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A cGMP-Dependent Protein Kinase Gene, foraging, Modifies Habituation-Like Response Decrement of the Giant Fiber Escape Circuit in Drosophila

Jeff E. Engel,1,3 Xian-Jin Xie,1 Marla B. Sokolowski,2 and Chun-Fang Wu1

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Protein kinases play key roles in the activity-dependent modulation of neuronal activity and morphology. Interest in the cGMP-dependent serine/threonine kinase, or PKG, has grown with the awareness of the diversity of biochemical pathways that involve cGMP (Koesling et al. 1991; Garbers 1992; Sheth et al. 1997; Wang and Robinson 1997; Moon et al. 1998; Simpson et al. 1999). PKG has been shown to influence characteristics involved in both functional and developmental plasticity of neural circuits (Zhuo et al. 1994, 1999; Lev-Ram et al. 1997; Wu et al. 1998b; Calabresi et al. 1999; Lewin and Walters 1999; Renger et al. 1999; Yawo 1999). Despite this, there has been little direct evidence that PKG actually affects learning (but see Bernabeu et al. 1997). Here, we have taken a genetic approach to show that altered levels of PKG are associated with the modulation of a simple form of response modification in an identified escape reflex pathway in intact flies.

In Drosophila, one form of PKG (known as dg2; Kalderon and Rubin 1989) is encoded by the foraging gene (Osborne et al. 1997), which takes its name from a behavioral phenotype, the degree of locomotion while feeding, indicated by larval and adult foraging trail lengths (Sokolowski 1980; de Belle and Sokolowski 1987; de Belle et al. 1989; Pereira and Sokolowski 1993). Two naturally occurring variants, forR (“rovers”, with long foraging trails) and forS (“sitters”, with short foraging trails), have high and low PKG levels, respectively (Osborne et al. 1997). The genetic dissection of learning and memory in the fly Drosophila melanogaster has given significant insights into molecular and cellular mechanisms that underlie neural and behavioral plasticity (Dudai 1988; Griffith et al. 1994; Tully et al. 1994; DeZazzo and Tully 1995; Heisenberg et al. 1995; Davis 1996; Wolf et al. 1998; Wu et al. 1998a). At least two classes of molecules, second messengers and ion channels, have been implicated (Wu et al. 1998a). The aforementioned studies have been based on laboratory-induced mutations that cause extreme modifications of specific molecules and severe defects in behavioral phenotypes. The study of more modest genetic variants, such as polymorphisms found in nature (Greenspan 1997; Sokolowski 1998), may give in-
sights that apply more directly to understanding the control of behavior in natural populations. This study used naturally occurring genetic variants along with mutated foraging alleles to examine the role of PKG in regulating the habituation-like response decrement of an escape response pathway in flies.

Habituation is a form of nonassociative learning in which a behavioral response is reduced or disappears with repeated stimulation (Thompson and Spencer 1966). Non-associative conditioning is of interest as a simple manifestation of physiological mechanisms that also may underlie more complex associative learning paradigms (e.g., Fitzgerald et al. 1990). Habituation may be mediated by a variety of mechanisms, including homosynaptic depression (Castellucci and Kandel 1974; Thompson and Glanzman 1976) and extrinsic inhibition (Krasne and Teshiba 1995). Habituation is phylogenetically widespread (Thompson and Spencer 1966; Castellucci and Kandel 1974; Boulis and Sahley 1988; Rankin et al. 1990; May and Hoy 1991; Krasne and Teshiba 1995) and has functional significance in modulating both the gain and sensitivity of behavioral responses (Fischer and Carew 1993; Bäßler and Nothof 1994; Engel and Hoy 1999).

The Drosophila giant fiber pathway that mediates the visually induced startle reflex, a jump-and-flight escape response, has been studied extensively at the levels of neural physiology and development (Koenig and Ikeda 1980; Tanouye and Wyman 1980; Strausfeld and Bassemir 1983; Wyman et al. 1984; Engel and Wu 1992; Sun and Wyman 1995; Trimarchi and Schneiderman 1995; Lin and Nash 1996; Allen et al. 1998; Blagburn et al. 1999; Kawasaki and Ordway 1999). The response can be evoked by electrical stimulation to the brain in an intact animal, and this has allowed us to bypass visual input and focus on central and motor stages of neural processing in an intact, behaviorally relevant circuit. The response likelihood diminishes with repeated electrical stimulation. This response decrement shows most of the typical characteristics of behavioral habituation (Thompson and Spencer 1966) including frequency dependence, strength dependence, habituation beyond zero response, spontaneous recovery, faster rehabilitation, dishabituation, and habituation of dishabituation (Engel and Wu 1996, 1998). Because electrical stimulation recruits the escape response circuit after initial stages of sensory processing, this report refers to modification patterns resembling “habituation” and “dishabituation” as “response decrement” and “evoked recovery,” respectively. Nevertheless, conformity to the characteristics of a widely studied learning paradigm makes the giant fiber response a useful model for genetic analyses of behavioral plasticity and its physiological correlates at the circuit level (Engel and Wu 1996, 1998). This approach has provided evidence that Drosophila mutants defective in associative learning paradigms (in genes affecting cAMP metabolism [Davis 1996; Dubnau and Tully 1998] and K+ channels [e.g., Griffith et al. 1994; Wu et al. 1998a]) also display abnormal response decrement of the giant fiber response in a habituation protocol (Engel and Wu 1996, 1998).

In this work, we found that the rate of response decrement is correlated with PKG activity and foraging behavior: decrement of the electrically induced response was most rapid in genotypes previously shown to have low PKG activity and sitter-like foraging behavior. We also found differences in spontaneous recovery from response decrement during a rest from stimulation and in dishabituation-like recovery evoked by a novel stimulus (a puff of air). Our data suggest that these differences in spontaneous recovery and evoked recovery may be secondary consequences of differing rates of response decrement. This indicates the interdependence of multiple processes of plasticity in stimulus-dependent response decrement of the giant fiber response.

The data further raise the possibility that two processes with different time courses contribute to the response decrement.

Overall, our results show that PKG affects habituation-like response decrement in an identified neural circuit of intact tethered flies. From this we can hypothesize that PKG also may be involved in other forms of learning. We previously showed that cAMP signaling pathways, which play an essential role in associative learning in flies (Davis 1996; Dubnau and Tully 1998), also affect stimulus-dependent decrement of the giant fiber response (Engel and Wu 1996). The present results suggest that modulation of the escape response could involve the counterbalancing of multiple second messenger systems. We have defined a new adult-stage phenotype of the foraging locus. Finally, we have shown that behaviorally relevant neural plasticity in an identified circuit can be influenced by a single-locus genetic polymorphism.

RESULTS

By using different kinds of electrical and visual stimuli, the giant fiber response can be triggered at different points in the pathway in intact tethered flies. As we have shown previously (Engel and Wu 1996), long-latency and short-latency responses are initiated by different electrical stimulus voltages (Fig. 1). The long-latency response shows response decrement and evoked recovery similar to habituation and dishabituation, respectively. These changes are attributable to afferent pathways in the brain. The short-latency response allows us to examine properties of signal conduction and transmission in identified neurons and synapses.

Stimulus-Dependent Response Decrement

We examined the response decrement of the long-latency giant fiber response induced by electrical stimulation,
which bypasses the initial stages of visual processing to recruit afferents to the descending giant fibers (Fig. 1; Engel and Wu 1996; Trimarchi and Schneiderman 1993). Rates of response decrement were strongly affected by allelic variation in the foraging gene (Figs. 2 and 3; Table 1). Sitter stocks showed more rapid response decrement than rovers in comparisons between the two artificially induced alleles or the two natural alleles. The most dramatic difference was between alleles generated artificially by P-element insertion and excision. for189Y showed more rapid response decrement than any other line in this study. The abundance of foraging PKG is quite low in for189Y (Osborne et al. 1997; Y. Ben Shahar and M.B. Sokolowski, unpubl.). In contrast, forE1 showed scarcely any response decrement at the standard stimulation frequency of 5 Hz (Fig. 2). In fact, some forE1 flies could be driven at stimulus rates of 30 Hz or higher without showing failures. forE1 arose by excision of the P-element from the foraging locus in for189Y; rover behavior and high abundance of PKG are restored in forE1 relative to for189Y.

More subtle differences were observed between the naturally occurring alleles. forR flies showed more rapid response decrement than forS (Fig. 3). Flies homozygous for each of the two foraging alleles forR and forS differ in their degrees of PKG activity (Osborne et al. 1997). forR is genetically dominant to forS for the larval foraging phenotype (de Belle and Sokolowski 1987) but intermediate for adult foraging (Pereira and Sokolowski 1993). As was the case for adult foraging behavior, heterozygous F1 progeny (forR/forS) showed a rate of response decrement intermediate between the parental stocks (Fig. 3; Table 1), suggesting semidominance for this response modification phenotype.

The experiments described in this article were conducted within a single year (1999). The forR and forS stocks also were tested in this habituation-like protocol in 1996. In these earlier tests, the absolute resistance to response decrement was greater for both genotypes than in 1999, but forS again showed more rapid response decrement than forR (data not shown). Similarly, repeated measurements of larval and adult foraging behavior have shown that it is the relative differences between rovers and sitters, not the absolute mean behavioral scores, that are maintained across tests performed at different times or in different laboratories (for discussion, see Sokolowski 1992).

Spontaneous Recovery and Recovery Evoked by a Novel Stimulus

We next observed differences in spontaneous recovery from decrement of the long-latency electrically induced response. Flies first were stimulated to a response decrement criterion of five consecutive failures (indicating a low response likelihood). One measure of spontaneous recovery is the response likelihood for the first stimulus given after 5 sec of rest (Fig. 4, initial values of dashed curves). A 5-sec rest period is ordinarily sufficient for the response likeli-
hood to return to nearly 100\%, even in genotypes with very rapid response decrement (Engel and Wu 1996, 1998). Full recovery of the response was observed for forR/fors flies as well as forR/fors heterozygotes (Fig. 4A). However, forR/fors flies did not recover fully in 5 sec (Fig. 4B).

A second measure of recovery is the resistance to response decrement within a subsequent stimulus episode. This was quantified as the number of responses evoked after a treatment (airpuff or sham) as a ratio of the first 20 stimuli of the bout, repeated 5x for each treatment (see Fig. 7). Evoked recovery indices were averaged across flies, including all trials (all flies) or only those trials in which the Test (airpuff) index was more than double the Control (sham treatment) index ($T > 2 \cdot C$).

**Table 1. Stimulus-Dependent Decrement and Evoked Recovery of the Electrically Induced Giant Fiber Response**

<table>
<thead>
<tr>
<th>Rate of decrement( ^a )</th>
<th>Evoked recovery index( ^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment $⇒$ Test (air puff)</td>
</tr>
<tr>
<td></td>
<td>All flies: $n$</td>
</tr>
<tr>
<td></td>
<td>(T $&gt; 2 \cdot C$: $n$</td>
</tr>
</tbody>
</table>

\( ^a \)Response decrement rate is indicated by number of stimuli to reach a criterion of five consecutive failures (5 Hz stimulus rate). Response decrement rates are shown as geometric means (i.e., log transformed) with 95% confidence interval (CI) and as medians with interquartile range.

\( ^b \)Evoked recovery index is the number of responses to 20 stimuli given after a treatment (airpuff or sham) as a ratio of the first 20 stimuli of the bout, repeated 5x for each treatment (see Fig. 7). Evoked recovery indices were averaged across flies, including all trials (all flies) or only those trials in which the Test (airpuff) index was more than double the Control (sham treatment) index ($T > 2 \cdot C$).

<table>
<thead>
<tr>
<th>for( ^{11} )</th>
<th>897.4 (6) (677.6–1185.8)</th>
<th>1000 (6) (1000–1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-test</td>
<td>0.0001</td>
<td>—</td>
</tr>
<tr>
<td>for( ^{189} )</td>
<td>18.1 (8) (4.5–72.8)</td>
<td>10 (8) (7.5–17.5)</td>
</tr>
<tr>
<td>for( ^{189} ) t-test</td>
<td>$p = 0.0001$</td>
<td>—</td>
</tr>
<tr>
<td>for( ^{189} )</td>
<td>84.9 (15) (41.0–176.2)</td>
<td>78 (15) (30.5–288.25)</td>
</tr>
<tr>
<td>for( ^{K} )</td>
<td>22.6 (7) (11.5–44.6)</td>
<td>23 (7) (15.5–33.75)</td>
</tr>
<tr>
<td>for( ^{t} )</td>
<td>48.9 (10) (27.4–87.3)</td>
<td>48.5 (10) (29.0–59.0)</td>
</tr>
<tr>
<td>ANOVA</td>
<td>$p = 0.04$</td>
<td>—</td>
</tr>
</tbody>
</table>

**Fiber Response**

Stimulus-Dependent Decrement and Evoked Recovery of the Electrically Induced Giant Fiber Response

Fiber response showed a similar ranking pattern, with the greatest degrees of recovery after 30- and 120-sec intervals being shown by rapidly decrementing genotypes.

The slight degree of spontaneous recovery between 30 and 120 sec (Fig. 5) suggests that, in addition to a short-term component of response decrement that recovers in less than 30 sec, there is also a long-term component of response decrement with slower onset and recovery kinetics that becomes stronger over multiple stimulus bouts and recovers with a time course exceeding 120 sec. In previous work, 30- or 120-sec recovery intervals were tested after a single prior stimulus bout (in different groups of flies), and with that protocol the recovery to first-bout response decrement rates was nearly complete (Engel and Wu 1996, 1998). In the present experiments, each fly received four stimulus bouts separated by intervals of 5, 30, and 120 sec, so that 30- and 120-sec recovery intervals were tested after two or three prior stimulus bouts (instead of one prior bout as in the earlier studies). It appears that additional prior stimulus bouts affected the state of the response pathway even though every bout ended with a consistent response decrement criterion of five failures.

A slowly developing component of response decrement could be most apparent in slowly decrementing flies, because they are exposed to a greater number of stimuli in the two or three bouts preceding the recovery interval. Consistent with this, the lowest 30- and 120-sec recovery indices were shown by the most slowly decrementing geno-
types (Fig. 5A, inset). To examine this relationship more directly, normalized recovery indices for individual flies of all genotypes were plotted against the total number of stimuli given in bouts before the recovery interval (Fig. 6). After 30- or 120-sec intervals, recovery indices were inversely related to the number of prior stimuli (Fig. 6B,C). This relationship was most evident for the range of 50 to 300 prior stimuli, suggesting that this slow component of response decrement became saturated after 300 stimuli and that other factors contributed more to response variation with fewer than 50 prior stimuli. After the shortest recovery interval of 5 sec, the relationship was weak (Fig. 6A). This suggests that recovery from a short-term process of response decrement is the predominant factor during the first 5 sec after the end of a bout.

The potential to distinguish multiple components of habituation-like response decrement in this system will require further study. Here, it is most important to note that for genotypes showed differences in recovery when tested under a consistent protocol (Figs. 4 and 5).

Recovery of the long-latency giant fiber response can be evoked by a novel stimulus such as an airpuff in a dishabituation protocol (Engel and Wu 1996, 1998). Clear evoked recovery could be shown in each strain except for 189Y (Fig. 7; Table 1). The number of responses for the 20 stimuli after an airpuff or “sham puff” (each averaged from five repetitions) was divided by the number of responses at the beginnings of bouts, giving test and control scores, respectively (Table 1). The operational criterion for evoked recovery was a test score greater than double the control score (test >2 controls). Evoked recovery was observed most often in slowly decrementing genotypes (forR and forR/fors; Table 1). Among flies that did show evoked recovery by this definition, the magnitude of recovery (the test score) was also greatest in slowly decrementing genotypes (Table 1).

Few forE1 flies showed response decrement to five-failure criterion at the standard stimulation frequency of 5 Hz (Fig. 2). However, with higher stimulus frequencies forE1 flies did display habituation-like response decrement, characterized by synchronous loss of responses in DLM (Dorsal Longitudinal Muscle) and TTM (Tergotrochanteral Muscle), spontaneous recovery, and recovery evoked by an airpuff (data not shown).

**Latency and Refractory Period**

Latency and refractory period are indicators of the integrity of neural connectivity and signal transmission in the giant fiber pathway (Gorczyca and Hall 1984; Baird et al. 1990; Nelson and Wyman 1990; Kawasaki and Ordway 1999). Two response classes, evoked by different stimulus voltages, give information about different parts of the circuit. Weak stimuli evoke a long-latency response by recruiting afferent neurons upstream of the giant fibers, whereas stronger stimuli trigger a short-latency response by directly activating the giant fibers (Fig. 1; Engel and Wu 1996). The long-latency response can reveal properties of connections in the brain that do not contribute to the short-latency response. The thoracic portion of the circuit (activated in both long- and short-latency responses) can give information about how mutations affect neural functioning within a network of identified neurons. The TTM branch has a single electrochemical neuronal synapse onto the TTM motoneuron (King and Wyman 1980; Allen et al. 1999; Blagburn et al. 1999), whereas the DLM branch includes two synapses, an apparent electrochemical synapse of the cervical giant fiber onto the peripherally synapsing interneuron (PSI) neuron (Blagburn et al. 1999) and cholinergic synapses of the PSI onto the DLM motoneurons (Gorczyca and Hall 1984; Fig. 1).

We found that response latencies differed between forE1 and for189Y for the long-latency response but not the...
short-latency one (Table 2). Latency (but not refractory period or response decrement in a habituation protocol) is significantly influenced by ambient temperature (Engel and Wu 1996). We tested the response latencies for forE1 and for189Y under similar temperature conditions and during the same period of days. Response latencies did not differ when other genotypes were compared (Table 2).

The twin-pulse refractory period of the short-latency response, mediated in the thoracic portion of the giant fiber pathway (Fig. 1), has proven to be a sensitive indicator of deficits in basic physiological properties such as transmitter processing and ion channel function (Gorczyca and Hall 1984; Nelson and Wyman 1990; Engel and Wu 1992). Short-latency response refractory periods were not significantly affected by allelic variation at the foraging locus (Table 2).


dated in the afferent portion of the pathway (Fig. 1), is an indicator of properties of the brain portion of the circuit (Engel and Wu 1996, 1998). The long-latency refractory period tended to be shorter in genotypes with slower stimulus-dependent response decrement. This is most clear when forE1 and for189Y are compared (Table 2).

It is interesting that forE1 and for189Y showed differences in response properties that were restricted to the afferent portion of the neural pathway, because these stocks showed an extreme difference in response decrement in the habituation protocol, which also is mediated in the afferent portion of the pathway. Despite these differences, it is clear that the giant fiber pathway is fundamentally sound in all the foraging genotypes tested. The extreme effects on response latency or short-latency refractory period that have been reported using mutations affecting ion channels or synaptic integrity (Gorczyca and Hall 1984; Nelson and Wyman 1990; Engel and Wu 1992; Kawasaki and Ordway 1999) were not found in genotypes differing in PKG activity.

**DISCUSSION**

**Stimulus-Dependent Response Decrement Is Modified by foraging**

The genetic dissection of learning and memory in the fly *D. melanogaster* has given significant insights into molecular and cellular mechanisms that underlie neural and behavioral plasticity (Dudai 1988; Griffith et al. 1994; Tully et al. 1994; DeZazzo and Tully 1995; Heisenberg et al. 1995; Davis 1996; Wolf et al. 1998; Wu et al. 1998a). At least two classes of molecules, second messengers and ion channels, have been implicated (Wu et al. 1998a). Our results strongly indicate that the foraging PKG affects habituation-like response decrement in the electrically induced giant fiber response.

Artificially induced alleles (forE1 and for189Y) defined the influence of PKG in response decrement of the giant fiber response, and more modest naturally occurring genetic variants (forR and forS) showed similar but more subtle effects. In comparisons between different genotypes at the PKG foraging locus, response decrement was slower in genotypes with more abundant PKG (forE1 and forR) than in genotypes with less abundant PKG (for189Y and forS). It is interesting that rate of response decrement, response latency, and refractory period were all more extreme in forE1 than the wild rover genotype forR (Tables 1 and 2). It is possible that imprecise excision of the Pele-
ment from for189Y resulted in a more highly expressing allele in forE1 than the original parental for allele from which for189Y arose. Sequencing of forE1, currently in progress, should help to resolve this possibility. Differences in rate of response decrement followed a semidominant mode of inheritance as shown by forE1/fors heterozygotes. Semidominant inheritance also has been reported for the adult rover and sitter foraging phenotypes (Pereira and Sokolowski 1993).

Spontaneous Recovery and Evoked Recovery Are Influenced by foraging

Recovery results (Figs. 4–6) indicate that foraging affects spontaneous recovery from stimulus-dependent response decrement. The results also imply the existence of distinct components of this habituation-like response decrement with different kinetics of onset and recovery that could partly account for genetic differences in recovery phenotypes. A long-term component of response decrement is suggested by the similarity of recovery indices after either 30- or 120-sec recovery intervals (Fig. 5A, inset). For those intervals, recovery of the resistance to subsequent response decrement is correlated with the number of stimuli that were given before the recovery rest interval (Fig. 6B,C).

Sitter genotypes with low PKG expression showed the greatest recovery of resistance to response decrement after 30- and 120-sec intervals (Fig. 5A, inset). However, these flies also showed more rapid response decrement in initial stimulus bouts (Figs. 2 and 3) and experienced fewer stimuli in all bouts before recovery testing (Fig. 6), and in consequence may have had less exposure to a long-term component of response decrement. Therefore, differences in rates of response decrement may have contributed indirectly to the observed genetic differences in recovery indices for 30 and 120 sec (Fig. 5A, inset). This would not preclude the possibility that PKG also could play a role in physiological processes that underlie spontaneous recovery per se.

Early recovery after stimulus-dependent response decrement appears to be dominated by a short-term component of response decrement. Recovery indices increased substantially between 5 and 30 sec after ending the preceding stimulus bout (Fig. 5A, inset), and response likelihood did not recover to 100% after 5 sec in some genotypes (Fig. 4B; Engel and Wu 1996). Response likelihood for the first stimulus following a 5-sec recovery interval showed complete recovery in forR and forE (Fig. 4A) but did not recover completely in for189Y flies (Fig. 4B), which showed the most rapid response decrement in this.
study (Fig. 2) and have low PKG expression (Osborne et al. 1997). In contrast to the recovery of resistance to subsequent response decrement (discussed above), this genetic effect could not be a consequence of differences in exposure to a long-term response decrement process, because for189Y flies actually experienced the smallest numbers of stimuli before the 5-sec recovery interval (Figs. 5A and 6A). This result suggests that PKG may facilitate recovery of the likelihood of responding to a single stimulus after prior response decrement.

Evoked recovery in a dishabituation protocol was weakest in the most rapidly decrementing foraging genotypes (Fig. 7; Table 1). These results may point to a direct involvement of PKG pathways in evoked recovery. Alternatively, a more rapid rate of response decrement in sitter genotypes could have reduced evoked recovery in an indirect manner as follows. Assuming an equivalent activation of recovery processes by an airpuff in all genotypes, more rapid response decrement after the puff could diminish the amount of recovery observed. Furthermore, because a standard decrement criterion of five consecutive failures preceded the puff in all genotypes (Fig. 7), a rapid rate of “latent” response decrement during the five criterion stimuli could induce a deeper level of response decrement for the circuit to recover from at the time of the airpuff.

Our results suggest that the foraging PKG could affect the observed levels of spontaneous recovery and evoked recovery in part through altering the rate of stimulus-dependent response decrement. Similar correspondences between response decrement rates, spontaneous recovery, and evoked recovery may be seen for cAMP metabolic mutants (see. Fig. 5 and Table 2 of Engel and Wu 1996). This highlights the interrelatedness of these three processes in the giant fiber system. One goal for the future is to determine the extent to which these phenomena can be altered independently by mutations and thus may involve independent molecular mechanisms.
Afferent Latency and Refractory Properties Are Affected by foraging Differences were seen in the response latencies and refractory periods of the forE1 and for189Y genotypes. These effects were seen in the long-latency response but not the short-latency response, indicating that they are mediated in the afferent or brain segment of the giant fiber pathway in which habituation-like response decrement also is mediated (Engel and Wu 1996, 1998). However, response decrement rate and long-latency refractory period may not be functionally related. Earlier studies with mutations affecting cAMP cascades (Engel and Wu 1996) and K+ channels (Engel and Wu 1998) have not shown a strong correlation between response decrement in a habituation protocol and refractory period. Moreover, flies bearing Shaker and ether a`go-go K+ channel mutations have refractory periods comparable to forE1 but show much more rapid response decrement (Engel and Wu 1998). Our previous studies (Engel and Wu 1992, 1996, 1998) indicated that the thoracic portion of the giant fiber pathway may have qualitatively normal characteristics even in genotypes with very rapid response decrement. Consistent with this, short-latency refractory periods and latencies did not differ between foraging genotypes, even the very rapidly decrementing genotype for189Y.

Involvement of PKG in Neural Function and Plasticity Our results associate high PKG expression with a slow rate of response decrement in a habituation protocol but do not indicate the mechanism underlying this association. PKG may play a direct role in plasticity, either by down-regulating a physiological process that underlies response decrement or by enhancing a concomitant process of response sensitization as in a dual process model (Groves and Thompson 1970). Alternatively, high PKG expression could influence response decrement in a less direct manner by modifying the physiological or developmental context in which it occurs. For instance, if PKG enhanced basic properties of neural conduction or synaptic transmission so that the neural signal were stronger to begin with, then it could take longer for normally functioning mechanisms underlying stimulus-dependent response decrement to lead to failed responses. Enhancement of neural response properties would be consistent with the forE1 phenotype of shortened latency and refractory period of the long-latency response (Table 1), parameters that are mediated in the afferent part of the giant fiber pathway just as habituation-like response decrement is.

PKG appears to affect such basic functional properties differently in different parts of the fly nervous system. Variation in foraging genotype did not affect latency or refractory period of the short-latency (giant-fiber-evoked) responses, for both DLM (flight) and TTM (jump) muscles. Refractory periods were also measured for long- and short-latency responses, and are expressed as geometric means with 95% confidence interval.

Table 2. Giant Fiber Response Parameters

<table>
<thead>
<tr>
<th></th>
<th>Response latencya</th>
<th>Refractory periodsb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LL response</td>
<td>SL response</td>
</tr>
<tr>
<td></td>
<td>DLM</td>
<td>TTM</td>
</tr>
<tr>
<td>forE1</td>
<td>3.21 (8)</td>
<td>2.74 (8)</td>
</tr>
<tr>
<td>±0.29</td>
<td>±0.21</td>
<td>±0.14</td>
</tr>
<tr>
<td>for189Y</td>
<td>3.51 (9)</td>
<td>3.17 (9)</td>
</tr>
<tr>
<td>±0.29</td>
<td>±0.32</td>
<td>±0.08</td>
</tr>
<tr>
<td>t-test (p value)</td>
<td>0.05</td>
<td>0.006</td>
</tr>
<tr>
<td>forR</td>
<td>3.54 (18)</td>
<td>2.98 (19)</td>
</tr>
<tr>
<td>±0.52</td>
<td>±0.30</td>
<td>±0.41</td>
</tr>
<tr>
<td>for+</td>
<td>3.53 (12)</td>
<td>3.17 (12)</td>
</tr>
<tr>
<td>±0.22</td>
<td>±0.19</td>
<td>±0.18</td>
</tr>
<tr>
<td>forR/for+</td>
<td>3.37 (15)</td>
<td>3.01 (15)</td>
</tr>
<tr>
<td>±0.22</td>
<td>±0.16</td>
<td>±0.15</td>
</tr>
<tr>
<td>ANOVA (p value)</td>
<td>0.39</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Latencies were measured for long-latency (brain-evoked) and short-latency (giant-fiber-evoked) responses, for both DLM (flight) and TTM (jump) muscles. Refractory periods were also measured for long- and short-latency responses, and are expressed as geometric means with 95% confidence interval.
observations suggest a widely distributed role for PKG in the nervous system of flies (Renger et al. 1999).

We have identified several gene loci that influence habituation-like decrement of the giant fiber response, with products that include adenylyl cyclase (rutabaga) and cAMP phosphodiesterase (dance; Engel and Wu 1996). K+ channel subunits with distinct physiological properties including voltage activation (Shaker, ether à go-go), calcium activation (slowpoke), and channel modulation (Hyperkineletic; Engel and Wu 1998), and now PKG (foraging). Like the cAMP pathway genes that affect learning (Nighorn et al. 1994; Davis 1996; Dubnau and Tully 1998), foraging has pleiotropic effects with potential fitness consequences (Hughes and Sokolowski 1996; Sokolowski et al. 1997; Wingoove and O’Farrell 1999). This pleiotropy is paralleled at the cellular level in which these gene products have diverse molecular targets and actions. PKG serine/threonine kinases have numerous targets that could affect neuronal function and growth, such as ion channels (Stockand and Sansom 1996; Carrier et al. 1997; Taguchi et al. 1997; Alioua et al. 1998; Han et al. 1998; Vaandrager et al. 1998; Wexler et al. 1998). ATPases (e.g., Uneyama et al. 1998), and regulators of gene expression (Gudi et al. 1997; Idriss et al. 1999). PKG may interact with other second messenger systems such as PKA, either by regulating such other systems (Moon et al. 1998) or by phosphorylating common targets (Lengyel et al. 1999). It is interesting that mutations of dance that increase cAMP abundance lead to more rapid stimulus-dependent response decrement (Engel and Wu 1996), opposite to the effect of increased PKG activity in foraging rover genotypes.

A picture thus has emerged in which the molecular mechanisms that underlie response decrement in a habituation paradigm, like other neural plasticity such as LTP, are influenced by multiple biochemical and genetic factors. The redundancy of pathways influencing response modification therefore could allow habituation of the escape behavior to be modified and fine-tuned over the course of generations for more adaptive matching to ecologically relevant stimuli. An important point is that the foraging locus is known to be polymorphic in wild populations. This suggests that habituation of escape could vary among flies in a natural population. The foraging locus may be part of the genetic architecture through which plasticity and sensitivity of the escape response have been fine-tuned over evolutionary time.

**MATERIALS AND METHODS**

**Fly Stocks**

We examined several naturally occurring and genetically altered alleles of foraging. Two naturally occurring alleles were tested: forE1 is the rover allele isolated and described initially (Sokolowski 1980; de Belle and Sokolowski 1987) and w/forr is a sitter stock used as a host for transformations in previous work (Osborne et al. 1997). for189Y, a sitter allele, resulted from a P-element insertion into the foraging locus (Osborne et al. 1997). The corresponding rover allele, for45+, arose from for189Y by excision of the P-element (Osborne et al. 1997). Thus, forE1 and forr lines harbor the natural allelic variations, and for189Y was derived from for45+ forr, for45+ and for189Y were all in a w (white) background, and for189Y also carried a minu+ insert. Giant fiber assays are performed in darkness to increase the consistency of the response (Engel and Wu 1996). Under these conditions, eye color does not appear to affect response decrement of the electrically induced giant fiber response or any of the other physiological parameters measured in the present study (J.E. Engel and C-F. Wu, unpubl.).

**Physiology and Behavior**

Methods for the giant fiber assay are described in Engel and Wu (1996). Briefly, adult flies were held on ice for 20–30 min before being tethered to a wire mount that was glued behind the neck of the fly; the legs then were waxed into flight position. Trials were performed in darkness. Stimulating voltage pulses (0.1 ms duration) were given with electrodes in the eyes, and action potentials in flight (DLM) and jump (TTM) muscles were recorded with tungsten electrodes in the thorax. The descending giant fibers conduct signals from sensory afferents in the brain to motor outputs in the thorax, recruiting the TTM motoneuron through an electrochemical synapse and the DLM motoneurons via a disynaptic pathway that includes the PSI interneuron (King and Wyman 1980; Tanouye and Wyman 1980). The giant fiber pathway can be triggered at different points by different stimulus voltages (Fig. 1), giving rise to response classes distinguished by latency (Engel and Wu 1996). Long-latency stimulus voltages were 0.4–0.6 V below the threshold for the next-shorter response latency class (intermediate latency or short latency; Engel and Wu 1996). Response latency and refractory period were measured as described previously (Engel and Wu 1992, 1996). The long-latency response refractory period can be influenced by stimulus voltage (J.E. Engel and C-F. Wu, unpubl.). Consequently, refractory periods reported here were measured at stimulus voltages 0.6–1.0 V below the ceiling of the long-latency stimulus range, as in previous work (Engel and Wu 1996, 1998).

Each fly was tested once using habituation, recovery, and dishabituation protocols, referred to here as “response decrement,” “spontaneous recovery,” and “evoked recovery.” Rates of response decrement were tested at a stimulus frequency of five pulses per sec. A stimulus bout ended when the fly attained a standardized response decrement criterion of five consecutive failures. Flies not attaining this criterion within 1000 stimuli were given a stimulusto-criterion score of 1000 (Engel and Wu 1996). In flies that did reach five failures, spontaneous recovery was tested by giving three additional stimulus bouts after recovery intervals of 5, 30, and 120 sec. Evoked recovery then was tested in 10 stimulus bouts separated by 30-sec intervals, beginning 30–120 sec after the last bout of the recovery test. In five of these bouts, an airpuff directed to the head was given after five-failure response decrement criterion (Engel and Wu 1996), followed by 20–40 additional stimuli to detect any evoked recovery. The other five bouts were sham controls with no airpuffs but with 20–40 stimuli after five-failure criterion.

**Statistics**

Data were analyzed with two-tailed ttest or ANOVA using StatView 5.0 for Macintosh (SAS Institute). Refractory periods and scores for number of stimuli to reach five-failure criterion were logtransformed before analysis to improve normality (Engel and Wu 1996).
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