

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

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18 **Abstract**

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5 20 Depressive-like forms of waking inactivity have been recently observed in laboratory
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7 21 primates and horses. We tested the hypotheses that being awake but motionless within
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10 22 the home-cage is a depression-like symptom in mice, and that in impoverished housing,
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12 23 it represents an alternative response to stereotypic behaviour. We raised C57BL/6
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14 24 ('C57') and DBA/2 ('DBA') females to adulthood in non-enriched (n = 62 mice) or
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17 25 enriched (n = 60 mice) cages, observing home-cage behaviour during the active (dark)
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20 26 phases. We predicted that being still but awake would be reduced by environmental
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22 27 enrichment; more pronounced in C57s, as the strain most prone to learned helplessness;
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25 28 negatively related to stereotypic behaviour; and positively related to immobility in
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27 29 Forced Swim Tests (FST). Compared to enriched mice, non-enriched subjects did spend
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30 30 more time spent being inactive but awake, especially if they displayed relatively little
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32 31 stereotypic behaviour. C57 mice also spent more time awake but motionless than DBAs.
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35 32 Furthermore, even after statistically controlling for housing type and strain, this
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37 33 behaviour very strongly tended to predict increased immobility in the FST, while high
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40 34 levels of stereotypic behaviours in contrast predicted low immobility in the FST. Being
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42 35 awake but motionless is thus a reaction to non-enriched housing that seems to be an
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45 36 alternative to stereotypic behaviour, and could reflect depression-like states.

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49 38 Key words: Mice; Depression; Inactivity; Forced Swim Test; Stereotypic Behaviour;
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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

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

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

76 *Cohort 1:* Ninety mice were randomly assigned into mixed strain trios of two C57
77 mice and one DBA mouse per cage (previously shown not to affect welfare or strain-
78 typical phenotypes: [15]), one C57 per cage being ear-notched after receiving Carprofen.
79 Half were housed in non-enriched ('NE') cages, half in larger enriched ('EE') cages, as
80 described in [16]. Enrichments were biologically relevant items (*e.g.* allowing hiding and
81 nest-building), selected from publications indicating they enhanced mouse welfare *e.g.*
82 [10]. In each cage, they included: a plastic igloo mouse house & 'fast-trac' running wheel
83 combo (Bio Serv®); a black polyvinyl chloride PVC tunnel (10cm x 4cm; also used for
84 handling, see below); two paper cups; a Nestlet; one tissue; two square pieces of cotton
85 fabric (each 4 x 4 cm); a pinecone (from one of several diverse conifer species); a sponge
86 (roughly 5 x 3 x 5 cm); a sock 'hammock' (a 12 x12 cm piece of sock hung from the cage
87 lid via cable ties); and two cotton balls. EE mice were also always handled using their
88 familiar PVC tunnel, not directly by hand, to further reduce stress [17]. Two mice were

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2 89 lost to malocclusion, such that final sample sizes were 58 C57s (29 EE, 29 NE) and 30
3 90 DBAs (15 EE, 15 NE).

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5 91 *Cohort 2:* These mice were part of another experiment that further validated mixed
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7 92 strain housing, here for trios consisting of one C57, one DBA and one BALB/c mouse per
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9 93 cage [18] (this last strain being excluded from our analyses as absent from Cohort 1).
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11 94 Housing is described elsewhere [18], but briefly, NE housing was as for Cohort 1, except
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13 95 that each cage contained a paper coffee cup [18] instead of a plastic shelter [15]; while
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15 96 EE cages were larger than for Cohort 1, and contained one extra running wheel (metal),
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17 97 a metal platform, and no pinecone [18]. Mice were again handled using either a PVC
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19 98 tunnel if EE or, if NE, using a paper cup. Due to some instances of malocclusion and
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21 99 severe barbering, our final sample sizes were 17 C57s (8 EE, 9 NE) and 17 DBAs (8 EE, 9,
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23 100 NE).
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30 101 In-cage behavioural data were collected via live scan-sampling (switching to 15 s
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32 102 focal observations for mice displaying SB or the 'still but awake' behaviour, the former
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34 103 being characterised by repetition and the latter by a lack of movement), using well-
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36 104 validated methods described elsewhere (Experiment 1; ref [18]; see Table 1 for
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38 105 ethogram). At the time of the observations, Cohort 1 mice were six months old and
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40 106 Cohort 2 mice, 3 months old.
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44 107 Forced Swim Tests (FSTs) were conducted as described elsewhere *e.g.* [13, 14],
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46 108 from 10:30am to 07:00 pm over 2 (Cohort 1) or 4 (Cohort 2) consecutive days. Mice
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48 109 were allowed to habituate to the testing room in their home-cage for 5 minutes. Ambient
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50 110 temperatures here were maintained at 29°C to reduce risks of wet mice developing
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52 111 hypothermia. Mice were placed individually in three side-by-side, visually separated
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54 112 glass cylinders (23cm h x 19cm w), filled with 18 cm water (25.38°C ± 0.29, 24.30-
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56 113 25.90°C). They were videotaped for 6 min (2 min of habituation, 4 min of test [13, 14])
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114 before being returned to their home-cages, and allowed to dry fully for 20 minutes
115 before returning to the colony room. Cylinders were cleaned with disinfectant
116 (CaviWipes™, Metrex), rinsed with water and re-filled between tests. Housing treatment
117 was counter-balanced between test days, test hours and the three cylinders. Every tape
118 was observed by CF and one assistant (blind to treatment and hypothesis) to score each
119 mouse's total duration of immobility (floating for at least 2 seconds with at least 3 legs
120 motionless [13]) in the 4min test period. Inter-observer reliability was excellent (Cohort
121 1: $F_{1, 214} = 2561.550$; $p < 0.0001$; Cohort 2: $F_{1, 154} = 1145.000$; $p < 0.0001$); data were
122 therefore averaged between observers.

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

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

139 0.04), and more than 75% of the mice displayed it at least once. Time spent still but
 140 awake was predicted by strain and housing type, being higher in C57s ($\bar{X} = 0.04 \pm 0.11$,
 141 $\bar{X}_{DBA} = 0.02 \pm 0.13$, $F_{1,56} = 6.993$; $p = 0.011$) and NE cages ($\bar{X} = 0.04 \pm 0.13$; $\bar{X}_{EE} = 0.02 \pm$
 142 0.13 ; $F_{1,34} = 20.369$; $p < 0001$). NE mice also performed more SB ($\bar{X}_{NE} = 0.10 \pm 0.02$; \bar{X}_E
 143 $= -0.003^b \pm 0.02$; $F_{1,38} = 21.5814$, $p < 0.0001$), while strain differences for SB were in the
 144 opposite direction than for awake inactivity, DBAs displaying more ($\bar{X}_{DBA} = 0.05 \pm 0.02$;
 145 $\bar{X}_{C57} = 0.04 \pm 0.01$; $F_{1,66} = 12.081$; $p = 0.0009$). Exploring relationships between awake
 146 inactivity and SB revealed significant interactions with housing type ($F_{1,105} = 4.582$; $p =$
 147 0.035) and strain ($F_{1,92} = 14.217$; $p = 0.0003$). Splitting data by housing revealed a
 148 negative relationship between the two behaviours in NE mice ($F_{1,54} = 20.532$; $p <$
 149 0.0001); in EE mice SB was too low for detectable effects: $F_{1,52} = 0.139$; $p = 0.7111$) (**Fig**
 150 **2**). Splitting data by strain, in contrast, revealed similarly significant negative
 151 relationships between 'still but awake' behaviour and SB in both strains (DBAs: $F_{1,39} =$
 152 11.220 ; $p = 0.0018$; C57s: $F_{1,60} = 10.037$; $p = 0.0024$).

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

159 Thus as predicted, enriched mice spent less of the dark phase being motionless
 160 although awake (consistent with [10], but with added controls to ensure effects were
 161 not caused by enrichments reducing visibility). Enriched mice also displayed less
 162 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)

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

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

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

188 intake (*e.g.* [24]); and evidence consistent with low mood, such as making more
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2 189 ‘pessimistic’ judgments about ambiguous situations or attending more to negative
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5 190 stimuli *e.g.* [25]. Further research should also assess whether being still but awake
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7 191 increases with other stressors that induce depression-like symptoms (*e.g.* chronic mild
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10 192 stress [8]) and diminishes after anti-depressant drug treatments [11]. Were such data to
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12 193 support this hypothesis, this would have great animal welfare implications, and would
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15 194 also reveal home cage time budgets as a convenient, non-invasive source of data for
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17 195 researchers interested in animal depression.
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204 **Figures legends**1
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206 *Figure 1: Relationship between duration of immobility in Forced Swim Tests (FST)*207 and: **A/** time spent still but awake in the home-cage, **B/** time spent displaying208 stereotypic behaviour (*SB*), statistically controlling in both cases for Housing type (non-

209 enriched/enriched), Cohort, Strain, water temperature and proportion of scans out of

210 sight. The presented values reflect the model taking into account these other factors,

211 which is why some values are negative.

212

213 *Figure 2: Relationship between time spent still but awake and stereotypic behaviour*214 (*SB*), controlling for Cohort, Strain, and proportion of scans out of sight. **A/** non-enriched215 housing; **B/** enriched housing. Again, presented values reflect the model taking into

216 account these other factors, which causes some values to be negative.

217 **Table**

1
2 218 *Table 1: Behaviours relevant to hypothesis under test (adapted from e.g. [10, 16])*

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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 (<i>bar mouthing</i>)
	Mouse runs along cage floor in a fixed pattern for three or more repetitions (<i>route tracing</i>)
	Route-tracing while hanging upside down from the cage lid in patterns for three or more repetitions (<i>patterned climbing</i>).
Out of sight	In nest/shelter, out of sight of experimenter

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Figure1

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Least square mean duration of immobility in the FST (square-root transformed)

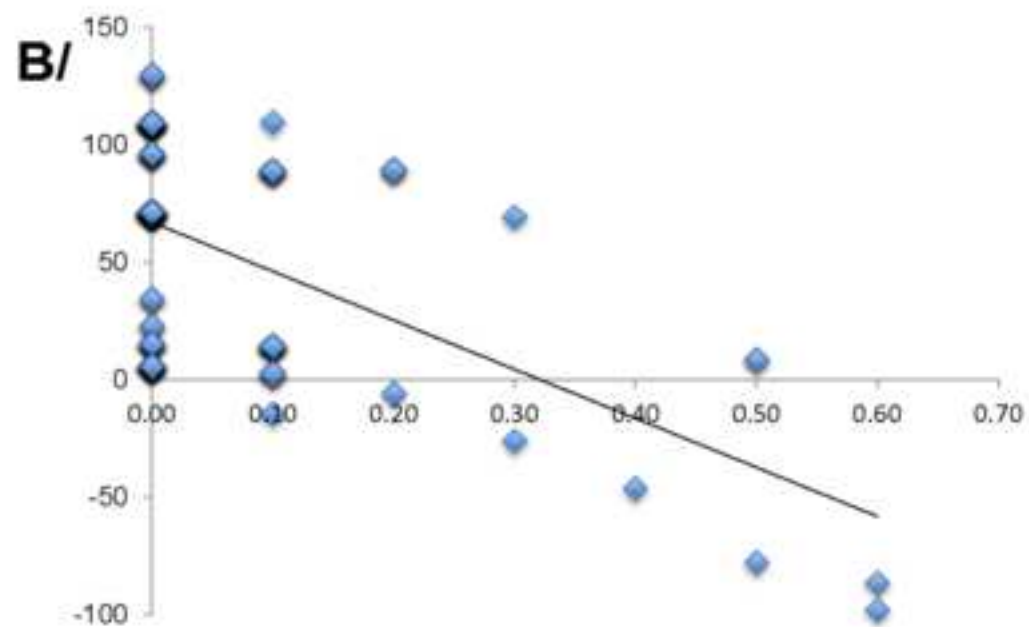
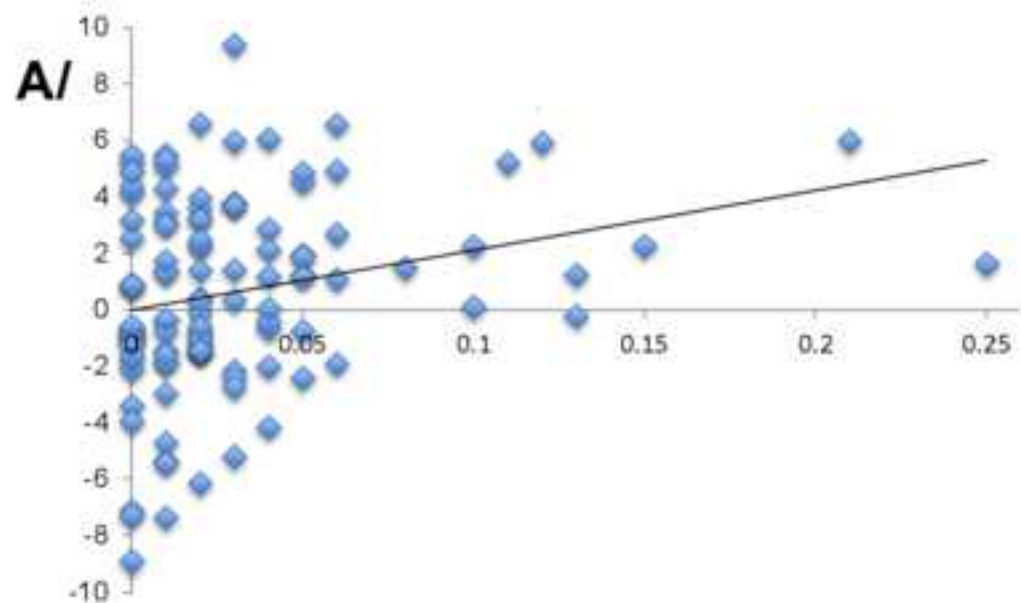
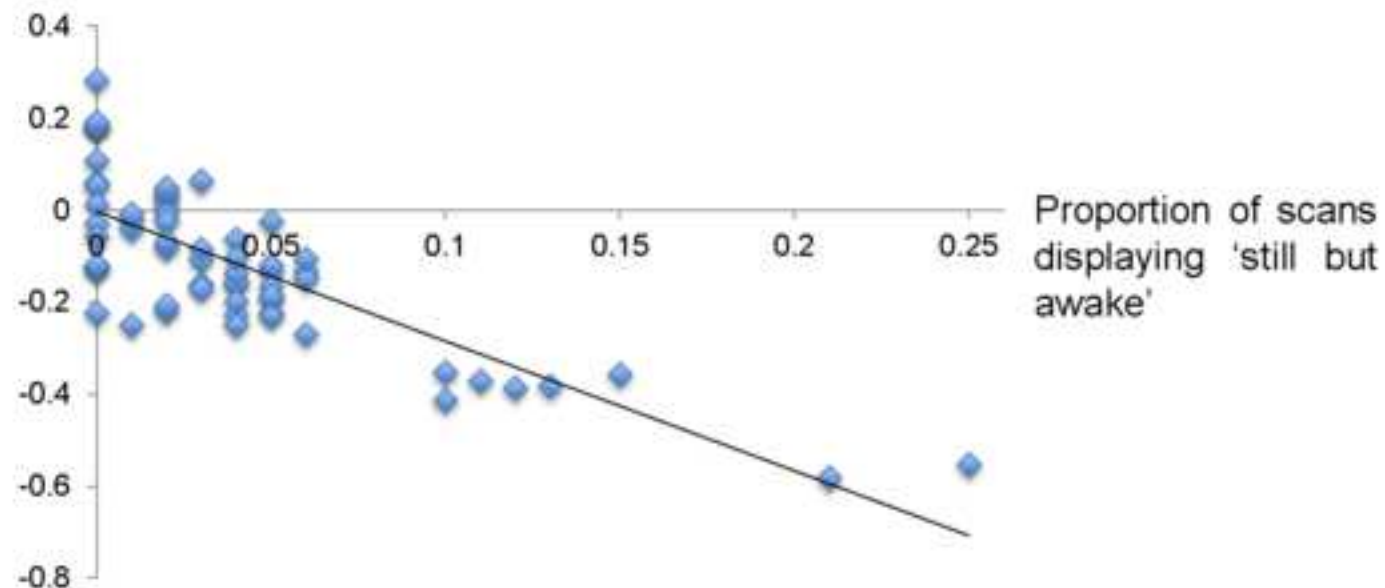


Figure2

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Least square
proportions of
displaying SB
mean
of
scans

A/



B/

