Reference: *Biol. Bull.* **216:** 1–6. (February 2009)

Oral Administration of Pharmacologically Active Substances to Squid: A Methodological Description

WILLIAM BERK[1], JAKE TEPERMAN[1], KERRY D. WALTON[1,2], KAZUNARI HIRATA[2], MUTSUYUKI SUGIMORI[1,2], AND RODOLFO R. LLINAS[1,2,*]

[1]*Marine Biological Laboratory, 7 MBL Street, Woods Hole, Massachusetts 02543;* [2]*Department of Physiology and Neuroscience, NYU School of Medicine, New York, New York 10016*

Abstract. The squid giant synapse is a well-defined experimental preparation for the study of ligand-dependant synaptic transmission. Its large size gives direct experimental access to both presynaptic and postsynaptic junctional elements, allowing direct optical, biophysical, and electrophysiological analysis of depolarization-release coupling. However, this important model has not been utilized in pharmacological studies, other than those implementable acutely in the *in vitro* condition. A method is presented for oral administration of bioactive substances to living squid. Electrophysiological characterization and direct determination of drug absorption into the nervous system demonstrate the administration method described here to be appropriate for pharmacological research.

Introduction

The squid *Loligo pealei* LeSueur, 1821 has long been a remarkable experimental model for exploration of fundamental questions in neuroscience. Many aspects of the action potential, synaptic transmission, and fast axonal transport have been worked out in detail in the squid (Young, 1939; Bulloch, 1948; Bloedel *et al.,* 1966; Katz and Miledi, 1967; Kusano *et al.,* 1967; Llinas *et al.,* 1981; Augustine *et al.,* 1985; Serulle *et al.,* 2007). The scale of the axon and the synaptic structures allows unparalleled access for experiments. However, the synaptic and axonal experiments in the squid have historically been limited to acute preparations, precluding use of experimental conditions that require prolonged exposure. Here we describe a method for administering water-soluble drugs and reagents parenterally to small squid suitable for use in synaptic physiology experiments. Protocols for administering a drug by intubation and absorption through the squid digestive system are described in some detail, and alternative approaches are considered. The method is suitable for treatments administered 1–2 days ahead of a challenge and was validated using the previously described acute 1-methyl-4-phenylpyridinium (MPP+) exposure paradigm prior to treatment with a neuroprotective agent. The level of the neuroprotective agent at the synapse at the time of electrophysiological analysis was determined and found to be in the effective range, and significant protective effect was documented.

The technique described here is part of a study concerning the neuroprotective properties that a compound (T-817) developed by Toyama Chemical Company, Japan, has against the toxic effect of intraxonal injection of MPP+, a substance known to produce synaptic transmission failure in squid (Serulle *et al.,* 2007).

Squid digestive system

The digestive system starts with a sharp parrot-like beak leading to an esophagus 8.6 cm long and 0.6 cm wide. Pharmaco-active substances to be administered are delivered directly into the muscular stomach *via* a cannula inserted down the esophagus, and are absorbed at the caecum level (see Fig. 1F).

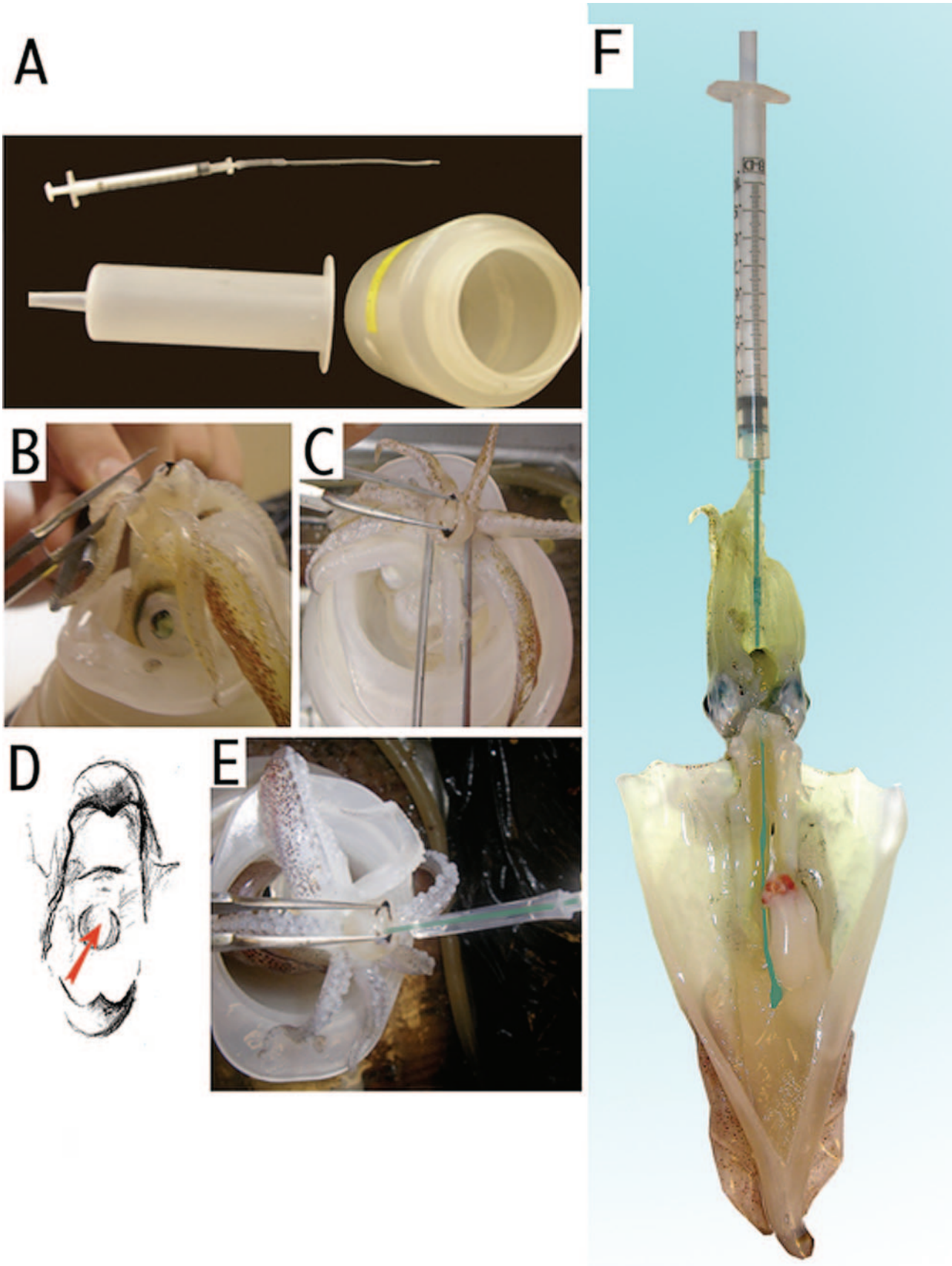
Anesthesia

Our initial attempts at oral administration of pharmaceuticals were performed while the animals were anesthetized or dormant. In the first case, 0.75 mmol l^{-1} of MS-222

Received 24 July 2008; accepted 26 September 2008.

* To whom correspondence should be addressed. E-mail: rodolfo.llinas@med.nyu.edu

Abbreviations: HPLC, high performance liquid chromatography; MPP+, 1-methyl-4-phenylpyridinium.


A
B
C
D
E
F

(tricaine mesilate) was added to the seawater (Frazier and Narahashi, 1975). MS-222 is a local anesthetic that prevents the generation and conduction of action potentials by blocking sodium conductance, rendering the squid immobile (Frazier and Narahashi, 1975). This method was less than optimal because squid survival was low and in all cases the squid displayed ink-ejecting behavior, a sign of extreme distress (Andrews and Tansey, 1981). In addition, some animals were made dormant by immersing them in an ice bath (Andrews and Tansey, 1981).

Although MS-222 was the only anesthetic administered in this study, there are other general anesthetics that can be used in these cephalopods. A 2% solution of ethanol and a 2%–3% solution of urethane in seawater have been cited (Messenger *et al.,* 1985) as useful anesthetics. However these anesthetics do generate a pre-anesthetic excited condition that traumatizes the squid, resulting in violent behavior and inking similar to that seen with MS-222. After immersion in these solutions, the animals stop breathing but quickly begin again when placed in regular seawater (Andrews and Tansey, 1981). Ice baths (at temperatures from 3–5 °C), in combination with ethanol or urethane, can also be used. Under these conditions the animals do not react violently, but the temperature must be carefully regulated: if it dips to 2 °C the animals will die, and if it rises above 5 °C the ice bath becomes useless (Messenger *et al.,* 1985), making the procedure on the whole very cumbersome to control.

The most successful long-term anesthesia is that afforded by 0.596 mol l^{-1} $MgCl_2$ administered at temperatures between 13 and 22 °C (Messenger *et al.,* 1985). There is little struggling or inking, showing that there is little trauma to the squid, and animals kept under optimal temperature and aquarium conditions survive for up to 5 days.. This mixture may be adjusted to meet different needs: a 1:1 dilution works as a surgical anesthesic, and a 1:9 can be used for long-term sedation for shipping (Messenger *et al.,* 1985). Ultimately, however, we decided that the best strategy was to intubate the animal without anesthesia, as the method is less stressful and rapid enough to afford only minimum discomfort to the squid.

Materials and Methods

Animals

The squid used in these experiments, *Loligo pealei*, were captured off the coast of Woods Hole in June and July of 2008. Female squid of about 65 g were used. They were kept in a 17 °C, 70-gallon tank for about 2 days, during which they were given either one or two doses of the experimental drug. The seawater was kept at a pH near 7.2. After 2 days, the stellate ganglion was isolated and the synapse prepared for electrophysiological and ultrastructural analysis.

Restraint and intubation apparatus

A squid was first gently placed into a 60-ml syringe tube. This tube was placed in a plastic bottle to hold the squid vertically (Fig. 1A, B). With the squid held in this position, there was direct access to the beak and good visualization for cannula insertion.

The cannula was made from a Tygon tube (0.76-mm diameter) with a smooth, bulbous tip made by attaching a fine elastic tube segment to the end of the Tygon tube. The cannula was attached directly to a 1-ml syringe, affording simple and fast gastric administration of a measured dose of medication. Intubation was rapid, and the viability of the squid was as good as that of the non-intubated controls. Neither the restraint nor the intubation apparatus appeared to hurt the squid.

Administration of pharmacologically active substances

Drugs were administered to the squid using an eight-step procedure.

1. Prepare the stand as described above (Fig. 1A).
2. Place a squid in the stand by gently letting it slide through your hands into the syringe tube, making sure that the squid's siphon is facing away from you.
3. Hook the top beak with fine forceps and gently pull upward (Fig. 1B).
4. Hold the bottom of the throat bulb with blunt forceps, keeping the fine forceps hooked on the upper beak (Fig. 1C).
5. Slide the fine forceps between the beaks and hold the mouth open.
6. Carefully slide the intubation canula down the esophagus (located next to the lower beak, which should be nearest to you if the siphon is pointing away from you). Slide the tube until its end is touching the opening of the squid's esophagus (Fig. 1D). If an esophageal spasm occurs while the tube is being pushed, carefully raise the beak. If this does not work,

Figure 1. Squid intubation (A). Upper panel, intubation canula (top). Lower left syringe body serving as squid holder (left); plastic jar (right) serving as the syringe body holder. (B) Detail of top beak being hooked with fine tweezers. (C) Image of blunt tweezers holding the bottom of the throat bulb and fine tweezers keeping the beak open. (D) Red arrow points to esophagus. (E) Sliding tube down esophagus till the tube stop reaches the beginning of the esophagus. (F) Image of canula path down esophagus and methyl-blue-stained fluid in gastric cavity.

remove the canula and return the squid to the holding tank.

7. Slowly empty the syringe contents into the stomach, take out the canula, and place the squid back in the holding tank.
8. If the squid has trouble swimming, gently squeeze the mantle to remove any air that may have entered the mantle hood cavity (Fig. 1E).

Pharmaceuticals

One group of 10 squid was fed 0.25 ml of 3.68 mmol l^{-1} T-817. A second group of squid was fed twice (at a 24-h interval) 0.25 ml of 36.8 mmol l^{-1} T-817. T-817 is a neuroprotective compound developed by Toyama Chemical of Japan (Hirata *et al.,* 2005).

MPP^+ presynaptic microinjection and visualization

Presynaptic electrodes were filled with 0.5 mmol l^{-1} MPP^+ dissolved in an electrode solution containing potassium acetate (0.625 mol l^{-1}), HEPES, and rhodamin dextran. The last is a fluorescent tracer used to track the movement of the injected material along the terminal. MPP^+ was introduced into the terminal using pressure injections.

Diffusion of the injected fluid was monitored using a Videcon CCD camera attached to a high-gain microchannel plate-image intensifier, allowing the imaging of the intraterminal fluorescence. The images were captured and analyzed on a Hamamatsu C 1966 VIM image analysis system (Llinas *et al.,* 1991).

Drug absorption determination

Two paradigms were used. In the first, synaptic transmission was tested 24 h after a single oral administration of T-817 (3.68 mmol l^{-1}) to the squid. In the second, synaptic transmission was tested after two oral administrations of T-817 (36.8 mmol l^{-1}), the first at 23 h and the second at 1 h prior to synapse isolation. After the animals were sacrificed,

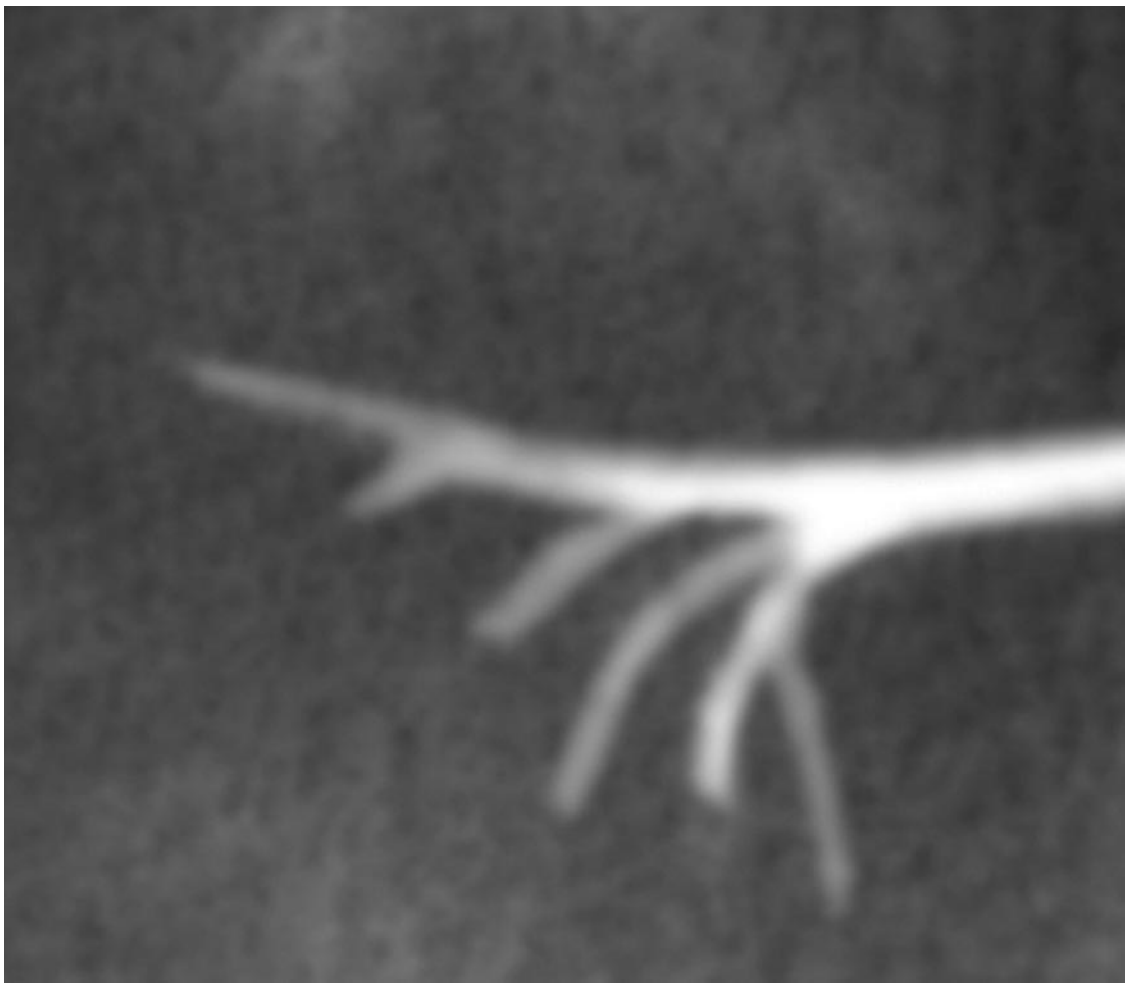


Figure 2. Image obtained 50 min after the microinjection of a mixture of MPP+, K acetate, HEPES, and rhodamin dextran into the presynaptic terminal.

the optic lobes were rapidly dissected from the animals and frozen at −70 °C. The procedure to determine drug concentration involved sonication (SEIKO Instruments, Chiba, Japan) and centrifugation to obtain a purified solid sample extracted according to the manufacturer's manual (Oasis HLB: Waters, Tokyo, Japan). The concentration of T-817 was measured at room temperature using HPLC.

Results

Mortality

Mortality rates were the same for intubated and non-intubated squid and were solely related to the initial condition of the squid.

Concentration of T817 in optic lobes

To test the absorption and final concentration of T-817 within the cephalopod nervous system, the optic lobes of control and fed squid were studied. In those squid fed one 3.68 mmol l^{-1} dose of T-817 and sacrificed 1 day later (first group), the final HPLC-determined concentration of T-817 was 0.21 μmol l^{-1} ($n = 13$), indicating that 1.49% of the compound was still present in the nervous system 1 day after administration of a low dose.

In the squid given two 36.8 mmol l^{-1} doses of T-817 24-h apart (second group), HPLC analysis indicated a final optic lobe concentration of 26.7 μmol l^{-1} ($n = 10$ squid). No T-817 was detected in 10 control squid.

MPP+ presynaptic microinjection

Intraterminal injection was performed after impalement at the presynaptic terminal with a sharp microelectrode. The total volume injected was determined to be 0.5 to 1 pL (Llinas *et al.,* 1991). A typical image of the presynaptic terminal 50 min after the initial injection is shown in Figure 2. The image indicates that the injected fluid had permeated the entire extent of the presynaptic terminal.

Electrophysiology

Simultaneous electrophysiological recordings were made from the presynaptic and postsynaptic terminals of the giant synapse, following our standard procedure (Llinas *et al.,* 1981). Injection of MPP+ into the terminal was monitored electrically as well as visually (Fig. 2) to assure terminal viability. Chemical transmission was tested using direct electrical stimulation of the preterminal axon with a bipolar electrode placed over the presynaptic axon bundle. This resulted in the generation of a presynaptic spike and a subsequent postsynaptic response. Under normal conditions, synaptic transmission will remain unchanged for 4 h or more. In these experiments, injection of MPP+, as previously reported (Serulle *et al.,* 2007), resulted in a rapid block of synaptic transmission (illustrated in Fig 3). After the oral administration of T-817 to the squid, the time for such a block was increased by a factor of 3, indicating that the drug had the expected neuroprotective effect (Fig. 3).

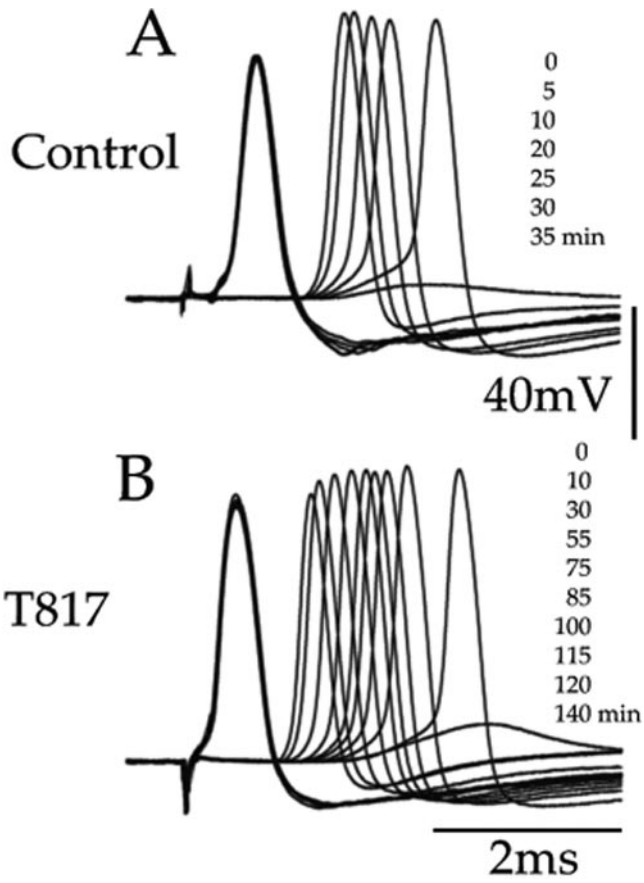


Figure 3. (a) The time course of chemical transmission failure in the "control" group. (b) The time course of synaptic transmission failure in squid having received two 36.8 mmol l^{-1} injections of T817 at a 24-h interval.

Discussion

The effect of MPP+ injected in the presynaptic terminal is illustrated in Figure 3A. Under such conditions, synaptic transmission lasts for about 30 min after injection, given pulse delivery once every 5 min. Synaptic transmission obtained from a squid fed T-817 before a MPP+ injection similar to that illustrated in Figure 3A is presented in Figure 3B. In these cases, transmission failure occurred 4 times more slowly than in the control experiment, indicating that oral feeding is successful as a drug delivery system. These results corroborate the drug concentration findings following chemical analysis of the brain tissue. The detailed electrophysiological and toxicological effects of T-817 will be published in a subsequent paper. The results presented in Figure 3 are only examples to show that the oral adminis-

tration method described here was effective in delivering the drug to the animals. Thus, this paper does not detail, nor is it a reference to, the actual effect of T-817.

In short, this simple technique affords new possibilities for the study of pharmacological medium-term effects on synaptic transmission in the squid giant synapse.

Acknowledgments

The studies were supported by National Institute of Health Grant NS13742 (to RLL and MS). We thank Ms. Sophie Cull-Candy for the drawing of the squid throat in Fig. 1D.

Literature Cited

Andrews, P. L. R., and E. M. Tansey. 1981. The effects of some anaesthetic agents in *Octopus vulgaris*. *Comp. Biochem. Physiol*. **70C:** 241–247.

Augustine, G. J., M. P. Charlton, and S. J. Smith. 1985. Calcium entry into voltage clamped presynaptic terminals of squid. *J. Physiol.* **367:** 149–162.

Bloedel, J. R., P. W. Q. Gage, T. Llinas, and D. M. J. Quastek. 1966. Transmitter release at the squid giant synapse in the presence of tetrodotoxin. *Nature* **212:** 49–50.

Bullock, T. H. 1948. Properties of a single synapse in the stellate ganglion of squid. *J. Neurophysiol.* **11:** 343–364.

Frazier, D. T., and T. Narahashi. 1975. Tricaine (MS-222): effects on ionic conductances of squid axon membranes. *Eur. J. Pharmacol*. **33:** 313–317.

Hirata, K., H. Yamaguchi, Y. Takamura, A. Takagi, T. Fukushima, N. Iwakami, A. Saitoh, M. Nakagawa, and T. Yamada. 2005. A novel neurotrophic agent, T-817MA [1-{3-[2-(1-benzothiophen-5-yl) ethoxy]propyl}-3-azetidinol maleate], attenuates amyloid β-induced neurotoxicity and promotes neurite outgrowth in rat cultured central nervous system neurons. *J. Pharmacol. Exp. Ther*. **314:** 252–259.

Katz, B., and R. Miledi. 1967. A study of synaptic transmission in the absence of nerve impulses. *J. Physiol.* **192:** 407–436.

Kusano, K., D. R. Livengood, and R. Werman. 1967. Correlation of transmitter release with membrane properties of the presynaptic fiber of the squid giant synapse. *J. Gen. Physiol.* **50**: 2579.

Llinas, R., I. Z. Steinberg, and K. D. Walton. 1981. Presynaptic calcium currents in squid giant synapse. *Biophysics J.* **33:** 289–321.

Llinas, R., J. A. Gruner, M. Sugimori, T. L. McGunness, and P. Greengard. 1991. Regulation by synapsin-I and Ca^{2+}- Calmodulin-dependant protein-kinase II of transmitter release in squid giant synapse. *J. Physiol. Lond.* **426:** 257–282.

Messenger, J. B., M. Nixion, and K. P. Ryan. 1985. Magnesium chloride as an anesthetic for cephalopods. *Comp. Biochem. Physiol.* **82:** 203–205.

Serulle, Y., G. Morfini, G. Pigino, J. E. Moreira, M. Sugimori, S. T. Brady, and R. Llinas. 2007. 1-methyl-4-phenylpyridinium induces synaptic dysfunction through a pathway involving caspase and PKC∂ enzymatic activities. *Proc. Natl. Acad. Sci.* USA **104:** 2437–2441.

Young, J. Z. 1939. Fused neurons and synaptic contacts in the giant nerve fibers of cephalopods. *Philos. Trans. R. Soc. Lond. B* **229:** 465–503.