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Bilateral multielectrode neurophysiological recordings coupled to local pharmacology in awake songbirds

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Here we describe a protocol for bilateral multielectrode neurophysiological recordings during intracerebral pharmacological manipulations in awake songbirds. This protocol encompasses fitting adult animals with head-posts and recording chambers, and acclimating them to periods of restraint. The adaptation period is followed by bilateral penetrations of multiple electrodes to obtain acute, sensory-driven neurophysiological responses before versus during the application of pharmacological agents of interest. These local manipulations are achieved by simultaneous and restricted drug infusions carried out independently for each hemisphere. We have used this protocol to elucidate how neurotransmitter and neuroendocrine systems shape the auditory and perceptual processing of natural, learned communication signals. However, this protocol can be used to explore the neurochemical basis of sensory processing in other small vertebrates. Representative results and troubleshooting of key steps of this protocol are presented. Following the animal's recovery from head-post and recording chamber implantation surgery, the length of the procedure is 2 d.

INTRODUCTION

As excitable cells, neurons process and propagate information within the nervous system through electrochemical signaling. In fact, the detection, analysis and interpretation of neuronal electrical discharges, or action potentials, that occur with various nervous system operations have become a cardinal means of elucidating basic properties of nervous system function and dysfunction. Extracellular electrophysiology in intact animals has been reliably and extensively used as a method for studying fundamental aspects of the functional organization of the nervous system. Since its refinement in the 1950s, the applications of this method have been numerous and diverse; electrophysiological recordings have been used to reveal basic functional properties of all sensory systems, a variety of motor networks, and have been central to uncovering complex, higher-order central nervous system capabilities including learning, memory formation and decision-making (1-10). Moreover, extracellular electrophysiology has been pivotal in establishing how many diseases and disorders impact the functionality of central circuits and has highlighted how pharmacological, cellular and genetic manipulations may ultimately be used therapeutically to recover or reinstate their functions.

Neurophysiological recordings in anesthetized and awake animals

A large fraction of extracellular electrophysiological studies available in the literature involve recordings obtained from experimental models that are heavily sedated (4-7,11,12). Historically, the use of anesthesia was, and continues to be, regarded as advantageous, as it alleviates animal distress, simplifies the stimulus-response relationship, reduces movement artifact to improve the signalto-noise ratio of recordings and enables local (intracerebral) infusions of agents of interest during the recording procedure. It is now known, however, that many brain systems operate very differently in alert and unconscious states (13-16). These observations have increasingly prompted researchers to seek new methods for obtaining and evaluating neuronal activity in animals that are awake. For instance, significant efforts were dedicated to develop and implement methods for recording neurophysiological responses in freely moving animals equipped with a series of implanted electrodes and a headstage preamplifier (17-21). These approaches contributed fundamental information on how the functionality of single units or neuronal ensembles is modulated in conscious animals, in real time and under more 'naturalistic' behavioral conditions. Experimentation in freely behaving animals, however, is often accompanied by an array of technical challenges that complicate experimental control, thus confounding the evaluation of neuronal responses in unpredictable and, sometimes, unidentifiable ways (17). Moreover, the integration of multiple methodologies, such as the carefully controlled injection of pharmacological agents during a recording session, is virtually precluded in a freely behaving preparation.

Multielectrode recordings in awake animals during local pharmacological manipulations

In this protocol, we detail how many of these barriers can be overcome in small animals, with an awake, restrained preparation that enables multielectrode neurophysiological recordings coupled to local pharmacological manipulations, bilaterally (Fig. 1). This method derives from and expands on previously established recording techniques carried out by other groups in the awake songbird. Notably, the first recordings in awake animals were often conducted with single electrodes, an approach that continues to be used (22-28). The multi-electrode recording configuration has also been implemented in either awake restrained or freely behaving songbirds (21,29-37). Finally, a combination of neurophysiological recordings and unilateral local pharmacological treatment in the avian brain has been used for several years (32,33,38).

Initially, we and our collaborators used multi-electrode recordings in the awake songbird to explore the functional organization of auditory cortical areas, and the contributions of different neurotransmitter systems to shaping the auditory processing of complex, behaviorally relevant communication signals (songs, a learned vocal signal) (32,39-41). Here we describe a method for bilateral multiple electrode recordings, with the added benefit of bilateral intracerebral pharmacological manipulations in awake songbirds. Our group recently used this approach to uncover the first direct evidence that a classic steroid hormone (i.e., estrogen) directly modulates auditory processing in the vertebrate brain by regulating local neurotransmission via nongenomic mechanisms (42).

[FIGURE 1 OMITTED]

This method involves implanting animals with a head-post and a recording chamber, under anesthesia. After recovery, awake animals are repeatedly adapted to the restraint procedure and sensory-driven neurophysiological recordings are obtained bilaterally (pre-drug session). Subsequently, two independent glass pipettes are lowered into each hemisphere, to converge on the recording electrodes. Vehicle is injected unilaterally and agents of interest are infused contralaterally, using independent, calibrated microinjectors; each animal serves as its own control. The microinjectors are also used to supply maintenance doses of both solutions during the repetition of the stimulus set (drug session). This approach provides a carefully controlled means of coupling bilateral intracerebral pharmacology with multielectrode recordings in animals that are awake, enables the rigorous assessment of the magnitude and breadth of the impact of a given agent on neural responses, and is useful to show the spatial and temporal spread of the agent's effects.

There are three main limitations of this method. First, under no experimental circumstances can the contributions of stress-related signaling be completely accounted for, given that, despite extensive adaptation, animals are kept restrained during this procedure. Second, recording in the absence of movement might constrain complex neuromodulatory interactions that may occur in a freely behaving animal. Finally, this approach has some of the common constraints of standard extracellular electrophysiological recording methods, such as low spatial resolution and potential electrode-mediated damage. Despite these limitations, this method provides a powerful means of locally manipulating the neurochemistry of neuronal ensembles to investigate directly its effect on neural function in alert animals. In this protocol, we provide solutions on how to troubleshoot common problems encountered in all steps of our method. Although these protocols were developed for use in songbirds, they can, in principle, be applied to any small vertebrate, as has been recently confirmed by our group in mice. The wide application of this approach is expected to help establish causal relationships between the neurochemical, molecular and cellular biological properties of neural circuits, and their functionality in the awake vertebrate brain.

BOX 1 | CONSTRUCTING ELECTRODE ARRAYS

Low-cost multielectrode arrays can be constructed for use with most

recording systems. We describe here an array designed for use with

the HS-16 preamplifier (Neuralynx) that uses an Omnetics nano NPD series connector (parts are compatible with Omnetics A-7518-001, empty connector shells and strips containing several gold pins can be purchased from Omnetics).

1. Cut electrodes to 1 cm in length.

2. Etch electrodes according to procedure described by Hofer and Klump43.

3. Scrape insulation from the blunt end of the electrode.

4. Connect blunt end of the electrode to a gold pin that fits within the connector stated above.

5. Crimp the stripped end of the electrode into the pin and repeat this step for all desired recording channels.

6. Measure impedance from the gold pin to verify connectivity.

7. Cut plastic connector shell to desired size (e.g., HS-16 requires 20 channels).

8. Insert each electrode into its respective slot in the plastic connector shell. Many arrays will be used with differential headstage amplifiers and therefore will likely also require the construction and placement of flexible narrow cables of near 0 Q resistance into the ground or reference positions.

* CRITICAL STEP To avoid a potential source of cross talk, ensure that no physical contact occurs between the bases of the inserted gold pins.

9. Insulate recording channels by applying plastic Epoxy between all electrodes.

* CRITICAL STEP The interelectrode distance required to eliminate detection of the same current source across multiple channels is a

function of a combination of variables including, but not limited to, cell sizes and electrode resistance. However, we have found that spacing electrodes by > 100 urn in the caudomedial nidopallium (NCM) eliminated cross talk for all arrays constructed by our group.

Materials

* Experimental animals of interest. We have used zebra finches (Taeniopygia guttata) and mice (Mus musculus), obtained from commercial suppliers. Our studies have been conducted in accordance with the National Institutes of Health guidelines and have been approved by the University of Rochester Committee on Animal Resources * CRITICAL This protocol describes methodologies used in adult animals of either sex.! CAUTION The use of animals must conform to institutional and national regulations.

* Isoflurane (Baxter Healthcare, cat. no. 1001936040)! CAUTION Toxic hazard; can irritate the respiratory pathways and cause headache in sensitive individuals; use in a well-ventilated room with a scavenger system in place.

* Nembutal (Sigma, cat. no. P3636)

* Surgical tools (Fine Science Instruments; scalpel handle, cat. no. 10003-12; blades, cat. no. 10060-00; metal spatula, cat. no. 10089-11; and fine forceps, cat. no. 11252-00)

* Jordan Canal Incision Knife (Storz, cat. no. N1704-03)

* Straight-handled razor blade (VWR, cat. no. 101132-582)

* Ortho-Jet dental acrylic (Lang Dental Manufacturing, cat. no. 1323) ! CAUTION Solvent can irritate skin and respiratory pathways; use only under well-ventilated conditions and with appropriate personal protective equipment.

* Head-post (Crown Bolt, cat. no. 148451) * CRITICAL Shank diameter 1.5 mm, diameter of (flat) nail head 3 mm.

* Lactated Ringer Solution (Hospira, cat. no. 0409-6664-02)

* Gold pins (Omnetics, cat. no. A9941-001)

* Contact insulators (Omnetics, cat. no. A9942-024)

* Fast setting Epoxy (Loctite)

* Plasticine (Roseart)

* Electrodes. Suitable electrodes used by our group: tungsten (0.0014", California Fine Wire, cat. no. H-ML, lot number 22776), 90% platinum-0% iridium (0.0015" Teflon insulation; A-M Systems, cat. no. 7750) and NiChrome (0.0015" with Formvar insulation; A-M Systems, cat. no. 761500)

* CRITICAL Electrode diameters should fall between 12 and 25 [micro]m for the songbird preparation. Suitability of electrodes must be determined by the end user (see Box 1).

* Glass pipettes (Drummond Scientific Company, tip i.d. ~20 [micro]m; cat. no. 5-000-1001) * CRITICAL Inner diameter of pipette should match the outer diameter of plunger for injections, but should move freely (see Box 2).

* Petroleum jelly (i.e., Vaseline)

* 6" Cotton-tipped applicators (Puritan, cat. no. 10806-005)

* Gauze pads (VWR, cat. no. 82030-638)

* 50-ml conical centrifuge tubes (VWR, cat. no. 21008-940) Useful nonessential items

* Electrical tape

* Liquid electrical tape (Micro-Tools, cat. no. 10-1762-F)

* Conductive silver paint (SPI, cat. no. 04998-AB)

* High vacuum grease (Dow Corning, cat. no. 05054-AB)

EQUIPMENT

* Vapomatic anesthetic vaporizer (A.M. Bickford, cat. no. 20010)

* Water bath (VWR, cat. no. 80086-982)

* Recording system with preamplifier and amplifier (Neuralynx, cat. no. Cheetah 32, 16-channel configuration and HS-16 CNR). This system is equipped with a 12-kHz low-pass filter in the final output stage to provide noise reduction and anti-aliasing functions. Sampling rate is typically at 30.3 kHz.

* Flexible fiber optic lighting (Chiu Technologies, cat. no. Ultra Compact FO-50)

* Surgical microscope (Wild Heerbrugg, cat. no. M7A)

* Parallel rail stereotaxic frame (Lab-Tronics, cat. no. G-109)

* Free stereotaxic base with single arm (Kopf Instruments)

* Minimum of five arm mount micromanipulators (Kopf Instruments, cat. no. 1460)

* Head-post adapter (custom made; see Box 3).

* Two hydraulic calibrated micropumps (Narishige, cat. no. MMO-220A)

* Microfil (World Precision Instruments, cat. no. CMF34GXXL)

* Lightweight cordless drill (Dremel)

* Small diameter drill bit (Caulk Dentsply, cat. no. 2 HP)

* Speakers (Altec Lansing, cat. no. VS2521) * CRITICAL Speakers used by us had a flat frequency in the relevant output range for songbird studies. However, confirmation of the flat frequency range should be verified by the investigators carrying out auditory physiology studies.

* Glass electrode puller (David Kopf Instruments, cat. no. 700C)

* Signal generator (Wavetek, cat. no. 184) * CRITICAL Signal generators are useful for troubleshooting sources of electrical noise and cross talk between recording channels.

* Impedance meter (World Precision Instruments, cat. no. SYS-OMEGAZ)

* Computer and monitor (Workstation, Dell, cat. no. T3400 and monitor, Dell, cat. no. G2210) * CRITICAL Recording and analyses of multichannel electrophysiological data are memory intensive. Performing recording and analysis on a system exclusively dedicated to electrophysiology work with at least 4 GB RAM are recommended.

* Soundproof recording room

* Sound-attenuation boxes that can accommodate a single cage

BOX 2 | LOADING AND SEALING GLASS PIPETTES

Before loading and sealing the glass pipettes with solutions of interest, it is necessary to calibrate the hydraulic microinjectors to ensure that adequate injection volumes are achieved. Although this procedure will vary for different brands of microinjectors, we calibrate our micropumps by measuring the number of spins of the hydraulic wheel (which vertically displaces the pump head and plunger) necessary to eject 1 ul of our solution of interest. Typically our working configuration requires five full spins to yield this volumetric displacement. Therefore, one full rotation of

the hydraulic wheel ejects 200 nl solution. Given that our hydraulic wheels are divided into 250 units, this system enables the ejection of 0.8 nl per unit.

1. Using capillary action, back-fill each pulled glass pipette with a solution of interest.

2. Tap the pipette to ensure that solution moves into the tip.

3. Place the plunger for the hydraulic pump into the glass pipette.

4. Seal the junction of the glass and plunger with petroleum jelly or grease.

5. Slightly depress the plunger to ensure that a small bead of fluid is ejected.

* CRITICAL STEP This bead will not likely be visible to the naked eye and will require the use of a dissecting scope or other

magnifying device and a light path targeted to the tip of the pipette. If no solution bead is observed, the tip of the pipette should be slightly widened by very lightly touching the tip with the base of a fine forceps. Optimal tip aperture should approximate 20 um and should not be larger than 30 um.

6. Rinse the pipette tip with a bead of saline after ejecting solutions from the pipette.

BOX 3 | CONSTRUCTING A HEAD-POST ADAPTER

The clamp for securing the head-post was custom made within our laboratory. The specific steps required to assemble this adapter may differ by stereotaxic frame and part availability, but generally involve the following:

1. Select a thumbscrew that has more than 2 cm threaded length. According to the thread selected, core a corresponding hole into the rostral end of the stereotaxic frame.

2. Cut a piece of aluminum or comparable alloy that is 5 mm in thickness, 4-5 cm in length and 2 cm width. Core a hole through one end of this metal piece that accommodates the threaded end of the thumbscrew. These steps are used to tighten the aluminum rectangle onto the stereotaxic frame.

3. Select/cut a stainless steel rod of approximately 6 cm in length and 5.5 mm in diameter. Thread one end of this post.

4. Using the aluminum alloy rectangle attachment to stereotaxic, cut a second threaded hole into the end of the rectangle that is opposite to the hole designated for the thumbscrew. The thread on the second hole must correspond and lock the thread machined onto the metal post. It can be noted that when secured together this adapter must stay firmly in place. The final organization of this setup will be an aluminum alloy arm that extends in from the base in parallel with the stereotaxic frame joined to a stainless steel post that extends vertically.

5. Acquire a Kopf micromanipulator clamp that is a modified c–clamp with an Allen screw on one end and a triangular form with two holes for thumbscrews on the opposite end. The c–clamp end of this piece is placed over the metal rod and secured in place by tightening the Allen screw.

6. Obtain two washers that are more than 2 cm in outer diameter and that have internal openings that will accommodate the diameter of the thumbscrew fitted to the c–clamp adapter. Generally the diameter of the inner hole on washers more than 2 cm will be larger than the head of the thumbscrew or Allen screw supplied with the Kopf clamp. The two washers are used to generate opposing pressure as the screw is tightened onto the c–clamp that secures the head–post into a final and fixed position.

PROCEDURE

Animal preparation (head-posting and creation of recording chamber) * TIMING ~30 min per animal

1| Anesthetize animals with isoflurane in oxygen. To this end, induce the anesthetic plane with isoflurane administered at 2 ml min - 1 in 0.8 ml min - 1 oxygen. Once deeply anesthetized, subjects will remain

unconscious if outflow is positioned under the beak at a flow rate of 0.5-2 ml min - 1 of anesthetic with no change in the oxygen flow rate. Gas anesthetic can be substituted by Nembutal (50 mg kg - 1).

2| Remove feathers from the dorsal surface of the head by gently pulling them against the natural direction of the calami.

3| Carefully and firmly place animal into the stereotaxic device. ! CAUTION Use of hollow plastic ear bars with rubber wide end adapter is recommended to avoid potential damage to the eardrum.

4| Make a midline incision from the level of the frontal telencephalon to the cerebellum. This incision begins roughly between the eyes, ~3 mm caudal to the upper beak insertion, and ends ~3 mm before reaching the muscle insertions at the caudal aspect of the skull. Retract loose skin.

5| Scrape the skull free of periosteum with cotton-tipped applicators and allow it to dry (3-5 min).

* CRITICAL STEP Dental acrylic will not adhere to a smooth or moist surface.

6| Using a razor blade, remove the upper bone layer of the skull to open a small oval window over the bifurcation of the midsagittal sinus, which should be visible through the remaining bone layer.

* CRITICAL STEP In songbirds, this landmark in the brain surface is used to measure stereotaxic coordinates. In our case, we recorded bilaterally from the caudomedial nidopallium (NCM), the songbird analogue of the mammalian auditory association cortex, which spans mediolaterally from 0.1 to 1.2 mm. Thus, our oval window spanned mediolaterally from approximately + 1.5 to - 1.5 mm, thereby uncovering the NCM of both hemispheres. Regardless of the recording area of interest, ensure that the opened window is slightly larger than the general area to be recorded, to allow for bilateral placement of glass pipettes during the recording session.

* CRITICAL STEP This step is not necessary for research conducted in rodents, given that stereotaxic coordinates can be achieved without opening of the skull (e.g., using bregma as a reference).

7| Drill a small opening over the frontal telencephalon, along the midline, to accommodate the flat head of the head-post. This opening should only be made through the superficial layer of bone, while maintaining the remaining, deeper layer intact.

8| Prepare the intact skull that surrounds both openings (oval window and head-post site) by completely drying the bone with a cotton-tipped applicator. Repeatedly and gently score the outer bone layer surrounding these openings.

* CRITICAL STEP Scoring the outer bone layer is central to ensure that the dental acrylic does not detach from the skull.

9| Apply dental acrylic to form a recording chamber around the open oval window. The walls of this chamber must be built up to a consistent height (at least 1 mm).

* CRITICAL STEP Dental acrylic that is used for well construction should be prepared into a consistency that is comparable with toothpaste. A spatula should be used to quickly and carefully apply the dental acrylic to the scored bone around the opening in the skull. These conditions will prevent the dental acrylic from invading the craniotomy.

10| Using one of the arms of the stereotaxic device, carefully lower the head-post to the opening over the frontal telencephalon, on the dorsal surface of the skull, directly above the caudal-most point of the orbital socket. The head-post should be oriented orthogonally to the brain surface.

11| Apply dental acrylic over the head-post section that is in contact with the skull. To ensure the integrity of the head-post attachment, spread dental acrylic beyond the skull opening to include surrounding, scored bone regions.

* CRITICAL STEP The head-post must remain perfectly still while drying dental acrylic; this typically corresponds to a period of approximately 15 min.

12| Remove animals from the stereotaxic device and allow them to recover from anesthesia.

* PAUSE POINT Animals should be allowed to recover for a minimum of 2 d. However, animals can be kept in a flight aviary or cage for days to weeks before proceeding to neurophysiological recording sessions.

Acclimation sessions * TIMING ~8 h

13| Move animals from the common aviary to the acoustically isolated recording room. In addition to the recording setup, a stereotaxic device and two calibrated microinjectors, this room contains three custom-made sound-isolation boxes. Each box is double-lined with soundproofing material and accommodates a single birdcage. These individual boxes are used to ensure that the experimental animals inside the acoustically isolated recording room will not hear each other.

14| Gently restrain the animal by placing it inside a body restraint tube, and fix it through the head-post to the adapter in the stereotaxic device.

* CRITICAL STEP As a body restraint tube we use a 50-ml plastic culture tube. To fabricate this device, cut a hole in the narrow end of the tube to create an opening for the head and neck. Next, cut the entire tube in half for its full length. Finally, to rejoin the tube, adhere a piece of tape across the incision for the full length of the tube on only one side. This configuration enables the tube to be easily opened and closed. To secure the tube in the closed position, place a second piece of tape orthogonally through the remaining incision.

* CRITICAL STEP To minimize discomfort and maximize fit, cover the inner aspect of the tube with standard gauze pads. In addition, holes can be punched into the sides of the restraint tube to facilitate core temperature regulation by the bird.

! CAUTION Do not restrain the bird too tightly to avoid suffocation.

* TROUBLESHOOTING

15| Maintain the animal restrained in the device for 30 min in the presence of investigator.

* TROUBLESHOOTING

16| Remove the animal from the stereotaxic device and body restraint tube, and place it inside an individual sound- isolation box. Maintain the animal in this isolation box for 1.5 h.

17| Repeat Steps 14-16 for a minimum of four additional times. After the last acclimation session, animals should be kept overnight in the individual sound-isolation boxes.

* CRITICAL STEP The number of acclimation sessions may be initially determined as the number of restraint trials required for an animal to abolish its natural resistance to placement in the body restraint tube and affixation to the stereotaxic device. On average, three sessions are required to adapt zebra finches to the restraint procedure. As a conservative measure, we subject animals to two additional restraint sessions to ensure that all animals are fully acclimated to the restraint procedure. Studies conducted in other species will require different numbers of acclimation sessions, to be determined by the investigator, which should also be defined based on behavioral indicators, and ensured by the addition of 2-3 supplemental restraint sessions.

18| The next day, subject animal to a final acclimation session, as detailed above, and return it to the individual isolation box. After this last acclimation session, animals are considered ready for the electrophysiological recording sessions.

* TROUBLESHOOTING

Craniotomy * TIMING ~30 min

19| Remove animal from the individual isolation box to the soundproof recording room.

20| Gently place animal in the body tube and fix it to the stereotaxic device through the head-post following precisely the same routine used during acclimation sessions.

* CRITICAL STEP Head position should be absolutely stable.

21| Place a small piece of plasticine on the underside of the restraint tube to promote stability during the recording session.

22| Under visualization with the dissecting scope and using a Jordan Canal Incision Knife, gently remove the remaining bone layer inside the recording chamber.

* CRITICAL STEP Bone removal must be performed gently to avoid bleeding and swelling. In addition, bone should be cut with the Jordan knife rather than broken, to avoid having sharp bone fragments accidentally perforate the underlying brain.

* TROUBLESHOOTING

Acute electrode placement * TIMING ~30-45 min

23| Make two midline incisions through the dura mater of approximately 2-3 mm in length over the target coordinate on either side of the midsaggital sinus carefully using the sharp end of a sterile, new 26 gauge 5/8 inch needle; slightly retract the dura mater to avoid contact with electrodes.

* CRITICAL STEP Once the dura mater is cut the brain must constantly be kept moist with warmed saline solution. To this end, fill the recording chamber with warm saline that has been preheated to 37.5 [degrees]C.

* TROUBLESHOOTING

24| Connect the electrode array containing the eight electrodes to the HS-16 preamplifier. The preamplifier should be mounted on an adapter attached to the micromanipulator arm.

25| Optional: dip electrodes in a solution containing a dye of interest for follow-up histology (DiI, 1% pontamine sky blue, wt/vol).

26| Remove saline solution from the recording chamber and, using the micromanipulator arm, position the reference electrodes directly over the bifurcation of the midsagittal sinus (zero point).

* CRITICAL STEP Ideally saline should never be fully removed, but rather only a thin film of solution should be left while positioning the array and/or pipette. As much as possible, the brain should not be exposed to air as the absolute minimum exposure is required to create optimal recording conditions.

27| Guide the electrode array into the desired stereotaxic coordinates with the micromanipulator arm. For NCM recordings, guide the most medial electrodes of the array to AP: 0.5 mm; ML: [+ or -]0.2 mm; and DV: 0.65 mm.

* CRITICAL STEP In our multielectrode configuration, we guide four electrodes in the control hemisphere, and an additional four electrodes in the experimental hemisphere (array recording).

* TROUBLESHOOTING

28| Stimulate birds with bursts of white noise (500 ms duration, 3 s interstimulus interval; 70 dB SPL (sound pressure level)) to locate responsive sites during the final approach to the desired location.

29| Using the fine manipulator, slowly advance the electrodes (5 (im min - 1) to maximize the number of channels exhibiting evoked single-unit activity.

* CRITICAL STEP It is unusual to detect hearing-driven single-unit activity across all channels with either array or independently movable electrode configurations. We typically proceed with the recording sessions if 75% (3/4) of our electrodes yield high-quality responses in each hemisphere.

* TROUBLESHOOTING

30| Once desired location and the number of responsive sites are achieved, refill the recording chamber with warmed saline solution and interrupt white noise presentation.

Electrophysiological recordings and local pharmacological manipulations * TIMING ~2-3 h

31| Stimulate animals pseudorandomly with four conspecific song segments (25 times per song; range of stimulus durations: 067-0.73 s; interstimulus interval: 5 s; 70 dB SPL). Spontaneous and song-evoked auditory activities should be recorded bilaterally (predrug session).

* CRITICAL STEP Microelectrode signals are amplified and filtered (low pass 5 kHz, high pass 500 Hz) and digitized with the acoustic stimuli.

* TROUBLESHOOTING

32| After completion of the pre-drug stimulation session, remove saline solution from the recording chamber and drive one glass pipette (tip i.d. ~20 ([micro]m) containing vehicle solution in the control hemisphere and a second pipette containing the pharmacological agent of interest in the contralateral (experimental) hemisphere.

* CRITICAL STEP Channels showing the most reliable and stable song-evoked responses should be targeted by the glass pipettes.

* CRITICAL STEP Pipettes should enter each hemisphere at a 30[degrees] angle. Linear distances from the target electrodes on the brain surface, and depth required to approach the electrode tips should be calculated trigonometrically.

? CRITICAL STEP In our preparations we typically target the tips of our glass pipettes to reach ~20 below the electrode tip of interest.

33| Refill the recording chamber with saline solution and allow the brain/electrode/pipette ensemble to settle for at least 5 min.

34| While in silence, inject a 20-nl loading dose of vehicle in the control hemisphere and 20 nl or the agent of interest in the experimental hemisphere, through the two Narishige microinjectors, over the course of 2 min. Collect spontaneous neural activity during and after the infusion of both the solutions (5-10 min).

* CRITICAL STEP Diffusion properties will be different across drugs and will depend on its concentration and choice of solvent. Thus, a careful quantification of diffusion of each drug of interest should be carried out before proceeding to electrophysiological recordings to determine appropriate loading and maintenance doses.

* TROUBLESHOOTING

35| Stimulate animals once again with the auditory stimulus set containing the four conspecific songs (as in Step 31) while recording both spontaneous and song-evoked activities.

36| Inject solution maintenance doses in both hemispheres every 2-5 min, each consisting of 5-10 nl, throughout the duration of the auditory stimulus trials. These maintenance doses are required to maintain steady-state levels of each solution within each NCM (32,42).

* CRITICAL STEP The interval and volume of maintenance doses will directly depend on the spread and degradation kinetics of each drug of interest. Thus, a carefully conducted drug titration curve should be performed in preliminary studies before carrying out definitive experiments.

* CRITICAL STEP We determined that a single 100-nl injection of vehicle in NCM covers an average radius of ~250 |am42, which ensures that the combination of loading and maintenance doses provides relatively broad coverage to impact most or all electrodes, while maintaining the diffusion of solutions restricted to NCM. As indicated above, however, diffusion properties will vary across drugs and should be carefully quantified before electrophysiological recordings.

37| Carefully and slowly retract each glass pipette.

38| Optional: conduct electrolytic lesions in both hemispheres (15 of current for 10 s through the electrodes) to confirm electrode placement through Nissl histochemistry (32,42).

* TIMING

Steps 1-12, animal preparation: 30 min per animal

Steps 13-18, acclimation sessions: 8 h

Steps 19-22, craniotomy: 30 min

Steps 23-30, acute electrode placement: 30-45 min

Steps 31-38, electrophysiological recordings and local pharmacological manipulations: 2-3 h

* TROUBLESHOOTING

General comments

(1) Although the protocol described here focused on electrode array recordings, we and our collaborators have also successfully carried out this method with independently movable electrodes (Thomas multielectrode microdrive), where we typically placed four electrodes in the experimental hemisphere and three electrodes in the control hemisphere (32,39-41).

(2) Damage to the midsagittal sinus is often fatal. This damage can occur as a function of direct trauma with a surgical instrument, but may occur more frequently by mechanical movement of the meninges, which causes the vessel to tear. Moderate bleeding, however, can usually be stopped by applying light pressure to the bleeding source for several minutes with a sterile cotton-tipped applicator. If such an intervention is required, the subject must be supplemented with fluids. A single, subcutaneous injection of 0.25 ml of Ringer's solution, placed in the interscapular region, should be conducted. After supplemental fluid injection, procedure can be resumed.

Troubleshooting advice can be found in Table 1.

ANTICIPATED RESULTS

Song-driven electrophysiological responses of NCM neurons are expected to be differentially modulated by dissimilar pharmacological agents. Our own studies involved the use of various agonists and antagonists targeting classic neurotransmitter and neuroendocrine systems in NCM. Examples of representative results obtained with our method are illustrated in Figure 2. The further development and implementation of the protocol detailed here is anticipated to continue to be a highly valuable means to uncover fundamental properties of how the vertebrate brain processes complex, behaviorally relevant communication signals and is expected to open new research avenues that aim to causally link how the brain's neurochemistry modulates sensory responses and alters neural network function in the awake state.

[FIGURE 2 OMITTED]

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AUTHOR CONTRIBUTIONS L.A.T., T.A.T. and R.P. extended the method; L.A.T., J.K.J. and R.P. refined the method; L.A.T. customized the stereotaxic equipment and collected the data presented in the paper; L.A.T., T.A.T., J.K.J. and R.P. wrote the paper.

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TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason

14, 15, 18	Head-post and recording chamber separate from the skull	Skull was not dry before dental acrylic application
		Bone surface was not rough before acrylic application
		Periosteum was not fully reflected from skull surface
		Head-post was not stable during the curing phase
15, 31	Birds overheat and lose consciousness	Restraint tube and gauze wrapping do not allow ventilation
22, 23	Brain swelling	Aggressive and hurried bone removal

		Depression or puncture of brain while removing the inner skull layer
		Depression or puncture of brain while cutting or reflecting the dura mater
		Use of improperly sterilized surgical instruments
27, 29	Electrodes bend or do not enter brain easily	Entry trajectory is not orthogonal to brain surface
		A thin inner layer of meninges is still present over brain surface
		Brain surface has dried

31	Periodic EMG occludes song-driven responses	Animal responding to outside noise
		Discomfort in restraint tube
34	Isolated cell disappears immediately with drug injection	Mechanical displacement of cell and/or electrode

Step	Solution
14, 15, 18	Reanesthetize animal, fully reflect periosteum, rescrape the surface of skull cutting shallow bone flaps into dorsal bone layer and reassemble recording chamber
	Reapply head-post and dental acrylic

15, 31	Cut series of 5 mm holes into the sides of the restraint tube
22, 23	Gently and slowly cut the inner skull layer
	Use additional care during bone removal; use alternative hand stabilization methods
	Use additional care while manipulating the dura mater; use instruments with newly sharpened cutting edges
	Autoclave instruments before use and bead-sterilize them between animals
27, 29	Reposition electrodes for perpendicular entry into the brain
	Gently stroke a fine gauge needle over the opening

in the dura mater to tear back any remaining tissue

Fill the chamber with saline solution and allow to hydrate for several minutes

31 Isolate recording room from laboratory traffic or adequately soundproof recording room

Loosen the gauze or restraint tube enough to increase comfort of subject

Loosen tape securing restraint tube closed

34 Place the pipette into target position before inserting electrodes

Inject solution more slowly

Improve the stability of pipette holder

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