

Video Article

Hippocampal Insulin Microinjection and *In vivo* Microdialysis During Spatial Memory Testing

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Abstract

Glucose metabolism is a useful marker for local neural activity, forming the basis of methods such as 2-deoxyglucose and functional magnetic resonance imaging. However, use of such methods in animal models requires anesthesia and hence both alters the brain state and prevents behavioral measures. An alternative method is the use of *in vivo* microdialysis to take continuous measurement of brain extracellular fluid concentrations of glucose, lactate, and related metabolites in awake, unrestrained animals. This technique is especially useful when combined with tasks designed to rely on specific brain regions and/or acute pharmacological manipulation; for example, hippocampal measurements during a spatial working memory task (spontaneous alternation) show a dip in extracellular glucose and rise in lactate that are suggestive of enhanced glycolysis^{1-3,4-5}, and intrahippocampal insulin administration both improves memory and increases hippocampal glycolysis⁶. Substances such as insulin can be delivered to the hippocampus via the same microdialysis probe used to measure metabolites. The use of spontaneous alternation as a measure of hippocampal function is designed to avoid any confound from stressful motivators (e.g. footshock), restraint, or rewards (e.g. food), all of which can alter both task performance and metabolism; this task also provides a measure of motor activity that permits control for nonspecific effects of treatment. Combined, these methods permit direct measurement of the neurochemical and metabolic variables regulating behavior.

Video Link

The video component of this article can be found at <https://www.jove.com/video/4451/>

Protocol

1. Surgery Preparation

1. Handling. Animals (most commonly, rats or mice, although the method for microdialysis and combination with behavioral testing is largely a general one with any required species-specific adaptations needed, e.g. for anesthesia) are handled for a minimum of 10 min/day for at least two days prior to surgery. Extensive handling has been shown to leave animals in an unstressed state at the time of testing, avoiding possible confound^{4-5,7,8}. We will use rats throughout this protocol as an example species. Handling must be done without stress caused by pulling on fur from e.g. latex or nitrile gloves: this can be best achieved using bare hands, in consultation with the facility veterinarian and employee health officials. If this is not possible, soft cotton gloves should be worn over barrier gloves to avoid pulling on the rats' fur.
2. Sterile Field Preparation. Prior to beginning surgery, the surgical area is prepared, a sterile field prepared around the stereotaxic apparatus and a sterile drape placed across the apparatus, covering a heating pad that is used to maintain rat body temperature. A circulating warm water pad with accurate thermostat is used to prevent overheating of the animal.
3. Anesthesia induction. The isoflurane vaporizer is checked to contain optimal levels of isoflurane and connected to the induction chamber. Connections are checked to confirm a closed loop. The air-handling vacuum system is checked to be operational. Isoflurane vaporization is switched on and the animal placed into the induction chamber using a 5% isoflurane in oxygen mix: that is, 5% isoflurane added into a stream of 100% oxygen and delivered to the induction chamber.
4. Placement into apparatus. Animals are secured in the stereotaxic apparatus using earbars and a tooth bar. Correct earbar placement results in an immobile head and ears that lie flat along the earbars. Animals are rapidly secured and the nose inserted into a purpose-designed anesthetic nosecone; delivery of vaporized isoflurane is switched from the induction chamber to the nosecone and the anesthetic mix adjusted to deliver 2-3% isoflurane into the oxygen stream going to the nosecone.
5. Confirmation of anesthesia. A surgical anesthetic plane is confirmed by delivering a hard pinch to the foot and a puff of air to the eye; neither should cause any response. These tests are repeated at roughly 15 min intervals throughout surgery to confirm maintenance of a surgical anesthetic plane. Hair is removed from the incision site prior to entering the sterile field.

2. Surgery

1. Animal initial treatment. Ophthalmic ointment is applied to each eyeball to prevent drying. 1 ml sterile saline is given s.c. to prevent any dehydration during surgery, and the body is covered with a sterile drape. Betadine is applied to the scalp, and swabbed from the center out with a cotton swab; 70% ethanol is swabbed similarly, and the two swabbing steps are repeated twice more to ensure an appropriate incision site. Skin contact time for betadine and alcohol should be at least 3 min prior to incision. Anesthesia is maintained and checked regularly throughout surgery; a single s.c. injection of carprofen 5 g/kg is given to initiate analgesia, and aseptic technique is used. Analgesia is given following induction of anesthesia to minimize any stress from injection.
2. Incision and skull preparation. A 3-4 cm cut is made sagittally in the center of the skull. A 1:1 mix of bupivacaine:epinephrine is applied topically to provide further analgesia and minimize bleeding. The scalp is held away from the incision using surgical clips and sterile swabs are used to remove overlying membranes from the skull. Coordinates for drilling are measured using bregma as a reference point, marked using a sterile drill bit (or, alternatively, a hand-held cautery device), and re-confirmed for accuracy before drilling starts. Coordinates for specific brain regions of interest (e.g. here the hippocampus) are determined using a brain atlas. For hippocampal microdialysis, we use a drill site at 5.6 mm posterior to bregma, +5.0 lateral, and 3.0 ventral from dura.
3. Drilling. Three holes are drilled through the skull, with care being taken to apply only minimal force such that trauma to the dura and underlying membranes is minimal or absent. One hole is at the measured site of cannula insertion; the other two are positioned as convenient for insertion of skull screws. Purpose-sized screws (e.g. 1.17 mm self-tapping screws; Fine Science Tools) are inserted into these holes without impacting the brain beneath, and are used as anchor points for subsequent dental cement application.
4. Cannula placement and closure. The cannula is positioned at insertion coordinates, which are re-confirmed to be the site of the hole drilled, and is then slowly lowered to the target depth. Once correctly positioned, the cannula is secured in place with dental cement. If needed, a single sterile surgical suture is used to close the wound. A stylet is inserted into the cannula to maintain patency. In this protocol we use a CMA12 probe and guide cannula (CMA/Microdialysis).
5. Acute post-surgical care. 3 ml sterile saline is given s.c. to continue hydration; a single s.c. injection of carprofen 5 g/kg is also given to initiate analgesia. Animals are removed from the apparatus and placed in a warmed recovery room, in a clean cage, and monitored until they are fully recovered from anesthesia. Full recovery is assessed by restoration of righting reflex and normal locomotion. Animals are then returned to their home cage and regular holding room.
6. Short-term follow-up care. Cages of animals that have undergone surgery are marked with the date of surgery. Animals are monitored at least once each day for at least three days following surgery, and given one carprofen chewable tablet (2 mg) on the day after surgery and each of the two following days. If animals do not consume the carprofen, injectable carprofen may be given to ensure adequate analgesia. Monitor both the overall health of the animal and the state of the wound site (for infection, redness, etc.) following institutional animal care guidelines and seeking assistance or advice from animal care staff or the veterinarian if needed. Importantly, note that appropriate handling and acclimation to the experimenter is essential: animals should be handled extensively, including manipulation of the cannula, until no sign of nervousness or stress remains when handled by the experimenter.
7. Subsequent animal care and treatment. Animals will follow appropriate testing and euthanasia procedures according to the approved protocol and their specific experimental group.

3. Microdialysis (mD)

1. Perfusate preparation. Artificial extracellular fluid (aECF) is made with 153.5 mM Na, 4.3 mM K, 0.41 mM Mg, 0.71 mM Ca, 139.4 mM Cl, 1.25 mM glucose, buffered at pH 7.4⁹. **NOTE** that accurate fluid composition is essential: inaccuracies or use of other, less physiological fluids such as Ringer's or PBS for microdialysis will result in markedly erroneous results⁹. Specifically, note that the ionic composition of the extracellular fluid (ECF) is NOT the same as that of the CSF, as we showed in a 2004 detailed study of the hippocampal ECF⁹. On the day of testing, bovine serum albumin (BSA) should be added at 2% weight/weight and fully dissolved; this reduces loss of peptides such as insulin from adherence to the tubing, and also reduces the risk of fluid loss (ultrafiltration) at the probe membrane. After preparation, the perfusate should be filtered through a .2 μ m filter.
2. If a treatment such as insulin is to be delivered to the brain region of interest (here the hippocampus), prepare this treatment using an aliquot of the prepared aECF with the appropriate drug concentration. For insulin, a concentration of 400 nM (66.7 μ U/ μ l) for delivery via reverse microdialysis has been shown to affect hippocampal metabolic and cognitive function¹⁰. Note that the resulting tissue concentration of insulin is not measured here and remains unknown.
3. Setting up mD probe and lines. Prepare a fresh microdialysis probe and line the day before testing. Create two separate lines for "inflow" and "outflow". Use PE50 tubing to connect two 1 meter long pieces of FEP tubing and ensure that there is minimal dead space between the lines. Connect the inflow tubing to a 1 ml Hamilton syringe filled with sterile-filtered, deionized H₂O (dH₂O), and then attach to the probe.
4. Microdialysis swivel. To allow for free animal movement while taking measurements, connect a liquid swivel to the inflow and outflow lines, close to the pump, using additional FEP tubing. Remember to take the internal volume of this swivel and tubing into account in considering timing of sample collection (below).
5. Setting up mD pump. Turn on the mD pump and run at 1.5 μ l/min until you see dH₂O exiting the outflow tube on the probe. Then, while the pump is turned off, connect the other line between the outflow tube and a sample collection tube. Run 5 ml through the tube and place the probe in a vial containing sterile dH₂O overnight; ensure that the probe tip always remains wet.
6. Pre-probing test subject. 24 hr before testing, remove the dummy stylet from the rat's head and insert an mD probe (used only for this purpose, not for sampling) for 10 min. Then replace the dummy stylet and put the rat back into its cage. This procedure is designed to minimize any effect of reactive gliosis on the day of testing¹¹⁻¹² and in our hands, this gives good results that match data from other techniques and appear to reflect hippocampal activation^{4-5,8,10,13}; others have used a similar approach that leaves the probe in place for 24 hr prior to measurement, which is also a good approach if damage to the probe overnight can be avoided.
7. Probe equilibration. On the day of microdialysis, fill the Hamilton syringe and scintillation vial with filtered aECF and pump through to equilibrate for 1 hr. If needed, fill a second Hamilton syringe with prepared treatment (for example, insulin-aECF) and place into the syringe pump.
8. Probe insertion. When equilibration is complete, remove the dummy stylet and gently insert the equilibrated mD probe into the rat's brain via the cannula. Place the rat into a clear plastic box that contains some of the rat's home bedding. Make sure to counterbalance the tubing:

attach a 1.6 ml microcentrifuge tube filled with water to the tubing outside the cage in such a way that gravity keeps the microdialysis lines unkinked but not taut. Let probe equilibrate in rat for 2 hr. At the start of this period, confirm that flow of perfusate is unblocked: this is most easily done by collecting perfusate outflow over a defined period and weighing the sample to confirm that the expected volume is exiting the system. Any probe that yields <90% of expected volume, and does not self-correct after removal and re-insertion, should be replaced (otherwise edema at the probe tip will result). If flow is persistently low or absent, check all connections for leakage; failing that, disconnect tubing in stages to see whether the blockage can be isolated. If the problem is not identified, replace the probe; if the problem does not resolve, it may be necessary to start afresh with new probe and lines from Section 3.3. The two-hour equilibration period permits the blood-brain barrier to reseal around the probe and avoid any acute effect of probe insertion.

9. Collecting samples. Ensure that the correlation between sample dialysis (from brain) and collection (into tube) is accurately calculated. For instance, using two meters of FEP tubing between swivel and probe, there is a total volume between probe and collection of 30 μ l and hence it takes 20 min at 1.5 μ l/min flow rate for sample to pass through the probe, tubing, connectors and swivel to reach the end of tubing for collection. When collecting samples ensure that you collect enough volume to have concentration necessary for your assays. Here, we will use 5 min sample bins, and thus collect 7.5 μ l of dialysate in each collection tube.
10. Baseline samples. Once equilibration is complete, begin sample collection. Collect at least three samples while the rat is at rest in the home chamber to establish a stable baseline of measurements.
11. Treatment timing. Take care to calculate required timing for initiation of treatment prior to the experiment: remember that just as there is a time lag between dialysis and sample collection, there is a lag (usually identical) between perfusate leaving the syringe and arriving at the animal's hippocampus. Hence, in our setup with a 30 μ l volume between syringe and probe, change the syringe to that containing treatment-perfusate 20 min before you wish the treatment to begin arriving at the hippocampus.
12. Changing syringes. A liquid switch can be used if desired but is not necessary: at the appropriate time, simply disconnect the inflow line from the control-perfusate syringe and rapidly connect to the treatment-perfusate syringe. This should take no more than 5 sec to avoid significant interruption to the perfusate flow. **NOTE** that this process should be reversed after the desired dosage has been delivered, if a time-limited delivery of treatment is desired. Alternatively, treatment may continue for the duration of sampling (see **Figure 2**). Air bubbles should not be introduced into the line during switching of syringes, as they may accumulate at the dialysis membrane and reduce probe efficiency.
13. Behavioral testing. If performing a behavioral task, follow that procedure (see section 4 below). **NOTE** that coordination with delivery of treatment to the hippocampus is important. For instance, delivery of insulin should be timed to occur 10 min prior to beginning testing¹⁴. Hence, the switch to an insulin-containing perfusate should occur 30 min prior to behavioral testing (20 min for perfusate to pass through the lines plus 10 min desired delivery time ahead of testing).
14. Completion of sample collection. After collecting the desired samples, gently remove the probe from the animal's head, again remembering to take into account time lag between dialysis and sample collection. Return the animal to its home cage and observe closely for any post-experimental change in health or behavior until the animal is killed and the brain removed to confirm correct probe placement. If sacrifice will not occur immediately, return the dummy stylet to the cannula to avoid any introduction of foreign material.
15. Analysis. The analytical methodology will vary depending on the analyte(s) of interest. Because dialysis sampling does not allow for complete analyte equilibration at the probe membrane, sample concentrations should be corrected to give ECF concentrations using the zero-net-flux method^{15,16}.

4. Behavioral Testing

1. Placement on maze. After baseline samples (*i.e.*, after at least three samples have completed dialysis, but may still be in the outflow tubing being collected), gently move the rat into the behavioral testing apparatus. Any appropriate task may be used; the data in **Figure 1** were collected using a four-arm plus-shaped maze and measuring spontaneous alternation, which is a measure of spatial working memory: the rat is initially placed in the center of the maze and allowed to explore freely^{13,17-18}. Because any behavioral test may be used, the focus of this protocol is not on the specific task used as an example here (which is detailed elsewhere^{13,19,20}) but in brief, animals are allowed to explore the maze (the period of the grey box in **Figure 1**) and use hippocampally-dependent processes to retain memory of which arms have recently been visited.
2. Dialysis tubing movement during testing. Hold the tubing such that it moves freely, neither hindering the rat's movement nor being permitted to move in front of the animal and distract it. Stay in place and minimize your own movement so that you do not affect the rat's behavior.
3. Continued sample collection. As during baseline, move the outflow tubing to a new collection tube every 5 min.
4. Alternation testing. Allow the animal to freely explore the maze for 20 min. Record the sequence and timing of arm entries either using a video recording or by hand for later task performance analysis^{13,20}.

5. Post-testing

1. Final sample collection. Following testing, remove rat gently from maze and return to control chamber. Continue microdialysis sample collection for at least four samples to cover the period of recovery from task performance.
2. Probe removal. After all samples have been collected, gently remove the probe from the animal's head and place into a storage vial; return the animal to its home cage.
3. Probe storage. After returning animal to the home cage and completing collection of any remaining dialysate, rinse the probe thoroughly with dH₂O and store in a scintillation vial filled with dH₂O and covered with parafilm. Flush the tubing by switching to a syringe containing a solution of 1:10,000 Kathon in dH₂O to prevent microbe growth. Probes can be re-used as long as they maintain good flow and have no damage to the membrane; with care this should routinely be in excess of ten uses.
4. Histology. Kill the animal and remove the brain. Slice on a cryostat and use standard histological techniques (*e.g.* cresyl violet staining) to confirm correct probe placement.

Representative Results

Rats should recover rapidly from surgery and be alert, motile and active within 30 min following cessation of anesthesia. The impact of the cannula cap should be minimal if the surgery is performed cleanly and minimal dental cement is used. If any sign of infection is noticed during

the post-operative monitoring, or the rat is in any way showing signs of distress or discomfort, immediately terminate the experiment; this should be extremely rare. During the handling prior to testing, animals should be alert, freely motile, friendly and inquisitive. Well-handled animals should have essentially no observable stress remaining from surgery, post-surgical recovery, or the presence of the experimenter; this can be confirmed if desired by measurement of plasma stress hormones (epinephrine, glucocorticoids) and/or glucose^{4-5,7,8}.

Brain ECF glucose is typically in the range 0.5 - 1.5 mM, varying by brain region, although higher values may be seen in diabetic animals; in the hippocampus, baseline glucose concentrations are on the close order of 1.25 mM^{9,16}. In the absence of any exogenous treatment, a dip in ECF glucose (and, commonly, a rise in ECF lactate) should be seen, during task performance, in brain regions involved in mediating that task (see **Figure 1** for example): this reflects the increased metabolic activity induced by the cognitive load^{13,19,21}. Similar changes may be taken as evidence that a particular treatment increases local metabolism, as seen for instance after acute administration of insulin via addition to the perfusate¹⁰ (**Figure 2**).

In general, a well-handled animal should perform behavioral testing with no sign of distress and without any sign of awareness of the microdialysis tubing: signs of excessive grooming, immobility, any indication of pain or attempts by the animal to remove the probe indicate likely insufficient handling and/or infection around the probe site, and should be taken as an indication to terminate that experiment.

Animals receiving insulin administration should, in addition to elevated hippocampal metabolism, show markedly enhanced spatial memory¹⁰. Control, untreated animals should have mean alternation performance on the 4-arm maze of between 65 and 75%^{10,13,19}.

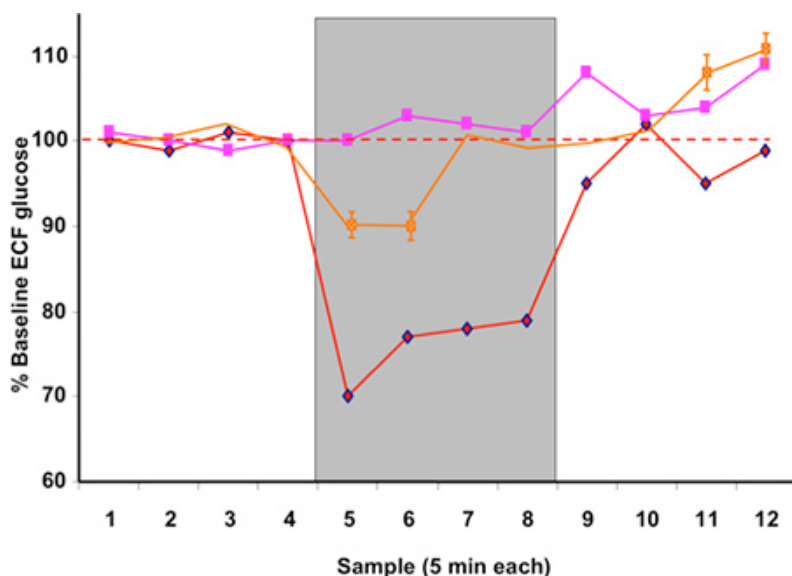


Figure 1. Task-associated dip in hippocampal ECF glucose (adapted from¹³). Grey box is the period of maze testing on spontaneous alternation (SA). Purple line shows hippocampal glucose in animals with no maze testing (but who were otherwise handled identically). Red line is measurements in animals performing the 4-arm SA task; orange line shows measurements in animals performing the easier 3-arm version of SA.

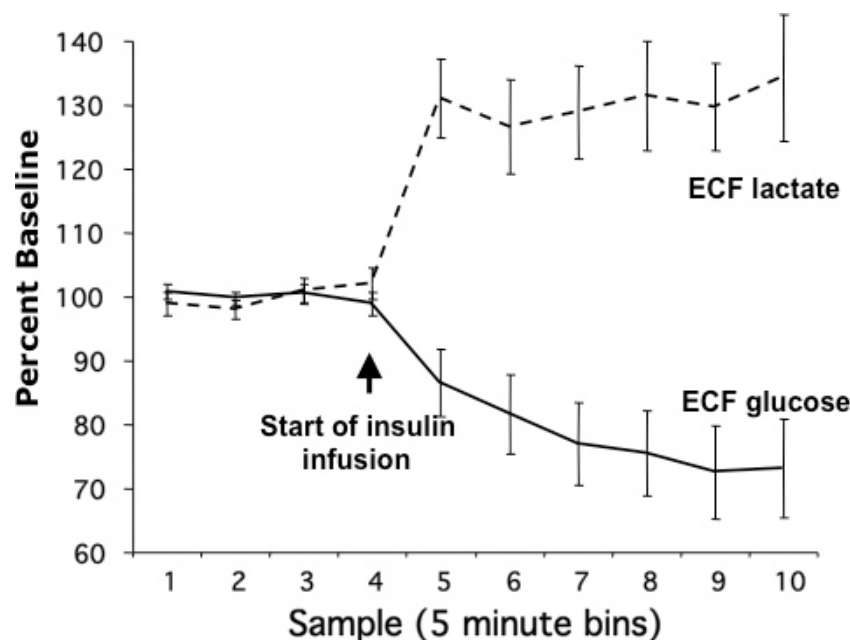


Figure 2. Alterations in hippocampal glucose and lactate after administration of insulin via inclusion in the perfusate (adapted from¹⁰). Insulin reaches the hippocampus at the point indicated by arrow and is administered continuously thereafter. Animals were tested in their home cages, with no behavioral manipulation.

Discussion

All solutions used in microdialysis should be filtered immediately prior to use, using a .2 μ m filter. After insertion of the probe into the guide cannula, observe to confirm that flow is unimpeded and sample collection is occurring. If flow stops after insertion, the most likely cause is damage to the probe membrane caused by insufficient care on insertion, and a fresh probe must be substituted.

As noted above, a key advantage to these methods is the lack of confound, permitting both behavioral and neurochemical measures in awake, freely moving animals: to take advantage of this, extensive handling prior to testing is essential in order that the animal be unstressed during measurements. The equilibration period is generally sufficient to provide a stable metabolic baseline, but food may be removed for 1-2 hr prior to testing if desired in order to ensure uniform blood glucose levels across animals.

The two most significant limitations of mD as a sampling technique are (i) restricted size of analyte and (ii) potential for loss of analyte due to adhesion in the tubing. The former can be alleviated to some extent by the use of mD probes with a larger molecular weight cutoff. Typical commercial probes permit cutoffs of up to 100 kD, so that molecules of up to perhaps 50 kD will pass through relatively unimpeded; however, use of lower cutoff membranes is recommended where possible to minimize any sample loss via ultrafiltration at the probe tip. Adhesion of target molecules to the tubing is an issue primarily in the cases of peptide measurements, many of which will tend to adhere to FEP tubing and hence reduce both analyte recovery and measurement accuracy. This problem can be minimized by (i) pre-treating the interior of the tubing using a perfusate to which 2% bovine serum albumin has been added, acting as a blocking agent, and (ii) by minimizing the length of the return tubing: if needed, a lightweight collection vial can be attached close to the outflow of the probe, although this poses additional challenges in switching collection tubes without disturbing the animal, especially during behavioral testing. One advantage of the technique is that samples are obtained in a form free of cellular debris or large molecules such as enzymes, and are generally suitable for direct analysis via injection into HPLC, MS or other analytical machinery as-is without the need for further purification; this also permits in many cases the analysis of multiple analytes in each sample (such as the glucose and lactate measurements shown in **Figure 2**).

A variation on the use of reverse microdialysis to deliver pharmacological treatments is to use dual microinjection-microdialysis probes, available from several sources. However, the bore of the injection port is generally very narrow and prone to blockage, and it can be difficult to accurately control the timing and/or volume of treatment delivery. This alternative is hence only recommended for treatments that are not amenable to delivery via inclusion in the perfusate.

Disclosures

No conflicts of interest declared.

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