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TITLE:

A Quantitative Autoradiographic Method for Determination of Regional Rates of Cerebral Protein Synthesis In Vivo

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SUMMARY:

Protein synthesis is a critical biological process for cells. In brain, it is required for adaptive changes. Measurement of rates of protein synthesis in the intact brain requires careful methodological considerations. Here we present the L-[1-^{14}C]-leucine quantitative autoradiographic method for determination of regional rates of cerebral protein synthesis *in vivo*.

ABSTRACT:

Protein synthesis is required for development and maintenance of neuronal function and is involved in adaptive changes in the nervous system. Moreover, it is thought that dysregulation of protein synthesis in the nervous system may be a core phenotype in some developmental disorders. Accurate measurement of rates of cerebral protein synthesis in animal models is important for understanding these disorders. The method that we have developed was designed to be applied to the study of awake, behaving animals. It is a quantitative autoradiographic method, so it can yield rates in all regions of the brain simultaneously. The method is based on the use of a tracer amino acid, L-[1-^{14}C]-leucine, and a kinetic model of the behavior of L-leucine in the brain. We chose L-[1-^{14}C]-leucine as the tracer because it does not lead to extraneous labeled metabolic products. It is either incorporated into protein or rapidly metabolized to yield $^{14}CO_2$ which is diluted in a large pool of unlabeled CO_2 in the brain. The method and the model also allow for the contribution of unlabeled leucine derived from tissue proteolysis to the tissue precursor pool for protein synthesis. The method has the spatial resolution to determine protein synthesis rates in cell and neuropil layers, as well as hypothalamic and cranial nerve nuclei. To

obtain reliable and reproducible quantitative data, it is important to adhere to procedural details. Here we present the detailed procedures of the quantitative autoradiographic L-[1-^{14}C]-leucine method for the determination of regional rates of protein synthesis *in vivo*.

INTRODUCTION:

Protein synthesis is an important biological process required for long-term adaptive change in the nervous system[1]. Inhibiting protein synthesis blocks long-term memory storage in both invertebrates and vertebrates[2]. Protein synthesis is essential for maintenance of the late phases of some forms of long-term potentiation (LTP) and long-term depression (LTD)[3], neuronal survival during development[4], and for general maintenance of the neuron and its synaptic connections[5]. Measurement of rates of brain protein synthesis may be an important tool with which to study adaptive changes as well as neurodevelopmental disorders and disorders related to learning and memory.

We have developed a method to quantify rates of cerebral protein synthesis *in vivo* in an awake animal that offers inherent advantages over other techniques that estimate rates in *ex vivo* or *in vitro* preparations of brain tissue[6]. Foremost is the applicability to measurements in the intact brain in an awake animal. This is a key consideration because it allows measurements with synaptic structure and function in place and without concerns about *post mortem* effects. Moreover, the quantitative autoradiographic approach that we employ achieves a high degree of spatial localization. Whereas the energy of ^{14}C is such that we cannot localize the tracer at the subcellular or cellular level, we can measure rates in cell layers and small brain regions such as hypothalamic nuclei, with approximately a 25 µm resolution[7].

One challenge of *in vivo* measurements with radiotracers is to ensure that radiolabel measured is in the product of the reaction of interest rather than unreacted labeled precursor or other extraneous labeled metabolic products[6]. We chose L-[1-^{14}C]-leucine as the tracer amino acid because it is either incorporated into protein or rapidly metabolized to $^{14}CO_2$, which is diluted in the large pool of unlabeled CO_2 in brain resulting from the high rate of energy metabolism[8]. Moreover, any ^{14}C not incorporated into protein exists primarily as free [^{14}C]-leucine, which over the 60 min experimental period, is almost entirely cleared from the tissue[6]. Proteins are then fixed to tissue with formalin and subsequently rinsed with water to remove any free [^{14}C]-leucine before autoradiography.

Another important consideration is the issue of the dilution of the specific activity of the precursor amino acid pool by unlabeled amino acids derived from tissue proteolysis. We have shown that in adult rat and mouse, about 40% of the precursor leucine pool for protein synthesis in the brain comes from amino acids derived from protein breakdown[6]. This must be included in the computation of regional rates of cerebral protein synthesis (rCPS) and must be confirmed in studies in which this relationship may change. The theoretical basis and the assumptions of the method have been presented in detail elsewhere[6]. In this paper, we focus on the procedural issues of the application of this methodology.

This method has been employed for the determination of rCPS in ground squirrels[9], sheep[10], rhesus monkeys[11], rats[12-21], a mouse model of Tuberous Sclerosis complex[22], a mouse model of fragile X syndrome[23-26], fragile X premutation mice[27], and a mouse model of phenylketonuria[28]. In this manuscript, we present the procedures for measurement of rCPS with the *in vivo* autoradiographic L-[1-^{14}C]-leucine method. We present rCPS in brain regions of an awake control mouse. We also demonstrate that *in vivo* administration of anisomycin, an inhibitor of translation, abolishes protein synthesis in the brain.

PROTOCOL:

All animal procedures were approved by the National Institute of Mental Health Animal Care and Use Committee and were performed according with the National Institutes of Health Guidelines on the Care and Use of Animals.

Note: An overview of the protocol is presented in **Figure 1**.

1. Femoral Vein and Artery Catheterization

Note: Complete surgery at least 22 h prior to administration of the tracer. Surgery requires about 1 h to complete.

1.1. Gather necessary materials: sterile surgical instruments (surgical scissors, micro-scissors, forceps, three surgical skin hooks), equipment for isoflurane anesthesia (isoflurane vaporizer, active gas scavenger, sealed anesthesia chamber, anesthesia nose cone), sterile surgery stage, fur clippers, 70% ethanol, sterile gauze, surgical tape, commercial hand warmers, surgical microscope, sterile 0.9% sodium chloride (saline), sterile heparin 100 USP units/mL in 0.9% sodium chloride (heparinized saline), five 20-cm strips of 6-0 absorbable suture, 25-cm strands of PE-8 and PE-10 polyethylene catheters with one end cut at 45°, 1 mL syringes, 32 gauge needle, cautery equipment, 15-20 cm hollow stainless steel rod (2.5 mm inside diameter, 3 mm outside diameter), and an animal enclosure with swivel appendage set-up (30 cm spring tether with button, swivel, swivel mount and arm, 20 × 13 cm clear cylindrical container).

1.2. Prepare animal for surgery.

1.2.1. Weigh the mouse.

Note: The animal must be at least 25 g for successful surgery.

1.2.2. Place the mouse inside a sealed plexiglass chamber and connect the chamber to the isoflurane anesthesia apparatus. Set the flow rate to 2.5 L/min for males and 3.0 L/min for females of 1.5% isoflurane in O_2. After roughly 2 min, ensure that the mouse is appropriately sedated by lack of a withdrawal reflex with a toe pinch.

1.2.3. Once sedated, remove the mouse from the chamber and lay it in a prone position with its face inside the anesthesia nose cone. Set up nose cone to receive gas from the vaporizer and to return gas to the gas scavenger which will capture isoflurane in a charcoal filter.

1.2.4. Use clippers to shave fur between the shoulder blades. Wipe off excess fur with 70% ethanol and gauze.

1.2.5. Flip the mouse over into a supine position keeping the face in the nose cone. Tape down the left leg onto the surgery stage and use clippers to shave fur from the left inner thigh to the upper left abdomen. Wipe off excess fur with 70% ethanol and gauze.

1.2.6. Slide an activated commercially available handwarmer, wrapped in gauze, under the mouse. Tape down the right leg onto the surgery stage.

1.3. Insert catheter into the left femoral vein.

1.3.1. With the aid of a surgical microscope, use surgical scissors to make a 1 cm incision from the upper medial portion of the left thigh rostrally towards the midline, revealing the femoral artery and vein.

1.3.2. Retract loose skin with surgical skin hooks above and on either side of the incision. Secure the skin hooks by taping them to the surgery stage.

1.3.3. Apply sterile 0.9% sodium chloride to exposed area to maintain adequate moisture.

1.3.4. Use forceps to blunt dissect, separating connective tissue around a small section of the femoral artery and vein. Carefully separate the artery and vein (**Figure 2**).

1.3.5. Use forceps to thread one strand of absorbable suture (Strand A) under both the femoral vein and artery at the most lateral point of the incision. Pull the suture halfway through so the ends are even.

1.3.6. At a more proximal point to the groin, use forceps to thread a second suture (Strand B) under only the femoral vein.

1.3.7. At a point between Strand A and Strand B, use forceps to thread a third suture (Strand C) under only the femoral vein. Gently tie a half knot that will be used to restrict blood flow. Be careful not to tear the vein.

1.3.8. Gently tug on Strand B to restrict blood flow. Tape the ends of Strand B to the surgery stage to maintain the restricted blood flow.

1.3.9. Connect the non-cut end of the PE-8 tubing to a 32 gauge needle and 1 mL syringe filled with heparinized saline. Flush catheter to remove air bubbles.

1.3.10. Cut a small hole in the restricted area of the femoral vein with micro scissors and carefully insert the angled end of the flushed PE-8 tubing, towards Strand B. Once inserted, release Strand B's tension and guide the catheter further up the vein. Tie Strand B around the vein containing the catheter.

1.3.11. Loosen Strand C's half knot and make a full knot around both the vein and the tubing. Make sure this knot does not capture the femoral artery.

1.3.12. Gently pull back on the syringe barrel to partially fill the tubing with blood to ensure that the catheter has been implanted properly.

1.4. Following the same procedure, insert a PE-10 catheter into the left femoral artery.

1.5. Complete surgical procedure.

1.5.1. Once both femoral vein and artery catheters have been secured, tie Strand A into a knot around both catheters.

1.5.2. Cut all excess sutures and remove skin hooks. Flush the arterial catheter with heparinized saline to prevent clotting. Cauterize the ends of both catheters to create a seal.

1.5.3. Place the mouse in the prone position and make a small incision at the base of the neck and apply saline to the exposed area.

1.5.4. Insert hollow metal rod subdermally from the neck incision to the femoral incision. Snake the catheters through the hollow rod and out of the neck incision. Remove the hollow rod.

Note: Implanting catheters subdermally will prevent the mouse from damaging the catheters.

1.5.5. Close the femoral incision with suture.

1.5.6. Snake the catheters through a 30-cm flexible hollow tube (spring tether) and suture the button of the spring tether under the skin.

1.5.7. Move the mouse into a clear cylindrical container (20 cm high, 13 cm diameter) with a swivel mount and arm to house the mouse during the recovery period. Place a hand warmer under the container to keep the mouse warm.

1.5.8. Screw the top of the spring tether to a swivel and secure the swivel to the swivel arm attached to the cylindrical container. Make sure the mouse has full range of motion and the catheters can be accessed.

1.5.9. Place a slotted lid that does not interfere with the swivel mechanism over the animal enclosure. Refer to **Figure 3** for the full set-up.

1.5.10. Allow the mouse to recover for at least 22 h.

2. Tracer Preparation

2.1. Prepare L-[1-^{14}C]-leucine solution for injection and 16% (w/v) 5-sulfosalicylic acid (SSA) dihydrate solution for deproteinizing plasma samples. In the SSA solution, also include 0.04 mM norleucine and 1 µCi/mL [H^3]-leucine as internal standards for amino acid analysis and analysis of tracer concentration in the acid-soluble plasma fractions, respectively. Store the SSA up to two months at 4 ˚C.

2.2. Purchase commercially available L-[1-^{14}C]-leucine (50-60 mCi/mmol), which is sold as a solution in 2% ethanol or 0.1 N HCl. Blow dry a known activity of the tracer under a gentle stream of nitrogen and reconstitute in a solution of sterile normal saline made up to a concentration of 100 µCi/mL.

3. Tracer Administration and Collection of Blood Samples

3.1. Gather necessary materials: 18 1.5 mL microtubes for deproteinizing plasma samples (add 70 µL of deionized water to each tube), 17 250 µL glass vial inserts (15 inserts for collection of arterial blood samples and 2 inserts for collection of dead space blood to be reinjected), 2 microcapillary tubes (32 × 0.8 mm, for hematocrit measurement), 1 heparin and lithium fluoride-coated microcentrifuge tube (to prevent clotting and glycolysis, respectively), hemostats (cover the tips with flexible polymer tubing so that clamps will not damage PE tubing), blood glucose monitor, blood pressure transducer, 1 mL sterile syringes (for saline flushes), and commercially available euthanasia solution for mice (diluted 1:1 in deionized water).

Note: To limit the collection of extra and unnecessary blood, small, thin glass vial inserts with tapered bottoms that allow for accessible pipetting of supernatant plasma is recommended.

3.2. Ensure that the mouse is in a normal physiological state at the outset of the experiment.

3.2.1. Clamp the arterial tubing about 2 cm from the end and cut off the tip, creating an opening for blood to flow. Then unclamp tubing and collect approximately 30 µL of dead space blood to collect any residual saline and/or blood from previous draws. Subsequently in a separate tube, collect a control sample (approximately 30 µL), hematocrit samples (about half of the capillary tube volume), and a glucose sample (approximately 20 µL).

3.2.2. Measure hematocrit by plugging one end with sealant putty and centrifuge for 1 min at 4500 x g. Measure the ratio of the volume of red cells to the total blood volume.

Note: If an animal has a hematocrit below 30%, do not continue the study.

3.2.3. Use a commercially available blood glucose monitor to measure the glucose level in a drop of blood.

3.2.4. Centrifuge the control sample for 2 min at 18,000 x g to separate plasma. Deproteinize plasma samples as follows: add 5 µL of plasma to 70 µL of deionized water in a 1.5 mL microtube, add 25 µL of the 16% SSA solution and vortex. Place on ice for 30 min before freezing on dry ice.

3.2.5. Return dead space blood to the animal through the venous line, followed by a heparinized saline flush to prevent excess blood loss.

3.2.6. Connect the arterial line to a blood pressure transducer to measure mean arterial blood pressure.

3.2.7. After taking the samples be sure to re-clamp the arterial line and to flush the line with a small volume (50 µL) of heparinized saline.

3.3. Administer tracer intravenously and collect timed arterial blood samples.

3.3.1. Use a Y-connector to attach one syringe with the tracer (100 µCi/kg) and one syringe with 50 µL sterile saline to flush the venous line after injection of tracer. Connect Y-connector to the venous line.

3.3.2. Initiate the study by simultaneously starting a stop watch and injecting the tracer. Flush the venous line with saline (c. 100 µL) immediately following injection.

3.3.3. Collect blood samples 1-7 continuously throughout the first 2 min of the experiment in the same manner. After collecting the 7th sample, collect 30 µL of dead-space blood before each remaining sample. Samples 8-14 are collected at 3, 5, 10, 15, 30, 45, and 60 min, respectively.

3.3.4. Process blood samples immediately after collection, as described for the control sample in step 3.2.4. If there is a delay, place the samples on ice. Carefully re-inject dead space blood into the artery *via* the arterial catheter and flush with heparin saline.

3.3.5. At some point during the experiment, process three internal standards by adding 25 µL of 16% SSA, 0.04 mM norleucine, and 1 µCi/mL [^{3}H]-leucine to 75 µL water, vortex and place on ice.

3.3.6. After collecting the 14th sample at 60 min, inject approximately 0.2 mL of B-euthanasia-D into the venous line to euthanize the animal. Record the time of death.

3.3.7. Unscrew the animal from the swivel mount and remove from the animal enclosure. Carefully remove the brain, place on aluminum foil, and freeze on dry ice. Store brain, samples, and internal standards at -80 ˚C until ready for processing.

Note: Do not freeze brains with liquid nitrogen as brains may crack. Processing can be performed at any point afterwards.

4. Analysis of Leucine and [^{14}C]-Leucine Concentrations in Plasma

4.1. Thaw samples and internal standards on ice, vortex, and centrifuge 18,000 x g for 5 min at 2 ˚C.

Note: The supernatant fraction will contain the free labeled and unlabeled leucine.

4.2. Transfer 40 µL of the supernatant to a liquid scintillation vial and add scintillation cocktail. Quantify disintegrations per min (DPM) of ^{3}H and ^{14}C by means of liquid scintillation counting and a quench curve designed for simultaneous double-label (^{3}H and ^{14}C) counting.

4.3. To quantify plasma leucine concentrations, use a high-performance liquid chromatography (HPLC) system with a sodium cation exchange column and post-column derivatization with o-phthaldehyde and fluorometric detection.

4.3.1. Set HPLC to the following specifications: fluorometer excitation of 330 nm and emission of 465 nm. The mobile phase consists of sodium eluant, pH 7.40, and sodium eluant + 5% sulfolane, pH 3.15. Set the buffer flow rate to 0.400 mL/min and the derivatization instrument flow rate to 0.300 mL/min. Set the column temperature to 48 ˚C and reactor temperature to 45 ˚C.

4.3.2. Calibrate the system with a range of amino acid concentrations (including norleucine) between 30 and 500 pmol/10 mL.

Note: The calibration curve is linear. The amino acid concentrations of the tested 10 µL injection samples fall within the ranges of this calibration curve.

5. Preparation of Brain Sections and Quantitative Autoradiography

5.1. Prepare brain sections 20 µm in thickness for autoradiography. Section brain by means of a cryostat at -20 ˚C.

5.2. Thaw mount serial brain sections on gelatin-coated slides. Air dry.

5.3. Wash slides in five changes of 10% formalin for 30 min per change, followed by a continuous flow of deionized water for 1 h. Cover the slides loosely with foil to avoid dust and allow to dry for 24 h.

5.4. Arrange slides in an X-ray film cassette along with a set of [^{14}C]methylmethacrylate standards that are pre-calibrated against tissue of known ^{14}C concentrations as described[29]. Under red safelight, place a piece of mammography film, emulsion side down, on top of the sections.

Note: Cassettes that fit 20 × 25 cm mammography films are recommended. Standards can be commercially purchased but ensure that these standards cover a range of 2-300 µCi/g of tissue and are calibrated against 20 µm tissue thickness.

5.5. Seal the cassettes and place in a black changing bag and store in a cabinet for 40-45 days.

5.6. Develop films according to manufacturer's directions.

Note: Automated film development is not recommended because the background may be uneven and can affect quantification.

6. Image Analysis

Note: A commercially available program for image analysis coupled with a charge-coupled device (CCD) camera and a fluorescent light box with even illumination is recommended. The relative optical densities in the illuminated film are detected by the CCD camera.

6.1. Construct a calibration curve of optical density (OD) *vs.* tissue ^{14}C concentration based on the ODs of the set of calibrated standards on the film. Fit these data (including the blank or background) to either a second or third degree polynomial equation.

6.2. To analyze specific brain regions, locate the region of interest (ROI) in six to eight sections by comparison with a brain atlas. Record the ODs of the pixels within a ROI in all sections and, based on the calibration curve, compute the tissue ^{14}C concentration in each pixel. Compute the average tissue ^{14}C concentration in the ROI.

7. Data Analysis

7.1. Compute rCPS in each ROI by means of the following equation:

$$R_i = \frac{P_i^*}{\lambda_i \int_0^T \left[\frac{C_p^*(t)}{C_p}\right] dt}$$

Where P*(T) is the weighted average tissue concentration of ^{14}C in the ROI, $C_p(t)$ and $C^*_p(t)$ are the arterial plasma concentrations of unlabeled and labeled leucine at time t, *T* is the time that the animal died (about 60 min), and λ is the fraction of leucine in the tissue precursor pool that comes from the plasma.

Note: Evaluation of λ is carried out in a separate experiment[6]. λ has been evaluated in wild type (WT), *Fmr1* knockout, *Tsc*$^{+/-}$, and PKU mice[6,22,25,28]. If an experiment involves either genetic or pharmacological changes that might affect rates of protein synthesis, degradation, or metabolism of leucine, λ should be evaluated under the new conditions.

REPRESENTATIVE RESULTS:

Here we show a representative experiment demonstrating the effects of prior administration of a protein synthesis inhibitor on rCPS. Anisomycin in normal saline was administered to an adult C57/BL6 male wild-type mouse subcutaneously (100 mg/kg) 30 min prior to initiation of rCPS determination. Effects of anisomycin treatment compared to a vehicle-treated control animal show that rCPS is almost undetectable in the anisomycin-treated mouse (**Figure 4**). These data represent a validation that the *in vivo* autoradiographic L-[1-^{14}C]-leucine method measures rates of protein synthesis in brain.

We present a figure of L-[1-^{14}C]-leucine autoradiograms at four levels of the brain to demonstrate the resolution of the method (**Figure 5**). Illustrated are the cell layers in the olfactory bulb (**Figure 5A** and **B**), the hippocampus (**Figure 5C**), and the cerebellum (**Figure 5G**). Nuclei in the hypothalamus (**Figure 5D**), the pons (**Figure 5E** and **F**), and the brain stem (**Figure 5H**) are also clearly seen in the autoradiograms. We also show the quantitative regional rates of protein synthesis in the frontal cortex (5.88 nmol/g/min) (**Figure 6A**) and dorsal hippocampus (5.35 nmol/g/min) (**Figure 6B**) of a typical control animal.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representing the steps of the entire rCPS protocol.

Figure 2: Image of exposed femoral artery and femoral vein. Laying parallel to one another, the femoral artery is shown above the femoral vein. The femoral vein also has a deeper red color than the femoral artery.

Figure 3: Image of recommended animal enclosure set-up for rCPS experiment. It utilizes a clear cylindrical animal enclosure with swivel appendage connected to a spring tether.

Figure 4: Representative images from a vehicle-treated animal (A) compared with an animal treated with anisomycin (100 mg/kg, subcutaneously) 30 min prior to administration of tracer (B). Rates of protein synthesis are proportional to the level of darkness in the image. Anisomycin drastically reduces the measured rates of protein synthesis indicating the specificity of this method. The scale bar in the upper right of A represents 1 mm and applies to both images.

Figure 5: Digitized autoradiograms from an awake behaving mouse at the level of the olfactory bulb (A, B), hypothalamus (C, D), pons (E, F), cerebellum (G), and brain stem (G, H). The darker regions have higher rCPS. The scale bar in panel G applies to panels A, C, E, and G. Autoradiograms on the right (B, D, F, and H) are enlarged images from the areas designated on the images on the left and the scale bar in panel H applies to panels B, D, F, and H. Abbreviations are as follows: FrA, frontal association cortex; OB, olfactory bulb; AO, anterior olfactory nucleus; Gl, glomerular layer; EPl, external plexiform layer; BLA, basolateral amygdala; py, pyramidal cell layer; dHi, dorsal hippocampus; DG, dentate gyrus; MHb, medial habenula; Rt, thalamic reticular nucleus; VMH, ventral medial hypothalamic nucleus; Arc, arcuate nucleus; EW, Edinger-Westphal nucleus; R, red nucleus; PN, pontine nucleus; ML, molecular layer; GL, granular layer; Pc, Purkinje cell layer; Cu, cuneate nucleus; AP, area postrema; 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus.

Figure 6: Digitized autoradiograms from an awake behaving control mouse at the level of the frontal cortex (A) and dorsal hippocampus (B). Rates of cerebral protein synthesis are color coded in the images according to the color bar shown on the right. The scale bar in the lower left of A represents 1 mm and applies to both images.

DISCUSSION:

We present a quantitative method for determination of regional rates of cerebral protein synthesis (rCPS) *in vivo* in experimental animals. This method has considerable advantages over existing methods: 1. Measurements are made in the awake behaving animal, so they reflect ongoing processes in the functioning brain. 2. Measurements are made by means of quantitative autoradiography affording the ability to determine rCPS in all regions and subregions of the brain simultaneously. 3. The kinetic model of the method takes into account the possibility of recycling of unlabeled amino acids derived from tissue protein degradation and its effect on the precursor pool for protein synthesis[6].

The primary limitation of this method is that it is time-consuming and demanding. Whereas it is tempting to employ simpler and higher throughput methods, the limitations of data obtained must be acknowledged.

Because of the complexity of measuring rCPS in an intact mouse, problems with maintenance of a normal physiological state, collecting adequate blood samples, and avoiding possibly interfering conditions may be encountered. Surgical implantation of the venous and arterial catheters is challenging. As with any surgical procedure, especially with the handling of delicate vasculature, there is an inherent risk for mortality of the animal. For us, it is rare (about 1%). During the ensuing 22 h recovery period, occasionally (about 4%) an animal will pull a catheter out. During the measurement, it is important that catheters are patent and that animals are in a normal physiological state. In our recent experience, arterial blood cannot be collected in about 2% of animals and about 1% of animals had a low hematocrit (< 40%) or low arterial blood pressure (< 85 mm Hg), suggesting blood loss during surgery and/or recovery.

In the preparation of brain sections for autoradiography, it is important to ensure that section thickness is 20 µm because that is the section thickness to which [^{14}C]methylmethacrylate standards have been calibrated. Use care to ensure good quality sections, *i.e.,* without tears, folds, or bubbles as these imperfections will interfere with the autoradiographic analysis. We develop autoradiographic films by hand rather than in an automated film processor because we find that background optical density can be uneven following automated processing, and this can affect the quantification.

In the equation for rCPS, we include a factor, lambda (λ), that is the fraction of leucine that comes from the arterial plasma, the remainder comes from the recycling of amino acids derived from tissue protein degradation[6]. We have evaluated λ in separate experiments in WT and *Fmr1* KO (fragile X model) C57Bl/6J mice and shown that its value is 0.603. The value of λ may vary depending on species, genetic background, or the presence of a genetic mutation. Therefore, if

designing protein synthesis experiments for other models, one will need to evaluate λ before an accurate measurement can be obtained.

Our work in genetic mouse models of neurodevelopmental disorders demonstrates that this methodology reveals changes in rCPS in these models and in some cases responses to pharmacological treatments[22,23,25,26]. It is also conceivable that the rCPS measurement may also monitor degenerative changes in brain in conditions such as models of Alzheimer's disease, Parkinson's disease, fragile X tremor ataxia syndrome, traumatic brain injury, *etc.* In these models, it might be possible to track early degenerative changes and possibly also responses to early interventions. The rCPS method can be used alongside immunohistochemistry in parallel sections to further examine specific brain changes[25]. In summary, the quantitative autoradiographic L-[1-^{14}C]-leucine method is ideal for accurate determination of rCPS values *in vivo*. It offers considerable advantages in terms of accuracy and applicability to *in vivo* conditions over existing methods.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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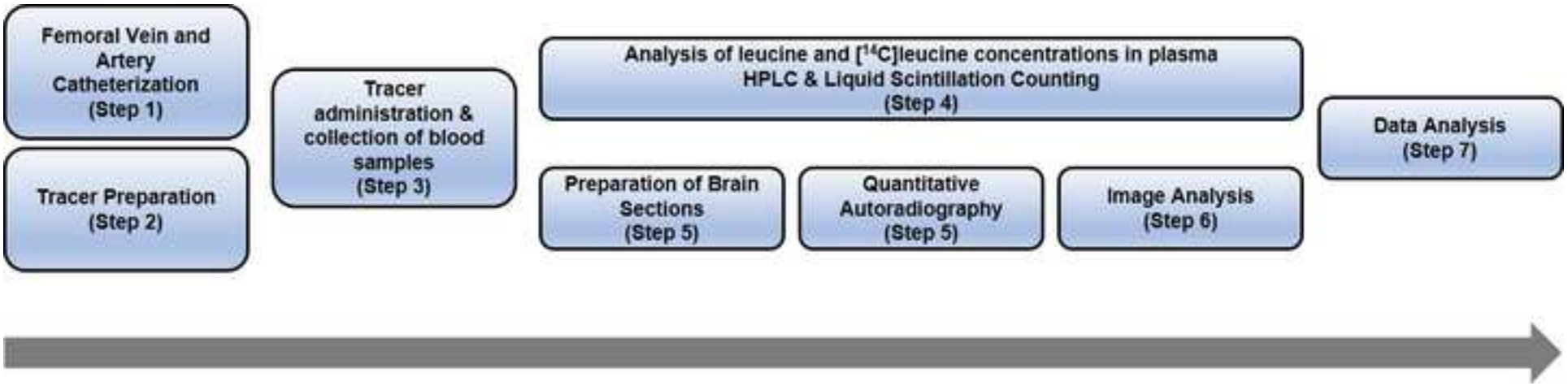

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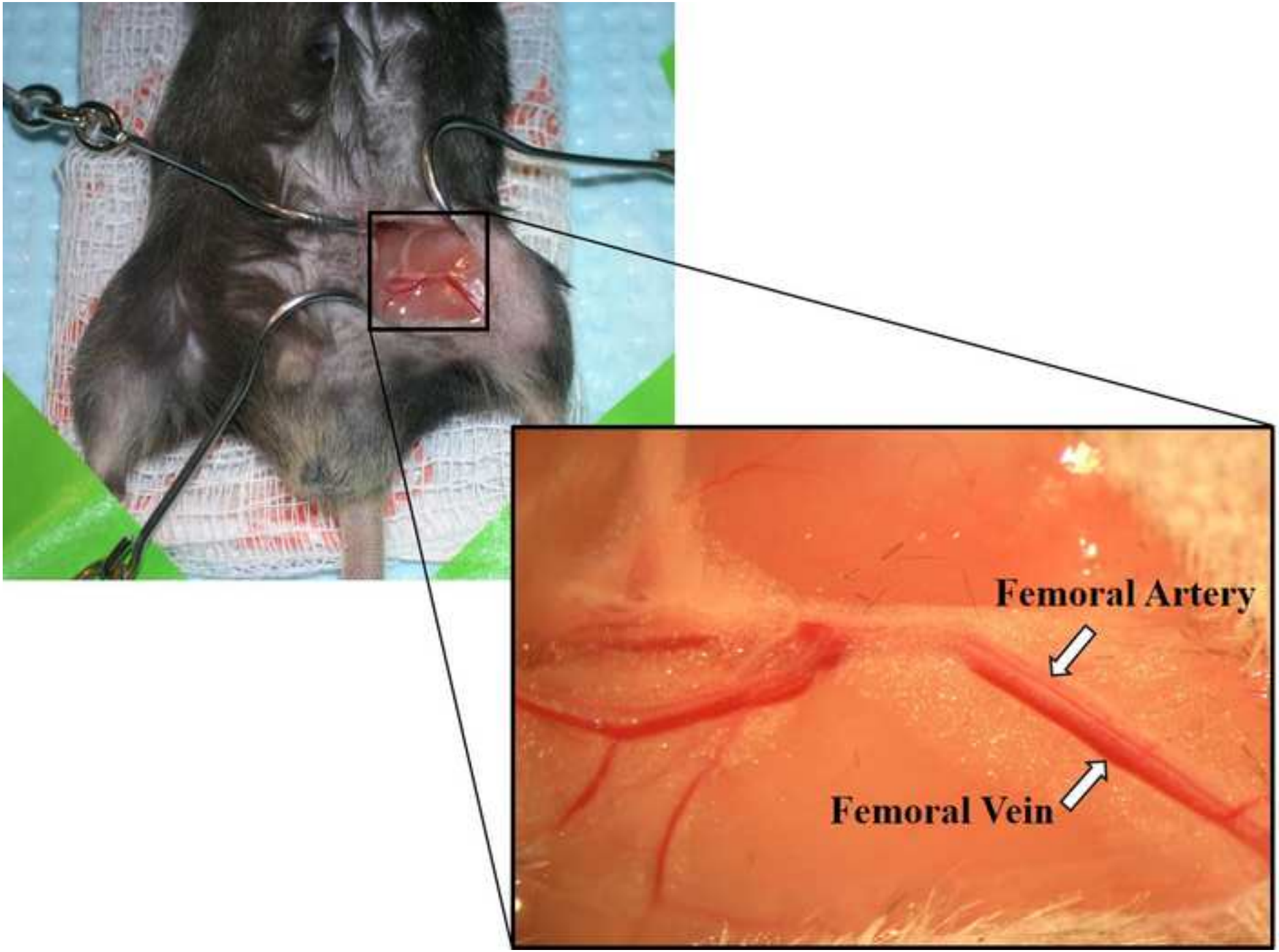

Figure 3

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Figure 4

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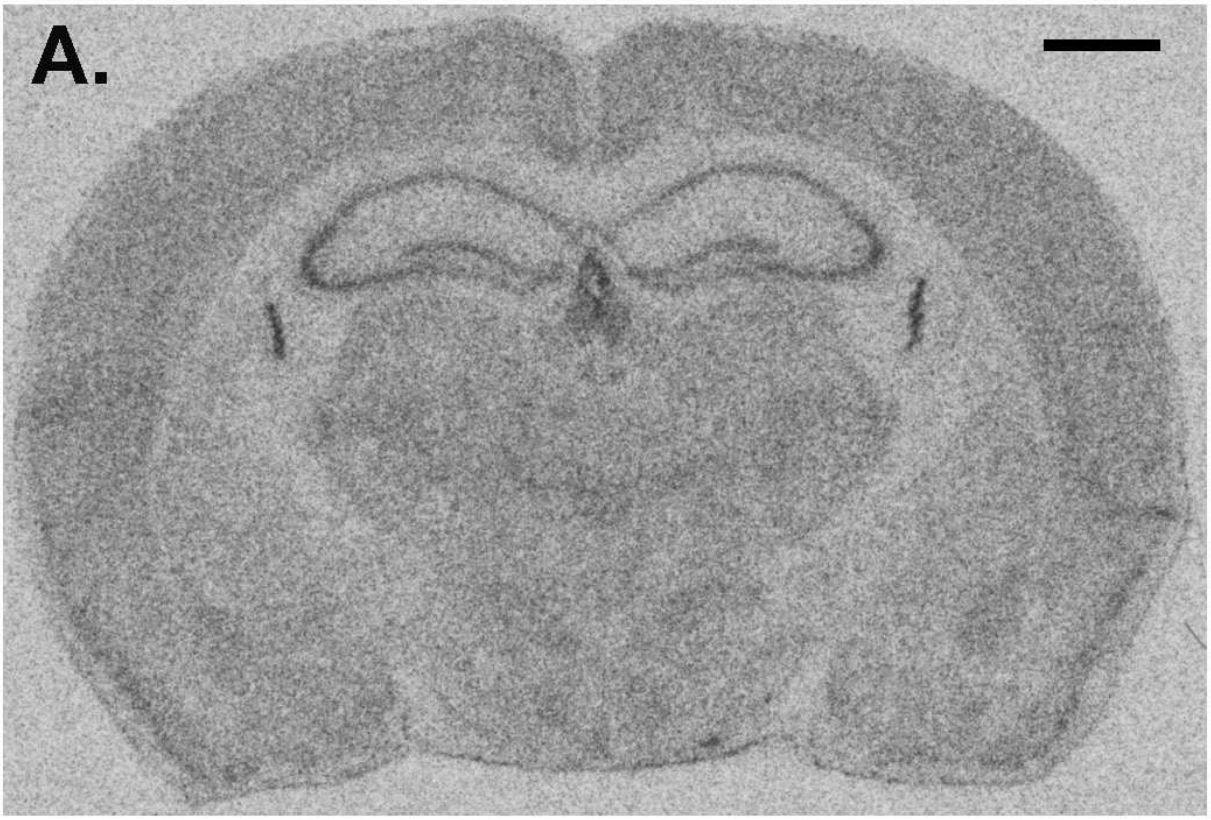

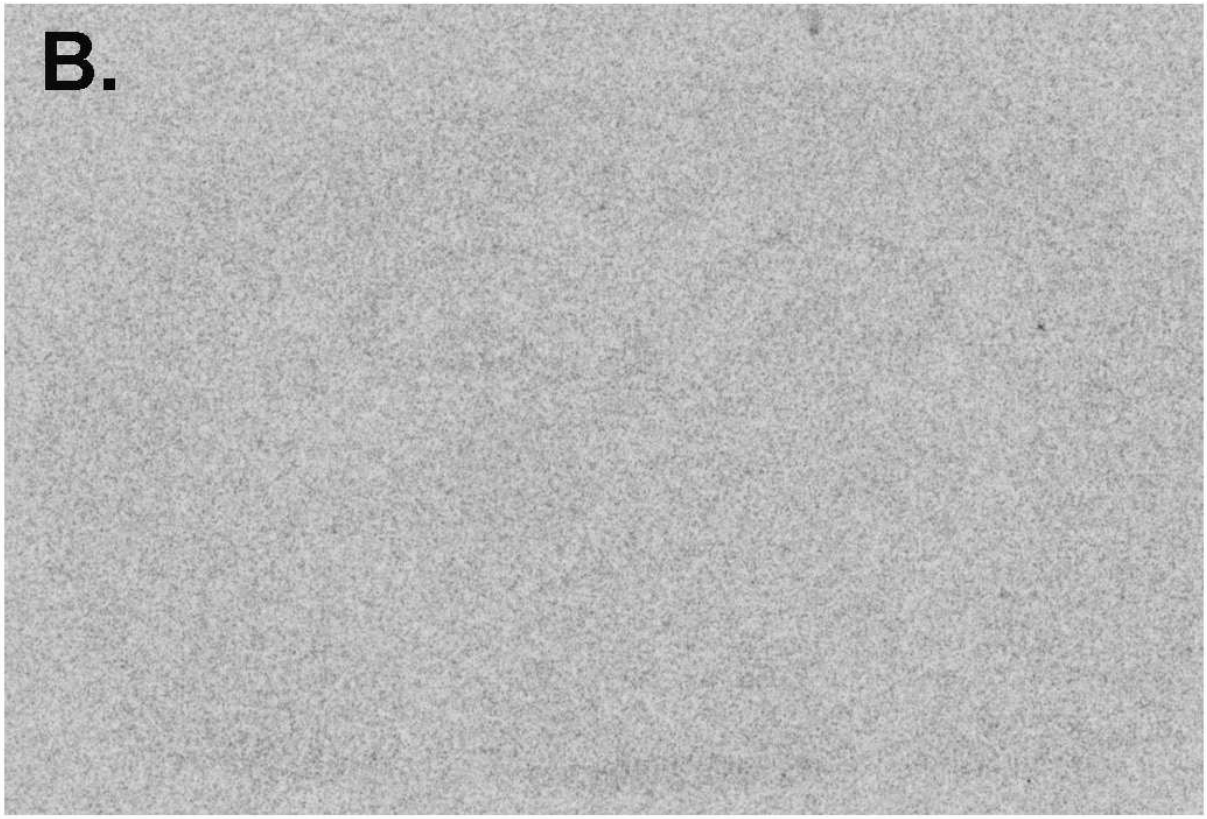

Figure 5 new

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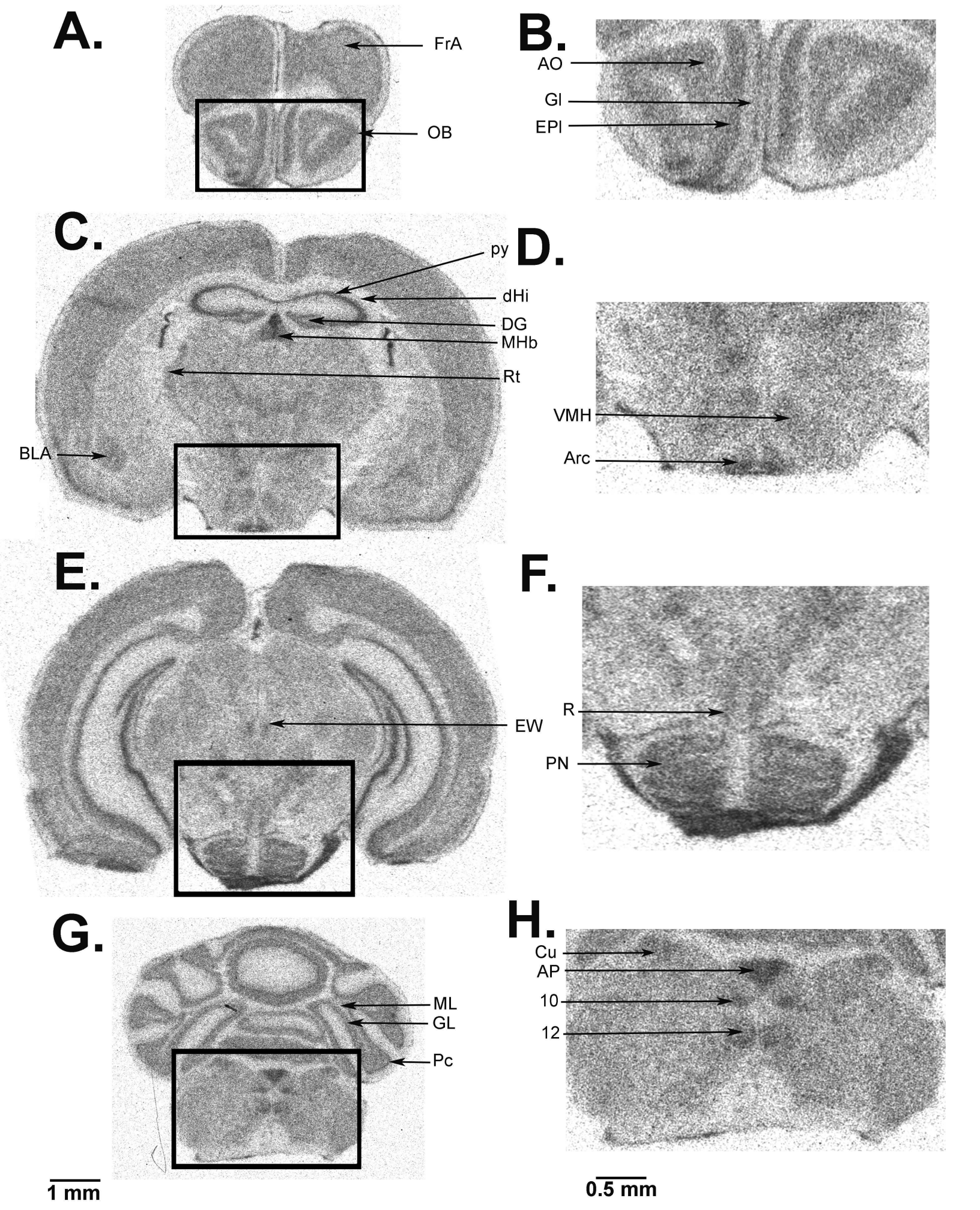

Figure 6

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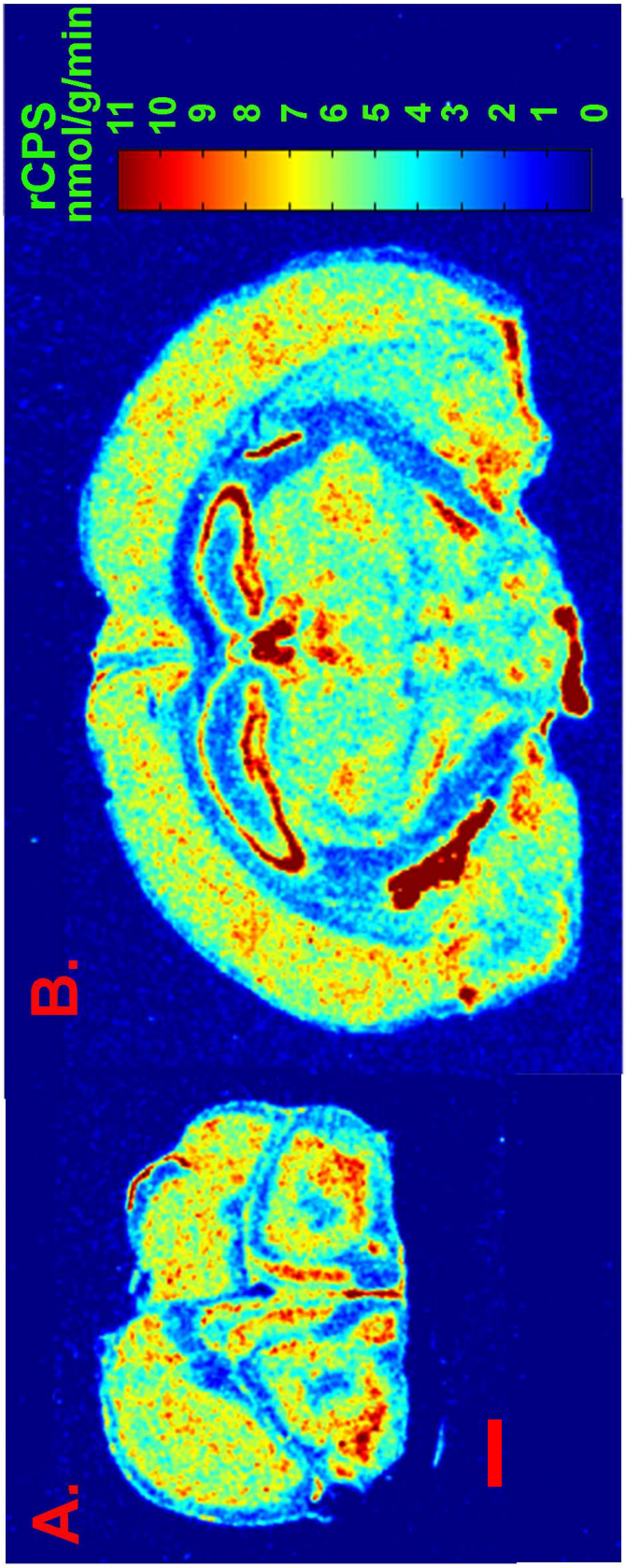

Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Mice	The Jackson Laboratory	003024	*Fmr1* knockout breeding pairs
Anisomycin	Tocris Bioscience	1290	
Microhematocrit Tubes	Drummond Scientific	1-000-3200-H	capillary tubes
Critoseal Capillary Tube Sealant	Leica Microsystems	39215003	sealant putty
Glass vial inserts	Agilent	5183-2089	used to collect blood samples
Digi-Med Blood Pressure Analyzer	Micro-Med Inc.	BPA-400	blood pressure analyzer
Bayer Breeze 2 Blood Glucose Monitoring System	Bayer Breeze	9570A	glucose meter
Gastight syringe	Hamilton Co.	1710	tuberculin glass syringe
HeatMax HotHands-2 Hand Warmers	HeatMax	Model HH2	warming pads
Heparin Lock Flush Solution	Fresenius Kabi USA, LLC	504505	heparin saline
Clear animal container	Instech	MTANK/W	animal enclosure
Spring tether	Instech	PS62	catheter tube/rodent attachment
Swivel	Instech	375/25	hooks to spring tether
Swivel arm and mount	Instech	SMCLA	hooks to swivel and animal enclosure
Tether button	Instech	VAB62BS/22	attaches to bottom of spring tether
Stainless steel tube	Made in-house	N/A	used to snake catheters through mouse
Matrx VIP 3000	Matrx	91305430	isoflurane vaporizer
Isoflurane	Stoelting Co.	50207	isoflurane/halothane adsorber
Clippers	Oster Finisher	Model 59	
Surgical skin hooks	Made in-house (??)	N/A (??)	
0.9% Sodium Chloride Saline	APP Pharmaceuticals LLC	918610	
Forceps	Fine Science Tools	11274-20	
Surgical scissors	Fine Science Tools	14058-11	
Microscissors	Fine Science Tools	15000-00	
UNIFY silk surgical sutures	AD Surgical	#S-S618R13	6-0 USP, non-absorbable
PE-8 polyethylene tubing	SAI Infusion Technologies	PE-8-25	
Syringe	Becton Dickinson and Co.	309659	1cc/mL
PE-10 polyethylene tubing	Clay Adams	427400	
MCID Analysis	Imaging Research Inc.	Version 7.0	optical density analysis

Gelatin-coated slides (75x25mm)	FD Neurotechnologies	PO101	
Cryostat	Leica	CM1850	
Super RX-N medical x-ray film	Fuji	47410-19291	
Hypercassettes (8x10 in)	Amersham Pharmacia Biotech	11649	
[1-^{14}C]leucine	Moravek	MC404E	
Microcentrifuge tube	Sarstedt Aktiengesellschaft & Co.	72.692.005	used to deproteinize blood samples
Glass pasteur pipette	Wheaton	357335	
Glass wool	Sigma-Aldrich	18421	
Nitrogen	NIH Supply Center	6830009737285	
Scintillation fluid	CytoScint	882453	
Liquid scintilllation counter	Packard Tri-Carb	2250CA	
Amino acid analyzer	Pickering Laboratories	Pinnacle PCX	
HPLC unit	Agilent Technologies	1260 Infinity	include 1260 Bio-Inert Pump
Surgical microscope	Wild Heerbrugg	M650	
Sulfosalicylic acid	Sigma-Aldrich	MKBS1634V	5-sulfosalicylic acid dihydrate
Norleucine	Sigma	N8513	
1.0 N HCl	Sigma-Aldrich	H9892	
[H^{3}]leucine	Moraevk	MC672	
Falcon tube	Thermo Scientific	339652	50 mL conical centrifuge tubes
Stopwatch	Heuer Microsplit	Model 1000	1/100 min
Euthanasia Solution	Vet One	H6438	
Northern Light Precision Illuminator	Imaging Research Inc.	Model B95	fluorescent light box
Micro-NIKKOR 55mm f/2.8	Nikon	1442	CDD camera

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Title of Article: In Vivo Quantitative Autoradiographic L-[1-14C]leucine Method for Determination of Regional Rates of Ce

Author(s): R. Michelle Saré, Anita Torossian, Michael Rosenheck, Tianjian Huang, Carolyn Beebe Smith*

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Reviewer's Comments

Reviewer #1

This is an interesting method for the measurement of protein synthesis using a quantitative autoradiography approach in mice by which all regions of the brain are measured simultaneously. The protocol is clearly described and easy to follow. However, the method is challenging and will therefore not be used widely. I have only minor comments for the revision:

1. Could the authors comment on the resolution? The authors should show images zooming into specific brain areas.

We have specified in the text that the resolution is approximately 25µm for this method. We also include a new figure (Figure 5) with some higher resolution images.

2. Is it possible to combine the method with immunohistochemistry?

We have added a mention that this method can be combed with immunohistochemistry and provided a relevant study where these methods were done together.

Reviewer #2

Manuscript Summary:

This is a well written paper detailing the application of a method for in vivo measurement of regional cerebral protein synthesis (rCPS), by autoradiographic assessment of the incorporation into proteins of radioactively (c14) labelled amino acid leucine. The method has been developed and championed by the Beebe Smith lab and it has been used to show alterations in rCPS in several animal models of ageing, developmental disorders, anaesthesia and environmental/lifestuyle modifications including exercise. A related method is available for measuring CPS in vivo in humans, by PET, making this an important method for translating preclinical into clinical findings. The autoradiography methodology is technically challenging as it involves mouse recovery surgery and the collection of blood samples whilst requiring their physiological stability. Moreover, specialist equipment and analytical knowledge, as well as ability to work with radioactivity, are required. To this end, this manuscript (and it video counterpart) should be endorsed as an important utility that will help share the method with other labs. It is hoped that Beebe Smith lab will continue to offer expert advice to other researchers if they contact them with questions about the methodology. I have only minor comments on the manuscript which I list below.

Minor Concerns:

-58: we can measure rates in cell layers and hypothalamic nuclei

-This is a bit misleading - why just list these regions, when it is possible to measure CPS in other nuclei, and in other areas of the brain?

This comment was clarified in the manuscript's introduction.

-65 Any remaining [14C]leucine is removed during the washing process with formalin before autoradiography

-Please explain the requirement for formalin fixation - this is unusual, why not just wash the sections in

water, to remove 14C-leucine? The authors cite ref 6, but this refers to another reference (Ingvar et al) in which a slightly different method was used (overnight in formalin vapour). Could it perhaps be that formalin fixation was required in order to perform histology on some sections? Either way, a clarification would be helpful.
We wash with liquid formalin followed by DI water. This process fixes protein to tissue, while washing away free amino acids. We would not advise washing solely with water as it may wash away smaller proteins.

-153 Cauterize the ends of both catheters to create a seal.
-Cautery equipment has not been listed.
Cautery equipment has been added to the list.

-229 remove the brain and place on dry ice to freeze
-How exactly, directly onto dry ice, or with e.g. a sheet of foil between the brain and ice? It might be worth warning not to freeze the brain in liquid nitrogen, as this is too cold for subsequent cutting (tissue becomes brittle)
The brain freezing process has been clarified in the protocol, as well as an additional comment cautioning against using liquid nitrogen.

-254 along with a set of [14C]methylmethacrylate standards, which were previously calibrated against tissue of known 14C concentrations as described 28
-Why not obtain commercially-available standards from American Radiolabelled Chemicals? Given the importance of standards for calculation, making own standards in a lab that isn't otherwise equipped for radioactive chemistry work, strikes me as a major limitation to being able to do this method. The authors should suggest to either make own, or purchase commercial (unless they disaprove of these - then an explanation in the discussion would be useful) and give the appropriate range of nCi/mg
This has been clarified in the manuscript. Standards can be commercially purchased from American Radiolabelled Chemicals and customized with a specific range (2-300 nCi/g of tissue).

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