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Wolbachia infection may improve learning and memory capacity of *Drosophila* by altering host gene expression through microRNA

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ABSTRACT

Wolbachia are endosymbiotic bacteria present in a wide range of invertebrates. Although their dramatic effects on host reproductive biology have been well studied, little is known about the effects of *Wolbachia* on the learning and memory capacity (LMC) of hosts, despite their distribution in the host nervous system, including brain. In this study, we found that *Wolbachia* infection significantly enhanced LMC in both *Drosophila melanogaster* and *D. simulans*. Expression of LMC-related genes was significantly increased in the head of *D. melanogaster* infected with the *w*Mel strain, and among these genes, *crebA* was up-regulated the most. Knockdown of *crebA* in *Wolbachia*-infected flies significantly decreased LMC, while overexpression of *crebA* in *Wolbachia*-free flies significantly enhanced the LMC of flies. More importantly, a microRNA (miRNA), *dme-miR-92b*, was identified to be complementary to the 3'UTR of *crebA*. *Wolbachia* infection was correlated with reduced expression of *dme-miR-92b* in *D. melanogaster*, and *dme-miR-92b* negatively regulated *crebA* through binding to its 3'UTR region. Overexpression of *dme-miR-92b* in *Wolbachia*-infected flies by microinjection of agomirs caused a significant decrease in *crebA* expression and LMC, while inhibition of *dme-miR-92b* in *Wolbachia*-free flies by microinjection of antagomirs resulted in a significant increase in *crebA* expression and LMC. These results suggest that *Wolbachia* may improve LMC in *Drosophila* by altering host gene expression through a miRNA-target pathway. Our findings help better understand the host-endosymbiont interactions and, in particular, the impact of *Wolbachia* on cognitive processes in invertebrate hosts.

1. Introduction

Wolbachia are gram-negative endosymbiotic bacteria that infect a wide range of arthropods and filarial nematodes. It is estimated that up to 40% of arthropod species are infected with *Wolbachia* (Zug and Hammerstein, 2012). *Wolbachia* are best known for their manipulation of host reproduction that serves to enhance their transmission through host populations (Zheng et al., 2011a; LePage et al., 2017; Beckmann et al., 2017), and it has also been shown that *Wolbachia* can influence host fitness traits, including physiology, immunity and pathogen interference (Hedges et al., 2008; Zug and Hammerstein, 2015; Ye et al., 2017; Ross et al., 2017; Teixeira et al., 2008). The influence of *Wolbachia* on fitness traits may be multidimensional, and a number of host genes and proteins modified by *Wolbachia* infection have been identified (Xi et al., 2008; Landmann et al., 2009; Caragata et al., 2017; Zheng et al., 2011b; Yuan et al., 2015; Christensen et al., 2016).

Many microorganisms have the ability to manipulate host behaviour to increase their successful transmission. Recent studies have revealed evidence for a link between some bacteria and the cognition of their hosts. For example, germ-free mice showed a defect in non-spatial and working memory tasks, and reduced expression of brain-derived neurotrophic factor (BDNF) in the hippocampus (Gareau et al., 2011), which is crucial for synaptic plasticity and cognitive function. Furthermore, disruption of the gut microbiota after antibiotic treatment also induces cognitive impairment accompanied by a significantly decreased BDNF level in the adult brain of mice (Desbonnet et al., 2015). Additionally, there are an increasing number of studies in animal models showing the effect of some probiotics on cognitive behaviour.

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Savignac et al. found that *Bifidobacteria longum 1714* can improve learning and memory as shown in the object recognition test, Barnes maze, and fear conditioning (Savignac et al., 2015). *Wolbachia* are known to accumulate in nervous tissues of hosts, including the brain (Albertson et al., 2013; Strunov et al., 2013, 2017). In *Drosophila*, the areas most infected by *Wolbachia* are the central brain and the sub-oesophageal ganglion (Albertson et al., 2009, 2013). The central brain includes the antennal lobes responsible for receiving input from the olfactory sensory neurons, and the mushroom bodies which provide sensory learning and memory capacity (Albertson et al., 2009; Strausfeld and Li, 1999; Zars et al., 2000). *Wolbachia* strains have been demonstrated to affect olfactory-cue performance, mating and male aggression in adult *Drosophila* (Peng et al., 2008; Peng and Wang, 2009; Liu et al., 2014; Rohrscheib and Brownlie, 2013). Our previous study reveal *Wolbachia* mediates the expression of dopamine related genes, and decreases the sleep quality of their insect hosts (Bi et al., 2018). Kishani Farahani et al. revealed that *Wolbachia*-infected wasps display shorter memory retention in new environments compared with uninfected wasps; in this way, they can increase the broadcast of *Wolbachia* by forgetting the trace connected with previous environments (Kishani Farahani et al., 2017). It was reported that *w*VulC *Wolbachia*-infected *Armadillidium vulgare*, a terrestrial isopod, had worse ability to learn and memorize the correct direction after training compared to a *Wolbachia*-free group. *Wolbachia* infection in *A. vulgare* may affect cognitive processes by decreasing host adaptation capacity (Templé and Richard, 2015); however, the mechanisms by which these changes are imposed were not explored.

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression post-transcriptionally (Zhang et al., 2013). They play important roles in development, cellular growth control, and organismal behaviour (Zhang et al., 2014). Prior studies have shown that *Wolbachia* may use host miRNA to manipulate host gene expression and facilitate colonization in the mosquito *Aedes aegypti* (Hussain et al., 2011). In addition, Yang et al. (2014) have revealed that *miRNA-133* could inhibit behavioural aggregation by controlling dopamine synthesis in locusts. Recently, Liu et al. (2018) have demonstrated that a conserved invertebrate *miRNA-14* plays an important role in ecdysteroid regulated development in the silkworm *Bombyx mori*.

To better understand the impact of *Wolbachia* infection on host cognitive ability, we investigated the influence of two *Wolbachia* strains, *w*Mel and *w*Ri, which are found to infect wild populations of *Drosophila melanogaster* and *D. simulans*, respectively, on the learning and memory capacity (LMC) of *Drosophila* hosts. Our results suggest that *Wolbachia* may use host miRNAs to regulate transcripts of LMC-related genes, resulting in altered cognitive ability in *Drosophila* hosts. As *Wolbachia*-infected arthropods and filarial nematodes can be intermediate hosts during infection of vertebrates, the behavioural and ecological consequences of arthropod infection may be of great importance in controlling pests and insect-borne diseases.

2. Materials and methods

2.1. Wolbachia strains, Drosophila fly stocks and S2 cells

D. melanogaster infected with *Wolbachia* strain *w*Mel (Brisbane nuclear background with introgressed *w*Mel from YW, namely Dmel *w*Mel) and *w*Ri-infected wild populations of *D. simulans* (Dsim *w*Ri) were kindly provided by Professor Scott L. O'Neill (Monash University, Australia). Wild-type flies w^{1118} were *Wolbachia*-free. Tetracycline treatments were performed as described previously (Hoffmann et al., 1986), to generate genetically paired fly lines that were *Wolbachia*-free, here referred to as Dmel T or Dsim T. We verified the tetracycline treatment by PCR using primers specific for the *Wolbachia wsp* gene (Fig. S1). Penicillin treatments were performed as described previously (Gotoh et al., 2007), here referred to as Dmel P or Dsim P. Gut flora was reconstituted using standard methods (Chrostek et al., 2013), and all experiments were conducted at a minimum of six generations post-antibiotic treatment. A transgenic *crebA* RNAi line (*crebA*-hp) was obtained from Tsinghua FlyCenter (Beijing) on the background of w^{1118}. The UAS-*crebA* line was purchased from the Bloomington *Drosophila* Stock Center. The *actGal4/Cyo-pscGFP* driver-line was obtained from Curie Institute, Paris, France and reared in standard medium in our laboratory. This *actGal4* with actin promoter can drive ubiquitous gene expression in flies. The *Wolbachia*-infected *actGal4* flies [*actGal4* (I*)] was the offspring through crossing *actGal4/Cyo-pscGFP* males and *Wolbachia*-infected balancer females (*Wolbachia* are maternally transmitted). These transgenic flies were treated with tetracycline if necessary, as described previously (Hoffmann et al., 1986), and demonstrated to be *Wolbachia*-free by PCR using primers specific for the *Wolbachia wsp* gene (Liu et al., 2014). All fly lines were reared on standard cornmeal-yeast-agar medium at 25 °C with a photoperiod of 14L: 10D (light: dark) under non-crowded conditions (200 ± 10 eggs per 50 mL vial of medium in a 150-mL conical flask) (Yamada et al., 2007). *Drosophila* S2 cells were cultured at 27 °C in 90% Schneider's insect medium (Sigma) and supplemented with 10% heat inactivated fetal bovine serum (FBS). S2 cells were transfected at densities ranging from 0.5 to 5 × 10^6 cells per mL.

2.2. Learning and memory capacity assays

The conditioning and testing protocol with sugar reward was as described previously, with minor modifications (Ichinose et al., 2015). Briefly, 4～7-day-old flies were starved for 16–20 h before experiment. A conditioned stimulus (CS+) tube was made by spreading saturated sucrose (allowed to dry before use) onto a filter paper that covered the entire training tube. Another tube representing the CS− was prepared, containing a filter paper soaked in water (and allowed to dry). The two odours used in the experiment were 3-octanol and 4-methylcyclohexanol, which were diluted with paraffin oil to 10%. Approximately 100 starved flies were loaded into the elevator section of a T-maze and trained as following: flies were transferred to the CS− tube and exposed to one odour for 2 min. After 30 s of clean air stream, they were transferred back into the elevator and into the sugar reward (CS+) tube, where they were exposed to the other odour for 2 min. We tested olfactory memory 24 h after training (Fig. 1A). The performance index (PI) was calculated by subtracting the number of flies running toward the unconditioned odour from the number of flies running toward the conditioned odour and dividing by the total. A single PI value is the average score from two groups (Groups 1 and Group 2 in Fig. 1A) of flies trained with the reciprocal CS+/CS− odour combination (3-octanol or 4-methylcyclohexanol). To reduce variation between experiments, all fly lines were tested in parallel in each experiment. In all LMC assays, the sample size was ～100, and replicate number was 12. LMC assays were performed and samples were collected at 8: 00–11: 30 in the morning.

2.3. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA enriched in small RNAs was isolated from adult flies using a miRNeasy extraction kit (TIANGEN catalogue no. DP501). Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Promega) and a miRNA first-strand cDNA synthesis kit (TIANGEN catalogue no. KR201, containing reverse primer) were used to prepare the Oligo(dT)-primed cDNA with a poly(A) tail. qRT-PCR was carried out using gene expression assays and SYBR Green miRNA (from the heads of flies) expression, respectively, according to the manufacturer's instructions (Tiangen), on a LightCycler 480 instrument (Roche). Gene-specific primers for LMC-related genes were designed (Table S1). As endogenous controls, U6 snRNA and the ribosomal protein gene *rp49* were used to quantify miRNA and mRNA expression levels, respectively. The relative expression of each gene was calibrated against the reference gene using $2^{-\triangle Ct}$ ($\triangle Ct = CT_{,\ target\ gene} - CT_{,\ reference\ gene}$).

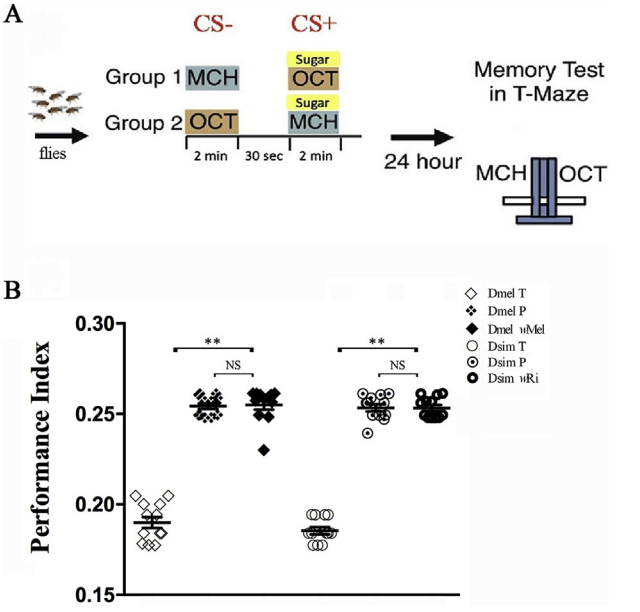

Fig. 1. *Wolbachia* infection significantly improves learning and memory capacity in *Drosophila*. (A) Design of the experiment for assaying the learning and memory capacity of *D. melanogaster*. For group 1, presentation of 3-octanol was paired with a sugar reward. The reciprocal group received sugar with 4-methylcyclohexanol. In the test situation, flies of each group were allowed to choose between 3-octanol and 4-methylcyclohexanol. (B) Effects of *Wolbachia* infection on LMC of *Drosophila*. Dmel T: *D. melanogaster* treated with tetracycline (*Wolbachia*-free); Dmel P: *D. melanogaster* treated with penicillin (*Wolbachia*-infected); Dmel *w*Mel: *D. melanogaster* infected with *w*Mel *Wolbachia*; Dsim T: *D. simulans* treated with tetracycline (*Wolbachia*-free); Dsim P: *D. simulans* treated with penicillin (*Wolbachia*-infected); Dsim *w*Ri: *D. simulans* infected with *w*Ri *Wolbachia*. (ANOVA, post-hoc Tukey's HSD; $^{**}p < 0.01$, NS: not significant. n = 12).

2.4. Prediction of miRNA targeting crebA

RNAHybrid and RNA22 software (IBM) were used to find potential miRNAs that might target *crebA* in *D. melanogaster*. BLASTN was performed to search homologous sequences of *crebA* and miRNA. Expression profiles of candidate miRNAs were confirmed by qRT-PCR analysis as described above.

2.5. Transfection of inhibitor and mimic of miR-92b into Drosophila S2 cell

The *miR-92b* inhibitor and mimic, as well as the control 'scramble' inhibitor and mimic (Table S1) were synthesized by GenePharma. Approximately 2 μg of inhibitor or mimic RNA was transfected into *Drosophila* S2 cells using Cellfectin according to the manufacturer's instructions (Promega). Cells were collected at 48 h after transfections, total RNA was extracted, and qRT-PCR analysis was performed with *crebA*-specific primers as described above.

2.6. Plasmid construction

Sense and antisense oligonucleotides of the *crebA* (NM_206374.2) 3'UTR fragment from 358 to 418 bp were synthesized (Integrated DNA Technologies), which contains an imperfect *miR-92b* target sequence and 5'-*Xho*I and 3'-*Not*I overhangs for cloning (Table S1). The synthesized 3'UTR was cloned to the downstream of *Renilla* luciferase in the *Xho*I/*Not*I restriction enzyme sites of the psiCHECK2 vector (Promega, Madison, WI, USA) to generate psi-*crebA* 3'UTR-wt, while psi-*crebA* 3'UTR-mut plasmids were created by mutating the seed region of the *miR-92b* site (GTGCAATT to GTAGCGTT) (Table S1).

2.7. Luciferase assay

Drosophila S2 cells were grown to approximately 80% confluence in 6-well plates and co-transfected with 0.1 μg psi-*crebA* 3'UTR-wt, psi-*crebA* 3'UTR-mut or psiCHECK2 empty vector, and 0.4 μg miR-*92b* mimic or mimic control using Fugene HD (Promega). Luciferase assays were performed 42 h later using a Dual-Luciferase reporter system (Promega); *Renilla* and firefly luciferase activities were measured with a Luminoskan Ascent (Thermo Labsystems) luminometer. For each sample, *Renilla* luciferase activity was normalized to firefly luciferase activity. Each experiment was repeated three times.

2.8. MiRNA inhibition and overexpression in vivo

Agomirs and antagomirs were purchased from Ribobio company. The miRNA agomir was a cholesterylated and chemically modified stable miRNA mimic which can induce target gene silencing when delivered *in vivo* with similar effects to those caused by the overexpression of endogenous miRNA (Wang et al., 2013). A chemically modified and cholesterol-conjugated single-stranded RNA analogue named miRNA antagomir is complementary to the miRNA and can specifically silence endogenous miRNAs (Krützfeldt et al., 2005). The thorax of ~3-day-old adult flies was microinjected with 50 pmol agomir-92b/NC or antagomir-92b/NC (100 μM in a volume of 0.5 μL) using a UMP3 Ultra Micro Pump (World Precision Instruments Inc). The mRNA levels of *CrebA* were detected 48 h post-injection, and these flies were used for the learning and memory assay as described above.

2.9. Statistical analysis

Four biological replicates were carried out for each experiment (unless otherwise noted) in this study. Results were presented as means ± SE (n = 4). Twelve biological replicates were carried out for each LMC experiment; results are presented as scatter plots (n = 12). The GraphPad Prism program (Prism 5, GraphPad Software) was used to analyse and graphically present all *in vitro* and *in vivo* data. Comparisons of the data series between two conditions were achieved by Student's *t*-test. Comparisons between more than two groups were made with one-way ANOVA, followed by Tukey's HSD comparisons between the experimental group and its controls. $p < 0.05$ indicates significant difference, and $p < 0.01$ indicates extremely significant difference; NS: not significant.

3. Results

3.1. Wolbachia infection improves the LMC of Drosophila

Prior to investigating the effect of *Wolbachia* on LMC in flies, we demonstrated that tetracycline treatment had no effect on LMC of w^{1118}, *D. melanogaster* and *D. simulans* (Table S2), as it has recently been shown that tetracycline not only depletes *Wolbachia*, but also massively shifts the composition of the gut microbiome (Ye et al., 2017). To confirm the effect of *Wolbachia* on the LMC of *Drosophila*, we also used penicillin, which does not affect *Wolbachia*, to eliminate other bacteria in Dmel *w*Mel and Dsim *w*Ri. We did not observe any effect of penicillin treatment on the LMC of either *D. melanogaster* or *D. simulans* (Table S2).

To examine whether *Wolbachia* infection can influence LMC, we trained flies, including *w*Mel-infected and *w*Mel-free *D. melanogaster* (Dmel *w*Mel and Dmel T), as well as *w*Ri-infected and *w*Ri-free *D. simulans* (Dsim *w*Ri and Dsim T), and then tested their LMC. We used the performance index (PI) to evaluate LMC, which was calculated by subtracting the number of flies running toward the unconditioned odour from the number of flies running toward the conditioned odour and dividing by the total. As shown in Fig. 1B, the PI of Dmel *w*Mel flies was significantly higher than that of Dmel T flies ($p < 0.01$). A similar

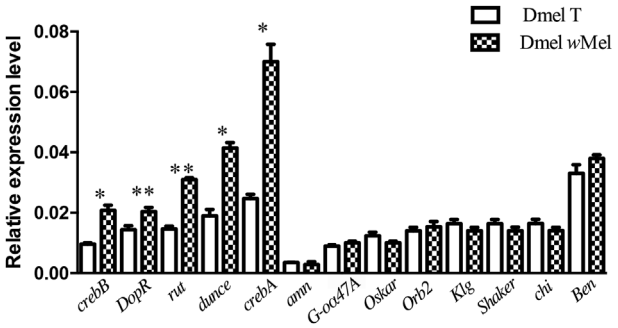

Fig. 2. *Wolbachia* infection increases the mRNA level of 5 out 13 LMC-related genes in the head of *D. melanogaster*. Student's *t*-test; $*p < 0.05$, $**p < 0.01$, n = 4.

result was found for *D. simulans* (Fig. 1B, $p < 0.01$). However, significant differences in PI were observed neither between Dmel *w*Mel and Dmel P, nor between Dsim *w*Ri and Dsim P. These results indicate that *Wolbachia* infection correlates with a significant increase in the LMC of their *Drosophila* hosts, this effect is independent of host background, and, furthermore, it is not other bacteria but *Wolbachia* that affect the LMC level of flies.

3.2. Wolbachia infection alters expression of some LMC-related genes

To dissect the molecular mechanism of LMC changes when there is a *Wolbachia* infection, we focused on the classical model organism *D. melanogaster*. We first decided to test the expression of 13 well-characterized genes known to be related to LMC in the head of *D. melanogaster*. qRT-PCR showed that among these genes, five of them: *crebB*, *DopR*, *rutabaga* (*rut*), *dunce* and *crebA* were significantly up-regulated in the head of Dmel *w*Mel flies compared to Dmel T flies (Fig. 2, $p < 0.05$ or 0.01). *CrebB* encodes Cyclic-AMP response element binding protein B, which plays a critical, evolutionarily conserved role in the conversion of short-term memory (STM) to long-term memory (LTM) (Zhang et al., 2015). *DopR* encodes D1-like dopamine receptor which is required for aversive and appetitive learning in *Drosophila* (Kim et al., 2007). *rut* encodes a calcium/calmodulin-dependent adenylyl cyclase and is associated with *Drosophila* memory formation (Kacsoh et al., 2015; Han et al., 1992; Levin et al., 1992). *Dunce* encodes a Cyclic-AMP specific phosphodiesterase, which has been revealed to be closely related to *Drosophila* memory formation (Kacsoh et al., 2015; Byers et al., 1981; Chen et al., 1986). *CrebA* encodes Cyclic-AMP response element binding protein A and has been shown to be involved in dendrite development in *Drosophila* (Iyer et al., 2013). Of the five up-regulated genes, *crebA* was up-regulated most in the presence of *Wolbachia*.

3.3. Wolbachia mediates LMC change through regulation of crebA

Since *crebA* manifested the highest degree of up-regulation (increased about three-fold) in Dmel *w*Mel flies when compared to Dmel T flies, we examined whether the improvement of LMC in Dmel *w*Mel was due to increased expression of *crebA* by *Wolbachia* infection. Hence, we knocked down *crebA* in *Wolbachia*-infected flies by using a *Wolbachia*-infected *actGal4* driver line [*actGal4* (I*)]. As shown in Fig. 3A, *actGal4* drove significant down-regulation of *crebA* in *actGal4*; *crebA-hp* (I*) flies when compared with *actGal4* (I*) control flies ($p < 0.01$), though it was still higher than in the *Wolbachia*-free control (*crebA-hp*) (here we did not get the *w*Mel-infected *crebA-hp* control). Then, *CrebA*-knockdown *Wolbachia*-infected flies [*actGal4*; *crebA-hp* (I*)] were tested for LMC after training. As expected, the PI of *crebA*-knockdown *Wolbachia*-infected flies was significantly lower than that in *actGal4* (I*) control flies ($p < 0.01$, Fig. 3B), indicating that knockdown of *crebA* in *Wolbachia*-infected flies significantly reduces LMC.

Here, to strengthen the initial observed LMC changes due to the *Wolbachia* infection in a second *D. melanogaster* genetic background, we compared the LMC between *actGal4* and *actGal4* (I*). As shown in Fig. S2, the PI for *actGal4* (I*) was higher than that for *actGal4*, further supporting that *Wolbachia* infection may increase the LMC of their *Drosophila* hosts.

To further investigate whether the up-regulation of *crebA* induced by *Wolbachia* causes an increase in LMC, we then overexpressed *crebA* in *Wolbachia*-free flies. Fig. 3C shows that *crebA* was significantly up-regulated in *actGal4*; *UAS-crebA* flies relative to control flies ($p < 0.01$). Correspondingly, the PI of flies overexpressing *crebA* was significantly higher than that in the two control fly lines ($p < 0.01$, Fig. 3D), suggesting that overexpression of *crebA* can indeed significantly improve LMC in *Drosophila*. We also tested the PI of uninfected flies knocking down *crebA* and of *Wolbachia*-infected flies overexpressing *crebA*. We found the consistent results that knockdown of *crebA* caused a reduction of LMC and overexpression of *crebA* improved LMC (Fig. S3). These results indicate that up-regulation of *crebA* due to *Wolbachia* infection is at least one of the reasons that *Wolbachia* infection increase the LMC of their *Drosophila* hosts.

3.4. CrebA is negatively regulated by dme-miR-92b

It has been reported that *Wolbachia* infection might alter transcription of host microRNAs (miRNAs) (Hussain et al., 2011; Zhang et al., 2013) to regulate expression of host genes, thereby affecting host fitness or reproduction. The up-regulation of *crebA* in *Wolbachia*-infected *Drosophila* led us to hypothesize that *Wolbachia* may regulate the expression of host miRNAs targeting *crebA*. Using *in silico* homology searches, a putative miRNA (*dme-miR-92b*) that may target *crebA* was identified. Target sequences with complete complementarity to the *dme-miR-92b* seed region were predicted in the 3'UTR of *crebA* at nucleotides 1557–1564 (Fig. 4A). Expression of *dme-miR-92b* was confirmed using qRT-PCR, with the expression level significantly lower in Dmel *w*Mel flies than in Dmel T flies (Fig. 4B). As *Wolbachia* infection is associated with an increase in the expression of *crebA* (Fig. 2) and a decrease in the expression of *dme-miR-92b*, these results suggest that *dme-miR-92b* acts as an inhibitor of *crebA* expression in *D. melanogaster*.

To further determine whether *crebA* mRNA is repressed by *dme-miR-92b*, we performed two independent experiments. First, *Wolbachia*-free *Drosophila* S2 cells were transfected with a specific synthetic mimic or inhibitor (reverse complementary sequence) of *dme-miR-92b*. Control cells were transfected with an unrelated miRNA sequence (mimic NC) or miRNA negative control (inhibitor NC). After 48 h, we observed a significantly lower transcript level of *crebA* in cells transfected with the miRNA mimic than in cells transfected with control transfections (Fig. 4C). In contrast, *crebA* expression was significantly up-regulated after *dme-miR-92b* inhibitor was transfected (Fig. 4D). These results confirm that *dme-miR-92b* acts as an inhibitor of *crebA* expression.

To test whether *crebA* is a direct target of *dme-miR-92b*, fragments of the 3'UTR seed region of wild-type *crebA* and *crebA* containing a 4-bp mutation in the seed region (Fig. 4A) were respectively cloned into the psiCHECK2 dual luciferase reporter plasmid. Luciferase reporters were co-transfected with either *dme-miR-92b* mimic or miRNA negative control (miRNA NC) into S2 cells. As shown in Fig. 4E, co-transfection of *dme-miR-92b* mimic with the *crebA* 3'UTR reporter resulted in an extremely significant decrease (more than 50%) in luciferase activity compared to other groups. No decrease in luciferase activity was observed when *dme-miR-92b* mimic was transfected together with the mutant reporter or null plasmid (Fig. 4E), indicating that the predicted site in *crebA* is a direct target of *dme-miR-92b*. Taken together, these results suggest that the *dme-miR-92b* might directly regulate *crebA* expression by targeting the 3'UTR of its transcript in *D. melanogaster*.

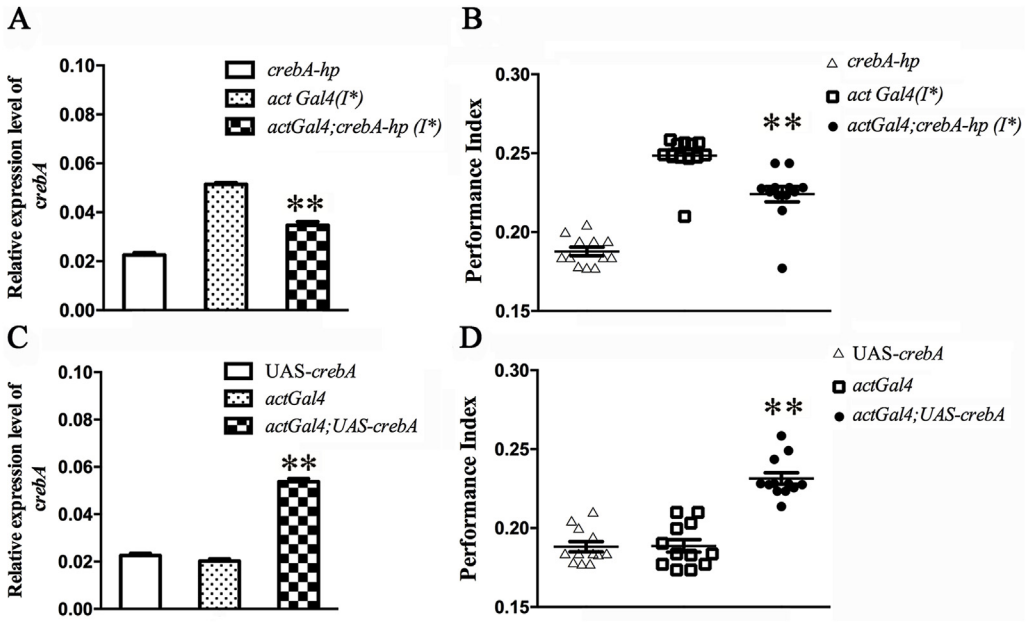

Fig. 3. LMC change is regulated by expression level of *crebA*. (A) *CrebA* expression level was significantly down-regulated in *actGal4*; *crebA-hp* (I*) flies relative to *actGal4* (I*) control flies. (B) LMC was significantly decreased in *crebA*-knockdown *Wolbachia*-infected flies when compared to *Wolbachia*-infected *actGal4* (I*) controls. I*: *Wolbachia*-infected. (C) *CrebA* expression level was significantly up-regulated in *actGal4; UAS-crebA* flies relative to control flies. (D) LMC was significantly increased in flies overexpressing *crebA* when compared to control flies. ANOVA, post-hoc Tukey's HSD; $^{**}p < 0.01$, n = 4 (for gene expression analyses) or n = 12 (for LMC assay).

3.5. Wolbachia infection improves LMC by altering crebA expression through regulation of dme-miR-92b

To further investigate the molecular mechanism by which *Wolbachia* infection improves LMC, we synthesized the agomir and antagomir of *dme-miR-92b*, and injected them into adult flies. The mRNA level of *crebA* in Dmel *w*Mel was significantly decreased after injection of *miR-92b* agomir; correspondingly the LMC was reduced significantly (Fig. 5A and B). In contrast, the mRNA level of *crebA* in Dmel T was significantly increased after injection of *miR-92b* antagomir, and the LMC was also improved compared to the control group (Fig. 5C and D). These results indicate that *Wolbachia* infection improves LMC by increasing *crebA* expression through down-regulation of *dme-miR-92b*.

4. Discussion

As symbionts, *Wolbachia* can manipulate their hosts, ranging from parasitism to mutualism (Cook and McGraw, 2010). While much is known about the effect of *Wolbachia* on host fitness, the underpinning molecular mechanisms are largely unknown (Hussain et al., 2011). Here, we examined the effect of *Wolbachia* on the LMC of adult *D. melanogaster* and *D. simulans*. We observed that the LMC of both *w*Mel-infected *D. melanogaster* and *w*Ri-infected *D. simulans* was significantly enhanced compared to that of *Wolbachia*-free flies. This is in contrast with the results reported by Templé and Richard, who observed a reduction of LMC in the *w*VulC-infected crustacean host *A. vulgare* when compared to *Wolbachia*-free individuals (Templé and Richard, 2015). These contrasting observations may be due to differences in experimental design: Templé and Richard tested the effect of *Wolbachia* on STM (memory tested < 1 h post-training), while our experiments examined the effects on LTM (memory tested 24 h after training). Another explanation is that different strain of *Wolbachia* may have distinct effects on their hosts, and a single *Wolbachia* strain also has various effects on different hosts (Dean, 2006; Chafee et al., 2011; Russell et al., 2018). In their study (Templé and Richard, 2015), they used pathogenic strain *w*VulC which induces feminization of the host *A. vulgare*. Here, we used *w*Mel and *w*Ri *Wolbachia* which induce sperm-egg cytoplasmic incompatibility in *D. melanogaster* and *D. simulans*, respectively. Furthermore, the different taxonomic groups of the hosts (Insecta and Crustacea) may also contribute to the difference seen in this study and that of Templé and Richard (2015). The crustacean *A. vulgare* is reported to have a gregarious lifestyle and a strong tendency for individuals to aggregate (Broly et al., 2013). This lifestyle carries a danger of being fully attacked by predators. The reduction of learning capacity in *A. vulgare* induced by *w*VulC infection may decrease the tendency of the isopod to aggregate, thus decreasing the risk of being fully preyed upon, which may also ensure the survival and transmission of *Wolbachia*. However, *Drosophila* is not gregarious. The improvement in LMC by *Wolbachia* may help them to find food and mates and to avoid danger more efficiently, which increase the fitness of the hosts and thus favour propagation of the bacteria.

Along with the increase in LMC performance, there was an increase in the expression of five LMC-related genes: *crebB*, *DopR*, *rut*, *dunce* and *crebA*, in *w*Mel-infected *D. melanogaster* relative to *w*Mel-free flies. Among the five genes, *crebA* showed the largest increase in gene expression, indicating that *crebA* might play a major role in the observed improvement in LMC of *Drosophila* associated with *Wolbachia* infection. Formation of LTM is associated with increased protein synthesis in the central nervous system (CNS) (Kandel, 2001). Regulation of gene transcription *via* the cAMP (cyclic adenosine 3′, 5′-monophosphate)-mediated second messenger pathway has been implicated in learning and memory. An increasing number of studies on organisms ranging from invertebrates to mammals have suggested that CREB (cAMP response element-binding protein) acts as the molecular switch that may be the core component controlling long-term synaptic plasticity and LTM (Zhang et al., 2015; Bourtchuladze et al., 1994; Mizuno et al., 2002). CaMKII (Calcium/Calmodulin-Dependent Protein Kinase II) has been shown to undergo CREB-dependent gene transcription and translation during LTM formation in mushroom bodies and dorsal anterior lateral neurons, the two key processing and memory forming structures within the *Drosophila* brain (Chen et al., 2012). Here, we demonstrated that knockdown of *crebA* can significantly attenuate LMC in *Drosophila*, which is consistent with the result in mice where targeted mutation of the CREB gene showed profound deficiency in studies of learning and memory (Bourtchuladze et al., 1994). *CrebA* has been demonstrated to be required for promoting high-order dendritic branching complexity by regulation of COPII secretory pathway genes (Iyer et al., 2013). As dendritic spines contribute to the synaptic plasticity underlying higher brain functions such as learning and memory, we therefore suggest that *Wolbachia* infection improves the LMC of *Drosophila* by increasing the *crebA* expression level and thus promoting dendrite development.

Based on our observations, *Wolbachia* infection appears to modulate *crebA* expression by manipulating host miRNA. *Wolbachia* are known to

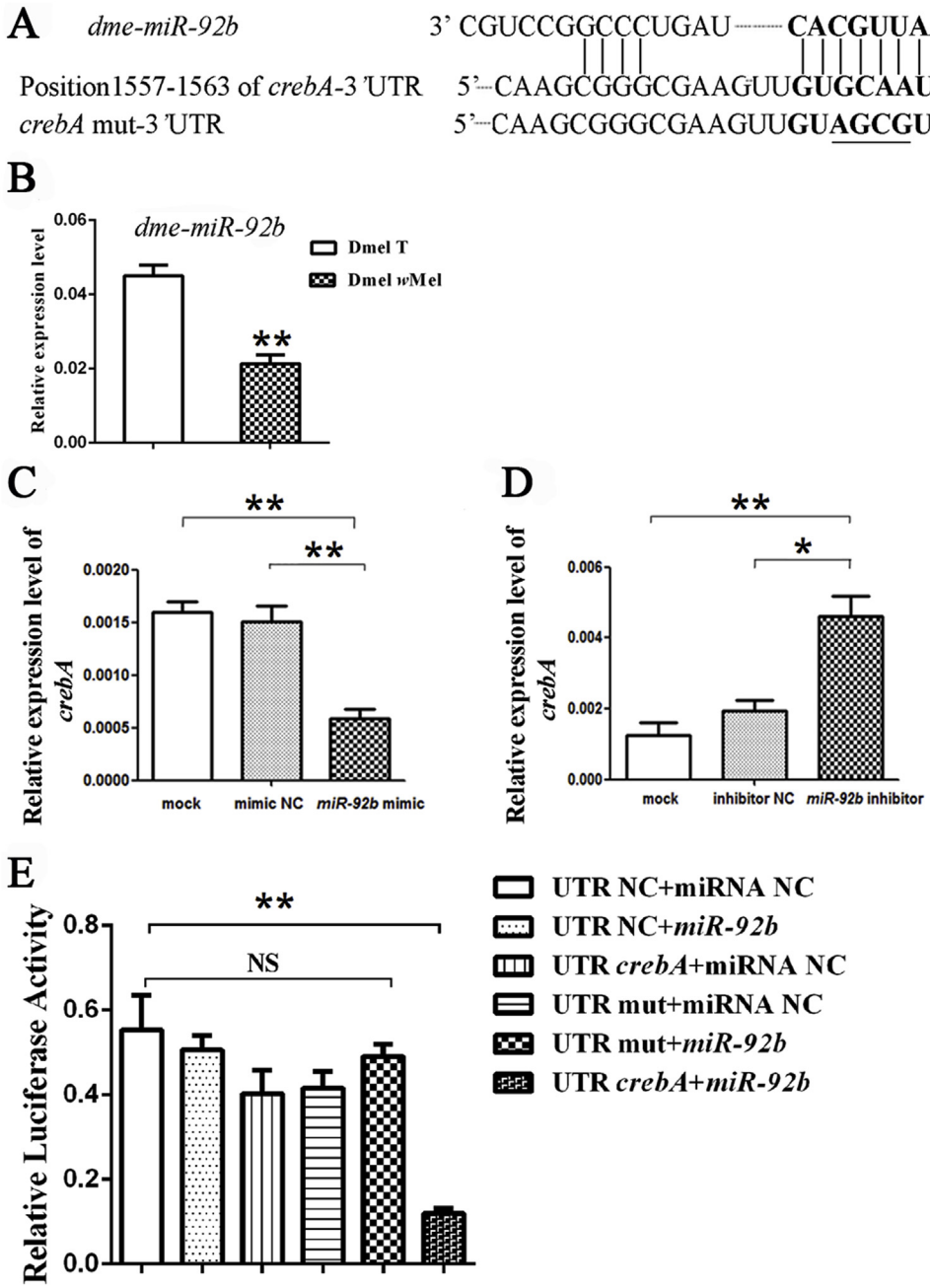

Fig. 4. *Dme-miR-92b* negatively regulates *crebA* expression through binding to the conserved *miR-92b* targeting sites of *crebA* 3'UTR in *D. melanogaster*. (A) The *Drosophila crebA* was predicted to be the potential target of *dme-miR-92b* with complete seed region complementarity (Bold). The target sequence was identified in the 3'UTR of *crebA*. The *crebA* 3'UTR mutant in the seed region was also indicated (underlined). (B) qRT-PCR analysis of *dme-miR-92b* in *w*Mel-free (Dmel T) and *w*Mel-infected *D. melanogaster* (Dmel *w*Mel). (C) The *crebA* expression level was decreased after *miR-92b* mimic transfection into *Drosophila* S2 cells. (D) The *crebA* expression level was increased after *miR-92b* inhibitor transfection. (E) Co-transfection of *miR-92b* and *crebA* 3'UTR in *Drosophila* S2 cells significantly inhibited luciferase activity. Mutation of *miR-92b* targeting sites largely abolished this inhibition. ANOVA, post-hoc Tukey's HSD; $*p < 0.05$, $**p < 0.01$, n = 4.

manipulate host miRNAs to control host gene expression to facilitate their own survival and transmission (Hussain et al., 2011; Zhang et al., 2013, 2014). Here, we identified the miRNA *dme-miR-92b*, which is complementary to the 3'UTR of *crebA* mRNA, and showed that its expression is significantly reduced in the presence of *Wolbachia*. Subsequently, we found that *dme-miR-92b* can negatively regulate *crebA* expression through binding to its 3'UTR region. Importantly, injection of *dme-miR-92b* agomirs in *Wolbachia*-infected flies caused a significant decrease in *crebA* expression level and LMC, while injection of *dme-miR-92b* antagomirs in *Wolbachia*-free flies resulted in a significant increase in *crebA* expression level and LMC. These results suggest that *Wolbachia* may regulate miRNA expression of their hosts, and thus affect the target genes of miRNAs, finally changing the host's LMC. Several studies in other systems have shown that miRNAs play an important role in neurological development as well as in learning and memory formation (Saab and Mansuy, 2014). Most recently, Busto et al. tested the potential involvement of 134 miRNAs in intermediate-term memory by silencing individual miRNAs through development and adulthood. They identified several different miRNAs important for olfactory memory formation in *D. melanogaster* (Busto et sl., 2015). Surprisingly, competitive inhibition of some miRNAs decreases memory formation, while inhibition of some others increases memory formation. Among these miRNAs, *miR-92a* can significantly modulate memory after miRNA 'sponging' in the CNS. *miR-92b* is highly conserved from *Drosophila* to humans, but its biological functions during development have not been studied in vertebrates. Using zebrafish as a model, researchers have found that *miR-92b* has a wide diversity of expression profiles in neural cells, including neural precursors and stem cells (Chen et al., 2005). Additionally, studies have shown that neuronally expressed *miR-92* is an endogenous fine regulator of contextual fear memory in mice (Vetere et al., 2014). Yuva-Aydemir et al. reported that *miR-92a* and *miR-92b* are highly expressed in the neuroblasts of larval brain in flies, and they play an important role in *Drosophila* neuroblasts (Yuva-Aydemir et al., 2015). These data suggest that *dme-miR-92b* might play

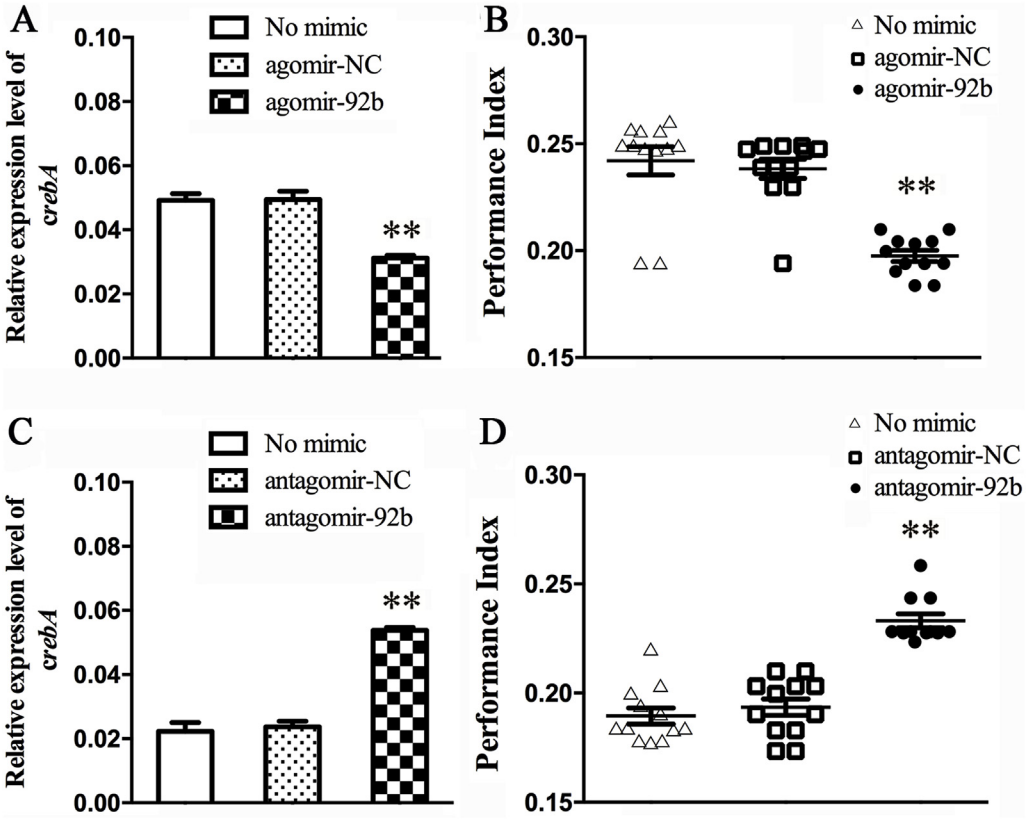

Fig. 5. *Dme-miR-92b* regulates LMC of flies by controlling *crebA* expression. (A) The *crebA* expression level was decreased after injection of *miR-92b* agomir into Dmel *w*Mel. (B) The LMC of Dmel *w*Mel was inhibited after injection of agomir. (C) The *crebA* expression level was increased after injection of *miR-92b* antagomir into Dmel T. (D) The LMC of Dmel T was improved after injection of antagomir. ANOVA, post-hoc Tukey's HSD; $^{**}p < 0.01$, n = 4 (for gene expression analyses) or n = 12 (for LMC assay).

an important role in LMC by regulating the expression of its target genes, such as *crebA*, thus influencing neuronal development and/or behaviour.

In conclusion, we have revealed that, through manipulating host miRNA, *Wolbachia* are able to modulate host gene expression and improve learning performance and LTM formation in adult *Drosophila*. By reducing expression of *dme-miR-92b* in *Wolbachia*-infected *Drosophila*, expression of *crebA*, an important gene that controls LMC in *Drosophila*, is increased, which is associated with enhanced LMC of *Drosophila*, probably by promoting dendrite development through the cAMP signalling pathway or secretory pathway. How *Wolbachia* control expression of miRNAs, including *dme-miR-92b*, remains the focus of further investigation and is emerging as a significant point of interaction between symbiont and host.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.11.007.

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