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Octopamine and tyramine respectively regulate attractive and repulsive behavior in locust phase changes

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Aggregative and solitary behaviors are universal phenomena in animals. Interestingly, locusts (*Locusta migratoria*) can reversibly transit their behavior between gregarious and solitary phase through conspecific attraction and repulsion. However, the regulatory mechanism of neurotransmitters underlying attraction and repulsion among locusts remains unknown. In this study, we found gregarious and solitary locusts were attracted or repulsed respectively by gregarious volatiles. Solitary locusts can transform their preference for gregarious volatiles during crowding, whereas gregarious locusts avoided their volatiles during isolation. During crowding and isolation, the activities of octopamine and tyramine signalings were respectively correlated with attraction- and repulsion-response to gregarious volatiles. RNA interference verified that octopamine receptor α (OARα) signaling in gregarious locusts controlled attraction-response, whereas in solitary ones, tyramine receptor (TAR) signaling mediated repulsion-response. Moreover, the activation of OARα signaling in solitary locusts caused the behavioral shift from repulsion to attraction. Enhancement of TAR signaling in gregarious locusts resulted in the behavioral shift from attraction to repulsion. The olfactory preference of gregarious and solitary locusts co-injected by these two monoamines displayed the same tendency as the olfactory perception in crowding and isolation, respectively. Thus, the invertebrate-specific octopamine-OARα and tyramine-TAR signalings respectively mediate attractive and repulsive behavior in behavioral plasticity in locusts.

Animals demonstrate remarkable behavioral plasticity in response to environmental changes, and they alter behavioral phenotypes while keeping their genotype unchanged[1,2]. Some animals attract to each other and live in group, whereas others avoid contacting with each other and prefer to live in a lone existence. Flocks of birds, schools of fish, herds of mammals, and swarms of insects are common examples of animal aggregation[3–5]. Some emerging properties of aggregated populations have adaptive advantages for these species, such as improved mobility, foraging[6,7] and defense against predators[3,8]. How these animals sense environmental changes and subsequently elicit a series of phenotypic changes is a topic of active research calling for detailed examination at molecular, genomic, biochemical and behavioral levels.

Interestingly, the migratory locust (*Locusta migratoria*) is an excellent model system, which can reversibly shift their behavior between gregarious and solitary phase in response to population density changes[9–12]. Locusts in gregarious phase aggregate with each other for active intraspecific interaction, whereas locusts in the solitary phase isolate themselves, remain cryptic to their conspecifics and prefer to avoid social communication. In laboratory condition, isolation (decreasing population density) and crowding (increasing population density) for a short term trigger the behavioral transition toward the solitary and gregarious phases, respectively[9,12]. Thus, a close examination of the behavioral phase change would allow us to better understand the mechanistic basis of locust polyphenism in aggregation.

Multiple sensory modalities are involved in sensing population density in locusts[9]. External stimuli, including olfactory, visual, and tactile cues, induce behavioral switches between solitary and gregarious phases of locusts[13]. Olfactory cues such as gregarious individual volatiles induce either locust attraction or repulsion that ultimately leads to phase change. Volatiles from gregarious locusts are causal factors for behavioral change in migratory locusts[14]. The chemosensory protein *CSPs* and *takeout* in antenna mediate attraction and repulsion respectively, confirming the important roles of chemosensory proteins in the peripheral system underlying phase change of the migratory locust[14]. However, in the central nervous system, the neurochemical signaling pathways that modulate

the properties of the olfactory system of locusts and alter odor perception in a phase-specific manner remain unknown.

Previous studies have provided insights into genetic, metabolic, and neurological mechanisms underlying phase change in the migratory locust[14–21]. Transcriptome analysis has indicated that the genes involved in tyrosine metabolism are up-regulated in fourth-stadium gregarious locusts[19]. The synthesis of catecholamine in tyrosine and dopamine metabolism is highly active in gregarious locusts and is involved in behavioral change regulation from the solitary to gregarious phase[15,19]. The phenylalanine and tyrosine are common precursor of tyramine and octopamine in the catecholamine pathway. Moreover, octopamine and tyramine, two tyrosine derivatives specifically synthesized in arthropods, reportedly regulate behaviors and neuronal responses in fruit flies and cockroaches[22,23]. In desert locusts, the octopamine receptors (OAR) comprising *SgOctαR* and *SgOctβR* show high expression levels in the brains of fifth-stadium gregarious locusts[24]. Tyramine, the precursor for octopamine production, is also considered as an independent neurotransmitter[25,26]. All these data seem to indicate that the action of biogenic amines are probably linked with the phenotypic changes of locusts, but how octopamine and tyramine regulate olfactory preferences through sensory pathways in phase change of the migratory locust have yet to be elucidated.

In this study, we studied the relationships among dynamics of octopamine and tyramine contents, expression of their receptors, and locust phase changes. Injection of octopamine and tyramine and pharmacological administration were used to investigate the effects of the two neurotransmitters on behavioral and olfactory response. RNA interference was applied to explore the coordinated actions of OAR and tyramine receptors (TAR) in regulating olfactory preferences during phase change of the migratory locust. We found that octopamine-OARα and tyramine-TAR signaling pathways respectively mediate the olfactory perception in behavioral changes between gregarious and solitary locusts.

Results

Olfactory perception during phase change. Because many behavioral traits changed in the arena (see Methods) during the mutual transition between solitary and gregarious locusts, we assigned a single probabilistic metric *P-solitary* (*P-sol*) to evaluate behavioral phase state of locusts from an overall point of view in the first measurement (Supplementary Table S1). *P-sol* = 0 indicates the gregarious phase, whereas *P-sol* = 1 indicates the solitary phase. Attraction index (AI), one of those behavioral parameters for calculating *P-sol*, can define attraction and repulsion to conspecifics in arena behavioral assay (see Methods). However, AI can't discriminate the effects of visual stimuli from olfactory stimuli in the arena, we applied Y-tube behavioral test as an additional evidence to directly detect olfactory preferences during behavioral phase change in the locusts. Thus, the second assay measured olfactory preferences of solitary and gregarious locusts to either volatiles of gregarious locusts or a clean air control by a Y-tube olfactometer.

As in our previous studies[14–19], the phase change, i.e., crowding of solitary locusts (CS) or isolation of gregarious locusts (IG), was clearly depicted from the distinct distributions of *P-sol*. In this study, solitary locusts showed a behavioral shift toward gregarious phase [Mann–Whitney *U* test (MWU), $U = 275$, $P < 0.001$, CS 32 h vs. solitary), but the crowded locusts displayed the distinguishable behavioral pattern from the typical gregarious locusts (MWU, $U = 309$, $P < 0.05$, CS 32 h vs. gregarious) (Supplementary Figure S1A). On the other hand, just after 1 h of isolation, the gregarious locusts shifted their behaviors toward solitary phase (MWU, $U = 148$, $P < 0.001$, IG 1 h vs. gregarious) and the behavioral patterns of these locusts were not different from those of the typical solitary locusts (MWU, $U = 540$, $P = 0.71$, IG 1 h vs. solitary) (Supplementary Figure S1B).

To describe attraction- and repulsion-response of locusts to their conspecifics during phase change, we analyzed the behavioral parameter AI of locusts with crowding or isolation treatment and compared them with that of the naive controls (Figure 1). The solitary locusts tended to approach gregarious conspecifics during entire process of crowding for 1, 4, 16 and 32 h (Kruskal-Wallis Test, $P < 0.001$), and after 16 h or 32 h of crowding, solitary locusts significantly increased their propensity toward gregarious locusts (MWU: 16 h, $U = 1269$; 32 h, $U = 840.5$; both $P < 0.001$) (Figure 1A). On the other hand, gregarious locusts displayed attraction to their conspecifics in the arena behavioral assay. After entire process of isolation for 1, 4, 16 and 32 h, gregarious locusts tended to avoid their conspecifics significantly (Kruskal-Wallis Test, $P < 0.001$). After 1 h of isolation, gregarious locusts showed the propensity of repulsion to the stimulus group (MWU, $U = 679.5$, $P < 0.01$) and then maintained relatively stable thereafter (Figure 1B). Thus, gregarious locusts displayed the propensity of repulsion to the stimulus group in isolation, whereas solitary locusts exhibited the propensity of attraction to the stimulus group in crowding.

Given the parameter AI can't discriminate visual and olfactory response of locusts in the arena behavioral assay, we detected olfactory preference of locusts during the crowding or isolation treatments in Y-tube olfactometer. The treated locusts were tested 1, 4, 16 or 32 h after training and their olfactory preference were compared with that of the naive locusts (Figure 1). The solitary locusts chose to go into the fresh air control arm ($G_1 = 22.03$, $P < 0.001$) and were repelled by the gregarious volatiles. After the entire range of crowding for 1, 4, 16 and 32 h, solitary locusts significantly increased their preference for gregarious volatiles ($G_8 = 35.10$, $P < 0.001$). After 1 h of crowding, solitary locusts increased the proportion of locusts preferring gregarious volatiles ($G_2 = 8.83$, $P = 0.0128$, CS 1 h vs. solitary). As compared with solitary controls, the preference for volatiles were also significantly higher at CS 4 h, CS 16 h, and CS 32 h (CS 4 h, $G_2 = 22.7$; CS 16 h, $G_2 = 21.85$; CS 32 h, $G_2 = 20.99$; all $P < 0.001$). The 4 h of crowding was sufficient to significantly increase the locusts' preference for gregarious volatiles. The preference for gregarious volatiles of the locusts with 32 h of crowding was similar to that of long-reared gregarious locusts ($G_2 = 4.61$, $P = 0.1$, CS 32 h vs. gregarious) (Figure 1C). Thus, after 32 h of crowding, solitary locusts show attraction-response comparable to gregarious locusts before displaying fully gregarious behaviors.

The gregarious locusts chose the volatile arm and were attracted by the volatiles from their conspecifics ($G_1 = 10.12$, $P = 0.001$). After the entire range of isolation for 1, 4, 16 and 32 h, the percentage of locusts choosing the fresh air was significantly increased ($G_8 = 36.25$, $P < 0.001$). When compared with the gregarious controls, the locusts with 1 h of isolation exhibited significantly higher preference for the fresh air control ($G_2 = 27.68$, $P < 0.001$, gregarious vs. IG 1 h), and the percentages of locusts preferring fresh air were also significantly higher at IG 4 h, IG 16 h, and IG 32 h (IG 4 h, $G_2 = 24.02$; IG 16 h, $G_2 = 22.27$; IG 32 h, $G_2 = 28.07$; all $P < 0.001$) (Figure 1D). Although the locusts with isolation for 1, 4 or 16 h did not show the preference for gregarious volatiles or the fresh air (IG 1 h, $G_2 = 1.34$; IG 4 h, $G_2 = 1.49$; IG 16 h, $G_2 = 0.07$; all $P > 0.05$), the gregarious ones isolated for 32 h preferred the fresh air control arm ($G_2 = 7.61$, $P = 0.006$). Thus, the gregarious locusts displayed solitary behavior after 1 h of isolation, but these insects displayed the shift of olfactory preference from attraction to repulsion after 32 h of isolation.

The correlation of OA-OARα and TA-TAR signaling pathways with olfactory preference. In the study, paralleling with the crowding or isolation of locusts for 0, 1, 4, 16 and 32 h to generate the gamut of olfactory behaviors from repulsion to attraction or vice versa, we measured the levels of octopamine and tyramine and the expressions of their receptor mRNAs to establish their correlations with olfactory preference.

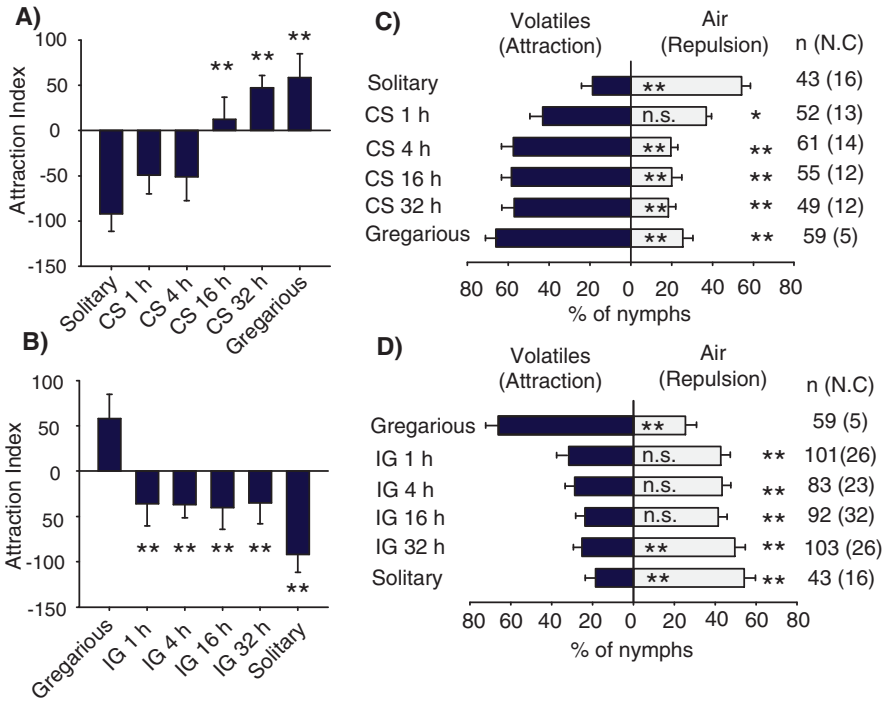

Figure 1 | The migratory locust shows olfactory preferences during phase change. (A) Arena behavioral assay indicates that solitary locusts show attraction-response to their gregarious conspecifics during crowding. (B) Arena behavioral assay finds that gregarious locusts display repulsion-response to their conspecifics during isolation. (C) Solitary locusts increase their preference for gregarious volatiles during the crowding process. (D) Gregarious locusts decrease their olfactory preference for gregarious volatiles in isolation. The asterisks (**) inside the strip indicate the significance of *G*-tests for goodness-of-fit in comparing individual numbers in each arm. The asterisks (* and **) outside the strip indicate the statistical significance of crowding and isolation as compared with naïve controls after Mann–Whitney *U* test (A and B) and *G*-tests for independence (C and D) (*, $P < 0.05$; **, $P < 0.01$). Error bars represent ± SEM.Abbreviations: CS, crowding of solitary locusts; IG, isolation of gregarious locusts; n.s., not significant; n, individual numbers with olfactory preference; N.C, individual numbers without olfactory choice.

The concentration of octopamine is higher in gregarious locusts [Student's *t*-test, $t_{(0.05/2,\ 10)} = 9.5$, $P < 0.001$] (Figure 2A), though the amount of octopamine was not significantly positively correlated with the extent of preferring gregarious volatiles across all entire stage of crowding [linear regression, $F_{(1,\ 35)} = 1.30$, $r^2 = 0.037$, $P = 0.261$]. Given the reference gene ribosome protein 49 (*RP–49*) expressed stably among the housekeeping genes we detected in brains (Supplementary Figure S2), we chose *RP–49* as the reference gene to examine expression levels of receptor genes in the locust brains. The levels of *OARα* mRNA were significantly positively correlated with the degree of preference for volatiles across the entire range of crowding intervals (Figure 2B), but the expression levels of another receptor *OARβ* did not display the same tendency [linear regressions; *OARα*, $F_{(1,\ 35)} = 0.119$, $r^2 = 0.119$, $P = 0.039$; *OARβ*, $F_{(1,\ 35)} = 0.119$, $r^2 = 0.003$, $P = 0.746$] (Supplementary Figure S3A). After 32 h of crowding, the solitary locusts with the most preference for gregarious volatiles displayed significantly higher level of *OARα* mRNA as compared with solitary or gregarious controls [Student's *t*-test; $t_{(0.05/2,\ 10)} = 3.49$, $P = 0.013$, CS 32 h vs. solitary; $t_{(0.05/2,\ 10)} = 3.31$, $P = 0.016$, CS 32 h vs. gregarious], suggesting that solitary locusts may show higher octopamine sensitivity during 32 h of crowding. Furthermore, during the isolation, decrease of the amount of octopamine is significantly positively correlated with the reduction of preference for gregarious volatiles across the entire range of isolation [linear regression, $F_{(1,\ 35)} = 14.672$, $r^2 = 0.367$, $P < 0.001$] (Supplementary Figure S4A). The mRNA levels of two receptors during isolation were not correlated with the reduction of the percentage of preferring volatiles [linear regressions; *OARα*, $F_{(1,\ 35)} = 2.97$, $r^2 = 0.08$, $P = 0.09$ (Supplementary Figure S4B); *OARβ*, $F_{(1,\ 35)} = 0.486$, $r^2 = 0.014$, $P = 0.491$ (Supplementary Figure S3B)].

During the entire process of isolation, the amount of tyramine in the brains increased and was significantly correlated with the extent of preference for fresh air [linear regression, $F_{(1,\ 35)} = 15.20$, $r^2 = 0.31$, $P < 0.001$] (Figure 2C). The level of tyramine is also higher in the brains of solitary locusts [Student's *t*-test, $t_{(0.05/2,\ 14)} = 5.1$, $P = 0.001$, solitary vs. gregarious] (Figure 2C). Although the expression tendency of *TAR* mRNA was not correlated with the trends of preferring fresh air during the isolation [linear regression, $F_{(1,\ 35)} = 15.20$, $r^2 = 0.0001$, $P = 0.949$], its expression level increased significantly after 1 h of isolation [Student's *t*-test, $t_{(0.05/2,\ 10)} = 2.53$, $P = 0.04$, IG 1 h vs. gregarious] (Figure 2D). Moreover, across the entire process of crowding, the fluctuations of the amount of tyramine and the levels of *TAR* mRNA did not correspond with the degree of preference for gregarious volatiles [linear regressions; TA, $F_{(1,\ 35)} = 1.13$, $r^2 = 0.032$, $P = 0.296$ (Supplementary Figure S4C); *TAR*, $F_{(1,\ 35)} = 0.351$, $r^2 = 0.01$, $P = 0.557$ (Supplementary Figure S4D)].

In addition, tyramine β hydroxylase (TβH) catalyzes the synthesis of tyramine into octopamine[25]. qPCR analysis found that the mRNA level of *TβH* increased significantly after 4 h of crowding and then decreased to the original level (One-Way ANOVA, $F_{(4,\ 25)} = 6.028$, $P = 0.003$) during the crowding. By contrast, its mRNA level remained unchanged during the isolation (One-Way ANOVA, $F_{(4,\ 25)} = 1.146$, $P = 0.358$) (Supplementary Figure S5). Moreover, across the entire process of crowding or isolation, the fluctuations of the expression of *TβH* did not correspond with the degree of preference for gregarious volatiles or fresh air, respectively [linear regressions; crowding, $F_{(1,\ 35)} = 0.94$, $r^2 = 0.027$, $P = 0.337$ (Supplementary Figure S6A); isolation, $F_{(1,\ 35)} = 0.008$, $r^2 = 0.002$, $P = 0.931$ (Supplementary Figure S6B)].

The interactions of receptors rely on co-hybridization in special tissues. We therefore performed *in situ* hybridization to detect the spatial expression of *OARα* and *TAR*. The results showed that *OARα*

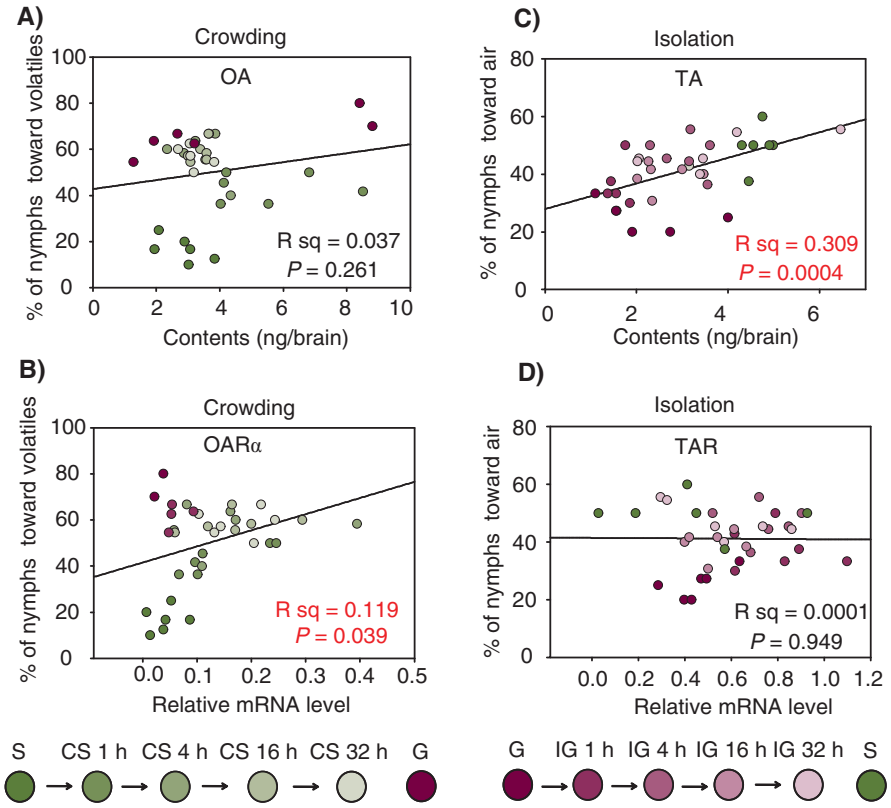

Figure 2 | The correlations of OA-OARα and TA-TAR signaling pathways with the extent of olfactory preferences for gregarious volatiles or the fresh air, respectively. (A and B) Relationships of the amount of octopamine (A) and the expression level of *OARα* (B) in the brains with the degree of preference for gregarious volatiles in crowding (n = 6 each). (C and D) Correlations of the amount of tyramine (C) and the expression level of *TAR* (D) with the extent of the preference for the fresh air in isolation (n = 6 each). Abbreviations: OA, octopamine; TA, tyramine; S, solitary locusts; CS, crowding of solitary locusts; G, gregarious locusts; IG, isolation of gregarious locusts.

and *TAR* were universally expressed in several olfactory centers, including antennal lobes, mushroom bodies, and higher centers of the superior protocerebrum (Supplementary Figure S7).

OARα signaling regulates olfactory preferences for the odor of gregarious locusts. Because the level of octopamine is higher in the gregarious locust brains, we firstly injected octopamine in thoracic cavities of solitary locusts to detect whether octopamine mediates their behavioral changes from solitary to gregarious phase. The results indicated that the solitary locusts injected with octopamine (100 μg) significantly shifted their behaviors toward gregarious phase (MWU, $U = 111$, $P = 0.015$) (Figure 3A) and induced the behavioral change from repulsion to attraction toward their gregarious conspecifics (MWU, $U = 115$, $P = 0.021$) (Figure 3B). The Y-tube assay showed that solitary controls injected with saline preferred the fresh air control arm and displayed repulsion-response to gregarious volatiles ($G_1 = 16.01$, $P < 0.001$). The injection of octopamine increased the proportion of solitary locusts choosing the volatiles ($G_1 = 6.13$, $P = 0.013$), compared with saline controls ($G_1 = 26.31$, $P < 0.001$, octopamine vs. saline) (Figure 3C). Thus, octopamine induces the preference of solitary locusts for gregarious volatiles.

Considering the increased expression level of *OARα* mRNA during the 32 h of crowding, we investigated whether or not *OARα* signaling is necessary in inducing behavioral change from solitary to gregarious phase. We injected ds*OARα* into the brains of solitary locusts, followed by 32 h of crowding or none, and then assessed their behaviors. RNAi knockdown caused a significant reduction of *OARα* mRNA in the brains [Student's *t*-test, $t_{(0.05/2,\ 14)} = 3.4$, $P < 0.01$] (Supplementary Figure S8A). The solitary locusts injected with ds*GFP* or ds*OARα* remained solitary behavior (Figure 3D right). After 32 h of crowding, the ds*GFP*-injected solitary locusts shifted their behavior toward gregarious phase (MWU, $U = 202$, $P < 0.01$, ds*GFP* with CS 32 h vs. ds*GFP*), but the ds*OARα*-injected solitary locusts still showed solitary behavior (MWU, $U = 211$, $P < 0.001$, ds*GFP* with CS 32 h vs. ds*OARα* with CS 32 h) (Figure 3D left). In addition, the analysis of AI showed that ds*GFP* or ds*OARα* did not change the repulsion-response of solitary locusts (Figure 3E). After 32 h of crowding, although dsGFP-injected locusts were attracted by the gregarious ones (MWU, $U = 238$, $P < 0.01$, ds*GFP* with CS 32 h vs. ds*GFP*), ds*OARα*-injected locusts were still repulsed by their gregarious conspecifics (MWU, $U = 357.5$, $P < 0.05$, ds*GFP* with CS 32 h vs. ds*OARα* with CS 32 h) (Figure 3E). These results confirmed that *OARα* mediates the gregarization of solitary locusts.

The Y-tube assay indicated that the solitary locusts injected with ds*GFP* or ds*OARα* showed the preference for the fresh air control (ds*GFP*, $G_1 = 9.14$; ds*OARα*, $G_1 = 10.76$; both $P < 0.01$) (Figure 3F). After ds*GFP* injection and 32 h of crowding, the solitary locusts increased their preference for gregarious volatiles ($G_1 = 6.76$, $P < 0.01$) as compared with ds*GFP*-injected solitary controls ($G_2 = 18.05$, $P < 0.001$, ds*GFP* with CS 32 h vs. ds*GFP*). However, ds*OARα*-injected solitary locusts increased their preference for the fresh air control ($G_1 = 5.44$, $P < 0.05$) as compared with the solitary ones exposed to injection of ds*GFP* and 32 h of crowding ($G_2 = 16.38$, $P < 0.001$, ds*GFP* with CS 32 h vs. ds*OARα* with CS 32 h) (Figure 3F).Thus, OARα mediates the olfactory preference for gregarious volatiles during the crowding of solitary locusts.

TAR signaling mediates repulsion-response to the gregarious volatiles. The titer of tyramine is higher in the solitary locust brains, implying that tyramine could link with the performances of solitary behavior. To verify this guess, we first injected tyramine

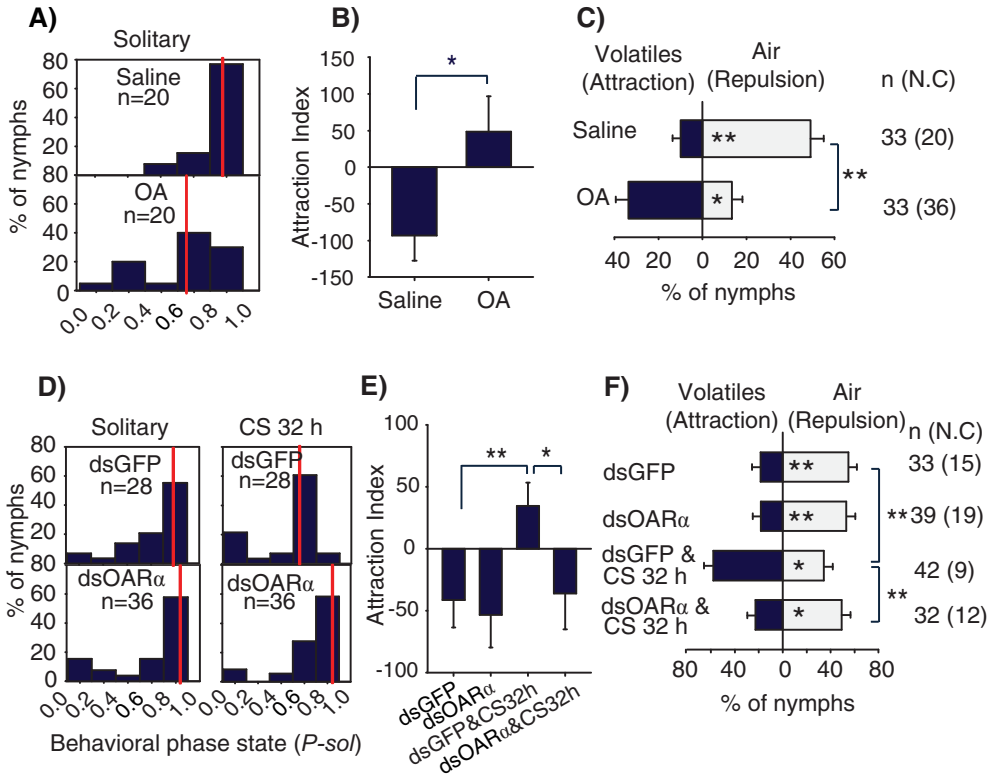

Figure 3 | OARα signaling induces olfactory responses pertaining to induction of locust gregariousness. (A) Injecting octopamine in solitary locusts induces a behavioral propensity toward gregarious phase (Mann–Whitney *U* test, $P < 0.001$). (B) Injecting octopamine in solitary locusts conduces to the propensity of locusts from avoidance to attraction to their conspecifics. (C) Injecting octopamine in solitary locusts causes the increase of preference for gregarious volatiles. (D)After *OARα* gene knockdown, the solitary locusts with 32 h of crowding do not shift their behaviors from solitary to gregarious phase (Mann–Whitney *U* test, $P < 0.01$). (E) During 32 h of crowding, *OARα* gene knockdown inhibits the propensity of locusts displaying attractive response to the stimulus conspecifics. (F) After 32 h of crowding, *OARα* gene knockdown causes a higher proportion of preference for the fresh air control. The asterisks (* and **) inside the strip indicate the significance of comparing individual numbers in each arm after *G*-tests for goodness-of-fit (*, $P < 0.05$; **, $P < 0.01$). The asterisks (* and **) outside the strip indicate the significance of treatment after Mann–Whitney *U* test (B and E) and *G*-tests for independence (C and F) (*, $P < 0.05$; **, $P < 0.01$). Red lines indicate median *P-sol* values. Error bars represent ± SEM. Abbreviations: OA, octopamine; CS, crowding of solitary locusts; n, individual numbers with olfactory preference; N.C, individual numbers without olfactory choice.

(100 μg) into thoracic cavities of gregarious locusts and detected its effects on gregarious behavior. The arena behavioral assay showed that, paralleling with the behavioral shifting of the tyramine-injected gregarious locusts toward solitary phase (MWU, $U = 262$, $P < 0.001$, saline vs. tyramine) (Figure 4A), the AI parameter indicated that the injected gregarious locusts were repulsed by their stimulus group (MWU, $U = 294$, $P < 0.001$, saline vs. tyramine) (Figure 4B).

After tyramine injection, the Y-tube assay revealed that the injected gregarious locusts increased their preferences for the fresh air control (tyramine, $G_1 = 9.05$, $P < 0.01$) as compared with saline-injected gregarious controls that preferred the gregarious volatiles (Saline, $G_1 = 8.18$, $P < 0.01$) ($G_2 = 20.65$, $P < 0.001$, saline vs. tyramine) (Figure 4C). These results indicated that tyramine mediates repulsion-response to gregarious volatiles.

Because the expression level of *TAR* increased after 1 h of isolation, we injected ds*TAR* in the brains of gregarious locusts and isolated them for 1 h to detect whether or not *TAR* signaling is related with the IG process. The qRT-PCR assay indicated that the expression level of *TAR* mRNA was significantly reduced after RNAi knockdown (Student's *t*-test, $t_{(0.05/2,\ 14)} = 5.495$, $P < 0.0001$) (Supplementary Figure S8B). The arena behavioral assay showed that, after 1 h of isolation, the ds*GFP*-injected gregarious locusts shifted their behavior toward solitary phase significantly (MWU, $U = 191$, $P < 0.0001$; *dsGFP* with IG 1 h vs. *dsGFP*) (Figure 4D), but the ds*TAR*-injected gregarious locusts still exhibited gregarious behavior even after 1 h of isolation (MWU, $U = 213$, $P < 0.0001$, *dsGFP* with IG 1 h vs. ds*TAR* with IG 1 h) (Figure 4D). Moreover, after 1 h of isolation, the ds*GFP*-injected gregarious locusts were repulsed by the stimulus group (MWU, $U = 257$, $P < 0.01$; *dsGFP* with IG 1 h vs. *dsGFP*), but the ds*TAR*-injected gregarious locusts were still attracted by their stimulus conspecifics after 1 h of isolation (MWU, $U = 294$, $P < 0.01$, *dsTAR* with IG 1 h vs. ds*GFP* & IG 1 h) (Figure 4E). Thus, TAR mediates the isolation of gregarious locusts.

The Y-tube assay revealed that, after 1 h of isolation, the ds*GFP*-injected gregarious controls increased their preference for the fresh air control as compared with ds*GFP*-injected gregarious controls preferring the arm with the gregarious volatiles (*dsGFP*, $G_1 = 9.67$, $P < 0.005$) ($G_2 = 20.08$, $P < 0.0001$, *dsGFP* with IG 1 h vs. *dsGFP*) (Figure 4F). After 1 h of isolation, the ds*TAR*-injected gregarious locusts decreased their preferences for the fresh air control ($G_1 = 10.24$, $P < 0.01$) as compared with ds*GFP*-injected gregarious ones ($G_2 = 20.49$, $P < 0.01$, *dsTAR* with IG 1 h vs. ds*GFP* with IG 1 h) (Figure 4F). These results indicated that TAR mediates the repulsion-response to gregarious volatiles during the isolation of gregarious locusts.

Interactions of octopamine-OARα and tyramine-TAR signaling pathways in olfactory preferences. The overlapped spatial distribution of *OARα* and *TAR* in antennal lobes and mushroom bodies suggest that octopamine and tyramine may coordinately regulate olfactory preference during locust phase change. To test this hypothesis, we simultaneously activated the OAR and TAR signaling pathways by

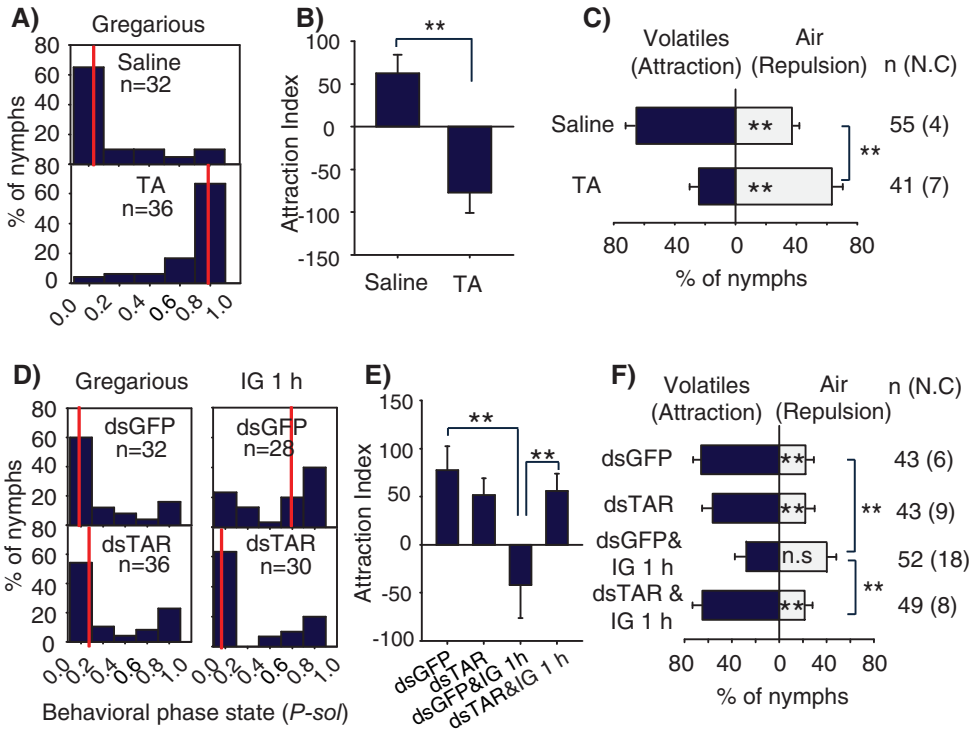

Figure 4 | TAR signaling mediates olfactory responses pertaining to induction of locust solitariness. (A) Injecting tyramine in gregarious locusts elicits solitary behavior (Mann–Whitney *U* test, $P < 0.01$). (B) Gregarious locusts injected with tyramine display repulsive response to their conspecifics. (C) Injecting tyramine in gregarious locusts increases the preference for the fresh air control. (D) *TAR* knockdown does not affect behavioral phase of gregarious locusts. After 1 h of isolation, *TAR* knockdown does not change the behavioral phase of gregarious ones (Mann–Whitney *U* test, $P > 0.05$). (E) Gregarious locusts with *TAR* knockdown and 1 h of isolation still show attractive response to their conspecifics. (F) Gregarious locusts with *TAR* knockdown and 1 h of isolation do not change the olfactory preference when compared with gregarious controls with ds*GFP* injection. The asterisks (**) inside the strip indicate the significance of comparing individual numbers in each arm after *G*-tests for goodness-of-fit ($P < 0.01$). The asterisks (**) outside the strip indicate the significance of treatments after Mann–Whitney *U* test (B and E) and *G*-tests for independence (C and F) ($P < 0.01$). Red lines indicate median *P-sol* values. Error bars represent ± SEM. Abbreviations: TA, tyramine; IG, isolation of gregarious locusts; n, individual numbers with olfactory preference; N.C, individual numbers without olfactory choice.

injecting a fixed amount of tyramine (100 μg) and a gradually increasing concentration of octopamine (0 μg to 200 μg) into the solitary locusts before the Y-tube assay. By compared with the solitary locusts solely injected with 100 μg of tyramine, we analyzed the volatile preference of the locusts with co-injection of tyramine and octopamine (Figure 5A). With the injection of a fixed amount of tyramine (100 μg), the volatile preference of locusts injected with 0, 50 and 100 μg of octopamine was not significantly different from that of solitary controls (50 μg, $G_2 = 0.52$; 100 μg, $G_2 = 2.59$; all $P > 0.05$). However, the preference for gregarious volatiles was significantly higher at 150 and 200 μg of octopamine (150 μg, $G_2 = 13.35$; 200 μg, $G_2 = 19.88$; all $P < 0.005$), suggesting that the pared injection of octopamine and tyramine significantly increased the locusts' preference for gregarious volatiles. This effect did not manifest immediately after octopamine and tyramine injection but instead increased gradually up to 150 μg of tyramine ($G_5 = 30.50$, $P < 0.001$). Thus, increasing octopamine concentration induces stronger attraction to gregarious volatiles once the octopamine concentration surpasses a threshold, whereas the lower octopamine concentrations did not change the attraction-response of solitary locusts.

Seeking clarification of the effects of octopamine and tyramine on olfactory preference during phase change, we analyzed correlations of olfactory preference between the solitary locusts with pared injections of two monoamines and the solitary ones with entire process of crowding intervals. After co-injection of a fixed amount of tyramine (100 μg) and a gradient concentration of 0, 50, 100, 150 and 200 μg of octopamine in solitary locusts, we found the extent of preferring gregarious volatiles in the injected solitary locusts is positively correlated with the degree of preference for volatiles in solitary ones with 0, 1, 4, 16 and 32 h of crowding [linear regression, $F_{(1, 28)} = 17.924$, $P = 0.0002$] (Figure 5B), suggesting that the effects of pared injections of a fixed amount of tyramine and a gradient concentration of octopamine on olfactory preference of solitary locusts are similar to that of the entire process of crowding.

In gregarious locusts, we simultaneously activated two pathways by injecting a fixed amount of octopamine (100 μg) and gradually increasing concentrations of tyramine (from 0 μg to 200 μg). The volatile preference of the locusts with pared injection of octopamine and tyramine was compared with that of the gregarious locusts solely injected with 100 μg of octopamine (Figure 5C). With the injection of a fixed amount of octopamine (100 μg), the air preference of locusts injected with 50 μg of tyramine did not significantly different from that of gregarious controls (50 μg, $G_2 = 1.43$, $P > 0.05$). By contrast, the locusts injected with 100 μg of tyramine and octopamine showed a significant higher preference for the fresh air control ($G_2 = 11.43$ $P < 0.01$). The preference for the fresh air control was also significantly higher at 150 and 200 μg of tyramine (150 μg, $G_2 = 14.94$; 200 μg, $G_2 = 20.19$; all $P < 0.005$). These pared injections significantly increased the locusts' preference for the fresh air control. This effect did not display immediately after octopamine and tyramine injection but instead increased gradually up to 100 μg of tyramine ($G_5 = 35.24$, $P < 0.0001$). Thus, increasing tyramine concentration elicits gradually stronger preference for fresh air control when the tyramine concentration is above a threshold, whereas the lower tyramine concentrations did not change the olfactory preference.

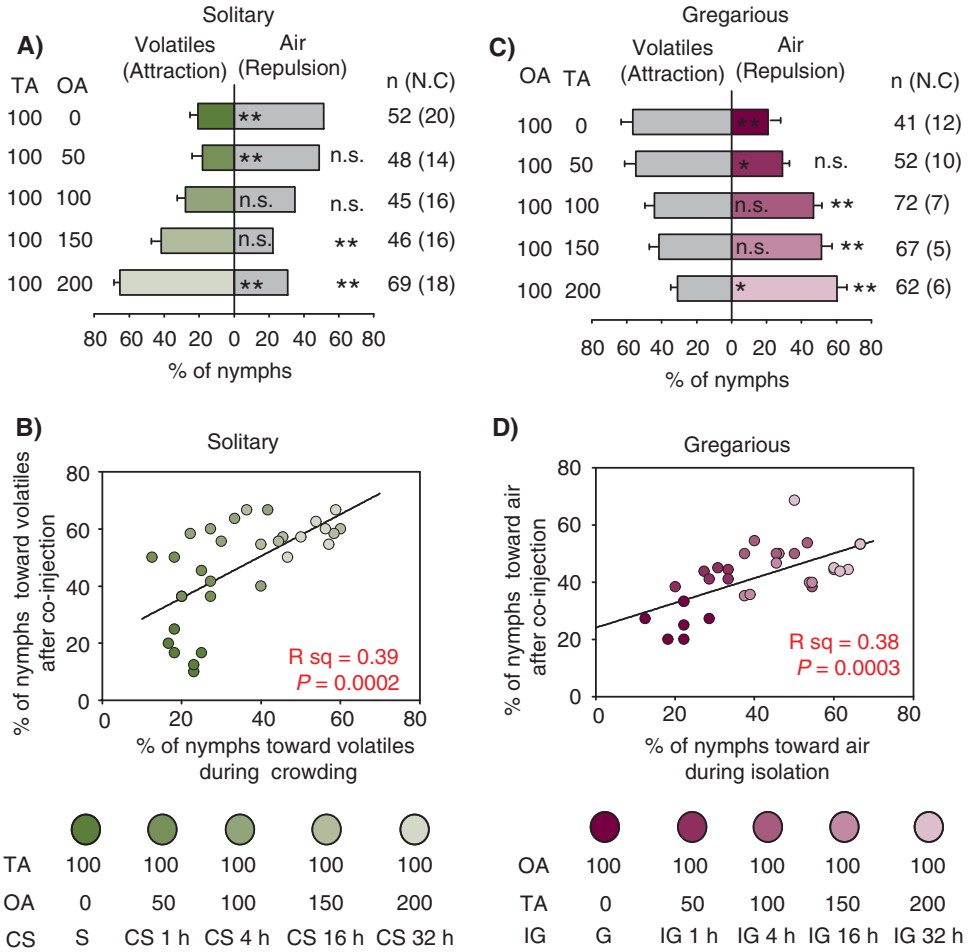

Figure 5 | OARα and TAR signaling pathways antagonistically regulate attractive and repulsive preferences in the migratory locust. (A) Effects of a fixed concentration of tyramine (100 μg) and a gradient concentration of octopamine (0 μg to 200 μg) on olfactory preferences of solitary locusts to gregarious volatiles. (B) The extent of propensity of solitary locusts for gregarious volatiles during crowding is closely correlated with the olfactory decisions of solitary ones after the combined injection of two monoamines. (C) Effects of a fixed concentration of octopamine (100 μg) and a gradient concentration of tyramine (0 μg to 200 μg) on olfactory preferences of gregarious locusts for gregarious volatiles. (D) In gregarious locusts, the degree of preference for fresh air control during isolation is significantly correlated with the preference of gregarious locusts for the fresh air after the combined injection. Error bars represent ± SEM. The asterisks (* and **) inside the strip indicate the significance of comparing individual numbers in each arm after *G*-tests for goodness-of-fit (*, $P < 0.05$; **, $P < 0.01$).The asterisks (* and **) outside the strip indicate the significance of treatments after *G*-tests for independence (*, $P < 0.05$; **, $P < 0.01$). Abbreviations: OA, octopamine;TA, tyramine; CS, crowding of solitary locusts; IG, isolation of gregarious locusts; n.s., not significant; n, individual numbers with olfactory preference; N.C, individual numbers without olfactory choice.

We further analyzed the relationship between the extent of air preference in gregarious locusts injected with two monoamines and degree of preferring fresh air in isolated locusts. After co-injection of a fixed amount of 100 μg octopamine and a gradient concentration of 0, 50, 100, 150 and 200 μg tyramine in gregarious locusts, we found the extent of preferring fresh air for gregarious locusts is positively correlated with the degree of volatile preference for solitary ones with 0, 1, 4, 16 and 32 h of isolation [linear regression, $F_{(1,\ 28)} = 17.371$, $P = 0.0003$)] (Figure 5D), suggesting that the effects of co-injections of a fixed amount of octopamine and a gradient concentration of tyramine on olfactory preference of gregarious locusts are similar to that of the entire process of isolation intervals.

Thus, the patterns of olfactory preference under the effects of monoamine co-injections in solitary and gregarious locusts are comparable to degree of preferring volatiles or fresh air control during crowding and isolation, respectively. The antagonistic effects of these two amines are clearly involved in regulating attraction- and repulsion-responses, and these two amines interact with each other to mediate olfactory preference during phase change.

Discussion

Our studies verified that octopamine-OARα and tyramine-TAR signaling pathways in the migratory locust mediate the attraction/repulsion to gregarious volatiles. The activation of octopamine-OARα signaling in solitary locusts induces a behavioral change from repulsion to attraction. By contrast, the activation of tyramine-TAR signaling in gregarious locusts causes a behavioral change from attraction to repulsion. Octopamine signaling facilitates attractive behavior in gregarious locusts that is balanced by tyramine signaling, which induces repulsive behavior in solitary locusts. These two pathways antagonistically modulate olfactory preference during phase change of the migratory locust.

In this study, the expression level of *OARα* mRNA is positively related with the change of attraction-response in crowding, whereas the level of tyramine is positively related with the change of repulsion-response in isolation (Figure 2). Pharmacological intervention of the two neurotransmitters and RNAi of *OARα* and *TAR* revealed a very consistent tendency with the extent of olfactory preference. These results implicate that attraction-response is modulated more by the expression of receptor OARα, whereas repulsion-response is

instead regulated by the change of tyramine titer during phase change. Octopamine has many functions in behavior and physiology through receptors OARα and OARβ[26,27]. In this study, OARα is confirmed to modulate the aggregative behavior of migratory locusts, whereas OARβ is not related to locust phase change. The other functions of octopamine in locusts besides in phase change may constrain the titer change of itself that possibly activates OARβ during phase change. Thus, increase of OARα mRNA expression may be more efficient than the titer change of octopamine in modulating phase change of the migratory locust. On the other hand, only one TAR is identified in the migratory locust until now. Modulation of phase change and olfactory behaviors by the fluctuation of tyramine might be easier and more efficient than that of the change of *TAR* expression. Moreover, modulation by OARα in crowding and mediation by tyramine in isolation might also be associated with different persistence of crowding and isolation, which may result in different speed between gregarization and solitarization. Thus, octopamine regulates attraction-response through the specific receptor OARα, whereas repulsion-response is modulated by the titer of tyramine in the brains of the locusts.

Here solitary and gregarious locusts perceive the same olfactory cue and make opposite behavioral decisions (attraction or repulsion) accordingly. The chemosensory protein *CSPs* in antenna mediates attractive behavior, whereas *takeout* modulates the repulsive behavior, respectively[14]. Octopamine-OARα signaling in gregarious locusts may process the neural and cellular signals emitted by *CSPs* in the peripheral nervous system (i.e., antennal and olfactory receptor neurons) after binding to attractive odorants in volatiles. Tyramine-TAR signaling in solitary locusts may deal with the neural and cellular signals emitted by *takeout* in the peripheral nervous system after binding to repulsive odorants in volatiles. Moreover, solitary locusts have more olfactory sensilla on the antenna than comparable gregarious insects[28]. Octopamine enhances olfactory response of sensilla in the antenna of cockroach to nonpheromone odorants[23]. Octopamine also mediates the sensitivity of the olfactory sensory system and promotes locomotion in insects[29–31]. Injection of this chemical in solitary locusts is expected to increase the response magnitude of olfactory neural circuits to external stimuli. In addition, tyramine and the receptor TAR regulate olfactory responses to repellent odorants in antennal lobes[31]. Therefore, octopamine and tyramine may change the sensitivity of olfactory sensory system to mediate behavioral preferences during phase change.

Interactions between octopamine and tyramine were found to affect the attractive or repulsive decision in a dose-dependent manner (Figure 5). The dose dependency of octopamine and tyramine revealed that the 2 : 1 ratio of one neurochemical to the other is required to meet the threshold to see a preference in corresponding direction (attraction/repulsion), indicating that the amount of octopamine or tyramine injected have surpassed the endogenous level of neurochemicals in locusts so that the direct response of injected octopamine or tyramine was observed. Although, combined injection in 2 : 1 ratio in induction of attraction or repulsion is independent of phase state of the individuals, the injection protocol confirmed the respective function of octopamine and tyramine in attraction-response and repulsion-response. Previous studies have confirmed that octopamine binds to OARα and then increase the intracellular calcium concentration $[Ca^{2+}]_i$, whereas tyramine binds to TAR coupled with the Gi protein to inhibit cAMP synthesis in insects[32–34]. Octopamine and tyramine modulate stress responses, pupation, sensory-mediated locomotion behavior, and the central pattern-generating network[23,35]. The stimulatory effects of octopamine and the inhibitory effects of tyramine on olfactory stimuli at the behavioral level were observed in the present study. Thus, octopamine-OARα and tyramine-TAR signaling pathways activate the respective downstream components in an amine-specific manner for olfactory perception in locust phase changes. To the best of our knowledge, this study is the first to demonstrate the existence of a bimodal system in regulating olfactory preferences in locust phase change.

Many biogenic amines have antagonistic functions in regulating animal behavior so that animals rapidly respond to the changing environment. Dopamine mediates the gregarization process[15], whereas serotonin modulates the solitariness of the migratory locust[14]. In this study, octopamine mediates the gregarization, whereas its precursor tyramine modulates the solitarization. In the migratory locust, there is a salvage pathway for octopamine synthesis[27]. In this pathway, dopamine is converted to tyramine by dopamine dehydroxylase, followed by β-hydroxylation of tyramine for synthesis of octopamine[27,36]. The increase of L-dopa results in a mark increase of tyramine and octopamine as well as dopamine in the migratory locust[37,38]. Thus, besides dopamine mediates gregarious behavior through its receptors, this chemical may act as a precursor of octopamine in the salvage pathway to induce gregarious behavior. On the other hand, tyrosine is the common precursor of dopamine, tyramine and octopamine[26]. Due to the similarity of chemical structures between dopamine and octopamine, dopamine may act as a non-specific pharmacological agonist to partially activate the two octopamine receptors. However, this non-specificity has no great effect on roles of octopamine receptors, or else dopamine and octopamine would not sophistically modulate insect behaviors through their corresponding receptors. Thus, the migratory locust can employ two strategies to ensure phase changes in response to local population density changes. The conserved neurochemicals dopamine and serotonin in animals mediate gregarization and solitarization, respectively, and the invertebrate-specific neurotransmitters octopamine and tyramine have similar roles with dopamine and serotonin involved in phase change of the migratory locust.

The migratory locust and desert locust have different traits in the rate of phase change: slow gregarization and quick solitarization in the migratory locust; quick gregarization and slow solitarization in the desert locust[9,14]. Although biogenic amines are suggested to regulate phase change of locusts[19,39], the neuroaminergic signals play different roles in phase change of the two locust species. In the migratory locust, the levels of dopamine and octopamine are higher in the brains of gregarious locusts, and these two chemicals mediate gregarization process[15]. Serotonin shows a surge after 32 h of isolation[16] and tyramine manifests a higher level in the brains of solitary locusts. These two chemicals mediate solitariness of the migratory locust. By contrast, in desert locust, serotonin displays a surge in thorax ganglia after 4 h of crowding and regulates swarm behavior and attraction to locust conspecifics[40,41]. The reason underlying the divergence of regulatory mechanism in phase change between the two species may partially attribute to species-specific traits, because the migratory locust and desert locust belong to different subfamilies of the Acridadae, Cyrtacanthacridinae and Oedipodinae, respectively[42]. Moreover, desert locusts mainly live in the African tropical zone desert in which the food plants are distributed as discontinuous patchiness[43]. Probably, desert locusts live in gregarious population for a long-term to make sure quick migration for food resources. By contrast, migratory locusts mainly occur in plains, river and lake basins, in which the distribution of food plants is more wide and sufficient[43]. Due to more adequate food resources than that for desert locusts, the solitary population of migratory locusts may be more beneficial for their long-term survival. For example, the population outbreaks of the migratory locust were recorded with 9–11 year intervals in Chinese history[44]. Thus, the deep exploration of molecular and neurobiological networks underlying behavioral phase change will be beneficial to explain the divergence and convergence in the regulatory mechanism between the two important species.

The olfactory preference in locust phase change is beneficial for the migratory locust to adapt the stress of high population density for homeostasis. The high expression of *OARα* and *TAR* increase their sensitivity to their corresponding ligands for the regulation of beha-

vioral plasticity during phase change. Given that octopamine–OARα and tyramine–TAR signaling pathways regulated the plasticity of the response to the volatiles of gregarious locusts, pheromonal information in volatiles of gregarious locusts may regulate other behaviors through the biogenic amine signaling. Our identification of the octopamine–OARα and tyramine–TAR signaling pathways sheds light on the complexity of regulatory networks underlying the sensory behavior in phenotypic plasticity of locusts and suggests that similar regulation might be present in the aggregation of other invertebrate animals.

Methods

Subjects. The locusts used in this study were derived from the colony maintained in the Institute of Zoology, Chinese Academy of Sciences, Beijing, China. Gregarious locusts were cultured at a density of 500 to 1000 insects in per container (40 cm × 40 cm × 40 cm) for at least three generations. Solitary locusts from the gregarious colony were cultured in white metal boxes (10 cm × 10 cm × 25 cm) and supplied with charcoal-filtered compressed air for at least three generations before the experiments. This colony was maintained under a 14 h light/10 h dark cycle at 30 ± 2°C and fed on fresh wheat seedlings and bran[20].

Isolation of gregarious locusts for qRT–PCR assay. After the fourth-stadium gregarious locusts were isolated and reared in solitude for 1, 4, 16 and 32 h, the brains (without optic lobe) of the isolated locusts were dissected and immediately put into RNAlater Solution (Ambion, Austin, Texas, USA) for qRT–PCR analysis. The brains of gregarious locusts were sampled as controls. Ten brains were collected as one biological replicate. All six biological replicates were sampled at the same time point to avoid circadian rhythm effects on gene expression in gregarious locusts. Every biological replicates contain equal numbers of male and female insects.

Crowding of solitary locusts for qRT–PCR assay. After 10 solitary locusts at the fourth stadium were reared in a plexiglass box (10 cm × 10 cm × 10 cm) with 20 gregarious locusts at the same stadium for 1, 4, 16, and 32 h of crowding, the brains (without optic lobe) of the crowded locusts were dissected and immediately put into RNAlater Solution (Ambion, Austin, Texas, USA) for qRT–PCR analysis. The brains of solitary locusts were sampled as the control group. Ten brains in solitary locusts were collected as one biological replicate. To avoid the effects of circadian rhythm on gene expression in solitary locusts, all six biological replicates were sampled at the same time point. Every biological replicate contains equal number of male and female insects.

RNA preparation and qRT-PCR assay. Total RNA was extracted from brain tissues following the protocol of RNA easy mini kit (Qiagen). DNAase was applied to eliminate DNA contamination in RNA samples. The details of reverse transcription and PCR amplification were referred to previous study[16]. We used standard curve method to measure the expression levels of the genes related with octopamine and tyramine. Before analyzing gene expression level, we screened the expression levels of housekeep genes: *GAPDH*, *Actin*, *EF1a*, and Ribosomal protein 49 (*RP–49*), to choose the reference gene with stable expression level. We found *RP–49* expressed stably during phase change and then chose it as the reference gene to normalize and calculate expression levels of target genes (Supplementary Figure S2). The primers for qRT-PCR assay were provided in Supplementary Table S2.

Phylogenetic analysis of octopamine and tyramine receptors. To confirm receptor subtypes, we cloned sequences of octopamine and tyramine receptors by referring to putative sequences in genome and transcriptome database of the migratory locust[45]. The other sequences for phylogenetic analysis were downloaded from the NCBI databases. The neighbor-joining analysis was performed in MEGA 5 with bootstrapping 1,000 replicates[46] (Supplementary Figure S9).

High-performance liquid chromatography (HPLC) with electrochemical detection (ECD). The concentrations of octopamine and tyramine in the brain (without optic lobe) were quantified using reverse-phase HPLC with ECD[16]. Ten brains per sample were homogenized using a mortar and pestle pre-cold with liquid nitrogen. Pulverized brain tissue was transferred to 1.5 ml Eppendorf tubes (Eppendorf International, Hamburg, Germany), and then lysed in 400 μL ice-cold 0.1 M perchloric acid (Sigma–Aldrich) on ice for 10 min. The homogenates were centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatants were passed through 0.45 μm filters (Millipore Corporation, Billerica, MA, USA), transferred to new Eppendorf tubes, and stored at −80°C until HPLC-ECD analysis. Forty microliter supernatants were automatically loaded onto a quaternary low-pressure pump (Waters Corporation, e2695, Milford, MA, USA) with a C18 reverse phase column (Atalantis™ dC18, 2.1 × 150 mm, 3 μm, Waters Corporation). The electrode potential in the electrochemical detector was set at 800 mV. The mobile phase (pH 3.00) was composed of 7% acetonitrile (J&K Scientific Ltd., Beijing, China), 90 mM monobasic phosphate sodium (Sigma–Aldrich), 50 mM citric acid (Sigma–Aldrich), 2 mM octanesulfonic acid (J&K Scientific Ltd., Beijing, China), 2 mM NaCl (Sigma–Aldrich), and 50 μM EDTA (Sigma–Aldrich). The flow rate was adjusted to 0.25 mL min^{-1}, and the temperature was set at 35°C. Data analysis was performed using Empower software (Waters Corporation). The octopamine and tyramine levels were quantified by referring to external standards. The standard curve was generated with serial dilutions of standard solution containing octopamine or tyramine (Sigma-Aldrich).

***In situ* hybridization of *OARα* and *TAR*.** We performed fluorescence *in situ* hybridization (FISH) to detect the spatial expressions of *OARα* and *TAR* in the locust brain. The *OARα* fragment (229 bp) and the *TAR* fragment (492 bp) were prepared for antisense and sense probes. We blasted these two fragments against genome sequences of the migratory locust to detect homologies and avoid non-specificity in hybridization[45]. The primers for *OARα* fragment were 5′–CGTATTCCTGTCGCACAAG and 5′–CGGGAAGCAGATGACGAAG. The primers for *TAR* fragment were 5′–GTCTCTTTCGTTTCGGGCTT–3′ and 5′–CAGTCTCCCAATCTGCCTCA–3′. After the dissected brains were fixed in 4% formaldehyde for 2 h at room temperature, they were washed 2 times for 15 min each in 0.1 M PBS (pH 7.4) and treated with 20 μg/ml Proteinase K in PBS for 2 h at 37°C. Then treated brains were washed 15 min in 0.1 M PBS (pH 7.4) for 3 times and refixed before being put in pre-hybridization solution (Boster, Wuhan, China) for 2 h at 65°C. We used pre-hybridization solution containing 3 μg/ml digoxigenin–labelled probes to do hybridization overnight in a humidified chamber at 65°C. Then, washing was carried out in 4×, 2×, 1×, 0.2 × SSC at 65°C for 60 min each. To detect the existence of hybrids, the brains were incubated with mouse anti–digoxigenin antibody conjugated with Dylight 488 (Jackson 1 : 300) overnight at 4°C. Hybridization was performed on the whole-mount brain, and the hybridized tissues were examined using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany).

Behavioral pharmacology of octopamine and tyramine in locusts. To determine the role of octopamine and tyramine in inducing behavioral shift in locust phase changes, we respectively injected octopamine (Sigma–Aldrich) (100 μg, 25 μg/μl) and tryamine (Sigma–Aldrich)(100 μg, 25 μg/μl) into thoracic cavities of the fourth-stadium solitary or gregarious locusts by using a micro syringe and then left these injected insects in solitude. Their behaviors were assessed 1 h later, respectively. The control group received the same volume of saline before behavioral assay.

***OARα* RNAi and behavior assay.** After designing the fragments of *OARα* sequence for RNAi, we blasted the target fragments against genome sequences of the migratory locust to detect sequence homologies[45]. We then chose the fragment with no homologies with other genes in genome database to avoid non-specificity in RNAi knockdown. Double-strand RNA (dsRNA) of green fluorescent protein (GFP) and *OARα* were prepared using the T7 RiboMAX Express RNAi system (Promega, Madison, USA). Before injecting 25 ng dsRNA of the *OARα* into the brains of fourth-stadium gregarious locusts, we placed the fourth-stadium locusts in a Kopf stereotaxic frame specially adapted for locust surgery[16]. The injected gregarious locusts were reared for 3 days before behavioral assay. To determine the role of *OARα* in crowding of solitary locusts, dsRNA-injected solitary locusts were directly assayed or crowded for 32 h with gregarious locusts (10 cm × 10 cm × 10 cm) before behavioral assay. The efficiency of RNAi on relative mRNA expression levels were investigated by qRT–PCR 3 days after injection[16].

***TAR* RNAi and behavior assay.** In order to avoid non-specificity in *TAR* RNAi knockdown, we designed the fragments of *TAR* for RNAi and blasted against genome sequences of the migratory locust to detect sequence homologies[45]. We chose the fragment with no homologies to prepare dsRNA. To examine the role of *TAR* during the isolation of gregarious locusts, we injected 25 ng ds*TAR* into gregarious locust brain and reared them for3 days. The injected locusts were isolated for 1 h before the behavioral assay. The efficiency of RNAi on the relative mRNA expression levels were investigated by qRT–PCR 3 days later. The primers for RNAi were provided in Table S3.

Behavioral assay in arena. In this study, locusts were recorded on EthoVision system (Noldus Inc. Wageningen, the Netherlands) for video recording and data extraction. After the locusts were gently transferred by a tunnel to the assay arena, they were recorded for 6 min and examined only once. The wall of the rectangular arena (40 cm × 30 cm × 10 cm) is opaque plastic and its top is clear. Both ends of assay arena are separated chambers (7.5 cm × 30 cm × 10 cm) and ends of the chamber were illuminated equally to prevent the formation of mirror images. Twenty fourth-stadium gregarious locusts were placed in one of the chamber as the stimulus group, and the other chamber with the same dimensions is left empty. Filter paper is in the floor of the open arena during the behavior assay. Behavioral phase state of locusts was determined in an established binary logistic regression model[16] encompassing three parameters: total distance moved (TDM), frequency of movement (FOM) and attraction index (AI, AI stands for the extent of tested animals attracted by the stimulus group. AI = total duration in stimulus area–total duration in opposite area)[14–16]. This established regression model in the migratory locust is similar to the one applied in the desert locusts[17,18,47]. The eleven behavioral parameters encapsulated in this model[16] were adjusted until the regression model discriminated the two phases at the optimum level according to the following equation: $P\text{-sol} = e^{\eta}/(1 + e^{\eta})$, where $\eta = \beta_0 + \beta_1 \cdot X_1 + \beta_2 \cdot X_2 + \ldots + \beta_k \cdot X_k$, $X_1, X_2, \ldots, X_k$ are the behavioral covariates. P-sol is the probability that locusts should be regarded as a member of the solitary phase population. The values of this probability range from 0 to 1, where 0 and 1 indicate that individuals display gregarious and solitary behavior, respectively

(Supplementary Table S1). The model was constructed by using the behavioral data from 100 solitary locusts and 100 gregarious locusts at the fourth stadium, and a forward stepwise approach was applied (Supplementary Table S1). This model correctly classified 89.2% and 91.2% of the solitary and gregarious model populations, respectively.

Behavioral assay in Y-tube. The olfactory behavior was examined in Y-tube olfactometer. Odorants were the volatiles (including volatiles from body and feces) from 30 fourth-stadium gregarious locusts. These volatiles were delivered to either arm to eliminate possible spatial bias. The air flow was set at 300 ml/min. Each locust was observed for 4 min and examined only once. Whenever moving more than 4 cm into the volatile or air arm within 4 min, individual locust was recorded and considered as the first choice for either arm. Every experiment encompassed at least 6 replicates, and every replicate contained at least 8 insects. To quantify the choice behavior, we directly calculated the percentage of locusts that chose either volatile arm or air arm.

Statistical analysis. The probabilistic metrics of solitariness (*P-sol*) for behavioral phase change were analyzed by Mann–Whitney *U* test to detect the significance of phase change. Octopamine, tyramine and expression levels of their receptors during crowding or isolation were analyzed by Student's *t*-test. The statistical analysis of olfactory preference was referred to the work about locust aversive learning[48]. In olfactory behavioral assay, *G-tests* for goodness-of-fit were used to determine the significance of the divergence from an expected 50% decision for the volatile arm or air control arm, and the counts of locusts preferring the volatile or air control were used for this analysis. The statistical comparisons between treatment and controls in volatile preference were analyzed by *G-tests* for independence, and quantities of all locusts tested were included for this analysis. When the counts of olfactory response are low, we chose Yate's continuity correction to avoid the overestimation of significance for volatile preference[49]. The standard error of locusts' volatile preference was calculated as $\sqrt{p(1\text{-}p)/n}$, where p is the proportion of locusts that were attracted or repulsed by the volatiles and n is the number of locusts that were tested[50]. We performed linear regressions to detect the correlation of neurochemcials and expression of their receptors with the extent of olfactory preference during the entire range of crowding or isolation. To clarify how octopamine and tyramine interact in regulating olfactory preferences, we performed linear regression analysis to predict the correlation of olfactory preference between locusts in phase change and locusts with co-injection of two monoamines.

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Author contributions

The author(s) have made the following declarations about their contributions. Z.M. and L.K. conceived and designed the experiments. Z.M., X.G. and T.L. performed the experiments. Z.M., X.G., H.L., S.H. and L.K. analyzed the data. Z.M., X.G., H.L. and L.K. wrote the manuscript.

Additional information

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