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10.1016/j.pbb.2021.173311

Link to publication record in Explore Bristol Research
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Do greater levels of in-cage waking inactivity in laboratory mice reflect a spontaneous depression-like symptom? A pharmacological investigation.

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Abstract

We previously identified in laboratory mice an inactive state [being awake with eyes open motionless within the home cage; inactive but awake, ‘IBA’] sharing etiological factors and symptoms with human clinical depression. We further test the hypothesis that greater time spent displaying IBA indicates a depression-like state in mice by investigating whether the antidepressant Venlafaxine, environmental enrichment, and their combination, alleviate IBA. Seventy-two C57BL/6J and 72 DBA/2J female mice were pseudo-randomly housed post-weaning in mixed strain-pairs in non-enriched (NE; 48 pairs) or in environmentally enriched (EE; 24 pairs) cages. After 34 days, half of the mice housed in NE cages were either relocated to EE cages or left in NE cages. For each of these conditions, half of the mice drank either a placebo or the antidepressant Venlafaxine (10mg/kg). The 48 mice housed in EE cages were all relocated to NE cages and allocated to either the placebo (n=24) or Venlafaxine (n=24).

IBA data were collected prior to and after environmental adjustment by trained observers blind to the pharmacological and environmental adjustment treatments. Data were analyzed using GLM models. NE cages triggered more IBA than EE cages (Likelihood-Ratio-Test Chi$^2$=53.501, p<0.0001). Venlafaxine and environmental enrichment appeared equally effective at reducing IBA (LRT Chi$^2$=18.262, p<0.001), and combining these approaches did not magnify their effects. Enrichment removal triggered IBA increase (LRT Chi$^2$=23.050, p<0.001), but Venlafaxine did not overcome the increase in IBA resulting from enrichment loss (LTR Chi$^2$=0.081, p=0.775). Theoretical implications for putative depression-like states in mice, and further research directions, are discussed.

Keywords: depression; mice; waking inactivity; behavioral indicator; Venlafaxine; environmental enrichment
1. Introduction

In humans, clinical depression is a common and debilitating mental illness, diagnosed by the co-occurrence of several affective, cognitive, behavioral and homeostatic symptoms, that can include a low mood, anhedonia, sleep and weight changes, psychomotor retardation or agitation, fatigue, feelings of worthlessness or guilt, difficulties in thinking or concentrating, and recurrent thoughts of death or suicide [1, 2]. Clinically depressed patients are also less active and engage in fewer social and non-social activities than their non-depressed counterparts e.g. [2-4]. Depressed people also show cognitive changes, including affect-related negative biases in information processing, e.g. they judge ambiguous situations more negatively e.g. [5, 6], as well as 'learned helplessness'. Learned helplessness is a 'giving-up' phenomenon, occurring when a desired outcome is perceived as being unlikely and negative outcomes as probable, regardless of one's action [7]. With regards to etiology, chronic stress commonly triggers depression, especially in vulnerable individuals with predisposing genetic factors and/or aversive experiences in early life e.g. [2, 8-10].

The symptoms and causes of depression are not unique to humans [11-13], and some depression-like features have been modelled in non-human animals (hereafter animals) for decades e.g. [14, 15], although a full diagnosis involving the co-occurrence of depressive criteria has yet to be confirmed in non-human animals [13]. Validated through their induction by stressors and alleviation by anti-depressants, these include anhedonia e.g. [16] and learned helplessness e.g. [17, 18]. Animals under chronic stress, such as those living in barren, socially and/or spatially restricted non-preferred environments, can also show negative ('pessimistic') judgements of ambiguity, just as with depressed patients [19, 20].

Considering the potential negative welfare implications of depression-like states for animals, further testing of the hypothesis that they can show housing-induced spontaneous depression-like states is worthwhile. Moreover, the ability to detect depression-like states in

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2 A term by which we mean 'major depressive disorder' or having 'depressive episodes’, to encompass Diagnostic Manual of Mental Disorders 5th edition & International Statistical Classification of Diseases & Related Health Problems terminologies.
animals used in research is important because such states may invalidate research where stress and depression are not of interest (violating the 3Rs Reduction [21], e.g. by altering neurological and other disease models with potential effects on processing of drugs/therapies that may lead to inaccurate information on bioavailability, dosage per body weight, toxicity profiles etc.) [22]. Conversely, validated measures of spontaneously occurring depression-like states in animals may represent new back-translational models of human depression [23].

If animals, or at least some animal species under certain conditions, can show states analogous to clinical depression, potential markers of such states should co-vary with a range of symptoms in animals similar to those described in clinically depressed people. Moreover, for animal and human illnesses to be deemed homologous, the processes by which individuals enter, and are cured from this state, must share similarities e.g. [23]. We propose that, in laboratory mice *Mus musculus*, greater time spent displaying a specific form of waking inactivity, *i.e.* being inactive but awake ‘IBA’ in the home cage during the active phase, might indicate a depression-like state. First, mice displaying greater IBA show symptomatic similarities with features of human depression. This includes elevated inactivity that may reflect psychomotor retardation [13, 24]; reduced preference for sucrose (Trevarthen *et al.* in preparation), *i.e.* a common proxy for anhedonia in animal model of depression; greater immobility in forced swim tests, *i.e.* a modelled feature of helplessness ([25], replicated in [26]), as well as homeostatic symptoms such as weight (increased Body Mass Index, [13]) and sleep changes (increased sleep during the active phase, [13], replicated in Trevarthen *et al.* in preparation). Furthermore, greater levels of IBA are triggered by some of the risk factors causing clinical depression, such as exposure to chronic stress (*e.g.* housing in relatively barren, non-preferred cages), and even more so in genetically vulnerable individuals. The current study aims to test further the hypothesis that greater time spent displaying IBA indicates a depression-like state in mice, by testing common curative factors between IBA and clinical depression, *i.e.* whether IBA levels are alleviated in mice by treatments clinically efficient in humans.
Therapeutic approaches to clinical depression in humans involve pharmacological interventions (of which antidepressant drugs are the first-line), non-pharmacological interventions (e.g. psychotherapy, education or disease management programmes promoting activities that encourage health and well-being), or their combination (especially for moderate and severe depressions) e.g. [27-31]. We therefore investigated whether IBA is alleviated by chronically administrating an antidepressant (Venlafaxine), by providing life conditions which promote better well-being (environmental adjustment to preferred, highly enriched, larger cages), and whether combining these therapeutic approaches magnifies their effects. Venlafaxine is a serotonin-noradrenaline reuptake inhibitor antidepressant that is commonly used to treat clinically depressed people e.g. [29, 31, 32]; that reverses depression-like features in mice e.g. [33, 34]; and the use of which supports Refinement, since clinically effective doses of the drug can be diluted in appetent solutions and administrated orally to the rodents (i.e. mice can be trained to drink from a syringe rather than being injected, [35]). Environmental adjustment in this study involved relocating mice from relatively barren ‘shoebox’ cages to larger cages with a varied set of enrichment items biologically relevant to mice e.g. [36]. Mice prefer, and are willing to pay a cost (e.g. push weighted doors) to access larger, highly enriched cages, which are also associated with signs of enhanced welfare and improved functioning such as reduced levels of stereotypic behaviors and aggression, strengthened immunity, longer life spans, and enhanced breeding performance [22, 37-41]. Since genetic predispositions can influence the development of depression-like states, we used C57BL/6J and DBA/2J mice, two common laboratory strains that differ in their tendencies to display both IBA and depression-like features [25]. C57BL/6Js are more prone to display depression-like profiles e.g. [25, 42, 43], although phenotypes may vary between laboratories e.g. [44], and greater IBA was observed in DBA/2Js in [45].

We predicted that IBA levels would be reduced by administrating Venlafaxine, by providing environmental enrichment, and that combining these approaches could magnify the effect on IBA (expecting the two strains not to differ in their reactions to these three treatment interventions). Additionally, IBA in mice has been shown to rise following environmental
enrichment removal from the home cage, compared to constant housing in relatively barren environment [45]. In rats [46], starlings [47] and pigs [48], removing environmental enrichment can induce ‘pessimistic’ judgements of ambiguity, a cognitive feature associated with human depression and used as a proxy for negative affective states in animals. We therefore tested a second prediction: that the antidepressant Venlafaxine would prevent the rise in IBA following environmental enrichment removal.

2. Methods

2.1. Ethical statement

This study was conducted from April to June 2019 at the University of Bristol under the UK Home Office Project License # P2556FBFE. The study design and the animal use and care were in accordance with the UK Animals (Scientific Procedures) Act 1986 (ASPA), the EU directive 2010/63/EU, the UK Home Office code of practice for the housing and care of animals bred, supplied, or used for scientific purposes, and the ARRIVE guidelines. All animals were regularly weighed and monitored throughout the study for any health issues (none observed at any phase of the study). At the end of the study, the mice were euthanized by skilled technicians (using concussion, immediately followed by cervical dislocation and confirmation of death; the use of both methods ensured rapid loss of consciousness).

2.2. Subjects and husbandry

In total, 72 female C57BL/6J (‘C57’) and 72 female DBA/2J (‘DBA’) mice Mus musculus (Charles River, France), equally spread across two batches of 36 C57s and 36 DBAs each, were studied. In humans, depression is more prevalent in women (1.5- to 3-fold higher rates [2]), so we focused on female mice following the recent urge to limit sex bias in neurosciences and biomedical research [49, 50] (although we would predict elevated levels of IBA to reflect
depression-like states in males as well; see discussion). Mouse estrous cycle was not assessed, since all the symptoms of clinical depression (except #9, related to suicidality) are expected to be present ‘nearly every day’ ([2] pp.160-161). Furthermore, assessing the estrus cycle comes with welfare costs to the mice, so this could conflict with Refinement, and not offer significant benefits, considering that recent reviews and meta-analyses have demonstrated that female rodents tested at random points in their estrous cycles are not more variable than males e.g. [49, 51]. Animal numbers were estimated, based on power analyses using data from [25] (power 80%, significance criterion 2-tailed 0.05, effect size 2%, replicated using several calculators). The mice were three-four weeks old on arrival at the laboratory. Upon arrival, each mouse was weighed and one C57 (black) and one DBA (brown) mouse of similar weight (+/- 2.5g) was pseudo-randomly allocated to each cage (mouse selected at random from mice of similar weights and allocated to the cage with a weight-matched cage-mate). This mixed-strain pair housing, immediately post-weaning, enabled individual identification within pairs without impairing mouse welfare, nor impacting each strain’s specificities [52, 53], whilst removing the need for invasive marking procedures. The pairs of mice were housed either in non-enriched shoebox cages (‘NE’, n = 96 mice) or in larger, environmentally enriched (‘EE’, n = 48 mice) transparent cages (Techniplast). Cages sizes and environmental enrichment provided are part of the experimental treatments and are detailed below in the housing condition section.

All cages were housed in a single colony room within three Scantainers (Scanbur BK), in a pseudo-random order that ensured an equal spreading of the experimental treatments between the three Scantainers, and within shelves for each Scantainer. NE cages were cleaned every week; EE cages were left undisturbed, since the treatment maximal housing duration in these cages did not exceed their routine cleaning frequency (every 4 to 5 weeks). All mice housed in NE cages were handled by the tail (as part of the experimental treatment), whilst EE mice were handled using their home-cage Perspex handling tunnel, following a validated method shown to reduce stress in laboratory mice [54]. Food (LabDiet) and water were available ad-libitum, and animals were kept under a 12hr reversed light-dark cycle (lights
on 1900-0700). Temperature (mean ± standard error 20.9± 0.5°C) and relative humidity were controlled at the room level (Scantainers used as shelving units with doors open) and were checked daily.

2.3. Experimental design (Figure 1)

On arrival, mice were pseudo-randomly allocated to either a NE or EE cage as described in section 2.2. NE cages (37cm L x 21cm W x 14cm H) contained sawdust (IPS), one handful of basic nesting material, a small piece of cardboard (approximately 8cm L x 8cm W) for gnawing and a transparent polycarbonate handling tunnel (13cm L, Ø 5cm, Datesand). The larger (44cm L x 34cm W x 20cm H) EE cages contained sawdust (IPS), three types of nesting material (basic nesting material, Sizzlenest, Datesand, and two cocoon nesting, Datesand), two nestlet squares (Ancare, USA) and tissues (Waitrose Basics or Kleenex), a plastic transparent shelter (Ø 15cm, 5.5cm H, Biopac UK), a red igloo with a running wheel (fast-trac, Datesand), a transparent polycarbonate handling tunnel (13cm L, Ø 5cm, Datesand), a flexible transparent plastic tunnel (Ø 6cm, 30cm L) which was attached to the cage lid, three aspen gnawing blocks (two small: 5cm L x 1cm W x 1cm H, one large: 10cm L x 2cm W x 2cm H, Datesand), a small pine cone, a hammock (roughly 12 cm x 12 cm) made from cotton attached to the lid, a sisal rope ladder, and one half of a coconut shell (12.7cm L x 7.6cm W x 5cm H, Little Cherry Ltd) attached to the lid with sisal rope. A millet spray (Pets at Home) was attached to the lid of each cage. From day 5 to day 13 post-arrival, the mice (irrespective of their experimental treatment) were habituated to two empty syringes being inserted through the cage lid and left for 3-4 hours, then individually trained within their home cage to consume 0.1ml dilute condensed milk (Nestlé Carnation Sweetened Condensed Milk) diluted at a ratio of 2 parts milk, 3 parts distilled water from a 1ml syringe (6-8 sessions). Mice were fed from syringes simultaneously within each cage to avoid competition between individuals.

2.3.1. Pre-environmental adjustment phase (Day 14 to Day 34 post-arrival)
Figure 1. Timeline of the experiment.

The mice remained in their original housing condition during this pre-environmental adjustment phase. Venlafaxine (or placebo) treatment started on Day 14. No therapeutic effect of Venlafaxine was expected to happen at this stage, since antidepressants commonly show delayed onset of actions, and their effects are usually evident after two to four weeks e.g. [29, 55]. Starting the chronic Venlafaxine administration during the 3-weeks pre-environmental adjustment phase, however, allowed us to match the timing of the pharmacological and non-pharmacological interventions happening during the subsequent phase. For each of the housing conditions (i.e. NE or EE), half of each strain of mice were randomly allocated to either a placebo \((n_{\text{in NE cages}} = 48 \text{ mice}, n_{\text{in EE cages}} = 24 \text{ mice})\) or the antidepressant Venlafaxine treatment \((n_{\text{in NE cages}} = 48 \text{ mice}, n_{\text{in EE cages}} = 24 \text{ mice})\). Mice in the same cage received the same treatment. Mice in the placebo group continued to orally receive 0.1ml condensed milk \((\text{Nestlé Carnation Sweetened Condensed Milk})\) diluted at a ratio of 2 parts milk, 3 parts distilled water and administered within the home cage from a 1ml syringe. Mice in the Venlafaxine group were orally administered (following [35]) Venlafaxine Hydrochloride \((Tocris, Bio-Techne)\).
Ltd, UK) according to mouse weight at a concentration of 10mg/kg. Mice were weighed weekly in advance of preparing the dose to ensure the target concentration was met as closely as possible. The Venlafaxine dose was mixed with a vehicle of dilute 0.1ml condensed milk at a ratio of 2 parts milk, 3 parts distilled water and was stored in a fridge at -4°C (dose preparation happening on Mondays for Monday and Tuesday doses, on Wednesdays for Wednesday and Thursday doses, and on Fridays for Friday, Saturday and Sunday doses), before being administered orally within the home cage from a 1ml syringe as during the training phase. Mice were still fed from their own syringe (to ensure correct dosage based on weight) simultaneously within each cage to avoid competition between individuals. The experimenters who were feeding the mice were blind to the syringe content (placebo or Venlafaxine). At the time of the delivery, all mice were carefully observed for any spills. Spills were minor (only one drop) and only happened 206 times out of 5184 administrations (4%) in total (of which 93 were in placebo- and 113 in Venlafaxine-treated mice). Once each mouse had been fed their individual dose (at least one hour later), the mice (regardless of their placebo or Venlafaxine treatment) were given a second untainted (unmedicated) dose containing 0.1ml dilute condensed milk, to avoid the Venlafaxine treatment groups developing a conditioned aversion to the milk (e.g. in case Venlafaxine induced nausea). Mice were observed live immediately after dosing (the researcher standing for ~1 minute in front of the cages screening each mouse) and again at least 1.5-2 hours following syringe contents administration to ensure no adverse effects occurred. Adverse effects were defined as endpoints in the ethical approval #P2556FBFE as per the UK Home Office health assessment criteria, and included screening for piloerection, pale color to ears, nose, and feet, hunched posture, eyes partially closed, unresponsiveness to the observer (e.g. the mouse does not interrupt its behavior to monitor its environment, e.g. no whiskers twitching, no air sniffing), aggressiveness to the cage mate, or any other features feeling atypical to the observer. Dosing continued daily for the remainder of the study during the same time period each day (13:00-15:30). Neither reluctance to drink from the syringes nor adverse effects were observed at any phases of the study.
2.3.2. Post-environmental adjustment phase (day 35 post-arrival to day 60, end of the experiment)

On day 35, half of the mice housed in NE cages were either relocated to EE cages or left in NE cages, balancing for pharmacological treatment (n_{NE-EE placebo} = 24 mice, n_{NE-EE Venlafaxine} = 24 mice, n_{NE-NE placebo} = 24 mice and n_{NE-NE Venlafaxine} = 24 mice). No EE-EE group was included in this study because previous works have robustly shown that this environment triggers less IBA than NE cages [25, 26, 45], and our primary interest here was triggering IBA behavior in order to investigate the effect of our interventions on IBA. All cages of mice were transferred to a clean cage regardless of whether their environment was being altered. The Perspex handling tunnel was transferred to the new cage to keep a familiar scent and aid transition. The 48 mice housed in EE cages (of which 24 were from the placebo and 24 from the Venlafaxine treatment) were all relocated to NE cages. The pharmacological treatment given to each mouse remained the same as the treatment given during the pre-environmental adjustment phase, with dosing continuing daily as above described in 2.3.1. until day 60 (end of the experiment).

2.4. Behavioral scan sampling

The behavior relevant to the hypothesis under investigation was being IBA, defined as ‘mouse motionless, muzzle in sight and eyes open, for at least 3s’ (adapted from [25, 26]). Behavior was recorded during the dark (active) phase, under red ambient light via live scan-sampling [56], switching from scan to 3s focal sampling to allow for differentiation between behaviors characterized by a lack of movement (e.g. IBA versus sleeping) as in e.g. [25]. The 3s cut-off duration was predetermined by an independent experimenter who took focal behavior observations from a different cohort of mice (Trevarthen et al, in preparation). Red light filters were superimposed on any recording device screens.
Behavioral scan sampling began on day 14 at the start of the pre-environmental adjustment phase. During the first three weeks of sampling for the first batch of mice, two experimenters were present during each scan to simultaneously record the behavior of each mouse to ensure reliability between observers. Since the observers reached a high degree of agreement (Cohen’s Kappa value: 0.85), all subsequent scan samples were split between two experimenters. Observations always took place during two 90-minute time blocks (block 1: 9:30-11:00, block 2: 11:00-12:30) and during each time block 8 scans were recorded per mouse. During the pre-environmental adjustment phase, behavior samples were taken over 3 consecutive weeks, and a further 3 weeks of observations were conducted during the post-environmental adjustment phase. We aimed to collect as many behavior samples as possible throughout the experiment, but due to time constraints, the number collected per mouse per week varied, giving a total of 15552 scan samples during the pre-environmental adjustment phase and 18432 during the post-environmental adjustment phase.

2.5. Statistical analyses

Data were analyzed in R Studio version 1.1.456 using the nlme package. We constructed general linear mixed models with random intercepts, but fixed slopes. The proportion of IBA (whilst in view during scan sampling) performed by each individual mouse during each phase was calculated and used as the dependent variable (unless stated otherwise below). Within each model, individual mouse was included as a repeated random effect.

We conducted analyses in three steps. First, we analyzed data from the pre-environmental adjustment phase only, in order to replicate previous results showing that NE cages trigger greater times spent displaying IBA compared to EE cages e.g. [25, 26, 45], therefore confirming a differential housing treatment effect on IBA behavior. For this model, treatment during the pre-environmental adjustment phase (i.e. ‘EE- Placebo’: mice initially housed in EE cages that will subsequently be moved to NE cages in the placebo condition; ‘EE-
Venlafaxine': mice initially housed in enriched cages that will be moved to NE cages in the Venlafaxine condition; ‘NE- Placebo': mice initially housed in NE cages that are all in the placebo condition and that will either remain in NE cages or be moved to EE cages; ‘NE-Venlafaxine': mice initially housed in NE cages that are all in the Venlafaxine condition and that will either remain in NE cages or be moved to EE cages), strain (C57 or DBA), and the relevant 2-way interaction were included as fixed factors. We expected a difference between NE vs EE but no difference between Venlafaxine and Placebo sub-groups due to the common antidepressants delayed onset of action. We constructed an additional model to investigate the timing-related development of IBA in enriched and non-enriched placebo treated mice by including (in addition to the above fixed factors) ‘week’ (1-3) and all the relevant interactions as additional factors.

We then tested the hypothesis that IBA triggered by NE cages is alleviated by Venlafaxine and by providing more enriched living conditions, and that combining such approaches might magnify their effects compared to using either approach in isolation. This model was constructed using the change in the proportion of IBA shown between the pre- and post-environmental adjustment phases as the dependent variable in order to simplify the model (i.e. accounting for phase directly in the dependant measure rather than added as an extra fixed factor). For this model, four treatment groups (i.e. NE-NE-Venlafaxine, NE-NE-Placebo, NE-EE-Venlafaxine, or NE-EE-Placebo), strain (C57 or DBA), and the relevant 2-way interaction were included as fixed factors.

Lastly, we investigated whether exposing mice to a reduction in home-cage environmental enrichment triggers IBA (as predicted from previous findings from [45]), and we tested the hypothesis that Venlafaxine would alleviate such an IBA increase. For this model, we used the proportion of IBA (whilst in view during scan sampling) performed by each mouse during each phase as the dependent variable, rather than change between phases. This was done to confirm that the Venlafaxine and Placebo groups did not differ during the pre-environmental adjustment phase (in this case, enrichment removal), and that such adjustment did trigger an increase in IBA. Therefore for this model, phase (i.e. pre- or post-environmental adjustment
phase), treatment (i.e. EE-NE-Venlafaxine or EE-NE-Placebo), strain (C57 or DBA), and the relevant 2-way and 3-way interactions were included as fixed factors.

For each model, the assumptions of parametric testing were checked using Shapiro-Wilk normality tests, Q-Q and normality plots of the model residuals. Likelihood ratio tests were used to compare each full model, with models excluding each non-significant term (which were removed iteratively) to generate a final model. This allowed us to determine the significance of the term by measuring the deviance from the full model, using Likelihood Ratio Test LRT \( \chi^2 \) tests. Interaction effects are presented regardless of their significance. Post hoc Tukey tests were used to measure differences between groups (with P value adjustment for multiple comparisons following the Holm method for the relevant number of tests) and the emmeans package allowed us to extract least square means (presented in the text together with standard error) for each variable.

3. Results

3.1. Do NE cages trigger greater levels of IBA compared to EE cages, confirming the differential housing treatment effect? (pre-environmental adjustment phase data only)

During the pre-environmental adjustment phase, mice spent, as predicted, a greater proportion of visible scans displaying IBA in the NE than in the EE cages (Likelihood Ratio Test ‘LRT’ \( \chi^2 = 53.501, p < 0.0001 \)), and as expected with no significant difference between mice under the two pharmacological conditions for either EE or NE housing conditions (Figure 2, see Table 1 in supplemental material for post-hoc comparisons). The levels of IBA were higher in the NE (vs EE) cages in weeks 2 and 3 of the pre-environmental adjustment phase, but not during week 1 (treatment*week LRT \( \chi^2_2 = 8.819, p = 0.0122 \), post-hoc comparisons week 1 t ratio \( t_{70} = -1.860, p = 0.3195 \); week 2 t ratio \( t_{70} = -5.388, p < 0.0001 \), week 3 t ratio \( t_{70} = -4.893, p < 0.0001, 0.3195 \)), suggesting that environmentally-induced IBA took at least one
week to become apparent. Neither strain displayed significantly more IBA overall than the other (LRT Chi$^2_1 = 0.210, p = 0.647$), nor did they differ in their response to housing condition (treatment*strain: LRT Chi$^2_3 = 0.801, p = 0.849$).

**Figure 2.** Bars show the least square means (+/-1 standard error) with individual raw data points of proportion of visible scans in which mice were inactive but awake (IBA) in enriched (EE) and non-enriched (NE) cages during the pre-environmental adjustment phase. 'EE- Placebo': mice initially housed in EE cages that will subsequently be moved to NE cages in the placebo condition; 'EE- Venlafaxine': mice initially housed in enriched cages that will be moved to NE cages in the Venlafaxine condition; 'NE- Placebo': mice initially housed in NE cages that are all in the placebo condition and that will either remain in NE cages or be moved to EE cages; 'NE- Venlafaxine': mice initially housed in NE cages that are all in the Venlafaxine condition and that will either remain in NE cages or be moved to EE cages. *** $p < 0.0001$, see Table 1 in supplemental material for post-hoc comparisons statistical details.

3.2. Are the greater levels of IBA triggered by NE cages alleviated by Venlafaxine, by increasing housing enrichment, and does combining these interventions magnify their effect?
As expected, we found a significant treatment effect (LRT $\chi^2_3 = 18.262, p < 0.001$; **Figure 3**, see Table 2 in supplemental material for post-hoc comparisons). As predicted, only individuals in the NE-NE-Placebo (control) group showed an increase over time in their proportion of visible scans displaying IBA, while all treatment groups (which had undergone either a pharmacological or environmental adjustment intervention, or both) displayed a decrease in IBA following treatments. No significant difference was observed in the change in proportion of IBA following treatment between the three NE-EE-Placebo, NE-EE-Venlafaxine and NE-NE-Venlafaxine treatment groups, suggesting that Venlafaxine and increasing environmental enrichment were equally effective at reducing IBA, and that combining these approaches did not magnify reduction in IBA. We found no significant difference between the two strains of mouse in their response to enrichment or Venlafaxine (treatment*strain: LRT $\chi^2_3 = 2.363, p = 0.501$) and similarly, neither strain displayed significantly more IBA overall than the other (LRT $\chi^2_1 = 1.010, p = 0.315$).

**Figure 3.** Bars show least square mean (+/- 1 SE) with individual raw data points of the change in the proportion of inactive but awake (IBA) behavior before and after the environmental adjustment and/or pharmacological manipulation. Positive values indicate that the proportion of visible scans in
which mice were displaying IBA increases, while negative values indicate a decrease in IBA levels post treatment. *** p < 0.001, * p < 0.05, • p = 0.07, see Table 2 in supplemental material for post-hoc comparisons statistical details.

3.3. Does environmental enrichment removal trigger greater levels of IBA, and does Venlafaxine alleviate such IBA increase?

As predicted, mice moved from the EE to the NE housing condition displayed a significant increase in IBA (LRT Chi$^2$ = 23.050, p < 0.001), however there was no significant difference in the increase shown by the EE-NE-Venlafaxine and EE-NE-Placebo groups (phase*drug: LRT Chi$^2$ = 0.081, p = 0.775; Main effect – drug: LRT Chi$^2$ = 0.019, p = 0.890, Figure 4). This suggests that the venlafaxine treatment did not provide an adequate therapeutic intervention to overcome the increase in IBA as a result of enrichment loss. We observed no significant differences between strains of mouse in the level of IBA displayed when housed in the EE-NE condition during either phase (phase*drug*strain: LRT Chi$^2$ = 0.483, p = 0.487, main effect – strain: LRT Chi$^2$ = 0.150, p = 0.669).

Figure 4. Bars display least square mean (+/- 1 SE) with the individual raw data points of the proportion of inactive but awake (IBA) behavior pre and post environmental enrichment removal for
mice in the EE-NE-Venlafaxine and EE-NE-Placebo treatment groups. As predicted, the two groups did not differ prior to environmental enrichment removal (post-hoc contrast: t = 0.142, p = 0.99), and both groups displayed a significant increase in IBA as a result of the shift from EE to NE housing (post-hoc contrasts: EE-NE-Drug pre – post enrichment removal: z = 3.326, p < 0.01; EE-NE-Control pre – post enrichment removal, z = 3.637, p < 0.01; EE-NE-Drug pre - EE-NE-Control post: z = -3.532, p < 0.01; EE-NE-Drug post - EE-NE-Control pre: z = 3.434, p < 0.01). However, no significant differences were seen between the Venlafaxine and placebo groups post enrichment removal (post-hoc contrast EE-NE-Drug - EE-NE-Control post enrichment removal, z = -0.248, p = 0.99). *** p < 0.001, ** p<0.01

4. Discussion

This study tested the hypothesis that greater time spent performing IBA indicates a depression-like state in mice, by investigating whether curative factors of human clinical depression are effective at reducing IBA in mice. We therefore investigated whether IBA was alleviated by chronically administrating an antidepressant (Venlafaxine), by providing preferred living conditions promoting better welfare (highly enriched, larger cages), and whether combining these therapeutic approaches magnifies their effects. As predicted, relatively barren, small cages triggered greater levels of IBA in mice compared to larger, enriched preferred cages, and the time mice spent displaying IBA decreased following Venlafaxine administration, environmental enrichment provision, and their combined intervention. The three treatments appeared equally effective at reducing IBA. This aligns with reported effects of such interventions on commonly assessed depression-like features in rodents. Indeed, both interventions in isolation have been shown to reduce immobility in forced swim tests (Venlafaxine: e.g. [33, 34]; environmental enrichment: e.g. [25, 57]). Furthermore, no additive nor synergistic effect on immobility in forced swim tests were observed when combining environmental enrichment promoting physical exercise (e.g. voluntary use of a running wheel, an item present in our enriched cages) with serotonin reuptake inhibiting pharmacological interventions [58]. This would suggest that such interventions may share
mechanisms [57, 58], such as a rise in extracellular serotonin produced by the pharmacological agents or reduction in serotonin transporter mRNA in running wheelequipped cages [58, 59]. Discussing common cellular effects remains speculative here, however, since the behavioral data collected in the current study do not allow us to assess this aspect of the pharmacological action. We also tested the prediction that Venlafaxine would prevent the rise in IBA following environmental enrichment removal that was previously observed by Trevarthen and colleagues [45]. As expected, mice moved from larger, enriched cages to relatively barren, small cages displayed a significant increase in IBA. Contrary to the prediction however, Venlafaxine did not overcome this increase in IBA resulting from the environmental enrichment loss.

Overall, the results align with previous studies, both with regards to triggering factors [25, 26, 40, 45], and to environmental enrichment interventions reducing IBA levels [40, 45], although the latter study did not correct for the mice being less visible in highly enriched larger cages (therefore reducing the reliability of the IBA frequencies recorded). That chronic administration of the antidepressant Venlafaxine reduces IBA further supports the hypothesis that greater levels of IBA could reflect depression-like conditions in mice, since Venlafaxine is of common clinical use in depressed people e.g. [29, 31, 32] and reverses depression-like features in rodents e.g. [33-35]. Greater levels of IBA in mice are thus triggered by some of the risk factors causing clinical depression, and reduced by interventions mimicking treatments of the human illness, further supporting our hypothesis that greater IBA can reflect depression-like conditions in mice.

One may cautiously suggest that greater levels of IBA might reflect a relatively mild form of a depression-like state in mice (‘mild’, ‘moderate’ or ‘severe’ being diagnosis specifiers in the DSM-V [2] P162), since combining Venlafaxine and environmental enrichment approaches did not magnify their effects. Indeed, combining interventions is usually required to alleviate symptoms of the illness primarily in people affected by moderate to severe forms of clinical depression e.g. [27, 28, 30]. That Venlafaxine did not overcome the increase in IBA resulting from the environmental enrichment loss could tentatively suggest that greater time displaying
IBA in this situation reflects a more severe form of the condition. This, however, is speculative since the full combination of pharmacological and non-pharmacological interventions is required to address that question.

Do these findings demonstrate with certainty that greater levels of IBA specifically reflect depression-like states in mice? They do not (although they do support the hypothesis), since, despite its advantages, our approach has drawbacks. First, although providing environmental enrichment favors better health and welfare in mice e.g. [22, 37-39], it does not encompass the diversity and the complexity of non-pharmacological therapeutic approaches used in humans, and the strength of its predictive validity might be questioned. Furthermore, that enriched preferred environments alleviate a given behavior exacerbated by non-preferred relatively barren environments does not demonstrate on its own that this behavior specifically reflects a depression-like condition. Indeed, one would predict to observe similar variations in IBA levels under NE and EE conditions if greater amounts of IBA were associated with other negatively valenced affective states, such as frustrated or bored-like states [24]. That the levels of IBA in mice are reduced by Venlafaxine shows enhanced predictive validity, but this approach also has weaknesses that we discuss below.

First, adverse side-effects of pharmacological interventions (reviewed in e.g. [29]), if present in the tested mice, could confound the results. A common adverse reaction that could confound mouse activity level is somnolence (incidence in Venlafaxine: ≥ 5% and at least twice the rate of placebo, [60]). If Venlafaxine-treated mice were more somnolent, they might have displayed reduced IBA levels, which would have been a by-product of increased sleeping/resting activity, rather than a demonstration of a putatively depressed-like symptom remission. Potential Venlafaxine-induced somnolence is unlikely to have confounded our results, however, since Venlafaxine-treated mice did not differ from the other treatment groups in their time spent sleeping or resting (Trevarthen et al, unpublished.). Another possible adverse effect of Venlafaxine is akathisia, i.e. a ‘subjectively unpleasant or distressing restlessness and need to move often accompanied by an inability to sit or stand still’ ([61], P19), which can develop within the first weeks of administration [29, 60, 62]). Should some of
the mice have developed akathisia, they would have displayed increased motor activity and, as a by-product, likely reduced IBA levels, with no improvement of their affective state. Akathisia is nevertheless a rare adverse effect of Venlafaxine in humans (≥1/10,000 to <1/1,000, [61-63]) and is thus unlikely to have confounded our results. Future studies should nonetheless include sampling methods that allow the assessment of, and control for, behavioral proxies of akathisia (e.g. continuous recording of motor activities allowing the quantification of increased non-stereotypic motor activity and postural changes frequencies).

Another limitation is that we have not directly verified whether NE mice displayed established symptoms of depression, and whether our Venlafaxine treatment induced remission from these symptoms (put differently, that our antidepressant ‘works’, although previous work would suggest it does e.g. [33-35]). Replication studies should therefore include testing whether our Venlafaxine treatment not only alleviates the amount of time the mice spend performing IBA, but also concomitantly changes known symptoms of depression, e.g. enhances sucrose consumption (proxy for reduced anhedonia), promotes more ‘optimistic’ processing of ambiguous information (cognitive proxy for improved mood), reduces changes in weight and sleep, elevates threshold in fatigue tests etc 3. Further investigations would also include replicating these results using a range of antidepressants; and assess the longer-term effect of the pharmacological and non-pharmacological interventions, since recommended durations of pharmacological treatments for clinically depressed usually vary from 6-9 to 12-24 months (depending on the severity of the illness, e.g. [29, 30]).

Lastly, depressive symptoms can be resistant to antidepressant actions in a high proportion (30-60%) of clinically diagnosed patients e.g. [55, 64, 65], and response to antidepressant is not a diagnostic criterion. Further testing of our hypothesis should therefore not only focus on common curative factors between IBA and depression in humans, but also investigate complete shared etiology with depression, including the interactions between exposure to stress later in life, exposure to developmental stress, and genetic susceptibility e.g. [8, 10, 23].

3 We believe that in depth discussion of how to operationalise symptoms of depression for application to non-humans falls beyond the scope of the current paper, but this is discussed in depth in 13.
Genetic-related investigations would involve *e.g.* testing sex differences, predicting lower levels of IBA in groups of male mice (since depression is higher in women, *e.g.* [2], although we would expect elevated levels of IBA in any individual male to reflect depression-like states as well). Strain effects on IBA levels should also be investigated further, including diversifying the number of strains studied, and strain-specific reactions to common environmental manipulations *e.g.* [66]. Indeed, no clear strain-related pattern has emerged yet, with levels of IBA observed to be higher in C57s in one laboratory (mouse supplier: Charles River North America, [25, 26]), DBA/2J in another laboratory [45], and not to differ between strains in this study (mice supplier: Charles River France). Since strain phenotypes may vary between laboratories *e.g.* [44], supplier and/or unidentified ‘local environment’ effects might be involved in this discrepancy, although this remains to be empirically tested.

Moreover, symptomatic similarities with other features of human clinical depression, covering the range of these symptoms and their co-occurrence (a key diagnostic criterion), should be tested further [13]. Indeed, greater levels of IBA have been shown to date to predict (as would be expected if reflecting depression-like states) signs of anhedonia (Trevarthen *et al.* in preparation), psychomotor changes and helplessness ([25], replicated in [26]), and two homeostasis symptoms, *i.e.* changes in weight [13] and sleep ([13], replicated in (Trevarthen *et al.* in preparation). Greater levels of IBA in mice therefore already ‘tick the box’ of the symptomatic similarities for four symptoms of human depression. However, whether elevated levels of IBA also predict the two remaining symptoms of the human illness that can be operationalized in animals, *i.e.* low mood (using cognitive affect-related biases as a proxy measure of mood) and diminished abilities to think or concentrate, has not been tested yet and remains to be investigated. So does the co-occurrence of greater levels of IBA with clusters of depressive symptoms [13]. Finally, further research should also investigate whether greater levels of IBA might be associated with other affective states. For instance, the result that Venlafaxine did not overcome the increase in IBA resulting from environmental enrichment loss could perhaps correspond to boredom-like reactions to impoverished environments *e.g.* [67].
In conclusion, our results show that greater levels of in-cage waking inactivity share curative factors with human depression, which, together with previous evidence of some symptomatic and aetiologic similarities between greater levels of IBA and human clinical depression, strongly support the hypothesis that mice displaying greater level of IBA are in depression-like conditions. Further research is warranted to demonstrate this further, which we encourage, considering the negative welfare and science quality implications that depression-like states are likely to have for laboratory mice.

5. Acknowledgments

We are grateful to Professor Emma Robinson and her team for advising on how to administrate Venlafaxine in a Refinement-friendly way; to Francesca Booth, Anna Garvey, Sarah Kappel, Kate Norman and Ellie Wigham for their assistance in delivering pharmacological treatment; and to the University of Bristol Animal Services Unit for taking care of the mice during the study.

6. Funding

This project was funded by a BBSRC grant to MM and CF (grant number BB/P019218/1). KB’s visiting period of study was funded by the Czech University of Life Sciences (Erasmus+, Faculty of Agrobiology, Food and Natural Resources) and the Institute of Animal Science Prague. The funders had no role in the study design, data collection, analyses and interpretation, decision to publish or preparation of the manuscript. The authors have nothing to disclose.

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