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# Early-Age Running Enhances Activity of Adult-Born Dentate Granule Neurons following Learning in Rats

## Early-age running and neurogenesis

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**Abstract**

Cognitive reserve, the brain's capacity to draw on enriching experiences during youth, is believed to protect against memory loss associated with a decline in hippocampal function, as seen in normal aging and neurodegenerative disease. Adult neurogenesis has been suggested as a specific mechanism involved in cognitive (or neurogenic) reserve. The first objective of this study was to compare learning–related neuronal activity in adult-born vs. developmentally-born hippocampal neurons in juvenile male rats that had engaged in extensive running activity during early development or reared in a standard laboratory environment. The second objective was to investigate the long-term effect of exercise in rats on learning and memory of a contextual fear response later in adulthood. These aims address the important question as to whether exercise in early life is sufficient to build a reserve that protects against the process of cognitive aging. The results reveal a long-term effect of early running on adult-born dentate granule neurons and a special role for adult-born neurons in contextual memory, in a manner that is consistent with the neurogenic reserve hypothesis.

**Significance Statement**

The role of adult neurogenesis in learning and memory is under active investigation, but the underlying mechanisms remain unclear. The present study found that early-age running led to enhanced associative learning and memory in adult rats and increased activity of adult-born granule neurons in the dentate gyrus during memory retrieval. This study demonstrates the long-term effect of early-age physical activity on learning and memory much later in life. The findings emphasize the involvement of adult-born

70 hippocampal neurons in neurogenic and functional cognitive reserve and show that

71 physical activity contributes to memory improvement.

72

73 **Introduction**

74 Cognitive reserve refers to the brain's capacity to draw on enriching experiences during

75 youth to protect against adverse effects of structural decline, as in normal aging, and

76 neuropathological damage resulting from accident or disease (Stern, 2002). A number of

77 factors, including physical exercise, education (Puccioni and Vallesi, 2012), occupation, and

78 lifestyle (Fratiglioni and Wang, 2007) contribute to cognitive reserve, which has been

79 viewed as a compensatory mechanism for optimizing function in the compromised brain.

80 The relationship between cognitive protection and brain plasticity is central to the concept

81 of cognitive reserve but our understanding of specific brain mechanisms involved is

82 limited.  One possibility may relate to adult neurogenesis, the capacity to produce new cells

83 in the hippocampus that become integrated into neuronal networks of learning and

84 memory. Several investigators have shown that physical exercise and other types of

85 environmental enrichment increase neurogenesis levels (van Praag et al., 1999) and

86 improve performance on hippocampus-sensitive tests of cognition (Farmer et al., 2004).

87 Based on these findings, Kempermann proposed a 'neurogenic reserve' hypothesis in which

88 the potential for adult neurogenesis is maintained. According to this hypothesis, the

89 continued exposure to stimulating events in young age leads to the increased production of

90 new hippocampal neurons to support cognitive function in old age (Kempermann, 2008).

91 In previous study we used voluntary running to increase the levels of hippocampal

92 neurogenesis in juvenile rats and examine neurogenesis levels at various time points, up to

93    9 months later (Merkley et al., 2014). Voluntary running was chosen as an enriching

94    activity because of its known effects on neurogenesis (van Praag et al., 1999), and

95    associated effects on learning and memory (van Praag et al., 2005; Abrous and Wojtowicz,

96    2016). Our study provided the first evidence that early life physical activity in rodents can

97    build a long-lasting neurogenic reserve later in life (Merkley et al., 2014).

98    The question addressed in the present study is whether voluntary running during early

99    development can contribute to such a process in later adulthood. To test whether

100    neurogenic reserve serves as a mechanism to improve memory, one group of rats was

101    given voluntary access to running wheels for 6 weeks and another group was housed in

102    standard laboratory cages. After 4 months rats were trained on a contextual fear (CF)

103    conditioning task and, 2 weeks later, tested for memory of the CF response. CF is known to

104    depend on the hippocampus and, in particular, new hippocampal neurons (Winocur et al.,

105    2006; Winocur et al., 2012). Rats received CF conditioning when they were 6-7 months old.

106    At that age, a potential reserve mechanism would be primed for possible use in case of

107    damage or disease, but not yet engaged in a compensatory manner.

108    Testing was done in the original conditioning context, a similar one, or a very different

109    context, to provide information on the quality of memories. The purpose of manipulating

110    context at test was to determine if these effects generalized to contexts that bore some

111    similarity to the conditioning chamber but were not associated directly with shock. A novel

112    aspect of the present study was the utilization of c-Fos-immunoreactivity to measure the

113    activity of adult born neurons in the DG in response to testing. Using immunohistochemical

114    methods with a mitotic marker CldU and an activity marker c-Fos we were able to

115 demonstrate the enhanced activity of adult-born dentate granule neurons in comparison to

116 developmentally-born neurons.

117

118 **Materials and Methods**

119 **Experimental Design**

120 One-month old (juvenile) male rats (n=80) were housed for 6 weeks in specially designed

121 cages with free access to running wheels, or in standard laboratory cages. After 1 week

122 acclimatization, the rats were divided into 2 groups, runners (n=40) and non-runners

123 (n=40), and kept in single cages throughout the study. Runners and non-runners were

124 maintained under the same housing conditions, including *ad libitum* access to food and

125 water, except that cages of runners were fitted with a running wheel (circumference:

126 1.07m). Running was monitored daily by means of a Vital View data acquisition system

127 (Mini-Mitter A Respironics Company, Inc., Bend, OR, USA).

128 After 6 weeks the running wheels were removed and the animals housed in pairs for 4

129 months in standard laboratory cages conditions for the remainder of the experiment (see

130 below). The running and non-running groups were subdivided into 4 sub-groups: 1. No

131 training and no test (controls), 2. Context A, 3. Context A' and 4. Context B. Three weeks

132 prior to CF conditioning, all rats received a CldU injection. When the rats were 6 – 7 months

133 old, they were trained on a standard form of CF conditioning (Winocur et al, 2006). Two

134 weeks later, memory for the fear response was tested in the original conditioning chamber

135 (context A), a similar chamber (context A'), or a very different chamber (context B).

136 Animals were sacrificed shortly after testing and adult neurogenesis in the dentate gyrus

137 (DG) of the hippocampus was analyzed. Training and testing environments, as well as

138    tissue collection, processing and immunohistochemistry, are described in detail below. The

139    experimental timeline is shown in Figure 1.

140

141    Figure 1 here

142

143    **Animals**

144    Long Evans rats (Charles River, St. Constant, Quebec, Canada) were used in this study. All

145    animals were maintained on a 12h light/dark cycle with lights on at 7am-7pm. Animal

146    weights were recorded regularly and animal procedures were in accordance with the

147    guidelines of the Canadian Council on Animal Care. The experimental protocol was

148    approved by the Animal Care Committee at the University of Toronto and Trent University.

149

150    **Running**

151    Running distances were monitored daily as well as the time of day that each animal spent

152    running. The records showed that the rats predominantly ran during the dark phase of

153    their cycle. Cumulative running distance or average distance traveled did not differ

154    between cohorts designated to testing and control groups but the distance covered across

155    the entire running period of 6 weeks increased progressively for all groups. Thus, rats ran

156    an average of 21.8(S.D.=14.8) km during week 1 and reached the maximum of

157    71.1(S.D.=57.9) km during weeks 5 and 6.

158

159    **Training and Testing Environments**

160 All rats received CF conditioning in a wooden chamber (50 cm x 40cm x18cm) that had

161 four walls made of clear Plexiglas, a hinged clear Plexiglas roof with holes to allow

162 ventilation, and a floor that consisted of metal rods, spaced 1.3 cm apart. The chamber was

163 placed on a table, 1.3 m above the floor, and situated in the centre of a standard laboratory

164 room.  The room contained standard furniture (eg., desk, table, bookshelf along one wall,

165 etc.), as well as pictures, light fixtures, etc. on the walls. Illumination was provided by

166 overhead fluorescent lights under rheostatic control.

167 Training procedures for CF conditioning were similar to those followed in previous studies

168 (Winocur et al, 2006; 2007). Each rat received one fear conditioning trial that began with

169 the rat being placed in the chamber and allowed to explore freely for 5 min. Near the end of

170 the exploration period and over a 64 s period, 8 observations of freezing behavior were

171 recorded every 8 s in order to obtain a pre-shock measure of freezing. Freezing was

172 defined by an immobilized crouching response in which the only detectable movement was

173 the rat's breathing.  Behavior was monitored by an overhead video camera connected to a

174 recorder and data processing system that recorded the time spent freezing. The rat then

175 received 10 tone-shock pairings at 2 min intervals (tone: 2000 Hz; 80 – 90 db, 30 s; shock:

176 1.5 mA; 1 s). The tone was presented through a centrally mounted speaker attached to the

177 box and the shock was delivered by TechServe (Model 452A shock generator). Beginning

178 30 s after the last shock and over a 64 s period, freezing behavior was recorded every 8 s (8

179 observations).  One minute later the rat was removed from the box and returned to its

180 home cage.

181 For testing, rats assigned to context A were tested in the same chamber and environment

182 as in CF conditioning.  Rats in context A' were tested in the same chamber but the

183  environment was slightly changed (eg., room objects re-arranged, lighting dimmed

184  slightly). Rats in the context B were tested in a smaller box (40 cm x30 cm x18cm), also

185  made of Plexiglas but with walls that were lined with opaque gray material.  The roof was

186  clear Plexiglas with ventilation holes and the floor consisted of metal rods, spaced 1.3 cm

187  apart. This test box was placed in a different room on a table that was situated against a

188  wall. Care was taken to ensure that the configuration of furniture, pictures, etc, was

189  different from that of the room in which fear conditioning took place. Testing procedures

190  were identical in all conditions. Testing consisted of a single trial in which the rat was

191  placed in the appropriate box for 8 min and, in the absence of the tone, the amount of time

192  spent freezing was recorded.  The rat was then removed from the box and returned to its

193  home cage.

194

195  **CldU injections**

196  Three weeks prior to CFC training, each rat was injected intraperitoneally with thymidine

197  analog 5-chloro-2'-deoxyuridine (CldU; 105478, MP Biomedicals). The injected solution

198  was prepared by dissolving CldU in saline at 10mg/ml and adjusting the pH to 7 with 0.5 μl

199  10N NaOH/ml saline. The injected dose was 85mg CldU/kg.

200

201  **Tissue Collection and Processing**

202  Ninety minutes after testing animals were deeply anesthetized with isoflurane inhalation

203  followed by transcardial perfusion with 300 mL ice-cold phosphate-buffered saline (PBS)

204  followed by 300 mL ice-cold 4% paraformaldehyde (PFA). Following decapitation, brains

205  were removed and placed in PFA for 24 h at 4°C. After post-fix, brains were placed in PBS

206  containing 0.1% sodium azide, and stored until further processing.

207

208  **Immunohistochemistry**

209  Brains were cut in half, and the hippocampus was dissected from the right hemisphere in

210  each animal. Isolated hippocampi were sectioned along the dorso-ventral axis using a

211  vibratome (VT1000S, Leica Microsystems, Heidelberg, Germany) into sections 30 μm thick.

212  The sections were stored in PBS containing 0.1% sodium azide at 4°C until staining. Twelve

213  sections were selected from each animal using a systematic random sampling procedure

214  previously described (Wojtowicz and Kee, 2006). All immunohistochemistry was

215  conducted on free floating sections. Importantly, sections were rinsed extensively in PBS

216  before processing and between each incubation. All primary and secondary antibody

217  incubations were conducted in a PBS solution containing 0.3% Triton X-100. In

218  experiments involving labeling of CldU, sections were incubated at 45°C for 30 min in HCL

219  (1.0 N) to denature DNA and unmask the antigen prior to incubation in primary antibody,

220  preceded and followed by extensive rinsing.

221

222  **Detection of c-Fos and CldU**

223  To identify cell survival and activity of newly formed cells, double-label

224  immunohistochemistry was conducted for CldU and immediate early gene (IEG) protein c-

225  Fos. Sections were incubated sequentially, with primary anti-c-Fos antibody (1:2000, 72 h

226  at $4^0$C; Millipore, ABE457), followed by secondary antibody donkey anti-rabbit IgG Alexa

227  568 (1:200, 2 h at RT, Life Technologies, A10042) in dark; afterward, to detect CldU, the

228  tissue was incubated with primary antibody rat anti-BrdU (1:1500, 24 h at 4$^0$C, AbD

229  Serotec, OBT0030), followed by secondary antibody goat anti-rat IgG Alexa 488 (1:200, 2 h

230  at RT, Life Technologies, A11006). This particular antibody can bind to CldU with high

231  affinity (Merkley et al., 2014). In all experiments, sections were mounted onto glass slides

232  using double-distilled water (ddH$_2$O), and coverslipped using PermaFluor (Thermo

233  Scientific, Freemont, CA, USA). Immunohistochemical controls included the omission of

234  primary antibodies, which resulted in lack of staining at the corresponding wavelength in

235  each instance.

236  **Quantification of Cells and Cell Counts**

237  All the single immunolabeled cells in the granule cell layer zone (GCL) of the DG were

238  counted using a fluorescent microscope (Nikon, Eclipse Ni). Double-labeled neurons were

239  visualized using Leica TCS SP5 confocal microscope (Leica Microsystems). The average

240  number of cells per sampling section was multiplied by the number of hippocampal

241  sections to yield the total number of cells per DG in each rat. Twelve sections were initially

242  sampled from each animal. The sampling was repeated twice or in some animals three

243  times in order to reach a minimum of at least 30 CldU+ cells per animal. On average

244  68.3(SD=34.3, n=80) CldU+ cells per animal were examined for double-labeling with c-Fos.

245

246  **Statistical Analysis**

247  For immunohistochemistry and behavioral data, differences between groups were

248  analyzed using Two-Way ANOVA with running and testing as variables. All pairwise

249  multiple comparisons were conducted using the Holm-Sidak post-hoc test or, in case of

250  behavioral data, the Bonferroni multiple comparisons procedure. All values were

251  expressed as mean ± SEM or S.D. when appropriate. Statistical analyses were performed

252  using Sigma Plot 12.0 software (Systat Software Inc). The level of statistical significance

253  was set at p≤0.05.

254

255  **RESULTS**

256  **Hippocampal Neurons are Activated During a Contextual Learning Task**

257  A necessary element of this study is a measure of overall activity in the hippocampal

258  neurons, especially those in the DG. For this purpose, we used a c-Fos protein detected by

259  immunohistochemistry as a measure of c-Fos gene expression. The results illustrated in

260  Figure 2, show c-Fos activity in the DG following test, which took place 2 weeks after CFC

261  training (see experimental timeline in Fig.1). As expected, only a small fraction of the

262  general granule cell population expressed c-Fos in control animals (Fig. 2A). A similar

263  result was obtained for the CA1 field of the hippocampus (Fig. 2-1, Extended Data).

264

265  Figure 2 here

266

267  A Two-way ANOVA comparing c-Fos activation within the DG in early runners vs. non-

268  runners shows a testing-induced increase in c-Fos (F=15.9, p<0.001) that was equal in

269  runners and non-runners $(F(1,79)=0.297, p=0.588)$ with no interactions among the

270  variables $(F(1,79)=0.063, p=0.979)$(Fig. 2B). Upon subsequent examination of different

271  contexts, the results showed that this testing-induced increase in the number of c-Fos+

272  cells generalized to all contexts, as shown by similar levels of activation in all tested sub-

273  groups in both running and non-running conditions (Fig. 2C). These results are a robust

274    indication that DG and the hippocampus as a whole participates in the CFC task and that

275    early running doesn't influence this activity.

276    Additional estimates of granule cell volumes revealed no significant differences among the

277    groups (Two-way ANOVA using running (p=0.116) and testing (p=0.883) as variables; data

278    not shown). Thus, the test-induced increase in the hippocampal c-Fos activity is a true

279    measure of the increased activity among the pre-existing neurons and not an effect on

280    hippocampal growth.

281

282    **c-Fos Activity is Related to Adult-Born Neurons and Enhanced by Running**

283    To determine whether the c-Fos activity was related to adult-born granule neurons rather

284    than to the pre-established general population, the densities of double-labeled cells (c-

285    Fos/CldU) were measured. The animals were injected with CldU three weeks prior to

286    training thus labeling a cohort of 5-week-old neurons at the time of testing. This age was

287    chosen to ensure a full maturity and functionality of the neurons in terms of IEG expression

288    (Snyder et al., 2009).

289

290    Figure 3 here

291

292    The estimates demonstrate that the proportion of active cells is approximately 3% in cage

293    controls, and up to 7% in tested runners (Fig. 3, B). In terms of absolute numbers the c-

294    Fos+/CldU+ cells (Non-runner controls, mean=20.4; SD=3.5/DG) were less numerous than

295    the c-Fos+ cells in the general DG population (compare to Fig.2). However, the % activation

296    numbers show that, not only are the adult-born neurons more active than a general

297    population but also their activity is significantly enhanced in early runners as compared to

298    non-runners in tested animals (Two-way ANOVA for running (F(1,79)=5.837,p=0.018),

299    testing (F(1,79)=13.946, p=0.001) and (F(1,79)=10.115, p=0.002) interactions) (Fig. 3B).

300    Next, c-Fos activity was examined in relation to specific tasks (A, A' or B) administered to

301    animals at the time of testing. Context A representing the environment in which the rats

302    were trained, A' representing a similar, and B a completely novel environment (Fig. 4).

303

304    Figure 4 here

305

306    Two-way ANOVA performed on the absolute numbers of the double-labeled cells shows

307    significant effects of running (F(1,79)=23.57, p<0.001), testing (F(3,79)=5.582, p=0.002)

308    and significant interaction (F(3,79)=2.872, p=0.042)(Fig. 4B). In particular, the three tested

309    groups were higher in comparison to controls (pairwise comparison at p<0.05). Also, the

310    cell numbers in tested runners were higher in comparison to those in tested non-runners.

311    There were no differences among any of the non-running groups. Thus, the early-age

312    running specifically enhances the activity of adult-born 5 week old neurons when rats are

313    tested in the same, familiar or novel environment. Importantly, the % c-Fos expression is

314    elevated in parallel to the absolute cells numbers. The increase shown in Figure 4 is not due

315    to enhanced cell survival in tested runners since the numbers of CldU+ cells did not differ

316    in any of the groups (Fig. 4-1, Extended Data).

317

318    **Memory Selectivity is Enhanced by Running**

319   The animals were administered CF conditioning in context A two weeks prior to perfusion

320   (see experimental timeline, Fig.1). The performance of all groups following CF conditioning

321   and testing are presented in terms of % time the animals spent freezing (Fig. 5). Two-way

322   ANOVA of freezing times and running showed a significant effect of running

323   $(F(1,54)=11.672, p<0.001)$ and testing $(F(2,54)=3.692, p=0.031)$. The interactions between

324   running and testing could not be adequately examined due to a low statistical power (0.05),

325   however a pairwise comparison with the Bonferroni t-test revealed a significant difference

326   between runners and non-runners within context A' (P=0.01) and B (P=0.049) but not

327   within A (P=0.219).

328   These effects were specific for memory testing since the conditioning (training) phase

329   showed no difference in learning. The % freezing scores in runners (84.2, SE=3.5) and non-

330   runners (78, SE=4.3) were not different (t=1.027, 58df, p=0.31). In summary, these

331   behavioral results show no effect of running on memory of the familiar context (A) but

332   reduced freezing in the similar (A') and completely different (B) contexts. The results

333   parallel the c-Fos activation in adult-born granule DG neurons showing selective activation

334   in runners but not in non-runners.

335

336   **Discussion**

337   The most striking result of this study is the effect of early-age running on memory-related

338   neuronal activity (Figs. 3, 4). This is the first evidence of such a long-term effect and a

339   confirmation of an animal model of cognitive reserve. The model was based on numerous

340   human studies which used a variety of forms of enrichment to induce the reserve

341   phenomenon reviewed in (Merkley and Wojtowicz, 2016). Running is known to reliably

342  induce increases in adult neurogenesis and enhance cognitive function in animals and in

343  humans (Dery et al., 2013; Hutton et al., 2015). The 6-week period of running was based on

344  a previous study and is sufficient to produce a maximal effect since running reached a

345  maximum at that period (see Materials and Methods). A relatively high 6-7% of c-Fos in

346  adult-born neurons, as shown in Figure 3, is far greater than the sparse activation of <1%

347  seen in the developmentally-born neurons in agreement with previous estimates (Chawla

348  et al., 2005; Ramirez-Amaya et al., 2006) and our data in Figure 2.

349  In the present study, rats in the running condition, that were found to exhibit enhanced

350  neurogenesis, also froze less than non-runners when tested in contexts A' and B indicating

351  better discriminability of the contextual environments and less generalization of the fear

352  response. There is considerable evidence that the ability to discriminate between

353  overlapping stimulus elements (pattern separation), is associated with hippocampal

354  function (Leutgeb et al., 2007) and, in particular, adult neurogenesis in DG (Clelland et al.,

355  2009; Abrous and Wojtowicz, 2016; McAvoy et al., 2016).  The present results extend

356  previous reports by showing that adult neurogenesis can be enhanced by early experience

357  and, as a mechanism underlying pattern separation, can be mediated through a build-up of

358  cognitive reserve.  The enduring effects of early running may have occurred at the level of

359  stem cells and/or the neurogenic milieu, presumably in the subgranular zone of the DG.

360  Hippocampal afferents are known to influence the neurogenic zone by stimulating distinct

361  phases of cellular growth and development (Wojtowicz and Tan, 2016) and epigenetic

362  influences on progenitors that could mediate such changes have been described (Ma et al.,

363  2009; Yao et al., 2016).

364    Runners tested in context A also exhibited more activity in adult-born cells than non-

365    runners, but the groups did not differ in freezing time.  This outcome was not necessarily

366    expected but is understandable when considering the dynamic changes that occur in

367    contextual fear memory over time (Winocur et al., 2010). Several studies have shown that

368    contextual fear memories, when initially encoded in the hippocampus, are highly context-

369    specific and do not generalize to other environments.  After a few days, these memories

370    transform into less specific schematic memories that can be evoked by a sample of the

371    original contextual cues or even by new contexts that bear only a slight similarity to the

372    original (Wiltgen and Silva, 2007; Winocur et al., 2007). In the normal animal, the context-

373    specific and non-specific versions can co-exist and the type of memory that is expressed

374    depends on several factors (Winocur et al., 2013). It is likely that, in the present study, the

375    runners, with elevated neurogenesis activity and enhanced hippocampal function, were

376    expressing hippocampus-dependent, context-specific memory of the fear memory, whereas

377    the non-runners were retrieving the more general, schematic version that is believed to be

378    represented in a network of cortical regions.

379    The influence of early life experiences on learning and memory in later life is a topic

380    needing more study (Stern, 2012). Numerous human studies point to beneficial effects of

381    educational and/or physical experiences during youth on symptoms of cognitive decline in

382    old age. The idea was publicized by the "Nun study" which described several examples of

383    such life-long effects (Iacono et al., 2009). As well, in line with the cognitive reserve

384    hypothesis, other survey-type studies have emphasized the impact of early age experiences

385    on cognitive performance in late adulthood (Scarmeas and Stern, 2003; Petrosini, 2009).

386    Qualitatively, cognitive reserve can be viewed as a buffer or reservoir of plasticity acquired

387 early in life, that is utilized later in response to normal age-related decay or pathological

388 damage. As such, cognitive reserve may constitute an important part of the compensatory

389 mechanisms frequently observed in the compromised brain(Wang et al., 2005).

390 There is considerable evidence that early physical and cognitive activity can delay the onset

391 of pathological changes that accelerate the process of cognitive decline in old age

392 (Valenzuela and Sachdev, 2006; Stern, 2012). In animal models of Alzheimer's disease (AD)

393 changes in the adult neurogenesis often precede appearance of pathological markers and

394 cognitive symptoms of AD (Hamilton et al., 2010; Krezymon et al., 2013). Thus, reduced

395 levels of adult neurogenesis may be a measure of early symptoms and a predictor of

396 subsequent disease.  If so, it follows that early life-style related interventions (eg., social

397 stimulation, physical exercise) that help to prevent such effects through the build-up of a

398 neurogenic reserve (Merkley and Wojtowicz, 2016), may provide a measure of protection

399 against early cognitive impairment.

400

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499          neurogenesis. Nat Rev Neurosci 17:537-549.

500

501 **Figure legends**

502 **Figure 1. Experimental timeline**

503 At one month of age a group of 40 rats was exposed to running wheel while the other group

504 was kept in standard cages for six weeks. All rats were trained on the CFC task in context A.

505 Two weeks after training 10 rats from each group were tested in context A, A' or B. The

506 remaining rats served as untested controls. Mitotic marker CldU was injected 3 weeks prior

507 to training. Ninety minutes after the test all animals were perfused for
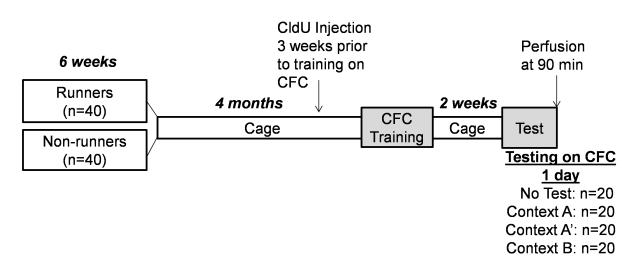
508 immunohistochemistry.

509

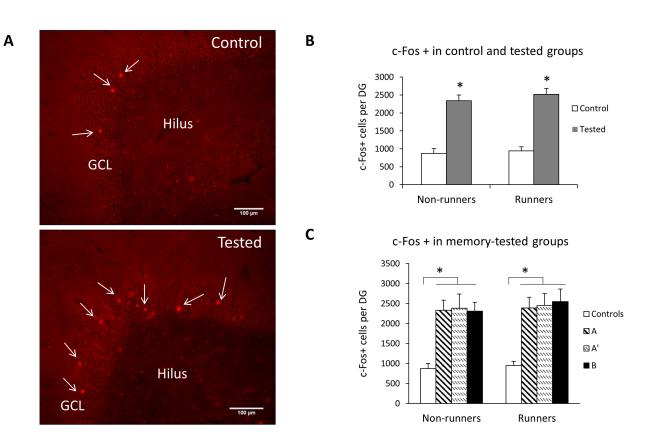510 **Figure 2. c- Fos activity in dentate gyrus neurons**

511 **A.**Fluorescent microscopic images showing c-Fos cells in the dentate gyrus (DG) of control

512 and tested rats. White arrows indicate c-Fos labeled cells in the granule cell layer (GCL) of

513 the DG. **B.** The number (mean ± SEM) of c-Fos labeled cells per DG. Tested animals showed

514 significantly more c-Fos+ cells per DG than controls (* $p < 0.05$). A similar result was

515 obtained for the CA1 field of the hippocampus (Fig. 2-1, Extended data). **C.** Running and

516 individual tested groups. Controls – non-tested cage controls. A – tested in the familiar

517 environment. A' – tested in the similar environment. B – tested in the novel environment.
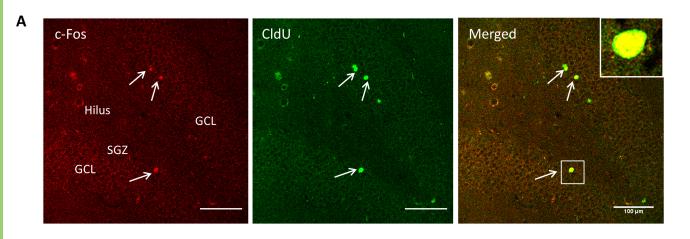
518   The number of c-Fos+ cells was greater in all tested groups compared to controls in

519   runners and non-runners (* p<0.05, n= 10 rats / group).

520

521   **Figure 3. Neuronal activity in adult-born neurons in runners and non-runners**

522   **A.** Confocal microscopic images showing c-Fos and CldU labeled cells within the

523   subgranular zone (SGZ) of the granule cell layer (GCL). White arrows indicate dual-labeled

524   c-Fos+/CldU+ cells in the GCL. Boxed area is enlarged in the inset showing the double-

525   labelled cell. Scale bars, 100 μm.

526   **B.** Graph showing % expression of c-Fos+ cells in CldU+ cells within dentate gyrus in

527   control and tested groups. There is a significant effect of running in memory tested group

528   (*p<0.05, n= 10 rats / control group; n=30 rats/memory tested group). No difference in cell

529   numbers between control and tested rats in non-runners.

530

531   **Figure 4. c-Fos activity in adult-born neurons is enhanced by running**

532   **A**. Representative images of c-Fos+/CldU+ labeled cells within the granule cell layer (GCL).

533   White arrow indicates dual-labeled cells. The inset shows a high magnification view of the

534   c-Fos+/CldU+ labeled cell.  Scale bars, 50 μm.

535   **B**. Absolute numbers of double labeled c-Fos+/CldU+ cells. Comparison of tested groups

536   (controls, context A,  A', B) within runners and non-runners.  All three tested groups have

537   significantly more cell numbers compared to controls (*p<0.05, n= 10 rats / control group;

538   n=30 rats/memory tested group). The numbers of CldU+ cells did not differ in any of the

539   groups (Fig.4-1, Extended data). **C.** Graph showing % expression of c-Fos in CldU cells.

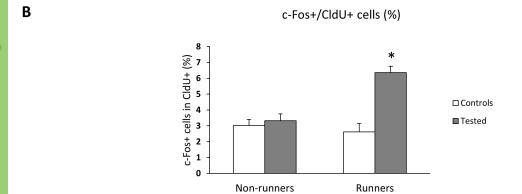540   Controls – non-tested cage controls. A – tested in the familiar environment. A' – tested in

the similar environment. B – tested in the novel environment. The % of c-Fos expression in

CldU cells was greater in all tested groups compared to controls in runners (* $p<0.05$, n= 10

rats / group).

**Figure 5. Results of CFC training and testing.** Early running did not affect acquisition of

the contextual fear response during training. There were no differences among the group A

(familiar environment) tested animals. The group A' (similar environment) showed

significantly (P=0.01**) less freezing in runners. The group B (different environment)

showed significantly (P=0.05 *) less freezing in runners.

**Figure 2-1. Activity-dependent regulation of c-Fos in the CA1 area of the hippocampus**

Number (mean ± SEM) of c-Fos labeled cells per CA1. Tested rats had significantly more

cFos+ cells per CA1 compared to controls. Two-way ANOVA shows an effect of testing

($F_{(3,79)}=10.838$, *p=0.001). There was no effect of running ($F_{(1,79)}=1.131$, p=0.291) and

no interaction ($F_{3,79}=2.151$, p=0.101).

**Figure 4-1. Cell survival in the dentate gyrus**

Graph showing number (mean ± SEM) of CldU labeled cells per DG. No difference in the

number of CldU+ cells was detected between groups. Two-way ANOVA shows no effect of

running ($F_{(1,79)}=1.749$, p=0.19), testing ($F_{(1,79)}=0.56$, p=0.453) and no interactions.
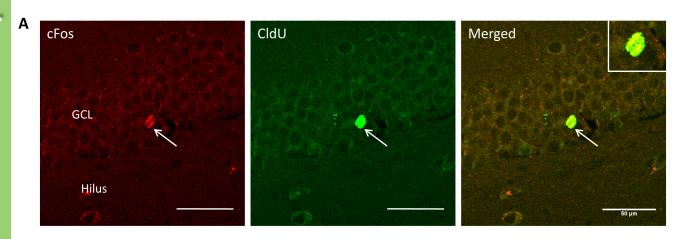
**Experimental Timeline**

CldU Injection
3 weeks prior
to training on
CFC

Perfusion
at 90 min

*6 weeks*

| Runners (n=40) |
| Non-runners (n=40) |

*4 months*

Cage

| CFC Training |

*2 weeks*

Cage

| Test |

**Testing on CFC**
**1 day**
No Test: n=20
Context A: n=20
Context A': n=20
Context B: n=20

**A**



**B**

c-Fos + in control and tested groups



**C**

c-Fos + in memory-tested groups

**A**



**B**

c-Fos+/CldU+ cells (%)
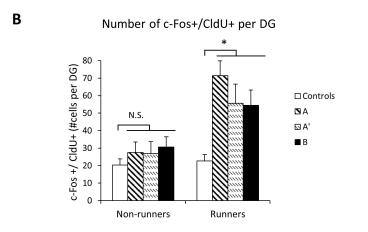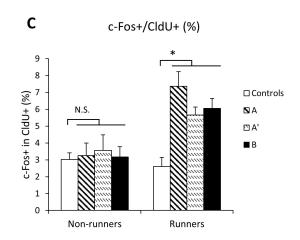
**A**



**B** Number of c-Fos+/CldU+ per DG



**C** c-Fos+/CldU+ (%)

**% Freeze in conditioning and testing**