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1 **Environmental enrichment rescues survival and function of adult-born neurons following early life**
2 **stress**

3 **Running title: early life stress, hippocampus and enrichment**

4

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22 **Abstract**

23 Adverse experiences early in life are associated with the development of psychiatric illnesses. The
24 hippocampus is likely to play pivotal role in generating these effects: it undergoes significant
25 development during childhood and is extremely reactive to stress. In rodent models, stress in the pre-
26 pubertal period impairs adult hippocampal neurogenesis (AHN) and behaviours which rely on this
27 process. In normal adult animals, environmental enrichment (EE) is a potent promoter of AHN and
28 hippocampal function. Whether exposure to EE during adolescence can restore normal hippocampal
29 function and AHN following pre-pubertal stress (PPS) is unknown. We investigated EE as a treatment
30 for reduced AHN and hippocampal function following PPS in a rodent model. Stress was administered
31 between post-natal days (PND) 25-27, EE from PND35 to early adulthood, when behavioural testing
32 and assessment of AHN took place. PPS enhanced fear reactions to a CS following a trace fear protocol
33 and reduced the survival of 4-week-old adult-born neurons throughout the adult hippocampus.
34 Furthermore, we show that fewer adult-born neurons were active during recall of the CS stimulus
35 following PPS. All effects were reversed by EE. Our results demonstrate lasting effects of PPS on the
36 hippocampus and highlight the utility of EE during adolescence for restoring normal hippocampal
37 function. EE during adolescence is a promising method of enhancing impaired hippocampal function
38 resulting from early life stress, and due to multiple benefits (low cost, few side effects, widespread
39 availability), should be more thoroughly explored as a treatment option in human sufferers of
40 childhood adversity.

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46 Introduction

47 Childhood adversity is associated with an increased risk of developing psychiatric illnesses¹.
48 Epidemiological studies have repeatedly shown that stressful early life experiences such as abuse and
49 neglect are associated with higher rates of schizophrenia, depression, borderline personality disorder,
50 anxiety and post-traumatic stress disorder. The hippocampus is a key target of the stress response,
51 being enriched for corticosteroid receptors, particularly in CA1 and dentate granule cells². Coupled
52 with significant post-natal maturation during childhood and adolescence, the hippocampus is
53 predicted to be especially vulnerable to the effects of early life stress (ELS). In support of this, meta-
54 analyses report significant associations between childhood adversity and reduced hippocampal
55 volume and impaired hippocampal function³⁻⁵. Animal models of ELS similarly report changes in
56 hippocampal-dependent learning and memory and associated molecular changes⁶⁻⁹. This has
57 relevance for psychiatric illness: the hippocampus is involved in cognitive and emotional functions,
58 and smaller hippocampal volumes and abnormal hippocampal-dependent behaviours are prevalent
59 in post-traumatic stress disorder, schizophrenia, anxiety and depression¹⁰⁻¹³.

60 On a neuronal level, rodent models demonstrate that stress early in life adversely affects the
61 generation of adult-born neurons in the dentate gyrus of the hippocampus (adult hippocampal
62 neurogenesis (AHN))⁸. These adult-born neurons are implicated in emotional regulation and
63 hippocampal-dependent behaviours, including trace and contextual fear conditioning, spatial
64 navigation, pattern separation and cognitive flexibility¹³⁻¹⁷. There is also evidence that AHN and
65 dentate gyrus volume are decreased in schizophrenic and depressed patients^{18,19}. Lasting impairments
66 in AHN resulting from ELS may therefore negatively affect behaviour and leave individuals vulnerable
67 to developing psychiatric illnesses.

68 Neuropsychiatric disorders are predicted to be the second highest cause of global disease
69 burden by 2020²⁰. Current treatments are ineffective in up to 30% of cases, often accompanied by
70 significant side effects, and many individuals relapse¹³. There is therefore a pressing need to develop

71 novel and improved treatments. Given the recent emphasis on links between dysregulated AHN and
72 psychiatric illness, this is a promising process to target, especially considering increased AHN is
73 postulated as one mechanism through which antidepressants exert their effects^(13,14,19,21).
74 Environmental enrichment (EE) is a robust method for improving AHN and hippocampal-dependent
75 cognition in adult animals²². In rodents, EE is a housing manipulation which increases exposure to
76 social and physical stimuli (e.g. larger social groups, toys and tunnels), promoting exploration, social
77 interaction and physical activity. During adolescence, EE has been shown to rescue social function,
78 attention and anxiety behaviours following early life stressors such as maternal separation²³⁻²⁷.
79 Interestingly, a recent study by Ardi *et al.* demonstrates that exposure to EE directly after pre-pubertal
80 stress, but not after an additional adult stressor, prevents stress vulnerability and normalises anxiety-
81 like behaviour after adult traumatic stress²⁸. However, the impact of EE in adolescence is not yet fully
82 explored or understood, and the potential benefits for rescuing ELS induced deficits in AHN and
83 hippocampal-dependent behaviour are unknown²⁹. This is a crucial area of exploration as EE holds
84 great potential for enhancing brain function in human populations, as highlighted in a recent review
85 (Kempermann, 2019)²², particularly in the context of neuropsychiatric or neurodegenerative diseases.
86 Cognitive, mental, nutritional, physical and social stimulation in humans have been categorised as EE
87 which may circumnavigate or compliment pharmaceutical treatments³⁰. EE based interventions are
88 particularly appealing due to low-risk of side effects, low cost and widespread availability, and have
89 already shown promise as an adjunct treatment for promoting functional recovery in stroke patients³¹.

90 The first aim of this study was to investigate how early life stress given in the pre-pubertal
91 phase (pre-pubertal stress (PPS), post-natal days 25-27, a time-point akin to human childhood³²)
92 impacted upon AHN and hippocampal-dependent behaviour. Secondly, we sought to determine
93 whether EE could reverse the effects of PPS on AHN and hippocampal dependent behaviour. We also
94 investigated whether PPS altered the number of adult-born neurons that were active during recall of
95 a conditioned stimulus (CS) following a trace fear protocol, and subsequent modulation by EE.

96

97 **Materials and Methods**

98 **Animals**

99 Experiments were approved by Cardiff University's Animal Welfare and Ethical Review Body and
100 adhered to UK Home Office Animals (Scientific Procedures) Act 1986 and European Regulations on
101 animal experimentation. Lister hooded rats were bred in house from 16 adult pairs (Charles River),
102 weaned at postnatal day (PND) 21 and housed in same-sex cages with littermates. Light was
103 maintained on a 12:12-h light/dark cycle, and food and water were provided *ad libitum*. Male rats
104 were used as PPS does not alter trace fear responses or neurogenesis in female animals⁸.

105

106 **Pre-pubertal stress & enrichment**

107 Based on previous experiments in our laboratory⁸ twenty-two animals served as controls and twenty-
108 four animals were given a short-term PPS between PND 25-27, originally described by Jacobson-Pick
109 and Richter Levin³³. Litters were allocated to treatment groups (control or stressed) based on order of
110 birth. Animals were given a 10 min swim ($25\pm 1^{\circ}\text{C}$) in an opaque swimming tank (25cm high, 34cm
111 diameter) on PND 25, restraint stress in plastic restraint tubes (15cm length, 5cm diameter) for 3
112 sessions of 30 minutes, separated by 30 minute breaks in the home cages on PND 26 and exposure to
113 an elevated platform (15x15cm, 115cm high) for three 30 minute sessions, separated by 60 minutes
114 in the home cage on PND 27. Animals were then returned to the holding room and housed in regular
115 cages (32cm x 50cm x 21cm, lined with wood shavings and a wooden stick, cardboard tube and
116 shredded paper provided as standard enrichment) in groups of three or four. On PND 35 half of the
117 animals (PPS and control) were moved into larger enriched cages (74cm x 59cm x 40cm) in groups of
118 six or seven (EE groups). The enriched cages contained a deep layer of sawdust bedding, platforms,
119 wooden sticks and a variety of toys including tubes, tunnels and hammocks, which were rotated

120 between cages every week. The remaining animals remained in regular cages described above (control
121 housed, or CH groups), and animals were left undisturbed, aside from cage cleaning, until early
122 adulthood.

123

124 **BrdU administration & behaviour**

125 Between PND 57-66 animals were given a single intraperitoneal injection of bromodeoxyuridine
126 (BrdU, 200mg/kg in 0.9% sterile saline solution), to label dividing neurons in the dentate gyrus. Four
127 weeks later (PND 83-97) animals were trained in a trace protocol. By the third week of life, 90% of
128 adult-born neurons express the mature neuronal marker NeuN and demonstrate electrophysiological
129 features of maturity, and by 4 weeks immature markers such as b-III-tubulin and doublecortin are no
130 longer detectable³⁴⁻³⁶.

131 **Apparatus:** Two modular test chambers (32cmx25.5cmx27cm, Sandown Scientific UK) with grid floors
132 (19 stainless steel rods, 1cm apart) and a stainless-steel pan were used for testing. Side walls were
133 stainless steel, ceiling, front and back walls clear plexiglass. Each chamber resided inside a sound
134 attenuating box, to which a speaker was attached on the inside. A ventilation fan provided a
135 background noise of 63dB and a video camera was attached to the inside of the door. A shock
136 generator was attached to the grid floor. Video recording, light, sound and shock were controlled by
137 computer interface. The two boxes provided distinct contexts, C1 and C2. C1 was illuminated by a
138 house light, the pan was filled with wood shavings, ceiling and walls decorated with black stars on a
139 white background. C2 was dark, an IR light bar allowed video recording, and was scented with a drop
140 of lavender oil in the pan. Between animals each box was cleaned with ethanol wipes, and lavender
141 scent/sawdust replaced. Half of the animals from each group (control and PPS) and treatment (EE and
142 CH) were trained in C1, half in C2. The unconditioned stimulus (US) was a 0.5s, 0.5mA scrambled
143 footshock, the conditioned stimulus (CS) a 15s, 75dB white noise.

144 **Protocol:** Animals were habituated by transport to the testing room and handling every day for three
145 days before testing began. On day 1, animals were placed into C1 or C2 for 120s. Rats then experienced
146 10 CS-US pairings, a 30 second stimulus free trace interval separated the offset of the CS from the
147 onset of the US. Intertrial intervals were 312s (+/-62s). This intertrial interval is optimal for producing
148 freezing to both context and cue^{8,37}. To assess contextual fear responses, twenty-four hours later
149 animals were returned to their original training chamber for 10 minutes. Forty-eight hours after
150 training, responses to the CS were determined by placing animals into the chamber they were not
151 trained in (i.e. trained in C1 placed into C2 and vice versa). A plastic insert covered the bars to aid in
152 context discrimination. After a 120s acclimation period, the CS was played for 360s, followed by a
153 stimulus-free 240s post-CS period. Freezing was used as a measure of fear response, defined as
154 immobility excluding movement due to respiration. It was sampled every 10s from video recordings
155 by an observer blind to group/treatment.

156

157 **Immunohistochemistry**

158 Thirty-five minutes after the start of trace recall, animals were deeply anaesthetised and transcardially
159 perfused with 0.01M PBS and 4% paraformaldehyde (PFA). Brains were removed and stored in PFA
160 overnight at 4°C, then transferred to 30% sucrose for cryoprotection, before being sliced coronally at
161 30µm on a freezing microtome (Leica RM2245). Sections were placed into cryoprotectant and stored
162 at -20°C. Sections were stained for: BrdU (labels dividing cells, marking 4-week-old neurons in this
163 study), neuronal nuclei (NeuN, mature neuronal marker) and cfos (immediate early gene, indirect
164 marker of neuronal activity). Unless otherwise stated, sections were washed between each step for 3
165 x 5 minutes in 0.01M Tris-buffered saline (TBS, pH 7.4) and carried out at room temperature. One in
166 every 12 sections throughout the entire extent of the hippocampus was denatured in 1M HCL for 30
167 minutes at 45°C, incubated for one hour in blocking solution (0.3% Triton-X in 0.01M TBS, 2% donkey
168 serum) then rat anti-BrdU (1:800, ab6326, abcam UK), mouse anti-NeuN (1:1000, MAB377, Merk UK)

169 and rabbit anti-cfos (1:5000, ABE457, Merk UK) in blocking solution for 24 hours at 4 °C, followed by
170 Alex Fluor secondary antibodies (donkey anti-rat 488, donkey anti-mouse 647 and donkey-anti rabbit
171 568, 1:200, Life Technologies UK) for 2 hours in the dark. Sections were then incubated with DAPI
172 (1:3000 in TBS, D9542, Sigma UK) for 5 minutes. Washed sections were then mounted on slides and
173 coverslipped with mounting medium (S3023, Dako UK). Slides were imaged using Axio Scan Z1 slide
174 scanner (Carl Zeiss Microscopy, Germany). The total number of cells double labelled with BrdU/NeuN
175 and triple labelled with BrdU/NeuN/cfos were counted bilaterally throughout the entire
176 infrapyramidal and suprapyramidal blades of the dentate gyrus in the dorsal (Bregma -1.72mm to -
177 5.28mm) and ventral (Bregma -5.28mm to -6.72mm) hippocampus, according to the atlas of Paxinos
178 and Watson (2009). As one in every 12 sections throughout the hippocampus was stained and
179 counted, the total number of labelled cells was estimated by multiplying total counts per area by 12³⁸.
180 Counts were analysed using Zen Blue (Carl Zeiss Microscopy, Germany).

181

182 **Data analysis**

183 JMP (statistical software, SAS Institute, Cary, NC, USA) was used for all data analysis. Homogeneity of
184 variance and normality of distribution were checked for all datasets, then data were analysed using
185 generalised linear models, with experimental treatment (control or PPS), enrichment (EE or CH) and
186 experimental treatment*enrichment fitted as factors. When analysing behavioural data from the
187 training day, shock number was added as a factor, and when analysing immunohistochemical data,
188 region (dorsal, ventral, infrapyramidal, suprapyramidal blade) was added. Where necessary, animal
189 was nested within litter and added as a random factor to account for multiple measurements on the
190 same animal. To account for the use of more than one animal per litter, litter was nested within group
191 and added as a random factor. Significant interactions were teased apart using post-hoc Tukey HSD
192 tests. Correlations between number of adult-born cells (all and active) and freezing to context and cue
193 were explored using Pearson's pairwise correlation.

194

195 **Results**

196 **Behaviour**

197 **Training day.** To investigate potential differences in encoding, freezing responses post CS and post US
198 were analysed on the training day. Freezing in the 30s post CS period was unaffected by PPS
199 ($F_{1,13.5}=0.03$, $p=0.85$) or enrichment ($F_{1,31.3}=2.9$, $p=0.09$). Animals froze progressively more as CS-US
200 stimuli were presented, with levels of freezing significantly higher following CS's 3-10 than 1 and 2
201 (shock: $F_{9,360}=35.82$, $p<0.0001$, Figure 1a). A similar pattern was observed following the US, with all
202 animals freezing significantly more after US's 8-10 than 1-7 (shock: $F_{1,360}=4.35$, $p<0.0001$). Following
203 US's 8-10, PPS resulted in lower levels of freezing (group*shock: $F_{9,360}=2.9$, $p=0.002$, Figure 1b).

204 **Context:** Results are shown for the first 120s of each period. Twenty-four hours after the trace fear
205 protocol, all animals demonstrated robust levels of contextual freezing (Figure 2a). Levels of freezing
206 were unaffected by PPS ($F_{1,127.75}=0.07$, $p=0.8$) or environmental enrichment ($F_{1,166.4}=0.15$, $p=0.7$).

207 **Cue recall.** Twenty-four hours after context recall, CS recall was performed. PPS enhanced freezing
208 during the first 90s of CS presentation, this was rescued by environmental enrichment
209 (group*enrichment: $F_{1,127.1}=5.1$, $p=0.03$, Figure 1b). There was no overall effect of PPS ($F_{1,14.82}=0.55$,
210 $p=0.47$) or enrichment ($F_{1,127.1}=3.92$, $p=0.06$) on freezing to the CS. There was no effect of PPS
211 ($F_{1,14.88}=0.74$, $p=0.4$) or enrichment ($F_{1,121.6}=0.63$, $p=0.43$) on freezing in the post-CS period. Baseline
212 freezing was low in all groups, and unaffected by PPS ($F_{1,14.84}=1.28$, $p=0.28$) or enrichment ($F_{1,34.99}=0.96$,
213 $p=0.33$).

214

215 **BrdU/NeuN**

216 PPS reduced the survival of adult-born neurons throughout the entire dentate gyrus, as measured by
217 BrdU/NeuN, and this effect was abolished by enrichment (group*enrichment: $F_{1,159.3}=11.61$, $p<0.001$,

218 Figure 3a). In all groups there were significantly more adult-born neurons in the suprapyramidal blade
219 of the dorsal hippocampus than any other region (region: $F_{3,149.7}=8.57$, $p<0.0001$). There was no overall
220 effect of PPS ($F_{1,14.34}=0.09$, $p=0.77$) or enrichment ($F_{1,159.3}=0.1$, $p=0.74$).

221

222 ***BrdU/NeuN/cfos***

223 PPS reduced the number of active adult-born neurons throughout the entire dentate gyrus post CS
224 recall, as measured by BrdU/NeuN/cfos staining, and this effect was rescued by enrichment
225 (group*enrichment: $F_{3,157}=18.4$, $p<0.001$, Figure 3b). In all groups there were significantly more active
226 adult-born neurons in the suprapyramidal blade of the dorsal dentate gyrus than any other region,
227 and more in the infrapyramidal blade of the dorsal than the ventral dentate gyrus (region:
228 $F_{3,149.6}=11.66$, $p<0.0001$). There was no overall effect of PPS ($F_{1,14.45}=0.19$, $p=0.67$) or enrichment
229 ($F_{1,157}=1.05$, $p=0.31$).

230

231 ***Proportion of BrdU/NeuN cells co-labelled with cfos***

232 There was a trend for PPS to reduce the proportion of active adult-born neurons post CS recall,
233 measured by dividing the number of cells co-labelled with BrdU/NeuN by those triple labelled with
234 BrdU/NeuN/cfos ($F_{1,22.9}=3.18$, $p=0.09$). Enrichment significantly increased the proportion of active
235 adult-born neurons in the stressed group (group*enrichment: $F_{1,156.4}=7.33$, $p<0.01$, Figure 3c). There
236 was a significantly higher proportion of active adult-born neurons in the dorsal compared to the
237 ventral hippocampus across all groups (area: $F_{3,149.2}=13.18$, $p<0.0001$). There was no overall effect of
238 PPS ($F_{1,13.99}=0.33$, $p=0.57$) or enrichment ($F_{1,156.4}=3.52$, $p=0.06$).

239

240 ***Correlations***

241 ***Adult-born neurons & context***

242 There was no correlation between freezing to context and number of adult-born neurons (con CH:
243 $r=0.36$, $p=0.8$; PPS CH: $r=0.5$, $p=0.6$; con EE: $r=0.41$, $p=0.81$; PPS EE: $r=0.4$, $p=0.79$), number of active
244 adult-born neurons (con CH: $r=0.46$, $p=0.85$; PPS CH: $r=-0.1$, $p=0.49$; con EE: $r=0.34$, $p=0.78$; PPS EE:
245 $r=0.42$, $p=0.8$) or proportion of active adult-born neurons (con CH: $r=0.28$, $p=0.77$; PPS CH: $r=-0.34$,
246 $p=0.29$; con EE: $r=0.04$, $p=0.63$; PPS EE: $r=0.3$, $p=0.74$) in any group.

247

248 ***Adult-born neuron & CS***

249 There was a significant positive correlation between CS-evoked freezing and number of adult-born
250 neurons (con CH: $r=0.85$, $p=0.002$; PPS CH: $r=0.73$, $p=0.007$, Figure 4a,b) and number of active adult-
251 born neurons (con CH: $r=0.62$, $p=0.05$; PPS CH: $r=0.73$, $p=0.007$, Figure 4c,d), but not proportion of
252 active adult-born neurons (con CH: $r=-0.35$, $p=0.32$; PPS CH: $r=0.19$, $p=0.55$) in control housed animals
253 only. This relationship disappeared in animals housed in an enriched environment (BrdU/NeuN. Con
254 EE: $r=0.37$, $p=0.24$; PPS EE: $r=0.09$, $p=0.8$, Figure 4a,b. BrdU/NeuN/cfos. Con EE: $r=0.36$, $p=0.77$; PPS
255 EE: $r=0.1$, $p=0.75$, Figure 4c, d. Proportion active. Con EE: $r=0.2$, $p=0.53$; PPS EE: $r=0.12$, $p=0.75$).

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263 **Discussion**

264 Early life stress (ELS) has been robustly and repeatedly associated with the development of psychiatric
265 illnesses. ELS can impinge upon adult hippocampal neurogenesis (AHN), and disrupted AHN is
266 postulated to play a role in psychopathology^{13,16}. This process provides a novel and accessible target
267 for improving hippocampal function and potentially preventing or alleviating psychiatric symptoms.
268 Given that current psychiatric treatments fail in up to 30% of cases, produce significant side effects
269 and high relapse rates, there is an urgent need to develop novel and improved treatments.
270 Environmental enrichment (EE) is an underexplored yet promising method of improving AHN and
271 hippocampal function in animals and humans.

272 We found that short-term stress exposure in the pre-pubertal phase produce decreased
273 survival of mature adult-born neurons in the hippocampus and altered behaviour in a hippocampal-
274 dependent task (trace fear conditioning). Early life stress (ELS) also decreased the number of adult-
275 born neurons that were active during recall of the fear conditioned stimulus cue. Experience of an
276 enriched environment during adolescence rescued all ELS-induced changes in adult hippocampal
277 neurogenesis (AHN), behaviour and neuronal activation.

278 Stress in adulthood has long been associated with impaired learning and memory in humans
279 and animals and with robust changes in stress-sensitive regions such as the hippocampus³⁹. Evidence
280 is mounting that stressful experiences early in life can have unique and permanent consequences for
281 the developing brain, adversely affecting behaviour and increasing risk for psychopathology. Meta-
282 analyses show that childhood abuse is consistently associated with smaller hippocampal volumes, and
283 hippocampal function is also affected⁴⁰⁻⁴³. Similar changes are found in animal models, where ELS
284 affects performance in hippocampal-dependent behaviours such as trace conditioning, pattern
285 separation, object recognition and spatial memory, as well as producing neuronal and molecular
286 alterations^{6,8,32,44}. AHN is particularly sensitive to the effects of pre-natal and early post-natal
287 stressors⁴⁵⁻⁵⁰, but less is known about the post-weaning, pre-pubertal phase, a time-point more akin

288 to human childhood³². In the present study we found that pre-pubertal stress significantly decreased
289 survival of mature (4-week-old) adult-born neurons throughout the dorsal and ventral hippocampus.
290 In a recent study we showed that pre-pubertal stress decreased production of adult-born neurons
291 (less than 24 hours old) and increased survival of immature adult-born neurons (birth-2 weeks,
292 neurons with the potential to survive and be incorporated into hippocampal networks) in the ventral
293 hippocampus only⁸. This demonstrates that different aspects of the neurogenesis process and
294 different regions of the hippocampus can react in a unique manner to stressful early life perturbations.
295 Future work should explore the effects of ELS on the developmental trajectories of adult-born neurons
296 in the hippocampus.

297 Neither PPS nor enrichment affected contextual fear responses, confirming results from our
298 recent study⁸. In an earlier study, we found that PPS decreased contextual fear responses in male
299 animals⁷. However, there were crucial differences between the training protocols. The previous study
300 administered only one shock during training, here we used 10 CS-US pairings, to ensure robust
301 encoding of the trace protocol. It is well known that increasing the number of shocks enhances
302 subsequent contextual freezing⁵¹, and it is interesting to note that increasing the severity of the
303 protocol is sufficient to overcome PPS induced deficits in contextual fear responses in our model.
304 Contextual fear responses require an intact hippocampus in one trial studies (i.e. one shock
305 administered) but can be acquired in the absence of a functioning hippocampus when multiple trials
306 are given⁵². Our results therefore support the hypothesis that PPS specifically impacts upon
307 hippocampal function in males in our model.

308 PPS impaired performance in a hippocampal-dependent task (trace fear). Gross hippocampal
309 lesions selectively impair performance on trace protocols^{53,54}, and ablation of AHN via infusion or
310 optogenetics has a similar effect^{52,55}. PPS reduced post-US freezing on the training day and increased
311 conditioned fear responses to the CS at recall. We recently demonstrated that PPS altered
312 performance in trace fear and pattern separation⁸, two tasks for which in-tact hippocampal function

313 and young adult-born neurons are crucial^{53,56}. Importantly, responses to a delay protocol (10 CS-US
314 pairings with no temporal gap, does not require intact hippocampal function^{53,54,57}), were not altered.
315 Interestingly, in a previous study we found that PPS *reduced* rather than enhanced freezing to the CS
316 in the 48-hour recall test following a trace protocol⁸. In the present study, animals were administered
317 BrdU in early adulthood to label mature adult-born neurons: this occurred 4 weeks before behavioural
318 testing, so these animals were significantly older than those in the previous study at testing. This raises
319 the intriguing possibility that age of testing in adulthood is important in determining the long-term
320 impact of ELS. This phenomenon has already been demonstrated in pre-pubertal vs. adult animals.
321 Perinatal stress increases hippocampal neurogenesis in pre-pubertal males yet causes a decrease in
322 adult males. In females, this stress decreases hippocampal neurogenesis in the pre-pubertal animal,
323 an effect that subsides in adulthood⁵⁸. It is currently unknown how the effects of ELS change as adult
324 animals age, and this should be the focus of further research.

325 In the present study, we investigated whether PPS altered the number of adult-born neurons
326 active during CS recall. We focussed on 4-week-old adult-born neurons, as these are functionally
327 relevant for behaviour. Once produced from dividing neural stem cells in the sub granular zone, adult-
328 born neurons migrate to the granule cell layer and become functionally integrated into circuitry.
329 Axonal projections extend towards the CA3 pyramidal layer, along the mossy fibre pathway, and
330 dendrites proceed towards the molecular layer^{59,60}. Before the formation of output synapses at two
331 weeks, adult-born neurons are not thought to contribute to hippocampal function⁶¹. Between 4 and
332 6 weeks of age, adult-born neurons are functionally and morphologically mature, although they
333 continue to develop for several months⁶⁰. 4-week old adult-born neurons display high sensitivity to
334 LTP induction due to high input resistance and low GABAergic inhibition⁶². Ablation of this population
335 of adult-born neurons, but not those of other ages, disrupts hippocampal-dependent behaviour^{62,63}.
336 PPS reduced the number of adult-born neurons active during CS recall. Other studies have found
337 changes in neuronal activity throughout the adult brain following ELS, but these have not been specific
338 to adult-born neurons. Variations in maternal care altered neuronal activity in the hippocampus

339 (increased) and paraventricular nucleus and periaqueductal grey (decreased) during a fear response
340 (shock-probe burying test)⁶⁴, and maternal separation results in higher neuronal activation throughout
341 the brain in adults exposed to stress⁶⁵⁻⁶⁸. To our knowledge, the present study presents the first
342 demonstration of decreased adult-born neuronal activity following ELS.

343 We also found that the number of adult-born neurons and number of active adult-born
344 neurons was strongly correlated with freezing to the CS but not the context in all animals housed in
345 control conditions, a relationship that was not affected by PPS. Several studies demonstrate a positive
346 relationship between levels of AHN and performance in hippocampal-dependent tasks⁶⁹⁻⁷¹, yet few
347 studies have investigated this relationship in animals exposed to ELS. Adults given limited nesting and
348 bedding (model of ELS) show reduced survival of adult-born neurons, and AHN similarly correlated
349 with performance in hippocampal-dependent, but not independent, tasks⁷². Unexpectedly, this
350 relationship was disrupted by EE, suggesting that additional mechanisms aside from AHN may
351 responsible for the beneficial effects of EE on behaviour. For example, alongside improving
352 performance on hippocampal-dependent tasks, EE also increases glutamic acid carboxylase
353 expression, as well as synaptic transmission and excitability in the hippocampus^{73,74}. Future research
354 should explore these potential mechanisms further.

355 Provision of environmental enrichment (EE) throughout adolescence rescued all ELS induced
356 alterations in AHN and behaviour and restored the number of adult-born neurons that were active
357 during CS recall. However, it had no effect in control animals. EE is a prominent method of improving
358 general wellbeing and rescuing stress-induced behavioural deficits in adult animals⁷⁵. EE also enhances
359 AHN in adult animals⁷⁶⁻⁷⁹. Based on this literature, we may predict that EE during adolescence would
360 be similarly beneficial for AHN and hippocampal-dependent behaviour. This was not the case in EE
361 control animals, which displayed similar behaviour, levels of AHN and new-born neuronal activation
362 to standard housed controls. The effects of EE during development, even in normal animals, are not
363 well understood²⁹, and it is possible that time of exposure (e.g. adolescence vs. adulthood) may

364 profoundly alter the effects of EE. Hippocampal neurogenesis is considerably higher in the developing
365 adolescent brain compared to adulthood in all species studied⁸⁰. This naturally higher rate of
366 hippocampal neurogenesis may be differentially affected by environmental experiences in a normally
367 developing organism. Support for this hypothesis come from a study comparing exercise in
368 adolescence vs adulthood. Adolescent initiated exercise increased the number of young (DCX positive)
369 adult-born neurons, yet this was not the case in adult-initiated exercise⁸¹. Conversely, adult-initiated
370 exercise enhanced fear learning, whereas adolescent-initiated exercise did not⁸².

371 EE during adolescence was effective in restoring normal hippocampal-dependent behaviour,
372 AHN and neuronal activation following ELS. A number of studies demonstrate the benefit of EE
373 throughout adolescence for restoring HPA axis function, learning and memory, anxiety, fear, social
374 performance, attention, depressive-like behaviours, amygdala activity and hippocampal LTP following
375 maternal separation or limited nesting and bedding in the early post-natal period^{23-25, 83-85}. One recent
376 study investigated the effects of exposure to EE during adolescence, directly following PPS, and found
377 this prevents stress vulnerability and normalises anxiety-like behaviour after adult traumatic stress.
378 Interestingly, the same effects were not found when EE was given in adulthood, suggesting there may
379 be an optimal window of opportunity in which to administer EE²⁸. However, to our knowledge this is
380 the first report examining the ability of EE to restore normal AHN and neuronal function following pre-
381 pubertal stress.

382 AHN has been demonstrated in humans and is believed to make meaningful contributions to
383 cognition and neural plasticity as well as contributing to hippocampal aspects of psychiatric illnesses^{13,}
384 ⁸⁶⁻⁸⁹. AHN is also implicated in the behavioural effects of antidepressants^{14,21}, making this an attractive
385 process to target in the treatment of neuropsychiatric disorders. Environmental enrichment in
386 humans (targeting cognitive, social and physical domains) may provide a novel route to improving
387 hippocampal function, however significant translational hurdles exist, and future work should aim to
388 more closely align pre-clinical and clinical studies³¹.

389 In conclusion, we show that stress in the pre-pubertal phase of life results in impaired
390 hippocampal-dependent behaviour, concomitant with a sustained decrease in survival and activity of
391 adult-born neurons in the hippocampus. Exposure to an enriched environment throughout
392 adolescence rescued behavioural performance and restored survival and activity of adult-born
393 neurons to normal levels. These findings provide important insights into the neural plasticity exhibited
394 by the hippocampus throughout development and demonstrate how environmental experiences can
395 impair and rescue hippocampal function. Environmental enrichment may provide a novel therapeutic
396 avenue for humans who have suffered trauma and are at elevated risk of developing neuropsychiatric
397 disorders. Here, as with animals, a multifactorial approach may provide the most effective
398 intervention.

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408

409 **Disclosures**

410 The authors declare no financial or other conflicts of interest.

411

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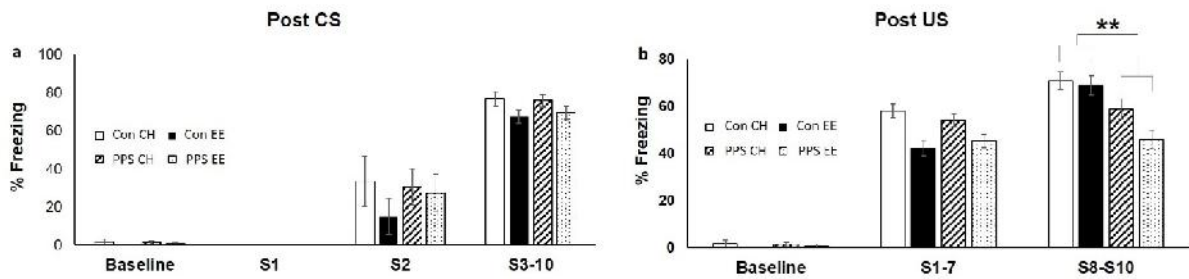
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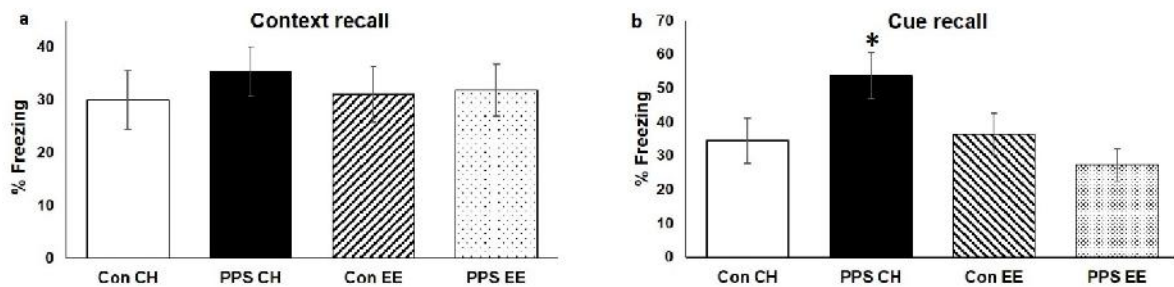
748 **Figure Legends**



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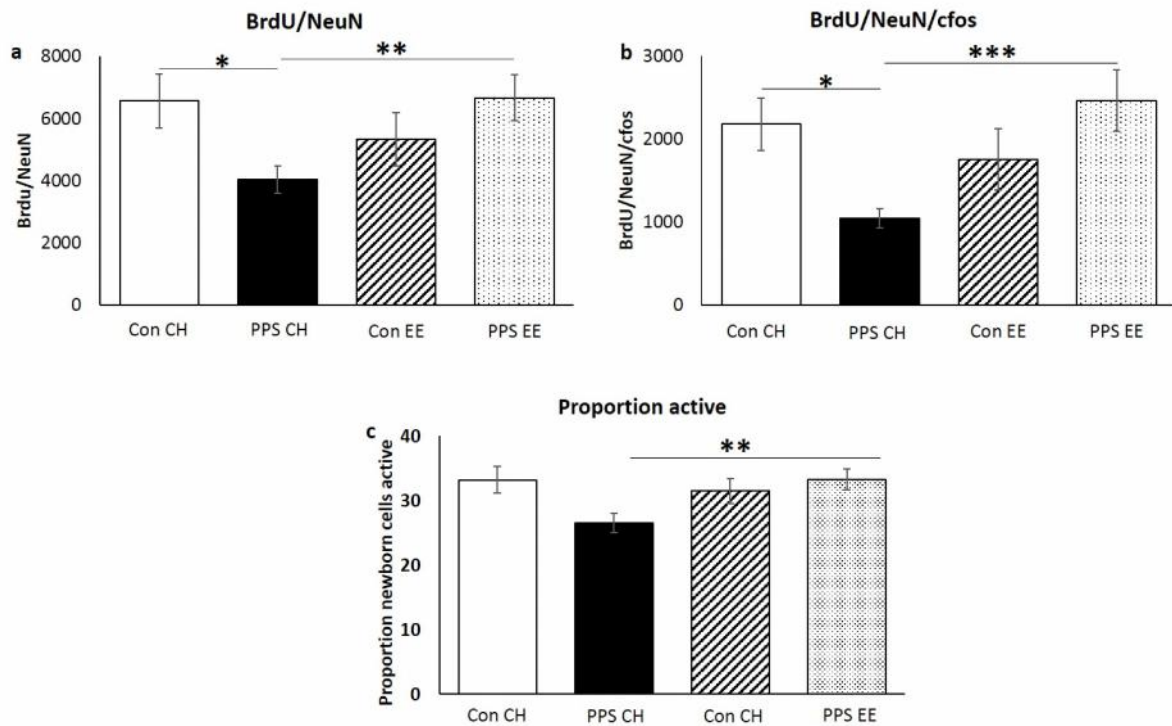
750 **Figure 1 a)** On the training day, animals froze progressively more in the post CS ‘trace’ interval as CS-
 751 US stimuli were presented, with levels of freezing significantly higher following CS’s 3-10 than 1 and
 752 2. **b)** A similar pattern was observed following the US, with all animals freezing significantly more after
 753 US’s 8-10 than 1-7. PPS resulted in lower levels of freezing post US’s 8-10. Con = control animals, PPS
 754 = pre-pubertally stressed animals, CH = control housing, and EE = enriched housing. Error bars
 755 represent 1 SE. **= $p < 0.01$. Bars joined by an asterisk are significantly different to one another.

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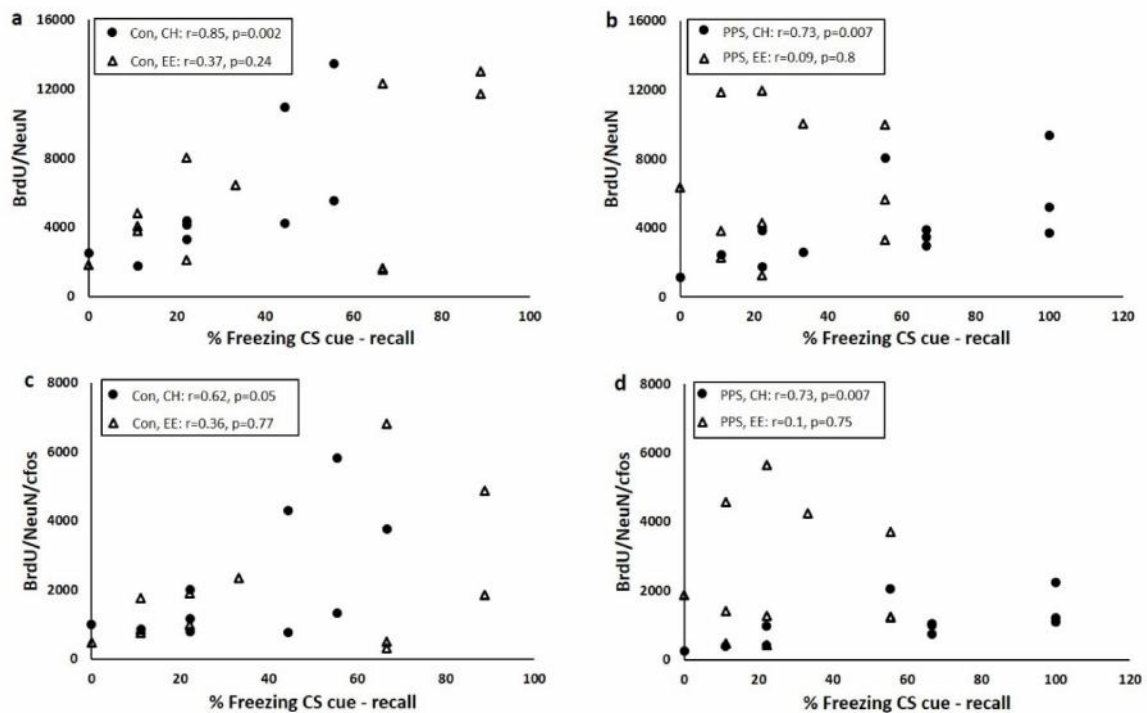
758 **Figure 2 a)** Neither PPS nor EE affected contextual freezing in the 24-hour recall test. **b)** Animals
 759 exposed to PPS and housed in control conditions (PPS CH) froze significantly more to representation
 760 of the CS 48 hours after conditioning. Levels of freezing in PPS animals were restored to control levels
 761 following EE. Con = control animals, PPS = pre-pubertally stressed animals, CH = control housing, and
 762 EE = enriched housing. Error bars represent 1 SE. *= $p < 0.05$. Bars marked with an asterisk are
 763 significantly different to all other groups.



764

765 **Figure 3.** PPS decreased **a)** survival of adult-born neurons, **b)** number of adult-born neurons active
 766 during CS recall and **c)** caused a trend for reduction in the proportion of adult-born neurons active
 767 during CS recall throughout the dorsal and ventral hippocampus. EE restored all measures. Con =
 768 control animals, PPS = pre-pubertally stressed animals, CH = control housing, and EE = enriched
 769 housing. Error bars represent 1 SE. *= $p < 0.05$, **= $p < 0.05$, ***= $p < 0.001$. Bars joined by an asterisk are
 770 significantly different to one another.

771



772

773 **Figure 4.** Freezing to the CS was positively correlated with number of adult-born neurons in **a)** control
 774 animals (Con CH) and **b)** PPS animals (PPS CH) housed in control conditions. Freezing to the CS was
 775 also positively correlated with number of adult-born neurons active during CS recall in **c)** control
 776 animals (CON CH) and **d)** PPS animals (PPS CH) housed in control conditions. This relationship was not
 777 observed in animals housed in enriched conditions (Con EE, PPS EE). Con = control animals, PPS = pre-
 778 pubertally stressed animals, CH = control housing, and EE = enriched housing.