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Title: An Efficient and Cost-effective Method of Generating Postnatal (P2 - 5) Mouse Primary Hippocampal Neuronal Cultures

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2 Primary Hippocampal Neuronal Cultures

3

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26 **Abstract**

27 **Background**

28 Primary culture of postnatal central neurons is a widely used methodology for
29 applications such as the investigation of neuronal development, protein
30 trafficking/distribution and cellular signalling. However, successful production and
31 maintenance of such cultures, particularly from postnatal animals, can be
32 challenging. In attempting to surmount these difficulties, several disparate culturing
33 methodologies have been developed. Such methodologies are centred on the
34 identification and optimisation of critical steps and, as such, the protocols and
35 reagents utilised can differ quite markedly from protocol to protocol, often with the
36 suggestion that the use of a (usually expensive) proprietary reagent(s), lengthy
37 substrate preparation and/or cell isolation techniques is/are necessary for successful
38 culture preparation.

39 **New Method**

40 Herein, we present a simple and inexpensive protocol for the preparation of primary
41 hippocampal neurons from postnatal (2 - 5 day old) mice, which remain viable for
42 experimental use for over one month.

43 **Results**

44 Neurons cultured using this method follow well established developmental norms
45 and display typical responses to standard physiological stimuli such as
46 depolarisation and certain pharmacological agents.

47 **Comparison with Existing Methods/Conclusion**

48 By using a novel trituration technique, simplified methodology and non-proprietary
49 reagents, we have developed a reliable protocol that enables the cost effective and
50 efficient production of high quality postnatal mouse hippocampal cultures. This

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51 method, if required, can also be utilised to prepare neurons both from other regions
52 of the brain as well as from other species such as rat.
53

54 **Keywords**
55 Primary neuronal culture; Hippocampal neurons; Postnatal; Trituration; Mouse.

56 **Abbreviations**

57 Postnatal (P), (S)-3, 5-dihydroxyphenylglyccine ((S)-DHPG), N-methyl-D-aspartic
58 acid (NMDA), days *in vitro* (DIV), group I metabotropic glutamate receptor (mGluR).

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59 ## 1. Introduction

60 ### *1.1 Uses of primary neuronal culture*

61 Primary neuronal culture enables the conversion of complex, three dimensional brain
62 tissue, which is difficult to study *in vivo,* into a two-dimensional monolayer of cells,
63 which allows easy access to, and visualisation of, individual neurons and synapses
64 (Nunez, 2008). As such, the technique is routinely utilised for a wide variety of
65 applications in neuroscience including, *inter alia,* pharmacological,
66 electrophysiological, immunohistochemical, neurotoxicological, developmental and
67 cell signalling studies (Beaudoin et al., 2012; Brewer and Torricelli, 2007; Nunez,
68 2008). However, in addition to ease of access to, and visualisation of, neurons and
69 synapses, this simplified monolayer system is also of use for several other purposes.
70 For example, neuronal cell culture eliminates, or at least reduces, potential
71 hormonal, vascular and/or inflammatory confounding influences that one might
72 encounter when using intact, or whole brain tissue, systems (Brewer and Torricelli,
73 2007), which may be important when investigating phenomena such as the
74 subcellular localisation and trafficking of neuronal proteins such as neurotransmitter
75 receptors (Chen et al., 2008). Conversely, one can easily and very specifically
76 manipulate the cellular environment of cultured neurons/glia to determine the effect
77 of a particular pharmacological or hormonal intervention in the absence of any other
78 confounding factors. Thus, careful and selective manipulation of the cellular
79 environment in this way provides more specific and reproducible information than
80 could be achieved by using whole tissue or *in vivo* animal studies in the same way
81 (Zhang et al., 2006).

82 Neuronal culturing also enables the preparation of multiple, separate neuronal
83 ‘populations’ which can be considered as identical replicates (Brewer and Torricelli,

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84 2007). As it is usually possible to seed a relatively large number of identical neuronal
85 populations from a single piece of brain tissue, this technique represents a highly
86 efficient method of increasing sample size, whilst reducing variability. Finally, but no
87 less importantly, given the *relative* long-term viability of cultured cells compared with
88 acutely dissected tissue such as acute brain slices, cell culture also allows for longer
89 term studies investigating toxicology and development (Chen et al., 2008; Zhang et
90 al., 2006).
91
92 ***1.2 Embryonic versus postnatal brain tissue***
93 The possible advantages of using cultured neurons/glia over intact brain tissue
94 notwithstanding, there can be significant practical limitations to producing viable
95 neuronal cultures as the technique itself is technically demanding and can be
96 hampered by a lack of reproducibility.
97 Variation in the quality of neuronal cultures can be ameliorated to some extent by
98 utilising embryonic, rather than postnatal, tissue. This is most likely due to the
99 proposed increased plasticity of embryonic, relative to postnatal, neurons. As such,
100 they have fewer complex neurites, lower inter-neuronal connectivity and a decreased
101 reliance on trophic support (Brewer and Torricelli, 2007; Kivell et al., 2000; Zhang et
102 al., 2006). Taken together, these factors are likely to bestow upon embryonic
103 cultures a higher resistance to, and higher survival rate following, chemical and
104 mechanical tissue dissociation. This technical advantage has led to the widespread
105 use of embryonic tissue, particularly that of mice, for the preparation of neuronal
106 cultures. Although, the use of embryonic neuronal culture may be relevant for studies
107 concerned with, for example, neuronal/glial development, disease etiology, or where,
108 due to genetic mutation(s), animals die at, or soon after, birth, it would often be much

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109 more appropriate to use more physiologically-relevant, postnatal tissue. For
110 example, when investigating the function and role of developed neurons, and for
111 studies linked to age-developed pathology such as Alzheimer's disease.
112 Unfortunately, however, in contrast to embryonic tissue, postnatal brain tissue is
113 relatively sensitive to the culturing process. This is thought to be due to the fact that
114 as neurons develop, they become more susceptible to glutamate-mediated
115 excitotoxicity (Brewer, 1998) and exhibit enhanced caspase activation and apoptosis
116 (Brewer et al., 2005). Physical degradation of neurons also removes intra-neuronal
117 trophic support, thereby decreasing cell viability still further (Brewer and Torricelli,
118 2007).
119 This susceptibility to culturing seems to be particularly true when one considers the
120 culturing of hippocampal and other central neurons derived from postnatal *mice*,
121 where there are limited documented successful culturing methodologies for either
122 very early postnatal (postnatal day (P) 0 - 1) (Ahlemeyer and Baumgart-Vogt, 2005;
123 Beaudoin et al., 2012) or adult mice (Brewer and Torricelli, 2007; Eide and
124 McMurray, 2005), relative to protocols published for producing postnatal rat neuronal
125 cultures (e.g. (Brewer, 1997; Drysdale et al., 2006; Hogins et al., 2011; Irving et al.,
126 2000; Kivell et al., 2000; Nunez, 2008; Rae et al., 2000; Rao and Sikdar, 2004;
127 Stoppelkamp et al., 2010; Ternaux and Portalier, 1993; Zhang et al., 2006). Given
128 that the vast majority of transgenic animal models that have been developed to date
129 are mice, this is a very significant issue. Thus, a reliable and cost-effective postnatal
130 mouse primary hippocampal culturing protocol could have widespread applications,
131 particularly in the fields of learning and memory, and neurodegeneration.
132

133 ***1.3 Previous studies***

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134 To this end, to date, opting to use postnatal tissue to prepare primary neuronal
135 cultures often “means accepting the fact that there will be some bad culture days”
136 Banker et al. (2007). Such “bad cultures” and lack of reproducibility are likely to arise
137 from subtle variations in reagents used (Beaudoin et al., 2012) and/or techniques
138 employed between labs. This has resulted in the utilisation and publication of several
139 distinct culturing protocols, with each containing highly specific, often very different,
140 and sometimes even conflicting, advice for each facet of the culturing protocol.
141 However, differences in protocol notwithstanding, a review of recent literature
142 reveals the widely held belief that preparation of postnatal mouse cultures
143 specifically necessitates the use of expensive, defined media and supplements,
144 namely, Neurobasal®-A (NB-A) and the HEPES-based Hibernate®A, which are
145 utilised in combination with a proprietary supplement solution, B-27® serum-free
146 supplement (B-27) (Ahlemeyer and Baumgart-Vogt, 2005; Beaudoin et al., 2012;
147 Brewer, 1997; Brewer and Torricelli, 2007; Drysdale et al., 2006; Eide and
148 McMurray, 2005; Hui et al., 2015; Kivell et al., 2000; Nunez, 2008; Rao and Sikdar,
149 2004; Stoppelkamp et al., 2010; Xie et al., 2000; Zhang et al., 2006). Indeed, based
150 upon a review of recent literature on the topic, one would be forgiven for thinking that
151 it is nigh on impossible to prepare postnatal cultures without using these proprietary
152 reagents and supplements (as well as lengthy protocols such as gradient separation
153 to further optimise the procedure (Brewer & Torricelli, 2007). However, not only are
154 these proprietary media and supplements much more expensive than standard, non-
155 proprietary, media preparations such as Dulbecco’s modified Eagle’s medium
156 (DMEM), there is also a concern in some quarters about variable quality and
157 uniformity between batches of B-27 (Chen et al., 2008) and certain, possibly
158 neurotoxic, ingredients contained within NB-A (Hogins et al., 2011).

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159 Therefore, it was the aim of the present study to determine the possibility of
160 producing consistent, successful postnatal primary mouse hippocampal neuronal
161 cultures utilising non-proprietary solutions and supplements whilst, at the same time,
162 also attempting to simplify the overall culturing protocol relative those published in
163 recent times (e.g. Brewer & Torricelli, 2007, Beaudoin et al., 2012) (Suppl. Table 1
164 provides a comparison of the main components of previously published postnatal
165 mouse hippocampal culture methods with our method).
166 Herein, we describe a simplified, technically straightforward and reliable method for
167 the production of postnatal (P2 - P5) mouse hippocampal cultures of consistently
168 high quality which remain viable and responsive to normal physiological stimuli for
169 over one month post-culture. We show that the critical element in the production of
170 successful neuronal cultures is the deployment of a novel, mild, tissue trituration
171 protocol which produces sufficient neuronal dissociation without overly deleterious
172 effects on neurons or the production of excessive amounts of cellular debris.
173 Significantly, good quality cultures can be produced using this method without the
174 need for the aforementioned expensive, proprietary media and supplement.

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175

176 ## 2. Materials and Methods

177 ### *2.1 Chemicals & Reagents*

178 • Mice (2 – 5 days old)

179 • Serum replacement 2 (SR2) (50×) (Sigma, cat. no. S9388).

180 • Glutamax™ supplement (ThermoFisher Scientific, cat. no. 35050038).

181 • L-glutamic acid (Sigma, cat. no. G1251).

182 • Penicillin-streptomycin (P-S) (Sigma, cat. No. P4333).

183 • Foetal bovine serum (FBS) (Sigma, cat. no. F7524). Storage limitations: store at -
184 80ºC and limit freeze thaw cycles to one.

185 • Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma, cat. no.
186 D5546).

187 • Papain (Worthington, cat. no. 3119) **(see *Reagent Setup*).**

188 • Poly-D-lysine hydrobromide (Sigma, cat. no. P6407) **(see *Reagent Setup*).**

189 • Dissection, trituration, plating and culture solutions **(see *Reagent Setup*).**

190 • Trypan blue solution (Sigma, cat. no. T8154) (optional).

191 • 70% ethanol solution for sterilisation of surfaces and hands.

192 • Deionised water (dH_2O).

193 • Immunocytochemistry reagents: Triton™ X-100 (Sigma, cat. no. X100),
194 paraformaldehyde (PFA) (Sigma, cat. no. P6148), sucrose (Sigma, cat. no. S7903),
195 glycine (e.g., Sigma, cat. no. G7126), bovine serum albumin (BSA) (Sigma, cat. no.
196 05470), mounting medium (Sigma, cat. no. F6182), microscope slides (25 x 75 mm)
197 (Sigma, cat. no. S8400). Primary antibodies: pan neuronal marker (mouse
198 monoclonal, Millipore, cat. no. MAB2300, Antibody Registry: AB_1587299), anti-
199 GFAP (rabbit monoclonal, Cell Signaling, cat. no. 12389, Antibody Registry:

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200 AB_2631098), anti-Synapsin I (rabbit polyclonal, Millipore, cat. no. AB1543, Antibody
201 Registry: AB_2200400), anti-NeuN (mouse monoclonal, Millipore, cat. no. MAB377,
202 Antibody Registry: AB_2298772). Secondary antibodies: FITC goat anti-mouse
203 (Jackson, cat. no. 115-095-003, Antibody Registry: AB_2338589), Cy5 goat anti-
204 rabbit (Abcam, cat. no. ab97077, Antibody Registry: AB_10679461), TRITC donkey
205 anti-rabbit (Jackson, cat. no. 711-025-152, Antibody Registry: AB_2340588).

206 • Drugs used for calcium imaging: (S)-3, 5-dihydroxyphenylglyccine ((S)-DHPG)
207 (Tocris, cat. no. 0805), caffeine (Sigma, cat. no. C0750), carbachol (Tocris, cat. no.
208 2810), N-methyl-D-aspartic acid (NMDA) (Sigma, cat. no. M3262)

209

210 ***2.2 EQUIPMENT***

211 • 35 mm cell culture dish (untreated) (Sigma, cat. no. CLS430588). Previous
212 studies have suggested cell culture treated dishes give rise to altered neuronal
213 morphology (Chen et al., 2011).

214 • 145 mm cell culture dish (Sigma, cat. no. Z652539)

215 • 13 mm glass coverslips, other sizes can be used as desired (TAAB, cat. no.
216 M160/1).

217 • 15 and 50 ml sterile polypropylene (PP) centrifuge tubes (Fisher Scientific, cat. no.
218 11849650 and 11512303 respectively). Previous studies have recommended the
219 routine use of polystyrene or polyethylene terephthalate (PET) tubes given reports of
220 toxicity from PP tubes (Brewer and Torricelli, 2007), but we found no adverse effects
221 from use of PP tubes.

222 • 15 ml and 30 ml syringes (Fisher Scientific, cat. no. 12931031and 10313015
223 respectively)

224 • Haemocytometer (optional).

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225 • Sterile syringe filter, 0.2 μm pore size (Sigma, cat. no. CLS431229).

226 • 150 mm Pasteur pipette (VWR, cat. no. 612-1701) **(see *Equipment Setup*).**

227 • 3.5 ml Plastic transfer pipette (Sarstedt, cat. no. 86.1171).

228 • Incubator, controlled 5% CO_2, humidified. Other studies have recommended the
229 use of an O_2 regulated incubator (Brewer and Torricelli, 2007). However, in our
230 experience these more costly incubators are not necessary for producing successful
231 cultures.

232 • Inverted microscope, phase contrast, with long working distance objective.

233 • Sterile laminar flow hood, HEPA-filtered air.

234 • Sterile dissection hood, HEPA-filtered air.

235 • Swinging bucket centrifuge compatible with the use of 15 ml tubes, ambient room
236 temperature, capable of speeds of 258 g.

237 • Water bath.

238 • Temperature-regulated orbital shaker, capable of incubating at 37 ^{O}C.

239 • Fine and course dissection tools.

240 • Osmometer.

241

242 ***2.3 REAGENT SETUP***

243 • **HEPES-buffered saline solution (HBSS)** of the following composition (in mM):
244 NaCl 130, HEPES 10, KCl 5.4, $MgCl_2$ 2, D-glucose 2 and $CaCl_2$ 0.5, pH 7.4, 310
245 mOsm (chosen to match the range of osmolarity (304-336 mOsm) of DMEM).

246 • **Dissection solution.** Prepare 8 ml of HBSS solution containing 0.5 mM
247 Glutamax, 0.025 mM L-glutamic acid, 1% P-S.

248 • **Trituration solution.** Prepare 15 ml of HBSS solution containing 10% FBS, 5 mM
249 Glutamax, 0.025 mM L-glutamic acid, 1% P-S. Place in water bath at 37ºC.

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250 • **Plating solution.** Prepare 2 ml of DMEM solution containing 10% FBS, 5 mM
251 Glutamax, 0.025 mM L-glutamic acid, 1% P-S. Place in incubator and allow CO_2 to
252 buffer solution.

253 • **Culture solution.** Prepare 35 ml of DMEM solution containing 2% SR2, 5 mM
254 Glutamax, 0.025 mM L-glutamic acid, 1% P-S. Place in incubator and allow CO_2 to
255 buffer solution.

256 • **Poly-D-lysine solution**. Dissolve at 0.1 g/ml in dH_2O. Store as frozen 2 ml
257 aliquots in 15 ml tubes.

258 • **Papain solution.** Immediately prior to beginning dissection, dissolve 2.5 mg/ml
259 papain in Dissection solution with a total volume of 2 ml (approximately 25 U $ml^{-}1$).
260 To ensure full dissolution of papain, the solution should be placed in a 37°C water
261 bath for approximately four minutes. Filter sterilise the solution into a 15 ml tube and
262 store on ice. Note: Papain has been found to be the most suitable protease for cell
263 culture (Brewer, 1997). Although in our hands papain from Worthington Scientific
264 was superior to that purchased from Sigma, the specific supplier does not appear to
265 be crucial for culture success. The merits of dissolving papain in Ca^{2+} and Mg^{2+} free
266 solution, to aid tissue digestion (Copray and Liem, 1993) are debated, due to the
267 damage that an absence of these cations may have directly on cells (Kivell et al.,
268 2000). It is recommended that papain be stored on ice for no longer than three hours
269 (Brewer and Torricelli, 2007).

270 • **Phosphate Buffered Saline (PBS)** of the following composition (in mM): NaCl
271 137, KCl 2.7, NA_2HPO_4 10, KH_2PO_4 2, pH 7.4.

272 • **Mice (2–5 days old).** The volumes provided in this protocol are designed for
273 hippocampi from three to four mice aged between 2–5 days old and would have to
274 be adjusted accordingly if one wished to use older animals. Animals were euthanized

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275 in accordance with the European Directive 2010/63/EU and experiments approved
276 by the Animal Experimentation Ethics Committee of University College of Cork.
277

278 ***2.4 Equipment Setup***

279 *2.4.1 150 mm Pasteur pipette*

280

281 Select pipettes with smooth tips and flame polish briefly (approximately two seconds)
282 to blunt, but not significantly reduce aperture size. Note: The use of siliconised glass
283 is recommended by some in the field for ease of trituration and transfer due to the
284 viscous nature of the dissociated cell suspension (Brewer and Torricelli, 2007).
285 However, in our hands standard glass pipettes were sufficient.
286

287 *2.4.2 Preparation of coverslips*

288 In a laminar flow hood, coverslips are transferred to a 35 mm cell culture dish
289 containing 2 ml of 70% ethanol for sterilisation. Two coverslips are then removed
290 and placed upright in each of sixteen 35 mm cell culture dishes and allowed to air
291 dry. Once dried, coverslips are placed horizontally on the base of each 35 mm dish.
292 Note: Cell substrate cleaning and coating are key steps in the culture process which
293 are utilised to ensure neuronal attachment and survival, as well as standard
294 development and maturation (Kaech and Banker, 2006). In terms of substrate
295 material, adult neurons do not grow well on plastic (Brewer and Torricelli, 2007) and
296 as such, glass coverslips are routinely used. Moreover, there are recommended
297 providers of cover slips with a proven reliability, without which cells will initially grow
298 but detach soon after, such as: Type “D” glass from Schott Desag, (Kaech and
299 Banker, 2006). In our hands, coverslips provided by TAAB have also been conducive

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300 to long-term reliable culturing. Treating these substrates with nitric acid is
301 discouraged by some (Brewer, 1997), but recommended by others, including a
302 lengthy ‘etching’ procedure with nitric acid, followed by thorough cleaning with
303 distilled H_2O, and then heat and/or radiation sterilization (Beaudoin et al., 2012;
304 Kaech and Banker, 2006). In our hands however, quick sterilization in 70% ethanol
305 was sufficient to allow for poly-D-lysine attachment and subsequent cell adherence,
306 without any adverse effects on neuronal survival, despite the assertion that solvents
307 leave a ruinous toxic residue (Brewer and Torricelli, 2007).

308

309 *2.4.3 Poly-D-lysine coating of coverslips*

310 One drop of sterile filtered poly-D-lysine solution was added to the centre of each
311 coverslip. The 35 mm cell culture dishes were then placed into two larger “holding”
312 culture dishes (145 mm) and left at ambient room temperature. After one hour, the
313 poly-D-lysine was aspirated and the coverslips rinsed twice with a drop of dH_2O.
314 Timing: This step can be conducted during the tissue incubation with papain. Note:
315 poly-D-lysine is superior in terms of consistent cell attachment and neurite
316 outgrowth, when compared with poly-L-lysine (Kivell et al., 2000). In the interest of
317 efficiency, we opted not to make poly-D-lysine fresh for each dissection as
318 recommended by Kaech and Banker (2006) and instead used frozen stocks, whilst
319 limiting freeze-thaw cycles to no more than two (Brewer and Torricelli, 2007).
320 Coverslips may be prepared in advance of the day of tissue preparation and have
321 been shown to be stable for up to seven days at 4 $^{\circ}$C (Beaudoin et al., 2012).
322 Although serum pre-treatment of coverslips is reported as being essential for cell
323 attachment, presumably linked with an initial stimulus for neurite growth which
324 facilitates attachment (Kivell et al., 2000), we opted to provide this stimulus through a

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325 serum-containing plating medium.

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2.5 Methods

Unless otherwise stated, all procedures were carried out in a sterile laminar flow hood, using equipment and reagents which, if not already contained in sterile packaging, were sterilised with 70% ethanol. Strict aseptic technique must be adhered to in order to avoid contamination.

2.5.1 Solution preparation

Approximately 3 ml of dissection solution was sterile filtered into each of two 35 mm dishes which were placed on ice in a dissection hood.

2.5.2 Tissue isolation and treatment

Hippocampi were isolated as described previously (Beaudoin 2012) and placed in the 35 mm dish on ice. The tissue was cleaned before being transferring to the second 35 mm dish on ice. The tissue was then chopped into smaller pieces with fine scissors and then transferred to a 15ml tube using a Pasteur pipette. This tube was placed in a water bath at 37ºC with the papain solution, separately, and both were allowed to equilibrate at this temperature for five minutes. The tissue was then carefully transferred into the papain solution and placed on an orbital shaker at 100 rpm at 37ºC for 45 minutes. The tissue was then gently removed from the papain solution and placed into another 15 ml tube containing 2 ml HBSS. This step was repeated two more times (to remove any residual papain), each time being careful to avoid trituration of the tissue at this stage. The tissue was then transferred into another 15 ml tube containing approximately 6 ml of trituration solution.

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351 *2.5.3 Trituration (see accompanying video)*

352 The tissue was allowed to settle to the bottom of the 15 ml tube containing the
353 trituration solution. The tip of a plastic transfer pipette, with the bulb fully depressed,
354 was firmly pushed against the bottom of the 15 ml tube. The solution containing the
355 tissue was then aspirated into the pipette such that the tissue experienced a certain
356 degree of friction as it moved between the edges of the bottom of the tube and the
357 pipette itself. This friction helped to dissociate individual hippocampal neurons from
358 the larger pieces. When all of the tissue had been aspirated into the pipette, the tip of
359 the pipette was then placed under the meniscus of the trituration solution and
360 forcefully expelled. Depending on the resulting tissue break up, this procedure was
361 repeated up to an absolute maximum of four times, allowing the tissue to settle to the
362 bottom of the tube between each aspiration. The supernatant was then placed into
363 another 15 ml tube, leaving behind as much as possible of the untriturated tissue.
364 This whole procedure should take no more than five minutes. The supernatant was
365 then centrifuged at 258g for two minutes at room temperature. Note: The trituration
366 procedure is the most critical step in determining the success of a culture. As this is
367 the point in the protocol where cells experience the main mechanical stress, a
368 balance is required between reaching optimal cell yield by sufficient tissue
369 dissociation, whilst minimising cell lysis. Such lysis and debris production can both
370 directly and indirectly, by altering the pH of the trituration solution and releasing
371 cytotoxic agents such as glutamate, hinder cell attachment, sprouting and viability
372 (Brewer, 1997; Eide and McMurray, 2005). Tip diameter is also crucial in determining
373 overall cell yield, with too small a tip diameter causing cell damage, and too large a
374 diameter resulting in insufficient breakdown of the tissue. We found that a plastic,
375 wide tip diameter (drop size 35–55 µl) transfer pipette (see equipment) was optimal

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376 for reaching this compromise. By using such a plastic pipette, the unreliable nature
377 of creating an optimally smooth glass pipette tip, which can result in such a small a
378 tip diameter that it damages cells, is circumvented (Beaudoin et al., 2012). Using
379 this novel trituration approach, we do notice some air bubble production does occur.
380 Although this is a phenomenon which has been actively discouraged by others
381 (Brewer and Torricelli, 2007), we have found that it does not significantly affect the
382 quality of our cultures in comparison to, say, over trituration which is much more
383 damaging.

384 **[insert trituration video here]**

385

386 *2.5.4 Cell seeding and culture*

387 After centrifugation, the supernatant was poured out of the tube, being careful not to
388 disturb the pellet of cells. Two drops of plating solution were then gently added and
389 subsequently removed in order to dilute any residual supernatant. The volume of
390 plating solution added (0.6 - 1 ml) is determined subjectively, depending on the size
391 of the pellet which, in turn, is dependent on the effectiveness of trituration. This
392 effectively results in a plating density from 1 – 1.5 x 10^5 cells/cm^2. The pellet was
393 carefully dislodged from the bottom of the 15 ml tube using a Pasteur pipette, and
394 the cells subsequently re-suspended by firmly finger vortexing 2 - 3 times, depending
395 on the level of resuspension. At this point, if an inexperienced operator has difficulty
396 objectively determining the amount of plating solution to be added, a sample can be
397 taken for cell counting using a hemocytometer, and the density altered thereafter. An
398 indication of successful technique up to this point can be determined by employing a
399 trypan blue staining and cell counting protocol (as per manufacturer's instructions). A
400 drop (approximately 25 μl) of the final plating solution was then added to each

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401 coverslip using a Pasteur pipette. The coverslips were then placed in the incubator,
402 where the cells were allowed adhere to the coverslips for one hour. Two ml of culture
403 solution was then very gently added to each 35 mm dish (to prevent damaging the
404 cells through sheer stress), before returning the dishes to the incubator for a further
405 two hours, after which the coverslips were inverted using sterile forceps. In order to
406 prevent cross contamination, forceps were flame sterilised between dishes.
407

408 *2.5.5 Immunocytochemistry*

409 Wash steps consisted of three changes of PBS, each lasting five minutes. Cells were
410 fixed with paraformaldehyde (4 %) & sucrose (4 %) in PBS, warmed to 37°C for ten
411 minutes. All subsequent steps, unless otherwise stated, were carried out at room
412 temperature. After washing, a permeabilisation solution (0.25 % Triton™X-100 in
413 PBS) was added for ten minutes. Cells were washed once more with PBS and a
414 blocking buffer (BSA 5 % & Glycine 0.3 M in PBS) was added for one hour. Primary
415 antibody incubation was carried out overnight at 4°C using the following antibodies:
416 Pan Neuronal Marker (1: 100), GFAP (1: 500), Synapsin I (1: 200). All antibodies
417 were diluted in an antibody buffer containing BSA 1 % in PBS. After washing, cells
418 were incubated in the corresponding secondary antibody at 1: 200 dilution for one
419 hour. Cells were again washed before being mounted on microscope slides.
420

421 *2.5.6 Determining the neuron vs glial cell composition of cultures*

422 The ratio of neuronal cells to glia was determined by counting the number of pan-
423 neuronal and GFAP -positive cells, respectively, under a confocal fluorescent
424 microscope. As described previously, when cells were initially added to cover slips
425 following their dispersal, a drop was placed in the centre of each coverslip. As a

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426 result, there is a non-uniform distribution and density of cells, with the highest density
427 of cells appearing in the centre of the coverslip and receding outwards towards the
428 perimeter of the initial cell droplet (see Suppl. Fig. 1.). As such, in order to obtain an
429 accurate representation of overall cell numbers, a central point was chosen and
430 three randomised points radiating from the middle to the edge of the immunostained
431 region of each coverslip were selected for counting (such regions contained an
432 average of 16 cells in an area of approximately 200 μm^2). Compositional analysis
433 was conducted upon coverslips fixed at 2, 5, 8, 11, 14, 17 and 20 days *in vitro* and
434 from three separate culture preparations (from three separate mouse litters),
435 harvested from 5 day old mice.
436
437 *2.5.7 Calcium Imaging*
438 Intracellular calcium measurements from the soma of neurons were carried out as
439 previously described (Rae et al., 2000). Briefly, using a conventional fluorescence
440 imaging system (Cairn Life technologies), neurons that had been preloaded for one
441 hour with the single wavelength, intensity modulating calcium indicator, fluo-2 AM
442 (excitation at 488 nm, emission at 550 nm) (4 μM), were imaged. Images were
443 acquired at one second intervals using the open source imaging software, Winfluor
444 (John Dempster, University of Strathclyde, Scotland) via an Olympus BX50 WI
445 microscope (20× objective). Winfluor calculated fluorescence values for each pixel in
446 the frame, after subtraction of background fluorescence intensities. Representative
447 traces were created off line using Graphpad Prism.
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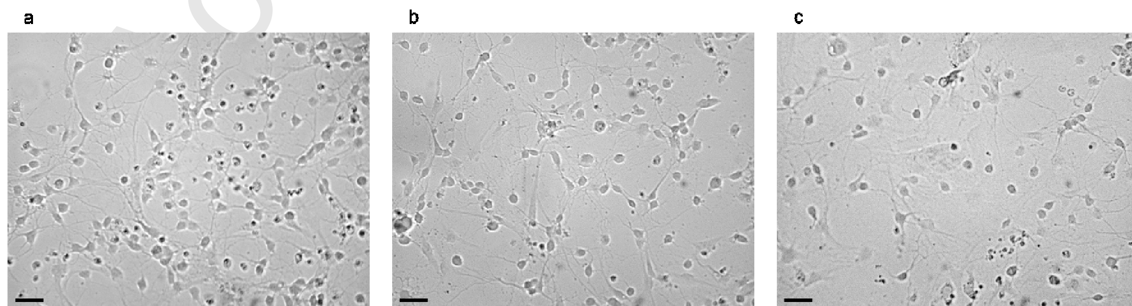
448

449 ## 3. Results

450 ### *3.1 Production and characterisation of postnatal primary mouse hippocampal
451 *cultures*

452 We have consistently produced postnatal hippocampal mouse cultures from animals
453 between 3 – 5 days old, which are routinely used for calcium imaging experiments.
454 Such cultures contain healthy, functional cells which respond appropriately to
455 physiological stimuli (see 3.2 Calcium imaging experiments). Cultures display normal
456 neuronal morphology, arborisation, synaptic connections and inter-cellular
457 associations from 2 days *in vitro* (DIV) onwards, evident from both bright field images
458 (Fig. 1 & 2) and from immunocytochemical images (Fig. 3 & 4 & Suppl. Fig. 2 for
459 corresponding phase contrast images) which were stained with a pan-neuronal
460 marker and with glial fibrillary acidic protein (GFAP) and synapsin I antibodies. In
461 order to characterise the development and composition of such cultures *in vitro* we
462 examined the neuron to glial cell ratio after fixing cultures at 2, 5, 8, 11, 14, 17 and
463 20 DIV (Fig. 5). Although there is a steady decline in the numbers of both glia and
464 neurons with increasing DIV, the neurons which do remain respond to standard
465 physiological stimuli for up to one month after initial culturing (*e.g.* Suppl. Fig. 3).



466

467 **Figure 1. Bright field (DIC) images showing cultured postnatal mouse**
468 **hippocampal neurons generated from 3 (a), 4 (b) and 5 (c) day old mice.** All
469 images were captured at 2 days *in vitro*. Scale bar 30 μm.

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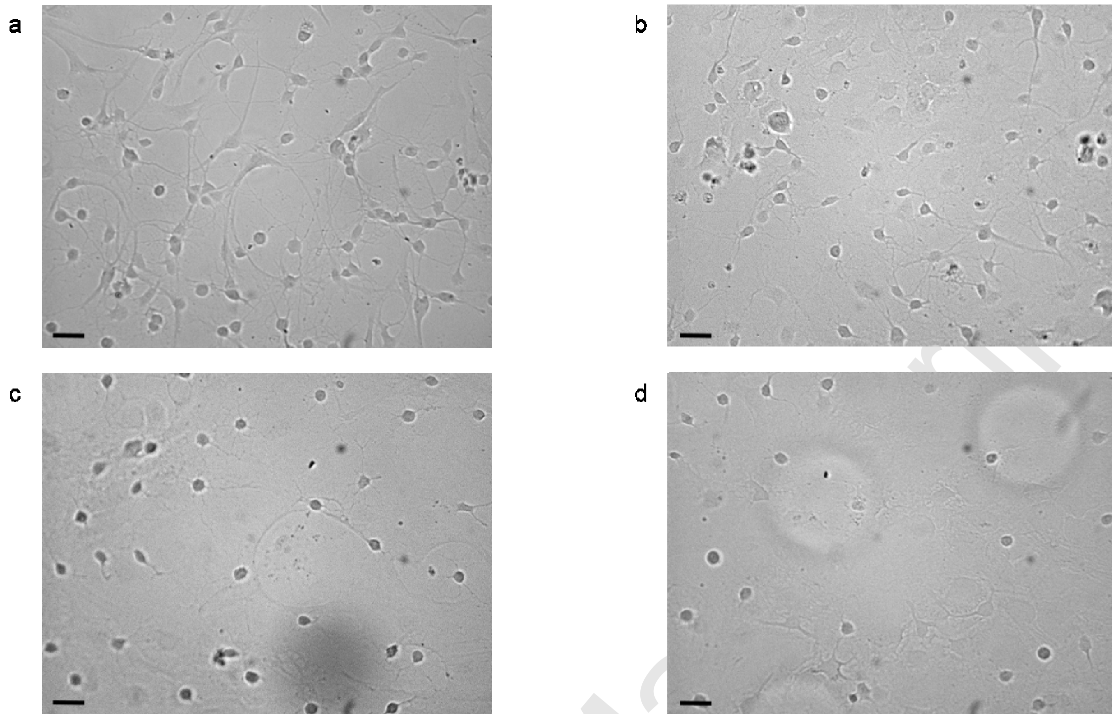
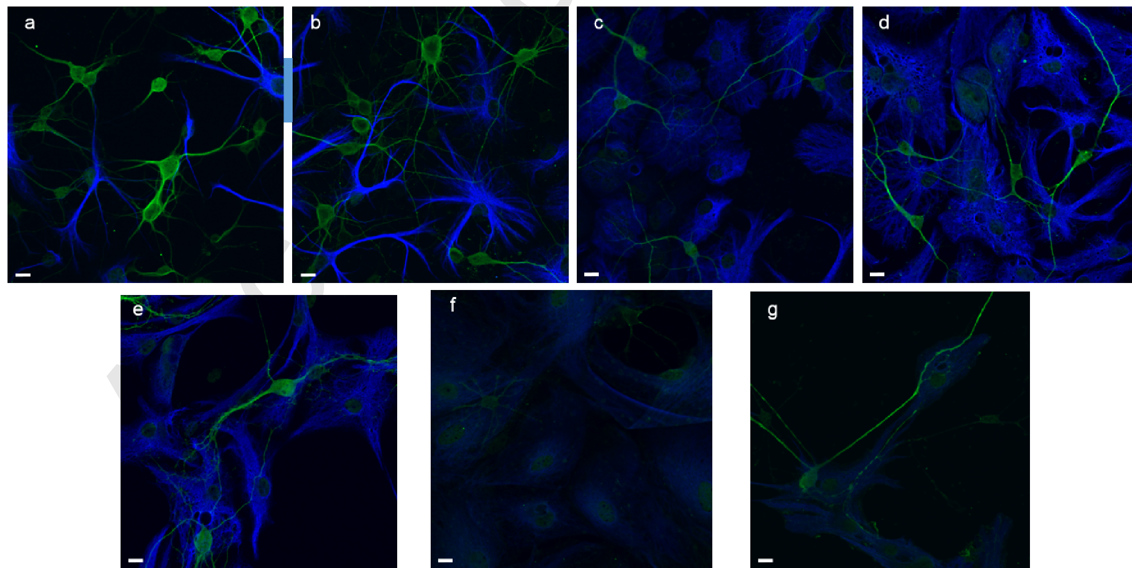


470

471 **Figure 2. Bright field (DIC) images of cultured mouse hippocampal neurons**
472 **taken at progressively older days *in vitro,* 2 (a), 4 (b), 10 (c), 13 (d)** All cultures
473 were generated from mice aged between 3 - 5 days of age. Scale bar 30 μm.



474

475 **Figure 3. Immunostaining of hippocampal cultures for neuronal and glial**
476 **markers. Images were captured from cultures fixed at progressively older days**
477 ***in vitro* 2 (a), 5 (b), 8 (c), 11 (d), 14 (e), 17 (f) and 20 (g).** Neuronal marker, pan-
478 neuronal stain containing NeuN, βii-tubulin, NF-H and MAP-2 (green). Glial marker,
479 GFAP (blue). All cultures were generated from 5 day old mice. Scale bars, 10 μM.
480

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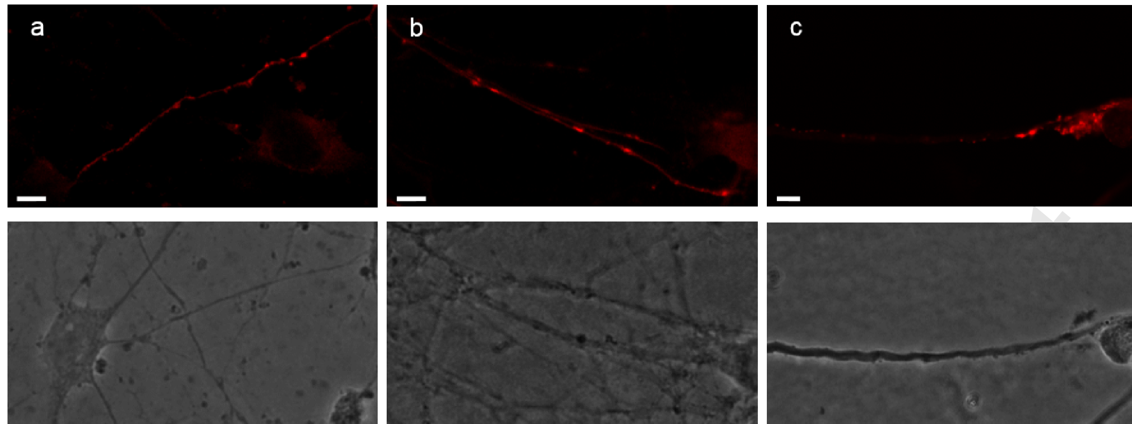
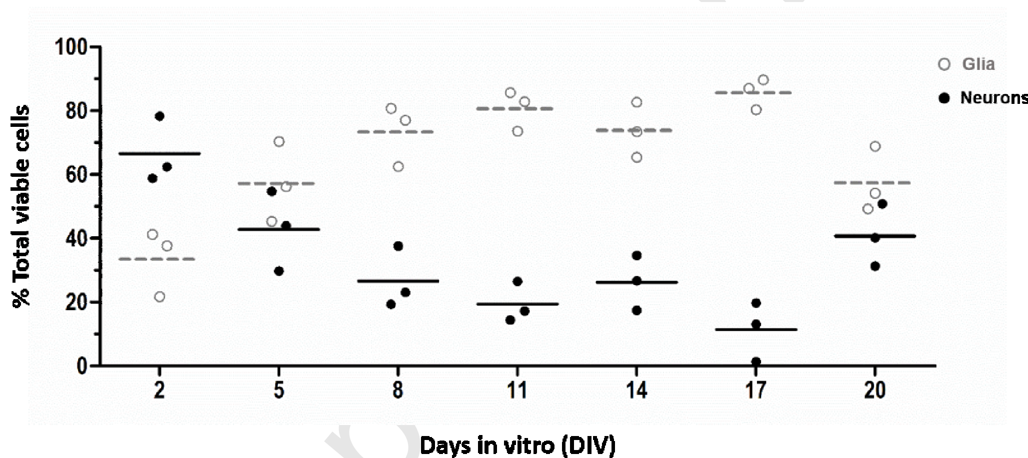


482 **Figure 4. Immunostaining of hippocampal cultures for synaptic marker**
483 **(synapsin I, red) with corresponding phase contrast images**. Images were
484 captured from cultures fixed at progressively older days *in vitro*, 2 (a), 10 (b) and 20
485 (c). All cultures were generated from 5 day old mice. Scale bars, 5 μM.
486



487

488 **Figure 5. Graphical representation of the neuron *versus* glial cell composition**
489 **of postnatal mouse hippocampal cultures at progressively older days *in vitro***
490 **(DIV).** The percentage of viable cells which are glia (grey, open circles) or neurons
491 (black, closed circles) are presented at 2 – 20 DIV. All cultures were generated from
492 5 day old mice.
493

494 ***3.2 Calcium imaging experiments***

495 Cultured neurons harvested from 3 - 5 day old mice are usually utilised up to
496 fourteen days *in vitro* for calcium imaging experiments (Kaar and Rae, 2016, 2014).
497 However, some experiments have also been carried out using neurons which had
498 been cultured for approximately one month in order to test long-term cell function

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(Suppl. Fig. 3). In the course of our calcium imaging investigations we have utilised various compounds which healthy and functional cultured hippocampal neurons would be expected to respond to. For example, we have conducted experiments in which these neurons have responded to physiologically relevant stimuli such as 15 mM and 50 mM K^+-containing HBSS (Fig. 6 a & b) (which depolarise neurons and thereby activate voltage-gated calcium channels (VGCCs) on the neuronal plasma membrane); the specific group I metabotropic glutamate receptor (mGluR) agonist, (S)-DHPG (50 µM), the muscarinic acetylcholine receptor agonist, carbachol (10 µM) and caffeine (20 mM), which all evoke calcium release from the endoplasmic reticulum (ER) (Fig. 6 c, d and f, respectively);, as well as the ionotropic glutamate receptor agonist, NMDA (1 µM; Fig. 6 d). All compounds were added to the superfusate.

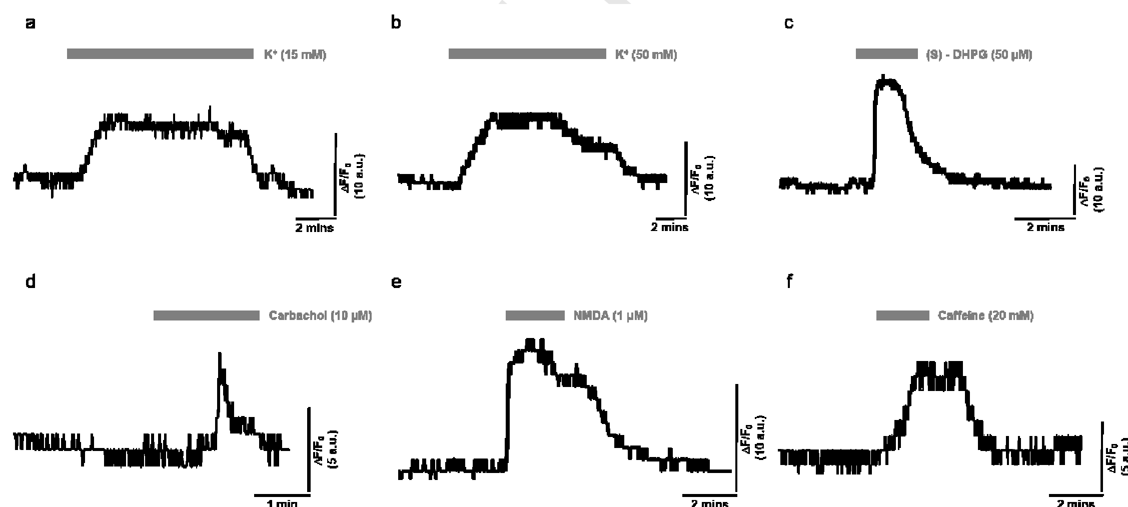

Figure 6. Representative traces showing changes in somatic [Ca^{2+}] levels within a cultured mouse hippocampal neuron in response to selected physiological stimuli. Representative traces display responses to the application of: K^+ (15 mM) (a), K^+ (50 mM) (b), (S)-DHPG (50 µM) (c), Carbachol (10 µM) (d), NMDA (1 µM) (e) and Caffeine (20 mM) (f). All compounds were added to the superfusate. All neurons were generated from 3 – 5 day old mice and imaged after 2 – 14 days *in vitro*.

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521 4. Discussion

522 The study of postnatal neurons, and therefore the use of postnatal cultures, is
523 important for the investigation of chronic and/or age-dependent conditions such as
524 neurodegeneration. Furthermore, as mentioned earlier, the importance and
525 widespread use of transgenic *mouse* models, but the inherent difficulty in culturing
526 postnatal mouse neurons relative to embryonic tissues (Brewer and Torricelli, 2007)
527 or those of the rat (Beaudoin et al., 2012), underpins the need for an easily
528 applicable and effective postnatal mouse culture protocol.
529 Herein we have described a novel, simplified protocol for the cost-effective
530 production of primary cultured neurones from postnatal mouse hippocampal tissue,
531 which remain viable for a minimum of one month post-culture. Hippocampal neurons
532 within such cultures display normal development and arborisation, observable
533 cellular inter-connection (including synapses) and association with glial cells. The
534 utilisation of either "pure" neuronal cultures, mixed cultures with or without glial
535 feeder layers is dependent upon the particular experiments one wishes to conduct.
536 However, given the role that glia play in supporting neuronal function, signalling,
537 synaptic plasticity, *etc.* (Araque and Navarrete, 2010; Auld and Robitaille, 2003;
538 Shaham, 2005) the presence, or otherwise, of glial cells in neuronal cultures will
539 almost certainly impact upon both culture development and phenotype. We have
540 opted not to use glial inhibitors, partly because they negatively impact upon culture
541 viability and synaptic development (Beaudoin et al., 2012; Pfrieger and Barres, 1997;
542 Ullian et al., 2001) but also because we feel the presence of such cells is more
543 reflective of the physiological environment which we are trying to model with these
544 cultures. However, depending upon the specific research question one wishes to

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545 investigate, the medium components suggested herein could be fine-tuned to
546 generate the particular final cellular composition one desires.

547 Importantly, the preparation and long-term survival of our neuronal cultures was
548 independent of any requirement for either proprietary media and/or supplements
549 (Ahlemeyer and Baumgart-Vogt, 2005; Beaudoin et al., 2012; Brewer and Torricelli,
550 2007; Kivell et al., 2000; Nunez, 2008; Zhang et al., 2006) or complex and/or lengthy
551 procedures such as glial feeder layers (Kaech and Banker, 2006) and gradient
552 isolation techniques (Brewer and Torricelli, 2007; Lee et al., 2009). Significantly,
553 even without using these 'essential' steps in the preparation of hippocampal cultures,
554 we have routinely utilised our neurons (between two and fourteen days *in vitro*) for
555 both calcium imaging, and whole-cell patch-clamp recording with simultaneous
556 calcium imaging experiments, in which normal physiological responses have been
557 recorded, indicating that the neurons remained viable and responsive even for this
558 extended time in culture.
559 Contrary to previous studies such as that of Eide and McMurray (2005), we have
560 found that the manner in which the tissue is treated following incubation with tissue
561 proteases, specifically the steps of trituration and subsequent re-suspension after
562 supernatant centrifugation, is absolutely critical to ensuring the production of
563 consistent and successful cultures. In this respect, mouse neurons seem to be much
564 more vulnerable and susceptible to mechanical stress than postnatal rat and
565 embryonic neurons at this point in the protocol. Thus, by using the novel trituration
566 technique described here, whereby we have reduced to an absolute minimum the
567 total number of triturations (<4) of the hippocampal tissue, and re-suspended the cell
568 pellet only using with finger vortexing, we believe that we have minimised the
569 mechanical stress experienced by the neurons. In turn, this results in the production

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570 of a cell suspension containing a very high percentage of viable cells (85 - 90%
571 viability as determined by trypan blue protocol).
572 To the best of our knowledge only three other papers have demonstrated effective
573 postnatal hippocampal culture from mouse nervous tissue, but all utilised the
574 aforementioned proprietary media and supplements. The method described by Eide
575 and McMurray (2005) using striatal and cortical mouse tissue is particularly
576 impressive given the age of the animals involved, 1–1.5 years old. Unfortunately, it is
577 unclear how these achieved this successful outcome as the paper lacks
578 methodological detail about the protocol that was employed to prepare the neurons.
579 Although the methods employed by both Beaudoin *et al.* (2012) and Brewer *et al.*
580 (2007), using early postnatal (P0–P1) and adult mouse hippocampal tissue
581 respectively, are well described and imply that good quality cultures can be
582 produced, they are much more laborious and expensive than the protocol described
583 here. Therefore, our technique would appear to offer several advantages over these
584 aforementioned studies, in that the methodology is simple and efficient, cultures can
585 be produced from older animals (routinely 3 - 6 day old mice, but numerous cultures
586 have been produced up to day 14 (unpublished data) and the protocol has relatively
587 lower costs of production (given the use of non-proprietary supplements).
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590 ## 5. Conclusions

591 In summary, we present here a simplified, economical and reliable method for
592 consistent production of primary hippocampal cultures from postnatal mouse
593 hippocampal tissue. Using this technique, we are able to reliably and reproducibly
594 produce hippocampal neuronal cultures of a consistently high standard.
595 Furthermore, the neurons within these cultures, in addition to displaying normal
596 characteristics of healthy neurons, also exhibit consistent and reproducible
597 responses to physiological stimuli. We propose that this method could also be
598 utilised to produce cultures of other postnatal mouse neuronal tissues.

599

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706

707 **Highlights**

708

709 1. Efficient and cost-effective method for postnatal mouse hippocampal culture

710 2. Novel trituration technique, simplified methodology and non-proprietary
711 reagents

712 3. Normal neuronal morphology and appropriate responses to physiological
713 stimuli

714 4. Neurons remain functional in an incubator for over one month

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