cGMP-Dependent Protein Kinase as a Modifier of Behaviour

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Contents

Abstract The importance of cGMP-dependent protein kinase (PKG) to the modulation of behavioural phenotypes has become increasingly clear in recent decades. The effects of PKG on behaviour have been studied in diverse taxa from perspectives as varied as ethology, evolution, genetics and neuropharmacology. The genetic variation of the *Drosophila melanogaster* gene, *foraging (for)*, has provided a fertile model for examining natural variation in a single major gene influencing behaviour. Concurrent studies in other invertebrates and mammals suggest that PKG is an important signalling molecule with varied influences on behaviour and a large degree of pleiotropy and plasticity. Comparing these cross-taxa effects suggests that there are several potentially overlapping behavioural modalities in which PKG signalling acts to influence behaviours which include feeding, learning, stress and biological rhythms. More in-depth comparative analyses across taxa of the similarities and differences of the influence of PKG on behaviour may provide powerful mechanistic explications of the evolution of behaviour.

Keywords: cGMP-dependent protein kinase · Behaviour · Natural genetic variation · Evolution · Pleiotropy · Plasticity · Foraging · Learning and memory · Stress · Rhythms

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1 A Natural History of PKG and Behaviour

The role of PKG in the modification of behavioural phenotypes arose initially out of ethological studies investigating natural populations of larval *Drosophila melanogaster*. Although *D. melanogaster* had been a model for the genetic dissection of phenotypic traits since the early 20th Century, it was not until the 1970s that investigations specifically addressed larval foraging behaviour (Sewell et al. 1975). By the late 1970s it became clear that larval behaviour had an important effect on resource utilization, competitive ability and fitness, prompting ethological and genetic descriptions of larval foraging strategies (Sokolowski 1980). Early studies showed that larvae differed in their foraging strategies displaying a phenotypic dimorphism (Sokolowski 1980; Bauer and Sokolowksi 1985). Individuals were dubbed either rovers, who traveled over a large area while foraging, or sitters, who covered a relatively smaller area. This distinction was quantitatively described by a bimodal distribution of the distance traveled while foraging (foraging trail-length). Later it was shown to arise from variation at a single locus named *foraging (for)* which encodes a cGMP-dependent protein kinase (PKG) (de Belle et al. 1989; Osborne et al. 1997). The polymorphism is not expressed when rovers and sitters are on a non-nutritive substrate (i.e. their foraging trail-lengths do not differ significantly on agar) suggesting a role for PKG signalling in the processing of a complex behaviour induced by a nutritive environmental stimulus (Douglas et al. 2005).

Several investigations indicated that the dimorphic behaviour exists in nature in the area of Toronto, Canada at about 70% rovers to 30% sitters (Sokolowski 1980, 1982; Sokolowski et al. 1997). Furthermore, when flies were collected from various microhabitats of a pear orchard, the rover and sitter variants were found foraging together within the fruit but preferred different pupation sites (Sokolowski 1986). While sitters pupated on the fruit, rovers were found to pupate off of the fruit.

The ecologically important process of density dependence was also found to be involved in changes of *for* allele frequencies (Sokolowski et al. 1997). In high density lab conditions rover larvae are preferentially selected whereas in low densities sitters predominate. More recent investigations suggest that the polymorphism may, in part, be maintained by balancing selection through a mechanism of negative frequency dependent selection under larval competition (Fitzpatrick et al. 2007).

Intriguingly, despite the strong association between the rover/sitter behaviour and *for*, the phenotypes are plastic when exposed to varying environmental parameters (Graf and Sokolowski 1989). For instance, expression of the behavioural polymorphism was found to be conditional on the distribution and abundance of food in the environment (Sokolowski et al. 1983; Kaun et al. 2007a). Specifically, food deprived rover larvae behave as sitters and exhibit sitter-like PKG activity (Kaun et al. 2007a). This is of particular interest since *D. melanogaster* larvae display habitat selection which has both heritable and plastic components (Rodriguez et al. 1992).

The first genetic analyses of larval foraging indicated that the behavioural polymorphism could be attributed to genetic properties on the second chromosome where the rover phenotype was found to be dominant to the sitter one (Sokolowski 1980). Subsequent analyses of isogenic rover and sitter strains indicated that the path

length phenotypes are autosomally inherited in a pattern indicative of a single gene influence (de Belle and Sokolowski 1987). Although a minor X-chromosome effect was observed in females, these analyses failed to detect other hereditary influences such as the Y-chromosome, transient maternal factors, permanent cytoplasmic factors or their interactions. Efforts to localize the genetic influence underlying the rover/sitter polymorphism were impeded by the modifying effects of phenotypic markers used in standard recombination mapping. To overcome this problem, de Belle and Sokolowski (1989) used compound reverse metacentric chromosomes which allowed for the localization of the allelic polymorphism to the left arm of the second chromosome of *D. melanogaster*. The lethal tagging technique was then used to genetically map the behaviour to chromosomal location 24A3-5 (de Belle et al. 1989, 1993). The technique involved mutagenizing rovers that were subsequently screened for the induction of both pupal lethality and sitter behaviour. The pupal lethality allowed for the subsequent mapping of the behavioural alteration. A number of mutations failed to complement for pupal lethality or sitter behaviour and they were readily mapped on the basis of their lethality to position 24A3-5 after which the behavioural alteration was also mapped to this position. The gene was named *foraging* (*for*) where rover alleles were designated as for^{R} and sitter alleles as for^{s}. Thus, the lethal tagging approach allowed for finer mapping of the behavioural polymorphism without the interference of marker genes and genetic backgrounds introduced in recombination mapping. The technique also provided mutant alleles for future studies.

The *for* gene was mapped within the region of a *Drosophila* cGMP-dependent protein kinase gene called *dg2*; cloning of *for* confirmed that it is synonymous with *dg2* (Osborne et al. 1997). Evidence for this includes the findings that wild-type for^{s} and sitter mutants, made on a rover background (for^{s2}), had lower PKG activity and *dg2* transcript levels in larval central nervous systems (CNS) and adult heads compared to wild-type for^{R} animals. Additionally, and perhaps most importantly, rover-type PKG activity and foraging behaviour resulted from increasing the level of *for/dg2* in transgenic sitter larvae (Osborne et al. 1997).

The natural allelic variation resulting in differing levels of PKG has allowed for the examination of variation in a single major gene affecting an ecologically relevant set of behavioural phenotypes from an evolutionary perspective (Douglas et al. 2005). Early studies were driven by unique lines of inquiry which have since had parallels in studies of other taxa such as nematodes, bees and ants (de Bono and Bargmann 1998; Ben-Shahar et al. 2002, 2003; Ingram et al. 2005). The implication of PKG in affecting diverse behavioural phenotypes in divergent species has brought increased attention to the role of PKG pathways in the evolution of behaviour (Fig. 1; Table 1).

The study of PKG in mammals, and in particular NO-cGMP-PKG signalling, preceded much of the work in invertebrates. However, many of the pharmacological and neuropharmacological studies only began to examine behavioural effects of PKG signalling more recently (Hofmann et al. 2006). The following sections will review these studies and consider the implications of their results for future research looking at the role of PKG signalling in the evolution and modulation of behaviour.

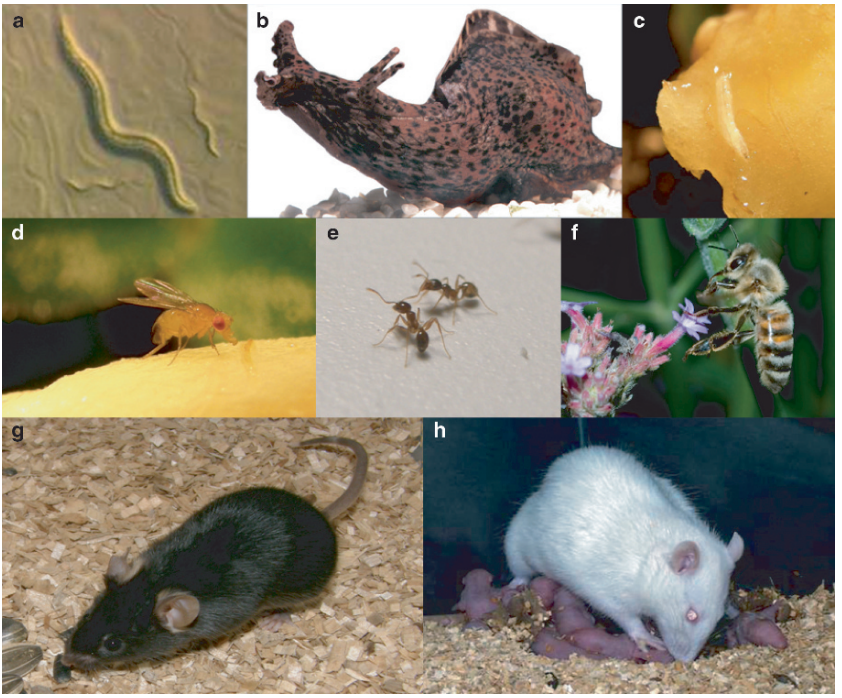

Fig. 1 cGMP-dependent protein kinase modulates behaviour in a variety of species. **a** *Caenorhabditis elegans* on a bacterial lawn **b** *Aplysia californica* **c** *Drosophila melanogaster* larva foraging on fruit **d** *Drosophila melanogaster* adult extending proboscis to feed **e** two worker ants of *Phidole pallidula* whose primary job is to forage for the colony **f** the honey bee, *Apis melifera* foraging for the hive **g** the lab mouse *Mus musculus* **h** the lab rat *Rattus norvegicus*. Photo credits: **a** Mario de Bono, **b** John H. Byrne, **c, d, e, g** Christopher J. Reaume, **f** Zachary Huang, Michigan State University, www.beetography.com, **h** Members of Alison Fleming's Lab

2 PKG and Food

The effects of *for* are not limited to larval foraging behaviour. Early descriptions of the rover/sitter polymorphism suggested that *for* is pleiotropic and that the allelic influences of PKG activity have resulted in various correlated traits including behavioural ones (Table 1). Initial investigations of these correlated effects demonstrated that rover females had no preference for oviposition site whereas sitters preferred to oviposite on sites that were inhabited by larvae (Sokolowski 1987). Descriptions of adult space-use and food-related behaviours showed that differences between for^R and for^s genotypes extend to life-history characteristics beyond the larval stage (Nagle and Bell 1987; Pereira and Sokolowski 1993). For instance, rovers were found to move farther away from a sucrose source following feeding.

More recent ecological and evolutionary studies have implicated the rover/sitter behaviours, and therefore PKG, in their investigations. For example, populations

Table 1 cGMP-dependent protein kinases (PKG) affect a variety of behaviours in diverse taxa

Species	Gene	Influence of PKG on Behaviour	References
C. elegans	*egl-4*	Food-related behaviours	Fujiwara et al. (2002); L'Etoile et al. (2002)
		Behavioural quiescence	Raizen et al. (2008)
Drosophila melanogaster	*for/dg2*	Larval forgaging path-length	Sokolowski (1980)
		Adult movement post-feeding	Nagle and Bell (1987); Pereira and Sokolowski (1993)
		Adult response to yeast	Shaver et al. (1988)
		Habituation	Engel et al. (2000)
		Sucrose response and habituation	Scheiner et al. (2004); Belay et al. (2007)
		Adult aversive olfactory associative learning and memory	Mery et al. (2007)
		Stress tolerance	Dawson-Scully et al. (2007)
		Larvae nutrient acquisition and absorption	Kaun et al. (2007a)
		Larval olfactory reward conditioning	Kaun et al. (2007b)
		Sleep	Raizen et al. 2008
		Visual pattern memory, operant learning	Wang et al. (2008)
Apis melifera	*Amfor*	Transition from nurse to forager	Ben-Shahar et al. (2002)
		Phototaxis	Ben-Shahar et al. (2003)
Pogonomyrmex barbatus	*Pbfor*	Worker and forager	Ingram et al. (2005)
Mus musculus	*cGKI*	nociception responses	Schmidt et al. (2002); Tegeder et al. (2004)
	cGKI	Cerebellar LTD	Feil et al. (2003)
	cGKI	Hippocampal L-LTP	Kleppisch et al. (2003)
	cGKI	Addiction	Jouvert et al. (2004)
	cGKII	Circadian rhythmicity	Oster et al. (2003)
	cGKII	Addiction and anxiety	Werner et al. (2004)
Rattus norvegicus	*cGKII*	Circadian rhythmicity	Tischkau et al. (2004)

selected for rapid development also expressed reduced foraging (Prasad et al. 2001). Investigations directed at examining adult space-use and habitat selection in which larval foraging distances were found to positively relate to lateral movements of adults also suggested that *for* may be involved in dispersal distances from food (Stamps et al. 2005). Given that an ever increasing number of pleiotropic effects of the *for* polymorphism were being discovered, the next obvious step was to try to understand what behavioural modalities and underlying circuitry PKG signalling was acting in to influence behavioural differences. Olfaction is an important and genetically well-studied modality involved in food-searching, foraging and feeding

behaviours. Shaver et al. (1998) asked whether rovers and sitters differed in their olfactory response (attraction). While larvae did not differ, sitter adults had a greater tendency to move towards a yeast source than did rover adults.

The *for* polymorphism affects larval food acquisition and, as with the foraging trail-length phenotype, its effects are sensitive to environmental variation (Kaun et al. 2007a). The behavioural effects of the natural variation in *for* may have an underlying metabolic basis since larvae with for^{R} and for^{s} alleles differ in their nutrient acquisition and absorption. For example, compared to for^{s}, for^{R} larvae have lower food intake, higher levels of glucose absorption and preferential allocation of glucose to lipids. Some of these differences can be modified by rearing larvae in conditions with lower food quality or availability. Such manipulations result in an overall rise in food intake in all strains but in these conditions rover intake no longer differs from that of sitters. Interestingly, for^{R} larvae maintain higher absorption efficiency but also have more rapid development and higher survivorship compared to for^{s} and for^{s2} when food is limited (Kaun et al. 2007a).

Recent studies have investigated members of the upstream signalling cascade thought to affect PKG-mediated behavioural phenotypes. For instance, Riedl et al. (2005) assayed the foraging behaviour of $dgc\alpha 1$ mutants. These mutants are deficient in a soluble guanylyl cyclase gene and were therefore predicted to produce less cGMP, leading to lower PKG activation and thus more sitter-like foraging behaviour. Contrary to expectations, the mutants expressed both increased PKG activity and greater foraging trail-lengths compared to controls, in both rover (for^{R}) and sitter (for^{s}) genetic backgrounds. Perhaps these results are not really surprising given that cGMP signalling has a substantial amount of inbuilt degeneracy with upwards of 12 genes with known or predicted guanylyl cyclase activity in *Drosophila* (Riedl et al. 2005). DNA microarray analyses of transcriptional differences between $dgc\alpha 1$ mutants and controls were performed and comparisons were made on both rover and sitter genetic backgrounds. Although many genes showed differential transcription, few differences were common to both backgrounds. Such results demonstrate the importance of genetic background when dissecting the signalling pathways involved in complex behavioural traits.

Complementary to reverse genetic approaches like that of Riedl et al. (2005), forward genetic approaches have been used to identify genetic modifiers of larval foraging behaviour (Pereira et al. 1995). *Chaser* (*Csr*), a dominant suppressor of for^{R}, was uncovered in a gamma mutagenesis screen of sitter flies. Although *Csr* has been mapped to regions 95F7-96A1 on the third chromosomes of *D. melanogaster*, the molecular basis of its modifying effects have not yet been determined. Shaver et al. (2000) used a mutagenesis screen to identify new genes that affect larval foraging behaviour. Once cloned, some of these genes may identify new members of the PKG signaling pathway important to larval foraging behaviour.

The influences of PKG on food-related behaviours are not restricted to *D. melanogaster* (Table 1). Investigations have been undertaken in several hymenopteran species which differ significantly in their life-history and social behaviour compared to *D. melanogaster* (Ben-Shahar et al. 2002; Ingram et al. 2005). In the honeybee, *Apis melifera*, PKG levels are associated with a temporal

polyethism which is important to the maintenance of the colony. Briefly, division of labour in *A. melifera* is described by the individual being a part of either a young cohort which act as nurses inside the hive or an older cohort (>3 weeks in age), which forage and defend outside the hive (Beshers et al. 2001). Ben-Shahar et al. (2002) found that transcript levels of the *for* orthologue, *Amfor*, as well as PKG activity were greater in foragers compared to the nurses. These effects were also causally demonstrated by pharmacological activation of cGMP (to increase PKG activity) which resulted in precocious foraging behaviour in young bees. Another study looking at red harvester ants (*Pogonomyrmex barbatus*) found similar, yet seemingly opposite, effects of the *for* orthologue of this species (*Pbfor*). In *P. barbatus*, mRNA levels of *Pbfor* were greater in young worker brains compared to those of foragers (Ingram et al. 2005).

Preliminary phylogenetic analyses suggest that the role of PKG in modulating food-related phenotypes may have a common evolutionary origin (Fitzpatrick and Sokolowski 2004). However, the specific circuitry and signalling underlying the effects of PKG expression and activity in diverse taxa are most likely dependent on aspects of the anatomy and life-history of the organism in question and so may provide interesting examples of evolutionary co-option from a behavioural perspective (Toth and Robinson 2007).

Another well-described organism that has provided insight into the role of PKG in the modification of food-related behaviour and physiology is the nematode *Caenorhabditis elegans* (Table 1). The *C. elegans* PKG mutant, *egl-4*, was first described phenotypically from an observed defect in egg-laying behaviour. Not unlike what has been found with *for* in *D. melanogaster, egl-4* has since been shown to affect diverse aspects of *C. elegans* biology including physiology, development, and behaviour.

Daniels et al. (2000) implicated PKG in the TGF signalling pathway which affects the dauer developmental stage of nematodes. Their results suggest that PKG signalling acts to modulate responses to sensory cues which are likely involved in both development and diverse behaviours including foraging, egg-laying, and stress responses. The effects of *egl-4* were directly implicated in the regulation of food-related behaviour by *in vivo* descriptions of neuronal signalling mechanisms (Fujiwara et al. 2002; L'Etoile et al. 2002).

C. elegans foraging behaviour is described by dichotomous behavioural states which are superficially similar to those found in *D. melanogaster* larvae. An individual will alter between two states called dwelling, in which speed is low and turning frequent, or roaming in which speed is high and turning infrequent. Ciliated sensory neurons involved in modulating these behavioural states, by inhibition of locomotion in the presence of food, were shown to have downstream effects partially mediated by PKG (L'Etoile et al. 2002). In contrast to expectations from observations in flies and bees, food-related locomotion and PKG are inversely related in *C. elegans*. Specifically, *egl-4*-PKG appeared to have a negative regulatory function in sensory neurons controlling this behaviour. The PKG signaling pathways involved in the behavioural effects of *egl-4* are not yet known. Nonetheless, a dominant mutation in *egl-4* causing increased gene activity has confirmed the role of

PKG in *C. elegans* locomotion and feeding and promises to be a valuable tool for elucidating the relevant signaling pathways (Raizen et al. 2006).

Overall, PKG affects feeding behaviours in a number of organisms but the exact details of these effects are likely to vary from species to species. However, a number of the developmental and behavioural effects of PKG in *C. elegans* appear to share commonalities with those of *D. melanogaster* (L'Etoile et al. 2002; Hirose et al. 2003; Raizen et al. 2006). Interestingly, as in *Drosophila*, behaviours affected by PKG display plasticity which may be modulated by environmental stimuli (Fujiwara et al. 2002).

3 PKG and Memory

Based on PKG's general neuromodulatory role, it is perhaps not surprising that it has been implicated in affecting phenotypes and mechanisms underlying learning and memory. There are many examples across divergent species of the interaction between learning, memory and food-related behaviours. For example, taxa as diverse as insects, birds, and mammals can anticipate the spatial and temporal availability of food in order to alter their foraging strategies (Shettleworth 2001; Boisvert and Shery 2006). In this section, we review studies in *D. melanogaster* as well as in mammalian models that suggest an important role for PKG signalling in the mechanisms of behaviours associated with learning and memory.

In *D. melanogaster, for*-PKG has been shown to affect habituation-related behaviours (Engel et al. 2000; Scheiner et al. 2004). Habituation is a well-studied form of non-associative learning in which a behavioural response is reduced or extinguished following repeated stimulation (Thompson and Spencer 1966; Castellucci and Kandel 1974). To assess the role of PKG in habituation and dishabituation, Engel et al. (2000) examined the stimulus-dependent response decrement of the long-latency giant fiber jump-and-flight escape response of intact tethered *for* genetic variants. While this escape response can be induced by a visual startle reflex, in this study the response pathway was electrically stimulated using electrodes implanted through the animal's eyes (Engel and Wu 1996). Levels of PKG were found to affect several parameters of the response. Specifically, rovers had a slower response decrement, a weaker reversal of the response decrement post-stimulus and stronger reversal of the response decrement evoked by a novel stimulus. Thus, natural genetic variation influencing PKG activity was shown to affect the neuronal plasticity of the giant-fiber circuit which underlies a non-associative sensorimotor behaviour (Engel et al. 2000). The precise mechanism or signalling pathways underlying these effects were not examined.

The role of *for*-PKG in habituation extends to a well-known phenotype in the invertebrate learning and memory literature: the proboscis extension response (PER). PER has long been used to assess sucrose responsiveness (SR) which itself correlates to other phenotypes such as odour preferences and phototaxis and is thought to be indicative of responsiveness in multiple behavioural modalities (Scheiner et al. 2004). Interestingly, SR correlates with the division of labour amongst foraging bees

(Scheiner et al. 2003). In these experiments, a drop of solution containing some stimulant, such as sugar, is applied to taste sensilla on the leg and the resultant proboscis extension response (PER) events are noted.

The for^{R}, for^{s} and for^{s2} strains, with their differing PKG activities, showed differences in sucrose responsiveness and habituation of the PER to repeated sucrose stimulations. The for^{R} line, with higher PKG activity, had higher levels of responsiveness to sucrose than did the natural sitter and the sitter mutant strains. Additionally, when flies with similar sucrose responsiveness were compared, rovers habituated more slowly and had less generalization of habituation than did sitter flies. The relationship of *for*-PKG to sucrose responsiveness has recently been supported using transgenic flies (Belay et al. 2007). A pan-neuronal driver (*elav-gal4*) was used to express *for* transcripts in neurons of the adult fly brain and this resulted in an increase of sucrose responsiveness in sitters.

Recently, rovers and sitters have been shown to differ in their short and long-term memory abilities in an associative olfactory learning paradigm (Mery et al. 2007). Specifically, Mery et al. (2007) found that sitters have poorer short-term memory but better long-term memory than rovers. Further, GAL4 drivers used to direct *for* expression to the mushroom bodies (MB) of for^{s} flies induced rover-like learning performance. The MB are central to olfactory learning processes in *D. melanogaster* (Heisenberg 2003) and the *for*-PKG protein has been localized in the MB (Belay et al. 2007). An earlier study of brain structure-function relationships of *for* expression found, however, that MB ablation in larvae did not affect the larval rover/sitter path-length phenotype (Osborne et al. 2001).

In larvae, Kaun et al. (2007b) showed that olfactory conditioning was also significantly influenced by *for* expression in the MB. Using an olfactory reward-conditioning paradigm, rover and sitter larvae were found to differ phenotypically. The for^{R} larvae, which exhibit greater PKG activity, showed faster memory acquisition and longer retention. Once again, the influence of PKG on the phenotype was sensitive to an environmental stimulus where in this case, an increase in the number of conditioning trials removed the phenotypic differences between the *for* genotypes (Kaun et al. 2007b).

Findings on *for*-related learning suggests that rovers and sitters may use different foraging tactics in nature and that *for* may be involved in an evolutionary trade-off between short-term learning performance and long-term memory (Mery et al. 2007). Because rovers and sitters are known to exploit different microgeographic habitat types in nature (Sokolowski 1986; Sokolowski and Carton 1989), it may be that *for*-PKG expression in the MB plays an important role in modulating decision-like processes that involve learning, memory and habitat selection. Indeed, it has been shown that flies without the MB are unable to suppress their memory from previous experience (Liu et al. 1999). Thus, the MB may be involved in the balance between maintaining an existing behaviour and switching to a new one. Taken together, these results and observations suggest that PKG signalling affecting several learning- and feeding-related behavioural phenotypes occur in separate and distinct neuronal circuits that underlie multiple modalities which can be modified by environmental stimuli.

Long-term potentiation (LTP) and long-term depression (LTD) are activity-dependent changes in synaptic transmission that are thought to play an important role in diverse forms of learning and memory. Briefly, these processes of synaptic plasticity can be described as the long-term increase (LTP) or decrease (LTD) of synaptic strength after activity-dependent changes in the strength and frequency of stimulation (Chen and Tonegawa 1997). Hippocampal LTP has been implicated in spatial and contextual learning, whereas cerebellar LTD is known to contribute to discrete motor learning. Research has suggested that hippocampal LTP and cerebellar LTD are both modulated through NO-cGMP-dependent signalling mechanisms (Chen and Tonegawa 1997; Boyden et al. 2004). As in *Drosophila*, vertebrate genomes contain two known PKG genes: cGKI (homologous to *Drosophila for/dg2*) and cGKII (homologous to *Drosophila dg1*). The mammalian PKG isoform, cGKIα, while having a variety of physiological (Hofmann et al. 2006) and neuronal (Feil et al. 2005) effects, exists in high concentrations in cerebellar Purkinje cells (PCs), which are involved in the modulation of various types of motor learning. PCs relay inhibitory signals from the cerebellum and receive a number of excitatory neuronal inputs. Both the induction and inhibition of LTD has been shown to be affected by upstream signalling components of PKG; however, it is thought that PKG serves a key function in cerebellar LTD. Additionally, pharmacological approaches using enzyme inhibitors with varying specificities suggested that PKG is involved in cerebellar LTD (reviewed in Feil et al. 2005).

A conditional knockout of cGKI in the PCs of mice showed that PKG is involved in cerebellar LTD (Feil et al. 2003). These experiments demonstrated that a lack of cGKI in the PCs led to a nearly complete loss of LTD at the synapses of PCs and granular layer parallel fibers (PF-PC synapses). Because the PCs are uniquely at the confluence of retinal and vestibular inputs, cerebellar LTD is thought to be involved in vestibular-ocular reflex adaptation (VOR). The exact mechanisms underlying cGKI's effect on cerebellar LTD has yet to be precisely elucidated (Feil and Kleppisch 2008).

The hippocampus is important for what might be a uniquely mammalian form of memory (Hawkins et al. 2006) known as explicit or declarative memory. Implicit memory is thought to underlie memory for motor skills and task-specific functions that do not invoke the need for conscious recall of a past experience, whereas explicit memory is thought to involve a more epistemic form of recall of, for example, specific places, objects, or past experiences.

Several upstream components of the NO-cGMP-PKG signalling pathway have been implicated in hippocampal LTP and, indeed, hippocampal neurons are known to predominantly express cGKIβ (Feil et al. 2005). Thus, a number of studies have been devoted to elucidating the role of NO-cGMP-PKG signalling in hippocampal LTP in hopes of gaining a better understanding of the mechanisms underlying explicit memory (reviewed in Feil et al. 2005; Hofmann et al. 2006) The role of PKG in hippocampal LTP was first implied by the observation that, following weak tetanic stimulation, hippocampal LTP was enhanced or suppressed in the presence of PKG activators and inhibitors, respectively (Zhuo et al. 1994). While mounting evidence suggests that retrograde signalling of NO via Ca^{2+}/calmodulin is important

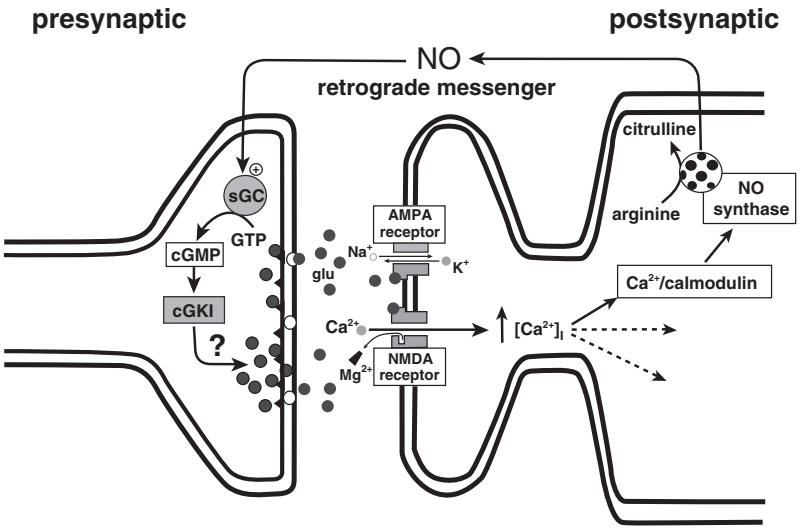

Fig. 2 A model of NO-cGMP-PKG signalling underlying LTP in Shaffer collateral/CA1 synapses of the hippocampus. Ca^{2+} influx into the postsynaptic neuron is mediated by NMDA receptors. An increase in Ca2+ concentrations in the postsynaptic neuron activates Ca^{2+}-calmodulin-dependent NOS. The retrograde action of NO then leads to increased neurotransmitter release in the presynaptic neuron via a NO-cGMP-PKG cascade (used with permission from Feil et al. 2005)

for the induction of LTP in Schaffer collateral/CA1 synapses of the hippocampus (Bolshakov and Siegelbaum 1995; Arancio et al. 2001), the downstream relationship to PKG has remained more elusive (Fig. 2). For instance, hippocampal LTP was found to be normal in mice lacking either one or both genes for cGKI and cGKII (Kleppisch et al. 1999).

More recent evidence suggests that PKG is an important target for NO/cGMP in this context given its effects on a protein synthesis-dependent late phase (L-LTP) form of LTP (Kleppisch et al. 2003). Using a hippocampus-specific cGKI knockout mouse line it was demonstrated that, while early-phase LTP (E-LTP) remained normal, L-LTP in the hippocampus was impaired following multiple episodes of strong theta burst stimulation. However, this synaptic effect does not appear to modulate the spatial learning and memory of the animal and L-LTP effects are only present in adult mice (Kleppisch et al. 2003). It remains to be seen, however, if this lack of correlation is due to the specific behavioural paradigms used in this study. That is to say, the effects of PKG on age-related L-LTP may extend to behaviours which have not yet been examined (Feil et al. 2005).

4 PKG and Stress

It is becoming increasingly clear that PKG signalling is important for modulating environmental stress. Although investigations of this type are emerging, PKG's roles in stress span developmental, physiological and behavioural phenotypes; however,

many of these biological influences may not be separable into discrete phenotypic outcomes. For instance, cGKIα expression patterns in the sensory cells of the dorsal root ganglion suggest that this isoform is involved in the development of sensory axons (Schmidt et al. 2002). Using cGKI-deficient mouse embryos, Schmidt et al. (2002) showed that PKG influences axonal branching of sensory neurons while also impairing nociceptive flexion reflexes. The influence of PKG signalling on nociceptive behaviour has focused primarily on NO and cGMP and the results have been mixed. Both positive and negative nociceptive responses have been attributed to NO-cGMP signalling (Tegeder et al. 2002; Vivancos et al. 2003). PKG has been more directly implicated in nociceptive behavioural responses in mammals using the response evoked by hind paw formalin injection. Conventional cGKI knockout mice were found to have lowered formalin-invoked licking behaviour than their wild-type counterparts (Tegeder et al. 2004). The elucidation of the precise role of NO-cGMP-PKG signalling in the nociceptive response is still in its infancy but the underlying mechanisms may be illuminated by existing research of *Aplysia* nociception (see Hofmann et al. 2006 for discussion).

Several lines of evidence have also implicated PKG in the behavioural effects of addictive drugs in mammals. Pharmacological manipulations which either stimulated cGMP production or activated PKG, as well as tissue specific overexpression of cGKIα by plasmid injection, were found to reduce cocaine-induced *egr-1* expression and also affect locomotor behaviour (Jouvert et al. 2004). These effects were reciprocally reversed by subsequent silencing or activation of PKG signalling following the initial treatment. Thus, NO-cGMP-PKG signalling is important for regulating cocaine-related effects on behaviour.

Similarly, NO-cGMP-PKG signalling has been implicated in anxiety-like behaviour as well as behavioural response to alcohol (Werner et al. 2004). When compared to wild-type mice, cGKII-deficient animals appeared to display augmented anxiety-like behaviour. Using a measure of the persistence of the hypnotic effect of ethanol, known as the loss of righting reflex, cGKII knockout mice were more resistant to the effects of ethanol. Furthermore, the cGKII mutants showed a greater preference for ethanol compared to wild-type mice (Werner et al. 2004).

Consistent with mammalian studies which suggest a role for PKG in stress resistance, recent studies of the *for* genetic variants in *D. melanogaster* suggest that a PKG pathway underlies synaptic response to thermal stress (Dawson-Scully et al. 2007). Cultured giant neurons and the larval neuromuscular junction (NMJ) of rovers and sitters were found to have dramatic physiologic polymorphisms (Renger et al. 1999). Particularly, cultured sitter neurons were found to have lower, more transient K^+ current conductance compared with the rover neurons. Pharmacological PKG inhibition of the rover neurons reduced K^+ conductance. Studies of heat shock-mediated protection of neural function in *Locusta migratoria* reported similar reductions in K^+ currents inducing thermotolerance of synaptic transmission (Ramirez et al. 1999) as well as central pattern generation (Newman et al. 2003).

Dawson-Scully et al. (2007) hypothesized that PKG acts as a regulator of neuronal thermotolerance and therefore mediates protection against heat-induced neuronal trauma. Both genetic and pharmacological manipulations indicate that

reductions in PKG or PP2A (protein phosphotase 2A) activity result in increased thermotolerance of synaptic transmission of the larval NMJ (Dawson-Scully et al. 2007). To assess the behavioural effects, *Drosophila* larvae were heated, along with their nutritive substrate, until cessation of larval moth hook movements was observed. Strains with lower PKG activity reached significantly higher temperatures before behavioural failure. Intriguingly, PKG or PP2A inhibition also provided strong thermotolerance of synaptic transmission in *D. melanogaster* and of a central circuit in *L. migratoria*, suggesting a conserved neuroprotective function of this pathway (Dawson-Scully et al. 2007) The role of PKG-PP2A signalling also appears to extend to other stress related pathways including hypoxia where low levels of PKG activity are, again, associated with increased tolerance to stress, in this case to anoxic conditions (Dawson-Scully pers. comm.).

5 PKG and Clocks

Circadian rhythmicity and molecular clock functions are highly conserved taxonomically and are important biological processes which interact with, or underlie, many physiological and behavioural processes (Dunlap et al. 2004). A number of studies have implicated kinases, including PKG, in the regulation of the circadian clock system (e.g. Comolli and Hastings 1999; Mathur et al. 1996; Tischkau et al. 2003; Agostino et al. 2007). In mammals, the suprachiasmatic nucleus (SCN), which is seated above the optic chiasm, receives retinal input from the optic nerve by means of the retinohypothalamic tract. The well-known feedback mechanism of the molecular clock is active in cells of the SCN where photosensory input entrains the circadian transcription/translation system (e.g. *Per, Tim, Clock, Bmal1*) to the organism's light/dark cycle.

NO-cGMP-PKG signalling has been implicated in circadian function by both pharmacological and genetic manipulations (reviewed in Golombek et al. 2004 and see also Hofmann et al. 2006). Indeed, significant phase delays of circadian rhythmicity can be caused by inhibition of clock-controlled increases in PKG activity (Tischkau et al. 2003). In mice, PKG appears to play a role in the modulation of the phase shift in which time-dependent exposure to light results in changes to the biological clock and of concomitant alterations in *mPer1* and *mPer2* expression (Oster et al. 2003). When compared to wild-type mice, differences in the onset of wheel running after a light pulse in cGKII-deficient mice suggested an aberration in circadian clock synchronization. While cGKII deficient mice were found to have 50% reductions in early night (CT14) phase delay compared to wild type mice, no difference in the late night (CT22) phase delay was found (Oster et al. 2003). Furthermore, Oster et al. (2003) found that the loss of cGKII resulted in a reduction of early night light-induced expression of *mPer2* while *mPer1* induction was elevated. cGKII was also implicated in phase advances of the diurnal domain of molecular clock function due to its observed effects on the positive feedback loop of the circadian transcription/translation system (i.e. *Bmal1* and *Clock*; Tischkau et al. 2004). SCN slice cultures were exposed to short-term cGKII inhibition using antisense

oligodeoxynucleotides which delayed rhythms of electrical activity as well as *Bmal1* mRNA production. Long-term inhibition of cGKII increased *Bmal1* mRNA and disrupted electrical activity rhythms. Pharmacological inhibition of cGKII resulted in the repetition of the last 3.5 hours of the cycle. Thus, PKG appears to be important for regulation and progression of the circadian cycle by influencing both the positive and negative arms of the clock feedback loop.

From a behavioural perspective, clock cellular mechanisms are an important part of the expression of various behaviours such as sleeping and feeding. Peripheral clocks, which reside outside of the SCN, can become asynchronous with the SCN due to environmental alterations. For example, when nocturnal animals are restricted to day-time feeding only, some peripheral clock organs become unsynchronized with the SCN and behaviour is altered to anticipate diurnal feeding times (Damiola et al. 2000; Stokkan et al. 2001; Horikawa et al. 2005). Interestingly, PKG has been implicated in the clock feedback mechanism of mammals, and in food intake in flies suggesting that food intake and circadian rhythms are intimately linked (Challet et al. 2003; Sarov-Blat et al. 2000; Lee et al. 2006; Mendoza et al. 2005; Challet 2007). Thus, PKG may play an important role in modulating complex composite behaviours through various signalling pathways of interacting circuits.

PKG has recently been implicated in clock-related behaviours in both *C. elegans* and *D. melanogaster* which have recently garnered a great deal of interest (Raizen et al. 2008). In mammals and flies it is called sleep; in worms it is called lethargus. The *C. elegans* homologue of *per* (LIN-42) is known to be expressed temporally with periods of lethargus. Raizen et al. (2008) demonstrated that lethargus has the crucial features which distinguish sleep, including reversibility, reduced-responsiveness, and homeostasis. Furthermore, they showed that PKG (*egl-4* and *for*) is a regulator of sleep-like behaviours in flies and worms. By comparing gain- and loss-of-function *egl-4* mutants, the authors demonstrated that PKG is associated with the extent of behavioural quiescence as well as its time-dependence. Raizen et al. (2008) then used the for^{R} and for^{s2} fly lines to ask whether the behavioural effect of PKG on sleep is evolutionarily conserved. And indeed, they found that the rover strain (for^{R}), with higher PKG activity, slept more than the sitter mutant on the rover genetic background (for^{s2}). Thus, in both species, PKG activity is positively associated with the amount of sleep that an animal displays.

6 Conclusion

While past studies of PKG biology were not as extensive as in other well-known kinases (Wang and Robinson 1997), recent research has shed light on its diverse roles in development, physiology and behaviour. Research into PKG and behaviour in invertebrate and mammalian models has progressed from rather divergent perspectives. Generally speaking, mammalian research has focused on elucidating neuronal PKG-related signalling pathways underlying behaviours. Concurrent invertebrate research has focused more on clarifying how natural genetic variation affecting PKG activity modifies behavioural phenotypes.

Commonalities emerging from many of these studies suggest that PKG is highly pleiotropic, acts through multiple modalities, and that its effects may be evolutionarily conserved. For instance, in flies, worms, and hymenopterans PKG affects food-related behaviours yet the phenotypic effects of PKG activity differs. This variation suggests that PKG function may have been utilized early on in metazoan evolution to affect food-related behavioural phenotypes while standing and/or new genetic variation has allowed for the specificities its effects to be modified in different taxa. Conversely, the effects of PKG on food-related behaviours may have evolved independently in these highly differentiated taxa. These hypotheses are becoming more testable with the increasing availability of genome sequences and bioinformatic tools.

The effects of PKG also appear to be modulated significantly by environmental context or stimuli, suggesting that this molecule is important for behavioural plasticity. This observation is supported by studies of the influence of PKG in the plasticity of caste-specific behaviours in hymenopterans (Ben-Shahar et al. 2002, 2003). Many interesting hypotheses emerge from these observations which are of interest to the evolution of behaviour and behavioural plasticity. For instance, one might ask whether biochemical properties of PKG distinguish it from other kinases allowing it to be more easily co-opted by selection for use in behavioural plasticity. One might also ask if the PKG-signalling that influences food-related behavioural plasticity functions along the same signalling pathways and homologous neural circuits in invertebrates.

Food-related behaviours present a good model for testing such hypotheses in invertebrates (Douglas et al. 2005). Nevertheless, comparisons across wider taxonomic gaps might also be approached by looking at PKG effects on learning and memory. PKG affects habituation, learning, and memory in flies, while also being involved in synaptic plasticity in mammals – i.e. processes that are thought to underlie learning and memory.

A model describing the role of PKG in modulating LTP in mammalian Schaffer collateral/CA1 synapses of the hippocampus suggests a critical role for NMDA receptors (NMDARs) (Fig. 2; Feil et al. 2005). Recently, NMDAR activation has been more directly implicated in mammalian learning through its effects on LTP and synaptic strengthening of the barrel cortex in the single-whisker experience protocol (Clem et al. 2007). Interestingly, NMDAR disruption in the ellipsoid body of *D. melanogaster*, a brain structure in which *for*-PKG is highly expressed, was shown to disrupt long-term memory consolidation of a Pavlovian olfactory learning paradigm (Wu et al. 2007). *for*'s presence in the ellipsoid body of *D. melanogaster*, a structure thought to be a control centre for a variety of behavioural outputs (Strauss and Heisenberg 1993), suggests that PKG may play an important role in the processing of information (Varnam et al. 1996; Belay et al. 2007). A recent paper examining operant learning indicated that PKG expression in the central complex is important for visual learning (Wang et al. 2008). Also, Mery et al. (2007) showed that PKG expression in the mushroom bodies is important for olfactory based associative learning. The tools used in these studies will allow investigators to determine whether PKG's influence on the cellular, synaptic and evolutionary bases of learning share common themes.

PKG affects development and physiology in both invertebrates and mammals but the implications of these effects for behaviour are still in early stages of understanding. Of primary importance to understanding the links between PKG and behaviour are descriptions of its interactive roles in development and of its distribution in tissues outside of the nervous system as well as in the peripheral and central nervous systems. Additionally, PKG may play important developmental roles in flies due to the lethality of several *for* alleles. Tools are now available to asses the relative influence of developmental and/or real-time gene expression of PKG on behaviour.

The use of increasingly well-known model organisms, advancements in sequencing and expression analyses, quantitative genetic approaches, neurophysiology, pharmacology and neuronal imaging are constantly advancing our understanding of behavioural effects of genetic architecture (Mackay 2004; Wagner et al. 2007), while great strides are being made in the understanding of the evolutionary processes which act on genetic variation for phenotypic traits (Mitchell-Olds et al. 2007). Exciting avenues for the future study of PKG and behaviour also include the functional analysis of enzymatic activity from an evolutionary perspective (Dean and Thornton 2007). Studies examining PKG signalling and behaviour in diverse taxa hold a great deal of potential in this regard.

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