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Overexpression of the *limk1* Gene in *Drosophila melanogaster* Can Lead to Suppression of Courtship Memory in Males

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Abstract—Courtship suppression is a behavioral adaptation of the fruit fly. When majority of the females in a fly population are fertilized and non-receptive for mating, a male, after a series of failed attempts, decreases its courtship activity towards all females, saving its energy and reproductive resources. The time of courtship decrease depends on both duration of unsuccessful courtship and genetically determined features of the male nervous system. Thereby, courtship suppression paradigm can be used for studying molecular mechanisms of learning and memory. p-Cofilin, a component of the actin remodeling signaling cascade and product of LIM-kinase 1 (LIMK1), regulates *Drosophila melanogaster* forgetting in olfactory learning paradigm. Previously, we have shown that *limk1* suppression in the specific types of nervous cells differently affects fly courtship memory. Here, we used Gal4 > UAS system to induce *limk1* overexpression in the same types of neurons. *limk1* activation in the mushroom body, glia, and *fruitless* neurons decreased learning index compared to the control strain or the strain with *limk1* knockdown. In cholinergic and dopaminergic/serotoninergic neurons, both overexpression and knockdown of *limk1* impaired *Drosophila* short-term memory. Thus, proper balance of the *limk1* activity is crucial for normal cognitive activity of the fruit fly.

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Keywords: *Drosophila*, LIM-kinase 1, courtship suppression, learning, memory and forgetting

INTRODUCTION

Changing behavioral strategies due to personal positive and negative experience is a form of adaptation, common to both invertebrates and vertebrates. Molecular processes regulating memory formation and consolidation appeared to be similar in the fruit fly and mammals [1]. About 75% of the genes associated with human diseases have structural analogues in *Drosophila*. Many of them are likely responsible for neurodegenerative and neurological dysfunctions [2]. This makes the fruit fly a perspective model object to study molecular mechanisms of higher nervous activity.

An example of behavioral adaptation in *Drosophila* is courtship suppression. Male courtship is a complex repertoire of innate reactions aimed to achieve reproductive success. It includes a series of successive stages – orientation and following a female, tapping the female with forelegs, licking, singing courtship song, and copulation attempt. All these processes are regulated by the orchestrated activity of specific brain neural circuits, receiving visual, taste, smell, and hearing sensory inputs from a female [3-5]. While the vir-

Abbreviations: CCSP, conditioned courtship suppression paradigm; CI, courtship index; KC, Kenyon cells; βL, β-lobes; γL, γ-lobes; LI, learning index; LIMK1, LIM-kinase 1; MB, mushroom body; PAM, protocerebral anterior medial cluster; STM, short-term memory.

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gin female usually accepts the male's courtship, ending in copulation, the mated female rejects it by actively avoiding the male.

To maximize their reproductive success, males should be able to discriminate receptive and non-receptive females, using some signal substances. One of them is *cis*-vaccenyl acid, a volatile male pheromone that is transferred to the female cuticle and genital tracts on mating, thus serving as a repellent for further males. The prolonged rejection of a male by a female increases the male's sensitivity to *cis*-vaccenyl acid, which results in temporal decrease of its courtship vigorousness [6]. This adaptive response depends on the activity of specific type of dopaminergic neurons, which belong to the protocerebral anterior medial cluster (PAM). Activity of the above neurons seems to represent unconditioned stimulus, inhibiting male courtship behavior. Courtship suppression also results from the counterconditioning of the initially attractive conditioned stimulus, such as the female age-specific cuticular pheromone, after its association with unconditioned stimulus [7, 8].

Failed attempts of pairing with a non-receptive female decrease the male courtship index (CI) compared to the naïve males – relative difference is expressed as the learning index (LI). LI stays high for a period of time that depends on the time of pairing, as well as on the male genotype. For example, after 1-h pairing session, the wild-type male showed CI decrease up to 6 h, while for the mutant *amnesiac* CI decline was not observed after 1 h [9]. Memory defects in the *amnesiac* were also revealed in the odor-shock association paradigm [10, 11]. Thus, conditioned courtship suppression paradigm (CCSP) can be used to test fly learning and memory abilities. CCSP is more natural and easier compared to the widely used paradigm of classical Pavlovian aversive olfactory learning with negative electroshock reinforcement.

Historically, olfactory learning was the first method to study genetic mechanisms of memory in fruit flies [10, 12, 13]. Mushroom body (MB) plays a central role in the *Drosophila* olfactory associative memory [14]. Activation of the specific pattern of the MB intrinsic neurons, Kenyon cells (KC), represents olfactory conditioned stimulus. Their main effectors, the MB output neurons (MBON), specify positive or negative memory valence and bias attraction or aversion behavior. MB > MBON synaptic contacts are regulated by the specific clusters of dopaminergic neurons, which represent either attractive or aversive unconditioned stimulus [15-17]. The non-linear system of interplay between these neural components can theoretically explain all known forms of the MB-dependent classical conditioning [18].

The role of fly brain structures and neurons in courtship learning and memory was revealed using the Gal4 > UAS binary expression system [19, 20]. MB are not involved in courtship learning. However, γd neurons of the MB ventral accessory calyx regulate short-term memory (STM) formation in CCSP [20]. These neurons receive visual inputs from optic lobes [21] and project to γ5 area [16]. PAM-γ5 (aSP13) dopaminergic neurons, which innervate γ5 area, are crucial for STM formation in CCSP [6]. M6-MBON serve as an output for aSP13, being required during memory acquisition and recall. Recurrent MBγ > M6-MBON > aSP13 interaction is essential for courtship STM [22]. Thus, neuronal basis of courtship memory in *Drosophila* resembles that for Pavlovian conditioning.

Along with the memory formation, storage, and retrieval, the brain is able to purposefully forget information that becomes unused [23]. It is arguable whether forgetting is critical to make room for new engrams. The number of individual memory patterns that can be stored using the Hopfield auto-associative neural network is αN, where N is the number of neurons and α is ~0.14 [24]. For a sparsely encoded associative memory, the number of patterns is even larger, being $\sim N^2 \log_2 N$ [25]. Since the human brain has ~100 billion neurons, that is theoretically enough to encode much more than people can experience during the whole life. This may explain the rare cases of extraordinary memory abilities, such as described in [26]. Having such photographic memory, however, is suboptimal in terms of survival. Transience of memory makes behavior flexible, erasing the reactions to irrelevant and outdated information, and provides generalization, preventing overfitting to specific signals. Thus, memory formation and forgetting together serve the purpose of decision-making optimization [27].

Recently, Pavlovian learning was applied to reveal molecular basis of forgetting in the fruit fly. In addition to being crucial for learning and memory formation, dopaminergic neurons contribute to forgetting of certain types of memory. Some of them belonging to the protocerebral posterior lateral 1 (PPL1) cluster, which innervate the MB peduncle and stalk, induce aversive memory forgetting. While memory acquisition is regulated by the dopamine receptor DopR1 (dDA1) coupled to the G_s–cAMP-dependent signaling pathway, forgetting is mediated by the dopamine receptor DopR2 (DAMB) coupled to the $G_{\alpha q}$–Ca^{2+} pathway [28, 29]. Activation of small GTPase Rac1 in the αβ and γ KC leads to STM forgetting in olfactory learning paradigm, probably acting through the PAK–cofilin pathway [30]. Interference-induced forgetting is induced in the αα' KC through the DAMB and Rac1–PAK3–cofilin pathway [31], which also regulates actin remodeling [32].

Much less is known about the functional role and mechanisms of forgetting in CCSP. This can be partly explained by the fact that CCSP is relatively less flexible experimental technique compared to the Pavlovian

learning, where it is easy to specify different learning parameters –modalities of stimuli, order of pairing, number and spacing of training trials, and others. Efficiency and steadiness of the courtship suppression depend on many factors, such as training duration, testing with virgin or mated females, chamber size, food presence/absence, etc. [33, 34].

The effects of actin-remodeling signaling cascade on the fruit fly courtship memory were first shown in the temperature-sensitive mutant agn^{ts3} [35, 36]. LIM-kinase 1 (LIMK1) phosphorylates *Drosophila* cofilin (twinstar) inhibiting its actin-depolymerizing activity [32]. In agn^{ts3}, both LIMK1 and p-cofilin levels are increased, while courtship learning and STM are absent. After heat shock, the LIMK1 and p-cofilin levels decrease to normal, becoming similar to those in the wild-type strain *Canton-S*, with concomitant learning and memory recovery [35].

While comparing the dynamics of LI in several strains with *limk1* neurospecific knockdown, we revealed that the knockdown effect depended on the neural types [37]. The Gal4 drivers, CHN and DAN/SRN, that induced *limk1* knockout in the cholinergic and dopaminergic/serotoninergic neurons, respectively, caused learning decrease and/or faster courtship memory decay compared to the control. This speaks against the role of LIMK1 and p-cofilin in the memory forgetting, but could also mean insufficient decrease of the LIMK1 protein level or that some optimal LIMK1 level is necessary for learning. On the contrary, the driver FRN that induced *limk1* knockdown in the *fruitless* neurons, increased the 30-60 min STM. Neurospecificity of the LIMK1 effects may be due to the different roles of these neuronal types in the courtship suppression or result from their initially different levels of LIMK1, being above or below the functional optimum.

In this research, we continue to study the effects of LIMK1 on the *Drosophila* courtship learning and memory, specifically activating extra copy of *limk1* in several neuronal types. In most cases, *limk1* activation led to the LI decrease compared to the control and/or *limk1* knockdown. This is in agreement with the known role of p-cofilin in active forgetting after olfactory learning.

MATERIALS AND METHODS

Fly strains. Flies were taken from the Biocollection of Pavlov Institute of Physiology, Russian Academy of Sciences, for the Study of Integrative Mechanisms of Nervous and Visceral Systems, Saint Petersburg, Russia. Detailed description of the strains is given in table. Females with Gal4 expression in the specific type of nervous cells were crossed to one of the following strains: #9116 expressing wild type LIMK1 strongly under UAS control (*limk1* "+"); #26294 expressing dsRNA under UAS control for RNA interference-dependent knockdown of *limk1* (*limk1* "–"); the host strain #36303 with genetic background of #26294 but without dsRNA (*limk1-C*, control). The following Gal4 driver strains were used: #6793, Gal4 and green fluorescent protein (GFP) are expressed in cholinergic neurons (CHN); #6794, Gal4 is expressed in some neurons of the mushroom body and in cortex glia (MB/Glia); #7009, Gal4 is expressed in dopaminergic and serotoninergic neurons (DAN/SRN); #30027, Gal4 is expressed in *fruitless*-positive neurons (FRN); Act-Gal4, Gal4 is expressed in all fly tissues. The numbers are given according to the Biocollection and Bloomington Drosophila Stock Center (BDSC).

Fly maintenance. Flies were kept on a standard yeast–raisin medium with 8 a.m.-8 p.m. daily illumination at 25 ± 0.5°C. 5-7-day-old males were used in experiments. For Act-Gal4 > 9116 cross, lethality was estimated as (1 – S) × 100%, where S is a relative share of the eclosed flies of the desired phenotype (straight wings, red eyes) to all eclosed flies. Both for males and females, lethality appeared to be 100% (n = 26 and 38 for surviving males and females without *limk1* transgene, respectively).

Antibodies. The following antibodies were used: primary antibodies: rat anti-Limk1 multi-specific (Enzo Life Sciences, USA, ALX-803-343-C100), rabbit anti-p-cofilin (MyBioSource, USA, MBS9458475), rabbit anti-beta-tubulin (Abcam, UK, ab179513); secondary antibodies: donkey anti-rat HRP-conjugated (Thermo Fisher Scientific, USA, A18745), donkey anti-rabbit HRP-conjugated (Abcam, UK, ab97064), donkey-anti-rat Alexa Fluor 594 (Thermo Fisher Scientific, A-21209), goat anti-rabbit Alexa Fluor 633 (Invitrogen, USA, A21071).

RNA extraction and reverse transcription. Ten 5-day-old male flies were homogenized in 300 µl TRI reagent (MRC, USA, TR 118). Total RNA was isolated using a Direct-zol RNA MiniPrep kit (Zymo Research, USA, R2050) with an on-column DNAse I treatment, according to the manufacturer's protocol. Concentration of RNA was measured using an Eppendorf BioPhotometer (Germany). Quality of RNA was estimated by electrophoresis in 1.5% agarose gel using an Agagel Mini system (Biometra, Germany). 1 µg RNA was reverse-transcribed using a MMLV reverse transcriptase (Evrogen, Russia, #SK022S), according to the manufacturer's protocol, using random decamer primers and RNAse inhibitor (Syntol, Russia, #E-055).

Semi-quantitative real-time PCR (sqPCR) analysis of *limk1* expression level. The reaction was performed using a StepOnePlus real-time PCR system (Applied Biosystems, USA) with a qPCRmix HS SYBR+LowROX (Evrogen, #PK156L). The level of *rpl32* and *EF1α2* expression served as an internal control. *limk1* primers (PP12636

Drosophila strains

Strain	Genotype	Phenotype
Canton-S (*CS*)	wild type strain	dark-red eyes, grey body, straight wings
Act-GAL4	w[1118]; P{w[+mC]=} 25FO1/CyO, y[+]. The genetic background of *CS*	light pink eyes, curled wings. GAL4 is expressed in all tissues of the fly organism
#6794	w[*]; P{w[+mC]=nrv2-GAL4.S}8 P{w[+mC]=UAS-GFP.S65T}eg[T10] chromosomes 1;3	GAL4 is expressed in some neurons of the mushroom body and in glia cells
#6793	w[*]; P{w[+mC]=ChAT-GAL4.7.4}19B P{w[+mC]=UAS-GFP.S65T} Myo31DF[T2] chromosomes 1;2	GAL4 is expressed in cholinergic neurons
#7009	w[1118]; P{w[+mC]=Ddc-GAL4.L}Lmpt[4.36] chromosomes 1;3	GAL4 is expressed in dopaminergic and serotoninergic neurons; light pink eyes
#30027	w[1118]; P{w[+mW.hs]=GawB}fru[NP0021] chromosomes 1;3	GAL4 is expressed in *fruitless* neurons
#9116	y[1] w[*]; P{w[+mC]=UAS-LIMK1.T:Ivir\HA1}M6 chromosomes 1;2	additional copy of *limk1* is expressed strongly under UAS control
#26294	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02063}attP2 chromosomes 1;3	dsRNA for RNAi is expressed under UAS control
#36303	y[1] v[1]; P{y[+t7.7]=CaryP}attP2 chromosomes 1;3	genetic background is the same as in #26294, but without UAS-dsRNA; bright red eyes

Numbers are shown for the strains taken from the Bloomington Drosophila Stock Center.

in FlyPrimerBank) bind all five *limk1* cDNA isoforms, including both premature and mature forms. Relative *limk1* transcript levels were calculated using the comparative ΔΔCt method, with the help of StepOne software v2.3 (Applied Biosystems). Primer sequences and sqPCR parameters are given in Online Resource 1.

Western blot analysis of LIMK1 level in *Drosophila* brains. For each sample, 12 brains (head ganglions) were homogenized in 18 µl of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1× Complete protease inhibitor cocktail (Sigma-Aldrich, USA, #11697498001), 1 mM phosphatase inhibitor Na_3VO_4, pH 8.0), kept at 4°C for 30 min, mixed with 9 µl of 3× Laemmli buffer, and heated at 95°C for 5 min. Samples were separated by denaturing SDS-electrophoresis in 10% polyacrylamide gel, using a Mini-Protean Tetra System electrophoresis chamber (Bio-Rad, USA).

Proteins were transferred from the gel to a PVDF membrane (Thermo Fisher Scientific, 88520; 0.2 µm), using wet transfer for 70 min at 100 V. The membrane was blocked for 40 min with 3% bovine serum albumin (BSA) in TBST (0,1% Tween-20 in TBS) at room temperature, washed, and incubated with one of the primary antibodies in TBST (anti-LIMK1 – 1 : 1000, anti-p-cofilin – 1 : 1000, anti-β-tubulin – 1 : 2000; 0.3% BSA) for 2 h with gentle shaking. After washing, the membrane was incubated with secondary HRP-conjugated antibodies in TBST (1 : 2000; 0.3% BSA) for 1 h with gentle shaking. Chemiluminescent detection was performed using a ChemiDoc XRS+ System (Bio-Rad). Average signal level was measured using ImageJ. The levels of LIMK1 and p-cofilin were normalized to the level of the reference protein β-tubulin, based on calibration curves.

Confocal microscopy. Experiments were performed as in [37], with some modifications. Briefly, brains were isolated in a chilled PBS buffer (pH 7.5), fixed in 4% paraformaldehyde in PBS for 1 h at RT, washed in TBS with 0.5% Triton X-100, and incubated in 0.2% Tween 20 – TBS with 5% BSA for 1 h at RT. Antibodies against LIMK1 and p-cofilin were diluted

in TBT (0.2% Tween 20, 0.5% BSA in TBS, pH 7.5) at the ratio 1 : 200, secondary antibodies – at the ratio 1 : 200. Incubation was performed at 4°C for 3 days with primary antibodies and overnight with secondary antibodies. Brains were covered by a Vectashield mounting medium containing DAPI (Vector laboratories, H-1200-10) and scanned with a laser scanning confocal microscopy (LSM 710 Carl Zeiss, Germany; Confocal microscopy Resource Center; Pavlov Institute of Physiology Russian Academy of Sciences, Saint Petersburg, Russia). Scanning was performed frontally with X63 objective at different depths (z-step 2 μm).

For each Gal4 driver, crosses with *limk1* "+", *limk1* "–", and *limk1-C* were stained and analyzed at the same day, using the same microscope settings. Fiji software was used for images analysis. The Manders' overlap coefficients [38] were calculated using Colocalization Threshold analysis to measure pairwise co-localization of GFP, LIMK1, and p-cofilin on z-stacks. tM1 and tM2 are the Manders' coefficients for channels 1-2 and 2-1 (proportion of the signal in the first channel co-localized with the second channel) above the calculated threshold level of fluorescence. All images were auto contrasted for figure preparation, which did not affect co-localization level.

Learning and short-term memory analysis. Learning and memory abilities of the Gal4 > UAS flies were tested using CCSP [9, 39, 40]. For training, a naïve male (without mating experience) was placed together with a mated female for 30 min. Then testing with a new mated female was performed for 5 min. Courtship index (CI) was percentage of the time that a male spent courting a female. Learning index (LI) were estimated at the following time points after training: 0 min (learning), 15 min, 30 min, 60 min, 120 min. LI was calculated as follows:

$$LI = [(CI_N - CI_T)/CI_N] \times 100\% = (1 - CI_T/CI_N) \times 100\%$$

where CI_N is the middle CI for the naive males, and CI_T is the middle CI for the males after training. The following criterion of learning and memory preservation was considered: LI differed from zero. Additional criterion for the memory preservation at the time X was absence of the statistically significant differences between LI(X) and LI (0 min). Change in LI over time represents dynamics of forgetting for a given *Drosophila* strain. Statistical analysis was performed using two-sided randomization test (significance level $\alpha < 0.05$, $n = 20$, 10,000 iterations), Drosophila Courtship Lite software [Nikolai Kamyshev, 2006; freely available from the author (nkamster@gmail.com) upon request]. Randomization test is better for LI comparison than the *t*-test [39]. For each *Drosophila* cross, forgetting curves were built as regression lines for LI values at various points of time.

RESULTS

***limk1* transcription level in fly crosses.** *limk1* expression in the *Drosophila* crosses was measured at the level of RNA and protein (Fig. 1). For the CHN driver and MB/Glia driver, we observed a pronounced increase in the *limk1* transcription after crossing with the strain carrying the extra *limk1* copy under UAS promotor (*limk1* "+") relative to the cross with the control strain (*limk1-C*). Difference between the *limk1* "+" crosses and the *limk1* "–" crosses (with *limk1* neurospecific knockdown) was significant for all drivers except FRN (Fig. 1a). Development at 25°C also caused 100% lethality for the Act-Gal4 > *limk1* "+" cross, in agreement with [41]. This clearly shows that the UAS-*limk1* transgenic construct is activated by Gal4. Out of ~100,000 *Drosophila* neurons, only 127 and 80 are dopaminergic and serotoninergic, respectively [42], and ~1500 are *fruitless*-positive [4]. This may explain slight difference or lack thereof in the expression of *limk1* measured throughout the body of the above crosses.

LIMK1 and p-cofilin protein levels in CHN crosses. Cholinergic neurons are the dominant neuron type in *Drosophila* [43]. CHN > *limk1* "+" cross showed the highest level of *limk1* activation. To reveal *limk1* expression changes more specifically, we measured the level of LIMK1 protein in the isolated *Drosophila* brains of the CHN crosses. Decrease in the LIMK1 total level was seen in the *limk1* "–" cross relative to the control. However, we did not observe any difference in the LIMK1 level between *limk1* "+" and *limk1* "–" crosses (Fig. 1, b and c). This could be the effect of compensatory LIMK1 translation decrease in the *limk1* "+" cross due to the *limk1* overexpression. There were no interstrain differences in the level of main LIMK1 product p-cofilin as well. The above corresponds to the results of previous study [44], where expression of the constitutively active LIMK1 did not affect the level of p-cofilin in the *Drosophila* nervous system, although leading to the increase in filamentous actin.

LIMK1 and p-cofilin distribution in the brains of CHN and MB/Glia crosses. Changes in the LIMK1 level could occur locally in the *Drosophila* brain, being more prominent in the structures expressing Gal4 and marked by GFP. We performed analysis of the LIMK1 and p-cofilin distribution in the brains of the crosses with the CHN driver using confocal microscopy (Fig. 2).

Generally, both LIMK1 and p-cofilin levels are low in the MB lobes and in the central complex (CC) [37]. Similar picture was observed here for the most of the *limk1* "–" and *limk1-C* crosses (Fig. 2a). Co-localization of LIMK1 and GFP was mostly observed in the neuropil structures, such as antennal lobe (AL) and superior medial protocerebrum (SMP), while the neuronal cell bodies were mostly LIMK1- and p-cofilin-positive (Fig. 2b). For the CHN > *limk1* "+" cross, we observed

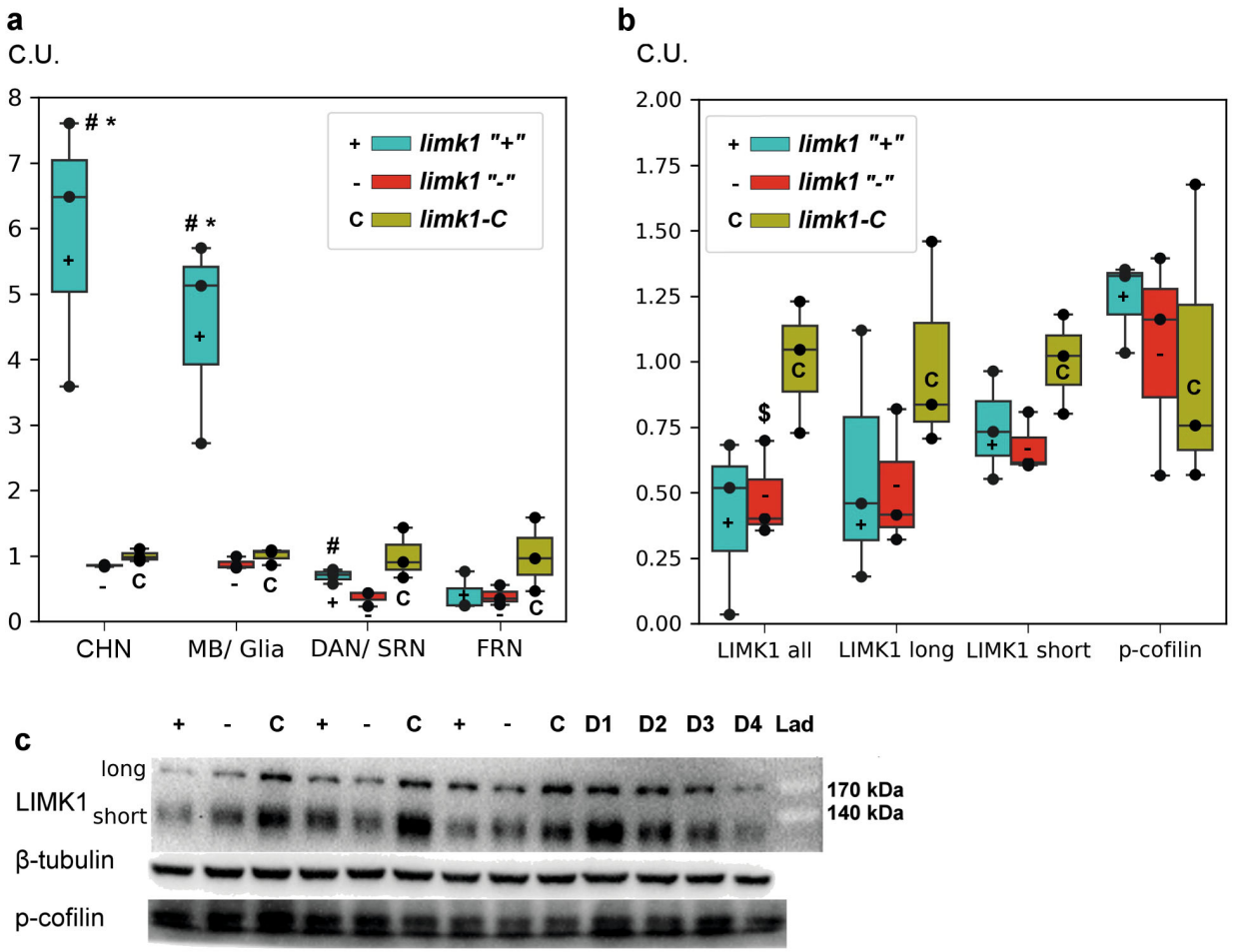

Fig. 1. *limk1* expression in the *Drosophila* Gal4 > UAS crosses. a) RNA level, the whole body (box plots). Statistical differences: * *limk1* "+" cross *vs* control (*limk1-C* cross); # *limk1* "+" cross *vs limk1* "–" cross (two-sided t-test; $p < 0.05$, $n = 3$). Medial is shown as a black line. b) Protein level, head ganglia of CHN > UAS crosses. Statistical differences: $ *limk1* "–" cross *vs limk1-C* cross (two-sided *t*-test; $p < 0.05$, $n = 3$). c) Protein bands for CHN > UAS crosses, Western blotting data. + *limk1* "+" cross; – *limk1* "–" cross; C, *limk1-C* cross (three independent samples are shown for each cross); D1-D4, calibration dilutions (1, 0.8, 0.6, 0.4, respectively). C.U., conventional units (equal to 1 for the mean normalized LIMK1 value for *limk1-C* crosses).

decrease in the LIMK1 and p-cofilin distribution contrast: their levels were elevated in the structures expressing Gal4, including β-lobes (βL) of MB and CC.

For the CHN driver, increase in the GFP–LIMK1 and GFP–p-cofilin co-localization was shown for the *limk1* "+" cross relative to the control. For the MB/Glia driver, the same effect was also observed relative to the *limk1* "–" cross (Fig. 3). Thus, *limk1* activation in cholinergic neurons and in MB/glia seems to increase the levels of LIMK1 and p-cofilin specifically in these types of cells.

Short-term memory and forgetting dynamics. For the strains with neurospecific alterations of *limk1* expression, learning and memory abilities were tested in CCSP paradigm. Courtship indices (CI) were calculated for the individual males (Fig. S1 in the Online Resource 1), and then learning indices (LI) were calculated for the male groups tested at a specific time after training (Fig. 4). For the CHN driver, LI of the *limk1* "+" cross significantly decreased with time relative to the initial value (LI(0)), being not different from zero after 30 min. Interestingly, the forgetting curve for *limk1* "+" and *limk1* "–" crosses were rather similar, located below the curve for the *limk1-C* cross with LI not showing a significant decrease for up to 120 min. For the MB/Glia driver, the *limk1* "+" curve was nearly the same as for the CHN driver, which could be caused by the similar spatial pattern of Gal4 expression (e.g., in MB). However, here the *limk1* "+" curve was much lower than those for the *limk1* "–" and *limk1-C* with LI differences observed after 15 min. The above corresponds to the fact that the LIMK1 and p-cofilin levels were increased in the MB/glia cells of the *limk1* "+" cross relative to both *limk1* "–" and *limk1-C* crosses, while for the CHN driver increase was observed only compared to the control cross. In all cases, neurospecific increase in the LIMK1 and p-cofilin levels seems to decrease courtship memory.

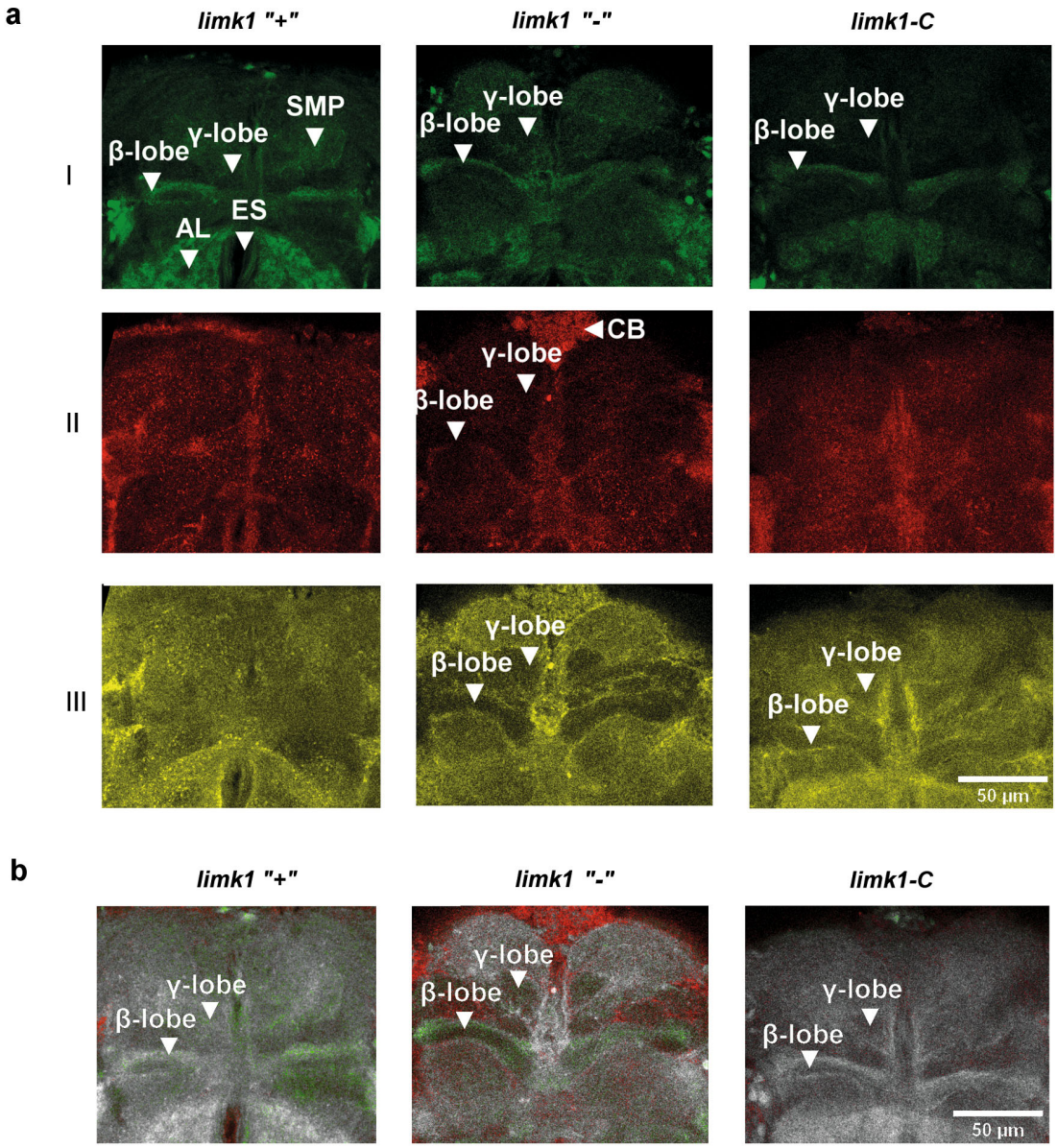

Fig. 2. LIMK1 and p-cofilin distribution in the *Drosophila* brain. a) LIMK1 and p-cofilin localization in the brain structures of the crosses with the CHN driver (γ-lobe level). Color scheme: green, GFP (I); red, p-cofilin (II); yellow, LIMK1 (III). b) Co-localization of GFP and LIMK1. Color scheme: white, area of co-localization; green, only GFP; red, only LIMK1. Structure names: AL, antennal lobes; CB, cell bodies; ES, esophagus; βL, β-lobes; γL, γ-lobes (γ5 area); SMP, superior medial protocerebrum.

For the dopaminergic/serotoninergic neurons driver (DAN/SRN), the *limk1* "+" cross was unable to store memory already 15 min after training. For the FRN driver, we also observed a significant difference between the *limk1* "+" and *limk1* "–" crosses. The first one had LI values about zero at most time points, while the *limk1* "–" cross preserved memory up to 60 min. Fluctuations of the CI and LI values were rather high for all strains, which may reflect uneven degree of the Gal4 > UAS system activity and LIMK1 change in the individual flies. Nevertheless, the *limk1* "+" crosses mostly showed decrease in LI relative to the control or the crosses with *limk1* knockdown. This effect was largely independent on the neuronal-specific driver. On the contrary, the effect of *limk1* knockdown was strain-specific, as described in [37]: its induction in the MB/glia cells and *fruitless* neurons resulted in the initially high LI, decaying to zero in about two hours.

DISCUSSION

In the nervous system, the processes of memory formation and forgetting compete with each other. Forgetting makes behavior more flexible, provides generalization, and prevents overfitting [27].

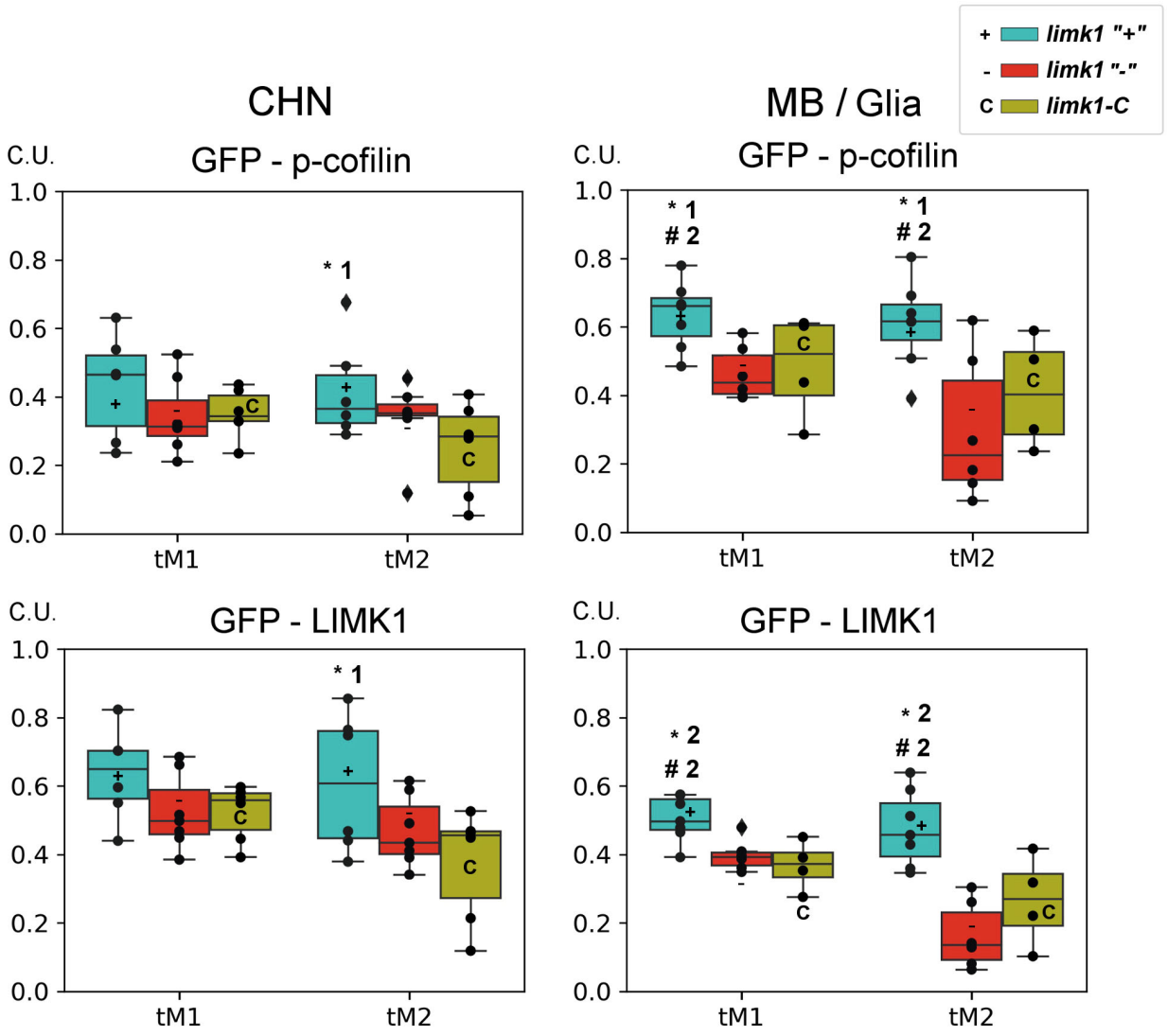

Fig. 3. Co-localization analysis of Gal4 (GFP), LIMK1, and p-cofilin. tM1 and tM2 are Manders' coefficients of the channels 1-2 and 2-1 overlap above the threshold level of fluorescence. Medial is shown as a black line. Outliers are shown as diamonds. Statistical differences: * from *limk1-C* cross; # from *limk1* "–" cross; *t*-test (1 – one-sided, 2 – two-sided), $p < 0.05$, $n = 4$-7.

There are different forms of active forgetting, such as interference-based forgetting, retrieval-induced forgetting, neurogenesis-based forgetting, and intrinsic forgetting. The latter is of particular interest, as it activates along with memory acquisition, preventing consolidation of non-specific memory traces [23]. Imbalance of these processes may significantly impair the animal's cognitive abilities.

Memory suppressing genes include genes for silencing RNAs, cell receptors, proteases, chromatin-modifying enzymes, and components of signaling cascades [45]. Phosphorylation of cofilin, which depends on LIMK1 [32], induces *Drosophila* intrinsic forgetting in olfactory learning paradigm [30]. The LIMK1-dependent phosphorylation of cofilin increases concentration of the filamentous actin, affecting the shape of dendritic spines. LIMK1 also regulates gene activity in the cofilin-independent way via the CREB transcription factor [46]. Both processes can influence neuronal morphology and effectiveness of synaptic transmission. In the rat hippocampus, translation of LIMK1 in the dendritic spines changes their morphology [47], which could be crucial for learning and memory storage. In mice, LIMK1 mediates long-term memory formation through CREB activation [48, 49]. However, to the best of our knowledge, there are no data in the literature on positive effect of the LIMK1 – p-cofilin signaling pathway on the *Drosophila* memory. Thus, in the fruit fly this pathway seems to specifically respond for memory erasure.

Here, we have shown that activation of the transgenic *limk1* in different types of neurons leads to the decrease of LI in CCSP. The decrease may occur just after training (for the FRN driver) or somewhat later.

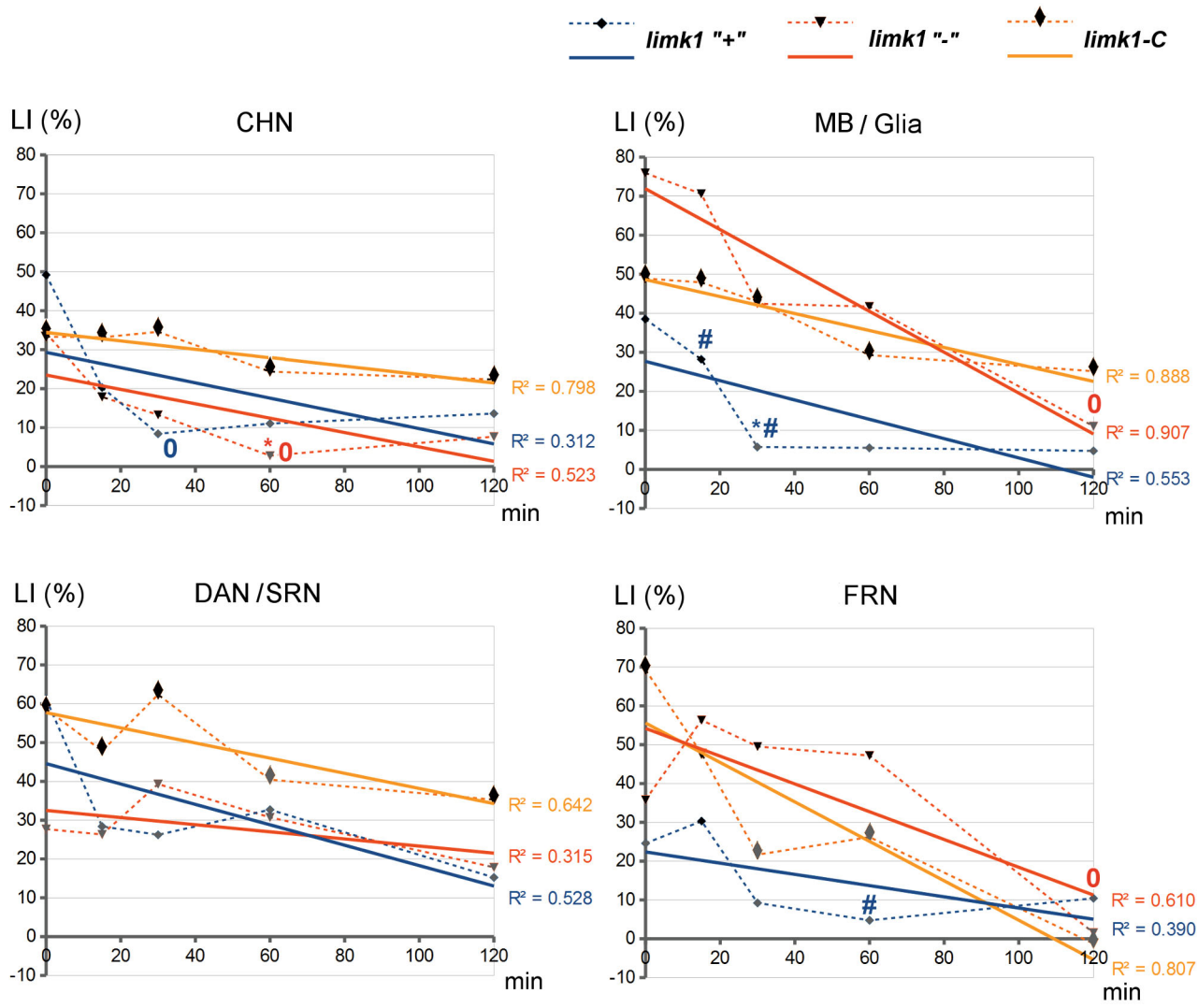

Fig. 4. Learning indices of *Drosophila* crosses with neuronal type-specific alteration of *limk1* activity. Lines: dotted, connects LI values for each cross at different time point; thick, regression line; R^2, coefficient of determination. Statistical differences: * from control (*limk1-C* cross); # from *limk1* "–" cross; 0, from LI at 0 min after training; black markers on curve, from zero (two-sided randomization test, $\alpha < 0.05$, $n = 20$).

In the first case, learning is initially impaired in flies, similar to that for agn^{ts3} [35, 50-52]. The rate of forgetting appears to be low, when LI is low just after training, and high when it drops soon. In both cases, the LIMK1-dependent signaling seems to prevent memory persistence.

We did not observe a significant difference of the p-cofilin level in the brains of the CHN > *limk1* "+" and CHN > *limk1* "–" crosses relative to the control cross. This could be caused by the activity of non-canonical PAK-independent signaling pathway, which includes Sickie factor counteracting the LIMK1-dependent cofilin phosphorylation via the Ssh phosphatase [44]. Interestingly, Sickie also impairs forgetting in *Drosophila*, acting in the PPL1-γ1pedc dopaminergic neurons and reducing their synaptic activity [53]. In addition to their role in forgetting, LIMK1 and cofilin regulate axon growth during the *Drosophila* neuronal morphogenesis [32]. Increased *limk1* activity in the whole nervous system causes fly death [41]. Hence, *limk1* expression in the fly organism must be tightly regulated. This probably explains why the pronounced increase in the *limk1* transcription in our study did not lead to the similar increase in its protein level, as well as the level of p-cofilin.

The paramount question is in which brain structures and/or cell types the LIMK1-dependent signaling cascade regulates forgetting. In olfactory learning paradigm, forgetting is induced by the Rac and p-cofilin in MB neurons [30]. The Rac1–SCAR/WAVE–Dia pathway in γKC activates forgetting of the short-term anesthesia sensitive memory, whereas the Cdc42–WASp–Arp2/3 pathway erases anesthesia-resistant memory [54]. γ5 KC are directly involved in the courtship mem-

ory formation [6]. γ-lobes (γL) outputs are involved in the STM expression, while the α/βL outputs mediate long-term memory expression [55]. Thus, it is reasonable to presume that the LIMK1 activation in γL and α/βL could induce courtship short- and long-term memory forgetting, respectively. In both CHN and MB/ Glia driver strains, Gal4 is mainly expressed in α/βL and less in γL (see Fig. 2 and [37]). In both γL and α/βL, the levels of LIMK1 and p-cofilin are generally low. Hence, fast forgetting induced by CHN and MB/Glia drivers is probably caused by the LIMK1 and p-cofilin increase in γL.

Remarkably, STM is also impaired by the *limk1* knockdown in the cholinergic neurons, but not in the MB/glia cells. This may be the effect of incomplete *limk1* knockdown in the cholinergic neurons, as its neurospecific levels of LIMK1 and p-cofilin did not differ from that in the *limk1* "+" cross. Different brain structures are involved in the above two cases as well. CC is known to regulate courtship memory [56]. All CC structures contain cholinergic neurons [57]. Gal4 level is increased in the CC of the CHN driver strain, but less so in the MB/ Glia driver strain [37]. Possibly, the *limk1* knockdown induced by the MB/Glia driver affects CC outputs to MB, resulting in the memory decrease. Glia cells also participate in regulation of the *Drosophila* courtship and memory processes [58].

Dopaminergic system is the key element regulating learning and memory processes in both olfactory learning and courtship conditioning paradigms. Dopaminergic neurons respond to courtship motivation [59], as well as associative memory formation and forgetting [29]. NO serves as a dopamine co-transmitter in the subset of dopaminergic neurons, including PAM-γ5 and PPL1-γ1pedc. Activation of the above neurons generally induces positive and negative associative odor memory, respectively, while NO changes their valence to the opposite [60]. Memory modality is also affected by the order of conditioned and unconditioned stimuli, changing from positive to negative and *vice versa* in the backward conditioning (the effect is dependent on Dop1R2) [61]. Final behavioral effect is determined by the dopaminergic plasticity rule, which specify the mode of dopaminergic neurons–KC–MBON interactions. Its effect is maximization of separation between the reinforced inputs synaptic weights, in agreement with the information maximization principle [62]. Serotoninergic neurons also affect courtship behavior [43] and regain mating motivation of the males after unsuccessful courtship attempts [63]. However, the role of this neuronal type in courtship learning and memory processes remains largely unknown.

aSP13, which are both dopaminergic and *fruitless*-positive neurons, govern memory formation in CCSP [4, 6]. We did not observe a significant increase in the *limk1* RNA for the crosses with DAN/SRN and FRN drivers, possibly because of the relative rarity of these cell types in *Drosophila*. However, both drivers impaired courtship memory in the *limk1* "+" crosses. Learning and memory were impaired after the *limk1* knockdown by DAN/SRN, as well as by CHN, possibly due to the systemic disorders of the brain cognitive processes. The most pronounced memory differences caused by *limk1* activation and suppression in the MB/ glia cells and *fruitless* neurons probably reveals specific role of these brain cells in the LIMK1-dependent CCSP forgetting.

Generally, intrinsic forgetting lags behind learning, making it possible for memory to form and consolidate. This lag may be absent in the studied crosses and agn^{ts3}, resulting in the LI decrease in the course of training or soon after. Fast memory decay was observed for both *limk1* activation and suppression: LI did not differ from zero 2 h after learning. In contrast, for the control cross, LI (120 min) was equal to zero only with the FRN driver. Hence, courtship memory preservation seems to require proper balance of the *limk1* activity in different brain structures. Decrease in the LIMK1 and p-cofilin levels in agn^{ts3} after heat shock is associated with the recovery of ability to learn and form courtship memory [35, 64]. Heat shock also affects the agn^{ts3} profile of microRNAs [36] and activity of the specific dopaminergic neurons responsible for aversive learning [65]. Thus, mechanisms of courtship memory impairments and recovery seem to be rather complex.

It is still unclear how the fly brain processes information about the failed courtship attempts, and which kind of signals affect the activity of aSP13. Most of the MB neurons involved in the courtship memory coincide with the neurons responsible for appetitive memory in Pavlovian conditioning [20]. Thus, we can expect similar mechanisms of forgetting in both CCSP and olfactory learning paradigm. As PAM neurons generally cause attractive behavioral effects, e.g., with sugar reward [66], we can propose an antagonism between the courtship and other behavioral programs, such as feeding or spontaneous locomotor activity. Choice between feeding and courtship is controlled by tyramine, which activates P1, the key brain structure regulating courtship process [4, 67]. Another control link could be the complex system of interconnected KC and MBON, regulated by dopaminergic neurons. PAM-γ5 (aSP13) secret NO, reversing their attractive effect to aversive [60]. Hence, activation of PAM-γ5 may serve as a trigger, switching off the male courtship, while its suppression or modality change leads to forgetting.

Another question is how LIMK1 and p-cofilin induce forgetting at the subcellular level. Forgetting of anesthesia sensitive and anesthesia resistant memory in olfactory learning paradigm is probably caused by formation of linear and branched actin, respectively, indicating the role of cytoskeletal morphology in these

processes [54, 68]. Lack of active cofilin leads to cytoskeletal defects and impairs release of synaptic vesicles [69]. Overactivation of the LIMK1 – cofilin pathway could lead to the significant rearrangements of cytoskeleton in PAM-γ5, which, in turn, could cause imbalance between the dopamine and NO release. The above can enhance the effects of NO, receipted by the nearest KC or MBON and lead to the return of high courtship motivation.

In summary, our data show involvement of LIMK1 and p-cofilin in the *Drosophila* brain in courtship memory decay. Changes in the *limk1* expression, as well as LIMK1 and p-cofilin distribution within the brain, were associated with interstrain differences of learning and memory. Thus, even local variation of the *limk1* expression in the *Drosophila* brain can drastically affect its learning and memory abilities. Using drivers with narrower pattern of Gal4 expression can help to reveal the role of specific neuronal subtypes in forgetting of courtship memory.

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