Shal and Shaker Differential Contribution to the K^+ Currents in the *Drosophila* Mushroom Body Neurons

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Shaker, a voltage-dependent K^+ channel, is enriched in the mushroom bodies (MBs), the locus of olfactory learning in *Drosophila*. Mutations in the *shaker* locus are known to alter excitability, neurotransmitter release, synaptic plasticity, and olfactory learning. However, a direct link of Shaker channels to MB intrinsic neuron (MBN) physiology has not been documented. We found that transcripts for *shab*, *shaw*, *shaker*, and *shal*, among which only Shaker and Shal have been reported to code for A-type currents, are present in the MBs. The electrophysiological data showed that the absence of functional Shaker channels modifies the distribution of half-inactivation voltages ($V_{1/2}$) in the MBNs, indicating a segregation of Shaker channels to only a subset (~28%) of their somata. In harmony with this notion, we found that approximately one-fifth of MBNs lacking functional Shaker channels displayed dramatically slowed-down outward current inactivation times and reduced peak-current amplitudes. Furthermore, whereas all MBNs were sensitive to 4-aminopyridine, a nonspecific A-type current blocker, a subset of neurons (~24%) displayed little sensitivity to a Shal-specific toxin. This subset of neurons displaying toxin-insensitive outward currents had more depolarized $V_{1/2}$ values attributable to Shaker channels. Our findings provide the first direct evidence that altered Shaker channel function disrupts MBN physiology in *Drosophila*. To our surprise, the experimental data also indicate that Shaker channels segregate to a minor fraction of MB neuronal somata (20–30%), and that Shal channels contribute the somatic A-type current in the majority of MBNs.

Key words: learning; memory; mushroom bodies; Shaker; K^+ channels; *Drosophila*

Introduction

Olfactory learning in *Drosophila melanogaster* depends on the structural and physiological integrity of the mushroom bodies (MBs) (Roman and Davis, 2001). In the fruit fly brain, MBs are specific structures composed of ~2500 MB intrinsic neurons (MBNs) or Kenyon cells, which receive inputs from olfactory and other sensory organs and send outputs to premotor brain regions (Stocker, 1994; Ito et al., 1998). Therefore, MBs are suited to integrate sensory experiences to bring about new behaviors in coherence with previous experience. Chemical ablation or mutations that alter the MB structure lead to the loss of associative olfactory learning (Heisenberg et al., 1985; de Belle and Heisenberg, 1994). Genes required for associative olfactory learning are preferentially expressed in the MBs (Roman and Davis, 2001). Although important information has been obtained from behavioral and molecular studies of olfactory learning in *Drosophila*,

the physiological basis of this process in the fly brain is essentially unknown. The available information on the physiological properties of MBNs has been derived essentially from studies on either acutely dissociated neurons or in primary culture (Wright and Zhong, 1995; Delgado et al., 1998).

Early evidence that ion channels are relevant to associative olfactory learning in the fruit fly was provided by Cowan and Siegel (1986), who found that this process is deficient in a *shaker* mutant. The *shaker* gene codes for voltage-dependent K^+ -selective channels (VDKCs) that, through alternative splicing, give rise to either rapidly inactivating A-type currents or noninactivating currents, both of which are blocked by 4-aminopyridine (4-AP) (Iverson et al., 1988; Timpe et al., 1988; Iverson and Rudy, 1990; Stocker et al., 1990). Immunohistochemistry studies revealed that Shaker channels are preferentially expressed in the MB neuropil (Schwarz et al., 1990; Rogero et al., 1997), suggesting that they play an important role in MB physiology. Shaker channels are important to membrane repolarization, and the lack of Shaker function alters excitability, neurotransmitter release, and synaptic plasticity (Jan et al., 1977; Tanouye et al., 1981; Delgado et al., 1994).

Although the available information favors a role for Shaker in MB function, we lack direct evidence documenting a physiological correlate to the *shaker* defect in MBNs of *Drosophila*. Here, we took advantage of an enhancer-detector fly line expressing green fluorescent protein (GFP) in the *Drosophila* MBs and combined the reverse transcriptase (RT)-PCR, patch-clamp recordings, and

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genetic and pharmacological inquiries to determine the identity of the VDKCs operating in MBs and to assess the contribution of Shaker channels to the A-type conductance. The results obtained reveal the following: (1) MBs express four genes coding for VDKCs, among which only *shaker* and *shal* code for A-type currents; (2) Shal channels are the main contributors to the somatic A-type current in the MBs; (3) Shaker channels conduct the A-type current in ~25% of dissociated MBNs; and (4) the absence of functional Shaker channels significantly modifies the whole-cell K^+ current profile of the *shaker*-expressing MBNs.

Materials and Methods

Fly stocks. The 7A2-Gal4 transgenic strain was generated using standard *Drosophila* gene transfer procedures (Rubin and Spradling, 1983; Pirrotta, 1988). The 7A2 construct was generated by cloning a 10 kb fragment that includes the transcription initiation site of the *fruitless* (*fru*) gene into the pPTGAL vector (Sharma et al., 2002). This fragment is flanked by *SalI* sites and was cloned into the *BamHI* site of pPTGAL rendered compatible after a 2 bp fill-in reaction (for a detailed map of the construct, see supplemental material, available at www.jneurosci.org). The pattern of expression was observed after crossing the resulting transgenic fly lines with upstream activating sequence (UAS)-GFP transgenic fly lines, and 7A2 was selected for its capacity to drive enriched expression of Gal4 into the MBs at several developmental stages (Fig. 1*A,B*). The 7A2 expression had no correlation with the endogenous expression of *fru*. For additional experiments, wandering male third-instar larvae were obtained from the mating of males from the homozygous line 7A2-Gal4 and females homozygous for a UAS-GFP transgene in the second chromosome ($w^{1118}; p[w(+mC)] = \text{UAS-EGFP}5a.2$; Bloomington Stock Center, Bloomington, IN). Additional information about the lines used in this study is provided in the supplemental material (available at www.jneurosci.org). For a *shaker*-mutant genotype, the GFP insertion on the second chromosome was transferred to a *sh*^{KS133} background, and female *sh*^{KS133}; UAS-GFP were used for mating. *sh*^{KS133} is an antimorphic allele in which the substitution of a valine for an aspartate residue in the selectivity filter yields a nonconducting protein (Lichtinghagen et al., 1990) (see Fig. 3*A*).

RT-PCR analysis. Fifteen to 18 larval brains were dissected in PBS, and ventral ganglia and imaginal discs were removed. Neuronal tissue was dispersed according to the study by Wu et al. (1983). Cerebral lobes were incubated for 25 min in 1 ml of PBS containing trypsin (0.0125%, type III; Sigma, St. Louis, MO) at 37°C. After two washes in Schneider's *Drosophila* medium (Invitrogen, Carlsbad, CA) supplemented with 20% of heat-inactivated fetal bovine serum (Invitrogen) and 50 $\mu\text{g}/\text{ml}$ gentamicin (Invitrogen), the tissue was resuspended in 180 μl of a modified *Drosophila* defined medium (DDM) (O'Dowd, 1995). Trypsinized tissue was passed 50 times through the tip of a beveled pipette. After achieving cell dissociation, the suspension was placed on a multiwell slide and inspected for clusters of GFP+ cells (see Fig. 2*A*). Four to five clusters were taken up into a broken-tipped patch pipette (see Fig. 2*B*), released into an Eppendorf (Eppendorf Scientific, Westbury, NY) tube, and processed for cDNA synthesis using the Cell-to-cDNA kit (Ambion, Austin, TX), according to the instructions of the manufacturer. A total of 2 μl of the cDNA solution was used as template for each PCR. Primers 5'-GCCGAAGAGGAGGATA-3' and 5'-CTGGCAAATATGGACAAC-3' were used to amplify a region of 535 bp common to all reported alternative transcripts of the *shaker* gene. Primers 5'-AAGCACGAATGCC-TACC-3' and 5'-GAAGACAAGGAAGCCAGTT-3' were designed to amplify a 666 bp fragment of the *shal* transcripts. Primers 5'-GCAGCATTGTCTCCTTCCATC-3' and 5'-TCTCCATCAGCGCTC-CCTC-3' were used to amplify a region of 651 bp of the long isoform of *shab* and a region of 560 bp of the short isoform. Primers 5'-GAC-TATTGGCGTGGTTTCGG-3' and 5'-GAAGTCGTTGTGCGGATT-GG-3' were used to amplify the *shaw* transcripts (562 bp). A total of 2 μl of each PCR was reamplified, lowering the temperature of annealing 5°C. PCR products were purified and sequenced to verify their molecular identity. Negative controls were treated equally, but reverse transcriptase

Figure 1. GFP+ neurons in the 7A2-Gal4/UAS-GFP line identify MBNs *in vivo* and *in vitro*. *A*, Adult brains were dissected in PBS, fixed in 4% paraformaldehyde, and slightly flattened by a glass coverslip. A bright-field image of a whole mount is shown at the left, whereas a maximal intensity confocal projection of the same preparation is shown at the right. The brain is oriented with the anterior face up and dorsal at the top. *B*, Larval brains were treated in the same manner as the adult brains and mounted with the ventral ganglion down. A bright-field image is at the left, whereas the confocal projection is at the right. Scaling is the same as in *A*. The inset shows a higher magnification of the larval brain. Two dorsally projecting lobes (arrows) and two medially projecting lobes (arrowheads) were stained. Neurons in the *par intercerebralis* are also marked (asterisk). *C*, In acutely dissociated larval brains, MBNs are identified as small GFP+ neurons (arrow). The image at the left is a bright-field view of a 7A2-Gal4/UAS-GFP dissociated brain within 12 h after plating. The image at the right shows the same field under epifluorescence illumination and viewed via FITC filters.

was omitted in the RT reaction. Under these conditions, no PCR products were observed.

Expression of recombinant *Drosophila* Shal channels. Stage VI *Xenopus* oocytes were prepared as described previously (Espinosa et al., 1999). *Shal* mRNA was synthesized *in vitro* from a clone of *dshal2* (a gift from L. Salkoff, University of Washington, St. Louis, MO) and injected (50 nl) into the oocytes at a concentration of ~1 ng/nl. Oocytes were incubated at 18°C for 6 d in ND-96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 sodium pyruvate, 5 HEPES, pH 7.6) saline solution containing 50 $\mu\text{g}/\text{ml}$ gentamicin before electrophysiological examination. Currents were recorded in ND-96 at ~20°C with a two-electrode voltage-clamp amplifier (GeneClamp 500B; Axon Instruments, Union City, CA). The electrodes had a resistance of 0.4–0.9 M Ω when filled with 3 M KCl. Leak currents were subtracted using a P/4 protocol, and the data were filtered at 1 kHz and acquired at 5 kHz.

Larval muscle recordings. The ventral lateral longitudinal fiber 6 of segments A2 and A3 of wandering third instar larvae was used for all experiments (Bate, 1993). The larval preparation was identical to that described previously (Jan and Jan, 1976). Larvae were dissected and kept in hemolymph-like 3 saline solution (Stewart et al., 1994) until electro-

physiological examination. This solution was developed to increase the morphological and physiological stability of *Drosophila* muscles at room temperature. K^+ currents of the larval muscles were recorded using the two-electrode voltage-clamp methodology from a holding potential of -65 mV. To eliminate the inward Ca^{2+} currents as well as the Ca^{2+} -dependent outward currents, a Ca^{2+} -free saline solution bathed the muscles during the experiments. This solution contained the following (in mM): 130 NaCl, 5 KCl, 18 $MgCl_2$, 36 sucrose, 0.5 EGTA, 5 HEPES, pH 7.2. High Mg^{2+} concentration was used to circumvent the problems of muscle surface potential change and membrane leakage increase caused by a Ca^{2+} -free solution (Wu and Haugland, 1985). Currents were filtered at 2 kHz and digitized at 10 kHz. Either a Warner OC-725 (Warner Instruments, Hamden, CT) or a Dagan CA-1 (Dagan, Minneapolis, MN) voltage-clamp amplifier was used. The linear components of the elicited currents were on-line subtracted using a P/4 protocol. Electrodes were filled with 3 M KCl. Tip resistances of voltage and current electrodes were in the range of 10–20 M Ω and 5–10 M Ω , respectively.

MB neuronal preparation for electrophysiological recordings. Dissociated larval neurons were obtained by treating larval brains exactly as described above (RT-PCR analysis) with the exception that the dissociated cells were plated on two 12 mm coverslips (Bellco Glass, Vineland, NJ) located in a Petri dish and allowed to settle for 1 h before flooding the dish with 2 ml of DDM. Cells were kept at 23–24°C for 2–24 h before electrophysiological examination. MBNs were identified on the coverslips as small-size GFP+ cells (Fig. 1C). A count of at least 10 random fields in five independent cultures yielded an average of $4.6 \pm 0.6\%$ GFP+ MBNs, comparable with previous estimates obtained in independent studies made with different transgenic lines expressing a reporter gene in the MBs (Wright and Zhong, 1995; Delgado et al., 1998; Su and O'Dowd, 2003) and with the fraction of MBNs in the CNS (Ito et al., 1998). Visual examination at 1 μ m resolution of 183 GFP+ cells yielded a modal size of 5 μ m. Cell capacitance averaged 0.42 ± 0.01 pF ($n = 116$). Assuming spherical shape and a specific cell capacitance of 1μ F/cm², average cell capacitance corresponds to a diameter of 3.6 μ m. Both estimates are in close agreement with the documented size of *Drosophila* MBNs *in vivo* (3.9 μ m) (Wang et al., 2001).

Electrophysiological recordings from neurons. The coverslip containing the cells was placed in a recording chamber mounted on an inverted microscope endowed with epifluorescence, and the chamber was superfused with an external saline solution composed of the following (in mM): 140 NaCl, 3 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 5 HEPES, pH 7.2, with NaOH (osmolality of 290–292). Experiments were done at room temperature (22–25°C). The patch pipettes were polished and filled with an internal solution composed of the following (in mM): 140 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, pH 7.2, with KOH (osmolality of 288–290; resistances of 4–7 M Ω). To block VDKCs, in some experiments, the KCl in the pipette solution was replaced for an equimolar concentration of CsCl and, in such cases, the pH was adjusted to pH 7.2 with CsOH. Junction potentials were cancelled immediately before the establishment of a high-resistant seal, and the pipette capacitance was compensated before achieving the whole-cell configuration. During the recording session, the cells were kept at a holding potential of -70 mV. Cell capacitance was determined integrating the average current elicited by five consecutive 5 mV hyperpolarizing pulses. Series resistance compensation was used at 60–70%. Experiments in which the input resistance was <1.0 G Ω or the series resistance higher than 30 M Ω were discarded. Thus, the clamp error during the recording of the largest currents obtained (~ 780 pA) amounted to <3.3 mV. 4-AP was purchased from Sigma, diluted at the appropriate concentration in the external saline solution, and superfused to the recording chamber. Phrixotoxin-2 (PaTx2; Alomone Labs, Jerusalem, Israel) was kept frozen for <2 months as 10 X stock aliquots and thawed immediately before use. After dilution in the appropriate recording solution, the toxin was bath applied to the muscle- or oocyte-recording chamber or pressure ejected (~ 10 psi) directly to the soma of the patch-clamped neuron. PaTx2 was delivered from a $\sim 5 \mu$ m diameter pipette connected to a Picospritzer II (General Valve Corporation, Fairfield, NJ) positioned $\sim 20 \mu$ m from the target neuron. Only visually confirmed toxin ejection was considered.

Data acquisition and analysis. Cells were voltage clamped using an

Axopatch 200 amplifier (Axon Instruments). Except for the capacitive currents that were filtered at 10 kHz and digitized at 100 kHz, signals were low-pass filtered at 5 kHz, and the sampling interval was set to 40 μ s/point. Data points were acquired and further analyzed with pClamp6 software. Input resistance was evaluated before each voltage paradigm using a 30 mV hyperpolarizing voltage step from the holding potential. The calculated input resistance was then used to leak-subtract all records linearly. Unless otherwise stated, the membrane was held at -100 mV for 1 s before any voltage protocol to remove inactivation from inactivating components of the current.

Steady-state inactivation data were obtained with a 1 s prepulse protocol ranging from -100 to -20 mV in 5 or 10 mV increments. The current remaining after the prepulse was elicited by a jump to $+40$ mV. The peak current was plotted as a function of the prepulse potential (V_p) and the resulting points fitted to a single Boltzmann distribution. To avoid changes in the voltage-operating range of VDKCs induced by whole-cell configuration (Baker and Salkoff, 1990; Hardie, 1991), all of the steady-state data reported here were taken within 2 min of establishing the whole-cell recording mode.

In our analysis of inactivation time constants, the decaying phase of the current elicited by an 80 ms depolarizing pulse to $+40$ mV was fitted to a sum of exponential terms plus a base line. The optimal number of coefficients was determined with a confidence of 99.9% according to the theory of nested models (Horn, 1987).

Data are presented as mean $\pm$ SEM, unless otherwise stated. Differences were treated as statistically significant when $p < 0.05$.

Results

RT-PCR analysis of voltage-dependent K^+ channels in the mushroom bodies

A Shaker-like current has been postulated to be the major component of the A-type current in honeybee MBNs in primary culture (Pelz et al., 1999). The presence of A-type currents is apparent in patch-clamp recordings from *Drosophila* MBNs acutely dissociated (Wright and Zhong, 1995) and in primary culture (Delgado et al., 1998). However, the identity of the ion channel(s) contributing to the A-type current of *Drosophila* MBNs has not been determined.

We searched for transcripts coding for VDKCs in the MBs by RT-PCR and found expression of *shaker*, *shal*, *shab*, and *shaw* (Fig. 2C). This result suggests that either *shaker* or *shal* could encode the somatic A-type current in MBNs. To address this issue, we investigated the properties of inactivating outward K^+ currents in these acutely dissociated neurons in control *wt* flies and in a *shaker* mutant using whole-cell recordings.

Properties of A-type currents in mushroom body neurons

A *shaker*-mutant line was obtained by mating homozygous $w^{1118}/y;7A2$ -Gal4 males with homozygous $sh^{KS133};UAS$ -GFP females. Only male offspring (called sh^{KS133}) were used for electrophysiological recordings and compared with control male *wt* neurons ($w^{1118}/y;7A2$ -Gal4/*UAS*-GFP). The sh^{KS133} allele is an ideal model to investigate the contribution of Shaker to A-type currents, because it codes for a nonfunctional protein that, although expressed, is unable to conduct ions (Lichtinghagen et al., 1990). We checked the phenotype of our lines by recording VDKCs in the larval ventrolateral muscle 6, where Shaker provides all of the A-type current component (Wu and Haugland, 1985). Figure 3A documents that control muscles expressed a prominent A-type current. In contrast, the A-type current was absent in the sh^{KS133} line. We next recorded outward currents in acutely dissociated MBNs. These cells were identified as small ($\sim 5 \mu$ m diameter) GFP+ cells (Fig. 1C) (for additional information, see Materials and Methods). A preliminary screening of such cells revealed the presence of robust, inactivating outward currents in all 17 neu-

Figure 2. Mushroom bodies express transcripts for *shaker*, *shal*, *shab*, and *shaw* channels. Clusters of GFP+ cells derived from the MBs recovered from the dispersal of larval brains were used for cDNA preparation. One of those clusters is shown before (**A**) and after (**B**) it had been taken up into a broken-tipped pipette. The left column depicts bright-field views, whereas the right column shows the same fields under epifluorescence illumination and viewed via FITC filters. **C**, cDNA prepared from four to five clusters of GFP+ cells was subjected to a double PCR run, and the expected products were resolved in a 1.5%-agarose gel stained with ethidium bromide. M, Molecular weight marker in bp. The intensity of the bands in the gel is not quantitative.

rons tested. A representative recording is shown in Figure 3B. Such whole-cell currents were carried by K^+ ions, because they were absent when Cs^+ replaced K^+ in the internal solution (Fig. 3B, inset) and were completely blocked by a mixture of 4-AP (2.5–10 mM) and quinidine (100 μ M) (data not shown). The bottom part of Figure 3B illustrates the average currents elicited by depolarizing pulses from -60 to $+40$ mV in 10 mV increments at the peak (filled circles), at the end of the depolarizing pulse (inverted triangles) and in experiments in which K^+ was replaced by Cs^+ in the internal solution (open circles). The relative contribution of the noninactivating component that remained at the end of the depolarizing pulse varied from neuron to neuron, ranging from 0 to 78 pA at $+40$ mV. It averaged 60 ± 10 pA/pF when normalized by cell capacitance and represented only a minor fraction of the current density (9%). Because we were only interested in investigating the properties of inactivating outward currents, in additional analysis, the contribution of the noninactivating outward current component was subtracted. All of the MBNs tested lacked inward currents. This was confirmed in studies where Cs^+ replaced internal K^+ , a condition that

should increase the prominence of Na^+ - and Ca^{2+} -inward currents (Fig. 3B, inset). Similar results were reported by Wright and Zhong (1995) and Delgado et al. (1998). Therefore, all recordings of K^+ -carried outward currents were performed in the absence of Na^+ - or Ca^{2+} -channel blockers.

At $+40$ mV, wt outward currents, normalized by cell capacitance, averaged 624 ± 67 pA/pF ($n = 33$) (Fig. 3C, bottom). Similar to results obtained in a different transgenic line expressing *lacZ* in MBNs (Wright and Zhong, 1995), the range of peak outward current density was broad, extending from 180 to 1917 pA/pF.

When we compared the recordings from wt and *sh*^{KS133} MBNs, we were surprised to find that genetic removal of functional Shaker channels did not cause a dramatic modification in the whole-cell current profile or a significant decrease in the current density. The top traces in Figure 3C are representative whole-cell currents obtained from a *sh*^{KS133} neuron; they are visually indistinguishable from the control (Fig. 3B). The bottom graph in Figure 3C shows average peak current density values at $+40$ mV obtained from 33 wt neurons and 33 *sh*^{KS133} neurons. Shaker clones encode either a delayed rectifier (Iverson and Rudy, 1990; Stocker et al., 1990) or a rapidly activating and inactivating current (Iverson et al., 1988; Timpe et al., 1988) when expressed in *Xenopus* oocytes. In cell division-arrested embryonic *Drosophila* neurons, noninactivating currents were attributed to the Shaker channels (Saito et al., 1993). However, in most *Drosophila* cells, including larval muscles (Wu and Haugland, 1985), pupal neurons (Baker and Salkoff, 1990), and pupal photoreceptors (Hardie, 1991), Shaker A-type currents have been described. Because the noninactivating current recorded from MBNs remained in the presence of Shaker-blocking 4-AP (see below), we inferred that any effects of the mutation at the *shaker* locus would mostly affect peak current. However, peak outward current density was diminished by only 15% in *sh*^{KS133} relative to wt, and the current amplitude difference was not significant at $p < 0.05$ (*sh*^{KS133}, 533 ± 56 pA/pF; wt, 624 ± 67 pA/pF; one-tail Student's *t* test). This result would seemingly point to Shaker being only a minor contributor to somatic A-type currents in MBNs in *Drosophila*. Alternatively, it is conceivable that somatic Shaker channels might segregate to a subset of these neurons. In additional work, we performed pharmacological and biophysical studies to differentiate between these two alternatives.

Comparison of the blockade of A-type currents by a Shal-specific toxin and by the A-type current blocker 4-AP

In the most parsimonious scheme, if Shaker does not conduct the somatic A-type current in the *Drosophila* MBNs, Shal does. To confirm this hypothesis, we analyzed the blocking effect of a Shal-specific toxin, PaTx2 (Alomone Labs), over the whole-cell current of wt MBNs. PaTx2 specifically blocks the mammalian VD-KCs Kv4.2 and Kv4.3 (Diochot et al., 1999), with an IC_{50} value of 650 nM (Alomone Labs technical sheet). The Kv4.2 and Kv4.3 channels are homologous to the *shal* gene of *Drosophila* and yield an A-type current in heterologous systems (Diochot et al., 1999). Although it is documented that PaTx2 does not block mammalian shaker-like channels expressed in *Xenopus* oocytes (Diochot et al., 1999), the toxin has not been tested on *Drosophila* Shal or Shaker channels. Therefore, we decided to analyze the blockade of PaTx2 on recombinant *Drosophila* Shal (dShal) channels expressed in *Xenopus* oocytes and *Drosophila* Shaker channels recorded in larval muscles. The top traces in Figure 4A show recombinant dShal K^+ current expressed in *Xenopus* oocytes before and after exposure to 1 μ M PaTx2. The average blockade

was $70 \pm 2\%$ ($n = 4$) (Fig. 4C). The bottom traces in Figure 4A show the Shaker A-type current recorded from larval muscles at the same potential. In this case, it is clear that PaTx2 had no effect on transient current amplitude (Fig. 4C).

In 76% of tested MBNs, PaTx2 blocked a rapidly inactivating outward current component (Fig. 4B, top traces). The extent of blockade averaged $45 \pm 3\%$ ($n = 41$) (Fig. 4C), and this effect was reversed after toxin washout. The above result led us to conclude that *shal* is functionally expressed in the somata of these neurons where it contributes significantly to the A-type current. Additionally, inspection of the extent of blockade of outward currents by PaTx2 in 41 MBNs revealed that not all neurons were equally sensitive to the toxin (Fig. 4B, compare top and bottom traces). In fact, in 10 neurons, the toxin had only little effect on peak current amplitude (Fig. 4D, bottom). This observation led us to suspect the presence of a subset of MBNs in which expression of Shal might be less conspicuous or even absent. Therefore, we compared the extent of PaTx2-mediated blockade of A-type currents to that produced by 4-AP, a K^+ channel blocker for both Shaker and Shal currents (Iverson et al., 1988; Wei et al., 1990). Figure 4D (top traces) shows a representative example of the blocking effect of 4-AP on outward current in MBNs. The bottom plot in Figure 4D shows that, in contrast to PaTx2, the extent of 4-AP block was quite homogeneous (average block, $55 \pm 3\%$; $n = 26$), revealing that the 4-AP-sensitive outward current contributes more or less evenly to the inactivating current in these cells. Moreover, in the PaTx2-sensitive MBN subset (31 of 41), the extent of block by 4-AP and by PaTx2 was equal (55 ± 3 vs $55 \pm 2\%$, respectively). Thus, we conclude that in such neurons, the somatic type-A current is contributed mostly by Shal. On such grounds, it would follow that the subset of neurons that were relatively resistant to PaTx2, but in which the A-type current was blocked by 4-AP, represents a subset of neurons in which the somatic A-type current is dominated by Shaker.

Analysis of the steady-state inactivation of outward currents in mushroom body neurons

In additional work, we analyzed the biophysical properties of outward currents in MBNs. We focused first on the steady-state inactivation, which for simplicity was modeled with a single Boltzmann distribution. This analysis, in a sample of 46 *wt* and 37 *sh^{KS133}* MBNs, yielded average midpoint voltage of inactivation ($V_{1/2}$) values that were not significantly different (*wt*, -76.4 ± 4.6 mV; *sh^{KS133}*, -77.0 ± 2.7 mV; $\pm$ SD). However, we noted that the range of $V_{1/2}$ values in *wt* was broader (-83.0 to -66.0 mV) than in *sh^{KS133}* (-83.0 to -71.0 mV), and an *F* test comparison

Figure 3. Shal dominates inactivating outward K^+ current in the *Drosophila* MBNs. **A**, K^+ currents recorded from the ventral lateral longitudinal fiber 6 of segments A2 and A3 of *wt* or *sh^{KS133}* wandering third-instar larvae, elicited by the voltage protocol shown below. Voltage increments were of 10 mV. Each trace represents average currents from six different muscles in four different preparations (for *wt*) or average currents from 18 different muscles from 11 different larvae (for *sh^{KS133}*). *sh^{KS133}* muscles lack prominent inactivating outward current contributed by Shaker channels. **B**, Top traces are representative control outward currents recorded from acutely dissociated *wt* MBNs, elicited by the voltage protocol shown below. Voltage increments were of 10 mV. Holding potential was -70 mV. Depolarizing pulses were preceded by a 1 s prepulse to -100 mV. Bottom graph shows average *I/V* plots (normalized by cell capacitance) of peak current (●), noninactivating current recorded after 80 ms of depolarization onset (○), and peak current when internal KCl was replaced by equimolar CsCl (○). For details of the recording solutions, see Materials and Methods. Each point represents the average of 17 neurons (for KCl records) or eight neurons (for CsCl records). The inset shows average current traces of eight consecutive neurons registered with either KCl or CsCl in the pipette. Calibration values are as described at the top. **C**, Top traces are representative outward currents recorded from acutely dissociated *sh^{KS133}* MBNs, elicited by the same voltage protocol as in **B**. Calibration values have the same dimensions as in **B**. The bottom plot depicts average peak current density, in which the sustained component has been subtracted, of 33 *wt* and 33 *sh^{KS133}* neurons. Values are 624 ± 67 and 533 ± 56 pA/pF, respectively.

of the SDs of the two samples revealed that, at $p < 0.005$, they were significantly different. Moreover, the steady-state inactivation curves in *wt* segregated into two groups: one with $V_{1/2}$ values more negative than -75 mV and another with $V_{1/2}$ values more positive than -72 mV (Fig. 5A). This impression was strengthened when we inspected the frequency distribution of $V_{1/2}$ data, grouping neurons according to their $V_{1/2}$ values using 1 mV intervals (Fig. 5B). As seen, the distribution strongly suggests that the *wt* sample segregates into two groups: one with a $V_{1/2} \approx -79$ mV and the other with a more depolarized $V_{1/2} \approx -70$ mV. The χ^2 test showed that a single Gaussian function constructed with the experimentally derived mathematical average and SD values was insufficient to account for the distribution of $V_{1/2}$ measurements (Fig. 5B, continuous line). In contrast, the distribution was well fitted by two Gaussian functions (Fig. 5B, discontinuous line) yielding $V_{1/2} = -78.0$ mV for a major component of the

Figure 4. A-type K^+ currents of the MBNs are differentially sensitive to a Shal-specific toxin. PaTx2 specificity was tested on the recombinant dShal channels expressed in *Xenopus* oocytes and in Shaker currents recorded in the ventral lateral longitudinal muscles of third-instar larvae. **A**, Superimposed control and PaTx2-exposed currents recorded from Shal-expressing oocytes (top) and muscle fibers (bottom). PaTx2 ($1 \mu\text{M}$) removed a significant fraction of the dShal currents but did not significantly block muscular Shaker channels. **B**, Superimposed traces of K^+ currents before and after exposure to $1 \mu\text{M}$ PaTx2 recorded from two different MBNs. In a toxin-sensitive cell (top traces), the blocked component is a rapid-activating rapid-inactivating current. The neuron recorded at the bottom was almost insensitive to the toxin. Calibration dimensions are the same as in the top traces. **C**, Summary of the results described in **A** ($n = 4$, for both recombinant dShal and muscular Shaker channels) and **B** ($n = 41$). The inset shows the percentage blockade by $1 \mu\text{M}$ PaTx2 of the peak current recorded in the dissociated MBNs as a function of time after plating (in hours). The line indicates the lowest blockade achieved by 4-AP. **D**, The top graph shows superimposed traces of K^+ current recorded from a MBN before and after exposure to 2.5 mM 4-AP, a general blocker of A-type currents. Calibration dimensions are the same as in **B**. The bottom plot shows the blockade efficiency of 4-AP and PaTx2 in each cell tested. The extent of 4-AP blockade was quite even ($55 \pm 3\%$), whereas blockade by PaTx2 was broader, stronger, and almost equal to 4-AP blockade in approximately two-thirds of the sample. Although each MBN expresses approximately the same amount of 4-AP-sensitive outward current, the PaTx2-sensitive Shal-contributed component is relatively weak in a subset of neurons.

distribution, which comprised 72% of the sample, and -70.0 mV for a minor component. For the sake of completeness, Figure 5E offers the distribution of $V_{1/2}$ data in *wt* in cumulative manner to further document the gross departure from a normal distribution. The continuous line joining the experimental data in Figure 5E was estimated using the parameters derived from fitting two Gaussian distributions to the data.

The separation of neurons into two populations was not apparent in the *sh^{KS133}* sample, which distributed around an average $V_{1/2} = -77.0 \pm 2.7 \text{ mV}$ (Fig. 5C). Figure 5D depicts the plot of the frequency distribution of $V_{1/2}$ values in *sh^{KS133}*. The line

Figure 5. Shaker channel adds a depolarized component to the whole-cell inactivating current. **A**, Steady-state inactivation data from 46 *wt* neurons were obtained by measuring peak current elicited by a depolarizing pulse to $+40 \text{ mV}$ (inset) after the conditioning prepulses (V_p) indicated in the x-axis. Data were normalized to maximal current and single Boltzmann distributions used to fit experimental data. Midpoint voltage of steady-state inactivation ($V_{1/2}$) averaged $-76.4 \pm 4.6 \text{ mV}$ ($\pm \text{SD}$). **B**, Histogram showing that *wt* MBNs can be divided into two groups according to their $V_{1/2}$. Bin size is 1 mV . Continuous line depicts Gaussian function built with the mathematical average and SD values obtained from the experimental data. Dashed line shows best Gaussian fit. Optimization yielded the following values: $V_{1/2} = -78.0 \text{ mV}$ for the major component (72% of the sample) and $V_{1/2} = -70.0 \text{ mV}$ for the minor component. **C**, Steady-state inactivation data from 37 *sh^{KS133}* neurons were obtained and analyzed exactly as described above. $V_{1/2}$ averaged $-77.0 \pm 2.7 \text{ mV}$ ($\pm \text{SD}$). **D**, Histogram of $V_{1/2}$ for *sh^{KS133}* neurons was constructed as described above. The Gaussian function described by the empirical average value of $V_{1/2}$ and its SD is shown as a continuous line. **E**, The distribution of $V_{1/2}$ in *wt* and *sh^{KS133}* neurons is shown in cumulative manner. Continuous lines are Boltzmann distributions constructed with the parameters derived from fitting Gaussian distributions to the data in Figure 5, **B** and **D**. Whereas *sh^{KS133}* closely matches a single Gaussian distribution, *wt* sample departs grossly. Inset, *wt* $V_{1/2}$ values (in millivolts) plotted as a function of time in culture (in hours). The line indicates -73 mV . **F**, Sensitivity of the currents to PaTx2 ($n = 20$) and 4-AP ($n = 16$) is plotted as function of $V_{1/2}$. The dashed line indicates average blockade by 4-AP ($56 \pm 3\%$). Calibration: **A**, **C**, 100 pA , 20 ms .

joining the experimental bars is a Gaussian distribution constructed with the average and SD parameters derived from the whole sample to document that the fitting yields a good estimate of the population properties. In Figure 5E, the distribution of $V_{1/2}$ data in *sh^{KS133}* neurons is presented also in cumulative manner to emphasize the match to a normal distribution. Note that in *sh^{KS133}*, the distribution reaches 95% of cumulative inactivation

at -73 mV. In contrast, in *wt*, a significant fraction ($\sim 30\%$) of the neurons had $V_{1/2}$ values more depolarized than -73 mV (Fig. 5B). Thus, the analysis of the steady-state inactivation data indicated the presence of two neuronal populations in the *wt* sample: a major one ($\sim 72\%$) displaying $V_{1/2}$ values of approximately -78 mV and a minor one displaying more depolarized $V_{1/2}$ values. This last subset of neurons is absent in *sh^{KS133}*. It is important to note that the percentage of neurons for which outward currents inactivated at more depolarized voltages ($\sim 28\%$) is similar to the percentage of neurons that displayed PaTx2-insensitive outward currents (24%) (Fig. 4) and were assumed in the previous section to represent a subset in which Shaker is a major contributor to the somatic A-type current. Thus, Shaker contributes a PaTx2-resistant A-type current, which inactivates at more depolarized voltages in a subset of MB neuronal somata in *wt* flies.

We further examined this hypothesis by analyzing the steady-state inactivation properties of the subgroups of MBNs showing either sensitivity or resistance to PaTx2. If the toxin-resistant current was contributed by Shaker, the same subgroup of neurons displaying resistance should also exhibit depolarized $V_{1/2}$ values. In 20 of the cells shown in the bottom panel of Figure 4D, the giga-seals lasted long enough (>4 min) to evaluate both steady-state inactivation and blockade by the toxin. The empty circles in the plot in Figure 5F show that in 16 of these neurons, the extent of block by PaTx2 was $\geq 40\%$, and these neurons had a $V_{1/2}$ more negative than -75 mV. The four neurons in which the toxin had little effect on peak current amplitude displayed more depolarized half-inactivation voltages. This result fits well with the idea that Shaker segregates to a small subset of MB neuronal somata where it contributes a PaTx2-resistant A-type current that inactivates at more depolarized voltages. To complete the argument, the triangles in the plot in Figure 5F document that the extent of A-type current block by 4-AP was quite even in a set of neurons that displayed a wide range of $V_{1/2}$ values.

The observation that Shaker segregates to a subset of dissociated MBNs in *wt* flies could also be explained by a time-dependent manufacture and transport of new channels for growing and maturing processes, because neurons recover from the axotomy performed during the dissociation procedure. In this instance, the toxin sensitivity and steady-state inactivation would vary during culture time, and the resistance to PaTx2 and depolarized $V_{1/2}$ values should segregate to the older neurons. As seen in the insets of Figures 4C and 5E, this was not the case, making this alternative explanation highly unlikely.

Shaker neuronal somata lack a rapidly inactivating outward current

The results in previous sections provide evidence that cells in the MBs express transcripts for four types of VDRCs and indicate that Shaker contributes to the somatic A-type current in only a subset of the intrinsic neurons. In additional experiments, we challenged this last notion by analyzing the kinetics of outward current inactivation. Outward currents in MBNs acutely dissociated (Wright and Zhong, 1995), or in primary culture (Delgado et al., 1998), display fast and slow inactivation. Shaker and Shal channels registered in larval (Tsunoda and Salkoff, 1995b) and pupal (Baker and Salkoff, 1990) neurons are known to encode for A-type currents exhibiting inactivation times <15 ms at voltages above $+20$ mV. Thus, both Shaker and Shal are competent to comprise the fast inactivating outward current component observed in MBNs. Therefore, if somatic Shaker channels segregate to a particular subset of neurons in which Shal makes little or no contribution, those neurons derived from the *sh^{KS133}* mutant

deprived of functional Shaker channels should lack the fast component of outward current inactivation.

We analyzed outward current inactivation using a nested model to establish the minimal number of exponential components that best account for the time course of inactivation (Horn, 1987). Figure 6A shows representative examples of the *wt* and *sh^{KS133}* currents for which inactivation kinetics was analyzed. The improvement in the correlation coefficient (r) when the number of terms in the sum of exponentials was increased is shown in Figure 6B. The analysis reveals that at most, two exponential components suffice to account for outward current inactivation (Fig. 6B). Moreover, in 58 of 60 *wt* neurons (97%), outward current inactivation was better accounted for in terms of two exponential components (Fig. 6C). In this majority of neurons, the time constant for the fast component of inactivation averaged 4.6 ± 0.3 ms, and the slow component averaged 22.0 ± 2.0 ms. The time constants for each individual neuron for which inactivation kinetics was fitted with two exponentials are displayed in the left panel of Figure 6D. The fast component contributed 66% of the peak current amplitude. In the two *wt* neurons in which a single exponential best fitted the time course of inactivation, the time constant was 22.0 and 21.0 ms, respectively.

The *sh^{KS133}* sample contained a significantly larger number of neurons in which the time course of inactivation was well fitted by a single exponential (12 of 61; $p < 0.005$; χ^2 test) (Fig. 6C). In these neurons, the time constant of inactivation averaged 19.0 ± 2.0 ms, and the range of inactivation time constants are shown in the right panel of Figure 6D. Within error, this value equals the time constant of the slow inactivation component in *wt* (22.0 ± 2.0 ms) and is significantly slower than the fast inactivation component (4.6 ± 0.3 ms) in the *wt* sample ($p < 0.0005$, one-tail Student's t test). Importantly, the peak-current density of MBNs lacking the fast component of inactivation in *sh^{KS133}* (432 ± 82 pA/pF; $n = 12$) was below the average peak current in *wt* neurons (602 ± 46 pA/pF; $n = 60$). This difference (28%) is marginally significant ($p = 0.056$; one-tail Student's t test). The above analysis indicates that in *sh^{KS133}*, there is a significant fraction of MBNs displaying single-exponential inactivation in which peak outward currents are diminished relative to those exhibiting double-exponential inactivation as well as to peak outward currents in *wt* neurons from this brain region. These observations agree with the hypothesis that Shaker channels code for a fast-inactivating outward current that segregates to 20–30% of MB neuronal somata in which it contributes approximately one-third of the peak current density. Notably, the minor subgroup of *sh^{KS133}* neurons displaying K^+ currents inactivating along a single exponential were only weakly blocked by the Shal-specific PaTx2 (blockade, $<23\%$) (Fig. 6E, left top traces and filled circles), indicating that their current is conducted mostly through a different channel set. This is evidence for the existence of a subset of MBNs that expresses few (if any) rapid-inactivating Shal channels. In the *wt* flies, this subgroup of neurons expresses Shaker as the major somatic A-type current component.

In that majority of neurons ($\sim 76\%$) from *sh^{KS133}* individuals displaying double-exponential inactivation, the time constant for the fast and slow components averaged 5.7 ± 0.5 and 27.0 ± 2.0 ms, respectively, and the fast component contributed 64% of peak-current density. The time constants for each individual *sh^{KS133}* neuron for which inactivation kinetics was fitted with two exponentials are displayed in the right panel of Figure 6D. In this subset of *sh^{KS133}* neurons, the peak-current density (595 ± 48 pA/pF; $n = 49$) nearly equals the peak-current density in the *wt* neuron population. They would represent that majority of MBNs

Figure 6. Shaker contributes to the rapidly inactivating current in a subset of MBNs. **A**, Transient K^+ currents from four different *wt* cells and four different *sh^{K5133}* cells are shown with fitted curves superimposed on current traces. Curves were fitted using a single or a double exponential function plus a base line. The minimal number of exponential components required to account for the time course of outward current inactivation was determined statistically according to the theory of nested models (see Materials and Methods). Calibration: (in all traces) 100 pA, 20 ms. **B**, Improvement of the coefficient of correlation (r) is shown as a function of incrementing terms in the fitted exponential functions of the traces shown in **A**. Some of the *wt* and *sh^{K5133}* neurons were already well fit by a single exponential (■, ●, □, ○), whereas others required two terms (▲, ▼, △, ▽). Symbols are related to the currents in **A**. **C**, Distribution of the analyzed *wt* ($n = 60$) and *sh^{K5133}* ($n = 61$) neurons according to the number of exponential terms for an adequate fitting. Whereas 97% of the *wt* neurons required two exponentials to account for the inactivation kinetics of the whole-cell current, only 80% of *sh^{K5133}* inactivated along a double exponential. Difference is statistically significant ($p < 0.005$; χ^2 test). **D**, Distribution of the fast and slow time constants of 58 *wt* and 49 *sh^{K5133}* MBNs, the inactivation kinetics of which were fitted with two exponentials. Squares in the right panel show the distribution of the 12 *sh^{K5133}* neurons inactivating along a single exponential. **E**, Left traces show examples of *sh^{K5133}* K^+ currents inactivating along a single (top) or a double (bottom) exponential before and after exposure to 1 μ M PaTx2. Only currents presenting a fast inactivating component were sensitive to the toxin. The right panel displays the results obtained in 22 *sh^{K5133}* neurons. Only four of them (filled circles) exhibited a single-exponential current and were slightly sensitive to the PaTx2.

in which Shal is solely responsible for carrying the somatic fast inactivating A-type current. In the *sh^{K5133}* neurons exhibiting a double-exponential current, the rapid-inactivating current was removed by application of the Shal-specific toxin PaTx2 (Fig. 6E, bottom left traces and inverted triangles) ($n = 18$) in an amount comparable with the PaTx2-sensitive current of the *wt* neurons (Fig. 4D).

The slowly inactivating current might be encoded by Shab

The slowly inactivating current that remained after 4-AP exposure (Fig. 4D) resembles the current reported previously in cultured larval neurons (Solc and Aldrich, 1988), embryonic myotubes (Zagotta et al., 1988), type III larval synaptic boutons (Martinez-Padron and Ferrus, 1997), and photoreceptors (Hardie, 1991). This current has been termed K_D and is possibly encoded by *shab* (Tsunoda and Salkoff, 1995a,b). Although we did not attempt to unambiguously identify the molecular nature of the 4-AP-resistant current in MBNs and it inactivates faster than K_D , by exclusion, we believe that Shab is indeed its major contributor. Because this current is resistant to 4-AP and removed by quinidine (data not shown) and is present in the *shaker^{K5133}* genotype, it is unlikely to be encoded by Shal or Shaker. Furthermore, although *shaw* transcripts were also found in the MB preparation for RT-PCR experiments, it is improbable that our recordings contain Shaw-conducted current. Shaw channels recorded in embryonic neurons or expressed in oocytes have extremely low voltage sensitivity and appear to function as a leak current (Wei et al., 1990; Tsunoda and Salkoff, 1995b). These currents would have been excluded from our analysis, because we linearly subtracted all of the traces according to the estimated input resistance of the cells. In addition, the slowly inactivating current was resistant to 4-AP (Fig. 4D), but native and recombinant Shaw channels are very susceptible to blockade by 4-AP (Wei et al., 1990; Tsunoda and Salkoff, 1995b).

Discussion

Ion channels expressed in the plasma membrane of neurons define their excitability (Hille, 2001). A total of 145 sequences coding for α and accessory ion channel subunits were identified in the *Drosophila* genome (Littleton and Ganetzky, 2000). From this repertoire, each neuron expresses a set of ion channels that endows it with particular excitability properties (Mandel, 1992; Serodio and Rudy, 1998; Hille, 2001).

MBNs are required for olfactory learning and retention in *Drosophila* (Roman and Davis, 2001). Although it is not yet possible to isolate *Drosophila* MBs because of the small brain region they occupy, transgenic lines expressing β -galactosidase or GFP highly preferentially in their intrinsic neurons has allowed their identification in whole-brain dispersions (Wright and Zhong, 1995; Delgado et al., 1998; Su and O'Dowd, 2003). Importantly, the expression of these proteins does not seem to alter the properties of the currents thus far recorded in the labeled neurons (Wright and Zhong, 1995; Su and O'Dowd, 2003). Here, we used GFP-labeled MBNs dissected from larval brains to characterize their VDKCs.

Olfactory learning is not adult specific. *Drosophila* larvae are capable of olfactory learning (Aceves-Piña and Quinn, 1979), which requires MB integrity (Heisenberg et al., 1985). A mutation in the gene *dunce* expressed both in larval and adult MB neuropil (Nighorn et al., 1991) perturbs learning acquisition at both stages (Aceves-Piña and Quinn, 1979). Moreover, under proper training, adult flies can recall what they were taught as larvae (Tully et al., 1994), even though axonal rearrangements

occur during metamorphosis (Armstrong et al., 1998; Lee et al., 1999). Among several molecular MB markers (Nighorn et al., 1991; Crittenden et al., 1998), the general Shaker-staining pattern is conserved in larval and adult brains (Rogero et al., 1997). Several of these proteins have been implicated in olfactory learning and memory (Dubnau and Tully, 1998; Roman and Davis, 2001). Thus, there is a strong suggestion that MBs serve the same learning functions in larvae and in adults.

Our results show that cells in the MBs express four genes coding for VDKCs. Because the isolated cell clusters used in the RT-PCR contain neurons and glia, probably in a ratio 10:1 (Klammt et al., 2001), we cannot determine which cell type is responsible for the signal. Nevertheless, these data are consistent with our electrophysiological results and support the following conclusions: Shal is the major somatic A-type current conductor in MBNs, somatic Shaker channels segregate to 20–30% of the dissociated MBNs, and the absence of functional Shaker channels modifies significantly the whole-cell current profile of this subset of neurons.

In *Drosophila*, the product of the *shal* gene (Wei et al., 1990; Covarrubias et al., 1991) and several of the splice variants of *shaker* (Iverson et al., 1988; Timpe et al., 1988) produce A-type currents when expressed in *Xenopus* oocytes. Shal behaves as delayed rectifier and Shaw operates as a leak channel (Wei et al., 1990; Covarrubias et al., 1991; Tsunoda and Salkoff, 1995a,b). In vertebrates, a Kv channel formed by an α subunit alone, which conducts a sustained current may behave as an A-type channel when an auxiliary β subunit is bound to it (Pongs et al., 1999). However, the only β subunit of *Drosophila* (Littleton and Ganetzky, 2000), Hyperkinetic (Hk), lacks the inactivating ball domain of the vertebrate $\beta 1$ subunit and is incapable of restoring rapid inactivation to a Shaker mutant channel that lacks N-type inactivation (Chouinard et al., 1995). Because coexpression experiments of Hk with *shab* and *shaw* are lacking, and inactivating splice variants have not been described for these genes, the possibility that these α subunits conduct an A-type current cannot be excluded, but this seems remote.

Because at the time of electrophysiological examination dissociated MBNs did not develop appreciable neurites (Fig. 1C), we recorded mainly from channels expressed in the cell body membrane. The cell capacitance averaged 0.42 ± 0.01 pF, which corresponds to a $3.6 \mu\text{m}$ diameter sphere, assuming $1 \mu\text{F}/\text{cm}^2$ of specific capacitance. This size is approximately the same as that reported for MB neuronal somata in intact brains ($3.9 \mu\text{m}$) (Wang et al., 2001). Therefore, we exclude the possibility of significant axonal current contribution to our reported data. We do not rule out the possibility that ion channel expression in MB neuronal axons could be different from the one we report here. In fact, Shaker seemed to be the major contributor to the A-type current recorded from honeybee dissociated MBNs that had developed axonal and dendritic arborizations (Pelz et al., 1999).

In our system, Shal seems to be the major A-type current contributor. This might be viewed as a common neuronal strategy, because in several experimental models (including *Drosophila* embryonic, larval, and pupal neurons), Shal channels underlie the somatic current (Solc et al., 1987; Baker and Salkoff, 1990; Tsunoda and Salkoff, 1995b; Song et al., 1998; Baro et al., 2000). In *sh^{KS133}* cells, inactivating outward currents half-inactivate at approximately -77 mV. Since first described, A-type currents activated at hyperpolarized voltages were proposed to have a functional role in determining interspike intervals (Connor and Stevens, 1971). In *Drosophila* embryonic neurons, 4-AP increased the firing frequency and shortened the latency to the

onset of spikes (Zhao and Wu, 1997). Genetic studies showed that the effect of 4-AP on the neuronal firing pattern of *Drosophila* neurons occurred because of Shal blockade (Tsunoda and Salkoff, 1995b). Thus, it is plausible that Shal determines the frequency coding capabilities of the MBNs.

Three morphological categories of MBNs associated with five sets of lobes have been described previously (Crittenden et al., 1998; Lee et al., 1999). Two types of neurons branch to give rise to a vertical and a median lobe (α/β and α'/β' , respectively). The third type composes the γ median lobe. Our results indicate that somatic Shaker channels are functionally expressed in a restricted subset of neurons, which represents $\sim 25\%$ of the GFP+ neurons. This value is a lower limit; we may have missed small Shaker contributions in the remaining cells. Whether this Shaker-expressing subgroup of neurons represents one of the three previously identified morphological classes remains to be established. *In situ* hybridization experiments failed to identify any preferred expression pattern of the *shaker* transcripts within adult MBNs (Pongs et al., 1988; Tseng-Crank et al., 1991), and they were undetected in larval brains possibly because of very low expression levels (Tseng-Crank et al., 1991). However, differential distribution among the three MBN subtypes has been reported for other proteins related to fly learning and memory, including metabotropic amine receptors, cAMP-related enzymes, and adhesion proteins (Crittenden et al., 1998). Distinct protein expression in the MBN subsets surely implies functional diversity. Several studies have addressed and supported this hypothesis by selectively modifying or disrupting defined lobes (O'Dell et al., 1995; Zars et al., 2000; McGuire et al., 2001; Pascual and Preat, 2001). Because a mutation in the *shaker* locus interferes with olfactory conditioning (Cowan and Siegel, 1986), its neuronal segregation might reveal the relevant circuits for this behavior.

Shaker expression in a subset of MB neuronal somata has a major impact in whole-cell current properties. In this neuronal subset, Shaker is the main contributor to the rapidly inactivating outward current ($\tau \approx 5$ ms), shifting their steady-state inactivation by approximately $+10$ mV. This contribution is physiologically relevant, because, as stated above, a *shaker* mutant performed poorly in an olfactory learning protocol that relies on the MBs (Cowan and Siegel, 1986). Shaker channels repolarize the action potential, and mutations in the *shaker* locus produce malfunctioning of the nervous system (Tanouye et al., 1981). The lack of Shaker function led to abnormal basal neurotransmitter release (Jan et al., 1977) and inhibited synaptic plasticity development (Delgado et al., 1994). This could account for part of the behavioral phenotype of *shaker* mutants. The Shaker-dependent currents recorded here stand out because they inactivate ~ 30 mV more negative than those reported in muscle or in oocyte expression studies (Wu and Haugland, 1985; Wei et al., 1990). A similar hyperpolarized $V_{1/2}$ was reported for somatic Shaker channels in *Drosophila* photoreceptors (Hardie, 1991). In these cells, inactivation of Shaker channels provides the membrane with an increased gain leading to amplification of the signal-to-noise ratio of the graded voltage signals (Niven et al., 2003). The work by Niven et al. (2003) provides evidence that the lack of Shaker channels alters the coding capabilities of photoreceptors. Additional work should establish the way in which Shaker helps to shape the electrophysiological properties of MBNs.

Ion channel modulation in the context of learning and memory is of major importance (Byrne and Kandel, 1996). PKA-dependent phosphorylation of Shaker channels at a C-terminal consensus sequence occurring in all functionally tested clones

induces an increase in inactivation velocity (Drain et al., 1994). cAMP metabolism is central to the cellular processes that underlie learning and memory in *Drosophila* (Dubnau and Tully, 1998; Roman and Davis, 2001), and it seems probable that Shaker channels are regulated by this signaling cascade. Wright and Zhong (1995) reported that a subset of MBNs express a rapidly inactivating current that is downregulated by permeable analogs of the cAMP. A comparative analysis of modulation in *wt* and *shaker* neurons should help to determine whether or not Shaker contributes to this component.

In summary, our findings lead to the notion that although *Drosophila* MBNs express more than two types of VDCC transcripts, Shal and Shaker channels segregate to different neuronal somata and that, surprisingly, lack of functional Shaker channels, which alters olfactory learning, modifies the electrophysiological profile of only a minor subset of MBNs.

References

- Aceves-Piña EO, Quinn WG (1979) Learning in normal and mutant *Drosophila* larvae. *Science* 206:93–96.
- Armstrong JD, de Belle JS, Wang Z, Kaiser K (1998) Metamorphosis of the mushroom bodies: large-scale rearrangements of the neural substrates for associative learning and memory in *Drosophila*. *Learn Mem* 5:102–114.
- Baker K, Salkoff L (1990) The *Drosophila* Shaker gene codes for a distinctive K^+ current in a subset of neurons. *Neuron* 4:129–140.
- Baro DJ, Ayali A, French L, Scholz NL, Labenia J, Lanning CC, Graubard K, Harris-Warrick RM (2000) Molecular underpinnings of motor pattern generation: differential targeting of *shal* and *shaker* in the pyloric motor system. *J Neurosci* 20:6619–6630.
- Bate M (1993) The mesoderm and its derivatives. In: *The development of Drosophila melanogaster* (Bate M, Martínez Arias A, eds), pp 1013–1090. Plainview, NY: CHSL.
- Byrne JH, Kandel ER (1996) Presynaptic facilitation revisited: state and time dependence. *J Neurosci* 16:425–435.
- Chouinard SW, Wilson GF, Schlimgen AK, Ganetzky B (1995) A potassium channel beta subunit related to the aldo-keto reductase superfamily is encoded by the *Drosophila hyperkinetic* locus. *Proc Natl Acad Sci USA* 92:6763–6767.
- Connor JA, Stevens CF (1971) Prediction of repetitive firing behaviour from voltage clamp data on an isolated neurone soma. *J Physiol (Lond)* 213:31–53.
- Covarrubias M, Wei A, Salkoff L (1991) Shaker, Shal, Shab, and Shaw express independent K^+ current systems. *Neuron* 7:763–773.
- Cowan TM, Siegel RW (1986) *Drosophila* mutations that alter ionic conduction disrupt acquisition and retention of a conditioned odor avoidance response. *J Neurogenet* 3:187–201.
- Crittenden JR, Skoulakis EM, Han KA, Kalderon D, Davis RL (1998) Tripartite mushroom body architecture revealed by antigenic markers. *Learn Mem* 5:38–51.
- de Belle JS, Heisenberg M (1994) Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science* 263:692–695.
- Delgado R, Latorre R, Labarca P (1994) *Shaker* mutants lack post-tetanic potentiation at motor end-plates. *Eur J Neurosci* 6:1160–1166.
- Delgado R, Davis R, Bono MR, Latorre R, Labarca P (1998) Outward currents in *Drosophila* larval neurons: *dunce* lacks a maintained outward current component downregulated by cAMP. *J Neurosci* 18:1399–1407.
- Diochot S, Drici MD, Moinier D, Fink M, Lazdunski M (1999) Effects of phrixotoxins on the Kv4 family of potassium channels and implications for the role of Ito1 in cardiac electrogenesis. *Br J Pharmacol* 126:251–263.
- Drain P, Dubin AE, Aldrich RW (1994) Regulation of Shaker K^+ channel inactivation gating by the cAMP-dependent protein kinase. *Neuron* 12:1097–1109.
- Dubnau J, Tully T (1998) Gene discovery in *Drosophila*: new insights for learning and memory. *Annu Rev Neurosci* 21:407–444.
- Espinosa F, Lopez-Gonzalez I, Serrano CJ, Gasque G, de la Vega-Beltran JL, Trevino CL, Darzon A (1999) Anion channel blockers differentially affect T-type Ca^{2+} currents of mouse spermatogenic cells, alpha1E currents expressed in *Xenopus* oocytes and the sperm acrosome reaction. *Dev Genet* 25:103–114.
- Hardie RC (1991) Voltage-sensitive potassium channels in *Drosophila* photoreceptors. *J Neurosci* 11:3079–3095.
- Heisenberg M, Borst A, Wagner S, Byers D (1985) *Drosophila* mushroom body mutants are deficient in olfactory learning. *J Neurogenet* 2:1–30.
- Hille B (2001) *Ion channels of excitable membranes*, Ed 3. Sunderland, MA: Sinauer.
- Horn R (1987) Statistical methods for model discrimination. Applications to gating kinetics and permeation of the acetylcholine receptor channel. *Biophys J* 51:255–263.
- Ito K, Suzuki K, Estes P, Ramaswami M, Yamamoto D, Strausfeld NJ (1998) The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* Meigen. *Learn Mem* 5:52–77.
- Iverson LE, Rudy B (1990) The role of the divergent amino and carboxyl domains on the inactivation properties of potassium channels derived from the *Shaker* gene of *Drosophila*. *J Neurosci* 10:2903–2916.
- Iverson LE, Tanouye MA, Lester HA, Davidson N, Rudy B (1988) A-type potassium channels expressed from *Shaker* locus cDNA. *Proc Natl Acad Sci USA* 85:5723–5727.
- Jan LY, Jan YN (1976) Properties of the larval neuromuscular junction in *Drosophila melanogaster*. *J Physiol (Lond)* 262:189–214.
- Jan YN, Jan LY, Dennis MJ (1977) Two mutations of synaptic transmission in *Drosophila*. *Proc R Soc Lond B Biol Sci* 198:87–108.
- Klamt C, Hummel T, Granderath S, Schimmelpfeng K (2001) Glial cell development in *Drosophila*. *Int J Dev Neurosci* 19:373–378.
- Lee T, Lee A, Luo L (1999) Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development* 126:4065–4076.
- Lichtinghagen R, Stocker M, Wittka R, Boheim G, Stuhmer W, Ferrus A, Pongs O (1990) Molecular basis of altered excitability in *Shaker* mutants of *Drosophila melanogaster*. *EMBO J* 9:4399–4407.
- Littleton JT, Ganetzky B (2000) Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron* 26:35–43.
- Mandel G (1992) Tissue-specific expression of the voltage-sensitive sodium channel. *J Membr Biol* 125:193–205.
- Martinez-Padron M, Ferrus A (1997) Presynaptic recordings from *Drosophila*: correlation of macroscopic and single-channel K^+ currents. *J Neurosci* 17:3412–3424.
- McGuire SE, Le PT, Davis RL (2001) The role of *Drosophila* mushroom body signaling in olfactory memory. *Science* 293:1330–1333.
- Nighorn A, Healy MJ, Davis RL (1991) The cyclic AMP phosphodiesterase encoded by the *Drosophila dunce* gene is concentrated in the mushroom body neuropil. *Neuron* 6:455–467.
- Niven JE, Vahasoyrinki M, Kauranen M, Hardie RC, Juusola M, Weckstrom M (2003) The contribution of Shaker K^+ channels to the information capacity of *Drosophila* photoreceptors. *Nature* 421:630–634.
- O'Dell KM, Armstrong JD, Yang MY, Kaiser K (1995) Functional dissection of the *Drosophila* mushroom bodies by selective feminization of genetically defined subcompartments. *Neuron* 15:55–61.
- O'Dowd DK (1995) Voltage-gated currents and firing properties of embryonic *Drosophila* neurons grown in a chemically defined medium. *J Neurobiol* 27:113–126.
- Pascual A, Preat T (2001) Localization of long-term memory within the *Drosophila* mushroom body. *Science* 294:1115–1117.
- Pelz C, Jander J, Rosenboom H, Hammer M, Menzel R (1999) I_A in Kenyon cells of the mushroom body of honeybees resembles shaker currents: kinetics, modulation by K^+ , and simulation. *J Neurophysiol* 81:1749–1759.
- Pirrotta V (1988) Vectors for P-mediated transformation in *Drosophila*. *Biotechnology* 10:437–456.
- Pongs O, Kecskemethy N, Muller R, Krah-Jentgens I, Baumann A, Kiltz HH, Canal I, Llamazares S, Ferrus A (1988) *Shaker* encodes a family of putative potassium channel proteins in the nervous system of *Drosophila*. *EMBO J* 7:1087–1096.
- Pongs O, Leicher T, Berger M, Roeper J, Bähring R, Wray D, Giese KP, Silva AJ, Storm JF (1999) Functional and molecular aspects of voltage-gated K^+ channel beta subunits. *Ann NY Acad Sci* 868:344–355.
- Rogero O, Hammerle B, Tejedor FJ (1997) Diverse expression and distribution of Shaker potassium channels during the development of the *Drosophila* nervous system. *J Neurosci* 17:5108–5118.
- Roman G, Davis RL (2001) Molecular biology and anatomy of *Drosophila* olfactory associative learning. *BioEssays* 23:571–581.

- Rubin GM, Spradling AC (1983) Vectors for P element-mediated gene transfer in *Drosophila*. *Nucleic Acids Res* 11:6341–6351.
- Saito M, Zhao ML, Wu CF (1993) Irregular activity in the giant neurons from *Shaker* mutants suggests that the *Shaker* locus may encode non-A-type K^+ channel subunits in *Drosophila*. *Ann NY Acad Sci* 707:392–395.
- Schwarz TL, Papazian DM, Carretto RC, Jan YN, Jan LY (1990) Immunological characterization of K^+ channel components from the *Shaker* locus and differential distribution of splicing variants in *Drosophila*. *Neuron* 4:119–127.
- Serodio P, Rudy B (1998) Differential expression of Kv4 K^+ channel subunits mediating subthreshold transient K^+ (A-type) currents in rat brain. *J Neurophysiol* 79:1081–1091.
- Sharma Y, Cheung U, Larsen EW, Eberl DF (2002) PPTGAL, a convenient Gal4 P-element vector for testing expression of enhancer fragments in *Drosophila*. *Genesis* 34:115–118.
- Solc CK, Aldrich RW (1988) Voltage-gated potassium channels in larval CNS neurons of *Drosophila*. *J Neurosci* 8:2556–2570.
- Solc CK, Zagotta WN, Aldrich RW (1987) Single-channel and genetic analyses reveal two distinct A-type potassium channels in *Drosophila*. *Science* 236:1094–1098.
- Song WJ, Tkatch T, Baranauskas G, Ichinohe N, Kitai ST, Surmeier DJ (1998) Somatodendritic depolarization-activated potassium currents in rat neostriatal cholinergic interneurons are predominantly of the A type and attributable to coexpression of Kv4.2 and Kv4.1 subunits. *J Neurosci* 18:3124–3137.
- Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF (1994) Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J Comp Physiol [A]* 175:179–191.
- Stocker M, Stuhmer W, Wittka R, Wang X, Muller R, Ferrus A, Pongs O (1990) Alternative *Shaker* transcripts express either rapidly inactivating or noninactivating K^+ channels. *Proc Natl Acad Sci USA* 87:8903–8907.
- Stocker RF (1994) The organization of the chemosensory system in *Drosophila melanogaster*: a review. *Cell Tissue Res* 275:3–26.
- Su H, O'Dowd DK (2003) Fast synaptic currents in *Drosophila* mushroom body Kenyon cells are mediated by alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors and picrotoxin-sensitive GABA receptors. *J Neurosci* 23:9246–9253.
- Tanouye MA, Ferrus A, Fujita SC (1981) Abnormal action potential associated with the *Shaker* complex locus of *Drosophila*. *Proc Natl Acad Sci USA* 78:6548–6552.
- Timpe LC, Schwarz TL, Tempel BL, Papazian DM, Jan YN, Jan LY (1988) Expression of functional potassium channels from *Shaker* cDNA in *Xenopus* oocytes. *Nature* 331:143–145.
- Tseng-Crank J, Pollock JA, Hayashi I, Tanouye MA (1991) Expression of ion channel genes in *Drosophila*. *J Neurogenet* 7:229–239.
- Tsunoda S, Salkoff L (1995a) The major delayed rectifier in both *Drosophila* neurons and muscle is encoded by Shab. *J Neurosci* 15:5209–5221.
- Tsunoda S, Salkoff L (1995b) Genetic analysis of *Drosophila* neurons: Shal, Shaw, and Shab encode most embryonic potassium currents. *J Neurosci* 15:1741–1754.
- Tully T, Cambiazo V, Kruse L (1994) Memory through metamorphosis in normal and mutant *Drosophila*. *J Neurosci* 14:68–74.
- Wang Y, Wright NJ, Guo H, Xie Z, Svoboda K, Malinow R, Smith DP, Zhong Y (2001) Genetic manipulation of the odor-evoked distributed neural activity in the *Drosophila* mushroom body. *Neuron* 29:267–276.
- Wei A, Covarrubias M, Butler A, Baker K, Pak M, Salkoff L (1990) K^+ current diversity is produced by an extended gene family conserved in *Drosophila* and mouse. *Science* 248:599–603.
- Wright NJ, Zhong Y (1995) Characterization of K^+ currents and the cAMP-dependent modulation in cultured *Drosophila* mushroom body neurons identified by *lacZ* expression. *J Neurosci* 15:1025–1034.
- Wu CF, Haugland FN (1985) Voltage clamp analysis of membrane currents in larval muscle fibers of *Drosophila*: alteration of potassium currents in *Shaker* mutants. *J Neurosci* 5:2626–2640.
- Wu CF, Suzuki N, Poo MM (1983) Dissociated neurons from normal and mutant *Drosophila* larval central nervous system in cell culture. *J Neurosci* 3:1888–1899.
- Zagotta WN, Brainard MS, Aldrich RW (1988) Single-channel analysis of four distinct classes of potassium channels in *Drosophila* muscle. *J Neurosci* 8:4765–4779.
- Zars T, Fischer M, Schulz R, Heisenberg M (2000) Localization of a short-term memory in *Drosophila*. *Science* 288:672–675.
- Zhao ML, Wu CF (1997) Alterations in frequency coding and activity dependence of excitability in cultured neurons of *Drosophila* memory mutants. *J Neurosci* 17:2187–2199.