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Measurement of Glutamate Uptake using Radiolabeled L-[3H]-Glutamate in Acute Transverse Slices Obtained from Rodent Resected Hippocampus
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1 **TITLE:**
2 **Measurement of Glutamate Uptake using Radiolabeled L-[³H]-Glutamate in Acute Transverse**
3 **Slices Obtained from Rodent Resected Hippocampus**
4
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22
23 **KEYWORDS:**
24 Acute hippocampal slices; hippocampal transverse section; excitotoxicity; glutamate
25 transporters.
26
27 **SUMMARY:**
28 This article describes a reliable and simple way to obtain ex vivo acute hippocampal transverse
29 slices from mice and rats using a tissue chopper. Slices obtained from resected hippocampi can
30 be submitted for functional glutamate uptake analysis to investigate glutamatergic system
31 homeostasis.
32
33 **ABSTRACT:**
34 Glutamate removal from the extracellular space by high-affinity Na⁺-dependent transporters is
35 essential to ensure that the brain's intrinsic connectivity mechanisms work properly and
36 homeostasis is maintained. The hippocampus is a unique brain structure that manages higher
37 cognitive functions, and is the subject of several studies regarding neurologic diseases. The
38 investigation of physiological and pathological mechanisms in rodent models can benefit from
39 acute hippocampal slice (AHS) preparations. AHS has the advantage of providing reliable
40 information on cell function since the cytoarchitecture and synaptic circuits are preserved.
41 Although AHS preparations are commonly used in neurochemistry laboratories, it is possible to
42 find some methodological differences in the literature. Considering that distinctive slice
43 preparation protocols might change the hippocampal regions analyzed, this current protocol
44 proposes a standard technique for obtaining transverse AHS from resected hippocampus. This

45   simple-to-perform protocol may be used in mice and rats' experimental models and allow
46   several ex vivo approaches investigating neurochemical dynamics (in dorsal, intermediate and
47   ventral hippocampus) in different backgrounds (e.g., transgenic manipulations) or after in vivo
48   manipulations (e.g., pharmacological treatments or suitable rodent models to study clinical
49   disorders). After dissecting the hippocampus from the rodent brain, transverse slices along the
50   septo-temporal axis (300 µm thick) were obtained. These AHS contain distinct parts of the
51   hippocampus and were subjected to an individual neurochemical investigation (as an example:
52   neurotransmitter transporters using their respective substrates). As the hippocampus presents
53   a high density of excitatory synapses, and glutamate is the most important neurotransmitter in
54   the brain, the glutamatergic system is an interesting target for in vivo observed phenomena.
55   Thus, the current protocol provides detailed steps to explore glutamate uptake in ex vivo AHS
56   using L-[^{3}H]-Glutamate. Using this protocol to investigate hippocampal function may help to
57   better understand the influence of glutamate metabolism on mechanisms of neuroprotection
58   or neurotoxicity.
59

60   **INTRODUCTION:**
61   The hippocampus, a brain structure buried deep in the medial temporal lobe of each
62   hemisphere, where high cognitive functions lie, is one of the most studied entities of the
63   central nervous system (CNS). The function of the hippocampus is strongly related to
64   declarative and spatial memory. This structure also plays a part in emotional behavior and in
65   the regulation of hypothalamic functions[1-4]. Ever since it was confirmed, important mechanisms
66   of memory formation and storage take place in this region and the field began to deeply
67   investigate the hippocampal region. Accordingly, the use of animal models that resemble
68   human cerebral disorders related to hippocampal functions, such as Alzheimer's disease,
69   epilepsy, major depression, and stress, continues to grow.
70

71   In rodents, the hippocampus is a curved-shaped structure starting from near the medial septum
72   towards the ventral temporal cortex. Along its longitudinal axis, the hippocampus can be
73   divided into three different regions, each one related to specific circuitry[1]. The upper part
74   constitutes the dorsal/septal hippocampus, the lower part constitutes the ventral/temporal
75   hippocampus, and the area between them is considered the intermediate hippocampus. There
76   is extensive literature covering the differences in cellular projections to each part, as well as
77   reports of specific cognitive aspects processed by each[5,6]. Regarding its internal organization,
78   the hippocampal regions can be separated by its functional areas. The Cornu ammonis (CA)
79   area is subdivided in CA1, CA2, and CA3 and extends through the superior part of the
80   hippocampus, above the dentate gyrus (DG) and the subiculum, which are the most internal
81   hippocampal parts (**Figure 1**). The synapses located in these regions undergo continuous
82   rearrangement, showing neurogenic and plastic processes throughout life[3]. Several studies
83   have already shown that distinct experimental manipulations in the hippocampus result in
84   cognitive disability[7]. Regarding the assessment of biochemical and molecular alterations,
85   techniques using acute brain slices are an excellent tool to improve the knowledge regarding
86   different aspects of the hippocampus.
87

88   Due to its precision and reproducibility, many studies that explored aspects of

89 neurotransmission-related phenomena (enzyme activity, uptake, or release) used transverse
90 AHS from resected hippocampus obtained by tissue chopper[8-12]. This slicing technique followed
91 by uptake assessment is suitable for sophisticated neurochemical experiments that require the
92 transporter activity from hippocampal tissue to be preserved. For that, the employment of a
93 tissue chopper is preferable, since it is faster than the vibratome and provides the AHS in a
94 proper time for experimental use with suitable accuracy.
95
96 The excitatory neurotransmission in the brain is accomplished by glutamate, the most
97 abundant neurotransmitter, including in the hippocampus, which is dependent on glutamate
98 signaling to a greater extent[13,14]. This neurotransmitter abundance is tightly controlled in the
99 extracellular environment. Inside intracellular vesicles, however, it can reach up to 100 mM[15].
100 Once released in the synaptic cleft, glutamate is not metabolized and needs to be removed in
101 order to avoid excitotoxicity, usually triggered as a response to an overload of glutamate[14,16].
102 The only mechanism separating toxicity from normal signaling is sodium-dependent transport
103 through the activity of proteins located in the plasma membranes of, majorly, glial cells[14,17-19].
104 These transporters [GLAST (EAAT1) and GLT-1(EAAT2)] tightly regulate extracellular glutamate
105 levels and can be modulated by a wide range of factors, such as DNA transcription, mRNA
106 splicing and degradation, protein synthesis and targeting, amino acid transport activity, and ion
107 channel activities[20-23]. Accordingly, their activity can be measured by the transport of
108 radiolabeled substrate, as glutamate.
109
110 The use of radiolabeled substrates represents a preferable method for quantifying transporter
111 activity since they allow tracing dynamic mechanisms such as transport across cell membranes.
112 Besides their high sensitivity and specificity, the advantages of radiotracer experiments include
113 their simplicity and small expense compared to competing technologies such as mass
114 spectrometry[24]. Also, by using only small amounts of tracer, physiological levels of substrates
115 are not altered, thus providing a more representative picture of the real metabolic activity
116 scenario.
117
118 The availability of ex vivo experimental approaches is critical to support basic research on
119 identifies novel molecular targets and drug discovery activities. Thus, considering the relevance
120 of the glutamate uptake for glutamatergic system homeostasis and the high predominance of
121 glutamatergic synapses in the hippocampus, this protocol demonstrates how to assess
122 glutamate uptake activity in a fast and easy-to-reproduce method using transverse AHS from
123 the resected hippocampus. This assay uses radiolabeled L-[^{3}H]-Glutamate, which allows for
124 quantitative comparisons and clear visualization of results, and can be modified for use with
125 specific or customized substrates, over a wide range of reactions conditions[25].
126
127 Acute brain slices present many advantages and have been used to support function change
128 under pharmacological and genetic manipulations[26-28]. Their use benefits from the following: (i)
129 the neurochemical functionality conservation and cell-to-cell interactions; (ii) the possibility to
130 perform numerous pharmacological and genetic manipulations to investigate pathways
131 underlying neuronal and glial functions; (iii) precise control of the extracellular environment;
132 and (iv) good experimental access to different hippocampal areas (such as CA1, CA3 or DG),

133 which are kept in the same slice depending on the slicing method. Considering that distinctive
134 slice preparation protocols might change the hippocampal regions exposed, this protocol
135 proposes a standard technique for obtaining transverse AHS from the resected hippocampus.
136 This simple-to-perform protocol may be used in rodent models and may allow several ex vivo
137 approaches investigating neurochemical dynamics in different backgrounds or after in vivo
138 manipulations[29,30] (**Figure 2**).
139
140 **PROTOCOL:**
141
142 All procedures were performed in accordance with the NIH Guide for the Care and Use of
143 Laboratory Animals and approved by the local Ethics Committee (project approval
144 # 33732/CEUA-UFRGS). All efforts were made to minimize discomfort and the number of
145 animals used in the experiments.
146
147 **1.   Preparing Hank's Balanced Salt Solution (HBSS)**
148
149 1.1.   In a 1 L beaker, add approximately 0.5 L of sterile or double-distilled water and begin
150 stirring vigorously on a magnetic stirrer. Add the following HBSS components (in mM): 137
151 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 5.55 D-
152 Glucose (see **Table 1**).
153
154 1.2.   Adjust the pH to 7.4 with NaOH or HCl before adding 1.26 mM $CaCl_2$ to avoid
155 precipitation. Add this salt very slowly and under agitation. Bring it up to a volume of 1 L with
156 sterile or double-distilled water. This buffer will be used to wash the tissue and to maintain the
157 AHS in a viable state during the experiment. It will provide inorganic ions while maintaining a
158 physiological pH and osmotic pressure.
159
160 NOTE: The addition of divalent cations is done after titration of pH to avoid precipitation.
161
162 1.3.   Separate two different fractions of HBSS: one will be kept in a water bath at 37 °C and
163 used during the uptake paradigm, while the other will be kept ice-cold (4 °C) and used for
164 washings before and after the experiment.
165
166 NOTE: It is possible to prepare stock solutions of each salt and keep them frozen until use, store
167 it in the freezer (-20 °C) and use it within a few weeks.
168
169 **2.   Preparing Sodium-Free HBSS**
170
171 2.1.   Before mixing the components for sodium-free HBSS, prepare in advance two other
172 solutions: Glucamine-HCl (137 mM) and Glucamine-HEPES (4.17 mM). Mix equimolar
173 concentrations of NMDG base and HCl to obtain the Glucamine-HCl solution. Do the same
174 procedure with 2.0 M HEPES free acid to obtain the NMDG-HEPES solution (see **Table 2**).
175
176 2.2.   In a 0.5 L beaker, add approximately 0.2 L of sterile or double-distilled water and begin

177 stirring vigorously in a magnetic stirrer. Add the following buffer components (in mM): 137
178 Glucamine-HCl, 4.17 Glucamine-HEPES, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $MgSO_4$, 0.41 $MgCl_2$, and
179 5.55 D-Glucose (see **Table 2**).

180

181 2.3.   Adjust the pH to 7.4 with KOH or HCl and keep stirring. Add 1.26 mM $CaCl_2$ very slowly
182 and under agitation. Bring the solution up to a volume of 0.3 L with sterile or double-distilled
183 water.

184

185 NOTE: This buffer will be used to measure non-sodium-dependent glutamate uptake, which will
186 allow the measurement of passive/non-specific glutamate uptake. After the experiment, this
187 parameter was subtracted from the total glutamate uptake to determine sodium-dependent
188 uptake.

189

190 2.4.   After preparation, this buffer can be stocked in the freezer (-20 °C) and used afterward
191 in subsequent experiments. The same goes for the NMDG solutions.

192

193 2.5.   During the experiment, always keep the sodium-free HBSS ice cold.

194

195 **3.   Organizing the material for hippocampus dissection from rats**

196

197 3.1.   Organize the dissection area next to the sink before starting the experiment. Position a
198 specific guillotine for rats (for mice, it will be possible to use sharp scissors) into the sink to keep
199 the bench clean.

200

201 3.2.   Arrange the surgical instruments and other materials for brain removal and
202 hippocampal dissection in the order of use and as close as possible to where they are needed
203 (see **Table of Materials**). Fill the Petri dish with ice. Also, keep a plastic bag nearby for disposal
204 of the carcass.

205

206 3.3.   Prepare both HBSS solutions at the recommended concentration for use. The sodium-
207 free HBSS can be frozen and thawed before the experiment.

208

209 3.3.1. Separate sodium-containing HBSS into two distinct portions, as previously
210 recommended: one heated at 37 °C and another at 4 °C that will be used for dissection. Thaw
211 sodium-free HBSS and keep at 4 °C.

212

213 3.4.   To induce anesthesia, use 3% isoflurane [31]. Before beginning the experiment, make sure
214 to verify the absence of corneal reflex and nociceptive stimulation of the hind paw.

215

216 NOTE: Be careful to choose the adequate anesthetic drug, as it should not interfere with any
217 parameters that will be under investigation. For this protocol, it is recommended the use of
218 inhalant anesthetic (e.g., isoflurane) [32].

219

220 **4.   Euthanizing the rat**

221

222   4.1.   Euthanize the rodent by using the approved methods by the Institutional Animal Care
223   and Use Committee (IACUC), as well as by the National Institutes of Health Guide for the Care
224   and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

225

226   4.2.   Using a guillotine, decapitate the rat immediately prior to the first cervical vertebra.

227

228   4.3.   Place the head on a clean paper towel moistened with cold HBSS buffer. Completely
229   remove the scalp with the surgical scissor size S, as well as the cutaneous muscle aiming to fully
230   exposing the sutures on the superior surface of the skull. Initiate this procedure carefully,
231   starting near the occipital bone and running posteriorly to the nasal bone.

232

233   NOTE: In case the experimental model is mice, use sharp scissors to remove the head. Be sure
234   to cut just behind the skull to exclude excess tissue.

235

236   5.   **Removal of the rat brain**

237

238   5.1.   Perform brain removal as quickly as possible. With an appropriate bone plier, make a
239   single cut between both eyeholes joining both orifices in the rat's skull. After, place one tip of
240   the surgical scissor size S into the foramen magnum and make a single cut laterally into the
241   skull. Repeat for the other side.

242

243   5.2.   Cut the skull plates from the occipital plate, then along the midline suture of the parietal
244   plates, and finally to the frontal plate towards the nose. Be sure that the rodent's head is held
245   firmly and that the pressure is directed away from the brain to prevent damaging the tissue
246   underneath.

247

248   NOTE: It is critically important that pressure is directed away from the brain to prevent
249   damaging the tissue underneath. In young rats and mice, it is possible to cut the skull with a
250   small surgical scissors.

251

252   5.3.   Gently remove the occipital and parietal plates from both hemispheres using a spatula
253   or forceps. Closely examine the skull-brain interface to remove any dura matter that may be
254   attached to the skull bones. Carefully inspect for any dura matter that may be attached to the
255   skull bones and stretched across the surface of the brain.

256

257   5.4.   Remove the brain immediately with the thin double-ended spatula and gently place the
258   whole brain on an ice-cold Petri dish covered with filter paper. With the plastic Pasteur pipette
259   dampen it with ice-cold saline solution. Immediately start to dissect the hippocampi from both
260   brain hemispheres as explained below.

261

262   5.5.   Carefully resect the cerebellum from the whole brain with the #11 scalpel blade. In
263   order to completely separate the cerebral hemispheres run a scalpel blade throughout the
264   intrahemispheric fissure. With the plastic Pasteur pipette, dampen it with ice-cold HBSS buffer

265 frequently to keep the tissue wet and preserved.

266

267 5.6.   Take each hemisphere separately, resect brain stem and midbrain from the overlying
268 cortex with an Iris scissors. Carefully, with the surgical scissor cut the
269 brainstem/midbrain/thalamus, while with a thin brush push down and pull these brain
270 structures. Below it will be possible to observe the hippocampus (medial surface) and the
271 lateral ventricle. The cortex with the hippocampus should now be free from the brainstem.

272

273 6.   **Hippocampi dissection from rats**

274

275 6.1.   To resect the hippocampus, put one brain hemisphere in the coronal plane with the
276 parietal cortex facing down. At this moment, it is possible to observe the white fimbria fibers
277 that form a shallow hyperbola at the bottom of the hippocampus.

278

279 6.2.   Very carefully, with the non-dominant hand, anchor the brain tissue with thin tweezers.
280 With your dominant hand, place other tweezers under the caudal tip of the hippocampus.

281

282 6.3.   Anchoring the medial white matter tracts, position the tweezer completely under the
283 hippocampus to separate it from the rest of the brain gently. Apply pressure while moving the
284 second tip slightly anteriorly and laterally. If done properly, the hippocampus should land on
285 the filter paper.

286

287 6.4.   To finish the hippocampus dissection, remove the spare tissue with the same tweezers
288 (usually any remaining cortex, blood vessels, and white matter). Repeat the same methodology
289 for the second hemisphere.

290

291 6.5.   Once both hippocampi are removed, transfer it to a Petri dish with HBSS placed on the
292 ice bucket and transfer to the cutting table area above the blade in the tissue chopper. Be sure
293 to properly identify the dorsal and ventral areas if the investigation requires analyzing them
294 separately.

295

296 NOTE: It is important to always distinguish the ventral and dorsal parts of the hippocampus
297 during dissection. By doing so, the anterior end will be facing upwards [33]. This part of the
298 hippocampus is slightly thinner than the posterior end.

299

300 **7.   Slices preparation**

301

302 NOTE: While arranging the surgical material on the bench (session 2), prepare the tissue
303 chopper to obtain the transverse slice from the resected hippocampus.

304

305 7.1.   Set the parameters in the tissue chopper before its use. The Chopper can cut slices from
306 100 to 400 µm; set it to 300 µm. Additionally, it is recommended a range of 60-80 strokes per
307 minute, as it maintains the tissue integrity during the slicing procedure.

308

309　7.2.　　After all the procedures performed in session 2, once the hippocampi were resected
310　from the brain and placed on the filter paper humidified with cold saline solution on top of a
311　smooth polypropylene support, align each hippocampus on the filter paper before submitting
312　them to the tissue chopper.
313
314　7.3.　　Place the hippocampus at a time on the circular stainless-steel cutting table from tissue
315　chopper, perpendicular to the cutting blades to obtain the transverse hippocampus slices along
316　the septo-temporal axis. Before starting the chopping process, lightly humidify the blade using
317　isopropyl alcohol. This procedure keeps the tissue from sticking on the blade, allowing a more
318　smooth and precise slicing.
319
320　7.4.　　After turning the equipment on, the blade in the chopping arm will be raised and
321　dropped, slicing the tissue in the selected thickness. This is a quick process, and normally the
322　tissue will be properly sliced after a minute. It is possible to chop more than one hippocampus
323　at the same time if they are kept properly separated on the cutting table.
324
325　7.5.　　To separate the slices, take each sliced sample from the tissue chopper carefully using
326　the double-ended spatula and plunge it into a Petri dish with ice-cold saline solution. Using two
327　thin brushes, gently pinch the hippocampus and separate the slices. Be careful to maintain slice
328　integrity through this process.
329
330　7.5.1.　Perform this experiment in triplicates, hence select 6 slices from each sample: 3 slices
331　for the total glutamate uptake and 3 for the unspecific uptake.
332
333　7.6.　　With the brush, gently transfer each slice from the Petri dish to a 24-well plate
334　containing: (i) 1 mL of HBSS; and (ii) 1 mL of HBSS sodium-free. Identify the well/plate with the
335　correspondent experimental groups and perform the analysis at least in triplicates.
336
337　NOTE: Due to a time-limited issue, if the experiment is performed in triplicates, it limits the
338　number of animals/groups to only 6 at a time (one hippocampus per animal in 5 min uptake).
339　This means that the L-[^{3}H]-Glutamate will be administered every 15 s until all slices are
340　incubated. To stop the reaction, the same interval should be observed to ensure that all
341　samples are exposed to a 5 min incubation period.
342
343　**8.　　Glutamate uptake assay**
344
345　8.1.　　During the slices stabilization in the respective buffer (HBSS or sodium-free HBSS),
346　prepare the radioactive-containing solution by diluting L-[^{3}H]-Glutamate in sterile water to a
347　final concentration of 4.5 μCi/mL in a final volume that allows 20 μL per slice plus some excess,
348　which will prevent the lack of radioactive input at the end of the batch, since it will be needed
349　to quantify how many disintegrations per minute (DPM) was offered to each slice in the
350　beginning.
351
352　NOTE: For each 24-well plate, it is recommended to prepare 500 μL of 4.5 μCi/mL L-[^{3}H]-

353   Glutamate due to the total incubation time needed for the L-[³H]-Glutamate uptake (5 min) and
354   the time to pipette each 20 μL aliquot (0.33 μCi in each well), as explained below. The stock
355   concentration of L-[³H]-Glutamate is usually 1.0 mCi/mL, thus prepare a 1:200 dilution to apply
356   on the slices.

357

358   8.2.   After 15 min of pre-incubation in HBSS, completely remove the buffer with a Pasteur
359   pipette and replace it by adding to the slices 280 μL of one of the following: (i) regular HBSS (37
360   °C) for total uptake; or (ii) sodium-free HBSS (4 °C) for unspecific uptake. It is important to
361   notice that total uptake must be performed on a heating plate at 37 °C, while the unspecific
362   uptake is performed on ice.

363

364   8.3.   Prepare one timer and set the beginning of the experiment when adding 20 μL input in
365   the first well containing a slice. Wait for 15 – 20 s (depending on the number of slices in the
366   plate) and add the radioactive input to the next well and so on until the end of the slice-
367   containing wells. Distribute 20 μL of input in all the wells always considering the total
368   incubation time (5 min).

369

370   8.4.   Once each of the wells containing slices in the plate are filled, wait for the first slice to
371   complete 5 min incubation with L-[³H]-Glutamate. Then, immediately remove the incubation
372   media to a proper flask for radioactive waste.

373

374   8.5.   Immediately after the removal, quickly add 300 μL of ice-cold HBSS and gently remove
375   it. Repeat this procedure two more times in each well, completing three washes for each slice.
376   Be sure to use the sodium-free HBSS for washing the slices devoted to the unspecific uptake
377   measurement. After washing all the slices in the plate, add 300 μL 0.5 M NaOH to each well and
378   store the plate protected with parafilm at 4 °C overnight.

379

380   **9.   Counting the radioactive L-[³H]-Glutamate**

381

382   9.1.   On the next day, label as many tubes as slices in the experiment and collect the content
383   of the well to each tube. Be sure that the slices are completely solubilized in the lysis solution. If
384   necessary, homogenize the sample using a 26-gauge needle coupled to a 1 mL syringe.

385

386   9.2.   Add 1.2 mL of scintillation liquid to a final volume of approximately 1.5 mL and
387   homogenize all samples in a vortex before reading them in a scintillation counter for 60 s each.

388

389   NOTE: Be sure that the detection parameters are set to tritium and the result will be delivered
390   in DPM.

391

392   **10.   Calculations**

393

394   10.1.   Convert the results given by the scintillation counter to the radioactivity emitted by the
395   reagent trapped within the slice and represents the amount of glutamate taken up. Deduct the

396 value obtained for the unspecific uptake from the result obtained with the regular HBSS. One
397 µCi is equivalent to 2.22×10^6 DPM.

398

399 10.2.   By knowing the amount of µCi and the original concentration of [³H]L-Glutamate
400 (information usually provided in the reagent's flask or datasheet), calculate the L-[³H]-
401 Glutamate captured by each slice.

402

403 **REPRESENTATIVE RESULTS:**
404 Glutamate uptake is one of the most important mechanisms controlling neurotransmission in
405 the brain. The hippocampus, specifically, is a critical place in glutamate signaling, being an
406 important hub connecting memory, cognition, and emotions in the brain. Following the
407 protocol, adult male Wistar rats were used to generate representative results. Animals were
408 anesthetized using isoflurane 3% until unconscious. After dissecting the brain, hippocampi were
409 removed and placed in the chopper table perpendicularly to the blade. Three hundred µm thick
410 slices were obtained at a rate of 60 per minute and collected to a petri dish containing ice-cold
411 saline solution. Slices were separated each in a well of a 24-well plate and L-[³H]-Glutamate
412 uptake was carried out for 5 min (unless stated otherwise) in sodium-free or regular HBSS, at 4
413 or 37 °C, respectively.

414

415 After an overnight period in lysis solution at 4 °C, samples were mixed with scintillation liquid
416 and read in a scintillation counter for 60 s. The results are given in DPM and the value of the
417 uptake carried out in sodium-free HBSS is deducted from the value obtained with regular HBSS.
418 One DPM corresponds to 0.000000000450 or $4,5 \times 10^{-10}$ Curie (Ci). By knowing the specific
419 radioactivity of the L-[³H]-Glutamate, is possible to establish a relationship between the result
420 in DPM and the amount of L-[³H]-Glutamate captured by each sample.

421

422 First, we carry out the uptake assay using 200 and 300 µm slices to demonstrate that the total
423 protein amount does not influence the uptake parameter. No differences in glutamate uptake
424 in 5 min were observed comparing slices with 200 and 300 µm (data not shown). Accordingly,
425 the researcher has to use slices obtained from the same hippocampal region of all animals to
426 prevent size bias. In summary, this protocol highlights the usual thickness (300 µm) used in
427 different experiments with slices from hippocampus, striatum or cortex [10,34,35]. Moreover, in
428 order to clarify if there was any difference in L-[³H]-Glutamate uptake in the three main
429 hippocampal regions, dorsal, intermediate and ventral regions were assayed separately. There
430 was no difference in L-[³H]-Glutamate between the three regions (**Figure 3A**, p = 0.638, one-
431 way ANOVA of repeated measures). This is not surprising since the experiments were
432 performed with naïve animals. However, the results may vary between regions when
433 considering different experimental models or pharmacological challenges.

434

435 To make sure that the experiment was not carried out in a saturation range, the uptake was
436 performed for 2.5, 5, 10 or 15 min. The results demonstrate that in a 10 min incubation time
437 the amount of L-[³H]-Glutamate taken up was significantly higher when compared with 5 min
438 (**Figure 3B**, p = 0.0061, F = 6.864, one-way ANOVA of repeated measures). Conversely, 10 and
439 15 min uptake results were not statistically different, suggesting a plateau was reached. This

440   data shows that a 5 min incubation is adequate to measure the uptake in most conditions.

441

442   To demonstrate that the method is sensitive enough to respond to a blockage in glutamate
443   uptake, 100 µM dihydrokainic acid (DHK), a specific GLT-1 blocker, was administered to the
444   AHS, 5 min prior the uptake. DHK was supplied in stabilization media during 5 min previous to
445   media replacement and [³H]L-Glutamate uptake start [36], according to the following timeline: t=
446   0: slice stabilization in 37 °C HBSS, t= 10 min: addition of DHK to specific slices, t= 15 min:
447   substitution of stabilization media by fresh 37 °C HBSS and beginning of [³H]L-Glutamate
448   uptake. As expected, DHK was able to decrease glutamate uptake (**Figure 3C**, p = 0.0034, r
449   squared = 0.9069, paired Student's t test) in the protocol conditions, indicating that other
450   pharmacological or genetic manipulations can be used to modulate glutamate uptake.

451

452   As a way to determine if the samples are stable enough to withstand a delay in the
453   measurement, some replicates were kept with no scintillation liquid at 4 °C (**Figure 3D**) or
454   frozen for 7 days (**Figure 3E**), having the scintillation of their counterparts measured in the next
455   day of the experiment. The data shows that there was no difference between the DPM values
456   of the samples tested 24 h after the experiment and the samples kept refrigerated or frozen for
457   7 days (p = 0.229 and p = 0.9623, respectively, paired Student's t test).

458

459   Considering that D-[³H]-Aspartate presents an index of glutamate uptake since it is metabolized
460   in a lesser extent than L-[³H]-Glutamate and the accumulation of D-[³H]-Aspartate in the slice
461   would provide another important index of transporter activity, it was performed D-[³H]-
462   Aspartate uptake in 300 µm hippocampal slices for 2.5 and 5 min in the same conditions than L-
463   [³H]-Glutamate. Different time points for the uptake of L-[³H]-Glutamate and D-[³H]-Aspartate
464   under basal conditions did not present any difference comparing both groups (**Figure 3D**; p =
465   0.0632 for radiolabeled substrate, 2-way ANOVA followed by Tukey).

466

467   Finally, to demonstrate that unlabeled glutamate in incubation media could hamper the
468   radiolabeled transport activity the uptake buffer was supplemented with different
469   concentrations of unlabeled glutamate (0, 50, 100 and 200 µM). Here, in **Figure 3G** it is
470   demonstrated that the presence of unlabeled glutamate in incubation media significantly
471   impact the L-[³H]-Glutamate uptake in a concentration-dependent manner in 300 µm
472   hippocampal slices (p = 0.02, repeated measures ANOVA).

473

474   **FIGURE AND TABLE LEGENDS:**

475

476   **Table 1. Hank's Balanced Salt Solution composition**

477

478   **Table 2. Sodium-Free HBSS composition**

479

480   **Figure 1. Schematic representation of the rodent brain and hippocampi location beneath the**
481   **temporoparietal and occipital cortices.** When resected, the hippocampus can be divided into
482   the dorsal, intermediate, and ventral parts, the first being oriented in the upper direction of the
483   brain. The hippocampal slices along the transverse axis contain the main regions (CA1, CA3 and

484   DG). CA1: Cornu ammonis 1, CA3: Cornu ammonis 3 and DG: dentate gyrus.

485

486   **Figure 2: Step-by-step flow of the experiment, from buffer preparation to result calculations.**

487

488   **Figure 3: Representative results of L-[³H]-Glutamate uptake performed in AHS of adult male**
489   **Wistar rats. A**) Different regions take up the same amount of L-[³H]-Glutamate under basal
490   conditions (p = 0.638, one-way ANOVA of repeated measures). **B**) The contact of L-[³H]-
491   Glutamate with the slices for 10 min increases the amount of L-[³H]-Glutamate captured,
492   compared with 5 min (p = 0.02, F = 6.864, one-way ANOVA). **C**) Dihydrokainic acid (DHK), a
493   specific GLT-1 blocker, decreases glutamate uptake (p = 0.0034, r squared = 0.9069, paired
494   Student's t test). Sample stability was measured after 7 days. Replicates were kept **D**) at 4 ˚C (p
495   = 0.229, paired Student's t test) and **E**) -20 ˚C (p = 0.9623, paired Student's t test) and compared
496   to its counterparts measured 24 h after the experiment. **F**) Different time points for the uptake
497   of L-[³H]-Glutamate and D-[³H]-Aspartate under basal conditions (p = 0.0632 for radiolabeled
498   substrate, 2-way ANOVA followed by Tukey); **G**) Different concentrations of unlabeled
499   glutamate (0, 50, 100 and 200 µM) supplemented in uptake buffer. GLT-1: Glutamate
500   transporter type 1. DPM: disintegrations per minute. The data in **A** and **B** are expressed as
501   minimum value, median and maximum value. The data in **C**, **D**, and **E** represent paired slices,
502   therefore they are unique values.

503

504   **DISCUSSION:**
505   The presented protocol shows an easy-to-perform glutamate uptake assessment using
506   hippocampal slices. The results demonstrate that AHS regularly takes up around 60 fmol of
507   radiolabeled L-[³H]-Glutamate, that the thickness of the slice (protein amount) did not influence
508   the L-[³H]-Glutamate uptake (data not shown), and that the dorsal, intermediate, and ventral
509   parts of the hippocampus exhibited similar performances when obtained from naïve adult male
510   Wistar rats (**Figure 3A**). It was also demonstrated, in a time-course experiment, that in 5 min
511   incubation the L-[³H]-Glutamate transport system was not saturated (**Figure 3B**), while adding
512   unlabeled glutamate to uptake media decreases the AHS L-[³H]-Glutamate uptake by an
513   important reduction in DPM index (**Figure 3G**). Moreover, it was shown that D-[³H]-Aspartate, a
514   radiolabeled excitatory neurotransmitter, could be also taken up in a similar way comparing to
515   L-[³H]-glutamate (**Figure 3F**). Finally, an example of pharmacological manipulation was
516   demonstrated by the incubation of DHK with AHS and as expected, a significant decrease in the
517   final L-[³H]-Glutamate uptake was verified (**Figure 3C**). To test the stability of the samples, some
518   replicates were kept at 4 ˚C or at -20 ˚C for seven days. The data indicate that the measurement
519   of DPM did not suffer significant alterations despite the gap of time (**Figure 3D**, for samples
520   kept at 4 ˚C; and **Figure 3E**, for samples kept at -20 ˚C).

521

522   The use of radiolabeled compounds to estimate transport across membranes is largely used in
523   neurochemical assessments[37,38]. This technique has the potential to disclose a cellular
524   mechanism underlying behavioral changes in preclinical models of several kinds of diseases.
525   Glutamate uptake is a crucial process for brain homeostase and an essential part of
526   neurotransmission, being able to dictate synaptic strength, length and timing. Glutamate, the
527   most ubiquitous neurotransmitter in the brain, is involved in several complex functions, such as

528 learning, memory, and behavior, most of which are directly related to hippocampus.
529 Glutamatergic neurotransmission is dampened in several neurodegenerative diseases, as well
530 as in peripheral conditions such as nutritional restrictions[39]. Astrocytes, glial cells responsible
531 for the most of glutamate uptake, may undergo changes in their functions in the course of
532 many diseases, which would impair their ability to take up glutamate at a physiological rate.
533 The condition elicited by excess glutamate in the extracellular space, referred to as
534 excitotoxicity, has the potential to cause extensive damage to neurons and other cells
535 surrounding the area.
536
537 Some important observations using the AHS protocol are related to the need to normalize the
538 L-[³H]-Glutamate uptake by the protein content. However, in acute brain slices, we hypothesize
539 that the glutamate uptake activity occurs on the surface of the slice, and the inner tissue
540 probably does not contribute to the amount of glutamate captured. In order to test this, we
541 carried out uptake experiments with L-[³H]-Glutamate comparing 200 and 300 μm slices from
542 the same animals for 5 min. It is clear to us that the use of 200 or 300 μm slices does not
543 present significant differences in the amount of L-[³H]-Glutamate transported. Additionally, the
544 use of 200 μm slices is experimentally more challenging since the slice is much more delicate
545 due to its reduced thickness. Preferably, when performing this technique, the researcher should
546 use slices obtained from the same hippocampal region of all animals.
547
548 Some studies use 5 or 7 min of uptake time[10]. Herein the results show a significant difference
549 comparing 5 with 10 min (**Figure 3B).** In addition, a plateau was observed comparing the 10 and
550 15 time points. It is strongly recommended that the entire procedure is performed quickly to
551 preserve metabolic function, reduce tissue stress and consequent damage related to the
552 technique. Given the proper adaptations, this method can be modified to fit other
553 neurotransmitter systems depending on the researchers' interest. AHS can be prepared from
554 non-treated, pharmacologically treated, and/or genetically modified animals. Additionally, it
555 allows ex vivo manipulations such as treatment of the tissue with compounds that modulate
556 neurotransmitter uptake. Combining these approaches allows testing a variety of research
557 applications[40]. As expected, unlabeled glutamate in incubation media decreases L-[³H]-
558 Glutamate uptake in a concentration-dependent manner (**Figure 3G**).
559
560 Considering that glutamate transporters can take up both D- and L-Aspartate, we performed an
561 experiment comparing the L-[³H]-Glutamate and D-[³H]-Aspartate uptake. No differences were
562 observed (**Figure 3F**); however, it is important to highlight that D-[³H]-Aspartate is metabolized
563 in a lesser extent than L-[³H]-Glutamate. Thus, the accumulation of D-[³H] -Aspartate in the slice
564 could provide another important parameter to measure transporter activity.
565
566 The present results reported the total amount of L-[³H]-Glutamate taken up by each AHS. It is
567 important to note, however, that one of the most common ways of reporting L-[³H]-Glutamate
568 is dividing the amount taken up by the time, thus reporting in fmol/min. Also, when using cell
569 culture to L-[³H]-Glutamate uptake, it is common to measure the protein of the sample and
570 report the results as fmol L-[³H]-Glutamate /mg protein/min. When using AHS or any other
571 brain slices, it is not recommended to report the results referring to the protein, since a 300 μm

572   slice contains much more protein than the protein that indeed may take up the L-[^{3}H]-
573   Glutamate.
574
575   Regarding this protocol, it is still important to clarify some important issues. i) HBSS will be used
576   to measure total specific glutamate uptake, as the glutamate transport is sodium-dependent. ii)
577   N-methyl-D-glucamine (NMDG) chloride is frequently used as a substitute for NaCl in systems to
578   maintain physiological solutions as isotonic without sodium. iii) The addition of HEPES to this
579   formulation may improve the AHS's resistance to brain slicing edema and provide stronger pH
580   buffering[38]. iv) The use of salts containing K$^+$ should be avoided since high concentrations of K$^+$
581   in the extracellular medium can interfere with membrane potential. Therefore, KCl should not
582   be used as an alternative to NaCl; and v- to induce unconsciousness, the rats were exposed to
583   isoflurane 3% before euthanasia for at least one minute. Considering that it has been previously
584   shown that isoflurane influences glutamate uptake[41], it is argued that the time taken between
585   isoflurane exposure and ex vivo glutamate uptake had no significant effect on the parameters
586   illustrated in this protocol.
587
588   One limitation of the technique is that it does not distinguish how much glutamate is taken up
589   by glial and neuronal transporters. Nevertheless, the contribution of neuronal transporters in
590   the glutamate uptake is rather modest, and most prominent transporters are located on
591   astrocytes surrounding synapses[14,17]. Also, the assessment of glutamate uptake in specific,
592   almost microscopic, hippocampal areas (e.g., CA3 exclusively) would pose an additional
593   challenge, since the dissection procedure would be much more delicate. It is possible to
594   decrease inter-experimental variation by performing the hippocampal slicing quickly (less than
595   3 minutes) and maintaining the tissue humidified with HBSS buffer or saline solution until
596   placing it in the plate for the assay. Another advantage of using this protocol is the possibility to
597   perform kinetics experiments, since it was chopped 3 whole hippocampi from adult rats and
598   counted between 34-36 slices from each one. This way, with a single adult rat it is possible to
599   obtain about 70 transverse hippocampal slices from both hippocampi and different areas
600   (ventral, intermediate and dorsal). Therefore, this protocol provides a complete picture of the
601   glutamate uptake by the slices obtained from resected hippocampus.
602

611
612   **DISCLOSURES:**
613   The authors have nothing to disclose.
614
615   **REFERENCES:**

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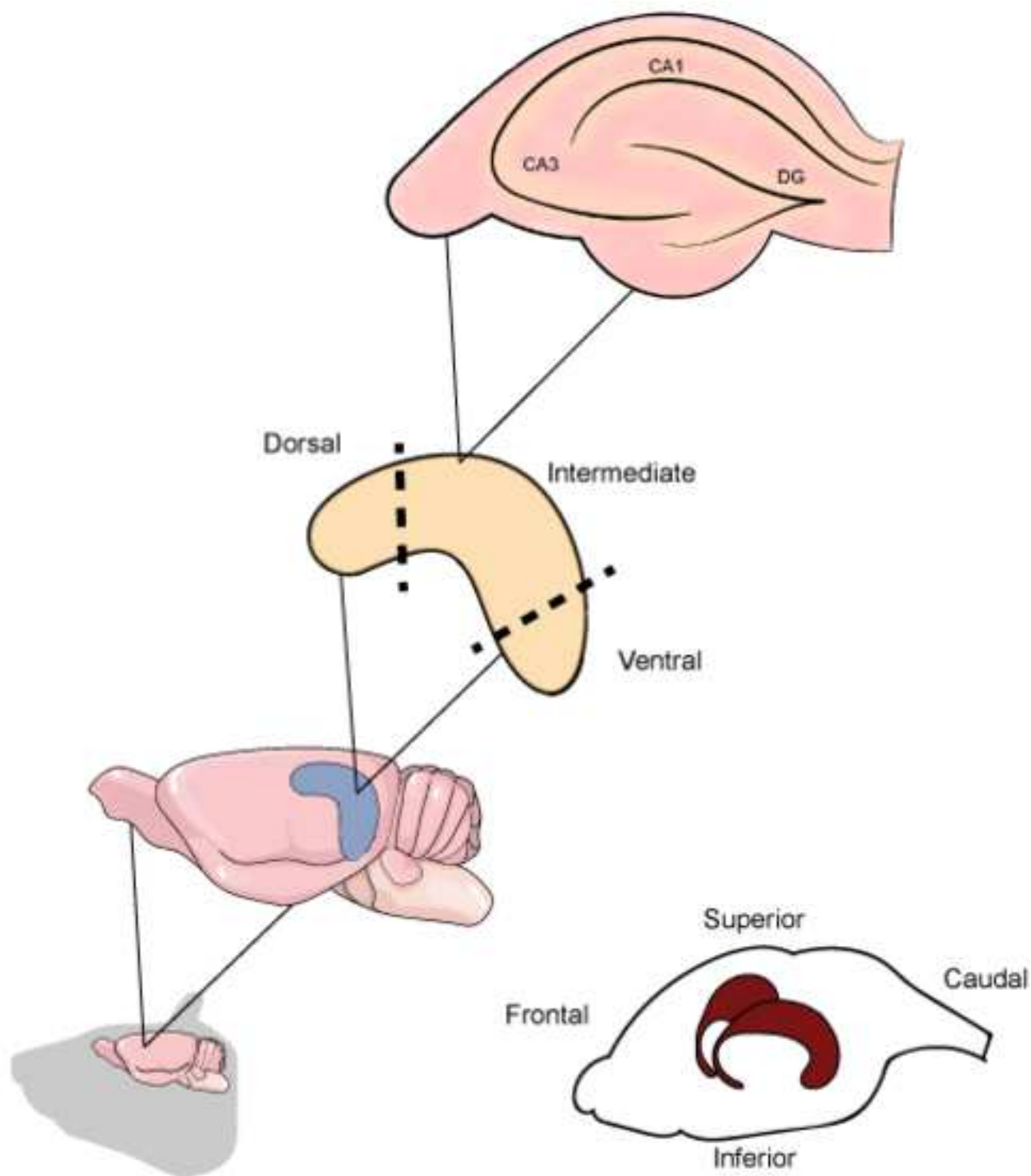
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CA1

CA3

DG

Dorsal

Intermediate

Ventral

Superior

Caudal

Frontal

Inferior

Prepare HBSS with and without sodium. Have separate HBSS portions at 4° and 37 °C. → Organize the bench with all the materials needed for dissection. Organize the tissue chopper materials. → Prepare the [³H]L-Glutamate input. → Euthanise the rodent according to the methods allowed by the Ethics Committee.

Separate the slices with a thin brush and place each slice in a well of a 24-well plate with 37 °C HBSS (for specific uptake) or 4 °C sodium-free HBSS (for unspecific uptake). ← Slice the hippocampi and collect the slices to a petri dish with ice-cold saline solution. ← Place the hippocampi in the tissue chopper table beneath the blade, oriented along the transverse axis (be sure to identify the specific regions if necessary). ← Carefully dissect the brain and remove both hippocampi.

Stabilize the slices for 15-30 min in a heating plate. Remove the stabilization media and add 280 µL of fresh 37 °C or 4 °C sodium-free HBSS. → Start the uptake by adding 20 µL of input to each well, every 15 - 20 s. Be sure to let the radioactive glutamate exactly 5 min in contact with each slice and then remove the media by aspiration. → Wash the slice 3 times with ice-cold HBSS and add 300 µL NaOH 0.5 M. Incubate overnight at 4 °C.

Use the DPM value and the specific radioactivity of the reagent to calculate [³H]L-Glutamate taken up. ← Add scintillation liquid to the microtube up to a volume of 1.5 mL. Homogenize by vortex and read in a scintillation counter for 60 s each sample. ← Collect the content of the well to a microtube. Make sure the solution is homogeneous, use a thin gauge needle to homogenize if necessary.

Table 1

Reagent	Final concentration (mM)
$CaCl_2$	1.26
D-Glucose	5.55
KCl	5.36
KH_2PO_4	0.44
$MgCl_2$	0.49
$MgSO_4$	0.41
NaCl	137
$NaHCO_3$	4.17
Na_2HPO_4	0.63

Table 2   ⬇   Click here to access/download;Table;Table 2.xlsx ⬇

Reagent	Final concentration (mM)
$CaCl_2$	1.26
D-Glucose	5.55
Glucamine-HCl pH 7.4 (adjust with concentrated HCl)	137
Glucamine-HEPES pH 7.4 (adjust pH with HEPES free acid 2 M)	4.17
KCl	5.36
KH_2PO_4	0.44
$MgCl_2$	0.49
$MgSO_4$	0.41

Table 3. Materials and equipment used in this protocol.

Name of Material/Equipment	Company	Catalog number	Comments/description
#11 scalpel blade	Swann-Morton	525	
100 mm glass petri dish	Common suppliers		
110 mm diameter Whatman Filter	Sigma Aldrich	WHA1001110	
42.5 mm diameter Whatman Filter	Sigma Aldrich	WHA1001042	
24-well cell culture plate	Falcon	353047	
Becker	Common suppliers		
Blades for the tissue chopper	Wilkinson	3241	
Bone rongeur	Erwin Guth	900,005	
$CaCl_2$	Sigma Aldrich	C4901	
D-[2,3-^{3}H]-Aspartic acid	PerkinElmer	NET581001MC	11.3 Ci/mmol (37 MBq)
D-Glucose	Sigma Aldrich	G8270	
N-Methyl-D- Glucamine	Sigma Aldrich	M2004	
HEPES	Sigma Aldrich	H3375	
Hidex 300 SL	Hidex Oy.	Super Low Level #425-020	
Iris scissors	Erwin Guth	800,040	
Isoflurane	Cristalia (São Paulo, Brazil)	410,525	1 mL/mL
KCl	Sigma Aldrich	P3911	
KH_2PO_4	Sigma Aldrich	P0662	
L-[3,4-3H]-Glutamic Acid	PerkinElmer	NET490005MC	49.7 Ci/mmol (185 MBq)
$MgCl_2$	Sigma Aldrich	M8266	
$MgSO_4$	Sigma Aldrich	M7506	
Na_2HPO_4	Sigma Aldrich	S9763	

NaCl	Sigma Aldrich	S9888	
NaHCO$_3$	Sigma Aldrich	S5761	
Plastic Pasteur pipette	Common suppliers		
Scintillation liquid	PerkinElmer	1200.437 for 1 x 5 Liter	Optiphase HiSafe 3
Small surgical scissors	Erwin Guth	800,040	
Small tweezers	Erwin Guth	600,131	
Spare chopping discs for the chopper	Common suppliers		
Standard scissors	Erwin Guth	800,010	
Thin brushes (size 0 or 2)	Common suppliers		
Thin double-ended spatula	Erwin Guth	470.260E	
Tissue Chopper	Ted Pella, Inc.	10180	

Ouro Preto, May 2021

Dear Editor Dr. Nam Nguyen, PhD

JoVE Manager of Review,

As recommended we have addressed the ethics statement to the MS, and hope that this revised version proves acceptable for publication.

Please see below the detailed responses to the Editor´s comments and changes in the MS.

Editorial and production comments:

Please provide an ethics statement for the animal work: "All animal studies were approved by ..."

In the revised version of the MS (line 607), we included the following ethics statement: "All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee (project approval # 33732/CEUA-UFRGS). All efforts were made to minimize discomfort and the number of animals used in the experiments."

We are very grateful for your attention. Thank you.