1   **A population of neurons that produce hugin and express the *diuretic hormone 44 receptor***

2   **gene projects to the corpora allata in *Drosophila melanogaster***

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23   Running title: *Drosophila* corpora allata-projecting neurons

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Abstract

The corpora allata (CA) are essential endocrine organs that biosynthesize and secrete the sesquiterpenoid hormone, namely juvenile hormone (JH), to regulate a wide variety of developmental and physiological events in insects. Previous studies had demonstrated that the CA are directly innervated with neurons in many insect species, implying the innervations to be important for regulating JH biosynthesis in response to internal physiology and external environments. While this is also true for the model organism, *Drosophila melanogaster*, which neurotransmitters are produced in the CA-projecting neurons are yet to be clarified. In this study on *D. melanogaster*, we aimed to demonstrate that a subset of neurons producing the neuropeptide hugin, the invertebrate counterpart of the vertebrate neuromedin U, directly projects to the adult CA. A synaptic vesicle marker in the hugin neurons was observed at their axon termini located on the CA, which were immunolabeled with a newly-generated antibody to the JH biosynthesis enzyme JH acid *O*-methyltransferase (JHAMT). We also found the CA-projecting hugin neurons to likely express a gene encoding the specific receptor for diuretic hormone 44 (Dh44). Moreover, our data suggested that the CA-projecting hugin neurons have synaptic connections with the upstream neurons producing Dh44. To the best of our knowledge, this is the first study to identify a specific neurotransmitter of the CA-projecting neurons in *D. melanogaster*, and to anatomically characterize a neuronal pathway of the CA-projecting neurons and their upstream neurons.

Key words:

Corpora allata, Diuretic hormone 44, *Drosophila melanogaster*, Hugin, Juvenile hormone

Introduction

49

50   An endocrine organ is responsible for biosynthesizing specific hormones to control animal

51   development and physiology. In general, hormone biosynthesis in the endocrine organ is

52   influenced by conditions of internal physiology and external environments, and regulates

53   organismal homeostasis. In vertebrates, most endocrine organs are innervated with sympathetic

54   and parasympathetic nerves, and the innervations play indispensable roles in the regulation of

55   hormone biosynthesis (Schmidt-Nielsen, 1997).

56   Insect endocrine organs are also regulated by neurons that directly innervate the organs.

57   One of the best examples is the prothoracic gland (PG), which biosynthesizes and secretes the

58   principal insect steroid hormone ecdysone (Niwa and Niwa, 2014a; Pan et al., 2020), while the

59   neurons produce prothoracicotropic hormone (PTTH) in the fruit fly *Drosophila melanogaster*

60   (Kannangara et al., 2021; Malita and Rewitz, 2021; McBrayer et al., 2007; Niwa and Niwa,

61   2014b). Two pairs of cell bodies of the PTTH neurons are located in the brain hemispheres,

62   extending their axons to the PG cells. PTTH is released at the synapses between PTTH neurons

63   and the PG cells, stimulating the biosynthesis and secretion of ecdysone to regulate the timing of

64   larval-to-pupal transition as well as body growth (McBrayer et al., 2007; Shimell et al., 2018). In

65   addition to PTTH neurons, a subpopulation of serotonergic neurons is known to directly

66   innervate the PG to regulate ecdysone biosynthesis in *D. melanogaster*. Cell bodies of the PG-

67   projecting serotonergic neurons, called $SE0_{PG}$ neurons, are located at the subesophageal zone

68   (SEZ) and extend their long axons to the PG. The $SE0_{PG}$ neurons are essential for controlling

69   developmental timing in response to nutritional availability (Shimada-Niwa and Niwa, 2014).

70   Recently, a subset of neurons producing the neuropeptide corazonin (Crz) has been reported to

71   project to the PG in *D. melanogaster*, although the role of Crz neurons in ecdysone biosynthesis

72   is still unclear (Imura et al., 2020).

73   Another endocrine organ in insects, the corpus allatum (plural: corpora allata (CA)), is

74   also innervated with certain neurons. The CA are crucial organs for the biosynthesis and

75   secretion of the sesquiterpenoid hormone named juvenile hormone (JH), which plays essential

76   roles in a wide variety of aspects of insect development and physiology, including the larval-to-

77   adult transition, oogenesis, epithelial morphogenesis, neuronal development, diapause, behavior,

78   and longevity (Martín et al., 2021; Riddiford, 2020). Previous studies had suggested that

79   neuronal inputs to the CA are essential for regulating JH biosynthesis and/or secretion, thereby

80   regulating the biological events. For example, in the acridid grasshopper *Gomphocerus rufus*,

81   and the locusts *Locusta migratoria* and *Schistocerca gregaria*, the CA are innervated with two

82   morphologically distinct types of brain neurons (Virant-Doberlet et al., 1994). In the cockroach

83   *Diploptera punctata*, nervous connections between the CA and brain are required for mitosis of

84   the CA cells (Chiang et al., 1995). In the northern blowfly *Protophormia terraenovae* and the

85   brown-winged green bug *Plautia stali*, neurons projecting to the CA are essential for inducing

86   reproductive dormancy in short-day and low-temperature conditions (Matsumoto et al., 2013;

87   Shiga et al., 2003). In case of *P. stali*, the CA-projecting neurons produce the neuropeptide

88   *Plautia stali* myoinhibitory protein (Plast-MIP), which is considered to directly suppress JH

89   biosynthesis in the CA (Hasegawa et al., 2020; Matsumoto et al., 2017). In *D. melanogaster*, at

90   least two types of neurons had been reported to directly innervate the CA (Siegmund and Korge,

91   2001). These neurons, which seem to highly express a gene encoding the cell adhesion molecule

92   Fasculin2, are involved in the epithelial movement of male genitalia by inhibiting JH

93   biosynthesis during the pupal stage (Ádám et al., 2003). All the accumulated evidence together

94   indicate direct neuronal innervation of the CA to be crucial for regulating JH biosynthesis in the

95   latter. However, in almost all cases, except Plast-MIP, the neurotransmitter produced in the CA-

96   projecting neurons has not been clarified. To further understand the neuroendocrine mechanisms

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97 of JH biosynthesis in the CA, clarification and characterization of the neurotransmitters would be

98 crucial.

99 Using *D. melanogaster*, a previous pioneering study had demonstrated that a subset of

100 neurons producing the neuropeptide hugin (Hug), the invertebrate counterpart of vertebrate

101 neuromedin U (Meng et al., 2002), extends the axons to the complex formed by the CA and

102 corpora cardiaca (CC) in the adult insect (Melcher and Pankratz, 2005). However, which

103 endocrine organ (the CA or CC) the Hug neurons project to still remains unclear. In this study,

104 we aimed to provide an anatomical evidence of the subset of Hug neurons that directly project

105 into the CA of *D. melanogaster*. The CA-projecting Hug neurons express a gene encoding the

106 specific receptor for diuretic hormone 44 (Dh44), namely *Dh44-R2*. The study also showed that

107 the CA-projecting Hug neurons may have a synaptic connection with the upstream Dh44 neurons.

108

109 **Materials and methods**

110 *Drosophila* **strains**

111 Flies were reared on 0.275 g agar, 5.0 g glucose, 4.5 g cornmeal, 2.0 g yeast extract, 150 μl

112 propionic acid, and 175 μl 70% butylparaben (in EtOH) in 50 ml water. All experiments, except

113 for the temperature shift experiment using *UAS-shibire^{ts}*, were conducted at 25 °C under a 12:12-

114 h light/dark cycle. For the temperature shift experiment, *UAS-shibire^{ts}* flies were first reared at

115 21 °C and transferred to 29 °C within 12 h after eclosion.

116 The following transgenic *D. melanogaster* strains were used in the study: *hug-YFP*

117 (Melcher and Pankratz, 2005), *R18A04-GAL4* (BDSC #48793), *R65C11-LexA* (BDSC #54089),

118 *R19D04-GAL4* (BDSC #48847), *hugS3-GAL4* (Melcher and Pankratz, 2005), *Dh44-R2-T2A-*

119 *GAL4* (Kondo et al., 2020), *PK2-R2-T2A-GAL4* (Kondo et al., 2020), *PK2-R1-2A-GAL4* (BDSC

120 # 84686), *LexAop-CD4::spGFP11,UAS-CD4::spGFP1-10* (BDSC #58755), *LexAop-mCherry*

121 (BDSC #52272), *UAS-DenMark,UAS-Syt1::GFP; In(3L)D,mirr,D / TM6C* (BDSC #33064),

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122 *UAS-GFP;UAS-mCD8::GFP* (Ito et al., 1998) (a gift from Kei Ito), *UAS-TurboRFP* (a gift from

123 A. Koto and M. Miura, The University of Tokyo), and *UAS-shibire^{ts}* (Kitamoto, 2001) (a gift

124 from Hiroshi Kohsaka, The University of Tokyo).

125

126 **Generation of guinea pig anti-*Drosophila melanogaster* JH acid *O*-methyltransferase**

127 **(JHAMT) antibody**

128 Guinea pig polyclonal anti-*D. melanogaster* JHAMT antibody was raised against the peptide

129 NH$_2$- MNQASLYQHANQVQRHDAK-COOH, which corresponds to 1–19 amino acid residues

130 of the mature JHAMT protein (Niwa et al., 2008).

131

132 **Immunohistochemistry**

133 Tissues were dissected in phosphate buffer saline (PBS) and fixed in 3.7% formaldehyde in PBS

134 for 30 to 60 min at room temperature (RT). The fixed samples were washed thrice in PBS, then

135 washed for 15 min with PBS containing 0.3% Triton X-100 (PBT), and finally treated with the

136 blocking solution (0.2% bovine serum albumin (Sigma A9647) in PBT) for 1 h at RT or

137 overnight at 4 °C. Thereafter, the samples were incubated with a primary antibody in the

138 blocking solution overnight at 4 °C. The primary antibodies used were as follows: mouse anti-

139 GFP antibody (Sigma G6539, 1:2000); rabbit anti-RFP antibody (Medical & Biological

140 Laboratories PM005, 1:2,000); rabbit anti-hugin-PK2 antibody (Schoofs et al., 2014) (1:500);

141 rabbit anti-hugin-γ antibody (Schoofs et al., 2014) (1:500); and guinea pig anti-JHAMT antibody

142 (this study, 1:1000-2000). After washing, the samples were incubated with the fluorophore

143 (Alexa Fluor 488, 546, or 633)-conjugated secondary antibodies (Thermo Fisher Scientific)

144 (1:200) in the blocking solution for 2 h at RT or overnight at 4 °C. After another round of

145 washing, all samples were mounted on the glass slides using FluorSave reagent (Merck Millipore

146 #345789).

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147

148 **Quantitative RT-PCR for *Krüppel-homolog 1* (*Kr-h1*) expression**

149 Total RNA was extracted from whole bodies of 4-day-old adult virgin female flies. RNA was

150 reverse transcribed using ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO)

151 and cDNA samples were used as templates for quantitative PCR using THUNDERBIRD SYBR

152 qPCR mix (TOYOBO) on a Thermal Cycler Dice Real Time System (Takara Bio Inc.). The

153 amount of target RNA was normalized to an endogenous control ribosomal protein 49 gene

154 (*rp49*), and the relative fold change was calculated. The expression level of *Kr-h1* gene was

155 compared using the $\Delta\Delta$Ct method. The following primers were used for *Kr-h1*, as previously

156 described (Kang et al., 2017): *kr-h1 F* (5□-TCACACATCAAGAAGCCAACT-3□) and *kr-h1 R*

157 (5□ -GCTGGTTGGCGGAATAGTAA-3□). The primers for *rp49* were *rp49 F* (5□ -

158 CGGATCGATATGCTAAGCTGT-3□) and *rp49 R* (5 □ -GCGCTTGTTCGATCCGTA-3□).

159

160 **Statistical analysis**

161 Sample sizes were chosen based on the number of independent experiments required for

162 statistical significance and technical feasibility. The experiments were not randomized, and

163 investigators were not blinded. All statistical analyses were performed using the "R" software

164 environment.

165

166 **Results**

167 **Some of the *Dh44-R2*-expressing neurons project to the corpora allata**

168 This study originally intended to identify candidate neurons projecting to the ring gland (RG),

169 comprised of the CA, CC, and PG, in *D. melanogaster* larvae, but not in the adult. We first

170 browsed the large collections of larval images in the FlyLight database of Janelia Research

171 Campus, Howard Hugh Medical Institute (https://flweb.janelia.org/cgi-bin/flew.cgi). The

7

172   FlyLight database provides large anatomical image data sets and highly characterized collections

173   of GAL4 lines that allowed us to visualize individual neurons in the *D. melanogaster* central

174   nervous system (Jenett et al., 2012). In the FlyLight images of each GAL4 line, fluorescent

175   proteins driven by the GAL4-UAS system (Brand and Perrimon, 1993) specifically visualize a

176   subset of larval neurons, whereas many images do not include the RG located on the dorsal side

177   of the two brain hemispheres. Nevertheless, some of the FlyLight images show neurons that

178   extend their axons toward the longitudinal fissure of the two brain hemispheres, which are

179   reminiscent of the axonal morphology of the identified RG-projecting neurons, such as PTTH

180   neurons (McBrayer et al., 2007; Siegmund and Korge, 2001). Therefore, we expected that

181   neurons that possess such characteristic axonal morphology are the RG-projecting neurons.

182   Based on this working hypothesis, we could successfully identify a GAL4 line labeling a subset

183   of the Crz neurons that projects to the prothoracic gland cells (Imura et al., 2020).

184   During the course of our FlyLight database search, we found not only the *Crz-GAL4* line

185   projecting to the prothoracic gland but also other GAL4 lines that visualize neuronal processes at

186   the longitudinal fissure of the two brain hemispheres (E.I, Y.M, Y.K., Y.S.N., R.N.,

187   unpublished). Next, we obtained the GAL4 lines, and crossed them with *UAS-mCD8::GFP*

188   strain to examine whether the GFP-labeled neurons project to the RGs in the 3rd-instar larvae.

189   These strains included *R18A04-GAL4*, in which *GAL4* expression was driven by a part of the

190   enhancer region of the G protein-coupled receptor gene *Dh44-R2* (Figure 1a). We confirmed that

191   *R18A04-GAL4* was active in neurons projecting to the central region of the RG, which seemed to

192   correspond to the CA (Figure 1b). To precisely examine whether the *R18A04-GAL4*-positive

193   neurons projected to the CA, by immunostaining, we generated a new guinea pig antibody

194   against JHAMT protein (Niwa et al., 2008). We found GFP signals in *R18A04-*

195   *GAL4>mCD8::GFP* larvae to be observable in the neurite terminals located at the CA, which

196   was JHAMT-positive (Figure 1b).

8

197   Notably, we found the projection of *R18A04-GAL4*-positive neurons to the CA to be

198   observable in the adult stage (Figure 1c). Till date, CA-projecting neurons had not been

199   identified and characterized in adult *D. melanogaster*. On the other hand, many recent studies

200   have reported JH biosynthesis to be adaptively regulated in response to internal physiological

201   conditions and external environmental cues in adult *D. melanogaster* (Lee et al., 2017;

202   Meiselman et al., 2017; Ojima et al., 2018; Reiff et al., 2015; Wu et al., 2018; Yamamoto et al.,

203   2013). Therefore, we decided to focus on the anatomical characteristics of *R18A04-GAL4*-

204   positive neurons in the adult stage.

205   Since the expression of *R18A04-GAL4* is regulated by the enhancer region of *Dh44-R2*,

206   we expected the *R18A04-GAL4*-positive CA-projecting neurons to express *Dh44-R2* gene. To

207   test this idea, we used the *Dh44-R2* knock-in T2A-GAL4 line (Kondo et al., 2020), and found a

208   group of neurite termini, labeled with GFP of *Dh44-R2-T2A-GAL4>UAS-mCD8::GFP*, to be

209   located at the CA in the adult stage (Figure 1d). These results clearly suggested that neurons

210   expressing *Dh44-R2* project to the CA.

211   Since Dh44-R2 is a receptor for the neuropeptide Dh44, CA-projecting *Dh44-R2*-

212   expressing neurons might receive a neuronal input from Dh44 neurons. To confirm the

213   anatomical relationship of the ligand-expressing neurons with receptor-expressing neurons, we

214   performed co-labeling experiment using *R65C11–LexA* and *R18A04-GAL4*. Since *R65C11–LexA*

215   is a fly strain that expresses *LexA* transgene under control of *Dh44* promoter, hereafter, we refer

216   to *R65C11–LexA* as *Dh44-LexA*. We found both *Dh44-LexA*-labeled and *R18A04-GAL4*-labeled

217   neurites to be in close proximity in the SEZ of the adult brain (Figure 2a). The GFP

218   Reconstitution Across Synaptic Partners (GRASP) method (Feinberg et al., 2008) was used to

219   confirm whether the neurons were close enough in proximity to form synapses. As expected, we

220   observed reconstituted GFP fluorescence at the sites where neurites were observed (Figure 2a,b).

221   These results suggested that the CA-projecting *Dh44-R2*-expressing neurons have direct synaptic

222   contact with the upstream Dh44 neurons.

223

224   **The corpora allata-projecting *Dh44-R2*-expressing neurons produce the neuropeptide**

225   **hugin**

226   We next investigated which neurotransmitter is utilized in the CA-projecting *Dh44-R2*-

227   expressing neurons. For this analysis, we focused on a subset of neurons producing the

228   neuropeptide Hug, which is synthesized in approximately 20 neurons, whose cell bodies are

229   located in the SEZ of the *D. melanogaster* brain (Melcher and Pankratz, 2005; Meng et al., 2002).

230   Previous studies had reported that there are several clusters of Hug neurons, each of which

231   projects to various regions, including not only the pars intercerebralis and ventral nerve cord but

232   also the CC-CA complex in the adults (King et al., 2017; Melcher and Pankratz, 2005). However,

233   the previous study had not clarified which endocrine organs the Hug neurons project to, the CA

234   or CC. Through our anatomical analysis of *R18A04-GAL4* and *Dh44-R2-T2A-GAL4* flies, we

235   hypothesized that overall morphology of the CA-projecting *Dh44-R2*-expressing neurons is

236   similar to that of the CA-projecting Hug neurons. To test this hypothesis, we simultaneously

237   labeled both *Dh44-R2*-expressing neurons and Hug neurons with *R18A04-GAL4-* or *Dh44-R2-*

238   *T2A-GAL4*-driven red fluorescence protein (RFP) and *hug* promoter-driven yellow fluorescence

239   protein (YFP), respectively. We found that, with either of *R18A04-GAL4* or *Dh44-R2-T2A-GAL4*,

240   GAL4-driven RFP signal was observed in the cell bodies of *hug*-promoter-driven YFP signal

241   (Figure 3a,c). Moreover, we observed both YFP and RFP signals in the identical axon terminals

242   located on the CA (Figure 3b,d). Together, the results suggest that the CA-projecting *Dh44-R2*-

243   expressing neurons are indeed Hug-positive.

244   To confirm whether the Dh44 neurons are located upstream of the Hug neurons, we

245   performed the same experiments as in Fig. 2 using *Dh44-LexA* along with *hugS3-GAL4* strain, in

246   which *GAL4* is expressed downstream of the *hug* promoter (Melcher and Pankratz, 2005). We

247   found the neurites of Hug neurons labeled with *hugS3-GAL4* to be closely located to those of the

248   Dh44 neurons labeled with *Dh44-LexA* (Figure 3e). Furthermore, GRASP experiments showed a

249   reconstituted GFP fluorescence at the sites where both Hug and Dh44 neurites were observed

250   (Figure 3f). The results, therefore, suggest that the CA-projecting Hug neurons have direct

251   synaptic contact with the upstream Dh44 neurons.

252

253   **The corpora allata-projecting hugin neurons may be dispensable for juvenile hormone**

254   **biosynthesis**

255   As shown above, *R18A04-GAL4* line was active in at least 14 Hug neurons in the SEZ (Figure

256   3a). Given the fact that Hug neurons divide into multiple subpopulations that project to distinct

257   regions (Schlegel et al., 2016), we expected a few, not all, of these Hug neurons to project to the

258   CA. This expectation was supported by our anatomical observation using another GAL4 line that

259   we identified during our FlyLight image screen, namely *R37F05-GAL4*, which is under the

260   control of a small enhancer region of retinal degeneration A gene. We found that, while *R37F05-*

261   *GAL4* was active in several neurons, only 2 pairs of the neurons were merged with *hug*

262   promoter-driven fluorescence signal (Figure 4a). Importantly, morphology of the *R37F05-GAL4*

263   neurite termini on the CA was identical to that of the CA-projecting Hug neurons (Figure 4b).

264   Moreover, *R37F05-GAL4*-driven synaptotagmin-GFP (Syt1::GFP), a synaptic vesicle marker,

265   was observed on the CA while *R37F05-GAL4*-driven dendritic marker DenMark was not (Figure

266   4c). These results strongly suggest that only the 2 pairs of Hug neurons, not the rest, project to

267   the CA in adults.

268   Further, we realized by chance that another GAL4 line, namely *R19D04-GAL4*, which is

269   under the control of a small enhancer region of *asense* gene, also labeled the 2 pairs of CA-

270   projecting Hug neurons in the adult (Figure 4d) while we did not focus on *R19D04-GAL4* line at

11

271   our initial FlyLight image screen. We confirmed that *R19D04-GAL4*-positive neurons indeed

272   project to the CA (Figure 4e).

273   Since *R19D04-GAL4* is active in fewer neurons than other GAL4 lines used in this study,

274   we utilized *R19D04-GAL4* to manipulate neuronal activity of the CA-projecting Hug neurons in

275   the following experiment. We expressed the gene encoding temperature-sensitive dynamin

276   (*shibire^{ts}*) specifically in the CA-projecting Hug neurons and inhibited their neuronal activity

277   specifically in the adult stage at a restrictive temperature (Kitamoto, 2001). We collected mRNA

278   from these flies and examined expression of the *Kr-h1* gene; *Kr-h1* is well known as a

279   downstream target of JH receptors and its expression level correlates well with hemolymph JH

280   titer level (Minakuchi et al., 2009). However, we found the inhibition of CA-projecting Hug

281   neurons to not significantly alter *Kr-h1* expression compared to that in the controls (Figure 5a).

282   We also took another approach to examine whether Hug signaling is involved in the

283   regulation of JH biosynthesis in the CA. A previous study had identified 2 specific receptors for

284   Hug, namely Pyrokinin 2 Receptor 1 (PK2-R1) and Pyrokinin 2 Receptor 2 (PK2-R2) (Park et al.,

285   2002). To examine whether these receptors are expressed in the CAs, we performed cell-specific

286   labeling using *PK2-R1-T2A-GAL4* and *PK2-R2-T2A-GAL4* lines. However, we could not

287   observe any significant *T2A-GAL4*-driven GFP fluorescence signal in the CA (Figure 5b,c). In

288   conjunction with our observation regarding *Kr-h1* expression (Figure 5a), these results suggested

289   the CA-projecting Hug neurons to be dispensable for JH biosynthesis; they may rather play a

290   role in systemic function of *D. melanogaster*, which will be discussed later.

291

292   **Discussion**

293   In this study, we anatomically characterized the CA-projecting Hug neurons in *D. melanogaster*.

294   Hug was originally identified as a myostimulatory and ecdysis-modifying neuropeptide (Meng et

295   al., 2002); further studies have demonstrated that it plays a central role in integrating external

296   and internal feeding-relevant information to control feeding behavior (Bader et al., 2007;

297   Hückesfeld et al., 2016; Melcher and Pankratz, 2005; Melcher et al., 2006; Melcher et al., 2007;

298   Schoofs et al., 2014; Surendran et al., 2017). More recently, Hug has been found to regulate

299   circadian rhythm in *D. melanogaster* (King et al., 2017). To understand the role of Hug in *D.*

300   *melanogaster* physiology and behavior, neuronal circuits and synaptic connections of Hug

301   neurons have been intensively studied (Bader et al., 2007; Melcher and Pankratz, 2005; Schlegel

302   et al., 2016). Regarding the anatomical relationship between Hug neurons and the CA, the former

303   have been reported to project to the complex including the CA and CC in the adult stage

304   (Melcher and Pankratz, 2005), and to the CC in the larval stage (Hückesfeld et al., 2020).

305   However, these studies have not precisely examined whether a part of Hug neurons project to the

306   CA. To the best of our knowledge, this is the first study to identify a specific neurotransmitter

307   that is produced in the CA-projecting neurons of *D. melanogaster*, and to anatomically

308   characterize a neuronal relay to the CA-projecting neurons from the upstream interneurons.

309   Based on our anatomical observation, we expected the CA-projecting Hug neurons to be

310   involved in the regulation of JH biosynthesis in the CA. However, our genetic analysis till date

311   has not validated this expectation. Particularly, to our surprise, either of the 2 identified Hug

312   receptor genes, *PK2-R1* and *PK2-R2*, appeared not to be expressed in the CA. Although it has

313   been reported that another receptor called PK1-R is potentially activated by Hug in a

314   heterologous cell culture experiment (Cazzamali et al., 2005), the half-maximal effective

315   concentration of hug for PK1-R is more than 10 μM (Park et al., 2002). Therefore, we have not

316   examined the involvement of PK1-R in this study. Alternatively, an unknown Hug receptor may

317   still possibly exist and its gene expressed in the CA. Nevertheless, our data implied that Hug

318   released from the CA-projecting Hug neurons is not locally received by the CA cells, and rather

319   received by other tissues apart from the CA. In this scenario, the CA-projecting Hug neurons

320   would secrete Hug from the synaptic termini on the CA to hemolymph. Such systemic release of

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321 neuropeptides from the CA is already known in the silkworm *Bombyx mori*, since *B. mori* PTTH

322 neurons project to the CA instead of the PG, and the secreted PTTH from the CA is circulated in

323 the hemolymph and then received by the PG cells (Mizoguchi et al., 1990). Whether the CA-

324 projecting neuron-derived Hug is released to hemolymph and, if so, how essential the secreted

325 Hug is for regulating *D. melanogaster* biological functions, other than JH biosynthesis, would be

326 worth investigating.

327 Our current data suggest that the CA-projecting Hug neurons have synaptic connections

328 with the upstream Dh44 neurons. Dh44 is a crucial neuropeptide that regulates the rest:activity

329 rhythms and sleep in *D. melanogaster* (Cavanaugh et al., 2014). In addition, Dh44 receptor 1

330 (*Dh44-R1*), though not *Dh44-R2*, is involved in the regulation of circadian locomotor activity

331 and is expressed in Hug neurons in the SEZ of *D. melanogaster* (King et al., 2017). Therefore, it

332 is feasible to think that, analogous to the neuronal relay from the Dh44 neurons to *Dh44-R1*-

333 expressing neurons, *Dh44-R2*-expressing neurons might be involved in the regulation of

334 circadian rhythms and/or sleep. Of note, JH is involved in the regulation of sleep behavior in *D.*

335 *melanogaster* (Wu et al., 2018) and of circadian rhythms in the bumble bee *Bombus terrestris*

336 (Pandey et al., 2020). Alternatively, since Dh44 neurons are also important for the sensing

337 mechanisms of dietary sugar and amino acids (Dus et al., 2015; Yang et al., 2018), the CA-

338 projecting Hug neurons might play a role in nutritional sensing and metabolism. JH biosynthesis

339 has also been suggested to be dependent on the nutritional status of some insects (Badisco et al.,

340 2013).

341 We should also note that the CA-projecting Hug neurons that we identified might be

342 different from the previously identified CA-projecting neurons, namely CA-LP1 and CA-LP2, in

343 *D. melanogaster* (Siegmund and Korge, 2001). While the cell bodies of the CA-projecting Hug

344 neurons are located in the SEZ, those of CA-LP1 and CA-LP2 are placed in the lateral

345 protocerebrum (Siegmund and Korge, 2001). Although the role of CA-LP1 and/or CA-LP2

14

346   might suppress JH biosynthesis in the CA to regulate epithelial morphogenesis of male genitalia

347   during pupal stage (Ádám et al., 2003), which neurotransmitters are produced in CA-LP1 and

348   CA-LP2 have not been elucidated yet. In future, identities of these CA-projecting neurons should

349   be analyzed, and the functional relationship across CA-LP1, CA-LP2, and Hug neurons should

350   be investigated to understand the molecular and neuronal mechanisms of JH biosynthesis.

351

Competing interests

353   The authors declare that the research was conducted in the absence of any commercial or

354   financial relationships that could be construed as a potential conflict of interest.

355

366

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519   1025.

520

521

522 **Figure legends**

523 **Figure 1**

524 *Dh44-R2*-expressing neurons project to the CA. (a) The enhancer region of *R18A04-GAL4* and

525 the construct of *Dh44-R2-T2A-GAL4*. Gray, blue, magenta, and green boxes indicate the

526 untranslated region, coding sequence, T2A peptide sequence, and *GAL4* sequence, respectively.

527 (b) The brain-RG complex (left) and the RG (right) of *R18A04-GAL4; UAS*-GFP, *UAS*-

528 *mCD8::GFP* in the 3rd-instar larval stage. Scale bar, 50 μm (left), 25 μm (right). (c) *R18A04-*

529 *GAL4*-positive neurons project to the CA in the adult stage. The brain-proventriculus (PV)

530 complex (left) and the CA (right). Scale bar, 100 μm (left), 25 μm (right). (d) *Dh44-R2-T2A-*

531 *GAL4*-positive neurons project to the CA. The brain-PV complex (left) and the CA (right). Scale

532 bar, 100 μm (left), 25 μm (right).

533

534 **Figure 2**

535 Dh44 neurons and *Dh44-R2* neurons have synaptic contact. (a) *Dh44-R2* neurons are adjacent to

536 Dh44 neurons. Left: Co-staining of Dh44 neurons (magenta) and *Dh44-R2* neurons (green).

537 Scale bar, 25 μm. Right: Schematic diagram showing the cell bodies and neurites of Dh44

538 neurons (magenta) and *Dh44-R2* neurons (green). (b) In GRASP analysis, *spGFP1-10::CD4* was

539 expressed by *R18A04-GAL4*, and *spGFP11::CD4* was expressed by *R65C11–LexA* (middle

540 panel). GRASP signal was detected between *Dh44-R2* neurons and Dh44 neurons (arrow). In

541 contrast, negative controls did not give signals, as shown in left and right panels. Scale bar, 25
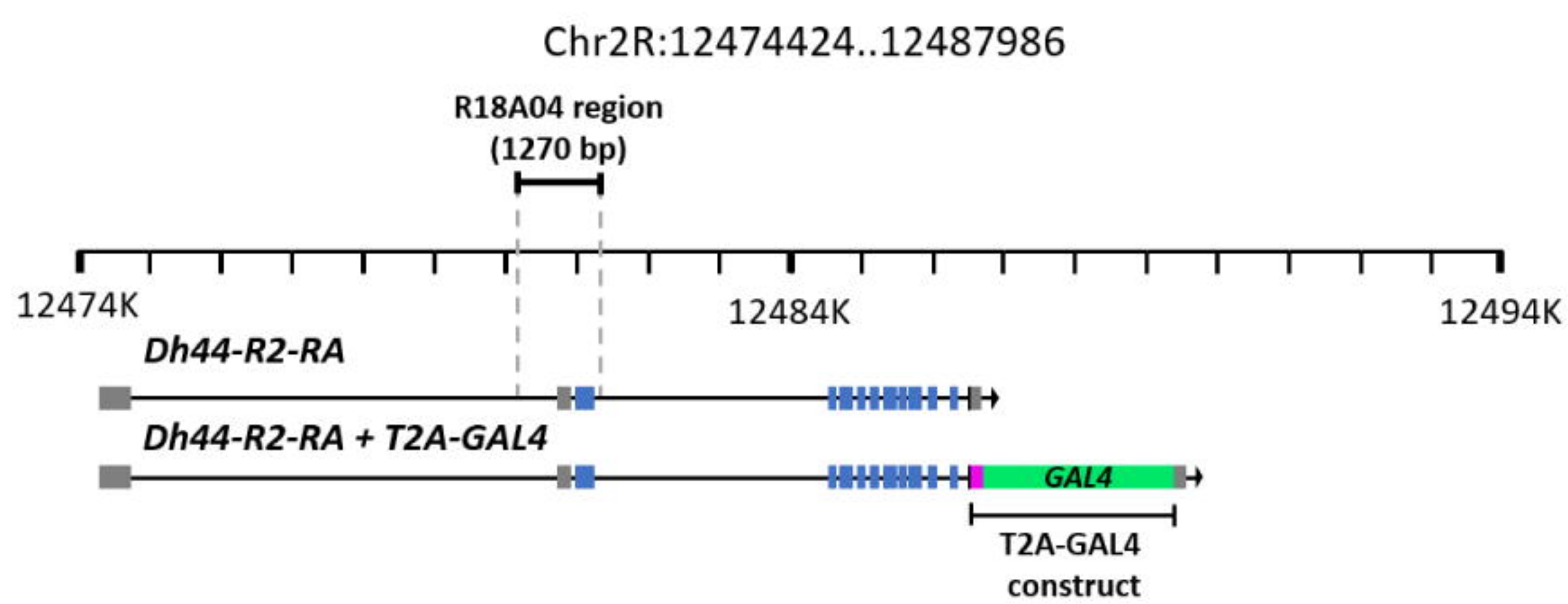
542 μm.

543

544 **Figure 3**

545 The CA-projecting *Dh44-R2*-expressing neurons produce Hug. (a) *R18A04-GAL4; UAS-*

546 *TurboRFP* signals (magenta) are detected in the cell bodies of Hug neurons (green). Scale bar,

23

547   25 μm. (b) RFP signals reflecting *R18A04-GAL4* expression are merged with Hug-YFP signals

548   (green) in the axon terminals. Scale bar, 10 μm. (c) *Dh44-R2-T2A-GAL; UAS-TurboRFP* signals

549   (magenta) are detected in the cell bodies of Hug neurons (green). Scale bar, 25 μm. (d) RFP

550   signals reflecting *Dh44-R2* expression are merged with Hug-YFP signals (green) in the axon

551   terminals. Scale bar, 10 μm. (e) Hug neurons are adjacent to Dh44 neurons. Left: Co-staining of

552   Dh44 neurons (magenta) and Hug neurons (green). Scale bar, 25 μm. Right: Schematic diagram

553   showing the cell bodies and neurites of Dh44 neurons (magenta) and Hug neurons (green). (f) In

554   GRASP analysis, *spGFP1-10::CD4* was expressed by *hugS3-GAL4*, and *spGFP11::CD4* was

555   expressed by *R65C11–LexA* (middle panel). GRASP signal was detected between Hug neurons

556   and Dh44 neurons (green, arrow). In contrast, negative controls did not give signals, as shown in

557   left and right panels. Scale bar, 25 μm.

558

559   **Figure 4**

560   Two pairs of Hug neurons project to the CA. (a) *R37F05-GAL4; UAS-TurboRFP* signals

561   (magenta) are detected in the cell bodies of Hug neurons (green). Scale bar, 25 μm. (b) RFP

562   signals reflecting *R37F05-GAL4* expression are merged with Hug-YFP signals (green) in the

563   axon terminals. Scale bar,10 μm. (c) A synaptic vesicle marker Syt1::GFP, but not a dendritic

564   marker Denmark, was observed on the CA. *Syt1::GFP* and *DenMark* were expressed by

565   *R37F05-GAL4*. Scale bar, 25 μm. (d) Cell bodies of the CA-projecting Hug neurons are

566   overlapped with those of *R19D04-GAL4*-positive neurons. Scale bar, 25 μm. (e) *R19D04-GAL4;*

567   *UAS-GFP,* and *UAS-mCD8::GFP* neurons (green) are merged with Hug neurons (magenta).

568   Anti-Hug antibody labeled not only the CA-projecting neurons but also neurons projecting to

569   regions other than the CA (arrowhead). Scale bar, 10 μm.

570

571   **Figure 5**

24

572   The CA-projecting Hug neurons might not be required for JH biosynthesis. (a) The *shibire^{ts}*-

573   mediated neuronal inhibition of CA-projecting Hug neurons could not change the expression

574   level of *Kr-h1*. Student's *t*-test with Bonferoni's collection was used for this data. ***p ≤ 0.001,

575   **p ≤ 0.01, and *p ≤ 0.05; n.s., non-significant (*p* > 0.05). (b, c) GFP signals reflecting *PK2-R1*

576   (b) and *PK2-R2* (c) expression are not detected in the CA. Scale bar, 25 μm.

Fig. 1

(a)

Chr2R:12474424..12487986

R18A04 region
(1270 bp)

12474K   12484K   12494K

Dh44-R2-RA

Dh44-R2-RA + T2A-GAL4

GAL4

T2A-GAL4
construct

(b)
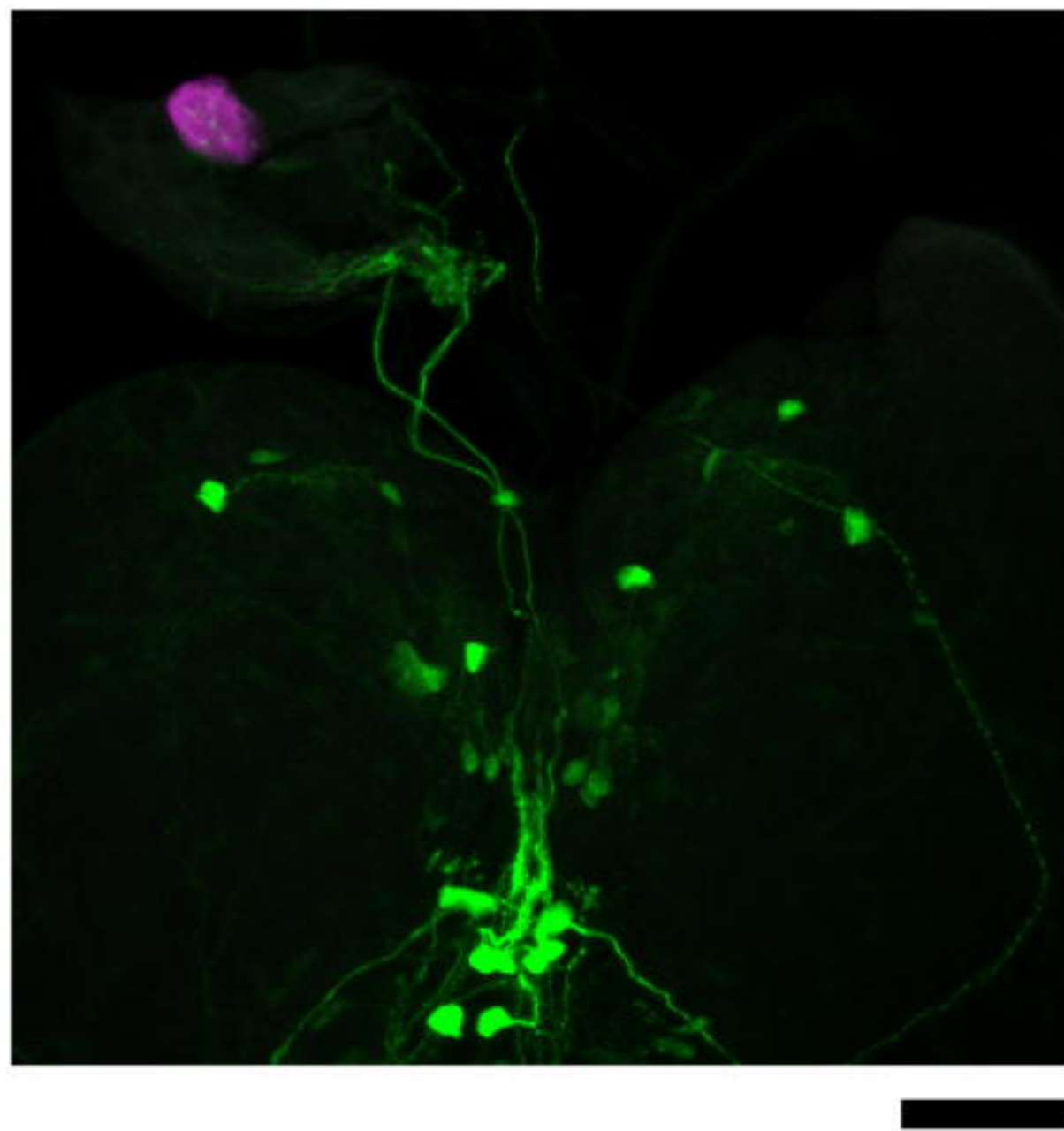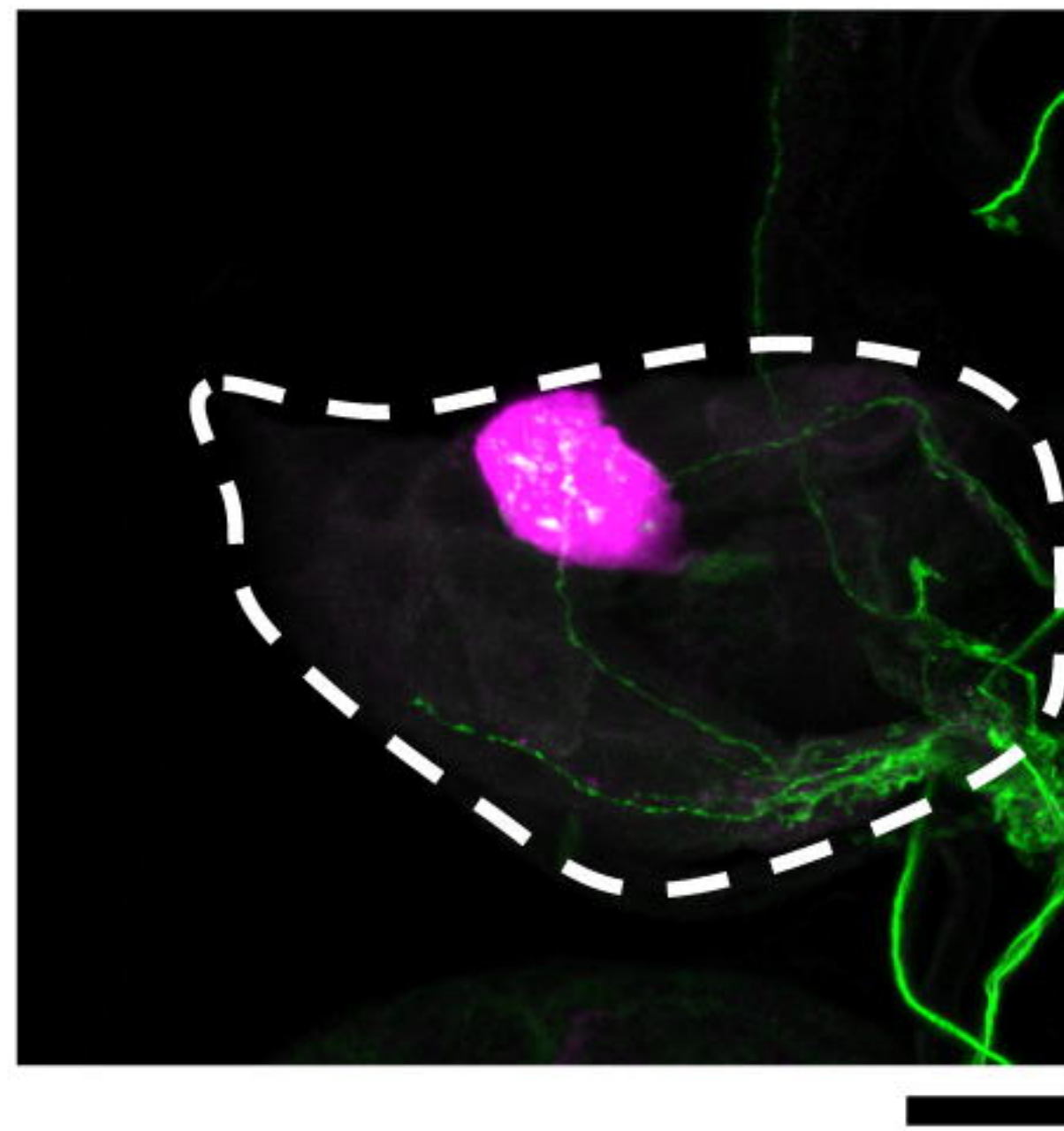
JHAMT GFP   JHAMT GFP

R18A04 > GFP, mCD8::GFP

(c)

JHAMT GFP   JHAMT GFP
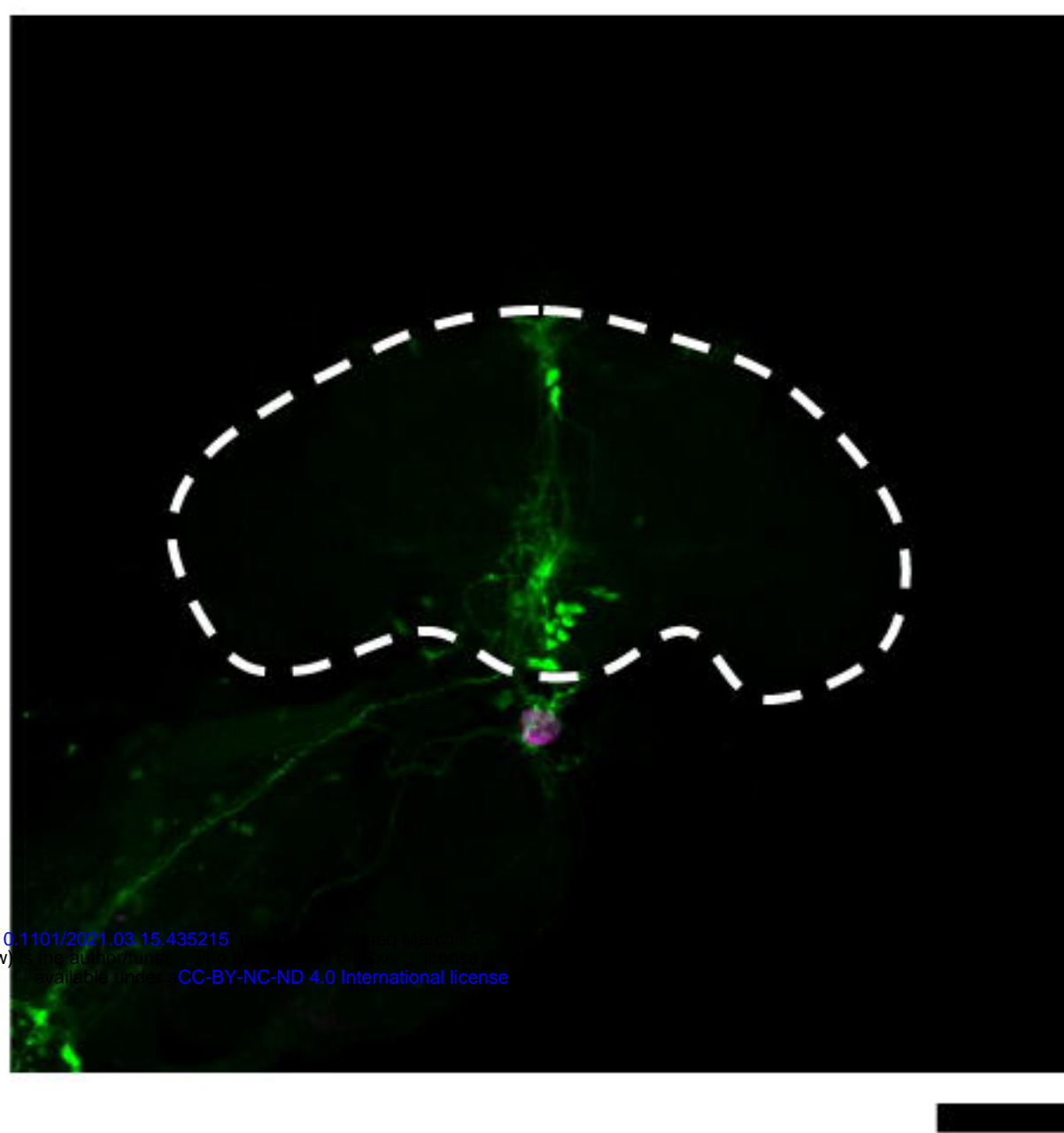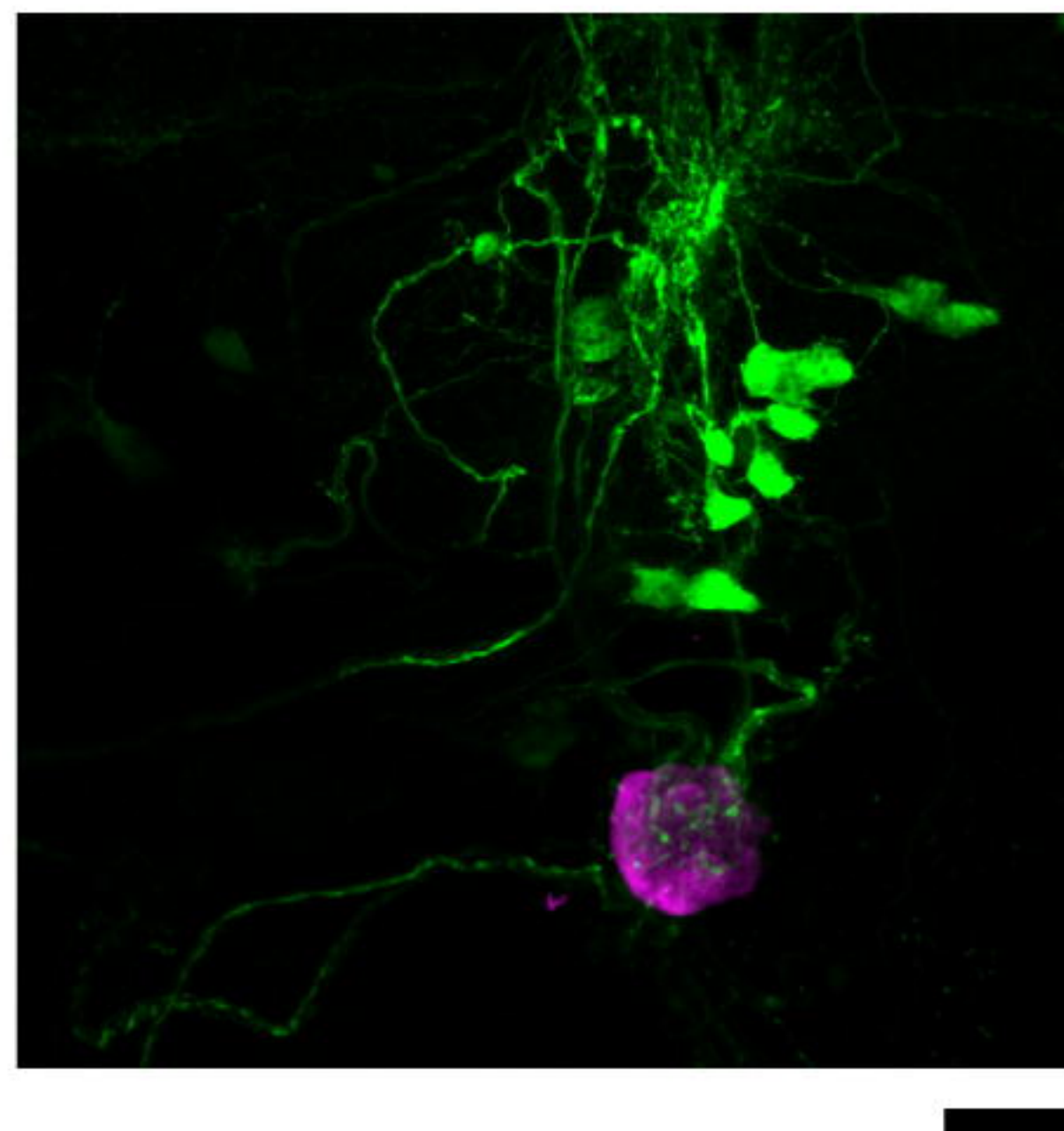
R18A04 > GFP, mCD8::GFP

(d)
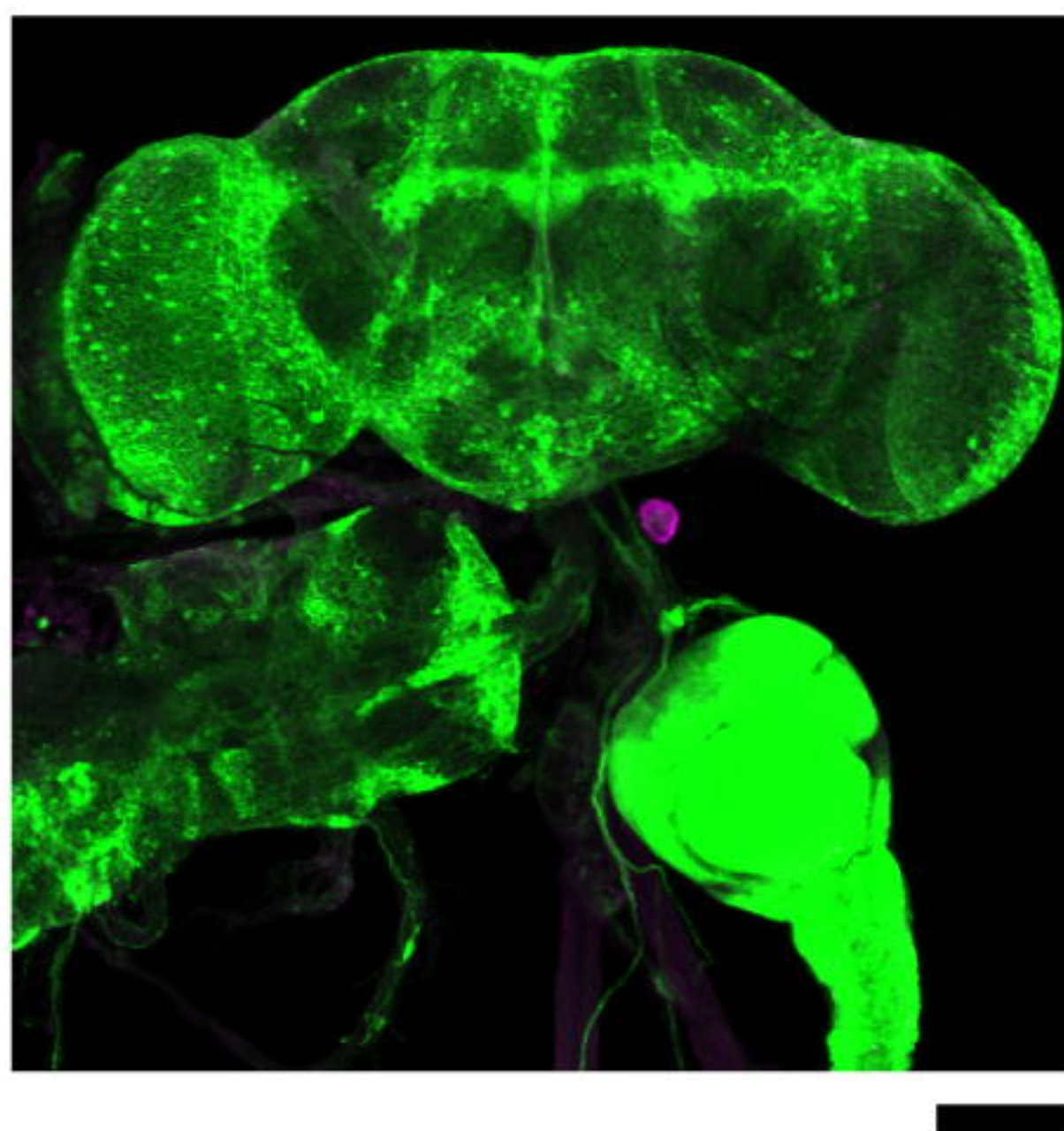
JHAMT GFP   JHAMT GFP
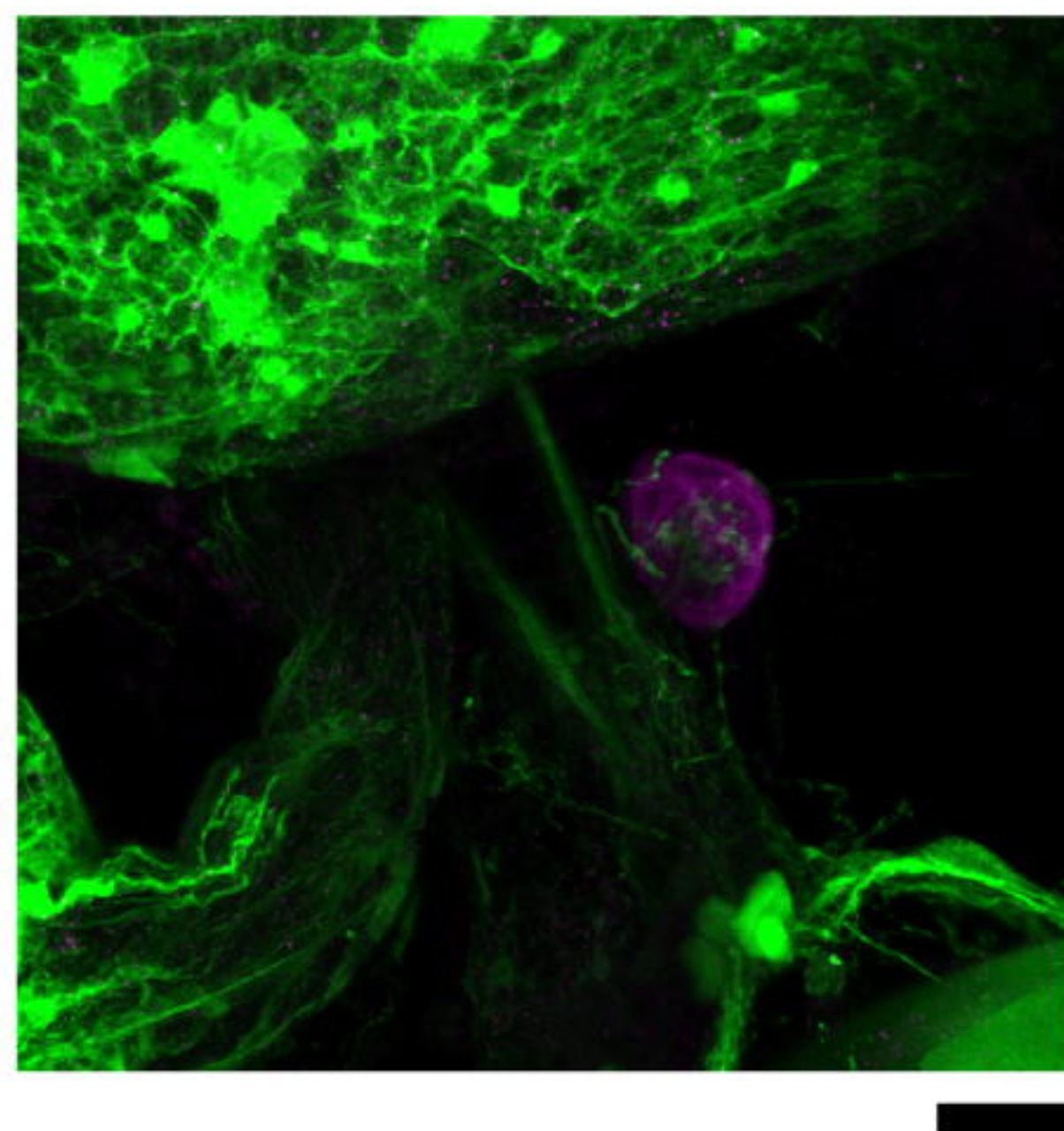
Dh44-R2-T2A > GFP, mCD8::GFP

Fig. 2

(a)

GFP mCherry

Dh44 neurons

Dh44-R2-expressing neurons
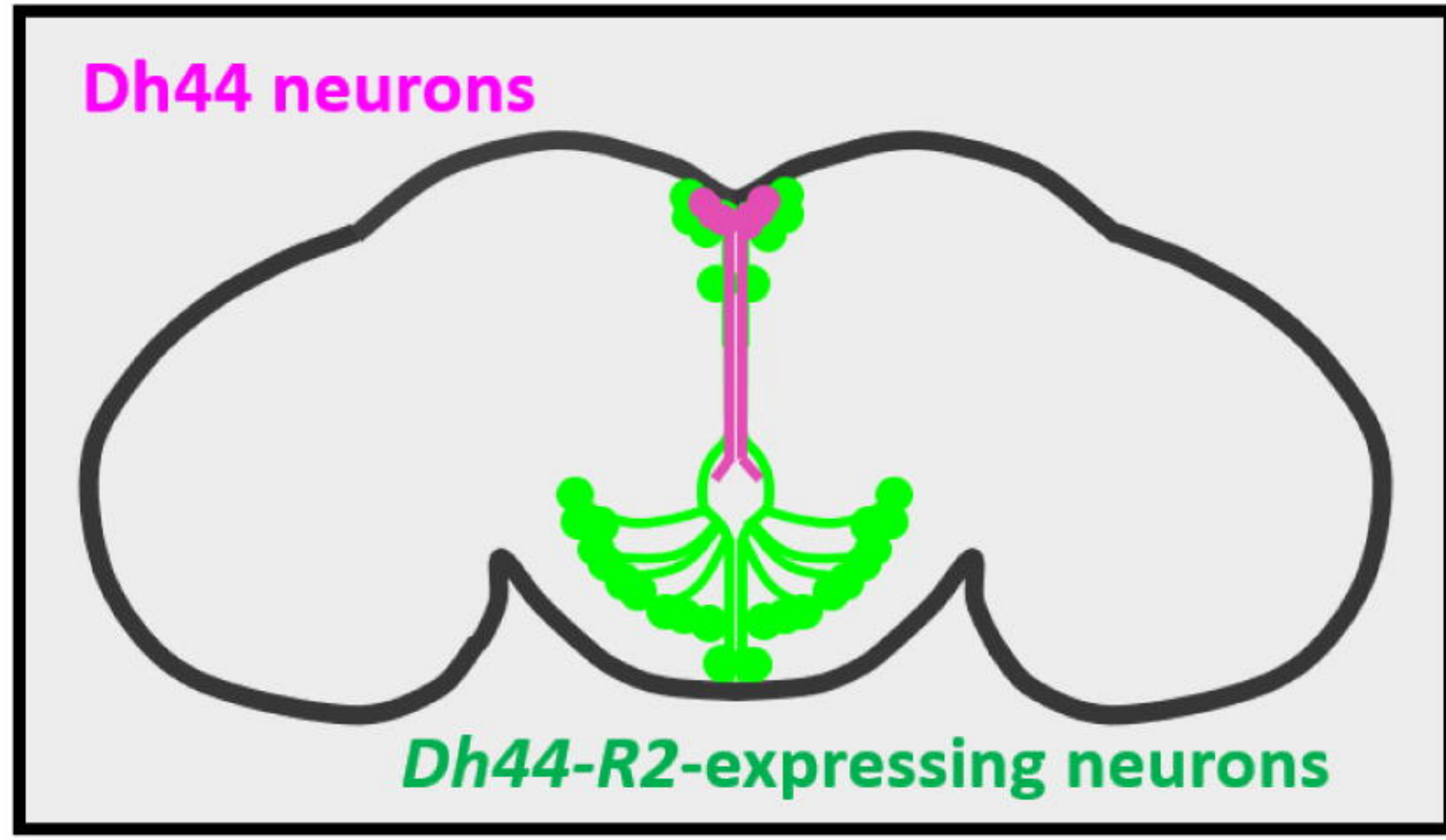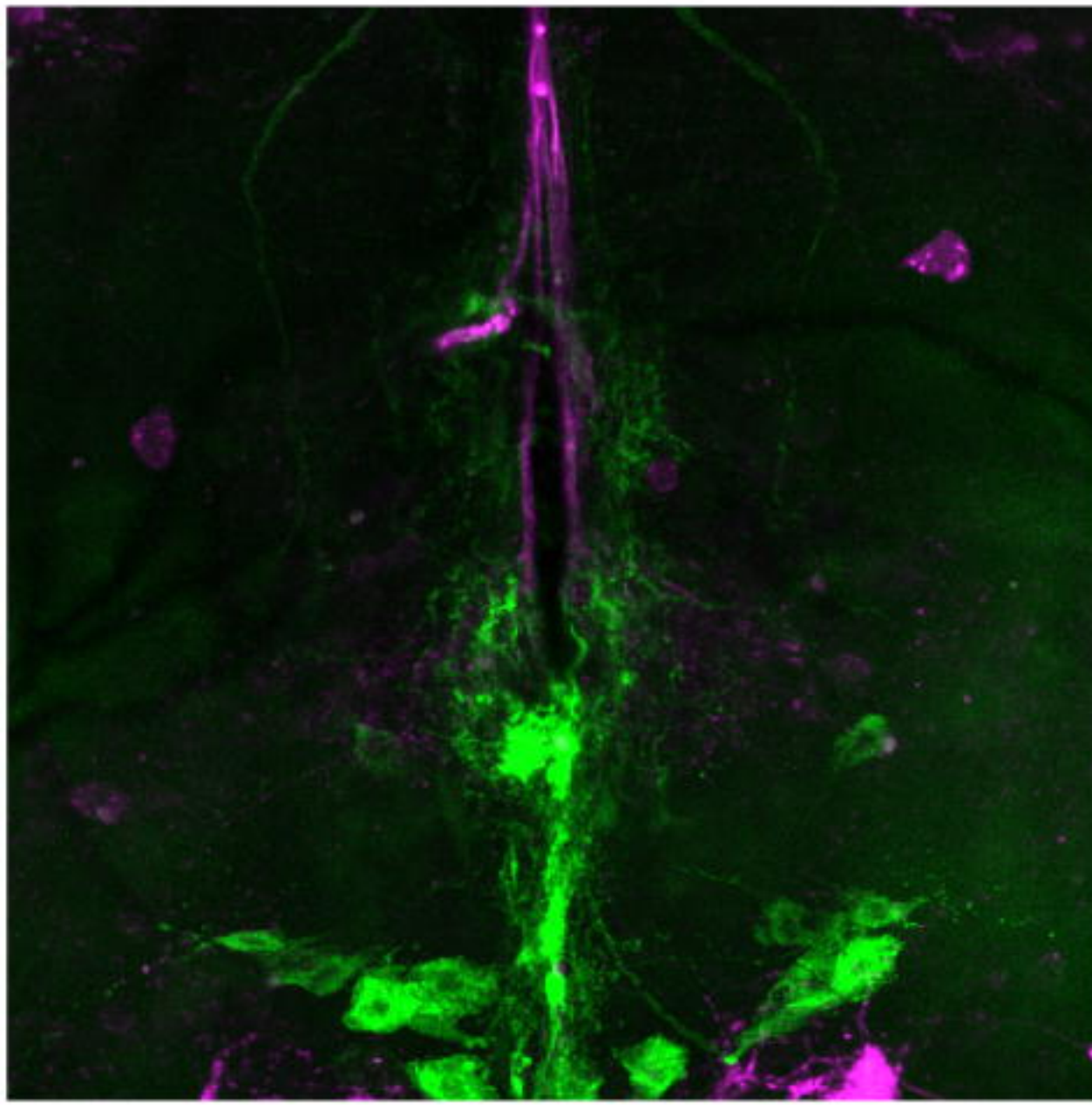
R18A04-GAL4 > UAS-mCD8::GFP, R65C11-LexA > LexAop-mCherry

(b)

GRASP
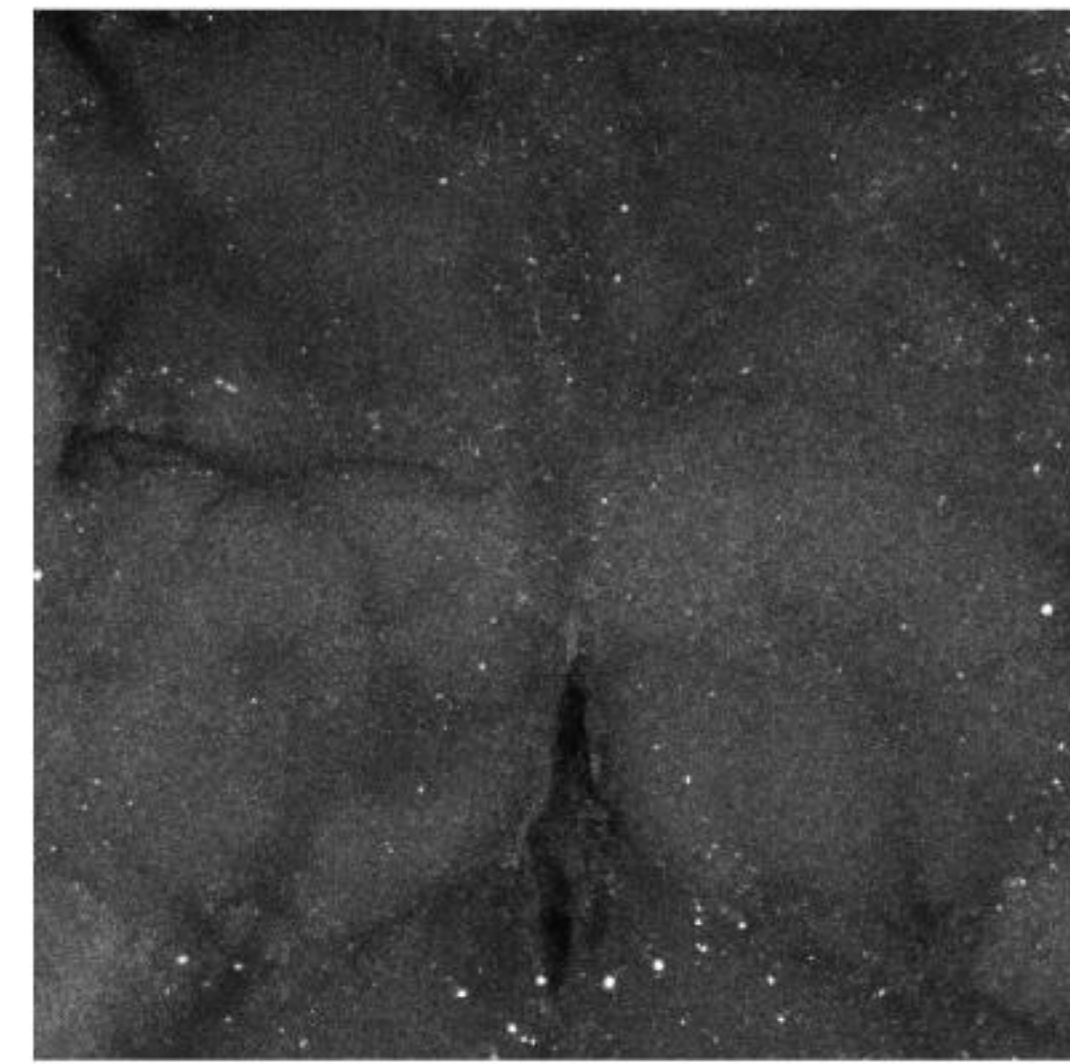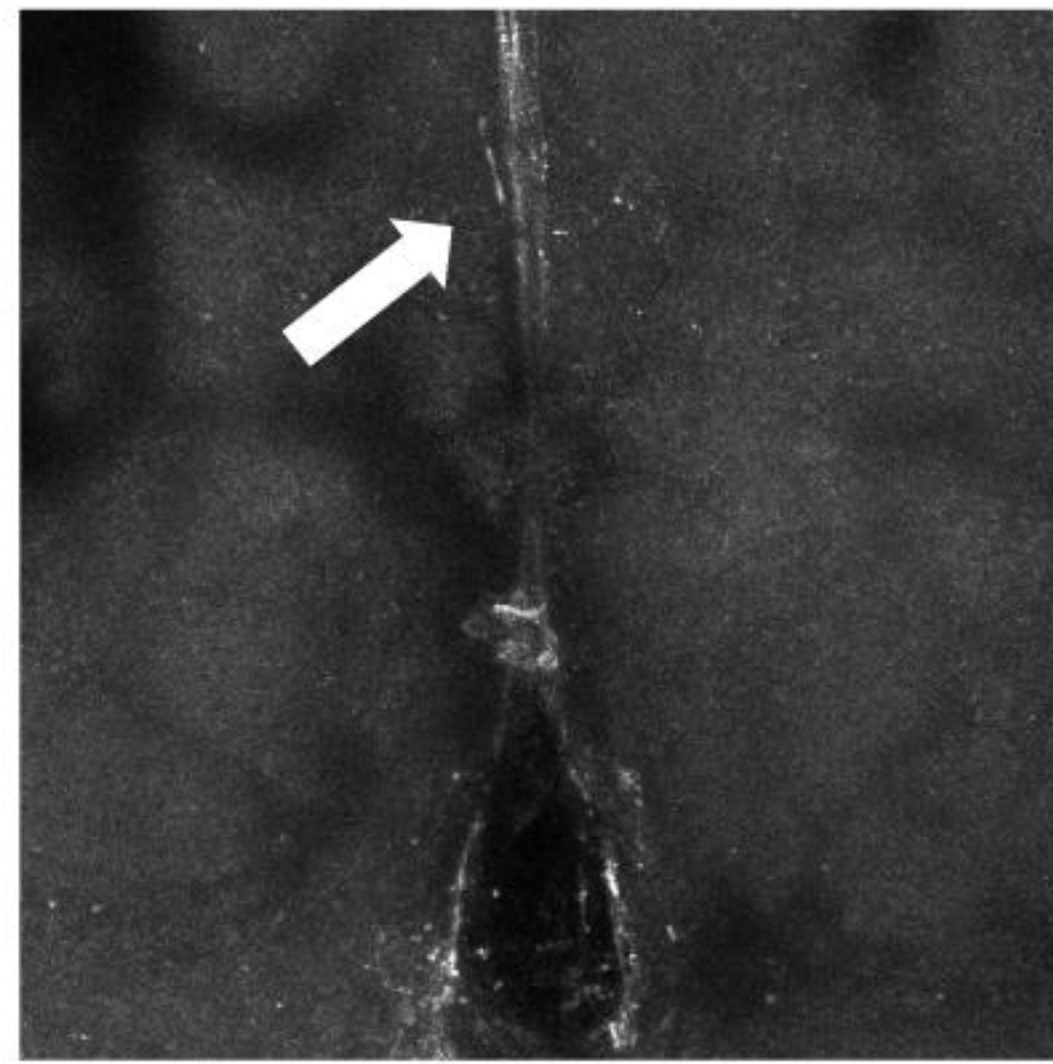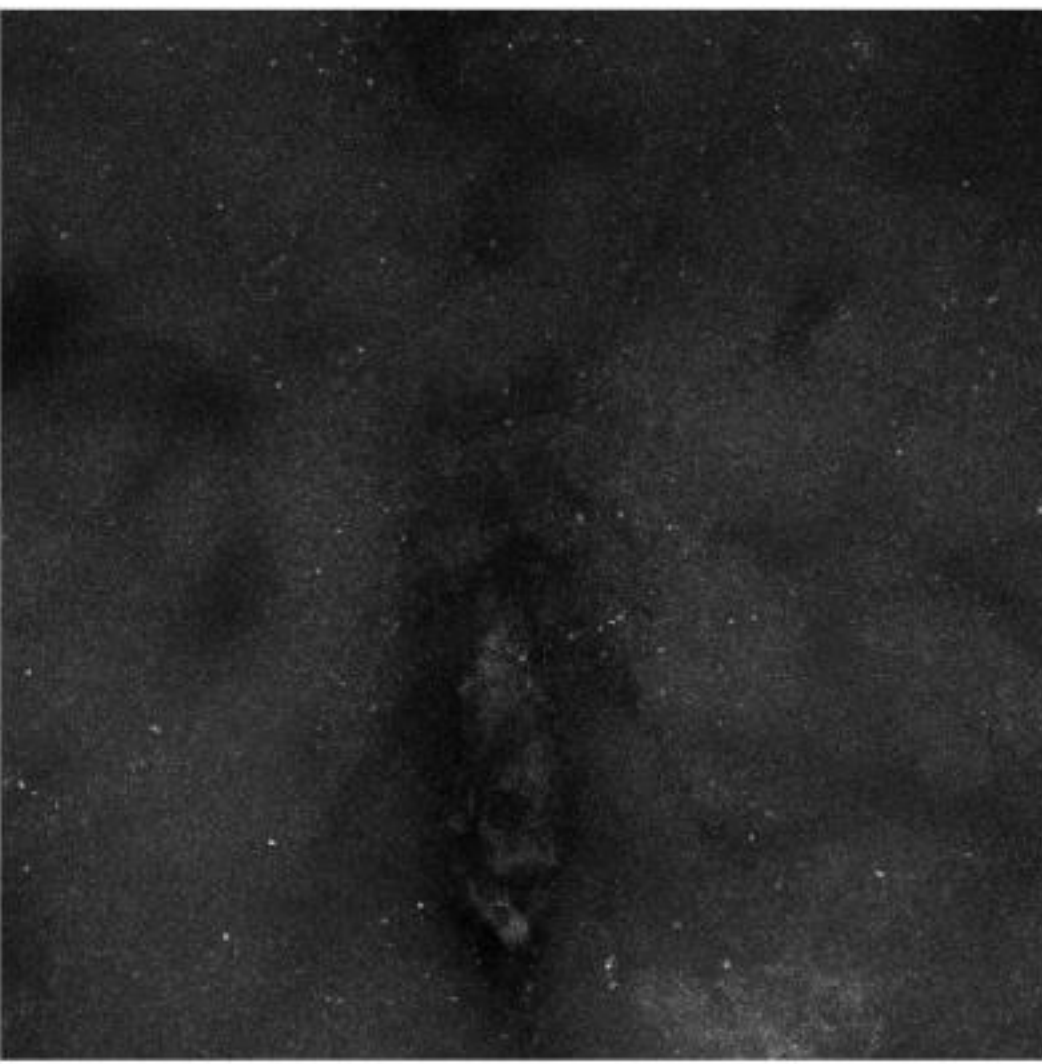
*R18A04-GAL4 >
UAS- CD4::sp1-10-GFP*

*R18A04-GAL4 >
UAS- CD4::sp1-10-GFP;
R65C11-LexA >
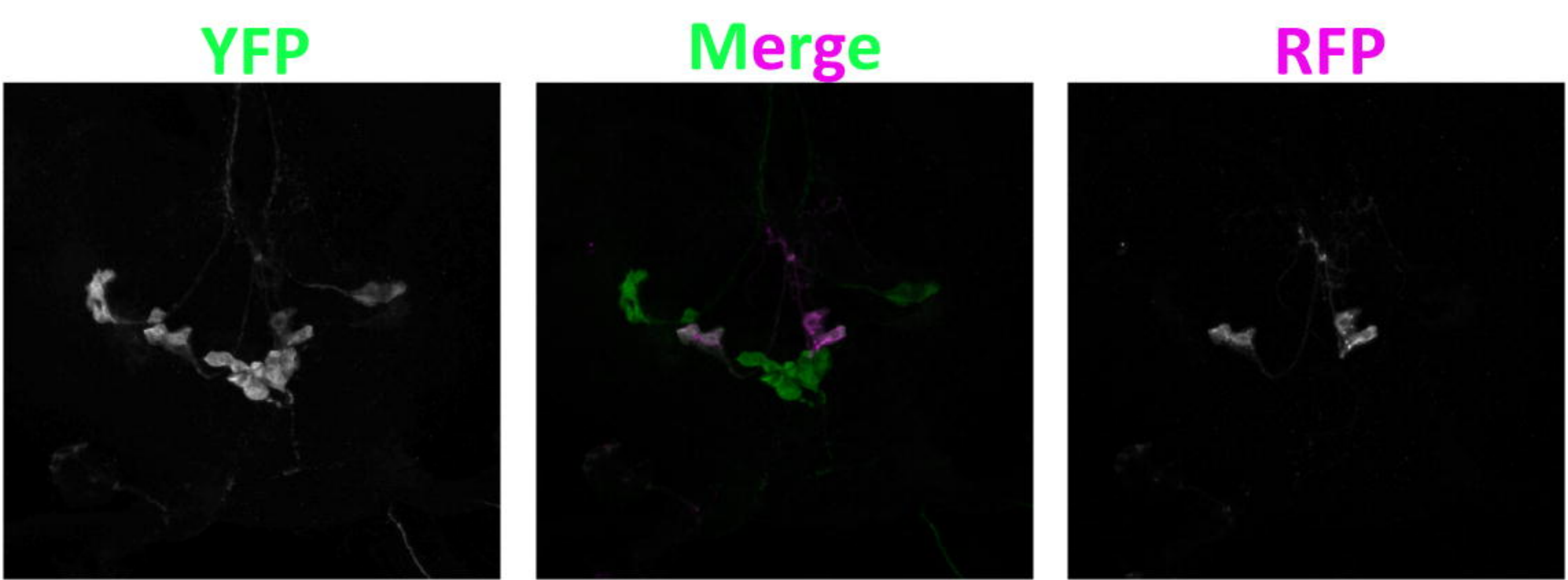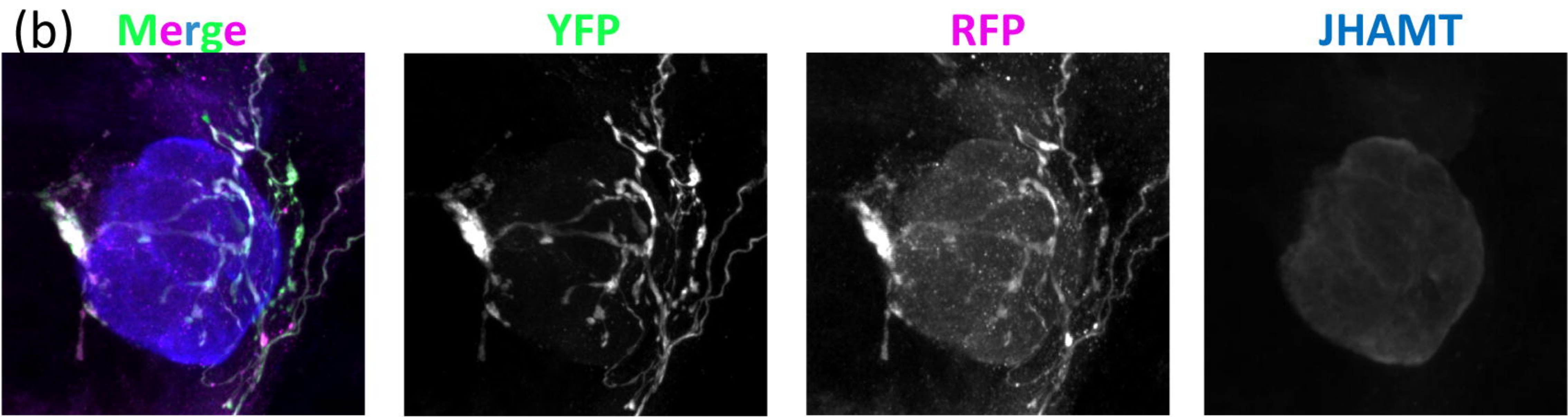LexAOP-CD4::sp11-GFP*

*R65C11-LexA >
LexAOP-CD4::sp11-GFP*

Fig. 3

(a)
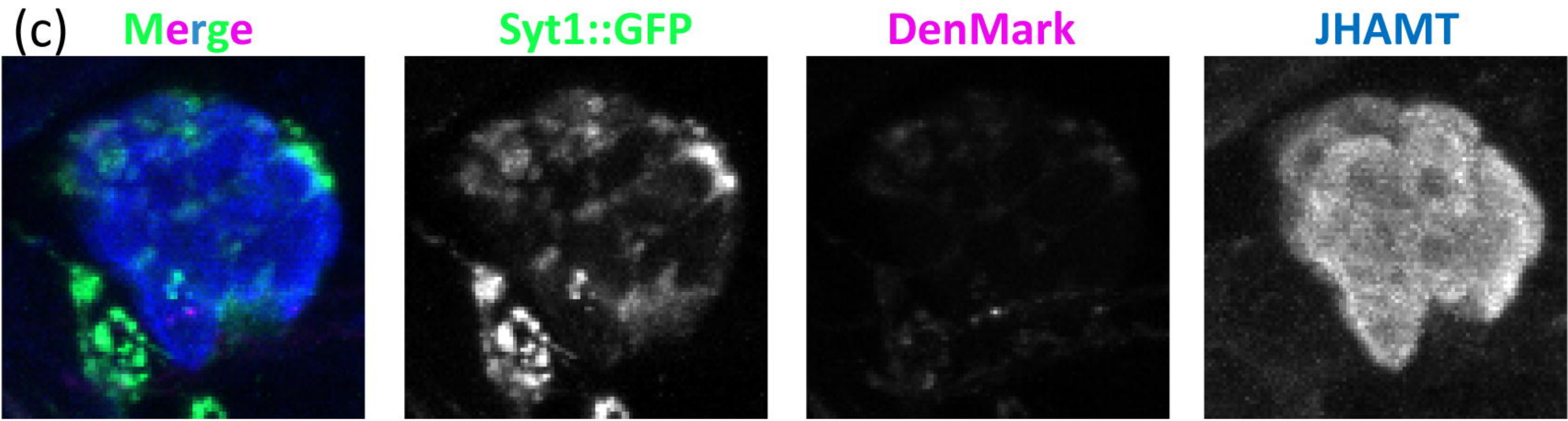

hug-YFP, R18A04 > TurboRFP

(c)

hug-YFP, Dh44-R2-T2A > TurboRFP

(b)

hug-YFP, R18A04 > TurboRFP

(d)

hug-YFP, Dh44-R2-T2A > TurboRFP

(e)

HugS3-GAL4 > UAS-mCD8::GFP, R65C11-LexA > LexAop-mCherry

(f)

HugS3-GAL4 >
UAS- CD4::sp1-10-GFP

HugS3-GAL4 >
UAS-sp1-10-GFP::CD4;
R65C11-LexA >
LexAop-sp11-GFP::CD4

R65C11-LexA >
LexAOP-CD4::sp11-GFP

Fig. 4

(a)

YFP Merge RFP

hug-YFP, R37F05 > TurboRFP

(b)

Merge YFP RFP JHAMT

hug-YFP, R37F05 > TurboRFP

(c)
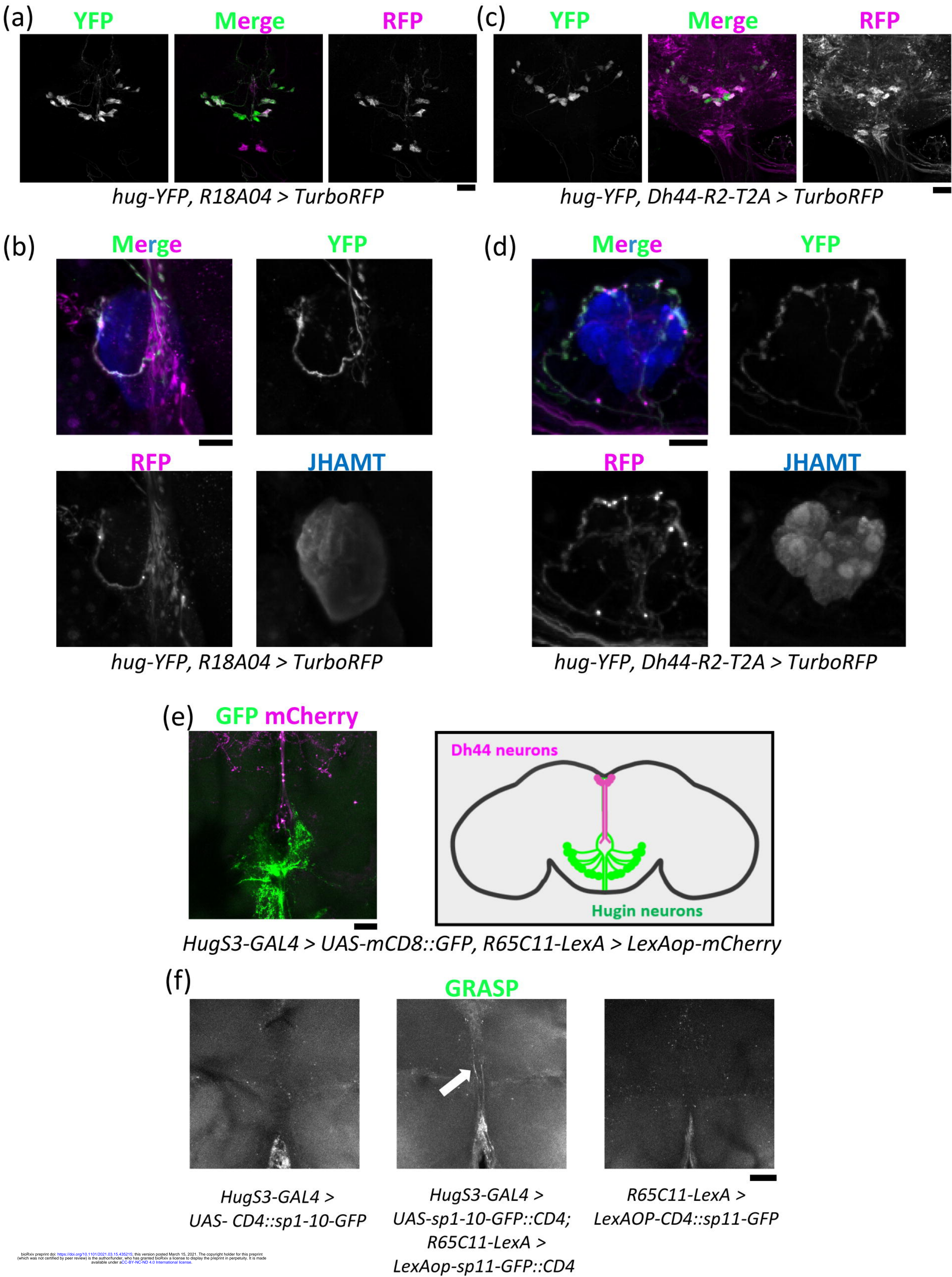
Merge Syt1::GFP DenMark JHAMT

R37F05 > Syt1::GFP, DenMark

(d)
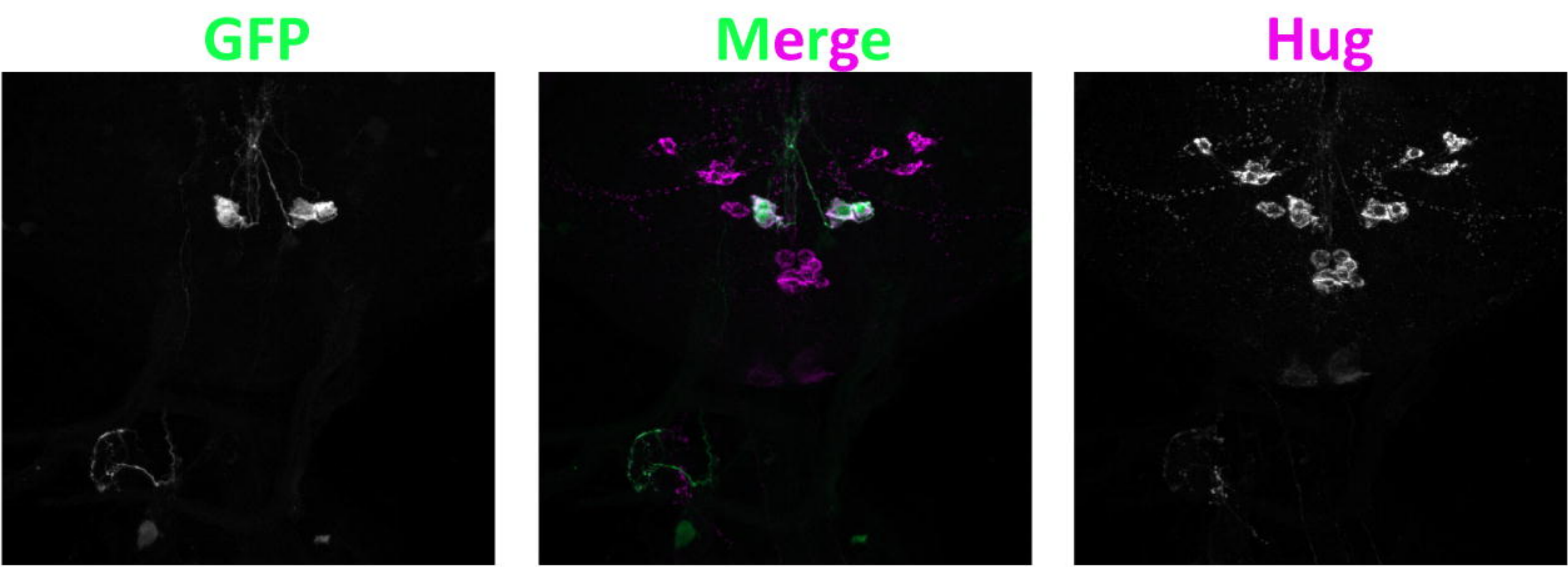
GFP Merge Hug

R19D04 > GFP, mCD8::GFP

(e)

Merge GFP Hug JHAMT

R19D04 > GFP, mCD8::GFP

Fig. 5

(a)

Kr-h1

n.s

n.s

Expression Level

2.0
1.5
1.0
0.5
0.0

R19D04 > ×   Shibire^ts / +   R19D04 > Shibire^ts
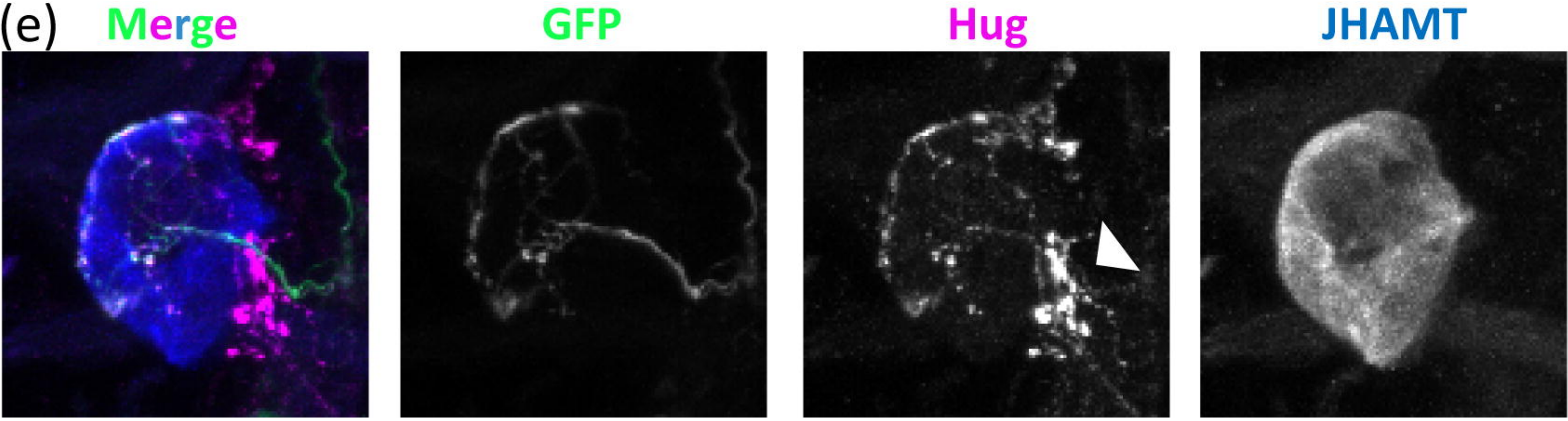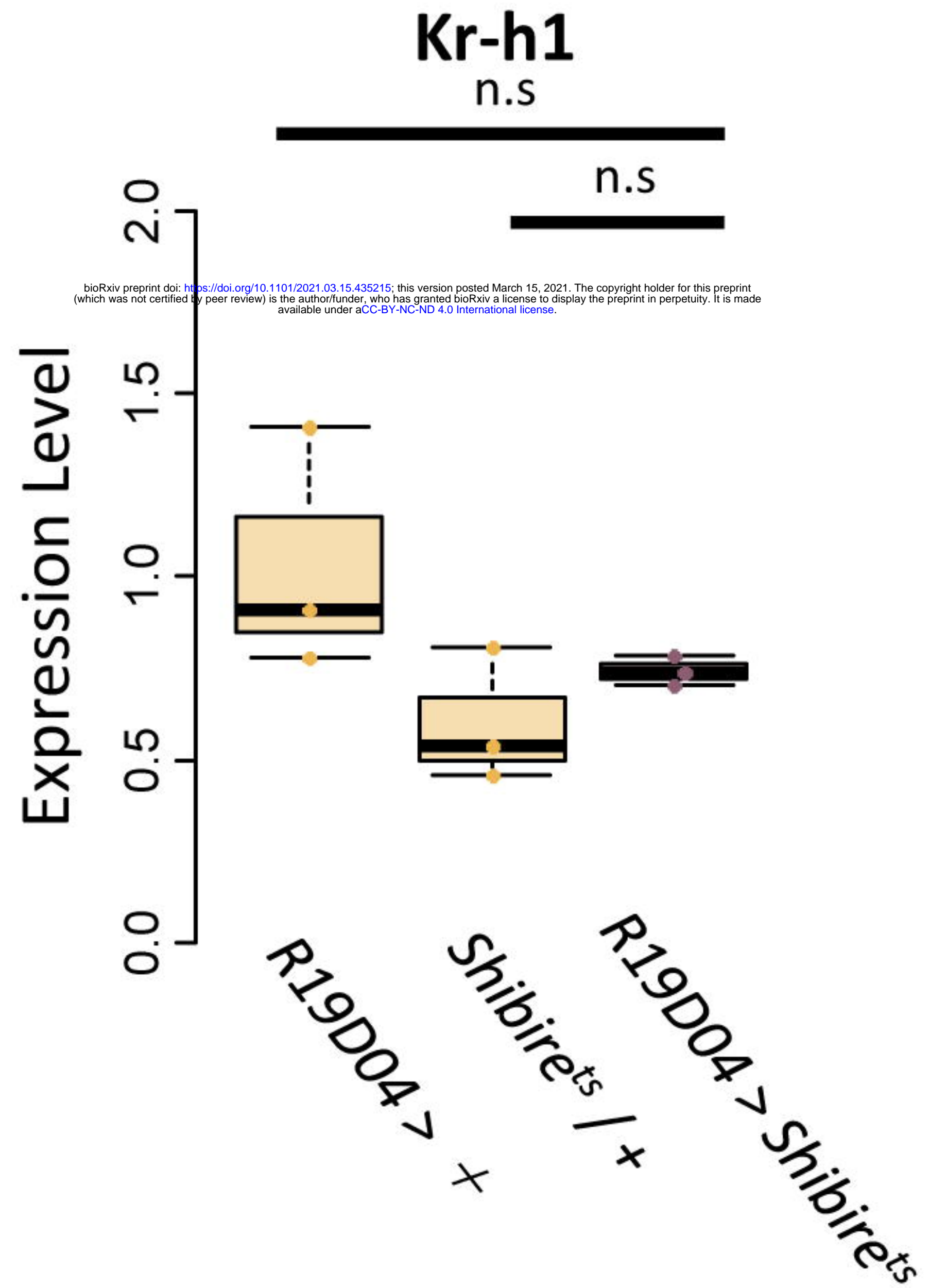
(b) GFP   Merge   JHAMT

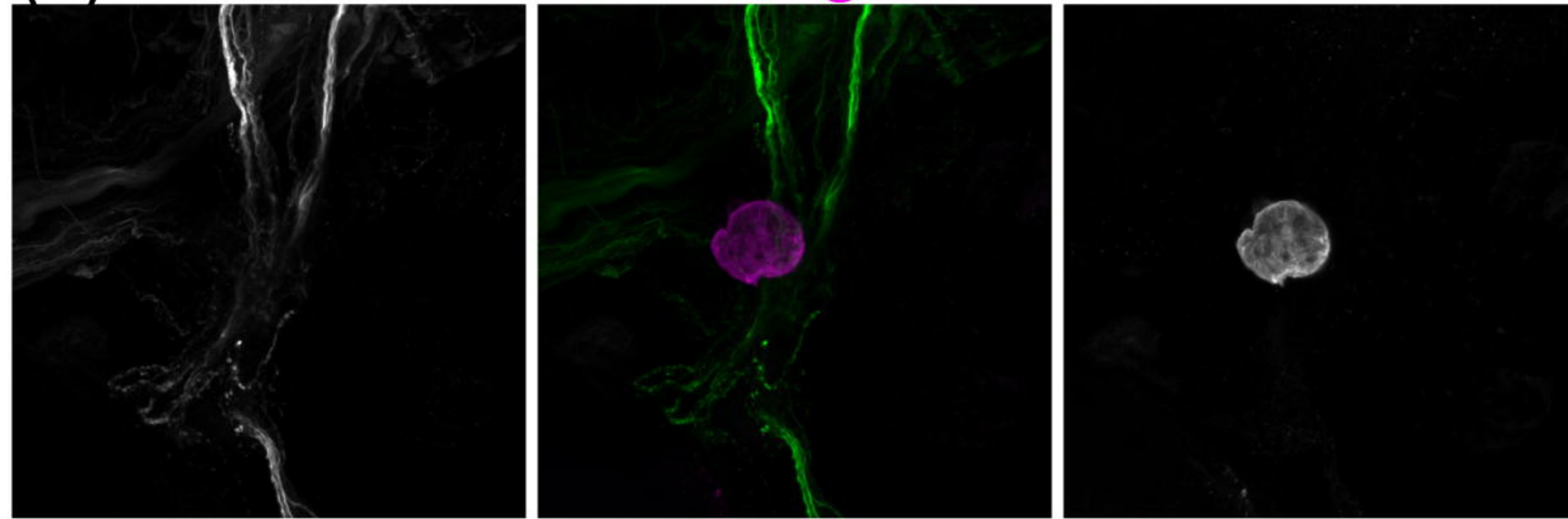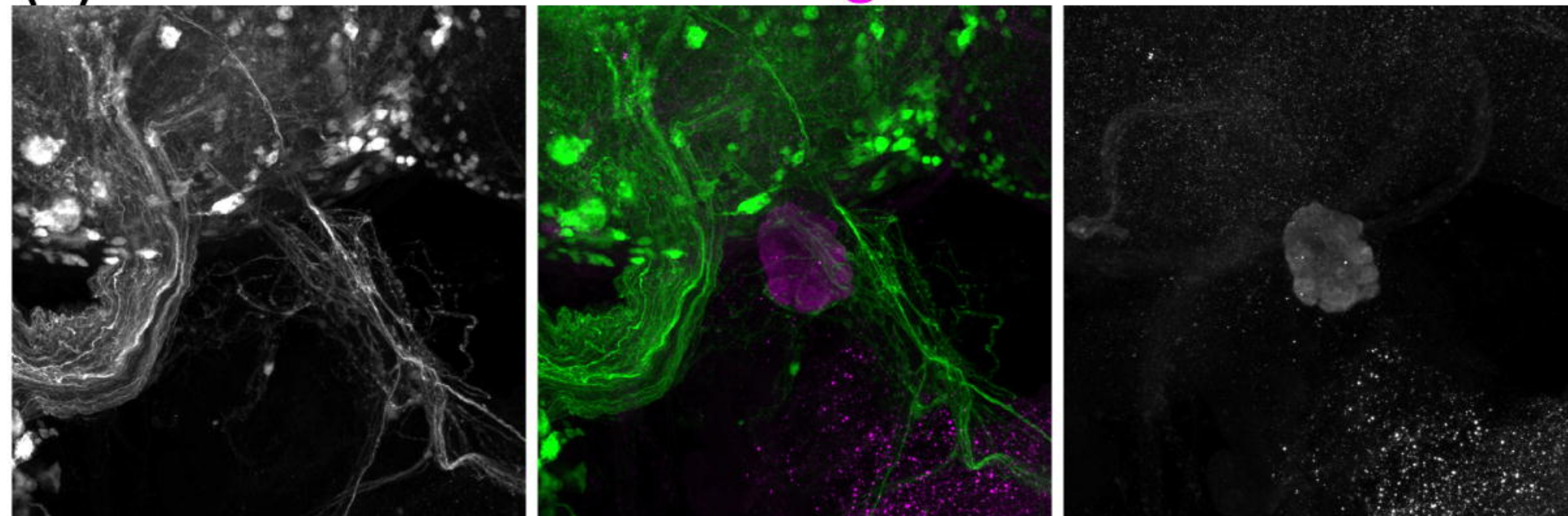*PK2-R1-2A > GFP, mCD8::GFP*

(c) GFP   Merge   JHAMT

PK2-R2-2A > GFP, mCD8::GFP