Title

In vivo detection of fluctuating brain steroid levels in zebra finches.

Journal

Cold Spring Harbor Protocols, 2014(12)

Authors

Ikeda, Maaya
Rensel, Michelle
Schlinger, Barney
et al.

Publication Date

2014-10-23

DOI

10.1101/pdb.prot084616

Peer reviewed

HHS Public Access

Author manuscript

Cold Spring Harb Protoc. Author manuscript; available in PMC 2015 December 01.

Published in final edited form as:

Cold Spring Harb Protoc. ; 2014(12): 1267–1272. doi:10.1101/pdb.prot084616.

In vivo detection of fluctuating brain steroid levels SHORT

Maaya Ikeda[1], **Michelle A. Rensel**[2], **Barney A. Schlinger**[2], and **Luke Remage-Healey**[1]

[1]Neuroscience and Behavior Program, Center for Neuroendocrine Studies, University of Massachusetts, Amherst, MA 01003

[2]Department of Integrative Biology and Physiology, University of California, Los Angeles, Terasaki Life Sciences Building, 610 Charles E. Young Dr. East, Los Angeles, CA 90095

Abstract

This protocol describes a method for in vivo measurement of steroid hormones in brain circuits of the zebra finch. In vivo microdialysis has been used successfully to detect fluctuating neurosteroids in the auditory forebrain (Remage-Healey et al., 2008; 2012; Ikeda et al., 2012) and in the hippocampus (Rensel et al., 2012; 2013) of behaving adult zebra finches. In some cases, the steroids measured are derived locally (e.g., 'neurosteroids' like estrogens in males) whereas in other cases the steroids measured reflect systemic circulating levels and/or central conversion (e.g., the primary androgen testosterone and the primary glucocorticoid corticosterone). We also describe the method of reverse-microdialysis ('retrodialysis') of compounds that can influence local steroid neurochemistry as well as behavior. In vivo microdialysis can now be used to study steroid signaling in the brain for a variety of experimental purposes. Furthermore, similar methods have been developed to examine changing levels of catecholamines in behaving zebra finches (e.g., Sasaki et al., 2006). Thus, the combined study of neurochemistry and behavior in a vocal learning species now has a new set of powerful tools.

MATERIALS

Reagents

aCSF (artificial cerebrospinal fluid)

*Our formulation of aCSF includes 1% BSA to increase solubility of lipophilic steroids, and is outlined in previous work (*Remage-Healey et al., 2008*, 2010).*

Reagents for aCSF are dissolved in Ultra pure ddH2O (~18 MΩ). The pH of aCSF should be adjusted to 7.4–7.6 and should be stored at 4°C in the dark for maximum of 4 weeks to avoid contamination. To increase detection of lipid-soluble molecules like steroids we add BSA (A1470, Sigma) to 50 ml aliquots and filter the final solution (0.2 micron syringe filters) to eliminate suspended particles. This can reduce the likelihood that particles will clog inside FEP tubing and probes.

CORRESPONDING AUTHOR: Luke Remage-Healey, 413-545-0772, healey@cns.umass.edu.

Validation experiments indicated that the addition of BSA caused interference with the ELISA for CORT. Current experiments with CORT use a perfusion medium that is BSA-free (Dulbecco's phosphate-buffered saline). It is always essential to test the perfusion medium on the hormone assay alongside dialysate from experiments.

General anesthetic:

Equithesin (IM):3.2 mg per kg of body weight)

Isofluorane can be used as an alternative

Local anesthetic (e.g., 2% lidocaine in ethanol)

Equipment

Acoustic attenuated chamber with one-way glass partition and 10 mm portal for microdialysis inflow/outflow tubing (Eckel Industries, Audiometric Booth AB08)

Microdialysis probe (CMA Microdialysis; CMA7, 1 mm cuprophane probe membrane)

This probe membrane has a 6 kDa cutoff, which allows the passive diffusion of small molecules and pharmacological agents, like steroids and enzyme inhibitors (~300 Da).

Microdialysis guide cannula (CMA7)

Tether / arm (modified sleeve of Fisher laboratory tape or Instech counterbalanced lever arm for mice) See Fig. 1.

Probe/guide clip (CMA)

Dissection scope (Zeiss OPMI 1)

Syringe pump (PHD 2000 Infusion; Harvard Apparatus)

Needles (22, 26, 30 gauges)

Stereotaxic apparatus

We use a custom-made stereotaxic by Herb Adams. Other labs have successfully employed the small animal version of David Knopf instruments for zebra finch stereotaxic surgeries.

Cotton-tipped applicators

Dental acrylic (Perm Reline and Repair Resin, Hygenic # H00358).

Cyanoacrylate (e.g. 'Vetbond')

Kimwipes

Laboratory tape

Hotplate/stirrer

DC Heating pad (FHC Neurocraft)

FEP tubing (CMA)

FEP tubing adaptors (CMA)

0.5 cc Syringe (for injections)

Swivel (dual-channel, quartz-lined, Instech 375/D/22QM)

Microdialysis tubing adaptors (for connection to swivel; Instech MC015)

Cage (csnstores.com BBX1047 Blue Ribbon Pet Arch Rosf Bird Cage in White/yellow)

Food dish and perches

Syringe for pump (1.0 cc set to infuse at 2.0 μl/min)

Syringe filters (0.22 μm)

Standard surgical tools

Refrigerated fraction collector (CMA 470;

This is optional but is very useful for collection of a large number of samples.

METHOD

Surgery (30 min – 1 hr)

1 Weigh the animal. Food-deprive the animal for ~40 min prior to anesthesia to allow crop emptying and to prevent aspiration pneumonia during surgery.

2 Heat equithesin in mild warm water bath to dissolve any crystals in the solution.

3 Administer one intramuscular injection of 50 μl of equithesin to breast muscle. For animals younger than 100 days old and animals weighing lower than 12g, inject smaller amount (30 ul–45ul).

Wait 20 min until the bird is deeply anesthetized.

For equithesin, breathing rates should be short and shallow. Labored breathing is a sign that the surgery should be immediately terminated. Note: if using isoflurane for anesthesia, induce anesthesia using 2–3% isoflurane with 0.4L/min oxygen. Monitor depth of anesthesia as described above; note that anesthetic will take effect more quickly (i.e., within ~5 min). Incrementally decrease isoflurane throughout procedure to prevent overdose while maintaining depth of anesthesia.

4 Wrap the bird in a kimwipe ‘tunic’ and secure the tape around the body loosely together using tape.

Animals during survival surgery are more comfortable during the surgery if they are lightly restrained in the kimwipe, similar to the ‘Principles of Low Stress Restraint’ outlined by Grandin, (2007).

5 Remove feathers from the head and around ear canal using scissors.

An alternative to this is brushing the feathers back gently with a cotton-tipped applicator soaked in 20% ethanol.

6 Secure the bird on the stereotaxic apparatus on top of a heating pad heated to ~34° C.

Set the angle of the head to ensure skull plane of the target area is perpendicular to the cannula entry point. For implantations targeting the caudal telencephalon this head angle is typically 45 degrees.

7 Inject 10–15 μl of 2% lidocaine subcutaneously into the scalp.

Smooth the lidocaine into the working area and confirm that the local anesthetic is effective by testing the scalp with light forceps.

8 Using iridectomy scissors make incisions to the skin along the midline and expose the skull. Make lateral incisions from this medial incision at both rostral and caudal ends, forming an 'I'.

9 Locate the bifurcation of the mid-sagittal sinus (point-of-origin).

This can be best accomplished by making a small window incision (1 mm × 1mm square, removed) in the upper leaflet of skull surrounding the sinus area. Leave the lower leaflet intact.

10 Secure a 26-gauge needle to a probe/gauge clip attached to a micromanipulator and place the tip of the needle on top of the point-of-origin ('zero point') without piercing the lower leaflet. Note the coordinates on the micrometer scales for x and y dimensions on the stereotaxic.

11 Using stereotaxic coordinates create a small hole through both upper and lower leaflets of skull using needle tips.

12 Carefully resect the dura mater inside the hole using a 30-gauge needle and expose the brain surface.

Shallow, lateral resection is essential here to avoid damaging underlying brain tissue.

13 Descend the guide cannula ventral to the target area using pre-established depth coordinates.

Leave the obdurator ("dummy probe"; as shipped in CMA cannula) in the guide cannula

14 Secure the guide cannula to the skull by applying a thin layer of cyanoacrylate around the opening, avoiding direct contact with the surface of the brain.

With subadult animals it is often advisable to use a # 11 scalpel blade to etch small, shallow lines in the skull area surrounding the cannula implantation site to to roughen the skull surface to encourage cyanoacrylate adhesion.

15 While the cyanoacrylate is still drying, apply dental acrylic on top of it using cotton swabs and layering up more cyanoacrylate if necessary.

Dental acrylic should cover the guide shaft of the cannula completely and at least 1/3 of the cannula housing itself.

16 Re-attach the scalp to all remaining exposed skull areas by carefully applying cyanoacrylate under the skin and securing it to the skull and dental acrylic.

17 After surgical recovery monitor and treat the animal for peri-operative pain as necessary with meloxicam or other NSAIDs.

Probe implantation (3–4 days after surgery; 1hr)

18 Fill FEP tubing and the swivel assembled to the microdialysis cage with aCSF.

19 Prime the probe by placing it in the probe clip and immersing it in 100% ethanol in a microcentrifuge tube. While it is still immersed, pump water (20 min) and then aCSF (8 μl/min) into the probe through the inlet tube and attach a microdialysis tether to the probe using a small piece of tape. After flushing, remove the probe from the microcentrifuge tube and implant in the guide cannula (step 20).

This transfer should be completed quickly (< 1 min) so that the microdialysis probe does not dry out and become damaged.

20 Carefully grasp the bird in one hand, stabilizing the head gently with fingertips. With #5 forceps, carefully remove the dummy probe from the guide cannula and replace it with the pre-filled microdialysis probe.

Implantation can be completed under light isoflurane anesthesia to minimize disturbance.

21 Apply small amount of cyanoacrylate to the exterior of the probe housing at the top of the cannula to keep the probe in place.

Make sure the cyanoacrylate is external to the cannula channel to not impact the CNS or probe, and double-check that aCSF is welling out from the outlet tubing after the implantation.

22 Connect the inlet and outlet tubing to rest of the microdialyis tubing inside the chamber.

23 Attach the tether to the arm as in Fig. 1.

Adjust the tether height and lever arm as necessary. Confirm that dialysate is flowing at the correct rate. Begin in vivo experiments 8–12 hr following implantation to allow implantation-induced neurochemical responses to subside.

TROUBLESHOOTING

Problem: aCSF dialysate is not flowing at correct rate.

Solution: To prevent clogging of aCSF perfusate in the FEP tubing and swivel inside the chamber when they are not in use, we recommend flushing ddH2O through the entire system (FEP tubing and swivel inlet and outlet as a chained loop) continuously at 8–10 μl/min for the first 60 min after every use, and to continue flushing ddH2O at 0.5–1.0 μl/min until the next experiment. Also, all aCSF perfusate should be continuously syringe-filtered (0.22 μm filters) before pumping it into the microdialysis setup to prevent particulate buildup.

Problem: FEP tubing is clogged (i.e., no flow through a section of the dialysis setup).

Solution:

1. Double-check that fluid is welling from the syringe tip at the desired flow rate.
2. Run at 8 μl/min for ~1 min to try to flush bubbles or particulate from tubing. Check whether fluid is coming out of each connection of FEP tubing. It is often useful to 'trim' the ends of the FEP tubing at each connection with a sharp razor blade. Clogs often occur at these connection points and trimming them can help avoid replacement of the entire section of tubing.

Problem: The clog is isolated to the bird (i.e., within the probe tubing).

Solution:

1 Make sure the FEP tubing in the tether are not tangled.

2 Remove the tape around the FEP tubing and reapply. At times the rotational torque on the tether can cause constriction of the tubing, disrupting flow through the system.

3 Unfortunately, if these steps are unsuccessful, the experiment is over with this probe.

Problem: The clog is within the swivel.

Solution:

4 Try running briefly at 20 μl/min (without incorporating the probe lead tubing in the loop – this flow rate should never be put through the microdialysis probe in vivo)

5 If a slight amount of perfusate is welling from the swivel, wait 5 min at 8 μl/min. At times, the swivel can be clogged with particulate and/or bubbles and this will flush them.

6 Swivels have a limited lifespan. If the above do not work, replace the swivel.

Problem: The clog is contained within a section of FEP tubing.

Solution:

7 Try running at 20 μl/min about 5–10 min (Just through the tubing; no bird, no swivel)

8 If these don't work, switch out the section of FEP tubing and continue with the experiment.

DETECTION OF STEROIDS

We have used commercially-available ELISA kits for detecting steroids in dialysate collected using the above protocol. For 17-beta-estradiol, we have validated the use of Cayman Chemical's E2 ELISA (www.caymanchem.com; catalog # 582251). Most ELISAs require at least 50 μl of sample stored in −80 °C. If the flow rate of the perfusate is 2 μl/min, collection time should be at least 30 min to obtain 50 μl of sample. For testosterone, we have validated the use of Assay Design's T ELISA (http://www.enzolifesciences.com; catalog # ADI-900-065). In most cases dialysate can be run directly on the ELISA plate since the composition of our aCSF w/ BSA is very similar to the composition of assay buffer. Always run 4–8 control wells for each ELISA that contain aCSF that has not been perfused through the dialysate system, to enable baseline comparisons. For corticosterone, we have validated the use of Cayman Chemical's corticosterone ELISA (www.caymanchem.com; catalog # 500655) with Dulbecco's phosphate buffered saline (Sigma) instead of aCSF.

RETRODIALYSIS

The procedures above can be adapted to reverse-microdialyze (retrodialyze) drugs and steroidogenic compounds in a variety of experimental preparations. When selecting candidate retrodialysis compounds it is important to consider their molecular weight. Typically, proteins and other large molecules are more likely to be bound up in microdialysis tubing, which is why most brain microdialysis research is focused on small neuromodulators that do not approach the molecular weight cutoff of the membrane (typically 6 kDa; but see Ulrich et al., 2013). In awake subjects, in vivo retrodialysis of the estrogen-synthesis inhibitor fadrozole (FAD) rapidly suppresses local neuroestrogen levels in the NCM of zebra finches (Remage-Healey et al., 2008; unpubl obs.). Typical retrodialysis experiments involve a collection of several serial baseline samples using aCSF perfusion, followed by switching the syringe to a new syringe prefilled with a drug of interest (such as FAD dissolved in aCSF) for a brief period (usually 30–60 min), followed by a period of washout in aCSF. When retrodialysis experiments are performed inside a sound-attenuation chamber, all of the syringe-changes can be conducted outside the chamber, allowing an experimental manipulation without disturbing the focal animal. Immediately following the switch to a new syringe solution it is advisable to allow 1–2 min of high-rate of flow (8–10 μl/min), to confirm that the perfusate is flowing and eliminate air bubbles from clogging the FEP tubing. Once flow is re-established the experiment can resume at the desired flow rate (i.e., 2 μl/min). Automated liquid switches (CMA Microdialysis) can also be employed for this purpose. It is important to account for the 'dead volume' of the microdialysis tubing and swivel, given the dimensions of FEP tubing and the total volume of perfusate passing through the swivel. Calculations of dead volume, factoring in the flow rate, allow for precise timing of sample collection during retrodialysis intervals.

Acknowledgments

The authors would like to thank anonymous reviewers for reading over an earlier version of the manuscript; Nigel Maidment and Hoa Lam for initial training and expertise in adapting rodent microdialysis for birds, and Randi Oyama, Andrew Chao, Stephanie Dong, Ashley Paon, Ify Arinze, and Tom L. Cao for technical assistance. Support from NINDS K99/R00NS066179, NIMH061994 and the University of Massachusetts.

References

Grandin, T. Livestock Handling and Transport. 3. CABI; 2007.

Ikeda, M.; McClure, JR.; Remage-Healey, L. Catecholaminergic regulation of estradiol concentrations in the zebra finch auditory forebrain. Society for Neuroscience Abstracts; 2012. 92.07/SS16

Remage-Healey L, Maidment NT, Schlinger BA. Forebrain steroid levels fluctuate rapidly during social interactions. Nat Neurosci. 2008; 11(11):1327–34. [PubMed: 18820691]

Remage-Healey L, Dong SM, Chao A, Schlinger BA. Sex-specific, rapid neuroestrogen fluctuations and neurophysiological actions in the songbird auditory forebrain. J Neurophysiol. 2012; 107(6): 1621–31. [PubMed: 22190616]

Rensel, MA.; Salwiczek, L.; Hsaio, CF.; Xia, S.; Remage-Healey, L.; Schlinger, BA. In vivo microdialysis reveals dynamics of estradiol production in the avian hippocampus. Society for Integrative and Comparative Biology, Annual Meeting; Charleston, SC. 2012.

Rensel, MA.; Schlinger, BA. Real-time measurement of hippocampal corticosterone in a songbird. Society for Integrative and Comparative Biology, Annual Meeting; San Francisco, CA. 2013.

Sasaki A, Sotnikova TD, Gainetdinov RR, Jarvis ED. Social context-dependent singing-regulated dopamine. J Neurosci. 2006; 26:9010–9014. [PubMed: 16943558]

Ulrich JD, Burchett JM, Restivo JL, Schuler DR, Verghese PB, Mahan TE, Landreth GE, Castellano JM, Jiang H, Cirrito JR, Holtzman DM. In vivo measurement of apolipoprotein E from the brain interstitial fluid using microdialysis. Mol Neurodegener. 2013; 8:13. [PubMed: 23601557]

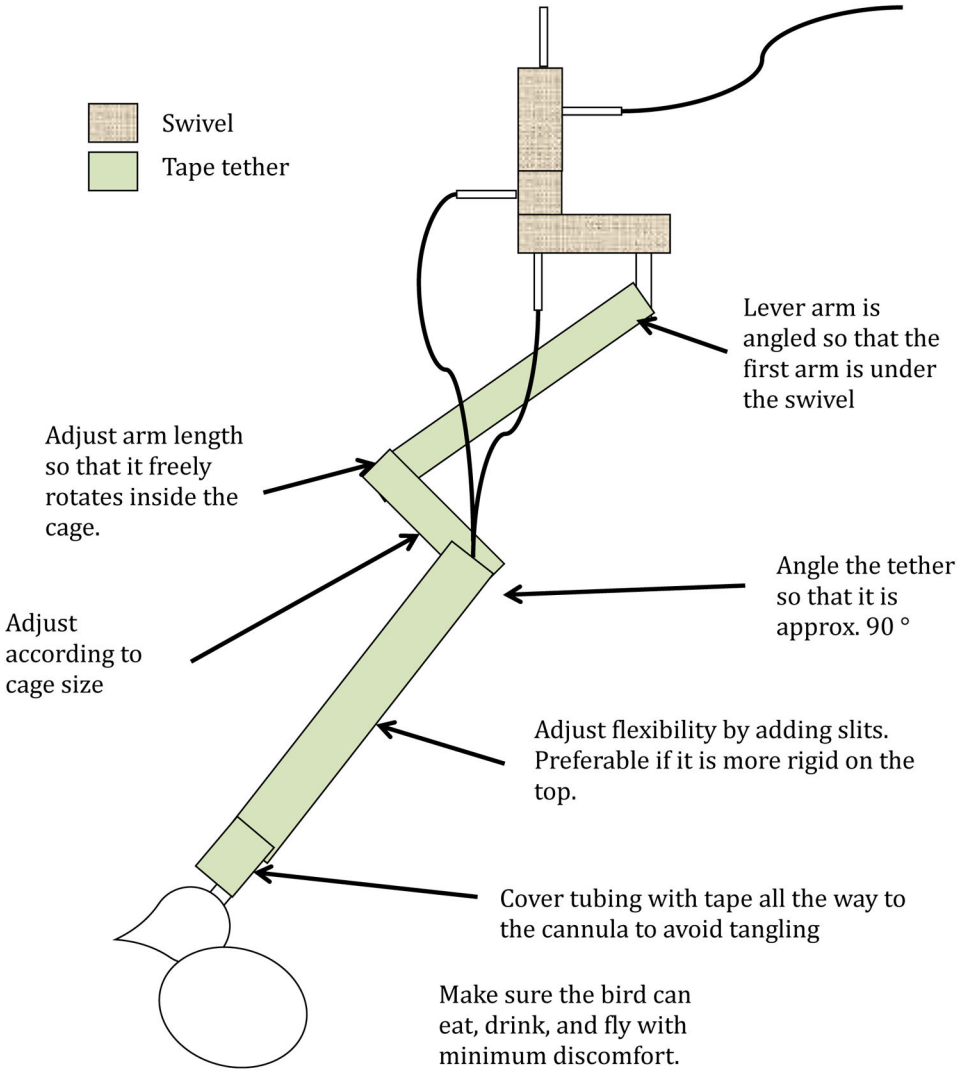

Figure 1.

Schematic of the customizable tether design used in zebra finch in vivo microdialysis. At top is the swivel connected to the cage with inlet and outlet FEP tubing. The tether is angled to provide a lever arm to translate rotational torque from the bird to the swivel while also allowing full freedom of movement (including flight) inside the cage. The top of the tether (lever arm) usually contains either a thin wire or plastic straw (such as can be found protecting CMA-7 probes during shipment) to enable lightweight rigidity, and is then enshrouded with tape. Multiple tape component stages ensure flexibility in addition to a lever arm.