# Socialization causes long-lasting behavioral changes

Beatriz Gil-Martí[1,2], Julia Isidro-Mézcua[1], Adriana Poza-Rodriguez[1], Gerson S. Asti Tello[3], Gaia Treves[1], Enrique Turiégano[2], Esteban J. Beckwith[3*] and Francisco A Martin[1,4*]

Affiliations:

[1] Cajal Institute, Spanish National Research Council (CSIC), Av Dr Arce 37, 28002 Madrid, Spain.

[2] Department of Biology, Autonomous University of Madrid, Madrid, Spain.

[3] Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE), UBA-CONICET, Buenos Aires, Argentina.

[4] Lead contact

* To whom correspondence should be addressed: ebeckwith@fbmc.fcen.uba.ar and famartin@cajal.csic.es

**SUMMARY**

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

Most 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 and sleep in response to social enrichment[6–8]. Key clusters of dopaminergic neurons are also essential components of learning and memory circuits[9], since they innervate the main *Drosophila* memory structure, the mushroom body (MB)[10].

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[11]. 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[11,12]. Social interaction causes structural changes in the MB, an effect that is abolished in mutant flies for memory-related genes like *rut* and *dnc*[13,14]. Furthermore, the function of such genes is necessary for immediate sleep changes triggered by social interaction[15,16].

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.

**Keywords**

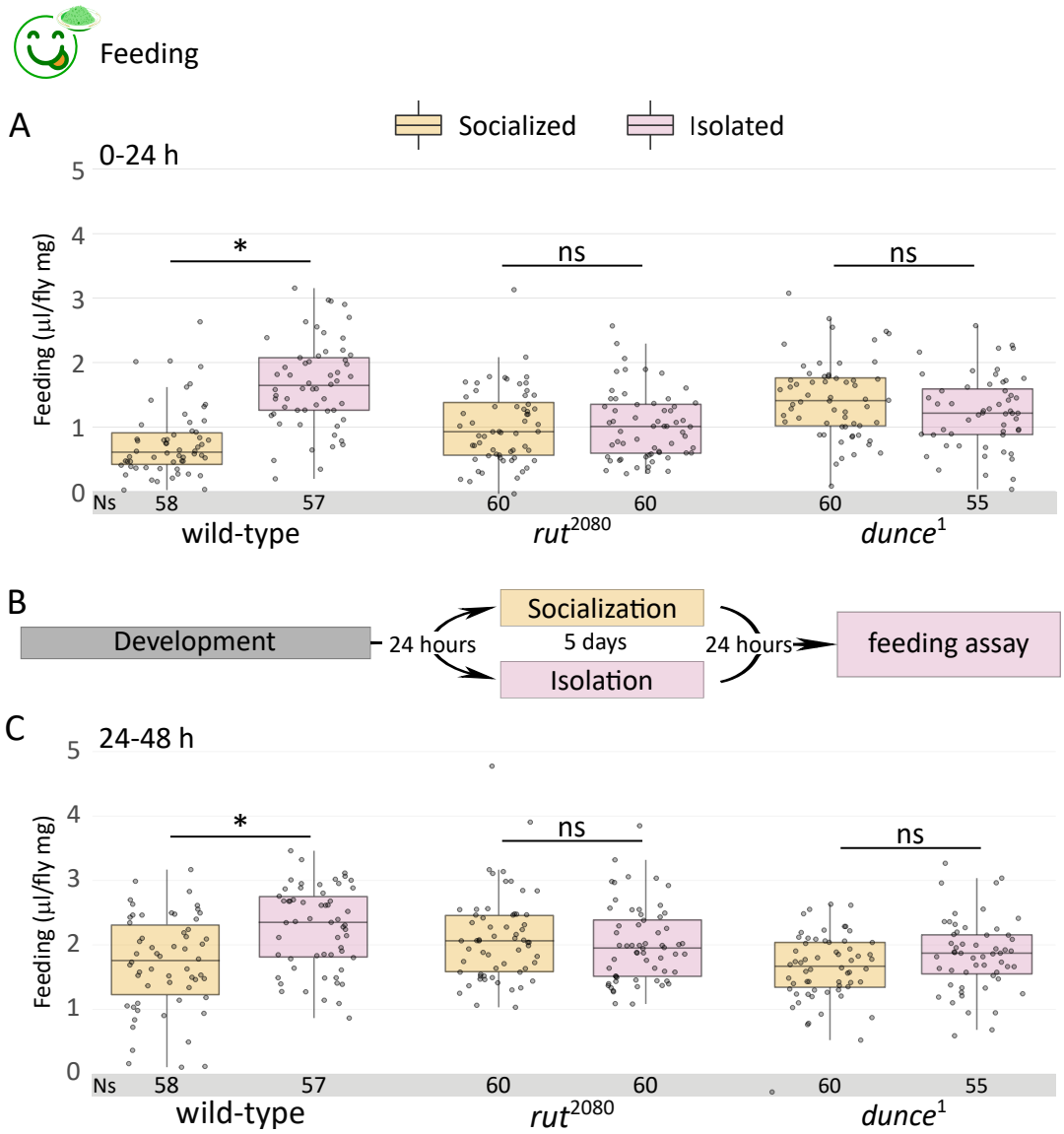
Social interaction, feeding behavior, sleep, aggression, synaptic plasticity, CREB, socialization awareness, *Drosophila*

## RESULTS

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

Flies that experienced social interaction show reduced food consumption when compared with flies that were socially reared and posteriorly isolated[17]. We used single-fly CApillary FEeding -sCAFE- assay (modified from[18]) to extend these findings. We compared grouped flies with animals singly reared since eclosion, meaning that they were socially naive. As expected, there was a significant decrease in food uptake of 5-day socialized flies when compared to individual flies in the immediate 24 hours (0 h-24 h time window) (fig 1A). Next, to determine if such feeding effect is maintained even in the absence of social interaction, we slightly modified the socially-enriched paradigm: flies were group- or single-reared for 5 days and then animals from both experimental groups were kept isolated for additional 24 hours previous to assessing feeding (fig 1B). Using this protocol, we also detected a decreased food consumption of grouped flies in the 24 h-48 h time window, confirming a long-lasting effect of social interaction on feeding behavior (fig 1C). We reasoned that the most plausible candidate genes to play a role for such long-lasting effect would be memory-related genes, such as *rutabaga* (*rut*)[19]. Despite their past experience, isolated *rut* mutant flies in the 24 h-48 h period after socialization showed no differences in food intake with solitaire animals since eclosion (fig 1C). Besides, *rut* mutant flies do not change their feeding behavior during the first 24 hours (0 h-24 h), suggesting a requirement of cAMP for this response (fig 1A). To confirm the involvement of cAMP signaling we repeated the sCAFE assay in animals mutant for *dunce* (*dnc*). Results were comparable to *rut* mutant: *dnc* mutant flies fail to modify their food consumption not

75 only during the first 24 hours after socialization (0 h-24 h) but also according to previous experience,
76 in the 24 h-48 h period (fig 1A, C). Strikingly, feeding behavior of memory-related mutant animals
77 lies in an intermediate state between socialized and isolated flies, maybe suggesting that their
78 basal food consumption is different from wild type strain (see below). We propose that this long-
79 lasting effect of social interaction on (feeding) behavior indicates a that animals have a socialization
80 awareness that lasts even after social interaction has ceased.




81

82 **Figure 1. Reduced food consumption induced by socialization depends on memory-related**
83 **genes.** (A) Quantification of food consumption of *wt*, *rut* and *dnc* mutant flies in socialized and

84 isolated conditions (single fly CAFE assay) in the 0-24 h time window (Kruskal-Wallis chi-squared
85 = 75.905, df = 5, p-value = 6.022e-15; *post hoc* Dunn comparisons: $wt^{social}|wt^{isolated}$ p = 6.24e-13,
86 $rut^{social}|rut^{isolated}$ p = 1.00, $dnc^{social}|dnc^{isolated}$ = 1.00). (B) Scheme of the socialization protocol:
87 recently eclosed animals were either grouped or isolated for five days, and subsequently isolated
88 for additional 24 hours before tested. C) Quantification of food consumption of *wt*, *rut* and *dnc*
89 mutant flies in socialized and isolated conditions (sCAFE) in the 24 h-48 h h time window (Kruskal-
90 Wallis chi-squared = 32.698, df = 5, p-value = 4.32e-06; *post hoc* Dunn comparisons:
91 $wt^{social}|wt^{isolated}$ p = 1.04e-03, $rut^{social}|rut^{isolated}$ p = 1.00, and , in fig S1, $dnc^{social}|dnc^{isolated}$ = 1.00).
92
93 LONG-TERM EFFECT OF SOCIALIZATION ON SLEEP IN ABSENCE OF INTERACTION
94 Sleep (particularly daytime sleep) is also regulated after social interaction. When flies are kept in
95 mixed-sex groups immediately at eclosion, sleep increases[15]. However, courtship experience
96 inhibits sleep in male flies [20,21], which lasts for several hours, proving a complex regulation of sleep
97 by social cues and experiences. Most published sleep studies employ *Drosophila* Activity Monitors
98 (DAMs), which only detect movement when the fly crosses a midpoint sensor in the housing tube[22],
99 overestimating actual sleep time[17]. The ethoscope was developed to unequivocally identify
100 immobility periods and assess sleep[23]. We confirmed that social interaction also induced animals
101 to sleep more[15] (fig 2A). To ensure consistency with previous works[17], we focused on the first four
102 hours after lights ON, where the effect is unambiguous and reproducible (i.e. 24 h-28 h time
103 window, ZT0-ZT4, fig 2A). Sleep quantification shows a significant difference between social-
104 enriched and lonely animals in this 24 h-28 h period (fig 2B), in line with previous publications[15,17].
105 Furthermore, *rut* mutant animals showed no significant difference in 24 h-28 h sleep time (fig 2A,
106 B), although socialized *dnc* mutants did exhibit a significant sleep difference in the 24 h-28 h, (fig
107 2C). Intriguingly, memory-related mutant flies sleep considerably more than their wt counterparts,
108 suggesting additional levels of sleep regulation related to cAMP signaling (fig 2A-C).

109 Our results with *dnc* and *rut* mutant flies were apparently contradictory with those previously
110 described using DAMs[15]. However, the ethoscope offers the possibility of analyzing data as they
111 were extracted from DAMs, thus depicting comparable results to published DAM data. This *virtual*
112 DAM analysis did render a significant difference between *rut* mutant grouped and single-reared
113 flies, whereas no sleep changes were apparent in *dnc* mutant animals, in agreement with previous
114 studies (fig S1)[15]. The differing results obtained depending on the type of analysis (regular or virtual
115 DAM) stem from the higher sensitivity of ethoscopes to movement. It also explains why the
116 increased sleep behavior of memory-mutant flies remained unnoticed until now, given that DAMs
117 cannot detect such changes ([24] and fig S2). Nevertheless, in either case, our data and previous
118 work support the idea that cAMP regulation, necessary for synaptic plasticity, is needed to sustain
119 long-lasting changes in sleep triggered by socialization awareness.

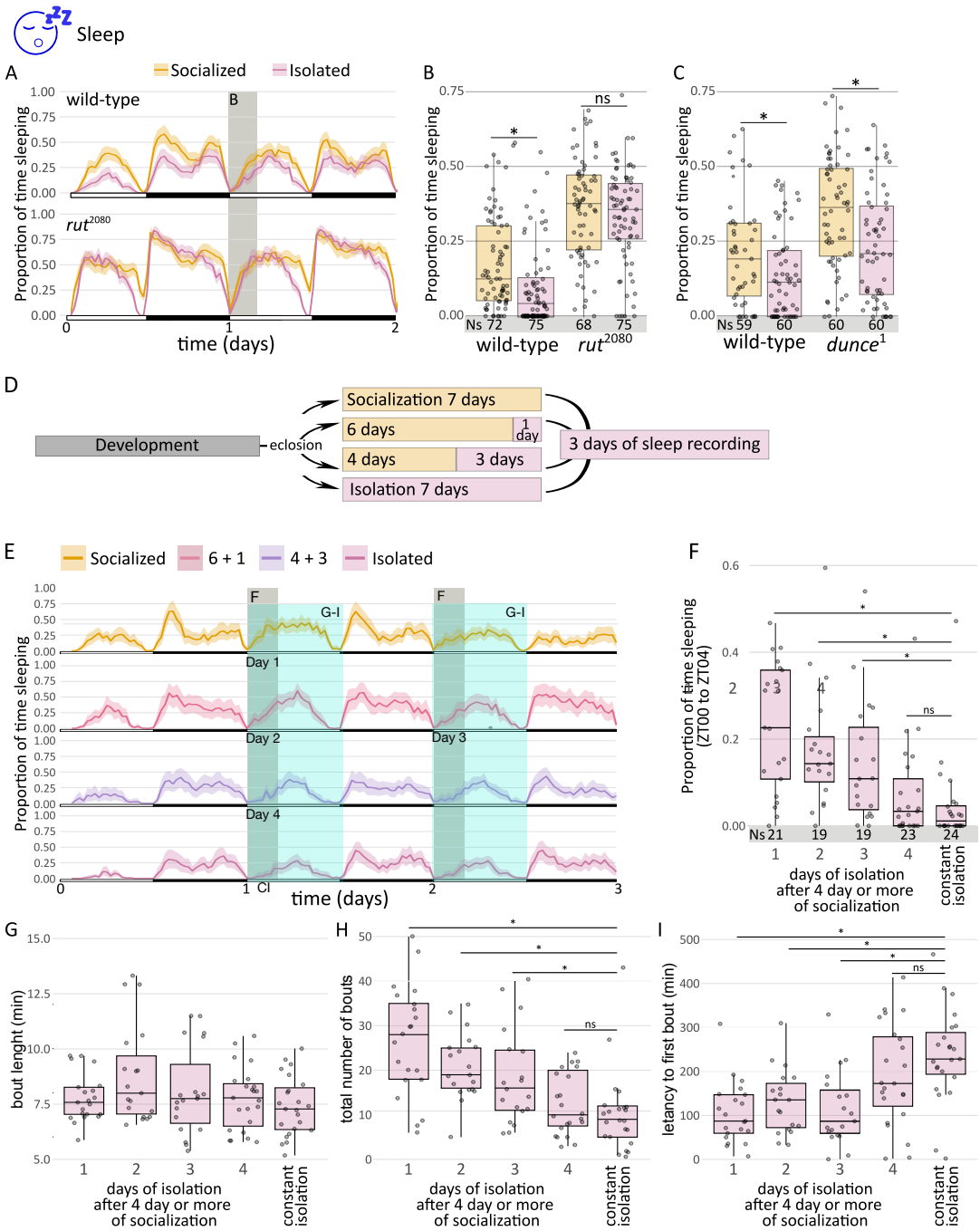



**Figure 2. Sleep behavior after socialization is long-term and relies on *rut*.** (A) Sleep profile and (B) sleep quantification of the 24 h-28 h time window for *wt* and *rut* mutant background (Kruskal-Wallis chi-squared = 94.165, df = 3, p < 2.2e-16; *post hoc* Dunn comparisons: $wt^{social}|wt^{isolated}$ p = 1.07e-02, $rut^{social}|rut^{isolated}$ p = 0.438). (C) Sleep quantification of the 24 h-28 h time window for *wt* and *dnc* mutant background (Kruskal-Wallis chi-squared = 36.476, df = 3, p-

126 value = 5.94e-08; *post hoc* Dunn comparisons: $wt^{social}|wt^{isolated}$ p = 3.43e-02, $dnc^{social}|dnc^{isolated}$ p =
127 3.48e-03). (D) Scheme of the sleep time course: flies were either isolated or grouped after eclosion
128 for 7, 6 or 4 days and subsequently isolated for 0, 1 or 3 days (named as socialized, 6+1, 4+3 and
129 constant isolation); after introducing them in ethoscopes, sleep behavior was recorded for 3 days.
130 (E) Sleep profile of animals isolated for 1 to 4 days, using isolated flies as control. Total number of
131 days in isolation for E-I is depicted in the panel. (F) Quantification of sleep from ZT0 to ZT4 for day
132 1-4 and flies under constant isolation (CI). (G-I) Analysis of bout length (G), total number of bouts
133 (H) and latency to first bout (I) from ZT0 to ZT12 for day 1-4 and animals under CI.
134
135 We wondered how long animals that experienced a socially-enriched environment maintained such
136 sleep behavior in absence of social interaction. To compare animals of the same age, we socialized
137 flies for 7, 6 or 4 days (which is enough socialization time in order to generate a sleep effect [15]) and
138 subsequently isolated them for 0, 1 or 3 additional days (named as socialized, 6+1 or 4+3,
139 respectively). Continuously isolated animals were used as control. Then, their sleep behavior was
140 recorded for the following 3 days (i.e. depicted in fig 2D). In the framework of this experimental
141 approach, we could compare continuously isolated flies with animals isolated for 1 to 4 days after
142 socialization (fig 2E). We could observe a progressive reduction of sleep time in the ZT0- ZT4 after
143 isolation, with significant decrease at day 4 that was comparable to continuous isolation (fig 2F).
144 Thus, 4 days of isolation are enough to modify sleep reaching similarly sleep levels than socially
145 naive flies, in contrast to the need of 5 days described previously using DAMs[17]. The ethoscope
146 also allows a detailed sleep analysis regarding bout length, the total number of bouts and the
147 latency to first bout in a 12-h analysis (ZT0-ZT12). There were no differences in the sleep bout
148 length amongst experimental groups. In contrast, isolated flies for 4 days reduced the number of
149 sleep bouts to similar levels than the ones from socially naive animals, despite we noticed a
150 progressive reduction but still statistically significative (fig 2H). Intriguingly, the latency to the first

151 bout in grouped flies remained similar up to day 3, where it raised sharply, similar to the latency of
152 isolated flies (fig 2l). We conclude that the effect of socialization lasts at least for 3 days, and
153 indeed, it can be considered as long-term.
154
155 SOCIALIZATION-INDUCED DIMINISHED AGGRESSIVENESS DEMANDS cAMP SIGNALING
156 In isolated flies that previously experienced social interaction, isolation signals starvation and, as a
157 consequence, increases feeding and decreases sleep, meaning that both behavioral changes are
158 reciprocally related[17]. We wondered if socialization awareness was also evident in a different social
159 behavior. Previous data showed that 5-day grouped male flies since eclosion were less aggressive
160 than their single-reared counterparts when tested immediately after the treatment[25]. This behavior
161 also allowed us to determine the progression of long-lasting effects, in order to compare the
162 temporal requirements of social interaction with those of classical learning and memory assays.
163 Thus, we employed animals from both experimental conditions and then evaluated aggression after
164 different isolation periods in a well-established paradigm[26]. Socially-experienced flies showed
165 reduced aggression (i.e. measured as the proportion of time lunging) at 1, 4, 8 and 24 hours after
166 isolation when compared with single-reared animals (fig 3), evidencing a behavioral change at
167 short- and long-term. Despite social interaction had ceased up to 24 h before, grouped flies still
168 spent considerably less time fighting than lonely flies (fig 3), confirming that socialization awareness
169 is a general feature of socialization. Critically, *rut* mutant flies did not show any difference according
170 to their previous experience (fig 3), reinforcing the role of memory-related genes. Besides, our data
171 revealed that at short-term (one hour), grouped *rut* mutant animals were significantly less
172 aggressive than *rut* single-reared flies, thus differentiating from *rut* requirements in classical
173 learning assays. However, this rut-independent effect disappeared after four hours, suggesting that
174 social interaction imprinted a temporary effect independently of cAMP, but long-term socialization

consequences in aggression depends on *rut* activity. Overall, *rut* mutant flies showed decreased levels of aggression, which makes comparison with *wt* animals difficult, as previously noticed[27].

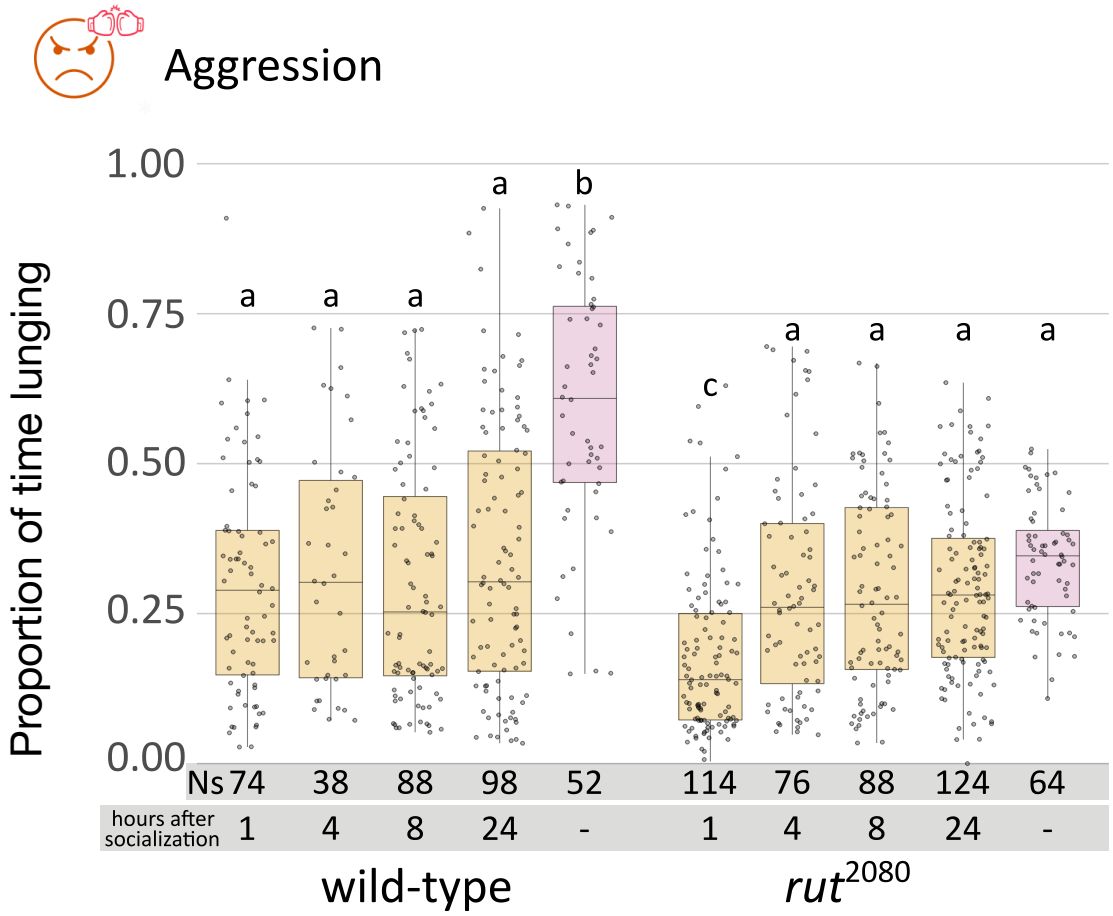
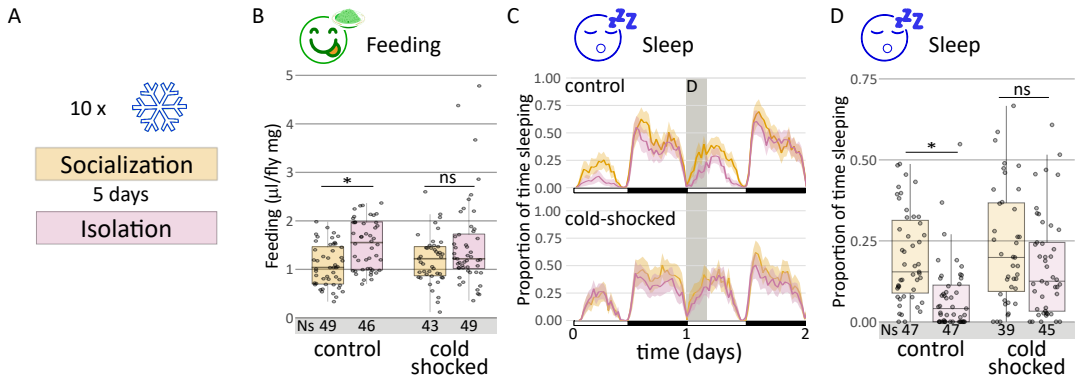



**Figure 3. Socialized-reduced aggression shows short- and long-term effect.** Quantification of proportion of time expend lunging after different times of re-isolation. Flies either *wt* or in a *rut* mutant background were grouped or isolated for 5 days and then socialized flies were tested after 1, 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

188 Anesthesia blocks long-term memory consolidation in most species[28,29]. In *Drosophila*, a 2-min
189 cold shock acts as anesthetics and is able to impede long-term memory in the classical aversive
190 olfactory conditioning assay[30]. We wondered if anesthesia was also able to block socialization
191 awareness. We exposed adult flies to 3-min cold shock two times per day to single and grouped
192 flies during the training period (fig 4A). Both experimental "cold-shocked" groups did not show any
193 significant differences in food consumption in the 24 h-48 h time window after isolation, in contrast
194 to non-shocked control animals (fig 4B). Given the reciprocal relationship between feeding and
195 sleep behavior regarding social interaction[17], we confirmed that sleep between lonely and
196 socialized animals in the 24 h-28 h time window also remained similar after cold shock (fig 4C, D).
197 As expected, in non-shocked animals the difference was statistically significant (fig 4C, D). In
198 summary, we found that socialization awareness relies on cAMP signaling and is blocked by
199 anesthesia, as it occurs in long-term memory.




200

201 **Fig 4. Anesthesia abolishes socialization effects on sleep and food consumption.** (A)
202 Scheme of the cold-shock protocol (twice per day). (B) Quantification of food consumption using
203 sCAFE (Kruskal-Wallis chi-squared = 15.954, df = 3, p-value = 1.16e-3; *post hoc* Dunn
204 comparisons: non-shocked$^{social}$|non-shocked$^{isolated}$p = 5.26e-3, shocked$^{social}$|shocked$^{isolated}$ p =
205 1.00). (C) sleep profile and (D) sleep quantification of the 24 h-28 h time window (Kruskal-Wallis
206 chi-squared = 31.184, df = 3, p-value = 7.78e-07; *post hoc* Dunn comparisons: non-

207 shocked$^{social}$|non-shocked$^{isolated}$ p = 3.05e-06, shocked$^{social}$|shocked$^{isolated}$ p = 0.116) of cold-

208 shocked socialized and isolated *wt* flies, together with non-shocked control *wt* flies.

209

210 SOCIALIZATION CORRELATES WITH INCREASED NEURONAL ACTIVITY AND SYNAPTIC

211 PLASTICITY

212 In *Drosophila*, LTM increased the number of CREB-activated neurons in the MB[10,31]. To evaluate

213 whether or not socialization also correlates with higher levels of CREB activity in the MB, we used

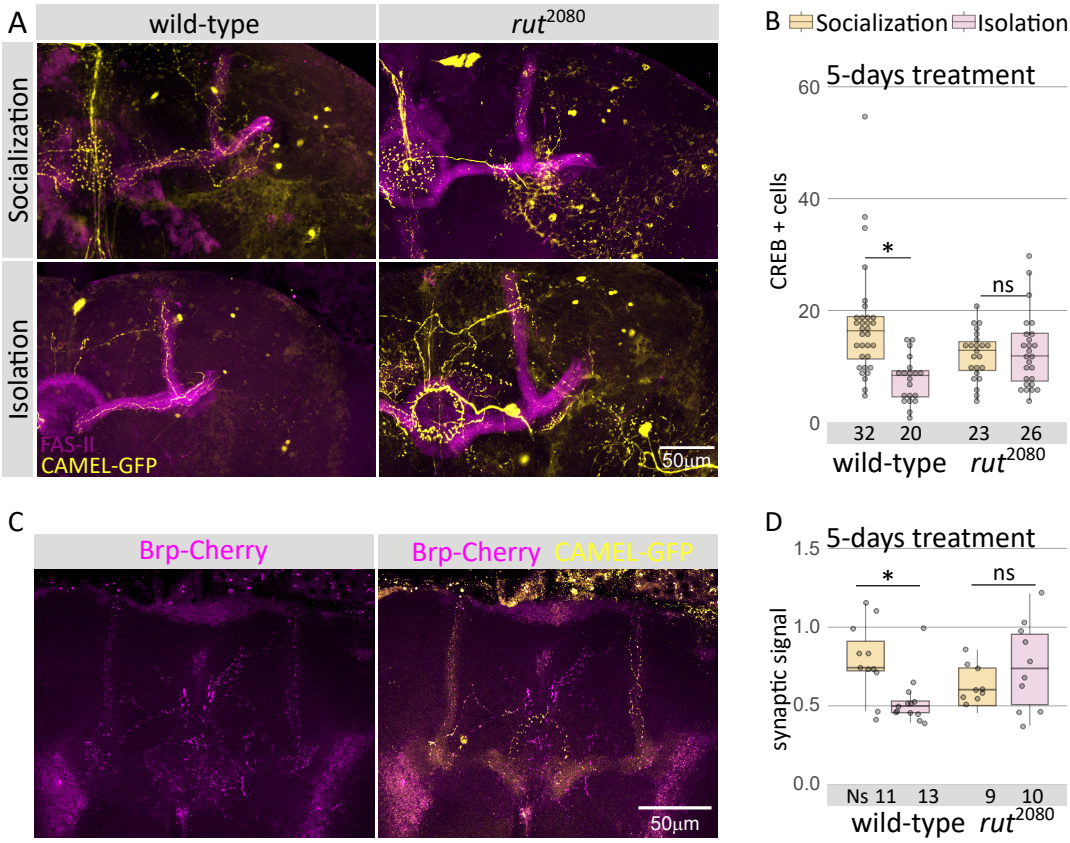
214 the CAMEL reporter tool after 5 days of socialization directly after eclosion. This tool bears a MB-

215 specific transgenic construct that responds to phosphorylated CREB with the production of GFP[31].

216 We quantified the number of GFP positive soma (fig 5A) in adult brains, observing an increase in

217 the number of CREB-positive cells in grouped vs single-reared animals (fig 5B). In contrast, this

218 CREB response was lost in *rut* mutant brains (fig 5B).




219

220 **Figure 5. Socialization correlates with cellular and synaptic plasticity.** A) Representative
221 confocal images of CAMEL tool for wt and *rut* mutant MB, either socialized or isolated. Only one
222 representative MB is shown. B) Number of CREB GFP-positive cells in the MB of socialized or
223 isolated wt and *rut* mutant animals after 5 days of socialization. Kruskal-Wallis chi-squared =
224 33.735, df = 3 p-value = 1.93e-05; *post hoc* Dunn key comparisons: 5 days: $wt^{social}|wt^{isolated}$ p =
225 6.01e-05, $rut^{social}|rut^{isolated}$ p = 1.00. C) Example of CAMEL tool (MB cells marked by GFP) combined
226 with the pre-sinaptic marker brp-cherry after 5 days of socialization for wt and *rut* mutant animals,
227 either socialized of isolated. D) Quantification of the number of synapses after either isolation or
228 socialization in both wt and *rut* mutant flies (see fig S5 for a detail on the quantification). Kruskal-
229 Wallis chi-squared = 9.7691, df = 3, p-value = 0.021; *post hoc* Dunn key comparisons:
230 $wt^{social}|wt^{isolated}$ p = 2.51 e-02, $rut^{social}|rut^{isolated}$ p = 1.00.
231
232 LTM formation using an appetitive conditioning paradigm increased the number of MB-input
233 synapses[32]. Thus, to determine if CREB-activated neurons after socialization also showed signals
234 of increased synaptic plasticity, we included in the CAMEL tool a second reporter, the presynaptic
235 marker BRP, fused with the RFP-variant cherry. This reporter combination allowed the visualization
236 of the presynaptic densities (fig 5C). We quantified the number of synapses per cell volume in
237 brains of 5-day grouped and single-reared animals (fig S2 shows an example of this quantification
238 technique, see M&M). There was a significant increase in the relative number of pre-synapses in
239 the MB of grouped flies compared to single-reared animals (fig 5D), similar to the synaptic plasticity
240 described in mammals after an experience[33]. In contrast, in a *rut* mutant background we could not
241 detect any difference in the number of MB pre-synapses, which was in agreement with the reduced
242 pre-synapse number in *rut* MB-input neurons after appetitive conditioning[32](fig 5D). Given that
243 intensity of fluorescence varies greatly depending on the region, for analytical purposes we divided
244 the MB in three areas, alpha, beta and the tip of beta. Interestingly, the former two showed only a

245 marginal increase that did not reach statistical significance, however the tip of the MB concentrated
246 most of the increase (fig S3). In summary, results showed a clear correlation of CREB-activated
247 neurons and increased synaptic plasticity with effective social interaction that is abolished in
248 memory impaired mutants, thus supporting a resemblance between socialization awareness and
249 LTM.

250 **DISCUSSION**

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

260 The classic view on sleep regulation indicates that this behavioral state is regulated by the circadian
261 clock and the internal sleep homeostat[34], but recent work in many species including *Drosophila*
262 show that sleep regulation goes beyond these two processes and includes temperature, starvation,
263 sexual arousal, and social context, among others[35]. Our data suggest that recalling a past social
264 experience may also regulate sleep in flies (fig 1), similar to what happens with psychophysiological
265 insomnia in humans[36].

266 In mammals, social isolation has profound effects on behavior and cognition, which is accompanied
267 by detectable alterations in brain structure and function at several levels[37]. For instance, the
268 hippocampus shows reduced dendritic spine density after either postnatal or juvenile social
269 isolation[38,39]. *The* hippocampus is the main structure related to long-term memory, analog to the

270 insect Mushroom Body[40]. In fact, it was previously described that socialization increases the fiber
271 number in the MB, an increase that is impeded by classic learning mutation such as rutabaga[13,14].
272 In addition, our results revealed that socialization also induces rut-dependent changes in synaptic
273 plasticity of the previously activated MB neurons. The increased synaptic densities in CREB-
274 positive neurons might be explained by the socialization-induced enhanced sleep, given that sleep
275 loss diminishes pre-synaptic densities in cholinergic neurons, including the MB neurons[41,42]. This
276 is unlikely because despite *rut* mutant animals do sleep much more (fig 2B), their MB presynaptic
277 densities do not reach levels of socialized *wt* animals, although they are higher than in isolated flies
278 (fig 5D). Besides, *rut* mutant flies did not reach enough sleep levels as to restore behavioral
279 plasticity[24], thus suggesting that *rut* increased synaptic activity might be due to the excess of sleep
280 but it is unable to rescue the effect of social interaction (fig 1-3). This reinforces the idea that
281 socialization awareness may induce behavioral plasticity by similar mechanisms to long-term
282 memory.
283 An apparent contradictory result was that memory-mutant animals did not behave as expected,
284 i.e., as *wt* socialized flies, resembling more to wt isolated flies (fig 1-3). There are several reasons
285 to explain this presumed inconsistency. The most obvious one is that the basal behavior of *rut* and
286 *dnc* mutant flies are different due to the lack of cAMP signaling, as previously described for
287 aggression[27]. Actually, the reduced basal behavioral response of wt isolated animals correlated
288 with their decreased number of CREB-positive cells and pre-sinapses when compared to rut mutant
289 animals, which showed intermediate levels. We might speculate that isolated animals would also
290 generate an "isolation awareness" that enhanced the effect of isolation by diminishing synaptic
291 plasticity due to a cAMP-related mechanisms and, as a consequence, the behavioral response.
292 Why do isolated flies that were previously socialized behave similar to single-reared flies since
293 eclosion[15]? Actually, chronic isolation displays starvation-like phenotypes in *Drosophila*[17] and
294 starvation disables aversive long-term memory[43], probably because increased metabolism in the

295 MB and glia are necessary[44,45]. It might well be that socialization awareness was prevented as a
296 consequence of the starvation signaling, and this would explain the similar phenotypes achieved
297 by isolation after socialization and isolation since eclosion, despite mechanistically they should be
298 different. Indeed, one might hypothesize that rescuing such starvation-like phenotype would reveal
299 differences between both experimental conditions.
300 Notably, socialization-induced behavioral changes are sexually dimorphic, since grouped and
301 single-reared females behave similarly[46]. In fact, male-specific P1 interneurons act as an internal
302 state regulatory hub for sleep, aggression, sleep and spontaneous locomotion[47]. Together with
303 *Diuretic hormone 44-* (*DH44*) and *Tachykinin-* (*TK*) expressing interneurons, P1 neurons form a
304 male-specific neural circuit that regulates spontaneous locomotion in response to social interaction,
305 thus suggesting a possible common mechanism for socially-induced behavioral changes[46].
306 Interestingly, P1 neurons directly activate a specific subset of dopaminergic neurons that innervate
307 the MB and it drives LTM appetitive olfactory memory formation[48]. The MB is not only a memory
308 regulatory center but also acts as a sleep and feeding regulatory center[49,50]. In this work we have
309 shown that social interaction correlates with increased synaptic plasticity in the MB itself (fig 4).
310 Thus, it is tempting to postulate that socialization awareness may use a general neural circuit
311 connecting P1 neurons, dopaminergic neurons and the MB in order to modify several behaviors
312 with long-lasting effects.
313

314 **MATERIALS**

315 **- Stocks and fly husbandry**

316 Flies were raised and experiment performed using standard food at 25ºC on a 12/12h light/dark
317 cycle. *rutabaga*$^{2080}$ (#9405), *dunce*$^{1}$ (#6020) and *Wild Type* (*Canton S* #64349) stocks were
318 obtained from Bloomington Drosophila Stock Center. The CAMEL tool is composed by 6xCRE-
319 splitGal4$^{AD}$, UAS-eGFP and R21B06-splitGal4$^{DBD}$, gently donated by Dr Jan Pielage[31]. *rut*$^{2080;}$

320 *6xCRE-splitGal4$^{AD}$* and *UAS-cherry-Brutchpilot; R21B06-splitGal4$^{DBD}$* stocks were combined in our
321 laboratory and are available under request.

322 **- Isolation/socialization Protocol**

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

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

332 The protocol from [18] was used with slight modifications. Males were placed in individual vials with
333 a wet filter paper at the bottom and a 5 μl capillary (Blaubrand, 708707) with 5% sucrose water
334 food. The capillary was introduced through a 5mm cut 200 μl pipet tip that goes through a wet plug
335 and sustained with an additional tip. After 24 h food intake is measured (0 h-24 h time window), the
336 capillary substituted by a new one and plugs are wet again to preserve moisture. 24 h later food
337 intake is measured again. Once the experiment has finished flies are weighted. Additional 3
338 individual tubes without flies were measured to control the evaporation rate.

339 **- Sleep**

340 For all experiments, flies were sorted into glass tubes [70 mm × 5 mm × 3 mm (length × external
341 diameter × internal diameter)] containing the same food used for rearing under a regime of 12:12
342 Light:Dark (LD) condition in incubators set at 25°C. Activity recordings were performed using
343 ethoscopes[23]. Behavioural data analysis was performed in RStudio (RStudio Team. RStudio:
344 Integrated Development for r. RSudio, Inc. Boston, MA; 2015. http://www.rstudio.com/) employing

345 the Rethomics suite of packages[51]. All sleep assays were repeated at least twice with 20–40
346 flies/treatment/experiment.

347 - **Aggression**

348 The protocol from[26] with slight modifications was used. Briefly, two flies were placed into each
349 chamber of the arena (4x3 mm grid) with food. One-to-one socialization was achieved by allowing
350 both flies to interact, whereas isolation was caused by a black divider that allowed physical
351 separation of flies. After 5 days, socialized flies were also separated by the divider for 1, 4, 8 or 24
352 h. After removing the divider, reunited flies were recorded for 20 minutes and agression analyzed
353 by means of the *FlyTracker* (MATLAB) software and the platform JAABA (*Janelia Automatic Animal*
354 *Behavior Annotator*), that identifies when the animal is lunging. The proportion of time fighting is
355 the number of frames in which a particular animal lunges divided by the total number of frames.

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

357 Adult brain preparations were stained following the same protocol as in[52]. Dissections were always
358 performed at ZT4-5 to avoid possible circadian-induced changes.

359 For CREB+ cells experiment, primary antibodies used were anti-GFP rabbit (1/200; DSHB???REF)
360 and anti-Fasciclin II mouse (1/50; DSHB). To quantify synapse number, primary antibodies used
361 were anti-GFP goat (1/200; DSHB), anti-RFP rabbit (1/200; DSHB) and anti-Fasciclin II mouse
362 (1/50; DSHB). Secondary antibodies used were Alexia 488, 568 or 680 (1/500; Life Technologies).
363 Images were taken by a Leica SP5 confocal microscopy re-using the same experimental
364 conditions, avoiding saturation. CREB+ cell images were taken using a 40X objective, with slices
365 of 3 um. Synapse quantification confocal images were taken the same day using a 63X objective,
366 slices of 0,8um. Posteriorly images were treated using Imaris 6.3.1 software. Axon volume was
367 rebuilt using the Volume tool and brutchpilot signal was quantified using the Spots tool. To adjust
368 brightness parameters accurately the MB was divided in three parts (alfa, beta and beta tip) (Fig.

369 Supp. 2). Synaptic density for each Mushroom Body is the summatory of spots/volume from each
370 part.

371 **- Statistical analysis**

372 For the behavioral and morphological experiments (figures 1, 2, 3, 4, 5, S1 and S3), the data was
373 analyzed in R (version 3.6.3) through Rstudio (Version 1.0.153), employing the Kruskal-Wallis non-
374 parametric test (library *stats*). When appropriate, we performed post hoc Dunn analyses (library
375 FSA) to identify specific differences between treatments. All assays were repeated at least twice
376 with sample sizes as indicated within the figure

377 **REFERENCES**


378 1. Donovan, N.J., and Blazer, D. (2020). Social Isolation and Loneliness in Older
379 Adults: Review and Commentary of a National Academies Report. American
380 Journal of Geriatric Psychiatry *28*, 1233–1244. 10.1016/j.jagp.2020.08.005.
381 2. Lee, C.R., Chen, A., and Tye, K.M. (2021). The neural circuitry of social
382 homeostasis: Consequences of acute versus chronic social isolation. Cell *184*,
383 1500–1516. 10.1016/J.CELL.2021.02.028.
384 3. Vora, A., Nguyen, A.D., Spicer, C., and Li, W. (2022). The impact of social
385 isolation on health and behavior in Drosophila melanogaster and beyond.
386 https://doi.org/10.26599/BSA.2022.9050016 *8*, 183–196.
387 10.26599/BSA.2022.9050016.
388 4. Chen, M., and Sokolowski, M.B. (2022). How Social Experience and
389 Environment Impacts Behavioural Plasticity in Drosophila. Fly (Austin) *16*, 68–
390 84. 10.1080/19336934.2021.1989248.
391 5. Yadav, R.S.P., Ansari, F., Bera, N., Kent, C., and Agrawal, P. (2024). Lessons
392 from lonely flies: Molecular and neuronal mechanisms underlying social
393 isolation. Neuroscience and biobehavioral reviews *156*.
394 10.1016/J.NEUBIOREV.2023.105504.
395 6. Kent, C., and Agrawal, P. (2020). Regulation of Social Stress and Neural
396 Degeneration by Activity-Regulated Genes and Epigenetic Mechanisms in
397 Dopaminergic Neurons. Molecular Neurobiology 2020 57:11 *57*, 4500–4510.
398 10.1007/S12035-020-02037-7.
399 7. Agrawal, P., Chung, P., Heberlein, U., and Kent, C. (2019). Enabling cell-type-
400 specific behavioral epigenetics in Drosophila: A modified high-yield INTACT
401 method reveals the impact of social environment on the epigenetic landscape in
402 dopaminergic neurons. BMC Biol *17*, 1–19. 10.1186/s12915-019-0646-4.
403 8. Agrawal, P., Kao, D., Chung, P., and Looger, L.L. (2020). The neuropeptide
404 Drosulfakinin regulates social isolation-induced aggression in Drosophila. The
405 Journal of experimental biology *223*. 10.1242/JEB.207407.
406 9. Kaun, K.R., and Rothenfluh, A. (2017). Dopaminergic rules of engagement for
407 memory in Drosophila. Current opinion in neurobiology *43*, 56–62.
408 10.1016/J.CONB.2016.12.011.

10. Davis, R.L. (2023). Learning and memory using Drosophila melanogaster: a focus on advances made in the fifth decade of research. Genetics *224*. 10.1093/GENETICS/IYAD085.
11. Kaldun, J.C., and Sprecher, S.G. (2019). Initiated by CREB: Resolving Gene Regulatory Programs in Learning and Memory: Switch in Cofactors and Transcription Regulators between Memory Consolidation and Maintenance Network. BioEssays *41*. 10.1002/bies.201900045.
12. Chowdhury, M.A.R., An, J., and Jeong, S. (2023). The Pleiotropic Face of CREB Family Transcription Factors. Molecules and cells *46*, 399–413. 10.14348/MOLCELLS.2023.2193.
13. Technau, G.M., and Technau, G.M. (2007). FIBER NUMBER IN THE MUSHROOM BODIES OF ADULT DROSOPHILA MELANOGASTER DEPENDS ON AGE, SEX AND EXPERIENCE. Journal of Neurogenetics *21*, 183–196. 10.1080/01677060701695359.
14. Balling, A., Technau, G.M., and Heisenberg, M. (2007). ARE THE STRUCTURAL CHANGES IN ADULT DROSOPHILA MUSHROOM BODIES MEMORY TRACES? STUDIES ON BIOCHEMICAL LEARNING MUTANTS. Journal of Neurogenetics *21*, 209–217. 10.1080/01677060701695383.
15. Ganguly-Fitzgerald, I., Donlea, J., and Shaw, P.J. (2006). Waking experience affects sleep need in Drosophila. Science (New York, N.Y.) *313*, 1775–1781. 10.1126/SCIENCE.1130408.
16. Donlea, J.M., Ramanan, N., and Shaw, P.J. (2009). Use-dependent plasticity in clock neurons regulates sleep need in Drosophila. Science (New York, N.Y.) *324*, 105–108. 10.1126/SCIENCE.1166657.
17. Li, W., Wang, Z., Syed, S., Lyu, C., Lincoln, S., O'Neil, J., Nguyen, A.D., Feng, I., and Young, M.W. (2021). Chronic social isolation signals starvation and reduces sleep in Drosophila. Nature 2021 597:7875 *597*, 239–244. 10.1038/s41586-021-03837-0.
18. Diegelmann, S., Jansen, A., Jois, S., Kastenholz, K., Escarcena, L.V., Strudthoff, N., and Scholz, H. (2017). The CApillary FEeder Assay Measures Food Intake in Drosophila melanogaster. Journal of visualized experiments : JoVE *2017*. 10.3791/55024.
19. Lee, D. (2015). Global and local missions of cAMP signaling in neural plasticity, learning, and memory. Frontiers in Pharmacology *6*, 1–7. 10.3389/fphar.2015.00161.
20. Beckwith, E.J., Geissmann, Q., French, A.S., and Gilestro, G.F. (2017). Regulation of sleep homeostasis by sexual arousal. eLife *6*. 10.7554/ELIFE.27445.
21. Machado, D.R., Afonso, D.J.S., Kenny, A.R., Öztürk-Çolak, A., Moscato, E.H., Mainwaring, B., Kayser, M., and Koh, K. (2017). Identification of octopaminergic neurons that modulate sleep suppression by male sex drive. eLife *6*. 10.7554/ELIFE.23130.
22. Konopka, R.J., and Benzer, S. (1971). Clock mutants of Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America *68*, 2112–2116. 10.1073/PNAS.68.9.2112/ASSET/7D2537AA-6B18-4329-86E9-9AFE9454904F/ASSETS/IMAGES/LARGE/2112FIG2.JPG.
23. Geissmann, Q., Garcia Rodriguez, L., Beckwith, E.J., French, A.S., Jamasb, A.R., and Gilestro, G.F. (2017). Ethoscopes: An open platform for high-

459 throughput ethomics. PLOS Biology *15*, e2003026.
460 10.1371/JOURNAL.PBIO.2003026.
461 24. Dissel, S., Angadi, V., Kirszenblat, L., Suzuki, Y., Donlea, J., Klose, M., Koch,
462 Z., English, D., Winsky-Sommerer, R., Van Swinderen, B., et al. (2015). Sleep
463 restores behavioral plasticity to drosophila mutants. Current Biology *25*, 1270–
464 1281. 10.1016/j.cub.2015.03.027.
465 25. Kravitz, E.A., and Fernandez, M. de la P. (2015). Aggression in Drosophila.
466 Behavioral Neuroscience *129*, 549–563. 10.1037/BNE0000089.
467 26. Chowdhury, B., Wang, M., Gnerer, J.P., and Dierick, H.A. (2021). The Divider
468 Assay is a high-throughput pipeline for aggression analysis in Drosophila.
469 Communications Biology 2021 4:1 *4*, 1–12. 10.1038/s42003-020-01617-6.
470 27. Trannoy, S., Penn, J., Lucey, K., Popovic, D., and Kravitz, E.A. (2016). Short
471 and long-lasting behavioral consequences of agonistic encounters between male
472 Drosophila melanogaster. Proceedings of the National Academy of Sciences of
473 the United States of America *113*, 4818–4823. 10.1073/PNAS.1520953113.
474 28. Wang, D.S., and Orser, B.A. (2010). Inhibition of learning and memory by
475 general anesthetics. Canadian Journal of Anesthesia/Journal canadien
476 d'anesthésie 2010 58:2 *58*, 167–177. 10.1007/S12630-010-9428-8.
477 29. Hemmings, H.C., Riegelhaupt, P.M., Kelz, M.B., Solt, K., Eckenhoff, R.G.,
478 Orser, B.A., and Goldstein, P.A. (2019). Towards a Comprehensive
479 Understanding of Anesthetic Mechanisms of Action: A Decade of Discovery.
480 Trends in Pharmacological Sciences *40*, 464–481. 10.1016/J.TIPS.2019.05.001.
481 30. Bourouliti, A., and Skoulakis, E.M.C. (2022). Cold Shock Disrupts Massed
482 Training-Elicited Memory in Drosophila. International Journal of Molecular
483 Sciences 2022, Vol. 23, Page 6407 *23*, 6407. 10.3390/IJMS23126407.
484 31. Siegenthaler, D., Escribano, B., Bräuler, V., and Pielage, J. (2019). Selective
485 suppression and recall of long-term memories in Drosophila. PLOS Biology *17*,
486 e3000400. 10.1371/JOURNAL.PBIO.3000400.
487 32. Baltruschat, L., Prisco, L., Ranft, P., Lauritzen, J.S., Fiala, A., Bock, D.D., and
488 Tavosanis, G. (2021). Circuit reorganization in the Drosophila mushroom body
489 calyx accompanies memory consolidation. Cell Reports *34*, 108871.
490 10.1016/j.celrep.2021.108871.
491 33. Rao-Ruiz, P., Visser, E., Mitrić, M., Smit, A.B., and van den Oever, M.C. (2021).
492 A Synaptic Framework for the Persistence of Memory Engrams. Preprint at
493 Frontiers Media S.A., 10.3389/fnsyn.2021.661476 10.3389/fnsyn.2021.661476.
494 34. A two process model of sleep regulation - PubMed
495 https://pubmed.ncbi.nlm.nih.gov/7185792/.
496 35. Beckwith, E.J., and French, A.S. (2019). Sleep in Drosophila and Its Context.
497 Frontiers in physiology *10*. 10.3389/FPHYS.2019.01167.
498 36. Espie, C.A., Broomfield, N.M., MacMahon, K.M.A., Macphee, L.M., and
499 Taylor, L.M. (2006). The attention–intention–effort pathway in the development
500 of psychophysiologic insomnia: A theoretical review. Sleep Medicine Reviews
501 *10*, 215–245. 10.1016/J.SMRV.2006.03.002.
502 37. Xiong, Y., Hong, H., Liu, C., and Zhang, Y.Q. (2023). Social isolation and the
503 brain: effects and mechanisms. Molecular psychiatry *28*, 191–201.
504 10.1038/S41380-022-01835-W.
505 38. Silva-Gómez, A.B., Rojas, D., Juárez, I., and Flores, G. (2003). Decreased
506 dendritic spine density on prefrontal cortical and hippocampal pyramidal neurons
507 in postweaning social isolation rats. Brain Research *983*, 128–136.
508 10.1016/S0006-8993(03)03042-7.

39. Ohta, K.I., Suzuki, S., Warita, K., Kaji, T., Kusaka, T., and Miki, T. (2017). Prolonged maternal separation attenuates BDNF-ERK signaling correlated with spine formation in the hippocampus during early brain development. Journal of neurochemistry *141*, 179–194. 10.1111/JNC.13977.
40. Menzel, R. (2014). The insect mushroom body, an experience-dependent recoding device. Journal of physiology, Paris *108*, 84–95. 10.1016/J.JPHYSPARIS.2014.07.004.
41. Weiss, J.T., and Donlea, J.M. (2021). Sleep deprivation results in diverse patterns of synaptic scaling across the Drosophila mushroom bodies. Current biology : CB *31*, 3248-3261.e3. 10.1016/J.CUB.2021.05.018.
42. Weiss, J.T., Blundell, M.Z., Singh, P., and Donlea, J.M. (2024). Sleep deprivation drives brain-wide changes in cholinergic presynapse abundance in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America *121*. 10.1073/PNAS.2312664121.
43. Plaçais, P.Y., and Preat, T. (2013). To favor survival under food shortage, the brain disables costly memory. Science *339*, 440–442. 10.1126/SCIENCE.1226018/SUPPL_FILE/PLACAIS.SM.PDF.
44. Silva, B., Mantha, O.L., Schor, J., Pascual, A., Plaçais, P.Y., Pavlowsky, A., and Preat, T. (2022). Glia fuel neurons with locally synthesized ketone bodies to sustain memory under starvation. Nature Metabolism 2022 4:2 *4*, 213–224. 10.1038/s42255-022-00528-6.
45. Plaçais, P.Y., De Tredern, É., Scheunemann, L., Trannoy, S., Goguel, V., Han, K.A., Isabel, G., and Preat, T. (2017). Upregulated energy metabolism in the Drosophila mushroom body is the trigger for long-term memory. Nature Communications 2017 8:1 *8*, 1–14. 10.1038/ncomms15510.
46. Zhao, H., Jiang, X., Ma, M., Xing, L., Ji, X., and Pan, Y. (2024). A neural pathway for social modulation of spontaneous locomotor activity (SoMo-SLA) in Drosophila. Proceedings of the National Academy of Sciences of the United States of America *121*, e2314393121. 10.1073/PNAS.2314393121/SUPPL_FILE/PNAS.2314393121.SAPP.PDF.
47. Jiang, X., and Pan, Y. (2022). Neural Control of Action Selection Among Innate Behaviors. Neuroscience bulletin *38*, 1541–1558. 10.1007/S12264-022-00886-X.
48. Shen, P., Wan, X., Wu, F., Shi, K., Li, J., Gao, H., Zhao, L., and Zhou, C. (2023). Neural circuit mechanisms linking courtship and reward in Drosophila males. Current Biology *33*, 2034-2050.e8. 10.1016/J.CUB.2023.04.041.
49. Sgammeglia, N., and Sprecher, S.G. (2022). Interplay between metabolic energy regulation and memory pathways in Drosophila. Trends in Neurosciences *45*, 539–549. 10.1016/J.TINS.2022.04.007.
50. Wu, L., and Liu, C. (2023). Integrated neural circuits of sleep and memory regulation in Drosophila. Current opinion in insect science *59*. 10.1016/J.COIS.2023.101105.
51. Geissmann, Q., Rodriguez, L.G., Beckwith, E.J., and Gilestro, G.F. (2019). Rethomics: An R framework to analyse high-throughput behavioural data. PLOS ONE *14*, e0209331. 10.1371/JOURNAL.PONE.0209331.
52. Jarabo, P., Barredo, C.G., de Pablo, C., Casas-Tinto, S., and Martin, F.A. (2022). Alignment between glioblastoma internal clock and environmental cues ameliorates survival in Drosophila. Communications Biology 2022 5:1 *5*, 1–12. 10.1038/s42003-022-03600-9.

558 **ACKNOWLEDGEMENTS**

559 We would like to thank Javier Gil Castillo for his invaluable help and advices in 3D printing. We
560 also appreciate flies and reagents from the Bloomington and VDRC stock centers. Special thanks
561 to our colleagues Prof Alberto Ferrús, Dr Sergio Casas-Tintó, Dr Pablo Méndez, Dr Abhijit Das and
562 Dr JL Trejo-Pérez for their helpful comments and suggestions on this manuscript. Special thanks
563 to Dr Pavan Agrawal and his lab for their critical reading and suggestions on BioRxiv manuscript.
564 We thank the support of the scientific image and microscopy unit (Cajal Institute).

565 FAM was a recipient of a RyC-2014-14961 contract (2016-2022). Grant RyC-2014-14961 funded
566 by MICIU/AEI/10.13039/501100011033 and by ESF Investing in your future. Grant CNS2022-
567 135223 funded by MICIU/AEI/10.13039/501100011033 and by European Union NextGeneration
568 EU/PRTR.

569 BG-M is a recipient of a FPI-UAM predoctoral fellowship, grant number SFPI/2020/00878. JIM was
570 a recipient of a JAE intro fellowship (grant number JAEINT_22_01271) funded by the Spanish
571 National Research Council (CSIC).

572 EJB is a member of the Argentine Research Council (CONICET), and he is funded by *Agencia*
573 *Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación*, Argentina,
574 through grants PICT-2020-SERIEA-01240, PICT-PRH-2021-00009, and CONICET through grant
575 PIP 11220200102510CO.

576 **Author contributions**

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

582 **Corresponding authors**

583 Correspondence to Esteban J Beckwith and Francisco A Martin.

584 **Competing interests**

585 The authors declare no competing interests.

586 **Supplemental Information**

587 Document S1. Figures S1-S3