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Integration of laboratory exercises in developmental biology and neurobiology courses using the *Xenopus* oocyte expression system

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This collaborative laboratory exercise integrates two upper division laboratory courses (Developmental Biology and Neurobiology) offered to biology majors at Wake Forest University. The laboratory exercise involves the use of the *Xenopus* oocyte expression system to study the function of specific membrane receptors and ligand-activated channels. cDNA or mRNA for receptor proteins is injected into *Xenopus* oocytes. The oocytes are assayed for expression of receptor proteins and two-electrode voltage clamping is done to determine whether the expressed proteins are functional in the oocyte system. This series of laboratory exercises is innovative in its interdisciplinary and collaborative approach to undergraduate teaching, and in its use of sophisticated molecular biological and physiological techniques in the undergraduate teaching laboratory. Students learn first-hand how these techniques have been used to achieve a new level of understanding of both development and neurobiology. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 353–358.

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Introduction

Over 25 years ago, John Gurdon and his colleagues showed that injection of globin mRNA into *Xenopus laevis* oocytes resulted in the synthesis of globin protein [5]. Subsequently, a wide variety of mRNAs have been injected into *Xenopus* oocytes, demonstrating the oocyte's ability not only to synthesize but to correctly assemble, post-translationally modify, and direct to the appropriate site in the cell, multi-subunit proteins [11].

In the 1980s, the value of this system for expressing nervous system receptors was recognized [7]. Messenger RNAs encoding neurotransmitter receptors and voltage-gated ion channels were expressed in *Xenopus* oocytes, allowing for electrophysiological study of the receptors in isolation from other proteins that are normally found in association with them. This was an important breakthrough, because cells in the nervous system have many receptors and channel proteins on their surface, and analysis of protein function can be clouded by the responses of other proteins to stimuli. Cloning of receptor and channel proteins allows the cDNA or mRNA for these proteins to be injected and expressed in large quantities in a system where no other nervous system proteins are expressed. Once expression occurs, the biochemistry and physiology of the proteins can be readily analyzed.

We used the *Xenopus* oocyte expression system to integrate the teaching laboratories of two courses, Developmental Biology and Neurobiology. The goal of these exercises was to introduce biotechnology into what were formerly very traditional course laboratories. Students learn that biotechnological tools are used in disparate areas of modern biological research. Each course enrolls 15 students, who are divided into five lab groups of three students each. The two courses are scheduled for the same time and labs meet once a week for 3 h. Ten lab periods are set aside to conduct the experiments on *Xenopus* oocytes. Lab groups from each course are paired and these six students work together for the semester. Several joint lectures during the semester help give students necessary background information in both subjects. At the end of the semester, each group of six students writes one scientific paper discussing all aspects of the project and makes an oral presentation to an audience of both classes.

The purpose of integrating two distinct laboratory courses is to show students that modern science is often interdisciplinary and collaborative. However, this laboratory project may also be appropriate for a single course in either discipline. In these exercises, students use sophisticated molecular biological and electrophysiological techniques and learn first-hand how these techniques have been used to achieve a new level of understanding of both development and neurobiology.

In the first part of the project, students from the Development class learn how to prepare oocytes for microinjection and how to microinject oocytes in an exercise in which they microinject the cDNA for Green Fluorescent Protein (GFP) into the nucleus of *Xenopus* oocytes. GFP was chosen because the success of microinjection can readily be ascertained by microscopic examination of the eggs with a fluorescence microscope, and verified by Western blotting with an antibody to GFP. Students take several weeks to master the techniques of microinjection. During this time, the students also isolate a cloned plasmid containing acetylcholine (ACh) or glycine (Gly) receptor cDNA from *Escherichia coli*, which will later be injected into oocytes.

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Finally, the class microinjects the cDNA for the ACh or the Gly receptor into the nucleus of *Xenopus* oocytes, and passes the oocytes on to the Neurobiology class for analysis. While the Developmental Biology class is learning microinjection, the Neurobiology class is examining endogenous calcium-activated chloride conductances in *Xenopus* oocytes to learn the two-electrode voltage clamp technique. The Neurobiology class then uses this technique to examine the expressed ACh or Gly receptors.

Materials and methods

Preparation of Xenopus oocytes

Numerous reviews are available with additional information about procedures for using the *Xenopus* oocyte expression system [3,10–12].

Xenopus laevis is commonly called the South African clawed frog or toad. The frogs are native to South Africa, Botswana, and Zimbabwe, but are readily obtained from American suppliers (eg *Xenopus* Express, Homosasso, FL, USA; Amphibians of North America, Nashville, TN, USA). In captivity they live an average of 8 years, but in the wild can live as long as 25 years. Although *Xenopus* breathes air, unlike most frogs, it is entirely aquatic and does not require a terrestrial habitat.

The ovaries of a mature female can contain over 100 000 oocytes. In order to obtain oocytes, a large mature female *Xenopus* is anesthetized by immersion in 1000 ml of 0.2% Tricaine (ethyl-*m*-aminobenzoate; this and all other chemicals listed were purchased from Sigma, St Louis, MO, USA) for approximately 10 min. The frog is sufficiently anesthetized when it no longer responds to a pinch on its foot, and can no longer right itself when turned ventral side up. All procedures with *Xenopus* have been approved by the Wake Forest University Animal Care and Use Committee.

The animal is placed on a tray of ice, dorsal side down. A small incision is made through the outer layer of skin with a scalpel (Figure 1). Another incision is made through the connective tissue, and then through the muscle layer. Cutting through the exposed muscle layer exposes the ovary. Using a pair of fine forceps, a section of the ovary

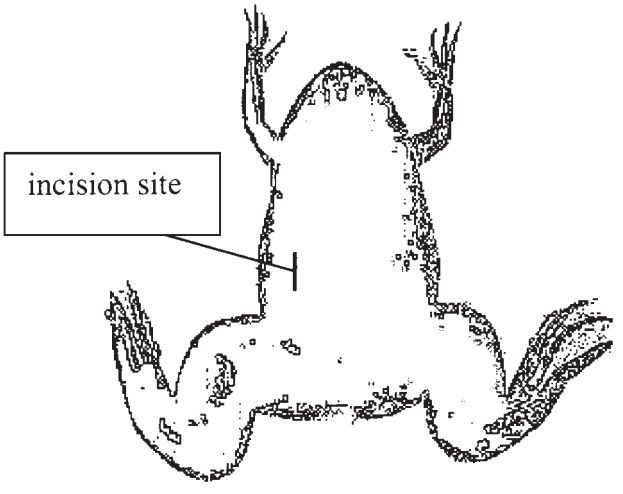

Figure 1 Incision site on abdomen of *Xenopus*.

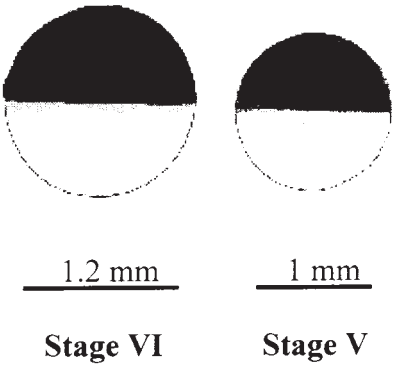

Figure 2 Stage V and VI oocytes.

is carefully teased out onto the surface of the frog. This section of ovarian tissue is separated from the rest of the ovary with scissors, and transferred to a Petri dish containing an ND96 solution (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES brought to pH 7.5 with NaOH) on ice. The tissue is repaired by tying 4–5 sutures on the inner muscle layer, and 5–6 sutures on the outer skin layer. *Xenopus* produces endogenous antibiotics on its skin, so no antibiotic treatment is necessary. The animal is placed in a container with shallow, room temperature tap water, with its head elevated to prevent drowning.

Once a portion of the ovary has been removed to buffer, the oocytes are staged and separated. The *Xenopus* ovary contains oocytes of differing stages of maturity all year [4]. It is possible to express exogenous RNA or DNA in all developmental stages of the oocyte, but the largest oocytes are best since they can be injected with the greatest amount of solution and may produce the biggest currents. The oocytes are categorized as stage I–stage VI, with the largest oocytes being stage VI (Figure 2). Stage I oocytes are small, 50–300 μm in diameter, and unpigmented. Stage VI oocytes are approximately 1.2 mm in diameter, and are characterized by a lightly pigmented ring around the equator (Figure 3). Stage V oocytes are slightly smaller, and also highly pigmented in the animal hemisphere, but lack the equatorial ring (Figure 2). Both stage V and VI eggs are used for microinjection. Eggs with patchy pigmentation or yolk leakage are discarded.

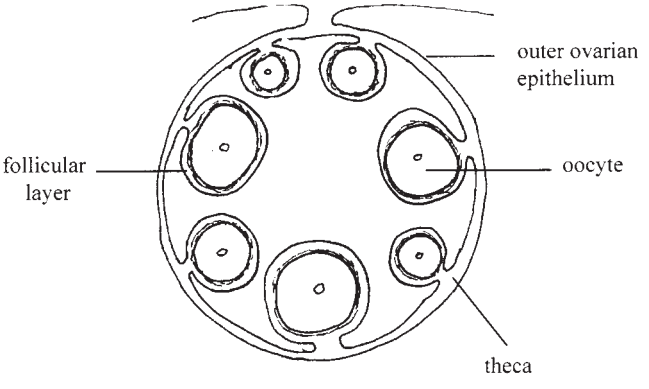

Figure 3 A cross section of an ovarian lobule. The oocytes are enclosed within a follicular layer, which is covered by a theca containing blood vessels, which in turn is encased in the ovarian epithelium.

The oocytes can be separated from the ovarian tissue in ND96 solution using sterile, fine forceps in a sterile Petri dish under a dissecting microscope. The oocytes are contained in sacs formed from the inner ovarian epithelium (Figure 3). A sac of oocytes is separated from the ovary with scissors or forceps by grasping the neck of the sac without touching the oocytes. The ovarian epithelium is opened so that the oocytes lie facing upwards on a single sheet of ovarian tissue. The oocytes can then be separated from one another using fine forceps.

The oocytes are enveloped in a follicular layer that may be removed before microinjection. Although removal of the follicular layer facilitates injection, defolliculated oocytes are more fragile and subject to damage. The follicular layer can be removed manually, with forceps, or by a gentle collagenase treatment. To remove the follicular layer manually, two pairs of forceps are held very close to the surface of the egg, and the follicular layer is gently peeled off. This is a challenge, and lots of eggs will be destroyed in the process. But once the technique is mastered, it is faster and safer than collagenase. To defolliculate the egg using collagenase, separated oocytes with their follicular membrane intact are placed in 1–2 mg ml^{-1} of collagenase A (0.2–0.5 units ml^{-1}) in a Ca^{2+}-free ND96 solution for up to 2 h at room temperature. Removal of the follicle cells is facilitated by gentle agitation during enzyme treatment. After treatment, the oocytes must be washed several times in ND96 to remove excess collagenase.

Microinjection of DNA coding for GFP

In order to demonstrate that microinjected cDNA is indeed being expressed in the oocytes, the Development class microinjects cDNA for GFP into the nucleus of *Xenopus* oocytes. GFP is a protein originally isolated from the bioluminescent jellyfish, *Aequorea victorea* [9]. It produces light in jellyfish when energy is transferred to it from the Ca^{2+}-activated photoprotein aequorin. When expressed in other eucaryotic or procaryotic cells and illuminated by blue or UV light, GFP yields bright green fluorescence in the absence of any other products or substrates from *A. victoria*. The GFP gene has been cloned, and has proved to be a very useful tool in cell and molecular biology [6].

We inject the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA). pEGFP-N1 encodes a variant of the wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells. This version of GFP is excited at wavelengths of 488 and 507 nm. pEGFP-N1 has been designed as a cloning vector, into which other genes can be inserted for cloning. Thus it has been engineered with a number of restriction sites. The vector also contains an SV40 viral origin for replication in eucaryotic cells and an origin for replication in *E. coli*. In addition, the vector has an SV40 promoter attached to a neomycin resistance gene and a bacterial promoter attached to a kanamycin resistance gene. Neomycin and kanamycin are antibiotics that allow for selection of plasmid-containing eucaryotic or procaryotic cells respectively.

In order to microinject oocytes, a stereo microscope, a micropipet puller (Sutter Instrument Co, Novato, CA, USA), a micromanipulator, a microneedle holder, and an injection apparatus (all obtained from WPI, Inc, Sarasota, FL, USA) are needed. Needles for microinjection are made out of thin-walled borosilicate sterile glass tubing (OD 1.2 mm, WPI, Inc). The needles are pulled to a tip of approximately 1 μm over a heated filament in a micropipet puller. A glass tube is placed into the spring-held mechanism in the needle puller and centered over the filament. As the filament heats, the glass softens, and begins to pull, thinning over the filament, until it breaks. A short tip is preferable to a long, drawn-out tip which would more readily clog. Needles are stored on a piece of wax or clay in a petri dish. If the needle tips are closed, they can be opened by breaking them against the sides of the petri dish.

The micromanipulator and microinjection apparatus must be secured adjacent to the microscope, to minimize vibrations. The needles are backloaded with mineral oil using a Hamilton syringe, and mounted in the micromanipulator. The eggs are placed on a nylon mesh grid (710 μm opening, Spectrum Laboratory Products, Houston, TX, USA) in ND96, and oriented with the animal pole up. An injection volume of approximately 50 nl is used. A drop of the DNA to be injected is placed on a piece of parafilm, and a small quantity sufficient for numerous injections is drawn up into the pipet tip.

When injecting DNA, the injection site must be in the nucleus, which is unfortunately not visible. Using the micromanipulator and microscope, the filled micropipet is inserted into the middle of the animal pole, and about 1/3 of the way down into the egg. This is where there is the greatest probability of encountering the nucleus. Injected eggs are placed into petri dishes in sterile ND96 solution and incubated for 48 h at 18°C. The injected eggs are examined under a dissecting microscope for fluorescence using the FITC filter set, or prepared for gel electrophoresis and Western blotting. GFP is visualized in gels via the normal Western blot procedure using commercially available antibody to GFP (Clontech).

Preparation and microinjection of the plasmid

The preparation and microinjection of the plasmid containing the gene for an ACh receptor (provided by Dr James Patrick at the Baylor College of Medicine) are described below. Similar procedures are used with other plasmids containing other receptor genes, including genes for a Gly receptor (provided by Dr John Mihic at the Wake Forest University School of Medicine).

Students in the Development class attempt to express in *Xenopus* oocytes a gene coding for the $\alpha 7$ subunit protein of the ACh receptor. Alpha 7 subunits are derived from neuronal cells and by themselves can assemble into a functional receptor, the presence of which can be detected by voltage clamping [2]. The gene has been inserted into a protein vector and cloned. The pc DNA I AMP plasmid vector into which the gene was cloned is a commercially available expression vector (Invitrogen, Carlsbad, CA, USA). It is a supercoiled plasmid that has been genetically engineered to contain an ampicillin resistance gene, Amp 8, an M13 bacteriophage origin of replication, which allows it to be replicated in bacteria such as *E. coli*, a ColEl origin of replication, which results in high copy number reproduction of the plasmid, an SV40 viral origin of replication, to allow eucaryotic expression, and the polylinker sequence

or cloning site. The polylinker sequence is an artificial sequence of DNA containing recognition sites for a number of restriction enzymes. It is flanked by two promoter sequences, the T7 and SP6 promoters, oriented in opposite directions. This allows the plasmid to be used for the cloning of DNAs for both sense and anti-sense *in vitro* transcription.

The cDNA for the α7 subunit of the ACh receptor was supplied in the pc DNA amp I in an XL blue *E. coli* bacterial stock (Stratagene, La Jolla, CA, USA) vector. The plasmid contains the ampicillin resistance gene to allow for use of the antibiotic ampicillin to select for those bacterial colonies that have incorporated the plasmid. The plasmid is provided in a X-gal blue bacterial stock. Only those colonies of bacteria that grow on ampicillin-containing agar contain the plasmid, but in order to detect which plasmids contain the amp gene, the agar will also contain a dye called X-gal. X-gal is colorless, but when acted upon by the enzyme β-galactosidase, it forms a blue precipitate. The bacteria that were used to incorporate the plasmid lack the β-galactosidase gene. The polylinker inserted into the plasmid is inserted into the middle of a β-galactosidase gene, but no nucleotides are deleted and the gene can still be transcribed to produce a functional product. However, if the sequence to be cloned inserts into the polylinker, the β-galactosidase gene becomes inactive. Thus, one can test for the presence of the gene-containing plasmid in bacterial colonies by growing the colonies on agar containing ampicillin, the substrate of β-galactosidase, X-gal, and the artificial inducer of the lac operon, IPTG. Recombinant colonies are white.

Overnight cultures of *E. coli* are grown in LB medium, and plasmid DNA is purified using the QIAprep Spin Miniprep Kit (Quiagen, Valencia, CA, USA). Purified cDNA is injected as described above. Injected eggs are placed into petri dishes in sterile ND96 solution and incubated for 48 h at 18°C before passing them on to the Neurobiology class.

Recording the endogenous Ca^{2+}-activated Cl^- conductance

A detailed description of this procedure can be found in Paul *et al* [8]. Uninjected stage VI *Xenopus* oocytes are obtained as described above and placed in Ca^{2+}-free ND96 containing 0.5 nM EGTA approximately 2 h before the lab. About 20 min before the lab, some of the oocytes are transferred to Ca^{2+}-free ND96 containing the calcium ionophore, A23187, which forms calcium channels in the membrane, allowing Ca^{2+} ions to move into the cell. The remaining oocytes, kept in the Ca^{2+}-free ND96 containing 0.5 nM EGTA, serve as controls.

Low-resistance microelectrodes (1–5 megohm, MΩ) are pulled from borosilicate glass capillaries (1.2 mm OD × 0.9 mm ID × 75 mm, Frederick Haer & Co, Bowdoinham, ME, USA) using the micropipet puller and filled with 3 M KCl. The microelectrodes are connected via holders (Axon Instruments, Foster City, CA, USA, HL-U) to headstages (Axon Instruments, HS-2A-x1LU: voltage; and Hs-2A-×10MGU: current) which, in turn, are connected to an amplifier (Axon Instruments, GeneClamp 500). The bath is grounded by means of Ag/AgCl wires connected to a bath clamp (Axon Instruments, VG-2A-x100). Data are recorded using data acquisition hardware and software (Biopac, Santa Barbara, CA, USA) and an IBM computer. A schematic diagram of the recording set up is shown in Figure 4.

Oocytes are placed in a perfusion chamber (Warner Instruments, Hamden, CT, USA) with a plastic mesh (710 μm opening) on the bottom. The perfusion system is pump driven (Control Company, Friendswood, TX, USA) and connects to four 60-ml syringes by way of a 5-way valve (Cole Parmer, Vernon Hills, IL, USA). In this way a constant flow of perfusion solution (Ca^{2+}-free ND96 in this case) from one of the syringes is perfused over the oocyte and this flow can be replaced by solutions in the other three syringes by switching the valve.

Under a dissecting microscope, the two microelectrodes (one for voltage and one for current) are carefully lowered until they dimple the oocyte. This is accomplished using micromanipulators (Newport, Irvine, CA, USA). Once the dimple appears, a gentle tap is delivered to the side of the micromanipulators allowing the microelectode to penetrate the oocyte. This results in a drop in the recorded potential, so that the oocyte's resting membrane potential can be determined. Cells with resting membrane potentials of less than approximately −30 mV are discarded.

Once a stable resting membrane potential is obtained, the amplifier is switched to the voltage clamp mode. A control solution of Ca^{2+}-free ND96 is then applied to the bath by switching from the background flow. After determining there is no response to the control solution, several concentrations of $CaCl_2$ (1, 2, and 5 mM) are perfused through the bath for 10 s at 10-min intervals. Following the concentration-response procedure, 5 mM $CaCl_2$ is perfused over the oocyte at different clamped voltages and the reversal potential is determined as that voltage at which no current flows across the membrane.

Recording from expressed ACh and Gly receptors

Oocytes which are injected with cDNA for ACh or Gly receptors, as described above, are given to the Neurobiology class by the Development class. The oocytes have been injected 2–4 days previously. The oocytes are placed in the perfusion bath as described above and constantly bathed in a solution of ND96. The procedures for two-electrode voltage clamping these oocytes are also the same as described above.

Once a stable resting membrane potential is obtained (≤−30 mV), the amplifier is switched to the voltage clamp mode and the experiment begins. The membrane is clamped at −70 mV and different concentrations of Gly or ACh (0, 20, 50, 75, and 100 μM) are perfused though the bath for 10 s at 10-min intervals. Then, the concentration of Gly or ACh is held constant and the clamped voltage is varied. Responses are recorded using the data acquisition hardware and software and computer. The reversal potential is also determined.

Expected results

Expression of GFP

Oocytes whose nuclei have been successfully injected with cDNA for GFP fluoresce, while uninjected control oocytes

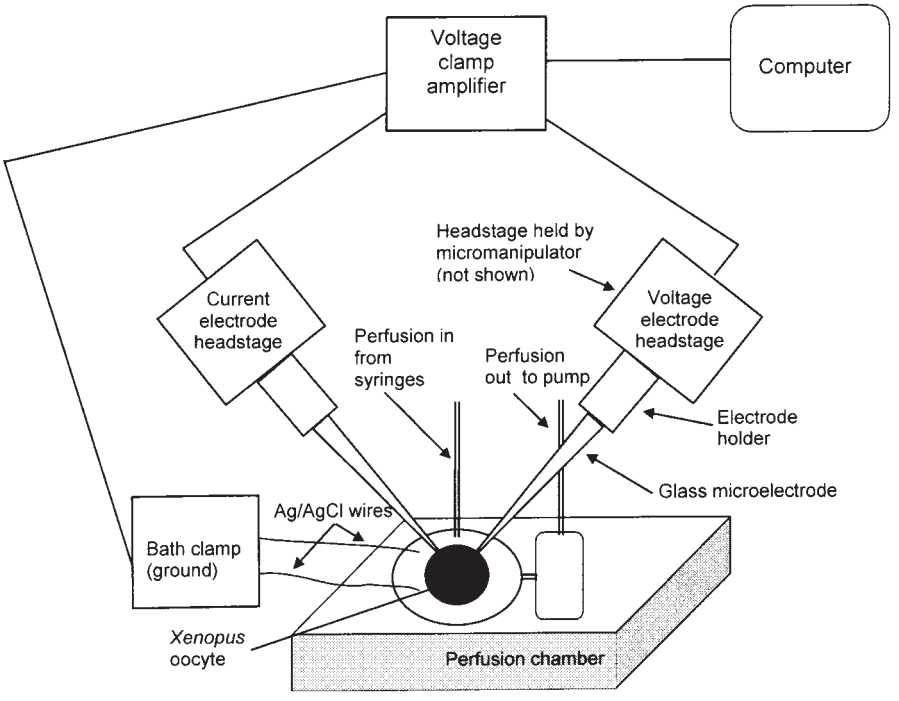

Figure 4 Schematic diagram of experimental set up.

show only limited background fluorescence. Western blots for GFP should show a band above background at 27 kDa, the molecular weight of GFP and its variants.

Recording the endogenous Ca^{2+}-activated Cl^- conductance

Students first determine the resting membrane potential for the oocyte. Resting membrane potentials are usually −30 to −50 mV. The magnitude of the response increases with increasing concentrations of $CaCl_2$. By keeping the concentration of $CaCl_2$ constant and varying the clamped potential, a current-voltage (IV) curve is constructed and a reversal potential is obtained. The reversal potential is the potential at which there is no net ion flow across the membrane. Current flows in opposite directions below and above this potential. Since the reversal potential is often the equilibrium potential for the ion species responsible for current flow it can be used to determine the ion species responsible for the current flow. For the endogenous Ca^{2+}-gated Cl^- channels in *Xenopus* oocytes, the reversal potential is around −25 mV [1]

Recording from expressed ACh and Gly receptors

Resting membrane potentials for oocytes injected with cDNA for ACh or Gly receptors were similar to those reported above. Increasing concentrations of ACh or Gly elicit increasing current flow though the membrane. Figure 5 shows responses to two different concentrations of Gly at a clamped voltage of −70 mV. A response-concentration curve can be determined from such data.

Figure 6 shows responses to 75 μM glycine at two different clamped voltages. By keeping the concentration of ACh or Gly constant and varying the clamped voltage, the reversal potential can be determined. The reversal potential in our recent experiments was -31.2 ± 2.4 mV ($n = 5$).

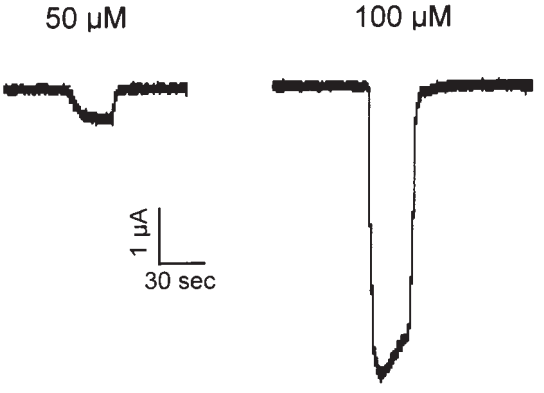

Figure 5 Responses to two concentrations of glycine from a *Xenopus* oocyte that had been injected with DNA for the glycine receptor (α1 subunit). The oocyte membrane was clamped at −75 mV.

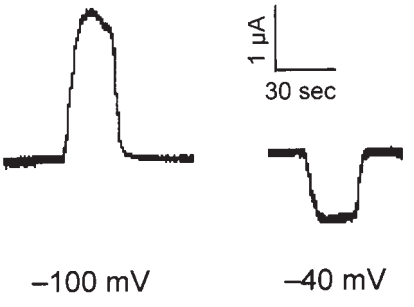

Figure 6 Responses to 75 μM glycine from a *Xenopus* oocyte that had been injected with DNA for the glycine receptor (α1 subunit). The membrane was clamped at two different voltages.

Discussion

We have described laboratory exercises that integrate the Developmental Biology and Neurobiology courses in the Department of Biology at Wake Forest University. We believe these exercises constitute a valuable alternative to traditional approaches to undergraduate experiences in teaching laboratories. While a third of the semester is devoted to more classic laboratory exercises, the final two-thirds of the semester is designed to introduce students to the most modern techniques. Students experience first hand how the biotechnology revolution has permeated all areas of biological study. The techniques learned by students in these courses can be applied to other areas of biotechnology and should help prepare our students for graduate school and the job market. This arrangement incorporates a group learning process, with students from both courses helping each other learn and understand the material. The exercises are linked, with one logically leading into the next as opposed to discrete, unrelated experiments. Using relatively sophisticated but reasonable techniques gives the students a better understanding of how current research is conducted. Combining the Neurobiology and Development classes also allows students to appreciate the interdisciplinary and collaborative nature of modern science.

Acknowledgements

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