

Neuronal Polymorphism among Natural Alleles of a cGMP-Dependent Kinase Gene, *foraging*, in *Drosophila*

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Natural variation in neuronal excitability and connectivity has not been extensively studied. In *Drosophila melanogaster*, a naturally maintained genetic polymorphism at a cGMP-dependent protein kinase (PKG) gene, *foraging* (*for*), is associated with alternative food search strategies among the allelic variants *Rover* (*for*^R; higher PKG activity) and *sitter* (*for*^S; lower PKG activity). We examined physiological and morphological variations in nervous systems of these allelic variants isolated from natural populations. Whole-cell current clamping revealed distinct excitability patterns, with spontaneous activities and excessive evoked firing in cultured *sitter*, but not *Rover*, neurons. Voltage-clamp examination demonstrated reduced voltage-dependent K⁺ currents in *sitter* neurons. Focal recordings from synapses at the larval neuromuscular junction demonstrated spontaneous activity and supernumerary discharges with increased transmitter release after nerve stimulation. Im-

munolabeling showed more diffuse motor axon terminal projections with increased ectopic nerve entry points in *sitter* larval muscles. The differences between the two natural alleles was enhanced in laboratory-induced mutant alleles of the *for* gene. The pervasive effects of the *for*-PKG on neuronal excitability, synaptic transmission, and nerve connectivity illustrate the magnitude of neuronal variability in *Drosophila* that can be attributed to a single gene. These findings establish the consequences in cellular function for natural variation in an isoform of PKG and suggest a role for natural selection in maintaining variation in neuronal properties.

Key words: *Drosophila*; foraging; neuronal polymorphism; natural variation; cultured neurons; neuromuscular junction; membrane excitability; K⁺ currents; cGMP-dependent protein kinase; focal recording; patch clamping

Variation in neuronal excitability and connectivity within natural populations demonstrated in breeding and selection studies (Bently and Hoy, 1970; Goodman, 1977) has not been linked to identified genes, although some naturally occurring behavioral variations have been attributed to single mendelian loci (de Belle and Sokolowski, 1987; de Belle et al., 1989; Sawyer et al., 1997; de Bono and Bargmann, 1998; Sokolowski, 1998). Recently, two naturally occurring alleles of a single gene, *foraging* (*for*), have been shown to confer distinct strategies of food-searching behavior in *Drosophila melanogaster* (Osborne et al., 1997). The *for* gene is one (*dg2*) of the two identified genes (Kalderon and Rubin, 1989) known to encode cGMP-dependent protein kinase (PKG) in *Drosophila* (Osborne et al., 1997). Larvae of the natural *sitter* allele and laboratory-induced *sitter* mutants travel shorter distances while feeding and have lower PKG enzyme activities in their nervous systems than do their *Rover* counterparts (Osborne et al., 1997). These behavioral differences suggest variation in physiological or morphological properties of neurons.

Such an evolutionarily maintained polymorphism in the *for* locus provides the opportunity to examine the extent to which natural variations in neuronal physiology and connectivity stem from allelic variation of a major gene. We applied whole-cell patch-clamp analyses to cultured embryonic central neurons to study effects of the *for*-PKG on neuronal excitability. We used the "giant" neuron culture, which offered an effective *in vitro* system for studying intrinsic membrane properties and enabled us to circumvent technical difficulties associated with physiological recordings from small *Drosophila* neurons (Wu et al., 1990; Saito and Wu, 1991; Zhao and Wu, 1997). Furthermore, *in situ* studies were performed on the well characterized larval preparation to evaluate differences in synaptic transmission and in stereotypical projection patterns of motor axons onto identified body wall muscle fibers (Jan and Jan, 1976a,b; Budnik et al., 1990; Zhong and Wu, 1991; Wang et al., 1994). Our results demonstrate that natural polymorphism in the *for* gene causes remarkable neuronal variation in membrane excitability and the underlying K⁺ cur-

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rents, as well as in synaptic activities and nerve terminal morphology. These differences in neuronal phenotypes found in naturally occurring alleles were further enhanced in laboratory-induced mutant *sitter* alleles.

Parts of this paper have been published previously in abstract form (Renger et al., 1997).

MATERIALS AND METHODS

Fly stocks. The *for* strains used in this work have been described previously (de Belle et al., 1989; Osborne et al., 1997). We found that the natural and all mutant *sitter* alleles display mild, but noticeable, ether-induced leg-shaking behaviors that were clearly distinguishable from *Rover*. *Sh^M* is a null *Sh* mutant (Zhao et al., 1995). The *ShD* transgenic lines used in this study were generated by P-element insertion of a vector containing *ShD* cDNA fused to a hsp-70 promoter into the first, second, or third chromosome in the *Sh^M* genetic background (Zhao et al., 1995). All stocks were grown on standard medium and were raised at 20–22°C.

Cell culture and whole-cell recording. The procedure for culturing *Drosophila* “giant” neurons and the whole-cell patch-clamp technique have been reported previously (Wu et al., 1990; Saito and Wu, 1991; Zhao and Wu, 1997; Yao and Wu, 1999a). Briefly, gastrulae embryos were homogenized and suspended in Schneider medium (Life Technologies, Grand Island, NY) supplemented with 200 ng/ml insulin (Sigma, St. Louis, MO), 20% fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin. After washing, cells were resuspended in the above medium containing 2 μg/ml cytochalasin B (Sigma) and plated on glass coverslips. Cultures were maintained in humidified chambers at room temperature for 2–3 d before recording. Recording bath solution (Jan and Jan, 1976a) contained (in mM): 128 NaCl, 2 KCl, 4 MgCl₂, 1.8 CaCl₂, and 35.5 sucrose, buffered with 5 HEPES at pH 7.1. Patch pipettes were filled with solution containing (in mM): 144 KCl, 1 MgCl₂, 0.5 CaCl₂, and 5 EGTA, buffered with 10 HEPES, pH 7.1. K⁺ currents were isolated by adding tetrodotoxin (TTX) (0.2 μM) and Cd²⁺ (0.2 mM) to the bath solution. In pharmacological experiments that test the PKG modulation on K⁺ currents, the PKG inhibitor guanosine 3',5'-cyclic monophosphothioate, 8-(4-chloro-phenylthio)-Rp-isomer (Rp-8-pCPT-cGMPS) (Calbiochem, La Jolla, CA), was applied to the bath after control recordings. All recordings were obtained at room temperature from isolated neurons with a patch-clamp amplifier (Axopatch 1B; Axon Instruments, Foster City, CA). Data acquisition and analysis were performed using pClamp software (Axon Instruments), and continuous data were stored on a frequency modulation tape recorder (Store 4D; Lockheed Electronics, Plainfield, NJ).

Heat-shock protocol for ShD current induction. Expression of transgenic *ShD* channels in cultured *Sh^M* host neurons was induced by exposure to 38.5°C for 30 min (Zhao et al., 1995). For isolation of the heat shock-dependent *ShD* currents, a twin-pulse protocol was used to extract the fast inactivating ($\tau < 20$ msec), slow recovering ($t_{1/2} \approx 5$ sec) A-type *ShD* current by subtracting the currents elicited by the second pulse from that elicited by the first pulse delivered 1 sec apart (Zhao et al., 1995).

Extracellular focal recording. Postfeeding third instar larvae were dissected in Ca²⁺-free bath saline (see above). The neuromuscular junction was visualized with differential interference contrast optics through a 40× water immersion objective on an upright compound microscope (Zeiss, Jena, Germany). Focal recording electrodes were pulled from glass capillary tubes (75 μl, 1.5 mm outer diameter; VWR Scientific, West Chester, PA) on a pipette puller (model pp-83; Narishige, Tokyo, Japan) and then polished and bent on a microforge (model de fonbrune; Aloe Scientific, St. Louis, MO). The recording bath solutions (Jan and Jan, 1976a) contained Ca²⁺ at concentrations specified for different experiments. Focal electrodes had an inner diameter from 4 to 8 μm and were filled with extracellular solution. Boutons recorded were from type I terminal branches on muscle 13, of abdominal segment 3 (Zhong et al., 1992; Wang et al., 1994). The segmental nerves were stimulated at the cut end with a suction electrode (5–10 μm inner diameter). Focal recordings (Dudel, 1977) were made with a loose patch-clamp amplifier (model 8510; Zeitz Instruments, Munich, Germany) and stored on video cassette recording tapes with a pulse code modulator (Neuro-Corder DR-384; Neuro Data, New York, NY). Seal resistances were determined to correct for attenuation in synaptic current amplitude caused by leakage at the pipette tip (Stühmer et al., 1983; Renger, 1997). Data analysis was performed with the software Axograph (versions 2.0 and 3.0; Axon Instruments), and for presentation, some traces were digitally filtered at 1 kHz.

Confocal microscopy. The procedures for immunostaining and confocal microscopy have been described previously (Wang et al., 1994). Postfeeding third instar larvae were dissected in Ca²⁺-free saline and immediately immersed in Bouin's fixative. Preparations were washed in 0.1 M phosphate buffer, pH 7.2, with 0.2% Triton X-100 (PBT) for 20 min, three to five repetitions. Rabbit anti-HRP conjugated to FITC (Cappel, Durham, NC) was applied at 1:100 in PBT to preparations overnight in a humidified chamber and were washed again in PBT for three repetitions of 20 min each. Preparations were then mounted on glass slides in Vectashield H-1000 (Vector Laboratories, Burlingame, CA). Confocal scanning laser microscopy was performed on a Bio-Rad (Hercules, CA) MRC model 600 or 1200 in conjunction with an upright microscope equipped with a 60× oil immersion lens. Serial images were overlaid using the accompanying Bio-Rad software.

RESULTS

Neuronal firing properties and K⁺ current amplitude in natural and mutant *for* alleles

We examined neuronal phenotypes of the two naturally occurring alleles, *for^R* and *for^S*, as well as a number of *sitter* mutant alleles of the *for* locus (Osborne et al., 1997). The giant neuron culture system was used to examine the extent of phenotypic variation in neuronal membrane properties between *Rover* and *sitter* neurons. A hallmark of neurons cultured from *sitter* variants, with reduced PKG activity, was membrane hyperexcitability. Spontaneous nerve firing occurring in the absence of stimulation was observed in 36% of *for^S* neurons examined (Fig. 1a). In addition, supernumerary, aftershock nerve spikes were evident after cessation of current injections in 18% of *for^S* neurons (Fig. 1b). Significantly, such events were absent in all *for^R* neurons examined (Fig. 1). The mutant allele *for^{S2}*, induced on a *for^R* genetic background, has a significantly lower PKG enzyme activity level than the natural *for^S* allele (Osborne et al., 1997). This mutant allele displayed even greater hyperexcitability than the naturally occurring *sitter* (*for^S*). Spontaneous and supernumerary action potentials were found in 42% of *for^{S2}* neurons (Fig. 1).

For an initial exploration of the ionic basis underlying the contrast in membrane excitability, we undertook voltage-clamp studies of voltage-activated outward K⁺ currents, which could be readily measured in cultured giant neurons. We found striking differences in K⁺ currents between *Rover* and *sitter* neurons. Inward Na⁺ currents were eliminated by TTX and inward Ca²⁺ currents and outward Ca²⁺-activated K⁺ currents were abolished by Cd²⁺ added to the saline (Saito and Wu, 1991; Zhao et al., 1995; Yao and Wu, 1999a). Depolarizing voltage pulses from a holding potential of −80 mV were used to elicit voltage-activated K⁺ currents, which demonstrate a transient peak of fast-inactivating current, followed by a sustained current plateau (Fig. 2a). Clear differences in both components of the K⁺ current were apparent between the *Rover* and *sitter* neurons (Fig. 2a,b). The more excitable *for^S* neurons demonstrated significantly lower levels of both peak and sustained outward currents compared with *for^R*. A more striking contrast in neuronal phenotypes was found when the natural *for^R* allele was compared with the mutant allele *for^{S2}* [maximum peak and sustained conductances in *for^R*, 523.9 ± 51.9 and 254.4 ± 28.8 pS/pF (mean ± SEM; n = 29); *for^S*, 410.0 ± 41.5 and 205.6 ± 20.2 pS/pF (n = 27); p < 0.05; *for^{S2}*, 347.0 ± 37.2 and 159.0 ± 13.2 pS/pF (n = 24); p < 0.05] (Fig. 2b). The half-activation voltages of the peak (but not sustained) K⁺ currents in *for^S* and *for^{S2}* also shifted significantly toward positive potentials compared with *for^R* (see half-activation voltages $V_{m1/2}$ in Fig. 2 legend). The slopes of voltage-dependent activation, however, were similar among the three alleles for both the peak and sustained currents (see V_{slope} in Fig. 2 legend).

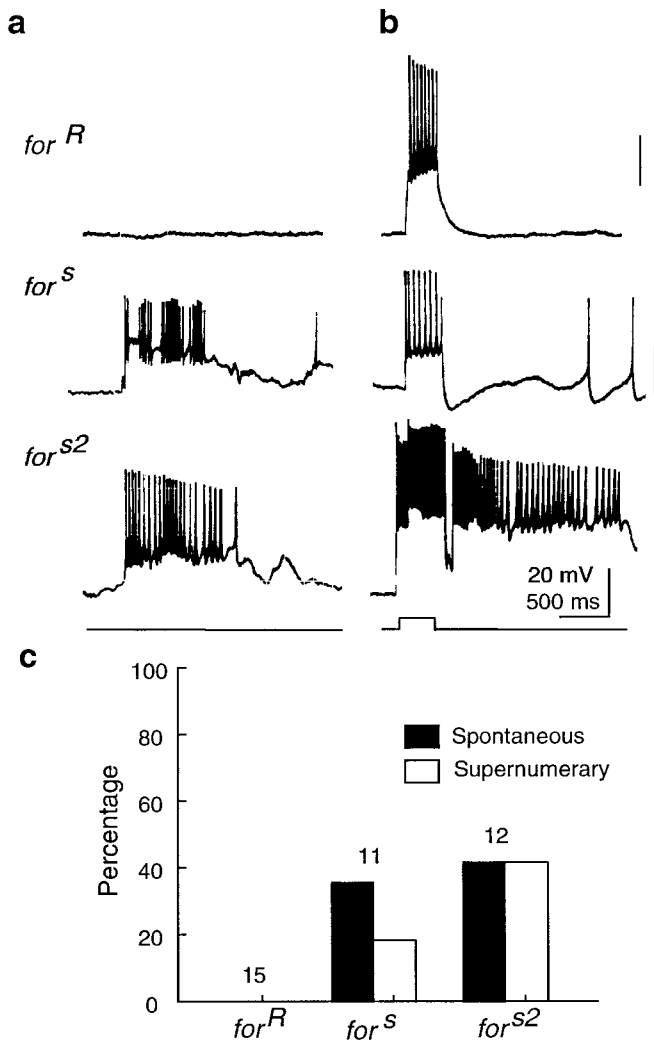


Figure 1. Variations in neuronal firing properties observed among alleles of the *for* gene, which encodes a PKG in *Drosophila*. *a*, Spontaneous bursts of action potentials in the absence of stimulation occurred in *sitter* (*for*^s and *for*^{s2}) neurons but not in *Rover* (*for*^R) neurons. *b*, Aftershock supernumerary spike activity, after the cessation of current injection pulses, appeared in neurons of the naturally occurring *for*^s allele and was more extreme in the *for*^{s2} mutant neurons. *c*, Comparison of spontaneous and aftershock supernumerary nerve activities among different alleles. Spontaneous action potentials occurred in *for*^s ($n = 4$ of 11) and *for*^{s2} ($n = 5$ of 12) but not in *for*^R ($n = 0$ of 15) neurons. Current injections evoked supernumerary action potentials that overlapped the current pulse in *for*^s ($n = 2$ of 11) and *for*^{s2} ($n = 5$ of 12) but not in *for*^R ($n = 0$ of 15) neurons. All recordings were made under whole-cell current-clamp conditions at rest (-50 to -60 mV). Current injection was 60 pA. Only cells capable of firing all-or-none spikes were included in the analysis.

Voltage-activated K^+ currents in *Drosophila* are encoded by several related genes, including *Sh*, *Shal*, *Shab*, and *Shaw*, generating currents with distinct kinetics and voltage dependence when expressed in heterologous systems (Butler et al., 1989). Available mutants and transgenic lines of the *Sh* gene enabled an examination of PKG modulation on *Sh* channels. A membrane-permeant PKG antagonist, Rp-8-pCPT-cGMPS ($10 \mu\text{M}$), was found to suppress both the peak and sustained K^+ currents in *Rover* (Fig. 2*c*) and other laboratory wild-type strains (data not shown). Significantly, neurons of a null allele, *Sh*^M, showed a markedly reduced sensitivity to this inhibitor (Fig. 2*d,f*). To further confirm

that identified *Sh* channels are sensitive to Rp-8-pCPT-cGMPS modulation, we used *ShD* transgenic lines in which a fast-inactivating, slow-recovering *Sh* current can be readily induced by heat shock (Zhao et al., 1995). The extremely slow recovery kinetics allowed extraction of the *ShD* currents by a twin-pulse protocol (see Materials and Methods). We found that *ShD* currents were significantly inhibited by Rp-8-pCPT-cGMPS (Fig. 2*e,f*). The above results suggest that the *Sh* product may be a major target for PKG modulation.

Variation in synaptic transmission at the neuromuscular junction in *for* alleles

The performance of neuronal circuits underlying complex behaviors is determined not only by intrinsic neuronal excitability but also through cell-cell communication at the synapse. The *Drosophila* larval neuromuscular junction has been widely used for analyzing the genetic control of synaptic transmission (Jan and Jan, 1976*a,b*; Budnik et al., 1990; Zhong and Wu, 1991; Wang et al., 1994). Extracellular focal recording has been shown to be an effective method to examine synaptic transmission at individual boutons of *Drosophila* larval neuromuscular junctions (Mallart, 1993; Kurdyak et al., 1994; Renger, 1997). Our results revealed considerable differences in both spontaneous and nerve-evoked excitatory junctional currents (ejcs) between the two natural alleles *for*^R and *for*^s. In the absence of nerve stimulation, we detected only spontaneous miniature ejcs with a small amplitude (<0.5 nA) at a low frequency (<2 Hz) in the natural *for*^R allele, whereas large spontaneous ejcs (up to 5 nA) occurred in both the natural *for*^s as well as mutant *for*^{s2}, *for*^{s189Y}, and *for*^{s92} *sitter* alleles (Fig. 3*a*). In response to each nerve stimulus (1 Hz), an ejc clearly time-locked to stimulation was seen in *for*^R. In contrast, significant supernumerary discharges after the time-locked ejcs were found in *for*^s as well as *for*^{s2}, *for*^{s189Y}, and *for*^{s92} alleles (Fig. 3*b*). Quantification of these data show significantly greater amplitudes of evoked ejcs that are time-locked to the stimulus (Fig. 3*d*). Furthermore, the spontaneous and supernumerary evoked ejcs observed in *sitter* alleles could be genetically suppressed to the *Rover* level in the *dg2*-cDNA transgenic rescue strain (Fig. 3), which contained four copies of the *for*^R version of *dg2* in the *for*^s background (Osborne et al., 1997), suggesting that *for*-PKG is responsible for the phenotypic variation observed.

As suggested by the observations on cultured neurons (Fig. 1), the enhanced neuromuscular transmission in *sitter* alleles may involve increased membrane excitability of the motor axons. This was examined through application of subthreshold doses of TTX to the larval neuromuscular junction (0.5 – 1 nM) (cf. Ganetzky and Wu, 1982). Low doses of TTX suppress excitability by blocking a portion of Na^+ channels and preventing repetitive neuronal firing. This effect is presynaptic because, as in other invertebrate species (Wu and Ganetzky, 1992), there are no Na^+ channels in the postsynaptic muscle membrane in *Drosophila*. The immediate effect observed after TTX application was the disappearance of the large spontaneous ejcs and the supernumerary discharges after the time-locked evoked ejc. Nevertheless, spontaneous miniature ejcs, similar to those seen in *for*^R, were not affected by TTX treatments. Figure 3*c* shows one example in this series of TTX experiments.

The suppression of supernumerary release by low doses of TTX corroborates observations in cultured neurons and demonstrates that the asynchronous synaptic discharge in *sitters* is caused, at least in part, by enhanced nerve membrane excitability. Although a modulatory effect of PKG on postsynaptic receptor

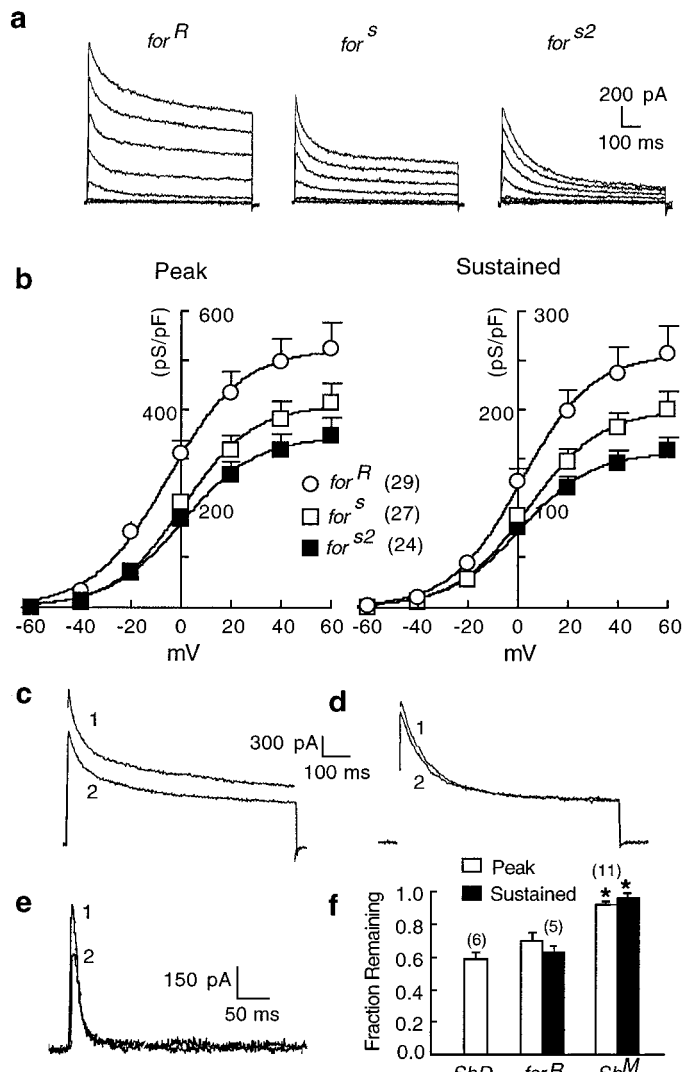


Figure 2. Reduced voltage-activated K⁺ currents in neurons of *for^S* and *for^{S2}* and altered PKG modulation of *Sh* currents. *a*, Both transient and sustained outward K⁺ currents were attenuated in *for^S* and *for^{S2}* compared with *for^R*. The voltage-activated K⁺ currents were elicited by depolarization steps (950 msec) from a holding potential of -80 mV to voltages between -60 and +60 mV in 20 mV increments. *b*, *G-V* curves of the peak and sustained K⁺ currents for the three *for* allelic variants. The membrane conductance *G* (in picosiemens per picofarads; mean ± SEM) was obtained using the formula $G = I/(V - V_r)$, where *I* is the current density and *V_r* the reversal potential of the K⁺ current (-75 mV). The current density (in picoamperes per picofarads) was determined by normalizing the K⁺ current to membrane capacitance. The conductance was fit to the Boltzmann relationship $G = G_o/(1 + \exp((V_{m1/2} - V)/V_{slope}))$, where *G_o*, *V_{m1/2}*, and *V_{slope}* are the maximum conductance, half-activation voltage, and limiting slope, respectively. *G_o* for both the peak and sustained currents were significantly larger in *Rover* than the *sitter* alleles ($p < 0.05$; see Results). *V_{m1/2}* of the peak K⁺ currents in *for^S* and *for^{S2}* shifted significantly toward positive potentials compared with *for^R* (*for^R*, -6.22 ± 1.71 mV; *for^S*, -0.34 ± 1.88 mV; $p < 0.01$; *for^{S2}*, -0.17 ± 2.07 mV; $p < 0.01$); *V_{m1/2}* of the sustained K⁺ currents was not significantly different ($p > 0.05$) among the three alleles (*for^R*, 1.75 ± 1.41 mV; *for^S*, 3.55 ± 2.41 mV; *for^{S2}*, 2.63 ± 2.11 mV). However, *V_{slope}* was similar for both the peak and sustained currents among the three alleles (*for^R*, 14.23 ± 0.55 and 13.56 ± 0.8 mV/e-fold; *for^S*, 12.66 ± 0.45 and 13.11 ± 0.64 mV/e-fold; *for^{S2}*, 13.3 ± 0.64 and 14.6 ± 1.2 mV/e-fold for peak and sustained K⁺ currents, respectively). *c-f*, Modulation of *Sh* K⁺ currents by Rp-8-pCPT-cGMPS, a PKG inhibitor. Addition of 10 μM Rp-8-pCPT-cGMPS to the bath resulted in suppression of voltage-activated K⁺ currents (compare traces 1 and 2 in each panel). *c*, Suppres-

function cannot be ruled out, our findings in cultured central neurons and peripheral motor axons suggest an important role for the *for* locus in regulating neuronal membrane excitability.

Variations in nerve terminal projection at the neuromuscular junction in *for* alleles

A clear association between neuronal activity and nerve terminal outgrowth has been demonstrated at the *Drosophila* larval neuromuscular junction (Budnik et al., 1990; Zhong et al., 1992; Wang et al., 1994; Jarecki and Keshishian, 1995), as well as in vertebrate systems (Cline, 1991). The body wall muscle fibers in third instar larvae are innervated by identifiable motor axons, and their branching patterns have been morphometrically characterized (Johansen et al., 1989; Budnik et al., 1990; Zhong et al., 1992; Chiba et al., 1993; Wang et al., 1994; Renger, 1997). Immunohistochemical staining of the motor axon terminals on muscles 12 and 13 (Budnik et al., 1990; Wang et al., 1994) within the natural *for^R* and *for^S* alleles and induced mutant *sitter* alleles revealed morphological variation correlated with differences in excitability (Fig. 4). The pattern of the motor terminal projections in *for^R* larvae resembled the stereotypical patterns described previously in several laboratory wild-type strains (Johansen et al., 1989; Budnik et al., 1990; Zhong et al., 1992; Wang et al., 1994; Stewart et al., 1996; Fig. 4*a*). The motor axon terminals from motor neurons RP1 and RP4, or RP5 and motor neuron V, branch out from the intersegmental nerve ISNa (Landgraf et al., 1997) to innervate the postsynaptic muscles 12 or 13, respectively, from a single entry point (Fig. 4*a*). In contrast, axon terminals of the more excitable *for^S* and *for^{S2}* alleles displayed ectopic nerve entry points on the muscle surface (Fig. 4*a*). Significantly, the number of ectopic nerve entry points correlated with the excitability levels of both central neurons and motor axons. This is supported by statistical comparisons of supernumerary nerve entry points found in the various *for* alleles (Fig. 4*b*). These data indicate that differences of PKG activity lead to variations not only in nerve membrane excitability but also in neuronal connectivity patterns of the nervous system.

DISCUSSION

This report demonstrates that a natural polymorphism of a single gene in wild populations can generate considerable variations in basic neuronal properties and that electrophysiology provides a sensitive probe to detect allelic variation. Natural variation of the *foraging* gene results in discrete patterns of food-searching behaviors, indicating that natural selection has acted to fine-tune the activity of *for*-PKG in response to the ecological and evolutionary history of *Drosophila* populations. Laboratory-induced mutant alleles of the *for* locus revealed extreme phenotypes in neuronal excitability and connectivity. This raises several questions about the developmental and functional roles of *for*-PKG and the mechanism by which it gives rise to the behavioral polymorphism.

sion of both the transient and sustained K⁺ currents in *for^R* neurons. *d*, The currents in neurons of a null mutant, *Sh^M*, were almost insensitive to Rp-8-pCPT-cGMPS. *e*, An identified *Sh* current expressed in *ShD* transgenic neurons was reduced by Rp-8-pCPT-cGMPS. This fast-inactivating current was induced by heat shock and was extracted by a twin-pulse protocol based on its extremely slow recovery kinetics (see Materials and Methods). All depolarization pulses in *c-e* were to +40 mV from a holding potential of -80 mV. *f*, Fraction of remaining transient and sustained currents after 10 μM Rp-8-pCPT-cGMPS treatment. All data are mean ± SEM for the number of cells indicated in parentheses. Student's *t* tests in *f* were performed between *for^R* and *Sh^M*; * $p < 0.001$.

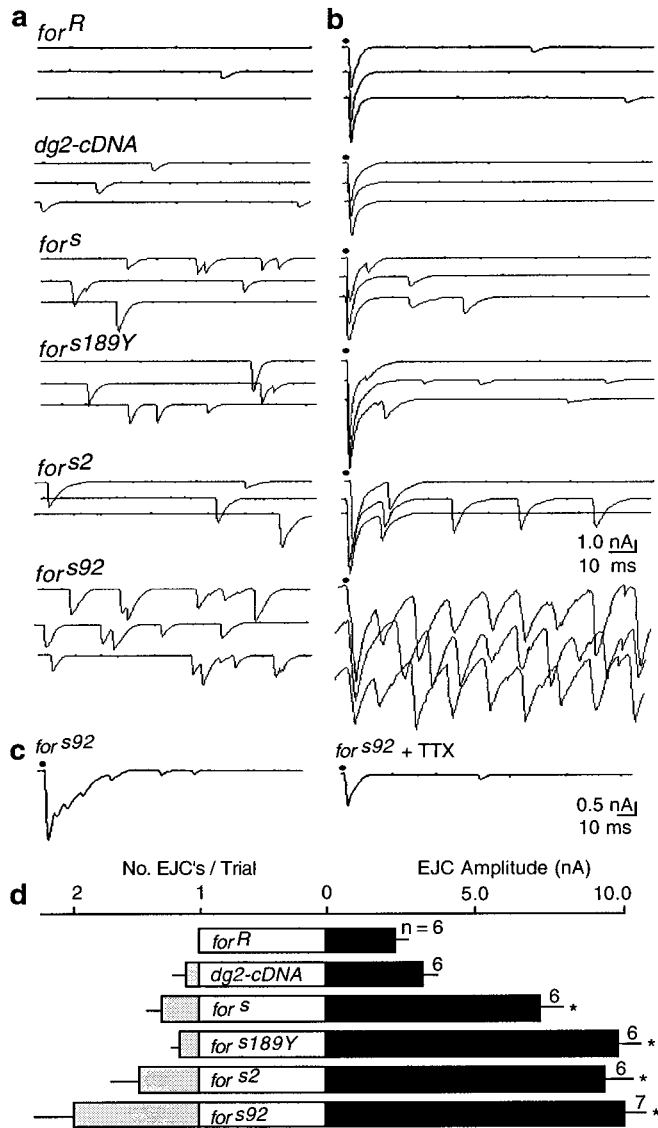


Figure 3. Focal loose patch-clamp recordings from individual synaptic boutons in larval neuromuscular junctions of different *for* alleles. *a*, Spontaneous ejcs in the absence of nerve stimulation. In the naturally occurring *Rover* (*for^R*) allele, only spontaneous miniature ejcs were seen. In contrast, the natural (*for^S*) and mutant *sitter* strains displayed spontaneous ejcs (0.5 mM Ca^{2+}). In the transgenic rescue line *dg2-cDNA* in which four copies of the *for^R* version of *dg2* are placed in the *for^S* background (Osborne et al., 1997), the larger spontaneous ejcs were suppressed. *b*, Supernumerary ejcs after nerve stimulation (filled dots) in saline containing 0.5 mM Ca^{2+} . Supernumerary discharges after the enhanced initial release were most extreme in the *sitter* mutant *for^{S92}* but were absent in the *for^R* and transgenic *dg2-cDNA* lines. *c*, Supernumerary ejcs in the *sitter* allele *for^{S92}* could be suppressed by reducing membrane excitability with subthreshold doses (~10 nM) of TTX. [Ca^{2+}]_o, 0.2 mM. *d*, Occurrence of ejcs was determined in a 125 msec poststimulus period (left; $n = 50$ or 100 trials, 1 Hz, 0.5 mM Ca^{2+}). Number of ejcs per trial period (mean \pm SEM) are shown. Shaded bars indicate excess ejcs with respect to the ejcs time-locked to the stimulus (unshaded bars). No supernumerary ejcs in excess of the time-locked eJC was observed in *for^R*, whereas *for^{S92}* had the most supernumerary ejcs among all *sitter* alleles. The amplitude and number of supernumerary ejcs were restored in transgenic rescue line *dg2-cDNA* to a level near that of *for^R*. The mean \pm SEM amplitude of the time-locked ejcs for each genotype are shown in the right (filled bars). One-way ANOVA shows that the *for^R* strain had significantly ($F_{(4,26)} = 3.95$; $p < 0.01$) lower mean amplitude than did *for^S*, *for^{S189Y}*, *for^{S2}*, and *for^{S92}* (Student–Neuman–Keuls test groupings; * $p < 0.05$).

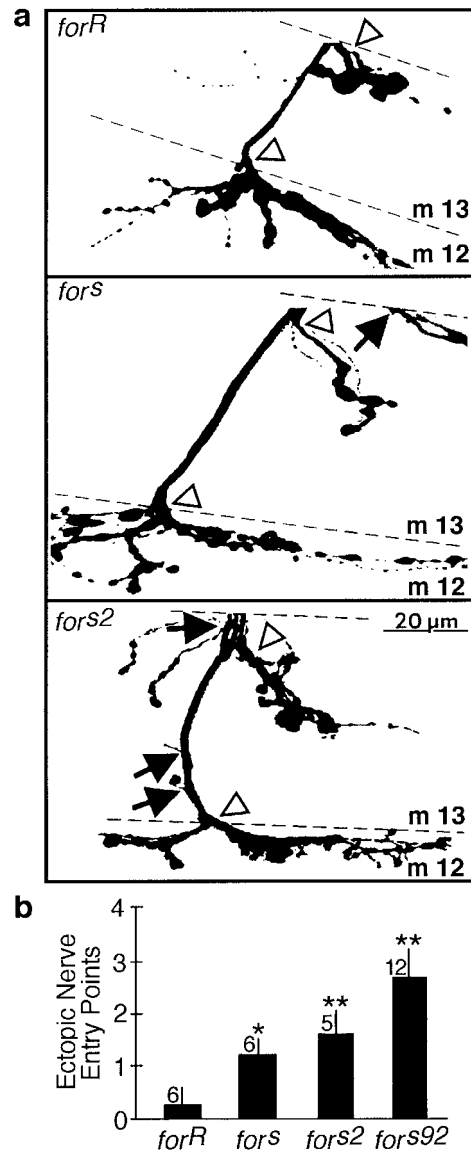


Figure 4. Neuronal polymorphism in synaptic terminal morphology among *Rover* and *sitter* alleles. *a*, Immunohistochemically stained third instar larval neuromuscular junctions. Anti-horse radish peroxidase staining demonstrated that *Rover* larvae displayed stereotypical branching patterns in muscles 12 and 13 of abdominal segment 3, similar to those previously described. However, larval muscles of the natural *for^S* and mutant *for^{S2}* alleles contained ectopic nerve entry points (filled arrows) associated with atypical branches that deviated from the usually restricted single nerve entry points (open triangles). *b*, Bar graph comparing the occurrence of ectopic nerve entry points found within the neuromuscular junction in *sitter* and *Rover* larvae. Occurrences of ectopic nerve entry points (mean \pm SD) found in muscles 12 and 13 of the third abdominal hemisegment were determined in the number of larvae indicated. One-way ANOVA showed significant differences ($F_{(3,24)} = 8.72$; $p < 0.0004$) in the mean number of nerve entry points (Student–Neuman–Keuls test groupings; * $p < 0.05$) with *for^R* having fewer nerve entry points than all of the *sitter* strains, *for^S*, *for^{S2}*, *for^{S189Y}*, and *for^{S92}*.

PKG isoforms and expression patterns

The upstream signaling systems that regulate enzymatic activity levels of PKG may include both nitric oxide (NO)-dependent and -independent mechanisms, which are mediated by the soluble and the membrane-bound guanylyl cyclase (GC), respectively (Koesling et al., 1991; Garbers, 1992; Simpson et al., 1999).

Recently, histochemical and immunochemical staining revealed expression of NO synthase and cGMP within the developing CNS and the larval neuromuscular junction in *Drosophila* (Gibbs and Truman, 1998; Wildemann and Bicker, 1999). Because the soluble GC is known to be a downstream target of NO for cGMP synthesis, these observations suggest potential roles for PKG in developmental and functional regulation of the nervous system. In addition to the *for* locus (*dg2*), at least one other putative PKG-encoding gene (*dg1*) has been identified in *Drosophila* (Kalderon and Rubin, 1989). It will be important to establish the functional distinction between these genes. So far, no mutations in *dg1* are available to help elucidate the function of this gene.

A subtle difference in PKG activity levels is found between *for^R* and *for^S* head homogenates (Osborne et al., 1997). One possibility for such a remarkably specific effect could be a restricted expression pattern of this enzyme in a particular subset of cells in the relevant neural circuits. Further investigations are required to determine the developmental timing, subcellular localization, and cell-specific expression of the *for* gene products (Sokolowski and Riedl, 1999). The connection between the observed hyperexcitability in *for^S* alleles (Figs. 1, 3) and their apparently less-active food-searching behavior (Osborne et al., 1997) compared with the *Rover* strains is not immediately obvious and somewhat counter-intuitive. Although explaining specific behavioral differences requires direct examination of activities in functionally relevant neural circuits, the multitude of neuronal phenotypes observed in *for* alleles provide a basis for further investigation into the role of PKG in regulating nervous system development and function.

K⁺ channels as potential targets for PKG modulation

The influence of PKG on neuronal excitability (Fig. 1) is likely mediated by modulation of downstream targets, e.g., ion channels. Several reports have described modulation of different K⁺ channels by PKG, including an inward rectifier (Kubokawa et al., 1998) and a Ca²⁺-activated maxi-K (Alioua et al., 1998) channel. However, mutations of the *slo* gene, which encodes a Ca²⁺-activated K⁺ channel subunit (Atkinson et al., 1991) mediating maxi-K⁺-like outward currents in *Drosophila* (Komatsu et al., 1990), do not replicate the *sitter* phenotypes but predominantly affect action potential duration (Saito and Wu, 1991), which does not vary among different *for* alleles.

Among different ionic currents in *Drosophila* neurons, the role of voltage-activated K⁺ currents in excitability patterns has been more extensively characterized in K⁺ channel mutants in *Drosophila*, allowing a comparison with those seen in different *for* alleles. Interestingly, the hyperexcitability patterns in the *sitter* alleles do not completely coincide with any of the K⁺ channel subunit mutants known to affect neuronal firing patterns, including *Hk*, *eag*, and *Sh* (Saito and Wu, 1991, 1993; Yao et al., 1998; Yao and Wu, 1999a). Nevertheless, some aspects of the K⁺ channel mutant phenotypes appeared to correspond to those of the hyperexcitability in *sitter* alleles. For example, the spontaneous bursting activity observed in *sitter* neurons is distinct from the rhythmic firings in *Hk* and *eag* neurons (Yao et al., 1998; Yao and Wu, 1999a) but resembles the spontaneous bursting in some *Sh* neurons (Yao and Wu, 1999a). Upon current injection, the supernumerary spikes can be seen in a substantial portion of *eag* neurons (Yao et al., 1998), reminiscent to those seen in *sitter*

neurons. Our results demonstrate variation in voltage-activated K⁺ currents among different *for* alleles and suggest Sh subunits to be a major target for PKG modulation (Fig. 2). However, a role of other K⁺ channel subunits in PKG signaling cannot be ruled out (cf. Zhong and Wu, 1993).

Rover and *sitter* larvae are distinct mainly in food-searching behaviors. Differences in their locomotion patterns are evident when traversing yeast-covered surfaces containing the food stimulus (Sokolowski and Riedl, 1999). In contrast, compared with wild type, all the above K⁺ channel mutant larvae show differences in locomotion pattern on agar-covered surfaces lacking the food stimulus (Wang et al., 1997; J. Wang and C.-F. Wu, unpublished observations). It is likely that regulation of behavior depends on fine-tuning of multiple K⁺ channel types through complex mechanisms. In addition, inward Na⁺ and Ca²⁺ currents play important roles in action potential generation. The potential modulatory effects of PKG on these currents need to be further investigated in *Drosophila* neurons.

Synaptic transmission, nerve connectivity, and the evolution of signaling pathways

Our data indicate that *for*-PKG regulates not only neuronal excitability but also synaptic transmission and nerve connectivity, which take part in regulating behavioral expressions. The spontaneous ejs and evoked supernumerary afterdischarges in *sitter* alleles are likely caused by increased motor axon excitability (Fig. 3). However, the increased evoked transmitter release in the time-locked ejs of *sitter* alleles indicates variation in transmitter release machinery per se. An NO-mediated cGMP cascade can influence long-term potentiation (Zhuo et al., 1994) and depression (J. Wu et al., 1998), and capsaicin-dependent enhancement of neurotransmitter release has been linked to PKG-dependent processes (Sluka and Willis, 1998) in vertebrate preparations. Indeed, variations in activity-dependent synaptic efficacy was found in the pair-pulse paradigm among *for* alleles (Renger, 1997).

The profound effects of allelic variation on the *for*-PKG activity are reminiscent of changes in neuronal and behavioral plasticity by mutational perturbations of the cAMP (Zhong and Wu, 1991; Zhong et al., 1992; Dubnau and Tully, 1998) and Ca²⁺/calmodulin-dependent protein kinase II (CaMK) (Griffith et al., 1994; Wang et al., 1994; Yao and Wu, 1999b) in cascades in *Drosophila*. In general, disruptions of both cAMP and CaMK pathways also alter neuronal excitability, synaptic transmission and facilitation, motor axon terminal projection, and behavioral plasticity. However, unique neuronal phenotypes result from genetic manipulations of each of the three pathways. For example, spontaneous activity in dissociated neurons and at the larval neuromuscular junction is far more striking in *sitter* mutants than in mutants defective of the cAMP or CaM kinase II cascade (Zhong and Wu, 1991; Wang et al., 1994; Zhao and Wu, 1997). The ectopic entry points of the motor neuron projection in *sitter* alleles are distinct from the increased nerve terminal branching in the cAMP cascade mutant, *dnc*, which displays ramification of higher order branches (Zhong et al., 1992). Thus, the three genetically separable signaling pathways might differentially regulate distinct functional aspects of activity-dependent neuronal properties that are relevant to specific behavioral tasks, e.g., foraging, learning, and memory (C.-F. Wu et al., 1998).

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