Socialization causes long-lasting behavioral changes

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In modern human societies, social isolation acts as a negative factor for health and life quality. On the other hand, social interaction also has profound effects on animal and human behaviors, reducing aggressiveness, feeding and loss of sleep. Here, we observe that in the fly *Drosophila melanogaster* these behavioral changes long-last even after social interaction has ceased, suggesting that the socialization experience triggers behavioral plasticity. We find that impairing long-term memory mechanisms either genetically or by anesthesia abolishes the expected behavioral changes in response to social interaction. Furthermore, we show that socialization increases CREB-dependent neuronal activity and synaptic plasticity in the mushroom body, the main insect memory center analogous to mammalian hippocampus. We propose that social interaction triggers socialization awareness, understood as long-lasting changes in behavior caused by experience with mechanistic similarities to long-term memory formation.

INTRODUCTION

Animals live in social contexts. In our modern human society, the feeling of loneliness is increasing despite the technological advances in social media and communication[1]. The prolonged absence of social interaction has detrimental effects on quality of life, lifespan and several health problems[2,3]. In *Drosophila melanogaster*, social interaction strongly modulates several behaviors, diminishing male-to-male aggression, decreasing food consumption and, depending on the context, increasing or decreasing sleep, among others[4]. Socialization impacts several parallel modulatory systems[5]. In particular, activity-regulated genes in dopaminergic neurons modulate aggression in response to social enrichment[6,7]. Key clusters of dopaminergic neurons are also essential components of learning and memory circuits[8], since they innervate the main *Drosophila* memory structure, the mushroom body (MB)[9].

At the molecular level, long-term memory (LTM) formation in the MB requires *rutabaga (rut- adenylate cyclase)* and *d*unce (*dnc- cAMP phosphodiesterase)* gene functions, in order to adequately regulate cAMP levels and ensure neuronal plasticity[10]. cAMP signaling mediates CREB (c-AMP response binding element) phosphorylation. CREB is a conserved transcription factor that is key to form long-term memory and synaptic plasticity, among many other processes[10,11]. Social interaction causes structural changes in the MB, an effect that is abolished in mutant flies for memory-related genes like *rut* and *dnc*[12,13]. Furthermore, the function of such genes is necessary for immediate sleep changes triggered by social interaction[14,15].

In this work, we inquired if socialization was able to generate long-lasting changes on behavior, and addressed how these changes are associated with synaptic plasticity. We showed that underlying mechanisms have similarities with LTM: an altered behavior in response to experience lasted for 8 or more hours after the training; it depended on cAMP levels and was blocked by anesthesia; and ultimately, it correlated with changes in number of CREB-responsive neurons and synapses. In summary, we propose that socialization awareness modifies long-term behavior sharing some underlying mechanisms that are characteristic of long-term memory processes.

51

52 **RESULTS**

53 LONG-TERM SOCIALIZATION-INDUCED FEEDING BEHAVIOR REQUIRES cAMP SIGNALING

54 Flies that experienced social interaction show reduced food consumption when compared with flies
55 that were socially reared and posteriorly isolated[16]. We used single-fly CApillary FEeding -sCAFE-
56 assay (modified from[17]) to extend these findings. We compared grouped flies with animals singly
57 reared since eclosion, meaning that they were socially naive. As expected, there was a significant
58 decrease in food uptake of 5-day socialized flies when compared to individual flies in the immediate
59 24 hours (0 h-24 h time window) (fig 1A). Next, to determine if such feeding effect is maintained
60 even in the absence of social interaction, we slightly modified the socially-enriched paradigm: flies
61 were group- or single-reared for 5 days and then animals from both experimental groups were kept
62 isolated for additional 24 hours previous to assessing feeding (fig 1B). Using this protocol, we also
63 detected a decreased food consumption of grouped flies in the 24 h-48 h time window, confirming
64 a long-lasting effect of social interaction on feeding behavior (fig 1C). We reasoned that the most
65 plausible candidate genes to play a role for such long-lasting effect would be memory-related
66 genes, such as *rutabaga* (*rut*)[18]. Despite their past experience, isolated *rut* mutant flies in the 24 h-
67 48 h period after socialization showed no differences in food intake with solitaire animals since
68 eclosion (fig 1C). Besides, *rut* mutant flies do not change their feeding behavior during the first 24
69 hours (0 h-24 h), suggesting a requirement of cAMP for this response (fig 1A). To confirm the
70 involvement of cAMP signaling we repeated the sCAFE assay in animals mutant for *dunce* (*dnc*).
71 Results were comparable to *rut* mutant: *dnc* mutant flies fail to change their feeding behavior not
72 only during the first 24 hours after socialization (0 h-24 h) but also according to previous experience,
73 in the 24 h-48 h period (fig 1A, C). We propose that this long-lasting effect of social interaction on
74 (feeding) behavior indicates a that animals have a socialization awareness that lasts even after
75 social interaction has ceased.

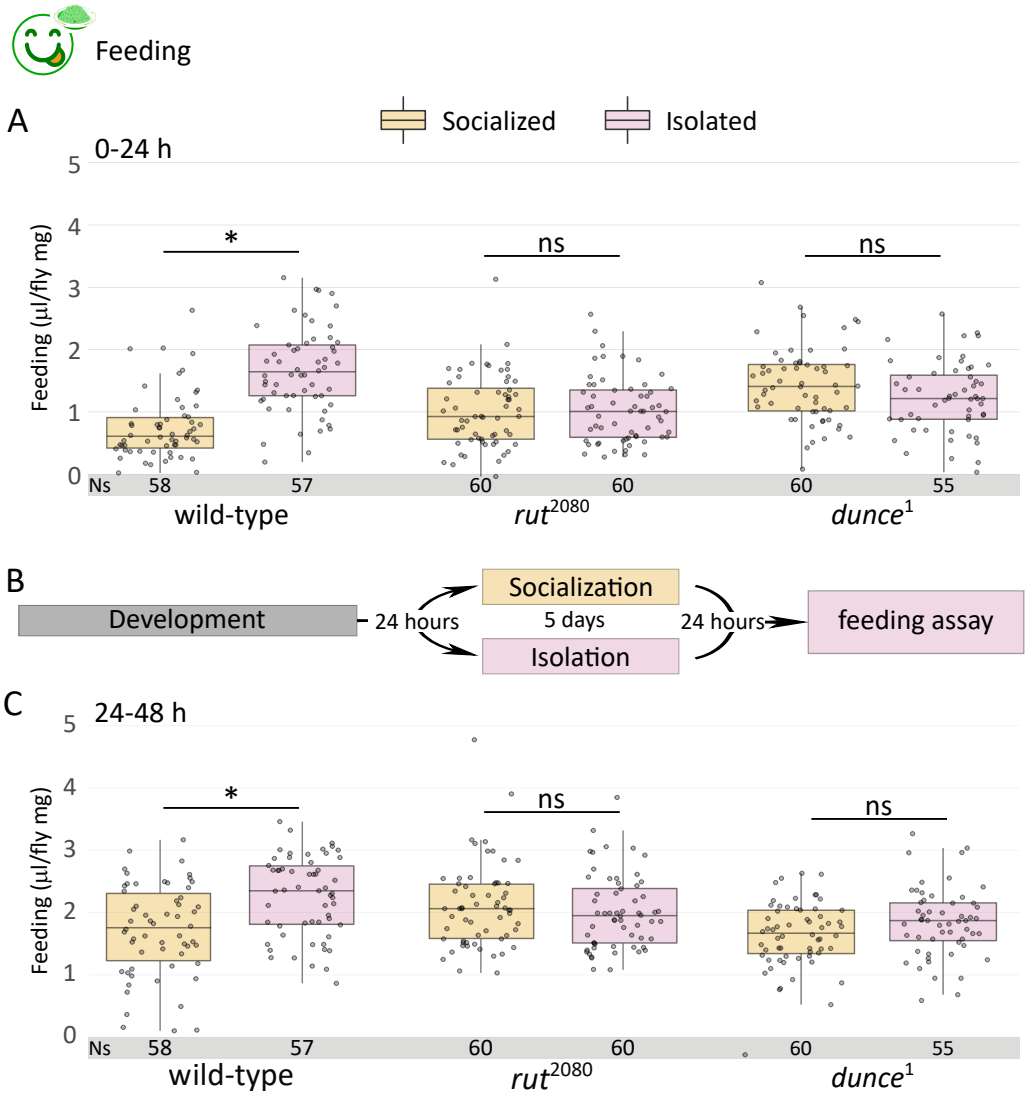


Figure 1. Reduced food consumption induced by socialization depends on memory-related genes. (A) Quantification of food consumption of *wt*, *rut* and *dnc* mutant flies in socialized and isolated conditions (single fly CAFE assay) in the 0-24 h time window (Kruskal-Wallis chi-squared = 75.905, df = 5, p-value = 6.022e-15; *post hoc* Dunn comparisons: $wt^{social}|wt^{isolated}$ p = 6.24e-13, $rut^{social}|rut^{isolated}$ p = 1.00, $dnc^{social}|dnc^{isolated}$ = 1.00). (B) Scheme of the socialization protocol: recently eclosed animals were either grouped or isolated for five days, and subsequently isolated for additional 24 hours before tested. C) Quantification of food consumption of *wt*, *rut* and *dnc* mutant flies in socialized and isolated conditions (sCAFE) in the 24 h-48 h h time window (Kruskal-

85 Wallis chi-squared = 32.698, df = 5, p-value = 4.32e-06; *post hoc* Dunn comparisons:
86 $wt^{social}|wt^{isolated}$ p = 1.04e-03, $rut^{social}|rut^{isolated}$ p = 1.00, and , in fig S1, $dnc^{social}|dnc^{isolated}$ = 1.00).
87
88 LONG-TERM EFFECT OF SOCIALIZATION ON SLEEP IN ABSENCE OF INTERACTION
89 Sleep (particularly daytime sleep) is also regulated after social interaction. When flies are kept in
90 mixed-sex groups immediately at eclosion, sleep increases[14]. However, courtship experience
91 inhibits sleep in male flies[19,20], which lasts for several hours, proving a complex regulation of sleep
92 by social cues and experiences. Most published sleep studies employ *Drosophila* Activity Monitors
93 (DAMs), which only detect movement when the fly crosses a midpoint sensor in the housing tube[21],
94 overestimating actual sleep time[16]. The ethoscope was developed to unequivocally identify
95 immobility periods and assess sleep[22]. We confirmed that social interaction also induced animals
96 to sleep more[14] (fig 2A). To ensure consistency with previous works[16], we focused on the first four
97 hours after lights ON, where the effect is unambiguous and reproducible (i.e. 24 h-28 h time
98 window, ZT0-ZT4, fig 2A). Sleep quantification shows a significant difference between social-
99 enriched and lonely animals in this 24 h-28 h period (fig 2B), in line with previous publications[14,16].
100 Furthermore, *rut* mutant animals showed no significant difference in 24 h-28 h sleep time (fig 2A,
101 B), although socialized *dnc* mutants did exhibit a significant sleep difference in the 24 h-28 h, (fig
102 2C). Our results with *dnc* and *rut* mutant flies were apparently contradictory with those previously
103 described using DAMs[14]. However, the ethoscope offers the possibility of analyzing data as they
104 were extracted from DAMs, thus depicting comparable results to published DAM data. This *virtual*
105 DAM analysis did render a significant difference between *rut* mutant grouped and single-reared
106 flies, whereas no sleep changes were apparent in *dnc* mutant animals, in agreement with previous
107 studies (fig S1)[14]. The differing results obtained depending on the type of analysis (regular or virtual
108 DAM) stem from the higher sensitivity of ethoscopes to movement. However, in either case, our

data and previous work support the idea that cAMP regulation, necessary for synaptic plasticity, is needed to sustain long-lasting changes in sleep triggered by socialization awareness.

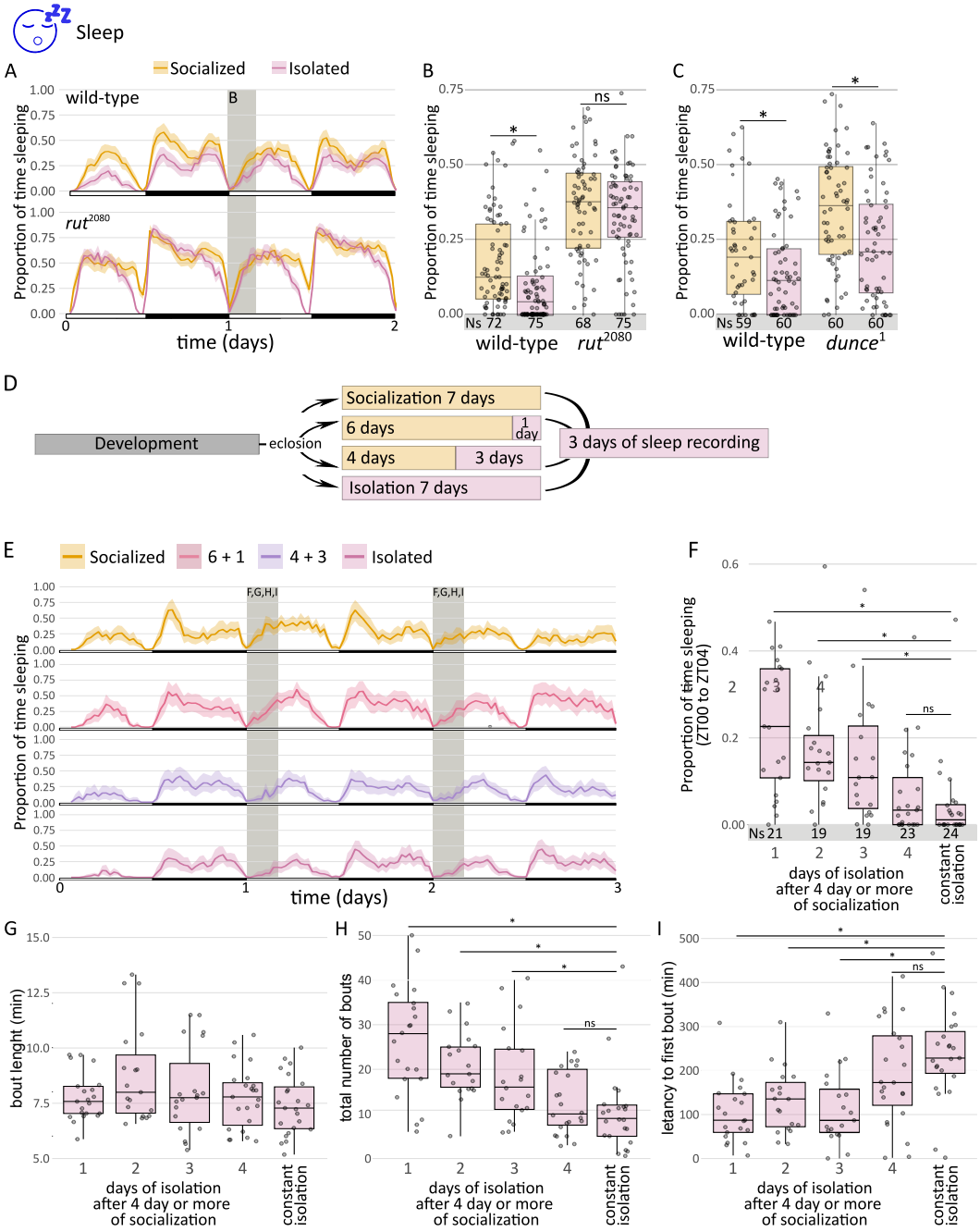


Figure 2. Sleep behavior after socialization is long-term and relies on *rut*. (A) Sleep profile and (B) sleep quantification of the 24 h-28 h time window for *wt* and *rut* mutant background (Kruskal-Wallis chi-squared = 94.165, df = 3, p < 2.2e-16; *post hoc* Dunn comparisons: $wt^{social}|wt^{isolated}$ p = 1.07e-02, $rut^{social}|rut^{isolated}$ p = 0.438). (C) Sleep quantification of the 24 h-28 h

116 time window for *wt* and *dnc* mutant background (Kruskal-Wallis chi-squared = 36.476, df = 3, p-
117 value = 5.94e-08; *post hoc* Dunn comparisons: $wt^{social}|wt^{isolated}$ p = 3.43e-02, $dnc^{social}|dnc^{isolated}$ p =
118 3.48e-03). (D) Scheme of the sleep time course: flies were either isolated or grouped after eclosion
119 for 7, 6 or 4 days and subsequently isolated for 0, 1 or 3 days (named as socialized, 6+1, 4+3 and
120 constant isolation); after introducing them in ethoscopes, sleep behavior was recorded for 3 days.
121 (E) Sleep profile of animals isolated for 1 to 4 days, using isolated flies as control. (F) Quantification
122 of sleep from ZT0 to ZT4 for day 1 (day 1 of socialized), day 2 (day 1 of 6+1), day 3 (day 2 of 6+1),
123 day 4 (day 1 of 4+3) and isolated flies (day 1 of constant isolation). (G-I) Analysis of bout length
124 (G), total number of bouts (H) and latency to first bout (I) from ZT0 to ZT4.
125
126 We wondered how long animals that experienced a socially-enriched environment maintained such
127 sleep behavior in absence of social interaction. To compare animals of the same age, we socialized
128 flies for 7, 6 or 4 days (which is enough socialization time in order to generate a sleep effect[14]),
129 subsequently isolated them for 0, 1 or 3 days (named as socialized, 6+1, 4+3 or constant isolation).
130 Then, their sleep behavior was recorded for the following 3 days (fig 2D). In the framework of this
131 experimental approach, we could compare continuously isolated flies with animals isolated for 1 to
132 4 days after socialization (fig 2E). We could observe a progressive reduction of sleep time in the
133 ZT0- ZT4 after isolation, with significant decrease at day 4 that was comparable to continuous
134 isolation (fig 2F). Thus, 4 days of isolation are enough to modify sleep reaching similarly sleep
135 levels than socially naive flies, in contrast to the need of 5 days described previously using DAMs[16].
136 The ethoscope also allows a detailed sleep analysis regarding bout length, the total number of
137 bouts and the latency to first bout. There were no differences in the sleep bout length amongst
138 experimental groups. In contrast, isolated flies for 4 days reduced the number of sleep bouts to
139 similar levels than the ones from socially naive animals, despite we noticed a progressive reduction
140 but still statistically significative (fig 2H). Intriguingly, the latency to the first bout in grouped flies

141 remained similar up to day 3, where it raised sharply, similar to the latency of isolated flies (fig 2I).
142 We conclude that the effect of socialization lasts at least for 3 days, and indeed, it can be
143 considered as long-term.

144 SOCIALIZATION-INDUCED DIMINISHED AGGRESSIVENESS DEMANDS cAMP SIGNALING

145 In isolated flies that previously experienced social interaction, isolation signals starvation and, as a
146 consequence, increases feeding and decreases sleep, meaning that both behavioral changes are
147 reciprocally related[16]. We wondered if socialization awareness was also evident in a different social
148 behavior. Previous data showed that 5-day grouped male flies since eclosion were less aggressive
149 than their single-reared counterparts when tested immediately after the treatment[23]. This behavior
150 also allowed us to determine the progression of long-lasting effects, in order to compare the
151 temporal requirements of social interaction with those of classical learning and memory assays.
152 Thus, we employed animals from both experimental conditions and then evaluated aggression in
153 a well-established paradigm after different isolation periods[24]. Socially-experienced flies showed
154 reduced aggression at 1, 4, 8 and 24 hours after isolation when compared with single-reared
155 animals (fig 3), evidencing a behavioral change at short- and long-term. Despite social interaction
156 had ceased up to 24 h before, grouped flies still spent considerably less time fighting than lonely
157 flies (fig 3), confirming that socialization awareness is a general feature of socialization. Critically,
158 *rut* mutant flies did not show any difference according to their previous experience (fig 3),
159 reinforcing the role of memory-related genes. Besides, our data revealed that at short-term (one
160 hour), grouped *rut* mutant animals were significantly less aggressive than *rut* single-reared flies,
161 thus differentiating from *rut* requirements in classical learning assays. However, this rut-
162 independent effect disappeared after four hours, suggesting that social interaction imprinted a
163 temporary effect independently of cAMP, but long-term socialization consequences in aggression
164 depends on *rut* activity.

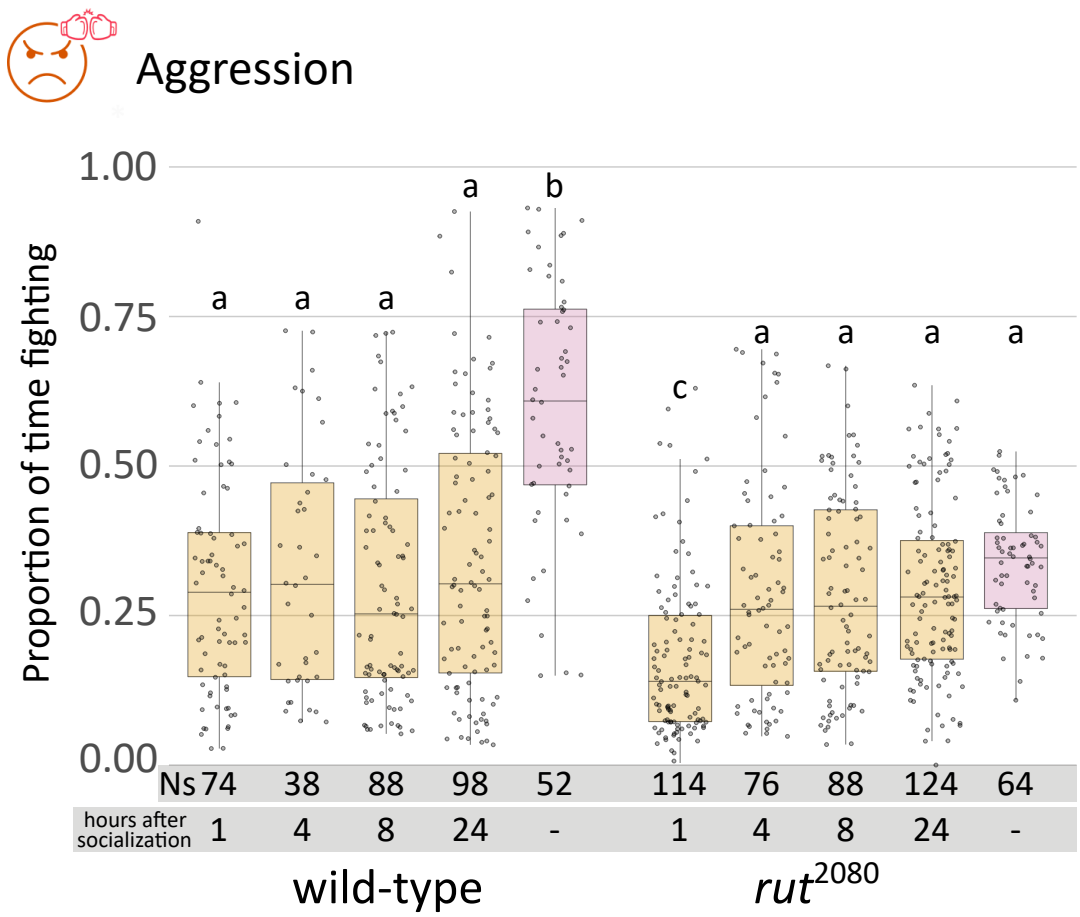
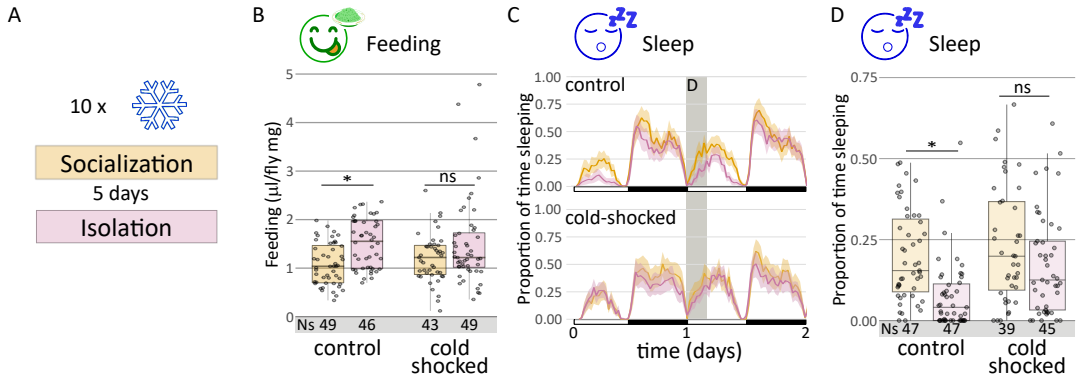


Figure 3. Socialized-reduced aggression shows short- and long-term effect. Quantification of proportion of time expend fighting after different times of re-isolation. Flies either *wt* or in a *rut* mutant background were grouped or isolated for 5 days and then socialized flies were tested after 1, 4, 8 or 24 hours after isolation (Kruskal-Wallis chi-squared = 139.99, df = 9, p-value < 2.2e-16, *post hoc* Dunn comparisons: $wt^{24h_after_social}|wt^{isolated}$ p = 1.08e-07, $wt^{8h_after_social}|wt^{isolated}$ p = 1.61e-10, $wt^{4h_after_social}|wt^{isolated}$ p = 1.88e-05, $wt^{1h_after_social}|wt^{isolated}$ p = 2.24e-09, $rut^{24h_after_social}|rut^{isolated}$ p = 1.00, $rut^{8h_after_social}|rut^{isolated}$ p = 0.815, $rut^{4h_after_social}|rut^{isolated}$ p = 0.598, $rut^{1h_after_social}|rut^{isolated}$ p = 7.52e-10).

ANESTHESIA ABOLISHES SOCIALIZATION EFFECTS

Anesthesia blocks long-term memory consolidation in most species[25,26]. In *Drosophila*, a 2-min cold shock acts as anesthetics and is able to impede long-term memory in the classical aversive

178 olfactory conditioning assay[27]. We wondered if anesthesia was also able to block socialization
179 awareness. We exposed adult flies to 3-min cold shock two times per day to single and grouped
180 flies during the training period (fig 4A). Both experimental "cold-shocked" groups did not show any
181 significant differences in food consumption in the 24 h-48 h time window after isolation, in contrast
182 to non-shocked control animals (fig 4B). Given the reciprocal relationship between feeding and
183 sleep behavior regarding social interaction[16], we confirmed that sleep between lonely and
184 socialized animals in the 24 h-28 h time window also remained similar after cold shock (fig 4C, D).
185 As expected, in non-shocked animals the difference was significant (fig 4C, D). In summary, we
186 found that socialization awareness relies on cAMP signaling and is blocked by anesthesia, as it
187 occurs in long-term memory.



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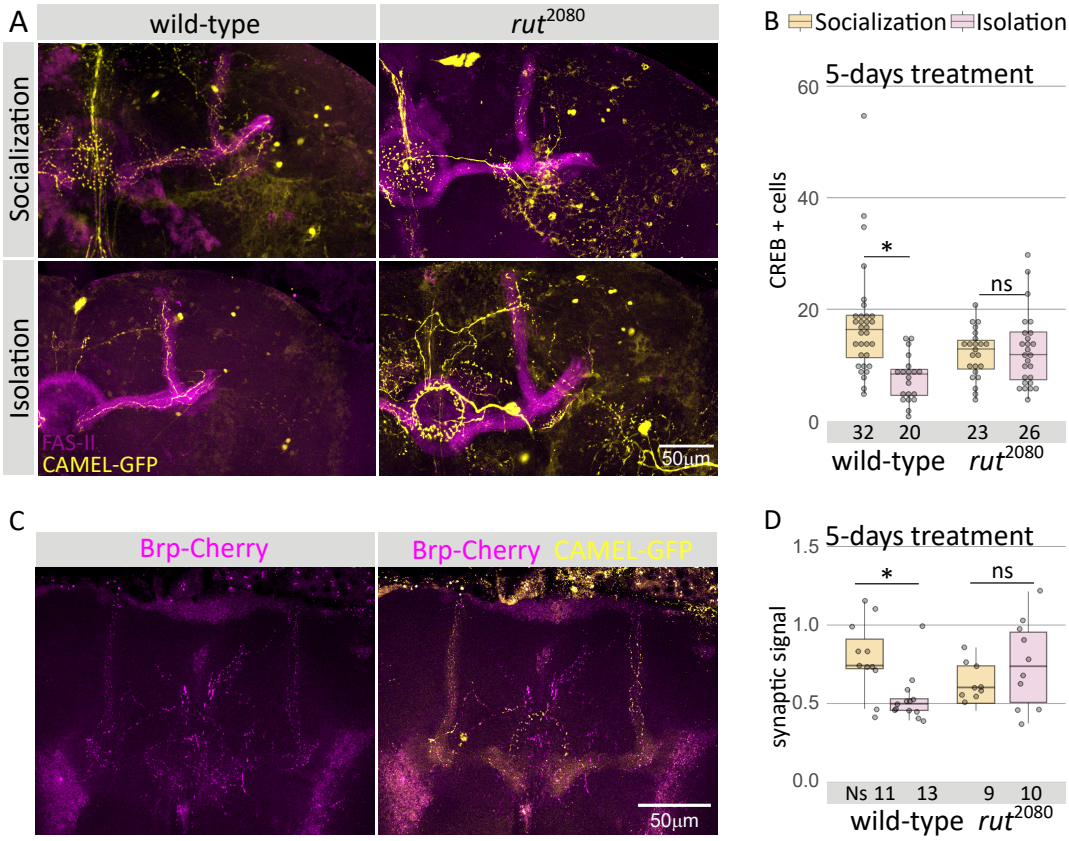
189 **Fig 4. Anesthesia abolishes socialization effects on sleep and food consumption.** (A)
190 Scheme of the cold-shock protocol (twice per day). (B) Quantification of food consumption using
191 sCAFE (Kruskal-Wallis chi-squared = 15.954, df = 3, p-value = 1.16e-3; *post hoc* Dunn
192 comparisons: non-shocked^{social}|non-shocked^{isolated}p = 5.26e-3, shocked^{social}|shocked^{isolated} p =
193 1.00). (C) sleep profile and (D) sleep quantification of the 24 h-28 h time window (Kruskal-Wallis
194 chi-squared = 31.184, df = 3, p-value = 7.78e-07; *post hoc* Dunn comparisons: non-
195 shocked^{social}|non-shocked^{isolated} p = 3.05e-06, shocked^{social}|shocked^{isolated} p = 0.116) of cold-
196 shocked socialized and isolated wt flies, together with non-shocked control wt flies.

197

198 SOCIALIZATION CORRELATES WITH INCREASED NEURONAL ACTIVITY AND SYNAPTIC
199 PLASTICITY

200 In *Drosophila*, LTM increased the number of CREB-activated neurons in the MB[9,28]. To evaluate
201 whether or not socialization also correlates with higher levels of CREB activity in the MB, we used
202 the CAMEL reporter tool after 5 days of socialization directly after eclosion. This tool bears a MB-
203 specific transgenic construct that responds to phosphorylated CREB with the production of GFP[28].
204 We quantified the number of GFP positive soma (fig 5A) in adult brains, observing an increase in
205 the number of CREB-positive cells in grouped vs single-reared animals (fig 5B). In contrast, this
206 CREB response was lost in *rut* mutant brains (fig 5B).



207

208 **Figure 5. Socialization correlates with cellular and synaptic plasticity.** A) Representative
209 confocal images of CAMEL tool for wt and *rut* mutant MB, either socialized or isolated. Only one
210 representative MB is shown. B) Number of CREB GFP-positive cells in the MB of socialized or
211 isolated wt and *rut* mutant animals after 5 days of socialization. Kruskal-Wallis chi-squared =

212 33.735, df = 3 p-value = 1.93e-05; *post hoc* Dunn key comparisons: 5 days: $wt^{social}|wt^{isolated}$ p =
213 6.01e-05, $rut^{social}|rut^{isolated}$ p = 1.00. C) Example of CAMEL tool (MB cells marked by GFP) combined
214 with the pre-sinaptic marker brp-cherry after 5 days of socialization for wt and *rut* mutant animals,
215 either socialized of isolated. D) Quantification of the number of synapses after either isolation or
216 socialization in both wt and *rut* mutant flies (see fig S5 for a detail on the quantification). Kruskal-
217 Wallis chi-squared = 9.7691, df = 3, p-value = 0.021; *post hoc* Dunn key comparisons:
218 $wt^{social}|wt^{isolated}$ p = 2.51 e-02, $rut^{social}|rut^{isolated}$ p = 1.00.
219
220 LTM formation using an appetitive conditioning paradigm increased the number of MB-input
221 synapses[29]. Thus, to determine if CREB-activated neurons after socialization also showed signals
222 of increased synaptic plasticity, we included in the CAMEL tool a second reporter, the presynaptic
223 marker BRP, fused with the RFP-variant cherry. This reporter combination allowed the visualization
224 of the presynaptic densities (fig 5C). We quantified the number of synapses per cell volume in
225 brains of 5-day grouped and single-reared animals (fig S2 shows an example of this quantification
226 technique, see M&M). There was a significant increase in the relative number of pre-synapses in
227 the MB of grouped flies compared to single-reared animals (fig 5D), similar to the synaptic plasticity
228 described in mammals after a memorable experience[30]. In contrast, in a *rut* mutant background we
229 could not detect any difference in the number of MB pre-synapses, which was in agreement with
230 the reduced pre-synapse number in *rut* MB-input neurons after appetitive conditioning[29](fig 5D).
231 Given that intensity of fluorescence varies greatly depending on the region, for analytical purposes
232 we divided the MB in three areas, alpha, beta and the tip of beta. Interestingly, the former two
233 showed only a marginal increase that did not reach statistical significance, however the tip of the
234 MB concentrated most of the increase (fig S3). In summary, results showed a clear correlation of
235 CREB-activated neurons and increased synaptic plasticity with effective social interaction that is

236 abolished in memory impaired mutants, thus supporting a resemblance between socialization
237 awareness and LTM.

238 **DISCUSSION**

239 Socialization induces several changes in animal behavior and here we show that such changes
240 are long-lasting, as a result of social interaction experience. Not surprisingly, socialization
241 awareness shows similarities with a long-term memory process: involvement of cAMP signaling
242 and processes of neuronal and synaptic plasticity. However, it presents differences with LTM. For
243 instance, the role of *rut* in short-term behavioral changes seems dispensable, at least for
244 aggression (fig 3A, B). It may indicate that short-term effect is independent of *rut*. A striking
245 peculiarity is its temporal dynamics since it would be hard to distinguish putative learning and
246 consolidation stages during socialization, while in long-term memory paradigms both phases are
247 clearly distinguishable (as, for example, in appetitive or aversive olfactory conditioning).

248 The classic view on sleep regulation indicates that this behavioral state is regulated by the circadian
249 clock and the internal sleep homeostat[31], but recent work in many species including *Drosophila*
250 show that sleep regulation goes beyond these two processes and includes temperature, starvation,
251 sexual arousal, and social context, among others[32]. Our data suggest that recalling a past social
252 experience may also regulate sleep in flies (fig 1), similar to what happens with psychophysiologic
253 insomnia in humans[33].

254 In mammals, social isolation has profound effects on behavior and cognition, which is accompanied
255 by detectable alterations in brain structure and function at several levels[34]. For instance, the
256 hyppocampus shows reduced dendritic spine density after either postnatal or juvenile social
257 isolation[35,36]. *The* hyppocampus is the main structure related to long-term memory, analog to the
258 insect Mushroom Body[37]. In fact, it was previously described that socialization increases the fiber
259 number in the MB, an increase that is impeded by classic learning mutation such as rutabaga[12,13].
260 In addition, our results revealed that socialization also induces rut-dependent changes in synaptic

261 plasticity of the previously activated MB neurons. The increased synaptic densities in CREB-
262 positive neurons might be explained by the socialization-induced enhanced sleep, given that sleep
263 loss diminishes pre-synaptic densities in cholinergic neurons, including the MB neurons[38,39]. This
264 is unlikely because despite *rut* mutant animals do sleep much more (fig 2B), their MB presynaptic
265 densities do not reach levels of socialized *wt* animals, although they are higher than in isolated flies
266 (fig 5D). Besides, *rut* mutant flies did not reach enough sleep levels as to restore behavioral
267 plasticity[40], thus suggesting that *rut* increased synaptic activity might be due to the excess of sleep
268 but it is unable to rescue the effect of social interaction (fig 1-3). This reinforces the idea that
269 socialization awareness may induce behavioral plasticity by similar mechanisms to long-term
270 memory.
271 Why do isolated flies that were previously socialized behave similar to single-reared flies since
272 eclosion[14]? Actually, chronic isolation displays starvation-like phenotypes in *Drosophila*[16] and
273 starvation disables aversive long-term memory[41], probably because increased metabolism in the
274 MB and glia are necessary[42,43]. It might well be that socialization awareness was prevented as a
275 consequence of the starvation signaling, and this would explain the similar phenotypes achieved
276 by isolation after socialization and isolation since eclosion, despite mechanistically they should be
277 different. Indeed, one might hypothesize that rescuing such starvation-like phenotype would reveal
278 differences between both experimental conditions.
279 Notably, socialization-induced behavioral changes are sexually dimorphic, since grouped and
280 single-reared females behave similarly[44]. Indeed, male-specific P1 interneurons act as an internal
281 state regulatory hub for sleep, aggression, sleep and spontaneous[45]. Together with Diuretic
282 hormone 44- (DH44) and Tachykinin- (TK) expressing interneurons, P1 neurons form a male-
283 specific neural circuit that regulates spontaneous locomotion in response to social interaction, thus
284 suggesting a possible common mechanism for socially-induced behavioral changes[44].
285 Interestingly, P1 neurons directly activate a specific subset of dopaminergic neurons that innervate

286 the MB and it drives LTM appetitive olfactory memory formation[46]. The MB is not only a memory
287 regulatory center but also acts as a sleep and feeding regulatory center[47,48]. In this work we have
288 shown that social interaction correlates with increased synaptic plasticity in the MB itself (fig 4).
289 Thus, it is interesting to speculate that socialization awareness may use a general neural circuit
290 connecting P1 neurons, dopaminergic neurons and the MB in order to modify several behaviors
291 with long-lasting effects.

292 **Materials**

293 **- Stocks and fly husbandry**

294 Flies were raised and experiment performed using standard food at 25ºC on a 12/12h light/dark
295 cycle. *rutabaga*^{2080} (#9405), *dunce*^{1} (#6020) and *Wild Type* (*Canton S* #64349) stocks were
296 obtained from Bloomington Drosophila Stock Center. The CAMEL tool is composed by 6xCRE-
297 splitGal4^{AD}, UAS-eGFP and R21B06-splitGal4^{DBD}, gently donated by Dr Jan Pielage[28]. *rut*^{2080}*;*
298 *6xCRE-splitGal4*^{AD} and *UAS-cherry-Brutchpilot; R21B06-splitGal4*^{DBD} stocks were combined in our
299 laboratory and are available under request.

300 **- Isolation/socialization Protocol**

301 Male virgin flies were collected under CO_2 anesthesia within 4 hours post-eclosion and isolated in
302 individual glass vials or socialized (25:25 male:female) in a plastic bottle. After 5 days of
303 socialization or isolation, all flies were isolated without using anesthesia for 24 h (except where
304 indicated) and then, behavioral experiments or dissections were performed.
305 In the case of cold shock, flies were ice-cold shocked twice a day (Zeitgeber Time 1 -ZT01- and
306 ZT9) during the five days of isolation/socialization protocol for 2-3 minutes (i.e. until flies fainted).
307 Glass vials were used to allow good cold transfer from ice. Afterwards vials were placed horizontally
308 in a RT surface to let flies recover.

309 **- single fly Capillary Feeding (sCAFE)**

310 The protocol from [17] was used with slight modifications. Males were placed in individual vials with
311 a wet filter paper at the bottom and a 5 μl capillary (Blaubrand, 708707) with 5% sucrose water
312 food. The capillary was introduced through a 5mm cut 200 μl pipet tip that goes through a wet plug
313 and sustained with an additional tip. After 24 h food intake is measured (0 h-24 h time window), the
314 capillary substituted by a new one and plugs are wet again to preserve moisture. 24 h later food
315 intake is measured again. Once the experiment has finished flies are weighted. Additional 3
316 individual tubes without flies were measured to control the evaporation rate.

317 **- Sleep**

318 For all experiments, flies were sorted into glass tubes [70 mm × 5 mm × 3 mm (length × external
319 diameter × internal diameter)] containing the same food used for rearing under a regime of 12:12
320 Light:Dark (LD) condition in incubators set at 25°C. Activity recordings were performed using
321 ethoscopes[22]. Behavioural data analysis was performed in RStudio (RStudio Team. RStudio:
322 Integrated Development for r. RSudio, Inc. Boston, MA; 2015. http://www.rstudio.com/) employing
323 the Rethomics suite of packages[49]. All sleep assays were repeated at least twice with 20–40
324 flies/treatment/experiment.

325 - **Aggression**

326 The protocol from[24] with slight modifications was used. Briefly, two flies were placed into each
327 chamber of the arena (4x3 mm grid) with food. One-to-one socialization was achieved by allowing
328 both flies to interact, whereas isolation was caused by a black divider that allowed physical
329 separation of flies. After 5 days, socialized flies were also separated by the divider for 1, 4, 8 or 24
330 h. After removing the divider, reunited flies were recorded for 20 minutes and agression analyzed
331 by means of the *FlyTracker* (MATLAB) software and the platform JAABA (*Janelia Automatic Animal*
332 *Behavior Annotator*), that identifies when the animal is lunging. The proportion of time fighting is
333 the number of frames in which a particular animal lunges divided by the total number of frames.

334 **- Immunolabeling, imaging and image analysis**

335 Adult brain preparations were stained following the same protocol as in[50]. Dissections were always
336 performed at ZT4-5 to avoid possible circadian-induced changes.
337 For CREB+ cells experiment, primary antibodies used were anti-GFP rabbit (1/200; DSHB???REF)
338 and anti-Fasciclin II mouse (1/50; DSHB). To quantify synapse number, primary antibodies used
339 were anti-GFP goat (1/200; DSHB), anti-RFP rabbit (1/200; DSHB) and anti-Fasciclin II mouse
340 (1/50; DSHB). Secondary antibodies used were Alexia 488, 568 or 680 (1/500; Life Technologies).
341 Images were taken by a Leica SP5 confocal microscopy re-using the same experimental
342 conditions, avoiding saturation. CREB+ cell images were taken using a 40X objective, with slices
343 of 3 um. Synapse quantification confocal images were taken the same day using a 63X objective,
344 slices of 0,8um. Posteriorly images were treated using Imaris 6.3.1 software. Axon volume was
345 rebuilt using the Volume tool and brutchpilot signal was quantified using the Spots tool. To adjust
346 brightness parameters accurately the MB was divided in three parts (alfa, beta and beta tip) (Fig.
347 Supp. 2). Synaptic density for each Mushroom Body is the summatory of spots/volume from each
348 part.
349 **- Statistical analysis**
350 For the behavioral and morphological experiments (figures 1, 2, 3, 4, 5, S1 and S3), the data was
351 analyzed in R (version 3.6.3) through Rstudio (Version 1.0.153), employing the Kruskal-Wallis non-
352 parametric test (library *stats*). When appropriate, we performed post hoc Dunn analyses (library
353 FSA) to identify specific differences between treatments. All assays were repeated at least twice
354 with sample sizes as indicated within the figure
355
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472

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Author contributions

FAM conceptualized and designed the project; FAM and EJB supervised the project; BGM, GT and JIM performed aggression experiments and cellular studies; BGM and APZ performed feeding assays; GSAT and EJB performed and analyzed sleep experiments; EJB analyzed all data; FAM and EJB wrote the original draft and made the figures; AD and ET made extensive editing to the manuscript and revised statistics; FAM and EJB were responsible of funding acquisition.

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Competing interests

The authors declare no competing interests.