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RESEARCH ARTICLE

Suppression of Locomotor Activity in Female C57Bl/6J Mice Treated with Interleukin-1 β : Investigating a Method for the Study of Fatigue in Laboratory Animals

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Abstract

Fatigue is a disabling symptom in patients with multiple sclerosis and Parkinson's Disease, and is also common in patients with traumatic brain injury, cancer, and inflammatory disorders. Little is known about the neurobiology of fatigue, in part due to the lack of an approach to induce fatigue in laboratory animals. Fatigue is a common response to systemic challenge by pathogens, a response in part mediated through action of the pro-inflammatory cytokine interleukin-1 beta (IL-1 β). We investigated the behavioral responses of mice to IL-1 β . Female C57Bl/6J mice of 3 ages were administered IL-1 β at various doses i.p. Interleukin-1 β reduced locomotor activity, and sensitivity increased with age. Further experiments were conducted with middle-aged females. Centrally administered IL-1 β dose-dependently reduced locomotor activity. Using doses of IL-1 β that caused suppression of locomotor activity, we measured minimal signs of sickness, such as hyperthermia, pain or anhedonia (as measured with abdominal temperature probes, pre-treatment with the analgesic buprenorphine and through sucrose preference, respectively), all of which are responses commonly reported with higher doses. We found that middle-aged orexin^{-/-} mice showed equivalent effects of IL-1 β on locomotor activity as seen in wild-type controls, suggesting that orexins are not necessary for IL-1 β -induced reductions in wheel-running. Given that the availability and success of therapeutic treatments for fatigue is currently limited, we examined the effectiveness of two potential clinical treatments, modafinil and methylphenidate. We found that these treatments were variably successful in restoring locomotor activity after IL-1 β administration. This provides one step toward development of a satisfactory animal model of the multidimensional experience of fatigue, a model that could allow us to determine possible pathways through which inflammation induces fatigue, and could lead to novel treatments for reversal of fatigue.

Introduction

Fatigue is a symptom associated with many neurological disorders, particularly multiple sclerosis and Parkinson's Disease. In the general population, the prevalence of fatigue increases with age, particularly in women and is estimated to cost U.S. employers \$136 billion annually through reduced productivity [1]. In diseases such as multiple sclerosis, prevalence may be as high as 85% [2]. Currently, researchers have little understanding of the neurobiological basis of disease-associated fatigue, and clinicians have few effective treatments to offer patients.

Fatigue is common with inflammation [3–5]. In response to an immune challenge, the organism mounts behavioral and physiological actions, which have been collectively termed “sickness behaviors”. These include fatigue, fever, social withdrawal, anorexia, anhedonia and muscle-ache [6]. Prior research has effectively utilized pathogens and related antigens that act on Toll-like receptors (TLRs) to bring about sickness behaviors. These include the bacterial endotoxin lipopolysaccharide (LPS) [7], viral mimetics such as polyinosinic:polycytidylic acid (Poly I:C) [8] and inoculation with the parasite *Trypanosoma brucei* [9]. Sickness behaviors are mediated through multiple pro-inflammatory cytokines, including interleukin-1 β (IL-1 β), interleukin-6, tumor necrosis factor- α and interferon [6].

How might one study fatigue separate from other sickness behaviors? We began these studies with the goal of trying to separate immune activation effects on locomotor activity from the wider array of sickness behaviors. We focused on wheel running behavior because it is a voluntary activity that can be intense and thus effortful, and might tap both lethargy as well as altered motivation associated with the symptom of fatigue. In animal studies, immune system activation or treatment with chemotherapy causes a reduction in wheel-running as well as general locomotor activity [8–12]. Some evidence suggests that IL-1 β in particular is linked to the reduction of activity. Peripherally administered IL-1 β reduces locomotor activity in mice [13,14] through activity on CNS endothelial cells [15,16]. Disruption of IL-1 β signaling in the CNS attenuates LPS effects on locomotor activity [17,18]. Furthermore, central administration of an interleukin-1 receptor antagonist or IL-1 β neutralizing antibody attenuates the reduction in wheel-running activity caused by peripheral administration of IL-1 β or Poly I:C [12,19,20].

Clinical studies support the direction of an inflammation-based animal model of fatigue. Cytokine induction in healthy individuals causes feelings of fatigue [21]. Cancer-related fatigue is correlated with markers of inflammation (C-reactive protein, IL1-receptor antagonist, IL-6) [22,23]. Both fatigue and inflammation increase with age [24]. Although an animal model will not replicate all the complex symptoms and correlates associated with fatigue in clinical populations [25], such a model may allow us to determine specific mechanisms by which inflammation induces fatigue in clinical populations.

It is still unclear in which brain regions immune activation induces suppression of locomotor activity. One hypothesis is that orexin-containing neurons of the lateral hypothalamus play an important role. Mice lacking orexins have reduced running-wheel activity [26]. Both inflammation and chemotherapy decrease orexin (hypocretin) neuron activity, with effects on wheel-use reversed by central administration of orexin-A [7,27,28].

In the studies reported below, we determined that IL-1 β can reduce locomotor activity in an age- and dose-dependent manner. We focused our subsequent studies on middle-aged female mice because our initial studies showed that young mice were less sensitive to these effects of IL-1 β and because fatigue is most highly reported in middle-aged women. To determine the possibility of this effect on locomotor behavior being separable from expected effects on a range of sickness behaviors, we conducted further studies with one dose to measure its effects on sleep, body temperature, anhedonia and pain in middle-aged female mice. To determine if IL-1 β effects are mediated by orexin, we tested the hypothesis that orexin gene knockout mice

will not show further reduced wheel-running activity after IL-1 β . Given the limited number of clinical treatments available to treat fatigue [2], we subsequently tested two potential pharmacological treatments to reverse these effects on locomotor activity. These included the CNS stimulant methylphenidate and a drug commonly prescribed to alleviate daytime sleepiness, modafinil. These drugs are both thought to block the reuptake of dopamine, a neurotransmitter strongly implicated in the signaling of motivated behaviors [29,30]. Effectiveness of either of these compounds would make sense in light of a current theory of dopamine circuit dysfunction underlying fatigue [31].

Methods

Animals

The Smith College Animal Care & Use Committee approved these studies, which were conducted in a facility certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Female C57Bl/6J mice were bred in-house at Smith College under a 12:12 hour light-dark cycle and provided with food (2014 Teklad Global 14% Protein Diet; Harlan Laboratories) and water *ad libitum*, with Harlan Tek-Fresh bedding. Our first experiment used young (3–5 months), middle-aged (6–12 months) and aged (18–24 months) mice; all subsequent experiments were conducted using middle-aged (6–12 months) mice. Heterozygous prepro-orexin knockout mice backcrossed more than 6 generations to C57Bl/6J [32] were crossed to produce homozygous prepro-orexin knockout and wildtype littermates. Offspring were genotyped as previously described [26,32] by PCR, using primers for the wildtype allele, 5' -GACGACGGCCTCAGACTTCTTGGG, 3' -TCACCCCTTGGGATAGCCCTTCC (1.5kb product) and the mutant knockout allele, 5' -TAGTTGCCAGCCATCTGTTG, 3' -ACTCTCCACCCACAGACAGG (2.1kb product). These mice were group housed until age 6 months.

Home cage behavioral measures

When animals reached the appropriate age range, they were housed individually in a cage (483mm x 267mm x 152mm, AnCare Corp. Bellmore, NY, USA) provided with a running wheel (120mm diameter) and motion sensor (K-940, Visonic, Bloomfield, CT, USA) for a minimum of two weeks to record daily wheel revolutions and general locomotor behavior respectively. Wheel and motion activity were collected in 1-minute bins using ClockLab Data Collection Software (Actimetrics Inc., Wilmette, IL, USA). Once activity levels were stable, mice were recorded for an additional 5 days to provide baseline activity in wheel revolutions and general locomotion prior to administration of IL-1 β . A 5-day baseline was chosen to adjust for variability associated with the estrous cycle in females. On day 6, mice were injected i.p. 30 minutes before lights off (ZT11.5) with recombinant mouse IL-1 β (carrier-free, BioLegend, San Diego, CA, USA) at doses ranging from 0–24 μ g/kg in 0.1ml volume. Control mice received a vehicle injection of 0.1ml pyrogen-free 0.9% saline (Besse Medical, Cincinnati, OH, USA). Activity recorded post-IL-1 β was analyzed as a percentage of the mean baseline activity for each animal and as absolute counts for time course analysis. Subjects received a maximum of two treatments, limited to one vehicle and one IL-1 β dose, separated by a minimum 8-day washout period. Mice were weighed before and after procedures (approx. every 10 days).

I.C.V. administration of IL-1 β

To administer IL-1 β directly to the brain, middle-aged female mice were surgically implanted with a stainless steel guide cannula (22 gauge, Plastics One, Roanoke, VA, USA) stereotaxically directed at the lateral ventricle. Mice were administered 0.05mg/kg buprenorphine (Reckitt

Benckiser Pharmaceuticals Inc., Richmond, VA, USA) and 5mg/kg ketoprofen (Fort Dodge Laboratories, Fort Dodge, IA, USA) analgesics subcutaneously 30 minutes prior to surgery. Under isoflurane anesthetic, the guide cannula was positioned relative to bregma (-0.4mm anterior-posterior, +1mm lateral and -3mm ventrally) and secured with a single screw anchor and dental cement (GC Fuji Plus, Patterson Dental, Rocky Hill, CT, USA). Mice were left to recover individually housed, with a dummy cap (Plastics One, Roanoke, VA, USA) preventing obstruction of the guide cannula for 7 days. Mice were closely monitored during recovery period with analgesics administered as needed. After recovery, a running wheel was placed into the animal's home cage. Mice were given two weeks to habituate to the running-wheel and produce stable wheel-running activity. After a 5-day baseline, mice were injected i.c.v. 30 minutes before lights off with 2, 10, or 50ng recombinant mouse IL-1 β (carrier-free, BioLegend, San Diego, CA, USA) prepared in artificial cerebrospinal fluid (aCSF; 2.5mM CaCl₂, 124mM NaCl, 3.3mM KCl, 1.2mM KH₂PO₄, 1mM MgSO₄, 25.5mM NaHCO₃, 10mM D-Glucose, 1.14mM Ascorbic Acid) in a volume of 500nL (n = 5–7 per dose). Doses were chosen based on previous studies [33,34]. Control mice received 500nL aCSF i.c.v.

Novel environment behavioral measures

Activity levels of middle-aged female mice were measured in a novel environment after treatment with 14 μ g/kg IL-1 β (n = 5) or vehicle (n = 5). Mice were injected at ZT11.5 and placed back in their home cage. After 4hrs (ZT15.5), a time when wheel-use and general motion are both low in the home cage, mice were placed in a novel cage of equal size without nesting material under dim red light for 5 minutes to habituate and for a further 15 minutes to assess activity. Activity was recorded using ANY-maze video tracking software (San Diego Instruments, San Diego, CA, USA). Maximum speed was measured as the shortest time required to move one body length over the duration of assessment.

Sleep analysis

To determine if IL-1 β alters sleep-wake behavior, middle-aged female mice (n = 12) were individually placed in a non-invasive Piezo Sleep Sensor Quad Cage (Signal Solutions LLC, Lexington, KY, USA) as described previously [35]. These cages are equipped with a Polyvinylidene Difluoride (PVDF) sensor that detects pressure changes on the cage floor in response to motion. These pressure changes were measured using SleepStatLab acquisition software (Signal Solutions LLC, Lexington, KY, USA), which used a computer algorithm to predict episodic sleep activity every 2–4 seconds based on periodic changes in pressure recorded from the PVDF floor generated by regular breathing observed when mice were sleeping. These predictions of sleep-wake activity correlated strongly with human observations of sleep-wake behavior. Mice could be observed at times to be awake but not moving, and the algorithm correctly distinguished this state of quiet resting from sleep during our observations in pilot studies. Mice were habituated in the sleep cages for 3 days before commencement of the study. Sleep-wake predictions were collected continuously over 5 days to establish a stable baseline. On day 6, mice were injected i.p. 30 minutes before lights off (ZT11.5) with 14 μ g/kg IL-1 β (shown to reduce activity to less than 25% baseline) or 0.9% saline vehicle. Two days after the first injection, all animals were counterbalanced to ensure they had received both vehicle and IL-1 β doses.

Abdominal temperature recordings

Peripherally administered IL-1 β has been previously shown to induce hyperthermic responses in mice under non-thermoneutral conditions [36]. To determine if the doses of IL-1 β that decreased locomotor activity were also inducing hyperthermia, middle-aged females were

surgically implanted with abdominal G2 E-Mitters (Starr Life Sciences Corporation, Oakmont, PA, USA) under isoflurane anesthesia. Mice were administered 0.05mg/kg buprenorphine (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA, USA) and 5mg/kg ketoprofen (Fort Dodge Laboratories, Fort Dodge, IA, USA) analgesics subcutaneously 30 minutes prior to surgery and left to recover for 7 days. After 5 days of baseline, mice were given 14 μ g/kg IL-1 β (n = 11), 16 μ g/kg IL-1 β (n = 4), or vehicle (n = 15) at ZT11.5. Over the course of the experiment, each animal received a total of two injections, one dose IL-1 β and one vehicle, with a minimum of 8 days between treatments. The order of injections was determined randomly. Probe data were detected by ER-4000 receivers placed under each cage and collected in 5-minute bins using the VitalView Data Acquisition System (Starr Life Sciences Corporation, Oakmont, PA, USA). All data were collected at an ambient temperature of 21°C.

Effects of analgesic pre-treatment on IL-1 β -induced fatigue

IL-1 β may trigger pro-inflammatory responses resulting in pain during wheel-running activity [37]. To exclude the possible contribution of pain on wheel-running behavior, middle-aged female mice were pre-treated with the analgesic buprenorphine. Mice were initially recorded for 5 days of baseline activity. On day 6, mice received either buprenorphine/vehicle (n = 10), or buprenorphine/ IL-1 β (n = 5). The analgesic buprenorphine was given at a dose of 0.05mg/kg subcutaneously 60 minutes before lights off (ZT11). Mice were injected with 14 μ g/kg IL-1 β i.p. 30 minutes after buprenorphine pre-treatment (ZT11.5). Control groups received 0.9% saline as vehicle in lieu of drug.

Sucrose preference

Middle-aged female mice (n = 19) were pre-exposed daily to a 4% sucrose solution (ThermoFisher Scientific Inc, Waltham, MA, USA) during the dark phase (ZT12-24) until an >80% preference over water was established. Once pre-exposure was complete, mice were given 14 μ g/kg IL-1 β or vehicle i.p. at ZT11.5 and provided with a 2.5% sucrose solution and water. To eliminate side preference, sucrose placement was randomly divided between preferred and less preferred sides for each animal and switched after 6 hours.

Pharmacological treatments

We tested the effects of two drugs that are occasionally used for the treatment of fatigue [2]. After 5 days of baseline recording, middle-aged females were co-administered with 14 μ g/kg IL-1 β and 150mg/kg modafinil i.p. (Sigma-Aldrich, St. Louis, MO, USA, n = 6) or vehicle (0.25% methylcellulose in saline, n = 7) at ZT11.5. This dose has previously been shown to increase locomotor activity in rodents [38]. In line with the study design by Antle et al., [39], a separate cohort received methylphenidate hydrochloride (0.8–1.6mg/ml, Sigma-Aldrich, St. Louis, MO, USA) in the drinking water during the 5 days of baseline activity and for 24hrs post-IL-1 β given on day 6 at ZT11.5. This concentration range has been previously shown to increase wheel-running behavior in mice [39]. Consumption was measured daily and the dose of methylphenidate (mg/kg) received during IL-1 β -induced fatigue was calculated post hoc. Control animals received either methylphenidate in the drinking water and a vehicle i.p. injection on day 6 (n = 8), or drinking water and 14 μ g/kg IL-1 β i.p. on day 6 (n = 10).

Data analysis

Behavioral activity (wheel rotations and motion around the cage) over the 5 baseline days and treatment day was binned into 30 minute counts and exported as time-series using ClockLab

Analysis Software (Actimetrics Inc., Wilmette, IL, USA). Nocturnal activity following IL-1 β was calculated as a percentage of the mean 5-day baseline nocturnal activity levels. Individual bouts of wheel-running activity were defined as periods with no more than 1 minute of inactivity.

Core body temperature was corrected for activity-dependent heat generation based on methods previously reported [40]. Briefly, core body temperature over the 5-day baseline period is normalized and plotted against activity (as measured by the abdominal G2 E-mitter probe). The slope of a least-squares regression plotted through these data calculated the additional heat generated by the previous 5 minutes of activity. The activity-dependent contribution was subtracted providing an activity-independent temperature change.

Changes in baseline activity with age and pre-treatment with buprenorphine were analyzed by one-way ANOVA with Tukey-Kramer multiple comparisons post hoc tests. The effects of IL-1 β over time, on core body temperature, sleep and sucrose preference were analyzed by two-way ANOVA (time x treatment) with Tukey-Kramer multiple comparisons post hoc test. Analyses of wheel-running bouts, velocity changes and effects of modafinil were performed by two-tailed Student's t-test. The correlation between increasing methylphenidate concentrations and wheel-running activity was determined by Pearson's correlation coefficient. For all experiments the experimental unit was an individual animal.

Results

IL-1 β decreases wheel-running and general locomotor behaviors in a dose-and age-dependent manner

Voluntary use of running wheels declined with age in female C57Bl/6J mice (Fig 1a). Analysis of baseline wheel-running activity by one-way ANOVA showed significant variation between the daily wheel use of young (3–5 months, 756 \pm 49 counts), middle-aged (6–12 months, 560 \pm 43 counts) and aged (18–24 months, 197 \pm 37 counts) female mice ($F_{(5,116)} = 27.09$, $p < 0.0001$).

Increasing doses of IL-1 β did not significantly attenuate wheel-running activity in young female mice within the range tested ($F_{(3,30)} = 1.217$, $p = 0.32$, one-way ANOVA). However, middle-aged and aged females showed sensitivity to IL-1 β starting at 10 μ g/kg (95 \pm 1% decrease from baseline, $F_{(7,75)} = 24.88$, $p < 0.0001$, one-way ANOVA) and 6 μ g/kg (65 \pm 7% decrease from baseline, $F_{(4,52)} = 19.77$, $p < 0.0001$, one-way ANOVA) respectively (Fig 1b). Similar responses to IL-1 β were seen in general locomotor activity of young ($F_{(3,30)} = 0.219$, $p = 0.88$, one-way ANOVA), middle-aged (76 \pm 13% decrease from baseline, $F_{(7,75)} = 16.68$, $p < 0.0001$, one-way ANOVA) and aged (39 \pm 5% decrease from baseline, $F_{(4,52)} = 20.84$, $p < 0.0001$, one-way ANOVA) females over the same dose range (Fig 1c).

Central administration of IL-1 β directly into the lateral ventricle resulted in dose-dependent decreases in wheel-running activity in middle-aged females (Fig 1d). Doses of 10ng and 50ng IL-1 β reduced wheel-running activity by 73 \pm 10% ($F_{(3,18)} = 18.54$, $p = 0.001$, one-way ANOVA) and 99 \pm 0.1% ($p < 0.0001$) respectively. General locomotor activity was also reduced in a dose-dependent manner with 50ng IL-1 β significantly suppressing activity by 88 \pm 8% ($F_{(3,18)} = 8.506$, $p = 0.0007$, one-way ANOVA, Fig 1e).

Middle-aged female mice given 14 μ g/kg IL-1 β 30 minutes prior to lights off show suppressed wheel-running activity throughout their active phase (Fig 2a). Analysis by two-way ANOVA indicated significant effects of time ($F_{(11,902)} = 16.71$, $p < 0.0001$) and treatment ($F_{(2,82)} = 37.07$, $p < 0.0001$) with a significant interaction ($F_{(22,902)} = 5.86$, $p < 0.0001$) on wheel-running activity. Post hoc analysis confirmed IL-1 β -induced changes in wheel-running activity until 12 hours post injection, when baseline activity decreased at the end of the active phase.

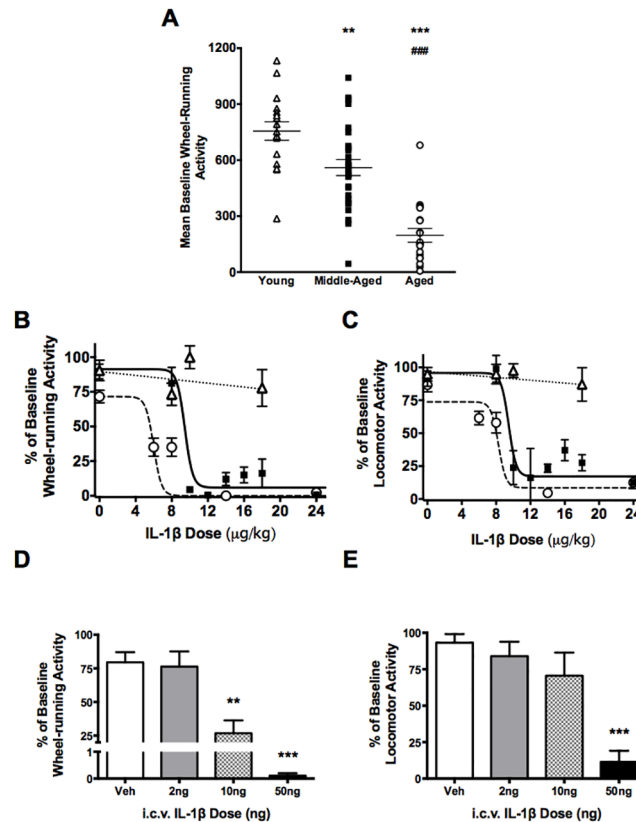


Fig 1. Dose- and age-dependent effects of IL-1MID: locomotor activity. (A) Spontaneous running-wheel use declines with age in female C57Bl/6 mice. Peripheral IL-1 β reduced (B) wheel-running and (C) general locomotor activity in middle-aged (black squares, $n = 2-15$) and aged (white circles, $n = 4-16$) female C57Bl/6 mice. IL-1 β given i.p. had little effect on wheel running in young (white triangles, $n = 4-8$) female mice. IL-1 β given centrally dose-dependently reduced (D) running-wheel and (E) general locomotor activity of middle-aged female mice ($n = 5-7$ per dose). Data shown as the percentage mean \pm SEM 24hr post-treatment activity relative to the mean activity of the previous 5 days ('baseline activity'), ** $p < 0.01$, *** $p < 0.001$ differences compared to young / vehicle, ### $p < 0.001$ differences compared to middle-aged.

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Central administration of 50ng IL-1 β also suppressed wheel-running activity throughout the night (Fig 2b). There were significant effects of time ($F_{(11,132)} = 13.79$, $p < 0.0001$), treatment ($F_{(2,12)} = 36.60$, $p < 0.0001$) and interaction ($F_{(22,132)} = 4.11$, $p < 0.0001$). Multiple comparisons post hoc analysis showed significantly lower activity until 11 hours post injection, when baseline activity decreased.

The suppression of locomotor activity in middle-aged female mice by IL-1 β were further measured in terms of frequency and duration (as indications of stamina) as well as velocity. Mice treated with IL-1 β showed a 66% reduction in the number of wheel-use sessions during the night (11 ± 2 sessions compared to 31 ± 1 sessions during baseline days, $p < 0.0001$, $t = 7.699$, unpaired t-test, Fig 2c). A single session was concluded when there was greater than 1 minute without wheel use. IL-1 β -treated mice also showed a 60% reduction in the duration of wheel-running sessions (4.14 ± 0.4 minutes compared to 10 ± 0.4 minutes during baseline days, $p < 0.0001$, $t = 6.596$, unpaired t-test, Fig 2d) and a 51% reduction in the maximum speed of wheel revolutions (33 ± 6 revolutions/min compared to 68 ± 4 revolutions/min during baseline days, $p < 0.0001$, $t = 5.161$, unpaired t-test, Fig 2e). The effects on speed were not restricted to the home cage and wheel-use. The maximum locomotor speed of mice placed into a novel

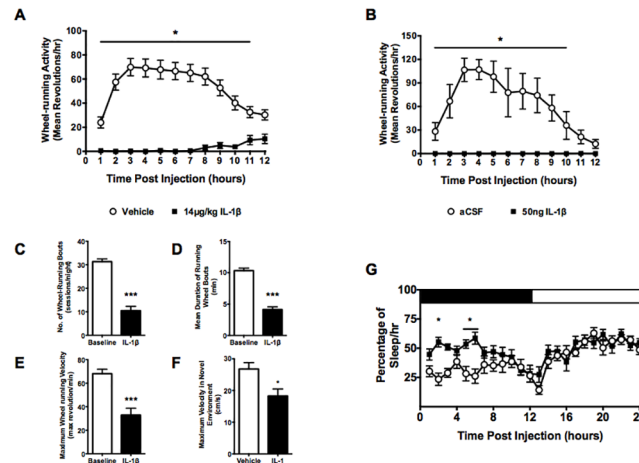


Fig 2. Time course of activity levels in middle-aged female mice. Administration of 14 μ g/kg IL-1 β (black squares, $n = 15$) suppressed (A) wheel-running activity during the active (dark) phase compared to vehicle (white circles, $n = 35$). (B) Similar reductions in wheel-running activity were seen in mice given 50ng IL-1 β i.c.v. ($n = 5$). (C) The total number of wheel-running sessions during the dark period was reduced by IL-1 β , with increased fatigability (measured as decreased bout duration) (D) seen during each session as compared to baseline levels. Individual wheel-running bouts were defined as any period of wheel use with no more than 1 minute of inactivity. Mice showed reduced maximum velocity in their movement, both (E) on the wheel and (F) when placed in a novel environment. (G) IL-1 β transiently increased sleep during the first 6hrs in Piezo Sleep Quad cages ($n = 12$). Bars show mean \pm SEM. * $p < 0.05$, *** $p < 0.001$.

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environment 4 hours after receiving 14 μ g/kg IL-1 β was 32% slower than vehicle treated mice (18 ± 2 cm/s compared to 27 ± 2 cm/s, $p = 0.0218$, $t = 2.841$ unpaired t-test, Fig 2f).

IL-1 β has been previously shown to have somnogenic effects in rodents when administered centrally [41]. To determine whether sleepiness might reduce wheel and locomotor activity, middle-aged females were placed into non-invasive Piezo Sleep Sensor Quad cages [35]. There were significant effects of treatment ($F_{(1, 11)} = 35.17$, $p < 0.0001$), time ($F_{(35, 385)} = 9.592$, $p < 0.0001$) and an interaction of both factors ($F_{(35, 385)} = 2.200$, $p = 0.0002$, repeated measures two-way ANOVA). Post hoc analysis showed that IL-1 β increased the number of sleep-associated recordings as detected by the computer algorithm. This increase was seen at 2 hours ($p = 0.0004$), 5 hours ($p = 0.0159$) and 6 hours ($p = 0.0003$) post injection compared to vehicle controls (Fig 2g). Over the first 6 hours post injection, vehicle control mice spent $29 \pm 2\%$ per hour in computer-scored sleep whereas IL-1 β treated mice spent $52 \pm 2\%$ per hour ($F_{(1, 71)} = 36.73$, $p < 0.0001$). However, there were no significant effects of IL-1 β on computer-scored sleep during the second 6 hours post injection with control mice spending $34 \pm 2\%$ per hour and IL-1 β -treated mice $40 \pm 2\%$ per hour ($p = 0.0952$).

Suppression of locomotor activity was induced independently of hyperthermia, pain or depressive-like symptoms

IL-1 β is a pleiotropic cytokine linked to many sickness behaviors and responses including fever [41], pain [37] and depression [42]. Middle-aged females had lower core body temperature between 2–5 hours after treatment with 14 μ g/kg IL-1 β (-0.4 to -0.7°C) compared to vehicle controls (Fig 3a). Temperature measures were corrected for differences in activity-dependent heat generation since IL-1 β reduces locomotor activity. At 14 μ g/kg, a hypothermic response was seen that was not accounted for by inactivity. These measures were taken in an ambient environment of 21°C and not under thermoneutral conditions. Furthermore, it is possible that the increased sleep occurring during the early night could contribute to the hypothermic

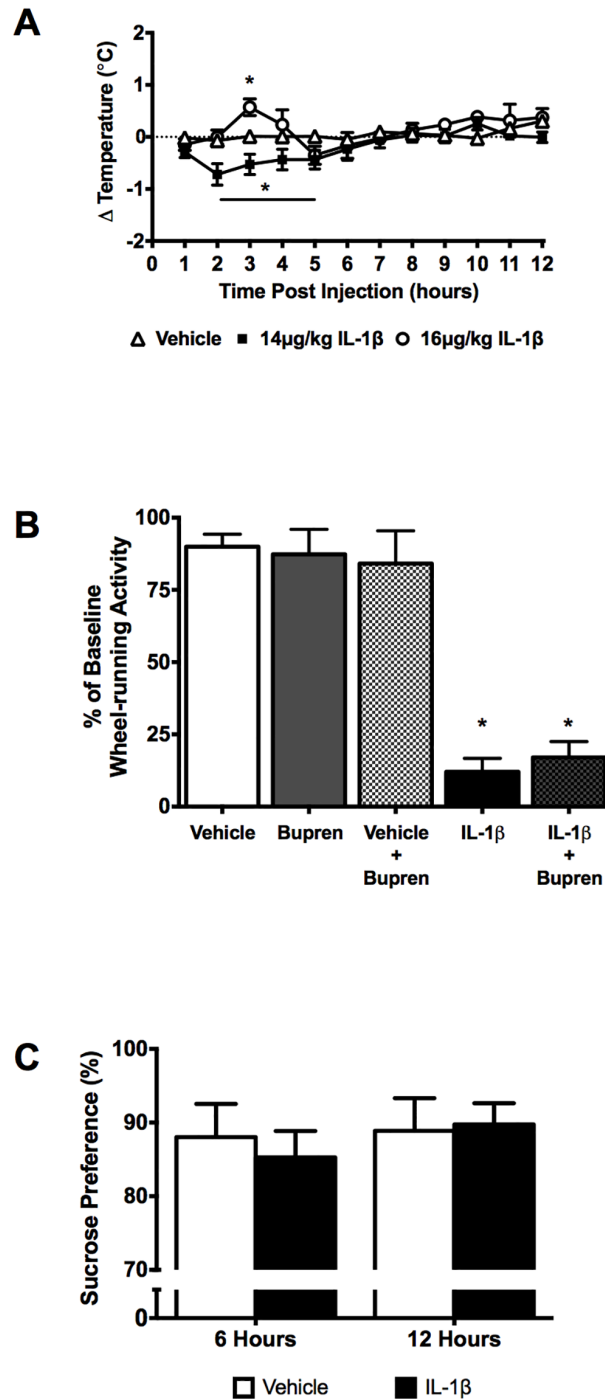


Fig 3. Effects of IL-1 β on sickness responses. (A) Middle-aged female mice given 14 μ g/kg IL-1 β showed mild hypothermic responses after correction for heat generated through activity (n = 11) when measured under non-thermoneutral conditions. However, higher doses of IL-1 β produced transient hyperthermia (n = 4). (B) 14 μ g/kg IL-1 β did not reduce wheel-running activity via systemic pain induction. Pre-treatment with the analgesic buprenorphine (0.05mg/kg) did not alter IL-1 β -induced reductions in wheel-running activity (n = 5). Bars show mean \pm SEM change in wheel-running activity over the 24hr post-treatment as a percentage of the average 5 day baseline activity. (C) Reduced wheel-running activity by IL-1 β was not caused by anhedonia. Sucrose preference was measured 6hrs and 12hrs into the active (dark) phase after IL-1 β treatment, with no changes in preference reported (n = 19). *p<0.05.

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response, as sleep has been shown to reduce core body temperature [43]. Despite this cooler environment, mice administered 16 μ g/kg IL-1 β showed a transient hyperthermic response 3 hours after injection (+0.6°C, $p = 0.0415$), also independent of changes in activity-generated heat. Two-way ANOVA showed significant effects of time ($F_{(11,297)} = 5.932$, $p < 0.0001$), treatment ($F_{(2,27)} = 4.022$, $p = 0.0296$) as well as an interaction ($F_{(22,297)} = 3.018$, $p < 0.0001$).

Subcutaneous administration of the analgesic buprenorphine prior to IL-1 β treatment was given to determine if pain contributed to the suppression of locomotor activity. Buprenorphine with vehicle did not alter running-wheel activity, compared to vehicle alone ($p = 0.9688$, one-way ANOVA) or buprenorphine alone ($p = 0.9988$, one-way ANOVA) (Fig 3b). Administration of 14 μ g/kg IL-1 β significantly reduced wheel-running activity by $88 \pm 5\%$ ($p < 0.0001$, one-way ANOVA), which was not significantly affected by pre-treatment with buprenorphine, resulting in a $83 \pm 5\%$ attenuation by 14 μ g/kg IL-1 β ($p = 0.9955$, one-way ANOVA). Buprenorphine pretreatment prior to IL-1 β resulted in significantly less wheel-running activity than control or buprenorphine alone ($p < 0.0001$).

Sucrose preference over water consumption was measured in vehicle and IL-1 β treated middle-aged mice after 6 and 12 hours post injection as a measure of anhedonia (Fig 3c). Two-way repeated measures ANOVA showed no significant effects of time ($F_{(1,18)} = 1.545$, $p = 0.2299$), treatment ($F_{(1,18)} = 0.06237$, $p = 0.8056$) or a significant interaction ($F_{(1,18)} = 1.001$, $p = 0.3303$) after 6 hours ($88 \pm 5\%$ for control, $85 \pm 4\%$ for IL-1 β treated, $p = 0.5033$) or after 12 hours ($89 \pm 4\%$ for control, $90 \pm 3\%$ for IL-1 β , $p = 0.9324$).

Orexins are not a necessary component of IL-1 β -induced fatigue

Decreased cellular activity of orexin-containing neurons was previously found to be associated with LPS-induced lethargy [27]. To determine if a change in orexin signaling is required to induce fatigue-like behaviors, orexin-deficient mice and their wildtype littermates were given 14 μ g/kg IL-1 β (Fig 4). As percentages of baseline, IL-1 β reduced wheel-running activity to comparable levels in both wildtype and orexin deficient mice (Fig 4b). However, when comparing total counts of baseline activity, wildtype females showed significantly more nocturnal use of the running wheel than orexin deficient mice (580 ± 56 counts in wildtype, 146 ± 36 counts in knockouts, $p < 0.0001$, $t = 6.268$, unpaired t-test). Middle-aged female wildtype littermates showed significant effects of time ($F_{(11,429)} = 22.99$, $p < 0.0001$), treatment ($F_{(2,39)} = 29.94$, $p < 0.0001$) and interaction ($F_{(22,429)} = 7.187$, $p = 0.0001$) with decreased wheel-running activity for the first 9 hours post injection (Fig 4c). Middle-aged orexin-deficient females also showed significantly decreased wheel-running activity after IL-1 β treatment during the first 6 hours (Fig 4d), with significant effects of time ($F_{(11,363)} = 2.610$, $p = 0.0033$) and treatment ($F_{(2,33)} = 6.554$, $p = 0.0040$) with the interaction term not making our cut-off of $p < .05$ but perhaps indicating a trend in that direction ($F_{(22,363)} = 1.504$, $p = 0.0687$).

Current pharmacological treatments have limited success in alleviating fatigue

Among the few pharmacological options available for patients suffering from fatigue with no identifiable cause are the stimulants modafinil and methylphenidate, which have limited success at alleviating the symptom [44,45]. Modafinil given alongside IL-1 β did not improve the reduced wheel-running activity caused by IL-1 β alone ($13 \pm 9\%$ of baseline compared to $6 \pm 3\%$ by IL-1 β alone, $p = 0.4035$, $t = 0.8689$, unpaired t-test) (Fig 5a). Analysis of the time course by two-way ANOVA (Fig 5b) confirmed a significant treatment effect of IL-1 β ($F_{(2,21)} = 85.10$, $p < 0.0001$) and interaction with time ($F_{(22,231)} = 4.013$, $p < 0.0001$) but no main effect of time

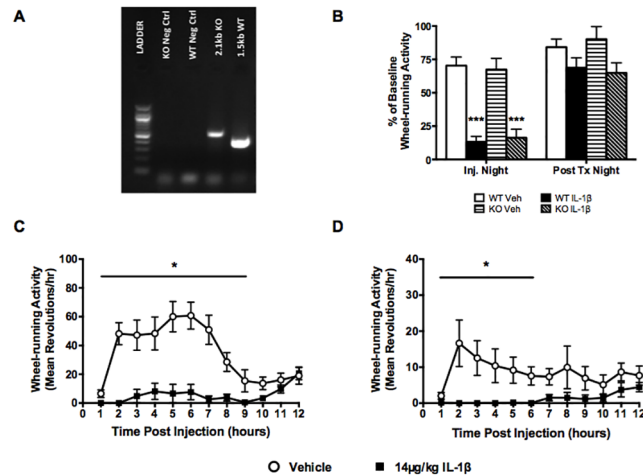


Fig 4. Orexins are not necessary to mediate IL-1 β -induced reductions in activity. (A) Representative gel of genotyping results, distinguishing the wild-type allele (1.5kb) from the mutant prepro-orexin knockout allele (2.1kb). (B) 14 μ g/kg IL-1 β reduced wheel-running activity in (C) wild type and (D) prepro-orexin knockout littermates to similar levels of baseline (n = 12–14). Data expressed as mean \pm SEM, *p<0.05, **p<0.01, ***p<0.001.

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($F_{(11,231)} = 1.643$, $p = 0.0879$). Post hoc analysis confirmed no improvement with modafinil co-administration.

Methylphenidate added to the drinking water provided a self-medicating regime. Daily dose was variable across subjects, dependent on both drug concentration and individual consumption of water (Fig 5c). When binned as 25mg/kg/day increments, one-way ANOVA confirmed a significant effect of treatment with 125-150mg/kg/day ($22 \pm 11\%$ compared with $6 \pm 2\%$ for untreated fatigued mice, $F_{(5,25)} = 2.928$, $p = 0.0325$). Analysis by Pearson’s correlation results in

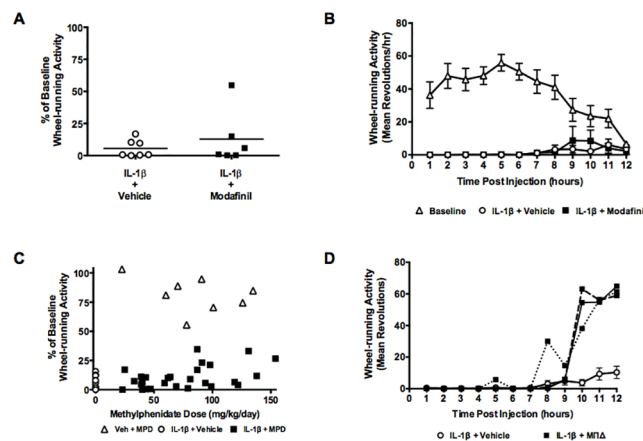


Fig 5. Effects of stimulants on IL-1 β -induced reductions in locomotor activity. (A, B) Pre-treatment with 150mg/kg modafinil did not significantly attenuate the effects of IL-1 β on wheel-running in middle-aged females (n = 6–7). (C) Increasing doses of methylphenidate, provided in the drinking water was correlated with increased wheel-running activity after IL-1 β . (A-C) IL-1 β + vehicle (white circles), IL-1 β + treatment (black squares), methylphenidate + vehicle (white triangles). (D) Example time courses of wheel-running activity for three mice, dosed with methylphenidate, with post-IL-1 β activity levels greater than 25% of baseline. IL-1 β + vehicle (white circles), IL-1 β + methylphenidate (black squares), different line styles indicate different animals.

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a coefficient $r = 0.4619$, $p = 0.0040$, suggesting that with increasing concentrations of methylphenidate, wheel-running activity was partially restored in some animals. Hourly time courses for these individuals indicate earlier restoration of activity towards the end of the night, approximately 10hrs post injection (Fig 5d).

Discussion

Our characterization of the effects of different doses of IL-1 β in mice of different ages exemplifies the critical importance of these variables in studies of cytokine-induced suppression of locomotor activity in mice. Young mice were uniformly resistant to these effects in the doses tested. After this initial characterization, we chose to conduct the bulk of our further studies with middle-aged (6-12mos) female mice. With this standardization of the subject characteristics, dose becomes the defining variable that determines if the mouse shows effects solely on locomotor activity, or (as dose is increased) if the mouse also shows other aspects of sickness behaviour, such as hyperthermia. We expect that with higher doses IL-1 β would induce the full range of sickness behaviors, and a key contribution of our work is the characterization of the dose-response curve for effects on locomotor activity. Our initial work followed prior studies [13,46,47] in administering a set nanogram amount of IL-1 β per animal, but our analysis and later studies demonstrated that we are better able to predict responses when the dose is adjusted for the animal's body weight. When given 14 μ g/kg IL-1 β , middle-aged females showed reduced locomotor activity throughout the night. Using the piezo sleep quad cage system, sleep-associated changes in breathing and motion were observed in the first half of the night; our methods cannot distinguish individual sleep stages, but IL-1 β has been previously reported to increase non-REM sleep [48]. We did not see other significant effects of this dose of IL-1 β such as pain-induction (as determined by pre-treatment with an analgesic) or depressive-like symptoms (assessed through sucrose preference) that may otherwise have accounted for the decrease in locomotor activity (Fig 3b and 3c)[49,50]. However, it is important to note that these effects might be present at a level that our tests were not sensitive enough to measure, and that with increasing doses of IL-1 β , these symptoms would be expected to emerge alongside the reductions in locomotor activity. Neuroinflammation is common in many neurological disorders that share the symptom of reductions in locomotor activity [5]. We have shown here that direct administration of IL-1 β into the brain may suppress behavioral activity in a similar dose-dependent manner as that produced by peripheral IL-1 β , supporting a centrally mediated mechanism of induction [46,51].

Fatigue, as described by patients, is a multifaceted symptom, with cognitive and emotional aspects as well as reductions in locomotor activity [52]. To investigate fatigue using laboratory animals, other researchers have also found measures of wheel-running useful [53–55]; it remains to be determined if these lab animals express cognitive and/or emotional aspects of fatigue reported in human populations. IL-1 β treated animals manifest not only an overall reduction in wheel-use, but changes in the pattern of wheel-use, including shorter bouts of exertion (Fig 2c–2f). These changes are common measures of increased fatigability, an important aspect of the symptom of fatigue [2].

A key rationale for these studies was to develop a method to allow determination of neurobiological mechanisms underlying inflammation-induced reduction in locomotor activity. Based on prior research suggesting a critical role for the orexin-expressing lateral hypothalamic neurons [7,27,28], we predicted that orexin-deficient mice would not show reduced locomotor activity following administration of IL-1 β . We replicated prior findings that the prepro-orexin knockout mice have reduced wheel-use [26]; yet, despite this lowered basal level, when given IL-1 β these mice showed further reductions in wheel-use, similar in proportion to that shown by wildtype littermates. Therefore, our experiment suggests that orexins are not, by themselves,

a necessary component in IL-1 β cytokine signalling decreased locomotor activity. On the other hand, it remains possible that these lateral hypothalamic neurons may play a role in signalling inflammation-induced changes in locomotor activity. Inflammation and chemotherapy-induced lethargy was associated with reduced cellular activity within orexin-containing neurons of the lateral hypothalamus [7,27,28]. These neurons are still present in the orexin-deficient mice used in the current study, and have been shown to contain several other neurotransmitters including dynorphin and glutamate [56,57], that may be involved in inflammation-induced lethargy. Our experiment has not ruled out an orexin-independent role for these neurons, nor a non-essential role for orexins in mediating reduced locomotor activity. These experiments have demonstrated that IL-1 β can induce suppression of locomotor activity even in mice that are more sedentary, as inferred by the baseline reduced wheel use.

In general, current medications produce little improvement in fatigue. We tested two commonly prescribed stimulants, modafinil and methylphenidate, tested for effect in patients with either Parkinson's Disease suffering from fatigue [2], or cancer-related fatigue [2,58]. Patients being treated with modafinil typically report improvement in excessive daytime sleepiness, measured by the Epworth Sleepiness Score, but no improvement in self-reporting of subjective fatigue (reviewed by [59]). We found no change in IL-1 β -induced decreased locomotor activity with co-administration of modafinil. We have not determined whether modafinil attenuated IL-1 β -induced increases in sleep, but our data concur with clinical findings that modafinil is ineffective in treating fatigue of this extent [45,60]. One study of cancer-related fatigue showed modafinil improved self-reported fatigue scores, but only in patients classified as having severe fatigue prior to the start of the study, with no improvement in mild or moderate patients [61]. The second treatment we tested was methylphenidate. Methylphenidate has been shown to improve self-report scores of fatigue in patients with Parkinson's Disease [62] and cancer-related fatigue [58,63], with dose-dependent effects [64]. In the current study, animals were exposed to methylphenidate in the drinking water, allowing them to self-medicate throughout the night after IL-1 β -administration, limiting the need for repeated handling. Higher doses of methylphenidate significantly increased wheel-running activity after IL-1 β . Animals responding to methylphenidate showed earlier recovery of wheel-running activity, but improvement with methylphenidate remained at less than 40% of baseline activity. A meta-analysis of methylphenidate use with cancer-related fatigue indicates improved benefits with longer exposure [44], offering an alternative approach for future investigation.

Methylphenidate is a stimulant that acts through blocking the reuptake of dopamine and norepinephrine, particularly relevant here in the prefrontal cortex and striatum [65,66]. There is some evidence suggesting a role for dopamine and deficits in reward signals playing a role in self-reported fatigue [31,67–69], and in effort-based tasks in laboratory animals [30]. Dopamine D₂ receptor antagonists reduce effort-based reward-seeking behaviors, which can be alleviated in part by an adenosine A_{2A} antagonist [70]. Recent work by Nunes et al. [71] has shown that IL-1 β can decrease effort-based motivational responses, which are restored by treatment with the same adenosine A_{2A} antagonist, hypothesized to be acting through potentiating dopamine signalling. Research using animal models of chronic pain indicates that changes in motivation to perform effortful tasks, a component of fatigue, is mediated in part by the neuropeptide galanin driving neural plasticity in the dopamine D₂ receptor-expressing nucleus accumbens neurons [72]. In addition to dopamine, changes in histamine and serotonergic signalling may also contribute to the mechanism of immune-mediated decreased locomotor activity. Gaykema et al. [73] described a reduction in several motivated behaviors in rats following LPS. These reductions were associated with attenuated c-fos induction within histaminergic neurons of the ventral tuberomammillary nucleus. Katafuchi et al. [74] reported increased expression of the serotonin transporter, together with reduced extracellular serotonin within

the prefrontal cortex following treatment with Poly I:C, suggesting a role for the 5HT_{1A} in mediating aspects of immune-activation reduced locomotor activity.

General comments

Fatigue is predominantly a subjective sensation, but it is accompanied by behavioural changes that should be able to be studied in mouse models. Fatigue is most prevalent in middle-aged women, and much of our research focuses on middle-aged female mice. Still, fatigue occurs in people of various ages and genders [75], and mouse models should thus examine mice of various ages and both sexes as the underlying mechanisms may vary. For example, immune system responsiveness changes with age; increased levels of circulating cytokines in the serum are found with age. Life history can impact these responses, as is shown from studies detailing how prior immune challenges can alter the immune system, for example, “priming” an exaggerated response to IL-1 β [76]. Neuro-inflammatory mechanisms for pain sensitivity vary between sexes [77], as may similar pathways underlying fatigue. Individuals vary in their susceptibility to fatigue, and we expect genetics would play a role as well. Studies using just one inbred strain of mice, as ours did, limits variation due to genetics but runs the risk of missing samples from individuals within the larger population that are more sensitive to these effects. Further studies using mice could test outbred strains.

We focused on responses to IL-1 β , given evidence linking this cytokine to fatigue. There is also good evidence linking several other cytokines to fatigue. For example, leucocytes from cancer patients with post-treatment fatigue showed increased expression from both IL-1 β and IL-6 genes [78]. Patients with chronic fatigue syndrome/myalgic encephalitis show increases in both IL-1 β and IL-6 [79]. In a sample of cancer patients prior to surgery, fatigue correlated with levels of IL-6 [80]; another study reported that fatigue that follows cancer treatment correlated with levels of IL-6 [23,81]. Cancer patients that show improvement in fatigue show decreases in IL-6 [82]. Thus, further studies might examine IL-6 as a potential cytokine that can alter behaviour of laboratory animals in a manner related to these patient populations. Models using different approaches to induce inflammation might allow insight into specific mechanisms able to mediate changes in behaviour and motivation as might underlie the experience of fatigue.

The need for an animal model for research related to fatigue is clear, given our lack of insight into the underlying biology and the lack of effective treatments. Here, we have ruled out a necessary role for the orexins in the IL1-induced decrease of locomotor activity. We have also demonstrated that methylphenidate treatment allowed some improvement in wheel-running activity, suggesting a possible role for dopaminergic signalling. While the precise mechanisms and brain areas involved in mediating fatigue remain unclear, the development of new tools that allow for its study will help address these questions in the future.

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Author Contributions

Conceived and designed the experiments: DRB HK CT AN AP GM-C TES MEH. Performed the experiments: DRB HK CT AN AP GM-C PCM. Analyzed the data: DRB HK CT AN AP GM-C MEH. Contributed reagents/materials/analysis tools: TES. Wrote the paper: DRB PCM TES MEH.

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