

## In Search of Food: Exploring the Evolutionary Link Between cGMP-Dependent Protein Kinase (PKG) and Behaviour<sup>1</sup>

MARK J. FITZPATRICK AND MARLA B. SOKOLOWSKI<sup>2</sup>

Department of Biology, University of Toronto at Mississauga, 3359 Mississauga Rd. N, Mississauga, Ontario L5L 1C6, Canada

**SYNOPSIS.** Despite an immense amount of variation in organisms throughout the animal kingdom many of their genes show substantial conservation in DNA sequence and protein function. Here we explore the potential for a conserved evolutionary relationship between genes and their behavioural phenotypes. We investigate the evolutionary history of cGMP-dependent protein kinase (PKG) and its possible conserved function in food-related behaviours. First identified for its role in the foraging behaviour of fruit flies, the PKG encoded by the *foraging* gene has since been associated with the maturation of behaviour (from nurse to forager) in honey bees and the roaming and dwelling food-related locomotion in nematodes. These parallels encouraged us to construct protein phylogenies using 32 PKG sequences that include 19 species. Our analyses suggest five possible evolutionary histories that can explain the apparent conserved link between PKG and behaviour in fruit flies, honey bees and nematodes. Three of these raise the hypothesis that PKG influences the food-related behaviours of a wide variety of animals including vertebrates. Moreover, it appears that the PKG gene was duplicated some time between the evolution of nematodes and a common ancestor of vertebrates and insects whereby current evidence suggests only the *for*-like PKG might be associated with food-related behaviour.

Both the sequence and function of many genes are conserved across the animal kingdom. This suggests that the functions of these genes are maintained despite a wide array of selection pressures experienced by different organisms. Many of these highly conserved genes are “vital” and often play crucial roles in development. As a result, mutations that strongly affect the function of their proteins are rarely propagated. Recent advances in the field of evolutionary developmental biology (evo-devo) have revealed that the now famous homeotic gene complex (Hox), which controls bilateral symmetry during development, is indispensable for the growth of most animals (Carroll, 1995; Gellon and McGinnis, 1998; Callaerts *et al.*, 2002). The conserved employment of Hox genes suggests an ancient evolution for the role of this gene family in development. Conservation of gene function on this grandiose scale is becoming generally accepted for some developmental genes but is this conservation also true for genes that influence behaviour?

Genes have been identified that affect behavioural traits such as foraging, courtship, learning and memory, and circadian rhythms (reviewed in Wahlsten, 1999; Baker *et al.*, 2001; Sokolowski, 2001; Bucan and Abel, 2002; Peixoto, 2002; Rankin, 2002). Most behaviour genetic analyses have been conducted on organisms with extensive genomic resources such as the nematode (*Caenorhabditis elegans*), the fruit fly (*Drosophila melanogaster*), and the mouse (*Mus musculus*). Research from model genetic organisms can inform us of candidate genes to be investigated in additional organisms. One requirement of this “candidate gene” approach is that the gene of interest, and its

function, is conserved across a number of species. The candidate gene approach is valuable when the gene’s function is conserved across model and non-model organisms. Indeed, it is important to perform a comparative analysis of gene function in behaviour since model genetic organisms alone do not adequately represent the diversity of species studied by animal behaviour researchers. Below we review examples of behaviours known to have a common genetic basis in multiple animals.

The *period* (*per*) gene first identified in the fruit fly *D. melanogaster* (Konopka and Benzer, 1971) has conserved function. Mutations in *per* cause various alterations in rhythmic behaviour (*e.g.*, aberrant circadian locomotion activity rhythms) when compared to the approximate 24-hour activity rhythms of normal flies. For example, the short mutant (*per<sup>s</sup>*) has a reduced period (19 hr), the long mutant (*per<sup>l</sup>*) has an extended period (28 hr), and *per<sup>o</sup>* mutants exhibit arrhythmic behaviour patterns. The insertion of a *per* transgene isolated from the house fly, *Musca domestica*, can restore normal circadian rhythmicity in *per* mutant fruit flies (Piccin *et al.*, 2000). *per* is also involved in the circadian behaviour of species such as mice (Zheng *et al.*, 1999), humans (Toh *et al.*, 2001), honey bees (Toma *et al.*, 2000; Bloch *et al.*, 2001), melon flies (Miyatake *et al.*, 2002), and tephritid flies (An *et al.*, 2002). Interesting evidence for the conservation of *per*’s role in behaviour also comes from human research. Toh *et al.* (2001) identified the genetic mutation contributing to a familial advanced sleep phase syndrome (FASPS). Individuals suffering from this affliction consistently fall asleep in the early evening and wake in the early hours of the morning regardless of their work schedule and life’s infringements. The authors use linkage analysis to identify the gene segregating in a family associated with this syndrome and

<sup>1</sup> From the Symposium *Recent Developments in Neurobiology* presented at the Annual Meeting of the Society for Integrative and Comparative Biology, 4–8 January 2003, at Toronto, Canada.

<sup>2</sup> E-mail: msokolow@utm.utoronto.ca

the FASPS phenotype was mapped to one of the human *period* genes (*hPer2*). Intriguingly, the mutation in *hPer2* is a single base change causing the substitution of serine with glycine at position 2106. Thus, this mutation in the human *per* homolog causes a phenotype that is strikingly similar to that of the *per<sup>s</sup>* mutant flies, implying a conserved structure and function.

Another example evaluates the functional significance of species-specific differences within a single gene. The gene *no-on-transient A* (*nonA*) is involved in the vision and male courtship song of *D. melanogaster* (see references in Sandrelli *et al.*, 2001). Transplanting the *nonA* gene from the related species *D. virilis* into *D. melanogaster* restores the visual phenotype of *nonA* demonstrating a conserved function for this gene in the vision of both species (Campesan *et al.*, 2001). Although the courtship song sung by these transgenic males resembles *D. melanogaster*, it also includes some characteristics of *D. virilis* song. Therefore, a single gene from one species (*D. virilis*) induces species-specific components of its courtship song in a second species (*D. melanogaster*) despite many underlying genetic differences between the two species. These results suggest that *nonA* serves similar functions in both species (vision and courtship song) and also conveys species-specific information in the song. The conservation of *nonA* function in other organisms remains to be determined.

Recent reports suggest that *foraging* (*for*) which encodes a cyclic GMP-dependent kinase (PKG), may have a conserved role in the food-related behaviours of various animals (briefly reviewed in Sokolowski, 1998, 2002; Schafer, 2002). Specifically, homologs of the PKG encoded by the *for* gene have been linked to food-related behaviours in fruit flies (Osborne *et al.*, 1997), honey bees (Ben-Shahar *et al.*, 2002), and nematodes (Fujiwara *et al.*, 2002). Below we introduce the known conserved functionality of PKG in the three aforementioned species. This is followed by evolutionary inferences using phylogenies constructed from various PKG proteins. We suggest PKG as a candidate for researchers interested in studying food-related behaviours in a variety of organisms.

#### PKG AND FOOD-RELATED BEHAVIOUR

##### *Fruit flies*

Sokolowski (1980) discovered that fruit fly larvae in nature exhibit one of two foraging strategies. "Rover" larvae travel long distances while feeding and often leave a food source in search of another. Conversely, "sitter" larvae travel shorter distances and remain on a food source once it is encountered. These behavioural differences reflect foraging and not general locomotion since rovers and sitters move similar distances in the absence of food. Adult flies are also dimorphic in their foraging behaviour since rovers walk further after eating than sitters (Pereira and Sokolowski, 1993). Differences in rover and sitter behaviour are attributable to a single major gene called

*foraging* (*for*) that is located on the 2nd chromosome at polytene position 24A3–5 (de Belle *et al.*, 1989). The rover allele (*for<sup>R</sup>*) is genetically dominant to the sitter allele (*for<sup>S</sup>*) (de Belle and Sokolowski, 1987). Both rovers and sitters are found in natural populations such that phenotypic variation is typically 70% rover (*for<sup>R</sup>* homozygotes and heterozygotes) and 30% sitter (*for<sup>S</sup>* homozygotes) (Sokolowski, 1980; Sokolowski *et al.*, 1997). Sokolowski *et al.* (1997) provide evidence for the evolution of the rover/sitter behavioural polymorphism via density-dependent selection. Rovers are favoured in high density environments and sitters are favoured in low density environments. During molecular characterization and cloning, Osborne *et al.* (1997) discovered that *for* is analogous to a previously described gene called *dg2* which encodes one of two PKG genes in *D. melanogaster* (Kalderon and Rubin, 1989). *for* is a large gene (approximately 40 kb of genomic DNA) and is alternatively spliced into three major transcripts (*for* T1, T2, & T3). A cGMP binding domain and a kinase domain are common to all transcripts of *for* such that the 5' regions make the transcripts unique (Kalderon and Rubin, 1989; Osborne *et al.*, 1997). The T1 and T3 transcripts contain dimerization and regulatory domains which are absent in T2. Rovers typically have higher transcript abundance and PKG activity levels relative to sitters (Osborne *et al.*, 1997). Using transgenic flies, sitters can be turned into rovers by providing more PKG (Osborne *et al.*, 1997). The spatial and temporal requirements for the expression of *for* in rover and sitter behaviour are currently under investigation. In summary, naturally occurring variation in the foraging behaviour of fruit flies can be explained by genetic variation at the *for* locus.

##### *Honey bees*

Ben-Shahar *et al.* (2002; 2003) extended the findings of Sokolowski and colleagues to the honey bee (*Apis mellifera*) by demonstrating a role for PKG in the behavioural transition from nurse to forager. Nurse bees, which distribute food within the hive, have lower PKG activity levels and lower abundance of *Amfor* RNA (the honey bee ortholog of *for*). This is similar to sitter fruit flies that forage close to home. In contrast, forager bees, which leave the hive in search of food, have higher PKG activity levels and higher RNA abundance much like rover flies that forage by moving from food patch to food patch. Nurses and foragers also differ in phototactic behaviour where nurses are negatively phototactic (avoid light) and foragers are positively phototactic (attracted to light) (Ben-Shahar *et al.*, 2003). Aside from their different tasks, nurses also differ from foragers with respect to their age. Nurses are much younger than foragers. However, the differences in RNA abundance and PKG activity leading to changes in division of labour are not age-dependent (Ben-Shahar *et al.*, 2002). Using single cohort colonies, nurse-aged bees can be induced to forage at an earlier age than normal (precocious foragers) and subsequently show an increase in RNA abundance and

PKG activity. Therefore, nurses and precocious foragers are the same age yet they have different levels of PKG activity and RNA abundance. This demonstrates a correlative relationship between PKG and a honey bee's task in the hive (nurse or forager).

To demonstrate a causal relationship between PKG and task, young bees fed 8-Br-cGMP, an activator of PKG, began to forage (Ben-Shahar *et al.*, 2002). The proportion of foragers is dependent on the dose of 8-Br-cGMP fed. This feeding treatment increases PKG activity levels showing that the change in behaviour is a PKG-specific phenomenon (control treatments with a cAMP analogue have no effect on the percentage of foragers or on PKG activity levels). In the honey bee head, *Amfor* is found in the optic lobes and mushroom bodies. Mushroom body expression is found primarily in certain Kenyon cell fibres known to play a role in honey bee vision.

The work of Ben-Shahar *et al.* (2002) offers two important contributions: 1) it shows that candidate genes from model organisms can be used to probe the genetic underpinnings of behavioural plasticity in additional organisms, and 2) it reveals that *for* is used differently in fruit flies than in honey bees. In the fly, rover and sitter behaviour arises from allelic variation in *for* likely maintained as a balanced polymorphism in natural populations. In the honey bee, *Amfor* is upregulated causing the transition from nurse to forager. Consequently, *for* is involved in two mechanisms of PKG-regulated behaviour—genetic variation in fruit flies and changes in gene regulation leading to behavioural plasticity in the honey bee. Despite potentially different modes of gene action, the *for* gene has a conserved role in food-related behaviours in both species. Evolutionary changes in gene regulation are widely recognized in developmental biology as causing major shifts in animal body plan and morphology (reviewed by Carroll, 2000). In a recent review, Robinson and Ben-Shahar (2002) suggest that changes in gene regulation might be involved in the evolution of social behaviour across the animal kingdom. This is supported by evidence showing that alterations in the regulation of *Amfor* result in the behavioural transition from nurse to forager (Ben-Shahar *et al.*, 2002). Whether functional genetic variation in *Amfor* in honey bees exists or whether *for* is differentially regulated during foraging across the life history of the fruit fly are both unknown.

### Nematodes

The PKG molecule encoded by the gene *egl-4* has recently been shown to influence food-related behaviours in the nematode worm, *C. elegans*. Nematode locomotion is categorized into two types: roaming and dwelling. Roaming is defined by long distances of uninterrupted locomotion and dwelling involves short distances and frequent stops. Using *egl-4* knock outs, Fujiwara *et al.* (2002) show that decreasing PKG causes an increase in roaming behaviour on food. The roamer and dweller phenotypes are reminiscent of ro-

ver and sitter fruit flies and nurse and forager honey bees. However in *C. elegans*, mutational analysis suggests that less PKG causes more roaming than dwelling. Hence, the regulation of foraging behaviour in fruit flies, honey bees, and nematodes differs, yet PKG weaves a common thread through these organisms. In a screen for suppressors of locomotion, the authors discover that reductions in roaming behaviour are correlated with a decrease in cilium structure leading to the hypothesis that sensory perception influenced by ciliated sensory neurons may be regulating the transition between roaming and dwelling. In another paper, *egl-4* is uncovered as a suppressor of mutations that affect deficiencies in olfactory adaptation behaviour in *C. elegans* (L'Etoile *et al.*, 2002). Here, mutants in *egl-4* which have reduced PKG expression show defects in the long-term regulation of olfactory behaviours. Specifically, olfactory adaptation involves pre-exposing the animals to an odour for one hour prior to testing olfactory behaviour (L'Etoile and Bargmann, 2000). Where normal worms respond by showing reduced chemotaxis to the adapted odour, *egl-4* mutants are indistinguishable from their pre-adaptation scores. Phosphorylation of a cGMP-gated ion channel encoded by *tax-2* partly rescues olfactory adaptation behaviour. Inserting a serine at position 727 of TAX-2 causes aberrant behaviour whereas changing the serine to alanine results in a full rescue of normal olfactory adaptation behaviour (L'Etoile and Bargmann, 2000). These mutational analyses in *C. elegans* have identified new functions for PKG.

There also exists a natural polymorphism in the feeding behaviour of *C. elegans*. Some individuals form aggregations while feeding (“social”) and others remain solitary (de Bono and Bargmann, 1998). Moreover, aggregating strains move slowly when feeding (like sitter flies) whereas strains that are solitary move faster (like rover flies). As with fruit flies, this behaviour is dependent on the presence of food. These naturally occurring behavioural differences are attributed to variation in the gene *npr-1*, which encodes a G protein-coupled receptor similar to the neuropeptide Y (NPY) receptors found in mammals. Natural aggregating strains differ from solitary strains by only a single amino acid at position 215 of NPR-1. Aggregating strains have a phenylalanine (NPR-1 215F) and solitary strains have a valine (NPR-1 215V). Insertion of an NPR-1 215V transgene into aggregating strains causes them to behave like solitary strains. By aligning the *C. elegans* NPR-1 protein sequence with three related *Caenorhabditis* species, it appears as though the phenylalanine is the ancestral state (Rogers *et al.*, 2003). Recent evidence suggests that solitary feeding is a result of the inhibition of aggregate feeding (Coates and de Bono, 2002). Expressed in neurons, the solitary NPR-1 215V isoform is thought to antagonize a cGMP-gated ion channel encoded by the genes *tax-2* and *tax-4*. Like mammalian NPY, nematode NPR is involved in the regulation of food related behaviours via the suppression of various neurons (Cowley *et al.*,

2001). de Bono *et al.* (2002) provide additional evidence regarding the neural mechanism contributing to this behaviour by demonstrating that ablation of the nociceptive neurons ASH and ADL in aggregating individuals causes them to remain solitary. Nociceptive neurons are involved in stress response and are hypothesized to induce the aggregate feeding behaviour when detecting toxic chemicals. Food-related behaviours in *C. elegans* involve aggregate *vs.* solitary feeding and roaming *vs.* dwelling. The former is regulated by a neuropeptide-Y-like receptor and the latter is influenced by PKG. It is not known whether *npr-1* and *egl-4* interact to affect foraging behaviour in *C. elegans* or whether *npf*, the *npv* homologue in fruit flies, known to play a role in feeding behaviour (Shen and Cai, 2001), interacts with *for* to affect rover and sitter behaviour.

#### THE cGMP-DEPENDENT PROTEIN KINASE

The cyclic GMP-dependent serine/threonine protein kinase is activated by cGMP (Butt *et al.*, 1993). The kinase region of PKG is very similar to that in PKA (a protein kinase mediated by cAMP). Much is known about the neural and genetic function of PKA (reviewed in Taylor *et al.*, 1990) whereas the role of PKG is much less resolved. This is thought, in part, to be due to the fact that cGMP is 10 to 100 times less abundant than cAMP making it more difficult to study (see Wang and Robinson, 1997). In general, the PKG protein consists of an N-terminal dimerization domain, a regulatory domain, one or two cGMP binding domains, and a kinase domain that contains an ATP binding site and a catalytic site. Mammals possess two PKG-encoding genes: type I (cGKI) and type II (cGKII). Although the kinase domains of these proteins are very similar, there are differences in the amino terminal residues (reviewed in Lohmann *et al.*, 1997). cGKI is soluble and is generally found in the brain along with smooth muscle cells, cardiac cells, endothelial cells, and lymphocytes with high expression in the Purkinje cells. In addition, many vertebrates contain two isoforms of cGKI— $\alpha$  and  $\beta$ . cGKII is membrane bound (insoluble) and is found in the intestinal mucosa, kidney, bone, and brain with a different brain expression patterns than cGKI. Mice lacking cGKII are dwarfed (Pfeifer *et al.*, 1996). Mice lacking cGKI suffer from vascular and gastric problems (Pfeifer *et al.*, 1998; Ny *et al.*, 2000), erectile dysfunction (Hedlund *et al.*, 2000), urinary problems (Persson *et al.*, 2000), and heart problems (Wegener *et al.*, 2002).

PKG genes have been isolated from various animals spanning a wide variety of taxa ranging from humans (Sandberg *et al.*, 1989) to even the malaria-causing protozoan *Plasmodium falciparum* (Gurnett *et al.*, 2002). Given the recent availability of these and other PKG sequences we decided to use phylogenetic analyses to explore the relationship between PKG and food-related behaviours. Even though at present we can only map the food-related behaviours of three spe-

cies onto the phylogeny, our results generate hypotheses for future research avenues.

#### EVOLUTIONARY ROLE OF PKG IN BEHAVIOUR

##### Methods

Using multiple search methods (BLASTp, t-BLASTx, and nomenclature searches) we obtained 32 PKG protein sequences nested within 19 species available as of March 2003 (Table 1). Protein sequence alignments from the conserved kinase domains and remaining carboxyl terminal residues (approximately 350 amino acids) were made using the default settings of CLUSTALX followed by minor editing and verification by hand (Higgins and Sharp, 1988; Thompson *et al.*, 1997). Kinase domains were determined with reference to that of the *D. melanogaster* FOR protein (also known as DG2; see Kalderon and Rubin, 1989). Evolutionary relationships were determined using phylogenetic distance methods. Neighbour joining trees (Saitou and Nei, 1987) were constructed in MEGA v 3.0 (Kumar *et al.*, 2001) using Amino: Poisson correction treating gaps as complete deletions. We also performed 5000 bootstrap replications to determine support for the nodes. Bootstrapping is a resampling technique that is used to estimate confidence for the placement of nodes in phylogenetic trees (Page and Holmes, 1998). Bootstrap values range from 0 to 100 where higher values indicate low sampling error and therefore higher support for those nodes.

#### RESULTS AND DISCUSSION

The resultant phylogeny is shown in Figure 1. There exist two major clades. The first contains protozoans (Apicomplexa) and the green alga (Viridiplantae) The second clade contains the metazoans ranging from hydra to humans and includes the three organisms known to use PKG in their food-related behaviours. This same topology was recently recovered in a large kingdom-level phylogeny constructed using combined protein sequences (Beldauf *et al.*, 2000). In order to better resolve the metazoan clade, we removed both the Apicomplexa and Viridiplantae and constructed a new alignment. A tree with similar topology as in Figure 1 was recovered and the bootstrap support values were generally increased (Fig. 2). Within the metazoans included in our tree, the PKG from hydra is the most ancestral followed by *C. elegans*. The remaining sequences (insects and vertebrates) together form a large clade that is itself separated into two subclades. This suggests that an ancestral PKG gene was duplicated some time following the evolution of *C. elegans* and before the radiation of insects and vertebrates (Jarchau *et al.*, 1994; this paper). Within each clade of duplicates there is a separation between the insects and vertebrates. All of the vertebrate cGKI sequences are most closely related to each other followed by the insect sequences including AMFOR from the honey bee and FOR from the fruit fly—both of which are implicated in behaviour. The vertebrate cGKII sequences group together and include DG1 from the fruit fly along with

TABLE 1. Information on all PKG sequences used in this analysis.

Common name <sup>a</sup>	Scientific name	Information and gene name <sup>a</sup>	GenBank accession number <sup>b</sup>
fruit fly	<i>Drosophila melanogaster</i>	PKG ( <i>for</i> -T1; <i>dg2</i> -T1)	FBpp0000504 <sup>c</sup>
fruit fly	<i>Drosophila melanogaster</i>	PKG G ( <i>dg1</i> )	M27114
honey bee	<i>Apis mellifera</i>	PKG ( <i>Amfor</i> )	AAL93136
mosquito	<i>Anopheles gambiae</i>	PKG (ebiP6403) (predicted type I)	EAA14900
mosquito	<i>Anopheles gambiae</i>	PKG (agCG54791) (predicted type II)	EAA10189
silkworm moth	<i>Bombyx mori</i>	PKG ( <i>PKG-Iα</i> )	AAL76256
silkworm moth	<i>Bombyx mori</i>	PKG ( <i>PKG-Iβ</i> )	AAL76255
silkworm moth	<i>Bombyx mori</i>	PKG ( <i>PKG-II</i> )	AAL76257
nematode worm	<i>Caenorhabditis elegans</i>	PKG ( <i>egl-4</i> ; <i>cgk-1</i> ), isoform a	AAD36954
human	<i>Homo sapiens</i>	PKG, type Iα ( <i>cgk-1α</i> )	Q13976
human	<i>Homo sapiens</i>	PKG, type Iβ ( <i>cgk-1β</i> )	S05702
human	<i>Homo sapiens</i>	PKG, type II ( <i>cgk-II</i> )	S68217
mouse	<i>Mus musculus</i>	PKG, type I ( <i>Prkg1</i> )	NP_035290
mouse	<i>Mus musculus</i>	PKG, type II ( <i>Prkg2</i> )	NP_032952
puffer fish	<i>Fugu rubripes</i>	PKG (predicted type I)	SIN FRUP00000066685 <sup>d</sup>
puffer fish	<i>Fugu rubripes</i>	PKG (predicted type II)	SIN FRUP00000077657 <sup>d</sup>
rabbit	<i>Oryctolagus cuniculus</i>	PKG, type Iα ( <i>PKG1α</i> )	AAC31192
rat	<i>Rattus norvegicus</i>	PKG, type II ( <i>Prkg2</i> )	NP_037144
rat	<i>Rattus norvegicus</i>	PKG, type I ( <i>Prkg1</i> )	XP_219805
cow	<i>Bos taurus</i>	PKG (isoform Iα) ( <i>cgk-1α</i> )	CAA34214
cow	<i>Bos taurus</i>	PKG (isoform I β) ( <i>cgk-1β</i> )	P21136
hydra	<i>Hydra oligactis</i>	PKG ( <i>hyGK</i> )	AAC23588
green alga	<i>Chlamydomonas reinhardtii</i>	PKG ( <i>CL-PK1</i> )	BAB18104
green alga	<i>Chlamydomonas reinhardtii</i>	PKG II ( <i>CL-PK2</i> )	BAB18105
	<i>Eimeria maxima</i>	PKG	AAM22643
	<i>Eimeria tenella</i>	PKG	AAM20900
	<i>Plasmodium falciparum</i>	PKG	AAM22644
	<i>Plasmodium falciparum</i> 3D7	PKG I, β isozyme	NP_702235
	<i>Plasmodium yoelii yoelii</i>	PKG-related ( <i>PY02304</i> )	EAA21741
	<i>Toxoplasma gondii</i>	PKG	AAM27174
	<i>Toxoplasma gondii</i>	PKG	AAM20901
	<i>Cryptosporidium parvum</i>	PKG	AAM20902

<sup>a</sup> = if available.

<sup>b</sup> = unless marked otherwise.

<sup>c</sup> = FlyBase (<http://flybase.net>).

<sup>d</sup> = *Fugu rubripes* 2.0 Genome Database (<http://genome.jgi-psf.org/fugu3/fugu3.home.html>).

a putative cGKII-like gene from the mosquito. Therefore, although PKG proteins in insects and vertebrates have evolved as expected in the tree of life, the cGKI-like proteins remain more similar to each other than any are to cGKII-like proteins and *vice versa*. For those species in the metazoan clade where only one copy of a PKG gene is shown we offer two explanations: 1) they have since lost one copy of the gene, or 2) the second copy has not yet been identified. It should be noted that a second PKG has been identified in *C. elegans* that is currently being cloned and characterized (see Stansberry *et al.*, 2001). In addition, our tBLASTx searches revealed what appear to be an additional PKG in *Bombyx mori* and two putative PKGs in *D. pseudoobscura*. Preliminary searches within the honey bee EST library have not revealed a second PKG (Y. Ben-Shahar, personal communication) but this will be further investigated once the honey bee genome sequencing project is completed.

Current evidence suggests the link between PKG and food-related behaviour is located within the metazoans. It is possible that prokaryotes and/or protozoans also show this link but it has not yet been investigated. Our PKG phylogenies imply five possible evolutionary histories that generate equally well the PKG-behaviour

association pattern shown here. Firstly, the PKG-behaviour link may have evolved once, in a common ancestor of nematodes, insects, and vertebrates (mode A in Fig. 2). This model predicts a widespread link between PKG and behaviour inherent to most vertebrates and invertebrates unless the association has been lost in some lineages. Secondly, the link may have evolved twice independently; once in nematodes, and again in an ancestor of vertebrates and insects (*i.e.*, before the duplication) (mode B in Fig. 2). Under this model we would predict that PKG would influence the behaviour of many vertebrates and insects unless some lineages have lost the behavioural association. Thirdly, the link may have again evolved twice independently; once in nematodes and then again in an ancestor of vertebrates and insects within only the cGKI copy (mode C in Fig. 2). Here, we would predict a widespread association between cGKI-type PKGs and behaviour in both vertebrates and insects. Fourthly, the link may have evolved twice; in nematodes and again in an ancestor of insects within cGKI (mode D in Fig. 2). Under this assumption, we predict food-related behaviours of many insects and possibly other arthropods to be influenced by PKG but not vertebrates. Finally, the link may have evolved completely independently

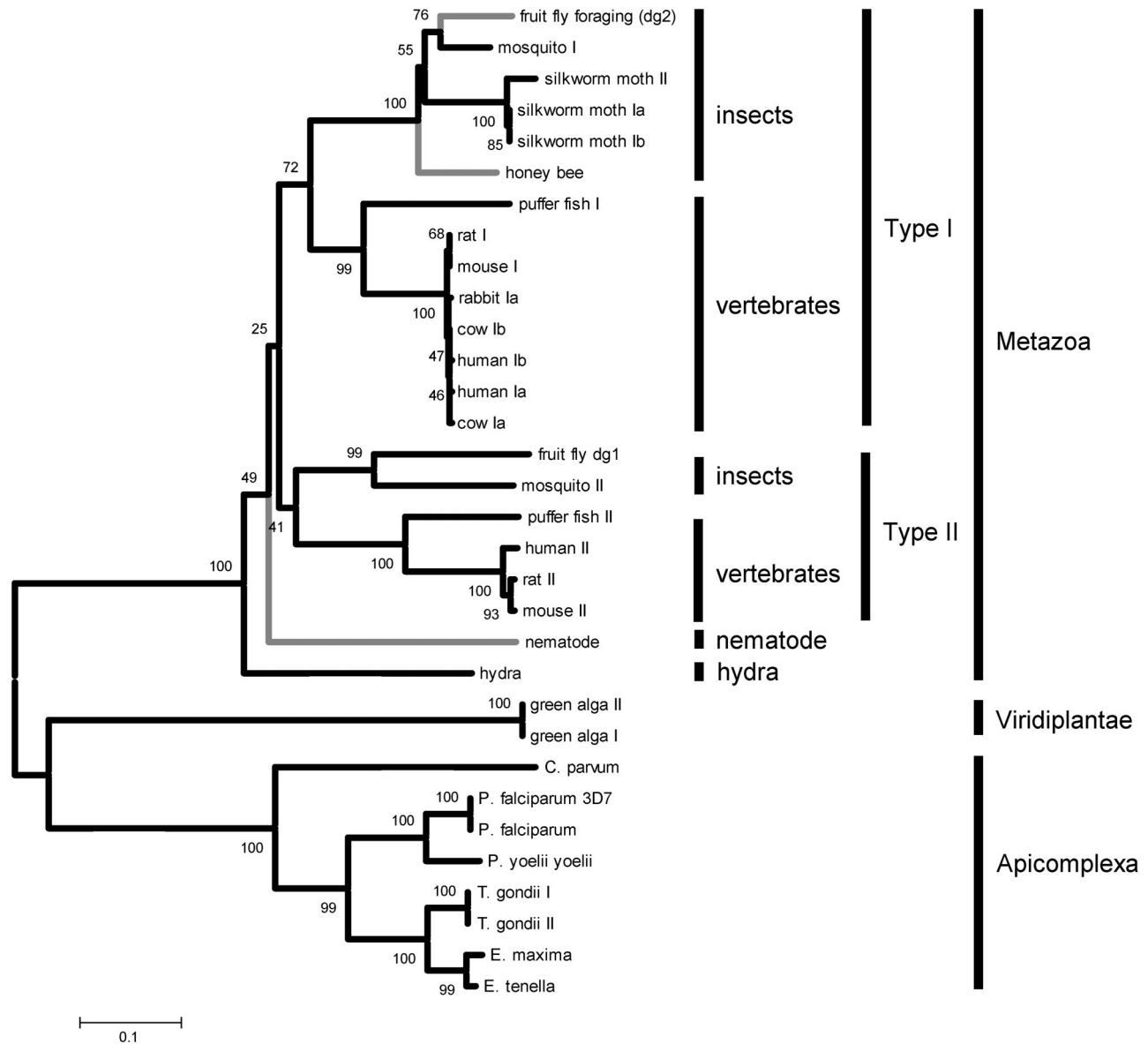


FIG. 1. Neighbour joining trees depicting the evolutionary relationships of 32 PKG kinase domain and C-terminal amino acid sequences spanning 19 species of protozoans and metazoans. Values at the nodes represent the results of 5000 bootstrap replications. Lineages with known behavioural links with PKG are indicated by grey branches.

within nematodes, honey bees, and fruit flies and is possibly unique to these lineages (mode E in Fig. 2). This would lead to a punctuated association between PKG and behaviour. While we cannot currently refute the hypothesis of a single evolutionary link between PKG and behaviour, partial evidence to support the proposal that the association evolved twice (modes B–D) can be taken from the recent findings in nematodes. Increased levels of PKG have completely opposite effects in *C. elegans* compared to the fruit fly or honey bee. In nematodes, increasing PKG causes decreased movement on food (Fujiwara *et al.*, 2002). Conversely, higher levels of PKG in fruit flies and honey bees cause an increase in food-related foraging behaviour. In support of the possible widespread association be-

tween PKG and behaviour (modes A–D), molecules such as nitric oxide, nitric oxide synthase, and cGMP (which act upstream in PKG signalling cascades) are known to influence food-related behaviours in numerous animals ranging from hydra (Colasanti *et al.*, 1997) to mouse (Morley *et al.*, 1996, 1999).

Efforts to untangle which of these evolutionary patterns is correct will require more data. Each hypothesis must be investigated preferably using an experimental approach (*e.g.*, natural variants, mutants, knockouts, transgenics, and pharmacological approaches such as artificially increasing or decreasing PKG using 8-Br-cGMP). More data on the association between PKG and behaviour in additional organisms within the metazoan clade will help resolve the evolutionary link

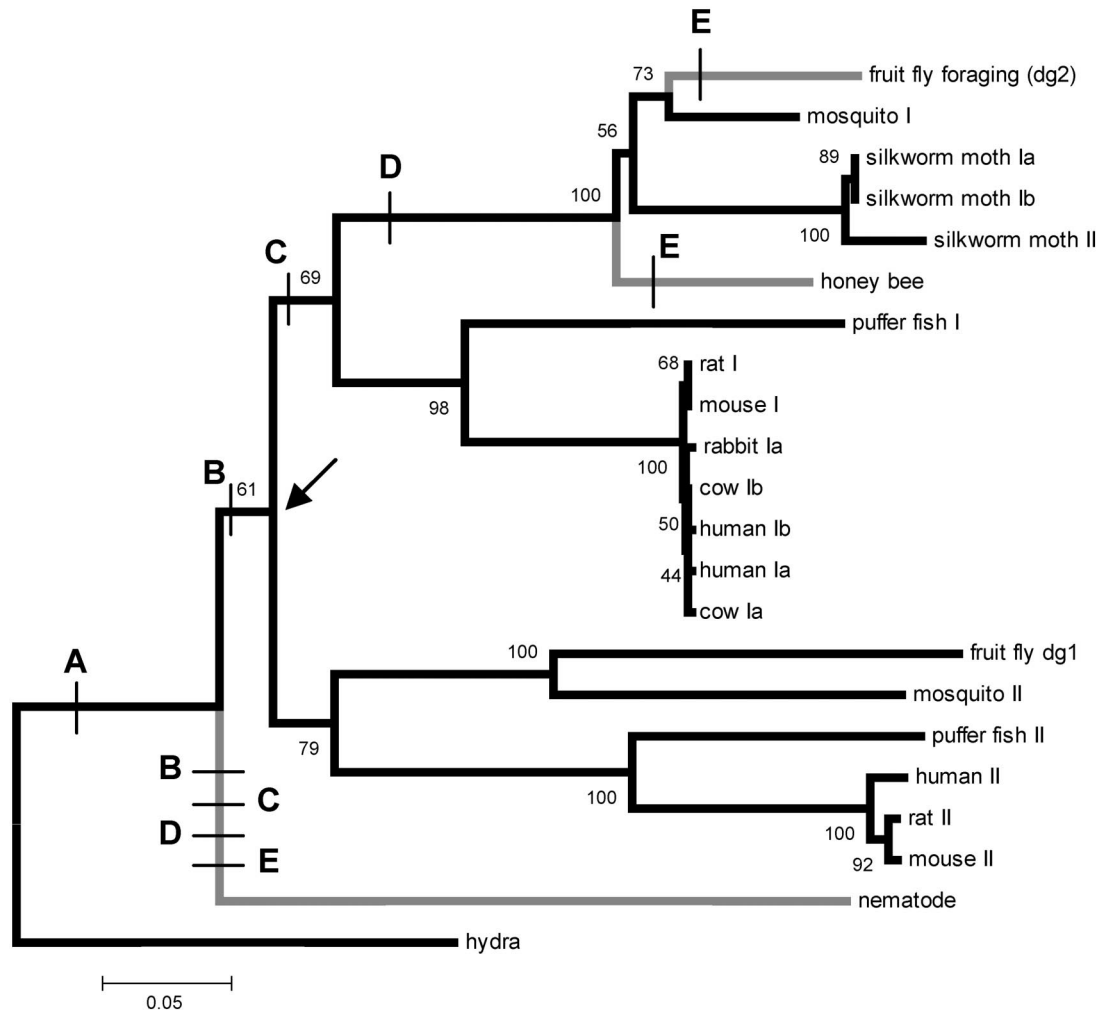


FIG. 2. Neighbour joining trees produced using PKG kinase domains from 12 metazoans species (22 sequences). Values at the nodes represent the results of 5000 bootstrap replications. Lineages with known behavioural links with PKG are indicated by grey branches. The PKG duplication is marked by an arrow. Letters represent the five possible evolutionary histories for the evolutionary link between PKG and behaviour.

between PKG and behaviour (*e.g.*, humans, mice, fish, birds, molluscs, and other arthropods). The role of cGKII-like PKGs on behaviour is unknown and this includes DG1 from *D. melanogaster*. We must also begin to investigate the food-related behaviours of mammalian PKG knockouts in both cGKI and cGKII. This will help us determine whether food-related behaviours are unique to the cGKI-like PKGs along with determining if the association is found in the vertebrate lineage. The inclusion of the second PKG from *C. elegans* will help us better understand whether nematode PKGs have experienced the same evolutionary history as insects and vertebrates. Only after these key experiments have been completed can we begin to decipher the mechanism of evolution and the extent of the behaviour associations. Nevertheless, it is apparent that genes encoding PKG are important evolutionary targets for food-related behaviours.

In our quest to understand the mechanisms of behaviour we must consider merging insights by com-

paring various animal groups to complete the story. The conservation of various genetic and neuronal mechanisms across different animals suggests this comparison is not outrageous. In addition, the integration of evidence from different species sheds light on the evolution of neurobehavioural and neurogenetic mechanisms. Taken separately, the current evidence regarding the food-related behaviours of fruit flies, honey bees, or nematodes is far from complete. For example, we do not fully understand the neural mechanism underlying foraging behaviour in fruit flies or honey bees whereas in nematodes this is much better understood. We have no evidence concerning the selection pressures leading to the evolution of natural food-related behavioural variation in nematodes, however, this has been well characterized in fruit flies. By drawing parallels we can begin to dissect the role of PKG in behaviour. If the link between PKG and behaviour was established early in the evolution of modern animals then it is reasonable to use PKG as a can-

didate gene for various food-related behaviours in other organisms. For example, PKG may influence behaviours such as the swarming of locusts and various other insects, the division of labour in other social animals (*e.g.*, ants), foraging in fish and birds, and perhaps even food-related behaviours in mammals including humans.

The mapping of phenotypes such as behaviour onto their underlying gene and/or protein phylogenies is an excellent tool for generating evolutionary hypotheses. Moreover, this approach reveals gaps in the current body of knowledge which can motivate further experimentation.

#### ACKNOWLEDGMENTS

We thank Peter Andolfatto, Bambos Kyriacou, Felix Breden, Amsale Belay, Karla Kaun, Craig Riedl, Kevin Judge, Travis Clark, Mike Myre, and an anonymous reviewer for valuable comments. We especially wish to thank Peter Andolfatto for assistance with the phylogenies. This work was supported by grants from the Natural Sciences and Engineering Research Council (NSERC), Canadian Institutes of Health Research, and Canada Research Chair in Genetics to M. B. S. along with an NSERC post graduate scholarship to M. J. F.

#### REFERENCES

- An, X., K. Wilkes, Y. Bastian, J. L. Morrow, M. Frommer, and K. A. Raphael. 2002. The *period* gene in two species of tephritid fruit fly differentiated by mating behaviour. *Insect Mol. Biol.* 11:419–430.
- Baker, B. S., B. J. Taylor, and J. C. Hall. 2001. Are complex behaviours specified by dedicated regulatory genes? Reasoning from *Drosophila*. *Cell* 105:13–24.
- Beldauf, S. L., A. J. Roger, I. Wend-Siefert, and W. F. Doolittle. 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* 290:972–977.
- Ben-Shahar, Y., A. Robichon, M. B. Sokolowski, and G. E. Robinson. 2002. Influence of gene action across different time scales on behaviour. *Science* 296:741–744.
- Ben-Shahar, Y., H.-T. Leung, W. L. Pak, M. B. Sokolowski, and G. E. Robinson. 2003. cGMP-dependent changes in phototaxis: A possible role for the *foraging* gene in honey bee division of labour. *J. Exp. Biol.* 206:2507–2515.
- Bloch, G., D. P. Toma, and G. E. Robinson. 2001. Behavioural rhythmicity, age, division of labor and *period* expression in the honey bee brain. *J. Biol. Rhythm* 16:444–456.
- Bucan, M. and T. Abel. 2002. The mouse: Genetics meets behaviour. *Nat. Rev. Genet.* 3:114–123.
- Butt, E., J. Geiger, T. Jarchau, S. M. Lohmann, and U. Walter. 1993. The cGMP-dependent protein kinase—gene, protein, and function. *Neurochem. Res.* 18:27–42.
- Callaerts, P., P. N. Lee, B. Hartmann, C. Farfan, D. W. Y. Choy, K. Ikeo, K.-F. Fischbach, W. J. Gehring, and H. G. de Couet. 2002. *Hox* genes in the sepiolid squid *Euprymna scolopes*: Implications for the evolution of complex body plans. *Proc. Natl. Acad. Sci. U.S.A.* 99:2088–2093.
- Campesan, S., Y. Dubrova, J. C. Hall, and C. P. Kyriacou. 2001. The *nonA* gene in *Drosophila* conveys species-specific behavioural characteristics. *Genetics* 158:1535–1543.
- Carroll, S. 1995. Homeotic genes and the evolution of arthropods and chordates. *Nature* 376:479–485.
- Carroll, S. 2000. Endless forms: The evolution of gene regulation and morphological diversity. *Cell* 101:577–580.
- Coates, J. C. and M. de Bono. 2002. Antagonistic pathways in neurons exposed to body fluid regulate social feeding in *Caenorhabditis elegans*. *Nature* 419:925–929.
- Colasanti, M., G. Venturini, A. Merante, G. Musci, and G. M. Lauro. 1997. Nitric oxide involvement in *Hydra vulgaris* very primitive olfactory-like system. *J. Neurosci.* 17:493–499.
- Cowley, M. A., J. L. Smart, M. Rubinstein, M. G. Cordan, S. Diano, T. L. Horvath, R. D. Cone, and M. J. Low. 2001. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411:480–484.
- de Belle, J. S. and M. B. Sokolowski. 1987. Heredity of rover/sitter: Alternative foraging strategies of *Drosophila melanogaster* larvae. *Heredity* 59:73–83.
- de Belle, S. J., A. J. Hilliker, and M. B. Sokolowski. 1989. Genetic localization of *foraging (for)*: A major gene for larval behaviour in *Drosophila melanogaster*. *Genetics* 123:157–163.
- de Bono, M. and C. I. Bargmann. 1998. Natural variation in a neuropeptide Y receptor homolog modifies social behaviour and food response in *C. elegans*. *Cell* 94:679–689.
- de Bono, M., D. M. Tobin, M. W. Davis, L. Avery, and C. I. Bargmann. 2002. Social feeding in *Caenorhabditis elegans* is induced by neurons that detect aversive stimuli. *Nature* 419:899–903.
- Fujiwara, M., P. Sengupta, and S. L. McIntire. 2002. Regulation of body size and behavioural state of *C. elegans* by sensory perception and the EGL-4 cGMP-dependent protein kinase. *Neuron* 36:1091–1102.
- Gellon, G. and W. McGinnis. 1998. Shaping animal body plans in development and evolution by modulation of *Hox* expression patterns. *BioEssays* 20:116–125.
- Gurnett, A., P. A. Liberator, P. Dulski, S. P. Salowe, R. G. K. Donald, J. W. Anderson, J. Wiltzie, C. A. Diaz-Saldana, G. Harris, B. Chang, S. J. Darkin-Rattray, B. Nare, T. Crumley, P. Blum, A. Misura, T. Tamas, M. Sardana, J. Yuan, T. Biftu, and D. Schmatz. 2002. Purification and molecular characterization of cGMP-dependent protein kinase from Apicomplexan parasites. A novel chemotherapeutic target. *J. Biol. Chem.* 277:15913–15922.
- Hedlund, P., A. Aszodi, A. Pfeifer, P. Alm, F. Hofmann, M. Ahmad, R. Fassler, and K. E. Andersson. 2000. Erectile dysfunction in cyclic GMP-dependent kinase I-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 97:2349–2354.
- Higgins, D. G. and P. M. Sharp. 1988. CLUSTAL: A package for performing multiple sequence alignment on a microcomputer. *Gene* 73:237–244.
- Jarchau, T., C. Hauser, T. Markert, D. Pohler, J. Vandekerckhove, H. R. De Jonge, S. M. Lohmann, and U. Walter. 1994. Cloning, expression, and *in situ* localization of rat intestinal cGMP-dependent protein kinase II. *Proc. Natl. Acad. Sci. U.S.A.* 91:9426–9430.
- Kalderon, D. and G. M. Rubin. 1989. cGMP-dependent protein kinase genes in *Drosophila*. *J. Biol. Chem.* 264:10738–10748.
- Konopka, R. J. and S. Benzer. 1971. Clock mutants of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 68:2112–2116.
- Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: Molecular evolutionary genetics analysis software. *Bioinformatics* 17:1244–1245.
- L'Etoile, N. D. and C. I. Bargmann. 2000. Olfaction and odor discrimination are mediated by the *C. elegans* guanylyl cyclase ODR-1. *Neuron* 25:575–586.
- L'Etoile, N. D., C. M. Coburn, J. Eastham, A. Kistler, G. Gallegos, and C. I. Bargmann. 2002. The cyclic GMP-dependent protein kinase EGL-4 regulates olfactory adaptation in *C. elegans*. *Neuron* 36:1079–1089.
- Lohmann, S. M., A. B. Vaandrager, A. Smolenski, U. Walter, and H. R. De Jonge. 1997. Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem. Sci.* 22:307–312.
- Miyatake, T., A. Matsumoto, T. Matsuyama, H. R. Ueda, T. Toyosato, and T. Tanimura. 2002. The *period* gene and allochronic reproductive isolation in *Bactrocera cucurbitae*. *Proc. Roy. Soc. London B* 269:2467–2472.
- Morley, J. E., V. B. Kumar, M. B. Mattammal, S. Farr, P. M. K. Morley, and J. F. Flood. 1996. Inhibition of feeding by a nitric oxide synthase inhibitor: Effects of aging. *Eur. J. Pharmacol.* 311:15–19.
- Morley, J. E., M. M. Alshaher, S. A. Farris, J. F. Flood, and V. B.

- Kumar. 1999. Leptin and neuropeptide Y (NPY) modulate nitric oxide synthase: Further evidence for a role of nitric oxide in feeding. *Peptides* 20:595–600.
- Ny, L., A. Pfeifer, A. Aszodi, M. Ahmad, P. Alm, P. Hedlund, R. Fassler, and K. E. Andersson. 2000. Impaired relaxation of stomach smooth muscle in mice lacking cyclic GMP-dependent protein kinase I. *Brit. J. Pharmacol.* 129:395–401.
- Osborne, K. A., A. Robichon, E. Burgess, S. Butland, R. A. Shaw, A. Coulthard, H. S. Pereira, R. J. Greenspan, and M. B. Sokolowski. 1997. Natural behaviour polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science* 277:834–836.
- Page, R. D. M., and E. C. Holmes. 1998. *Molecular evolution: A phylogenetic approach*, pp. 219–222. Blackwell Science, London.
- Peixoto, A. A. 2002. Evolutionary behavioural genetics in *Drosophila*. *Adv. Gen.* 47:117–149.
- Pereira, H. S. and M. B. Sokolowski. 1993. Mutations in the larval foraging gene affect adult locomotory behaviour after feeding in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 90:5044–5046.
- Persson, K., R. K. Pandita, A. Aszodi, M. Ahmad, A. Pfeifer, R. Fassler, and K. E. Andersson. 2000. Functional characteristics of urinary tract smooth muscles in mice lacking cGMP protein kinase type I. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 279:R1112–R1120.
- Pfeifer, A., A. Aszodi, U. Seidler, P. Ruth, F. Hofmann, and R. Fassler. 1996. Intestinal secretory defects and dwarfism in mice lacking cGMP-dependent protein kinase II. *Science* 274:2082–2086.
- Pfeifer, A., P. Klatt, S. Massberg, L. Ny, M. Sausbier, C. Hirneiss, G. X. Wang, M. Korth, A. Aszodi, K. E. Andersson, F. Krombach, A. Mayerhofer, P. Ruth, R. Fassler, and F. Hofmann. 1998. Defective smooth muscle regulation in cGMP kinase I-deficient mice. *EMBO J.* 17:3045–3051.
- Piccin, A., M. Couchman, J. D. Clayton, D. Chalmers, R. Costa, and C. P. Kyriacou. 2000. The clock gene *period* of the housefly, *Musca domestica*, rescues behavioural rhythmicity in *Drosophila melanogaster*: Evidence for intermolecular coevolution. *Genetics* 154:747–758.
- Rankin, C. H. 2002. From gene to identified neuron to behaviour in *Caenorhabditis elegans*. *Nat. Rev. Genet.* 3:622–630.
- Robinson, G. E. and Y. Ben-Shahar. 2002. Social behaviour and comparative genomics: New genes or new gene regulation? *Genes Brain Behav.* 1:197–203.
- Rogers, C., V. Reale, K. Kim, H. Chatwin, C. Li, P. Evans and M. de Bono. 2003. Inhibition of *Caenorhabditis elegans* social feeding by FMRFamide-related peptide activation of NPR-1. *Nature Neurosci.* 6:1178–1185.
- Saitou, N. and M. Nei. 1987. The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 13:399–411.
- Sandberg, M., V. Natarajan, I. Ronander, D. Kalderon, U. Walter, S. M. Lohmann, and T. Jahnsen. 1989. Molecular cloning and predicted full-length amino acid sequence of the type IB isozyme of cGMP-dependent protein kinase from human placenta. *FEBS Lett.* 251:191–196.
- Sandrelli, F., S. Campesan, M. Rossetto, C. Benna, E. Zieger, A. Megighian, M. Couchman, C. Kyriacou, and R. Costa. 2001. Molecular dissection of the 5' region of *no-on-transientA* of *Drosophila melanogaster* reveals *cis*-regulation by adjacent *dGpiI* sequences. *Genetics* 157:765–75.
- Schafer, W. R. 2002. PKG and the neural basis for behavioural phenotypes. *Neuron* 36:991–993.
- Shen, P. and H. N. Cai. 2001. *Drosophila* neuropeptide F mediates integration of chemosensory stimulation and conditioning of the nervous system by food. *J. Neurobiol.* 47:16–25.
- Sokolowski, M. B. 1980. Foraging strategies of *Drosophila melanogaster*: A chromosomal analysis. *Behav. Gen.* 10:291–302.
- Sokolowski, M. B. 1998. Genes for normal behavioural variation: Recent clues from flies and worms. *Neuron* 21:463–466.
- Sokolowski, M. B. 2001. *Drosophila*: Genetics meets behaviour. *Nat. Rev. Genet.* 2:879–890.
- Sokolowski, M. B. 2002. Social eating for stress. *Nature* 419:893–894.
- Sokolowski, M. B., H. S. Pereira, and K. Hughes. 1997. Evolution of foraging behaviour in *Drosophila* by density-dependent selection. *Proc. Natl. Acad. Sci. U.S.A.* 94:7373–7377.
- Stansberry, J., E. J. Baude, M. K. Taylor, P.-J. Chen, S.-W. Jin, and M. D. Uhler. 2001. A cGMP-dependent protein kinase is implicated in wild-type motility in *C. elegans*. *J. Neurochem.* 76:1177–1187.
- Taylor, S. S., J. A. Buechler, and W. Yonemoto. 1990. cAMP-dependent protein-kinase: Framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.* 59:971–1005.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876–4882.
- Toh, K. L., C. R. Jones, Y. He, E. J. Eide, W. A. Hinz, D. M. Virshup, L. J. Ptacek, and Y.-H. Fu. 2001. An *hPer2* phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* 291:1040–1043.
- Toma, D. P., G. Bloch, D. Moore, and G. E. Robinson. 2000. Changes in *period* mRNA levels in the brain and division of labor in honey bee colonies. *Proc. Natl. Acad. Sci. U.S.A.* 97:6914–6919.
- Uhler, M. D. 1993. Cloning and expression of a novel cyclic GMP-dependent protein kinase from mouse brain. *J. Biol. Chem.* 163:17632–17637.
- Wahlsten, D. 1999. Single-gene influences on brain and behaviour. *Annu. Rev. Psychol.* 50:599–624.
- Wang, X. and P. J. Robinson. 1997. Cyclic GMP-dependent protein kinase and cellular signaling in the nervous system. *J. Neurochem.* 68:443–456.
- Wegener, J. W., H. Nawrath, W. Wolfsgruber, S. Kuhbandner, C. Werner, F. Hofmann, and R. Feil. 2002. cGMP-dependent protein kinase I mediates the negative inotropic effect of cGMP in the murine myocardium. *Circ. Res.* 90:18–20.
- Zheng, B. H., D. W. Larkin, L. Albrecht, Z. S. Sun, M. Sage, G. Eichele, C. C. Lee, and A. Bradley. 1999. The *mPer2* gene encodes a functional component of the mammalian circadian clock. *Nature* 400:169–173.