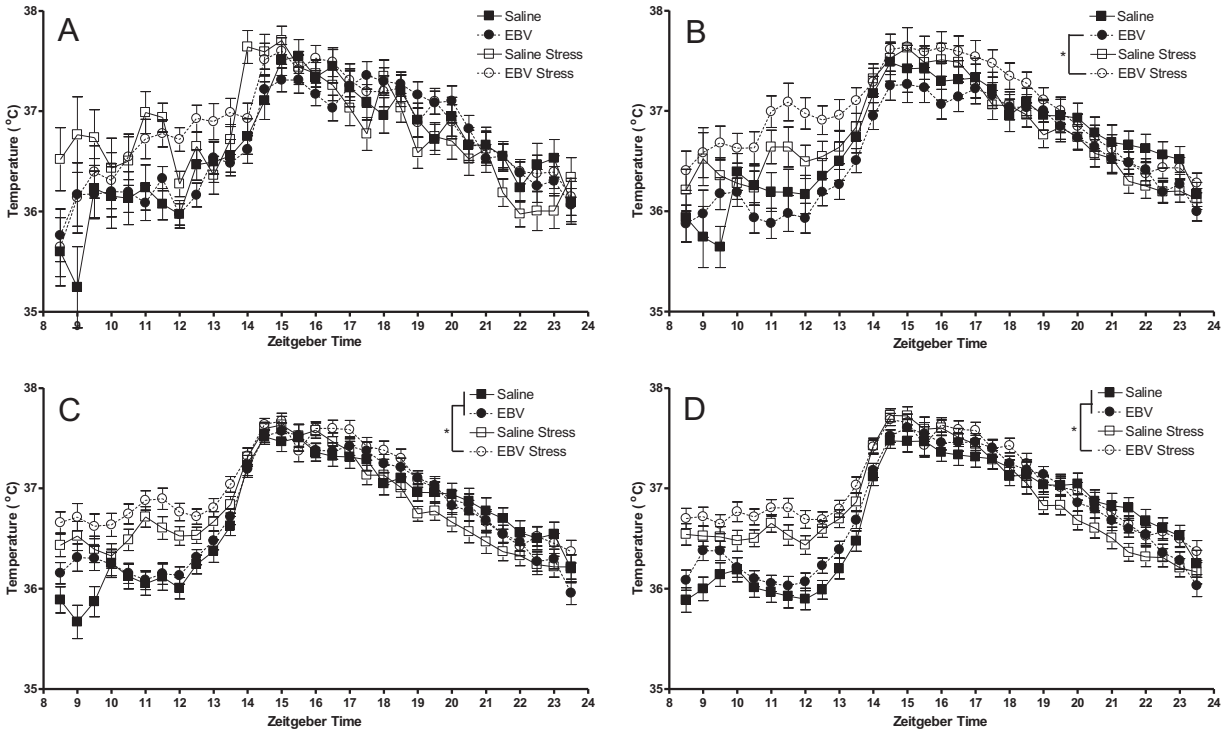

Fig. 1. Body temperature (±standard error of the mean (SEM)) was increased by EBV-encoded dUTPase injections and restraint. Zeitgeber time (ZT) 0 is lights on and injections occurred between ZT 8 and 9. Change in body temperature following injection on day one (A), averaged for days 1–4 (B), days 1–7 (C), and days 1–14 (D). Significant differences $p < 0.05$ indicated by *.

($p > 0.05$). Body temperature averaged over 7 and 14 days displayed a significant interaction between restraint and EBV-encoded dUTPase ($F_{1,1363} = 1.64$, $p < 0.01$; $F_{1,1363} = 2.18$, $p < 0.01$; Fig. 1, C and D); there was no interaction averaged over four days ($p > 0.05$). EBV-encoded dUTPase injected and restrained mice had increased body temperature compared to EBV-encoded dUTPase-injected- and unrestrained mice averaged over four days ($p < 0.05$). Body temperature on day 2 and averaged over 7 and 14 days was increased in EBV-encoded dUTPase-injected and restrained mice compared to EBV-encoded dUTPase- and saline-injected, unrestrained mice ($p < 0.05$).

3.2. Activity

Activity levels during days 1 and 2 of injections were decreased by restraint ($F_{1,1363} = 7.10$, $p < 0.01$; $F_{1,1363} = 7.77$, $p < 0.01$; Fig. 2, A). Further, EBV-encoded dUTPase injections decreased activity levels on days 1 and 2 compared to saline injections ($F_{1,1363} = 1.37$, $p < 0.05$; $F_{1,1363} = 1.95$, $p < 0.01$; Fig. 2, A). There was an interaction of restraint and EBV-encoded dUTPase on activity levels on days 1 and 2 ($F_{1,1363} = 1.73$, $p < 0.01$; $F_{1,1363} = 2.11$, $p < 0.01$; Fig. 2, A). Activity levels averaged over the first 4, 7, and 14 days of injections were decreased by restraint ($F_{1,1363} = 8.11$, $p < 0.01$; $F_{1,1363} = 10.45$, $p < 0.01$; $F_{1,1363} = 13.87$, $p < 0.01$; Fig. 2, B, C and D). Activity levels averaged over the first 4, 7, and 14 days were also decreased by 10 μg of EBV-encoded dUTPase injection compared to saline ($F_{1,1363} = 1.61$, $p < 0.01$; $F_{1,1363} = 2.07$, $p < 0.01$; $F_{1,1363} = 2.16$, $p < 0.05$; Fig. 2, B, C and D). There was an interaction of restraint and EBV-encoded dUTPase averaged over days 7 and 14 of activity levels ($F_{1,1363} = 1.49$, $p < 0.05$; $F_{1,1363} = 1.54$, $p < 0.05$; Fig. 2, C and D). Saline-injected, unrestrained mice increased activity compared to all other groups on days 1 and 2 and averaged over the first 4 and 7 days ($p < 0.5$). Saline-injected, unrestrained mice increased activity compared to restrained mice regardless of injection type averaged over 14 days ($p < 0.5$). EBV-encoded dUTPase-injected, unrestrained mice increased activity on days 1 and 2 and averaged over the first 4, 7, or 14 days compared to restrained mice regardless of injection ($p < 0.05$).

3.3. Body mass

Restraint decreased the rate of body mass gain during the experiment compared to unrestrained mice ($F_{1,33} = 35.46$, $p < 0.01$; Fig. 3, A). Saline and EBV-encoded dUTPase-injected restrained mice gained less weight than unrestrained mice regardless of injection ($p < 0.05$). Weight gain did not differ between saline and EBV-encoded dUTPase injections nor was there an interaction of restraint and injection type ($p > 0.05$).

3.4. Food intake

Restraint decreased total food intake over the entire experimental period ($F_{1,33} = 5.66$, $p < 0.05$; Fig. 3, B). Restraint and injection type did not interact to affect food intake over the experimental period ($p > 0.05$).

3.5. Milk intake

Milk intake averaged across days 4, 7, and 14 decreased in restrained mice ($F_{1,33} = 16.48$, $p < 0.01$; $F_{1,33} = 14.64$, $p < 0.01$; $F_{1,33} = 8.49$, $p < 0.01$; Fig. 4, A, B and C). Average milk intake for the first 4 and 7 days was decreased by restraint regardless of injection compared to mice injected with EBV and unrestrained mice ($p < 0.05$). Restrained and EBV-encoded dUTPase injected mice decreased percent of milk intake averaged over 14 days compared to EBV-encoded dUTPase-injected and unrestrained mice ($p < 0.05$). Injection type and restraint did not interact to alter milk intake averaged over the first 4, 7, or 14 days ($p < 0.05$). The percentage of milk intake was decreased across days

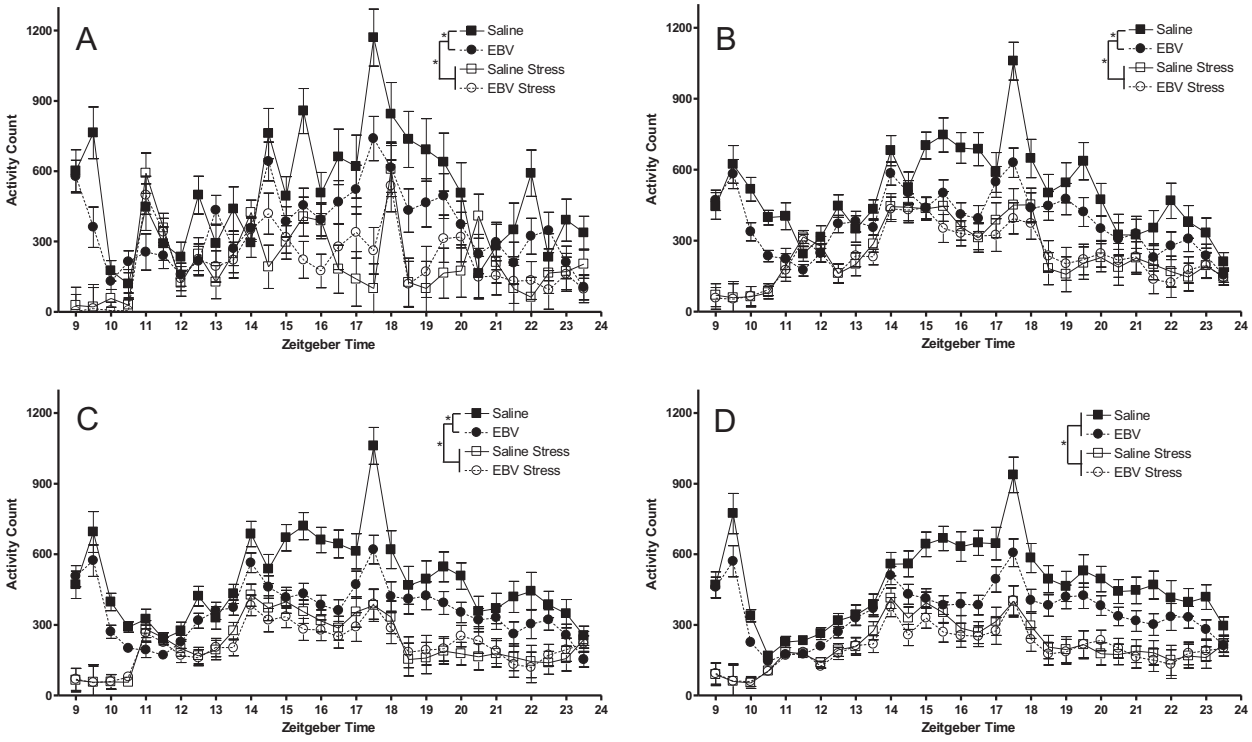

Fig. 2. Home cage activity was reduced by EBV-encoded dUTPase injections and restraint. Zeitgeber time (ZT) 0 is lights on and injections occurred between ZT 8 and 9. Change in activity (±SEM) following injection on day 1 (A), averaged for days 1–4 (B), days 1–7 (C), and days 1–14 (D). Significant differences $p < 0.05$ indicated by *.

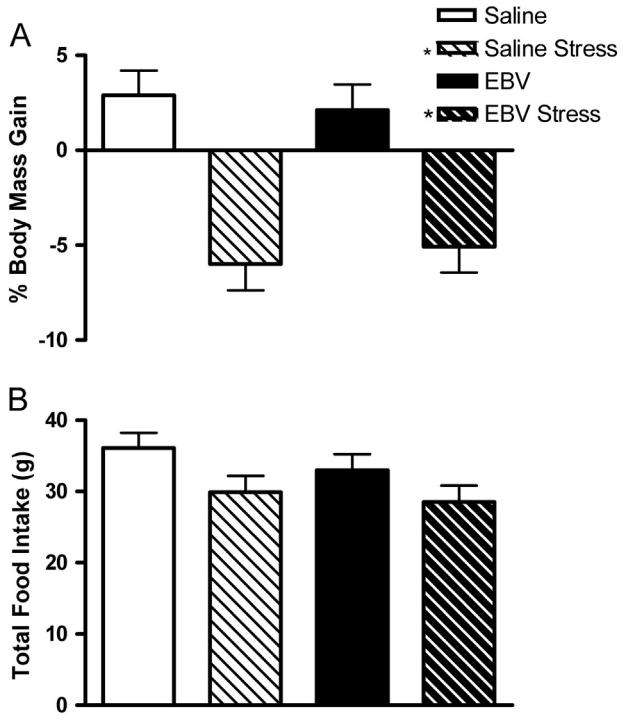

Fig. 3. Percent of body mass gained (±SEM) over 14 days of EBV-encoded dUTPase injections was decreased by restraint (A) as was total food intake (B). Significant differences $p < 0.05$ indicated by *.

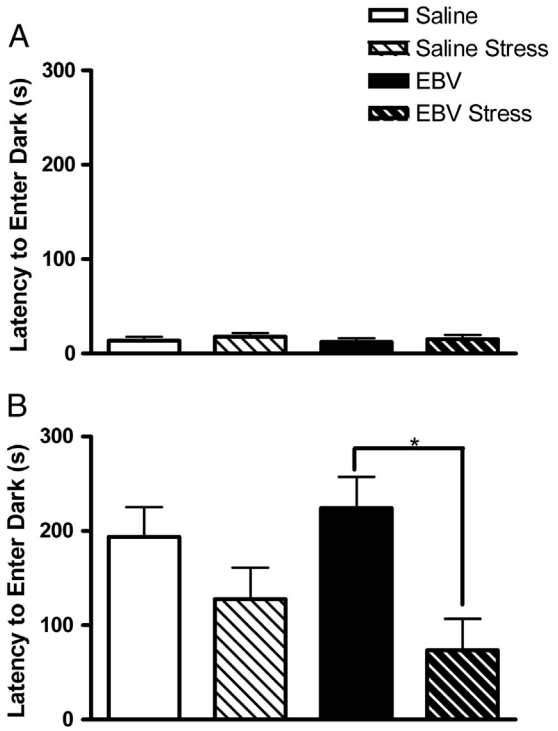

Fig. 5. The passive avoidance test assesses differences in learning and memory. Mice had similar latencies to enter the dark chamber on day 1 (A). Restraint decreased latency (±SEM) to enter the dark chamber on day 2 (B), indicating impaired learning and memory; this reduction in latency was driven by the restrained and EBV-encoded dUTPase-injected mice. Significant differences $p < 0.05$ indicated by *.

in restrained animals ($F_{1,363} = 3.14$, $p < 0.01$; Fig. 4, D). EBV-encoded dUTPase- and saline-injected mice drank similar percentages of milk across days ($p > 0.05$). There was an interaction of restraint and EBV-encoded dUTPase on rate of milk intake across days ($F_{1,363} = 1.91$, $p < 0.05$; Fig. 4, D), such that restrained EBV-encoded dUTPase-injected mice decreased milk intake across days compared to unrestrained mice injected with EBV-encoded dUTPase ($p < 0.05$).

3.6. Passive avoidance

There were no differences in latency to enter the dark chamber on day 1 ($p > 0.05$; Fig. 5, A). On day 2, restraint stress decreased the latency to enter the dark chamber compared to unrestrained mice

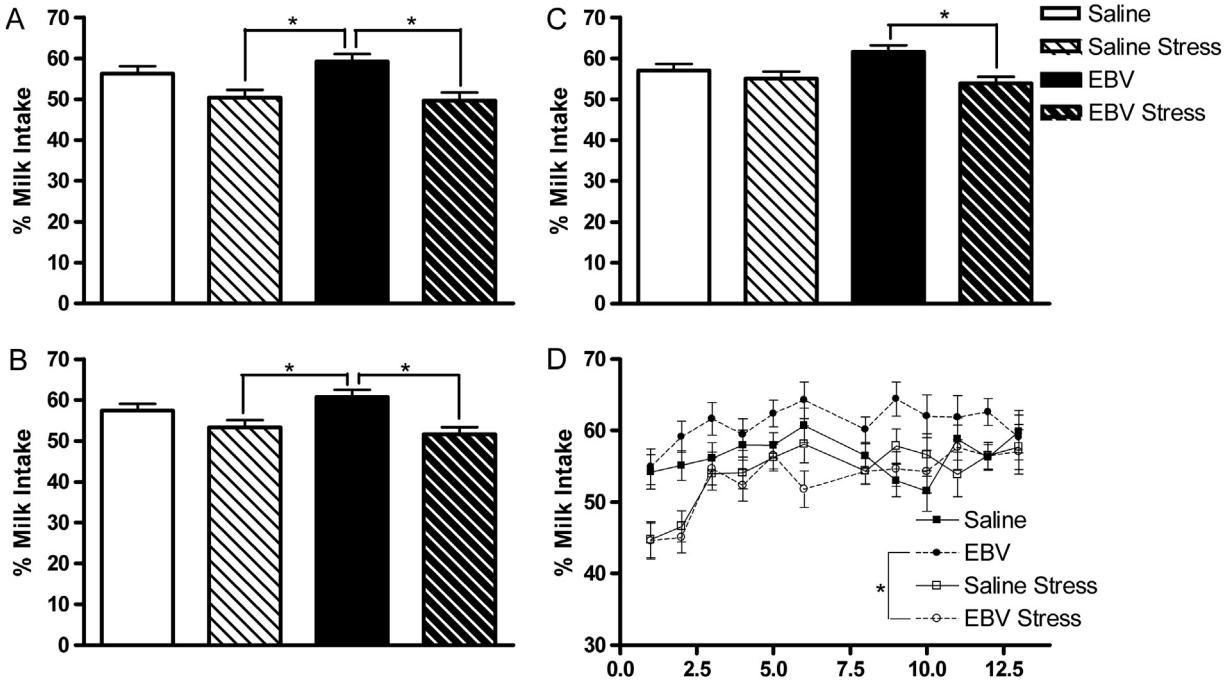

Fig. 4. Percent of milk intake (±SEM) averaged to baseline intake, restraint reduced percent of milk intake indicating an anhedonic-like response. Percent of milk intake averaged over first 4 days (A), 7 days (B), and 14 days (C), and percent milk intake across days (D). Significant differences $p < 0.05$ indicated by *.

($F_{1,33} = 10.83$, $p < 0.05$; Fig. 5, B). Restraint in dUTPase-treated mice decreased latency to enter the dark chamber on day 2 compared with EBV-encoded dUTPase unrestrained mice ($p < 0.05$). Latency to enter the dark chamber on day 2 did not differ among any other groups ($p > 0.05$; Fig. 5, B). Injection and restraint did not interact to alter latency to enter the dark side of the chamber on day 2 ($p > 0.05$; Fig. 5, B).

4. Discussion

Persistent EBV infections exist in the vast majority of the adult population; maintenance of persistence requires the periodic reactivation of latent virus [1]. The EBV genome encodes proteins that form an early antigen complex important for viral replication [14]. Administration of individual EBV-encoded proteins, such as EBV-encoded dUTPase, allows the effects of EBV to be studied independent of infectious viral replication. We hypothesized that EBV-encoded dUTPase would induce a sickness response and that restraint stress would augment this response. This was supported by both reduced cage activity and increased body temperature in EBV-encoded dUTPase-injected mice that was exacerbated by the addition of restraint, a well-characterized stressor. These data suggest that restraint stress combined with a specific virus encoded protein induces a sickness response and implicates the EBV-encoded dUTPase protein in the pathophysiology of EBV. EBV-encoded dUTPase is expressed during lytic and abortive-lytic replication of the virus, and thus stressor-induced viral reactivation of latent EBV may elicit and augment the sickness response.

EBV-encoded dUTPase induces a cytokine-like sickness response as observed previously in humans and mouse systems [14,24,25]. The EBV-encoded dUTPase protein increased body temperature and reduced home cage activity. Body temperature was increased by restraint at all time points and by EBV-encoded dUTPase up to day 7. Body temperature was highest for mice that experienced restraint and EBV-encoded dUTPase treatments. Similarly, prior exposure to an inescapable foot shock increased core body temperature; this was further increased following exposure to an LPS challenge [26]. Total locomotor activity was decreased by both restraint and EBV-encoded dUTPase treatments, consistent with lethargy observed during sickness [14]. Similarly, the effects of LPS following chronic unpredictable stress increased the sickness response, including lethargy relative to non-stressed animals [27]. These data indicate that the EBV-encoded dUTPase protein can induce a sickness response independent of viral replication. As observed in human populations, prolonged stressors increase the likelihood of developing infection and the associated responses [28]. Additionally, restrained EBV-encoded dUTPase-injected mice reduced intake of sweetened condensed milk compared to EBV-encoded dUTPase-treated, unrestrained mice. Reduced intake of sweetened condensed milk is interpreted as an index of anhedonia, as well as an increased depressive-like response [22]. These data indicate a role for prolonged exposure to restraint stress in enhancing the sickness response following an immune challenge. Therefore, stress may play a role in inducing EBV reactivation and thus increasing the production of the EBV-encoded dUTPase which contributes to the pathophysiology associated with EBV infections [10–13].

During a sickness response learning and memory is impaired, a response that may be enhanced by stress [29]. Stressors reduce learning and memory in a passive avoidance test, a validated test for learning and memory [30]. Rats that are subjected to chronic psychological stressors display impaired spatial learning and memory [31]. The effect of restraint on passive avoidance in the present study reflects decreased latency in mice injected with EBV-encoded dUTPase and restrained compared to unrestrained, EBV-encoded dUTPase-treated mice, suggesting that restraint stress impaired memory concomitant with virus infection. IL-1β given during a learning and memory task, such as fear conditioning, impairs context-dependent learning and memory, whereas blocking IL-1β inhibits the memory impairment [32]. Our previous studies have demonstrated that the EBV-encoded dUTPase also induces the production of IL-1β [10,13]. Because circulating concentrations of cytokines or other biomarkers were not assessed in this study, we cannot conclusively identify what elicited the observed sickness response or changes in behavior. However, there appears to be a combined role for restraint stress and EBV-encoded dUTPase protein in altering the anxiety-like responses likely through the production of IL-1β.

5. Conclusion

In conclusion, restraint stress interacts with EBV-encoded dUTPase to exacerbate the sickness response observed in this mouse model. Data from our earlier study showed that this viral protein can induce sickness behavior in mice [14]. The data from this study confirm and extend that initial observation. Considered together, the data suggest a combined role for EBV-encoded dUTPase and restraint stress in modulating the pathophysiology of an EBV infection.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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