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A novel mechanical dissociation technique for studying acutely isolated maturing *Drosophila* central neurons

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Abstract

A novel mechanical method, for studying acutely isolated maturing *Drosophila* central neurons, has been developed. Electrophysiological experiments have been carried out to assess the quality of these acutely dissociated neurons. The mechanically dissociated *Drosophila* central neurons were divided into three categories depending on their size and morphological features. Four types of whole-cell K^+ currents were identified in these neurons, based on their kinetic properties. Moreover, the K^+ currents in the new preparation were found to have similar electrophysiological and pharmacological properties to those reported in the cultured neurons. The new technique, however, was more rapid and convenient. Furthermore, this new system was successfully applied to the isolation of neurons from the adult *Drosophila*, a process that is extremely difficult by routinely used methods. Thus, this new preparation would be very reliable and applicable to preparing *Drosophila* central neurons for biophysical and physiological studies.

Keywords: *Drosophila*; Maturing neurons; Mechanical dissociation; Whole-cell recording; K^+ currents

1. Introduction

Drosophila have been successfully used for studying many cellular processes in the central nervous system (CNS). To facilitate the electrophysiological studies of *Drosophila* CNS neurons, primary embryonic cultures, prepared from the whole *Drosophila* gastrulae, have often been used (Petersen et al., 1977; Sakai et al., 1989). The whole embryo, however, is highly heterogeneous, and embryo cells differentiate into form several cell types in addition to neurons in vitro (Salvaterra et al., 1987). Although this may interfere with electrophysiological experiments in *Drosophila* neurons, cultures of embryonic *Drosophila* cells have provided an unique opportunity to examine directly the electrophysiological properties of, and synaptic transmission between, *Drosophila* central neurons (Lee and O'Dowd, 2000). Wu et al. (1983) successfully dissociated and cultured neurons from *Drosophila* larval CNS. This cell culture system avoids the interference of other cells and renders easy access to the analysis of normal or mutant neuronal properties. Several years later, they succeeded in differentiating the giant *Drosophila* neurons from cytokinesis-arrested embryonic neuroblast (Wu et al., 1990). With their larger sizes (>10 μm), the giant *Drosophila* neurons become a more accessible model system for the study of a variety of basic physiological and cell biological processes, which were normally limited by the small cell size of *Drosophila* neurons (Satio and Wu, 1991; Zhao et al., 1995; Zhao and Wu, 1997; Yao and Wu, 1999). All these methods are still used to date in the electrophysiological studies of *Drosophila* neurons (Alshuaib and Byerly, 1996; Delgado et al., 1998; Yao et al., 2000). Several other preparations have also been used, such as intact embryonic nerve cord preparation (Braines and Bate, 1998) and *Drosophila* giant neurons-*Xenopus* myocytes co-culture system (Yao et al., 2000). However, since neuronal differentiation (such as arborization and neurotransmitter production) occurs during culture, the properties of these neurons might be changed compared with those of in vivo (Huff et al., 1989; Wu et al., 1990). Moreover,

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enzymatic treatments usually have negative effects on the proteins which have a portion protruding of the external side of the cell membrane (Lee et al., 1977).

In this study, we developed a novel mechanical dissociation technique that yielded a large number of acutely isolated *Drosophila* larval CNS neurons that retained their electrophysiological and pharmacological properties. Compared with the previous enzymatic dissociation and subsequent culturing methods, this procedure was more rapid and convenient. This new method made the preparation of small *Drosophila* neurons much easier and would be very reliable and applicable to preparing *Drosophila* central neurons for biophysical and physiological studies. Thus, the new system would further facilitate the exploration of the gene functions of the CNS of normal flies and *Drosophila* mutants. Furthermore, the new system was successfully extended to obtain acutely dissociated adult *Drosophila* central neurons that are otherwise difficult to obtain.

2. Materials and methods

2.1. Fly stocks

Stocks of *Drosophila melanogaster* flies were maintained on standard media at room temperature (22–25 °C). Professor Chun-Fang Wu (Department of Biological Sciences, University of Iowa, Iowa, USA) kindly supplied the Canton-S and Oregon-R wild type strains.

2.2. Dissociation of neurons

Several third-instar larva of *Drosophila* were collected on a slide. After being rinsed three times with distilled water, they were dissected with a pair of sharp needles in Ca^{2+} and Mg^{2+} free *Drosophila* saline (800 mg of NaCl, 20 mg of KCl, 5 mg of NaH_2PO_4, 100 mg of $NaHCO_3$ and 100 mg of glucose in 100 ml of distilled water) as described previously (Rizzino and Blumenthal, 1978). Antenna discs, eye discs and ring gland which adhered to the two brain hemispheres were carefully removed to keep the larval CNS intact. For acute dissociation of the adult *Drosophila* central neurons, 2–4 days old adult *Drosophila* were decapitated under CO_2 anaesthesia and their heads were dissected to expose the brains. After the gnathite and the two compound eyes were removed, the protocerebrum (brain) (Fig. 5Aa) of the adult *Drosophila* was picked out carefully with a needle in Ca^{2+} and Mg^{2+} free *Drosophila* saline. Thereafter, the dissected *Drosophila* larval or adult's CNS was transferred into a culture dish (NUNC™ Brand Products, Denmark) which contained 3 ml of recording solution (to be described).

To mount the *Drosophila* CNS onto the bottom of the culture dish, a U-shape grid made of metal filament and nylon fibers was used. To make the U-shape grid, a segment of metal filament (1 mm in diameter) was curved into a 'U' shape. Five to 10 Nylon fibers, glued in parallel (approximately 100–200 μm apart from each other), were arranged on the U-shape metal filament forming a grid (Fig. 1). Mechanical dissociation was carried out with the apparatus illustrated in Fig. 1. The vibrator with an attached dissecting pipette was fixed on a manipulator and the dish with mounted tissue was placed on the stage of the dissecting microscope (XTL-II, Tech Instrument Co. Ltd, Beijing, China). The pipette was pulled from a glass capillary with an outer diameter in 1.5 mm and an inner diameter in 0.9 mm on a two-stage puller (PP-830, Narishige, Tokyo, Japan). The tip of the pipette was fire-polished and sealed. The tip of the dissecting pipette (about 6 μm in diameter) was lightly placed on the surface of the larval or adult's *Drosophila* CNS with the manipulator. A continuous (5–10 Hz) electrical square pulse was applied to the vibrator using a pulse generator (EE 1641B, Nanjing, China), producing a series of horizontal movements of about 100 μm of the dissecting glass pipette. The vibration-dissociation lasted for about 3 min. Under visual control, many small dissociated fragments could be seen clearly. The acutely isolated neurons attached to the bottom of the culture dish within 10 min.

2.3. Electrophysiology

Whole-cell recordings were carried out under voltage-clamp conditions at room temperature (22–25 °C) to assess the quality of the neurons. Patch pipettes were pulled from glass capillaries with an outer diameter of

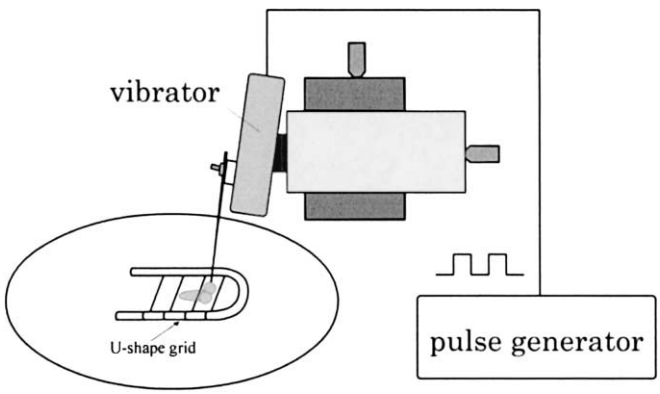

Fig. 1. Schematic diagram of the apparatus used for the mechanical dissociation of *Drosophila* CNS neurons (not drawn to scale). The *Drosophila* CNS was mounted under a U-shape grid. A continuous (5–10 Hz) electric square pulse was applied to the vibrator using a pulse generator, producing a series of fast horizontal movements of about 100 μm of the dissecting glass pipette.

1.5 mm (Narishige, Tokyo, Japan) on a two-stage puller (PP-830, Narishige, Tokyo, Japan). The resistance between the recording electrode, filled with the pipette solution, and the reference electrode was 10–12 MΩ. The liquid junction potentials were 3–4 mV, and they were used to calibrate the holding potential (V_H). Data were collected with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) which was connected to a Pentium III computer equipped with Digidata 1320A and Clampex and Clampfit software (version 8.0.1, Axon Instruments, Foster City, CA) for data acquisition and analysis. The series resistance, estimated from optical cancellation of the capacity transient, was 10–30 MΩ. In most experiments, 70–90% series resistance compensation was applied. The currents were sampled at 2 kHz and filtered at 1 kHz (4-pole Bessel filter). Unless otherwise noted, the membrane potential was held at −70 mV in the voltage-clamp studies.

2.4. Solutions

The ionic composition of the internal solution medium was (mM): 140 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA and 5 HEPES with pH adjusted to 7.2 with Tris–base. Stocks of adenosine triphosphate (stored at −20 °C) were dissolved in the intracellular solution shortly before use to a final concentration of 2 mM. The recording solution contained (mM): 130 NaCl, 6 KCl, 2.5 $CaCl_2$, 5 $MgCl_2$, 5 HEPES, 10 glucose. 3×10^{-7} M tetrodotoxin (TTX) and 2×10^{-4} M $CdCl_2$ were added into the recording solution to block voltage-gated Na^+ and Ca^{2+} channels, respectively. The pH of the recording solution was adjusted to 7.4 with Tris–base.

2.5. Drugs application

All drugs used throughout the experiment were from Sigma. 4-amino-pyridine (4-AP) and triethyl ammonium (TEA^+) were dissolved in the recording solution at concentrations of 5 and 10 mM, respectively. And they were freshly prepared each day. Application of the drugs was performed via the 'Y-tube' method as described elsewhere (Xu et al., 2000). The tip of the drug tube was positioned 50–100 μm away from the patched neurons. This system allows a complete exchange of external solution surrounding a neuron within 20 ms (Xu et al., 2000).

3. Results

3.1. Morphology of mechanically dissociated neurons of larval CNS

The present vibration-isolation system produced acutely isolated larval CNS neurons with sufficient numbers of cells to satisfy the needs of typical patch clamp experiments. These mechanically dissociated neurons survived over 10 h at room temperature (22–25 °C) even in the recording solution without any culture medium. The acutely isolated larval CNS neurons could be divided into three categories according to their morphology and size, which were similar to those reported in the cultured system (Wu et al., 1983). Fig. 2 shows the typical types of the acutely isolated larval CNS neurons. Type I consisted of large cells (diameter >8 μm) which comprised approximately 15% of the total population (31 of 200 neurons). Nearly all of these cells had no processes, and most were accompained by some smaller cells (Fig. 2A), as previously reported (Wu et al., 1983). Some of the type I cells were changing their morphology constantly. They were neuroblast that underwent asymmetric divisions (White and Kankel, 1978). Type II cells comprised approximately 75% of the total population (147 of 200 neurons). They were very small (3–4 μm in diameter) and some of them had long processes (Fig. 2B). The type III cells

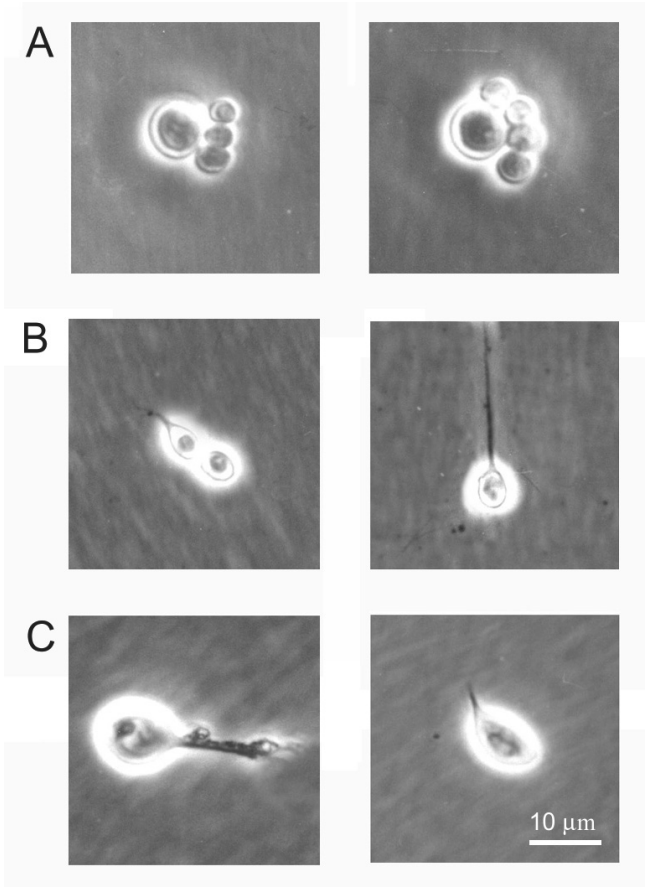

Fig. 2. Neurons mechanically dissociated from larval CNS could be divided into three categories. (A) Type I consisted of large cells and most of them were accompanied with smaller cells; (B) Type II cells were smallest and some of them had short or long processes; (C) Type III cells were of the intermediate size and had characteristic oval- or spindle-shaped cell body. All images were taken 1 h after dissociation.

had the intermediate size (4–10 μm in diameter) which comprised about 10% of the total population (22 of 200 neurons). They had characteristic oval or spindle-shaped cell body and some of them exhibited short and thick processes (Fig. 2C). Glia cells were not considered in the above categories because of their much lesser distribution in insect CNS (Wu et al., 1983).

3.2. Whole-cell K^+ currents in mechanically dissociated larval CNS neurons

In the present experiment, the acutely isolated larval type I cells were often undergoing asymmetric divisions after dissociation (White and Kankel, 1978), making the gigaohm seal rather difficult. Therefore, the type II and III neurons, which comprised the largest population of the acutely dissociated cells, were selected for the study of membrane currents.

Consistent with the previous reports in the cultured neurons (Delgado et al., 1998; Yu et al., 1999), the majority of the larval CNS neurons studied in the present experiment exhibited only net outward currents in response to depolarizing step pulses even in the absence of TTX and $CdCl_2$. When the K^+ in recording solution was replaced with TEA^+ and the K^+ in pipette solution was replaced with Cs^+, the outward currents disappeared completely (data not shown). This result showed that the outward currents were carried by K^+.

Heterogeneity of whole-cell K^+ currents in peak amplitude and inactivation time constants had been reported in the cultured *Drosophila* neurons (Delgado et al., 1998; Solc and Aldrich, 1988). Based on the kinetic properties, the whole-cell K^+ currents in the present neurons were also heterogeneous. Analysis of the decay phase of the currents allowed the classification into four phenotypes (Fig. 3). Some neurons (14/50) exhibited primarily fast transient K^+ currents (Fig. 3A) while the others (22/50) showed slower transient K^+ currents (Fig. 3B). A minority of neurons (4/50) were dominated by a sustained current (Fig. 3C). The rest of the neurons (10/50), however, expressed both the transient and slow inactivating components (Fig. 3D). The inactivation time coflnstants (τ) of the currents could be fitted with one component exponential (currents in Fig. 3B and C) or two components exponential (currents in Fig. 3A and D). The averaged τ of the four current phenotypes at $+30$ mV is shown in Fig. 3E. There is no obvious correlation between cell morphology and current phenotypes.

3.3. Pharmacological separation of the K^+ currents in larval CNS neurons

To separate the whole-cell K^+ currents in larval CNS neurons, electrophysiological and pharmacological methods were used (Singh and Wu, 1989; Tsunoda and Salkoff, 1995). Fig. 4 shows that in the neurons exhibiting both transient and sustained components, the transient component was completely inactivated when the prepulse was changed to -40 mV. A sustained, non-inactivating component was then revealed. The transient component had kinetics very similar to the I_A in previous reports (Salkoff, 1983; Byerly and Leung, 1988; Baker and Salkoff, 1990). The sustained component isolated by the prepulse method has usually been called I_K and it did not inactivate for the duration of the pulse (Salkoff, 1983; Tsunoda and Salkoff, 1995). The existence of these two components was further supported by pharmacological studies. When 5 mM 4-AP, a drug known to block transient K^+ currents (Solc and Aldrich, 1988), was applied to the patched neurons, the transient current was completely abolished (I_A, Fig. 4B). The 4-AP-insensitive component was comparable to the sustained component retained in response to the -40 mV prepulse inactivation protocol described above (I_K). On the other hand, 10 mM TEA^+, which is another K^+ channel blocker (Solc and Aldrich, 1988), blocked the I_K and had little effect on I_A (Fig. 4C). The peak currents were decreased in all cells treated with 5 mM 4-AP or 10 mM TEA^+, but the reduction varied among cells. These results suggest that several different channel types may underlie the whole-cell K^+ currents in the *Drosophila* larval CNS neurons (Solc and Aldrich, 1988; Zhao et al., 1995; Martinez-Padron and Ferrus, 1997; Zhao and Wu, 1997).

3.4. Whole-cell K^+ currents in neurons of adult Drosophila brains

Next, an attempt was made to extend the vibration-isolation system to obtain acutely dissociated neurons from the adult *Drosophila* brains, which proved difficult to acquire by routinely used enzymatic methods. It was found that the procedure described above was applicable to isolating neurons from the adult *Drosophila* brains. Fig. 5Aa shows the whole mount of a dissected adult *Drosophila* brain and the neurons acutely isolated from it by the present system. Most of the acutely dissociated neurons from the adult *Drosophila* brain were small (3–6 μm in diameter) and resembled the type II neurons in the larval CNS. Whole-cell voltage-clamp recordings from the acutely isolated adult neurons were performed and four phenotypes of outward currents could also be classified according to their kinetic characteristics (Fig. 5). The majority of the neurons (59 of 80 cells) had not only the inactivating currents but also a sustained component (Fig. 5Ba). Some neurons (12 of 80 cells) had K^+ currents with a slow inactivation time course (Fig. 5Bb). A small number of cells (four of 80 cells) exhibited primarily a fast inactivating K^+ current that turned on and inactivated

rapidly in response to depolarizing voltage steps (Fig. 5Bc). Few cells (five of over 80 cells) contained primarily the sustained component (Fig. 5Bd).

4. Discussion

In the present study, we established a novel mechanical method to isolate maturing *Drosophila* CNS neurons acutely. We have previously used this technique to successfully dissociate neurons from the rat sacral dorsal commissural nucleus (SDCN), even acquiring 'synaptic bouton preparation' in which spontaneous synaptic currents were recorded (Xu, 1999). Since there are much fewer synaptic connections in invertebrates than in vertebrates (Canal et al., 1994), we did not observe any spontaneous synaptic currents in the present preparation. Compared with the traditional cul-

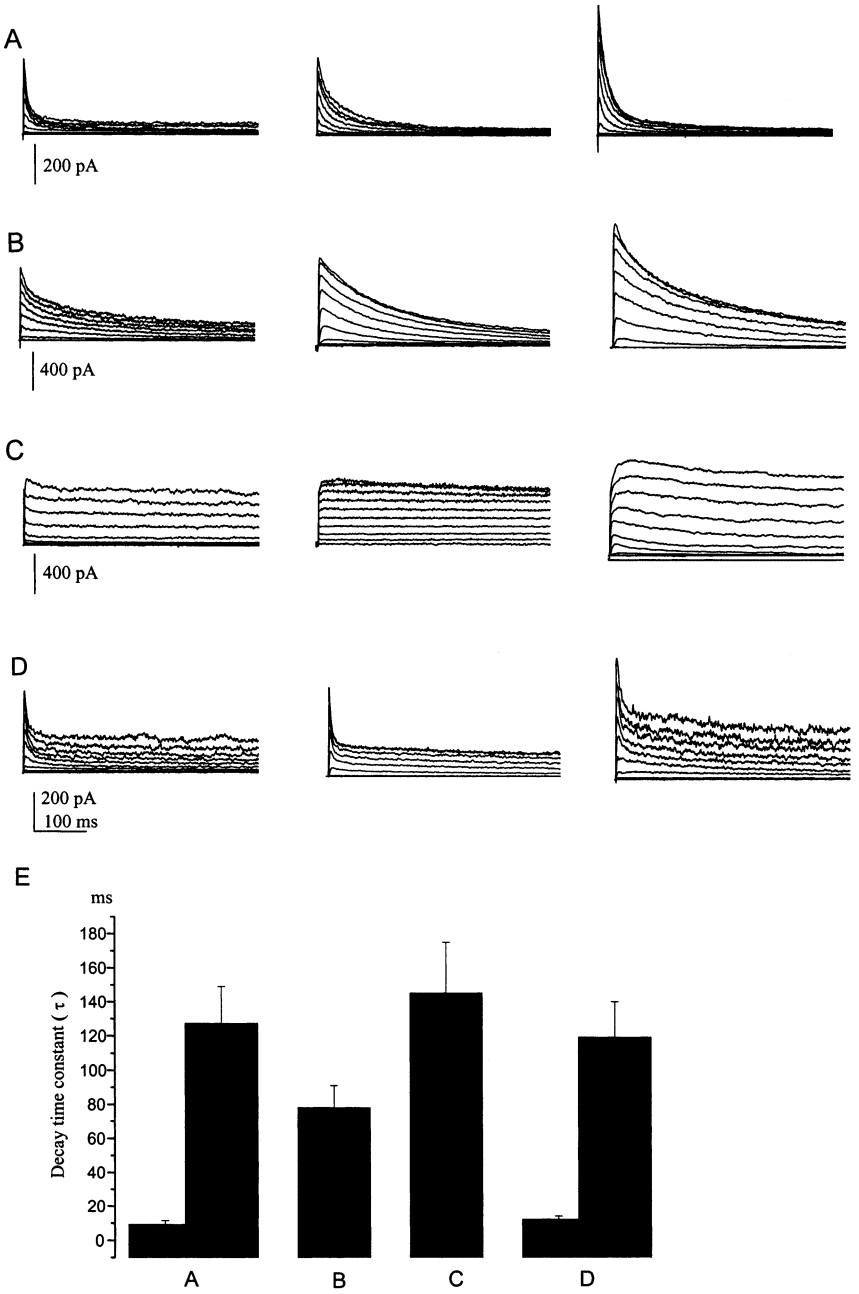

Fig. 3. K^+ current phenotypes in the larval CNS neurons. Holding potential was -70 mV. After hyperpolarizing to -90 mV for 200 ms, outward currents were elicited by 20 mV depolarizing steps for 500 ms, from -90 to 50 mV. Whole-cell currents exhibited primarily the fast transient K^+ component (A); the slower transient K^+ component (B); the sustained component (C) and the combination of the transient and sustained component (D). (E) The averaged decay time constants (τ) of the four currents phenotypes at $+30$ mV ($n = 10$ for each type except that $n = 4$ in C).

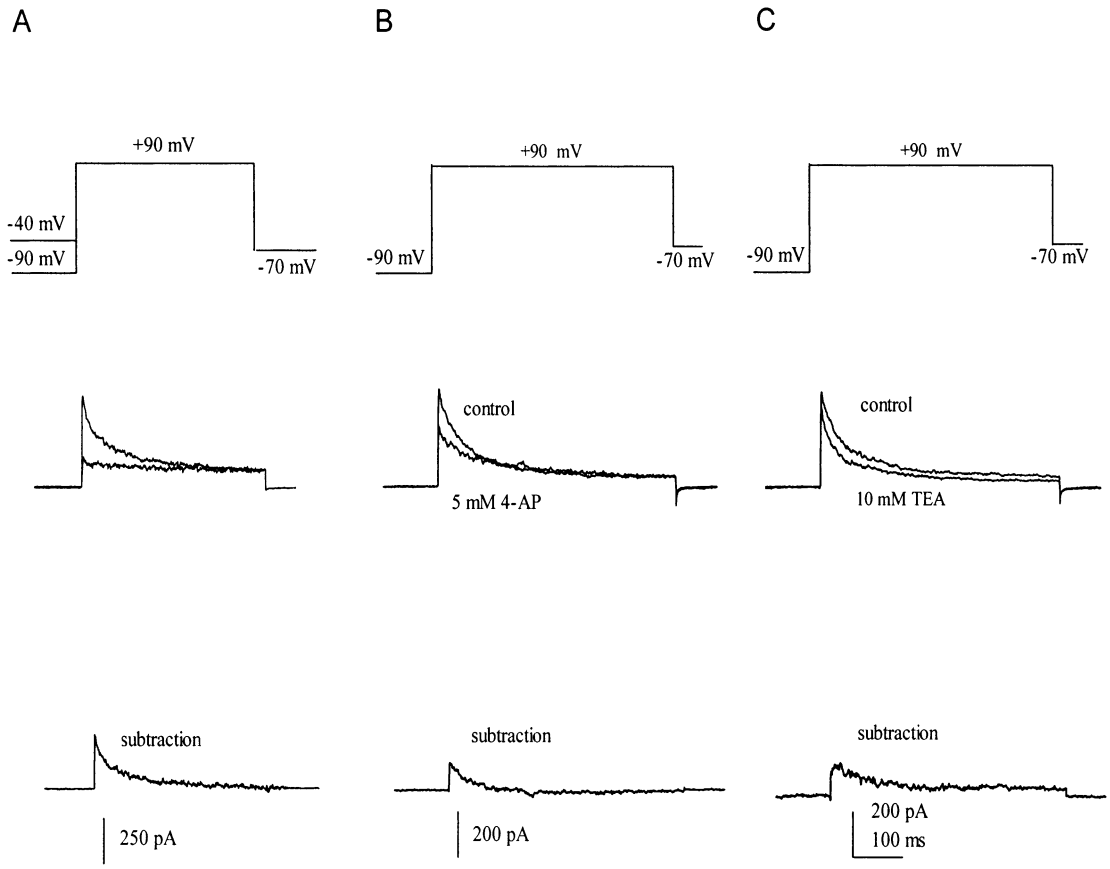

Fig. 4. Separation of I_A and I_K by prepulse protocol (A) and pharmacological tools (B, C).

ture methods or the previously reported mechanical methods (Kay and Wong, 1986), the new technique was not only more rapid and convenient, but also excluded any enzyme treatment. Since the patch clamp recording could be taken as soon as the neurons were dissociated, the sterilization and culturing were not needed. Furthermore, poly-lysine coating was not necessary as the neurons settled down well in the culture dish, and the density of the cells could be easily manipulated. Because the neurons had short (or even no) processes, the space clamp problems for voltage clamp studies could also be avoided.

The morphology and whole-cell K^+ current phenotypes were comparable to those observed in cultured *Drosophila* CNS neurons (Solc and Aldrich, 1988; Wright and Zhong, 1995; Delgado et al., 1998; Yu et al., 1999). The mechanically dissociated larval cells were divided into three categories by their size and morphological characteristics (Fig. 2). The most prevalent type II and III neurons were selected for patch clamp recording. Four types of whole-cell K^+ currents were identified in these neurons based on the kinetic properties (Fig. 3). Furthermore, the K^+ currents in the present preparation had electrophysiological and pharmacological properties (sensitivities to depolarizing prepulse and K^+ channel blockers) similar to those reported in cultured neurons (Solc and Aldrich, 1988; Wright and Zhong, 1995; Delgado et al., 1998; Yu et al., 1999). More importantly, the new method was successfully applied to the dissociation of adult *Drosophila* CNS neurons. To our knowledge, the electrophysiological characteristics of adult *Drosophila* neurons have never been reported before, due to the technical difficulties in obtaining adult neurons. Thus, the isolation of adult *Drosophila* neurons might provide a new area in studying the physiological properties of adult *Drosophila* CNS neurons. In addition, the studies of adult *Drosophila* neurons will compliment the results from embryonic neurons (Sakai et al., 1989; Lee and O'Dowd, 2000), larval neurons (Wright and Zhong, 1995; Delgado et al., 1998) as well as pupal neurons (Baker and Salkoff, 1990) in accomplishing a full understanding of the development of ion channels in the *Drosophila* CNS.

As all the genes in *Drosophila* have been recently identified (Adams et al., 2000), our method provides an opportunity to combine genetic, molecular and electro-

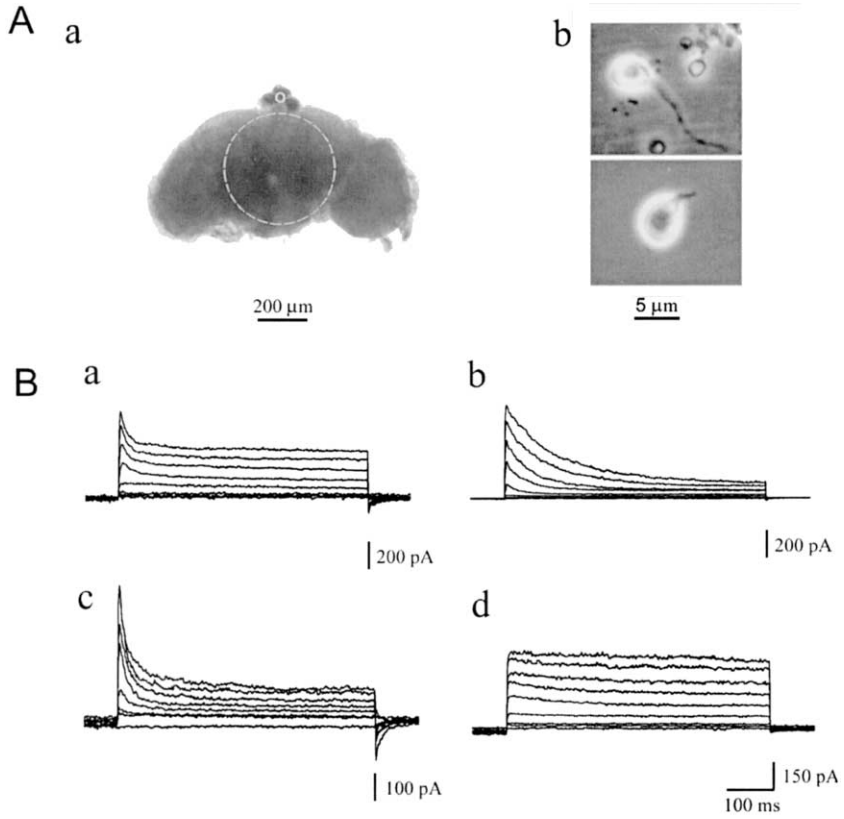

Fig. 5. K^+ current phenotypes in the adult *Drosophila* CNS neurons A. The protocere-brum (brain) dissected from an adult *Drosophila* (a) and the neurons acutely isolated from the adult *Drosophila* brains (b). The dotted circle in Aa indicates the part of the *Drosophila* CNS under vibration. O, ocelli. B. Representative current traces shown: (a) the combination of the transient and sustained current; (b) the slow transient current; (c) the fast transient current; (d) the sustained current. The test pulses were the same as in Fig. 3.

physiological data to study for example the mechanisms of learning and memory (Davis, 1996; Alshuaib and Mathew, 1998; Lee and O'Dowd, 2000), anesthetics (Nishikawa and Kidoworo, 1999), and synapse formation. The present vibration-isolation system will add an important means to the exploration of the gene functions of the CNS of normal flies and *Drosophila* mutants. Although this vibration-isolation method is not a cell culture system, and therefore is not suitable for long-term developmental and cell biological observations, it is useful for the isolation of both larval and adult neurons for acute electrophysilogical recordings.

Acknowledgements

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