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Complementation of Physiological and Behavioral Defects by a *Slowpoke* Ca^{2+}-Activated K^+ Channel Transgene

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Abstract: The *Drosophila slowpoke* gene encodes a large conductance calcium-activated potassium channel used in neurons, muscle, and some epithelial cells. Tissue-specific transcriptional promoters and alternative mRNA splicing generate a large array of transcripts. These distinct transcripts are thought to tailor the properties of the channel to the requirements of the cell. Presumably, a single splice variant cannot satisfy the specific needs of all cell types. To test this, we examined whether a single *slowpoke* splice variant was capable of complementing all *slowpoke* behavioral phenotypes. Null mutations in *slowpoke* cause animals to be semiflightless and to manifest an inducible "sticky-feet" phenotype. The well-characterized *slowpoke* transcriptional control region was used to direct the expression of a single *slowpoke* splice variant (cDNA H13) in transgenic flies. The endogenous gene in these flies had been inactivated by the slo^4 mutation. Action-potential recordings and voltage-clamp recordings demonstrated the production of functional channels from the transgene. The transgene completely complemented the flight defect, but not the sticky-feet phenotype. We conclude that distinct *slowpoke* channel isoforms, produced by alternative splicing, are not interchangeable and are required for proper function of different cell types. **Key Words:** Potassium channel—*slowpoke*—*Drosophila*—mRNA splicing—Behavior—Voltage clamp.
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Large conductance (maxi-K) Ca^{2+}-activated K^+ channels are a unique class of ion channel that respond to both voltage and Ca^{2+}. These channels are expressed in a broad range of excitable and nonexcitable tissues and have diverse physiological roles. In situ recordings have identified a large number of maxi-K-type currents with distinct electrophysiological properties (Latorre et al., 1989; Haylett and Jenkinson, 1990; McManus, 1991; Art et al., 1995). The channels that conduct these currents all appear to be products of the *slowpoke* gene. Channel diversity is thought to arise, in part, from alternative mRNA splicing and from the use of tissue-specific transcriptional promoters (Atkinson et al., 1991; Adelman et al., 1992; Brenner et al., 1996), both of which can alter the sequence of the encoded protein.

In oocytes or cell culture expression systems, alternative exons have been shown to affect channel conductance, activation and deactivation kinetics, and apparent Ca^{2+} sensitivity (Lagrutta et al., 1994; Tseng-Crank et al., 1994; Saito et al., 1997). In the chick and turtle cochlea, *slowpoke* alternative splice products are differentially distributed in the hair cells along the basilar papilla where they are proposed to participate in electrical tuning (Navaratnam et al., 1997; Rosenblatt et al., 1997; Jones et al., 1998; Fettiplace and Fuchs, 1999).

It is generally assumed that alternative splicing enables cells to express maxi-K channels with the specific properties required for cellular function. The qualities imparted by a particular splice variant are thought to be important for the normal operation of the animal. Although reasonable, this assumption is difficult to test. Heterologous expression systems do not permit one to determine how a splice variant affects the function of a cell in situ or the performance of the animal itself.

We are using *Drosophila* as a model to determine how alternative *slowpoke* transcripts affect cellular properties and animal behavior. Null mutations in the gene cause electrophysiological defects and multiple behavioral abnormalities. These animals exhibit an inducible sticky-feet phenotype, a constitutive flight defect, and an altered mating song (Elkins et al., 1986; Peixoto and Hall, 1998; Atkinson et al., 2000). The *Drosophila slowpoke* gene has five tissue-specific transcriptional promoters and five sites where the transcript is alternatively spliced (Adelman et al., 1992; Bohm et al., 2000). It has been shown that a loss of neuronal expression is correlated with the inducible sticky-feet phenotype and that muscle expression is sufficient to complement the flight defect (Atkinson et al., 2000). Here we show that a single *slowpoke*

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Abbreviations used: 4-AP, 4-aminopyridine; maxi-K, large conductance.

splice variant, expressed as a transgene by the *slowpoke* transcriptional control region, complements some, but not all, of the behavioral phenotypes. The strong conservation of *slowpoke* between invertebrates and vertebrates suggests that results obtained with *Drosophila* are useful for understanding the role of *slowpoke* channels in higher organisms (Butler et al., 1993).

MATERIALS AND METHODS

Stocks

Stocks were maintained using standard *Drosophila* husbandry techniques. A w^{1118} stock was transformed with the M131A transgene. Genetic crosses were used to move the transgene into a homozygous slo^4 genetic background. The slo^4 mutations have been shown to eliminate the Ca^{2+}-activated K^+ current, I_{CF} (Komatsu et al., 1990; Atkinson et al., 1991). A P element transposon vector, p[w^+; M131], which carries the w^+ eye marker gene and the M131 *slowpoke* transgene, has been inserted into the second chromosome. The genotype of this stock M131 is w^{1118}; P[w^+, M131]; slo^4. Heterozygous slo^4 animals were *y w;* slo^4/y^+ TM3 *Sb e Ser*. In all experiments, w^{1118} served as the wild-type (slo^+) control.

Germline transformation

Germline transformations were performed as described (Spradling, 1986). The plasmid pπ25.7wc (200 ng/ml) and the promoter transformation constructs (0.5–1 mg/ml) were microinjected into w^{1118} embryos.

Construction of transgene M131

The cDNA H13 was isolated from an adult head cDNA library (a kind gift from Thomas Schwarz, Stanford University School of Medicine) and has been described previously (Atkinson et al., 1991; Becker et al., 1995). The M131 transgene was constructed in pCaSperAUGβgal (Thummel et al., 1988). In this vector, the insert is flanked by P-element ends and can be used to transform *Drosophila*. The cloned *slowpoke* transcriptional control region (*Kpn*I to *Apa*I; plasmid 125) (Brenner et al., 1996) was fused, in translational frame, to the cDNA H13 (Becker et al., 1995). Plasmid 185 contains the full-length H13 cDNA followed by an HSV epitope tag (QPELAPEDPED) and a stop codon (Novagen, Inc., Madison, WI, U.S.A.). A *Kpn*I/*Apa*I restriction fragment that contains most of the *slowpoke* transcriptional control region was removed from plasmid 125 and ligated into plasmid 185 linearized with *Kpn*I/*Apa*I. The *Apa*I site was within exon C3 (Fig. 1A) of *slowpoke*, and the ligation event recreated exon C3. The ligation product, plasmid 196, contains most of the transcriptional control region in register with the H13 cDNA. The remainder of the transcriptional control region was reconstituted as follows. The vector pCaSperAUGβgal was digested with *Eco*RI and *Xba*I, and the lacZ-encoding DNA fragment was removed and replaced with a *Xho*I/*Kpn*I/*Not*I polylinker. Both the *Eco*RI and *Xba*I sites were destroyed in this process. The resultant plasmid is called pCaSperΔgal. The portion of the *slowpoke* control region missing from plasmid 196 was contained in the *Xho*I/*Kpn*I fragment from plasmid 125. This fragment was inserted into pCaSperΔgal (cut within its polylinker with *Xho*I/*Kpn*I) to produce plasmid 207. A *Kpn*I/*Not*I fragment from plasmid 196, which contained most of the *slowpoke* transcriptional control region and the H13 cDNA, was then inserted into a *Kpn*I/*Not*I digested plasmid 207. Transcription termination and polyadenylation sites were provided by SV40 sequences found in the vector. Following transformation into flies, the resultant plasmid, M131, expresses a *slowpoke* mRNA whose alternative exon splice pattern is found naturally in muscle. In muscle, this transgene expresses a *slowpoke* transcript that begins with alternative exon C2 and includes alternative exons 1a, 2b, 3a, and 4bf. At splice site 5, the alternative exon is skipped, which is referred to as splice pattern 5b (Fig. 1A).

RT-PCR

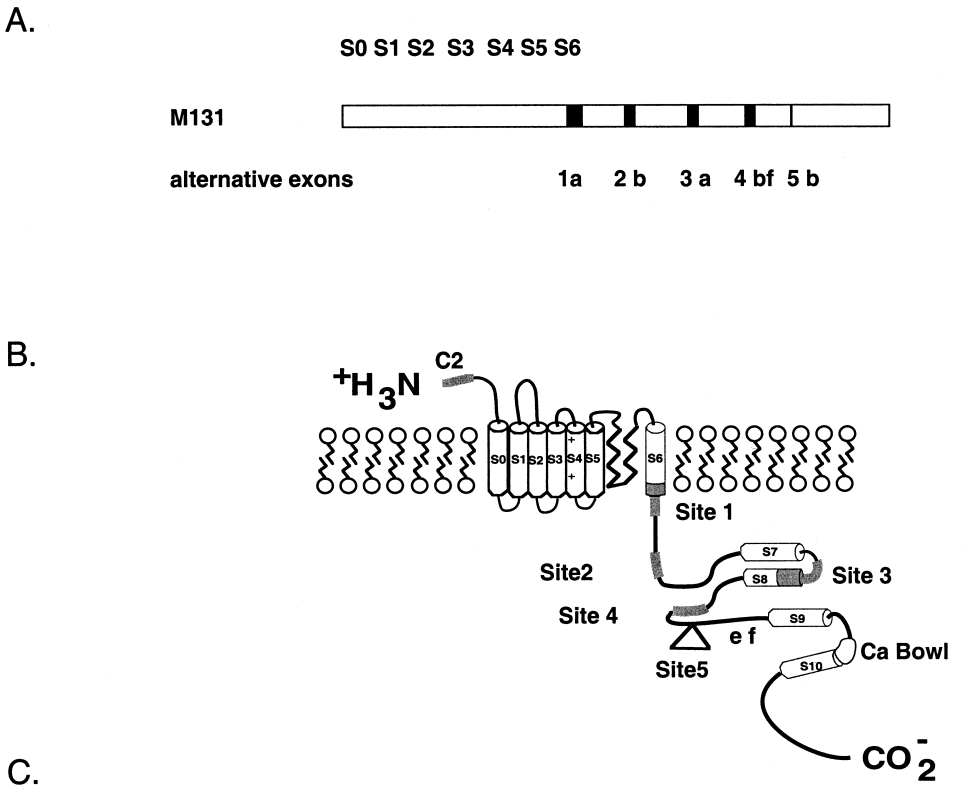
For RT-PCR, RNA was purified from 200 w^{1118} flies (wild-type for *slowpoke*), slo^4 flies, and w^{1118}; M131; slo^4 animals using RNAwiz (Ambion Inc.). One microgram of RNA was reverse-transcribed in a 25-μl volume with Superscript RT reverse transcriptase (GibcoBRL) and 1.25 pmol of primer SLO1891 (5′ CATCGTCGCCCTGTTTCCAATC 3′) at 40°C for 45 min. The solution was adjusted to 100 μl with water and heated at 65°C for 5 min. Ten microliters of the reverse transcription product was used to as template for the PCR reaction. A hot-start 30-cycle PCR amplification was carried out in a PCR machine (Perkin–Elmer, Inc.) using standard PCR reaction buffer with 2 m*M* $MgCl_2$, 0.125 m*M* dNTPs, and 1 μM concentrations of both PCR primers. To detect transcripts produced by *slowpoke* Promoter C0, the PCR was performed using the GAMMA5 primer (5′ ATTGTATACGCTGCTGACGAGA 3′, anneals to neural-specific exon C0) and primer SLO1462 (5′ GTTCTCTTTTCAGTTCACCGCC 3′), which anneals at approximately nucleotide 1,462. Annealing temperatures were determined using the OLIGO program (National Biosciences, Inc.). The products were separated on an agarose gel and visualized with ethidium bromide. The 1-kb molecular weight ladder from New England Biolabs was used as a molecular weight standard.

Flight assay

The relative capacity of the animals to fly was determined, with minor modifications, as described by Elkins et al. (1986) and Benzer (1973). The walls of a 15 cm × 62 cm pipette jar were coated with mineral oil. Flies were dropped through a funnel fixed on a platform at the top of the jar. The falling animals flew toward the jar walls and became embedded in the mineral oil. The position of each animal was marked, and the distance of each mark from the bottom of the jar was determined. During the time course of the experiment, the flow of the oil toward the bottom of the jar did not significantly alter the position of the flies. Furthermore, animals were quickly killed (smothered) by the oil and did not crawl once they made contact. Animals that flew well clustered near the top of the jar, whereas animals that flew poorly clustered nearer the bottom. Dead flies fell into the bottom of the jar and were not counted. Flies found within the first 2.54 cm of the column were also not counted, because it could not be ascertained that they did not crawl from the funnel, across the platform, to this position. This test readily distinguished wild-type animals from animals homozygous for a mutant *slowpoke* allele (Elkins et al., 1986). For each genotype, 500–1,000 flies were tested.

Sticky-feet behavioral assay

The sticky-feet behavior was evoked by exposing the flies to a brief heat pulse. Adults (3–5 days old) were trapped at the bottom of an empty glass fly vial using a cotton plug and incubated at 40°C for 7 min (the time required to elicit the behavior varies with the season, but not within a season). Storing the animals at 20°C for 24 h before the assay seemed to enhance reliability. Positive (wild-type animals) and negative (slo^4 mutant animals) internal controls were always performed.



C.

Splice site

This paper	1a	1b	1c	2a	2b	3a	3b	4bdef	4bf	4a	4aef	4af	4cef	4b	4c	4f	4bdf	4bef	5a
Atkinson et al (1991)								A2			A1								B
Adelman et al (1992)	A1	A2	A3	C1	C2	E1	E2	G1	G2	G3	G4	G5	G6						I
number of amino acids	30	30	30	35	35	37	37	62	29	13	49	24	61	18	25	12	37	54	22

FIG. 1. M131 transgene. **A:** The diagram represents the alternatively spliced exons in *slowpoke* cDNA H13 as expressed by the M131 transgene. The black boxes are sites of alternative exon use, and open boxes represent regions that are not alternatively spliced. The entries S0–S6 denote the relative positions of the putative transmembrane domains. **B:** A model of the topology of *slowpoke* protein in the plane of the cell membrane. This model is based on hydropathy plots of the cDNA from multiple species (Atkinson et al., 1991; Butler et al., 1993; Meera et al., 1997). **C:** Table for converting between the various names used to identify alternatively spliced exons. In this article, we have chosen to adopt a nomenclature similar to that used to describe alternatively spliced exons in the vertebrate *slowpoke* gene (Tseng-Crank et al., 1994). In this nomenclature, splice sites are identified by a number and the alternative exon by a letter. Splice site 4 is more complicated and includes multiple letters. This reflects the fact that what had been treated as alternative exons at this site are actually discrete combinations of six tiny exons (a–f; J. Y. Yu and N. S. Atkinson, unpublished observations). The letters identify the combination of smaller exons used in each combination. Not shown are the names of other alternative exons that result from differential promoter utilization, which have been called C1, C1b, C1c, and C2 (Becker et al., 1995; Brenner and Atkinson, 1996, 1997; Brenner et al., 1996; Thomas et al., 1997).

Following the heat pulse, the animals were gently transferred to the tabletop and left unmolested for ~15 s. A pencil was then used to push on the animals. Flies homozygous for a *slowpoke* mutant allele hold onto the surface and allow themselves to be pushed over. Flies heterozygous for a *slowpoke* mutant allele walk or fly away from the stimulus. Wild-type flies take flight and leave the area.

Action-potential recordings

Dorsal longitudinal flight muscle action potentials were recorded largely as described by Elkins and Ganetzky (1988). Adults were lightly anesthetized with ether and glued along their ventral midline to the edge of a 20-mm^2 coverslip using Superglue. The coverslip was held by a vertically mounted alligator clip such that the dorsal surface of the thorax was up and facing the recording electrode. The flies were allowed to recover for 1 h before recordings. The brain of the animals was stimulated using a Grass Instruments S88 stimulator and two uninsulated tungsten electrodes. Each stimulation activates the giant fiber pathway and causes the production of action potential in the dorsal longitudinal flight muscles. An uninsulated tungsten electrode inserted into the abdomen was used to provide a reference potential. The recording electrode consisted of a glass electrode pulled to a resistance of 2–5 MΩ and filled

with 1 *M* sodium chloride that connected to the headstage via a silver pellet half-cell. Action-potential spikes were passed through a WPI Electrometer Intra 676 and captured using a Mac Adios 8ain analog-to-digital converter (69.4 ms/point), filtered with a 14-kHz single-pole low-pass filter, and recorded to disk using the program Superscope. To record action potentials, the recording electrode was advanced from the ventral side through the thoracic cuticle and into dorsal longitudinal flight muscle. Electrode penetration of the muscle became apparent as a 40–80 mV potential drop. Following muscle impalement, the brain was stimulated for 0.1-ms pulses beginning with a nominal voltage (2 V) and increasing the voltage until an action potential was evoked. After a threshold voltage was determined, 0.2 V above threshold was used for the remainder of the experiment. Usually, dorsal flight muscles a and b had a hyperpolarizing afterpotential and a higher resting membrane potential. Muscles c–f, however, did not hyperpolarize after the action potentials and had a lower resting membrane potential (Engel and Wu, 1992). For all experiments, data were analyzed for only muscles c–f. One measure that we used was the half-height width of the action potential. The half-height width is determined by measuring the width of the action potential at one-half maximum amplitude. The amplitude of the action potential is measured with respect to the baseline immediately preceding the spike. Flies were viable for long periods of time and showed consistent electrophysiological responses for several hours after mounting.

Voltage-clamp recordings

Two-electrode voltage clamp was performed on third instar *Drosophila* larval body wall ventral longitudinal muscles numbers 6 and 7 as has been described previously (Singh and Wu, 1990). Recordings were performed at room temperature (22°C) using HL3 solution (Stewart et al., 1994) with 20 m*M* $CaCl_2$ (instead of the usual 5 m*M* $CaCl_2$). The increased Ca^{2+} has been reported to make the *slowpoke* currents more reproducible (Singh and Wu, 1990). Voltage and current recording electrodes were pulled to a resistance of 8–12 MΩ. The voltage electrode was filled with 3 *M* KCl, and the current electrode was filled with 4 *M* potassium acetate. Before each test pulse, a prepulse was delivered: −80 mV for 2 s, −50 mV for 50 ms, −80 mV for 500 ms, −50 mV for 50 ms. The −50-mV steps during this prepulse did not trigger any active currents (Singh and Wu, 1990). Leak current measured at the prepulse was used to subtract linear leak currents, and the capacitative transient current was measured and used to determine the fiber capacitance, which is proportional to cell size. All current recordings were normalized for fiber capacitance. Following the prepulse, the cells were returned to −80 mV for 500 ms and then stepped to a test potential of +10 mV. The pure *slowpoke* current, called I_{CF}, was isolated by subtraction. The traces generated by *slo*^{4} mutant muscle were subtracted point by point from recordings from wild-type, M131, and *slo*^{4} muscles. The *slo*^{4} mutation is a chromosomal inversion that eliminates expression of *slowpoke* protein (Becker et al., 1995). This approach has been used previously to isolate the *slowpoke* current (Komatsu et al., 1990). Subtracted averages were made using six wild-type, five M131, and five *slo*^{4} animals. Averages and standard error measurements were done using a "Jackknife" approach to ensure that the error was accurately propagated (Efron, 1982). The tau for the decay of current traces was calculated using the simplex method as implemented by the program Clampfit (Axon Instruments). Single and double exponential decays were best fit using the equations: $I(t) = A\exp(-t/\text{tau}) + \text{Offset}$ and $I(t) = A\exp(-t/\text{tau}_{fast}) + B\exp(-t/\text{tau}_{slow}) + \text{Offset}$, where I is current in picoamperes, t is time in milliseconds elapsed between two data points, A and B are the peak in picoamperes, Offset is final value for the decay curve, and tau is the time constant (millisecond).

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   exon C2
exon C2      MASGLIDTNFSSTLAN

   Site 1
exon 1a      AMFASSIPEIIELVGSGNKYGGELKREHGK

   Site 2
exon 2b      KPPDLELEGLFKRHFTTVEFFQGTIMNPIDLQRV

   Site 3
exon 3a      SPDTQAWQNDYLQGTGCEMYTETLSPSFTGMTFPQAS

   Site 4
exon 4bf     tgATFRKGVRAVQMVGRASDITRDREDTNc

   Site 5    exon excluded in this splice variant
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FIG. 2. Amino acid sequence of the exons present at each alternative splice site in the M131 transgene. The protein encoded by M131 is 1,197 amino acid residues. The uppercase letters refer to amino acid residues. The lowercase letters shown in the site 4 exon are deoxynucleotide residues. It was necessary to specify the sequence in this manner because the site 4 splice acceptor and donor sites occur within a codon. Splice site 5 either incorporates a small exon or skips this small exon entirely. We refer to the former case as splicing pattern 5a and the latter as pattern 5b. M131 uses pattern 5b.

4-Aminopyridine (4-AP) treatment

Flies were allowed to feed overnight on a yeast paste containing 25 m*M* 4-AP (Sigma) in yeast paste. This was the lowest concentration that phenocopies the *Sh*^{KS133} ether-induced leg shaking. The *Sh*^{KS133} mutation eliminates the *Shaker* encoded channel that conducts the current I_A.

RESULTS

Construction of *slowpoke* transgene

The *slowpoke* transcriptional control region has at least five distinct transcriptional promoters (Brenner and Atkinson, 1996; Thomas et al., 1997). The tissue specificity of these promoters has been reported (Brenner et al., 1996). Promoter C2 has been shown to be responsible for muscle and tracheal cell expression. This promoter is also active in a small number of neurons in the larval brain (Brenner et al., 1996; Chang, 1998). All promoter C2 transcripts begin with exon C2. A full-length cDNA that began with exon C2 was isolated from a cDNA library. This cDNA is called H13.

The H13 cDNA has alternative exons 1a, 2b, 3a, and 4bf (Fig. 1A). The unusual nomenclature of the site 4 alternative exon (4bf) reflects the fact that the sequences at this site, originally treated as a single exon (Adelman et al., 1992), are actually generated by 11 discrete combinations of six different tiny exons (exons a–f; unpublished observations). At splice site 5, alternative splicing either includes or skips a tiny exon. The absence of this

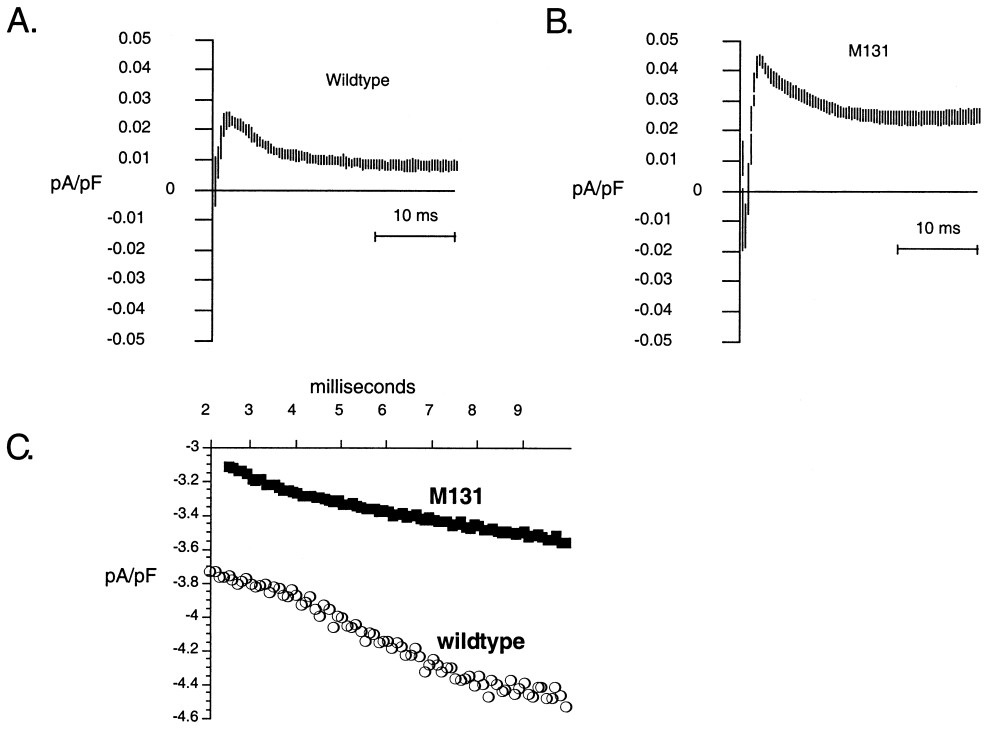

FIG. 3. Isolation of the I_{CF} by two-electrode voltage clamp of larval body wall muscles. Cells (ventral longitudinal larval muscle number 6 and 7) in HL3 buffer with 20 m*M* Ca^{2+} were subjected to a prepulse to measure leakage currents and cell capacitance (−80 mV for 2 s, −50 mV for 5 ms, −80 mV for 500 ms, −50 mV for 5 ms, −80 mV for 500 ms) and then stepped to +10 mV, and total cellular currents were recorded. Leak currents have been subtracted from all traces and the resultant normalized to cell capacitance. Normalized cellular currents recorded from *slo*^{4} homozygotes were then subtracted to reveal I_{CF}. **A** and **B:** Averaged currents. The vertical lines represent the SEM. In *Drosophila* muscle, *slowpoke* channels have been shown to be largely voltage-insensitive and to be activated as a function of Ca^{2+} concentration (Komatsu et al., 1990). Currents from wild-type muscle (A) have a peak current of 0.024 ± 0.002 pA/pF and decay with a time constant of 4.7 ms. The currents from M131-expressing muscle (B) have a peak current of 0.044 ± 0.001 pA/pF and a time constant of 5.4 ms for its decay. **C:** The decay from the peak current has been replotted to illustrate the similarities in the decay kinetics of the wild-type (tau = 4.7 ms) and M131 currents (tau = 5.4 ms).

exon is referred to as the 5b splice pattern. The sequence of the alternative exons in cDNA H13 is shown in Fig. 2. To drive expression in the appropriate tissues and at the correct level, we fused cDNA H13 to the complete *slowpoke* transcriptional control region. The resulting minigene construct is called M131.

The M131 transgene was transformed into flies using P element-mediated *Drosophila* transformation (Spradling, 1986). For our studies, the transgene was crossed into a *slo*^{4} mutant background. We chose the *slo*^{4} allele for all of our studies because the evidence that it is a null allele is very strong: first, this mutation is a chromosomal inversion with one end point near the 5′ end of the gene; second, electrophysiological assays have shown a complete lack of I_{CF} in flight muscle (Atkinson et al., 1991); and finally, neither in situ hybridization nor immunohistochemical staining with an antibody directed against the carboxy-terminal tail of the protein detected any expression products from the gene (Becker et al., 1995). Therefore, in the *slo*^{4} background it is possible to replace the products of the endogenous gene with the product of the transgene. All physiological assays make use of animals homozygous for the *slo*^{4} null mutation.

Two-electrode voltage-clamp analysis of *slowpoke* currents

To verify that the transgene expressed functional *slowpoke* channels, we used two-electrode voltage clamp to isolate the *slowpoke* current, I_{CF}, in larval body wall muscle. Subtraction was used to isolate I_{CF} (see Materials and Methods). These muscles are virtually isopotential and therefore can be easily voltage-clamped (Jan and Jan, 1976; Wu and Haugland, 1985); furthermore, in the filleted and eviscerated larvae, the surface of the cell is easily accessible. There is some animal-to-animal variation in the absolute size of a given muscle cell, which affects the absolute magnitude of the recorded currents, but not the kinetics. However, in these cells, we and others have found that current densities are consistent between cells of different size. Membrane capacitance is proportional to the membrane surface, and therefore the differences in cell size can be accounted for by normalizing the currents against cellular membrane capacitance (Wu and Haugland, 1985; Singh and Wu, 1989).

As shown in Fig. 3, wild-type muscle expresses a *slowpoke* current that is rapidly and transiently activated. The transgene is well expressed in muscle and generates

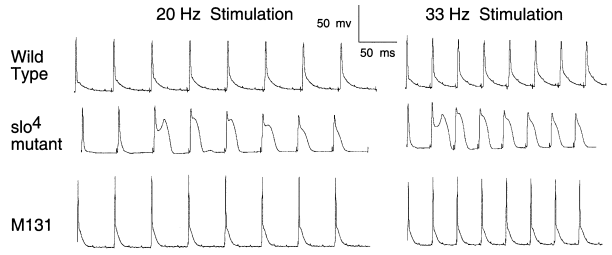

FIG. 4. Indirect flight muscle action potentials. Muscle action potentials were evoked by stimulation of the giant fiber pathway at 20 and 33 Hz and recorded using an intracellular electrode. The wild-type control is w^{1118}, which was also used as a transformation stock. The negative control is homozygous for the slo^4 null mutation and therefore does not express the Ca^{2+}-activated K^+ channels that conduct the current called I_{CF}. This stock is unable to repolarize its cell membrane properly and produces abnormally shaped action potentials. M131 refers to a *slowpoke* transgene that expresses a single *slowpoke* splice variant. The currents produced by the channels were recorded in a slo^4 mutant background. The trace labeled M131 was obtained from a w^{1118}; M131; slo^4 stock. The M131-expressing muscle produces sharp action potentials, indicating that this transgene complements the slo^4 electrophysiological phenotype.

about twice the peak current expressed by the wild type. Both the wild-type gene and the transgene produce currents with similar single exponential decay kinetics (tau = 4.7 ms versus 5.4 ms, respectively).

Action-potential recordings from mutant and transgenic muscle

At least four different K^+ currents exist in flight muscle; they are I_{CF}, I_A, I_K, and I_{CS} (Salkoff, 1983, 1985; Salkoff and Wyman, 1983; Elkins et al., 1986). The *slowpoke* gene encodes the channel that conducts the fast-activating, Ca^{2+}-dependent current that in *Drosophila* is called I_{CF} (Elkins et al., 1986; Atkinson et al., 1991). The *Shaker* gene produces the channel that conducts the fast-activating voltage-dependent current called I_A (Baumann et al., 1987; Kamb et al., 1987; Papazian et al., 1987). In larval muscle, the *Shab* gene has been shown to encode a channel that contributes to I_K (Hegde et al., 1999; Singh and Singh, 1999). I_{CS} is a slow-activating Ca^{2+}-dependent current (I_{Kc} in Wei and Salkoff, 1986). The gene that encodes the I_{CS}-conducting channel has not yet been identified.

Mutations in the *slowpoke* gene, which eliminate I_{CF}, cause muscle membranes to have grossly abnormal electrical properties. During repetitive stimulation, abnormally broad and Bactrian camel-shaped action potentials are produced (Elkins et al., 1988; Fig. 4). Typically, the first few action potentials are of normal breadth and shape (Fig. 4, discussed below).

What is the cause of the gradual increase in spike width?

Single action-potential spikes produced by slo^4 mutant muscle are uniformly sharp, but the subsequent spikes in a train are on average much broader (Figs. 4 and 5). A hypothesis put forth by Elkins and Ganetzky (1988) to explain data collected using constant current depolarization of slo^1 mutant flight muscle appears to account for this phenomenon. To understand this phenotype, one should consider the properties of two currents, I_A and I_{CF}. Both I_A and I_{CF} activate with the same time course, and either one appears sufficient to repolarize rapidly the flight muscle membrane (Salkoff, 1985; Elkins et al., 1986; Elkins and Ganetzky, 1988). However, the I_A conducting channels undergo long-lasting voltage-dependent inactivation, and during repetitive stimulation an increasing number of these channels would be predicted to enter the inactivated state (Elkins and Ganetzky, 1988). In contrast, *Drosophila slowpoke* channels have not been observed to exhibit intrinsic inactivation in *Drosophila* muscle or when expressed in *Xenopus* oocytes (Komatsu et al., 1990; Gorczyca and Wu, 1991; Lagrutta et al., 1994; Wei et al., 1994). In slo^4 mutants, which do not express I_{CF}, there is an increasing incidence of spike broadening with each successive action potential. In addition, the incidence of broadening increases at higher stimulation frequencies (compare slo^4 data in Figs. 4 and 5A and B). This is postulated to take place because of the gradual inactivation of the population of *Shaker* channels that conduct I_A. Hence, in *slowpoke* mutants, action-potential broadening is thought to occur because some of the *Shaker* channels do not recover from the inactivation before the arrival of the next action-potential spike. As the stimulation frequency increases, more and more *Shaker* channels remain inactivated and are unable to participate in spike repolarization.

To determine if this explanation accounts for the broadening of synaptically evoked action potentials, we used the *Shaker* mutation, Sh^{KS133}, and the drug 4-AP. Sh^{KS133} genetically eliminates the channel that conducts I_A and 4-AP is a blocker of *Shaker*-encoded K^+ channels (Salkoff and Wyman, 1983). Sh^{KS133} animals do not show action-potential broadening (Fig. 5), confirming that the other current that shares its time course, I_{CF}, must be sufficient to repolarize the membrane rapidly. We also recorded action potentials produced by slo^4 homozygous muscle that had been treated with 4-AP. Under these conditions, each and every spike produced by the flight muscle is extremely broad (Fig. 5). This indicates that I_A is involved in producing the gradual broadening of action potentials observed in slo^4 mutant muscle. At all stimulation frequencies (10, 20, 33, and 50 Hz), the half-height width of each and every action-potential spike in the presence of 4-AP is between 8 and 10 ms. This is very similar to the broadening end point eventually produced by slo^4 muscle in the absence of the 4-AP treatment. At frequencies >10 Hz, this end point is reached by the fourth or fifth stimulation. We suggest that by this time all of the *Shaker* encoded channels have become inactivated and fail to reactivate during the time course of the experiment.

M131 complements action-potential defect

We wished to determine if the channels encoded by the M131 transgene could completely substitute for na-

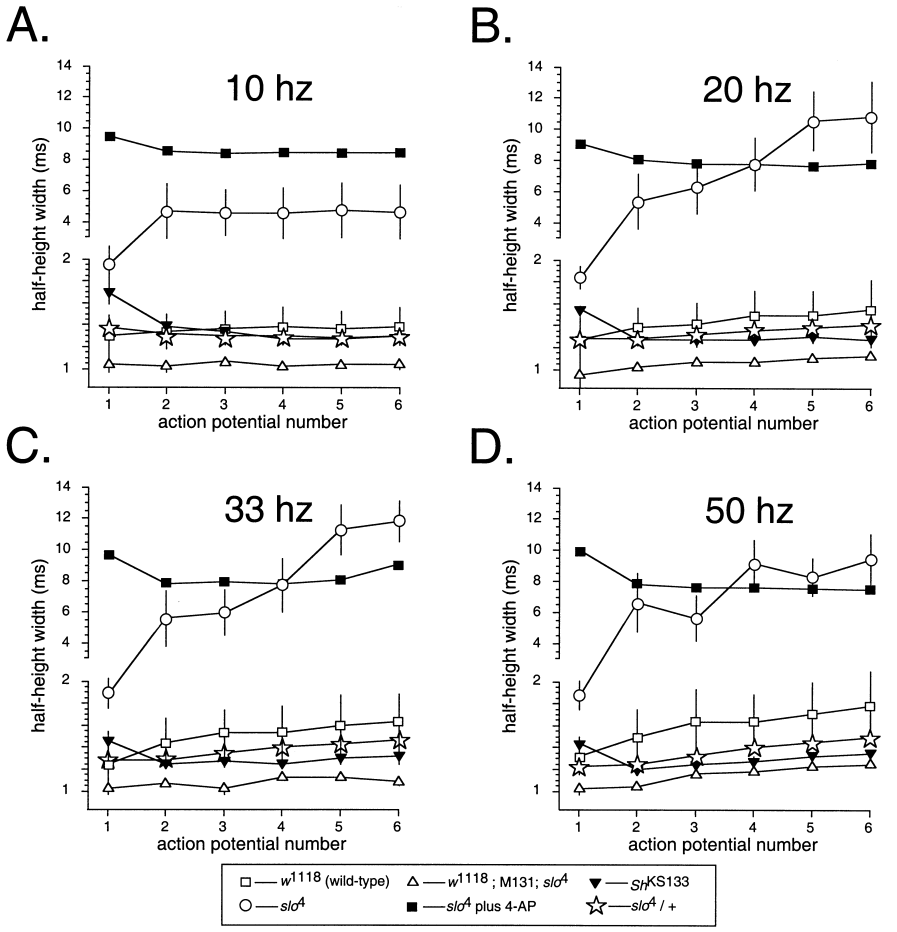

FIG. 5. Quantitative measure of action-potential broadening. Action-potential trains produced by indirect flight muscle were evoked by stimulation of the giant fiber pathway. To determine the extent and time course of action-potential broadening, we measured the half-height width of the first six action potentials. Action potentials were collected from muscle homozygous for the *slo*^{4} mutant allele (○); from w^{1118} wild-type control (□); from w^{1118}; M131; *slo*^{4} animals that express the *slowpoke* channel encoded by the M131 transgene (△); from *slo*^{4} homozygous muscle that had been exposed to 25 m*M* 4-AP (■), and from muscle carrying the Sh^{KS133} mutant allele (▼) and muscle heterozygous for *slo*^{4} (☆). **A–D** are different stimulation frequencies. The data shown are averages of three to 10 traces except that the *slo*^{4} plus 4-AP data represent a single iteration. In this assay, *slo*^{4} heterozygotes were indistinguishable from wild type (data not shown). Vertical lines represent SEM, and in many cases the symbol used to identify the line obscures the error bar.

tive *slowpoke* channels with respect to this electrophysiological phenotype. Figure 4 shows sample recordings collected at a stimulation frequency of 20 and 33 Hz. At these frequencies, as well as all other frequencies tested, the M131 transgene apparently complements the *slo*^{4} broad action-potential phenotype. To quantify the action-potential broadening that occurs in response to repetitive stimulation, we measured the average half-height width of the action potentials. Action-potential trains were evoked at 10, 20, 33, and 50 Hz and the half-height width of the first six spikes determined. Figure 5 shows the effect of repetitive stimulation on action-potential width for wild-type animals, heterozygous *slo*^{4} animals, homozygous *slo*^{4} animals, M131 *slo*^{4} homozygotes, and Sh^{KS133} homozygotes. At these stimulation frequencies, wild-type animals and heterozygotes produce trains of sharp action potentials (~1.3 ms half-height width). Muscle homozygous for the *slo*^{4} mutation, however, broadened with a maximal increase in spike width of 100, 486, 527, and 403% at 10, 20, 33, and 50 Hz, respectively. In these recordings, the first action potential produced by *slo*^{4} muscle characteristically has a normal shape and duration; however, the subsequent action potentials in a train show a gradual increase in spike width (Figs. 4 and 5). At frequencies >10 Hz, the width achieves a maximal value by the fourth or fifth stimulation.

At 10, 20, and 33 Hz stimulation frequency, M131 action potentials show no broadening and, in fact, are somewhat sharper and taller than wild-type muscle action potentials (Fig. 5A–C). At 50 Hz stimulation frequency, the M131 action potentials are the same width as those observed in wild-type muscle.

M131 complements the flight defect

Animals homozygous for null mutations in the *slowpoke* gene have a compromised ability for flight (Elkins et al., 1986). We used a flight column assay to test the ability of the M131 transgene to complement the *slowpoke* flight defect. This assay measures the relative distance that the animals fall in a column (see Materials and Methods) and was used by Elkins et al. (1986) to measure the flying ability of a *slowpoke* mutant. Figure 6 compares the relative flight capacity of wild-type, *slo*^{4} animals, and *slo*^{4} animals carrying the M131 transgene. Animals that fly well tend to accumulate near the top of the column, whereas animals that fly poorly accumulate nearer the bottom. In this assay, wild-type and *slo*^{4} animals are easily distinguished. Animals expressing the M131 transgene fly extremely well and tend to accumu-

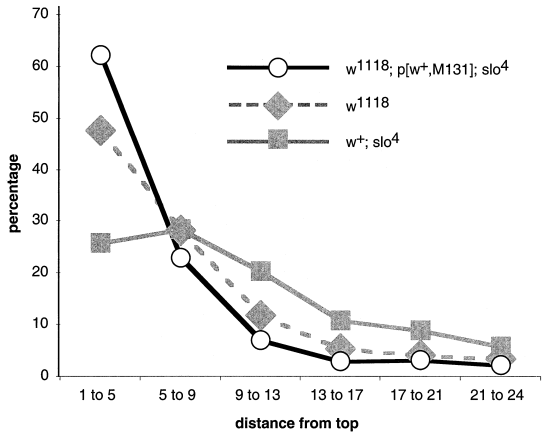

FIG. 6. Flight testing of wild-type, *slo*^{4}, and transgenic animals. Flies were dropped into the center of a 15 cm × 62 cm pipette jar whose walls had been coated with mineral oil. The falling animals fly toward the jar walls and become imbedded in the mineral oil. The position of each animal is marked and the distance traveled down the column determined. This assay has been used previously to quantify the relative capacity of *Drosophila* for flight (Benzer, 1973; Elkins et al., 1986). The graph shows the percentage of animals found in fractions 1–5, 5–9, 9–13, 13–17, 17–21, and 21–24. Fraction 1 is near the column top, and fraction 24 is near the column bottom. Each fraction is ~2.54 cm. All stocks are related to the w^{1118} stock. We used this stock as an example of an animal carrying a wild-type copy of the *slowpoke* gene. Wild-type animals (gray dotted line, data points marked with a diamond, 952 animals tested) fly well, and the majority of the flies are found in the first fraction. Animals homozygous for the *slo*^{4} mutation (gray lined marked with boxes, 828 animals tested) fly poorly, and most animals are found in the second fraction. The w^{1118}; p[w^+, M131]; *slo*^{4} flies (black line, open circles, 528 animals tested) fly well and accumulate near the column top.

late near the very top of the column. In fact, they appear to perform better than their wild-type counterparts. These results clearly indicate that the M131 transgene complements the *slo*^{4} flight defect.

M131 does not complement the sticky-feet behavioral defect

Mutant alleles of *slowpoke* also cause a second behavioral phenotype, the sticky-feet phenotype (Elkins et al., 1986). The behavior was evoked by exposure to a 40°C heat pulse. The animals are then placed on a countertop where they behave as if their feet are stuck to the surface. Identifying those with sticky-feet can be simplified by pushing on them with a flattened pencil point (Atkinson et al., 2000). Wild type and heterozygotes do not show this behavior and mosey or fly away from the area. The penetrance of this phenotype is ~90% for the *slo*^{4} mutant allele. Of 246 flies tested from the w^{1118}; M131A; *slo*^{4} stock, 230 manifested the sticky-feet phenotype and 16 did not. The *slo*^{4} stock showed similar ratios: of 258 flies tested, 230 were scored as having sticky-feet and 28 were scored as not having sticky-feet. In an independent control, 474 *slo*^{4} heterozygote animals were mixed with *slo*^{4} homozygotes and scored. In this assay, only eight heterozygotes were classified as having the sticky-feet phenotype. The sticky-feet phenotype has been shown to result from a loss of expression in the neural tissue (Atkinson et al., 2000). Therefore, it is incumbent on us to demonstrate that the transgene is active in the *Drosophila* CNS. RT-PCR was used to demonstrate that the transgene was producing neural-specific transcripts (Fig. 7).

The animals carrying the M131 transgene appear to recover more rapidly from the heat treatment than their *slo*^{4} counterparts. However, we do not know if this represents partial complementation of the phenotype or if it is merely a function of genetic background. In any case, it is clear that the M131 transgene does not significantly affect the fraction of animals manifesting the sticky-feet behavioral defect and, in this sense, can be said to fail to complement.

DISCUSSION

In heterologous expression systems, alternative splicing of ion-channel transcripts affects the biophysical properties of some channels. An obvious inference is that alternative splicing tailors the channel to the needs of the cell (Navaratnam et al., 1997; Rosenblatt et al., 1997; Saito et al., 1997). Selection for such an elaborate regulatory mechanism requires that the change in properties caused by alternative splicing be enough to affect the performance of the animal. We have used transgenic *Drosophila* and the *slowpoke* Ca^{2+}-activated K^+ channel gene to test this hypothesis. *Slowpoke* transcripts undergo extensive alternative splicing (19 alternative exons in five sites of alternative splicing), at least some of which is tissue-specific. Elimination of *slowpoke* expression by mutation causes both behavioral and physiological defects. Our approach was to eliminate expression from the endogenous gene using the *slo*^{4} mutation, and then to determine if a single *slowpoke* splice variant expressed from a transgene can complement defects caused by the mutation.

We took care in the design of the *slowpoke* transgene to ensure that it would reproduce the expression of the

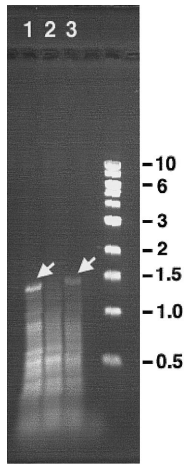

FIG. 7. The M131 transgene expresses a neural-specific transcript. Products of the neural-specific promoter C0 all begin with exon C0. Transcripts expressed by this promoter were detected by RT-PCR using exon-specific primers and visualized on an agarose gel. A 1.4-kb PCR fragment (identified by the arrows) is diagnostic for expression from neuronal promoter C0. It is present in the wild-type samples (lane 1), missing in the *slo*^{4} samples (lane 2), and present in samples from the M131 transgenic animals (lane 3). Lane 4 contains the molecular weight standard. Sizes of standard bands in kb are marked to the right of the standard lane. The M131 transgene is in a *slo*^{4} background; therefore, the endogenous gene does not contribute to the amplification product.

endogenous gene in all ways with the exception of alternative splicing. To drive expression, we used the cloned *slowpoke* transcriptional control region. This control region has been well characterized (Becker et al., 1995; Brenner and Atkinson, 1996, 1997; Brenner et al., 1996; Thomas et al., 1997) and mimics the expression pattern of the endogenous gene. This DNA contains all five of the *slowpoke* tissue-specific transcriptional promoters (Fig. 1 and Thomas et al., 1997), each of which generates a unique 5′ exon. The control region ends with an exon common to all *slowpoke* transcripts. A cDNA was fused in-frame to this exon. This design results in a product in which promoter choice generates the appropriate tissue-specific 5′ exon, but which is locked into the splicing pattern encoded in the cDNA.

A well-known phenotype of *slowpoke* null mutants is their extremely broad flight muscle action potentials. In slo^4 mutant muscle, the expression of the M131 transgene complements the physiological defect and the cell produces well-formed action potentials. It is interesting that these action potentials are taller and substantially sharper than wild-type (w^{1118}) action potentials. This could occur if the transgene had increased the repolarizing K^+ current density above and beyond that found in the wild-type. Indeed, the two-electrode voltage-clamp data in Fig. 3 show that transgenic muscle displays a *slowpoke* current density nearly twice the size of normal muscle.

We suggest the following explanation. An increased K^+ current density both sharpens and enhances spike amplitude. A larger K^+ current more quickly repolarizes the spike and leads to sharpening, and the increased spike amplitude results from fewer voltage-gated Ca^{2+} channels existing in the inactivated state. In *Drosophila* muscle, these Ca^{2+} channels underlie the rising phase of the action potential. The rising phase of the action potential is produced by a voltage-activated Ca^{2+} current. A reduction in the duration of the spike would reduce the time that the Ca^{2+} channels are exposed to the depolarized state and therefore reduce the activity-dependent inactivation of this current. An increased macroscopic Ca^{2+} current could lead to a taller spike.

An increase in K^+ current density might result from an increase in channel number, single channel conductance, or Ca^{2+} sensitivity. We speculate that the observed differences most likely result from differences in the biophysical properties of the native and transgenic channels resulting from alternative mRNA splicing rather than from an increase in the number of channels. This could result from alternative splicing. For instance, alternative splicing at splice site 1 can cause a 1.5-fold change in channel conductance, whereas changes at alternative splice site 3 can cause up to a 100-fold difference in Ca^{2+} sensitivity (Lagrutta et al., 1994).

Loss of *slowpoke* expression results in behavioral changes. Two that we examined were the sticky-feet phenotype and the flight defect [*slowpoke* mutations also affect the courtship song (Peixoto and Hall, 1998); however, we did not examine this aspect]. The sticky-feet phenotype is correlated with a loss of neuronal expression, and the flight defect can be complemented by muscle expression (Atkinson et al., 2000). Behavioral assays showed that the transgene failed to complement the sticky-feet phenotype, but clearly complemented the muscle flight defect. Apparently, the transgene-encoded protein is similar enough to substitute for the normal muscle product(s), or this tissue is quite forgiving in its requirements.

What accounts for the inability to complement the sticky-feet phenotype? One possibility is that the transcriptional control region fails to express the gene in a critical subset of cells. However, previous studies using a lacZ reporter indicate that this control region faithfully reproduces the *slowpoke* expression pattern. A more likely explanation is that a different splice variant or more than one splice variant is required for complementation.

Our results indicate that at least some of the *slowpoke* splice products are not interchangeable, and that substitution of inappropriate splice variants causes behavior defects affecting the performance of the animal. Clearly, alternative splicing of ion-channel genes must be carefully regulated to insure that each cell manifests an electrical character appropriate for its function.

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