

1 **Modeling human age-associated increase in Gadd45y expression leads to spatial**
2 **recognition memory impairments in young adult mice**

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26 **Abstract**

27 Aging is associated with the progressive decay of cognitive function. Hippocampus-
28 dependent processes, such as the formation of spatial memory, are particularly vulnerable to
29 aging. Currently, the molecular mechanisms responsible for age-dependent cognitive decline
30 are largely unknown. Here, we investigated the expression and function of the growth arrest
31 DNA damage gamma (Gadd45 γ) during aging and cognition. We report that Gadd45 γ
32 expression is increased in the hippocampus of aged humans and that Gadd45 γ
33 overexpression in the young adult mouse hippocampus compromises cognition. Moreover,
34 Gadd45 γ overexpression in hippocampal neurons disrupted CREB signaling and the
35 expression of well-established activity-regulated genes. This work shows that Gadd45 γ
36 expression is tightly controlled in the hippocampus and its disruption may be a mechanism
37 contributing to age-related cognitive impairments observed in humans.

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39 **Keywords:** Activity-regulated gene expression, age-related cognitive deficits, CREB,
40 Gadd45 γ , object location memory.

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51 **1. Introduction**

52 Age-related cognitive decline in humans affects about 40% of individuals aged 65 years or
53 older (Aigbogun et al., 2017), even though deterioration of cognitive functions may start
54 earlier (Singh-Manoux et al., 2012). Long-term memory formation requires activity-regulated
55 signaling that results in *de novo* gene expression. These genomic responses are known to
56 be disrupted in the aged hippocampus (Stefanelli et al., 2018). Therefore, age-associated
57 changes that dysregulate the coupling between neuronal activity and gene transcription likely
58 underlie age-related cognitive deficits.

59 Recently, we and others showed that the growth arrest DNA damage gamma (*Gadd45y*) is
60 required for memory formation in the prelimbic prefrontal cortex (Li et al., 2018) and the
61 hippocampus (Brito et al., 2020). Moreover, we found that aging reduces *Gadd45y*
62 expression in the mouse hippocampus and that mimicking this reduction in young adult mice
63 induces age-like memory impairments (Brito et al., 2020). At the molecular level, *Gadd45y* is
64 required for cAMP response element-binding protein (CREB) activation in response to
65 neuronal activity and associated gene expression (Brito et al., 2020). In the current study, we
66 found that in *postmortem* human hippocampal tissue from aged individuals, *Gadd45y*
67 expression levels are increased relative to young donors. Furthermore, we showed that
68 increasing *Gadd45y* levels in the mouse hippocampus led to impairments in memory
69 formation and disrupted CREB activation and memory-related gene expression in cultured
70 hippocampal neurons.

71 Overall, this work together with our previous findings (Brito et al., 2020), demonstrate a
72 requirement for tight regulation of neuronal *Gadd45y* levels in gene expression regulation
73 and cognitive abilities. Thus, dysregulation of *Gadd45y* expression might be an underlying
74 mechanism involved in age-related cognitive impairments observed in mice and humans.

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76 **2. Materials and Methods**

77 **2.1. Subjects.** The use of human samples was conducted in accordance with the
78 Helsinki Declaration as well as national ethical guidelines. Protocols were approved by the
79 Local Ethics Committee and the National Data Protection Committee. The biospecimens
80 were obtained 36h *postmortem* from healthy aged (60–65 years old) and young (21–22 years
81 old) individuals (Temido-Ferreira et al., 2018). The tissue was processed and preserved for
82 molecular analyses as previously described (Pliassova et al., 2016). Young adult male
83 C57BL/6N mice (Charles River, Sulzfeld, Germany) were 3-months-old at the time of
84 behavior experiments. Mice were group-housed (3-4 mice per cage) on a 12h light/dark cycle
85 ($22 \pm 1^\circ\text{C}$, $55 \pm 10\%$ relative humidity) with *ad libitum* access to water and food. All
86 behavioral experiments took place during the light phase. All procedures were carried out in
87 accordance with German guidelines for the care and use of laboratory animals and with the
88 European Community Council Directive 86/609/EEC.

89 **2.2. Recombinant adeno-associated virus (rAAVs).** Viral particles were
90 produced and purified as described previously (Gulmez Karaca et al., 2020). Overexpression
91 of Gadd45y was achieved by using a viral vector that contained the mouse CamKII α
92 promoter upstream of the Gadd45y full-length mouse cDNA sequence. As a control vector,
93 we used a construct containing the CamKII α promoter driving the expression of GFP. For
94 each virus batch, toxicity was analyzed on primary hippocampal cultures before the start of
95 the experiments. For this, different regions of the coverslip were imaged using identical
96 microscope settings and the number of dead cells was quantified using Fiji (Schindelin et al.,
97 2012) on day *in vitro* (DIV) 10.

98 **2.3. Primary hippocampal cultures.** Hippocampal cultures from newborn
99 C57Bl/6N mice (Charles River, Sulzfeld, Germany) were prepared and maintained as
100 previously described (Gulmez Karaca et al., 2020). rAAV infection of cultures occurred on
101 DIV 4. Experiments were performed on DIV 10. To induce action potential bursting, cultures
102 were treated with 50 μM bicuculline (Enzo Life Sciences, Germany).

103 **2.4. Stereotaxic surgery.** rAAVs were injected into the dorsal hippocampus at the
104 following coordinates relative to Bregma: – 2 mm anteroposterior, ± 1.5 mm medio-lateral, –

105 1.7, – 1.9 and – 2.1 mm dorsoventral. A total volume of 1.5 μ l was injected per hemisphere
106 at 200 nl/min. Following injections at each individual site, the needle was left in place for 60s.
107 Behavioral experiments started 2 weeks after rAAVs delivery. After behavioral testing,
108 histological analysis was performed to confirm tissue and cellular integrity.

109 **2.5. Behavioral testing.** Before behavioral testing started, mice were habituated
110 to the experimenter and behavioral room by handling for 3 consecutive days, 1 minute per
111 mouse. Object-location test and contextual fear conditioning were performed as previously
112 described (Oliveira et al., 2012; Oliveira et al., 2016). The open field test was carried out
113 within the first session of the object-place recognition training as previously described
114 (Gulmez Karaca et al., 2018).

115 **2.6. Quantitative reverse-transcription PCR.** Total RNA from human tissue was
116 extracted and cDNA produced as previously described (Temido-Ferreira et al., 2018). For
117 RNA isolation from mouse hippocampal tissue, the tissue was rapidly dissected, placed in
118 RNAlater (Sigma, Munich, Germany) and isolated using the RNeasy Plus Mini Kit (Qiagen,
119 Hilden, Germany) with additional on-column DNase I digestion, according to the
120 manufacturer's instructions. cDNA production and quantitative reverse-transcription PCR (q-
121 RT-PCR) was performed as previously described (Brito et al., 2020). The following TaqMan
122 probes were used: *Arc* (Mm00479619_g1), *c-Fos* (Mm00487425_m1), *FosB*
123 (Mm00500401_m1), *Gadd45 α* (Mm00432802_m1), *Gadd45 β* (Mm00345123_m1), *Gadd45 γ*
124 (Mm00442225_m1), *Egr1* (Mm00656724_m1) and *Npas4* (Mm00463644_m1). For human
125 genes the following TaqMan probes were used: *Gadd45 α* (Hs00169255_m1), *Gadd45 β*
126 (Hs00169587_m1), *Gadd45 γ* (Hs00198672_m1). Expression levels of target genes were
127 normalized to the expression of the housekeeping gene *GusB* (Mm00446953_m1) or β -actin
128 (Hs01060665_g1) for mouse or human genes, respectively. Controls were used to exclude
129 the possibility of DNA or RNA contaminations.

130 **2.7. Western blotting.** Western blotting was performed as previously described
131 with minor modifications (Brito et al., 2020; Gulmez Karaca et al., 2020). Briefly, hippocampal
132 cultures infected on DIV 4 were lysed on DIV 10 in SDS sample buffer. In the case of

133 western blotting of tissue samples, the dorsal hippocampus was microdissected from mouse
134 brain and homogenized in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium
135 deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with 1% protease inhibitor
136 cocktail (Sigma-Aldrich, Munich, Germany) and 1% phosphatase inhibitor cocktail II and III
137 (Sigma-Aldrich, P5726, P0044), the whole procedure was performed at 4°C. Protein
138 concentration was measured by Bradford assay and 20 µg of protein was loaded on a 10%
139 polyacrylamide gel after being denatured at 95 °C for 5 min. After SDS-PAGE, gels were
140 blotted onto a nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) and later
141 blocked in 5% milk and probed with the following antibodies: phospho-CREB (1:6000,
142 Millipore #05-667), total-CREB (1:5000, Cell Signaling, #4820), β-Actin (1:1000, Santa Cruz,
143 #SC-47778) or α-Tubulin (1:40000, Sigma-Aldrich, #T9026). Antibodies were diluted in 5%
144 milk in PBS-T (total-CREB, α-Tubulin and β-Actin) or in 5% bovine serum albumin in PBS-T
145 (phospho-CREB). Next, the membranes were incubated with horseradish peroxidase-
146 conjugated secondary antibodies and later analyzed using a ChemiDoc™ Imaging System
147 (Bio-Rad). Data is presented as ratio of phosphorylated/total protein normalized internally to
148 each uninfected condition.

149 **2.8. Statistical information.** For normally distributed data sets, two-tailed
150 unpaired Student's t test or one-way ANOVA were used to compare two or more groups
151 respectively (significant data is marked with *). Two-tailed Mann-Whitney test was used to
152 compare two distinct groups for non-Gaussian distribution (significant data is marked with #).
153 Correlation analysis was performed using Pearson correlation coefficient or Spearman
154 correlation for normally distributed or non-parametric data, respectively. The sample size was
155 determined based on similar experiments carried out in the past. All plotted data represent
156 mean ± SEM. Statistics were performed using GraphPad Prism for Mac OS X, version 8. For
157 behavioral experiments the investigators were blind to group allocation during data collection
158 and analysis. For *in vitro* experiments no blinding was performed since the outcome was
159 dependent on software analysis and not manual scoring.

160

161 **3. Results**

162 **3.1. Aging increases Gadd45 γ expression in the human hippocampus.**

163 Aberrant gene expression patterns are an evolutionarily conserved hallmark of aging.
164 However, no overall correlation between age-associated gene expression in mice and
165 humans has been detected (Zahn et al., 2007). We asked whether Gadd45 γ expression in
166 human aged hippocampus would be compromised as observed in mice (Brito et al., 2020).
167 We analyzed the expression of Gadd45 family members in young and aged human
168 hippocampi as we previously described (21–65 years old) (Temido-Ferreira et al., 2018)
169 (Figure 1A). We did not find any correlation between age and *Gadd45 α* expression.
170 Interestingly, we found that hippocampal *Gadd45 β* and *Gadd45 γ* levels were increased (~4.8
171 and ~8.6 fold, respectively) as age progressed. This result, together with our previous
172 findings in aged mouse tissue (Brito et al., 2020), suggests that age-related Gadd45
173 expression changes in the hippocampus may not be conserved in mice and humans.

174 **3.2. Gadd45 γ overexpression leads to impairments in spatial recognition**

175 **memory.** Next, we sought to model the human aging-associated Gadd45 γ increase in the
176 mouse hippocampus and determine the cellular and behavioral consequences of neuronal
177 Gadd45 γ overexpression. Given that previous studies showed a selective function for
178 Gadd45 γ in memory formation (Brito et al., 2020; Li et al., 2018), we focused on Gadd45 γ .
179 We stereotaxically delivered a viral vector containing the mouse CamKII α promoter driving
180 the expression of Gadd45 γ , or GFP as a control, into the dorsal hippocampus (dHPC) of
181 young adult mice (Figure 1B,C). We validated viral expression in the dHPC of injected
182 animals by assessing GFP expression and *Gadd45 γ* mRNA levels (Figure S1A-C). Neither
183 group showed anatomical or histological brain abnormalities. Two weeks after stereotaxic
184 surgery, before assessing cognitive function, we performed an open field test (Figure 1C) to
185 verify whether Gadd45 γ overexpression affects locomotor activity or anxiety-like behavior.
186 Total distance travelled and the percentage of the time spent in the central zone were similar
187 between groups (Figure 1D-F). Next, we assessed long-term memory in the object-place
188 recognition test and contextual fear conditioning. Increasing Gadd45 γ expression in the

189 dHPC of young mice impaired preference for the displaced object 24h after learning (Figure
190 1G). This impairment was not due to altered habituation patterns during the training trial
191 sessions or altered object exploratory behavior (Figure S1D,E). In contrast, Gadd45yOE
192 mice showed intact long-term memory in contextual fear conditioning (Figure 1H). Both
193 groups presented similar responses to shock administration (Figure S1F).

194 **3.3. Gadd45y overexpression disrupts activity-dependent CREB activation**

195 **and gene expression.** Considering that Gadd45y regulates CREB activity (Brito et al.,
196 2020), we next investigated whether Gadd45y overexpression would impact CREB
197 regulation. First, we assessed whether Gadd45y overexpression in the hippocampus of
198 young adult mice (Figure 2A) affects the levels of phosphorylated CREB in baseline
199 conditions. In agreement with a role for Gadd45y in signaling pathways that regulate CREB
200 activation (Brito et al., 2020), overexpression of Gadd45y resulted in increased levels of
201 phosphorylated CREB (Figure 2B,C). Next using primary hippocampal cultures (Figure
202 S1G,H) we analyzed the phosphorylation of CREB in baseline conditions and in response to
203 increased neuronal activity (Figure 2D). Similar to our *in vivo* findings (Figure 2A-C),
204 Gadd45y overexpression in primary hippocampal cultures led to increased levels of
205 phosphorylated CREB in basal conditions. Moreover, this effect appeared to blunt a
206 response to neuronal activity; CREB phosphorylation in response to neuronal activity did not
207 reach controls levels (Figure 2E,F). We next assessed the expression of the CREB-
208 dependent genes *Arc*, *FosB*, *c-Fos*, *Egr1* and *Npas4* (Impey et al., 2004; Rao-Ruiz et al.,
209 2019) in basal conditions and upon neuronal activity (Figure 2G). Hippocampal neuronal
210 cultures infected with rAAV-Gadd45yOE revealed disrupted CREB-dependent gene
211 expression in response to increased neuronal activity compared to control conditions (Figure
212 2H-L). This set of experiments shows that increasing Gadd45y above physiological levels in
213 hippocampal neurons disrupts CREB phosphorylation and gene expression required for
214 memory formation. Taken together, these findings demonstrate that an increase in
215 hippocampal Gadd45y levels disrupts CREB regulation, the expression of memory-related
216 genes and cognitive function.

217

218 **4. Discussion**

219 This study suggested that human aging is associated with increased hippocampal Gadd45y
220 expression. Together with our previous findings (Brito et al., 2020) we showed that
221 bidirectional dysregulation of hippocampal Gadd45y levels in young adult mice negatively
222 impacts cognitive function, CREB regulation and the expression of memory-related genes.
223 Thus, implicating a requirement for tight control of Gadd45y levels in brain function.

224 We observed that mimicking the human aging-related increase in Gadd45y expression in the
225 mouse hippocampus or in dissociated hippocampal neurons, promoted memory deficits and
226 impairments in CREB-dependent gene transcription, respectively. Both the reduction or
227 chronic enhancement of CREB function is known to lead to spatial memory deficits (Li et al.,
228 2015; Pittenger et al., 2002; Viosca et al., 2009), as described in studies using CREB-
229 deficient mutants (Pittenger et al., 2002) or mouse models that express constitutively active
230 forms of CREB such as VP16-CREB (Viosca et al., 2009). Moreover, constitutive CREB
231 activation has been identified as a possible contributing mechanism involved in Alzheimer's
232 disease (Muller et al., 2011). In our study Gadd45y overexpression induced increased levels
233 of phosphorylated CREB in basal conditions both in the mouse hippocampus and in
234 hippocampal cultures. Although this remains to be investigated, the observed increase in
235 Gadd45y expression levels in the aged human hippocampus, may also result in chronic
236 elevations in the activated form of CREB. Our experiments in hippocampal cultures indicate
237 that increased levels of phosphorylated CREB already in basal conditions impair a response
238 to neuronal activity and result in deficits in the expression of plasticity-related genes. Thus,
239 providing a possible mechanism for impaired cognitive function in conditions of elevated
240 Gadd45y expression like in our model and possibly in the hippocampus of aged individuals.
241 Together with our previous findings, which showed that Gadd45y knockdown leads to
242 impairments in CREB activation and associated gene expression (Brito et al., 2020), this
243 data suggests that proper cellular function requires the tight regulation of Gadd45y levels.

244 These findings are in agreement with another study showing that either Gadd45y loss- or
245 gain-of-function disrupts neural development (Kaufmann and Niehrs, 2011).

246 The deficits in memory were task-specific; young adult mice expressing Gadd45y above
247 physiological levels presented selective long-term memory impairments in object place-
248 recognition memory but not in contextual fear conditioning. Intriguingly, similar results have
249 been found in aged mice and humans. It has been described that aged mice (Kennard and
250 Woodruff-Pak, 2011) and humans (Battaglia et al., 2018; Foster et al., 2012; Leal and Yassa,
251 2015) are more likely to display deficits in forms of recognition memory than in contextual
252 fear conditioning. The reasons for the selective impairment may be attributed to the
253 characteristics of the tasks; despite hippocampal dysfunction in response to aging or
254 Gadd45y overexpression, mice may still be able to form and store the association between a
255 highly salient stimulus (novel context) and a foot-shock. Similar findings have been described
256 for other models of impaired hippocampal function. Namely, in a mouse model of Rett
257 syndrome (Gulmez Karaca et al., 2018) and Alzheimer's disease (Corcoran et al., 2002). In
258 the later, contextual fear conditioning impairments were only present when the salience of
259 the context was reduced.

260 Aberrant gene transcription patterns occur as a consequence of aging in the hippocampus
261 (Burger, 2010; Janov et al., 2017; Verbitsky et al., 2004). These changes do not overly
262 correlate across species (Bishop et al., 2010; Loerch et al., 2008; Zahn et al., 2007), thus
263 limiting the translational potential of animal models. Studies comparing cross-species
264 alterations in gene expression generally focus on shared changes. The similar
265 consequences of bidirectional dysregulation of Gadd45y expression levels suggest that this
266 approach may neglect functionally relevant and seemingly disparate age-associated
267 transcription changes. Using *in vivo* and *in vitro* models we show that hippocampal levels of
268 Gadd45y are tightly regulated and that either a decrease (Brito et al., 2020) or an increase in
269 Gadd45y can dysregulate plasticity-associated gene expression and cause cognitive
270 impairments. Notably, previous studies that performed transcriptomic analysis of human
271 hippocampal tissue throughout adulthood also suggested an age-associated increase in

272 Gadd45 γ expression (Kang et al., 2011; Pletikos et al., 2014). Accordingly, our findings
273 illustrate a scenario in which diverging age-related transcriptional programs in mice and
274 humans result in converging phenotypes.
275 Taken together, our results demonstrate the requirement for tight control of Gadd45 γ levels
276 in memory formation and further implicate Gadd45 γ as a molecular candidate that may
277 underlie cognitive impairments in aging-associated pathological conditions.

278

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285

286 **Disclosure statement**

287 The authors declare no conflict of interest.

288

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387

388 **Figure legends**

389 **Figure 1.** Human hippocampal *Gadd45γ* expression is increased during aging and
390 overexpressing *Gadd45γ* in the mouse hippocampus impairs object location memory. **A)**
391 Correlational analysis between the expression of *Gadd45α*, *Gadd45β* and *Gadd45γ* in
392 human *postmortem* hippocampal tissue and donors' age (N=6). Correlation analysis was
393 performed using Pearson correlation coefficient or Spearman correlation. **B)** Schematic
394 representation of the viral constructs used. The viral vector contains a *CamKIIα* promoter
395 driving *Gadd45γ* overexpression (*Gadd45γ*OE) or GFP as a control (GFP). **C)** Schematic
396 representation of the experimental design for behavioral tests. **D)** Representative exploration
397 patterns of all groups during open field test. **E)** Locomotion analysis of the different groups
398 measured as the total distance travelled during the open field test (N=8-9). **F)** Anxiety-like
399 behavior analysis measured as percentage of time spent in the center of the arena during the
400 open field test (N=8-9). **G)** 24h object location memory test of young adult mice expressing
401 GFP or *Gadd45γ*OE in the dHPC (N=13-15). Dashed line represents equal preference for
402 either object (chance preference). **H)** 24h contextual fear memory test of young adult mice
403 expressing GFP or *Gadd45γ*OE in the dHPC (N=9). ns: not significant; ***p*<0.01 by two-tailed
404 unpaired Student's t-test. Error bars represent SEM.

405

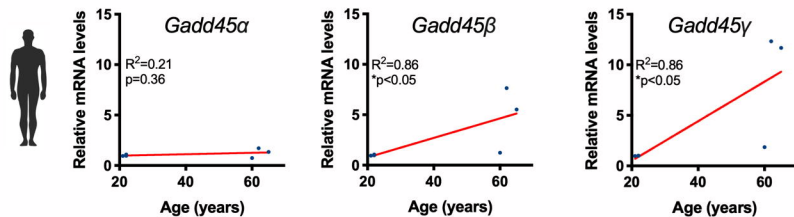
406 **Figure 2.** Increased *Gadd45γ* expression dysregulates hippocampal CREB phosphorylation
407 and activity-dependent gene expression. **A)** Schematic representation of the experimental

408 design for western blot analysis of CREB activation *in vivo*. **B)** Representative immunoblot
409 scans of hippocampal tissue from young adult mice infected with rAAVs expressing GFP or
410 Gadd45 γ OE using phosphospecific (pCREB) and total CREB (tCREB) antibodies. **C)**
411 Immunoblot quantification shown as ratios of phosphorylated/total protein normalized to GFP
412 control (N=5-6). **D)** Schematic representation of the experimental design for western blot
413 analysis of CREB activation in mouse hippocampal cultures. **E)** Representative immunoblot
414 scans of hippocampal cultures infected with rAAV expressing GFP or Gadd45 γ OE using
415 phosphospecific and total CREB antibodies. **F)** Immunoblot quantification shown as ratios of
416 phosphorylated/total protein normalized to uninfected control in baseline or bicuculline-
417 treated conditions (left and middle graphs) or pCREB fold increase (bicuculline/baseline) for
418 each condition and normalized to uninfected control (right graph) (N=7-8 independent cell
419 preparations). **G)** Schematic representation of the experimental design for qRT-PCR analysis
420 of the expression of CREB-dependent genes (N=5-6 independent cell preparations) **H)** *Arc*,
421 **I)** *c-Fos* **J)** *FosB* **K)** *Egr1* and **L)** *Npas4* in hippocampal cultures. Hippocampal cultures were
422 harvested at baseline conditions or after 2h, 4h, or 6h of bicuculline treatment. *p<0.05,
423 **p<0.01, ***p<0.001 and ****p<0.0001 by two-tailed Student's t-test. ns: not significant. Error
424 bars represent SEM.

425
426

Figure 1

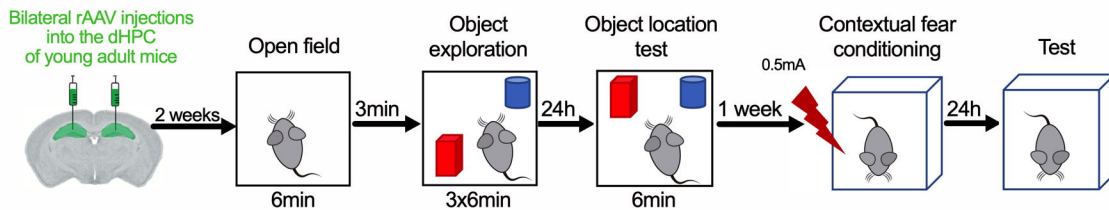
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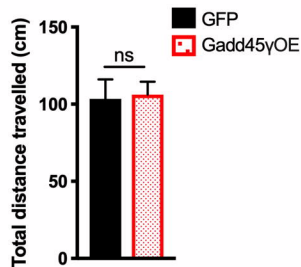
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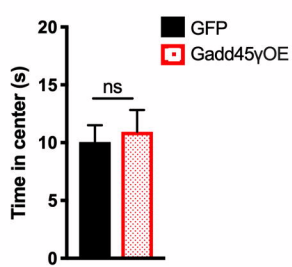
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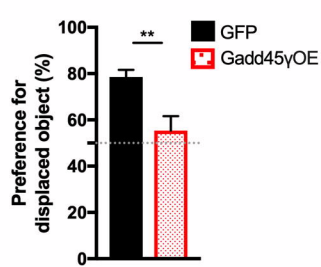


F



G

Object location test



H

Contextual fear conditioning

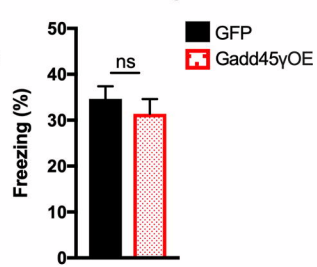
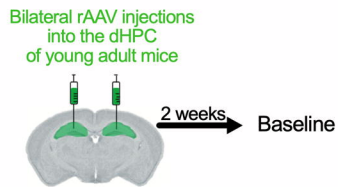
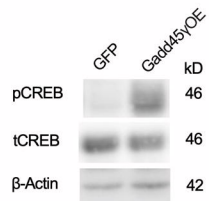


Figure 2

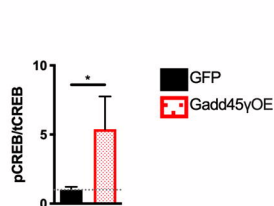
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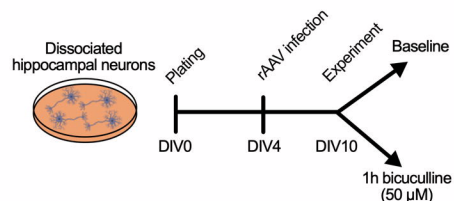
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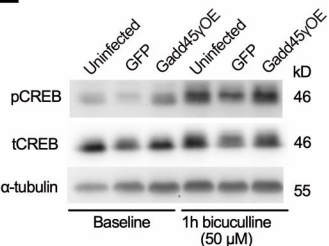
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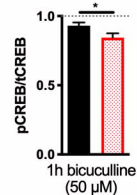
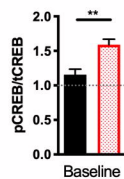
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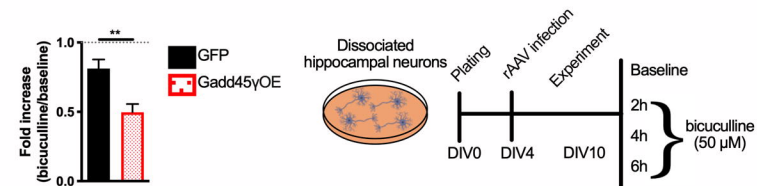
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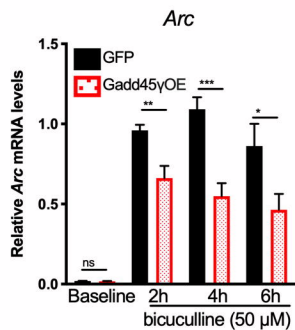
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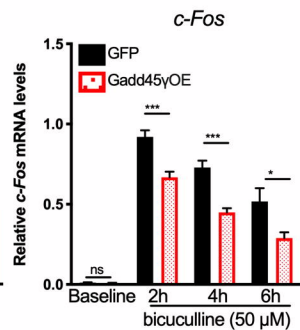
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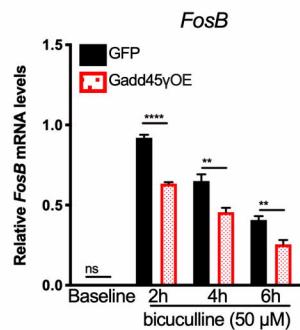
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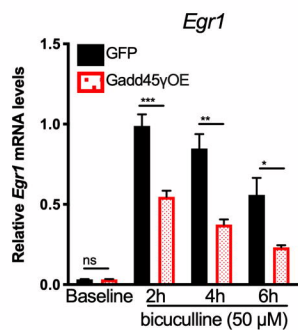
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