Molecular basis for changes in behavioral state in ant social behaviors

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A hallmark of behavior is that animals respond to environmental change by switching from one behavioral state to another. However, information on the molecular underpinnings of these behavioral shifts and how they are mediated by the environment is lacking. The ant *Pheidole pallidula* with its morphologically and behaviorally distinct major and minor workers is an ideal system to investigate behavioral shifts. The physically larger majors are predisposed to defend the ant nest, whereas the smaller minors are the foragers. Despite this predisposition, majors are able to shift to foraging according to the needs of the colony. We show that the ant foraging (*ppfor*) gene, which encodes a cGMP-dependent protein kinase (PKG), mediates this shift. Majors have higher brain PKG activities than minors, and the spatial distribution of the PPFOR protein differs in these workers. Specifically, majors express the PPFOR protein in 5 cells in the anterior face of the ant brain, whereas minors do not. Environmental manipulations show that PKG is lower in the presence of a foraging stimulus and higher when defense is required. Finally, pharmacological activation of PKG increases defense and reduces foraging behavior. Thus, PKG signaling plays a critical role in *P. pallidula* behavioral shifts.

cGMP-dependent protein kinase | defense | foraging

Major genes for normal individual differences in behavior are now familiar in a variety of species (1). However, an important feature of behavioral traits is that they are responsive to environmental change. Animals are able to switch from one behavioral state to another, yet little is known about the molecular basis of these behavioral shifts and how they are mediated by the environment. Eusocial insects are excellent models for studying these gene–environment interdependencies because their social organization relies on individuals who belong to behaviorally specialized castes. Wilson (2) showed that, despite this behavioral specialization, individuals of one caste can rapidly modify their behavior, depending on colony requirements; he called this the flexibility of behavioral castes. These quick changes in caste behavioral repertoires provide enough flexibility in colony response to maintain the colony when the environment goes through rapid changes. For example, ants whose role is to defend the nest are able to switch to foraging, depending on the needs of the colony. How might this switch from one behavioral state to another be accomplished?

The ant *Pheidole pallidula* provides us with an excellent system to investigate the molecular underpinnings of flexibilities in behavioral predispositions. This species has 2 morphologically distinct worker castes, called majors and minors (3), that we will refer to here as subcastes. Both reach the same stage of maturity and work outside the nest. Majors have large heads and mandibles and specialize in colony defense; they guard the nest, patrol outside the nest entrance, and kill intruders. Minors are smaller and perform foraging behaviors, including food-search and prey retrieval. However, majors who are built for defense can switch their behavioral state from defense to foraging, depending on the needs of the colony, thereby demonstrating flexibility in their behavioral repertoires (2, 4). Physical differences between majors and minors initiate in developmental pathways that diverge during the larval stage of development. Cues originating from nutrition fed to the larvae and from the social environment are integrated by the endocrine system to initiate the relevant developmental pathway (2, 5, 6).

Here, we investigate the molecular underpinnings of behavioral flexibility in *P. pallidula* workers ants, which are highly specialized both morphologically and behaviorally. We show that the activity of the enzyme cGMP-dependent protein kinase (PKG) encoded by the ant foraging (*ppfor*) gene functions in the change from one behavioral state to another and that this is correlated with differences in the spatial expression of the PPFOR protein in the brains of the 2 worker subcastes. Our study also provides evidence that PKG plays a role in defense.

Results

PKG Activity Is Subcaste Specific. We cloned the *for* ortholog, *ppfor*, from *P. pallidula*. To investigate subcaste-specific differences in PKG enzyme activity, PKG assays were performed on adult brains (7). Majors had significantly higher PKG enzyme activity than minors in all colonies (Fig. 1*A*). This suggested a correlation between brain PKG activity and these physical and behavioral castes.

PPFOR Differs Spatially in Subcaste Brains. We were successful in visualizing PPFOR expression in ant brains. We used an antibody made to regions of the *Drosophila melanogaster* FOR protein that had high homology to the PPFOR protein isoform encoded by the longer *ppfor* transcript T1 (8) (see *SI Text* on brain biometry; controls for the FOR antibody are in Fig. 3 and in Fig. S2). We found that *P. pallidula* has 3 main PKG-immunoreactive regions: one cluster dorsal and on the internal face of the lobula, another posterior and ventral to the mushroom body peduncles, and a third cluster on the anterior face of the subesophageal ganglion (Fig. 2). Notably, we observed expression in several regions of the anterior brain in majors but not minors. Specifically, brains of majors, but not minors, display strong immunoreactivity in approximately 5 cells clustered on the anterior face external to the mushroom body peduncles (Fig. 2*E*). An intriguing possibility is that this difference in subcaste PPFOR protein expression may be related to their different behavioral profiles. Such a link would reflect a similar case where social behaviors exhibited by montane and prairie voles are linked to variation in the distributions of vasopressin receptors in their brains (9).

PKG Activity and Environmental Manipulations of the Colony Environment. We investigated the flexibility of the behavioral predispositions in majors and minors by manipulating the colony envi-

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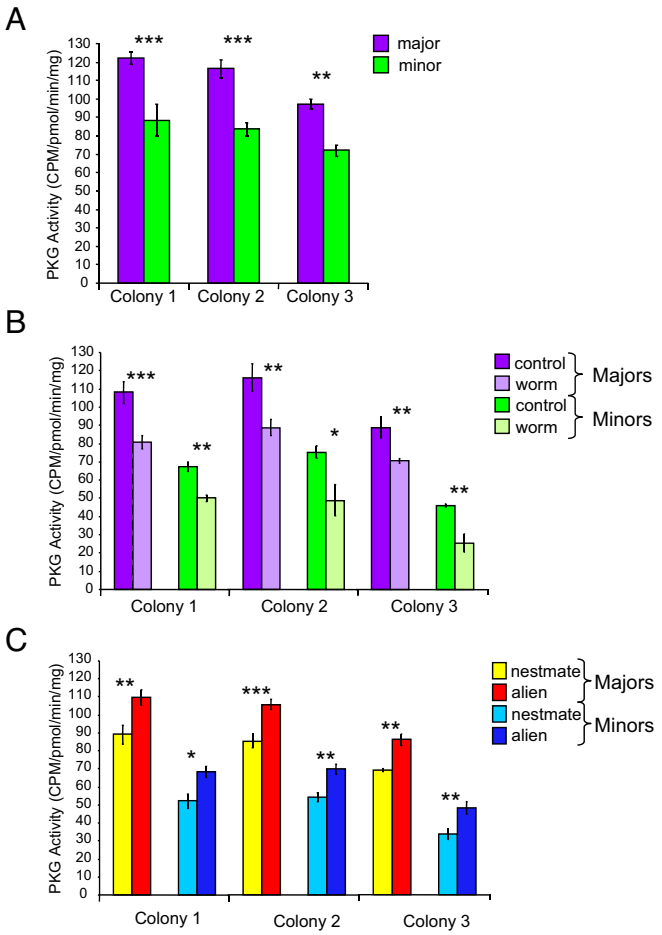

Fig. 1. PKG activity of major and minor *P. pallidula* worker ants (mean ± SE). (*A*) Brain PKG activities of majors and minors differ (nested ANOVA, $F_{(3,29)} = 19.04$, $P < 0.001$). (*B*) PKG activity of majors and minors was lower in the presence of the mealworm (foraging stimulus) compared with the control ($F_{(3,45)} = 24.13$, $P < 0.001$); majors had higher PKG activity than minors ($F_{(3,45)} = 73.41$, $P < 0.001$). (*C*) Majors and minors had higher PKG activity in the presence of aliens (defense stimulus) compared with nestmate intruders ($F_{(3,41)} = 23.07$, $P < 0.001$); majors had higher PKG activity than minors ($F_{(3,41)} = 103.19$, $P < 0.001$). For *B* and *C*, the significance of all within-colony comparisons not indicated on the figure was $P < 0.001$. In all figures, *P* levels of $P < 0.001$, $P < 0.01$, and $P < 0.05$ are represented as ***, **, and *, respectively.

ronment. We first introduced either a live mealworm or a mealworm-shaped plastic model (control). In the presence of the mealworm prey, foraging minors rapidly (<5 min) recruit majors to help them cut up the prey and transport it to the nest (4). This successful recruitment of majors for food-related activities confirms that majors are behaviorally flexible. Under certain circumstances and notwithstanding their disposition for defense behaviors, majors will perform foraging tasks. Because minors, which are specialized foragers, have lower PKG activity than majors, we predicted that PKG activities would be lower in majors performing foraging behaviors compared with those in control experiments. Consistent with this prediction, brain PKG activities of both majors and minors, collected from 3 independent colonies, were significantly lower than those of controls when exposed to a foraging stimulus: the mealworm prey (Fig. 1*B*). Another way to stimulate foraging behavior is food deprivation (3). We food-deprived the colonies for 3 weeks, and as expected, PKG activity was lower than that in well-fed controls (Fig. S1) as was found in the mealworm prey experiment (Fig. 1*B*). Together, these results suggest that the mealworm prey is acting as a foraging stimulus for the majors and minors.

Majors have higher PKG activity than minors and are predisposed to act in colony defense. Therefore, second, we asked whether the link between PKG activity and behavioral flexibility extends to colony defense. Methods developed for nestmate recognition experiments were used to assay colony defense (10). We introduced intruder ants who were either alien (from another colony) or nestmates (from the home colony) into an arena placed into a test colony. Ants contact the intruders through small openings in the side of the arena. In all colonies, brain PKG activity was elevated in both subcastes when exposed to alien intruders compared with nestmate controls (Fig. 1*C*), suggesting a possible role for PKG in defense.

Pharmacological Manipulation of PKG Activity Changes Subcaste Behaviors. Data presented thus far demonstrate a correlation between PKG enzyme activity and subcaste-specific behaviors, foraging and defense, and suggest that PKG may modulate the probability of performing these behaviors. To initiate testing a causal link, we pharmacologically manipulated PKG activity and measured foraging and defense behavior in majors and minors. We treated ants with a specific PKG activator, 8Br-cGMP, as in Ben-Shahar et al. (11). As expected, the mean specific brain PKG activity of majors and minors was higher after 8Br-cGMP treatment ($F_{(2,20)} = 16.76$, $P < 0.001$): majors, 84.85 ± 3.21 for control and 101.25 ± 4.55 for treatment; minors, 36.64 ± 2.27 for control and 50.51 ± 2.57 for treatment [units are pmol of ^{32}P-labeled PKG substrate/(min/mg of protein)]. Thus, PKG activities were elevated by the treatment, and this increase was within the normal range (Fig. 1).

To assess the behavioral effect of 8Br-cGMP treatment on foraging behaviors, we quantified the percentage of workers that interacted with a mealworm when treated with the PKG activator as compared with the control. As expected, the pharmacological treatment resulted in fewer majors and minors responding to the presence of the mealworm (Fig. 4*A*), suggesting that this increase in PKG was sufficient to change the probability of responding to the foraging stimulus. The relative decrease in the foraging response of majors was greater than that in minors ($F_{(3,27)} = 69.22$, $P < 0.001$), suggesting that majors may be more flexible than minors. We next tested the effects of the pharmacological treatment on defense behaviors. Consistently, majors showed a significantly higher defense response to the aliens in response to the pharmacological treatment relative to the control (Fig. 4*B*), showing that the treatment was sufficient to increase the probability of responding to the defense stimulus. The pharmacological treatment, however, did not affect the response of the minors to the alien intruders (Fig. 4*B*). Because the pharmacological treatment was administered for a number of days, we cannot yet distinguish the effects of chronic or acute changes in PKG activity on the behaviors of majors and minors.

Discussion

Physical castes provide a great model for understanding the molecular basis of individual differences in behavior because their discrete alternative phenotypes result in striking behavioral flexibility. One interpretation of our data is that the behavioral differences between majors and minors are due to shifts in levels of response for task-related stimuli (12) and that this occurs in part by tuning the PKG system. In this model, major and minor PKG activity in undisturbed colonies reflects developmentally established predispositions to perform defense and foraging behaviors, respectively. The mealworm and alien intruder experiments suggest that the PKG system can tweak the response, eliciting flexible behavioral responses especially in majors. Shifts in behavioral responses are further shown by using the PKG activator, which was fed to these ants for several days before the behavioral assay. This pharmacological treatment implies a causal relationship among PKG, behavioral flexibility, and the

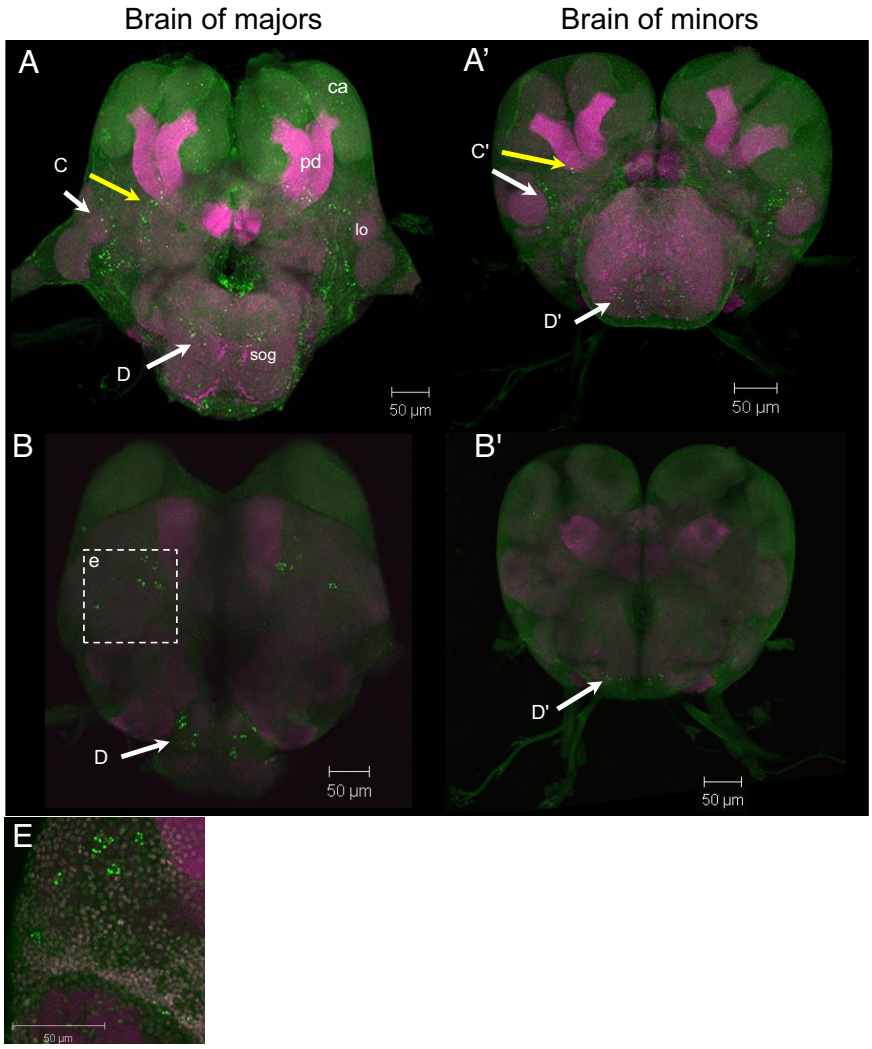

Fig. 2. FOR expression patterns differ in the brains of major and minor *P. pallidula* worker ants. All brain images are 3D reconstructions of 1-μm optical sections shown in a posterior view with dorsal part at the top. PKG immunoreactivity (PKG-IR) is indicated in green, and magenta indicates brain neuropiles (mouse antibody, nc82). (*A* and *A′*) Whole brain reconstruction of majors (*A*) and minors (*A′*) shows 3 main PKG-IR regions: a cluster dorsal and on the internal face of the lobula (*C* and *C′*, white arrows), a cluster posterior and ventral to the peduncles of the mushroom bodies (*C* and *C′*, yellow arrows), and a cluster on the anterior face of the subesophageal ganglion (*D* and *D′*) (enlargement of details is shown in Fig. 3). (*B* and *B′*) Three-dimensional reconstruction of subsections (40 sections) of the whole brains (*A* and *A′*), spanning the posterior part of the brain of majors (*B*) and minors (*B′*), respectively, shows a cluster of PKG-IR cells found in the brain of majors (*E*, 30 sections) but not in minors. This specific cluster is localized on the anterior face external to the peduncles of the mushroom bodies; negative controls using a preabsorption step to abolish PKG-IR and blank controls using only the secondary antibody are in Fig. 3. ca, Calyx; pd, peduncles; lo, lobula; sog, subesophageal ganglion.

environmental stimulus (mealworm or alien intruder). The pharmacological treatment, however, did not affect the response of the minors to the alien intruders. These results may indicate a limit, perhaps established in larval or early adult development, in the minors' ability to perform defense behavior, which could be tied to the differences in FOR spatial patterning in major and minor brains. The morphological specialization found in these physical castes could, for example, involve differential spatial patterning of major and minor brains.

The possible mechanistic similarity between developed predispositions (caste differences in behavior) and adult flexibility (within-caste behavioral shifts) is implicit in our findings. The same mechanisms that cause majors to defend and minors to forage could also underlie behavioral flexibility within a fully developed, morphologically differentiated caste. Certainly many genes and pathways are involved in the development and performance of foraging and defense behaviors in ants; however, we have shown here that it is sufficient to alter PKG activity to affect behavioral outcomes.

The behavioral effects of *for*-PKG were originally described in *D. melanogaster*, where allelic variation in *for* affects larval and adult foraging behaviors (13, 14). Individuals with a "rover" allele express greater foraging locomotion and eat less than those with only "sitter" alleles (7). In this system, the allelic variation in *for* is important for rover or sitter behavior; however, *for* also affects plasticity; it modulates these behaviors in response to environmental input (7, 15, 16).

A link between *for* and long-term behavioral maturation has been described in social insects where *for* up-regulation in individual workers leads to maturation from performing nursing activities within the colony to foraging outside the honey bee colony (11, 17). Nurses work inside the nest and become foragers who work outside the nest as they mature. Up-regulation of PKG in nurse bees appears to increase the readiness of honey bees to forage. In these honey bees, both age and place of work differ between nurses and forager bees. In *P. pallidula*, major and minor ants reach the same stage of maturity, and both work outside of the nest.

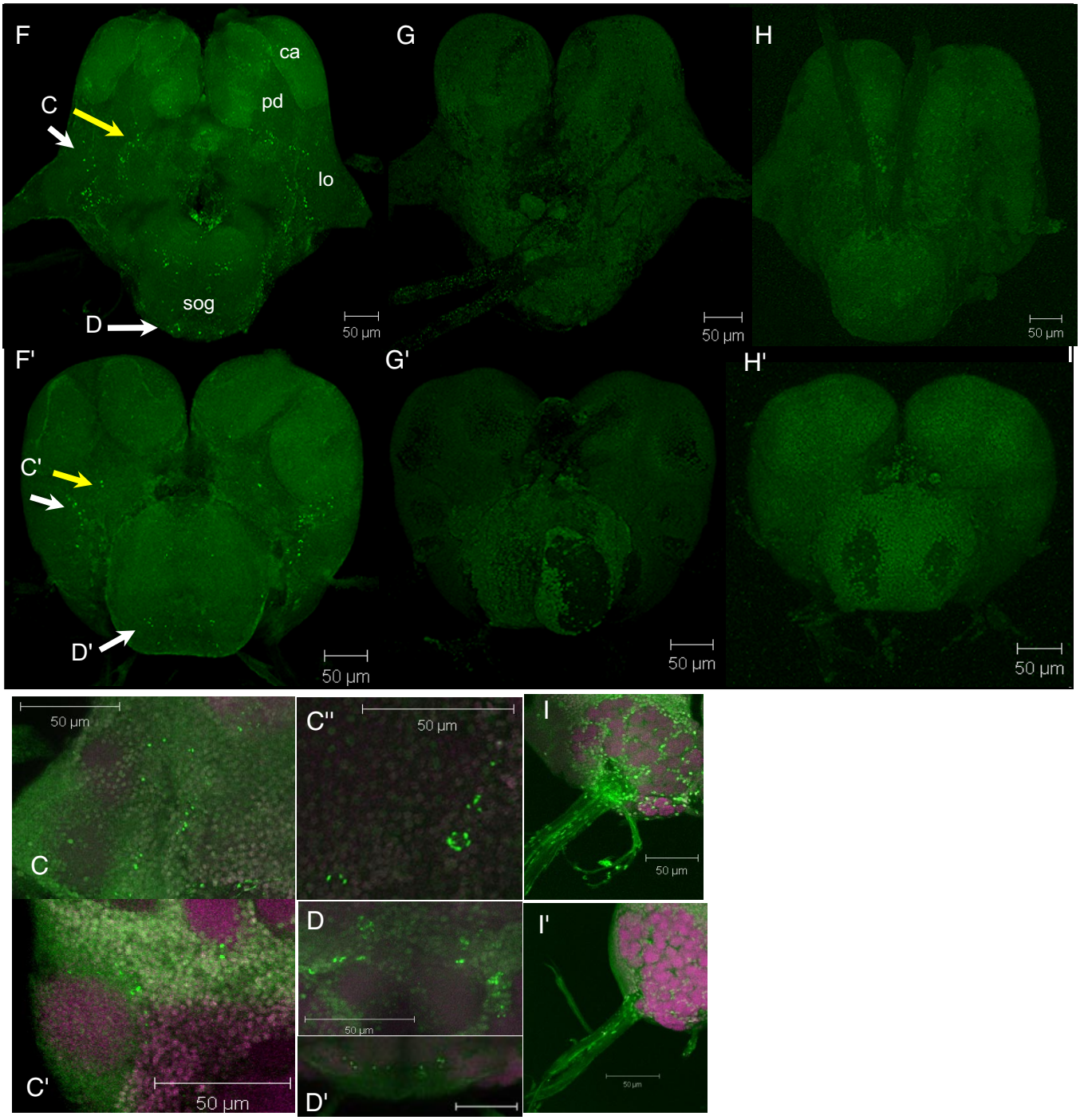

Fig. 3. Negative controls (posterior view) and details of PPFOR antibody clusters. Colors are the same as those in Fig. 2. All brain images are 3D reconstructions of 1-μm optical sections shown in a posterior view with dorsal part at the top. (*F*–*H*″) Anti-PKG staining of major (*F*) and minor (*F*′) whole brains; negative controls using a preabsorption step abolish PKG immunoreactivity in majors (*G*) and minors (*G*′); controls using only the secondary antibody in major (*H*) and minor whole brains (*H*′); 2 clusters, 1 dorsal and on the internal face of the lobula (lo) (white arrows) and 1 posterior and ventral to the peduncle (yellow arrows) of majors (*C*, 20 sections) and minors (*C*′, 20 sections); cluster on the anterior face of the subesophageal ganglion of majors (*D*, 20 sections) and minors (*D*′, 20 sections); (*C*″, 10 sections) single sections taken at higher magnification in the lobula region suggest that PKG is localized to the plasma membrane, cytosol, or both; PKG is also expressed in fiber-like projections in antennal nerves in majors (*I*, 25 sections) and minors (*I*′, 25 sections). ca, Calyx; pd, peduncles; lo, lobula; sog, subesophageal ganglion.

A correlative study on the *for* gene in the ant *Pogonomyrmex barbatus* compares *for* mRNA levels of nurse and forager ants who are different ages and work inside and outside the nest, respectively (18). As is the case in honey bees, foraging gene expression in *P. barbatus* ants is linked to the temporal polyethism (11). The present study provides a role for PKG in defense and caste polyethism.

There are many interesting contrasts and parallels between the behavioral roles of *for*-PKG in *D. melanogaster* and *P. pallidula*. For example, in *D. melanogaster*, genetic polymorphism contributes to the predisposition to behave as a rover or sitter (13). By contrast, in *P. pallidula*, differential feeding of the larvae and social cues influence the development of majors or minors physical caste and behavioral predispositions (6, 19). However,

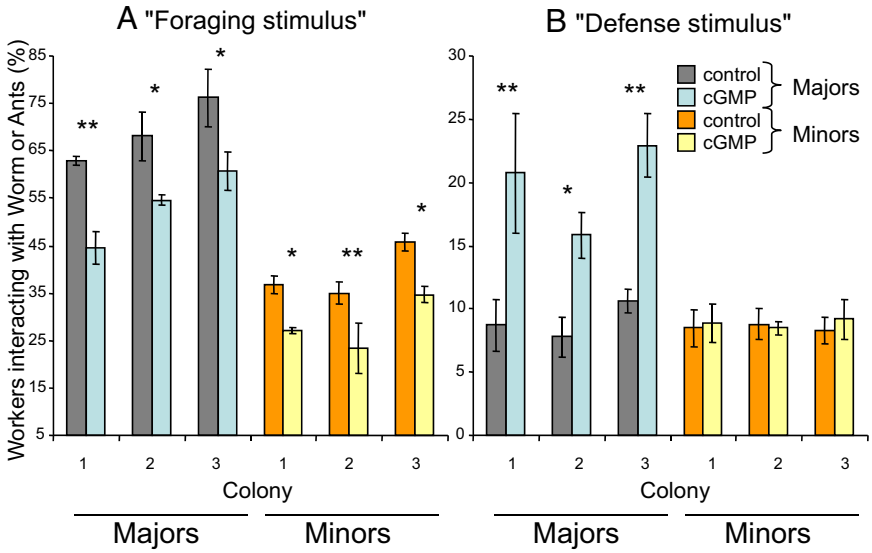

Fig. 4. Effect of a PKG activator (8Br-cGMP) on foraging and defense behaviors. (*A*) 8Br-cGMP treatment resulted in a lower percentage of majors ($F_{(3,12)} = 8.05$, $P = 0.003$) and minors ($F_{(3,12)} = 8.06$, $P = 0.003$) interacting with the mealworm compared with the control. Majors respond more to the presence of the worm than minors ($F_{(3,27)} = 69.22$, $P < 0.001$). (*B*) 8Br-cGMP treatment resulted in a higher percentage of majors responding to the presence of aliens ($F_{(3,12)} = 9.15$, $P = 0.002$) but had no significant effect on the percentage of minors interacting with the alien intruders ($F_{(3,12)} = 0.11$, $P = 0.95$). All data are mean percentages ± SE.

even with such predispositions, individuals of either species can exhibit flexible behavioral responses to environmental stimuli. Specifically, rover flies express more sitter-like foraging when they undergo long-term nutrient deprivation (7), and major ants can rapidly exhibit minor-like foraging behaviors in the presence of a prey item and according to the needs of the colony.

The behavioral influence of *for* is conserved across phyla. However, the directional affects of PKG on foraging-related behaviors differs between species: The rover "forager" type arises from higher *for* gene activity in the fly (13) and honey bee (11) and lower activity in *Caenorhabditis elegans* (20) and ants (18). These data suggest that natural selection has harnessed the same gene in different species in somewhat different ways for related behavioral phenotypes despite large differences in their life histories. That spatial differences in expression of PPFOR were found in the *P. pallidula* brain and not in the rover and sitter fly (8) or nurse and forager honey bee brains (11) supports this hypothesis.

Materials and Methods

Cloning. The *P. pallidula for* ortholog *ppfor* was cloned using degenerate primers designed to conserved sequence (sense primer, GTGAACTKAT-CAARGCWGCCATWTTGG; antisense primer, CGATGGCCCAYAGSTKG-CAMTCGG) after alignment of several other species. mRNA was extracted using 50 heads with an Amersham Biosciences kit. Specific cDNA was amplified using a RACE kit from Clontech and cloned with a TOPO TA Cloning kit from Invitrogen. We identified 2 transcripts, 3,924 and 1,836 bp in length (GenBank accession nos. EF999975 and EF999976). The longer *P. pallidula* (T1) transcript has 70% identity with the longest *D. melanogaster for* transcript and 81% identity with the honey bee *Amfor* transcript. The longer *ppfor* transcript resembles a typical PKG (21), sharing the same protein domains found in all of the foraging gene sequences, including: a kinase domain (with a catalytic site), 2 cGMP-binding domains, and a regulatory domain. The short *ppfor* (T2) transcript differs from T1; it only has half of the second cGMP-binding domain and lacks the catalytic site of the kinase domain. Northern blot analyses confirmed the number and size of the *ppfor* transcripts in ant heads.

PKG Enzyme Assays. PKG enzyme assay was modified from Kaun et al. (7). Ants were collected, flash frozen in liquid nitrogen, and kept at −80 °C until dissection. Five brains per sample were dissected and homogenized in 25 mM Tris (pH 7.4), 1 mM EDTA, 2 mM EGTA, 5 mM 2-mercaptoethanol, 0.05% Triton X-100, and protease inhibitor mixture (Roche). After 5 min of centrifugation, supernatants were quantified for total amount of protein and analyzed for PKG activity. The final reaction mixture contained 40 mM Tris·HCl (pH 7.4), 20 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP (250–500 cpm/pmol) (Amersham Biosciences), 1 mM EDTA, 2 mM EGTA, 143 μg/mL of a heptapeptide substrate highly specific to the PKG (RKRSRAE) (Promega), 3 μM cGMP, and 92 nM highly specific cAMP-dependent protein kinase inhibitor (K_i 50% = 2.3 nM) (5–24, Calbiochem). The reaction mixture was incubated for 10 min at 30 °C and terminated by spotting 70 μL onto Whatman P-81 filters. The specific PKG activity was expressed as pmol of ^{32}P-labeled PKG substrate/(min/mg of protein). PKG assays were performed on 5 groups of 5 dissected brains per subcaste per colony with 3 colonies.

Immunohistochemistry. Immunohistochemistry was as in Belay et al. (8). Briefly, brains of majors and minors were dissected in PBS (0.1 M, pH 7.4), fixed in 4% paraformaldehyde, and blocked in 4% normal goat serum (Jackson ImmunoResearch) in 0.5% Triton X-100/PBS. Individuals were collected from different colonies, and no differences in expression were found between colonies. The specific guinea pig antibody called anti-FOR, described in Belay et al. (8), was used at 1:100; the neuropile marker mouse mAb nc82 was used at 1:20 (22, 23). Incubation was for 24–48 h at 4 °C. After incubation, brains were washed several times in 0.5% Triton X-100/PBS before adding a goat Cy2-conjugated anti-mouse and a Cy5-conjugated anti-guinea pig Ig (1:100, Jackson ImmunoResearch) for 24 h at 4 °C. For negative controls, brains were incubated in only secondary antibody, in the absence of primary antibody or in preadsorbed anti-FOR serum (Fig. 3). Brains were washed several times, mounted, and examined with a Zeiss LSM 510 confocal laser scanning microscope. The specificity of the primary antibody, anti-FOR antibody generated in guinea pig (8), was measured by using Western blot immunodetection (24) (Fig. S2).

Ant Collection and Rearing. *P. pallidula* were collected from the field in southern France in the summer of 2003. This species is monoandrous (only 1 male inseminates a female) and polygynous (2 or more queens per nest) (25). In our experiments, only 1 queen was kept per nest, and experiments were conducted after the ants had been reared in the laboratory for 12 months. All nestmates within a colony shared the same mother and father, which maintained relatedness of the workers around 3/4. Ants were reared and tested under standard laboratory conditions (45% relative humidity, 27–28 °C, under a 12:12 light cycle with lights on at 0900). All colonies were fed the same food on the same schedule (ants were fed water ad libitum, honey, and ≈18 mealworms twice weekly).

Statistical Analyses. All data were analyzed byusing nested analyses of variance to take into account the effect of colony of origin (26). For the behavioral analyses, no effect of the repeated testing of each colony was found using a likelihood ratio test. This factor was then removed from analyses. All means are given ± SE. In all figures, *P* levels of $P < 0.001$, $P < 0.01$, and $P < 0.05$ are represented as ***, **, and *, respectively. The number of dead ants was not included as part of the totals.

Foraging Assays. The PKG activities of majors and minors given a live mealworm (*Tenebrio* spp., Ward's Natural Science) or a mealworm-shaped plastic model (control) were compared for ants from 3 colonies. Three-hundred minors and 50 majors (the approximate ratios of minors and majors found in our colonies) were collected 4 days before the experiment and placed in a test colony, which consisted of a new plastic Petri dish (140 mm in diameter by 20 mm in height, coated with Teflon on the inner dish wall) containing a watering tube (75 mm, containing 3 mL of distilled deionized H_2O, cotton sealed), a nest tube (100 mm, containing 5 mL of distilled deionized H_2O, cotton sealed and covered in dark plastic wrap), and a test arena. The test arena, composed of a small (35 mm in diameter by 10 mm in height) Petri dish, was placed in the center of the foraging area of the test colony. For the foraging assays, this arena was constructed so that ants could move freely in and out of the arena with a live mealworm. With a heated needle, 5 equally spaced slits were made around the wall of the Petri dish arena, 1 mm from the bottom of the dish. Each slit was 20 mm long, 1.5 mm wide, and 2 mm spaced apart. The entire outside surface of the dish was painted with white acrylic paint for optimal video recording contrast and counting of individuals within the arena. The colony lid was removed for filming, which began after a 5 min acclimatization period. The mealworm or a control was placed into the center of the arena for 35 min. At the end of this time period, ants that were inside the arena were collected, flash-frozen in liquid nitrogen, and kept at −80 °C until dissection. All colonies were fed 3 mealworms and 6 drops of concentrated honey daily for the 4 days before the experiment.

Defense Assays. The brain PKG activity of majors and minors exposed to either alien or nestmate intruder ants (control) were compared for ants from 3 colonies. All alien intruder ants came from the same colony. Test colonies were established as for the foraging assays with 300 minors and 50 majors. The defense arena was constructed so that intruder ants were contained within an area while ants from the colony could encounter the intruders through small slits in the arena. With a heated blade on a dissecting scalpel, 4 rows of 12 equally spaced slits were made around the wall of the Petri dish arena, 1 mm from the bottom of the dish and 1 mm from each successive row. Each slit was 8 mm long, 0.5 mm wide, and 1 mm spaced apart. The slits were made in a staggered brick pattern. The bottom of the arena dish was painted with white acrylic paint for optimal recording contrast. A primary cover was placed over the arena, and the outer wall side surface of the cover was coated with Teflon to prevent outside individuals from climbing over the arena and cover. A 20-mm circular aperture was burned through the top surface of the dish cover for easy insertion of the intruder alien or nestmate ants. A secondary dish cover was made to cover the surface aperture of the primary dish cover constructed from the bottom portion of a Petri dish. Two-hundred minors and 25 majors were collected from an alien source (individuals from another colony) or nestmate source (individuals from the home colony) and introduced into the defense arena. The colony lid was removed for filming, which began after a 5 min acclimatization period. The alien or nestmate intruder ants were placed into the center of the arena for 35 min. At the end of this time period, ants that were touching the side of the arena were collected, flash-frozen in liquid nitrogen, and kept at −80 °C until dissection. All colonies were fed 3 mealworms and 6 drops of concentrated honey daily for the 4 days before the experiment.

Pharmacological Treatment. A solution of 2 mM 8Br-cGMP was added daily to the mealworms and honey for the 4 days before the experiment. Treated water with activator was available ad libitum. The control received water, mealworms, and honey without the PKG activator. Feedings were performed in semidark testing room conditions, and test colonies were maintained under a cardboard cover to prevent photodegradation of the PKG activator. No differences were found in the number of dead between the different treatments for the foraging assay ($F_{(3,24)} = 1.23$, $P = 0.32$) and for the defense assay ($F_{(3,24)} = 0.12$, $P = 0.95$).

Behavioral Effect of Pharmacological Treatment on Foraging and Defense. The effect of the activator on foraging and defense was measured by using the assays described above. Behavior was recorded with a camera (DM-GL2; Canon). Two spotlights were used for illumination of the recording area (75-W BR30 Plant Light). The colony lid was removed for filming, then a 5-min acclimatization period was given. The stimulus, mealworm or alien ant, was placed in the test colony for 35 min. Data were quantified from the movies by counting the number of test minors and majors interacting with the stimulus every minute for 35 min for each of the 3 colonies. The mean number of ants responding to the stimulus for the last 10 min was used for each experiment. Each colony was tested 3 times over a 4-week period. Ants were scored as responding to the stimulus when they were touching the side of the arena for defense experiments or when they had entered the arena for foraging experiments. At the end of the 35 min, individuals responding to the stimulus were collected and counted under CO_2, flash-frozen in liquid nitrogen, and stored in a −80 °C freezer. The numbers of dead individuals were counted daily and at the end of the experiment; no differences in the number of dead ants were found between treatments.

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