1 # CREB repressor in mushroom body enhances *Drosophila* LTM formation

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Abstract

Long-term memory (LTM) requires learning-induced synthesis of new proteins allocated to specific neurons and synapses in a neural circuit. Not all learned information, however, becomes permanent memory. How the brain gates relevant information into LTM remains unclear. In *Drosophila* adults, a single training session in an olfactory aversive task is not sufficient to induce protein synthesis-dependent LTM. Instead, multiple spaced training sessions are required. Here, we report that initial learning induces neural activity in the early α/β subset of Kenyon cells of the mushroom body (MB), and output from these neurons inhibits LTM formation. Specifically in response to spaced training, Schnurri activates CREBB expression which then appears to suppress the inhibitory output from MB. One training session can enhance LTM formation when this inhibitory effect is relieved. We propose that learning-induced protein synthesis and spaced training-induced CREBB act antagonistically to modulate output from early α/β MB neurons during LTM formation.

61 **MAIN TEXT**

62 **Introduction**

63   *Drosophila* continues to demonstrate its utility as a model system to study memory, more

64 than four decades after the first mutant was described (*Dudai et al., 1976*). Genetic dissection of

65 olfactory aversive memory formation using various single-gene mutants has revealed at the

66 behavioral level several distinct temporal phases, including short-term memory (STM), middle-

67 term memory (MTM), anesthesia-resistant memory (ARM) and long-term memory (LTM) (*Tully*

68 *et al., 1994*; *Tully et al., 1990*; *Tully, 1996*; *Quinn and Dudai, 1976*). The initial learning event

69 (acquisition) after a single training session (1x) appears to induce STM, MTM and ARM, while

70 spaced training (10 training sessions with 15 min rest intervals between each, 10xS) appears

71 uniquely required to induce LTM consolidation. Manipulations of several of these "memory genes"

72 also have established cases where memory formation is either impaired or enhanced, revealing bi-

73 directional biochemical modulation of memory formation (*Yin et al., 1994*; *Yin et al., 1995a*; *Ge et*

74 *al., 2004*; *Presente et al., 2004*; *Wu et al., 2007*; *Pavlopoulos et al., 2008*; *Huang et al., 2012*; *Tubon*

75 *et al., 2013*; *Fropf et al., 2014*; *Lee et al., 2018*; *Scheunemann et al., 2018*).

76   As the neural substrates of olfactory memory formation are elucidated in flies, a remarkable

77 "memory circuit" is emerging. Olfactory information delivered from the antennal lobe (AL) by

78 projection neurons (PN) and foot shock reinforcement delivered by dopaminergic neurons (DAN)

79 both converge on mushroom body (MB) neurons in the central brain where their coincidence

80 triggers cascading cellular events that underlie learning (*Dubnau and Chiang 2013*; *Perisse et al.,*

81 *2013*; *Davis, 2015*; *Cognigni et al., 2018*). MBs play a predominant role in subsequent memory

82 formation, together with several groups of extrinsic MB neurons. Sequential genetically-defined

83 memory phases map onto distinct subpopulations of these neurons. STM involves

84 γ, α′/β′ and α/β neurons and two classes of MB output neurons (MBON: MB-M4, MB-M6) (*Blum*

85 *et al., 2009*; *Scheunemann et al., 2012*; *Bouzaiane et al., 2015*). MTM involves neural activity in γ,

86 α/β and MB-V2 neurons (*Blum et al., 2009; Scheunemann et al., 2012; Bouzaiane et al., 2015*).

87 ARM requires neural activity in MB γ, α′/β′, α/β neurons, dorsal paired medial (DPM) neurons,

88 anterior paired lateral (APL) neurons, DAN and four different MB output neurons (MB-M4, MB-

89 M6, MB-V2, MBON-β2β′2a) (*Lee et al., 2011; Knapek et al., 2011; Placais et al., 2012; Wu et al.,*

90 *2013; Bouzaiane et al., 2015; Yang et al., 2016; Scholz-Kornehl and Schwärzel, 2016; Kotoula et*

91 *al., 2017; Shyu et al., 2019*). LTM involves neural activity in late MB α/β neurons with output from

92 DPM, serotonergic projection neurons (SPN) and three classes of MBONs (MB-V3, MB-M4,

93 MBON-γ3,γ3β′1). Cyclic AMP response element binding protein (CREB)-dependent consolidation

94 of LTM also requires activity in dorsal anterior lateral (DAL) neurons (*Chen et al., 2012; Pai et al.,*

95 *2013; Tonoki and Davis 2015; Bouzaiane et al., 2015; Wu et al., 2017; Scheunemann et al., 2018*).

96 Finally, memory retrieval depends on neural activity in DAL, pioneer α/β neurons and four classes

97 of MBONs (MB-V2, MB-V3, MB-M4, MBON-γ3,γ3β′1) (*Séjourné et al., 2011; Chen et al., 2012;*

98 *Pai et al., 2013; Bouzaiane et al., 2015; Wu et al., 2017*).

99   Here, we describe another enlightening property of olfactory memory in *Drosophila*: inhibition

100 of LTM formation at the circuit level. Output from the early α/β subpopulation of MB neurons

101 appears initially to inhibit LTM formation, but with spaced training, transcription of *crebB* (*dcreb2*,

102 repressor) is induced therein, apparently reducing neural output therefrom and thereby enabling

103 LTM formation. Thus, persistent olfactory memory formation appears modulated or "gated" at the

104 level of neural activity in early α/β MB neurons. These observations presage the need for a more

105 general deconvolution of biochemical mechanisms into distinct neuronal subtypes within a memory

106 circuit.

107

108 **Results**

109 **Learning inhibits LTM**

110　Sixty of the single-gene mutants mentioned above were generated using transposon mutagenesis

111　and were screened for impairments of memory one day after 10xS training (*Dubnau et al., 2003*).

112　Twenty-two of these lines carried *P-Gal4* enhancer traps, which enabled us to drive targeted

113　inducible expression of a temperature-sensitive *Ricin^{CS}* transgene and then block protein synthesis

114　after 10xS (*Chen et al., 2012*; *Pai et al., 2013*; *Wu et al., 2017*). With protein synthesis inhibited in

115　this manner, we found impairments of 1-day memory in nine of these lines (figure supplement 1A-

116　C; table supplement 1) (*Tully et al., 1994*; *Yin et al., 1994*). Remarkably, GFP was expressed in

117　DAL neurons in all nine cases (figure supplement 1B), an observation that contributed to our

118　characterization of DAL neurons extrinsic to the MB as *bona fide* "LTM neurons" (*Chen et al.,*

119　*2012*). Two of the enhancer-trap memory mutants that we screened were particularly informative.

120　In *umnitza* flies, GFP was expressed in DAL neurons but very weakly in MB, and 1-day memory

121　after 10xS was impaired. Conversely in *norka* flies, GFP was expressed in MB but not in DAL

122　neurons and 1-day memory after 10xS was normal.

123　　What then might be going on in the seven enhancer-trap mutants with normal memory and with

124　GFP expression in both MB & DAL neurons (Figure 1A; figure supplement 1A)? First, we

125　confirmed that active Ricin^{CS} inhibition of protein synthesis in different subsets of MB neurons did

126　not impair 1-day memory after 10xS (figure supplement 2A-B and 3). In contrast, 1-day memory

127　after 10xS was impaired by active Ricin^{CS} in DAL or MB-V3 neurons (*Chen et al., 2012*; *Pai et al.,*

128　*2013*; *Wu et al., 2017*) but was normal after massed training (10x training sessions with no rest

129　intervals; 10xM) or in control flies with inactive Ricin^{CS} (18 °C) (figure supplement 3).

130　　We next tested the hypothesis that inhibition of protein synthesis in MB might enhance LTM

131　formation, thereby off-setting the impairment produced by blocking protein synthesis in DAL

132　neurons. Using *cry-Gal80* or *MB-Gal80*, we blocked transgenic Ricin^{CS} expression outside (i.e.

133　DAL neurons) or inside of MB, respectively, in these seven enhancer-trap memory lines (Figure

134   1B-C) and then subjected them to suboptimal 3xS training. Surprisingly, LTM formation was

135   *enhanced* in all seven lines by *cry-Gal80* subtraction (Figure 1D-F).

136   The MB is composed of approximately 2,500 intrinsic neurons (Kenyon cells; KCs)

137   developmentally derived from four neuroblasts and distinguished by their projections that form the

138   γ, α′/β′ and α/β lobes (*Ito et al., 1997; Zhu et al., 2003; Lin et al., 2007*). We looked among these

139   neuronal subpopulations to identify where LTM enhancement might reside (Figure 2; figure

140   supplement 2A). Active Ricin was expressed in all KCs or in γ, α′/β′, or α/β neurons separately.

141   Enhanced LTM was observed after 3xS only in α/β neurons, which were previously shown to have

142   a role in LTM formation (*Blum et al., 2009; Yu et al., 2006*) (Figure 2A).

143   The α/β neurons are subdivided further into three types: pioneer α/β, early α/β and late α/β

144   neurons based on their birth sequences (*Zhu et al., 2003; Lin et al., 2007; Tanaka et al., 2008; Aso*

145   *et al., 2014*). When active Ricin was expressed in these three subpopulations separately, we

146   observed enhanced LTM after 3xS only when protein synthesis was blocked in early α/β neurons

147   (Figure 2A, left). Enhanced LTM lasted for at least 4 days (Figure 2A, right) and was not observed

148   in control flies (inactive Ricin^{CS}) after 3xS training or in flies with active Ricin^{CS} after 3xM (figure

149   supplement 2C). Blocking protein synthesis in early α/β neurons enhanced 1- and 4-day memories

150   even after only 1x training (Figure 2B). Notably, LTM enhancement after 1x training required two

151   copies of transgenic Ricin^{CS}. Together, these results indicate that inhibition of protein synthesis in

152   early α/β neurons yields a *bona fide* enhancement of LTM formation.

153   We next inquired about the most effective time after training when inhibition of protein synthesis

154   would enhance LTM. Ricin^{CS} in early α/β neurons was activated for 12 h in a series of time

155   windows staggered by 2 h during the first 24 h after 1x training (*Chen et al., 2012; Wu et al., 2017*).

156   LTM was enhanced when protein synthesis was blocked beginning from 0- to 4-h but not from 6-

157   to 12-h after training (Figure 3A). Shortening the inhibition period to 3 h, we resolved the window

158   of protein-synthesis-dependent LTM inhibition to the first 6 h after training (Figure 3B).

159

Output from early α/β neurons inhibits LTM

161   To address whether the inhibitory effect on LTM in early α/β lobes depends on their neural output,

162   we blocked synaptic transmission from pioneer α/β, late α/β or early α/β neurons using *UAS-shi^{ts}*

163   (*Dubnau et al., 2001*; *McGuire et al., 2001*). In these experiments we found that 1- and 4-day

164   memory after 3xS training were enhanced by this manipulation in early α/β (Figure 4A-B), but not

165   in pioneer or in late α/β neurons (figure supplement 4A). Interestingly, we also established that

166   LTM after 3xS was enhanced when synaptic transmission was blocked from early α/β during the

167   first 8 h period after training but not 9-24 h after training or during 1-day memory retrieval (Figure

168   4A). Thus, this temporal requirement for synaptic transmission from early α/β neurons corresponds

169   to the requirement for protein synthesis (Figure 3). We confirmed these results using two additional

170   *Gal4* drivers expressing specifically in early α/β neurons (Figure 4C; figure supplement 2A) and

171   observed normal 1-day memory after 3xM (Figure 4B, middle) as in control flies carrying *UAS-*

172   *shi^{ts}* alone, *Gal4* alone (Figure 4B, right) or at the permissive temperature for *shi^{ts}* (Figure 4A, left

173   and 4C, right). Finally, we established that LTM after 10xS training was not impaired when neural

174   output from early α/β neurons was blocked (figure supplement 4B). Together, these results indicate

175   that, in the absence of spaced training, neural output from early α/β neurons inhibits LTM formation.

176   To examine the influence of early α/β neuron membrane excitability on memory, we used ectopic

177   expression of either transgenic hyperexcitors (*UAS-Shaw^{DN}*, a dominant-negative Shaw potassium

178   channel and *UAS-NaChBac*, a sodium channel) or hypoexcitors (*UAS-Shaw*, a Shaw potassium

179   channel and *UAS-Kir2.1::GFP*, an inward-rectifying potassium channel *Venken et al., 2011*).

180   Temporal control of these transgenes was enabled using a *tub-Gal80^{ts}* transgene (conditional

181   expression of Gal80 suppresses Gal4 expression at 18 °C but not at 30°C *McGuire et al., 2003*).

182   We found that increasing membrane excitability of early α/β neurons impaired 1-day memory after

183   10xS training (Figure 5A, left) without affecting (1) memory after 1x training (fig. S5A), (2) 1-day

184   memory after 10xM training (figure supplement 5B) or (3) 1-day memory after 10xS training when

185   flies were kept at permissive temperature (18°C) (figure supplement 5C). This inhibitory effect

186   appears to be complete, because inhibition of protein synthesis by feeding flies cycloheximide

187   (CXM) did not further reduce 1-day LTM after 10xS training (Figure 5A, right). Decreasing

188   membrane excitability of early α/β neurons with ectopic expression of hypoexciter transgenes, on

189   the other hand, enhanced both 1- and 4-day memory after 1x training (Figure 5B, left and middle),

190   whereas enhancement of 1-day memory was not observed in control transgenic flies kept at the

191   permissive temperature (18°C) (Figure 5B, right). We also found normal 1-day memory in these

192   transgenic flies after 10xS training (Figure 5C). Thus, sufficient spaced training appeared to occlude

193   the enhancing effects on LTM formation of decreased membrane excitability in early α/β neurons.

194   These data further support the notion that neural activity from early α/β neurons inhibits LTM

195   formation downstream (*Dubnau and Chiang 2013*; *Pai, et al., 2013*; *Wu et al., 2017*).

196

197   **cAMP signaling in early α/β neurons enhances LTM**

198   Neural excitability is suggested to be modulated by cAMP signaling (*Davis et al., 1998; Baines,*

199   *2003*), which in MB is also involved in LTM formation (*Blum et al., 2009*). Accordingly, we

200   inducibly overexpressed *rutabaga⁺* (*rut⁺*) adenylyl cyclase (AC) or constitutively active *cAMP-*

201   *dependent protein kinase* (*Pkaᵃᶜᵗ¹*) transgenes in early α/β neurons and found that 1-day memory

202   after 1x training was enhanced to levels normally seen after 10xS in both cases (Figure 6A and 7A).

203   Moreover, inducible RNAi knockdowns of these genes impaired 1-day memory after 10xS (Figure

204   6b and 7b; figure supplement 6). Together, these results suggest that LTM formation is also

205   modulated by cAMP in early α/β neurons.

206

207   **CREBB in early α/β neurons enhances LTM**

208 Protein synthesis-dependent LTM formation also depends on CREBB transcription factors, and

209 expression of CREBB protein is thought to be dependent on the expression level of protein kinases

210 involved in cAMP signaling (*Lee et al., 2018*). Consistent with our Rutabaga and PKA knockdown

211 results, we induced RNAi knockdown of CREBB in early α/β neurons and observed impairment

212 of 1-day memory after 10xS training. Further impairment was not seen in combination with

213 systemic protein synthesis inhibition by feeding CXM (Figure 8C). Because inhibition of protein

214 synthesis in early α/β neurons instead produced an enhancing effect on LTM formation, we

215 inducibly expressed *crebB* repressor-transgenes (*Zhang et al., 2019*) in these neurons only, with the

216 expectation that the manipulations would lead to enhanced memory. Indeed, expressing *crebB*

217 enhanced 1-day memory after 1x training to levels normally seen after 10xS training (Figure 8A-

218 B; figure supplement 6).

219

Spaced training induces *crebB* transcription

221 We next generated a *crebB* promoter-driven *Gal4* transgene containing an 11-kb 5′ genomic

222 sequence just upstream of CREBB (see Methods) (*Yin et. al., 1995b*). This *crebB-Gal4* drives GFP

223 expression in most glia cells and brain neurons, including most MB neurons, though higher levels

224 of expression can be seen in α′/β′ compared to α′/β′ or γ neurons (Figure 9A). By photo converting

225 pre-existing green KAEDE to red prior to training (*Chen et. al., 2012*), we measured significantly

226 more newly synthesized *crebB-Gal4* green KAEDE in the MB α-lobe during 24-h intervals after

227 5xS or 10xS training, but not after 1x training or 10xM training in comparison with naïve control

228 flies (Figure 9B-C). This training-induced increase in *crebB* KAEDE appeared specific to the MB

229 neurons because spaced training did not significantly change the levels of new *crebB* KAEDE in

230 ellipsoid body (EB) or glia (Figure 9B, right). These results demonstrate that multiple sessions of

231 spaced training increases CREBB expression in early α/β neurons. Our finding that inhibition of

232   protein synthesis in early α/β neurons enhanced LTM formation (Figure 1-3) suggests that *crebB*

233   gene products function to repress protein synthesis.

234

235   **Schnurri (Shn) regulates CREBB-dependent LTM formation**

236   How does spaced training induce CREBB expression? To address this question, we sought to

237   identify positive regulators of *crebB* transcription during LTM formation. Yeast two-hybrid and

238   chromatin immunoprecipitation experiments previously revealed several such candidates that bind

239   in the *crebB* promoter region (data not shown). Prominent among these were (1) CREB family

240   protein CREBA, a leucine-zipper transcription factor (*Smolik et al., 1992*) and (2) Shn, a zinc finger

241   C2H2 transcription factor encoded by the *shn* gene (*Marty et al., 2000*) which also was identified

242   in a transposon mutagenesis screen for impairment of 1-day memory after 10xS as the *umnitza*

243   mutant (*Dubnau et al., 2003*) (described above, see figure supplement 1).

244   Together, these findings implicated CREBA and Shn as candidate regulators of LTM formation

245   through transcriptional activation of *crebB*. Interestingly, we inducibly overexpressed each in early

246   α/β neurons and found enhanced 1-day memory after 1x or 3xS training with this manipulation of

247   the shn^+ transgene but not $crebA^+$ (Figure 10A; figure supplement 7A-B). We also observed

248   strongly elevated CREBB protein expression in transgenic shn^+ flies, but not in transgenic rut^+ or

249   Pka^{act1} flies (figure supplement 8). Moreover, inducible RNAi knockdowns of *shn* but not *crebA*

250   impaired 1-day memory after 10xS (Figure 10B; figure supplement 7C), and further impairment

251   was not seen with systemic protein synthesis inhibition after CXM feeding (Figure 10B, right).

252   Memory after 10xS was fully rescued in *shn* knockdown flies by *crebB* co-expression (Figure 10C)

253   and was enhanced relative to controls after 1x (Figure 10D). Taken together, these results show that

254   CREBB expression in early α/β neurons in response to spaced training is Shn-dependent.

255

256   **Discussion**

257 Our data suggest that MB neurons provide a compelling cellular gating mechanism for LTM

258 formation. A single training session is sufficient to increase early α/β neuronal excitability, the

259 output from which produces a downstream inhibitory effect on LTM formation. After spaced

260 training, cAMP signaling regulates neural excitability and/or Shn increases CREBB expression, the

261 net effects of which we suggest then represses further protein synthesis, thereby reducing early α/β

262 output and relieving the inhibitory effect on LTM formation. Remarkably, our observations

263 emerged from a screen of enhancer trap memory mutants using Ricin^{CS} protein synthesis inhibition

264 (figure supplement 1). 1-day memory after 10xS was impaired in nine lines, eight of which showed

265 expression in both MB & DAL neurons. Curiously, another seven lines were not impaired in 1-day

266 memory after 10xS training – but they, too, showed enhancer expression patterns in MB & DAL

267 neurons. We hypothesized that blocking protein synthesis in DAL neurons impaired LTM but doing

268 so in (some) MB neurons might actually enhance LTM, negating the inhibitory effects in DAL

269 neurons.

270 We tested this idea by maintaining Ricin^{CS} expression in MB while blocking Ricin^{CS} expression

271 outside of MB using *cry-Gal80* (Figure 1). Surprisingly, LTM now was enhanced in all seven of

272 these enhancer trap lines (Figure 1). We then identified early α/β as the subset of MB neurons

273 responsible for this enhancing effect (Figure 2). Inhibition protein synthesis in early α/β neurons

274 during the first 6 h, or blocking synaptic transmission from early α/β neurons during the first 8 h

275 after training was sufficient to enhance LTM (Figure 3 and 4). Increasing excitability of early α/β

276 neurons impaired LTM, but decreasing excitability again enhanced LTM (Figure 5). We next asked

277 whether these neural excitability-dependent effects were also cAMP dependent. RNAi mediated

278 knockdown of Rutabaga or PKA in early α/β impaired LTM, while overexpression of a *rut$^+$* or

279 *Pka^{act1}* transgene enhanced LTM (Figure 6 and 7). CREBB expression is suggest to be

280 synergistically and post-transcriptionally regulated by protein kinases responding to cAMP

281 signaling (*15*) and accordingly, our RNAi mediated knockdown of CREBB in early α/β impaired

282   LTM, while overexpression of a *crebB* transgene enhanced LTM (Figure 8). Finally, using a *crebB*

283   promoter driven *Gal4* transgene, we show that CREBB transcription increases after 5xS or 10xS

284   spaced training but not after 1x training (Figure 9). Thus, spaced training-dependent expression of

285   CREBB repressor proteins in early α/β neurons blocks this inhibitory output from early

286   α/β neurons, thereby allowing LTM formation (downstream) to proceed.

287   An enhancing role associated with Shn-induced expression of CREBB repressor is a novel aspect

288   of this LTM gating mechanism (Figure 10 and figure supplement 8). Previous reports have claimed

289   that chronic expression of a CREBB repressor or RNAi transgenes in all α/β neurons impaired 1-

290   day memory after spaced training (*Yu et al., 2006; Lee et al., 2018*). *Chen et al., (2012)* documented,

291   however, that these chronic disruptions of CREBB produced developmental abnormalities in MB

292   structure. In contrast, acute induced expression of active Ricin^{CS} or CREBB repressor only in adult

293   α/β neurons did not impair 1-day memory after spaced training (and did not produce structural

294   defects). Using a different inducible system (MB247-Switch) to acutely expresses CREBB in γ and

295   α/β neurons, *Hirano et al., (2016)* showed a mild impairment of 1-day memory after spaced training.

296   More interestingly, they used various molecular genetic tools to show that interactions among

297   CREBB, CREB Binding Protein (CBP) and CREB Regulated Transcription Coactivator (CRTC)

298   in MB clearly were involved in LTM formation or maintenance, respectively. Using the same

299   inducible gene switch tool, *Miyashita et al., (2018)* showed a fascinating positive regulatory loop

300   between Fos and CREBB in MB during LTM formation – but they did not show behavioral data

301   pertaining to manipulation of CREBB *per se-* and they did not restrict their experiments to early

302   α/β neurons.

303   A recent study that features *cyclic AMP-response element* (*CRE*)-driven transgenes is pertinent

304   to this report. *Zhang et al., (2015)* expressed a *CRE-luciferase* transgene in different subpopulations

305   of MB neurons and then monitored luciferase activity in live flies at various times after spaced

306   training. Immediately after spaced training, they showed in some cases luciferase expression

307   decreased (*OK107* expressing in all MB neurons; *c739* expressing in all α/β neurons; *1471*

308   expressing in γ neurons), in others expression increased (*c747* and *c772* expressing variably in all

309   MB neurons) or in some no changes were detected (*c320* expressing variably in γ α′/β′ and α/β

310   subpopulation, *17d* expressing primarily in late α/β and in early α/β neurons). Indeed, these authors

311   point out that, because *CRE-luciferase* was expressed in more than one subpopulation of MB

312   neurons, only net effects of CREB function could be quantified. Obviously, such a conclusion must

313   be drawn from any behavioral data collected after CREBB manipulations in multiple

314   subpopulations of MB neurons. Our study provides a dramatic example of this point. By restricting

315   our manipulation only to the early α/β neurons and only in adult stage animals, we show that acute

316   overexpression or knockdown of CREBB enhances or impairs LTM formation, respectively (Figure

317   8) and that spaced training serves to increase the expression of CREBB in these neurons (Figure 9).

318   Of particular relevance to our future studies is the curious discovery that output from early α/β

319   neurons specifically *inhibits* LTM formation. We find no evidence of inhibitory transmitter (*i.e.,*

320   GABA) synthesis or signaling in early α/β neurons, however, and others have suggested that

321   memory-relevant MB output synapses are cholinergic (*Barnstedt et al.,2016*). Thus, we presume

322   that inhibition of LTM lies somewhere downstream in the memory circuit. Furthermore, we note

323   that ARM appears to involve α/β neurons (*Lee et al.,2011*; *Knapek et al.,2011*; *Scholz-Kornehl and*

324   *Schwärzel, 2016*; *Kotoula et al., 2017*; *Shyu et al., 2019*) and to inhibit LTM formation (*Isabel et*

325   *al., 2004*; *Placais et al., 2012*). Thus, a molecular link between ARM and LTM may reside in early

326   α/β neurons.

327   More generally, our results underscore the need to study behavior-genetic relations in each of the

328   seven MB neuronal subpopulations (*Aso et al., 2014*) separately before drawing firm conclusions

329   about a role for MB in specific memory phases or in the dynamics of a larger memory circuit

330   involving neurons intrinsic and extrinsic to MB. With the more complex circuitries in vertebrate

331   animal models, such deconstruction of memory formation into specific neuronal subtypes will be

332   even more critical and enlightening.

333

334   **Materials and Methods**

335   A collection of *Drosophila P-Gal4* transposon insertions were previously selected in an enhancer

336   trap mutagenesis screen for long-term memory phenotypes (*Dubnau et al., 2003*). The resultant

337   Gal4 expression patterns in seven of these mutants were leveraged to drive and temporally control

338   cold-sensitive Ricin^{CS} activity to block protein synthesis in the identified neuron subsets. In addition,

339   we spatially restricted Ricin^{CS} activity by inhibiting Gal4 with MB or DAL neuron-specific

340   expression of Gal80. We used an automated olfactory aversive learning task (*Tully et al., 1994*) and

341   assessed LTM after blocking protein synthesis, inhibiting consolidation in these temporally and

342   spatially restricted domains to identify the subsets of neurons critical for this task. Blocking

343   transmission from these neurons with Gal4-targetted temperature-sensitive Dynamin^{ts} after training

344   was used to test the implicated roles of these neurons in LTM consolidation (*Dubnau et al., 2001;*

345   *McGuire et al., 2001*). Spatial and temporal regulation of K^+ and Na^+ channel activity with

346   transgene overexpression and RNAi knockdown within these neurons was used to assess the

347   downstream impacts of signaling valence on LTM. Similarly, restricted expression of transgenes

348   was used to examine the training-responsive effects on LTM. We evaluated training-responsive

349   CREBB expression with confocal microscopy using a *Gal4*-targeted UV-sensitive KAEDE reporter

350   system (*Chen et al., 2012*). In various experiments, flies were fed CXM to provide a systemic level

351   of protein synthesis inhibition. Detailed procedures for all methods are described in the

352   supplementary materials.

353

354   **Flies**

355 Fly stocks were maintained on standard corn meal/yeast/agar medium at 25 ± 1 °C or 18 ± 1 °C and

356 70% relative humidity on a 12:12-h light:dark cycle. All genotypes and sources are listed in table

357 supplement 2.

358

359 **Behaviour**

360 Olfactory associative learning was evaluated by training 6- to 7-day-old flies in a T-maze apparatus

361 with a Pavlovian olfactory conditioning procedure (*Tully and Quinn, 1985*) as described previously

362 (*Chen et al., 2012*; *Pai et al., 2013*; *Wu et al., 2017*). All experiments were conducted in the dark

363 in an environment-controlled room at the required temperatures and 70% relative humidity. The

364 odours used were 3-octanol (OCT) and 4-methylcyclohexanol (MCH). Each experiment consisted

365 of two groups of approximately 100 flies, each of which was conditioned with one of the two odours.

366 Flies were exposed sequentially to two odours that were carried through the training chamber in a

367 current of air (odours were bubbled at 750 ml/min). In a single training session, flies first were

368 exposed for 60 s to the conditioned stimulus (CS^+), during which time they received the

369 unconditioned stimulus (US), which consisted of 12 1.5-s pulses of 60 V dc electric shock presented

370 at 5-s interpulse intervals. After the presentation of the CS+ condition, the chamber was flushed

371 with fresh air for 45 s. Then flies were exposed for 60 s to the unpaired CS^-. To evaluate memory

372 retention immediately after single-session training (acquisition), flies were gently tapped into an

373 elevator-like compartment immediately after training. After 90 s, the flies were transported to the

374 choice point of a T-maze, in which they were exposed to two converging currents of air (one

375 carrying OCT, the other MCH) from opposite arms of the maze. Flies were free to choose between

376 and walk toward the CS^+ and CS^- for 120 s, at which time they were trapped inside the respective

377 arms of the T-maze (by sliding the elevator out of the register), anesthetised, and counted. Flies that

378 chose to avoid the CS^+ ran into the T-maze arm containing the CS^-, whereas flies that chose to avoid

379 the CS^- ran into the arm containing the CS^+. For each experiment, a performance index ($PI_{1,2}$) =

380 $(N_{CS-} - N_{CS+})/(N_{CS-} + N_{CS+})$ was calculated and averaged over these two complementary

381 experiments, with the final PI = $(PI_1 + PI_2)/2$. Averaging of the two reciprocal scores eliminated

382 any potential biases originating from the machine, naïve odour preferences, or non-associative

383 changes in olfaction. For 24-h memory experiments, flies were subjected to single-session training,

384 training massed together without rest, or training spaced out with 15-min rest intervals. For these

385 training protocols, robotic trainers were used. All genotypes were trained and tested in parallel and

386 rotated among all of the robotic trainers to ensure a balanced experiment. The genetic backgrounds

387 of all fly strains were equilibrated to the "Canton" wild-type background by five or more

388 generations of backcrossing. In *tub-Gal80^{ts}* experiments, flies raised at 18 °C were transferred to

389 30 °C for at least five days before the experiments.

390

391 **Pharmacological treatment**

392 To block protein synthesis, flies were fed 35 mM cycloheximide (Sigma) in 5% glucose 1 day

393 before training until immediately before the test (*Tully et al., 1994*).

394

395 ***crebB* promoter construct**

396 To engineer the *crebB* promoter construct, polymerase chain reaction (PCR) was performed using

397 genomic DNA from the wild-type *Canton-S w^{1118}* (*iso1CJ*) fly line as the template together with

398 the forward primer 5′GAAAAGTGCCACCTGCTGCATGTCTACCAACAGTTCGAG 3′ and the

399 reverse primer 5′CCGGATCTGCTAGCGGTTCCAGCTGCTGTCTGTATGAC 3′. A 11.6-kb

400 PCR product was generated and inserted into the pBPGAL4.2Uw-2 vector, was digested with AatII

401 and KpnI using In-Fusion® cloning system (Clontech). The promoter construct was injected into

402 *attP^{40}*-containing fly strains to obtain the transgenic fly lines.

403

404 **KAEDE measurement**

405 KAEDE is a photoconvertible green fluorescent protein, irreversibly changing its structure to a red

406 fluorescent protein upon ultraviolet irradiation (*Ando et al., 2002*). Taking advantage of circadian

407 transcription and protein synthesis in the lateral clock neurons, we previously validated *de novo*

408 KAEDE synthesis in *per-Gal4>UAS-kaede* flies, in which it faithfully reports the cyclic

409 transcriptions of the *period* gene. Feeding cycloheximide also suppressed green KAEDE synthesis,

410 while not affecting the already-converted red KAEDE (*Chen et al., 2012*). To measure the amount

411 of newly synthesised KAEDE in MB neurons, we used procedures adapted from a previous study

412 (*Chen et al., 2012*). Briefly, pre-existing KAEDE proteins were photoconverted into red fluorescent

413 proteins by 365–395 nm UV irradiation generated from a 120-W mercury lamp. For behavioural

414 testing, approximately 15–20 flies kept in a clear plastic syringe were directly exposed to UV light

415 at a distance of 5 cm for 1 h. Individual neurons expressing KAEDE were directly visualised

416 through an open window in the fly's head capsule. Living samples were used because the signal-

417 to-noise ratio of green to red KAEDE is greatly reduced after chemical fixation. KAEDE neurons

418 were located in less than 5 s by a fast pre-scanning of red KAEDE excited by a 561-nm laser, to

419 avoid unnecessary fluorescence quenching of green KAEDE during repeated scanning. A single

420 optical slice through the MB α-lobe tip was imaged at a resolution of 1024×1024 pixels under a

421 confocal microscope with a 40× C-Apochromat water-immersion objective lens (N.A. value 1.2,

422 working distance 220 μm). All brain samples in the experiment were imaged with the same optical

423 settings maximised for green and red KAEDE immediately before and after photoconversion,

424 respectively. In all cases, both green KAEDE (excited by a 488-nm laser) and red KAEDE (excited

425 by a 561-nm laser) were measured. By using the amount of red KAEDE as an internal standard to

426 calibrate individual variation, we calculated the rate of increase in green KAEDE synthesis after

427 photoconversion with the formula $(\Delta F) = \%(F_{t_1} - \text{average } F_{t_0})/\text{average } F_{t_0}$, where F_{t_1} and F_{t_0} are

428 the ratios of the averaged intensities of green (G) to red (R) KAEDE (G_{t_0}/R_{t_0}) immediately after

429 photoconversion (t_0) and at a later specific time point (t_1), respectively.

430

Spatiotemporal inhibition of protein synthesis

431

432 Ricin^{CS}, a mutated Ricin A chain, inactivates eukaryotic ribosomes by hydrolytically cleaving the

433 N-glycosidic bond (A4324) of the 28S ribosomal RNA subunit at high temperatures (30°C), but

434 not at low temperatures (18°C) (*Endo et al., 1987; Endo and Tsurugi, 1987; Moffat et al., 1992;*

435 *Allen et al., 2002*). We previously validated the spatiotemporal effect of Ricin^{CS} inhibition in the

436 *Drosophila* brain using lateral clock neurons. We found that Ricin^{CS} can effectively inhibit ~80%

437 of protein synthesis at a permissive temperature (30°C), which is quickly reversed to normal levels

438 after shifting to a restrictive temperature (18°C) (*Chen et al., 2012*). This suggests a quick

439 restoration of ribosomal synthesis once Ricin^{CS} becomes inactive. While active Ricin^{CS} is a potent

440 cytotoxin for inhibiting protein synthesis, it tends not to be lethal, as Ricin^{CS} eventually inhibits its

441 own synthesis (*Chen et al., 2012, Moffat et al., 1992; Allen et al., 2002*). In the current experiments,

442 two copies of Ricin^{CS} was used to block protein synthesis. All flies were raised at 18°C to keep

443 Ricin^{CS} inactive. Before or after training at 18°C, the *Gal4> UAS-ricin^{CS};UAS-ricin^{CS}* flies were

444 transferred to 30°C for 24 h to activate Ricin^{CS}, and then shifted back to 18°C for 1 h to inactivate

445 Ricin^{CS} before the experiments. Temporal control of Ricin^{CS} activation is indicated in the figures

446 for the relevant experiments.

447

Immunohistochemistry

448

449 Brains were dissected in phosphate-buffered saline (PBS), fixed with a commercial microwave

450 oven (2,450 MHz, 1100 Watts) in 4% paraformaldehyde on ice for 60 s three times, and then

451 immersed in 4% paraformaldehyde with 0.25% Triton X-100 for 60 s three times. After being

452 washed in PBS for 10 min at room temperature, brain samples were incubated in PBS containing

453 2% Triton X-100 (PBS-T) and 10% normal goat serum, and then degassed in a vacuum chamber to

454 expel tracheal air for four cycles (depressurizing to −70 mmHg and then holding for 10 min). Next,

455   brain samples were blocked and penetrated in PBS-T at 4 °C overnight, and then incubated in PBS-

456   T containing (1) 1:40 mouse 4F3 anti-DLG antibody (Developmental Studies Hybridoma Bank,

457   University of Iowa) to label Disc large proteins, and (2) 1:500 mouse anti-CREBB α657 antibody

458   (from Jerry Yin (*Tubon et al., 2013*)) at 4 °C for 1 day. Samples were subsequently washed in PBS-

459   T three times and incubated in PBS-T containing 1:200 biotinylated goat anti-mouse IgG

460   (Molecular Probes) as the secondary antibody at 25 °C for 1 day. Brain samples were then washed

461   and incubated with 1:500 Alexa Fluor 635 streptavidin (Molecular Probes) at 25 °C for 1 day.

462   Finally, after extensive washing, immunolabeled brain samples were directly cleared for 5 min in

463   *FocusClear*, an aqueous solution that renders biological tissue transparent (*Chiang et al., 2001*),

464   and mounted between two cover slips separated by a spacer ring with a thickness of ~200 μm.

465   Sample brains were imaged under a Zeiss LSM 780 or 880 confocal microscope with a 40× C-

466   Apochromat water-immersion objective lens (N.A. value 1.2, working distance 220 μm).

467

Statistics

469   All raw data were analysed parametrically with SigmaPlot 10.0 and SigmaStat 3.5 statistical

470   software. All the data including the behaviour Performance Index (PI) or KAEDE image (ΔF) were

471   evaluated via unpaired *t*-test (two groups) or one-way analysis of variance (ANOVA) (> two

472   groups). Data were evaluated with the Mann-Whitney Rank Sum Test in cases of unequal variances.

473   Data in all figures are presented as the mean ± SE. Experiments were replicated using multiple *Gal4*

474   drivers with equivalent expression patterns, and multiple effector genes and reagents that impact

475   shared cellular functions.

476

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486   **Author contributions:**

487   Conceived the project, analysed the data, and wrote the manuscript: C.C.C., J.S.D.,

488   T.T. and A.S.C.

489   Imaging experiments: H.W.L.

490   Behavioural experiments: C.C.C. and F.K.L.

491   Generated *creb2-Gal4* transgenic flies: R.Y.J. and L.C.

492

493   **Competing interests:** All other authors declare they have no competing interests.

494

495   **Data and materials availability:** All data are available in the main text or the supplementary

496   materials.

497

498   **References**

499   Allen, M.J., O'Kane, C.J., and Moffat, K.G. (2002). Cell ablation using wild-type and cold-

500   sensitive ricin-A chain in *Drosophila* embryonic mesoderm. Genesis 34, 132-134.

501   Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H., and Miyawaki, A. (2002). An optical

502   marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proc Natl

503   Acad Sci U S A 99, 12651-12656.

504   Aso, Y., Hattori, D., Yu, Y., Johnston, R.M., Iyer, N.A., Ngo, T.T., Dionne, H., Abbott, L.F.,

505   Axel, R., Tanimoto, H., et al. (2014). The neuronal architecture of the mushroom body provides a

506   logic for associative learning. Elife 3, e04577.

507   Baines, R.A. (2003). Postsynaptic protein kinase A reduces neuronal excitability in response to

508   increased synaptic excitation in the *Drosophila* CNS. J Neurosci 23, 8664-8672.

509   Barnstedt, O., Owald, D., Felsenberg, J., Brain, R., Moszynski, J.P., Talbot, C.B., Perrat, P.N.,

510   and Waddell, S. (2016). Memory-Relevant Mushroom Body Output Synapses Are Cholinergic.

511   Neuron 89, 1237-1247.

512   Blum, A.L., Li, W., Cressy, M., and Dubnau, J. (2009). Short- and long-term memory in

513   *Drosophila* require cAMP signaling in distinct neuron types. Curr Biol 19, 1341-1350.

514   Bouzaiane, E., Trannoy, S., Scheunemann, L., Placais, P.Y., and Preat, T. (2015). Two

515   independent mushroom body output circuits retrieve the six discrete components of *Drosophila*

516   aversive memory. Cell Rep 11, 1280-1292.

517   Chen, C.C., Wu, J.K., Lin, H.W., Pai, T.P., Fu, T.F., Wu, C.L., Tully, T., and Chiang, A.S.

518   (2012). Visualizing long-term memory formation in two neurons of the *Drosophila* brain. Science

519   335, 678-685.

520   Chiang, A.S., Liu, Y.C., Chiu, S.L., Hu, S.H., Huang, C.Y., and Hsieh, C.H. (2001). Three-

521   dimensional mapping of brain neuropils in the cockroach, *Diploptera punctata*. J Comp Neurol

522   440, 1-11.

523   Cognigni, P., Felsenberg, J., and Waddell, S. (2018). Do the right thing: neural network

524   mechanisms of memory formation, expression and update in *Drosophila*. Curr Opin Neurobiol

525   49, 51-58.

526 Davis, G.W., DiAntonio, A., Petersen, S.A., and Goodman, C.S. (1998). Postsynaptic PKA

527 controls quantal size and reveals a retrograde signal that regulates presynaptic transmitter release

528 in *Drosophila*. Neuron 20, 305-315.

529 Davis, R.L. (2015). SnapShot: Olfactory Classical Conditioning of *Drosophila*. Cell 163, 524-524

530 e521.

531 Dubnau, J., and Chiang, A.S. (2013). Systems memory consolidation in *Drosophila*. Curr Opin

532 Neurobiol 23, 84-91.

533 Dubnau, J., Chiang, A.S., Grady, L., Barditch, J., Gossweiler, S., McNeil, J., Smith, P., Buldoc,

534 F., Scott, R., Certa, U., et al. (2003). The staufen/pumilio pathway is involved in *Drosophila* long-

535 term memory. Curr Biol 13, 286-296.

536 Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. (2001). Disruption of neurotransmission in

537 *Drosophila* mushroom body blocks retrieval but not acquisition of memory. Nature 411, 476-480.

538 Dudai, Y., Jan, Y.N., Byers, D., Quinn, W.G., and Benzer, S. (1976). dunce, a mutant of

539 *Drosophila* deficient in learning. Proc Natl Acad Sci U S A 73, 1684-1688.

540 Endo, Y., Mitsui, K., Motizuki, M., and Tsurugi, K. (1987). The mechanism of action of ricin and

541 related toxic lectins on eukaryotic ribosomes. The site and the characteristics of the modification

542 in 28 S ribosomal RNA caused by the toxins. J Biol Chem 262, 5908-5912.

543 Endo, Y., and Tsurugi, K. (1987). RNA N-glycosidase activity of ricin A-chain. Mechanism of

544 action of the toxic lectin ricin on eukaryotic ribosomes. J Biol Chem 262, 8128-8130.

545 Fropf, R., Zhang, J., Tanenhaus, A.K., Fropf, W.J., Siefkes, E., and Yin, J.C. (2014). Time of day

546 influences memory formation and dCREB2 proteins in *Drosophila*. Front Syst Neurosci 8, 43.

547 Ge, X., Hannan, F., Xie, Z., Feng, C., Tully, T., Zhou, H., Xie, Z., and Zhong, Y. (2004). Notch

548 signaling in *Drosophila* long-term memory formation. Proc Natl Acad Sci U S A 101, 10172-

549 10176.

550 Hirano, Y., Ihara, K., Masuda, T., Yamamoto, T., Iwata, I., Takahashi, A., Awata, H., Nakamura,

551 N., Takakura, M., Suzuki, Y., et al. (2016). Shifting transcriptional machinery is required for

552 long-term memory maintenance and modification in *Drosophila* mushroom bodies. Nat Commun

553 7, 13471.

554 Hodge, J.J., Choi, J.C., O'Kane, C.J., and Griffith, L.C. (2005). Shaw potassium channel genes in

555 *Drosophila*. J Neurobiol 63, 235-254.

556 Huang, C., Zheng, X., Zhao, H., Li, M., Wang, P., Xie, Z., Wang, L., and Zhong, Y. (2012). A

557 permissive role of mushroom body alpha/beta core neurons in long-term memory consolidation in

558 *Drosophila*. Curr Biol 22, 1981-1989.

559 Isabel, G., Pascual, A., and Preat, T. (2004). Exclusive consolidated memory phases in

560 *Drosophila*. Science 304, 1024-1027.

561 Ito, K., Awano, W., Suzuki, K., Hiromi, Y., and Yamamoto, D. (1997). The *Drosophila*

562 mushroom body is a quadruple structure of clonal units each of which contains a virtually

563 identical set of neurones and glial cells. Development 124, 761-771.

564 Joiner, W.J., Crocker, A., White, B.H., and Sehgal, A. (2006). Sleep in *Drosophila* is regulated by

565 adult mushroom bodies. Nature 441, 757-760.

566 Knapek, S., Sigrist, S., and Tanimoto, H. (2011). Bruchpilot, a synaptic active zone protein for

567 anesthesia-resistant memory. J Neurosci 31, 3453-3458.

568 Kotoula, V., Moressis, A., Semelidou, O., and Skoulakis, E.M.C. (2017). Drk-mediated signaling

569 to Rho kinase is required for anesthesia-resistant memory in *Drosophila*. Proc Natl Acad Sci U S

570 A 114, 10984-10989.

571 Krashes, M.J., Keene, A.C., Leung, B., Armstrong, J.D., and Waddell, S. (2007). Sequential use

572 of mushroom body neuron subsets during *Drosophila* odor memory processing. Neuron 53, 103-

573 115.

574 Lee, P.T., Lin, G., Lin, W.W., Diao, F., White, B.H., and Bellen, H.J. (2018). A kinase-dependent

575 feedforward loop affects CREBB stability and long term memory formation. Elife 7.

576 Lee, P.T., Lin, H.W., Chang, Y.H., Fu, T.F., Dubnau, J., Hirsh, J., Lee, T., and Chiang, A.S.

577 (2011). Serotonin-mushroom body circuit modulating the formation of anesthesia-resistant

578 memory in *Drosophila*. Proc Natl Acad Sci U S A 108, 13794-13799.

579 Lin, H.H., Lai, J.S., Chin, A.L., Chen, Y.C., and Chiang, A.S. (2007). A map of olfactory

580 representation in the *Drosophila* mushroom body. Cell 128, 1205-1217.

581 Marty, T., Muller, B., Basler, K., and Affolter, M. (2000). Schnurri mediates Dpp-dependent

582 repression of brinker transcription. Nat Cell Biol 2, 745-749.

583 McGuire, S.E., Le, P.T., and Davis, R.L. (2001). The role of *Drosophila* mushroom body

584 signaling in olfactory memory. Science 293, 1330-1333.

585 McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., and Davis, R.L. (2003). Spatiotemporal

586 rescue of memory dysfunction in *Drosophila*. Science 302, 1765-1768.

587 Miyashita, T., Kikuchi, E., Horiuchi, J., and Saitoe, M. (2018). Long-Term Memory Engram

588 Cells Are Established by c-Fos/CREB Transcriptional Cycling. Cell Rep 25, 2716-2728 e2713.

589 Moffat, K.G., Gould, J.H., Smith, H.K., and O'Kane, C.J. (1992). Inducible cell ablation in

590 *Drosophila* by cold-sensitive ricin A chain. Development 114, 681-687.

591 Pai, T.P., Chen, C.C., Lin, H.H., Chin, A.L., Lai, J.S., Lee, P.T., Tully, T., and Chiang, A.S.

592 (2013). *Drosophila* ORB protein in two mushroom body output neurons is necessary for long-

593 term memory formation. Proc Natl Acad Sci U S A 110, 7898-7903.

594 Pavlopoulos, E., Anezaki, M., and Skoulakis, E.M. (2008). Neuralized is expressed in the

595 alpha/beta lobes of adult *Drosophila* mushroom bodies and facilitates olfactory long-term

596 memory formation. Proc Natl Acad Sci U S A 105, 14674-14679.

597 Perisse, E., Burke, C., Huetteroth, W., and Waddell, S. (2013). Shocking revelations and

598 saccharin sweetness in the study of *Drosophila* olfactory memory. Curr Biol 23, R752-763.

599   Placais, P.Y., Trannoy, S., Isabel, G., Aso, Y., Siwanowicz, I., Belliart-Guerin, G., Vernier, P.,

600   Birman, S., Tanimoto, H., and Preat, T. (2012). Slow oscillations in two pairs of dopaminergic

601   neurons gate long-term memory formation in *Drosophila*. Nat Neurosci 15, 592-599.

602   Presente, A., Boyles, R.S., Serway, C.N., de Belle, J.S., and Andres, A.J. (2004). Notch is

603   required for long-term memory in *Drosophila*. Proc Natl Acad Sci U S A 101, 1764-1768.

604   Quinn, W.G., and Dudai, Y. (1976). Memory phases in *Drosophila*. Nature 262, 576-577.

605   Scheunemann, L., Jost, E., Richlitzki, A., Day, J.P., Sebastian, S., Thum, A.S., Efetova, M.,

606   Davies, S.A., and Schwarzel, M. (2012). Consolidated and labile odor memory are separately

607   encoded within the *Drosophila* brain. J Neurosci 32, 17163-17171.

608   Scheunemann, L., Placais, P.Y., Dromard, Y., Schwarzel, M., and Preat, T. (2018). Dunce

609   Phosphodiesterase Acts as a Checkpoint for *Drosophila* Long-Term Memory in a Pair of

610   Serotonergic Neurons. Neuron 98, 350-365 e355.

611   Scholz-Kornehl, S., and Schwarzel, M. (2016). Circuit Analysis of a *Drosophila* Dopamine Type

612   2 Receptor That Supports Anesthesia-Resistant Memory. J Neurosci 36, 7936-7945.

613   Sejourne, J., Placais, P.Y., Aso, Y., Siwanowicz, I., Trannoy, S., Thoma, V., Tedjakumala, S.R.,

614   Rubin, G.M., Tchenio, P., Ito, K., et al. (2011). Mushroom body efferent neurons responsible for

615   aversive olfactory memory retrieval in *Drosophila*. Nat Neurosci 14, 903-910.

616   Shyu, W.H., Lee, W.P., Chiang, M.H., Chang, C.C., Fu, T.F., Chiang, H.C., Wu, T., and Wu,

617   C.L. (2019). Electrical synapses between mushroom body neurons are critical for consolidated

618   memory retrieval in *Drosophila*. PLoS Genet 15, e1008153.

619   Smolik, S.M., Rose, R.E., and Goodman, R.H. (1992). A cyclic AMP-responsive element-binding

620   transcriptional activator in *Drosophila melanogaster*, dCREB-A, is a member of the leucine

621   zipper family. Mol Cell Biol 12, 4123-4131.

622   Stoleru, D., Peng, Y., Agosto, J., and Rosbash, M. (2004). Coupled oscillators control morning

623   and evening locomotor behaviour of *Drosophila*. Nature 431, 862-868.

624   Tanaka, N.K., Tanimoto, H., and Ito, K. (2008). Neuronal assemblies of the *Drosophila*

625   mushroom body. J Comp Neurol 508, 711-755.

626   Tonoki, A., and Davis, R.L. (2015). Aging impairs protein-synthesis-dependent long-term

627   memory in *Drosophila*. J Neurosci 35, 1173-1180.

628   Tubon, T.C., Jr., Zhang, J., Friedman, E.L., Jin, H., Gonzales, E.D., Zhou, H., Drier, D., Gerstner,

629   J.R., Paulson, E.A., Fropf, R., et al. (2013). dCREB2-mediated enhancement of memory

630   formation. J Neurosci 33, 7475-7487.

631   Tully, T. (1996). Discovery of genes involved with learning and memory: an experimental

632   synthesis of Hirschian and Benzerian perspectives. Proc Natl Acad Sci U S A 93, 13460-13467.

633   Tully, T., Boynton, S., Brandes, C., Dura, J.M., Mihalek, R., Preat, T., and Villella, A. (1990).

634   Genetic dissection of memory formation in *Drosophila* melanogaster. Cold Spring Harb Symp

635   Quant Biol 55, 203-211.

636   Tully, T., Preat, T., Boynton, S.C., and Del Vecchio, M. (1994). Genetic dissection of

637   consolidated memory in *Drosophila*. Cell 79, 35-47.

638   Tully, T., and Quinn, W.G. (1985). Classical conditioning and retention in normal and mutant

639   *Drosophila melanogaster*. J Comp Physiol A 157, 263-277.

640   Venken, K.J., Simpson, J.H., and Bellen, H.J. (2011). Genetic manipulation of genes and cells in

641   the nervous system of the fruit fly. Neuron 72, 202-230.

642   Wu, C.L., Shih, M.F., Lee, P.T., and Chiang, A.S. (2013). An octopamine-mushroom body circuit

643   modulates the formation of anesthesia-resistant memory in *Drosophila*. Curr Biol 23, 2346-2354.

644   Wu, C.L., Xia, S., Fu, T.F., Wang, H., Chen, Y.H., Leong, D., Chiang, A.S., and Tully, T. (2007).

645   Specific requirement of NMDA receptors for long-term memory consolidation in *Drosophila*

646   ellipsoid body. Nat Neurosci 10, 1578-1586.

647   Wu, J.K., Tai, C.Y., Feng, K.L., Chen, S.L., Chen, C.C., and Chiang, A.S. (2017). Long-term

648   memory requires sequential protein synthesis in three subsets of mushroom body output neurons

649   in *Drosophila*. Sci Rep 7, 7112.

650   Yang, C.H., Shih, M.F., Chang, C.C., Chiang, M.H., Shih, H.W., Tsai, Y.L., Chiang, A.S., Fu,

651   T.F., and Wu, C.L. (2016). Additive Expression of Consolidated Memory through *Drosophila*

652   Mushroom Body Subsets. PLoS Genet 12, e1006061.

653   Yin, J.C., Del Vecchio, M., Zhou, H., and Tully, T. (1995a). CREB as a memory modulator:

654   induced expression of a dCREB2 activator isoform enhances long-term memory in *Drosophila*.

655   Cell 81, 107-115.

656   Yin, J.C., Wallach, J.S., Del Vecchio, M., Wilder, E.L., Zhou, H., Quinn, W.G., and Tully, T.

657   (1994). Induction of a dominant negative CREB transgene specifically blocks long-term memory

658   in *Drosophila*. Cell 79, 49-58.

659   Yin, J.C., Wallach, J.S., Wilder, E.L., Klingensmith, J., Dang, D., Perrimon, N., Zhou, H., Tully,

660   T., and Quinn, W.G. (1995b). A *Drosophila* CREB/CREM homolog encodes multiple isoforms,

661   including a cyclic AMP-dependent protein kinase-responsive transcriptional activator and

662   antagonist. Mol Cell Biol 15, 5123-5130.

663   Yu, D., Akalal, D.B., and Davis, R.L. (2006). *Drosophila* alpha/beta mushroom body neurons

664   form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning.

665   Neuron 52, 845-855.

666   Zhang, J., Tanenhaus, A.K., Davis, J.C., Hanlon, B.M., and Yin, J.C. (2015). Spatio-temporal in

667   vivo recording of dCREB2 dynamics in *Drosophila* long-term memory processing. Neurobiol

668   Learn Mem 118, 80-88.

669   Zhang, S.X., Rogulja, D., and Crickmore, M.A. (2019). Recurrent Circuitry Sustains *Drosophila*

670   Courtship Drive While Priming Itself for Satiety. Curr Biol 29, 3216-3228 e3219.

671 Zhu, S., Chiang, A.S., and Lee, T. (2003). Development of the *Drosophila* mushroom bodies:

672 elaboration, remodeling and spatial organization of dendrites in the calyx. Development 130,

673 2603-2610.

674

675

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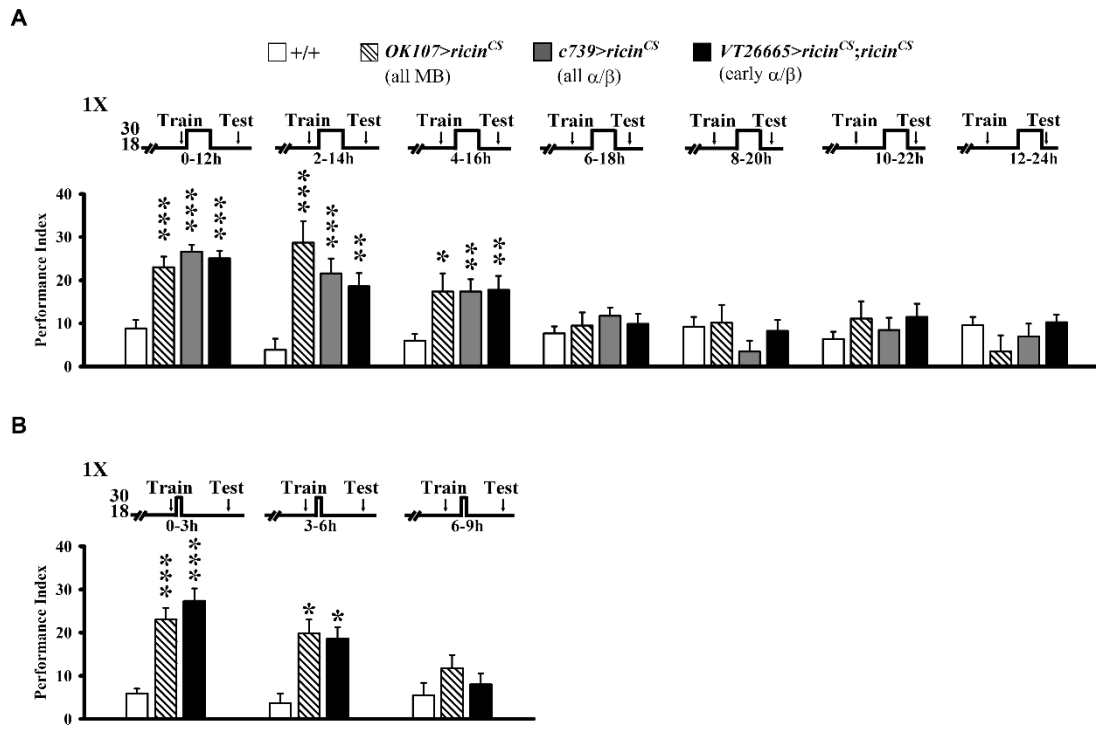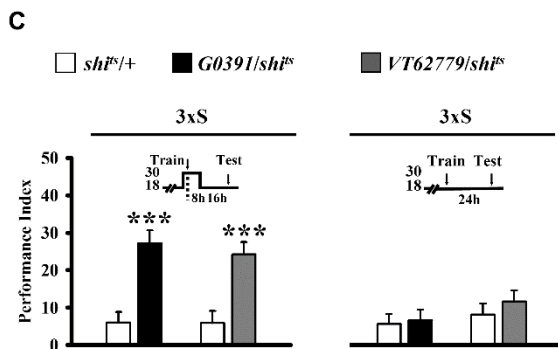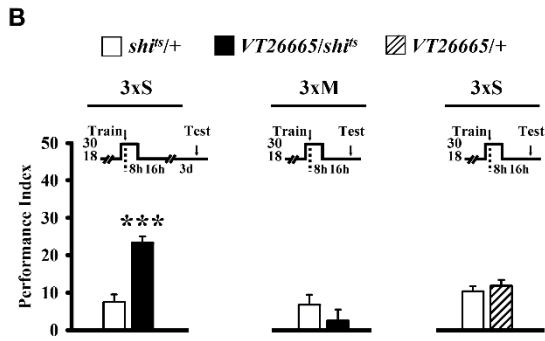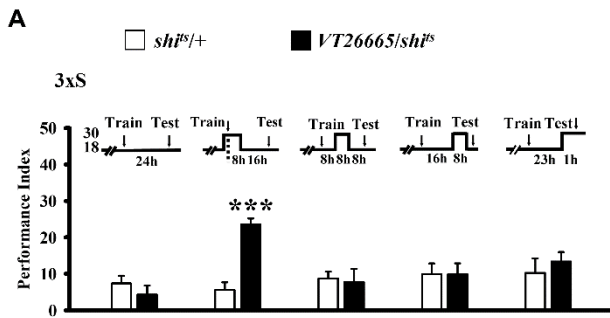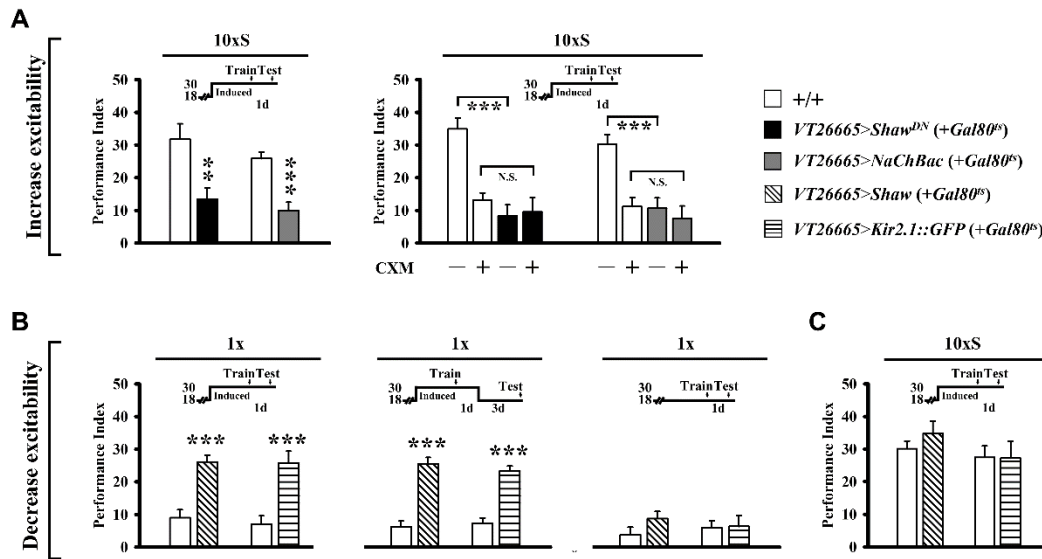678

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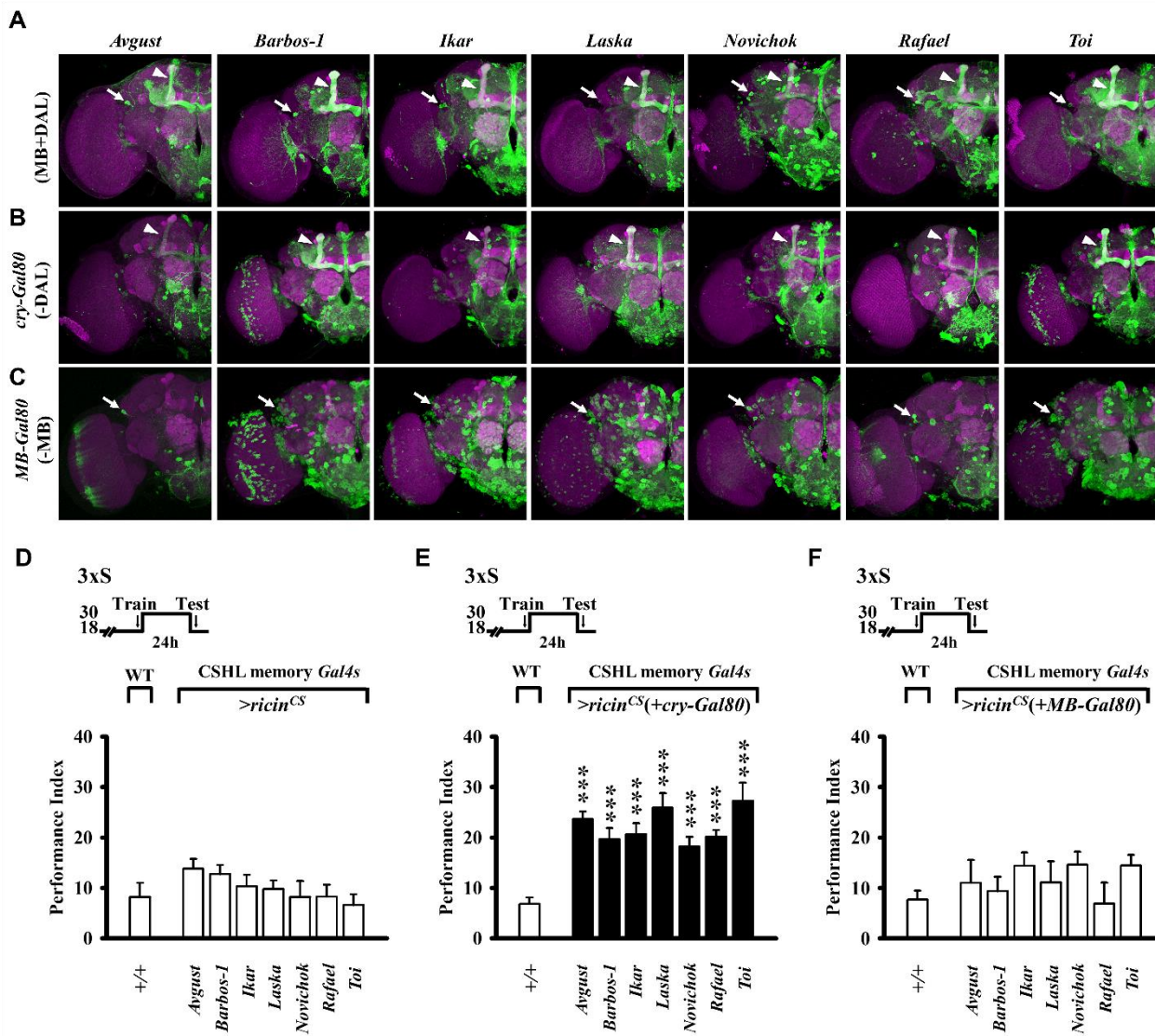
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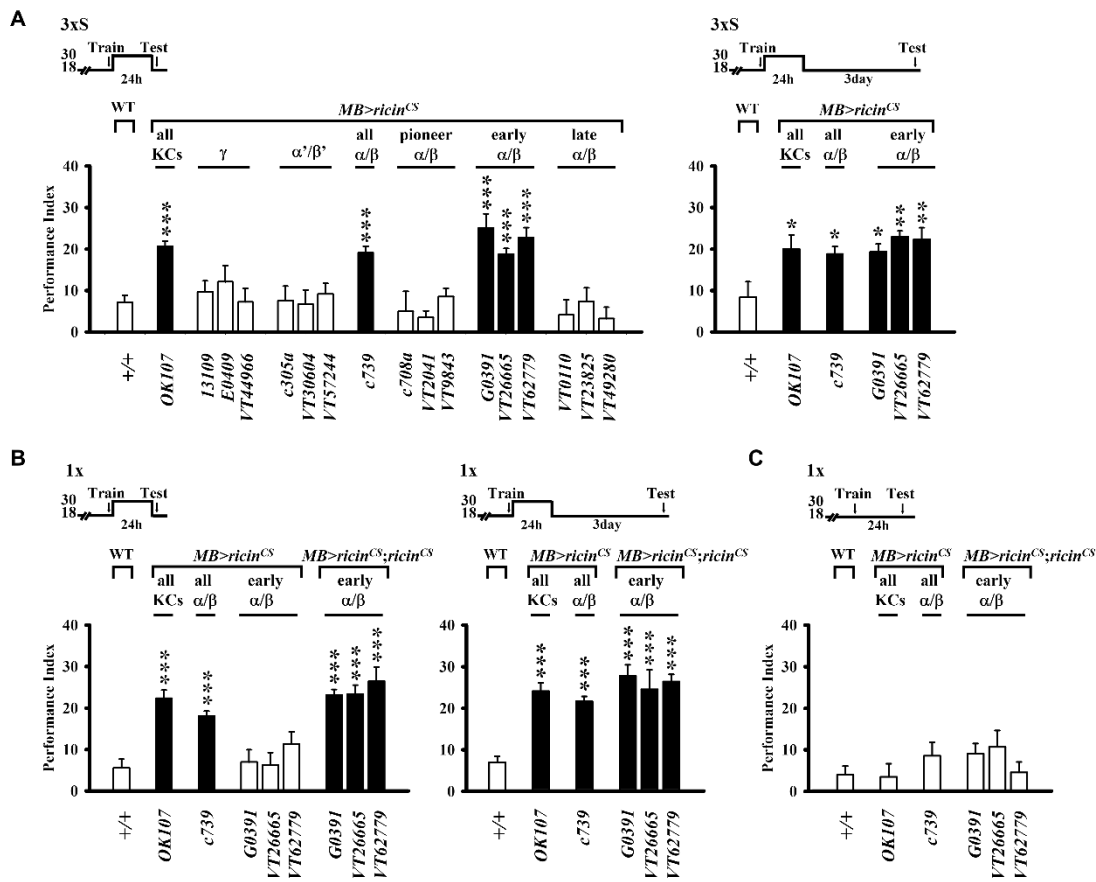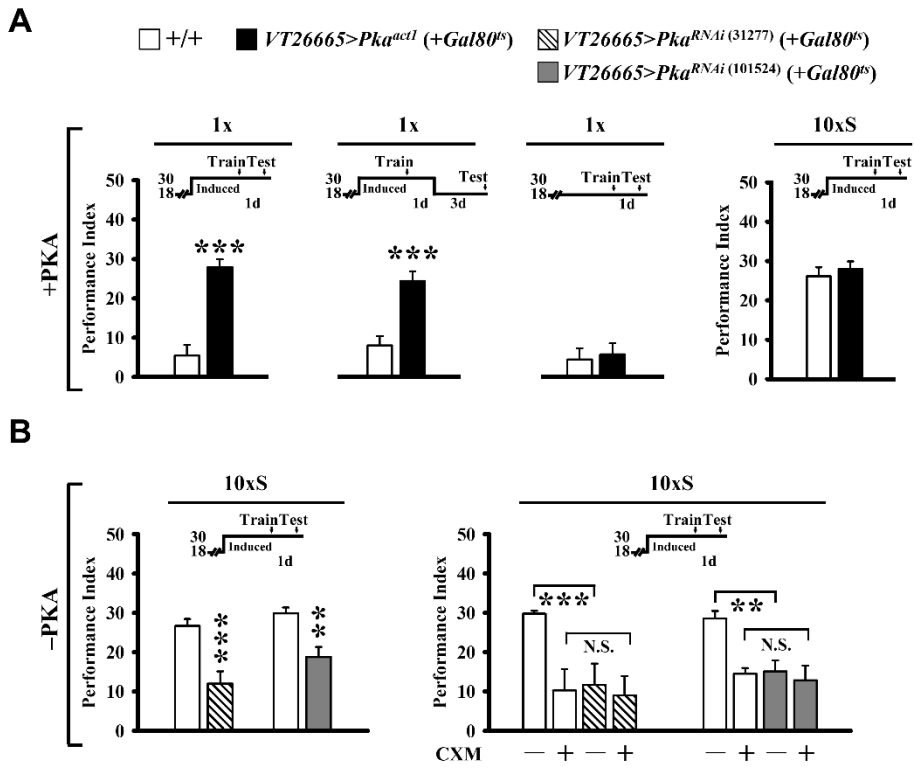
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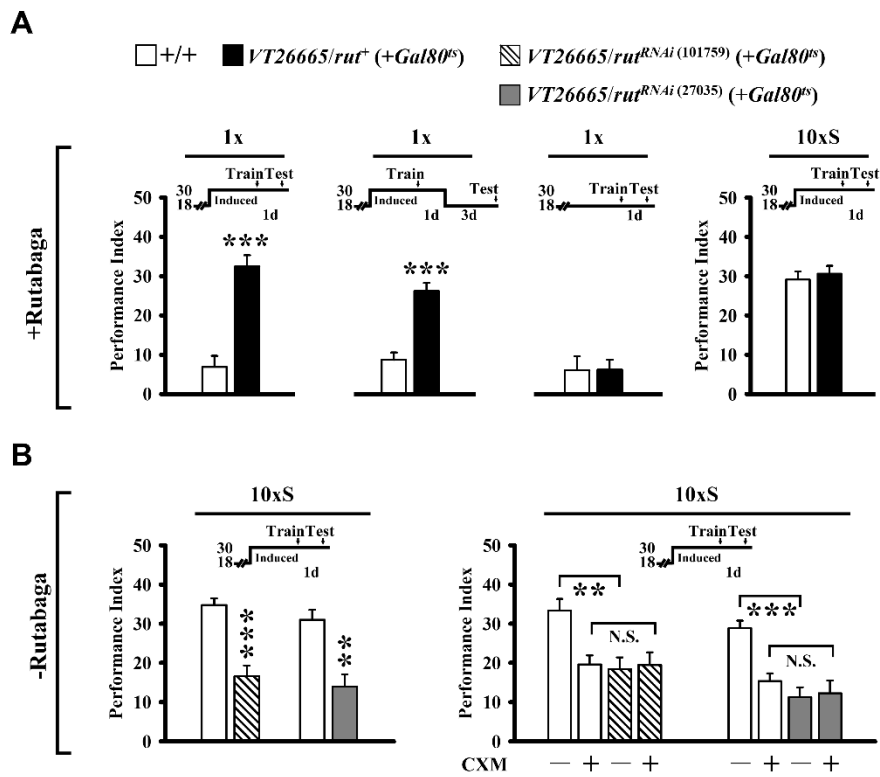
Figure 2

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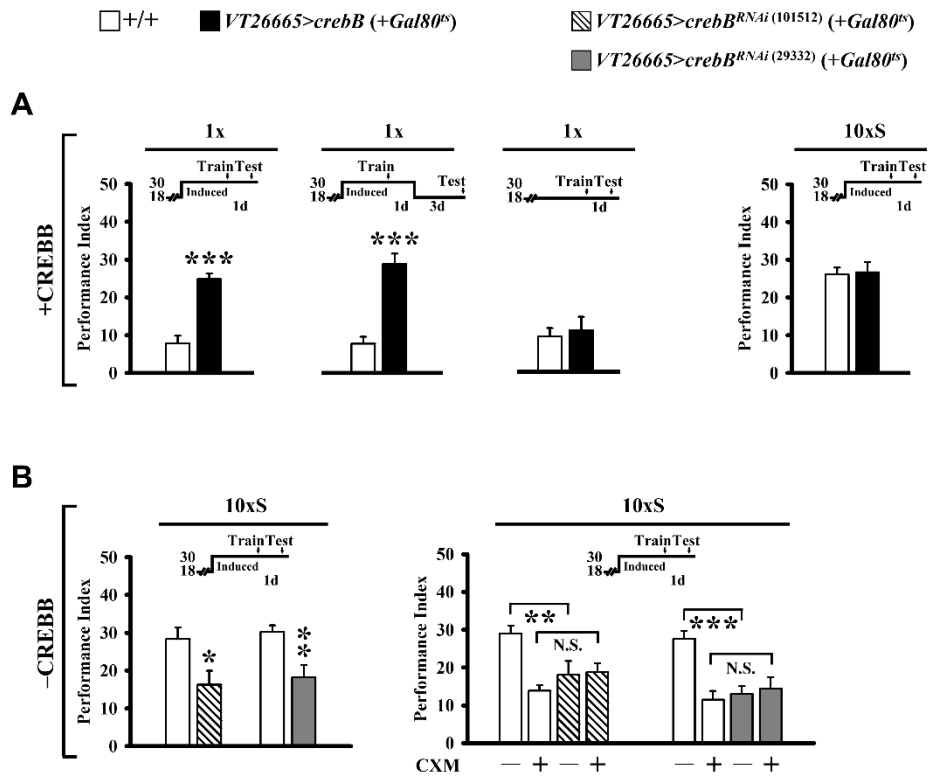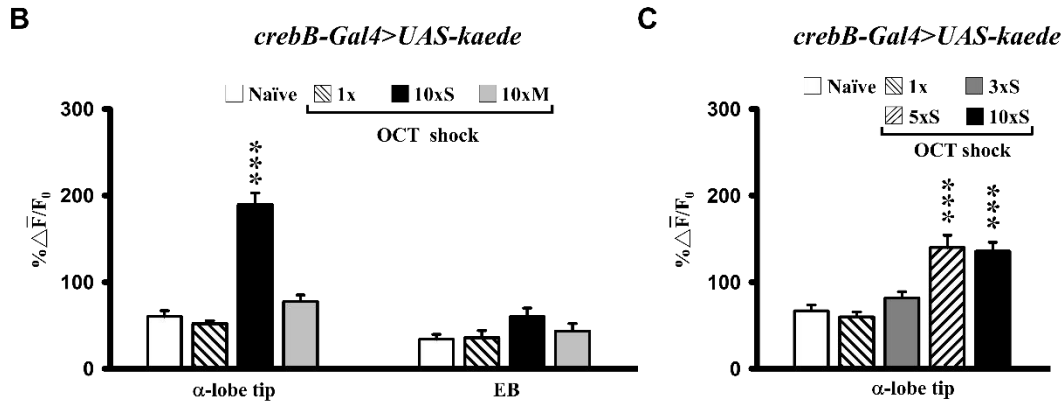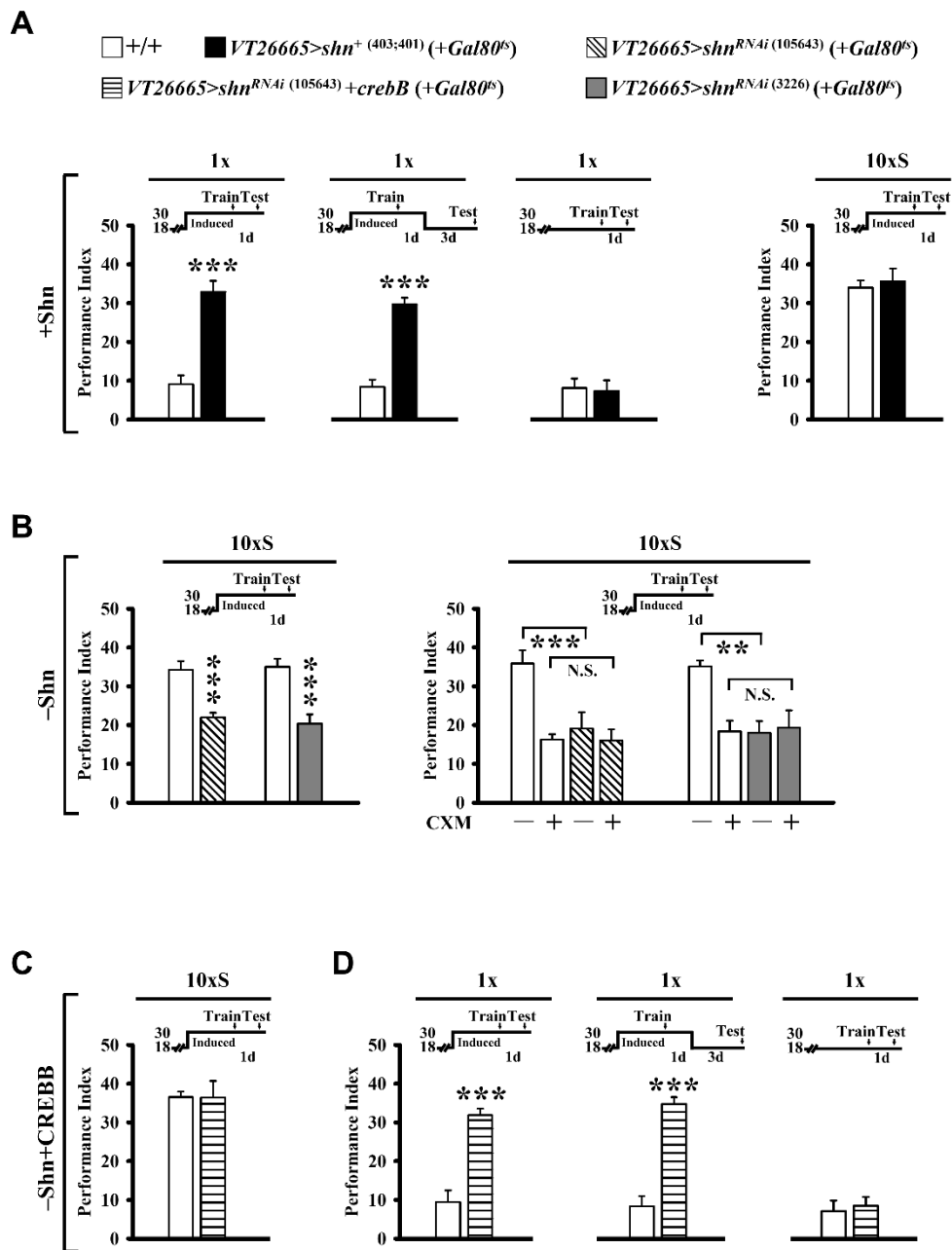**Figure 5**

Figure 6

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Figure 7

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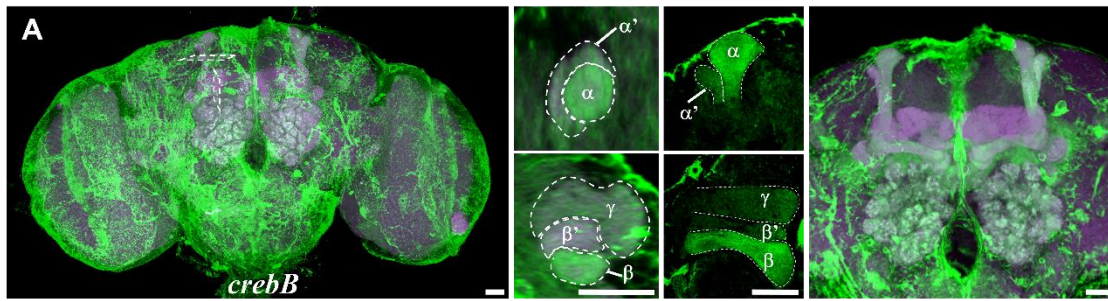
Figure 9

Figure 10

707 **Figure Legends**

708 **Figure 1. CSHL memory *Gal4* patterns in which protein synthesis inhibition has no net**

709 **effect on LTM formation.** (**A**) Full expression patterns that include MB and DAL neurons (top).

710 (**B**) Expression restricted from DAL neurons by *cry-Gal80* inhibition of Gal4 (center). (**C**)

711 Expression restricted from MB neurons by *MB-Gal80* inhibition of Gal4 (bottom). DAL (arrow)

712 and MB (arrowhead). (**D-F**) Protein synthesis inhibition in MB neurons enhances LTM

713 formation. Effects of memory circuit *Gal4*-targeted Ricin^{CS} on 1-day memory after three spaced

714 training cycles (3xS), compared with wild-type (+/+) controls. Cold-sensitive Ricin^{CS} blocks

715 protein synthesis at the permissive temperature (30 °C) between training and testing. (**D**) Protein

716 synthesis inhibition in all Gal4-expressing elements of the memory circuit has no net effect on

717 LTM. (**E**) Ricin^{CS} expression and blocking of protein synthesis in MB but not in DAL neurons

718 (where *cry-Gal80* inhibits Gal4), enhances 1-day memory in all seven *Gal4* genotypes. (**F**) By

719 contrast, Ricin^{CS} expression and blocking of protein synthesis in DAL but not in MB neurons

720 (where *MB-Gal80* inhibits Gal4) has no effect on LTM. In all figures, temperature control

721 schedules are indicated (top). Memory performance indices are calculated as the normalised

722 percent avoidance of shock-paired odour. Bars represent mean ± SE, *n* = 8/bar unless stated

723 otherwise. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. All genotypes are listed in table supplement

724 2.

725 **Figure 2. Protein synthesis inhibition in early α/β MB neurons enhances LTM formation.**

726 Effects of MB *Gal4*-targeted Ricin^{CS} on memory after sub-threshold training compared with wild-

727 type controls (see MB *Gal4* expression patterns in figure supplement 2A) (**A**) Protein synthesis

728 inhibition in MB *Gal4* patterns that include early α/β neurons enhance 1-day (left) and 4-day

729 (right) memory after 3xS training. (**B**) Two copies of Ricin^{CS} expressed in early α/β neurons are

730 necessary to enhance 1-day (left) and 4-day (right) memory after 1x training, whereas only one

731 copy of Ricin^{CS} is insufficient. (**C**) Expression of inactive Ricin^{CS} in α/β neurons at the restrictive

732 temperature (18 °C) has no effect on memory after 1x training.

733 **Figure 3. Protein synthesis in early α/β neurons after subthreshold training antagonizes**

734 **LTM formation.** Blocking protein synthesis in all MB (*OK107*), all α/β (*c739*), and early

735 α/β (*VT26665*) neurons 0-6 h after 1x training enhances 1-day memory compared with wild-type

736 (*+/+*) controls (related to Figure 2; also compare with Figure 4A). (**A**) Memory enhancement is

737 significant in all groups with Ricin^{CS} activity 0-12 h, 2-14 h and 4-16 h after training but not for

738 later time windows. (**B**) Memory enhancement is significant in all groups with Ricin^{CS} activity 0-

739 3 h and 3-6 h but not 6-9 h after training.

740 **Figure 4. Blocking early α/β neuron signaling enhances LTM formation.** Effects of early

741 α/β *Gal4*-targeted Dynamin^{ts} (*shi^{ts}*) on 1-day memory after 3xS training. (**A**) Blocking signaling

742 in the first 8-h after training enhances 1-day memory, whereas blocking signaling during

743 subsequent 8-h windows has no effect, compared with the unexpressed *shi^{ts}/+* control (also see

744 figure supplement 4). (**B**) Similarly, blocking signaling in the first 8-h after 3xS training enhances

745 4-day memory (left), but not after 3xM training (center) or after 3xS training of the *Gal4* driver or

746 *shi^{ts}* transgene alone (right). (**C**) Blocking early α/β signaling using two additional *Gal4* patterns

747 confirms this inhibitory effect and enhancement of 1-day memory (left). Memory is unaffected in

748 flies held at the permissive temperature (18 °C) after 3xS training (right).

749 **Figure 5. Neural membrane excitability in early α/β neurons bi-directionally regulates LTM**

750 **formation.** MB early α/β *Gal4*-targeted expression of K$^+$ and Na$^+$ channel proteins is induced at

751 the restrictive temperature for the *tub-Gal80^{ts}* inhibitor (30 °C) from five days before training

752 until testing. (**A**) *Shaw^{DN}* and *NaChBac* overexpression increase neural activity and impair 1-day

753 memory after 10xS training (left). The same LTM is similarly blocked by systemic protein

754 synthesis inhibition induced by CXM feeding (right) (also see figure supplement 5). (**B**) In

755   contrast, *Shaw* and *Kir2.1::GFP* overexpression decrease neural activity and enhance1-day

756   memory after only 1x training (left), which endures for at least 4 days (center). Memory is

757   unaffected in these flies held at the permissive temperature for *tub-Gal80^{ts}* (18 °C) after 1x

758   training (right). (**C**) Decreasing neural activity as in (**B**) does not affect 1-day memory after 10xS

759   training.

760   **Figure 6. Modulation of Rutabaga (AC) in early α/β neurons bi-directionally regulates LTM**

761   **formation.** (**A**) Overexpressing AC in early α/β neurons enhances 1-day memory after only 1x

762   training (left) and lasts at least four days (left center). Memory is unaffected in these flies held at

763   the permissive temperature for *tub-Gal80^{ts}* (18 °C) after 1x training (right center). One-day

764   memory is unaffected in these flies held at 30 °C after 10xS training (right). *Gal4*-targeted *rut^{+}*

765   overexpression is induced at the restrictive temperature for *tub-Gal80^{ts}* (30 °C) from five days

766   prior to training until testing. (**B**) By contrast, adult-stage specific RNAi down-regulation of AC

767   in early α/β neurons *(two independent RNAi lines) impairs* 1-day memory after 10xS training

768   (left) (also see figure supplement 6). The same LTM is similarly blocked by systemic protein

769   synthesis inhibition induced by cycloheximide (CXM) feeding (right).

770   **Figure 7. Modulation of PKA in early α/β neurons bi-directionally regulates LTM**

771   **formation.** (**A**) Overexpressing constitutively active PKA^{act1} in early α/β neurons enhances 1-day

772   memory after only 1x training (left) and lasts at least four days (left center). Memory is unaffected

773   in these flies held at the permissive temperature for *tub-Gal80^{ts}* (18 °C) after 1x training (right

774   center). One-day memory is unaffected in these flies held at 30 °C after 10xS training (right).

775   *Gal4*-targeted PKA^{act1} overexpression is induced at the restrictive temperature for *tub-Gal80^{ts}* (30

776   °C) from five days prior to training until testing. (**B**) By contrast, adult-stage specific RNAi

777   down-regulation of PKA in early α/β neurons *(two independent RNAi lines) impairs* 1-day

778   memory after 10xS training (left) (also see figure supplement 6). The same LTM is similarly

779   blocked by systemic protein synthesis inhibition induced by cycloheximide (CXM) feeding

780   (right).

781   **Figure 8. Modulation of CREBB protein in early α/β neurons bi-directionally regulates**

782   **LTM formation.** (**A**) Overexpressing CREBB proteins in early α/β neurons enhances 1-day

783   memory after only 1x training (left) and lasts at least four days (center). *Gal4*-targeted *crebB*

784   overexpression is induced at the restrictive temperature for *tub-Gal80^{ts}* (30 °C) from five days

785   before training until testing. Memory is unaffected in these flies held at the permissive

786   temperature for *tub-Gal80^{ts}* (18 °C) after 1x training (right). One-day memory is also unaffected

787   in these flies held at 30 °C after 10xS training. (**B**) By contrast, adult-stage specific RNAi down-

788   regulation of CREBB proteins in early α/β neurons *(with two independent RNAi constructs)*

789   *impairs* 1-day memory after 10xS training (left) (also see figure supplement 6). The same LTM is

790   similarly blocked by systemic protein synthesis inhibition induced by CXM feeding (right).

791   **Figure 9. Spaced training activates *crebB* transcription.** (**A**) CREBB expression visualized in

792   dissected brains with *crebB-Gal4* driven *UAS-mCD8::GFP* (green), counterstained with DLG-

793   antibody immunostaining (magenta), and viewed under a confocal microscope. Cross sections of

794   vertical and horizontal MB lobes (center) show more prominent expression in α/β neurons than in

795   α'/β' and γ neurons (labeled). Scale bar = 10 μm. (**B-C**) Promotor activation of *crebB* 24 h after

796   training reported by *de novo* Kaede synthesis, estimated by the ratio of new (green, 488 nm) and

797   preexisting (red, 561 nm) protein (% Δ F/ $\overline{F}_0$). For each brain, single optical slices through the

798   MB α-lobe tip or ellipsoid body (EB) were imaged under identical conditions. (**B**) Spaced

799   training stimulates *crebB* activity preferentially in the α-lobe, in comparison with EB controls.

800   (**C**) A minimum of 5xS training cycles are necessary to observe Kaede synthesis reflecting *crebB*

801   activity. Bars represent mean ± SE, $n \geq 8$.

802 **Figure 10. Shn in early α/β neurons regulates CREBB dependent LTM formation.** (**A**)

803 Overexpressing Shn proteins in early α/β neurons enhances 1-day memory after only 1x training

804 (left) and lasts at least four days ((left center). *Gal4*-targeted *shn⁺* overexpression is induced at the

805 restrictive temperature for *tub-Gal80ᵗˢ* (30 °C) from five days before training until testing.

806 Memory is unaffected in these flies held at the permissive temperature for *tub-Gal80ᵗˢ* (18 °C)

807 after 1x training (right center) (also see figure supplement 7). One-day memory is also unaffected

808 in these flies held at 30 °C after 10xS training (right). (**B**) In contrast, adult-stage specific RNAi

809 down-regulation of Shn in early α/β neurons *(with two independent RNAi constructs) impairs* 1-

810 day memory after 10xS training (left) (also see figure supplement 6). The same LTM is similarly

811 blocked by systemic protein synthesis inhibition induced by CXM feeding (right). (**C**) Down-

812 regulating Shn but co-overexpressing CREBB in early α/β neurons does not impair 1-day

813 memory after 10xS training. (**D**) One-day memory is enhanced after only 1x training (left) and

814 lasts at least four days (center). Memory is unaffected in flies held at the permissive temperature

815 for *tub-Gal80ᵗˢ* (18 °C) after 1x training (right).

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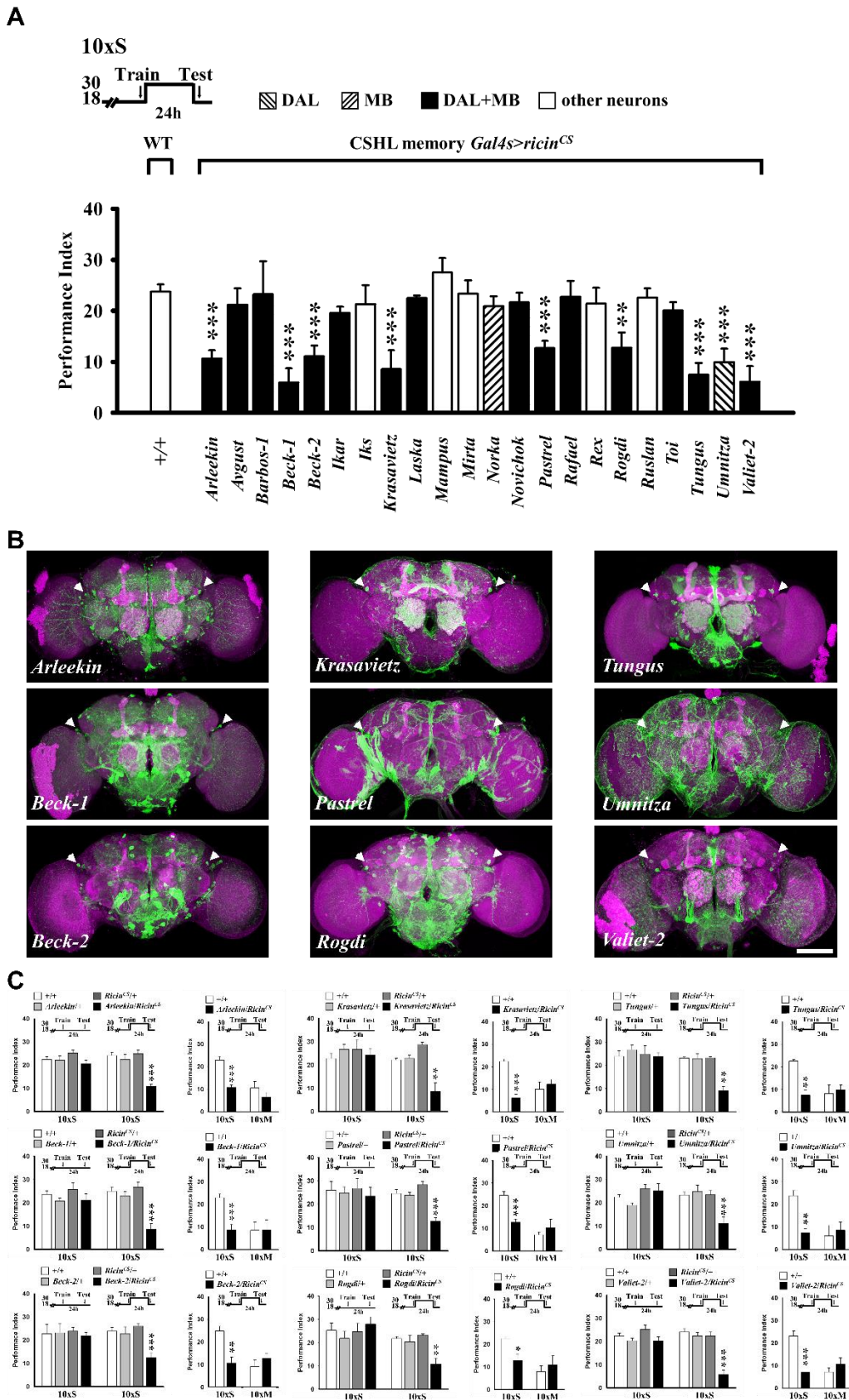
Figure supplement 1. Protein synthesis inhibition in MB and DAL neurons blocks LTM formation. (**A**) Effects of memory circuit *Gal4*-targeted Ricin[CS] on 1-day memory after ten spaced training cycles (10xS), compared with wild-type (+/+) controls. Cold-sensitive Ricin[CS] blocks protein synthesis at the permissive temperature (30 °C)

835   between training and testing. Inhibition of protein synthesis in eight of 22 *Gal4*-expressing patterns that include both

836   MB and DAL neurons and one that includes DAL but not MB neurons (*Umnitza*) blocks 1-day memory.

837   Interestingly, inhibition of protein synthesis in seven other *Gal4*-expressing patterns that include both MB and DAL

838   neurons (related to Figure 1) and one that includes MB but not DAL neurons (*Norka*) have no net effect on LTM. (**B**)

839   Nine Gal4 expression patterns in which protein synthesis inhibition blocks 1-day memory (**A**). (**C**) We observe no

840   LTM effects after Ricin^{CS} expression in patterns shown above (**B**) at the restrictive temperature (18 °C) or in flies

841   expressing the nine *Gal4* drivers or *ricin^{CS}* transgene alone (left). Ricin^{CS} expression in all nine patterns has no effect

842   on 1-day memory after 10xM training (right).

843   In all Extended Data figures, temperature control schedules for behavior experiments are indicated (top). Memory

844   performance indices are calculated as the normalized percent avoidance of shock-paired odor. Bars represent mean ±

845   SE, $n = 8$/bar unless otherwise noted. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. All images of dissected brains show

846   Gal4-driven *UAS-mCD8::GFP* (green), counterstained with DLG-antibody immunostaining (magenta), as viewed

847   under a confocal microscope. Scale bar = 50 μm unless otherwise noted. All fly genotypes are listed in table

848   supplement 1.

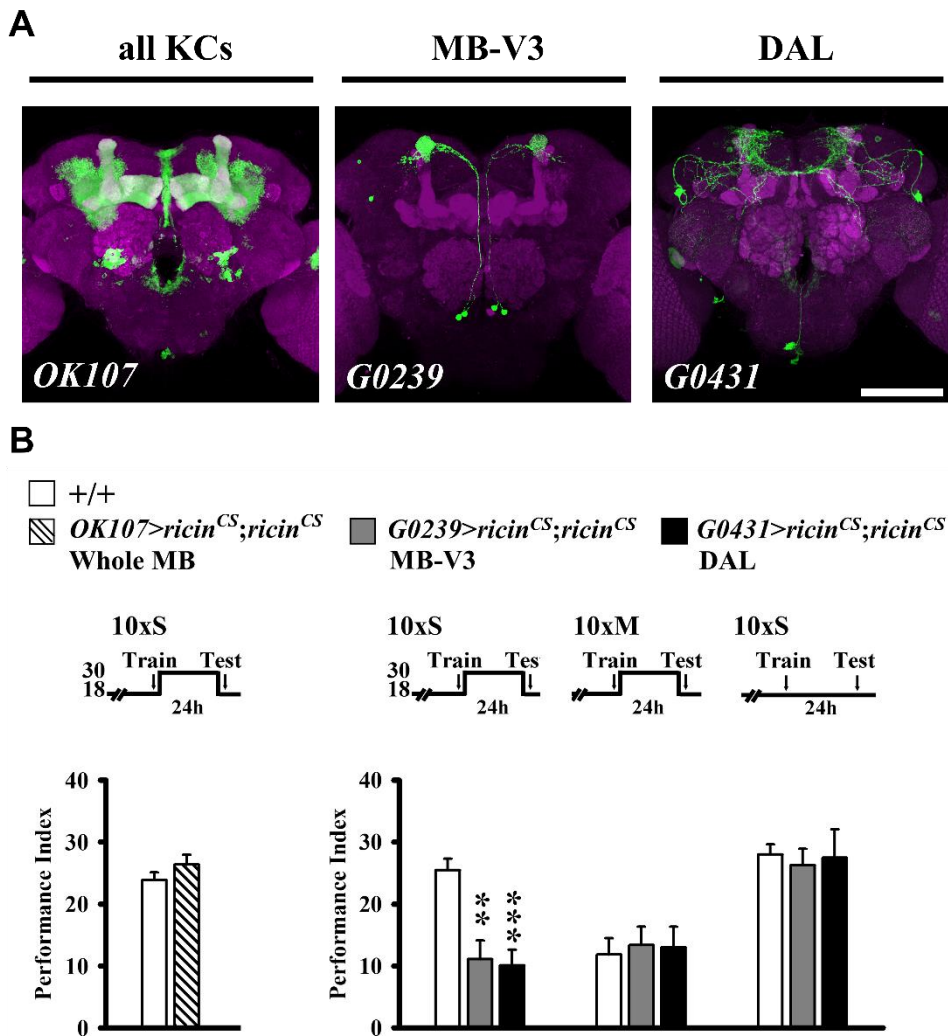849
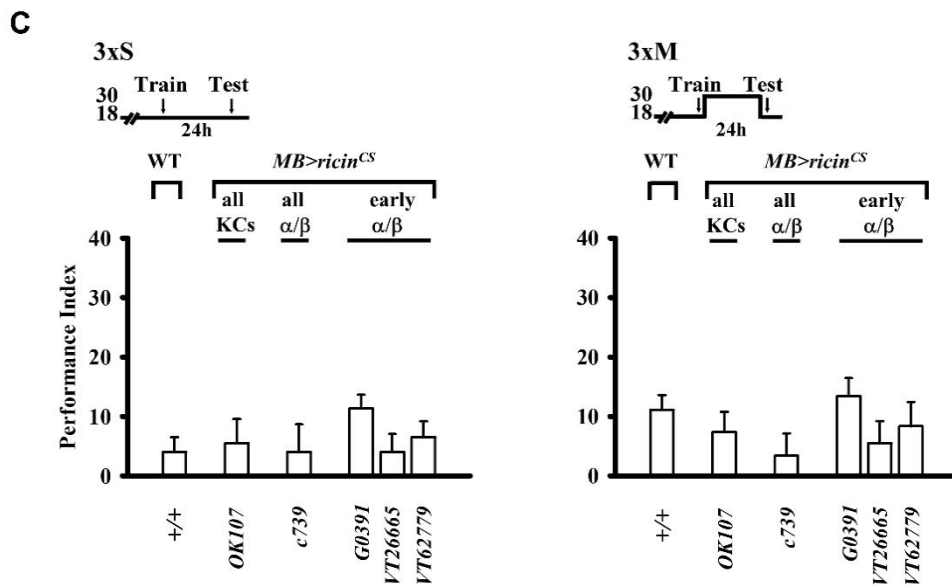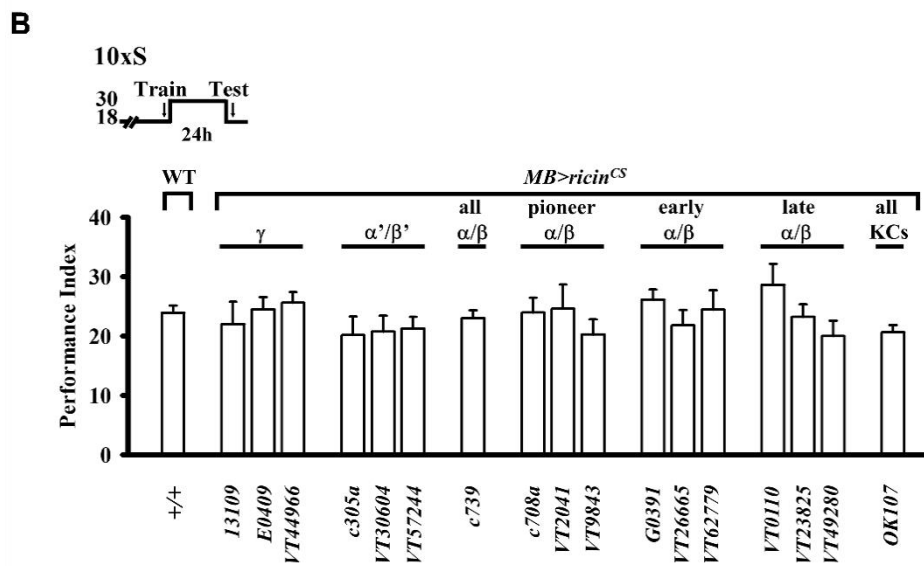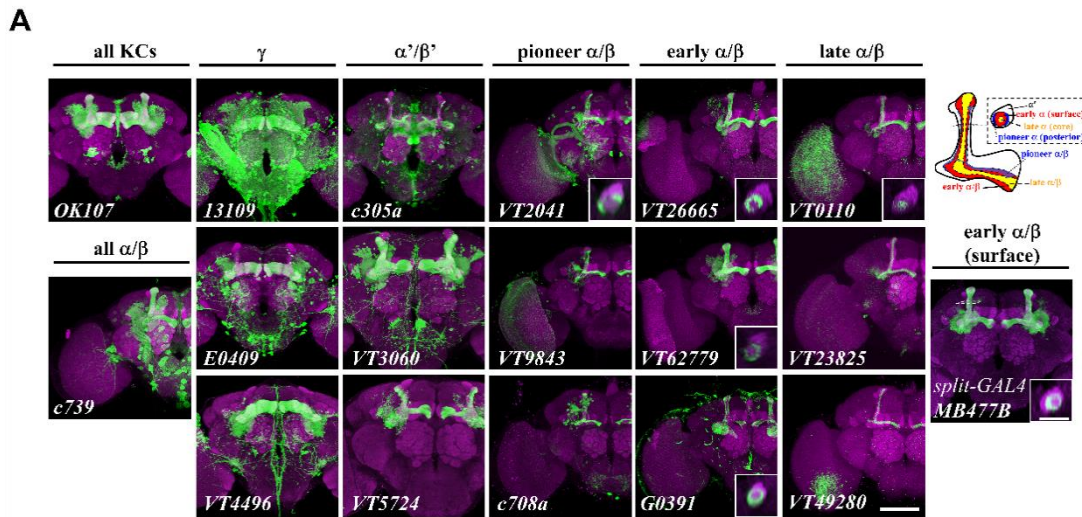
Figure supplement 2. Protein synthesis inhibition in MB neurons does not affect LTM formation after 10xS

training. (**A**) *Gal4* expression patterns that delineate five genetically and developmentally distinct MB neuron

852   subtypes. Spatial distributions of three α/β neuron subtypes shown in a schematic representation (right) and in cross

853   section at the vertical lobes (inset). MB *split-Gal4 MB477B* shows specific expression in early α/β (surface) neurons.

854   Scale bar (inset) = 10 μm. (**B**) Effects of MB *Gal4*-targeted Ricin^{CS} on memory compared with wild-type controls

855   (related to Figure 2). Blocking protein synthesis in MB neurons after 10xS training has no effect on LTM (compare

856   with the memory enhancing effects of protein synthesis inhibition in early α/β neurons after 3xS and 1x training,

857   Figure 2). (**C**) Ricin^{CS} expression in MBs neurons has no effect on memory at the restrictive temperature (18 °C) after

858   3xS training (left), or at the persmissive temperature (30 °C) after 3xM training (right).

859

Figure supplement 3. Protein synthesis inhibition in specific extrinsic MB neurons blocks LTM formation. (**A**) *Gal4* expression patterns that delineate intrinsic MB neurons and extrinsic MB-V3 and DAL neurons. (**B**) Blocking protein synthesis in MB neurons with two copies of active Ricin^{CS} has no effect on LTM after 10xS training (left). By contrast, blocking protein synthesis in MB-V3 or DAL neurons inhibits LTM after 10xS training (right), but not after 10xM training or after 10xS training when flies are held at the restrictive temperature (18 °C).
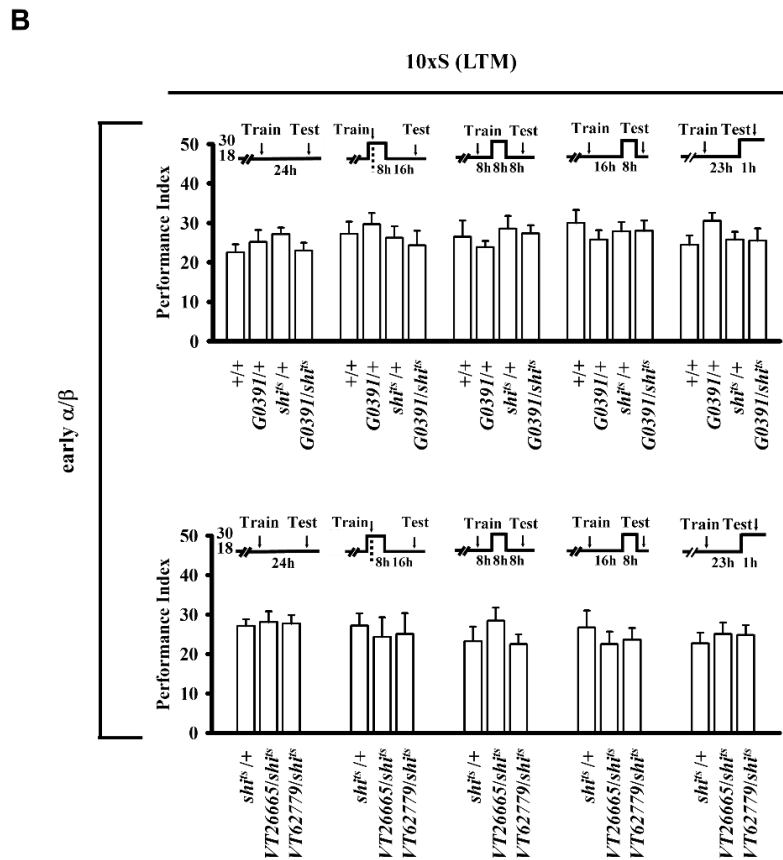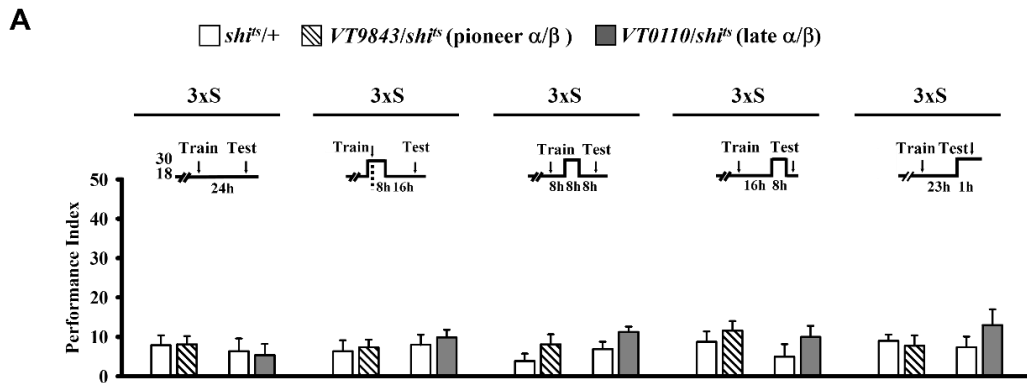
Figure supplement 4. Blocking transmission from MB neurons and effects on LTM formation. Effects of α/β *Gal4*-targeted Dynamin^{ts} (*shi^{ts}*) on 1-day memory. (**A**) Blocking neural transmission from pioneer and late α/β neurons during sequential 8-h time windows after subthreshold 3xS training has no effect on memory. (**B**) Blocking neural transmission from early α/β neurons during sequential 8-h time windows after 10xS training has no effect on memory. By comparison, blocking transmission from early α/β neurons after 3xS training enhances memory (Figure 4).

A

□ +/+ ■ *VT26665> Shaw^{DN}* (+*Gal80^{ts}*)

▨ *VT26665>NaChBac* (+*Gal80^{ts}*)
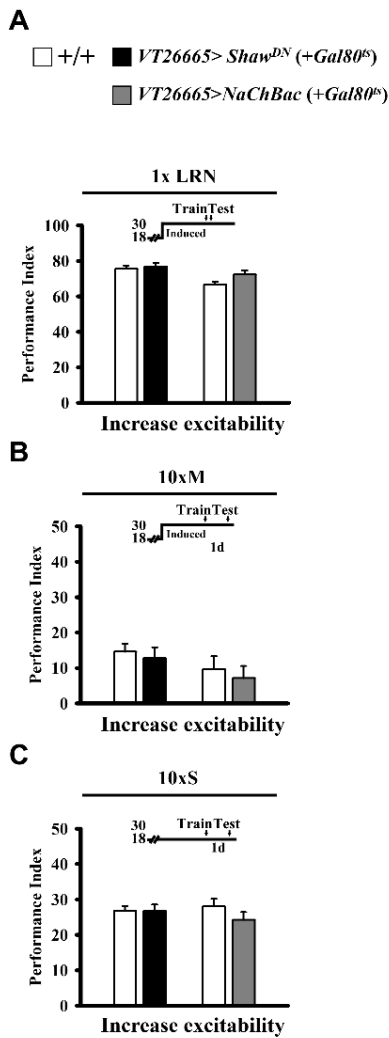
Figure supplement 5. Increasing membrane excitability in early α/β neurons and effects on memory formation.
Early α/β *Gal4*-targeted expression of K^+ and Na^+ channel proteins is induced at the restrictive temperature for the *tub-Gal80^{ts}* inhibitor (30 °C) from five days prior to training until testing. Experimental groups are compared with wild-type (+/+) controls. (**A**) *Shaw^{DN}* and *NaChBac* overexpression increase neural activity in early α/β neurons but have no effects on immediate memory after 1x training. (**B**) Similarly, elevated neural activity has no effect on 1-day memory after 10xM training. By comparison, elevated neural activity impairs 1-day memory after 10xS training (Figure 5). (**C**) Channel proteins are not induced in flies maintained at the permissive temperature for the *tub-Gal80^{ts}* inhibitor (18 °C) and show no differences in memory after 10xS training.
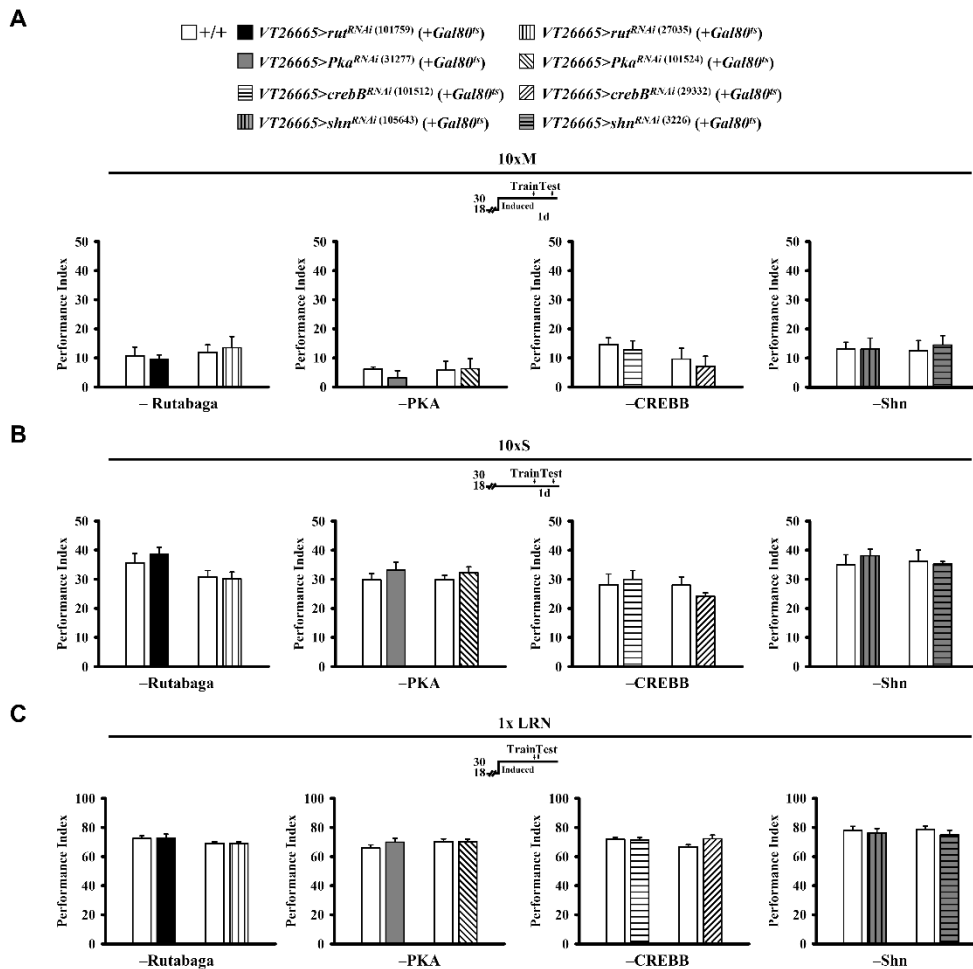
Figure supplement 6. Down-regulation of CREBB, Rutabaga (AC), PKA and Schnurri (Shn) in early α/β neurons and effects on memory formation. (**A**) Adult-stage specific RNAi down-regulation of these proteins (encoded by *rut*, *Pka*, *crebB* and *shn*) in early α/β neurons (two RNAi constructs each) have no effects on 1-day memory after 10xM training. *Gal4*-targeted RNAi specific for each gene is induced at the restrictive temperature for *tub-Gal80^{ts}* (30 °C) from five days prior to training until testing. Experimental groups are compared with wild-type (+/+) controls. (**B**) Proteins are not down-regulated in flies maintained at the permissive temperature for the *tub-Gal80^{ts}* inhibitor (18 °C) and there are no differences in memory in comparison with control flies after 10xS training. (**C**) Down regulation of all proteins in early α/β neurons has no effect on immediate memory after 1x training. By comparison, overexpressing AC, PKA, CREBB and Shn in early α/β neurons enhances 1-day memory after only 1x training (Figure 6-8 and 10).

□ +/+   ▨ *VT26665/crebA$^+$ (+Gal80^{ts})*   ■ *VT26665/shn$^{+ (403;401)}$ (+Gal80^{ts})*

▤ *VT26665>crebA$^{RNAi (110650)}$ (+Gal80^{ts})*
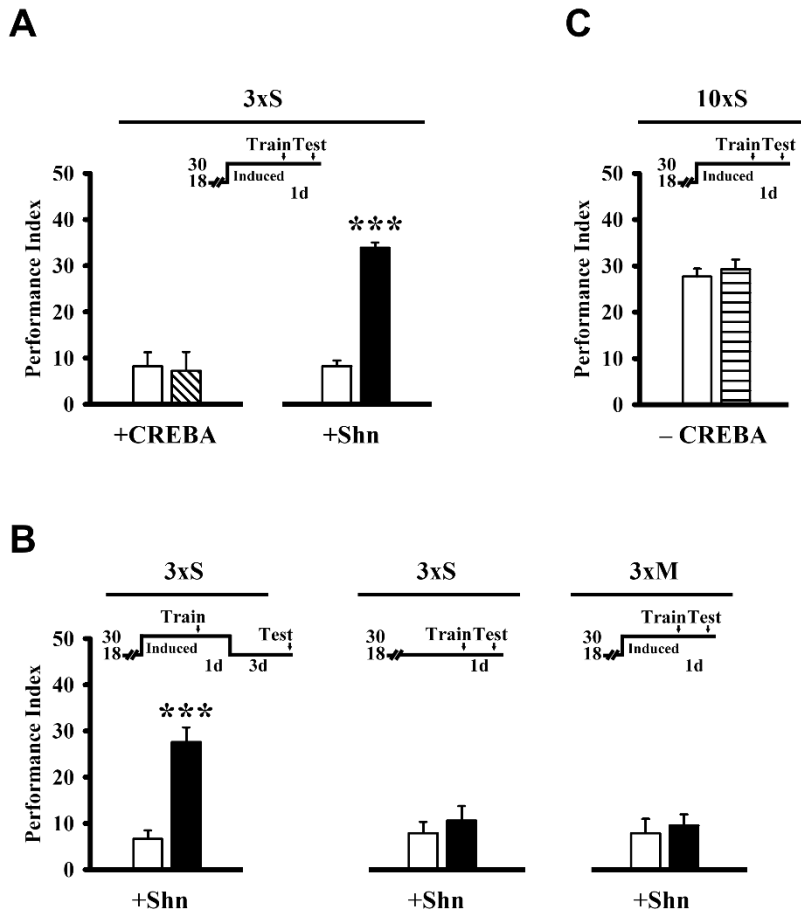
Figure supplement 7. Overexpression of Shn but not CREBA protein in early α/β neurons enhances LTM formation. (**A**) Overexpressing Shn but not CREBA in early α/β neurons enhances 1-day memory after 3xS training. *Gal4*-targeted *crebA* or *shn* overexpression is induced at the restrictive temperature for *tub-Gal80^{ts}* (30 °C) from five days prior to training until testing. (**B**) Enhanced memory lasts at least four days (left). Memory is unaffected in these flies held at the permissive temperature for *tub-Gal80^{ts}* (18 °C) after 3xS training (center), or at the restrictive temperature (30 °C) after 3xM training (right). (**C**) Adult-stage specific down-regulation of CREBA protein in early α/β neurons *has no effect on* 1-day memory after 10xS training.

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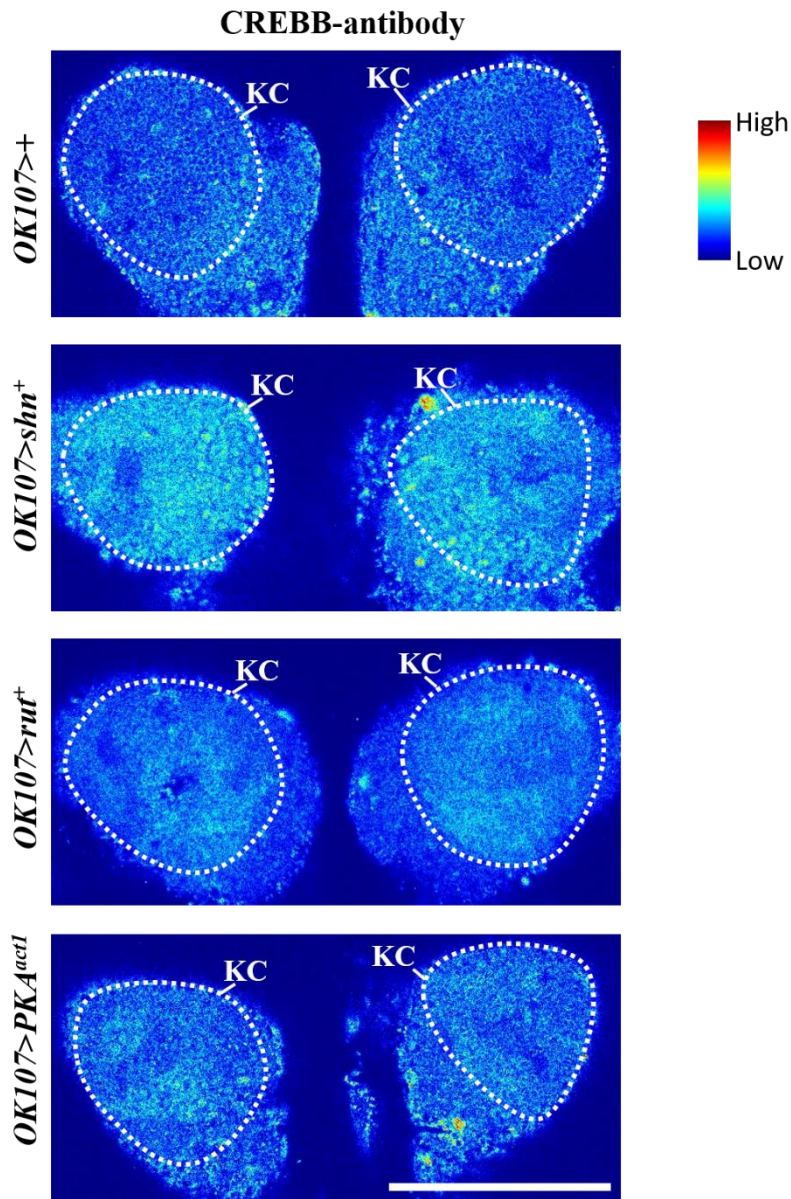
Figure supplement 8. Transgene over-expression and CREBB protein levels in MB Kenyon cells. *Gal4-OK107-*
driven *shn* over-expression led to elevated CREBB protein levels (second from top) in comparison with the control
(top). Over-expression of *rut* and *PKA* had only minor impact on CREBB expression (second from bottom and
bottom, respectively). Confocal images of *Drosophila* MB Kenyon cell bodies (encircled by the white dotted line)
show the intensity of CREBB immunostaining in a jet colormap.

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	γd	γm	α'/β'ap	α'/β'm	α/β pioneer (posterior)	α/β early (surface)	α/β late (core)	DAL	EB
Avgust	-	+	-	-	-	++	+++	+++	-
Barbos-1	-	+	-	-	-	+++	+++	+++	+
Ikar	-	+	+	+	-	+++	+++	+++	+++
Laska	-	+	+	+	-	+++	+++	+++	++
Novichok	-	+	-	-	-	+++	+++	+++	+
Rafael	-	++	-	-	-	+++	+++	+++	-
Toi	-	+	-	-	-	+++	+++	+++	+
Arleekin	-	-	++	++	-	++	+++	+++	+
Beck-1	-	-	-	-	-	-	+	+++	+++
Beck-2	-	-	-	-	-	-	+	+++	+++
Krasavietz	-	+	+	+	-	+++	+++	+++	+
Pastrel	-	+	-	-	-	+++	+++	+++	-
Rogdi	N.D.	+++	+++	+++	-	+	-	+++	+++
Tungus	++	+	-	-	-	+++	-	+++	+++
Umnitza	+	+	-	-	-	+	-	+++	+
Valiet-2	-	-	-	-	-	-	++	+++	-

Table supplement 1. CSHL memory *Gal4* patterns. Table shows Gal4 targeted GFP intensity was graded as strong (+++), intermediate (++), weak (+), absence (-) or non-distinguishable (N.D.). DAL, dorsal anterior lateral neuron; EB, ellipsoid body.

Fly genotypes utilized in experiments

Symbol	Genotype & Chromosome	Description	Source
+/+	*Canton-S w1118 (iso1CJ)*	Wild-type	Tully et al.,1994
Gal4/Gal80 Transgenes			
VT44966	*P{VT44966-Gal4}attP2* on Chr 3	*Gal4* driver, strong in γ neurons	VDRC[a]
VT30604	*P{VT30604-Gal4}attP2* on Chr 3	*Gal4* driver, specific in α′/β′ neurons	VDRC[a]
VT57244	*P{VT57244-Gal4}attP2* on Chr 3	*Gal4* driver, specific in α′/β′ neurons	VDRC[a]
VT2041	*P{VT2041-Gal4}attP2* on Chr 3	*Gal4* driver, specific in pioneer α/β neurons	VDRC[a]
VT9843	*P{VT9843-Gal4}attP2* on Chr 3	*Gal4* driver, specific in pioneer α/β neurons	VDRC[a]
VT26665	*P{VT26665-Gal4}attP2* on Chr 3	*Gal4* driver, specific in early α/β neurons	VDRC[a]
VT62779	*P{VT62779-Gal4}attP2* on Chr 3	*Gal4* driver, specific in early α/β neurons	VDRC[a]
VT0110	*P{VT0110-Gal4}attP2* on Chr 3	*Gal4* driver, specific in late α/β neurons	VDRC[a]
VT23825	*P{VT23825-Gal4}attP2* on Chr 3	*Gal4* driver, specific in late α/β neurons	VDRC[a]
VT49280	*P{VT49280-Gal4}attP2* on Chr 3	*Gal4* driver, specific in late α/β neurons	VDRC[a]
OK107	*P{GawB}OK107* on Chr 4	*Gal4* driver, strong in all MB neurons	BDSC[b]; Lin et al., 2007
c739	*P{GawB}c739* on Chr 3	*Gal4* driver, strong in all α/β neurons	BDSC[b]; Lin et al., 2007
c305a	*P{GawB}c305a* on Chr 1	*Gal4* driver, strong in α′/β′ neurons	BDSC[b]; Lin et al., 2007
c708a	*P{GawB}c708a* on Chr 1	*Gal4* driver, specific in pioneer α/β neurons	BDSC[b]; Lin et al., 2007
E0409 (*103496*)	*P{GawB}fru[NP0021]* on Chr 3	*Gal4* driver, strong in γ neurons	DGRC[c] ; Lee et al., 2011
Arleekin	*P{GawB}Arleekin* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Avgust	*P{GawB}Avgust* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
Barbos-1	*P{GawB}Barbos-1* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
Beck-1	*P{GawB}Beck-1* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
Beck-2	*P{GawB}Beck-2* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
Ikar	*P{GawB}Ikar* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Iks	*P{GawB} Iks* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Krasavietz	*P{GawB}Krasavietz* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Laska	*P{GawB}Laska* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Mampus	*P{GawB}Mampus* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
Mirta	*P{GawB}Mirta* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Norka	*P{GawB}Norka* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Novichok	*P{GawB}Novichok* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Pastrel	*P{GawB}Pastrel* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Rafael	*P{GawB}Rafael* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Rex	*P{GawB}Rex* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
Rogdi	*P{GawB}Rogdi* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Ruslan	*P{GawB}Ruslan* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Toi	*P{GawB}Toi* on Chr 3	CSHL memory *Gal4* driver	Dubnau et al., 2003
Tungus	*P{GawB}Tungus* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
Umnitza	*P{GawB}Umnitza* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
Valiet-2	*P{GawB}Valiet-2* on Chr 2	CSHL memory *Gal4* driver	Dubnau et al., 2003
13109 (*G0451*)	*P{GT1}BG00015* on Chr 1	*Gal4* driver, strong in γ neurons	BDSC[b]; Lin et al., 2007
G0391 (*12795*)	*P{GT1}BG02525* on Chr 3	*Gal4* driver, specific in early α/β neurons	BDSC[b]; Lin et al., 2007
G0239 (*12639*)	*P{GT1}BG01228* on Chr 2	*Gal4* driver, specific in MB-V3 neurons	BDSC[b]; Pai et al., 2013
G0431 (*12837*)	*P{GT1}BG02822* on Chr 3	*Gal4* driver, specific in DAL neurons	BDSC[b]; Chen et al., 2012
MB477B	*P{R44E04-p65.AD}attP40* on Chr 2; *P{R26E07-GAL4.DBD}attP2* on Chr 3	Split-*Gal4* driver, specific in early α/β neurons	Aso et al., 2014
crebB	*P{crebB-Gal4}attP40* on Chr 2	*crebB* promoter *Gal4* driver	This study

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cry-Gal80	P{cry-Gal80} on Chr 3	Inhibit Gal4 with DAL-specific (cryptochrome promoter) expression of Gal80	Stoleru et al., 2004; Chen et al., 2012
MB-Gal80	P{mb247-GAL80} on Chr 2	Inhibit Gal4 with MB-specific (mb247 promoter) expression of Gal80	Krashes et al., 2008
tub-Gal80^{ts}	P{tubP-GAL80^{ts}}20 on Chr 2	Inhibit Gal4 with tubulin promoter drive temperature-sensitive Gal80	BDSC[b]

UAS Transgenes

GFP	P{UAS-mCD8::GFP}LL5, P{UAS-mCD8::GFP}2 on Chr 2; P{UAS-mCD8::GFP.L}LL6 on Chr 3	membrane-targeted green fluorescent protein (GFP)	BDSC[b]
Kaede	P{UAS-kaede} on Chr 3	photoconvertible fluorescent protein to report de novo protein synthesis	Chen et al., 2012
ricin^{CS}	P{UAS-ricin^{CS}} on Chr 2; P{UAS-ricin^{CS}} on Chr 3	cold-sensitive ribosome-inactivating toxin to acutely inhibit protein synthesis	Pai et al., 2013; Chen et al., 2012; Wu et al., 2017; Moffat et al.,1992; Allen et al., 2002
shi^{ts}	P{UAS-shi^{ts}} on Chr 3	temperature-sensitive dominant-negative form of Dynamin to block synaptic transmission	Dubnau et al., 2001
Shaw^{DN}	P{UAS-Shaw^{DN}} on Chr 2	dominant-negative K+ channel	Hodge et al., 2005
NaChBac	P{UAS-NaChBac} on Chr 2	bacterial Na+ channel	Joiner et al., 2006
Shaw	P{UAS-Shaw} on Chr 2	K+ channel	Hodge et al., 2005
Kir2.1::GFP	P{UAS-Kir2.1::GFP} on Chr 3	inward-rectifying K+ channel; PIP2 dependent	Joiner et al., 2006
crebB	P{UAS-crebB} on Chr 3	cAMP response element binding protein (crebB)	BDSC[b]
rut$^+$	P{UAS-rut}2 on Chr 2; P{UAS-rut}1 on Chr 3	wild type rutabaga$^+$, adenylyl cyclase (AC)	BDSC[b]
Pka^{act1}	P{UAS-Pka^{act1}} on Chr 2	constitutively active subunit of cAMP-dependent protein kinase (PKA)	Joiner et al., 2006
crebA$^+$		cAMP response element binding protein (crebA)	BDSC[b]
shn$^+$	P{UAS-shn}403 on Chr 2; P{UAS-shn}401 on Chr 3	wild type schnurri$^+$, zinc finger C2H2 transcription factor	Marty et al., 2000
v101512	P{KK108927}VIE-260B on Chr 2	crebB RNA interference	VDRC[a]
v101759	P{KK109441}VIE-260B on Chr 2	rutabaga (AC) RNA interference	VDRC[a]
v101524	P{KK108966}VIE-260B on Chr 2	Pka RNA interference	VDRC[a]
v105643	P{KK101278}VIE-260B on Chr 2	schnurri RNA interference	VDRC[a]
v3226	P{GD1644}v3226 on Chr 3	schnurri RNA interference	VDRC[a]
29332	P{TRiP.JF02494}attP2 on Chr 3	crebB RNA interference	BDSC[b]
27035	P{TRiP.JF02361}attP2 on Chr 3	rutabaga (AC) RNA interference	BDSC[b]
31277	P{TRiP.JF01218}attP2 on Chr 3	PKA RNA interference	BDSC[b]

[a] Vienna Drosophila Resource Center; [b] Bloomington Drosophila Stock Center; [c] KYOTO Stock Center.

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921 **Table supplement 2. Fly genotypes used in this study.** Table shows all fly genotypes, descriptions and sources used

922 in this study, grouped by distinct types of transgenes. References are listed in Methods.

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