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Research

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

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The *Drosophila* giant fiber jump-and-flight escape response is a model for genetic analysis of both the physiology and the plasticity of a sensorimotor behavioral pathway. We previously established the electrically induced giant fiber response in intact tethered flies as a model for habituation, a form of nonassociative learning. Here, we show that the rate of stimulus-dependent response decrement of this neural pathway in a habituation protocol is correlated with PKG (cGMP-Dependent Protein Kinase) activity and foraging behavior. We assayed response decrement for natural and mutant rover and sitter alleles of the *foraging* (*for*) gene that encodes a *Drosophila* PKG. Rover larvae and adults, which have higher PKG activities, travel significantly farther while foraging than sitters with lower PKG activities. Response decrement was most rapid in genotypes previously shown to have low PKG activities and sitter-like foraging behavior. We also found differences in spontaneous recovery (the reversal of response decrement during a rest from stimulation) and a dishabituation-like phenomenon (the reversal of response decrement evoked by a novel stimulus). This electrophysiological study in an intact animal preparation provides one of the first direct demonstrations that PKG can affect plasticity in a simple learning paradigm. It increases our understanding of the complex interplay of factors that can modulate the sensitivity of the giant fiber escape response, and it defines a new adult-stage phenotype of the foraging locus. Finally, these results show that behaviorally relevant neural plasticity in an identified circuit can be influenced by a single-locus genetic polymorphism existing in a natural population of Drosophila.

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.

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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, for^R ("rovers", with long foraging trails) and for^s ("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-

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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). Nonassociative 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 rehabituation, 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,

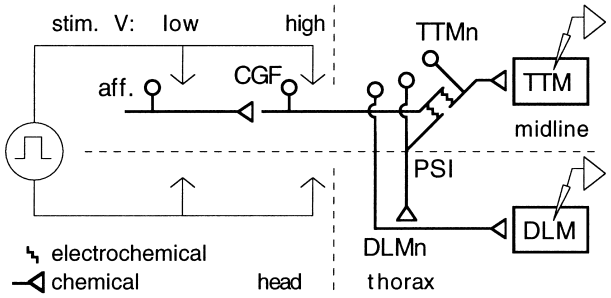

Figure 1 Schematic of giant fiber pathway (one side shown). High-voltage electrical brain stimulation (high) excites the cervical giant fiber (CGF) to evoke a short-latency response, whereas low-voltage stimulation (low) excites brain afferents (aff.) to trigger a long-latency response. (TTM) Tergotrochanteral muscle; (TTMn) TTM motoneuron; (DLM) dorsal longitudinal muscle; (DLMn) DLM motoneuron; (PSI) peripherally synapsing interneuron.

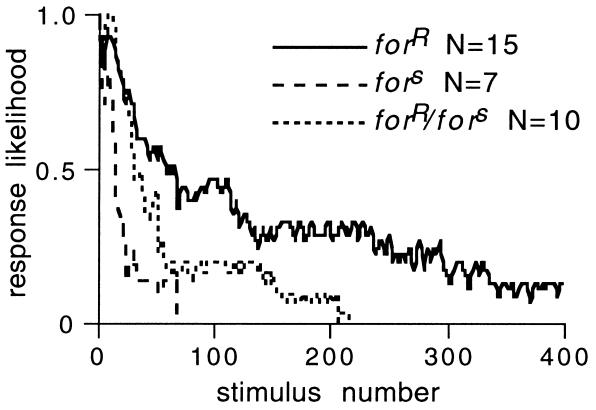

Figure 3 Response decrement in for^R, for^s, and for^R/for^s heterozygotes.

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. for^{189Y} showed more rapid response decrement than any other line in this study. The abundance of *foraging* PKG is quite low in for^{189Y} (Osborne et al. 1997; Y. Ben Shahar and M.B. Sokolowski, unpubl.). In contrast, for^{E1} showed scarcely any response decrement at the standard stimulation frequency of 5 Hz (Fig. 2). In fact, some for^{E1} flies could be driven at stimulus rates of 30 Hz or higher without showing failures. for^{E1} arose by excision of the P-element from the *foraging* locus in for^{189Y}; rover

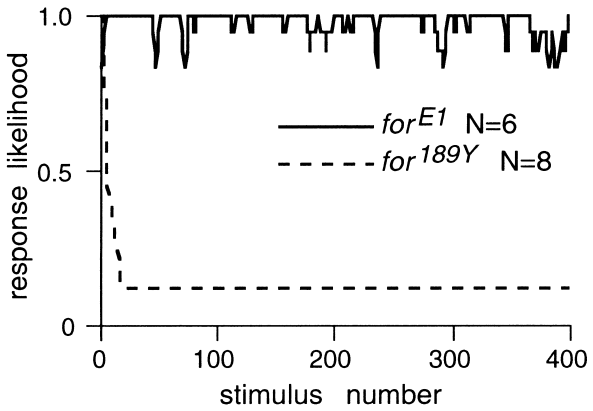

Figure 2 Giant fiber response decrement in a habituation paradigm in for^{E1} and for^{189Y} flies. Long-latency response likelihood declined as a function of stimulus number (5-Hz stimulation). Response likelihoods were averaged among flies and smoothed (running average, three-stimulus window).

behavior and high abundance of PKG are restored in for^{E1} relative to for^{189Y}.

More subtle differences were observed between the naturally occurring alleles. for^s flies showed more rapid response decrement than for^R (Fig. 3). Flies homozygous for each of the two *foraging* alleles for^R and for^s differ in their degrees of PKG activity (Osborne et al. 1997). for^R is genetically dominant to for^s 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 F_1 progeny (for^R/for^s) 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 for^R and for^s 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 for^s again showed more rapid response decrement than for^R (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-

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

	Rate of decrement[a]		Evoked recovery index[b]		
	Geo. mean (n) 95% CI	Median (n) quartiles 1–3	Treatment ⇒ All flies: n (T > 2 · C): n	Test (air puff) Mean ± S.D. Mean ± S.D.	Control (sham) Mean ± S.D. Mean ± S.D.
for^{E1}	897.4 (6) (677.6–1185.8)	1000 (6) (1000–1000)	ND	ND	ND
for^{189Y}	18.1 (8) (4.5–72.8)	10 (8) (7.5–17.5)	4	0.02 ± 0.04	0.02 ± 0.05
t-test	$p = 0.0001$		0	—	—
for^{R}	84.9 (15) (41.0–176.2)	78 (15) (30.5–288.25)	9 4	0.27 ± 0.24 0.35 ± 0.25	0.20 ± 0.22 0.11 ± 0.10
for^{s}	22.6 (7) (11.5–44.6)	23 (7) (15.5–33.75)	5 1	0.08 ± 0.09 0.21	0.03 ± 0.05 0.00
for^{R}/for^{s}	48.9 (10) (27.4–87.3)	48.5 (10) (29.0–59.0)	5 3	0.19 ± 0.18 0.30 ± 0.11	0.03 ± 0.02 0.03 ± 0.01
ANOVA	$p = 0.04$				

[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 5× 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 ($T > 2 \cdot C$).

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 for^{R} and for^{s} flies as well as for^{R}/for^{s} heterozygotes (Fig. 4A). However, for^{189Y} 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 before reaching the five-failure decrement criterion during stimulus bouts delivered after different recovery intervals (Fig. 5). The resistance to response decrement integrates performance over the entire stimulus bout rather than just the first stimulus. Note that Figure 4 shows the kinetics of response decrement after 5-sec recovery, for the same data as Figure 5. The *for* genotypes showed clear differences in their degree of recovery to initial rates of response decrement (Fig. 5). Absolute postrecovery response numbers were highest for for^{R}, the most slowly decrementing stock, and were progressively lower for more rapidly decrementing genotypes (Fig. 5A). However, when mean response numbers were divided by first-bout response numbers to give normalized recovery indices (Fig. 5A, inset), this ranking was reversed: the highest recovery indices were shown by rapidly decrementing sitter genotypes, particularly after 30- and 120-sec recovery intervals. When postrecovery response scores were log-transformed, effectively normalizing the results within genotypes while retaining scale differences between genotypes (Fig. 5B), the kinetic profiles of recovery 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-

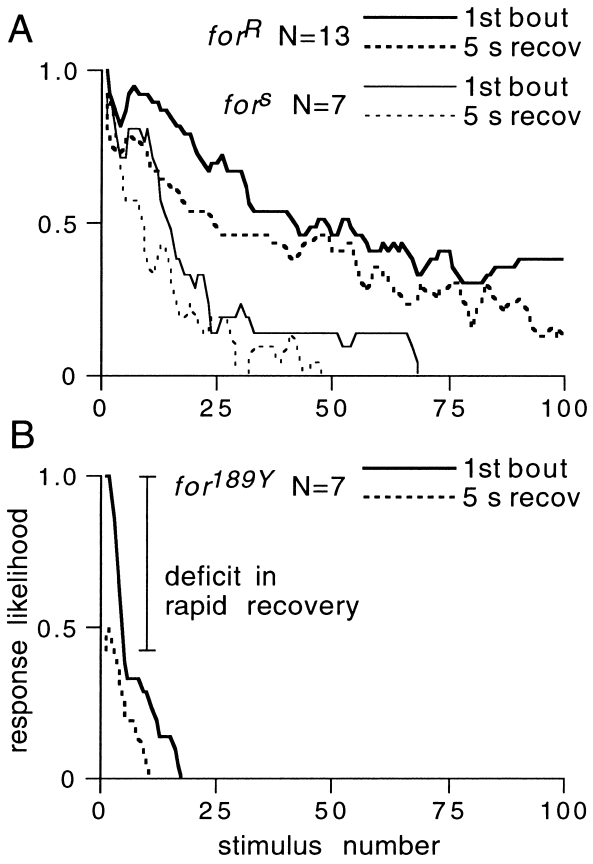

Figure 4 Spontaneous recovery from response decrement in *for-aging* strains. After response decrement to five-failure criterion (first bout), each fly rested for 5 sec before resuming stimulation (5 s recov). The response likelihood for the first stimulus of the recovery bout approached 100% in for^R and for^s (*A*) but not for^{189Y} (*B*). The for^{E1} stock was not tested for recovery because few of those flies attained response decrement to five-failure criterion with 5-Hz stimulation.

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 · control). Evoked recovery was observed most often in slowly decrementing genotypes (for^R and for^R/for^s; 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 for^{E1} flies showed response decrement to five-failure criterion at the standard stimulation frequency of 5 Hz (Fig. 2). However, with higher stimulus frequencies for^{E1} 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 for^{E1} and for^{189Y} for the long-latency response but not the

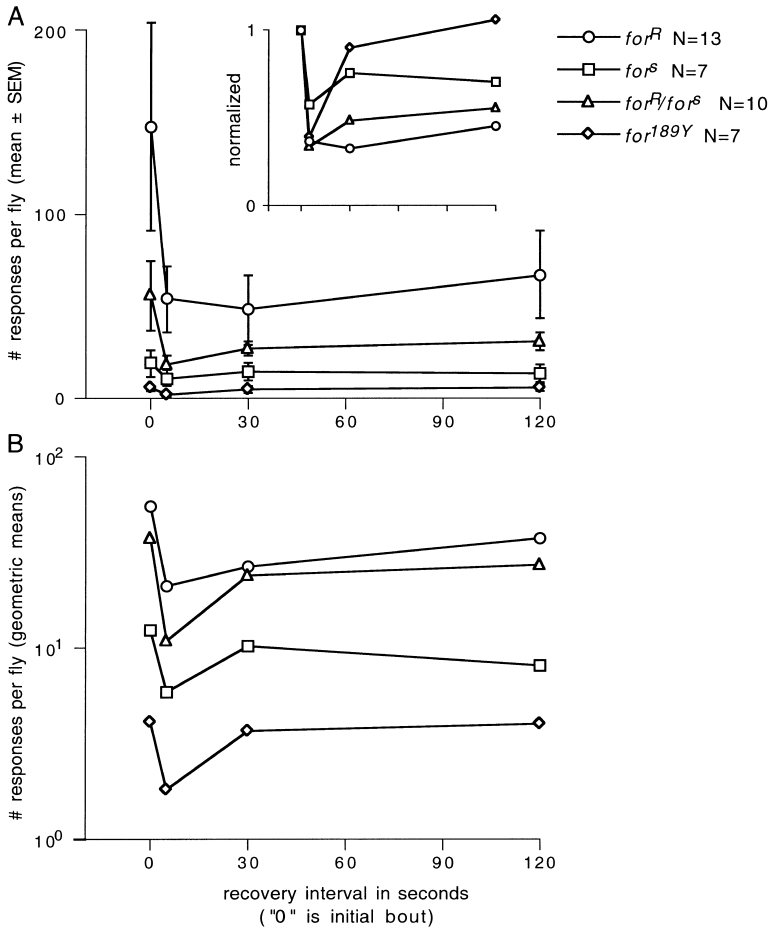

Figure 5 Spontaneous recovery quantified as the number of responses before reaching response decrement criterion in four successive bouts separated by 5-, 30-, and 120-sec recovery intervals. (*A*) Arithmetic means with SEM. Inset shows the same means, normalized to initial bout values. It is clear that proportional recovery after 30 and 120 sec was greatest in the flies with most rapid response decrement. (*B*) Log transformed data (i.e., geometric means). Before log transformation, flies that displayed no responses after 5-sec recovery (three for^{189Y} and one for^{R}) were assigned scores of 1 response.

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 for^{E1} and for^{189Y} 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). The refractory period of the long-latency response, mediated 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 for^{E1} and for^{189Y} are compared (Table 2).

It is interesting that for^{E1} and for^{189Y} 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 (for^{E1} and for^{189Y}) defined the influence of PKG in response decrement of the giant fiber response, and more modest naturally occurring genetic variants (for^{R} and for^{s}) 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 (for^{E1} and for^{R}) than in genotypes with less abundant PKG (for^{189Y} and for^{s}). It is interesting that rate of response decrement, response latency, and refractory period were all more extreme in for^{E1} than the wild rover genotype for^{R} (Tables 1 and 2). It is possible that imprecise excision of the P-ele-

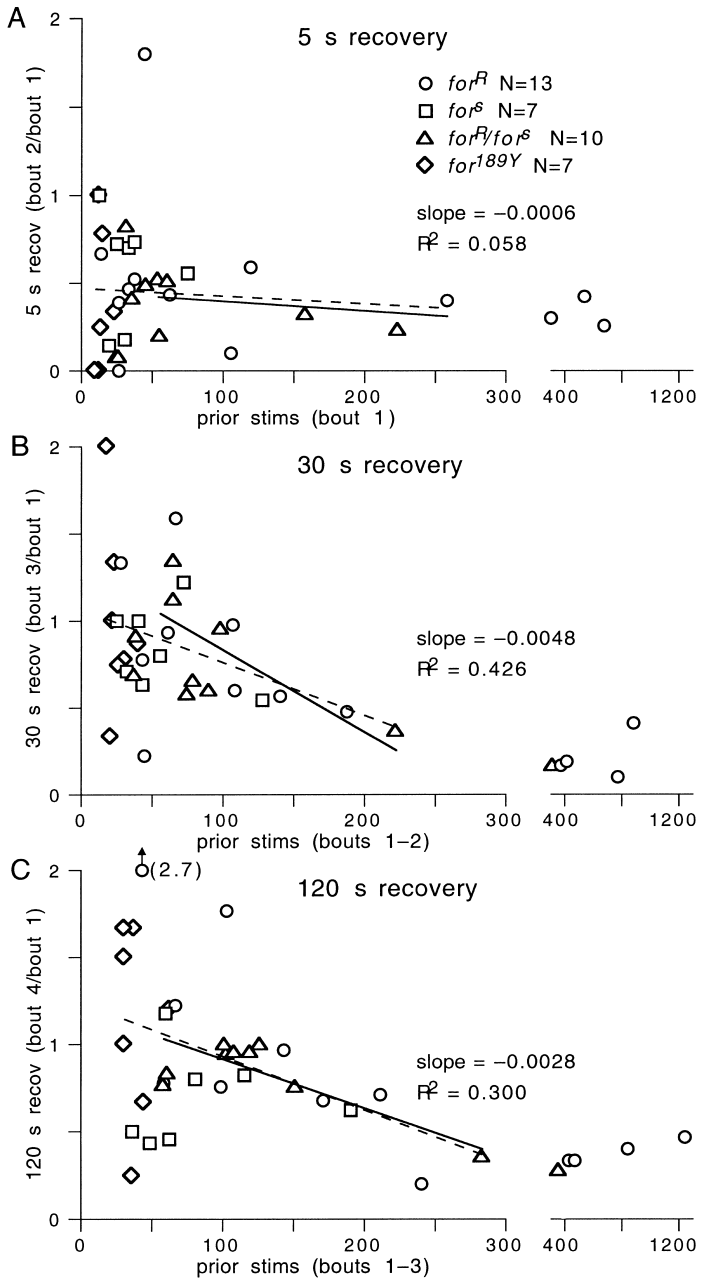

Figure 6 Spontaneous recovery from response decrement was affected by the number of stimuli given previously, for recovery intervals of 30 or 120 sec (s). Each fly was tested once for each of three recovery intervals: bout 1 was the initial stimulus bout of a trial, and bouts 2, 3, and 4 followed after 5-, 30-, and 120-sec recovery intervals (same trials as Fig. 5). Vertical axis shows normalized recovery (number of responses in recovery bout divided by initial bout, as for Fig. 5*A* [inset] but plotting individual flies). Horizontal axis is the cumulative number of stimuli delivered in all bouts before the recovery interval being tested. (*A*) Recovery after 5 sec. (*B*) Recovery after 30 sec. (*C*) Recovery after 120 sec. Lines show best-fit regressions, combining all four genotypes to emphasize the effect of stimulus number independent of genotype. (Solid lines) 50–300 prior stimuli; slopes and R2 are shown; (dashed lines) 0–300 prior stimuli; slopes and R2 as follows: −0.0005, 0.006 (*A*); −0.0031, 0.157 (*B*); −0.0031, 0.160 (*C*).

ment from for^{189Y} resulted in a more highly expressing allele in for^{E1} than the original parental *for* allele from which for^{189Y} arose. Sequencing of for^{E1}, currently in progress, should help to resolve this possibility. Differences in rate of response decrement followed a semidominant mode of inheritance as shown by for^R/for^s 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 for^s and for^R (Fig. 4A) but did not recover completely in for^{189Y} flies (Fig. 4B), which showed the most rapid response decrement in this

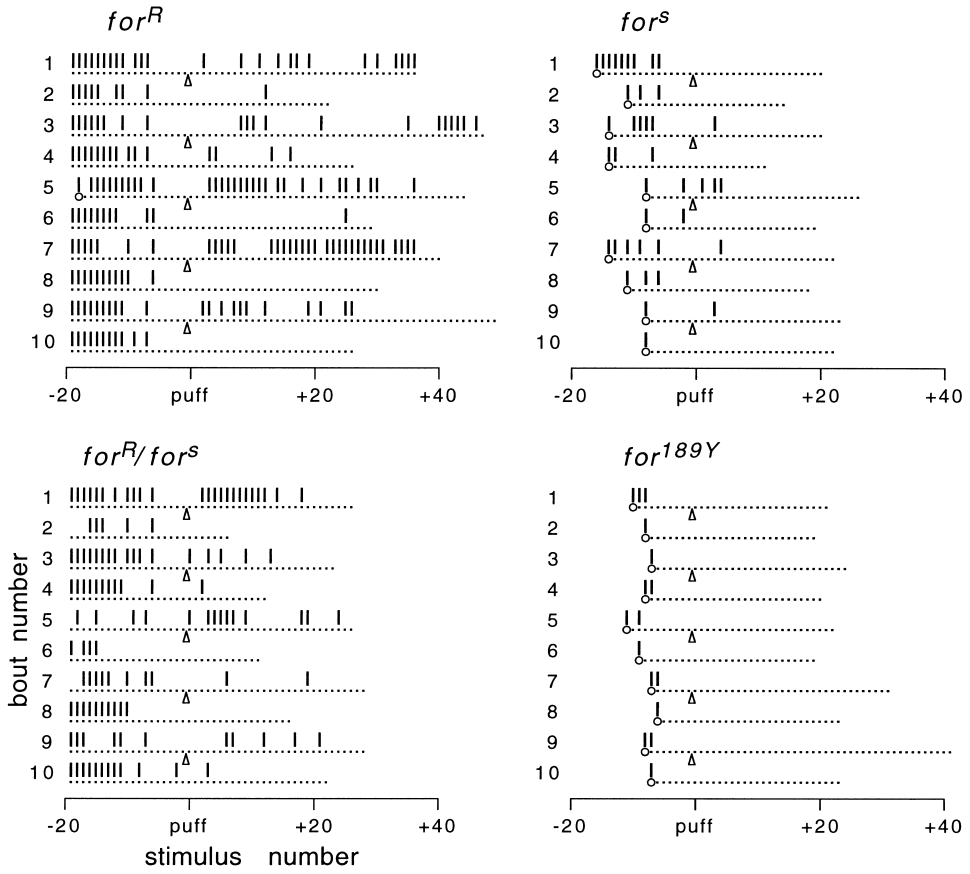

Figure 7 Evoked recovery induced by airpuffs in a dishabituation paradigm. Each example is from a single fly tested ten times (30 sec between tests). Dots indicate stimuli and ticks show long-latency responses. Circles indicate the first stimulus of the bout; in the more slowly decrementing *for*^{R} and *for*^{R}/*for*^{s}, most bouts began to the left of these plots. In odd numbered bouts, airpuffs (arrows) were given after response decrement to five failures; even numbered bouts serve as sham controls. For comparison, each sham bout is aligned with the preceding test bout according to the last response before response decrement criterion. After response decrement to criterion, each bout was allowed to continue for 20–50 stimuli to allow any evoked recovery to be seen. The examples shown were among the strongest for each genotype (see Table 1).

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 *for*^{189Y} 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.

Table 2. *Giant Fiber Response Parameters*

	Response latency[a]				Refractory periods[b]		
	LL response		SL response			SL response	
	DLM	TTM	DLM	TTM	LL response (both muscles)	DLM	TTM
for^{E1}	3.21 (8) ±0.29	2.74 (8) ±0.21	1.13 (8) ±0.14	0.70 (8) ±0.14	29.6 (6) 16.4–53.3	2.72 (3) 1.72–4.30	3.78 (4) 2.07–6.87
for^{189Y}	3.51 (9) ±0.29	3.17 (9) ±0.32	1.08 (9) ±0.08	0.76 (9) ±0.07	97.5 (7) 46.1–205.6	2.61 (5) 2.08–3.28	3.24 (6) 2.95–3.56
t-test (*p* value)	0.05	0.006	0.40	0.32	0.01	0.78	0.35
for^{R}	3.54 (18) ±0.52	2.98 (19) ±0.30	1.29 (18) ±0.41	0.77 (19) ±0.14	59.7 (10) 34.1–104.7	3.89 (10) 3.40–4.46	3.44 (9) 3.08–3.84
for^{s}	3.53 (12) ±0.22	3.17 (12) ±0.19	1.27 (12) ±0.18	0.86 (12) ±0.14	89.3 (9) 58.8–148.3	3.66 (8) 2.80–4.79	3.11 (9) 2.67–3.62
for^{R}/for^{s}	3.37 (15) ±0.22	3.01 (15) ±0.16	1.19 (15) ±0.15	0.80 (15) ±0.08	36.2 (10) 25.4–51.6	4.88 (8) 4.10–5.81	3.16 (9) 3.07–3.27
ANOVA (*p* value)	0.39	0.10	0.60	0.17	0.02	0.06	0.30

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.

Afferent Latency and Refractory Properties Are Affected by *foraging*

Differences were seen in the response latencies and refractory periods of the for^{E1} and for^{189Y} 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 à go-go* K_+ channel mutations have refractory periods comparable to for^{E1} 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 for^{189Y}.

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 for^{E1} 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 response (Table 1), parameters that are mediated in the thoracic portion of the pathway (Fig. 1). Moreover, reduced PKG activity in sitter genotypes is associated with hyperexcitability and enhanced nerve terminal sprouting at larval neuromuscular junctions and with reduced K_+ currents and increased membrane excitability in a significant population of neurons in dissociated embryonic cultures (Renger et al. 1999). These

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 (*dunce*; 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 (*Hyperkinetic*; 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; Wingrove 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 *dunce* 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: for^{R} is the rover allele isolated and described initially (Sokolowski 1980; de Belle and Sokolowski 1987) and *w*;for^{s} is a sitter stock used as a host for transformations in previous work (Osborne et al. 1997). for^{189Y}, a sitter allele, resulted from a *P*-element insertion into the *foraging* locus (Osborne et al. 1997). The corresponding rover allele, for^{E1}, arose from for^{189Y} by excision of the *P*-element (Osborne et al. 1997). Thus, for^{R} and for^{s} lines harbor the natural allelic variations, and for^{E1} was derived from for^{189Y}. for^{s}, for^{E1}, and for^{189Y} were all in a *w* (*white*) background, and for^{189Y} also carried a *miniw*+ 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 stimuli-to-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 *t*-test or ANOVA using StatView 5.0 for Macintosh (SAS Institute). Refractory periods and scores for number of stimuli to reach five-failure criterion were log-transformed before analysis to improve normality (Engel and Wu 1996).

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