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Kevin N Hascup

Erin R Hascup

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Overview

Electrochemical Techniques for Subsecond Neurotransmitter Detection in Live Rodents

Kevin N Hascup and Erin R Hascup*

Alterations in neurotransmission have been implicated in numerous neurodegenerative and neuropsychiatric disorders, including Alzheimer disease, Parkinson disease, epilepsy, and schizophrenia. Unfortunately, few techniques support the measurement of real-time changes in neurotransmitter levels over multiple days, as is essential for ethologic and pharmacodynamic testing. Microdialysis is commonly used for these research paradigms, but its poor temporal and spatial resolution make this technique inadequate for measuring the rapid dynamics (milliseconds to seconds) of fast signaling neurotransmitters, such as glutamate and acetylcholine. Enzymatic microelectrode arrays (biosensors) coupled with electrochemical recording techniques have demonstrated fast temporal resolution (less than 1 s), excellent spatial resolution (micron-scale), low detection limits (≤200 nM), and minimal damage (50 to 100 µm) to surrounding brain tissue. Here we discuss the benefits, methods, and animal welfare considerations of using platinum microelectrodes on a ceramic substrate for enzyme-based electrochemical recording techniques for real-time in vivo neurotransmitter recordings in both anesthetized and awake, freely moving rodents.

Abbreviations: DOPAC, 3,4-dihydroxyphenylacetic acid; FAST, fast analytical sensing technology; GABA, γ-aminobutyric acid; MEA, microelectrode array.

Alterations in neurotransmission have been implicated in numerous CNS disorders including Alzheimer disease, Parkinson disease, Huntington disease, amyotrophic lateral sclerosis, depression, anxiety, addiction, epilepsy and schizophrenia.[13] As the number of transgenic animal models designed to mimic human pathophysiology of CNS disorders exponentially increases, it has become paramount to quantify changes in neurotransmitter levels during the progression from healthy to diseased phenotype. Unfortunately, few techniques support the measurement of real-time changes in neurotransmitter levels over multiple days, as is essential for ethological or pharmacotherapy studies. Microdialysis typically is used for these research paradigms, but its poor temporal and spatial resolution, makes this technique inadequate for measuring the rapid dynamics (milliseconds to seconds) of fast signaling neurotransmitters, such as glutamate and acetylcholine.[37,63,66] As such, advances in enzymatic biosensors coupled with electrochemical recording techniques have closed the gap in our understanding of neurotransmission.[16,19,24,25,27,30,32,35,36,41,47,49,67] Here we discuss the benefits, methods, and animal welfare considerations of using platinum microelectrodes on a ceramic substrate for enzyme-based electrochemical recording techniques for real-time in vivo neurotransmitter recordings in both anesthetized and awake, freely moving rodents.

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Departments of Neurology and Pharmacology, Center for Alzheimer's Disease and Related Disorders, Southern Illinois University School of Medicine, Springfield, Illinois.
**Corresponding author. Email: ehascup@siumed.edu*

Microdialysis Compared with Electrochemical Biosensors

Since the early 1980s, microdialysis has been used routinely to sample neurotransmitters and neuromodulators in the extracellular space of the CNS. This technique uses a semipermeable membrane that acts as a capillary to control the diffusion of extracellular neurotransmitters along their concentration gradients,[17,37,65] which subsequently are measured using offline techniques such as HPLC coupled with electrochemical detection. Because microdialysis is a sample collection system, the entire interstitial fluid around the probe is obtained to enable offline determination of multiple analytes at femtomolar concentrations. Typically, microdialysis samples are collected every 5 to 20 min, although advances in the detection methods coupled with online HPLC have allowed for subminute sampling rates.[38,40,57,64] However, these approaches are complicated, labor-intensive, and expensive and therefore are impractical for routine use by many laboratories.

Although minute sampling rates may be sufficient to study most catecholaminergic neurotransmissions, the temporal resolution of microdialysis is too slow to detect the millisecond release and uptake of many excitatory or inhibitory neurotransmitters. This is the first of several limitations of microdialysis. Second is the low spatial resolution of the dialysis probes. A typical dialysis probe has a diameter of 150 to 400 µm with an average length of 1 to 4 mm— significantly larger than the synaptic cleft—thereby precluding analyte sampling close to the synapse. The large size of the dialysis probe coupled with the rapid uptake of glutamate into astroglial transporters hampers accurate measurement of

glutamate release, clearance, and resting levels by microdialysis.[18,25,63] Third, the constant perfusion of artificial cerebral spinal fluid into the CNS is speculated to dilute neurotransmitter levels.[25] Fourth, implantation of the microdialysis probe results in short- and long-term cellular damage, as evidenced by the histologic, physiologic, biochemical, and neurochemical changes in CNS tissue that can occur within a 2.8-mm circumference around the implant site[5,12,23] and result in aberrant neurotransmitter release and uptake as far as 220 µm from the probe.[4,68]

As such, electrochemical techniques have been developed to address the limitations of microdialysis. In vivo electrochemistry is a simple yet powerful means for real-time, online monitoring of neurotransmitter overflow in the extracellular space.[1,42] A potentiostat controls an applied potential at the working microelectrode (typically an inert material such as platinum, iridium, or carbon) compared with a Ag/AgCl reference. With a sufficient potential, molecules are either oxidized or reduced, depending upon their intrinsic electrochemical properties, directly at the working electrode surface. The currents generated from Faradaic reactions are linear with regard to the concentration of the electroactive molecule(s) in the tissue surrounding the microelectrode. For a more detailed explanation of the electrochemistry involved, we refer the reader to the literature.[1,21,26,28,31,42,45]

These microelectrodes initially were single-carbon fiber wires used for the detection of dopamine, serotonin, and norepinephrine; however, they have had limited success for monitoring nonelectroactive neurotransmitters, such as γ-aminobutyric acid (GABA), glutamate, and acetylcholine. A new generation of microelectrodes therefore was developed. By using photolithographic techniques, researchers have been able to reproducibly pattern multiple recording sites onto a single biocompatible substrate, thereby creating a microelectrode array (MEA). This development has become extremely important, considering that MEA measure analytes only from small foci (microns) of tissue directly surrounding the recording surface. In addition, electrodes with multiple recordings sites can be arranged geometrically to measure analyte concentrations from 2 or more distinct brain regions[28] (Figure 1 A through C). MEA designed by using photolithographic techniques have reproducible high spatial (microns) resolution and minimal damage to tissue (50 to 100 µm).[23] When coupled with constant-potential amperometry, the measurement of current at a constant, fixed potential, neurochemical events can be monitored as rapidly as every millisecond or less[21,45] over multiple days[29,59] with the ability to simultaneously detect single-unit activity and local field potentials.[23,69,70]

In conjunction with constant-potential amperometry, MEA are not without their pitfalls. They have poor chemical resolution, such that it is difficult to distinguish between multiple electroactive molecules that are present in vivo. To further complicate matters, several interfering compounds (ascorbic acid, 3,4-dihydroxyphenylacetic acid [DOPAC], 5-hydroxyindoleacetic acid, and uric acid) exist and can be measured at oxidation potentials similar to those of biogenic amines. Fortunately, these limitations can be addressed by using MEA surface modifications (exclusion layers and enzymes) as well as self-referencing techniques that allow for selective monitoring of specific neurochemicals, including dopamine, glutamate, choline, acetylcholine, glucose, lactate, and adenosine.

MEA Surface Modifications

Prior to in vivo studies, MEA are subjected to numerous cleaning and coating procedures to ensure successful detection of specific neurotransmitters. These procedures have been thoroughly detailed elsewhere.[28,31] Few neurochemicals are electrochemically active at potentials of ≤ +0.7 V on platinum surfaces compared with a Ag/AgCl reference electrode, and those that are detectable are often not the molecule of interest. Furthermore, compounds such as ascorbic acid, DOPAC, 5-hydroxyindolacetic acid, and uric acid are present in the extracellular fluid in 100- to 1000-fold higher concentrations than are biogenic amines and amino-acid transmitters.[1,42,43] To discriminate against these molecules and improve the selectivity for the analyte of interest, various protective films can be applied onto the MEA surface. Nafion is an anionic Teflon derivative whose negatively charged sulfonic acid groups repel anionic interferents (such as ascorbic acid, DOPAC, 5-hydroxyindolacetic acid, and uric acid) and concentrate cationic analytes (such as dopamine, serotonin, and norepinephrine) at the platinum recording surfaces. The preparation of Nafion on the MEA makes this film a reliable choice of measuring neurotransmitters over multiple days (7 to 10 d) in awake, freely moving rats and mice.[29,59] However, because Nafion slightly concentrates biogenic amines to the recording surface,[1] m-phenylenediamine can be used in CNS regions with high tissue levels of monoamines. m-Phenylenediamine is electropolymerized onto the MEA, and the selectivity is likely achieved by forming a size exclusion layer that prevents larger molecules (ascorbic acid, dopamine, and DOPAC) from reaching the recording surface, whereas smaller molecules (nitric oxide and hydrogen peroxide, H_2O_2) are able to pass through the matrix.[20,41] Finally, when measuring analytes that exist in millimolar concentrations (glucose and lactate), an additional polyurethane layer is applied. Polyurethane improves the linearity of the MEA for high concentrations of glucose or lactate yet provides an additional exclusion layer that prevents the diffusion of biogenic amines, thereby making the MEA nearly interferent free.[9]

Enzymes provide a means to convert a molecule that is not inherently electroactive (and thus not measurable with this technique) into a reporter molecule (such as H_2O_2) that is oxidized at the platinum recording surfaces. The current measured from the oxidation of H_2O_2 generated during the enzymatic breakdown is directly proportional to the analyte concentration.[28,31] Table 1 provides a list of available enzymes and their potential uses. Some compounds require multiple enzymes to convert them to a reporter molecule, such as acetylcholine, adenosine, and GABA. A chemical crosslinking procedure is used to immobilize the enzymes to the MEA recording surface, thereby stabilizing the enzymes and prolonging their activity. Researchers have used several of these enzymes, including L-glutamate oxidase,[3,6-8,11,14,15,19,24,25,27,29,34,39,46-48,50,55,56,59-62] acetylcholinesterase,[10,22,44] choline oxidase,[8,50-54] L-lactate oxidase[9] and L-glucose oxidase[6] for in vivo neurochemical measurements. Although uncoated MEAs have a long shelf life, we recommend using a coated MEA within 2 wks.

Self-Referencing

The development of MEA with multiple, uniform recording surfaces that are patterned in a precise geometrical configuration allows researchers to apply similar films or enzymes on 2 separate, yet spatially adjacent, recording surfaces. This coating procedure allows for recognition of possible interfering agents and their removal from the analyte signal. Essentially, a self-referencing MEA can be considered the electrochemical equivalent

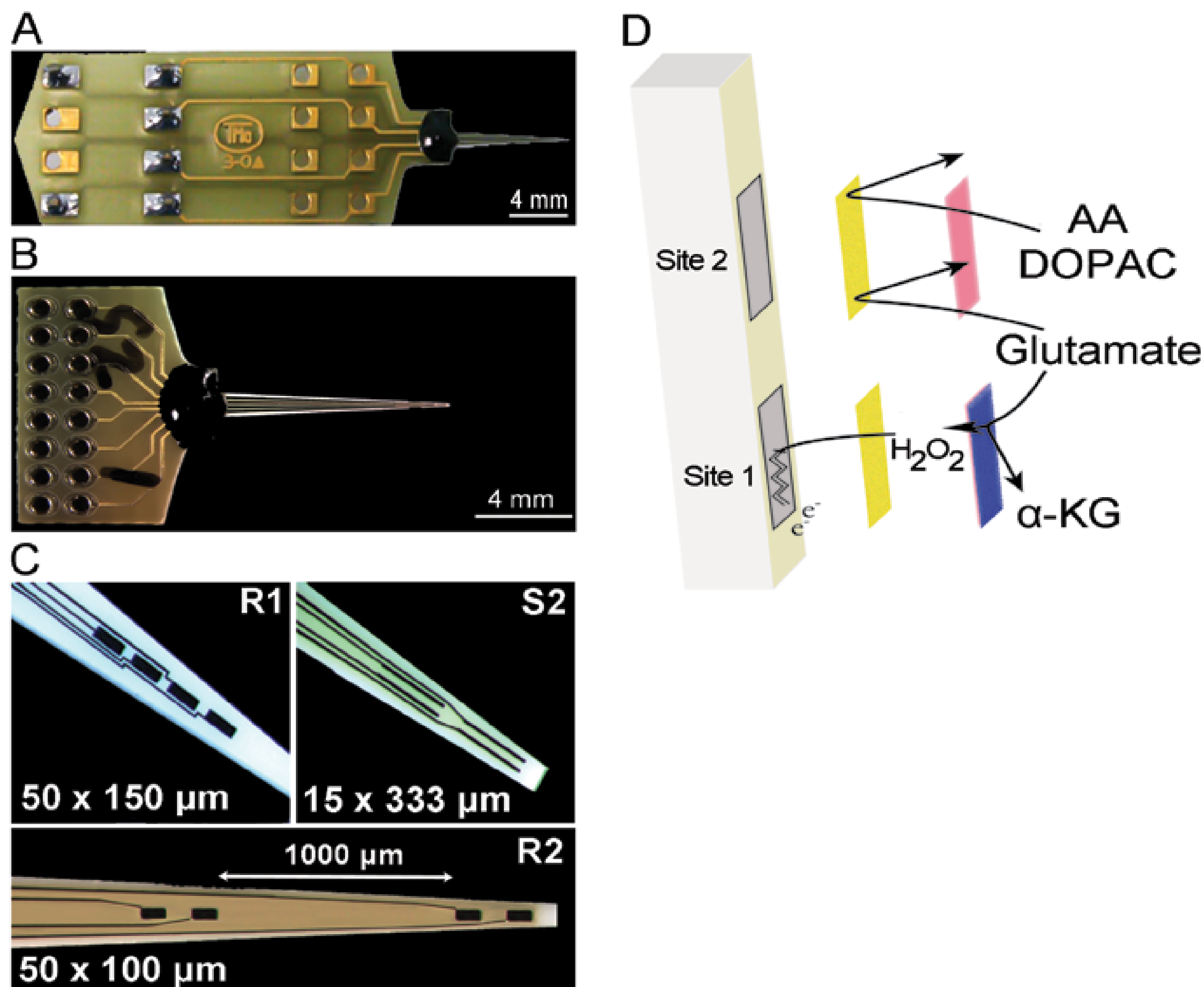

Figure 1. MEA designs and enzyme coating. Photographs of the fully fabricated (A) anesthetized and (B) freely moving MEA. (C) Magnified images of tips with several electrode recording sites patterned in unique geometrical configurations. The name of each tip is shown in the upper right, whereas the size of the recording sites is shown at the lower left. Where applicable, the distance between groups of recording sites is labeled. (D) Self-referencing schematic of an MEA coated with an exclusion and an enzyme layers for measurement of glutamate. Site 1 is an active recording site with the glutamate oxidase –BSA–glutaraldehyde coat in blue, and site 2 is a sentinel site with the BSA–glutaraldehyde coat in pink for the R2 MEA. Yellow indicates the exclusion layer, either Nafion or m-phenylenediamine.

of performing double-beam spectroscopy. Ideally, the only difference between the 2 sites is that one responds to the analyte of interest whereas the other does not. This difference is accomplished by coating an enzyme specific to an analyte of interest on one recording site and applying a chemically inactive protein to the other recording site (Figure 1 D). This chemically inactive site is often referred to as the control or sentinel site. The enzyme-coated site detects the analyte of interest in addition to everything else that the control site detects. Applying an inactive protein layer to the control site is necessary to minimize differences between the diffusional properties of molecules reaching the different recording surfaces.[6,8] Without this coating, the control sites may respond faster to interferents than do the analyte-detecting sites. Ensuring similar response times of the recording surface is imperative to remove the background and interfering signals from the analyte signal during offline subtraction. Using these offline subtraction methods, we are able to eliminate unknown artifacts from the analyte signal and enhance the signal-to-noise ratio. This procedure is often referred to as 'self-referencing,' and demonstrations of these principles are provided elsewhere.[6,8,11]

In addition to the ability to remove interferents that contribute to an analyte signal, self-referencing recordings can remove periodic or random noise and account for sensor drift, which may occur over prolonged implantation time. This ability provides an obvious advantage because smaller changes in current (lower detection limits) can be achieved in vivo.[6] In addition, self-referencing is useful during real-time (subsecond) monitoring of analytes in the brain. If the analyte-detecting site responds in the absence of a response on the control sites, the signal is considered to be due to analyte. For this situation, the control site is not used to quantify the analyte but rather to determine whether an interfering signal is present.[6]

Table 1. Enzymes

	Substrate	Product
Acetylcholinesterase	Acetylcholine	Choline, acetic acid
Alcohol oxidase	Alcohol, O_2	H_2O_2, aldehyde
Ascorbate oxidase	Ascorbate, O_2	Dehydroascorbate
Aspartate oxidase	Aspartate, O_2	H_2O_2, NH_4^+, oxaloacetate
Catalase	H_2O_2	O_2
Cholesterol oxidase	Cholesterol, O_2	H_2O_2, 4-cholesten-3-one
Choline oxidase	Choline, O_2	H_2O_2, betaine
Galactose oxidase	D-galactose, O_2	H_2O_2, D-galacto-hexodialose
Glucose oxidase	Glucose, O_2	H_2O_2, gluconic acid
L-glutamate oxidase	L-glutamate, O_2	H_2O_2, α-ketoglutarate
GABase	GABA, α-ketoglutarate	Succinic semialdehyde, L-glutamate, NADPH, H^+
Glutaminase	Glutamine	NH_4^+, L-glutamate
Glycerol kinase	Glycerol, ATP	Glycerol-3-phosphate, ADP
Glycerol-3-phosphate oxidase	Glycerol-3-phosphate, O_2	H_2O_2, dihydroxyacetone phosphate
Hexokinase	ATP, glucose	Glucose-6-phosphate, ADP
Horseradish peroxidase	H_2O_2	H_2O, O_2
Lactate oxidase	Lactate, O_2	H_2O_2, pyruvate
Lysine oxidase	Lysine, O_2	H_2O_2, NH_4^+, 6-amino-2-oxohexanoic acid
Pyruvate oxidase	Pyruvate, O_2, phosphate	H_2O_2, CO_2, acetyl phosphate
Sarcosine oxidase	Sarcosine, O_2	H_2O_2, glycine, formaldehyde
Xanthine oxidase	Xanthine, O_2	H_2O_2, uric acid

MEA Calibration

Microfabricated MEA generally have highly reproducible recording surfaces; however, manufacturing procedures may cause slight variations to each recording surface that could alter their responses to analytes. In addition, current-exclusion and enzyme-coating procedures can result in different layer thicknesses; therefore, each MEA must be calibrated in vitro prior to experimentation to determine standard curves. Essentially, the calibration is used to equate a change in current from the oxidation of H_2O_2 to a proportional change in analyte concentration from the oxidase enzyme generating H_2O_2 at physiologic temperature (37 °C) and pH (7.4). The current, in picoamperes, is measured by a potentiostat, such as the Fast Analytical Sensing Technology (FAST) 16mkIII system (Quanteon, Nicholasville, KY), and generates a standard curve for each recording site. In addition, known interferents such as ascorbic acid, are added during the calibration to test the selectivity of the recording sites to the analyte of interest compared with interferents. Finally, compounds used for pharmacodynamic studies should be tested in vitro to ensure they are not inherently electrochemically active, a situation that would falsely contribute to neurotransmitter levels during recordings. Once calibration is complete, the standard curve is used to determine the concentration of the measured analyte from the change in current during in vivo experimentation.

MEA Implantation and Neurotransmitter Recording

These MEA were designed for routine recordings of neurotransmission in the CNS of anesthetized or awake, freely moving rats and mice. The following section outlines the procedure for the preparation and surgical implantation of the MEA for in vivo CNS recordings. All protocols should be approved by the IACUC prior to proceeding. The procedures we outline here are based on methods used by the authors at several different institutions in the United States, Europe, and Canada. All procedures were approved with their respective IACUC.

Nonsurvival surgical procedure for recordings in anesthetized rodents Recording in anesthetized animals are ideal for performing dose–response studies in multiple CNS regions of a single animal. MEA can be reused multiple times for these experiments, helping to reduce costs. However investigators must remember that anesthetics can directly affect receptors. For example, ketamine, a noncompetitive N-methyl-D-aspartate receptor antagonist, would not be an ideal anesthetic for glutamatergic studies.[2,58]

Rodents are anesthetized with urethane (1.25 g/kg IP) or another IACUC-approved anesthetic, such as isoflurane. When the animal no longer responds to sensory stimuli, it is placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA) and monitored for responsiveness throughout the experiment approximately every 10 to 15 min. A Deltaphase Isothermal Pad (Braintree Scientific, Braintree, MA) is placed between the metal frame and the animal to maintain its body temperature at 37 °C. In the case of mice, these heating pads are bulky, so a water pad connected to a heated water bath (Stryker, Kalamazoo, MI) can be used to maintain body temperature. All surgical tools must be sterilized prior to surgery, typically by autoclave. The fur over the skull is shaved, wiped with a povidone–iodine solution, and a heat-sterilized scalpel is used to make a small incision along the midline of the scalp. The skin is reflected by using bulldog clamps (Fine Science Tools, Foster City, CA). Once the skull is exposed, a rotary tool (Dremel, Mount Prospect, IL) with bit size 107 (rats) or 105 (mice) is used to perform a craniotomy large enough to lower the MEA (Quanteon) into the CNS region of interest. With heat-sterilized forceps, the overlying dura is pulled laterally to expose the surface of the brain. Finally, a small hole is drilled in

a remote location from the recording site for an Ag/AgCl reference electrode (A-M Systems, Sequim, WA). The coated tip of the Ag/AgCl reference electrode (disinfected in 70% ethanol prior to coating) is inserted into the brain and held in place by using dental acrylic (Lang Dental MFG, Wheeling, IL). The MEA is attached to the stereotaxic arm via an electrode manipulator (Kopf Instruments), and a micromanipulator (Narishige International, East Meadow, NY) can be used to precisely raise or lower the MEA in the CNS.

Throughout the experiment, the animal should be evaluated for anesthetic depth and physiologic functions, and appropriate actions should be taken to prevent animal discomfort. Depending on the length of the surgery or experiment—typically 1 to 6 h depending on the anesthesia used and experimental design—fluid replacement with a subcutaneous injection of Ringers solution might be necessary. Once the experiment has concluded, the animal should be euthanized according to approved IACUC protocols.

Survival surgical procedure for recordings in awake, freely moving rodents Survival surgeries for long-term (multiple days) neurotransmitter recordings typically use chronically implanted MEA[23-25,29,30,59] and are necessary for ethologic testing to correlate phenotypic and neurotransmitter alterations during behavior-related tasks. Some behavioral paradigms, such as the Morris water maze, are ill-suited for these types of recordings. In our experience, MEA have successfully been implanted in rodents without noticeable changes in normal behavior (eating, grooming), with minimal damage to the surrounding tissue,[23] and without infection or irritation at the implant site for as long as 12 mo. However, in vivo MEA viability varies depending on the characteristic being tested. For example, glutamate has been reliably measured for at least 14 d after implantation.[23] Positive responses to local application of H_2O_2 (tests MEA viability but not enzyme-coating function) have been observed for at least 90 d after implantation.[59] Finally, electrophsyiologic studies have used MEA to record local field potentials for as long as 180 d after implantation.[23] The data indicate that the enzyme coating on the MEA deteriorates long before the MEA stops functioning.

During the week prior to surgery, rodents are acclimated to the recording chamber for 1 h each on 5 separate days to help prevent stress or anxiety due to introduction to novel stimuli during recordings. On the day of surgery, the MEA is calibrated and prepared for chronic implantation. All surgical instruments are autoclaved prior to initial use and heat-sterilized in a glass-bead sterilizer during surgery, and the operative work site is disinfected with 70% ethanol. All surgeries are performed in a Vertical Laminar Flow Workstation with HEPA filtration (Microzone, Ottawa, Canada). Rats or mice are anesthetized with approximately 2% isoflurane and placed in a stereotaxic apparatus fitted with a gas anesthesia head holder and mask and nonrupture ear bars (Kopf Instruments). Animal body temperature is maintained at 37 °C by using a Deltaphase isothermal heating pad (BrainTree Scientific), and the animals' eyes are lubricated with artificial tears (The Butler Company, Columbus, OH) to help maintain moisture and prevent infection. Prior to incision, the fur directly over the skull is shaved, and the skin directly on top of the animal's head is wiped with povidone–iodine solution to clean the incision area and to reduce the likelihood of infection. A scalpel is used to make a single incision along the midline, and the skin is reflected using bulldog clamps (Fine Science Tools). Then 3 small holes are drilled in the skull in the quadrants adjacent to the MEA implantation site, for placement of stainless steel skull screws. A fourth hole is drilled contralateral from the recording site for insertion of the Ag/AgCl reference electrode wire. Next, 3 small stainless steel screws (Small Parts, Logansport, IN; disinfected in 70% ethanol) are threaded into the skull to serve as anchors, and care is taken so that the screw tips do not touch brain tissue or dura. A small (maximum, 2 mm × 2 mm) craniotomy is performed over the recording area, and a calibrated MEA pedestal assembly is implanted based on stereotactic coordinates. The assembly is secured with approximately 4 layers of dental acrylic (Lang Dental MFG), with care taken to cover as much of the pedestal assembly as possible. The dental acrylic should have a smooth texture, and excess acrylic should be removed from the skin surface to avoid postsurgical irritation, which may cause the animal to scratch its head frequently and potentially damage the implant. Depending on the length of the surgery, fluid replacement with a subcutaneous injection of Ringers solution might be necessary.

Once the dental acrylic has dried, the rodent is removed from anesthesia, and oral acetaminophen (mice: 120 mg/kg PO every 4 h; rats: 100 mg/kg PO every 4 h)[33] is given to help alleviate postoperative pain. The described survival surgeries typically last approximately 30 to 45 min. Because of this short duration, analgesics can be given immediately preoperatively or postoperatively. Because acetaminophen is given orally, rodent access to postoperative analgesics can be maintained if necessary, in accordance with approved dosing schedule for the analgesic. However, subsequent doses of acetaminophen or other analgesics rarely are necessary with this procedure, as the rodents usually do not show signs of pain or distress (lack of eating, drinking, or grooming; hunched posture) after initial administration of acetaminophen. Animals are returned to their home cages and placed on a heating pad until fully recovered from anesthesia. Food and water are provided ad libitum. During this recovery phase, trained personnel should monitor continuously for signs of distress, and appropriate measures should be taken to alleviate such symptoms. Once fully recovered from anesthesia and in the absence of signs of distress, animals are returned to their housing facility and allowed 72 h to recover prior to initiation of behavioral testing. Although infection or inflammation is possible near the implantation site, we have had great success with the procedures outlined and have had no apparent incidence of infection or inflammation in chronically implanted rodents. At any time after surgery, animals developing signs of pain or distress that cannot be alleviated should be euthanized according to approved IACUC protocols.

Rodents are typically group housed before surgery, but are single-housed after surgery. This is done to avoid interactions with other animals that may interfere with healing of the skin surrounding the implant site. After surgery, rodents are housed in traditional solid-bottom rodent cages with bedding, and no changes in housing are necessary, including those involving the feeder and wire-bar lid. Additional bedding or enrichment can be added if deemed necessary for the experiment or for animal well being, but is not necessary for recovery.

Conclusion

Electrochemistry is a powerful technique for studying online, real-time neurotransmission in anesthetized and awake freely moving rats and mice. This technique has helped researchers elucidate altered neurochemical signaling in numerous models of

CNS disorders, leading to improved translational therapeutic outcomes. Good surgical technique and animal care are essential to the collection of valid data using this approach.

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The authors declare no conflicts of interest.

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