Research

Reduced Delayed-Rectifier K⁺ Current in the Learning Mutant *rutabaga*

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In the *Drosophila* mutant *rutabaga*, short-term memory is deficient and intracellular cyclic adenosine monophosphate (cAMP) concentration is reduced. We characterized the delayed-rectifier potassium current (IK_{DR}) in *rutabaga* as compared with the wild-type. The conventional whole-cell patch-clamp technique was applied to cultured *Drosophila* neurons derived from embryonic neuroblasts. IK_{DR} was smaller in *rutabaga* (368 ± 11 pA) than in wild-type (541 ± 14 pA) neurons, measured in a Ca²⁺-free solution. IK_{DR} was clearly activated at ~0 mV in the two genotypes. IK_{DR} typically reached its peak within 10–20 msec after the start of the pulse (60 mV). There was no difference in inactivation of IK_{DR} for wild-type (14 ± 3%) and *rutabaga* (19 ± 3%). After application of 10 mM TEA, in wild-type, IK_{DR} was reduced by 46 ± 5%, whereas in *rutabaga*, IK_{DR} was reduced by 28 ± 3%. Our results suggest that IK_{DR} is carried by two different types of channels, one which is TEA-sensitive, whereas the other is TEA-insensitive. Apparently, the TEA-sensitive channel is less expressed in *rutabaga* neurons than in wild-type neurons. Conceivably, altered neuronal excitability in the *rutabaga* mutant could disrupt the processing of neural signals necessary for learning and memory.

The *Drosophila* mutations *rutabaga* and *dunce* affect learning and memory due to defects in cAMP metabolism. The *dunce* mutant has a high intracellular cAMP concentration owing to cAMP-specific phosphodiesterase (PDE) disruption (Byers et al. 1981). The *rutabaga* mutant has a low intracellular cAMP concentration due to elimination of a calcium/cadmodulin-responsive adenylyl cyclase. The ability of the cyclase catalytic subunit to interact with calcium/cadmodulin may be affected in *rutabaga* (Livingstone et al. 1984; Levin et al. 1992). *dunce* and *rutabaga*, originally identified for affecting learning and memory (Dudai et al. 1976) have been shown to alter synaptic plasticity (Zhong and Wu 1991), disrupt habituation (Engel and Wu 1996), and reduce growth cone motility (Kim and Wu 1996).

cAMP-dependent modulation of ion channels has been shown to modify impulse activity of neurons (Kaczmarek and Kauer 1983). Modulation of neuronal electrical properties can change the operation of neural networks, it may be an important cellular mechanism for activity-dependent conditioning of behavior. Classical conditioning of the Aplysia siphon and tail-withdrawal reflex is believed to rely on presynaptic facilitation (Kandel et al. 1983). However, this form of simple learning, which depends on the synapse, cannot occur in complete isolation from the cell body, because changes in neuronal function can impact synaptic function. K⁺ current is one of the fundamental factors that regulate neuronal excitability (Klee et al. 1995) and neuronal function (Le Masson et al. 1993). Investigation of somal

K⁺ current is required to elucidate possible changes in neuronal function of the *Drosophila* learning mutants. The learning deficit in *rutabaga* has been demonstrated in the adult fly, nonetheless, the embryonic cell culture system provides an excellent preparation that can be manipulated easily in electrophysiological studies. Furthermore, embryonic neurons can be used to show whether the K⁺ current is altered in early life of the fly.

It has been shown previously that short-term (10 min) treatment with dibutyryl (db) cAMP does not affect neuronal K⁺ current (Alshuaib and Byerly 1996), whereas long-term (2 d) treatment with db-cAMP enhances neuronal K⁺ current (Alshuaib and Mathew 1998). Moreover, neuronal K⁺ current was shown to be greater in *dunce* than in wild-type neurons (Alshuaib and Mathew 1998). *dunce* and *rutabaga* were shown to display altered firing patterns in giant (cleavage-arrested) cultured neurons (Zhao and Wu 1997). However, neuronal K⁺ current of normal embryonic cultures has not been investigated in the *rutabaga* mutant. In the present study, we compared the delayed-rectifier K⁺ current (IK_{DR}) in wild-type and *rutabaga* neurons from normal embryonic cultures. IK_{DR} was reduced in *rutabaga* neurons as compared with the wild-type, a defect that can impact neuronal excitability and, ultimately, learning and memory.

RESULTS

Our purpose was to compare the delayed-rectifier K⁺ current between wild-type and *rutabaga* neurons using the conventional patch-clamp technique. Figure 1 shows neurons typical of those studied in the two genotypes. The diameters of the cells ranged from 4–7 μm, and each cell

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Figure 1 Photographs of cultured embryonic *Drosophila* neurons from wild-type and *rutabaga*. Cells were cultured (2 d) in a *Drosophila* medium, they represent typical examples of neurons that were studied. All photographs were taken using Carl Zeiss bright-field optics at the same magnification (X400). Scale bar (25 μ m) applies to the two photographs.

had one to three neurites. In general, neurons with isolated cell bodies (not in contact with other cells) were chosen for study, because they were easier to describe and to approach with the patch electrode.

Electrophysiological Properties

The electrophysiological properties of wild-type and *rutabaga* neurons are shown in Table 1. We calculated the total capacitance (C) for each cell by integrating the capacitive current flowing in response to a 50-mV hyperpolarizing step. The cell capacitance was essentially the same for wild-type (7.83 ± 1.26 pF, $n = 60$) and *rutabaga* (8.14 ± 1.12 pF, $n = 60$) neurons. This indicates that the membrane area of wild-type and *rutabaga* neurons is similar. There was no difference in resting membrane potential (RMP) between wild-type neurons (79.5 ± 0.1 mV, $n = 20$) and *rutabaga* neurons (79.3 ± 0.2 mV, $n = 22$). The whole-cell resistance (R_{in}) was measured by stepping the membrane potential from -60 mV to -110 mV and dividing this 50-mV step by the measured current amplitude between 90 and 100 msec. R_{in} was similar for wild-type (8.02 ± 0.89 G Ω , $n = 17$) and *rutabaga* (7.52 ± 0.76 G Ω , $n = 22$) neurons. This suggests that the specific resistances of wild-type and *rutabaga* neurons are similar.

Table 1. Electrophysiological Properties of Wild-Type and *rutabaga* Neurons

Property	Wild-type	<i>rutabaga</i>
C (pF)	7.83 ± 1.26 ($n = 60$)	8.14 ± 1.12 ($n = 60$)
RMP (mV)	79.5 ± 0.1 ($n = 20$)	79.3 ± 0.2 ($n = 22$)
R_{in} (G Ω)	8.02 ± 0.89 ($n = 17$)	7.52 ± 0.76 ($n = 22$)

All values are means $\pm$ SEM; the means were compared using a two-tailed independent Student's *t*-test. There was no significant difference between wild-type and *rutabaga* neurons in any of these properties.

Comparison of IK_{DR} in Wild-Type and *rutabaga* in 6K/0Ca Tris Solution

IK_{DR} was measured in a Ca-free *Drosophila* external solution, because inward calcium current can affect IK_{DR} (Alshuaib et al. 2001). Figure 2 shows typical examples of IK_{DR} recorded from neurons at potentials from -40 to $+60$ mV. IK_{DR} was calculated between 90 and 100 msec (steady state) of the pulse to exclude any possibility of A-current contribution to the measured amplitude. K^+ currents recorded at $+60$ mV were smaller in *rutabaga* neurons (368 ± 11 pA, $n = 80$) than in wild-type neurons

(541 ± 14 pA, $n = 83$) ($P < 0.001$) (Combined mean of stocks w23,w24,w25 versus combined mean of stocks r33, r34, r35; see Table 2). The IK_{DR} phenotype was consistent within wild-type (stocks w23,w24,w25) and within *rutabaga* (stocks r33, r34, r35) neurons.

All wild-type and *rutabaga* neurons displayed the delayed-rectifier (non-inactivating) K^+ current. However, in almost all neurons, the A-type (inactivating) K^+ current was not observed even with the voltage protocol (-120 mV prepulse, $+20$ mV test pulse) that usually elicits this transient current. For this reason, we focused mainly on the delayed-rectifier K^+ current. We applied a voltage protocol that maximizes the delayed-rectifier K^+ current and diminishes the A-type K^+ current (holding potential, -80 mV; test pulses, -40 to $+60$ mV) (Byerly and Leung 1988; Saito and Wu 1991; Alshuaib and Mathew 1998). IK_{DR} was clearly activated at 0 mV, but only weakly activated at -20 mV in wild-type and *rutabaga* neurons. IK_{DR} typically reached its peak within 10–20 msec after the start of the pulse (60 mV) in both wild-type and *rutabaga*. The time course of inactivation was quantified by calculating the percentage of the peak current that had inactivated at 100 msec. There was no statistically significant difference in values of IK_{DR} inactivation, which were $14 \pm 3\%$ ($n = 18$) for wild-type neurons, and $19 \pm 3\%$ ($n = 19$) for *rutabaga* neurons (60 mV pulse; Fig. 2). The current-voltage relations are shown in Figure 2 for wild-type and *rutabaga* neurons. The regression coefficient (the slope between -20 mV and $+60$ mV) is smaller for *rutabaga* ($B = 3.90$ pA/mV) than that for wild-type neurons ($B = 5.66$ pA/mV). At each of the clamp voltages between 0 and $+60$ mV, the current is significantly smaller in *rutabaga* than in wild-type neurons.

Effect of External Ca^{2+} on IK_{DR}

To investigate the effect of Ca^{2+} on potassium current, IK_{DR} was measured in a Ca^{2+} -containing *Drosophila* solution and

Figure 2 Current-voltage (I-V) relations of wild-type and *rutabaga* neurons in 6K/0Ca Tris *Dros.* saline (Ca^{2+} -free) and in a Ca^{2+} -containing *Dros.* saline. K^+ currents were recorded from wild-type and *rutabaga* neurons during six 100-msec clamp potentials between -40 mV (bottom trace) and $+60$ mV (top trace) in steps of 20 mV. Holding potential was -80 mV. Current traces are from individual neurons. Data points are mean $\pm$ SEM. The mean IK_{DR} was determined at steady-state (90–100 msec of the pulse). In the Ca^{2+} -free solution, $n = 83$ for wild-type; $n = 80$ for *rutabaga*. In the Ca^{2+} -containing solution, $n = 16$ for wild-type; $n = 17$ for *rutabaga*. The pipette contained potassium aspartate solution.

the current amplitude was compared with that measured in the 6K/0Ca Tris solution. For wild-type neurons, IK_{DR} measured in the Ca^{2+} -containing solution (399 ± 36 pA, $n = 16$) was smaller than that measured in the 6K/0Ca Tris solution (541 ± 14 pA, $n = 83$) ($P < 0.001$). Similarly, for *rutabaga* neurons, IK_{DR} measured in the Ca^{2+} -containing solution (236 ± 30 pA, $n = 17$) was smaller than that measured in the 6K/0Ca Tris solution (368 ± 11 pA, $n = 80$) ($P < 0.001$). IK_{DR} typically reached its peak within 10–20 msec in both wild-type and *rutabaga* neurons. There was no significant difference in values of IK_{DR} inactivation, which were $11 \pm 5\%$ ($n = 4$) for wild-type neurons and $20 \pm 8\%$ ($n = 4$) for *rutabaga* neurons (60 mV pulse). Figure 2 shows the I-V

relations of IK_{DR} measured in the Ca^{2+} -containing solution for both wild-type and *rutabaga* neurons. IK_{DR} is clearly activated at 0 mV, but only weakly activated at -20 mV in both wild-type and *rutabaga* neurons. At each of the clamp voltages between 0 and $+60$ mV, the current amplitude was significantly smaller in *rutabaga* than in wild-type neurons. The regression coefficient (the slope between -20 mV and -60 mV) was smaller for *rutabaga* neurons ($B = 2.35$ pA/mV) than that for wild-type neurons ($B = 4.08$ pA/mV).

Population Studies of Blockage of IK_{DR} With TEA

IK_{DR} was measured in a 10 mM TEA-6K/0 Ca Tris *Drosophila* saline (10 min), for both wild-type and *rutabaga* neurons. Comparing the two genotypes after blockage with TEA, IK_{DR} amplitude was not significantly different in wild-type (356 ± 44 pA, $n = 18$) and *rutabaga* (338 ± 38 pA, $n = 27$) (Fig. 3). This indicates that the blockage of IK_{DR} was greater in wild-type than in *rutabaga* neurons, making IK_{DR} amplitude similar in wild-type and *rutabaga* neurons. Nonetheless, the contribution of variability due to sampling cannot be completely excluded in such comparisons of limited samples from the population.

This TEA-blocked IK_{DR} was compared with the control IK_{DR} (without TEA) obtained from other cells in 6K/0Ca Tris saline (see above). Within the wild-type genotype, the IK_{DR} reduction with TEA was statistically significant (356 ± 44 pA Vs. 541 ± 14 pA, $P < 0.001$). Within the *rutabaga* genotype, there was no statistically significant difference with TEA (338 ± 38 pA) and without TEA (368 ± 11 pA) ($P = 0.303$) (Fig. 3).

Single-Cell Studies of Blockage of IK_{DR} With TEA

The best experimental treatment is obtained when comparing the subject with itself, rather than comparing independent samples. Therefore, to exclude variability due to sampling, we measured IK_{DR} from the same cell before and after application of 10 mM TEA-6K/0Ca Tris *Drosophila* saline (10 min). In wild-type, IK_{DR} was reduced by $46 \pm 5\%$ (before TEA, 575 ± 38 pA, $n = 14$; after TEA, 310 ± 52 pA, $n = 14$, $P < 0.001$), whereas in *rutabaga*, IK_{DR} was reduced by $28 \pm 3\%$ (before TEA, 417 ± 36 pA, $n = 14$; after TEA, 302 ± 42 pA, $n = 14$, $P = 0.048$, marginally significant) (see Fig. 4).

In these single-cell studies, after IK_{DR} blockage with TEA, IK_{DR} amplitude was not significantly different in wild-type (310 ± 52 pA, $n = 14$) and *rutabaga* (302 ± 42 pA, $n = 14$) neurons. This experimental approach confirmed that the blockage of IK_{DR} was greater in wild-type than in *rutabaga* neurons, due to a difference in delayed-rectifier K^+ channels in the two genotypes.

Table 2. Delayed-Rectifier K^+ Current (IK_{DR}) of Several Wild-Type and *rutabaga* Fly Stocks

Wild-type IK_{DR} (pA)	<i>rutabaga</i> IK_{DR} (pA)
533 ± 20 pA (stock w23, $n = 43$)	370 ± 17 pA (stock r33, $n = 40$) ^a
559 ± 28 pA (stock w24, $n = 20$)	353 ± 23 pA (stock r34, $n = 20$) ^a
541 ± 28 pA (stock w25, $n = 20$)	378 ± 22 pA (stock r35, $n = 20$) ^a
Combined mean	Combined mean
541 ± 14 pA ($n = 83$)	368 ± 11 pA ($n = 80$) ^a

IK_{DR} is presented as mean ± SEM. n is the number of neurons from 10–30 different cultures (1–5 neurons per culture). To control the possible accumulation of unidentified autosomal mutations (modifiers) that might contribute to the phenotypes examined, the following stocks were used: *rutabaga* phenotype; Stock r35, a homozygous *rutabaga/rutabaga* stock was reconstructed (see Materials and Methods). Stocks r33 and r34, these are copies of the parental *rutabaga/rutabaga* stock. IK_{DR} amplitude is essentially the same in the reconstructed *rutabaga* (r35) and parental *rutabaga* (r33, r34). Wild-type phenotype; Stock w25, a heterozygous wild-type/*rutabaga* stock was generated for examination (wild-type *rutabaga* allele is dominant over mutant *rutabaga* allele). Stocks w23 and w24, these are copies of the parental wild-type/wild-type stock. IK_{DR} amplitude is essentially the same in the wild-type/*rutabaga* stock (w25) and parental wild-type (w23, w24). The means were compared using a two-tailed independent Student's t -test and the difference is presented as follows: ^aSignificantly different from wild-type value in the same row ($P < 0.001$).

Effect of 4-AP on IK_{DR}

IK_{DR} was measured in a 5 mM 4-aminopyridine (4-AP)-6K/0Ca Tris *Drosophila* saline (10 min), for both wild-type and *rutabaga* neurons. In the 4-AP saline, IK_{DR} was smaller in *rutabaga* (371 ± 56 pA, $n = 11$) than in wild-type (516 ± 22 pA, $n = 13$, $P < 0.02$). These IK_{DR} amplitudes were not significantly different from those measured in the control 6K/0Ca Tris saline, which were 541 ± 14 pA for wild-type and 368 ± 11 pA for *rutabaga* (as already mentioned above). Thus, 4-AP did not have any significant effect on IK_{DR} in both genotypes.

DISCUSSION

Neuronal potassium current has not been characterized completely in *rutabaga*, a mutation with a low intracellular cAMP concentration due to complete elimination of a calcium/cadmodulin-responsive adenylyl cyclase. The second messenger cAMP has been found to reduce K^+ current in Aplysia sensory neurons (Siegelbaum et al. 1982) and increase both the Ca^{2+} current (Alshuaib and Byerly 1996) and the K^+ current (Alshuaib and Mathew 1998) in *Drosophila* neurons. A larger number of *rutabaga* larval neurons showed reduction of K^+ current by 8-bromo-cAMP than wild-type neurons (Yu et al. 1999). In *rutabaga* larval muscle, pituitary adenylyl cyclase-activating polypeptide (PACAP)-induced enhancement of K^+ current was abolished (Zhong 1995), whereas IK_{DR} was unchanged compared with that of the wild-type (Zhong and Wu 1993). Therefore, it was necessary to characterize the K^+ current in *rutabaga* neurons.

Reduced IK_{DR} in *rutabaga*

The present study shows for the first time that the delayed rectifier K^+ current is reduced in *rutabaga* (368 ± 11 pA) as compared with wild-type (541 ± 14 pA) normal culture neurons. This is in agreement with the reduction of K^+ current reported previously for cleavage-arrested giant *rutabaga* neurons (Zhao and Wu 1997). The reduced current mainly included a non-inactivating component that was sustained throughout the 100 msec. IK_{DR} amplitude was the same in the wild-type phenotype (wild-type/*rutabaga*, stock w25) and parental wild-type (stock w23, stock w24). IK_{DR} amplitude was the same in the reconstructed *rutabaga* (stock r35) and parental *rutabaga* (stock r33, stock r34).

The steady-state inactivation that was determined here (range 6%–20%) is typical of the delayed-rectifier K^+ current (Saito and Wu 1991; Alshuaib and Mathew 1998). Although the majority of cells did not display the transient A-type current, pharmacological experiments were carried out to test whether 4-AP affects the difference in K^+ current between the two genotypes.

Because IK_{DR} remained smaller in *rutabaga* (371 ± 56 pA) than in wild-type (516 ± 22 pA) neurons after 4-AP application, this confirms that the difference between the two genotypes is independent of the transient A-type current. For both wild-type and *rutabaga* neurons, IK_{DR} was smaller in the Ca^{2+} -containing solution than in the Ca^{2+} -free solution. This is probably due to contamination of the outward IK_{DR} by the inward Ca^{2+} current, and this justifies the measurement of IK_{DR} in a Ca^{2+} -free external solution, which was the approach used in our study. This result is also in agreement with a previous report (Alshuaib and Byerly 1996) that the neuronal K^+ current in *Drosophila* does not have a Ca^{2+} -dependent component. At any rate, IK_{DR} reduction in *rutabaga* is independent of Ca^{2+} effects on the K^+ current.

IK_{DR} Reduction is Due to *rutabaga* Mutation

Our results confirmed that the observed IK_{DR} was not derived from unidentified second-site mutations other than *rutabaga*. First, the w25 stock was a result of blending genetic backgrounds from the parental wild-type and *rutabaga* stocks. Because the w25 embryos were the F1 generation (wild-type/*rut*^{1f}) of a cross of wild-type males with *rut*^{1f} females, they are heterozygous at all autosomal loci (bearing one copy from each parental stock). Female w25 embryos are also heterozygous at all X-linked loci. This provides evidence for attributing the physiological phenotype to *rutabaga*, in spite of the fact that one copy of each

Figure 3 Comparison of IK_{DR} in wild-type and *rutabaga* neurons, population studies. Neurons in the control saline (6K/0Ca Tris) and the 10-mM TEA saline were not the same. The K^+ currents were elicited by stepping to +60 mV (holding potential was -80 mV). Currents were measured between 90 and 100 msec after the beginning of the pulse. The internal solution was potassium aspartate. Values are means $\pm$ SEM. In the control solution, $n = 83$ for wild-type; $n = 80$ for *rutabaga*. In the TEA solution, $n = 18$ for wild-type; $n = 27$ for *rutabaga*. The means were compared using a two-tailed independent Student's t -test and the difference is presented as follows: (*) significant difference between wild-type and *rutabaga* ($P < 0.001$); (†) significant difference within the same genotype ($P < 0.001$).

autosomal gene was from the *rut^{1f}* parental stock, the phenotype was perfectly wild-type (Table 2). In a large sample of embryos, female w25 embryos would be *rut^{1f}/rut^{1f}+*, but male w25 embryos would be *rut^{1f}/Y* (mutant physiological phenotype). With the small sample of embryos used (10–30 cultures), it appears that only female embryos were used in our cell culture.

Second, the r35 stock was a result of partial replacement of the genetic background of the parental *rutabaga* stock. Female *rut^{1f}* were crossed with male wild-type (this produced the F1 generation), and then a homozygous *rut^{1f}/rut^{1f}* stock was reconstituted. The perfect accord of the r35 result with the r33 and r34 phenotypes (Table 2), in spite of the mixing of wild-type autosomal alleles, suggests that the reduction of IK_{DR} is due to the *rutabaga* mutation. F2 flies were selected for *forked*. *rutabaga* is nearly 20 map units from the marker locus *forked* and crossing over could only have occurred in F1 females (there is ~0.2 probability of crossing over for each F2 fly selected). Fortunately, the perfect agreement of the r35 embryos with the r33 and r34 phenotype is consistent with avoidance of any crossover recombination between *rutabaga* and *forked*.

Mechanism of IK_{DR} Reduction in *rutabaga*

The amplitude of IK_{DR} and the slope of the I-V relation (Fig. 2) were both reduced in *rutabaga* neurons compared with wild-type neurons. This may be due to a smaller number

(density) and/or altered properties of K^+ channels. With respect to kinetic properties of the K^+ channel, it is plausible that the low level of intracellular cAMP in *rutabaga* neurons reduces phosphorylation of the K^+ channel in the long term, altering the channel open-time, single-channel conductance, or resistance to blockage by TEA. The present study was an investigation of the whole-cell K^+ current; it demonstrated that IK_{DR} activation time and steady-state inactivation were unchanged in *rutabaga* neurons, but clearly, single-channel studies are required to determine any change in kinetics.

With respect to the number of K^+ channels, we must first specify the type of channel. Our results suggest that IK_{DR} is carried by two different types of channels, one which is TEA-sensitive, whereas the other is TEA-insensitive. Concerning the TEA-sensitive channel, the single-cell studies of blockage of IK_{DR} with TEA demonstrated that the percentage of IK_{DR} blockage is greater in wild-type ($46 \pm 5\%$, $P < 0.001$) than in *rutabaga* ($28 \pm 3\%$, $P = 0.048$) neurons (Fig. 4). Apparently, the TEA-sensitive channel is less expressed in *rutabaga* neurons than in wild-type neurons. The low intracellular cAMP concentration in *rutabaga* may cause a specific reduction in the expression of

Figure 4 Comparison of TEA sensitivity in wild-type and *rutabaga* neurons, single-cell studies. The K^+ current was measured from the same cell, first in the control saline (6K/0 Ca Tris) and then in the 10-mM TEA saline. For wild-type, $n = 14$; for *rutabaga* $n = 14$. The K^+ currents were elicited by stepping to +60 mV (holding potential was -80 mV). The internal solution was potassium aspartate. In the wild-type neuron, 55% of the K^+ current was blocked; In the *rutabaga* neuron, only 30% of the K^+ current was blocked (values were measured between 90 and 100 msec after the beginning of the pulse).

TEA-sensitive channels. It is also plausible that CREB (cAMP-response element binding protein)-dependent gene expression is altered in *rutabaga* neurons, and that the expression of the TEA-sensitive channel may be CREB dependent.

Concerning the TEA-insensitive channel, the population studies of IK_{DR} blockage with TEA demonstrated that IK_{DR} amplitudes become similar in wild-type (356 ± 44 pA) and *rutabaga* (338 ± 38 pA) neurons after the block (Fig. 3). Furthermore, the single-cell studies of IK_{DR} blockage with TEA also demonstrated that IK_{DR} amplitudes become similar in wild-type (310 ± 52 pA) and *rutabaga* (302 ± 42 pA) neurons after the block. This suggests that the TEA-insensitive channels are essentially the same in wild-type and *rutabaga*. In summary, the difference in IK_{DR} between wild-type and *rutabaga* neurons may be explained by a smaller number of TEA-sensitive channels in *rutabaga*. Studies using pharmacological and immunohistochemical tools are required to determine the type and density of channels in *rutabaga* neuronal membrane.

Functional Implications of IK_{DR} Reduction in *rutabaga*

The reduced IK_{DR} in *rutabaga* neurons contrasts the increased IK_{DR} in *dunce* giant neurons (Alshuaib and Mathew 1998). Altered somal IK_{DR} in *dunce* and *rutabaga* can impact presynaptic facilitation that has been proposed to mediate learning (Kandel et al. 1983). Moreover, this altered IK_{DR} in *dunce* and *rutabaga* is in general agreement with the abnormal spontaneous spikes and altered firing patterns in *dunce* and *rutabaga* cleavage-arrested giant neurons (Zhao and Wu 1997). Voltage-dependent K^+ currents have been shown to be crucial in the regulation of neuronal firing patterns in many species (Hille 1992). For example, blockage of IK_{DR} with TEA broadened the duration of the action potential and reduced the rate of repolarization (Zhao and Wu 1997). In fact, spontaneous spikes, erratic firing, and long-lasting plateau action potentials were more abundant in *rutabaga* than in *dunce* giant neurons. Thus, our results of reduced IK_{DR} in normal *rutabaga* neurons coupled with the aberrant firing patterns in giant *rutabaga* neurons (Zhao and Wu 1997) suggest the possibility of defective frequency coding in *rutabaga* normal culture neurons. Conceivably, such frequency coding might be important for learning, because neuronal activity is often modulated by previous neuronal activity. In other words, modulation of neuronal excitability may be a mechanism underlying learning and memory.

MATERIALS AND METHODS

Genotypes and Construction of Fly Stocks

The *Drosophila melanogaster* (Oregon-R) strain was used as control wild-type. The homozygous *rutabaga^f* (*rut^f*) mutant allele in an Oregon-R genetic background was kindly supplied by Yi Zhong (Cold Spring Harbor Laboratory). *f* (*forked*) is a morphological

marker. *rutabaga* is an X-linked recessive, single-gene mutation that was isolated originally using ethyl methane sulfonate (EMS) mutagenesis (Livingstone et al. 1984). To control the possible accumulation of unidentified autosomal mutations (modifiers) that might contribute to the phenotypes examined, we reconstructed a homozygous *rut^f/rut^f* stock (stock r35, below). In addition, a heterozygous wild-type/*rut^f* stock (stock w25, below) was generated for examination. The characteristics of the different fly stocks are summarized below.

Wild-Type

Stocks w23 and w24 are two copies of the wild-type/wild-type stock. They have been maintained separately for 13 mo.

Stock w25. This stock of wild-type *rutabaga* phenotype is a heterozygous stock made by crossing the wild-type and *rut^f* stocks. In the parental generation, wild-type (w24) males were crossed with *rut^f* (r34) females to produce the F1 generation, wild-type/*rut^f*. Embryos were harvested for neuron cultures from the F1 generation (the first generation of progeny). Thus, wild-type/*rut^f* embryos were used to study wild-type IK_{DR} phenotype (wild-type *rutabaga* allele is dominant over mutant *rutabaga* allele).

rut^f

Stocks r33 and r34 are two copies of the *rut^f/rut^f* stock. They have been maintained separately for 13 mo.

Stock r35. This stock of mutant *rutabaga* phenotype was outcrossed and reconstructed. In the parental generation, wild-type (w23) males were crossed with *rut^f* (r33) females to produce the F1 generation. The product of this breed (wild-type/*rut^f*) were crossed with each other to produce the F2 generation. The F2 homozygous flies (*rut^f/rut^f*) were crossed with each other (F2 flies were selected for *forked*), and embryos were harvested for neuron cultures from the F3 generation.

Preparation of Cultures

Eggs were collected over a 1.5 hr period from *Drosophila* flies maintained in pint milk bottles at 26°C. Each culture was prepared from the cells of 1–3 gastrulating embryos in a modified Schneider's *Drosophila* medium (DM) (Salvaterra et al. 1987). Five hours after the beginning of egg collection, the embryos were placed in a 50% ethanol/50% Clorox solution for 2 min to sterilize and dechorionate them. The embryos were then repeatedly washed with DM. Two or three embryos were transferred to a drop of DM on a 35-mm tissue culture dish (Falcon 3001). Each embryo was impaled by a hand-held micropipette (tip diameter, ~100 μ m), the cells were collected by suction, and blown onto the surface of the dish. The cells were further dispersed by repeated passage through the tip of a smaller pipette (tip diameter, 50 μ m). The cells adhered to the surface of the dish within minutes of dispersal. The culture dish containing the embryonic cells (in a single drop of DM) was kept in a humid container at room temperature (23°C). All cell cultures were studied electrophysiologically 2 d (43–49 h) later at room temperature. The culture dish was used as the recording chamber with a Sylgard form insert in the dish to confine the extracellular solution to a small volume (0.3 mL). Cells were viewed using Carl Zeiss bright-field optics.

Patch-Clamp Techniques

The conventional whole-cell patch-clamp technique (Hamill et al. 1981) was used to study the membrane currents of neurons. A patch-clamp amplifier measures the membrane current while keep-

ing the membrane potential at a specific level. Electrodes were pulled from 100- μ L micropipettes (VWR), coated with Sylgard resin near the tip, and polished to a bubble number (Corey and Stevens 1983) of 3.0–4.0. When filled with potassium aspartate solution, these electrodes had resistances of 6–12 M Ω . The application of patch-clamp to cultured embryonic *Drosophila* neurons has been described in detail previously (Alshuaib and Byerly 1996). Typically, pipette potential was nulled, gigaohm seal was formed using gentle suction, pipette capacitance was compensated, and the whole-cell configuration was obtained with the application of further suction.

Experiments were performed with an Axopatch 200 A-patch-clamp amplifier (Axon Instruments). Data acquisition and analysis were performed using Digidata 1200 (Axon Instruments) and pCLAMP software (version 5.5, Axon Instruments) on a 486 HP personal computer. Current recordings were filtered (four-pole Bessel) at 5 kHz (capacitive currents) or 1 kHz (ionic currents), and digitized at 20 or 200 μ s intervals, respectively. Passive (leakage) currents, determined from negative pulses of one-quarter the amplitude of the test pulse ($-P/4$), were subtracted from all of the ionic currents. None of the voltages given in the Results have been corrected for the liquid junction potential that existed between the KAsp-filled electrode tip and the *Drosophila* saline bath solution. The nominal zero potential level is taken to be the level that gives zero current at the beginning of the experiment. By use of a 3 mole/l KCl reference electrode, the junction potential between the KAsp internal solution and the *Drosophila* saline external solution was -10.5 mV (Byerly and Leung 1988). Thus, the true somal membrane potential was actually 10.5 mV more negative than that given. In addition, there are errors due to uncompensated series resistance of ~ 5 mV per 1 nA.

Solutions

K⁺ currents were measured in an external 6K/0Ca Tris *Drosophila* saline, which contained (in mmole/L) 6 KCl, 10 MgCl₂, 140 TrisHCl, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with TrisOH. To test the effect of Ca²⁺ on the K⁺ current, a Ca²⁺-containing external solution was used in other experiments, it contained (in mmole/L) 125 NaCl, 6 KCl, 5 CaCl₂, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with NaOH. For these two external solutions, the osmolarity was ~ 296 mOsm/L (measured using a 3 MO micro-osmometer, Advanced Instruments). The external solution was changed during experiments by pipetting 3 mL of the new solution into the 0.3-mL bath; excess solution was removed by a continuous, vacuum-powered exhaust. The pipette internal solution was potassium aspartate, it contained (in mmole/L) 3 KCl, 139 L-aspartic acid, 1 MgCl₂, 10 HEPES, 0.1 CaCl₂, and 1 EGTA (final Ca²⁺ concentration is ~ 10 mmole/L). The internal solution was adjusted to pH 7.3 with KOH (final K⁺ concentration is ~ 156 mmole/L). The osmolarity of the internal solution was $\sim 10\%$ lower than that of the external solution to improve seal formation (Hamill et al. 1981).

Statistical Analysis and Data Presentation

It is possible to detect the effect of a mutation only if it causes a change in membrane current that is large compared with the inherent variability of cultured neurons (Alshuaib and Byerly 1996). Throughout the Results, population data are presented as the mean $\pm$ SEM. The means of the two populations were compared by use of a two-tailed Student's *t*-test for independent samples. A difference was considered statistically significant if the probability that

both samples came from the same distribution was at least $<5\%$ ($P < 0.05$). Graphics were generated with Excel (Microsoft) and SigmaPlot (Jandel Scientific) software packages.

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