Short communication

Stereotypic behaviour in standard non-enriched cages is an alternative to depression-like responses in C57BL/6 mice

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Abstract

Depressive-like forms of waking inactivity have been recently observed in laboratory primates and horses. We tested the hypotheses that being awake but motionless within the home-cage is a depression-like symptom in mice, and that in impoverished housing, it represents an alternative response to stereotypic behaviour. We raised C57BL/6 ('C57') and DBA/2 ('DBA') females to adulthood in non-enriched (n = 62 mice) or enriched (n = 60 mice) cages, observing home-cage behaviour during the active (dark) phases. We predicted that being still but awake would be reduced by environmental enrichment; more pronounced in C57s, as the strain most prone to learned helplessness; negatively related to stereotypic behaviour; and positively related to immobility in Forced Swim Tests (FST). Compared to enriched mice, non-enriched subjects did spend more time spent being inactive but awake, especially if they displayed relatively little stereotypic behaviour. C57 mice also spent more time awake but motionless than DBAs. Furthermore, even after statistically controlling for housing type and strain, this behaviour very strongly tended to predict increased immobility in the FST, while high levels of stereotypic behaviours in contrast predicted low immobility in the FST. Being awake but motionless is thus a reaction to non-enriched housing that seems to be an alternative to stereotypic behaviour, and could reflect depression-like states.

Key words: Mice; Depression; Inactivity; Forced Swim Test; Stereotypic Behaviour; Environmental Enrichment

Clinically depressed patients often show reduced activity, taking less exercise, and engaging less in both social and non-social activities [1, 2]. They can also show learned helplessness, wherein 'highly desired outcomes are believed improbable or highly aversive outcomes are believed probable, and the individual comes to expect that no response in his repertoire will change their likelihood' [3]. Aetiologically, a common trigger is chronic stress (*e.g.* aversive life events or chronic pain/illness [4, 5]). Such symptoms may well not be unique to humans [6, 7]; indeed, modelling them in rodents and primates is common in biomedical research [8]. Furthermore, depression-like inactivity has been reported in some animals after aversive life events and/or chronic stress (*e.g.* horses, dogs, cats, elephants, non-human primates; reviewed [9]).

This study aimed to test the hypothesis that a specific form of inactivity in mice is a depression-like symptom: being still but awake in the home-cage during the active (dark) phase, thus apparently alert but nonetheless motionless. This behaviour was previously observed in C57BL/6 (henceforth 'C57') mice, especially in standard, non-enriched laboratory cages compared to large, enriched, highly preferred enclosures [10] (although this study did not correct for the enriched mice being harder to observe). Unusual forms of inactivity, that proved reversible with imipramine, were also reported in C57 mice exposed to repeated defeat stress [11]. Furthermore, C57s appear prone to helplessness (*e.g.* in Forced Swim Tests and after exposure to inescapable shocks), in contrast to DBA/2 mice for example (henceforth 'DBAs') which instead react to stress with hyper-activity and stereotypy [12].

We tested the hypothesis that this specific form of inactivity is a depression-like symptom by assessing whether 'still but awake' behaviour is diminished by stress-reducing enrichment, even after statistically controlling for time spent out of sight; is performed more by C57s than by DBAs; and positively co-varies with immobility in

Forced Swim Tests, a well-accepted indication of helplessness [8, 13, 14]. We also investigated its relationship with stereotypic behaviour (SB) to test a second hypothesis: that it and SB are alternative behavioural responses to chronic stress [12].

All procedures were approved by the University of Guelph Animal Care Committee (AUP 1398) and complied with Canadian Council on Animal Care guidelines. Unrelated adult female C57 and DBA mice in two cohorts were purchased from Charles River Laboratories (Quebec), and differentially housed from three to five weeks into adulthood. Food (Harlan® Teklad Global Diet, Mississauga, ON, Canada) and water were *ad libitum*. Rooms were kept at 21°C and 48% relative humidity, on a 12-hour reverse light schedule (with lights out at 10 am).

Cohort 1: Ninety mice were randomly assigned into mixed strain trios of two C57 mice and one DBA mouse per cage (previously shown not to affect welfare or strain-typical phenotypes: [15]), one C57 per cage being ear-notched after receiving Carprofen. Half were housed in non-enriched ('NE') cages, half in larger enriched ('EE') cages, as described in [16]. Enrichments were biologically relevant items (*e.g.* allowing hiding and nest-building), selected from publications indicating they enhanced mouse welfare *e.g.* [10]. In each cage, they included: a plastic igloo mouse house & 'fast-trac' running wheel combo (Bio Serv®); a black polyvinyl chloride PVC tunnel (10cm x 4cm; also used for handling, see below); two paper cups; a Nestlet; one tissue; two square pieces of cotton fabric (each 4 x 4 cm); a pinecone (from one of several diverse conifer species); a sponge (roughly 5 x 3 x 5 cm); a sock 'hammock' (a 12 x12 cm piece of sock hung from the cage lid via cable ties); and two cotton balls. EE mice were also always handled using their familiar PVC tunnel, not directly by hand, to further reduce stress [17]. Two mice were

lost to malocclusion, such that final sample sizes were 58 C57s (29 EE, 29 NE) and 30 DBAs (15 EE, 15 NE).

Cohort 2: These mice were part of another experiment that further validated mixed strain housing, here for trios consisting of one C57, one DBA and one BALB/c mouse per cage [18] (this last strain being excluded from our analyses as absent from Cohort 1). Housing is described elsewhere [18], but briefly, NE housing was as for Cohort 1, except that each cage contained a paper coffee cup [18] instead of a plastic shelter [15]; while EE cages were larger than for Cohort 1, and contained one extra running wheel (metal), a metal platform, and no pinecone [18]. Mice were again handled using either a PVC tunnel if EE or, if NE, using a paper cup. Due to some instances of malocclusion and severe barbering, our final sample sizes were 17 C57s (8 EE, 9 NE) and 17 DBAs (8 EE, 9, NE).

In-cage behavioural data were collected via live scan-sampling (switching to 15 s focal observations for mice displaying SB or the 'still but awake' behaviour, the former being characterised by repetition and the latter by a lack of movement), using well-validated methods described elsewhere (Experiment 1; ref [18]; see Table 1 for ethogram). At the time of the observations, Cohort 1 mice were six months old and Cohort 2 mice, 3 months old.

Forced Swim Tests (FSTs) were conducted as described elsewhere *e.g.* [13, 14], from 10:30am to 07:00 pm over 2 (Cohort 1) or 4 (Cohort 2) consecutive days. Mice were allowed to habituate to the testing room in their home-cage for 5 minutes. Ambient temperatures here were maintained at 29°C to reduce risks of wet mice developing hypothermia. Mice were placed individually in three side-by-side, visually separated glass cylinders (23cm h x 19cm w), filled with 18 cm water (25.38°C ± 0.29, 24.30-25.90°C). They were videotaped for 6 min (2 min of habituation, 4 min of test [13, 14])

before being returned to their home-cages, and allowed to dry fully for 20 minutes before returning to the colony room. Cylinders were cleaned with disinfectant (CaviWipes™, Metrex), rinsed with water and re-filled between tests. Housing treatment was counter-balanced between test days, test hours and the three cylinders. Every tape was observed by CF and one assistant (blind to treatment and hypothesis) to score each mouse's total duration of immobility (floating for at least 2 seconds with at least 3 legs motionless [13]) in the 4min test period. Inter-observer reliability was excellent (Cohort 1: $F_{1, 214} = 2561.550$; $p < 0.0001$; Cohort 2: $F_{1, 154}=1145.000$; $p<0.0001$); data were therefore averaged between observers.

Data were analysed using general linear mixed models (GLM) (JMP®12; SAS Institute Inc., Cary, NC, USA). Cage was set as a random factor [19]; data were also blocked for strain, housing type, cohort and their interactions. Two-way interactions between strain, housing and 'still but awake' behaviour were always included, as were observations spent 'out of sight' [16] to avoid this confounding housing type effects. When investigating relationships between time spent 'still but awake' and FST immobility, water temperature was added as an additional control, because it can influence mouse motility during these tests [14]. Type 1 sums of squares were used (because continuous variables caused non-orthogonality), the term of interest being placed last in each model [19]. Data were always checked for normality and non-homogeneity of variance, being transformed (*e.g. via* square-root or logit) when necessary to meet these assumptions. Two-tailed tests were used to be conservative (despite directional predictions). Descriptive statistics are least square means ($\bar{X}$) followed by standard errors.

The overall proportion of scans (termed 'time spent' hereafter) displaying 'still but awake' behaviour varied across individual mice from 0 to 0.25 of scans ($\bar{X}$ = 0.03 ±

0.04), and more than 75% of the mice displayed it at least once. Time spent still but awake was predicted by strain and housing type, being higher in C57s ($\bar{X}$ = 0.04 ± 0.11, $\bar{X}_{DBA}$ = 0.02 ± 0.13, $F_{1, 56}$ = 6.993; p = 0.011) and NE cages ($\bar{X}$ = 0.04 ± 0.13; $\bar{X}_{EE}$ = 0.02 ± 0.13; $F_{1, 34}$ = 20.369; p < 0001). NE mice also performed more SB ($\bar{X}_{NE}$ = 0.10 ± 0.02; $\bar{X}_{E}$ = -0.003[b] ± 0.02; $F_{1, 38}$ = 21.5814, p < 0.0001), while strain differences for SB were in the opposite direction than for awake inactivity, DBAs displaying more ($\bar{X}_{DBA}$ = 0.05 ± 0.02; $\bar{X}_{C57}$ = 0.04 ± 0.01; $F_{1, 66}$ = 12.081; p = 0.0009). Exploring relationships between awake inactivity and SB revealed significant interactions with housing type ($F_{1, 105}$ = 4.582; p = 0.035) and strain ($F_{1, 92}$ = 14.217; p = 0.0003). Splitting data by housing revealed a negative relationship between the two behaviours in NE mice ($F_{1, 54}$ = 20.532; p < 0.0001); in EE mice SB was too low for detectable effects: $F_{1, 52}$ = 0.139; p = 0.7111) (**Fig 2**). Splitting data by strain, in contrast, revealed similarly significant negative relationships between 'still but awake' behaviour and SB in both strains (DBAs: $F_{1, 39}$ = 11.220; p = 0.0018; C57s: $F_{1, 60}$ = 10.037; p = 0.0024).

NE mice were immobile for longer in FSTs ($F_{1, 39}$ = 4.83, p = 0.034), as were C57s compared to DBAs ($\bar{X}_{C57}$ = 84.67 ± 0.59, $\bar{X}_{DBA}$ = 4.43 ± 0.70, $F_{1, 68}$ = 117.225, p < 0.0001). Furthermore, mice spending the most time 'still but awake' strongly tended to display more FST immobility ($F_{1, 100}$ = 3.849, p = 0.052, **Fig 1A**). Conversely, a negative relationship was observed between SB and FST immobility ($F_{1, 101}$ = 7.378, p = 0.0078, **Fig 1B**).

Thus as predicted, enriched mice spent less of the dark phase being motionless although awake (consistent with [10], but with added controls to ensure effects were not caused by enrichments reducing visibility). Enriched mice also displayed less immobility in FSTs. Our results thus join previous suggestions that typical laboratory

[b] Presented means are not the *standard means*, but *least squares means* generated by the GLM model, taking into account all other factors in the model (which is why some values here and in the figures are negative)

cages are depressogenic *e.g.* [20], adding to evidence for other species that standard husbandry can induce depression-like states (horses [6]; rhesus monkeys [21]). Furthermore, strain effects were as expected: C57s spent more time than DBAs being still but awake, and were also more immobile in FSTs (consistent with *e.g.* [22]). Thus genotypes and housing conditions that predisposed mice to learned helplessness also increased the time they spent standing still doing nothing in the home-cage. Furthermore, even after controlling for these group level effects, individual mice spending the most time in this waking inactivity strongly tended to be more immobile in FSTs (our 2-tailed test here being conservative), so strongly suggesting depression-like states.

Non-enriched cages also promoted SB (cf. *e.g.* [10, 15, 23]). Furthermore, within these non-enriched cages, mice with low levels of SB spent more time still but awake, both across the two strains and within them. This supports Cabib's hypothesis [12] that SB reflects one of two alternate behavioural reactions to chronic stress: hyperactivity, as opposed to its alternative: hypo-activity. Testing Cabib's hypothesis that such effects reflect differential mesoaccumbens dopamine functioning and meso-corticolimbic neuroplasticity [12] would be revealing. Further research could also include videotaping the behaviour to phenotype it more precisely (*e.g.* are NE mice and E mice 'still but awake' in exactly the same ways but to different extents, or are they also qualitatively different, in *e.g.* posture or ease of interruption?).

As for whether this distinctive inactivity in awake mice, especially non-enriched C57s, truly indicates depression-like states, more research is needed to address this. Human clinical depression is complex, characterised by the *co-existence* of several symptoms. Future work should therefore investigate whether these mice show anhedonia, a key depressive symptom [2] often modelled in rodents *via* reduced sucrose

intake (*e.g.* [24]); and evidence consistent with low mood, such as making more 'pessimistic' judgments about ambiguous situations or attending more to negative stimuli *e.g*. [25]. Further research should also assess whether being still but awake increases with other stressors that induce depression-like symptoms (*e.g.* chronic mild stress [8]) and diminishes after anti-depressant drug treatments [11]. Were such data to support this hypothesis, this would have great animal welfare implications, and would also reveal home cage time budgets as a convenient, non-invasive source of data for researchers interested in animal depression.

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Figures legends

Figure 1: Relationship between duration of immobility in Forced Swim Tests (*FST*) and: **A/** time spent still but awake in the home-cage, **B/** time spent displaying stereotypic behaviour (*SB*), statistically controlling in both cases for Housing type (non-enriched/enriched), Cohort, Strain, water temperature and proportion of scans out of sight. The presented values reflect the model taking into account these other factors, which is why some values are negative.

Figure 2: Relationship between time spent still but awake and stereotypic behaviour (*SB*), controlling for Cohort, Strain, and proportion of scans out of sight. **A/** non-enriched housing; **B/** enriched housing. Again, presented values reflect the model taking into account these other factors, which causes some values to be negative.

Table

Table 1: Behaviours relevant to hypothesis under test (adapted from *e.g.* [10, 16])

Behaviour	Description
Still but awake	Mouse is immobile, with eyes open.
Stereotypic behaviour (SB)	Mouse holds cage bar in mouth for 1s or longer while moving mouth along the bar *(bar mouthing)*
	Mouse runs along cage floor in a fixed pattern for three or more repetitions *(route tracing)*
	Route-tracing while hanging upside down from the cage lid in patterns for three or more repetitions *(patterned climbing*).
Out of sight	In nest/shelter, out of sight of experimenter

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Figure1

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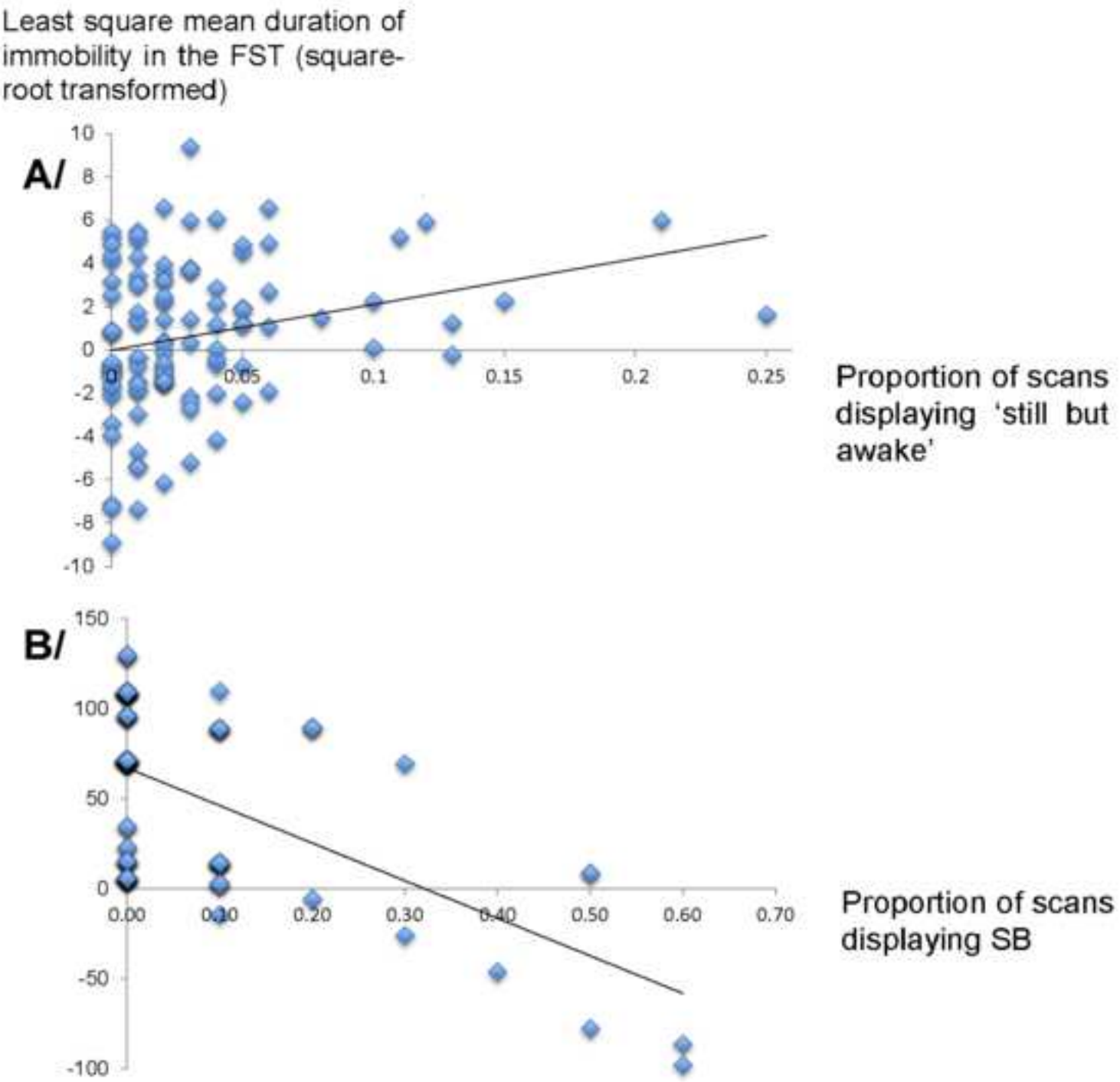

Figure2

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