Video Article

Local Application of Drugs to Study Nicotinic Acetylcholine Receptor Function in Mouse Brain Slices

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Abstract

Tobacco use leads to numerous health problems, including cancer, heart disease, emphysema, and stroke. Addiction to cigarette smoking is a prevalent neuropsychiatric disorder that stems from the biophysical and cellular actions of nicotine on nicotinic acetylcholine receptors (nAChRs) throughout the central nervous system. Understanding the various nAChR subtypes that exist in brain areas relevant to nicotine addiction is a major priority.

Experiments that employ electrophysiology techniques such as whole-cell patch clamp or two-electrode voltage clamp recordings are useful for pharmacological characterization of nAChRs of interest. Cells expressing nAChRs, such as mammalian tissue culture cells or *Xenopus laevis* oocytes, are physically isolated and are therefore easily studied using the tools of modern pharmacology. Much progress has been made using these techniques, particularly when the target receptor was already known and ectopic expression was easily achieved. Often, however, it is necessary to study nAChRs in their native environment: in neurons within brain slices acutely harvested from laboratory mice or rats. For example, mice expressing "hypersensitive" nAChR subunits such as α4 L9′A mice [1] and α6 L9′S mice [2], allow for unambiguous identification of neurons based on their functional expression of a specific nAChR subunit. Although whole-cell patch clamp recordings from neurons in brain slices is routinely done by the skilled electrophysiologist, it is challenging to locally apply drugs such as acetylcholine or nicotine to the recorded cell within a brain slice. Dilution of drugs into the superfusate (bath application) is not rapidly reversible, and U-tube systems are not easily adapted to work with brain slices.

In this paper, we describe a method for rapidly applying nAChR-activating drugs to neurons recorded in adult mouse brain slices. Standard whole-cell recordings are made from neurons in slices, and a second micropipette filled with a drug of interest is maneuvered into position near the recorded cell. An injection of pressurized air or inert nitrogen into the drug-filled pipette causes a small amount of drug solution to be ejected from the pipette onto the recorded cell. Using this method, nAChR-mediated currents are able to be resolved with millisecond accuracy. Drug application times can easily be varied, and the drug-filled pipette can be retracted and replaced with a new pipette, allowing for concentration-response curves to be created for a single neuron. Although described in the context of nAChR neurobiology, this technique should be useful for studying many types of ligand-gated ion channels or receptors in neurons from brain slices.

Video Link

The video component of this article can be found at https://www.jove.com/video/50034/

Protocol

1. Preparation of Solutions for Brain Slice Preparation and Electrophysiology

1. Solutions for preparation of brain slices were previously described [3, 4]. Prepare N-methyl D-glucamine (NMDG)-based cutting and recovery solution of the following composition (in mM): 93 n-methyl D-glucamine, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na^+ ascorbate, 2 thiourea, 3 Na^+ pyruvate, 10 $MgSO_4$•$7H_2O$, 0.5 $CaCl_2$•$2H_2O$. Adjust to 300-310 mOsm with NMDG. Adjust pH to 7.3-7.4 with 10 N HCl.
    a. Dissolve $MgSO_4$•$7H_2O$ and $CaCl_2$•$2H_2O$ in Millipore water to make 2 M stock solutions of each.
    b. Weigh out other components in the order listed and add to a volumetric flask partially filled with Millipore water while stirring to dissolve. Once everything is added, fill volumetric flask with Millipore water.
    c. Measure the pH and adjust with 10 N HCl.
    d. Measure the osmolarity and adjust with NMDG if necessary.

2. Prepare HEPES holding artificial cerebrospinal fluid (aCSF) of the following composition (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 5 Na^+ ascorbate, 2 thiourea, 3 Na^+ pyruvate, 2 $MgSO_4$•$7H_2O$, 2 $CaCl_2$•$2H_2O$. Adjust to 300-310 mOsm with sucrose. Adjust pH to 7.3-7.4 with 1 N HCl or NaOH.

a. Weigh out components in the order listed and add to a volumetric flask partially filled with Millipore water while stirring to dissolve. Once everything is added, fill volumetric flask with Millipore water.
b. Measure the pH and adjust with NaOH or HCl if needed.
c. Measure the osmolarity and adjust with sucrose if necessary.

3. Prepare standard aCSF recording solution of the following composition (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 24 $NaHCO_3$, 12.5 glucose, 2 $MgSO_4 \cdot 7H_2O$, 2 $CaCl_2 \cdot 2H_2O$. Adjust to 300-310 mOsm with sucrose. Adjust pH to 7.3-7.4 with 1 N HCl or NaOH.

2. Preparation of Acute Brain Slices

1. Brain slice cutting and recovery are done as previously described [3, 4]. Bubble two crystal dishes filled with NMDG recovery solution with 95% oxygen/5% CO_2 gas (carbogen), one dish on ice, the other at 33 °C.
2. Bubble one crystal dish filled with HEPES holding aCSF with carbogen at room temperature.
3. Anesthetize adult mouse (aged 2 to 12 months) with Na^+ pentobarbital (200 mg/kg, i.p.). Proceed with procedure when the animal has no response to a toe-pinch.
4. If necessary, cut a tail tissue sample for later genotyping of the animal.
5. Open the chest cavity, expose the heart, and clamp the descending aorta. Lacerate the right atrium.
6. Transcardially perfuse animal with 5-10 ml of 0-4 °C NMDG-recovery solution.
7. Decapitate animal, remove the brain and place it in 4 °C NMDG-recovery solution for 1 min.
8. On an ice-chilled metal plate, trim the brain in the desired orientation (coronal, parasagittal, horizontal, etc.) to include the area of interest.
9. Affix the brain block with superglue to the dry stage surface of a vibrating slicer (DSK-Zero 1; Dosaka), submerge the brain block in 4 °C NMDG-recovery solution, and continuously bubble the solution with carbogen.
10. Cut slices of the brain area of interest. Slice thickness is 200-400 µm, depending on the specific brain area of interest, animal age, and experiment to be performed.
11. Immediately after cutting, incubate desired slices in carbogenated, 33 °C NMDG-recovery solution for exactly 12 min, then transfer to carbogenated, room temperature HEPES holding solution for an initial period of 60 min. After this 60 min incubation, slices can be used for recording. Slices are maintained in HEPES holding solution throughout the day until they are used for recording.

3. Patch Clamp Recording from Neurons in Brain Slices

1. Pull a standard patch micropipette with a programmable Flaming-Brown puller (Sutter P-97 or equivalent vertical puller). Pipettes that are optimal for giga-ohm seal formation typically have a resistance of 4-6 MΩ
2. Transfer one slice to the recording chamber (RC-27L; Warner Instruments) on the stage of an upright microscope (Nikon FN-1). Continuously superfuse (1.5 - 2.0 ml/min) the slice with carbogenated, standard recording aCSF heated and maintained at 32 °C. Alternatively, slices can be maintained at room temperature.
3. Locate and center the brain area of interest using a 10X air objective (Nikon Plan Fluor 10X; NA 0.3; WD: 16 mm).
4. Visualize individual neurons using a 40X near-infrared (IR) water immersion objective (Nikon APO 40XW; NA: 0.80; WD: 3.5 mm) connected to IR-differential interference contrast (DIC) optics and a high-speed near infrared charged coupled device (CCD) video camera (Hamamatsu C-7500). Under IR-DIC observation, healthy neurons exhibit a smooth, light-grey plasma membrane.
5. Fill a micropipette with a suitable intracellular recording solution. For most applications, we use the following solution (in mM): 135 potassium gluconate, 5 EGTA, 0.5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 2 Mg-ATP, and 0.1 GTP (pH adjusted to 7.25 with Tris base, osmolarity adjusted to 290 mOsm with sucrose). Establish a whole-cell recording from a visualized neuron.

4. Local Application of Drugs to Neurons in Slices

1. Pull a standard patch clamp micropipette as described above. Using a pipette puller such as a Sutter P-97 that produces two identical "sister" tips from the same piece of glass is advantageous when multiple drug solutions are to be used on the same cell. Tip sizes that would have a resistance of ~5 MΩ if they were filled with the intracellular solution described above are optimal.
2. Backfill the micropipette with a solution of drug diluted in carbogenated, standard recording aCSF. Point the tip downward, and flick the drug-filled micropipette to remove any air bubbles trapped in the column of solution.
3. Mount the drug-filled micropipette in a pipette holder connected with tubing to a suitable pressure ejection system, such as a Picospritzer III (General Valve Co.). The pipette holder should be mounted onto a micromanipulator with the same resolution as manipulators commonly used for patch clamp recording (for example, Sutter MP-285). Additionally, the manipulator should allow for diagonal movement into and out of the slice. Manually eject pressure 3 to 4 times into the micropipette in order to build up adequate pressure behind the column of fluid.
4. Using the micromanipulator controls, lower the drug pipette into the slice while visualizing the tip under IR-DIC optics. Ideally, one should visually identify and center a neuron to be recorded from, followed by positioning of the drug pipette near the cell prior to lowering the recording micropipette into the slice. Positioning the drug pipette can cause movement of tissue in the vicinity of the recorded cell that could disrupt the recording if the drug pipette is moved into position after establishing a gigaohm seal.
5. Using IR video, position the drug micropipette at the top surface of the tissue slice. Execute one pressure ejection and monitor 1) the vicinity of the tip for movement of debris related to the ejection, and 2) the micropipette tip for any signs that the tip is clogged or blocked. If the tip is blocked/clogged, retract the pipette and replace it with a new one.
6. Approach the recorded cell diagonally from the top of the slice such that when in final position, the tip of the drug-filled pipette is 1) in the same focal plane (or slightly below) as the recorded cell and the tip of the recording electrode, and 2) 10 to 40 µm from the recorded cell. If the drug-filled pipette tip is above the recorded cell, the force from the pressure ejection could disrupt the gigaohm seal.
7. Record the desired cellular response while holding the cell in voltage clamp mode or current clamp mode. We routinely record acetylcholine and/or nicotine-elicited cellular currents while holding neurons in voltage clamp mode. Although pressure ejection can be triggered manually

when using a Picospritzer III, for more reproducible results it is advisable to trigger the pressure ejection using the acquisition system (Axon Digidata 1440 and pClamp 10.3) with a digital TTL pulse.

5. Controlling the Drug-filled Micropipette with a Piezoelectric Translator

1. If possible, mount the drug-filled pipette holder to a single-dimension piezoelectric translator (*e.g.* Piezojena PA-100 or Burleigh PZM-150) that is capable of accepting an analog voltage input (again, from the acquisition system), which is then mounted onto the high-precision micromanipulator (Sutter MP-285). A piezoelectric translator is useful because the movement of the drug-filled pipette into and out of the slice, including the timing and speed of entry/exit, can be more easily controlled and reproduced from experiment to experiment. When studying nAChRs, the desensitizing effects of nicotine leakage from the drug-filled pipette, should they ever occur, are minimized when the pipette is only moved near the cell with a piezoelectric translator for the moment of pressure ejection.
2. Using the manually-controlled potentiometer on the piezoelectric voltage control amplifier, dial the voltage to its maximal value.
3. Using the micromanipulator, position the drug-filled pipette in the slice where the pipette will eject its contents onto the recorded cell, and retract the pipette by manually reducing the voltage to zero on the piezoelectric voltage control amplifier.
4. Using an analog output signal from the recording acquisition system, move the drug-filled pipette into the slice over a period of 250 msec starting 300 msec before the pressure ejection TTL pulse occurs. Retract the pipette over a period of 250 msec, starting 50 msec after the TTL pulse ends.

Representative Results

In our experiments, we routinely record from dopamine (DA)-producing neurons of the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). In voltage-clamp mode, pressure application of acetylcholine or nicotine to these cells will typically result in a rapid, inward cation current that reaches peak within 100-200 msec (**Figure 1A-B**). Decay of the current is largely dictated by diffusion of the drug from the site of action, and whether enzymes in the slice are present to metabolize the drug into an inactive form. For example, ACh is rapidly hydrolyzed by acetylcholinesterases that reside on the surface of cells in areas of the brain that are rich in DA neurons [5]. This results in a rapid decay of the evoked current (**Figure 1A**). In contrast, nicotine is not hydrolyzed and nicotine-evoked currents do not decay as rapidly as ACh-evoked currents (**Figure 1B**), which allows it to strongly activate and desensitize nAChRs [6].

Pressure ejection systems allow the operator to vary the time of the pressure pulse. This feature is useful in studying nAChRs on the surface of neurons within brain slices, as it allows the investigator to determine whether the maximum evoked current has been achieved in response to a pulse of drug. For example, the two traces in **Figure 2** show that 250 msec of drug application (grey trace) may or may not reveal a definitive maximum-evoked current, whereas 1,000 msec of drug application (black trace) yields a clear maximum current plus some steady-state desensitization. Resolving such "peak" currents is crucial when constructing an accurate concentration-response curve, or when studying nAChR desensitization.

DA neurons in the ventral midbrain often fire spontaneous action potentials when recorded within acute brain slices [7, 8]. α6 L9′S mice, which express nAChRs that respond strongly to 10 to 100-fold lower concentrations of nicotine or ACh [2, 9-11], exhibit increased locomotion in response to nicotine injections. To correlate neuronal activity (action potential firing) with behavior, it is useful to apply nicotine to α6 L9′S DA neurons recorded in current-clamp mode. Typically, local application of nicotine (1 μM) to α6 L9′S DA neurons will result in a rapid and transient acceleration of firing that decays back to baseline within 30-40 sec [2] (**Figure 3**, upper panel). Such responses to nicotine are easily quantifiable (**Figure 3**, lower panel) with appropriate analysis software (*e.g.* pClamp; Molecular Devices Corp.).

Experiments in cultured cells or *Xenopus* oocytes with ectopically expressed nAChRs enjoy the advantage that a series of drug concentrations can be applied to each recorded cell [12]. In neurons within brain slices, typically a concentration-response curve is not possible, and responses to a single concentration of drug are often reported [1]. However, a drug-filled pipette as described in this paper can be repeatedly changed to allow for multiple concentrations of drug to be applied to the same recorded cell within a brain slice [2, 8, 13, 14]. **Figure 4** shows representative data from a single recorded DA neuron that was stimulated with three concentrations of ACh. Experience of the investigator, combined with a highly stable micromanipulator holding the drug-filled pipette, are critical factors leading to success in this type of experiment. When successful, this approach provides valuable pharmacological information about native nAChRs.

Neurons in brain slices, including uncharacterized cell types or those present in brain areas with a heterogeneous population, are often classified using various basic electrophysiological and/or morphological measures [15]. For example, the expression of I_h current, resting membrane potential, spontaneous firing rate, and cell size are typical measures used to characterize DA neurons [2, 7, 8, 16, 17]. Using our method, it is also possible to characterize neurons based on their response to locally-applied nicotine or ACh [18, 19]. For example, the superior colliculus (SC) is a heterogeneous and relatively uncharacterized brain area with extremely high expression of various nAChRs, including those that contain α6 subunits [20]. In brain slices from α6 L9′S mice, we recorded from several SC neurons and applied nicotine (1 μM) using pressure ejection with a nicotine-filled pipette. Two distinct response types were observed: 1) activation of inhibitory postsynaptic currents (IPSCs) (**Figure 5**, Type I responses), and 2) large inward cation currents with some induction of excitatory postsynaptic currents (EPSCs) (**Figure 5**, Type II responses).

The use of a piezoelectric translator offers the advantage that the drug-filled pipette can be held in a stationary position at least 100 μm away from the recorded cell until it is rapidly moved adjacent to the cell for the pressure ejection. This is useful because the pipette movement can be automated and is consistent from cell to cell and on day to day. It is also useful when high concentrations of nicotine (which desensitize nAChRs) are applied to the recorded cell, as it minimizes the effect of nicotine leakage from the drug-filled pipette should it ever occur. To demonstrate the consistency of nAChR currents when using a piezoelectric translator, we recorded from VTA DA neurons in brain slices from α6 L9′S mice. **Figure 6** shows consecutive responses in the same α6 L9′S VTA DA neuron in response to concentrations of nicotine that strongly activate hypersensitive α6* nAChRs (1 μM: **Figure 6A**; 10 μM: **Figure 6B**). If allowed to continuously leak onto the recorded cell, these concentrations of nicotine would be expected to desensitize nAChRs on the cell surface, and the second response would be attenuated relative to the first response.

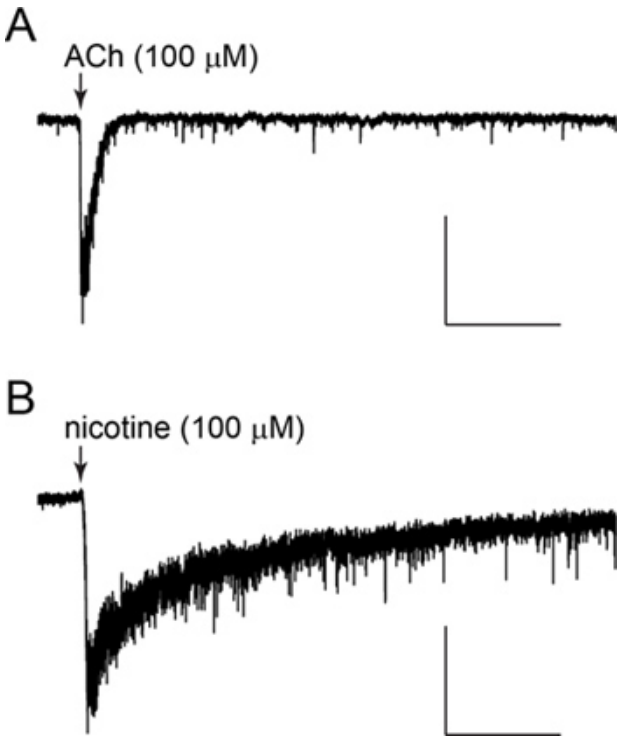

Figure 1. Representative nAChR responses in DA neurons. **A**. A DA neuron in a WT mouse brain slice was voltage clamped, followed by local application of ACh (100 μM) via pressure ejection (250 msec) using a second drug-filled pipette. Scale bar: 100 pA, 3 sec. **B**. Experiment was performed as described in *A*, except that nicotine (100 μM) was applied to the DA neuron. Scale bar: 60 pA, 3 sec.

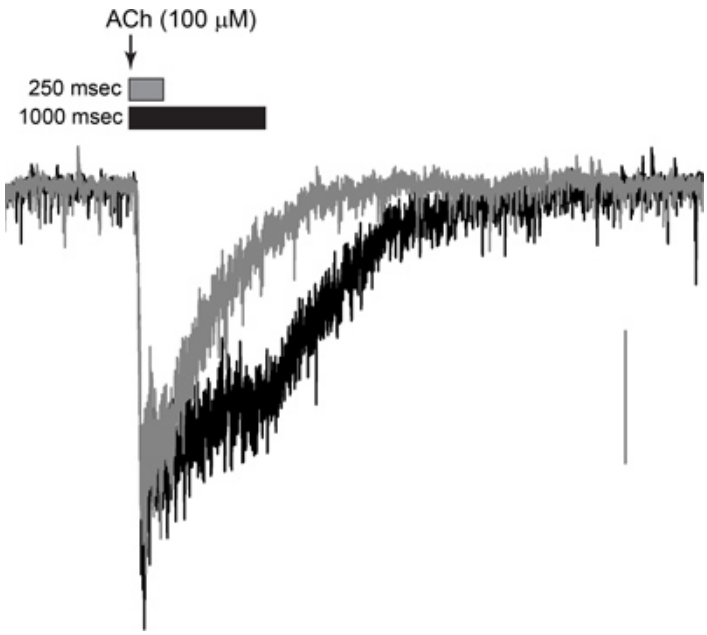

Figure 2. Resolving peak currents by varying drug application time. A DA neuron in a WT mouse brain slice was voltage clamped, followed by local application of ACh (100 μM). Two responses are shown for the same recorded cell, where ACh was ejected from the drug-filled pipette for 250 msec (grey trace) or 1000 msec (black trace). Scale bar: 50 pA.

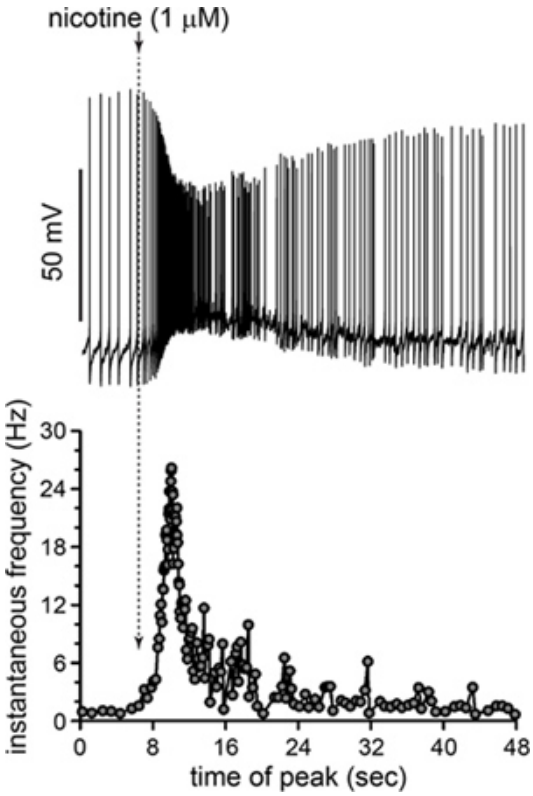

Figure 3. Studying action potential firing following local drug application. A DA neuron in a α6 L9′S mouse brain slice was recorded in current clamp (I = 0) mode, and spontaneous action potentials were observed. Nicotine (1 μM) was applied using pressure ejection (250 msec), and changes in action potential firing rate and resting membrane potential were observed. Using pClamp software (threshold search), instantaneous firing rate was derived and is shown in the lower panel.

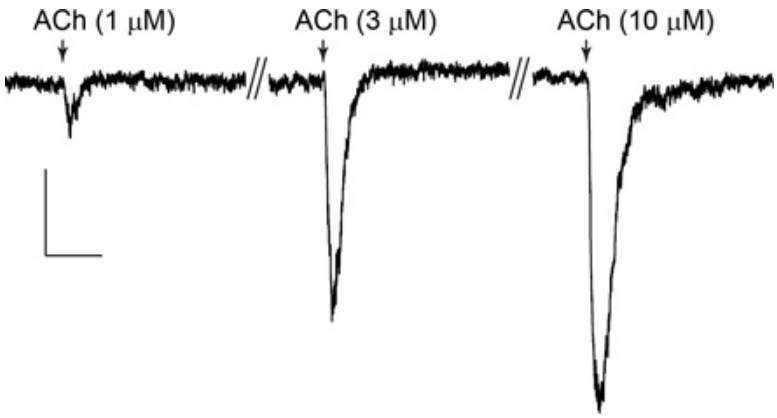

Figure 4. Multiple drug applications to the same recorded cell. A DA neuron in a α6 L9′S mouse brain slice was recorded in voltage clamp mode. A drug-filled pipette was used to locally apply ACh (1 μM; 250 msec). While continuing to record from the cell, the drug-filled pipette was withdrawn from the slice and replaced with another pipette filled with 3 μM ACh. A response to 3 μM ACh was recorded, and the process was repeated again for 10 μM ACh. Scale bar: 100 pA, 1.5 sec.

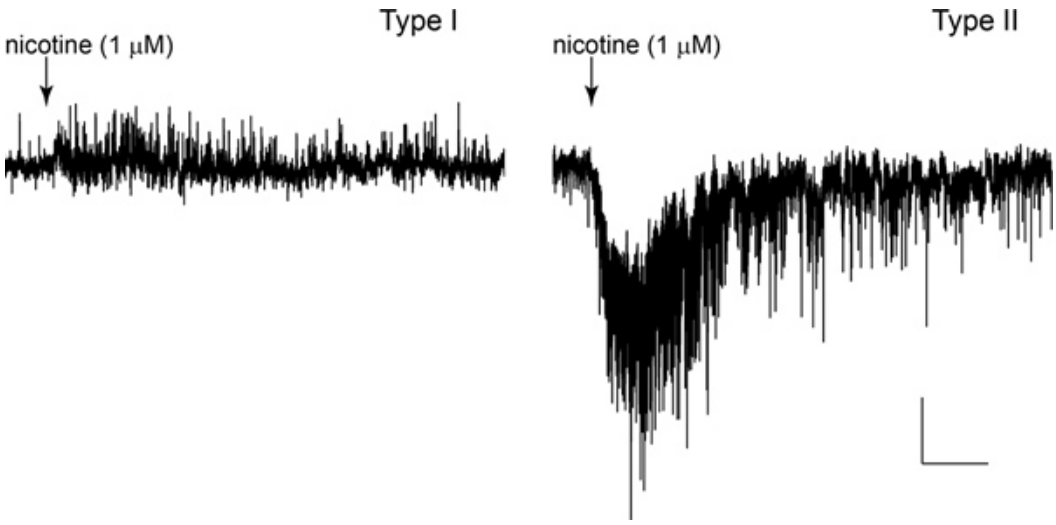

Figure 5. Classification of neurons by nAChR response type. A group of superior colliculus (SC) neurons in a α6 L9′S mouse brain slice were voltage clamped, followed by local application of nicotine (1 μM) via pressure ejection. Type I neurons exhibited increased IPSCs following nicotine application. Type II neurons exhibited large inward currents and increased EPSCs in response to nicotine application. Scale bar: 20 pA, 3 sec.

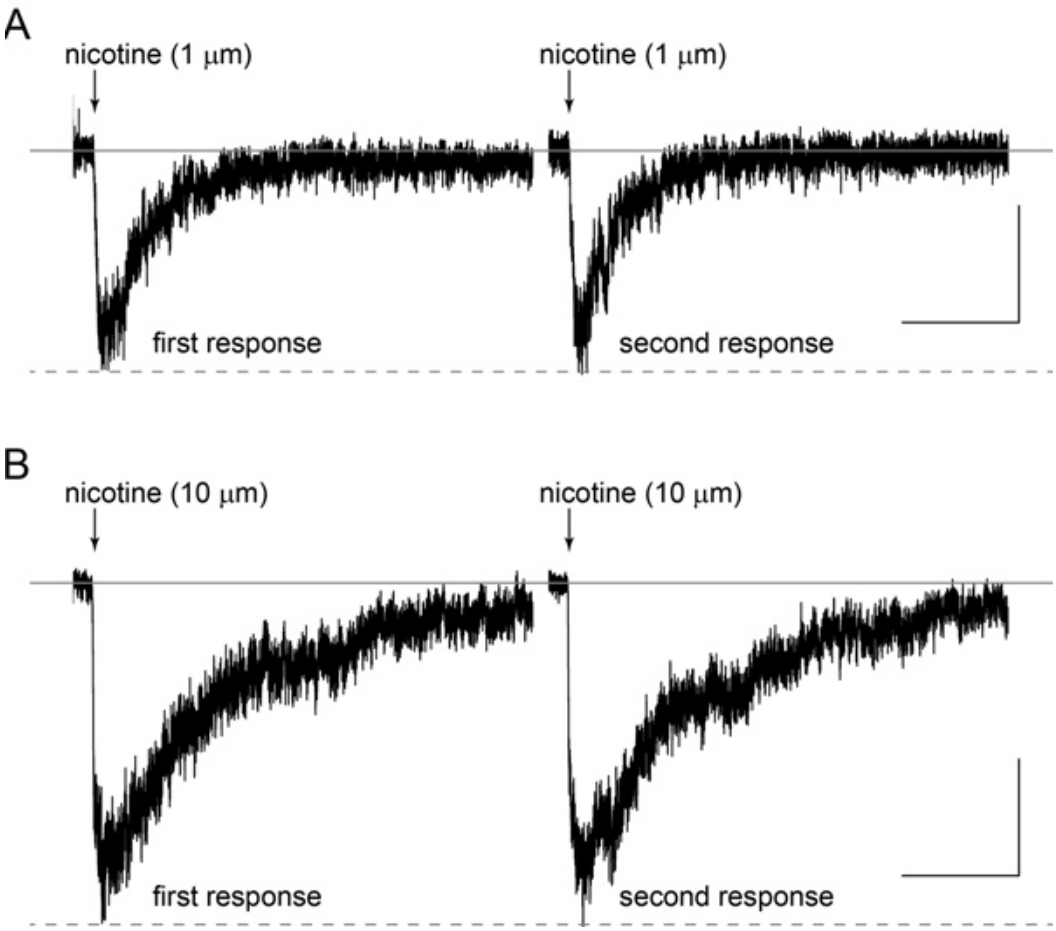

Figure 6. Piezoelectric translator offers consistency and protects from drug leakage. **A**. A VTA DA neuron in a brain slice from a α6 L9′S mouse was recorded in voltage clamp mode during application of 1 μM nicotine. The nicotine-filled pipette was maneuvered into final position before the pressure ejection and retracted after the ejection using a piezoelectric translator. A second response was recorded 2 min after the first to demonstrate that no nAChR desensitization had occurred. Scale bar: 100 pA, 8 sec. **B**. nAChR responses in α6 L9′S VTA DA neurons were recorded as in *A*, but 10 μM nicotine was applied. Scale bar: 100 pA, 8 sec.

Discussion

The method presented in this paper is broadly useful for studying ligand-gated ion channel function in brain slice preparations. However, there are a number of factors that will significantly affect the quality and reproducibility of experimental data that result from utilizing this method. For example, evoked currents are very sensitive to the diameter of the tip of the drug-filled pipette. Small tips will cause difficulty with ejecting the drug solution, and large tips with low resistance will be more likely to disrupt the gigaohm seal between the recorded cell and the recording electrode. One other limitation of the method presented is that cells within 10 µm of the recorded cell may be exposed to drugs from the drug-filled pipette, which may increase their activity and could potentially affect the recorded cell.

When applying drug to a cell multiple times with the same drug pipette, it is crucial that the pipette be returned to precisely the same position in the slice for each application. Even small deviations (*e.g.* 1-2 µm) in placement from one response to the next will often result in substantial differences in the observed peak response. A programmable piezoelectric translator (as described above) or robotic micromanipulator is advantageous for positioning the drug pipette. Another key consideration is the depth of the recorded cell within the slice. Drug will often diffuse away from a cell on the surface of the slice more rapidly, whereas drug can be "trapped" within the slice when ejected onto a cell that is located deep within the slice. This is often a key consideration when applying nicotine, which readily desensitizes nAChRs when exposure times are longer than 100-200 msec. Similarly, the pressure (in psi) that is ejected into the drug-filled pipette and the amount of time that pressure is applied (**Figure 2**) are important considerations for interpreting nAChR current data. We routinely use 10-12 psi of pressure, and we find that 250 msec is sufficient time to resolve nAChR peak currents without causing extensive receptor desensitization.

A key advantage of the method we describe is the ability to exchange drug-filled pipettes tips so that multiple drug concentrations can be applied to the same neuron. Key considerations when exchanging pipettes are 1) variation in pipette tip location from one concentration to the next, and 2) variability in pipette tip diameter. Pipette tip location can typically be adequately controlled with a high-resolution near-IR video camera and a precise micromanipulator. Variability in pipette tip diameter can be controlled by using "sister pipettes" whenever possible, and by using a pipette puller that pulls micropipettes with a high degree of accuracy and consistency.

Overall, the drug-filled pipette method described in this paper is a very useful approach when studying native nAChRs expressed in neurons found in acute brain slices. In addition to being useful for studying the neurobiology of nicotine dependence and nicotinic cholinergic biology, this method is easily applicable to other ligand-gated ion channels. 5-HT_3 receptors, $GABA_A$ receptors, and glycine receptors are several of the other "cys-loop" receptors that can be studied using these techniques [21].

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