Critical role for protein kinase A in the acquisition of gregarious behavior in the desert locust

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The mechanisms that integrate genetic and environmental information to coordinate the expression of complex phenotypes are little understood. We investigated the role of two protein kinases (PKs) in the population density-dependent transition to gregarious behavior that underlies swarm formation in desert locusts: the *foraging* gene product, a cGMP-dependent PK (PKG) implicated in switching between alternative group-related behaviors in several animal species; and cAMP-dependent PK (PKA), a signal transduction protein with a preeminent role in different forms of learning. Solitarious locusts acquire key behavioral characters of the swarming gregarious phase within just 1 to 4 h of forced crowding. Injecting the PKA inhibitor KT5720 before crowding prevented this transition, whereas injecting KT5823, an inhibitor of PKG, did not. Neither drug altered the behavior of long-term gregarious locusts. RNAi against *foraging* effectively reduced its expression in the central nervous system, but this did not prevent gregarization upon crowding. By contrast, solitarious locusts with an RNAi-induced reduction in PKA catalytic subunit C1 expression behaved less gregariously after crowding, and RNAi against the inhibitory R1 subunit promoted more extensive gregarization following a brief crowding period. A central role of PKA is congruent with the recent discovery that serotonin mediates gregarization in locusts and with findings in vertebrates that similarly implicate PKA in the capacity to cope with adverse life events. Our results show that PKA has been coopted into effecting the wide-ranging transformation from solitarious to gregarious behavior, with PKA-mediated behavioral plasticity resulting in an environmentally driven reorganization of a complex phenotype.

phase change | phenotypic plasticity | *Schistocerca gregaria*

How genetic information is integrated with environmental cues to control and coordinate complex phenotypes is a fundamental question in biology (1, 2). Environmental input can cause concerted changes in multiple traits to produce distinct phenotypic syndromes that are adaptive in particular conditions. Phase polyphenism in desert locusts (*Schistocerca gregaria*) is an extreme example. Locusts can reversibly transform between a solitarious phase and a gregarious phase that differ profoundly in morphology, physiology, and behavior (3–5). Solitarious locusts occur at low population densities and actively avoid conspecifics; they are cryptic in appearance and behavior; walk with a slow, creeping gait; and have restricted dietary preferences. The gregarious phase is characterized by increased activity and locomotion, an upright posture and gait, aposematic coloration, a broad dietary range, and, most critically, attraction to other locusts. Phase change is driven by huge changes in population density and is an adaptation to arid habitats where rains are infrequent and erratic. Transitory periods of verdure support rapid population growth, but after the rains cease, large numbers of solitarious locusts compete for dwindling patches of resources (6, 7). The resultant crowding causes a rapid transition to gregarious behavior by exposing solitarious locusts to specific sensory stimuli from conspecifics: repeated touch to the hind femur and the combination of visual and olfactory cues (8–10). Gregarious behavior ensures further exposure to other locusts and thereby sets up a positive feedback loop that drives further phenotypic changes accruing over the locusts' lifetimes and even across generations. This behavior is also what makes locusts notorious pests, as highly mobile groups of gregarized locusts can further coalesce to escalate into enormous swarms that devastate crops and pastures.

Knowledge about the extensive differences in gene expression between the fully established phases has increased dramatically in recent years, but the molecular mechanisms that switch between the two extreme phenotypes have yet to be fully established (11–13). The discovery of a central role for the biogenic amine serotonin [5-hydroxytryptamine (5-HT)] in inducing behavioral gregarization (14) suggests parallels with mechanisms underlying classical forms of neuronal and behavioral plasticity (15). In solitarious locusts, gregarizing stimuli cause an increase in 5-HT in the thoracic ganglia, but with prolonged crowding, 5-HT decreases to lower than solitarious levels (16). This indicates that, after induction, gregariousness is maintained by other means, echoing the transient role of 5-HT in classical forms of learning. Aminergic signaling has since been confirmed as central to behavioral phase change in the migratory locust (*Locusta migratoria*); in this insect, which is only distantly related to *Schistocerca*, 5-HT appears to act in synergy with dopamine (12).

A prominent effector mechanism in aminergic signaling is via adenylyl cyclase, resulting in the activation of cAMP-dependent protein kinase [protein kinase A (PKA)] (17–20). Adenylyl cyclase/PKA signaling plays a central role in diverse forms of plasticity, including reflex sensitization, contextual fear conditioning, appetitive and aversive conditioning, and addiction (17, 18, 21–25). PKA can phosphorylate both key proteins of the existing neuronal machinery (e.g., ion channels and synaptic proteins) to effect rapid but labile changes in behavior, and regulators of gene expression to effect changes that are slower but long-lasting. PKA is therefore potentially well placed to enact both the rapid and long-term effects of gregarizing stimuli. cGMP-dependent protein kinase [protein kinase G (PKG)] has similarly wide roles in neural and behavioral plasticity (26). In *Drosophila*, larvae that carry a "rover" allele of the *foraging* gene (for^R), one of two PKG-encoding paralogues, have elevated PKG

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activity and move longer distances while feeding compared with *for*^{S}-homozygous "sitters" (27). Sitters and rovers differ in nutrient uptake and feeding strategy, in their metabolic and transcriptional responses to the nutritional environment, in learning and memory formation, and more (28–31). In eusocial insects, activation of PKG can switch workers between tasks according to the nutritional needs of the colony (32–34). A fundamental question is whether such strong pleiotropy presents an adaptive and possibly conserved mechanism for orchestrating many traits to tailor the phenotype to one of two alternative lifestyles. Rearing *Drosophila* larvae at high densities selects for rovers (35), suggesting that the polymorphism buffers against fluctuations in population density and resource distribution. Phase change in locusts is a prime example of a nexus between the social and the nutritional domain, with intraspecific competition for ephemeral resources driving the transition to group living and foraging. The brains of long-term gregarious locusts contain more PKG activity than those of solitarious locusts (36), raising the possibility that the locust orthologue of *for* has been coopted into controlling behavioral phase state. Here we investigated whether PKA or PKG modulate the propensity of locusts to acquire and express gregarious behavior.

Results

Inhibition of PKA Interferes with Behavioral Gregarization. Solitarious desert locusts acquire key behavioral characters of the gregarious phase within less than 4 h of crowding (14, 37). To test for a role for PKA activity in this transition, we injected solitarious locusts (n = 23) with KT5720, a staurosporine/K525-type inhibitor that competes for the ATP-binding site on the PKA catalytic subunits (38). Matched control locusts were injected with only the vehicle (n = 22). To test for a role for PKG activity, we injected a separate cohort with KT5823 (n = 25), an ATP-competitive K525-type inhibitor of PKG (38), or vehicle alone (n = 26). After injection, locusts in all four treatment groups were crowded for 1 h with 30 gregarious locusts and then observed individually in an arena, which contained a group of 30 locusts behind a perforated Perspex partition at one end (37). One hour of crowding was chosen because the effect of inhibitors injected into the hemolymph may wear off over longer periods (39). An established binary logistic regression model comprising four behavioral variables (14) (*SI Appendix*, 4.1) was used to estimate each locust's propensity for gregarious behavior, which can be expressed as either the probability or the odds that a locust behaved gregariously. Probabilities (P_{greg}) range from 0 (i.e., fully solitarious) to 1 (i.e., fully gregarious), whereas odds range from 1:∞ to ∞:1 and are more amenable to parametric statistical analysis when log-transformed (log-odds or logits; *SI Appendix*, Fig. S1).

As expected, control-injected locusts were very likely to behave gregariously in the two experiments [median P_{greg} ($\tilde{P}_{greg}$) = 0.712 and 0.747 in the KT5823 and KT5720 cohorts, respectively; Fig. 1*A* and *SI Appendix*, Fig. S1*A*]. Locusts injected with KT5823 were as likely to show gregarious behavior as their matched controls ($\tilde{P}_{greg}$ = 0.723; t_{49} = 0.5964, P = 0.554), but those injected with KT5720 remained much more solitarious ($\tilde{P}_{greg}$ = 0.358; t_{43} = –3.0044, P = 0.00443). These results suggested that activation of PKA is critical for the transition to gregarious behavior. However, this experiment did not address whether PKA or PKG activity is required for the continued manifestation of gregariousness after the initial transition has occurred. Furthermore, we needed to rule out that KT5720 had a nonspecific toxicity that caused pseudosolitarious behavior. We therefore repeated the experiment on long-term gregarious locusts (Fig. 1*B* and *SI Appendix*, Fig. S1*B*). These locusts behaved very gregariously irrespective of whether they had been injected with KT5823, KT5720, or vehicle, indicating that neither kinase is important in the long-term expression of gregarious behavior when it has been consolidated through prolonged crowding ($\tilde{P}_{greg}$ = 0.973 vs. $\tilde{P}_{greg}$ = 0.990 for KT5823 vs. matched controls, t_{30} = –0.2437, P = 0.809, n = 16 each; $\tilde{P}_{greg}$ = 0.965 vs. $\tilde{P}_{greg}$ = 0.989 for KT5720 vs. matched controls, t_{40} = –0.6463, P = 0.5218, n = 21 each).

To confirm that locust PKA was inhibited by KT5720 but not by KT5823, we measured PKA activity in brain homogenates in the presence of 0 to 50 μM KT5720 or KT5823 (Fig. 1*C*). KT5720 resulted in a potent concentration-dependent inhibition (t = –4.717, P = 2.65 × 10^{-5}), whereas KT5823 had no effect (t = 0.763, P = 0.45; *SI Appendix*, 4.2). To summarize, the pharmacological results indicated that PKA but not PKG is required specifically in the transition to gregarious behavior. Kinase inhibitors may, however, show off-target effects and low in vivo efficacy. We therefore sought to support these conclusions by gene-specific reduction of kinase expression by RNAi.

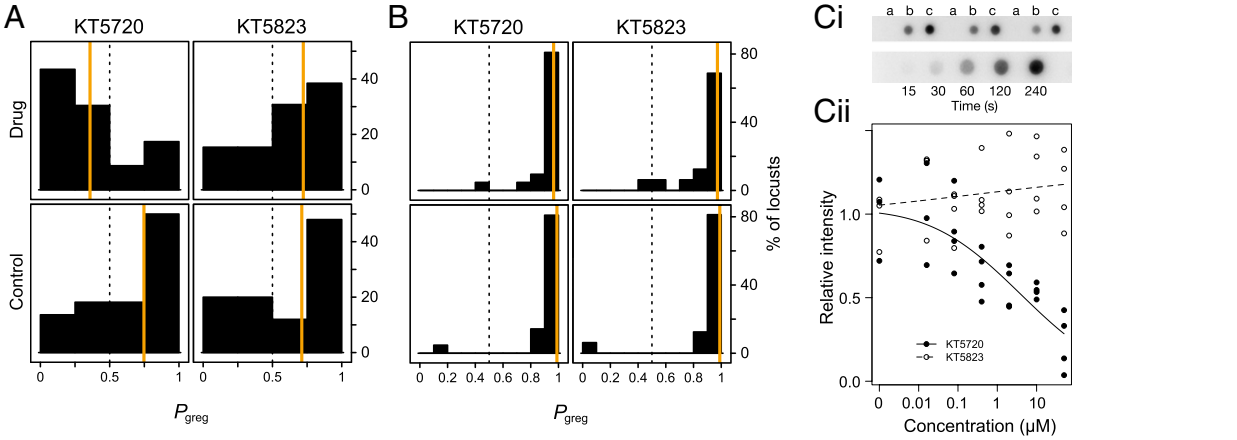

Fig. 1. Inhibition of PKA interferes with the transition to gregarious behavior. Histograms show the probability of gregarious behavior (P_{greg}); orange lines indicate medians. (*A*) Solitarious locusts were injected with the PKA inhibitor KT5720, the PKG inhibitor KT5823, or vehicle control, and then crowded for 1 h. Locusts injected with KT5720 (n = 23) remained solitarious compared with their matched controls (n = 22), whereas KT5823-injected (n = 25) and control-injected (n = 26) locusts were equally strongly gregarized. (*B*) Neither drug affected P_{greg} in gregarious locusts (KT5720, n = 16; control, n = 16; KT5823, n = 21; control, n = 21). (*C*) Inhibition of locust PKA activity as measured by dot blot immunoassay. *i*, The signal in the assay depends on 8-Br-cAMP and ATP [*Upper*, three repeats of (*a*) 100 μM ATP but no 8-Br-cAMP, (*b*) 5 μM 8-Br-cAMP but no ATP; and (*c*) both] and increases with reaction time (*Lower*). *ii*, KT5720 inhibits locust PKA activity in a concentration-dependent manner whereas KT5823 has no effect; lines represent a log-normal fit.

Reduced Expression of FOR Does Not Affect Gregarious Behavior. We identified a 1,392-bp contig of the *for* gene orthologue (36) in an *S. gregaria* CNS GenBank Expressed Sequence Tags (EST) database (13) and used it as a template to generate a 271-bp dsRNA construct (*SI Appendix*, Fig. S2). To characterize the effect of this construct on *for* mRNA expression, gregarious locusts were systemically injected with 0, 0.5, 1.25, or 5 μg *for* dsRNA and, 1, 3, 5, or 10 d later, *for* mRNA was quantified separately in the brain and the thoracic ganglia (*SI Appendix*, Fig. S3). We observed a strongly dose- and time-dependent reduction in *for* mRNA in both parts of the CNS [dose–time interaction, likelihood ratio (LR) statistic $LR_9 = 26.27$, $P = 0.0018$; no tissue differences, $LR_{16} = 16.33$, $P = 0.43$; *SI Appendix*, 4.3]. The effect was strongest after 3 to 5 d, with a nearly full recovery after 10 d. With a dose of 5 μg, the reduction reached 79.2% after 3 d relative to the average in controls over all days [95% confidence interval (CI) = −65.2 to −87.6].

To quantify the RNAi effect on FOR protein expression, we probed Western blots of CNS with an antibody against the conserved C-terminal sequence of human PKG 1α (*SI Appendix*, Fig. S2, blue). The blots showed four distinct bands in the expected molecular mass range (40): a double band at 73/77 kDa, and single bands at 107 kDa and 97 kDa (Fig. 2*A*). An intense 37-kDa band and a number of faint bands were also present. Three days after injecting 5 μg *for* dsRNA, the intensity of the 73-/77-kDa double band was reduced by 58.1% (95% CI = 43.7–68.8; $n = 5$ dsRNA, $n = 5$ control; $t_8 = -6.783$ on log-transformed values, $P = 0.00014$). The other bands probably represent products of genes other than *for* that cross-reacted with the antibody.

To assess the effect of reduced FOR expression on the transition to gregarious behavior, we injected solitarious locusts with 5 μg *for* dsRNA ($n = 18$) or a vehicle control ($n = 18$) and, 3 d later, we assayed their behavior before and after 2 h of crowding (Fig. 2*B* and *SI Appendix*, Fig. S1*C*). Crowding greatly increased the propensity for gregarious behavior in both dsRNA-treated and control-treated locusts (effect of crowding, $LR_1 = 62.2$, $P < 0.0001$; *SI Appendix*, 4.4). Both groups were equally solitarious before crowding ($t = 0.393$, $P = 0.697$), and there was no evidence that the dsRNA treatment modulated the degree of gregariousness achieved on crowding (crowding–treatment interaction, $LR_1 = 0.665$, $P = 0.415$; before crowding, $\tilde{P}_{greg} = 0.0651$ vs. $\tilde{P}_{greg} = 0.0217$ for dsRNA vs. control; after crowding, $\tilde{P}_{greg} = 0.911$ vs. $\tilde{P}_{greg} = 0.791$ for dsRNA vs. control). Also, injection of *for* dsRNA into long-term gregarious locusts had no significant effect on their strong propensity for gregarious behavior (Fig. 2*C* and *SI Appendix*, Fig. S1*C*), suggesting that the behavioral phenotype does not depend on FOR expression ($t_{28} = -0.879$, $P = 0.387$; $\tilde{P}_{greg} = 0.972$ vs. $\tilde{P}_{greg} = 0.985$ for dsRNA vs. control, $n = 15$ each). This is consistent with our finding earlier that injection of KT5823 does not affect the behavioral phase state of long-term gregarious locusts.

Reduction in PKA C1 Subunit Expression Correlates with Reduced Gregarious Behavior. In fruit flies and honeybees, the catalytic C1 subunit of PKA (PKAC1) is critically involved in associative memory formation (21, 41, 42). We cloned a 347-bp cDNA fragment of the *S. gregaria pkac1* gene and used it as a template to generate a 203-bp dsRNA construct (*SI Appendix*, Fig. S4). Systemic injections of this dsRNA into gregarious locusts caused a dose- and time-dependent reduction of *pkac1* mRNA in brain and thoracic ganglia (dose–time interaction, $LR_6 = 33.7$, $P < 0.001$; *SI Appendix*, 4.5 and Fig. S5). The decrease reached a maximum after 3 d, recovered slightly after 10 d, and was marginally more pronounced in the thoracic ganglia. Three days after injecting 5 μg dsRNA, *pkac1* mRNA expression was reduced by 94% (95% CI = 91.3–95.7) in the brain, and by 95% (95% CI = 92.8–96.4) in the thoracic ganglia, compared with control-injected locusts across all days.

Western blots of brain extracts probed with an antiserum against *Drosophila melanogaster* PKAC1 protein (anti-DC0) (43) showed a single intense band at 40 kDa (Fig. 3*A*), matching the apparent molecular weight in *D. melanogaster* (41). This band was slightly weaker 2 d after dsRNA injection. The high specificity of anti-DC0 in locust brain permitted immuno-dot blotting to quantify PKAC1 from six spots per brain for increased accuracy (Fig. 3*B*). Unlike Western blotting, this technique can readily be scaled up to larger sample sizes. After 2 d, the brains of dsRNA-injected locusts contained significantly less PKAC1 ($n = 15$ dsRNA, $n = 15$ control; $P = 0.0001$; *SI Appendix*, 4.6), but the mean reduction was only 15.9% and highly variable (SD, 15.3%). We therefore sought to achieve a stronger reduction in PKAC1 protein by extending the time after dsRNA injection from 2 d to 5 d ($n = 19$ dsRNA, $n = 17$ control). Each locust was then observed three times in the arena: before crowding and after 1 and 4 h of crowding. Immediately after the last observation, the brains were dissected out to quantify PKAC1 (Fig. 3*B*). The average reduction in dsRNA- vs. control-injected locusts was indeed significantly stronger than 2 d after injection ($P = 0.0072$; no significant change in controls over time, $P = 0.13$), but still relatively moderate and with considerable variability (mean, −30%; SD, 11.8%). However, quantification of behavioral phase state and brain PKAC1 expression in the same animals permitted us to directly relate the two. We contrasted

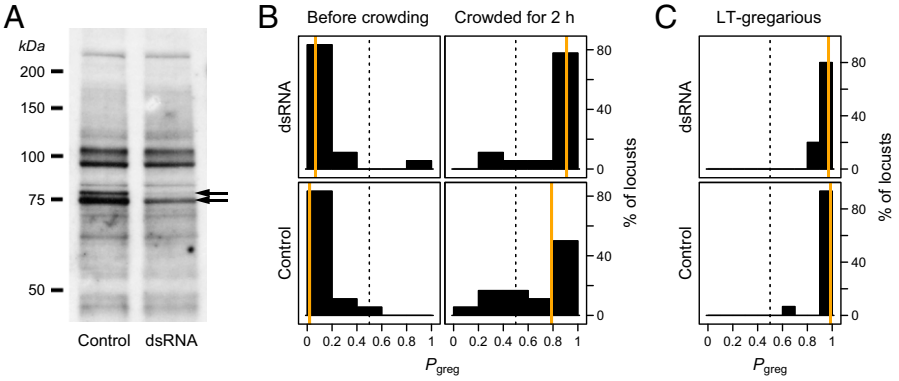

Fig. 2. RNAi-mediated reduction in FOR protein does not affect gregarious behavior. (*A*) Western blot of locust CNS probed with an antibody against the C-terminal region of human PKG 1α. A 73-/77-kDa double band (double arrows) is greatly reduced 3 d after systemic injection of *for* dsRNA. (*B*) Injecting *for* dsRNA ($n = 18$) into solitarious locusts did not affect their P_{greg} (orange lines, medians) before or after 2 h of crowding, compared with vehicle-injected controls ($n = 18$). (*C*) The P_{greg} of long-term gregarious locusts was likewise unaffected by injection with *for* dsRNA ($n = 15$; controls, $n = 15$).

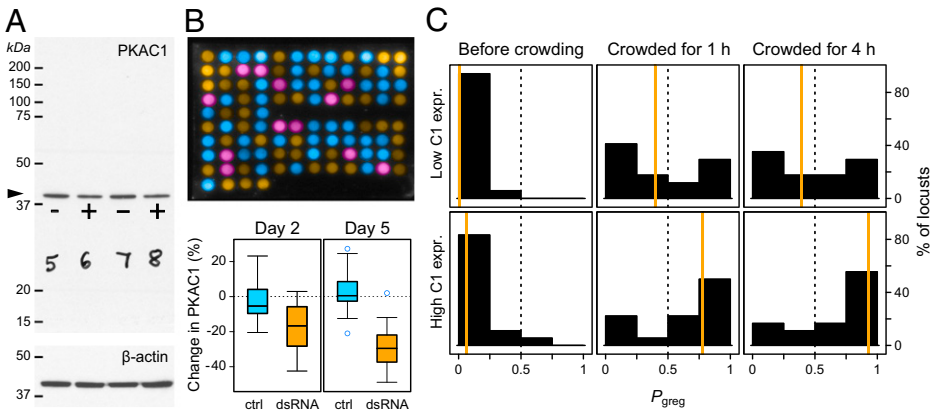

Fig. 3. RNAi-mediated reduction in PKAC1 expression interferes with behavioral gregarization. (*A*) Western blot of four brains probed with antibody DC0 against *Drosophila* PKAC1 shows a single band at 40 kDa (arrowhead); the annotations (i.e., numbers 5–8) are the identification numbers of the four individual locusts shown. The intensity of the 40-kDa band was slightly reduced 2 d after systemic injection of *pkac1* dsRNA (+) into locusts 6 and 8 compared with injection of vehicle control (–) into locusts 5 and 7 (band densities relative to mean of controls are 1.01, 0.76, 0.99, and 0.78 for locusts 5–8, respectively). *Lower:* Blot reprobed for β-actin. (*B*) Quantification of PKAC1 in individual brains by immuno-dot blot. *Upper:* Blot with colors overlaid to indicate treatment (cyan, control-injected; yellow, dsRNA-injected; magenta, reference samples). *Lower:* PKAC1 in the brain on day 2 (n = 15) or day 5 (n = 19) after injection of *pkac1* dsRNA, relative to the mean in controls across day 2 (n = 15) and day 5 (n = 17). (*C*) Solitarious locusts were injected with dsRNA (n = 19) or vehicle control (n = 17). Five days later, P_{greg} was measured before crowding and after 1 h and 4 h of crowding, followed by quantification of PKAC1 expression in the brain. Locusts with PKAC1 protein expression below the median had a significantly lower propensity for gregarious behavior (orange lines, medians).

the behavior of locusts that had low PKAC1 expression (i.e., lower than the median) with locusts in which expression was high (Fig. 3*C* and *SI Appendix*, Fig. S1*D*). This revealed that, although all locusts became increasingly gregarious during the 4 h of crowding (LR_2 = 66.31, P < 0.0001; *SI Appendix*, 4.7), locusts with low PKAC1 expression were significantly less likely to behave gregariously throughout the experiment (LR_1 = 9.048, P = 0.0026): they behaved more solitariously during their first encounter with conspecifics ($\tilde{P}_{greg}$ = 0.00602 vs. $\tilde{P}_{greg}$ = 0.0618 for low vs. high PKAC1 expression), and after crowding for 1 h ($\tilde{P}_{greg}$ = 0.398 vs. $\tilde{P}_{greg}$ = 0.779) and 4 h ($\tilde{P}_{greg}$ = 0.393 vs. $\tilde{P}_{greg}$ = 0.929).

RNAi Knockdown of Regulatory R1 Subunit Promotes Gregarization. Inactive PKA is a complex of two catalytic C and two inhibitory regulatory R subunits that dissociates upon binding of cAMP to the R subunits to release active C subunits. Degradation of the R subunits can then result in sustained PKA activity that outlasts the cAMP signal (44). RNAi against *PKAR1A* has recently been found to increase basal and stimulated PKA activity in HEK293 cells (45). We reasoned that, if PKA were the effector of a gregarizing 5-HT signal, RNAi against R1 might induce a shift toward increased gregariousness. We identified a 1,009-bp contig of *S. gregaria pkar1* in the EST database (13) and used it as a template for a 309-bp dsRNA construct (*SI Appendix*, Fig. S6). The efficacy of this construct was confirmed by quantifying *pkar1* mRNA expression 3 d after systemic injection with 5 μg dsRNA or vehicle; expression in brain and thoracic ganglia was reduced by 94.4% (95% CI = 96.3–91.5) in the dsRNA-treated locusts compared with vehicle-injected controls (LR_1 = 24.82, P < 0.0001; Fig. 4*A* and *SI Appendix*, 4.8), with no significant difference between the two tissues (LR_1 = 1.453, P = 0.2281). We then injected solitarious locusts with *pkar1* dsRNA (n = 20) or vehicle (n = 20) and, 3 d later, assayed their behavior before and after 2 h of crowding (Fig. 4*B* and *SI Appendix*, Fig. S1*E*). Crowding greatly increased the propensity for gregariousness (LR_1 = 71.20, P < 0.0001; *SI Appendix*, 4.9), irrespective of with what the locusts had been injected (LR_1 = 0.0404, P = 0.84), but dsRNA-treated locusts were significantly more likely to behave

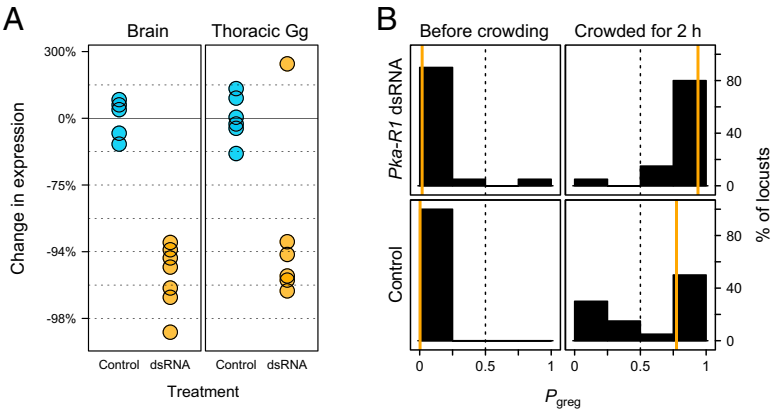

Fig. 4. RNAi interference against PKA regulatory subunit R1 promotes behavioral gregarization. (*A*) Systemic injection of *pkar1* dsRNA greatly reduced *pkar1* mRNA expression in the CNS. Points represent tissue pools from three locusts. The *y* axis gives the percent change on a log-scale relative to vehicle-injected controls. (*B*) Solitarious-phase locusts were injected with *pkar1* dsRNA (n = 20) or vehicle control (n = 20) and, 3 d later, their P_{greg} was quantified before and after 2 h of crowding. The dsRNA-treated locusts were significantly more likely to behave gregariously before and after crowding (orange lines, medians).

gregariously ($LR_1 = 10.20$, $P = 0.0014$), both during their first encounter with conspecifics ($\tilde{P}_{greg} = 0.0187$ vs. $\tilde{P}_{greg} = 0.00293$ in dsRNA-treated vs. control) and after crowding ($\tilde{P}_{greg} = 0.938$ vs. $\tilde{P}_{greg} = 0.775$ in dsRNA-treated vs. control).

Discussion

We have found several independent lines of evidence that identify PKA as a major effector of the serotonergic signaling initiated by gregarizing stimuli from other locusts (14). Injecting solitarious locusts with KT5720 strongly inhibited behavioral gregarization in the face of intense gregarizing stimuli, whereas the chemically closely related KT5823 was ineffective. In honeybees, an inhibition of PKA-dependent long-term memory formation has been reported with comparable doses of KT5720 but not of KT5823 (23). We used RNAi against *pkac1* and *pkar1* to independently confirm the involvement of PKA in gregarization. Partial RNAi-induced reductions in PKAC1 protein have previously been found to impair long-term memory formation in honeybees (42). We observed an RNAi-mediated reduction of 94% in *pkac1* mRNA, but PKAC1 protein was reduced on average by only 30% after 5 d, suggesting that the turnover of PKAC1 is slow. The size of the behavioral effect was also less dramatic than in the pharmacological experiments. Nevertheless, low PKAC1 expression in the brain was significantly associated with a reduced propensity for gregarious behavior when locusts were crowded. A critical role for PKA in gregarization is further supported by the more rapid shift in behavior that we observed in locusts treated with dsRNA against *pkar1* and subjected to a brief crowding experience. Increased gregarization after RNAi against *pkar1* derives from the inhibitory function of R subunits in regulating catalytic activity. In the absence of cAMP, C and R subunits assemble into inactive R_2C_2 complexes; active C subunits are released upon binding of cAMP to R. In this equilibrium reaction, a decreased R:C ratio leads to the release of more catalytic activity for a given concentration of cAMP (46), as recently demonstrated by RNAi against *PKAR1A* in HEK293 cells (45). In *Aplysia* neurons, 5-HT induces cleavage of the R1 subunit but not the R2 subunit (47). A reduction in PKAR1 would thus potentiate the effect of any 5-HT released in response to gregarizing sensory stimuli, resulting in the increased gregariousness we observed. The combined evidence from pharmacological and RNAi interventions indicates that activation of PKA has a substantial role in driving the transition to the gregarious behavioral state. This should not necessarily imply that PKA is the only effector active at this stage of phase change; studies of the molecular mechanisms underlying learning implicate parallel activation of other signaling systems including PKC and/or calcium/calmodulin-dependent kinase II in conjunction with PKA (48–50).

Injecting KT5720 into long-term gregarious locusts did not cause them to behave solitariously, indicating that gregarious behavior, when it has been consolidated through prolonged crowding, does not depend on PKA activity. That newly acquired and fully consolidated gregarious behaviors are mechanistically distinct also tallies with the finding that 5-HT is elevated only transiently, during the first few hours of crowding (16), and therefore cannot maintain gregariousness in the longer term. Activation of PKA may thus provide the link between a transient 5-HT signal and a later phase of consolidation that entails changes in gene expression (11, 13). However, previous studies have demonstrated that, after as much as 24 h of crowding, the newly acquired gregarious state is rapidly lost upon reisolation (51), suggesting a prolonged period after 5-HT levels have decreased during which gregarization is still realized through labile modifications of neuronal properties. PKA may contribute directly to this labile stage through phosphorylation of synaptic proteins or ion channels.

We obtained no positive evidence for an involvement of *for* in the transition to, or manifestation of, gregarious behavior in the arena. KT5823, an inhibitor of PKG previously used in *S. gregaria* (36, 52), did not significantly affect behavioral phase state after acute crowding, nor did it cause long-term gregarious locusts to behave less gregariously. Injection of dsRNA against *for* was likewise ineffective despite causing a 60% reduction in FOR protein expression in the CNS. By comparison, the behavioral differences between rovers and sitters in *Drosophila* arise from only a minor difference in *for* expression (53). We conclude that the pleiotropy of *for* does not extend to a modulation of behavioral phase state in *S. gregaria*. The previously reported higher PKG activity in the brains of gregarious desert locusts (36) may therefore be related to other biologically relevant differences between phases, including differences in foraging and nutritional regulation (54, 55). Our data do not preclude that PKG is involved in phase transitions in locust species other than *S. gregaria*, although the recent study implying dopamine and 5-HT in phase change in *L. migratoria* (12) is suggestive of a role for PKA in this species as well.

Half a century ago, Ellis (56, 57) wrote that "locusts learn to aggregate socially." Learning, if defined as experience-driven behavioral change, is indeed synonymous with phenotypic plasticity of behavior (2, 58). Our finding of a critical role for PKA shows that behavioral gregarization and classical forms of learning have coopted overlapping mechanisms from a conserved molecular toolbox for the transduction of environmental stimuli, in this case sensory cues from conspecifics, into phenotypic responses (15). Phase change in locusts, subordinate behavior following defeat (59, 60), and clinical depression in humans (61) are all manifestations of a fundamental capacity for behavioral remodeling in response to social experience in which biogenic amines and altered PKA signaling have been strongly implicated. They encompass changes in the individual's interaction with the environment, including its social domain; this altered interaction in turn feeds back through the social environment onto the individual to reinforce the new behavioral state (62). In locusts, the need for resources such as food outweighs the aversion solitarious locusts have for each other, exposing them to social cues from other locusts. This sets up just such a positive feedback loop whereby the initial behavioral change ensures that newly gregarized locusts will continue to be bombarded by stimuli from other locusts, reinforcing and driving on further phenotypic change. Our identification of a central role for PKA in this process shows that the similarities between phase change and other socially induced transitions between behavioral states extend beyond the phenotypic level to the cooption of common molecular mechanisms. Nevertheless, downstream components of PKA signaling in locusts may have sufficiently distinct genetic sequences and protein structures to present targets for new, locust-specific control strategies that interfere with the transition to swarming behavior.

Methods

Animals. Desert locusts (*S. gregaria*) were obtained from laboratory colonies at the University of Cambridge and the University of Leuven. The effects of dsRNA treatment on mRNA expression were characterized in both strains, and the effects on protein expression in the Cambridge strain. All behavioral experiments were performed on final instar nymphs of Cambridge stock. Gregarious-phase locusts were taken from a colony that had been maintained under crowded conditions for many generations. Solitarious-phase locusts were generated from this gregarious stock by isolation for two to three generations (37).

Behavioral Assay. Locusts were recorded for 500 s on digital video and their behavior tracked from playback in manual event recording software (37) by an independent observer. Behavioral phase state was determined in an established binary logistic regression model (14) that includes four behavioral characters: the time a locust spent near the stimulus group, the frequency with which it groomed, the speed at which it walked, and the time it spent motionless. The model was defined by using data from 100 gregarious-phase and 120 solitarious-phase locusts (*SI Appendix*, 4.1), and correctly

classified 91% and 89% of the solitarious and gregarious model populations, respectively. Treatment effects on the behavioral state of experimental locusts were analyzed by fitting linear models to the log-odds of gregariousness (i.e., logits of P_{greg}) predicted by the logistic regression model. Repeated observations were analyzed in linear mixed-effect models (R software package *nlme*).

Behavioral Pharmacology. KT5720 and KT5823 (Tocris Bioscience) were dissolved in DMSO as 2-mM stock solutions. Working solutions were prepared by diluting the stocks 1:20 in physiological saline solution, yielding 100 μM inhibitor and 5% DMSO. Locusts were injected with 15 $\mu L \cdot g^{-1}$ body weight of drug solution (1.5 $\mu mol \cdot kg^{-1}$ drug) or 5% DMSO in saline solution (control). Solitarious locusts were then returned to their individual cages for 30 min before crowding them for 1 h with 30 long-term gregarious final instar nymphs. Long-term gregarious locusts were returned to a crowded cage immediately after injection and observed 1.5 h later.

PKA Inhibition Assay. The effect of KT5720 and KT5823 on the activity of PKA in locust brain homogenates was measured by using phosphatase inhibitor-1 (I1; LAE Biotech International) as an exogenous substrate (63). Phosphorylation of I1 was detected with antibody sc-14267R (Santa Cruz Biotechnology) against the PKA substrate site of human I1 phosphorylated at threonine residue 35. The assay was adapted from an established radioassay for PKA activity in insect brain (63) and is described in *SI Appendix*.

Sequences of *for*, *pkac1*, and *pkar1*. Partial cDNA sequences of *for* and *pkar1* were identified in an EST database of *S. gregaria* CNS (13). The partial cDNA sequence of *pkac1* was obtained by PCR amplification with degenerate primers and subsequent cloning as described in *SI Appendix*. The sequences have been submitted to GenBank (*for*, accession no. HM363020; *pkac1*, accession no. HM363018; *pkar1*, accession no. HM363019).

Generation of dsRNA. dsRNAs were generated from the cDNA of the target genes using the Ambion MEGAscript RNAi Kit (Applied Biosystems/Ambion) as recommended by the manufacturer. In short, two successive PCR reactions (primer sequences are provided in *SI Appendix*, Table S1) were used to amplify a region within the cDNA (*SI Appendix*, Figs. S2, S4, and S6, red) and to ligate *KspAI* restriction sites to the 3′ ends of both the sense and the antisense strand, which later served as transcription termination sites for the RNA polymerase. The cDNA fragment was inserted into a TOPO4 vector with a T7 promoter site (Invitrogen). The vector was multiplied in TOP10 *Escherichia coli* cells (Invitrogen), isolated (GenElute HP Plasmid Miniprep Kit; Sigma-Aldrich), and sequenced on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) according to the protocol recommended by the manufacturer to confirm the identity and correct orientation of the insert. This vector served as a template to transcribe, in vitro, two cRNA strands that were hybridized to form dsRNA. DNA and single-stranded RNA were removed by nuclease digestion, and proteins and mono/oligonucleotides by solid-phase adsorption purification.

Quantification of mRNA Expression. Total RNA was extracted by using the RNAqueous Micro Kit (Ambion), which includes a DNase treatment to eliminate genomic DNA contamination. Gene-specific mRNA transcripts were quantified by quantitative real-time RT-PCR relative to two reference gene transcripts, β-actin and GAPDH (primer sequences shown in *SI Appendix*, Table S2), as described in *SI Appendix*.

Western Blotting. CNS extracts were reduced and denatured, electrophoresed on NuPAGE Bis-Tris minigels (Invitrogen), and transferred to PVDF membrane. FOR was detected with antibody KAP-PK005 (Stressgen) against the C-terminal sequence of human PKG 1α (antigen shown in blue in *SI Appendix*, Fig. S2). PKAC1 was detected with antibody DC0 against the orthologue in *D. melanogaster* (43). The detailed protocols are given in *SI Appendix*.

Quantification of PKAC1 Protein by Immuno-Dot Blot. Amersham Hybond-P PVDF membrane (0.45-μm pore size; GE Healthcare Bio-Sciences) was wet in methanol and floated on Tris-buffered saline (TBS) solution. Protein extracts from individual brains (*SI Appendix*) were diluted to 0.2 $g \cdot L^{-1}$ protein in TBS solution, and 10-μL droplets were spotted on the membrane in a grid pattern at random row and column positions. Each extract was spotted twice per membrane on three membranes, for a total of six replicates. After 1 h, the TBS solution was aspirated from the trays. The membranes were kept in a damp chamber overnight at 2 °C, air-dried, and then probed with antibody DC0 in the same way as Western blots. Chemiluminescence was captured on a Kodak IS4000R digital image station and quantified in Carestream Molecular Imaging software (Carestream Health). Best linear unbiased predictors of PKAC1 expression in individual brains were calculated by fitting a random-effect model to the six spot intensities per locust (R package *lme4*; *SI Appendix*, 4.6). To test whether dsRNA treatment and the time since the injection affected PKAC1 expression, the model was extended with treatment and time as fixed effects. Contrast estimates and associated *P* values were obtained by Markov-chain Monte Carlo estimation (R package *languageR*, version 1.0).

Statistical Analyses. All statistical analyses were performed in R, version 2.11.1, as detailed in *SI Appendix*.

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1. Pigliucci M (2001) *Phenotypic Plasticity: Beyond Nature and Nurture* (Johns Hopkins Univ Press, Baltimore).
2. West-Eberhard MJ (2003) *Developmental Plasticity and Evolution* (Oxford Univ Press, Oxford, UK).
3. Uvarov BP (1966) *Grasshoppers and Locusts: A Handbook of General Acridology* (Centre for Overseas Pest Research, London).
4. Verlinden H, Badisco L, Marchal E, Van Wielendaele P, Vanden Broeck J (2009) Endocrinology of reproduction and phase transition in locusts. *Gen Comp Endocrinol* 162:79–92.
5. Pener M, Simpson S (2009) Locust phase polyphenism: An update. *Adv Insect Physiol* 36:1–272.
6. Collett M, Despland E, Simpson SJ, Krakauer DC (1998) Spatial scales of desert locust gregarization. *Proc Natl Acad Sci USA* 95:13052–13055.
7. Despland E, Rosenberg J, Simpson S (2004) Landscape structure and locust swarming: a satellite's eye view. *Ecography* 27:381–391.
8. Roessingh P, Bouaïchi A, Simpson SJ (1998) Effects of sensory stimuli on the behavioural phase state of the desert locust, *Schistocerca gregaria*. *J Insect Physiol* 44: 883–893.
9. Simpson SJ, Despland E, Hägele BF, Dodgson T (2001) Gregarious behavior in desert locusts is evoked by touching their back legs. *Proc Natl Acad Sci USA* 98:3895–3897.
10. Rogers SM, et al. (2003) Mechanosensory-induced behavioural gregarization in the desert locust *Schistocerca gregaria*. *J Exp Biol* 206:3991–4002.
11. Kang L, et al. (2004) The analysis of large-scale gene expression correlated to the phase changes of the migratory locust. *Proc Natl Acad Sci USA* 101:17611–17615.
12. Ma Z, Guo W, Guo X, Wang X, Kang L (2011) Modulation of behavioral phase changes of the migratory locust by the catecholamine metabolic pathway. *Proc Natl Acad Sci USA* 108:3882–3887.
13. Badisco L, et al. (2011) Transcriptome analysis of the desert locust central nervous system: Production and annotation of a *Schistocerca gregaria* EST database. *PLoS ONE* 6:e17274.
14. Anstey ML, Rogers SM, Ott SR, Burrows M, Simpson SJ (2009) Serotonin mediates behavioral gregarization underlying swarm formation in desert locusts. *Science* 323: 627–630.
15. Kandel ER (2001) The molecular biology of memory storage: A dialogue between genes and synapses. *Science* 294:1030–1038.
16. Rogers SM, et al. (2004) Substantial changes in central nervous system neurotransmitters and neuromodulators accompany phase change in the locust. *J Exp Biol* 207:3603–3617.
17. Bernier L, Castellucci VF, Kandel ER, Schwartz JH (1982) Facilitatory transmitter causes a selective and prolonged increase in adenosine 3′:5′-monophosphate in sensory neurons mediating the gill and siphon withdrawal reflex in *Aplysia*. *J Neurosci* 2: 1682–1691.
18. Castellucci VF, Nairn A, Greengard P, Schwartz JH, Kandel ER (1982) Inhibitor of adenosine 3′:5′-monophosphate-dependent protein kinase blocks presynaptic facilitation in *Aplysia*. *J Neurosci* 2:1673–1681.
19. Müller U, Carew TJ (1998) Serotonin induces temporally and mechanistically distinct phases of persistent PKA activity in *Aplysia* sensory neurons. *Neuron* 21:1423–1434.
20. Huang YY, Kandel ER (2007) 5-Hydroxytryptamine induces a protein kinase A/mitogen-activated protein kinase-mediated and macromolecular synthesis-dependent late phase of long-term potentiation in the amygdala. *J Neurosci* 27:3111–3119.
21. Skoulakis EM, Kalderon D, Davis RL (1993) Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. *Neuron* 11:197–208.
22. Abel T, et al. (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. *Cell* 88:615–626.

23. Müller U (2000) Prolonged activation of cAMP-dependent protein kinase during conditioning induces long-term memory in honeybees. *Neuron* 27:159–168.
24. Michel M, Kemenes I, Müller U, Kemenes G (2008) Different phases of long-term memory require distinct temporal patterns of PKA activity after single-trial classical conditioning. *Learn Mem* 15:694–702.
25. Sanchez H, Quinn JJ, Torregrossa MM, Taylor JR (2010) Reconsolidation of a cocaine-associated stimulus requires amygdalar protein kinase A. *J Neurosci* 30:4401–4407.
26. Kleppisch T, Feil R (2009) cGMP signalling in the mammalian brain: Role in synaptic plasticity and behaviour. *Handb Exp Pharmacol* 191:549–579.
27. De Belle JS, Sokolowski MB (1987) Heredity of rover/sitter: Alternative foraging strategies of *Drosophila melanogaster* larvae. *Heredity* 59:73–83.
28. Kaun KR, et al. (2007) Natural variation in food acquisition mediated via a *Drosophila* cGMP-dependent protein kinase. *J Exp Biol* 210:3547–3558.
29. Kaun KR, Hendel T, Gerber B, Sokolowski MB (2007) Natural variation in *Drosophila* larval reward learning and memory due to a cGMP-dependent protein kinase. *Learn Mem* 14:342–349.
30. Wang Z, et al. (2008) Visual pattern memory requires foraging function in the central complex of *Drosophila*. *Learn Mem* 15:133–142.
31. Kent CF, Daskalchuk T, Cook L, Sokolowski MB, Greenspan RJ (2009) The *Drosophila* foraging gene mediates adult plasticity and gene-environment interactions in behaviour, metabolites, and gene expression in response to food deprivation. *PLoS Genet* 5:e1000609.
32. Ben-Shahar Y, Robichon A, Sokolowski MB, Robinson GE (2002) Influence of gene action across different time scales on behavior. *Science* 296:741–744.
33. Ingram KK, Oefner P, Gordon DM (2005) Task-specific expression of the foraging gene in harvester ants. *Mol Ecol* 14:813–818.
34. Lucas C, Sokolowski MB (2009) Molecular basis for changes in behavioral state in ant social behaviors. *Proc Natl Acad Sci USA* 106:6351–6356.
35. Sokolowski MB, Pereira HS, Hughes K (1997) Evolution of foraging behavior in *Drosophila* by density-dependent selection. *Proc Natl Acad Sci USA* 94:7373–7377.
36. Lucas C, et al. (2010) The locust foraging gene. *Arch Insect Biochem Physiol* 74:52–66.
37. Roessingh P, Simpson SJ, James S (1993) Analysis of phase-related changes in behavior of desert locust nymphs. *Proc Biol Sci* 253:43–49.
38. Hidaka H, Kobayashi R (1992) Pharmacology of protein kinase inhibitors. *Annu Rev Pharmacol Toxicol* 32:377–397.
39. Müller U (1996) Inhibition of nitric oxide synthase impairs a distinct form of long-term memory in the honeybee, *Apis mellifera*. *Neuron* 16:541–549.
40. Belay AT, et al. (2007) The foraging gene of *Drosophila melanogaster*: Spatial-expression analysis and sucrose responsiveness. *J Comp Neurol* 504:570–582.
41. Li W, Tully T, Kalderon D (1996) Effects of a conditional *Drosophila* PKA mutant on olfactory learning and memory. *Learn Mem* 2:320–333.
42. Fiala A, Müller U, Menzel R (1999) Reversible downregulation of protein kinase A during olfactory learning using antisense technique impairs long-term memory formation in the honeybee, *Apis mellifera*. *J Neurosci* 19:10125–10134.
43. Lane ME, Kalderon D (1993) Genetic investigation of cAMP-dependent protein kinase function in *Drosophila* development. *Genes Dev* 7(7A):1229–1243.
44. Hegde AN, Goldberg AL, Schwartz JH (1993) Regulatory subunits of cAMP-dependent protein kinases are degraded after conjugation to ubiquitin: A molecular mechanism underlying long-term synaptic plasticity. *Proc Natl Acad Sci USA* 90:7436–7440.
45. Ragazzon B, et al. (2009) Inactivation of the Carney complex gene 1 (protein kinase A regulatory subunit 1A) inhibits SMAD3 expression and TGF beta-stimulated apoptosis in adrenocortical cells. *Cancer Res* 69:7278–7284.
46. Brostrom CO, Corbin JD, King CA, Krebs EG (1971) Interaction of the subunits of adenosine 3′:5′-cyclic monophosphate-dependent protein kinase of muscle. *Proc Natl Acad Sci USA* 68:2444–2447.
47. Kurosu T, Hernández AI, Schwartz JH (2007) Serotonin induces selective cleavage of the PKA RI subunit but not RII subunit in *Aplysia* neurons. *Biochem Biophys Res Commun* 359:563–567.
48. Kelly MT, Crary JF, Sacktor TC (2007) Regulation of protein kinase Mζ synthesis by multiple kinases in long-term potentiation. *J Neurosci* 27:3439–3444.
49. Lorenzetti FD, Baxter DA, Byrne JH (2008) Molecular mechanisms underlying a cellular analog of operant reward learning. *Neuron* 59:815–828.
50. Jin I, Kandel ER, Hawkins RD (2011) Whereas short-term facilitation is presynaptic, intermediate-term facilitation involves both presynaptic and postsynaptic protein kinases and protein synthesis. *Learn Mem* 18:96–102.
51. Roessingh P, Simpson SJ (1994) The time-course of behavioral phase change in nymphs of the desert locust, *Schistocerca gregaria*. *Physiol Entomol* 19:191–197.
52. Armstrong GA, Rodgers CI, Money TG, Robertson RM (2009) Suppression of spreading depression-like events in locusts by inhibition of the NO/cGMP/PKG pathway. *J Neurosci* 29:8225–8235.
53. Osborne KA, et al. (1997) Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science* 277:834–836.
54. Despland E, Simpson SJ (2005) Food choices of solitarious and gregarious locusts reflect cryptic and aposematic antipredator strategies. *Anim Behav* 69:471–479.
55. Simpson SJ, Raubenheimer D, Behmer ST, Whitworth A, Wright GA (2002) A comparison of nutritional regulation in solitarious- and gregarious-phase nymphs of the desert locust *Schistocerca gregaria*. *J Exp Biol* 205:121–129.
56. Ellis PE (1959) Learning and social aggregation in locust hoppers. *Anim Behav* 7: 91–106.
57. Ellis PE (1963) The influence of some environmental factors on learning and aggregation in locust hoppers. *Anim Behav* 11:142–151.
58. Geva N, Guershon M, Orlova M, Ayali A (2010) Memoirs of a locust: Density-dependent behavioral change as a model for learning and memory. *Neurobiol Learn Mem* 93:175–182.
59. Hofmann HA, Stevenson PA (2000) Flight restores fight in crickets. *Nature* 403:613.
60. Stevenson PA, Dyakonova V, Rillich J, Schildberger K (2005) Octopamine and experience-dependent modulation of aggression in crickets. *J Neurosci* 25:1431–1441.
61. Shelton RC, Sanders-Bush E, Manier DH, Lewis DA (2009) Elevated 5-HT 2A receptors in postmortem prefrontal cortex in major depression is associated with reduced activity of protein kinase A. *Neuroscience* 158:1406–1415.
62. Sloman L, Farvolden P, Gilbert P, Price J (2006) The interactive functioning of anxiety and depression in agonistic encounters and reconciliation. *J Affect Disord* 90:93–99.
63. Hildebrandt H, Müller U (1995) PKA activity in the antennal lobe of honeybees is regulated by chemosensory stimulation in vivo. *Brain Res* 679:281–288.