The Journal of Neuroscience, February 1995, 15(2): 1025–1034

Characterization of K^+ Currents and the cAMP-Dependent Modulation in Cultured *Drosophila* Mushroom Body Neurons Identified by *lacZ* Expression

Nicholas J. D. Wright and Yi Zhong

Cold Spring Harbor Laboratory, Center for Learning and Memory, Cold Spring Harbor, New York 11724

Electrophysiological analysis of cultured neurons provides a potential approach toward understanding the physiological defects that may contribute to abnormal behavior exhibited by mutants of the fruit fly *Drosophila*. However, its application has been restricted by an inability to identify a particular functional or anatomical subpopulation of neurons from the CNS. To study neurons composing the CNS mushroom body proposed as a center for insect olfactory learning, we utilized a *Drosophila* enhancer detector line that expresses a *lacZ* reporter gene in these neurons and identified them in acutely dissociated larval CNS cultures by vital fluorescent staining. The patch-clamp analysis suggests that whole-cell voltage-activated K^+ currents can be classified into two types in identified mushroom body neurons. Type 1 current comprises a TEA-sensitive slowly inactivating current and a noninactivating component while type 2 current contains a 4-AP–sensitive transient A-current and a noninactivating component. Application of cAMP analogs induced distinct modulation of type 1 and type 2 currents. Our results demonstrate that the expression of the *lacZ* gene and the subsequent staining do not significantly alter the different types of K^+ currents. This initial characterization provides a basis for further analysis of mutations that impair learning and memory resulting from an abnormal cAMP cascade preferentially expressed in the mushroom body.

[Key words: K^+ current, mushroom body,* Drosophila, *cAMP, modulation, lacZ]

A large number of *Drosophila* mutants have been isolated based on defects in various types of behavior such as locomotion, mating, learning, and memory (Benzer, 1973; Hall, 1982). The molecular nature and expression pattern of many of these mutated genes have been characterized. For instance, mutants *dunce* (*dnc*) and *rutabaga* (*rut*) have been isolated based on their abnormal performance in a classical conditioning paradigm (for reviews, see Dudai, 1988; Davis, 1993). Although *dnc*, encoding a phosphodiesterase, and *rut*, encoding an adenylyl cyclase are expressed throughout the fly, both are expressed at high levels in the mushroom body, an anatomic region considered as a center for olfactory related learning in insects (Davis, 1993; deBelle and Heisenberg, 1994). Genetic or pharmacological ablation of this region abolishes the olfactory-associated learning and memory. (Heisenberg et al., 1985; deBelle and Heisenberg, 1994). Despite two decades of genetic, molecular, anatomical, and behavioral analyses of learning and memory mutants, it has not been possible to determine the functional defects of these learning and memory mutants in the CNS, particularly in mushroom body neurons. A large part of the problem has been due to the difficulty in recording from the small *Drosophila* brain and neurons composing it. As an alternative but indirect approach, analyses of neuromuscular junctions have demonstrated that synaptic facilitation, posttetanic potentiation, activity-dependent synaptic arborization and modulation of muscle membrane K^+ currents are altered in *dnc* and *rut* mutants (Zhong and Wu, 1991, 1993; Zhong et al., 1992).

Patch-clamp analysis of cultured neurons has provided a potential approach for the study of some aspects of the CNS physiology of *Drosophila*. Membrane currents in embryonic (Byerly and Leung, 1988; O'Dowd and Aldrich, 1988; O'Dowd et al., 1989; Saito and Wu, 1991), larval (Sun and Wu, 1985; Solc et al., 1987; Solc and Aldrich, 1988), and pupal neurons (Baker and Salkoff, 1990) have been examined in culture, but a particular subpopulation of neurons were not identifiable. The present work describes a preparation which allows us to identify fluorescently stained mushroom body neurons in acutely dissociated larval CNS cultures and to patch clamp these neurons. We used a *Drosophila* enhancer–detector line that expresses a *lacZ* reporter gene almost exclusively in mushroom body neurons to identify these neurons in culture. These transgenic flies are generated by introducing a piece of foreign transposable DNA that carries a *lacZ* reporter gene into the host genome while the expression of the *lacZ* gene is determined by its position in the host DNA (Bellen et al., 1989; Wilson et al., 1989). Our patch-clamp analysis of the physiological and pharmacological properties of voltage-activated K^+ currents in these identified neurons demonstrated that the expression of the *lacZ* gene and the subsequent staining do not alter significantly the different types of K^+ currents. Interestingly, these K^+ currents can be modulated by cAMP-dependent mechanisms. Thus, our approach promises to contribute to a better understanding of electrophysiological defects in the CNS of learning and memory mutants.

Materials and Methods

Fly stocks. Stocks of *Drosophila* flies were maintained on standard media at room temperature (19–22°C). In this study the strains Canton S

Received Mar. 21, 1994; revised June 23, 1994; accepted Aug. 15, 1994.

We thank Dr. R. Davis for kindly providing the enhancer trap line 221, which was essential for this study, and thank Dr. C.-F. Wu for comments on the manuscript.

Correspondence should be addressed to Yi Zhong, Cold Spring Harbor Laboratory, Center for Learning and Memory, P.O. Box 100, Cold Spring Harbor, NY 11724.

(as wild type) and enhancer trap line 221 were used. The strain 221 has been isolated in R. Davis laboratory. X-Gal staining has shown that the expression of the reporter gene *lacZ* is almost exclusively in both the adult and larval mushroom body (R. Davis, personal communication; also see Fig. 1).

Cell culture. The larval CNS culture was adapted from the method described by Wu et al. (1983). After dissection and washing, five brains and ganglia were pooled in the "divalent cation free" *Drosophila* normal saline (see below) containing collagenase at a concentration of 0.5 mg/ml (Sigma, type 1a) for 1–2 hr at room temperature. The softened tissue was then passed gently through the tip of a beveled pipette tip 50 times in order to completely dissociate the cells. The resulting suspension was then centrifuged for 2 min at 3750 rpm. The pellet was resuspended in 375 μl of medium prior to plating out on three plastic tissue culture petri dishes (25010, Corning). After incubation for 3–4 hr at room temperature the plates were very carefully flooded with *Drosophila* normal saline containing NaCl (128 mM), KCl (2 mM), sucrose (35.5 mM), $MgCl_2$ (4 mM), CaCl2 (1.8 mM), HEPES (5 mM, buffered at pH 7.1) prior to electrophysiological examination.

Vital fluorescent marking of neurons expressing lacZ *in culture.* After dissociating the cells (see Cell culture) the resulting suspension was centrifuged for 2 min at 3750 rpm. The pellet was then resuspended in 100 μl of medium diluted 40–50% with distilled water and containing 0.2 mM of the fluorogenic β-galactosidase substrate analog 5-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG, Molecular Probes Inc.). After 1 min tonicity was restored by the addition of 250 μl of undiluted medium and the suspension was centrifuged as before. The cells were washed in fresh medium again and respun and finally resuspended in 375 μl of medium prior to plating out on three plastic tissue culture petri dishes. After incubation for 3–4 hr at room temperature the plates were very carefully flooded with *Drosophila* normal saline. Neurons expressing *lacZ* were marked by the fluorescent product of β-galactosidase activity and were identified by using a Zeiss Axiovert 35 inverted microscope with a standard UV light source and FITC filters.

Whole-cell patch-clamp techniques. Whole-cell recordings were made using the Axopatch 200A (Axon Instruments) and stored on a IBM-type PC for analysis. The patch pipettes were pulled on a two-stage vertical puller (Narishige) and typically had resistances of between 5–7 MΩ when filled with the intracellular solution composed of KCl (144 mM), $MgCl_2$ (1 mM), $CaCl_2$ (0.5 mM), EGTA (5 mM), HEPES (10 mM) and pH 7.1. Typically gigaohm seals were obtained using gentle suction and the whole-cell configuration obtained with the application of further suction. Pipette potential was compensated for immediately prior to seal formation after which pipette capacitance was also balanced. Whole-cell capacitance was adequately compensated for by manual adjustment achieved by monitoring a test pulse at subthreshold voltages. Series resistance compensation was unnecessary with these cells. Any leak currents were compensated for manually and this confirmed by an initial subthreshold voltage step in all protocols. All recordings were filtered initially with the four-pole low pass Bessel filter set to 5 kHz included in the Axopatch 200 A and sampled at 100 kHz. Results were analyzed using pCLAMP software (Axon Instruments) and displayed using commercial graphics software.

Drug application. TEA, 4-AP, 8-bromo-cAMP, and cAMP solutions were prepared fresh each day in normal *Drosophila* saline. The solutions were applied by pressure ejection using the Picospritzer system (model 2; General Valve Corp.) via a microelectrode with a tip diameter of 1–2 μm. Pressure was set between 2–10 psi and the microelectrode positioned between 50 and 100 μm away from the patched neuron. Only visually confirmed drug ejection was considered.

β-Galactosidase staining. The larval CNS was fixed in 2% glutaraldehyde (Sigma) in phosphate-buffered saline (PBS) which contains NaCl 130 mM, Na_2HPO_4 7 mM, NaH_2PO_4 3 mM (Sigma) and pH 7 for 10 min. After washing in two changes of PBS for 5 min each the tissue was transferred into the staining buffer which contains NaH_2PO_4 10 mM, $K_4(Fe(II)(CN)_6)$ 3.1 mM, $K_3(Fe(II)(CN)_6)$ 3.1 mM, NaCl 150 mM, and $MgCl_2$ 1 mM (Sigma) for 1 min. The tissue was then incubated for 2 hr at 37°C in the staining buffer containing 0.2% 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (Sigma). The stained brain was mounted with glycerol. β-Galactosidase expression is visualized as dense black staining under normal light microscopy.

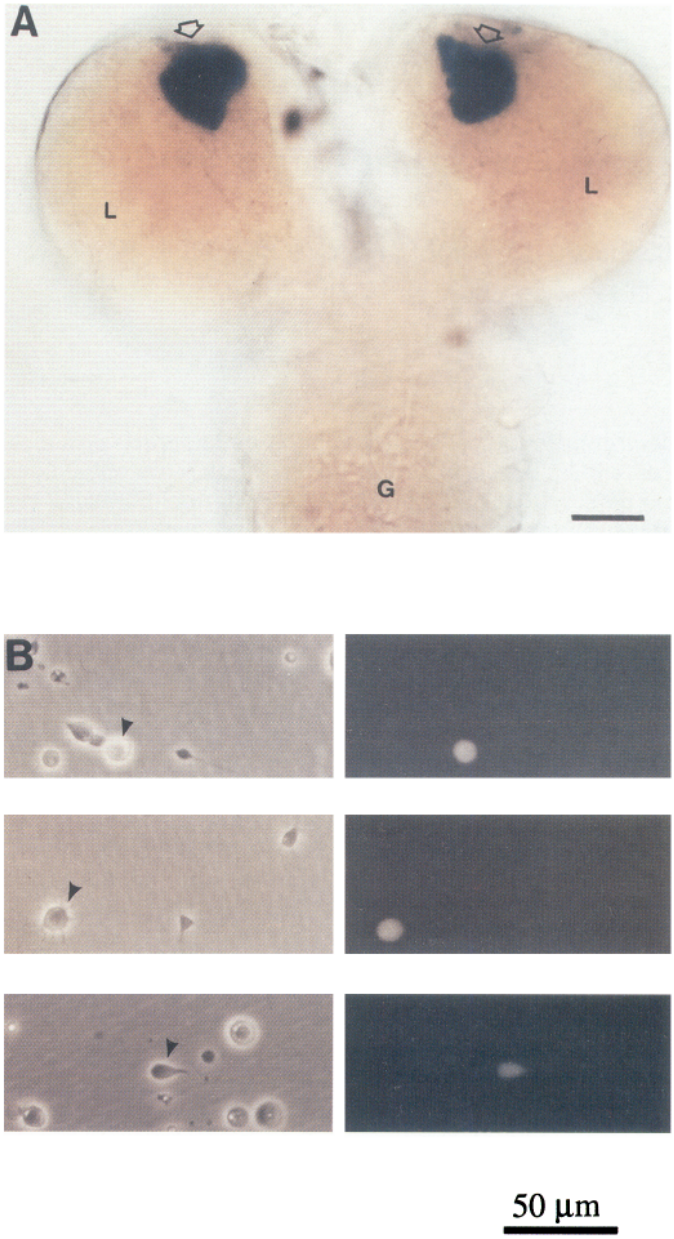

Figure 1. Vital fluorescent staining of larval mushroom body neurons expressing *lacZ* in culture. *A*, Whole-mount of *lacZ* staining of the brain lobes (*L*) and ventral ganglion (*G*) in a 3rd instar larva of the strain *221*. The dark blue staining is concentrated in the mushroom body region of the brain lobes (*arrows*). Scale bar, 50 μm. *B*, The *left column* is a selection of bright-field views of cultured *221* larval neurons 4 hr after plating. The *right column* shows the same corresponding fields but illuminated with a UV light source and viewed via FITC filters. The neurons expressing *lacZ* are marked with *arrowheads*.

Results

Mushroom body neurons identified in larval CNS cultures

The adult fly and third instar larval mushroom body consists of about 2500 neurons and is located in the dorsal and posterior cortex of each brain lobe (Technau and Heisenberg, 1982). The enhancer–detector line *221* (isolated in R. Davis' laboratory, Baylor University) used in this study expresses the *lacZ* gene almost exclusively in both adult and larval mushroom bodies (R. Davis, personal communication). Figure 1*A* shows the *lacZ*

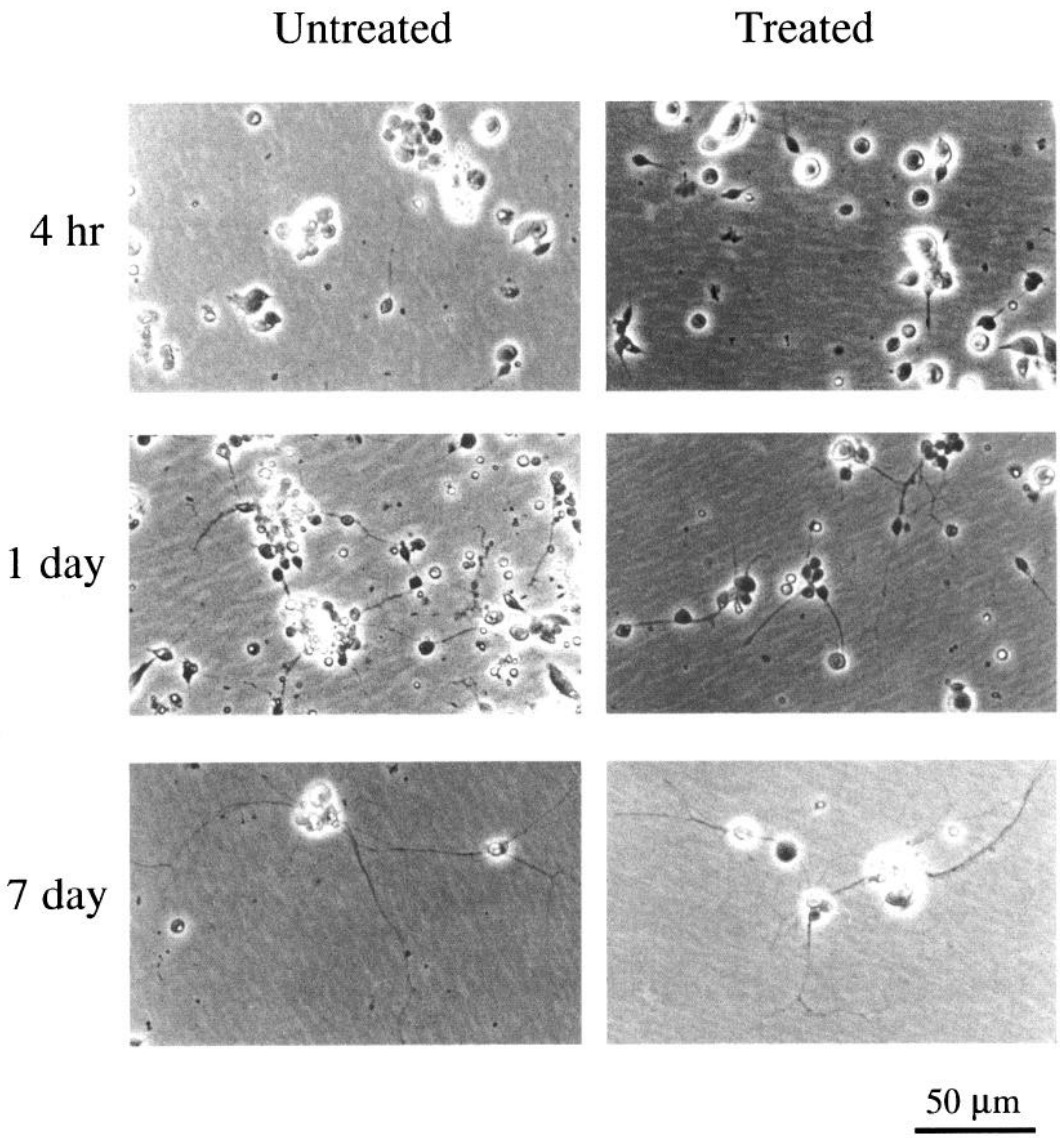

Figure 2. Growth and differentiation of larval neurons subject to hypotonic shock and subsequent loading of the β-galactosidase substrate. The *left column* illustrates the time course of the growth and differentiation of larval *221* neurons in regular, untreated cultures. The *right column* shows larval *221* neurons in cultures after hypotonic shock and loading of the fluorogenic β-galactosidase substrate CMFDG. After treatment the neurons were still able to grow and differentiate over a time course similar to that seen in regular cultures. Note the extensive axonal growth in 7 d old cultures.

expression pattern in the larval CNS of enhancer detector line *221*. The staining is almost exclusively localized in the region of the mushroom body. This transgenic strain does not exhibit any significant defects in the structure of the mushroom body (Davis, personal communication). When the larval CNS is dissociated in culture, individual mushroom body neurons can be identified by fluorescent staining.

The larval CNS culture has been well established (Wu et al., 1983; Kim and Wu, 1987; Solc and Aldrich, 1988). A major modification from the previously described culture protocols was the application of a half to one minute hypotonic shock to the dissociated cells in order to load the fluorogenic substrate 5-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG, Molecular Probes Inc.) of β-galactosidase (see Materials and Methods). Chloromethylfluorescein becomes fluorescent after cleavage from CMFDG by β-galactosidase activity in cells that express the *lacZ* gene. Four hours after plating, the fluorescence was strong enough to be visualized in the cell body (Fig. 1*B*) and could last for over 20 hr. About 5% of the cells were stained in the larval CNS cultures of *221* which is comparable to the fraction of mushroom body neurons in the whole CNS. The morphology of these marked mushroom body neurons varies and their diameters range from 3 to 12 μm (also see Fig. 5). In the early stages of these cultures (4–6 hr) most of the stained neurons appear to be spherical in shape although small mono- and multipolar cells are also seen (Fig. 1*B*). As a control, the wild-type CNS cultures did not show any appreciable fluorescence when treated similarly (data not shown). The cell density was estimated to be around 10^{-6}/ml which agrees with the recommendation of Fiering et al. (1991) to minimize the possible accumulation of fluorescent product in the medium.

Neurons subject to treatment by hypotonic shock and loaded with the fluorogenic substrate of β-galactosidase were still able to develop in culture (Fig. 2). Axonal outgrowth and branching

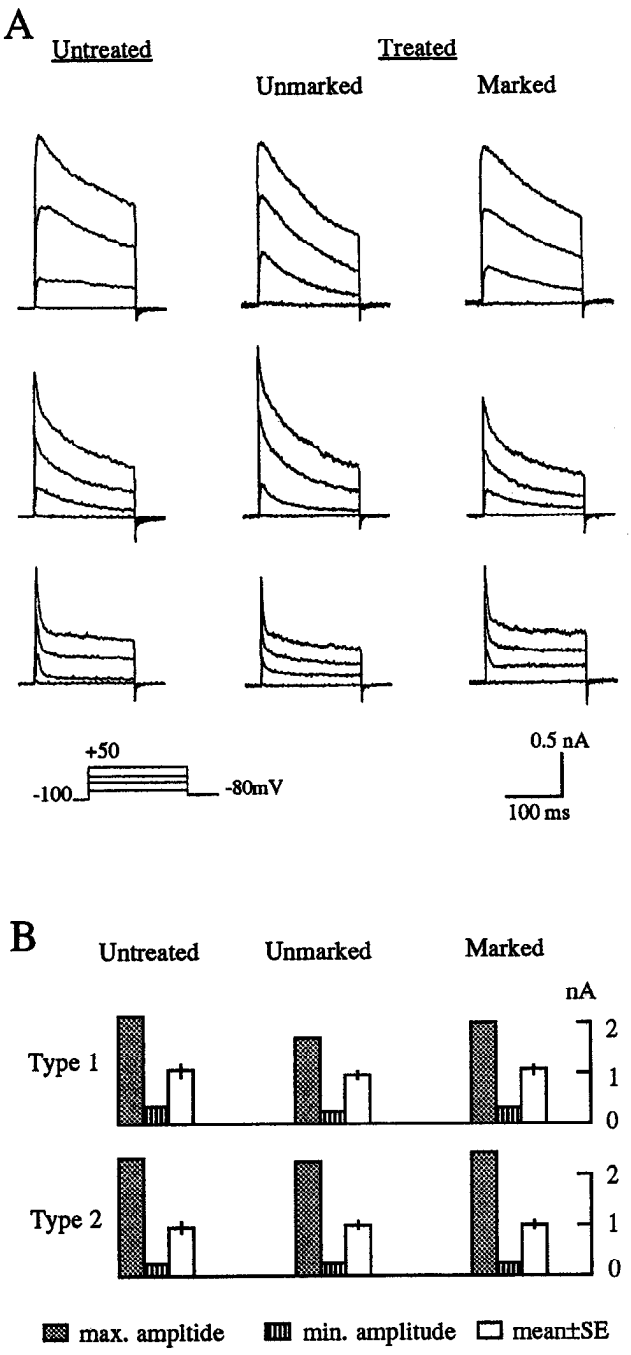

Figure 3. Voltage-activated whole-cell K^+ currents recorded from untreated, treated but unmarked and stained larval neurons in acutely dissociated cultures. *A*, Representative current traces. All recordings presented were made from dissociated *221* neurons. The family of current traces were elicited by depolarizations to −60, −20, 20, 50 mV following a 1 sec prepulse of −100 mV (see voltage traces shown). The examples in the *left column* were recorded from cells not subject to hypotonic shock. The *middle column* shows examples recorded from cells subjected to the hypotonic shock and loaded with CMFDG but unmarked due to the lack of expression of *lacZ*. The *right column* shows examples recorded from cells which are fluorescently labeled resulting from the *lacZ* expression. The marked neurons are derived from the mushroom body. *B*, The distribution of the amplitudes of K^+ currents recorded from untreated, treated but unmarked and stained neurons. Type 1 includes those with kinetics similar to that shown in the top two rows in *A*. Type 2 represents those with kinetics similar to that shown in the bottom row in *A*. Ten representative recordings of each current type in each category were analyzed. The maximum, minimum and mean peak amplitudes of each category are presented. There is no significant difference in the distribution of the current amplitude among untreated, treated but unmarked and stained neurons. These examples are chosen from over 250 recordings.

in these treated neurons exhibited a similar pattern and time course to those in the regular cultures (Fig. 2). Extensively branched processes produced by treated neurons could be observed in 7 d old cultures. In general, the treated cultures appeared to contain a slightly reduced number of surviving neurons. This was seen more clearly in older cultures. However, the membrane physiology examined in acutely dissociated neurons exhibited no significant differences among untreated, treated but unmarked and marked neurons.

Whole-cell K^+ currents in acutely dissociated CNS neurons

The characterization of the whole-cell membrane currents in acutely dissociated larval CNS neurons (4 hr after plating) showed that the types of currents and their properties are very similar to those observed from larval CNS neurons in up to week old cultures (Solc et al., 1987; Solc and Aldrich, 1988). There appeared to be no appreciable voltage-activated Na^+ currents expressed in the soma of these neurons even after blocking out the most K^+ currents by internal perfused cesium (data not shown). Occasionally, a small sustained inward current was observed with voltage steps in the range of −30 to −50 mV, which is probably a Ca^{2+} current (Solc et al., 1987; Solc and Aldrich, 1988). Therefore, we could examine the K^+ currents in isolation without having to block any inward currents. These whole-cell K^+ currents exhibited a range of peak amplitudes from less than 100 pA to over 2 nA and large variations in inactivation time courses. However, recordings from over 200 neurons in acutely dissociated cultures indicated that all whole-cell K^+ currents contain transient components. This is different from those observed in week old cultured neurons in which a small fraction of neurons showed only noninactivating K^+ currents (Solc and Aldrich, 1988).

In order to asses any possible effect of the treatment by hypotonic shock and the β-galactosidase substrate as well as the expression of β-galactosidase on membrane currents, a comparison was made from recordings of 68 untreated, 87 treated but not stained, and 115 stained neurons (for representative examples, see Fig. 3*A*). The results indicate that the three groups exhibit a similar range of variation in amplitude (Fig. 3*B*), time course of activation and inactivation, voltage dependence of activation and inactivation, reversal potentials and sensitivity to the drugs 4-aminopyridine (4-AP) and tetraethylammonium (TEA). In particular recordings of the fastest transient K^+ currents, termed A_2 by Solc and Aldrich (1988) which appeared the most labile under these recording conditions, could persist for up to 30 min which is similar to those seen in regular cultures. This suggests that the staining did not significantly affect neuronal membrane physiology, certainly as far as voltage-sensitive K^+ currents are concerned. In the following sections we will focus on the characterization of these voltage-sensitive K^+ currents recorded from stained mushroom body neurons.

Heterogeneity of K^+ currents in mushroom body neurons

Potassium currents recorded from all stained neurons contained at least some inactivating components. Analysis of the decay

←

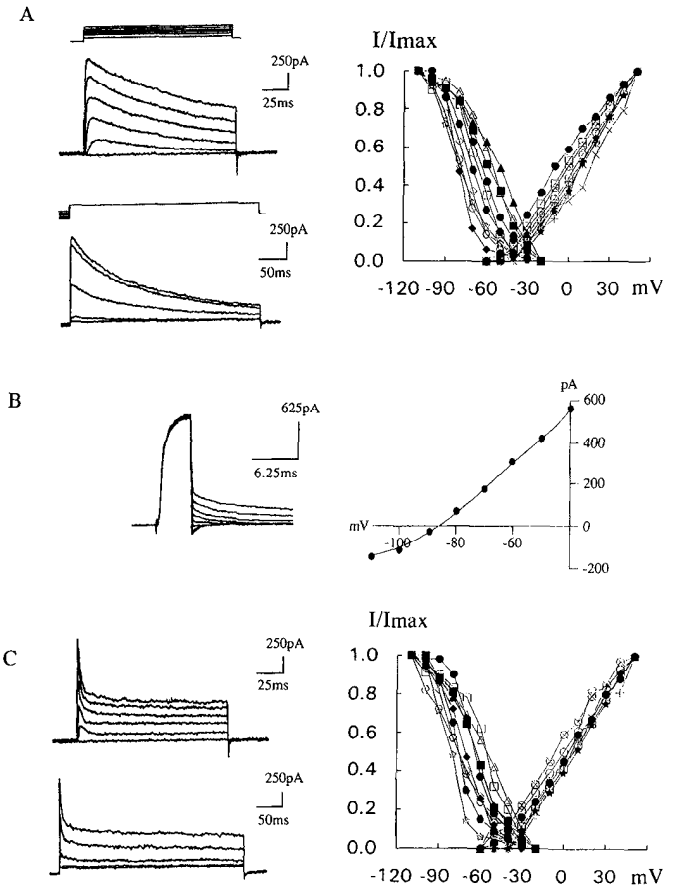

Figure 4. Voltage dependency of activation and inactivation of K^+ currents in identified mushroom body neurons. *A*, The top family of traces show an example of a type 1 current activated by depolarizations to −50, −30, −10, 10, 30, 50 mV in a mushroom body neuron. The lower family of traces show steady-state inactivation of the same current by 1 sec prepulses to −110, −90, −60, −50, −30 mV. To the right are *V–I* curves which illustrate the voltage dependency of both activation and steady-state inactivation of the type 1 current recorded from 10 mushroom body neurons. The peak current value is normalized to the maximum current at +50 mV. Note the wider variation of inactivation as compared to activation for the same 10 neurons. *B*, Tail currents from a mushroom body neuron that expressed a type 1 current (see *A*). The voltage was stepped to 50 mV for 5 msec following a 1 sec prepulse of −100 mV and then to postpulse voltages of −40, −50, −60, −70, −80, −90, −100, −110 mV. The current reverses at −86 mV. *C*, The voltage dependency of activation and inactivation of a type 2 current shown in the families of current traces and *I–V* curves. About a half of the 115 identified neurons recorded express type 1 K^+ currents while the rest were type 2. The examples presented cover the whole range of variation observed among all recordings.

phase of these inactivating components allowed us to classify the whole-cell currents recorded from marked neurons into two basic types. Type 1, as shown in Figure 4*A*, consisted of those whose inactivating components exhibited a slower time course and the decay phase could be fitted by a one component exponential curve (not shown). Type 2 consisted of principally a rapidly inactivating and a noninactivating component (Fig. 4*C*). Recordings from over 100 stained neurons resulted in roughly equal numbers of each type. The distributions of the decay-time constants of the type 1 current and the two components of the type 2 current are plotted respectively in Figure 5.

The reversal potentials for the inactivating components of both types are similar at about −70 to −90 mV (see Fig. 4*B*), which is typical for transient K^+ currents (Solc and Aldrich, 1988; Baker and Salkoff, 1990). Current–voltage (*I–V*) curves shown in Figure 4*A* illustrate the voltage dependency of both activation and steady-state inactivation of type 1 currents. Each curve represents recordings from a single identified neuron. In these *I–V* curves, the amplitude of the peak current at each voltage step is normalized to that of the largest amplitude (elicited by the +50 mV step) in all the data presented. It can be seen from these curves that these currents are activated at around −60 to −40 mV. The variation in the voltage dependency of the steady-state inactivation is more extensive than that observed in the voltage dependency of activation. The 50% values of the steady-state inactivation range from −80 to −50 mV. From the representative examples shown in both Figure 4, *A* and *C*, the variation in voltage dependency of peak current of both activation and inactivation of type 2 currents is almost identical to that seen in type 1 currents. The cell diameter and whole-cell membrane capacitance also showed a similar range of values in either type of neuron (Fig. 6).

As can be seen from Figure 6, the peak current density could vary tremendously among neurons exhibiting type 1 or type 2 currents. Again, a similar range of variation is observed in neurons exhibiting either current type.

Physiological and pharmacological separation of the different components of voltage-activated K^+ currents

The above analysis is based on the characteristics of the transient components. Further analysis indicates that each whole-cell current is made up of at least two components, one transient and another noninactivating, in both type 1 and type 2. These components can be at least partially separated by both physiological and pharmacological methods.

As shown in Figure 7, the transient K^+ current elicited by a voltage step from a holding potential of −80 mV, via a 1 sec prepulse of −100 mV to minimize voltage-sensitive inactivation, to +20 mV is almost completely inactivated when the prepulse is changed to −30 mV. A sustained, noninactivating components was then revealed. The amplitude of this noninactivating component varies from neuron to neuron. The distribution of the amplitudes of such noninactivating K^+ currents of 32 representative examples of both type are shown in a scatterplot (Fig. 7). This demonstrates that the variation in the amplitude of the noninactivating component is similar in neurons exhibiting either type of whole-cell current. The existence of such noninactivating K^+ currents was also further supported by pharmacological studies.

When 10 mM 4-AP, a drug that can block transient K^+ currents (Solc and Aldrich, 1988), was pressure-ejected from a micropipette onto patched neurons, the transient current in type 2 neurons was completely abolished (Fig. 8). A minute after the drug application, the transient current could be seen to recover fully in most cases. In contrast, the same concentration of 4-AP only slightly reduced the type 1 current (Fig. 8). Conversely, a 10 mM concentration of another K^+ channel blocker TEA (Solc and Aldrich, 1988) strongly inhibited the type 1 current while the type 2 current appeared to be relatively unaffected (Fig. 8). However, the component inhibited by TEA appeared to be a slowly inactivating current (see Subtraction in Fig. 8). As shown in the bottom traces (Fig. 8), the combined application of 10 mM 4-AP and 20 mM TEA blocked a large portion of both type 1 and type 2 whole-cell currents while

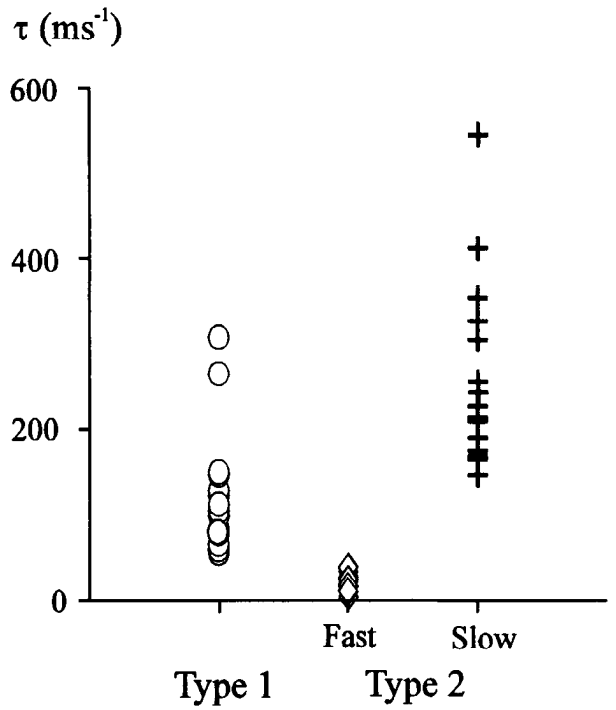

Figure 5. The distribution of the decay-time constants of type 1 and type 2 currents. The exponential decay (measured by time constant τ) of the K^+-currents with time were obtained using pCLAMP software. The type 1 current could be fitted with a single exponential function as confirmed by semilog plots, which gave virtually straight lines for the decay phase of the currents. The type 2 current is clearly made up of two components: an rapidly inactivating component and a noninactivating component. The currents analyzed were elicited by a step depolarization to +40 mV. Fifteen representative examples of each type of currents are presented.

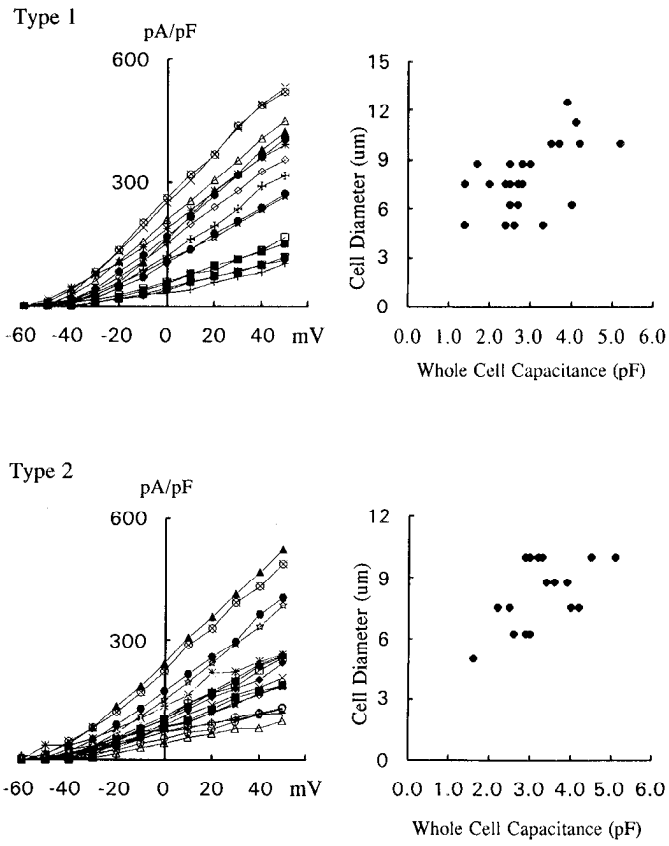

Figure 6. The distribution of cell diameters and current density in type 1 and type 2 neurons. *Left, I–V* curves of peak type 1 and type 2 current density in identified mushroom body neurons. Examples of 15 type 1 and 17 type 2 currents are shown which cover the whole range of variations observed from over a hundred recordings. The peak currents were normalized to the whole-cell capacitance to determine the current density. The voltage paradigm was the same as that shown in Figure 4. *Right,* The distribution of the range of whole-cell membrane capacitance and cell body diameter in identified mushroom body neurons. Data from 23 type 1 and 17 type 2 identified neurons is shown. Cell diameter was measured by using a calibrated graticule. Membrane capacitance was read directly from the Axopatch 200A after manual compensation achieved by monitoring a test pulse at subthreshold membrane voltages. The distribution is similar for cells exhibiting both types of whole-cell current.

leaving a drug-insensitive, noninactivating component (Fig. 8). The size of this insensitive component is comparable to that of the noninactivating component retained in response to the −30 mV prepulse inactivation described above. Further studies confirmed that the drug insensitive component could not be inactivated by a prepulse to −30 mV. Therefore, there is a sustained K^+ current that is insensitive to both 4-AP and TEA and is not inactivated by depolarization to −30 mV in addition to the transient components found in both type 1 and type 2 neurons.

cAMP-dependent modulation of K^+ currents

Since several genes encoding components of the cAMP cascade are preferentially expressed in the mushroom body (Davis, 1993), we therefore examined whether K^+ currents recorded from identified mushroom body neurons are subject to cAMP-dependent modulation. Figure 9 reveals that both type 1 and 2 currents can be modulated by application of 8-bromo cAMP, a membrane permeable cAMP analog.

Five micromolar 8-bromo cAMP was applied by pressure ejection via a microelectrode placed near the patched neuron. To observe the modulation, the whole-cell current was recorded immediately after ceasing perfusion of 8-bromo cAMP. Perfusion was usually for 10–60 sec. As compared to the control which was recorded prior to perfusion of 8-bromo cAMP, the amplitude of type 1 current is markedly reduced (Fig. 9*A*). A longer time of perfusion usually led to a stronger reduction in the amplitude of type 1 current (Comparing the upper panel with the lower panel in Fig. 9*A*). The modulation appears to be voltage dependent, for example, the reduction in the amplitude is stronger at lower membrane voltages. Thus, the threshold of activation of type 1 current is shifted by about +40 mV (Fig. 9*B*). All type 1 currents examined (n = 9) responded to application of 8-bromo cAMP in a very similar way. The current was normally seen to recover 2–4 min after stopping perfusion of 8-bromo cAMP (Fig. 9*A,C*).

Perfusion of 10 mM cAMP also reduced type 1 current (data not shown), but the reduction was much less as compared to that induced by 5 mM 8-bromo cAMP. This is probably due to reduced membrane permeability of cAMP as compared to 8-bromo cAMP.

The modulation of type 2 current is not as profound as that seen in type 1 current (see Fig. 9*C*). In most cases examined, the sustained component appears not to be altered by application of 8-bromo cAMP while the transient is only slightly reduced in the amplitude. A representative example (all four type 2 neurons examined responded similarly) is presented in Figure 9*C*.

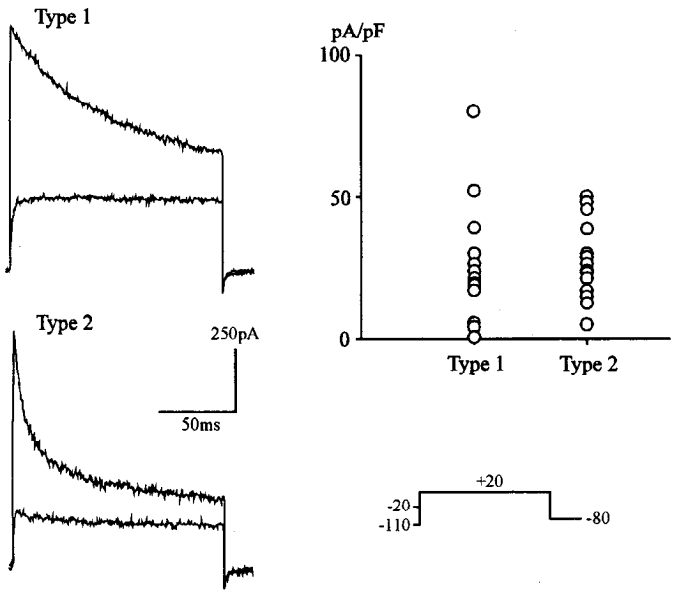

Figure 7. Physiological separation of the transient and sustained components of K^+ currents in identified mushroom body neurons. The traces indicate that the transient component seen in both type 1 and type 2 currents elicited by a test pulse (+20 mV) could be inactivated by a 1 second prepulse to −30 mV while a sustained component remained. The maximum amplitude of this sustained component is presented in the scatterplots from 17 type 1 and 15 type 2 neurons. This data suggests that almost all the identified neurons express a noninactivating, sustained K^+ current. The amplitude of this component as revealed by prepulse inactivation ranges from 0 to 250 pA and the distribution is similar for neurons expressing either type of current.

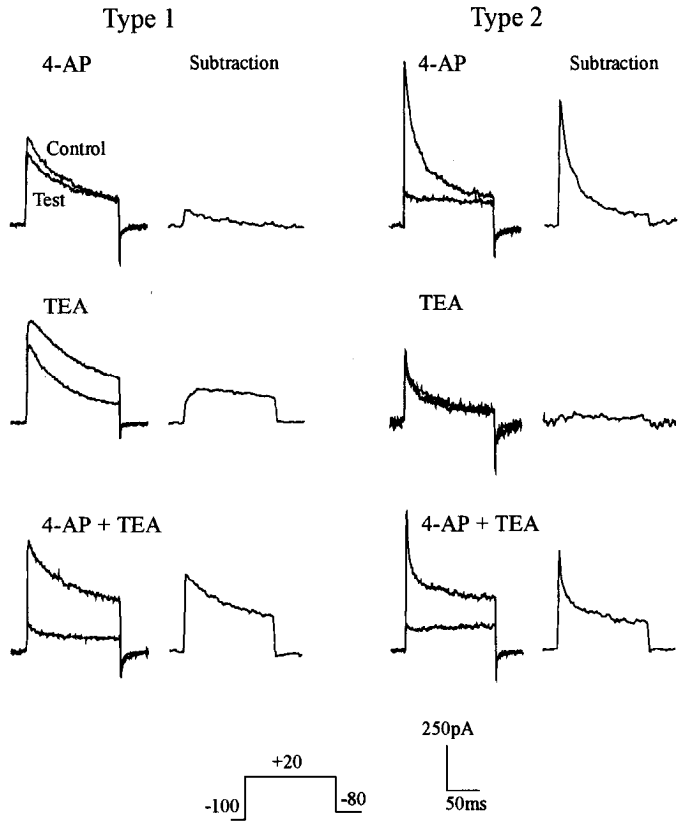

Figure 8. Pharmacological separation of the transient and sustained components of K^+ currents in identified mushroom body neurons. Current traces on the left illustrate the effects of the drugs 4-AP and TEA on type 1 current. The *top traces* show that 10 mM 4-AP pressure ejected via a micropipette onto the patched neuron blocked only a small portion of transient K^+ current. The *middle traces* show that 10 mM TEA inhibited a very slowly inactivating current as seen from the corresponding subtraction. However, 20 mM TEA together with 10 mM 4-AP eliminated most of transient current but still left a sustained component as shown in the bottom traces. Current traces on the right illustrate the effect of drugs on type 2 current. The *top traces* show that 10 mM 4-AP could completely block the transient current. The blocked transient current normally recovered fully within 1 min after ceasing perfusion of 4-AP (not shown). The *middle traces* show that 10 mM TEA had very little effect. The *bottom traces* show that a sustained component remains even after treatment by 20 mM TEA and 10 mM 4-AP applied together. The voltage paradigm is shown at the bottom. Twenty-two cells expressing type 1 and 16 cells expressing type 2 currents were examined for the effects of these drugs.

Recovery is normally observed soon after ceasing the perfusion (within 120 sec).

Discussion

A preparation for studying genetically marked neurons

The above experiments describe a preparation developed for studying identified *Drosophila* CNS neurons in acutely dissociated culture. A previous report has shown that *Drosophila* embryonic neurons expressing *lacZ* can develop normally in culture after isolation by means of similar fluorescent staining (Krasnow et al., 1991). To determine whether the hypotonic shock and the loading of the β-galactosidase substrate disrupt the K^+ channel physiology, we compared recordings from neurons in regular cultures with those subject to the treatment. It was demonstrated that the range of amplitudes, the kinetics, the voltage dependency of activation and inactivation, the drug sensitivity and the cAMP-dependent modulation of whole-cell K^+ currents were very similar in all three groups of cultured neurons: regular cultured, treated but unstained, and stained neurons (see Fig. 3). Furthermore, the type of K^+ currents and their properties observed in stained neurons (see Figs. 4–8) are also very similar to those recorded from week old neurons in regular culture (Solc and Aldrich, 1988; also see next section). To verify further whether the insertion of a P-element enhancer detector in *221* induces mutation that affects these K^+ currents, stained neurons from the heterozygous *221* strain were also examined, which indicated no significant difference from that of the homozygous (data not shown). We therefore conclude that β-galactosidase staining does not appreciably alter the voltage-activated K^+ currents of these neurons.

In addition, our preliminary data indicated that not only the cAMP-dependent modulation but also neuropeptide-induced modulation of the K^+ currents was similar for both the control and stained neurons (Wright and Zhong, unpublished data). This suggests that the potassium channels, their modulations and signal transduction mechanisms seem to remain functionally intact in neurons subjected to this technique. Since acutely dissociated neurons are used, this method should be applicable to the analysis of embryonic, pupal, and possibly even adult neurons of *Drosophila.*

A large number of *Drosophila* enhancer detector lines have been isolated in which various patterns of *lacZ* expression in the CNS are found (Bellen et al., 1989; Wilson et al., 1989). Many of these lines also carry a mutational phenotype. The preparation presented here will allow electrophysiological analysis of mu-

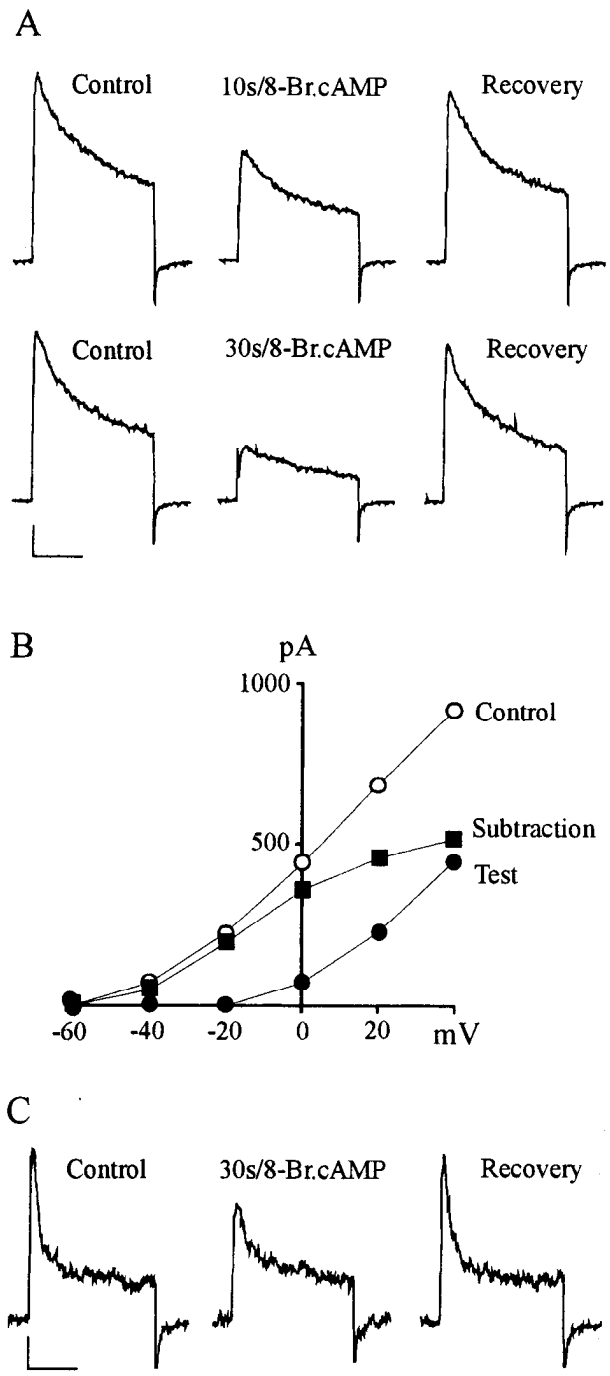

Figure 9. The cAMP-dependent modulation of type 1 and type 2 currents; 5 mM 8-bromo cAMP was pressure ejected via a micropipette over a patched neuron for 10–60 sec. Recordings were obtained immediately after ceasing ejection and every minute thereafter. *A*, Modulation of type 1 currents. For this representative example, the test recording (8-bromo cAMP) was obtained immediately after a 10 (*upper panel*) or 30 (*lower panel*) second perfusion. The reduction in the amplitude of K^+ currents induced by 8-bromo cAMP was often completely reversed within 2–4 min after ceasing perfusion. A longer time of perfusion leads to stronger reduction (The current traces elicited by +40 mV depolarization in the both *upper* and *lower panels* were recorded from the same neuron). All nine type 1 neurons examined responded to the application of 8-bromo cAMP in a very similar way. *B*, The *I–V* curves illustrate the voltage dependency of the amplitude of the peak current of the control, 8-bromo cAMP and the subtracted current. The recording was obtained from the same neuron as that in *A*, but with a 60 sec perfusion. *C*, Modulation of type 2 current. The experimental current trace is recorded after a 30 sec perfusion while the recovery current trace was recorded 120 sec after ceasing perfusion of 8-bromo cAMP. Since the volume of the perfusate, 8-bromo cAMP in saline, was so small that passive diffusion was adequate to observe the recovery. Calibration: *A*, 250 pA, 50 msec; *C*, 40 pA, 50 msec.

tational effects in any genetically defined subpopulation of neurons.

K^+ currents in acutely dissociated neurons

Similar types of K^+ currents were found in both regular and stained neurons in these acutely dissociated neurons. They can be classified into type 1 or 2 based on the time course of inactivation (Fig. 4) and the drug sensitivity (Fig. 8). The type 1 current has relatively slow inactivation kinetics and is mainly sensitive to TEA. The type 2 current inactivates more rapidly and can be blocked by 4-AP. In addition to the transient components, both types of neuron express a drug-insensitive, noninactivating component (see Figs. 7, 8).

Solc and Aldrich (1988) have described three different types of K^+ currents including A_2, K_D, and a sustained component in week old cultured larval neurons. Comparison of the kinetics and pharmacological properties suggests that the transient current from identified type 2 neurons may correspond to the A_2 current while the transient in identified type 1 neurons may be principally the K_D current. The noninactivating components in these identified neurons appears similar to the sustained current. Thus, the type of K^+ currents and their properties are very similar between acutely dissociated and week old neurons in the larval CNS culture.

There was no appreciable voltage-activated Na^+ currents recorded from the soma of acutely dissociated larval CNS neurons. The same result has also been observed in week old cultured larval neurons (Solc and Aldrich, 1988). In contrast, inward Na^+ currents have been recorded in *Drosophila* embryonic and pupal neurons in culture (O'Dowd et al., 1989; Baker and Salkoff, 1990; Saito and Wu, 1991).

Heterogeneity of K^+ currents in mushroom body neurons

As indicated in Figures 4–7, the current density, inactivation kinetics and voltage dependency of steady-state inactivation of K^+ currents in identified mushroom body neurons exhibit a large range of variation. The variation is similar to that seen in recordings randomly obtained from the whole CNS population. This heterogeneity of K^+ currents has been observed in larval (see Solc and Aldrich, 1988), embryonic, and pupal CNS neurons (Baker and Salkoff, 1990; Saito and Wu, 1991). Up to now, six genes encoding distinct K^+ channel subunits have been cloned (Kamb et al., 1988; Pongs et al., 1988; Schwarz et al., 1988; Atkinson et al., 1991; Covarrubias et al., 1991; Warmke et al., 1991). In addition, differential splicing of these genes and possible heteromultimeric assembly of distinct subunits into functional channels (Isacoff et al., 1990; McCormack et al., 1990) provide enormous potential for the formation of a diversity of K^+ channels.

The complexity of K^+ channels expressed has made it difficult for quantitative analysis of mutational effects in the CNS. One of the reasons for establishing this preparation is to enable us study a more homogenous group of K^+ currents in this subpopulation of CNS neurons instead of the large variation observed. One potential approach for further im-

←

provement is to use enhancer–detector lines that express *lacZ* in an even more restricted subpopulation of neurons. However, the heterogeneity observed in identified mushroom body neurons may not simply reflect genetic diversity of K^+ channels expressed in these neurons. Our preliminary observation indicates that some neuropeptides can modulate a type 1 current into a type 2–like current (Wright and Zhong, unpublished observation). It raises an intriguing idea that type 1 and type 2 currents might be conducted by the same group of K^+ channels, but that these channels are modulated to a different extent in type 1 and type 2 neurons. This idea can be further pursued by genetic analysis of K^+ channel subunits involved in type 1 and type 2 currents.

Although the kinetics and the voltage dependency of inactivation exhibit a range of variation within each type of K^+ currents, both type 1 and type 2 currents subject to modulation by cAMP (see Fig. 9) or several neuropeptides (Wright and Zhong, unpublished observation) in a fairly consistent manner. All type 1 currents show a strongly cAMP-dependent modulation while only the transient component is slightly inhibited by cAMP in all type 2 currents. Thus, this preparation provides an opportunity for the genetic and pharmacological analyses of mechanisms underlying modulation of membrane currents in mushroom body neurons in spite of the variation observed in each type of K^+ currents.

Modulation of K^+ currents in mushroom body neurons

We demonstrated that the amplitude of type 1 and type 2 K^+ currents in identified neurons could be modulated by cAMP. It has been reported that the amplitude of a voltage-activated transient A-current and the open probability of a voltage-independent K^+ channel in *Drosophila* larval muscle are increased by cAMP (Delgado et al., 1991; Zhong and Wu, 1993). In *Aplysia* sensory neurons, an S-type K^+ current is closed by cAMP-dependent phosphorylation (Kandel and Schwartz, 1982). In addition, it is known that cAMP may modulate K^+ channels by either directly binding to channels or by cAMP-dependent phosphorylation (Levitan, 1988). A voltage independent K^+ channel found in larval muscle is reported to be gated by direct cAMP binding (Delgado et al., 1991). It remains to be determined what mechanism is used in the modulation of type 1 and type 2 currents in mushroom body neurons.

Mutants *dnc* and *rut* have been isolated based on their poor performance in associative learning (for review see Dudai, 1988). The mutant larvae also show impaired learning and the *dnc* gene has also been shown to be expressed at a high level in the larval mushroom body (for review, see Davis, 1993). However, the cellular function of this cAMP cascade or its targets has not been identified in mushroom body neurons. Modulation of a K^+ current mediated by the cAMP cascade in *Aplysia* has been suggested as a key mechanism underlying synaptic facilitation which is considered as a cellular process involved in learning and memory (Kandel and Schwartz, 1982). Since K^+ currents in identified mushroom body neurons can be modulated by cAMP, it would be of great interest to investigate whether and how such modulation is altered in these mutants.

References

Atkinson NS, Robertson GA, Ganetzky B (1991) A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. Science 253:551–554.

Baker K, Salkoff L (1990) The *Drosophila Shaker* gene codes for a distinctive K^+ current in a subset of neurons. Neuron 2:129–140.

Bellen HJ, O'Kane CJ, Wilson C, Grossniklaus U, Pearson RK, Gehring W (1989) P-Element mediated enhancer detection: a versatile method to study development in *Drosophila*. Genes Dev 3:1288–1300.

Benzer S (1973) Genetic dissection of Behavior. Sci Am 229:24–37.

Byerly L, Leung H-T (1988) Ionic currents of *Drosophila* neurons in embryonic cultures. J Neurosci 8:4379–4393.

Covarrubias AM, Wei A, Salkoff L (1991) *Shaker, Shal, Shab* and *Shaw* express independent K^+ current systems. Neuron 7:763–773.

Davis R (1993) Mushroom bodies and *Drosophila* learning. Neuron 11:1–14.

de Belle JS, Heisenberg M (1994) Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. Science 263:692–695.

Delgado R, Hidalgo P, Diaz F, Latorre R, Labarca P (1991) A cyclic AMP-activated K^+ channel in *Drosophila* larval muscle is persistently activated in dunce. Proc Natl Acad Sci USA 88:557–560.

Dudai Y (1988) Neurogenetic dissection of learning and short-term memory in *Drosophila*. Annu Rev Neurosci 11:537–563.

Fiering SN, Roederer M, Nolan GP, Micklem DR, Parks DR, Herzenberg LA (1991) Improved FACS-Gal: flow cytometric analysis and sorting of viable eukaryotic cells expressing reporter gene constructs. Cytometry 12:291–301.

Hall JC (1982) Genetics of the nervous system in *Drosophila*. Q Rev Biophys 15:223–497.

Heisenberg M, Borst A, Wagner S, Byers D (1985) *Drosophila* mushroom body mutants are deficient in olfactory learning. J Neurogenet 2:1–30.

Isacoff EY, Jan Y-N, Jan LY (1990) Evidence for the formation of heteromultimeric potassium channels in *Xenopus* oocytes. Nature 345:530–534.

Kamb A, Tseng-Crank J, Tanouye MA (1988) Multiple products of the *Drosophila Shaker* gene contribute to potassium channel diversity. Neuron 1:421–430.

Kandel ER, Schwartz JH (1982) Molecular biology of learning: modulation of transmitter release. Science 218:433–443.

Kim Y-T, Wu C-F (1987) Reversible blockage of neurite development and growth cone formation in neuronal cultures of a temperature-sensitive mutant of *Drosophila*. J Neurosci 7:3245–3255.

Krasnow MA, Cumberledge S, Manning G, Herzenberg LA, Nolan GP (1991) Whole animal cell sorting of *Drosophila* embryos. Science 25:81–85.

Levin IB (1988) Modulation of ion channels in neurons and other cells. Annu Rev Neurosci 11:119–136.

McCormack K, Lin JW, Ramaswami M, Tanouye M, Iverson L, Rudy B (1990) Heteromultimer formation can produce a large number of distinct K channels. Biophys J 57:209a.

O'Dowd DK, Aldrich RW (1988) Voltage-clamp analysis of sodium channels in wild-type and mutant *Drosophila* neurons. J Neurosci 8:3633–3643.

O'Dowd DK, Germeraad SE, Aldrich RW (1989) Alterations in the expression and gating of *Drosophila* sodium channels by mutations in the *para* gene. Neuron 2:1301–1311.

Pongs O, Kecskemethy N, Muller R, Krah-Jentgens I, Baumann A, Kiltz HH, Canal I, Llamazares S, Farrus A (1988) *Shaker* encodes a family of putative potassium channel proteins in the nervous system of *Drosophila*. EMBO J 7:1087–1096.

Saito M, Wu C-F (1991) Expression of ion channels and mutational effects in giant *Drosophila* neurons differentiated from cell division-arrested embryonic neuroblasts. J Neurosci 11:2135–2150.

Schwarz TL, Tempel BL, Papazian DM, Jan Y-N, Jan LY (1988) Multiple potassium components are produced by alternative splicing at the *Shaker* locus in *Drosophila*. Nature 331:137–142.

Siegelbaum SA, Tsien RW (1983) Modulation of gated ion channels as a mode of transmitter action. Trends Neurosci 6:307–313.

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 analysis reveal two distinct A-type potassium channels in *Drosophila*. Science 236:1094–1098.

Sun Y-A, Wu C-F (1985) Genetic alterations of single channel potassium currents in dissociated central nervous system neurons of *Drosophila*. J Gen Physiol 86:16a.

Technau GM, Heisenberg M (1982) Neural reorganization during metamorphosis of the corpora pedunculata in *Drosophila* melanogaster. Nature 295:404–407.

Warmke J, Drysdale R, Ganetzky B (1991) A distinct potassium channel polypeptide encoded by the *Drosophila eag* locus. Science 252:1560–1562.

Wilson C, Pearson RK, Bellen HK, O'Kane CJ, Grossniklaus U, Gehring WJ (1989) P-Element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. Genes Dev 3:1301–1313.

Wu C-F, Suzuki N, Poo M-M (1983) Dissociated neurons from normal and mutant *Drosophila* larval central nervous system in cell culture. J Neurosci 3:1888–1899.

Zhong Y, Wu C-F (1991) Altered synaptic plasticity in *Drosophila* memory mutants with defective cAMP cascade. Science 251:198–201.

Zhong Y, Wu C-F (1993) Differential modulation of potassium currents by cAMP and its long-term and short-term effects: dunce and rutabaga mutants of *Drosophila*. J Neurogenet 9:15–27.