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Measuring in vivo changes in extracellular neurotransmitters during naturally rewarding behaviors in female Syrian hamsters

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Corresponding Author:	Kelsey M. Moore University of Minnesota Twin Cities Minneapolis, MN UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	kmmoore@umn.edu;kelsey.mccall.moore@gmail.com
Corresponding Author's Institution:	University of Minnesota Twin Cities
Corresponding Author's Secondary Institution:	
First Author:	Kelsey M Moore, B.S.
First Author Secondary Information:	
Other Authors:	Brett T Himmler
	Benjamin A Teplitzky
	Matthew D Johnson
	Robert L Meisel
Order of Authors Secondary Information:	
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TITLE:

Measuring *In Vivo* Changes in Extracellular Neurotransmitters During Naturally Rewarding Behaviors in Female Syrian Hamsters

AUTHORS & AFFILIATIONS:

Kelsey M. Moore[1], Brett T. Himmler[1], Benjamin A. Teplitzky[2], Matthew D. Johnson[2,3], Robert L. Meisel[1]

[1]Department of Neuroscience, University of Minnesota, Minneapolis, MN, USA
[2]Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, USA
[3]Institute for Translational Neuroscience, University of Minnesota, Minneapolis, MN, USA

E-MAIL ADDRESSES:

Kelsey Moore (kmmoore@umn.edu)
Brett Himmler (bhimmler@umn.edu)
Benjamin Teplitsky (ben.teplitzky@gmail.com)
Matthew Johnson (john5101@umn.edu)
Robert Meisel (meisel@umn.edu)

CORRESPONDING AUTHOR:

Kelsey Moore (kmmoore@umn.edu)
Tel: (612)-625-0784

KEYWORDS:

Female sexual behavior, male copulatory behavior, reward, nucleus accumbens, dopamine, glutamate, Syrian hamsters, fixed-potential amperometry, enzymatic biosensor.

SHORT ABSTRACT:

This paper details the use of fixed-potential amperometric recordings using carbon fiber electrodes and enzymatic biosensor technology to measure the release of dopamine and glutamate with high temporal resolution during natural rewarding behavior in the female hamster.

LONG ABSTRACT:

The ability to measure neurotransmitter release on a rapid time scale allows patterns of neurotransmission to be linked to specific behaviors or manipulations; a powerful tool in elucidating underlying mechanisms and circuitry. While the technique of microdialysis has been used for decades to measure nearly any analyte of interest in the brain, this technique is limited in temporal resolution. Alternatively, fast scan cyclic voltammetry is both temporally precise and extremely sensitive; however, because this technically difficult method relies on the electroactivity of the analyte of interest, the possibility to detect nonelectroactive substances (*e.g.*, the neurotransmitter glutamate) is eliminated. This paper details the use of a turn-key system that combines fixed-potential amperometry and enzymatic biosensing to measure both electroactive and nonelectroactive neurotransmitters with temporal precision. The pairing of

these two powerful techniques allows for the measurement of both tonic and phasic neurotransmission with relative ease, and permits recording of multiple neurotransmitters simultaneously. The aim of this manuscript is to demonstrate the process of measuring dopamine and glutamate neurotransmission *in vivo* using a naturally rewarding behavior (*i.e.*, sexual behavior) in female hamsters, with the ultimate goal of displaying the technical feasibility of this assay for examining other behaviors and experimental paradigms.

INTRODUCTION:

The ability to measure neurotransmitter release in awake behaving animals allows researchers to link specific behaviors with spatial and temporal patterns of neurotransmission—a powerful tool to investigate mechanisms and circuitry underlying both natural and operant behaviors in real-time. Historically, microdialysis has been employed to measure both electrically reactive and nonreactive substances in the extracellular milieu of the brain[1]. This technique uses a continuous flow of an aqueous solution of similar ionic composition to the extracellular fluid, through a microdialysis probe composed of a small shaft with a tip made of a semipermeable hollow fiber membrane[2]. After insertion of the probe, neurotransmitters or other analytes of interest can cross the semipermeable membrane by passive diffusion before being collected at intervals for subsequent analysis by high-performance liquid chromatography (HPLC), an analytical chemistry technique commonly utilized to separate, identify, and quantify components in a heterogeneous mixture[3].

Although microdialysis is a sensitive technique that can be used to measure virtually any analyte of interest, the temporal resolution is low, with maximum sampling rates on the order of minutes to tens of minutes[1-2]. The invention of fast scan cyclic voltammetry (FSCV), a technique that relies on the redox potential of electroactive species, can elucidate near instantaneous concentrations of the analyte of interest in the extracellular fluid. In brief (see Robinson *et al.*[4] for an extensive review), an electrode is applied to raise and lower the voltage in a triangular wave fashion on a fast time scale[4]. When the voltage is in the correct range, the compound of interest is repeatedly oxidized and reduced. This oxidization and reduction results in a movement of electrons that creates a small alternating current. Scan rates take place on the sub-second scale with oxidization and reduction of compounds occurring in microseconds. By subtracting the background current created by the probe from the resulting current, one can generate a voltage vs. current plot unique to each compound. Since the time scale of the voltage oscillations is known, these data can be used to calculate a plot of the current as a function of time. Thus, the relative concentrations of the compound may be determined as long as the number of electrons transferred in each oxidation and reduction reaction is known[4].

This chemical specificity and high temporal resolution make FSCV a powerful technique for detecting changing chemical concentrations *in vivo*. However, despite these manifold advantages, this technique requires extensive technical expertise and expensive equipment and setup. Further, nonelectroactive neurotransmitters (*e.g.*, glutamate) cannot be measured using this technique. Fortunately, technological advancements in the field of electrochemistry[5], as well as commercialization of these inventions, has introduced a relatively simple approach to measure non-electroactive neurotransmitters in awake behaving animals without

compromising temporal precision—a technique known as enzymatic biosensor technology. This technique uses enzymatic conversion of the nonelectroactive neurotransmitter of interest into two substrates, one of which is electroactive hydrogen peroxide that is detected as an amperometric oxidation current generated by an applied potential[5]. Commercially available biosensor probes (see **Figure 1**) selectively measure analytes of interest by competitively reducing the contribution of endogenous interferents. In the case of glutamate, the contribution of the common interferent ascorbic acid (AA) is competitively reduced to the measured current by co-localizing AA oxidase onto the active enzymatic surface of the sensor, converting AA to non-electroactive dihydroascorbate and water. In addition, a negatively charged Nafion polymer layer present under the enzyme layer excludes endogenous anionic compounds.

This same biosensor experimental setup can measure electroactive neurotransmitters as in FSCV, but instead it employs a fixed-potential recording[6]. In contrast to the oscillating voltage applied in FSCV, in a fixed-potential recording the voltage is kept at the redox potential for the analyte of interest. Although it is less chemically selective than FSCV as multiple neurotransmitters may have the same redox potential, in brain areas that overwhelmingly skew towards one neurotransmitter, the turn-key nature of this approach outweighs the lack of chemical specificity.

The ability to measure both electroactive and nonelectroactive neurotransmitter release in near real-time and link it to specific behavioral events provides an opportunity to examine converging neurotransmitter release. This manuscript details the use of this system to interrogate both dopamine and glutamate neurotransmission in response to natural reward in awake behaving hamsters. The aim of this paper is to detail the process of measuring this neurotransmitter release during sexual behavior in female hamsters, with the goal of demonstrating its feasibility for examining other behaviors and experimental paradigms.

Hamsters are an ideal model for use in electrochemical recordings

Historically, rat and mice models have been employed in the study of sexual behavior. These rodent species engage in a dynamic copulatory sequence, involving numerous female solicitation behaviors that include hopping, darting, and ear wiggling to entice the male to chase and ultimately mount the female[7]. The mounting by the male (with or without vaginal penetration) lasts only a few seconds, during which the female engages in her sexual behavior posture (termed *lordosis*) also only for a few seconds before resuming active solicitation behaviors. This pattern of behavior, composed of high levels of activity interspersed with brief periods of immobility, is problematic for measuring neurotransmission in behaving animals. First, there can be movement artifacts in the amperometric recordings that are unrelated to neural activity. Second, the locomotion is associated with the release of particular neurotransmitters in certain brain regions. For example, dopamine release has been coupled to locomotor activity in the dorsal and ventral striatum[8-9], a finding that formed the basis for microdialysis measurements of dopamine following psychostimulant administration[10]. Because the female-typical solicitation behaviors in most rodents involve high levels of locomotor activity, and are represented by the bulk of a 10 minute sexual behavior test, this makes it

difficult to ascribe changes in neurotransmission to the explicit components of sexual behavior that collectively last only minutes.

To analyze the neurochemical profile of female sexual behavior, this lab sought out a species in which there is minimal locomotor activity accompanying sexual behavior. The copulatory sequence in Syrian hamsters (*Mesocricetus auratus*) is ideal for neurochemical recordings due to the lack of solicitation behaviors typically seen in rats and mice[11]. As a consequence, female hamsters will enter and maintain the lordosis posture for upwards of 9 minutes out of a 10 minute testing session[12]. With the lack of extraneous locomotor movements by the female, *in vivo* electrochemical recordings that can be associated with components of sexual interactions with the male can be obtained.

Copulatory bouts in hamsters

After the introduction of a male stimulus animal into the testing chamber, the male will initially engage in anogenital investigation (AI) of the female before mounting her (**Figure 2A**). In order for the male to mount, the female must assume a receptive sexual posture known as lordosis, in which she arches her back and deflects her tail so that the mounting male can gain penile access to her vagina. The male will mount the female, clasping her hindquarters with both paws (**Figure 2B**), and begin thrusting in an attempt to gain penile intromission (**Figure 2C**). The male will mount the female (without insertion) as well as intromit a number of times before eventually achieving ejaculation. This sequence of mounts and intromissions leading to ejaculation is termed a "copulatory bout". Males will have several copulatory bouts within a single session.

PROTOCOL:

All procedures described here were approved by the Institutional Animal Care and Use Committee (IACUC) of The University of Minnesota, and are in accordance with The Guide for the Care and Use of Laboratory Animals[13].

1. Animals and Cannulation Surgery

1.1. Obtain Syrian hamsters from a common animal supplier at approximately 55 days of age.

Note: Although the age of animals will vary due to constraints of various experimental paradigms, for sexual behavior it is important to obtain sexually mature animals that are all approximately the same age.

1.2. House animals in a controlled temperature (22 °C) and lighting (14 h light followed by 10 h dark with lights out at 13:00 h) environment, with food and water available *ad libitum* except during periods of experimental testing.

Note: Since hamsters seasonally reproduce, this 14:10 light/dark cycle mimics their natural breeding conditions.

1.3. Following a 1-week acclimation to the laboratory, perform aseptic bilateral ovariectomies and intracranial stereotaxic cannulations under general anesthesia (*e.g.*, pentobarbital), and administer appropriate institutionally-approved post-operative analgesic and antibiotic treatment as previously described[14-15].

Note: For the study of sexual behavior, ovariectomies are necessary to remove the major endogenous source of sexual hormones so that the animals can be uniformly induced to sexual receptivity with exogenous hormone in accordance with the experimental timeline.

1.3.1. For both dopaminergic and glutamatergic probe implantation, stereotaxically implant guide cannulae (0.7 mm diameter; **Figure 3**) into the area of interest (*e.g.*, this lab targeted the right nucleus accumbens (NAc)) as described in stepwise detail elsewhere[15].

Note: Although the data presented are from single sensor recordings of one glutamate or dopamine probe per animal, this system allows for simultaneous recording of up to 4 sensors in a single animal; thus, 1–4 cannulae could be implanted depending on desired experimental design and outcomes.

1.3.1.1. Determine the implant coordinates of the region of interest using a stereotaxic atlas (*e.g.*, *The Golden Hamster Brain* by Morin & Wood), keeping in mind that the sensor extends 1 mm below the cannula into brain tissue.

1.3.2. After a cannula is stereotaxically lowered to the desired dorsal-ventral location, affix it to the skull using a cap constructed from dental acrylic, secured with stainless steel bone screws and a plastic head mount (**Figure 3**; see[15] for more details).

1.3.3. For carbon fiber testing, insert a reference electrode (350 μm diameter, 7.5 mm total length) into the contralateral hemisphere.

Note: A specific location or distance from the cannula is not required; reference electrodes may be placed anywhere in the contralateral hemisphere that is convenient.

2. Biosensor and Carbon Fiber Testing

2.1. Following 1-week of recovery from surgery, administer the hormone priming regimen to induce sexual receptivity.

2.1.1. Inject 10 μg of estradiol benzoate in 0.1 mL of cottonseed oil subcutaneously (s.c.), approximately 48 h and 24 h prior to the sexual behavior testing, to induce sexual receptivity towards the male[11].

2.1.2. Inject 500 μg of progesterone in 0.1 mL of cottonseed oil, s.c., 4 h prior to the introduction of the male to induce sexual receptivity[11].

2.2. Test all animals at the start of the dark phase of their daily cycle, as social behaviors of rodents are subject to alteration under white light and red light testing conditions[16-18].

2.3. For enzymatic biosensor glutamate testing, calibrate the sensors *in vitro* (**Figure 4**) before use as described previously[15,19].

2.3.1. Make the calibration solutions fresh on the day of testing in 20 mL glass centrifuge tubes. For the analyte solution, dissolve 7.4 mg of L-glutamic acid into 10 mL of ultra-pure H_2O by gently inverting the tube. Make the interferent solution by dissolving 176.1 mg AA in 10 mL of ultra-pure H_2O.

2.3.2. Set up for the calibration by placing a magnetic stirrer on the base of a basic lab ring stand. Place a 20-mL jacketed beaker on top of the magnetic stirrer, and clamp in place using a medium 2-prong clamp.

2.3.2.1. Add a magnetic stir bar and 20 mL of 100 mM phosphate buffered saline (PBS) into the jacketed beaker. Connect the jacketed beaker to a circulating water bath to heat the buffer solution to 37 °C.

Note: The enzymatic biosensor calibrations must be performed at body temperature since enzymatic activity is influenced by temperature.

2.3.3. Place the sensor calibration holder on top of the jacketed beaker, and set a 4-channel calibration preamplifier on top, securing in place using a right-angle clamp. Connect the preamplifier to a data conditioning and acquisition device to record calibration data.

2.3.4. Test the sensitivity of each enzymatic biosensor to glutamate (or other analytes of interest for other commercially available biosensor probes *e.g.*, glucose) before the experimental recording by placing the probe into the calibration holder atop a jacketed beaker, submerging the sensing cavity and some portion of the silver chloride (AgCl) reference wire in the buffer solution.

2.3.4.1. Before connecting each sensor (up to 4) being tested to a port on a 4-channel calibration preamplifier, initiate a new recording on the computer interface utilizing free downloadable acquisition software. Allow the sensors to reach a stable baseline.

2.3.5. Begin adding the analyte injections when the sensors have reached a stable baseline. Use the hole in the calibration holder to introduce a pipette tip to the buffer solution. Use the quick key annotation tool in the recording software to annotate when an injection is made.

2.3.5.1. Make 10 µM additions of glutamate by pipetting 40 µL of the analyte solution into the buffer solution. Wait for the sensor to stabilize between additions. Add 3 injections of analyte solution before adding a single injection from interferent AA solution (50 µL injection volume

for 250 μM change in concentration).

Note: The calibration allows for conversion of the changes in the amperometric signal (seen during the enzymatic experimental recording in step 2.12) to changes in glutamate concentration, if desired. See reference[15] for stepwise instruction. The dopaminergic probes used by this lab are calibrated during the manufacturing process by the commercial provider. Glutamate probes must be calibrated at the time of use as their sensitivity can decrease over time as the enzymatic components degrade[19]. Because carbon fiber electrodes do not undergo degradation, the calibration performed by the company prior to shipment is sufficient along as there is an increased likelihood of damaging the thin carbon fiber (350 μm diameter) through additional handling.

2.4. Line the test chamber with bedding taken from the animal's home cage.

Note: This increases the female's familiarity with the testing chamber, as well as exposes the male to sexually stimulating olfactory mating cues that were distributed by the female in response to the estradiol treatment.

2.5. Lightly anesthetize the animals prior to insertion of the probe by either isoflurane or another volatile anesthetic in an induction chamber.

2.6. Remove the occlusive obdurator from the guide cannula, and insert either the carbon fiber electrode or enzymatic probe through the guide shaft.

2.7. After the probe insertion, place the animal in the testing chamber.

Note: This lab uses a 10-gal glass aquarium (24.5 x 48.9 x 29.4 cm), but round chambers of equal area may also be employed.

2.8. Begin recording the video and time-locked amperometric signal in a commercially available software program, described in detail elsewhere[15].

2.9. Connect the sensor to the recording system: the potentiostat via an electrically shielded cable and an electrical swivel. Stabilize the sensor connection by screwing a pin attachment onto the head mount. Reinforce the connection using lab film if necessary.

Note: A customized connector is needed to attach both the carbon fiber electrode and reference electrode to the potentiostat, while the enzymatic glutamate sensors can be directly connected to the potentiostat.

2.10. Allow the sensor to equilibrate in the brain before experimental testing.

Note: Equilibration times differ depending on the recording sensor (*e.g.*, enzymatic sensors require 2–4 h equilibration, while carbon-fiber electrodes only require about 30 min). If both

types of sensors are implanted, equilibrate for the longer enzymatic sensor time.

2.11. After sensor equilibration, introduce a stimulus male into the testing chamber.

2.12. Following the first mount with penile insertion (termed intromission) by the stimulus male, continue recording for an additional 10–30 min, depending on the experimental goals.

2.13. Following behavioral testing, disconnect the female's sensor.

2.14. Remove both hamsters from the testing chamber. Remove the bedding and clean the chamber using 70% ethanol.

Note: Due to the brief equilibration required for carbon fiber recordings, steps 2.3–2.14 can be repeated to test multiple animals in the same day. In contrast, because the enzymatic sensors require about 4 h of equilibration, test only one animal per day to limit possible inter-subject variation due to differences in the animals' circadian time at testing.

3. Sacrifice and Perfusion

3.1. Following the conclusion of the testing period, deeply anesthetize the female test subject with a euthanizing agent (*e.g.*, this lab uses an intraperitoneal injection of 0.2 mL phenytoin and pentobarbital solution).

3.2. Transcardially perfuse the animal with 25 mM PBS, pH 7.6, to remove all circulating blood, followed by a 20 min fixation using 4% paraformaldehyde-PBS.

3.3. Remove the brain and postfix in ~ 30 mL of the 4% paraformaldehyde solution in a 50-mL conical tube overnight.

3.3.1. Store the brain in a 10% sucrose-PBS solution until ready to serial section.

Note: Brains may be stored up to 2-weeks, with weekly sucrose solution changes to avoid potential growth of contaminants.

3.4. Serial section the brain in 40 µm slices through the implanted region with either a freezing microtome or cryostat as previously described[19].

3.5. Mount slices serially on commercially available adhesive-coated slides (or see[20] to make). Allow to dry, at least overnight.

3.6. Stain the slides with cresyl violet dye, clear, and coverslip as previously described[20].

3.7. Image the slides using bright field microscopy to confirm the anatomical placement of the sensor.

4. Behavioral Coding

4.1. View and annotate the videos using free commercially downloadable software (see **Table of Materials**) in slow motion to precisely code behaviors time-locked to the amperometric signal.

Note: To use the MATLAB code in the analysis (see below*)*, annotate the *start* and *end* of each of the female's and male's behaviors.

4.1.1. Annotate *startLordosis* in the frame when the female initiates a dorsoflexion of her back and defects her tail upwards. Annotate *endLordosis* when the female terminates this posture, which often occurs when the female readjusts to another location in the testing chamber.

4.1.2. Annotate *startAI* when the female is in the lordosis posture and the male moves his snout towards the female's anogenital region, where sniffing, licking, or nuzzling of her perineal region may occur. Annotate *endAI* when the male removes his snout from close proximity to the female's anogenital region.

4.1.3. Annotate *startMount* when the male approaches and places his forepaws on the female in a mounting posture, regardless of the orientation of the mount attempt (*e.g.*, side, rump).

4.1.4. Annotate *startIntromission* when successful thrusting gains the mounted male penile access to the female's vagina.

Note: An intromission is behaviorally distinguishable from the mounted thrusting that occurs prior to intromission, as the male visibly pulls his upper limbs towards his body, thrusts his pelvis forward, and curls his tail upwards, indicating penetration (see **Figure 4C**).

4.1.4.1. When applicable, annotate *ejaculation*.

Note: At the end of a mating bout and in conjunction with a successful intromission, the male will ejaculate. Although one is unable to visualize the actual emission, male hamsters have a characteristic treading motion with their hind foot during an intromission[21], thus annotate the ejaculation at the occurrence of the foot movement.

4.1.5. Annotate *endIntromission* and *endMount* simultaneously in the frame when the male has removed his front two paws and thus, has no contact with the female. Due to the frequent inability to visualize his withdrawal, simultaneous code these two behaviors to allow for consistency and reliability across mating bouts.

Note: A mating bout either follows a successful ejaculation or is when the female breaks the lordosis posture following at least one *mount.*

5. Example Data Analysis

Note: The use of fixed-potential carbon fiber and enzymatic recordings for dopamine and glutamate, respectively, allows for the ability to measure both tonic and phasic patterns of electrochemical release. This lab uses a free, commercial acquisition system and software to record and convert recorded signals from analog to digital (bias 0.600 V, sampling rate = 1 Hz).

5.1. Description of example data analysis protocol

5.1.1. Although other programs can be used, quantify the tonic and phasic (transient) changes in the recorded electrochemical signal using MATLAB.

5.1.1.1. For the presented data, calculate the tonic signal using a 50-point moving average filter. A transient-only normalized representation of the signal was calculated by subtracting the tonic signal from the raw data. When identifying phasic peaks, there are various algorithmic options (*e.g.*, labeling peaks as changes in the signal more than 1 SD above the mean).

5.1.1.2. Identify the locations of these peaks, *i.e.*, local maxima, from the normalized signal using the MATLAB function *peakfinder* with the minimum peak amplitude set to the root mean square of the signal. The location of peaks detected from the normalized signal was used to extract the amplitude from each peak. The ability to detect peaks allows for the identification of any time-locked behaviors that may be coinciding with, or driving the relative peak in the neurochemical under investigation.

5.1.2. Segment the processed data in accordance with the behavioral annotations (see step 3); a Student's *t*-test was used to evaluate differences in the tonic neurotransmitter level, the occurrence of transients, and the amplitude of transients during the pre-mating bout lordosis versus lordosis during mating bouts. To measure changes to tonic patterns of electrochemical release, the data points occurring during the *Lordosis* position directly before a mating bout (*i.e.*, pre-mating bout lordosis) were compared to the data points during a mating bout session using a t-test.

5.2. Preparation of raw data for described data analysis protocol

Note: As stated, although other programs or analyses may be equally viable options, in order to use the MATLAB code described above, the raw data must be prepared in the following way:

5.2.1. Export the annotation and voltage files. To do this, under the 'File' tab, select the 'Export' function and select the 'Annotations' tab to save the annotations. Under the 'File' tab, select the 'Export' function and choose the "TSV' tab to save the voltage measurements as a TSV extension file.

5.2.2. Open each file with a spreadsheet program and save as 'xls/xlsx' to make the files compatible with MATLAB.

Note: For the 'Annotation' file, there are additional annotations that must manually added post-export in a spreadsheet program to dictate the *start* and *end* of both the pre-mating bout lordosis (annotated as *PreMBlordorsis*) and the mating bout so that these time points can be directly compared as mentioned in step 5.1.2.

5.2.3. Annotate the start of the pre-mating bout lordosis (*startPreMBlordosis*) at the same time as a lordosis behavior begins (*startLordosis*) that also includes a *mount* behavior, and also at the start of a new mating bout if the female remains in the lordosis posture.

5.2.4. Annotate the end of the pre-mating bout lordosis (*endPreMBlordosis*) when the first *mount* begins.

5.2.5. Annotate the start of a mating bout (*startMB*) at the same time of the endPreMBlordosis, as this signifies the start of a mating bout.

5.2.6. Annotate the end of a mating bout (endMB) at either the end of a *mount* that includes an *ejaculation* (see step 4.1.4.1), or following the female's termination of the lordosis posture (*endLordosis*).

REPRESENTATIVE RESULTS:

Using the electrochemical and behavioral coding methodology described above, this lab has begun to characterize both tonic and phasic fluctuations in both dopamine and glutamate during *in vivo* recordings of sexual behavior. Due to the temporally-precise manner of this methodology, we can more accurately characterize neurotransmission during sexual behavior; as well as ascribe specific changes in release patterns to corresponding tonic changes during mating bouts, and corresponding transient fluctuations during the female's receipt of individual copulatory behaviors (*e.g.*, intromission, ejaculation) from the mounting male.

Specifically, we used this methodology to focus on measuring the release patterns of dopamine and glutamate in the NAc of separate animals. The NAc is a critical region involved in reward processing[22] and has been described as an integral region in the reward aspects of sexual behavior[23].

We observed that during sexual behavior, females demonstrate a tonic increase in dopaminergic levels in the NAc core during mating bouts (**Figure 5**). While these tonic changes are shown for all mating bouts, the methodology described above can measure tonic changes between each mating bout, and thus increase the temporal identification of overall changes to tonic levels of neurotransmitter release throughout the entire session. Further, this described methodology can pinpoint phasic changes and temporally map the behaviors likely associated with these phasic neurotransmitter fluctuations. Specifically, these preliminary results suggest that during mating bouts, dopaminergic transients in the NAc arise during vaginal insertion (intromission) by the male (**Figure 6**). Furthermore, this association with intromission is specific, with negligible dopaminergic responses to other copulatory behaviors, such as the AI.

A similar pattern has been observed in other animals with regard to glutamate release, with rapid transients that correspond to individual intromissions in the dorsal NAc core during a copulatory bout (**Figure 7**). This pattern is region specific, with no discernable signal occurring in the NAc shell or medial caudate of other animals.

FIGURE AND TABLE LEGENDS:

Figure 1: Enzymatic biosensor technology.

Standard fabrication of commercially available enzymatic biosensor probes that detect neurotransmitters via enzyme-mediated processing (left image). In the case of glutamatergic sensors, glutamate oxidase is employed in the enzymatic layer (right image) to convert the nonelectroactive neurotransmitter to electroactive hydrogen peroxide (H_2O_2) that is detected by oxidation at the platinum-iridium (Pt-Ir) electrode. Ascorbic acid (AA), a common interferent that is oxidized at the same potential, is excluded via an addition of AA oxidase at the enzymatic layer that converts the interferent to nonelectroactive water. Other negative electroactive interferents present in the brain are excluded via a passive selective membrane.

Figure 2: Components of a hamster mating bout.

In hamsters, a mating bout consists of a sequence of copulatory behaviors, during which the female remains in the immobile lordosis posture while the male anogenitally investigates **(A)**, mounts **(B)**, and achieves intromission **(C)**. Note an intromission is behaviorally distinguishable from mounting as the male pulls his upper limbs towards his body, thrusts his pelvis forward, and curls his tail upwards, indicating penetration.

Figure 3: Skull cap construction.

Guide cannulae, bone screws, and dental acrylic are used in the construction of a skull cap to allow insertion of carbon fiber or enzymatic probe (see[15] for stepwise details). This figure depicts a single cannula implant over the right NAc for an enzymatic sensor (no implanted reference electrode) and plastic head mount for sensor stability.

Figure 4: Glutamate biosensor calibration.

The sensitivity of each biosensor to glutamate is tested *in vitro* before experimental recording by 10 µm additions of glutamate (green) at 37 °C (since enzymatic activity is influenced by temperature). Each sensor is confirmed to be non-responsive to ascorbic acid (AA; red) or dopamine (DA; blue). This calibration allows for the conversion of changes in the amperometric signal seen during experimental recording to changes in the glutamate concentration.

Figure 5: Tonic increases in dopamine during a mating bout.

A. Stylized example of premating bout lordosis signal vs. mating bout lordosis signal**.** To measure tonic dopamine release, the amperometric responses to dopamine levels during lordosis before a mating bout (MB) were compared to those during a MB. Gray boxes indicate lordosis before a MB. Blue boxes indicate lordosis during a MB. **B.** Representative quantification from one animal comparing the mean voltage between preMB lordosis and MB lordosis. Error bars indicate standard error of the mean (SEM), and asterisk indicates significant difference (p-value < 0.05).

Figure 6: Representative dopamine trace.
Phasic release of dopamine is time-locked to intromissions from the male in the dorsal NAc core.

Figure 7: Representative glutamate trace.
Glutamate transients correspond to individual intromissions from the male in the dorsal NAc core during an extended sexual experience.

DISCUSSION:
Although relatively straightforward, some issues can arise when employing this technique. First, the stereotaxic placement of the probes must be precise: unlike microdialysis that samples a wider radius of the extracellular milieu surrounding the probe, this technique only allows the measurement of a neurotransmitter that comes into direct contact with the probe. Second, in the case of the carbon fiber recording, due to the small width of fiber, breakage can occur, and the probe must be inserted with deliberate care. In the case of glutamatergic biosensors, enzymatic degradation can occur if the probe is not used within the guaranteed time-frame of 3-weeks. Fortunately, all of these issues are addressable, and can be eliminated with proper care and attention to detail.

A potentially problematic caveat arises in the specificity of fixed-potential carbon fiber recordings. Because the applied potential for dopamine coincides with other monoamines, such as norepinephrine, if the recordings are made in areas that release both neurotransmitters, than this lack of chemical specificity may limit the interpretation of the results. In the NAc this does not pose a major problem, with dopamine being the primary catecholamine transmitter[24]. Because roughly 98% of the cells in NAc are medium spiny neurons that respond to dopaminergic neurotransmission[24], the signal is biased towards detection of dopamine and facilitates the use of this more turn-key approach.

Another benefit of this enzymatic biosensor and fixed-potential carbon fiber recording system is that dual-probe recordings can be performed, so that both electroactive and nonelectroactive neurotransmitters such as dopamine and glutamate can be measured in the same animal concurrently. Although the data presented here come from recordings in separate animals, these techniques can be employed concurrently such that one can evaluate the convergence of neurotransmission in multiple brain regions or in the same region bilaterally. Combination of recordings within the same animal, or comparison of recordings across animals as shown here, are both powerful tools in elucidating mechanisms behind network connections and signaling.

In sum, this paper demonstrates the powerful techniques of enzymatic biosensing and fixed-potential recording to measure multiple electroactive and nonelectroactive neurotransmitters utilizing a single experimental setup. Although the data presented here come from individual neurotransmitter recordings across multiple animals, this system provides the capability to record from up to 4 sensors in one animal concurrently[15]. The sample results presented

collectively provide insight into rapid neurotransmission in the female hamster resulting from specific patterns of copulatory stimulation from the male. Further, these experiments demonstrate time-locked responses of dopaminergic and glutamatergic transients in the NAc core of the female to intromission from the male, suggesting that this release may be responsible for encoding distinct rewarding properties of sexual behavior. Our goal is to continue utilizing the mating pattern in female Syrian hamsters to develop a more comprehensive picture of the relation of *in vivo* dopamine and glutamate release and the underlying circuitry to individual components of copulatory stimuli from the male. We believe that the capability to characterize the patterns of these neurotransmitters is not only beneficial from a basic science perspective[23], but also in elucidating potential therapeutic approaches for pathological forms of reward behavior such as drug addiction[25].

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DISCLOSURES:
The authors have nothing to disclose.

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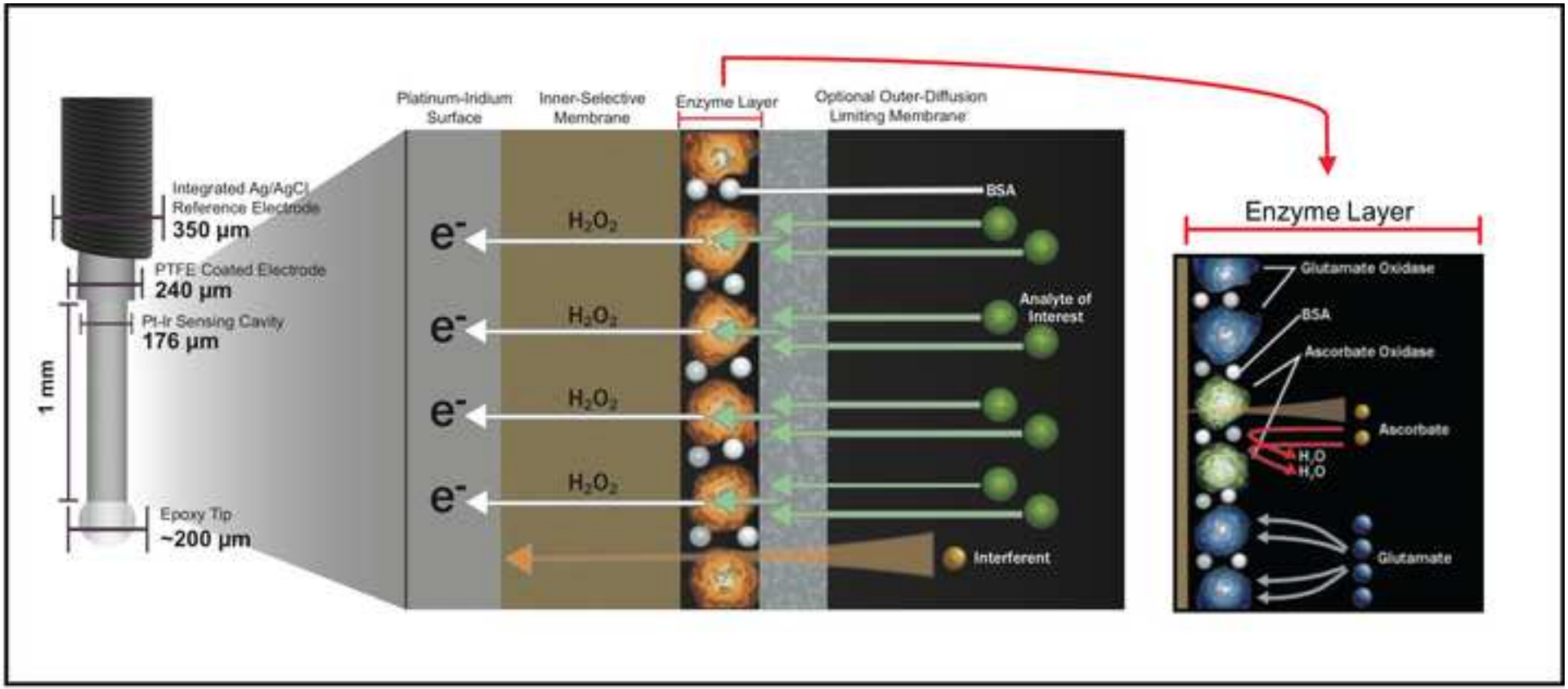

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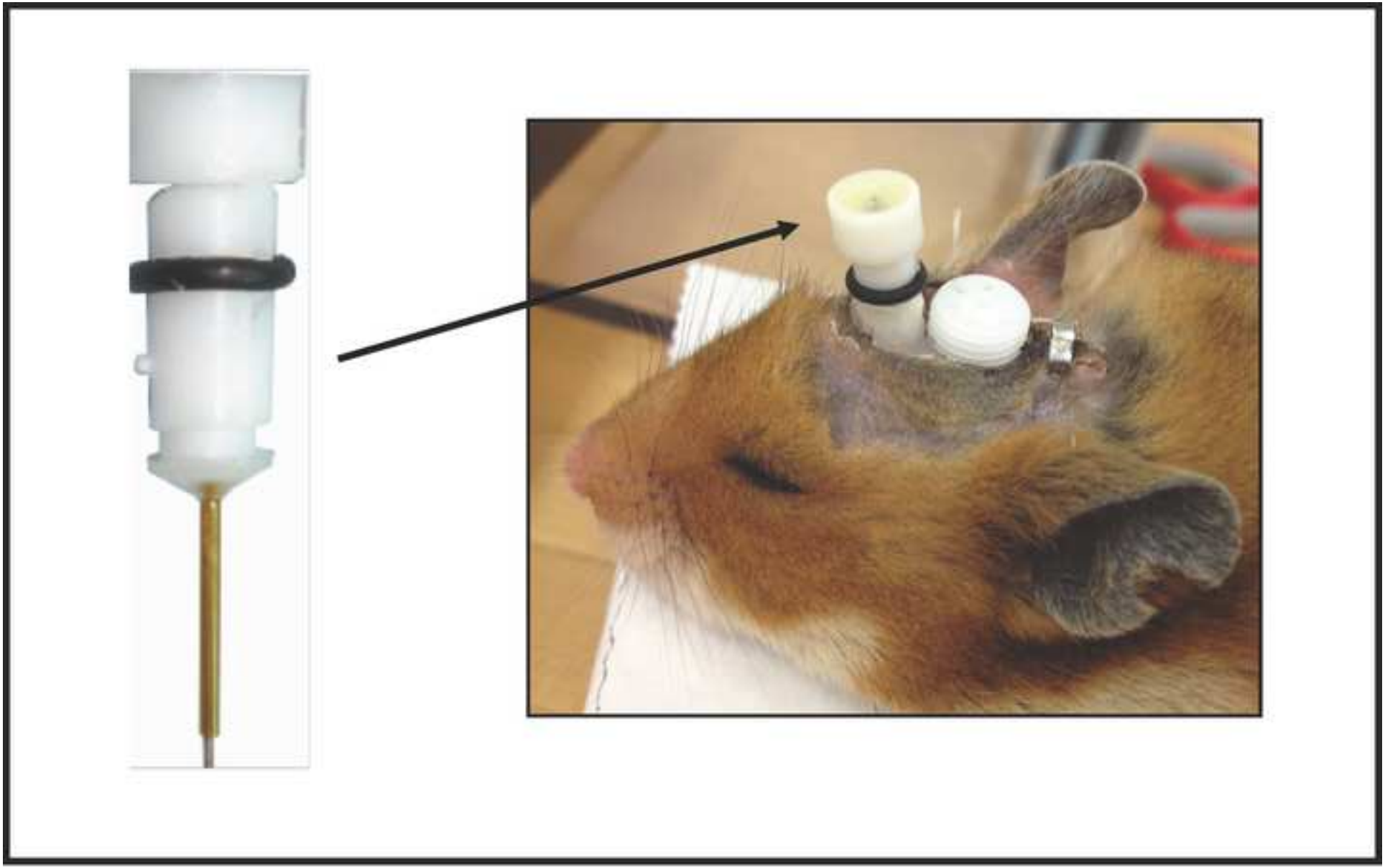

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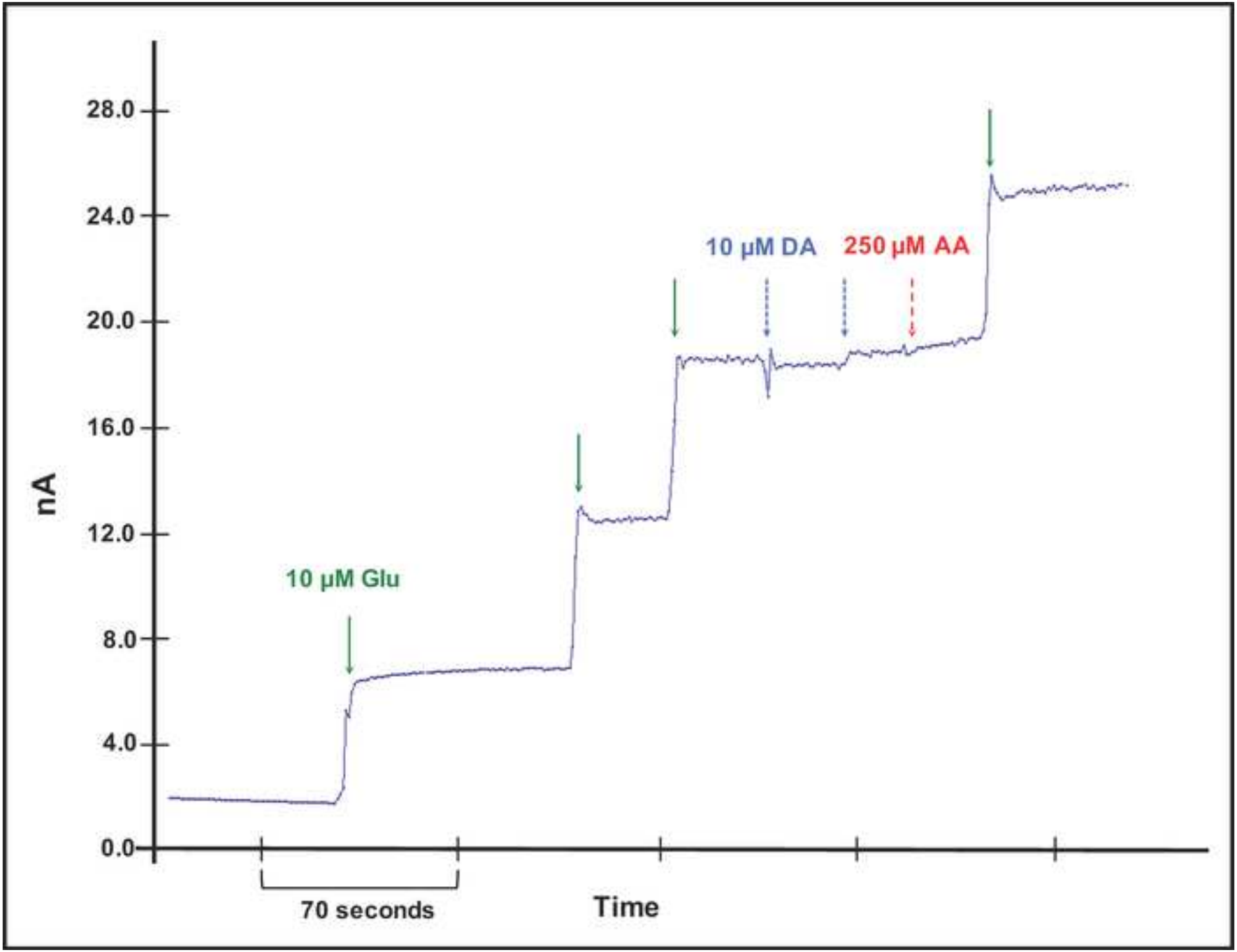

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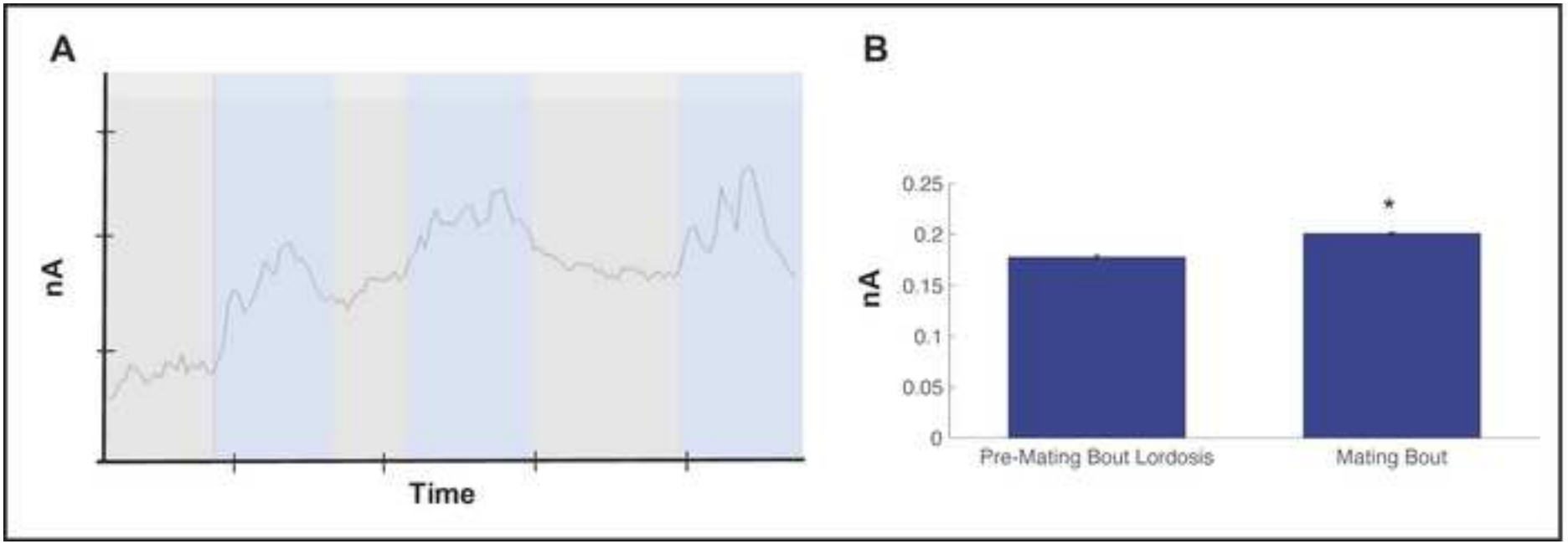

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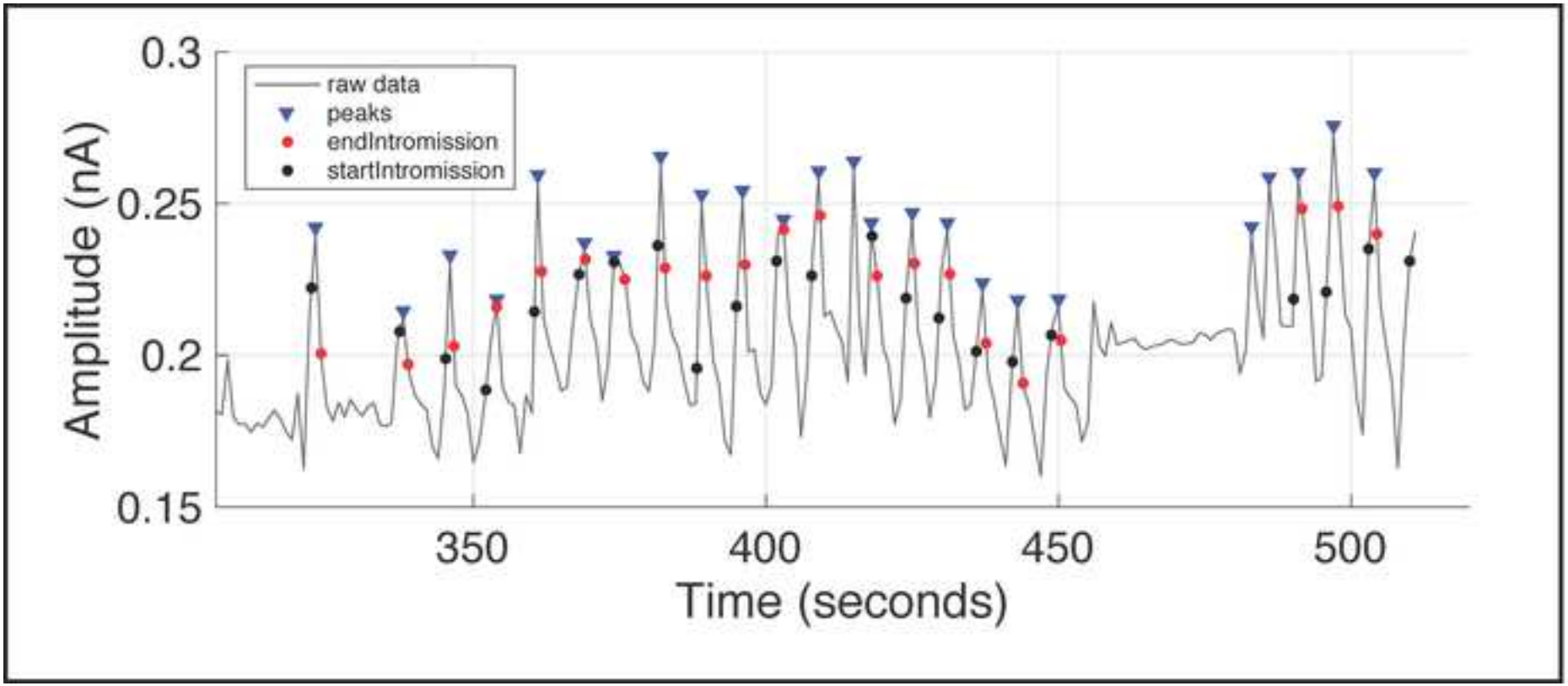

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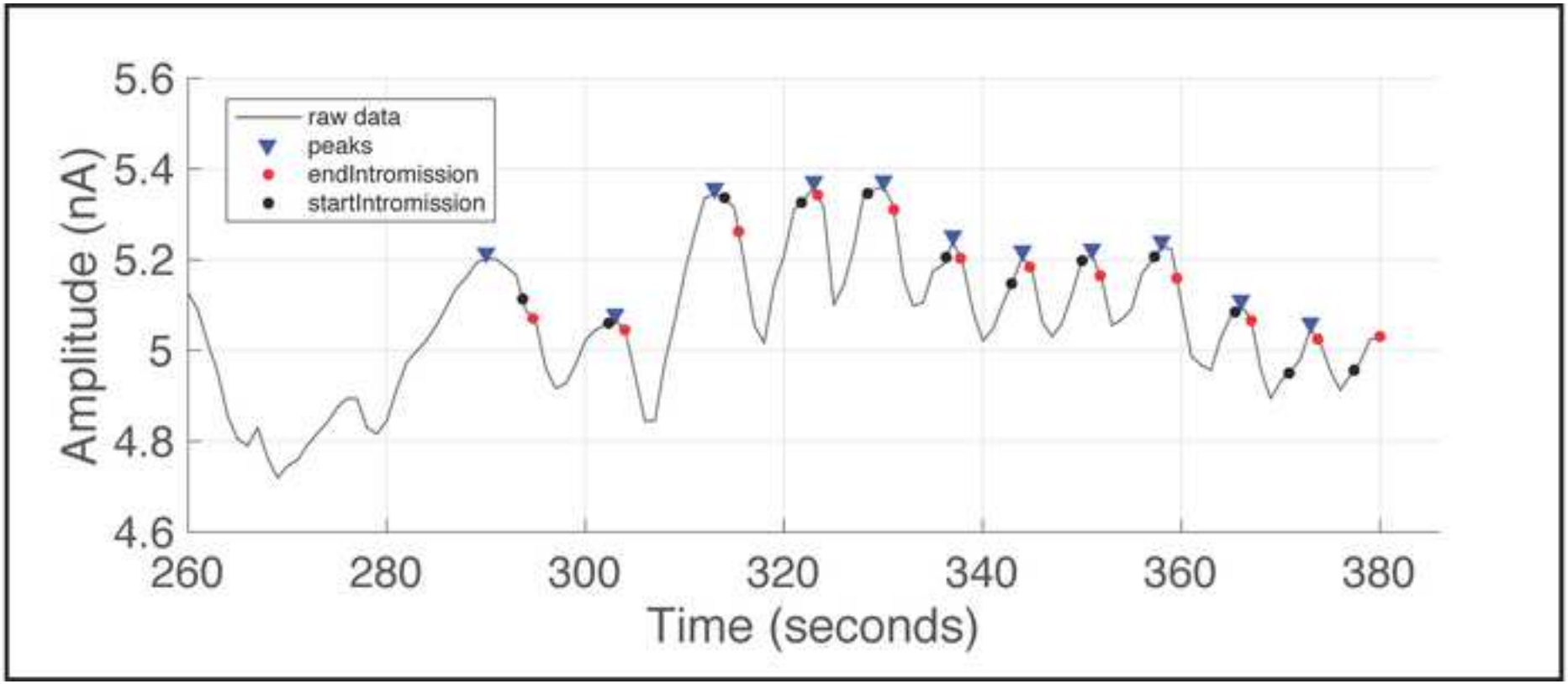

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Loxicom analgesic	Norbrook Laboratories
Enroflox antibiotic	Norbrook Laboratories
Beuthanasia-D	Merck Animal Health
Bone screws	Pinnacle Technologies, Inc.
Dental acrylic (Bosworth Duz-All)	Bosworth
Hardware biosensor setup	Pinnacle Technologies, Inc.
Base video computer package	Pinnacle Technologies, Inc.
Video EQ700 EverFocus camera package	Pinnacle Technologies, Inc.
Sirenia Acquisition software	Pinnacle Technologies, Inc.
Tethered rat *in vitro* calibration kit	Pinnacle Technologies, Inc.
Stir plate	Corning
Water bath capable of closed loop circulation	PolyScience
Carbon fiber sensor with BASi rat cannulae	Pinnacle Technology, Inc.
Ag/AgCl reference electrode	Pinnacle Technology, Inc.
Glutamate biosensors	Pinnacle Technology, Inc.

BASi guide cannulae	Pinnacle Technologies, Inc.
BASi cannula plastic headpiece for rats	Pinnacle Technologies, Inc.

Catalog Number
76478-501-50
6451603670
5552915411
00061047305
8111-16
166261C
8400-K2
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Free--available to download from pinnaclet.com
7000-K2-T-BAS
6795-410D
8006A11B
7002-CFS
7065
7001

7030
7011

Comments/Description
Pentobarbital sodium injection, USP. This lab uses 8.5mg/100g body weight, injected intraperotineally.
NSAID antinflammatory and analgesic used for post-operative pain control. Generic: meloxicam.
Fluoroquinolone antibiotic for post-operative infection prevention. Generic: Enrofloxacin.
Pentobarbital Sodium, Phenytoin Sodium euthanasia agent.
1/8" bone screw (Pkg. of 16) used to affix skull cap to skull.
Self curing dental acrylic is used in construction of a skull cap to affix cannula and head mount to skull.
Pinnacle offers complete hardware kits for new users of our tethered biosensor system for rats. Kits include a commutator, preamplifier, and data conditioning and acquisition system
The base computer package includes a preconfigured computer with ample hard disk storage, a high-definition monitor, a keyboard and mouse, an uninterruptible power supply, and all necessary cables.
EQ700 night vision capable box camera with independent IR source was obtained as part of Pinnacle video computer package. Dome camera (9000-K9) and HD camera (9000-K11) options are also available.
Sirenia Acquisition provides a single platform for recording data from any Pinnacle hardware system. The software features synchronization of all data streams, user-configurable settings, data consolidation, and multiple export options. In addition, the software includes basic review and analysis modules for biosensor recordings. Sirenia delivers free ll-in-one software that is ideal for data acquisition and review.
In order to relate the current changes measured by a biosensor to actual changes in analyte concentration, it is necessary to calibrate the biosensor prior to implantation into the animal. The process also confirms the integrity and selectivity of the sensors. Calibration kit includes 20 mL jacketed beaker (#7058), 1/2" by 1/8" magnetic stir bar (#7059), right angle clamp (#7056), 2 prong single-adjustment clamp (#7055), 4-channel calibration preamplifer (#7053), and calibration holder (#7051).
Corning digital Stirrer, 5" x 7", 120 VAC used to spin magnetic stirrer in jacketed beaker during *in vitro* calibration of glutamate biosensors.
PolyScience 8006A11B 6L Standard Digital Heated Circulating Bath, 120VAC water bath was used with plastic tubing to heat jacketed beaker to physiological temperature.
Carbon fiber electrode used for recording dopamine neurotransmission.
Necessary for carbon fiber recordings.
Enzymatic biosensor probe used for recording glutamatergic neurotransmission.

Guide cannulae implanted into brain region of interest to guide probe.
Headmount stabilizes probe and attaches to potentiostat.

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        vi. **23 I could not locate a doi**

5. Please define all abbreviations before use.
    a. **The entire document was reviewed and ensured that all abbreviations were defined before use.**

6. Please use focused images of uniform size/resolution (at least 300 dpi).
    a. **All figures were exported as TIFF files with uniform resolution of 600 dpi.**

7. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
    a. **The table has been revised to be more complete.**

8. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Please rewrite the text to avoid plagiarism (including self-plagiarism). Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. These sections are lines: 10-19, 27-36, 50-53.
    a. **Although we had attempted to vary sentence construction and word choice from sources in describing the techniques of microdialysis (10-19), voltammetry (27-36), and biosensing (50-53), we went back through each of these sections and made additional changes to structure and verbage.**

9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products

should be sufficiently referenced in the Table of Materials and Reagents.
For example: Pinnacle sensors in Figure 1, Pinnacle in the manuscript text, Sirenia, etc.

a. **Because this experimental technique relies so heavily on commercially available sensors from Pinnacle, we initially included commercial language with regards to products obtained from this company. The document has been reviewed and any reference to specific products or companies have been removed.**

10. Please include a Short Abstract to clearly describes the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
11. Please include a Long Abstract between 150-300 words.
12. Please provide at least 6 keywords or phrases.
13. Please provide all author affiliations in the manuscript text.
    a. **Our apologies. We initially misunderstood that if these sections were submitted in the different submission boxes through the submission portal that they were also required to be included in the manuscript document. We have added these sections into the manuscript document proper.**

14. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
    a. **We have gone through the protocol section and ensured that no "could be," "should be," or "would be" phrases were used. We took any text within the protocol that was not in the imperative tense and added them as separate notes as instructed.**

15. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
    a. **Notes to the reader that were not direct imperative commands were separated as notes. In particular, if the editor is referencing the paragraph about the use of hamsters at the beginning of the protocol, and the description of a copulatory bout in section 4, these sections were moved to the introduction so that the protocol only contains action items (aside from separated notes).**

16. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
    a. **Additional details were provided where necessary, and reference to the original source was made more apparent (e.g. in the case of ovariectomies and stereotaxic surgeries, which are well-established techniques, and we do not want to be stepwise filmed).**

17. Please use SI abbreviations for time: h, min, s, (Figure 2, etc).
    a. **The document was reviewed for time abbreviations, and changes were made where necessary (hr to h; wk to week since there no SI abbreviation for week or days and the SI website instructs to spell these words out in their entirety).**

18. 1.3: How is the anesthesia done? How are the ovariectomies and cannulations done? If this is to be filmed, we need explicit step wise details.
    a. **Details regarding anesthetic were provided. Reference to sources re: ovariectomies and cannulations were made apparent, and additional details regarding cannulations were given. We are not intending to have these filmed as they are previously established techniques.**

19. 1.3.1: Implant how? What is the area of interest?
    a. **Additional details regarding implantation and determination of coordinates for area of interest were added.**

20. 1.3.2: Please ensure you are using the correct abbreviation for microns (lower-case m).
    a. **We thank you bringing out attention to this oversight; we have since corrected the incidences of using a capitalized M for microns. The two incidences of the use of microns in the manuscript were changed to lower case m.**

21. 3.1: What is used and how much?
    a. **A sentence detailing euthanizing agent, dose, and method of administration was included.**

22. Please revise step 4 to be in the imperative tense throughout.
    a. **All stepwise instruction portions for step 4 were made imperative tense.**

23. Please note that we can only film step 4 if there are stepwise details at each step and there is a graphical user interface with all user input commands: File | Save | etc.
    a. **The data analysis portion of the protocol is not intended to be stepwise, but rather an overall explanation of how this lab utilizes one method for analyzing the output data from this technique. We have attempted to break it down into more stepwise detail, utilizing the use of non-imperative "notes" as instructed. We hope that these changes will be sufficient, but are willing to make additional accomodations as the editor sees fit.**

24. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The highlighted steps should form a cohesive narrative with a logical flow from one highlighted step to the next. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
    a. **We have highlighted key portions of the protocol to be filmed.**

25. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
    a. **We have ensured that the highlighted steps form a cohesive narrative with logical flow and complete sentences.**

26. Please define the error bar and asterisks in Figure 5.
    a. **A sentence explaining that error bars indicate SEM and asterisk indicates significance of $p < 0.05$ has been added to the figure legend.**

Response to reviewers' comments

Reviewer #1:

1. Line 82: "Trivially" is out of place here. Movement artifacts are not trivial. They are actually a major reason for using hamsters instead of other rodents.
    a. **Thank you for this observation. This word is definitely not the right fit. Indeed, these artifacts are one of the main reasons we use hamsters instead of other rodents for these experiments. "First" has been substituted for "Trivially" as the first word to that sentence.**

2. Lines 94-96: This sentence does not make sense.
    a. **In the editing process the words "is ideal for chemical recordings" was somehow removed in this sentence and have been replaced. We hope the sentence: *"The copulatory sequence in Syrian hamsters (Mesocricetus auratus) is ideal for neurochemical recordings due to the lack of solicitation behaviors typically seen in rats and mice[11]."* now makes sense.**

3. Line 99: Why is it "necessarily" associated? Seems like too strong of a claim unless additional justification is included.
    a. **"can be" has been substituted for "necessarily" in this sentence.**

4. Lines 152-153: What dose/type of anesthesia?
    a. **This sentence lists that isoflurane in an induction chamber is used to lightly anesthetize the animals.**

5. Lines 162-164: Is bedding really added after the animal is placed in the arena?
    a. **This section of the protocol has been rearranged to appear before the section listing the animal being added to the arena.**

6. Line 166: Is recording really performed during the 2-4 equilibration? Or is this line out of place?
    a. **Recording is performed during the equilibration period to ensure proper sensor function and equilibration.**

7. Lines 256-258: Better pictures of the various behaviors related to intromission would be useful. It is difficult to see many of these in the picture provided in Figure 4.
    a. **We have made a new figure that shows more detail and have added further clarification in the figure legend.**

8. Figure 2 legend: Missing. It would be useful to provide details of the skull cap construction.
    a. **The figure 2 legend was expanded, and reference to the explicit stepwise manual was made.**

9. Figure 1: Has the company given permission to use their figure? If available, a better figure may be one that focuses solely on the neurotransmitters described in the article.
    a. **This image was provided by the company and we have obtained permission to use it in this publication. The generalized image is representative of other sensors that readers may be interested in (e.g. glucose, ethanol, etc), but we have expanded the figure to include the enzymatic layer for glutamate, and added additional description in the legend.**

10. Figure 4: It is very difficult to see the differences between mounting and intromission. One way of fixing this would be to provide multiple pictures of intromission. Another would be to label the pictures with the specific details of each behavior (i.e., what to look for). A non-specialist would be hard pressed to describe the differences between these behaviors based solely upon these images. Perhaps this can also be directly addressed in the movie clips associated with this article?
    a. **We have made a new figure with a more zoomed in image that we hope is more clear in the distinction between these behaviors. In addition, this protocol is meant to detail the use of enzymatic and carbon fiber recording for utilization in multiple paradigms, sexual behavior is presented as an example that our lab uses.**

11. Figure 5A: Missing axis numbers and units.
    a. **This image is intended to be a stylized representative example so the reader can see the overall tonic increases in dopamine during a mating bout.**

Reviewer #2:

1. In the abstract, the investigators claim the biosensors permit recording of multiple neurotransmitters simultaneously. In the introduction, they state that the ability to measure both electroactive and non-electroactive molecules provides the opportunity to examine converging neurotransmitter release (line 63-65). Nevertheless, it is difficult to understand whether dopamine and glutamate levels are measured simultaneously in this manuscript. If the authors are not measuring multiple neurotransmitters in the same animal in this manuscript I suggest they not make these claims in the abstract and introduction. Rather, they might discuss future directions in the discussion section. Below are a few cases where the measurement of multiple neurotransmitters should be further clarified.
    - Line 121-123. More detail is needed on probe implantation. Are both dopamine and glutamate probes inserted in the same animal?
        - **A note explaining that the data presented are from single cannula implants, but that the system allows up to 4 sensors to be implanted and recorded from simultaneously has been added.**
    - Figure 2 needs a caption that explains what probes, cannulas, and/or reference electrodes are implanted. It appears that the rostral probe in the BASi guide cannula, although the caudal probe in unclear and should be identified.
        - **We have attempted to clarify this figure by adding additional description.**
    - Line 126-127. More detail is needed about implanting a reference electrode. Is the reference electrode needed only for the dopamine probe? Does it require an additional stereotaxic implant? Can it be included on the skull if both dopamine and glutamate probes are implanted?
        - **A note providing further clarification on each of these points was added.**
    - The methods appear to be written for the measurement of a single neurotransmitter per animal. How would the multiple stereotaxic implants and differential equilibrium times affect the simultaneous measurement of both dopamine and glutamate?
        - **A sentence describing what to do in this situation has been added.**
    - Line 386-391. Again, the investigators mention dual-probe recordings and the convergence of neurotransmission in multiple brain regions. Were dual-probe recordings used in this study?
        - **No, single probe recordings were used in this study and we have attempted to clarify that in the text.**

2. The authors use the term sex behavior and sexual behavior interchangeably. I think they should use one term consistently and believe sexual behavior is the more appropriate term.
    a. **We agree and have changed all instances of "sex behavior" to "sexual behavior."**

3. Line 94-96. This is an incomplete sentence.
    a. **In the editing process the words "is ideal for chemical recordings" was somehow removed in this sentence and have been replaced. We hope the sentence: *"The copulatory sequence in Syrian hamsters (Mesocricetus auratus) is ideal for neurochemical recordings due to the lack of solicitation behaviors typically seen in rats and mice[11]."* now makes sense.**

4. Line 113-115. The authors should briefly explain that hamsters are housed in a 14:10 light/dark cycle to maintain seasonal reproductive condition.
    a. **This is an important clarifying point, thank you for the suggestion. We have added a note that explains this.**

5. The table of materials and equipment is not formatted properly in the PDF file. The excel file is okay, but the table needs to be reformatted for publication.
    a. **We are unsure how the documents are compiled into the single PDF file through the submission portal; we are only able to control the formatting of the excel file that we upload.**

6. Line 142-143. In the manuscript the authors explain that the enzymatic biosensor should be calibrated before use. However, the table of materials says that it is necessary to calibrate the biosensor at the conclusion of an experiment. Should biosensors be calibrated at both the start and end of an experiment? Please clarify.
    a. **Thank you for pointing out this discrepancy. Although the company suggests both pre- and postcalibration, our lab precalibrates only because we prefer to have exact anatomical placement of our sensors confirmed and thus perfuse with the sensor implanted as removal prior to perfusion makes it more difficult to locate the very small 1mm track of the probe from the bottom of the cannula.**

7. Line 145-150. The sentence about carbon fiber electrodes is confusing and should be clarified. Is damage to the thin carbon fiber a problem for calibration performed by the company?
    a. **Due to the delicate nature of the carbon fibers, we limit the amount of handling that occurs in the laboratory. Since the company does calibrate the sensors before shipping them and because the carbon fibers sensors do not undergo any degradation, by using the company's calibrations, we can reduce the risk of breaking the carbon fiber.**

8. Line 181. Delete 'is' in "a stimulus male is into the testing chamber".
    a. **Deleted.**

9. Line 210. Insert space between lines.
    a. **A space has been inserted.**

10. Line 321-322. The investigators mention that there are negligible dopaminergic responses to other copulatory behaviors such as anogenital investigation. It would be useful to create an additional figure showing that AI start and AI end do not overlap with the dopaminergic peaks.
    a. **We removed this sentence as not to induce confusion.**

11. Figure 1. The legend describes an additional enzymatic layer of AA oxidase that converts the interferent to non-electroactive water. It appears to me this layer of AA oxidase is not shown in the figure and should be identified as 'not shown' in the figure legend.
    a. **We have added an additional image to this figure that demonstrates how the glutamate sensor functions specifically, including demonstrating the AA oxidase present.**

12. Figure 3. I do not understand how glutamate, dopamine, and ascorbic acid are applied to the biosensor. Is this performed in vitro? More detail is needed here.
    a. **In vitro was added for clarification as well as elaboration and stepwise detail of this calibration procedure.**

13. Figures 6 and 7. Figure legends that describe the data shown should be provided. The investigators should indicate that these plots are from representative animals. Also, they should indicate if the dopamine and glutamate data were obtained from the same animal (i.e. the issue of dual-probe recordings discussed in #1 above).
    a. **These changes have been made to provide clarification.**

Reviewer #3:

1. Grammar: lns 40-43, 57-61, 80, 94-96, 218, 293, 368-371
    a. **40-43: A dash was substituted for the semicolon since these were not two complete sentences**
    b. **57-61: The extra common was removed**
    c. **94-96: “is ideal for” was added**
    d. **218: “is” was removed**
    e. **293: “verses” was changed to “versus” and “lordosis” was also added for clarification**
    f. **368-371: This sentence was reworded for clarification**

2. Surgery: Why was this age chosen (ln 111), and what does L:10 hr D mean (ln 113)?
    a. **A note explaining the decision for the age chosen as well as spelling out “light” and “dark” instead of “L” and “D” was added.**

3. Biosensor testing: Please be specific about what insertion means. It is penile insertion. ln 183.
    a. **“Penile” was added for clarification.**

4. Behavioral coding: define intromission (ln 228), and remove the word "gently" on ln 240.
    a. **“Penile” was added and “gently” was removed.**

5. Legends: for figure 4 please include more information, as there are 3 panels.
    a. **More information was included, including the addition of panel labels for clarification.**

6. Figures:
    a. 5: the time is missing.
        i. **This image is intended to be a stylized representative example so the reader can see the overall tonic increases in dopamine during a mating bout.**

    b. 6-7: please include the mount and ejaculatory behaviors on this too. My understanding is that these behaviors will not be associated with the peaks. Is this only one animal?
        i. **Because this is a methods paper describing the technique of enzymatic biosensing/carbon fiber recording and not meant to be a detailed report discussing scientific findings from sexual behavior in hamsters, we chose to present data with one specific behavior (intromission) that is locked to dopamine and glutamate as an easy-to-read example. When this data is published for scientific dissemination information on other copulatory behaviors will also be included.**

Reviewer #4:
This reviewer had no comments on the manuscript.