Behavioral and Electrophysiological Analysis of Ca-activated K-channel Transgenes in Drosophila[a]

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ABSTRACT: The *slowpoke* gene of *Drosophila melanogaster* encodes a Ca-activated K channel. This gene is expressed in neurons, muscles, tracheal cells, and the copper and iron cells of the midgut. The gene produces a large number of alternative products using tissue-specific transcriptional promoters and alternative mRNA splicing. We have described in great depth how transcription is regulated and are now cataloging the tissue-specificity of different splice variants. It is believed that the diversity of products serves to tailor channel attributes to the needs of specific tissues. Electrophysiological and behavioral assays indicate that at least some of these products produce channels with distinct properties.

The range of electrical properties that a cell can produce is ultimately limited by the suite of ion channel genes that it expresses. For the cell this is not a simple decision. For instance, a Drosophila neuron, in theory, is capable of expressing thousands of different potassium channels. However, it will typically express less than 10 different K channels.[1–4] We are using the Drosophila *slowpoke* gene as a model system to understand how ion channel gene expression is regulated and the consequences of this regulation.

The Drosophila *slowpoke* gene was the first Ca-activated K-channel gene to be cloned.[5] Since then it has been used to clone Ca-activated K channels from a variety of organisms.[6–8] In vertebrates, the gene encodes the so-called BK type Ca-activated K channel. BK-type channels are high-conductance K channels that, in practice, require Ca to open, but then modulate their open probability as a function of membrane potential.[9]

BK-type channels are involved in a variety of processes in disparate cell types. In neurons and muscles, Ca-activated K channels participate in setting the cell's electrical excitability and its firing pattern;[10–14] in smooth muscle they act to modulate myogenic tone,[15] whereas in epithelial cells they assist in the control of osmotic pressure and in lymphocytes they help generate Ca oscillations that are associated with mitogen binding.[16–18]

It may seem surprising that a single K channel could contribute to such a wide variety of processes. However, the *slowpoke* gene appears well suited for such a role. The gene produces a startling number of different products using alternative exons whose expression is controlled in a tissue-specific manner. The regulation of *slowpoke* gene expression appears to permit the properties of Ca-activated K channels to be fine-tuned to meet the specific needs of a wide variety of different cells.

[a]This work was supported by the following grants: National Science Foundation IBN-9724088, American Heart Association, Texas Affiliate 96G-414, and The Council for Tobacco Research 4760.

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CONTRIBUTION OF TRANSCRIPTIONAL REGULATION TO CHANNEL DIVERSITY

The *slowpoke* transcriptional control region was cloned in 11 kilobases (kb) of genomic DNA.[19] This DNA is diagrammed in FIGURE 1A. To date, we have identified five *slowpoke* transcriptional promoters which differ in tissue specificity. (A promoter is a DNA sequence that acts as a landmark to identify where transcription should begin and the direction that transcription should proceed.) The tissue specificity and level of expression is typically determined by control elements that interact with the promoter. We have used immunohistochemical staining, RT-PCR, RNase protection assays, and *in situ*

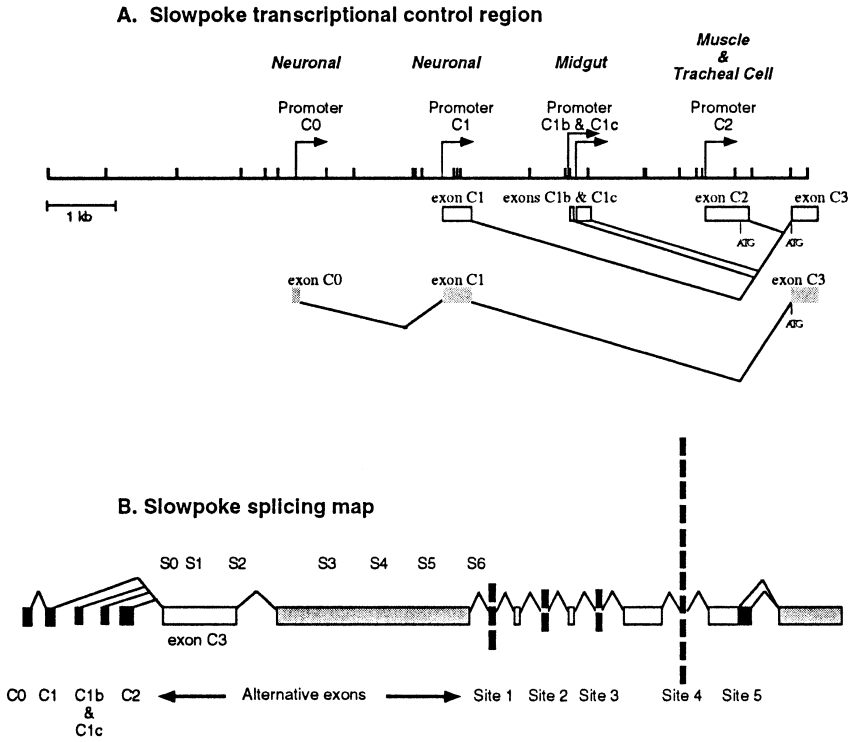

FIGURE 1. (**A**) Map of the *slowpoke* transcriptional control region. The horizontal line represents the genomic DNA. The arrowheads represent transcriptional promoters, and the tic marks represent common restriction enzymes. The tissue specificity of each promoter is indicated above the promoter. The boxes below the line are the alternative 5′ exons generated by each tissue-specific promoter. The connecting lines identify the splicing pattern. A lacZ reporter gene inserted into exon C3 has been used to monitor promoter activity in transgenic flies. (**B**) Contribution of alternative splicing to *slowpoke* product diversity. Open and gray boxes represent exons used in all mRNAs. Black boxes are alternative exons that arise from alternative promoter use (exons C0, C1, C1b, C1c and C2) or alternative splicing (exons at sites 1, 2, 3, 4, and 5). The boxes at site 4 actually represent 11 distinct splicing patterns produced by combinations of six smaller exons.[26] Gray boxes are regions that are not alternatively spliced but for which the number of exons is not certain. Connecting lines represent the splicing pattern. The labels S0, S1, S2, S3, S4, S5, and S6 represent areas that encode putative transmembrane domains.[5,33]

hybridization to determine the expression pattern of the endogenous gene.[19,20] In flies, the *slowpoke* gene is expressed in the central and peripheral nervous systems, musculature, midgut, and tracheal cells.

To determine the tissue-specificity of the *slowpoke* promoters, we performed deletion mapping using a transgene in which the *slowpoke* transcriptional control region drives expression of a lacZ reporter gene. The lacZ gene is inserted after the control region such that expression from any *slowpoke* promoter causes expression of the lacZ product, the enzyme β-galactosidase. The presence of this enzyme can easily be detected using the chromogenic substrate Xgal. Using this method, the expression pattern of the deletion-bearing transgenes was determined in sectioned animals and intact organs.

An advantage of transgenic animals for this type of analysis is that all cells of the animal carry a copy of the mutated transgene. Therefore, one can determine how the deletion affects the activity of the transgene in each and every tissue type. Furthermore, all of the tissues and organs are present in their natural context. In this manner, we showed that promoters C0 and C1 are active in the nervous system, that muscle and tracheal cell expression arises from promoter C2, and midgut expression from a DNA fragment carrying promoters C1b and C1c[20–23] (FIGS. 1A and 2).

It appears that one function served by the multiple promoters is to enable distinct tissues to express the gene at widely different levels.[24] Of course, because alternative promoters effectively choose the 5′ exon of a transcript, they influence the amino-terminal sequence of the protein. In fact, transcripts expressed from *slowpoke* promoter C2 include

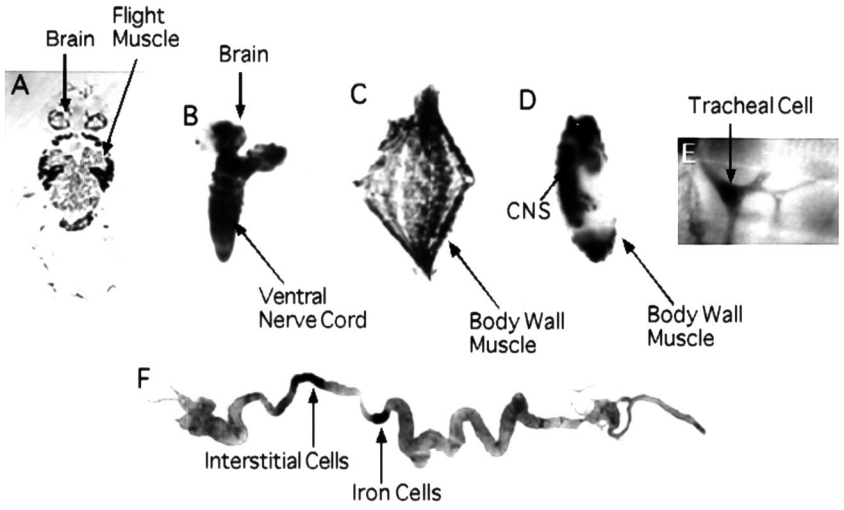

FIGURE 2. The *slowpoke* gene is expressed in a large number of different tissues during development.[20] Expression from a *slowpoke* transgene is used to document expression. (**A**) In the adult brain, expression arises from promoter C0 and C1. In adult flight muscle, promoter C2 is active. (**B**) Larval brain expression results from promoter C0 and C1 activity. (**C**) Using filleted and gutted larvae, expression of promoter C2 is documented in the larval body wall muscles. (**D**) In the embryonic central nervous system (CNS) expression is from promoter C0 and C1, while body wall expression is from promoter C2. (**E**) Promoter C2 is also active in tracheal cells as documented by immunohistochemical detection of the native protein. (**F**) *Slowpoke* is expressed in the interstitial cells of the copper cell region and in the iron cell region. This expression arises from a promoter fragment containing promoters C1b and C1c.

a 5′ exon that introduces a new translation start site. Its use appends 17 codons onto the 5′ end of the message's open reading frame. The activity of promoter C2 is limited to muscle and tracheal cells, which means that in these tissues *slowpoke* channels contain an additional 17 amino-terminal amino acids not present in neurons or midgut cells.[20] It is not known how these 17 amino acids affect the biophysical properties of the channel.

CONTRIBUTION OF ALTERNATIVE SPLICING TO CHANNEL DIVERSITY

The *slowpoke* gene also uses alternative mRNA splicing to choose among mutually exclusive alternative exons.[5,25] The splicing map of the mRNA is shown in FIGURE 1B. To date, we have documented the presence of 14 alternatively spliced exons. In theory, alternative splicing in the 3′ end of the message can produce 1,512 distinct splice variants. Using RT-PCR we have documented the expression of 132 variants that are produced in a tissue-specific manner.[26] This diversity is in addition to that generated by the alternative promoters. In this manner the gene can produce a dizzying array of distinct products. One should also consider the fact that Ca-activated K channels, like voltage-activated K channels, are thought to be tetramers.[27,28] If the formation of heterotetrameric channels is permitted, then the potential number of distinct *slowpoke* channels is astronomical.

ELECTROPHYSIOLOGICAL ANALYSIS OF DROSOPHILA TRANSGENES

It is often assumed that tissue-specific mRNA splicing is used to tailor a product to the specific needs of a cell. However, it is usually very difficult to test this hypothesis. To determine the functional consequences of *slowpoke* alternative splicing we used our transgenic expression system.[29] We built transgenic animals which expressed, in the musculature, either a neuronal or muscle mRNA splice variant. Expression from the endogenous bona fide *slowpoke* gene was prevented using the slo^4 mutation, which eliminates all expression from the gene.

The B52 transgene expresses a neuronal splice variant, and the M131 transgene expresses a common muscle variant. These two transgenes differ at the sites 3, 4, and 5 alternative splice sites (FIG. 1B and TABLE 1). These changes alter only 64 amino acids in the approximately 1,200 amino acid protein. All of these changes are in the carboxy terminal tail of the protein near a putative calcium sensor.[30,31]

The first parameter to be examined was the action potential waveform produced by the adult animal's flight muscle. Action potentials are recorded as described in FIGURE 3A. FIGURE 3B shows a comparison of the action potentials produced by normal animals and animals carrying the slo^4 mutation. The slo^4 mutation eliminates all expression of Ca-activated K channels from the gene. As shown, normal animals produce sharp action potentials regardless of stimulation frequency (10 to 50 Hz tested). In contrast, slo^4 mutant

TABLE 1. Differences in the Alternative Exons Carried in the *Slowpoke* Transgenes.

	Alternative Splice Sites				
Transgene	Site 1	Site 2	Site 3	Site 4	Site 5
B52	1a	2b	3b	4bdef	5a
M131	1a	2b	3a	4bf	5b

NOTE: The position of the splice sites referred to in this table are documented in FIGURE 1B. B52 is a transgene that expresses a brain-specific mRNA splice variant, and M131 is a transgene that expresses a muscle-specific mRNA splice variant. The abbreviations 1a, 2b, 3a, 3b, 4bdef, 4bf, and 5a refer to the alternative exons present in alternative splice sites 1 through 5.

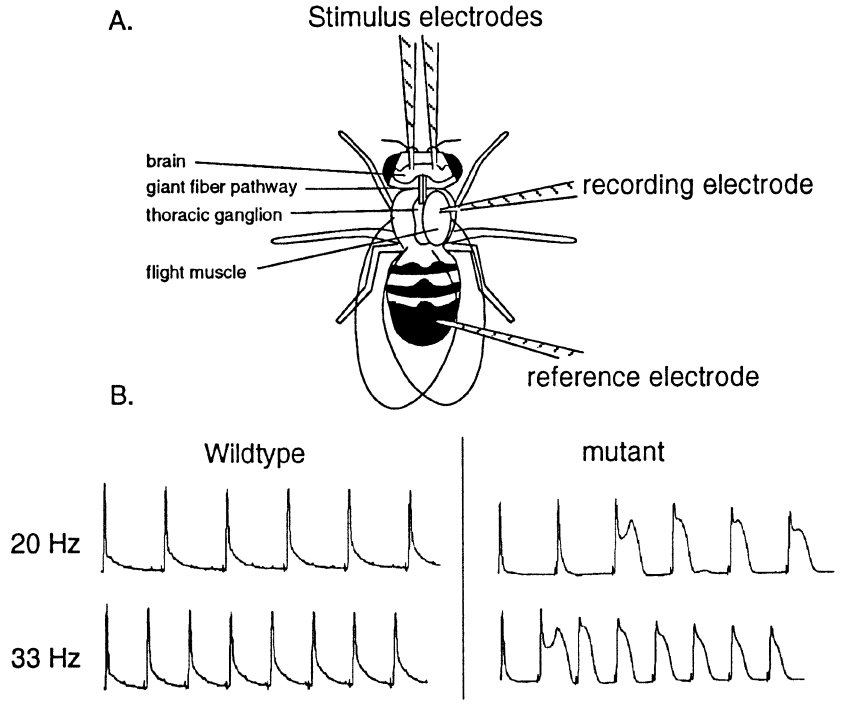

FIGURE 3. Action potentials produced by the flight muscles of wild-type and *slo*[4] mutant flies. (**A**) The brain of an immobilized fly is stimulated using tungsten electrodes which results in the production of action potentials in the flight muscle. These are recorded using a NaCl (1M) filled glass electrode. Muscle response is measured relative to a tungsten electrode inserted into the abdomen. (**B**) Action potentials produced by wild-type and *slo*[4] mutant animals when stimulated at 20 and 33 Hz. Notice the abnormal shape of the action potentials produced by mutant animals.

animals produce abnormally broad action potentials. The *slowpoke* channel plays a major role in the rapid repolarization of the muscle action potential.[12,32]

The severity of this phenotype is proportional to stimulation frequency. The first few action potentials are typically of normal width. It is believed that during this time voltage-gated K channels are capable of repolarizing the cell membrane. The broadening of the subsequent action potentials is thought to arise because of long-lasting inactivation of the voltage-gated K channels.

FIGURE 4 shows a quantitative measure of this effect and the capacity of the transgenes to complement it. The width at half height of the sixth action potential in a train has been measured and expressed as a percentage of the width of the first action potential. Wild-type muscle does not show significant action potential broadening while *slo*[4] mutant muscle shows substantial broadening. The transgenes expressing the muscle (M131) and brain (B52) splice variants differ in their ability to complement this phenotype.[29]

The muscle transgene complements the action potential broadening phenotype at all stimulation frequencies, whereas the brain variant complements at low-stimulation frequencies but not at high frequencies (FIG. 4). This effect is not due to poor expression of the brain variant in muscle cells; therefore, it must arise from a difference in biophysical properties of the channel.

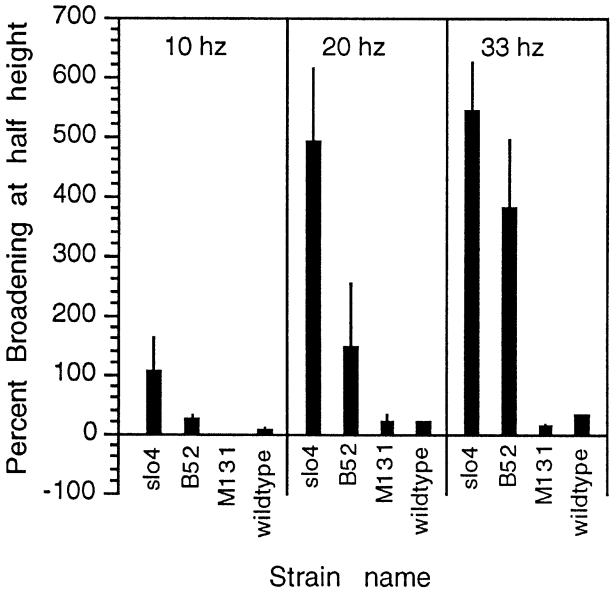

FIGURE 4. Action potential broadening during repetitive stimulation. The width of evoked action potentials in flight muscle were measured at half-height. The width of the sixth action potential is expressed as a percentage of the width of the first action potential. The first action potential is uniformly sharp. Mutant *slo*^{4} animals show substantial broadening at all stimulation frequencies. Mutant *slo*^{4} animals do not express any *slowpoke* channels; B52 expresses only a brain splice variant; M131 expresses only a muscle variant; and wild-type animals express the normal complement of *slowpoke* channels.

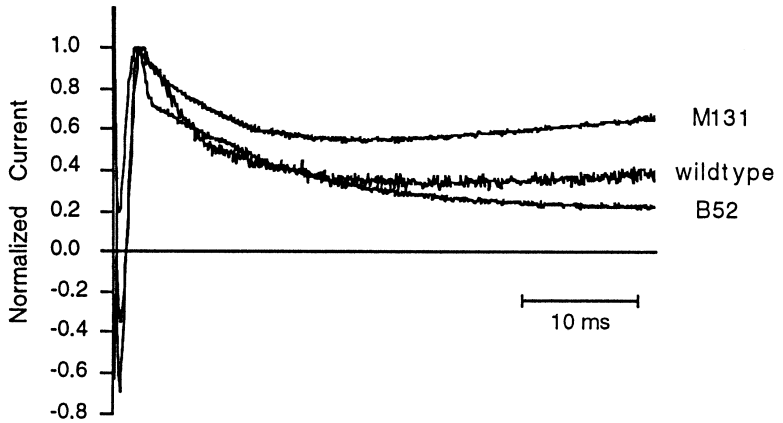

FIGURE 5. BK currents conducted by *slowpoke* channels expressed from the B52 and M131 transgenes or by the intact wild-type gene. Currents were recorded by two-electrode voltage clamp of larval body wall muscle. Currents have been normalized to peak height and cell capacitance so that the differences in kinetics can be easily compared. Currents were evoked by a voltage step from –80 to +10 mV. In transgenic animals expression from the endogenous gene is eliminated by use of the *slo*^{4} null mutation.

To determine whether the B52 and M131 channels had different biophysical properties, a two-electrode voltage clamp was used to monitor current flowing through the *slowpoke* channels. This assay was performed on larval body wall muscle became it more readily lends itself to this procedure. Although the brain variant produced a large initial current in muscle fibers, its current decayed more rapidly than the current produced by the muscle variant (FIG. 5).

In all cases, the *slowpoke* channel is responding to the concentration of calcium. It is believed that the primary source of this calcium is influx through the voltage-gated calcium channel in the cell membrane. Therefore, the difference in the current traces produced by the *slowpoke* brain and muscle variants must arise due to differences in their calcium sensitivity. This interpretation is bolstered by previous work which shows that changes in the carboxy terminal tail affect the calcium sensitivity of channels expressed in Xenopus oocytes.[30,31]

BEHAVIORAL ASSAYS CONFIRM THAT ALTERNATIVE SPLICE VARIANTS ARE NOT FUNCTIONALLY INTERCHANGEABLE

This subtle difference in electrical character of the brain and muscle splice variants also affects the flying ability of the animals. Animals that do not express *slowpoke* channels are poor fliers.[32] This is a muscle, not a neuronal, phenotype. We compared the flying ability of wild-type animals, *slo*^{4} mutant animals and *slo*^{4} mutant animals carrying either the B52 or M131 transgene using a very simple behavioral assay.

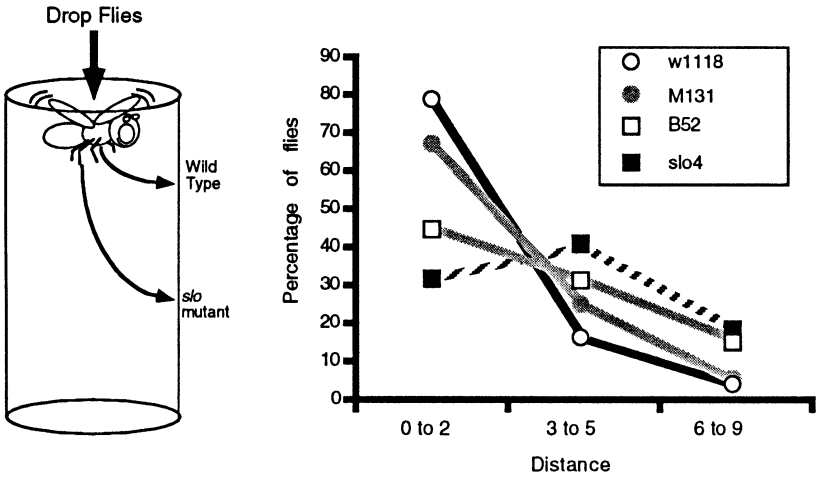

FIGURE 6: The muscle splice variant but not the neuronal splice variant restores normal flight. To measure flying ability, animals were dropped into a cylinder coated with mineral oil. Wild-type animals fly immediately and are primarily found embedded in the oil near the top of the cylinder. Animals that do not fly well tend to fall much further into the cylinder. The graph shows the distribution of flies. Wild-type (*open circles*) animals and those that express only the M131 muscle splice variant (*closed circles*) are found near the top of the column. Animals that express only the neuronal B52 splice variant (*open boxes*) or do not express any *slowpoke* channels (*slo*^{4} mutants; *closed boxes*) fall deeper into the column.

Flying ability was measured by dropping flies into the center of a pipette jar whose walls were coated with mineral oil. As the flies fall, they fly toward the walls of the jar and become embedded in the oil. Animals that fly well are typically found near the top of the jar, whereas the animals whose flying ability has been compromised are more evenly distributed between top and bottom halves of the jar (see FIG. 6).

Transgenic animals carrying the M131 transgene (muscle variant) are able to fly as well as wild-type. However, animals carrying the B52 transgene fly as poorly as animals that do not express any *slowpoke* channels. Therefore, the subtle difference in channel properties has been translated into a large difference in functional properties of the muscle.

CONCLUSIONS

The regulation of BK channel expression in flies is extremely complex. Gene expression is regulated at the level of transcription and mRNA splicing. The five *slowpoke* promoters and the 14 alternatively spliced exons can generate a remarkable number of different products. Alternative exon use is regulated in a tissue-specific manner presumably so that different cell types can manifest distinct electrical properties.

Experiments with transgenic flies have shown that the different splice variants are not functionally interchangeable. It is clear that the subtle differences in the polypeptide wrought by alternative mRNA splicing can cause a large change in the electrical properties of a cell. Furthermore, behavioral assays in which flight was measured have shown that these differences in electrical character dramatically affect the performance of the cell and the animal's capacity for flight.

We expect that the use of transgenic Drosophila will lend itself to the exploration of the activity-dependent regulation of electrical properties. One can envision that the use of an electrical circuit could change the expression level or splicing pattern of a channel gene and thereby cause a long-lasting change in a cell's electrical character.

ACKNOWLEDGMENTS

We thank Gwen Gage and Kristina Schlegel for their help with the figures.

REFERENCES

1. GORCZYCA, M. G. & C.-F. WU. 1991. Single-channel K^+ currents in Drosophila muscle and their pharmacological block. J. Membr. Biol. **121:** 237–248.
2. SINGH, S. & C.-F. WU. 1990. Properties of potassium currents and their role in membrane excitability in Drosophila larval muscle fibers. J. Exp. Biol. **152:** 59–76.
3. O'DOWD, D. K. 1995. Voltage-gated currents and firing properties of embryonic drosophila neurons grown in a chemically defined medium. J. Neurobiol. **27:** 113–126.
4. MARTINEZ-PADRON, M. & A. FERRUS. 1997. Presynaptic recordings from Drosophila: Correlation of macroscopic and single-channel K^+ currents. J. Neurosci. **17:** 3412–3424.
5. ATKINSON, N. S., G. A. ROBERTSON & B. GANETZKY. 1991. A component of calcium-activated potassium channels encoded by the Drosophila slo locus. Science **253:** 551–555.
6. BUTLER, A., S. TSUNODA, D. P. MCCOBB, A. WEI & L. SALKOFF. 1993. mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. Science **261:** 221–224.
7. PALLANCK, L. & B. GANETZKY. 1994. Cloning and characterization of human and mouse homologs of the Drosophila calcium-activated potassium channel gene, slowpoke. Hum. Mol. Genet. **3:** 1239–1243.

8. DWORETZKY, S. I., J. T. TROJNACKI & V. K. GRIBKOFF. 1994. Cloning and expression of a human large-conductance calcium-activated potassium channel. Brain Res. Mol. Brain Res. **27:** 189–193.
9. BLATZ, A. L. & K. L. MAGLEBY. 1987. Calcium-activated potassium channels. Trends Neurosci. **10:** 463–467.
10. HILLE, B. 1992. Ionic Channels of Excitable Membranes. 2nd edit. Sinauer Associates, Inc. Sunderland, MA.
11. RUDY, B. 1988. Diversity and ubiquity of K channels. Neuroscience **25:** 729–749.
12. ELKINS, T. & B. GANETZKY. 1988. The roles of potassium currents in Drosophila flight muscles. J. Neurosci. **8:** 428–434.
13. NAVARATNAM, D. S., T. J. BELL, T. D. TU, E. L. COHEN & J. C. OBERHOLTZER. 1997. Differential distribution of Ca^{2+}-activated K^+ channel splice variants among hair cells along the tonotopic axis of the chick cochlea. Neuron **19:** 1077–1085.
14. ROSENBLATT, K. P., Z. P. SUN, S. HELLER & A. J. HUDSPETH. 1997. Distribution of Ca^{2+}-activated K^+ channel isoforms along the tonotopic gradient of the chicken's cochlea. Neuron **19:** 1061–1075.
15. FAY, F. S. 1995. Calcium sparks in vascular smooth muscle: Relaxation regulators. Science **270:** 588–589.
16. SHEPPARD, D. N. & F. GIRALDEZ. 1988. Kinetics of voltage- and Ca^{2+} activation and Ba^{2+} blockade of a large-conductance K^+ channel from Necturus enterocytes. J. Membr. Biol. **105:** 65–75.
17. TURNHEIM, K., J. CONSTANTIN, S. CHAN & S. G. SCHULTZ. 1989. Reconstitution of a calcium-activated potassium channel in basolateral membranes of rabbit colonocytes into lipid bilayers. J. Membr. Biol. **112:** 247–254.
18. LEWIS, R. S. & M. D. CAHALAN. 1995. Potassium and calcium channels in lymphocytes. Annu. Rev. Immunol. **13:** 623–653.
19. BECKER, M. N., R. BRENNER & N. S. ATKINSON. 1995. Tissue-specific expression of a Drosophila calcium-activated potassium channel. J. Neurosci. **15:** 6250–6259.
20. BRENNER, R., T. O. THOMAS, M. N. BECKER & N. S. ATKINSON. 1996. Tissue-specific expression of a Ca^{2+}-activated K^+ channel is controlled by multiple upstream regulatory elements. J. Neurosci. **16:** 1827–1835.
21. BRENNER, R. & N. ATKINSON. 1996. Developmental and eye-specific transcriptional control elements in an intronic region of a Ca^{2+}-activated K^+ channel gene. Dev. Biol. **177:** 536–543.
22. THOMAS, T., B. WANG, R. BRENNER & N. S. ATKINSON. 1997. Novel embryonic regulation of Ca^{2+}-activated K^+ channel expression in Drosophila. Invertebr. Neurosci. **2:** 283–291.
23. BOHM, R., R. BRENNER & N. ATKINSON. 1998. Transcriptional control of Ca-activated K channel expression: Identification of a second, evolutionarily-conserved, neuronal promoter. Submitted.
24. BRENNER, R. & N. S. ATKINSON. 1997. Calcium-activated potassium channel gene expression in the midgut of Drosophila. Comp. Biochem. Physiol. B **118:** 411–420.
25. ADELMAN, J. P., K. Z. SHEN, M. P. KAVANAUGH, R. A. WARREN, Y. N. WU, A. LAGRUTTA, C. T. BOND & R. A. NORTH. 1992. Calcium-activated potassium channels expressed from cloned complementary DNAs. Neuron **9:** 209–216.
26. YU, J. Y. & N. S. ATKINSON. 1998. Unpublished data.
27. SHEN, K. Z., A. LAGRUTTA, N. W. DAVIES, N. B. STANDEN, J. P. ADELMAN & R. A. NORTH. 1994. Tetraethylammonium block of Slowpoke calcium-activated potassium channels expressed in Xenopus oocytes: Evidence for tetrameric channel formation. Pfluegers Arch. Eur. J. Physiol. **426:** 440–445.
28. WU, J. V., T. J. SHUTTLEWORTH & P. STAMPE. 1996. Clustered distribution of calcium sensitivities: An indication of hetero-tetrameric gating components in Ca^{2+}-activated K^+ channels reconstituted from avian nasal gland cells. J. Membr. Biol. **154:** 275–282.
29. BRENNER, R., J. Y. YU, P. SUBRAMONY, J. WILBUR & N. S. ATKINSON. 1998. Complementation of physiological and behavioral defects by a slowpoke Ca^{2+}-activated K^+ channel transgene. In preparation.
30. WEI, A., C. SOLARO, C. LINGLE & L. SALKOFF. 1994. Calcium sensitivity of BK-type KCa channels determined by a separable domain. Neuron **13:** 671–681.
31. SCHREIBER, M. & L. SALKOFF. 1997. A novel calcium-sensing domain in the BK channel. Biophys. J. **73:** 1355–1363.

32. ELKINS, T., B. GANETZKY & C.-F. WU. 1986. A Drosophila mutation that eliminates a calcium-dependent potassium current. Proc. Natl. Acad. Sci. USA **83:** 8415–8419.
33. MEERA, P., M. WALLNER, M. SONG & L. TORO. 1997. Large conductance voltage- and calcium-dependent K+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus. Proc. Natl. Acad. Sci. USA **94:** 14066–14071.