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MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

Mushroom body neuronal remodelling is necessary for short-term but not for long-term courtship memory in *Drosophila*

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Abstract

The remodelling of neurons during their development is considered necessary for their normal function. One fundamental mechanism involved in this remodelling process in both vertebrates and invertebrates is axon pruning. A well-documented case of such neuronal remodelling is the developmental axon pruning of mushroom body γ neurons that occurs during metamorphosis in *Drosophila*. The γ neurons undergo pruning of larval-specific dendrites and axons at metamorphosis, followed by their regrowth as adult-specific dendrites and axons. We recently revealed a molecular cascade required for this pruning. The nuclear receptor *ftz-f1* activates the expression of the steroid hormone receptor *EcR-B1*, a key component for γ remodelling, and represses expression of *Hr39*, an *ftz-f1* homologous gene. If ectopically expressed in the γ neurons, HR39 inhibits normal pruning, probably by competing with endogenous FTZ-F1, which results in decreased *EcR-B1* expression. The mushroom bodies are a bilaterally symmetric structure in the larval and adult brain and are involved in the processing of different types of olfactory memory. How memory is affected in pruning-deficient adult flies that possess larval-stage neuronal circuitry will help to explain the functional role of neuron remodelling. Flies overexpressing *Hr39* are viable as adults and make it possible to assess the requirement for wild-type mushroom body pruning in memory. While blocking mushroom body neuron remodelling impaired memory after short-term courtship conditioning, long-term memory was normal. These results show that larval pruning is necessary for adult memory and that expression of courtship short-term memory and long-term memory may be parallel and independent.

Introduction

The remodelling of neural circuits as the brain acquires new functions is a general feature of maturing brains in both vertebrates and invertebrates (Luo & O'Leary, 2005; Williams & Truman, 2005). In holometabolous insects, the difference in lifestyle is particularly apparent between the larval and the adult stages and the neuronal remodelling occurring during this developmental period is expected to be necessary for the normal functioning of the new circuits.

Drosophila mushroom bodies (MBs) form a bilateral and symmetrical structure in the adult brain. Each MB consists of ~ 2000 neurons arising from four neuroblasts per hemisphere (Ito *et al.*, 1997; Lee *et al.*, 1999; Aso *et al.*, 2009). These neurons project axons into two (α and α') vertical lobes and three (β, β' and γ) medial lobes (Crittenden *et al.*, 1998). Individual neuroblasts sequentially generate three distinct classes of neurons projecting into the different lobes. The MB neurons are classified as γ neurons (born during late embryonic and early larval stages), $\alpha'\beta'$ neurons (born during the late larval stage) and $\alpha\beta$ neurons (born during the pupal stage). Initially every γ neuron extends an axon which bifurcates into two major branches, one projecting medially and the other dorsally. Both branches are pruned during early metamorphosis and subsequently only the medial branch re-grows to form the adult γ neuron (Lee *et al.*, 1999). γ Neuron remodelling requires the expression of the ecdysone receptor *EcR-B1*, which is activated by TGF-β signalling (Lee *et al.*, 2000; Zheng *et al.*, 2003; Zhu *et al.*, 2003). We have recently shown that the orphan nuclear receptor *ftz-f1* plays a major role in γ neuron pruning and is required (i) to activate expression of the *EcR-B1* and (ii) to repress expression of *Hr39*, a *ftz-f1* homologous gene (Boulanger *et al.*, 2011). If inappropriately expressed in the γ neurons HR39 inhibits normal pruning, probably by competing with endogenous FTZ-F1, resulting in decreased *EcR-B1* expression. MB neuronal remodelling is thus tightly regulated. However, as was recently pointed out (Campbell & Turner, 2010), the precise functional consequences of this pruning remain as yet unclear.

MBs are essential for several forms of learning and memory (Heisenberg, 2003; Davis, 2005; Keene & Waddell, 2007; Keleman

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et al., 2007; Krashes & Waddell, 2008; Colomb *et al.*, 2009; Griffith & Ejima, 2009; Akalal *et al.*, 2010). In courtship conditioning, memory can be observed when males reduce their courtship behavior towards females after having been in contact with mated females (Mehren *et al.*, 2004). MBs are required for both short- and long-term courtship memory (STM and LTM, respectively; McBride *et al.*, 1999). Analyzing how memory is affected in pruning-deficient adult animals which display immature larval-stage neuronal circuitry may help reveal the functional role of neuron remodelling. In other words, what part does wild-type MB pruning play in courtship memory? It is possible to address this question by testing adults with γ pruning defects as *Hr39* overexpressors are viable into adulthood.

Materials and methods

All strains were maintained in standard culture medium at 25 °C. Except where otherwise stated, alleles have been described previously (http://flystocks.bio.indiana.edu). We routinely used *P{Mae-UAS.6.11}-Hr39^{C13} P* replacement to overexpress *Hr39*$^{+}$ (abbreviated as *Hr39^{C13}*) as previously described (Boulanger *et al.*, 2011). Furthermore, we identified one *UAS* insertion located 1.5 kb upstream of the *Hr39* initiator ATG codon, *P{GS:9939},* in the Kyoto stock collection (DGRC; abbreviated as *Hr39^{GS}*). Both *UAS* insertions were shown to express *Hr39* by RT-PCR when driven by the *GAL4-OK107* MB driver (Redt-Clouet, C. & Dura, J.-M., unpublished observations). All the GAL4 lines used in this study have been thoroughly described elsewhere (Aso *et al.*, 2009). Concerning the expression specificity of the GAL4 lines used in the present work, in the MB neurons: *GAL4-OK107* is strongly expressed in all the MB neurons (γ, $\alpha'\beta'$ and $\alpha\beta$); *GAL4-201Y* is strongly expressed in the γ neurons and in the $\alpha\beta$-core, a small subset of the $\alpha\beta$ neurons; *GAL4-H24* is expressed in the γ neurons*; GAL4-c739* is strongly expressed in all of the $\alpha\beta$ neurons; and *GAL4-17d* is strongly expressed in a large subset of the $\alpha\beta$ neurons. In order to block the synaptic transmission, we used flies carrying a single insertion of the *UAS-TNTE* transgene on the second chromosome (Sweeney *et al.*, 1995). The association of *UAS-TNTE* and *GAL4-H24* or *GAL4-c739* is lethal. We therefore used *GAL4-201Y* to block the γ neurons and *GAL4-17d* to block the $\alpha\beta$ neurons.

Immunohistochemistry

After dissection and fixation, adult brains were washed twice in PBS containing 0.5% Triton X-100 for 1 h. Then, 1% BSA was added to the samples before their incubation for 1 h. Primary antibody anti-Fasciclin II, 1D4, (1 : 10) was provided by the Developmental Studies Hybridoma Bank, Iowa, USA and was applied overnight. Cy3-conjugated secondary antibody against goat antimouse (Jackson Immunoresearch Laboratory, West Grove, PA, USA) was used at 1 : 300. Secondary antibody was diluted with 1% BSA and 0.5% Triton X-100 in PBS for 3 h. After several washes in PBS containing 0.5% Triton X-100, specimens were mounted with liquid VectaShield (Vector Laboratories, West Grove, PA, USA). All the steps were performed at 4 °C.

Microscopy and image processing

Images were acquired at room temperature using a Zeiss LSM 780 laser scanning confocal microscope (MRI Platform, Institute of Human Genetics, Montpellier, France) equipped with a 40× PLAN apochromatic 1.3 oil-immersion differential interference contrast objective lens. The immersion oil used was immersol 518 F. The acquisition software was Zen 2010. Settings were optimized for detection without saturating the signal. Contrasts and relative intensities of the green (green-fluorescent protein; GFP) and red (Cy3) were processed with the ImageJ software.

Mutant pruning phenotypic range

Quantifications of the γ axon phenotype were performed using a fluorescence microscope (Leica, DM6000) with anti-FASII staining. FASII is expressed strongly in the larval yet weakly in the adult γ neurons, and strongly in the adult $\alpha\beta$ neurons. Unpruned adult γ axons show a strong FASII labeling as they remain larval. Unpruned γ axons are also in another focal plane to the adult normally pruned γ axons (Boulanger *et al.*, 2011). These two specificities were used to assess the presence of unpruned γ axons in each of the scored MBs. A γ axon phenotype was denoted wild-type when no unpruned γ axons were observed (Fig. 1A). A phenotype was considered 'weak' (Fig. 1B) when a few unpruned individual axons were observed in the dorsal lobe but no apparent unpruned γ axons were observed in the medial lobe. The lack of visualization of the few unpruned γ axons in the medial lobe was probably due to their masking by the β axons. A phenotype was considered 'strong' (Fig. 1C) when a mix of pruned and unpruned γ axons was observed for which the unpruned γ axons were located out of the plan of the normally pruned adult γ axons. The percentage of unpruned axons was estimated as the width of the corresponding medial bundle relative to the width of the medial pruned axon bundle, and required visualization of the same MB at several different focal planes. We estimated that in the strong phenotype ≥50% of the γ axons are unpruned. In the dorsal lobe, these unpruned γ axons were always present and often organized into thick bundles (Fig. 1C and H–J). The complete mutant phenotype contained no visible pruned axons (asterisk in Fig. 1D). The brain anatomy was assessed using flies that had not previously been tested for memory.

Courtship conditioning paradigm

Courtship conditioning and memory tests were carried out in visible light essentially as previously described (Kamyshev *et al.*, 1999). We used the retraining test with fertilized females rather than the retention test with virgin females (McBride *et al.*, 1999) because it elicits more robust memory performances (Kamyshev *et al.*, 2002). The courtship index (CI) was calculated for each male as the percentage of time spent in courtship over a 300-s period. Independent samples of naive males of the same stock served as a control. On the same day, an equal number of naive or trained males from the same stock were individually tested under identical conditions. The performance index (PI) was calculated as follows: PI = 100% × (CI naive−CI trained)/CI naive, where CI naive and CI trained are the mean courtship indexes for independent samples of naive and trained males, respectively. At least 20 naive and 20 trained males were used to calculate each PI value. A PI of 100 (the upper limit for memory performance) corresponds to complete courtship inhibition after training, while a PI of 0 represents no conditioned inhibition (no memory). For short training, a 4- to 5-day-old male was placed into an experimental chamber together with a mated female and was allowed to court her for 30 min (training). Then, the male was left in isolation until testing. After a certain period of time (0 or 3 h), the trained male was placed in a fresh chamber with another mated female for the memory test (STM). To examine LTM, a 4- to 5-day-old male was placed with a mated female in one of our regular food vials for 5 h according to an

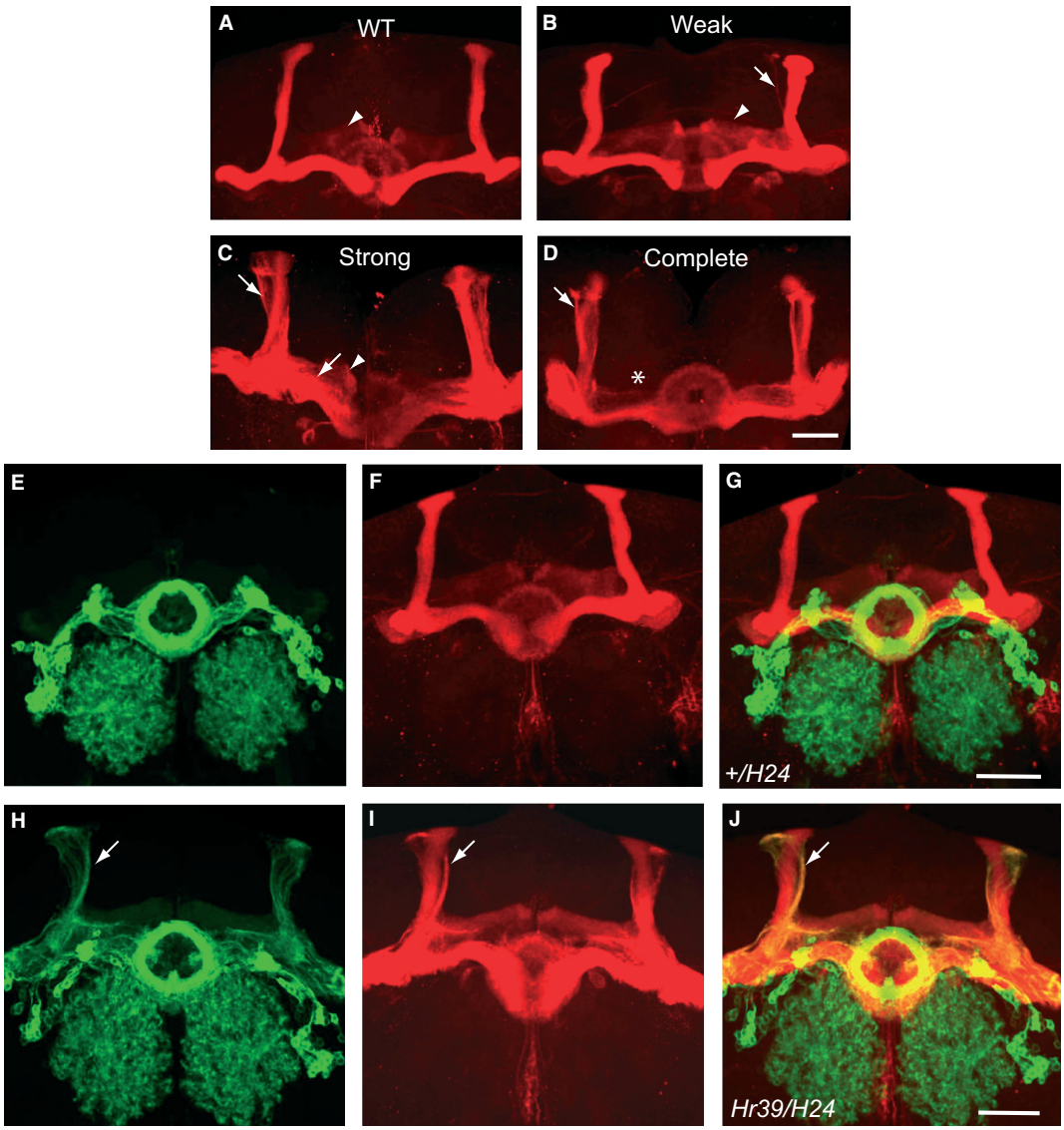

FIG. 1. γ Axon phenotypes observed in different genetic backgrounds. Images are confocal z-stacks in which red staining shows FASII expression and green represents *GAL4–H24*-driven GFP. Arrowheads indicate normally pruned γ axons and arrows unpruned γ axons. In (D) the asterisk indicates the areas in which wild-type remodelled γ axons should be located. (A–D) Four different γ axon phenotypes were observed as follows: (A) wild-type (WT) phenotype, (B) 'weak' phenotype, (C) 'strong' phenotype and (D) 'complete' phenotype. Note that no unpruned γ axons were observed in WT phenotypes and that progressive amounts of unpruned γ axons were visualized from the weak to the complete phenotype where no normally pruned γ axons were apparent (asterisk). (E–G) No unpruned γ axons were observed in the control *+/GAL4–H24* individuals. (H–J) In contrast, *Hr39* overexpression exclusively in the MB γ neurons in *Hr39/GAL4–H24* individuals resulted in a 'strong' unpruned γ axon phenotype. G and J are merged images of E + F and H + I respectively. Scale bar, 40 μm. Genotypes:
(A) *Hr39/+*: y^+ w^+ (Canton-S)/Y; $Hr39^{C13}$/+.
(B) *Hr39/c739*: y^+ w^+ (Canton-S)/Y; $Hr39^{C13}$/*GAL4-c739 UAS-mCD8-GFP*.
(C and D) *Hr39/201Y*: y^+ w^+ (Canton-S)/Y; $Hr39^{C13}$/*UAS-mCD8-GFP GAL4-201Y*.
(E–G) *+/H24*: y^+ w^+ (Canton-S)/Y; +/+; *UAS-mCD8-GFP, GAL4-H24/+*.
(H–J) *Hr39/H24*: y^+ w^+ (Canton-S)/Y; $Hr39^{C13}$/+; *UAS-mCD8-GFP, GAL4-H24/+*.

established protocol (McBride *et al.*, 1999). The male was then tested immediately (0 h), 3 h, 24 h, 2 days or 8 days later. Trained and age-matched naive males were tested at the specified times for each experiment. One particular male, either trained or naïve, was tested only once. Therefore, different males were used for different memory tests at different time points.

Statistics

No standard errors are presented with the PIs. See also Keleman *et al.* (2007). Details of the CIs with their corresponding standard errors are provided in Tables 2–5. In order to compare two PIs, statistical comparisons were made using the randomization test (Kamyshev *et al.*, 1999) with a significance level of $P \leq 0.05$. Briefly, for the comparison of two PIs, individual CI values from four independent samples (naive and trained males for each of the two PI values) are mixed and redistributed among four virtual samples of the same size as real samples. On each iteration (here we choose 10 000) the program calculates the virtual value of the difference

$PI_1 - PI_2 = [1 - CI_{trained1}/CI_{naive1}]. 100 - [1 - CI_{trained2}/CI_{naive2}]. 100 = [CI_{trained2}/CI_{naive2} - CI_{trained1}/CI_{naive1}]. 100$ and compares it with the PI_1–PI_2 difference obtained in the real experiment. The

TABLE 1. Quantification of the unpruned γ neuron phenotype of the different genotypes tested for memory

		Mutant pruning phenotype			
Genotype	*N*	WT	Weak	Strong	Complete
Hr39/+	102	102	0	0	0
Hr39/OK	110	0	0	110	0
Hr39/201Y	102	0	0	98	4
Hr39/H24	98	0	0	98	0
GS/+	108	108	0	0	0
GS/H24	90	0	0	90	0
Hr39/c739	140	118	22	0	0
Hr39//OK	120	120	0	0	0
[*GAL80^{ts}*@18 °C]					
TNT/+	116	116	0	0	0
TNT/201Y	116	116	0	0	0
TNT/17d	102	102	0	0	0

N is the number of scored mushroom bodies. Full genotypes are given in the legends to Figs 2, 4 and 5.

test gives the probability of rejecting H_0: $PI_1 = PI_2$ against H_1: $PI_1 > PI_2$. All other details of the technique and the statistical analysis including the programs used for behavior registration and primary treatment of the data have been described previously (Kamyshev *et al.*, 1999).

Results

We quantified the unpruned γ neuron phenotype in all the different genotypes tested for memory (Table 1). This quantification was based on the anti-FASII pattern of the γ axons and was therefore independent of the presence or not of a *GAL4* line associated with *UAS-mCD8-GFP* and, when present, potentially complicating the visualization of the γ neurons due to its neuronal specificity of expression. The unpruned γ axon phenotype was ranked into four categories of expression – wild-type, weak, strong and complete (Fig. 1 and see Materials and Methods). This quantitative analysis of the unpruned phenotype showed that, within a particular genotype, the variability of the classification of unpruned γ axons was low or absent. A strong or complete unpruned phenotype was apparent when *Hr39* was overexpressed in the γ neurons (*Hr39/OK*, *Hr39/201Y*, *Hr39/H24* and *GS/H24*). A wild-type (84% of the mushroom bodies) or a weak (16% of the mushroom bodies) phenotype was apparent when *Hr39* was expressed in the αβ neurons with the *GAL4-c739* line. Although strongly expressed in the pupal αβ and specific to the adult αβ neurons, *c739* is also weakly expressed in the pupal γ neurons (Nicolai *et al.*, 2003). This transient γ expression is probably at the origin of this minority weak unpruned γ phenotype that seems to have no consequence on memory (see below).

To address the possible impact of blocking γ axon pruning on memory, we used the conditioned courtship suppression paradigm (Siegel & Hall, 1979). As established previously, we used a 30-min training period with a mated female to induce memory after short-term training in males, and a 5-h training period to induce memory after long-term training (McBride *et al.*, 1999). In wild-type Canton-S flies, memory after short-term training was still robust 3 h after training. However, when *Hr39* was ectopically expressed in the entire MB (*Hr39^{C13}/+; GAL4-OK107/+*), memory after short-term training was severely impaired after 3 h, even though memory performance was similar to wild-type immediately after the training (Fig. 2A and B); $P < 0.02$ for H_0: $PI_{0\ h} = PI_{3\ h}$ against H_1: $PI_{0\ h} > PI_{3\ h}$ (one-sided randomization test) in *Hr39/OK* individuals. Memory after long-term training in these flies was not affected up to 24 h (Fig. 2A). At the developmental level, only γ neurons seem affected by *Hr39* overexpression in the adult MB neurons (Boulanger *et al.*, 2011). Nevertheless, as *Hr39* was expressed in the above experiment in all the MB

TABLE 2. Courtship indices shown in Fig. 2

	0 h			3 h			
	CI naive	CI trained	PI	CI naive	CI trained	PI	*N*
STM							
Canton-S	44.0 ± 2.8	14.2 ± 1.6	67.8	52.0 ± 2.7	25.8 ± 2.0	50.4	20
Hr39/OK107	35.5 ± 2.3	13.9 ± 1.7	60.8	38.6 ± 2.4	35.3 ± 3.1	8.5	20
Hr39/201Y	42.0 ± 2.4	16.5 ± 2.0	60.3	40.4 ± 2.5	36.6 ± 3.3	9.3	20
Hr39/H24	33.4 ± 3.0	10.2 ± 1.8	69.4	32.8 ± 3.0	29.1 ± 3.9	11.4	30
GS/H24	41.1 ± 2.3	22.9 ± 4.4	44.2	40.0 ± 2.6	42.7 ± 5.3	−6.9	20
Hr39/c739	29.3 ± 2.1	7.4 ± 1.3	74.9	36.7 ± 2.2	14.9 ± 1.2	59.5	20
+/OK107	34.8 ± 0.3	14.3 ± 1.9	59.1	44.2 ± 2.7	20.5 ± 1.6	53.6	20
+/201Y	31.7 ± 2.5	7.4 ± 1.1	76.7	30.4 ± 2.5	11.0 ± 1.9	63.8	20
+/H24	51.8 ± 4.8	8.1 ± 1.6	84.3	38.7 ± 3.2	6.6 ± 2.2	83.0	20
Hr39/+	45.7 ± 1.8	16.5 ± 2.0	63.9	44.9 ± 2.3	19.6 ± 1.5	56.5	20
GS/+	52.8 ± 3.8	24.1 ± 2.5	54.3	51.6 ± 4.9	28.4 ± 3.1	44.9	20
LTM							
Canton-S	44.0 ± 2.8	15.3 ± 2.6	65.2	53.8 ± 1.5	23.6 ± 1.6	56.1	20
Hr39/OK107	42.5 ± 2.2	13.7 ± 1.7	67.8	39.9 ± 2.4	16.1 ± 1.7	59.6	20
Hr39/201Y	46.4 ± 1.9	15.0 ± 1.3	57.9	46.4 ± 1.9	25.6 ± 1.5	44.9	20
Hr39/H24	32.5 ± 3.8	11.7 ± 2.3	64.2	26.3 ± 3.1	9.2 ± 1.9	65.1	30
GS/H24	33.1 ± 3.2	9.2 ± 2.7	72.3	32.0 ± 3.7	13.7 ± 1.9	57.0	20
Hr39/c739	30.8 ± 2.1	6.9 ± 0.9	77.7	32.5 ± 1.7	12.6 ± 1.6	61.3	20
+/OK107	42.2 ± 2.8	20.0 ± 2.3	52.6	38.3 ± 2.7	18.4 ± 2.0	52.0	20
+/201Y	29.6 ± 2.6	7.4 ± 1.5	74.8	28.8 ± 2.8	9.9 ± 1.6	65.8	20
+/H24	55.4 ± 3.5	9.9 ± 2.0	83.9	42.7 ± 4.2	17.7 ± 3.2	58.6	20
Hr39/+	44.6 ± 1.9	19.8 ± 2.3	55.7	42.7 ± 2.9	21.4 ± 1.9	49.9	20
GS/+	44.5 ± 2.5	9.1 ± 2.5	79.6	48.2 ± 4.1	26.4 ± 3.3	45.2	20

CI, courtship index; PI, Performance index; *N*, number of tested males. Data for CI are mean ± SEM.

TABLE 3. Courtship indices shown in Fig. 3

		CI naive	CI trained	PI	*N*
Canton-S	LTM 3 h	42.8 ± 2.3	20 ± 3.2	53.3	20
Hr39/OK107	LTM 3 h	36.2 ± 2.6	18.5 ± 2.7	49.0	20
Canton-S	LTM 2 days	62.2 ± 3.1	33.0 ± 2.1	47.0	20
Hr39/OK107	LTM 2 days	45.8 ± 2.1	24.5 ± 1.9	46.5	20
Canton-S	LTM 8 days	65.3 ± 2.4	35.3 ± 1.5	45.9	20
Hr39/OK107	LTM 8 days	37.5 ± 3.3	21.3 ± 2.3	43.2	20

CI, courtship index; PI, Performance index; *N*, number of tested males. Data for CI are mean ± SEM.

TABLE 4. Courtship indices shown in Fig. 4

		CI naive	CI trained	PI	*N*
18 °C → 18 °C	STM 0 h	50.8 ± 5.9	30.4 ± 5.2	41.9	20
18 °C → 29 °C	STM 0 h	51.6 ± 5.9	30.0 ± 5.6	40.3	20
18 °C → 18 °C	STM 3 h	42.8 ± 4.1	24.0 ± 4.4	43.6	20
18 °C → 29 °C	STM 3 h	48.8 ± 5.6	24.4 ± 6.4	50.0	20
18 °C → 18 °C	LTM 0 h	35.6 ± 5.1	11.6 ± 2.0	67.4	20
18 °C → 29 °C	LTM 0 h	54.0 ± 3.9	15.6 ± 3.7	70.8	20
18 °C → 18 °C	LTM 24 h	62.4 ± 5.1	41.2 ± 4.7	33.9	20
18 °C → 29 °C	LTM 24 h	58.8 ± 5.1	30.8 ± 4.1	47.4	20

CI, courtship index; PI, Performance index; *N*, number of tested males. Data for CI are mean ± SEM.

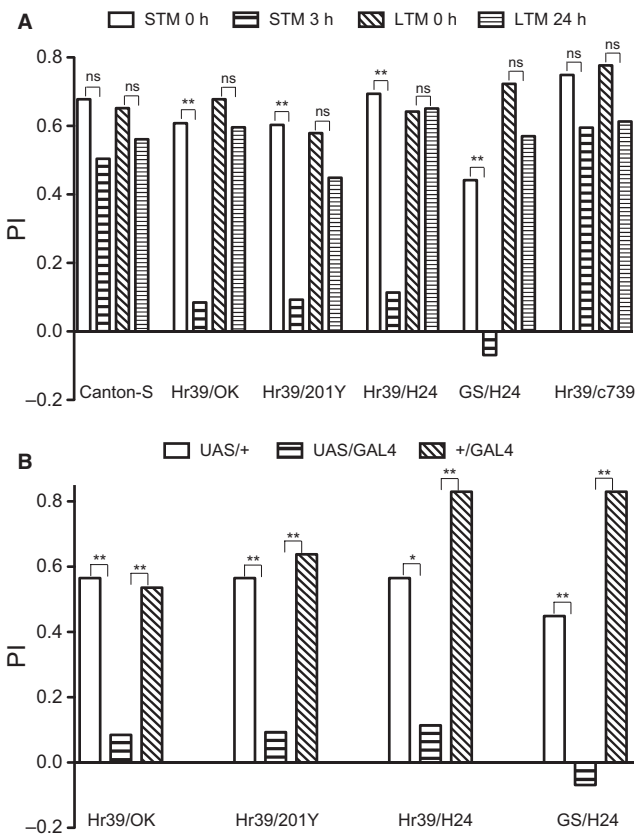

FIG. 2. Blocking neuronal remodelling of *Drosophila* MB γ neurons profoundly impaired STM while leaving LTM unaffected. (A) STM (0 and 3 h) and LTM (0 and 24 h) after courtship conditioning in different contexts of *Hr39* overexpression. We used *GAL4-OK107* for expression in the entire MB, *GAL4-201Y* to induce γ and $\alpha\beta$-core neuron expression, *GAL4-H24* for expression only in the γ neurons and *GAL4-c739* for expression only in the $\alpha\beta$ neurons (Aso *et al.*, 2009). By 3 h after short-term training all flies expressing *Hr39* in γ neurons (*OK107*, *201Y* and *H24*) had impaired memory (**$P < 0.02$, one-sided randomization test) when compared with flies tested at 0 h after short-term training. The flies overexpressing *Hr39* exclusively in the $\alpha\beta$ neurons (*c739*) had intact STM (ns, not statistically significant, randomization test). LTM at 24 h in all of these flies was normal. For *Hr39* expression, $Hr39^{C13}$ was used with all GAL4 lines. Another *Hr39*-expressing *UAS* line, $Hr39^{GS}$, was used with *H24* in addition to $Hr39^{C13}$ leading to similar results. Genotypes:
Hr39/OK: y^+ w^+ (Canton-S)/Y; $Hr39^{C13}$/+; *UAS-mCD8-GFP/+*; *GAL4-OK107/+*.
Hr39/201Y: y^+ w^+ (Canton-S)/Y; $Hr39^{C13}$/*UAS-mCD8-GFP GAL4-201Y*.
Hr39/H24: y^+ w^+ (Canton-S)/Y; $Hr39^{C13}$/+; *UAS-mCD8-GFP, GAL4-H24/+*.
GS/H24: y^+ w^+ (Canton-S)/Y; $Hr39^{GS}$/+; *UAS-mCD8-GFP, GAL4-H24/+* and
Hr39/c739 – y^+ w^+ (Canton-S)/Y; $Hr39^{C13}$/*GAL4-c739 UAS-mCD8-GFP*.
(B) UAS/+ and +/GAL4 control flies tested at 3 h after short-term training and compared with the corresponding UAS/GAL4 memory-deficient flies; *$P < 0.05$, **$P < 0.02$, one-sided randomization test.

neurons it was possible, although unlikely, that neurons other than γ neurons were also involved in the memory defect. Therefore, we subsequently expressed *Hr39* in different subsets of MB neurons. By 3 h after short-term training, mutant flies overexpressing *Hr39* in their γ neurons had impaired memory while the flies overexpressing *Hr39* in their $\alpha\beta$ neurons had intact memory (Fig. 2A); $P < 0.02$ for H_0: $PI_{0\ h} = PI_{3\ h}$ against H_1: $PI_{0\ h} > PI_{3\ h}$ (one-sided randomization test) in *Hr39/201Y*, *Hr39/H24* and *GS/H24* individuals. A clear correlation between impaired 3-h memory after short-term training and unpruned γ neurons phenotype became apparent (compare *Hr39/OK*, *Hr39/201Y*, *Hr39/H24*, *GS/H24* and *Hr39/c739* in Table 1 and Fig. 2). Memory after long-term training in these flies was normal up to 24 h.

As memory was affected at 3 h after the short training protocol we wondered whether memory would be unaffected if tested at this same time point but after the long training protocol. Memory after long-term training was found to be normal at 3 h, 2 days and even 8 days after the long training protocol in flies where neuronal remodelling had been blocked (Fig. 3). The retraining test used here, in contrast to the retention test previously used (McBride *et al.*, 1999), elicits more robust memory performances (Kamyshev *et al.*, 2002). This very probably explains why no decay in LTM performance was observed over several days in our study. Concordantly, no decay in LTM performance was described previously between 30 min and 24 h when using the retraining test (Keleman *et al.*, 2007).

Ecdysone signalling regulates the formation of LTM in adults (Ishimoto *et al.*, 2009). In the $Hr39^{C13}$/*GAL4* flies, *Hr39* was overexpressed not only during development but also during the adult stage, leading one to consider the possibility that the memory defect observed was not a consequence of the developmental defect but instead was due to the adult overexpression of the nuclear receptor. We took advantage of the TARGET technique which allows the temporal control of GAL4 regional expression (McGuire *et al.*, 2003). With the temperature-sensitive $GAL80^{ts}$ repressor we produced flies devoid of *Hr39* overexpression during their development but overexpressing *Hr39* during adulthood. *Hr39/OK* (*GAL80ts*@18 °C) mushroom bodies displayed no mutant pruning phenotype (100% 'wild-type' compared to the 100% 'strong' mutant pruning observed without $GAL80^{ts}$ in Table 1). The memory of these flies was no different from their sibling controls within which *Hr39* was overexpressed during neither development nor adult life, and both groups had robust 3-h memory after short-term training similar to wild-type flies (Fig. 4). All together our results indicate that larval neuron

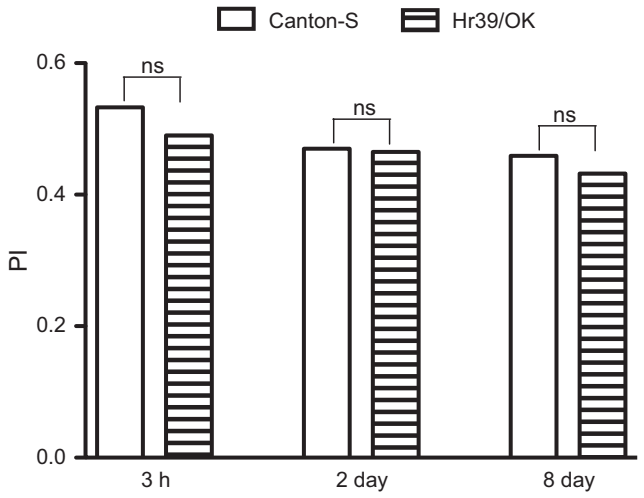

FIG. 3. LTM expression was normal for up to 8 days in flies within which neuronal remodelling has been blocked. Memory of Hr39/OK flies, after the long training protocol, at 3 h, 2 days (2 days) and even 8 days (8 days) was not affected (ns, not statistically significant, randomization test) when compared with the wild-type Canton-S flies. Genotypes:
Canton-S and
Hr39/OK: *y* w^{67c23} (Canton-S)/Y; $Hr39^{C13}$/+; *UAS-mCD8-GFP*/+; *GAL4-OK107*/+.

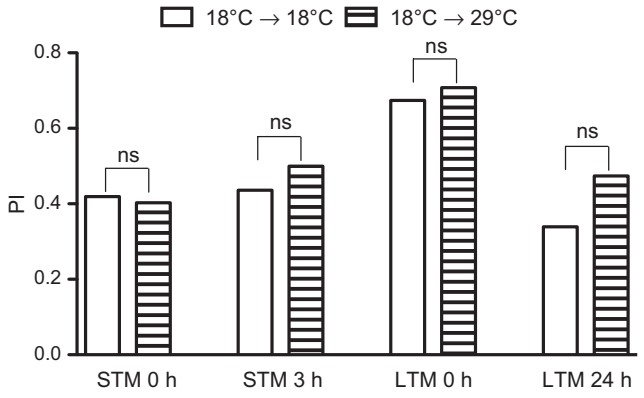

FIG. 4. Expression of *Hr39* in adult MBs had no effect on memory. Flies were grown at a $GAL80^{ts}$-permissive temperature (18 °C). Active GAL80 inhibits the expression of *UAS-Hr39* during MB development and hence γ axon pruning progresses normally. At adult hatching, a pool of flies was transferred to 29 °C, the restrictive temperature for $GAL80^{ts}$ (18 °C → 29 °C), while the other pool was left at 18 °C (18 °C → 18 °C). Flies at 29 °C expressed *UAS-Hr39* as can be monitored by GFP expression in the brain. The two pools of flies had similar PIs (ns, not statistically significant, randomization test) for STM (0 and 3 h) and LTM (0 and 24 h). Genotype:
$GAL80^{ts}$9/Y; $Hr39^{C13}$/+; *UAS-mCD8-GFP*/+; *GAL4-OK107*/+.

remodelling is a compulsory developmental process for the establishment of a functioning adult STM.

The blocking of the γ neuron remodelling is likely to produce γ neurons in the adult brain that are not correctly connected and/or that are physiologically inactive because of remaining in their larval status within an adult brain. This being the case, a similar memory phenotype should occur if the synaptic transmission of γ axons is prevented. To address this question, we blocked synaptic transmission of the γ neurons using a tetanus toxin transgene which did not affect γ neuron remodelling (*TNT/201Y* in Table 1) and assessed the impact on STM and LTM. Similar to that observed in flies with unpruned γ neurons, the 3-h memory after short-term training was abolished leaving memory at 24 h after long-term training unaffected (Fig. 5).

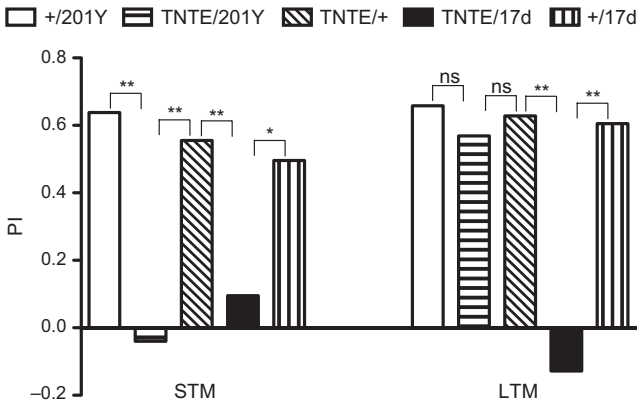

FIG. 5. STM required synaptic transmission in both the γ and the $\alpha\beta$ neurons while γ synaptic transmission was dispensable for LTM. Flies were tested at 3 h for STM and at 24 h for LTM. STM was abolished in TNTE/201Y flies while LTM was unaffected. Both STM and LTM were impaired in TNTE/17d flies. $*P < 0.05$, $**P < 0.02$; ns, not statistically significant, one-sided randomization test. Performance indexes in all cases at 0 h STM and at 0 h LTM were not affected (see Table 5). Genotypes:
+/201Y: y^+ w^+ (Canton-S)/Y; *UAS-mCD8-GFP GAL4-201Y*/+.
TNTE/201Y: w^{1118} (Canton-S)/Y; *UAS-TNTE/UAS-mCD8-GFP GAL4-201Y*.
TNTE/+: y^+ w^+ (Canton-S)/Y; *UAS-TNTE*/+.
TNTE/17d: w^{1118} (Canton-S)/Y; *UAS-TNTE/GAL4-17d* and
+/17d: y^+ w^+ (Canton-S)/Y; +/*GAL4-17d*.

For STM, $P < 0.02$ for H_0: $PI_{+/201Y}$ (or $PI_{TNT/+}$) = $PI_{TNT/201Y}$ against H_1: $PI_{+/201Y}$ (or $PI_{TNT/+}$) > $PI_{TNT/201Y}$ (one-sided randomization test). In contrast, $\alpha\beta$ neuron transmission was required for both STM and LTM (Fig. 5). For STM, $P < 0.02$ for H_0: $PI_{TNT/+}$ = $PI_{TNT/17d}$ against H_1: $PI_{TNT/+} > PI_{TNT/17d}$ (one-sided randomization test) and $P < 0.05$ for H_0: $PI_{+/17d} = PI_{TNT/17d}$ against H_1: $PI_{+/17d} > PI_{TNT/17d}$ (one-sided randomization test). For LTM, $P < 0.02$ for H_0: $PI_{+/17d}$ (or $PI_{TNT/+}$) = $PI_{TNT/17d}$ against H_1: $PI_{+/17d}$ (or $PI_{TNT/+}$) > $PI_{TNT/17d}$ (one-sided randomization test). In conclusion, we have clearly shown that when the remodelling of γ neurons is disturbed or their synaptic transmission blocked, STM of courtship memory is abolished whereas the LTM is unaffected.

Discussion

Axon pruning is a general mechanism used in the construction of the vertebrate and invertebrate nervous systems (Luo & O'Leary, 2005). It is presumed that developmental pruning is highly important for the wiring specificity of neuronal circuitry. Our results allow us to correlate larval-specific neuronal reorganization into adult-specific neurons with adult-specific behavior, namely conditioned courtship suppression in males.

We have shown that the same specific memory phenotype, an impaired STM and intact LTM, is obtained either by blocking the MB developmental remodelling which involves only the γ neurons or by blocking the synaptic transmission of normal γ neurons. One could argue here that there may be more subtle defects than the lack of pruning, in for example the synaptic connectivity of γ neurons that are inactive throughout development. While we cannot rule out this hypothesis, we should stress out that inactive γ neurons undergoing a normal pruning process also showed this STM versus LTM memory phenotype. Our results involving adult γ axons and STM expression correlate well with previous studies on courtship memory (Joiner & Griffith, 1999). Unexpectedly, however, they also revealed the dispensability of an intact set of mature adult γ neurons for LTM

TABLE 5. Courtship indices shown in Fig. 5

	0 h			3 h			
	CI naive	CI trained	PI	CI naive	CI trained	PI	*N*
STM							
TNTE/+	56.0 ± 3.9	21.6 ± 2.6	61.5	58.0 ± 3.9	25.6 ± 3.6	55.5	20
TNTE/201Y	61.6 ± 4.6	25.2 ± 3.7	59.1	44.4 ± 4.2	46.4 ± 3.7	−4.0	20
TNTE/17d	49.2 ± 4.8	29.6 ± 4.4	50.3	68.8 ± 4.6	62.4 ± 5.6	9.5	20
+/17d	54.0 ± 5.1	22.0 ± 4.8	59.5	50.0 ± 4.2	25.2 ± 3.3	49.6	20
LTM							
TNTE/+	62.4 ± 4.6	22.8 ± 3.9	63.1	63.2 ± 4.8	23.6 ± 3.8	62.8	20
TNTE/201Y	60.0 ± 5.2	10.0 ± 2.7	83.0	66.0 ± 4.0	28.4 ± 4.4	56.9	20
TNTE/17d	33.2 ± 3.7	16.4 ± 3.9	50.5	57.2 ± 6.2	64.6 ± 4.36	−12.8	20
+/17d	39.2 ± 4.2	12.8 ± 2.9	67.4	44.8 ± 5.1	17.6 ± 3.1	60.5	20

CI, courtship index; PI, Performance index; *N*, number of tested males. Data for CI are mean ± SEM. For +/ 201Y indices see Table 2.

expression. Indeed, LTM expression could be obtained independently of STM expression, which had been eliminated by altering the normal phenomenon of axon pruning during metamorphosis. This observation might reflect the description, in certain transgenic flies, of the dissociation of the learning of a task from its immediate performance (Kane *et al.*, 1997).

Using the courtship memory paradigm, the *orb2* gene has been shown to be required for LTM (Keleman *et al.*, 2007). The ORB2 protein belongs to the CPEB family proposed to act as key regulators of local protein synthesis. The *orb2* mutant LTM defect can be rescued by the forced expression of $orb2^{+}$ in the γ neurons. One way to reconcile these results with ours would be to propose the provocative hypothesis that some information (RNA and/or protein) may be transmitted from the γ neurons to the $\alpha\beta$ neurons for the LTM expression to occur. This information transfer would take place even when the γ neurons are not remodelled or when their synaptic transmission is blocked. Interestingly a recent study, using the olfactory aversive paradigm, reported a long-term memory trace by functional optical imaging in the γ lobes (Akalal *et al.*, 2010). Whether this γ neuron memory trace is specific to the aversive olfactory paradigm or could be extended to the courtship memory paradigm and linked to the *orb2* mutant LTM defect observed in the γ neurons is yet to be explored.

Two different models can be proposed for the relationship between the memory phases. First, STM and LTM expression could be two parallel and independent memory systems. This hypothesis has been recently proposed for aversive olfactory memory (Blum *et al.*, 2009) as well as for appetitive olfactory memory (Trannoy *et al.*, 2011) and thus could be extended to courtship memory. The appetitive olfactory memory paradigm is particularly interesting because both STM and LTM are generated after the same training protocol and not by two different training protocols as is the case for both the aversive olfactory and the courtship memory paradigms. In this appetitive paradigm, a continuous blockade of γ neuron neurotransmission with the tetanus toxin abolishes STM but leaves LTM unaffected (Trannoy *et al.*, 2011). Thus, flies trained with a single protocol show no appetitive STM at 1 h but normal LTM at 24 h. A second hypothetic model would be STM and LTM existing as sequential processes where LTM formation is built on the short-term memory trace (McGaugh, 1966). Based on this notion, in the courtship conditioning used here we can propose that STM could be formed in both γ and $\alpha\beta$ neurons but transformed into LTM only in the $\alpha\beta$ neurons. Therefore, whilst LTM would not be independent of STM, the γ neurons would not be necessary for LTM formation. Independently of the relationship between the memory phases, one might propose that γ neurons are sufficient although not necessary for LTM expression. However, and in opposition to this hypothesis, in this study we observed a lack of both STM and LTM when the tetanus toxin transgene was expressed specifically in a subset of the $\alpha\beta$ neurons with the *GAL4-17d* line. Finally, alternative ways of producing and/or expressing LTM might be triggered when pruning in the γ lobes is disturbed, as expected if compensatory processes take place during development.

Whatever the relationship between the memory phases, this study highlights the importance of the maturation of the larval nervous system into the adult nervous system to ensure adult-specific behavior. The creation of complex patterns of connectivity often requires the pruning of axon branches to supernumerary or temporary targets. Since the pioneering work in vertebrates (Hubel & Wiesel, 1959, 1962, 1963, 1968), a number of studies have shown that disturbing the maturation of neural circuits, in particular axon pruning, can impact the function of the mature neural network and subsequently adult behavior (Paus *et al.*, 2008). The need for these remodelling events to facilitate the establishment of complex neuronal networks is now appreciated (Insel, 2010). Indeed, there is some indirect evidence that pruning defects might cause neurological disorders such as chronic epilepsy, schizophrenia and autism including the fragile-X syndrome (Swann *et al.*, 1999; Churchill *et al.*, 2002; Eigsti & Shapiro, 2003; Frith, 2003; Roberts *et al.*, 2005). An excess of immature synapses could explain the disorganized and hyperexcitable brains and the behavioral and cognitive dysfunctions of these patients. However, related clinical studies only provide indirect evidence of the role of pruning for normal adult behavior. Here we have demonstrated the memory consequence of a defect in developmental axon pruning.

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Abbreviations

CI, courtship index; GFP, green-fluorescent protein; LTM, long-term memory; MB, mushroom body; PI, Performance index; STM, short-term memory.

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