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Levels and activity of cyclic guanosine monophosphate-dependent protein kinase in nurse and forager honeybees

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Abstract

Age-dependent division of labour in honeybees was shown to be connected to sensory response thresholds. Foragers show a higher gustatory responsiveness than nurse bees. It is generally assumed that nutrition-related signalling pathways underlie this behavioural plasticity. Here, one important candidate gene is the *foraging* gene, which encodes a cyclic guanosine monophosphate-dependent protein kinase (PKG). Several roles of members of this enzyme family were analysed in vertebrates. They own functions in important processes such as growth, secretion and neuronal adaptation. Honeybee *foraging* messenger RNA expression is upregulated in the brain of foragers. *In vivo* activation of PKG can modulate gustatory responsiveness. We present for the first time PKG protein level and activity data in the context of social behaviour and feeding. Protein level was significantly higher in brains of foragers than in those of nurse bees, substantiating the role of PKG in behavioural plasticity. However, enzyme activity did not differ between behavioural roles. The mediation of feeding status appears independent of PKG signalling. Neither PKG content nor enzyme activity differed between starved and satiated individuals. We suggest that even though nutrition-related pathways are surely involved in controlling behavioural plasticity, which involves changes in PKG signalling, mediation of satiety itself is independent of PKG.

Keywords: honeybee, division of labour, sucrose responsiveness, foraging gene/PKG.

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Introduction

The honeybee (*Apis mellifera*) has long been a model organism in neuroethology, because of its complex behavioural organization. In a honeybee colony, many tasks, such as cleaning, nursing, guarding and foraging, are coordinated to result in greater efficiency (Oster and Wilson, 1978). Age-dependent division of labour is an important way to ensure optimal task allocation within the hive. Whereas young bees work in the centre of the hive, older bees work in the periphery and later leave the hive to forage for pollen, nectar or water. The most dramatic behavioural changes occur when young nurse bees, which feed the larvae inside the hive, transition to forager bees (Seeley, 1995). A widely accepted hypothesis explaining this division of labour is the 'response threshold hypothesis'. This states that individuals with different tasks differ in their responsiveness for task-associated stimuli. The individual with the highest responsiveness is the first to perform the associated task (Robinson, 1992; Theraulaz *et al.*, 1998). Indeed, honeybee workers performing different tasks show distinct sensory responses. Forager bees show a higher sucrose responsiveness than nurse bees do (Thamm and Scheiner, 2014; Scheiner *et al.*, 2017a; 2017b). One candidate gene for the modulation of sucrose responsiveness within the context of division of labour is the *Apis mellifera foraging* gene (*Amfor*), which encodes a cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG; Ben-Shahar *et al.*, 2002; Hunt *et al.*, 2007). The expression of one isoform of this gene is upregulated in the brain of forager bees (Ben-Shahar *et al.*, 2002; 2003; Thamm and Scheiner, 2014), and its pharmacological activation increases sucrose responsiveness (Thamm and Scheiner, 2014). Finally, the protein encoded by this gene, AmForα-PKG, is located in the

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mushroom bodies and the gnathal ganglion (GG; Thamm and Scheiner, 2014). Because these neuropils have important roles in processing gustatory information in the brain (Haupt and Klemt, 2005; Nisimura *et al.*, 2005), this suggests a function of PKG in gustatory processing and behavioural modulation. In the fruit fly *Drosophila melanogaster*, individuals with the rover *for* allele have higher PKG enzyme activity than flies carrying the sitter *for* allele (Osborne *et al.*, 1997). This results in longer foraging trails in rover larvae than in sitters (Sokolowski, 1980). Furthermore, rover adults show a higher sucrose responsiveness (Scheiner *et al.*, 2004; Belay *et al.*, 2007) and store fewer carbohydrates in their fat body (Kaun *et al.*, 2008). If rovers are food deprived, the higher PKG activity causes decreasing haemolymph glucose levels and thus modulates food intake (Kaun *et al.*, 2008). These results suggest that a connection between nutrition and nutrition-related sensory responsiveness exists. In fact, nurse bees and forager bees display differences in their metabolism. While nurse bees invest in large lipid stores, forager bees show a stable loss resulting in small lipid stores (Ament *et al.*, 2010). Furthermore, the differential nutritional status of nurse bees and foragers may contribute directly to the regulation of the transition process, because worker bees start flying out significantly earlier when their colony is food deprived compared with bees from well-fed colonies (Schulz *et al.*, 1998). Furthermore, the timing of transition between both tasks depends on social and nutritional factors (Toth *et al.*, 2005). It is therefore assumed that nutrition-associated signalling pathways are involved in the regulation of honeybee division of labour (Ament *et al.*, 2010).

Based on our earlier results, we hypothesize that AmForα-PKG protein content and/or PKG activity correlate with different tasks in honeybees. To test this hypothesis, we compared the AmForα-PKG protein content and basal PKG activity in nurse bees and forager bees. Furthermore, we investigated whether PKG has a function in mediating satiety by comparing PKG protein content and basal PKG activity in fed and food-deprived honeybees.

Results

Sucrose responsiveness

Nurse bees and foragers differed significantly in their sucrose responsiveness, with foragers being more responsive. They displayed a significantly higher gustatory response score (GRS; Fig. S1 A: $P < 0.0001$, $Z = -6.102$, Figure S1 B: $P = 0.0012$, $Z = -3.16$, Mann–Whitney U test). Sucrose responsiveness was higher in food-deprived nurse bees (Fig. 1, nurse $P < 0.001$, Mann–Whitney U test) and foragers (Fig. 1, $P = 0.0049$, Mann–Whitney U test) than in fed bees.

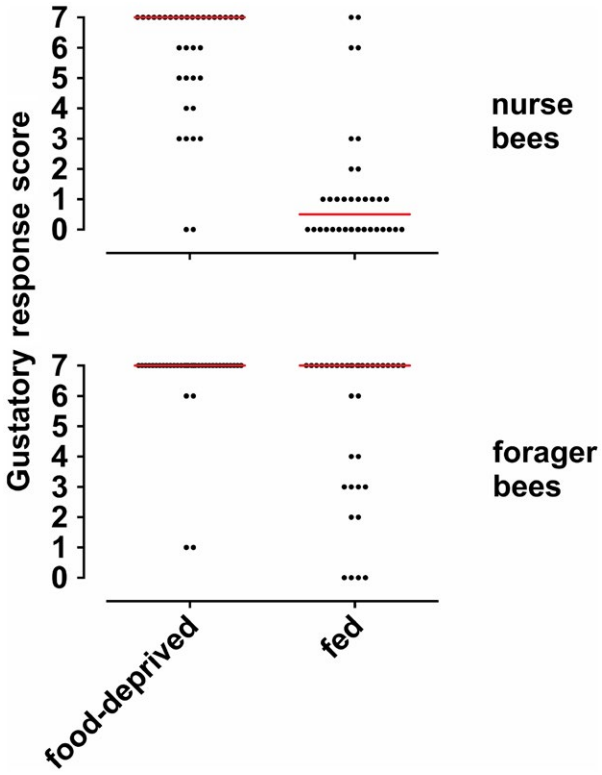

Figure 1. Sucrose responsiveness in fed and food-deprived bees. Sucrose responsiveness in nurse bees and foragers is affected by the feeding status as indicated by significant differences in gustatory response scores. The gustatory response scores are displayed as individual data points (black circles) and medians (red lines). Food-deprived bees were more responsive to sucrose than satiated bees were (nurse bees: $P < 0.001$, forager bees $P = 0{,}0049$, Mann–Whitney U test); 36 individuals were tested in each group. [Colour figure can be viewed at wileyonlinelibrary.com]

AmForα-PKG protein content in nurse and forager bees

AmForα-PKG protein content correlates with the task of the bees. Foragers had a significantly higher AmForα-PKG protein content than nurse bees did in their central brain (CB), optic lobes (OL) and GG (Fig. 2; CB: $t = 2.86$, $n_{\text{nurse bees}} = 9$, $n_{\text{foragers}} = 10$, $P = 0.01$; OL: $t = 2.09$, $n_{\text{nurse bees}} = 9$, $n_{\text{foragers}} = 12$, $P = 0.05$; GG: $t = 2.11$, $n_{\text{nurse bees}} = 10$, $n_{\text{foragers}} = 13$, $P = 0.04$, t-test). Only in their antennal lobes (AL) did foragers not differ in their AmForα-PKG content from nurse bees (Fig. 2; $t = 0.85$, $n_{\text{nurse bees}} = 13$, $n_{\text{foragers}} = 8$, $P = 0.408$, t-test), but they showed the same trend.

AmForα-PKG protein content and satiety

Feeding status did not correlate with AmForα-PKG protein level in the brain. There were no differences in the AmForα-PKG content between food-deprived bees and satiated bees in the CB, OL, GG or AL (Fig. 3; CB: $t = 0.21$, $n_{\text{food-deprived}} = 15$, $n_{\text{fed}} = 15$, $P = 0.83$; OL: $t =$

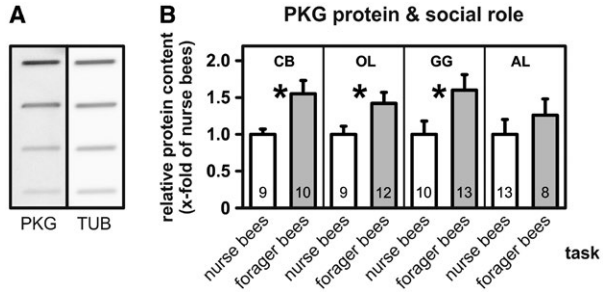

Figure 2. AmForα-PKG protein levels in nurse and forager bee brains. Protein levels of AmForα-PKG were analysed in slot blot measurements. (A) Representative slot blot analysis of the dilution series of the central brain fraction of a nurse bee for Amforα-PKG (left, PKG) and tubulin (right, TUB). (B) Different brain compartments of nurse bees (white) and foragers (grey) were compared: CB, central brain; OL, optic lobes; GG, gnathal ganglion; AL, antennal lobes. AmForα-PKG content is given relative to that of the reference protein tubulin. In each group, levels in nurse bees was set to one. Means and standard errors are displayed. Significant differences between groups are indicated by asterisks (* $P < 0.05$). Number of samples is indicated for each bar.

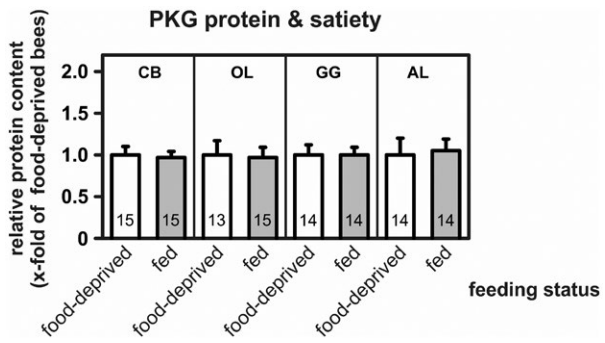

Figure 3. AmForα-PKG protein levels and satiety. Protein levels of AmForα-PKG were analysed in slot blot measurements. Different brain compartments of food-deprived (white) and fed bees (grey) were compared: CB, central brain; OL, optic lobes; GG, gnathal ganglion; AL, antennal lobes. AmForα-PKG content is given relative to that of the reference protein tubulin. In each group, levels in food-deprived bees was set to one. Means and standard errors are displayed. No significant differences were found between groups ($P > 0.05$). Number of samples is indicated for each bar.

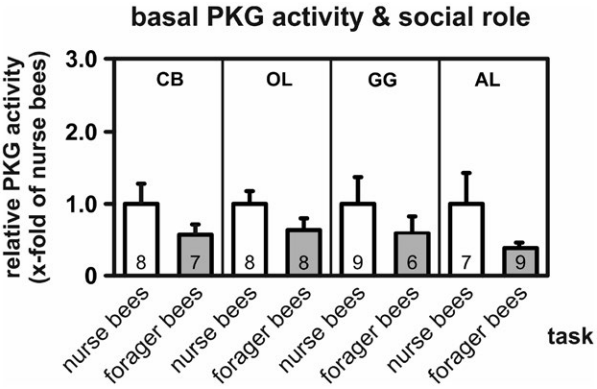

Figure 4. Basal cyclic guanosine monophosphate-dependent protein kinase (PKG) activity in nurse and forager bee brains. Basal PKG activity was measured using phosphotransferase assays in different brain compartments of nurse bees (white) and forager bees (grey): CB, central brain; OL, optic lobes; GG, gnathal ganglion; AL, antennal lobes. In each group, nurse bee PKG activity was set to one. Means and standard errors are displayed. No significant differences were found between groups ($P > 0.05$, Student's *t*-test). Number of samples is indicated for each bar.

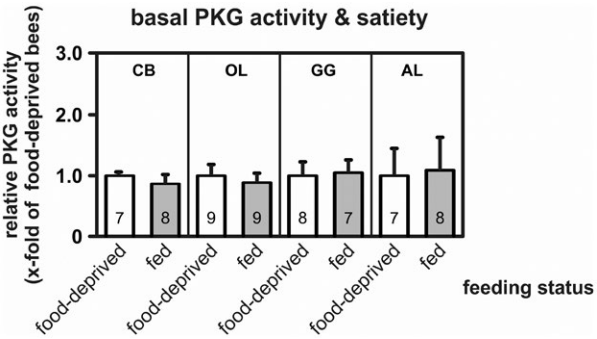

Figure 5. Basal cyclic guanosine monophosphate-dependent protein kinase (PKG) activity and satiety. Basal PKG activity was measured using phosphotransferase assays in different brain compartments of food-deprived (white) and fed bees (gray): CB, central brain;, OL, optic lobes; GG, gnathal ganglion; AL, antennal lobes. In each group, food-deprived bee PKG activity was set to one. Means and standard errors are displayed. No significant differences were found between groups ($P > 0.05$, Student's *t*-test). Number of samples is indicated for each bar.

0.16, $n_{\text{food-deprived}} = 13$, $n_{\text{fed}} = 15$, $P = 0.87$; GG: $t = 0$, $n_{\text{food-deprived}} = 14$, $n_{\text{fed}} = 14$, $P = 0.99$; AL: $t = 0.19$; $n_{\text{food-deprived}} = 14$, $n_{\text{fed}} = 14$, $P = 0.85$; *t*-test).

PKG activity in nurse and forager bees

We further wanted to know whether basal PKG activity correlates with the task of a bee. Basal PKG activity was measured using a radiolabelled phosphotransferase assay without adding an exogenous PKG activator (eg cGMP). To test our assay, we applied different cGMP concentrations and showed that PKG activity increases with increasing cGMP concentrations (Fig. S2). We did not detect any differences in the basal PKG activity between nurse bees and foragers in all brain regions (Fig. 4; CB: $t = 1.29$, $n_{\text{nurse bees}} = 8$, $n_{\text{foragers}} = 7$, $P = 0.22$; OL: $t = 1.49$, $n_{\text{nurse bees}} = 8$, $n_{\text{foragers}} = 8$, $P = 0.16$; GG: $t = 0.99$, $n_{\text{nurse bees}} = 9$, $n_{\text{foragers}} = 6$, $P = 0.24$; AL: $t = 1.59$, $n_{\text{nurse bees}} = 7$, $n_{\text{foragers}} = 9$, $P = 0.14$; *t*-test).

PKG activity and satiety

Feeding status and PKG activity in the different brain regions did not correlate. Food-deprived bees did not differ from satiated bees in their basal PKG activity (Fig. 5; CB: $t = 0.74$, $n_{\text{food-deprived}} = 7$, $n_{\text{fed}} = 8$, $P = 0.47$; OL: $t = 0.45$, $n_{\text{food-deprived}} = 9$, $n_{\text{fed}} = 9$, $P = 0.66$; GG: $t = 0.16$, $n_{\text{food-deprived}} = 8$, $n_{\text{fed}} = 7$, $P = 0.88$; AL: $t = 1.29$, $n_{\text{food-deprived}} = 7$, $n_{\text{fed}} = 8$, $P = 0.89$).

Discussion

Division of labour correlates with AmForα-PKG level but not with basal PKG activity

Our results confirm that sucrose responsiveness correlates with different tasks of honeybees (Fig. S1). Like in earlier studies (Behrends *et al*., 2007, Thamm and Scheiner, 2014; Değirmenci *et al*., 2017, Scheiner *et al*., 2017a; 2017b). Foragers were more responsive to sucrose than nurse bees were, supporting the hypothesis that nutrition-related pathways are possibly involved in the regulation of task allocation. Because the *foraging* gene has been shown to be involved in the regulation of nutrition (Kaun *et al*., 2007; Kent *et al*., 2009), we hypothesized that cGMP-dependent protein kinase, which is encoded by the *foraging* gene, might be involved in nutrition-related signalling and possibly in age-dependent polyethism of honeybees. We expected differences in the AmForα-PKG protein levels of nurse bees and foragers, particularly in neuropils associated with division of labour, ie the mushroom bodies, GG, OL and AL. These neuropils differ in their *Amfor* gene expression between nurse bees and foragers (Thamm and Scheiner, 2014).

Consistent with this hypothesis, we detected significantly higher AmForα-PKG protein levels in the brains of forager bees than in those of nurse bees (Fig. 2). These findings directly support data on *Amfor* messenger RNA expression in these behavioural groups (Ben-Shahar *et al*., 2002; 2003; Thamm and Scheiner, 2014). Among the brain parts with differential PKG protein content are the GG and the CB (including the mushroom bodies), which exhibit high AmForα-PKG protein levels (Thamm and Scheiner, 2014). The GG is directly involved in the processing of gustatory and tactile information received via sensory neurons in the antennae. These neurons project either to the dorsal lobe or to the GG (Brockmann and Robinson, 2007; Haupt, 2007). In the GG, about 40 motoneurons are involved in controlling the proboscis extension response (Rehder, 1989). In the mushroom bodies, several sensory modalities, including gustatory and mechanosensory information, converge (Schröter and Menzel, 2003). Experiments with blowflies suggest that these structures are involved in the feeding threshold determination (Nisimura *et al*., 2005). In addition, activation of PKG increases sucrose responsiveness (Thamm and Scheiner, 2014). Furthermore, PKG protein contents differed in the OL, where visual information is processed. Intriguingly, we did not find any effect of PKG activation on visual responsiveness in earlier experiments (Thamm and Scheiner, 2014), whereas Ben-Shahar *et al.* (2003 showed contradictory results using another assay. But in our previous experiments, bees treated with the PKG activator 8-Br-cGMP went significantly faster (Thamm and Scheiner, 2014), suggesting a role of PKG in locomotion, even though phototaxis was not affected by treatment (Thamm and Scheiner, 2014). These findings suggest that their motivation to move toward a light source may have been reduced by the activation of 8-Br-cGMP and could compensate for the faster locomotor behaviour. Surely, more experiments with local enhancement of PKG activity or knockdown of *Amfor* gene expression in specific brain neuropils are required to determine the function of PKG in vision and visual responsiveness.

Interestingly, although we found higher AmForα-PKG protein levels in forager bee brains than in nurse bee brains, basal PKG activity did not differ between brains of foragers and nurse bees (Fig. 4). Remarkably, PKG is not the only example with inconsistent protein and activity levels. Similar results were reported from protein kinase A (PKA). Here, protein levels were different in the CB between newly emerged bees and 5-day-old bees, whereas basal PKA activity levels were similar (Humphries *et al*., 2003).

Our results suggest that the majority of the differentially expressed AmForα-PKG protein is present in an inactive form and remains in this form when nurse bees turn into forager bees. As we focused on basal differences in PKG activity in this investigation, we did not measure the differences between nurse bees and foragers with respect to stimulus-induced PKG activation. Differing amounts of enzymes, however, can considerably affect their activation characteristics. In bees with a high PKG amount within cells (ie foragers), a transient stimulus-induced elevation of cGMP would reach more enzyme molecules and thus activate more PKG within the critical time window, thus making these animals more sensitive to the stimulus (eg sucrose). The next obvious step is to measure PKG activation in the brains of nurse bees and foragers following stimulation with sugar water or compounds that release or inhibit foraging behaviour.

Critical for PKG activation is cGMP, which can be provided by soluble guanylyl cyclases via nitric oxide (NO) signalling (Katsuki *et al*., 1977; Friebe and Koesling, 2003). The distribution of NO synthase (Watanabe *et al*., 2007) and NO synthase activity (Müller, 1997) in the same brain areas as AmForα-PKG (Thamm and Scheiner, 2014) indicates that AmForα-PKG activation via this pathway is conceivable. Which kind of guanylyl cyclases are expressed at certain time points in the honeybee brain and thus may be necessary for PKG signalling must be examined in future studies. In addition to the direct activation via cGMP, other mechanisms, like post-translational modifications, possibly may be required to convert AmForα-PKG into its active form. For instance, proteolytic cleavage was shown to be necessary to obtain a PKG protein that lacks its regulatory domain and is thus insensitive for cGMP and translocates to the nucleus (Sugiura *et al*., 2008). Moreover, to strongly reduce the possibility that other

kinase enzymes interfere with our phosphotransferase assay, we inhibited the most likely candidate PKA (see Experimental Procedures), because this enzyme can be activated not only by cAMP but also by cGMP (Leboulle and Müller, 2004). Furthermore, we do not think that we measured activity from another PKG than AmForα-PKG. Indeed, the honeybee genome harbours an additional gene that encodes for cGMP-dependent protein kinase (Gene ID: 551714; https://www.ncbi.nlm.nih.gov/gene), but we did not find evidence that this gene is expressed in the brain (Fig. S3).

Our results indicate that a putative link between PKG signalling, gustatory responsiveness and polyethism is highly complex. Although we found correlations of sucrose responsiveness and PKG protein amount in brains of bees performing different tasks, we were unable to resolve a precise function of PKG in mediating division of labour, since PKG activity does not correlate with behavioural differences.

Satiation correlates with gustatory responsiveness but not with AmForα-PKG protein content and basal AmForα-PKG activity

In *Caenorhabditis elegans*, PKG was shown to be involved in the mediation of starvation (You *et al*., 2008). PKG loss-of-functions-mutants, for example, never stopped feeding. We asked whether PKG would have a similar function in the honeybee and compared PKG content in satiated and food-deprived bees, since changes in nutritional status are hypothesized to be involved in regulating the transition from nursing to foraging (Schulz *et al*., 1998; Ament *et al*., 2010). Nurse bees and forager bees strongly differ in their stored amounts of triglycerides, in their metabolism and in their gustatory responsiveness (Scheiner *et al*., 2001; 2001; Toth and Robinson, 2005; Thamm and Scheiner, 2014). Satiation or starvation strongly affects individual gustatory responsiveness, which, in turn, can be modulated by activation of PKG (Thamm and Scheiner, 2014). Starvation for the duration of 1 h already makes bees more responsive to sucrose independent of their task (Fig. 1). However, this effect is much stronger in nurse bees than in forager bees. We therefore expected differential AmForα-PKG protein levels between satiated and food-deprived bees, particularly in neuropils associated with taste perception and mediation of starvation, ie the GG and the CB (Haupt, 2007; Marella *et al*., 2012; Tsao *et al*., 2018).

In contrast to our expectation, we did not detect a correlation between starvation and PKG signalling. Neither AmForα-PKG protein content nor AmForα-PKG activity were different in starved and satiated bees in our experiments (Figs 3 and 5). This is partially in contrast to findings from *Drosophila*. Here, a link between PKG activity and sucrose responsiveness was demonstrated. Rover flies, which have a higher sucrose responsiveness than sitter flies when starved for 2 or 24 h (Scheiner *et al*., 2004), also exhibited a higher brain PKG activity (Belay *et al*., 2007). Furthermore, increasing PKG activity in flies of a sitter background enhances sucrose responsiveness (Belay *et al*., 2007). These results indicate that the molecular pathways involved in the mediation of individual feeding state can differ grossly between various insects, such as flies and bees.

PKG signalling was shown to have several important functions in animal behaviour, including aggressive and sexual behaviour in mammals (for a review, see Hofmann *et al*., 2006), food-searching behaviour in insects and mediation of nutritional status in *C. elegans*. In social insects, this enzyme furthermore seems to have a pivotal role in the modulation of the transition between different behavioural states (Pereira and Sokolowski, 1993; Fujiwara *et al*., 2002; Ingram *et al*., 2005; Garabagi *et al*., 2008; Lucas *et al*., 2010; Tobback *et al*., 2011). Our results provide evidence that PKG protein content but not PKG enzyme activity correlates with the nurse–forager transition in honeybees, which seems to be independent of nutritional state.

Experimental Procedures

Animals

European honeybees (*A. mellifera carnica*) were collected from our departmental apiary at the University of Würzburg. Nurse bees were identified as bees sticking their heads into brood cells containing larvae for at least 10 s (Değirmenci *et al*., 2017; Scheiner *et al*., 2017a; 2017b). Foragers were identified by huge pollen loads at their hind legs and were caught when returning to the nest entrance.

Quantification of gustatory responsiveness

Bees were immobilized on ice immediately after collection and harnessed in small holders (Scheiner *et al*., 2013). For the 'nurse vs. foragers experiment', individuals were fed with 10 μl of a 30% sucrose solution. For the experiment 'hungry vs. satiated', the satiated groups were fed *ad libitum* with 30% sucrose solution until the bees pulled in their probosces. Gustatory responsiveness was quantified by presenting sequentially water and a series of sucrose concentrations (0.1, 0.3, 1, 3, 10 and 30% w/v) to both antennae of each bee (for details, see Scheiner *et al*., 2013). The sum of proboscis extension responses to the stimulations with water and six different sucrose concentrations constitutes the GRS of a bee (Scheiner *et al*., 2001; 2003; 2004; 2014). It serves as a measure for its gustatory responsiveness.

Microdissection of brain tissues

Fixed head capsules were opened by cutting a hole between the ocelli, the eyes and antennae. Trachea and glands were removed. Afterwards, whole brains were excavated and separated into the four major regions: CB (mainly consisting of the mushroom bodies, but also involving other parts, like the central complex), OL, AL and GG (suboesophageal ganglion, nomenclature according to Ito *et al*., 2014). For slot blot measurements, tissue samples of two bees of the same group that showed the same GRS were pooled.

AmForα-PKG protein content measurements

Tissue samples were homogenized in 35 µl of cold phosphate-buffered saline: 140 mM sodium chloride (NaCl), 2.7 mM potassium chloride, 10 mM sodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.3. A 5 µl sample of each protein was used to quantify the protein concentration using Bradford measurements. For each brain region, the protein concentrations were adjusted to the value of the sample with the lowest amount. Serial 1 : 2 dilutions of each sample were prepared using phosphate-buffered saline containing methanol (20% v/v) and sodium dodecyl sulphate (0.5% v/v). Afterwards, these were transferred on a polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany) using a slot blot machine (48-well; SCIE-PLAS, Cambourne, UK). Subsequently, blots were blocked 30 min with 5% dried milk (AppliChem GmbH, Darmstadt, Germany) in Tween buffer [10 mM tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl), 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5]. Incubation together with primary antibodies against AmFORα-PKG (1 : 660, rabbit anti-AmFORα-PKG; see Thamm and Scheiner, 2014 and Fig. S4) or tubulin (1 : 6600, mouse anti-tubulin DM1A, T9026; Sigma-Aldrich, St Louis, MO, USA) were applied for 60 min at room temperature. Afterwards, blots were washed: three times for 5 min with Tween buffer, 3 min urea buffer [2 M urea, 0.1 M glycine, 1% (v/v) Triton X-100], 5 min in Tween buffer. After 60 min incubation with peroxidase-conjugated secondary antibodies (1 : 6600, goat anti-rabbit, 111-035-003 or goat anti-mouse, 115-035-003; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) blots were washed again: three times for 5 min with Tween buffer, short rinsing with double-distilled water. Then, the blots were incubated with a mixture (1 : 1) of enhanced chemiluminescence (ECL) solution 1 [2.5 mM luminol, 0.4 mM coumaric acid, 0.1 M Tris, pH 8.5] and ECL solution 2 [0.02% (v/v) hydrogen peroxide, 0.1 M Tris, pH 8.5] for 5 min. Finally, the binding of secondary antibodies was visualized using an ECL Chemocam Imager (Intas Science Imaging Instruments GmbH, Göttingen, Germany) with an exposure time of 3 min and 1 × 1 pixel binning. AmForα-PKG content was determined as a function of the slope of the levels of grey and normalized using the appropriate tubulin level.

PKG activity measurements

The basal catalytic activity of PKG in homogenates of honeybee brain tissues was measured using a [γ-^{32}P]-adenosine-5-triphosphate (ATP) kinase assay (Wolfertstetter *et al*., 2015). Tissues were microdissected, homogenized in 100 µl (CB, OL) or 40 µl (GG, AL) of extraction buffer (20 mM Tris-HCl, 100 mM NaCl, pH 8.0) and immediately frozen in liquid nitrogen. Homogenates were stored at −80 °C until use. From each sample, 10 µl was used for protein amount quantification using a Bradford assay. By adding 20 µl of the homogenate to 80 µl of the reaction mixture [50 mM 2-[*N*-morpholino)ethanesulphonic acid, 0.4 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N,N*-tetraacetic acid, 1 mM magnesium acetate, 10 mM NaCl, 0.1% (w/v) bovine serum albumin, 10 mM dithiothreitol, 40 µM substrate peptide VASPtide (sequence: RRKVSKQE), 2 mM cAK-inhibitor peptide, 0.1 mM [γ-^{32}P]-ATP (100 cpm/pmol), pH 6.9] the kinase reaction was started. Phosphorylation via PKA was inhibited by using the cAK-inhibitor peptide (AS5-24), which is a potent inhibitor of cAMP-dependent protein kinases (Cheng *et al*., 1986). The reaction was carried out at 30 °C for 5 min. Afterwards, 50 µl of the reaction mixture were transferred to Whatman P-81 filter papers (Sigma-Aldrich, Darmstadt, Germany) which then were immediately transferred into 75 mM phosphoric acid (H_3PO_4). After additionally washing for three times for 2 min in 75 mM H_3PO_4 and for one time for 5 min in acetone (100% v/v), the filter papers were dried and transferred in 10 ml Rotiscint scintillation liquid (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Counts per minute (cpm) were measured using a β-counter (Tri Carb 2800TR Liquid Scintillation Analyzer, Perkin Elmer, Rodgau, Germany). Individual bees were measured in duplicate, and PKG activity was calculated from corrected cpm (subtract individual cpm from values of control samples without addition of brain lysate) per microgram of protein.

Statistical Analysis

GSRs were compared using Mann–Whitney *U*-tests, because data were not distributed normally. PKG protein levels and basal PKG activity were compared between different groups using two-tailed *t*-tests, because data were distributed normally. Statistics were performed with SPSS 22 (IBM, Armonk, NY, USA).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Sucrose responsiveness in honeybee workers. GRS were measured in nurse bees and foragers. Medians (red line) and individual data points are displayed. Foragers are significantly more responsive to sucrose than nurse bees. A: PKG content experiment. ***$P < 0.001$; $Z = -6.10$; Mann-Whitney U test. Number of bees tested: nurse bees = 87; foragers = 88. B: PKG activity experiment. **$P = 0.002$; $Z = -3.16$; Mann-Whitney U test. Number of bees tested: nurse bees = 22; foragers = 22.

Figure S2. Activation of PKG by cGMP. Example graph out of three independent experiments in which honeybee brain lysate was treated with increasing concentrations of cGMP. Half maximal stimulation was achieved at 1.5 μM ($\log EC_{50} = -5.825 \pm 0.1605$). Data were expressed as x-fold activity when stimulated with 10^{-3} M cGMP. Error bars indicate mean ± SEM of three independent experiments.

Figure S3. PCR experiment to investigate mRNA expression of two PKG genes in the honeybee brain. A) Using brain cDNA as a template, expression of *Amfor*, *Amef1α*, and *Amrpl32* was confirmed. For the second PKG gene, no PCR products appear, indicating that this gene is not expressed in the brain. Primer sequences: *Amfor*: forward 5′-ggAATCgACgCTATAgAATTCCCTAg-3′, reverse 5′-AATTAAAACCATCgAACCATTTgTgT-3′; *AmPKGII*: forward 5′-GAGTCGATTTATTATCGCTTGC-3′, reverse 5′-GCTTGAAAGGGTGTTTTATTTTC-3′; *Amef1α*: forward: 5′-gAACATTTCTgTgAAAgAgTTgAggC-3′, reverse: 5′-TTTAAAggTgACACTCTTAATgACgC-3′; *Amrpl32*: forward:5′-AgTAAATTAAAgAgAAACTggCgTAA-3′, reverse:5′-TAAAACTTCCAgTTCCTTgACATTAT-3′. B) Control PCR with the *AmPKGII* primers on gDNA. The resulting PCR product corresponds to a 398 bp genomic fragment of the second PKG gene. Sequencing results on the right: white letters/black background: primer binding sites, black uppercase letters: exon, grey lowercase letters: intron.

Figure S4. Western blot analysis. Serial dilutions of honeybee brain homogenates were analysed in Western blotting using the antibody against AmFORα-PKG (1:660) or tubulin (1:6,600). For Western blotting methodology see Thamm and Scheiner (2014).