Video Article

Sex Differences in Mouse Hippocampal Astrocytes after *In-Vitro* Ischemia

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URL: https://www.jove.com/video/53695
DOI: doi:10.3791/53695

Keywords: Neuroscience, Issue 116, Hypoxia, ischemia, hippocampus, astrocyte culture, GFAP, OGD-REOX, neonate

Date Published: 10/25/2016

Citation: Chanana, V., Tumturk, A., Kintner, D., Udho, E., Ferrazzano, P., Cengiz, P. Sex Differences in Mouse Hippocampal Astrocytes after *In-Vitro* Ischemia. *J. Vis. Exp.* (116), e53695, doi:10.3791/53695 (2016).

Abstract

Astrogliosis following hypoxia/ischemia (HI)-related brain injury plays a role in increased morbidity and mortality in neonates. Recent clinical studies indicate that the severity of brain injury appear to be sex dependent, and that the male neonates are more susceptible to the effects of HI-related brain injury, resulting in more severe neurological outcomes as compared to females with comparable brain injuries. The development of reliable methods to isolate and maintain highly enriched populations of sexed hippocampal astrocytes is essential to understand the cellular basis of sex differences in the pathological consequences of neonatal HI. In this study, we describe a method for creating sex specific hippocampal astrocyte cultures that are subjected to a model of *in-vitro* ischemia, oxygen-glucose deprivation, followed by reoxygenation. Subsequent reactive astrogliosis was examined by immunostaining for the Glial Fibrillary Acidic Protein (GFAP) and S100B. This method provides a useful tool to study the role of male and female hippocampal astrocytes following neonatal HI, separately.

Video Link

The video component of this article can be found at https://www.jove.com/video/53695/

Introduction

Astrocytes are one of the most important key players in the central nervous system (CNS). Growing body of evidence indicates that the roles of astrocytes are more than providing neuronal support. In fact, the roles of astrocytes under physiological conditions can be very complex, such as guiding the migration of the developing axons[1], regulating CNS blood flow[2], maintaining the pH homeostasis of the synaptic interstitial fluid[3], and participating in the blood brain barrier[4] and synaptic transmission[5]. Under pathological conditions, astrocytes respond to injury with a process called reactive astrogliosis in which the morphology, number, location, topography (with respect to distance from insult) and function of the astrocytes may change in a heterogeneous way[6,7]. Astrogliosis seen following neonatal hypoxic ischemic encephalopathy maybe contributing to the morbidity and mortality of neonates[8].

Recent clinical and experimental studies indicate that the severity of brain injury appears to be sex-dependent and that the male neonates are more susceptible to the effects of hypoxia/ischemia (HI)-related brain injury, resulting in more severe neurological outcomes as compared to females with comparable brain injuries[9-11]. Although the localization of the injury depends on the gestational age and the duration and the severity of the insult, hippocampus is one of the most commonly effected regions in the CNS after term neonatal HI, and increased hippocampal astrogliosis has been confirmed by up-regulation of the Glial Fibrillary Acidic Protein (GFAP) 3 d after the neonatal HI[7,10,12,13]. Sex differences in astrocyte function were shown in both neonates and adult rodents after cerebral ischemia[14,15]. In addition, male astrocytic susceptibility to *in-vitro* ischemia was shown by increased cell death compared to female cortical astrocytes in culture[16].

Sex differences start *in-utero* and continue until death[17]. Over the last decade, the importance of including the sexes in experimental conditions in cell culture and *in-vivo* studies have been the emphasis of the Institute of Medicine and NIH to seek fundamental knowledge in the sex differences seen in physiological and pathological conditions[17,18]. Development of reliable methods to isolate and maintain populations of sexed hippocampal astrocytes is essential to understand the cellular basis of sex differences in the pathological consequences of neonatal HI. The present study was designed to provide the techniques to prepare enriched sex-specific hippocampal astrocyte cultures from newborn mice in order to assess the roles of GFAP-immunoreactive astrocytes following Oxygen/Glucose Deprivation (OGD) and reoxygenation (REOX), inducing HI in cell culture environment. This technique can be used to test any hypothesis pertaining to hippocampal astrocytes in neonatal males and females under normoxic and ischemic conditions.

Protocol

NOTE: This study was conducted in accordance with the recommendation of the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The animal protocol was approved by the University of Wisconsin-Madison, Institutional Animal Care and Use Committee.

Primary Astrocyte Culture protocol that is presented here is adopted from the protocols presented by Zhang Y[19] *et al.* and Cengiz P[20] *et al.* with some modifications.

1. Hippocampal Dissection and Astrocyte Culture

1. Prepare all necessary reagents and materials including surgical scissors, smooth fine forceps, flat tip forceps, paper towels, waste bag, 70% ethanol and 2 dissecting dishes (3.5 cm diameter) on ice filled with 2 mL Hank's Balanced Salt Solution (HBSS) each. Make sure the surgical equipment's are sterile (autoclave) prior to procedure and use all other materials (Astrocyte plating medium: DMEM + 5% horse serum + 1% penicillin-streptomycin, horse serum, HBSS, 0.25% trypsin, glass coverslips, Petri dishes, 15/50 mL tubes, T25 flasks, *etc.*) as pre-sterile.
2. Gently hold and spray the head and neck of mouse pup with 70% ethanol and decapitate using sharp sterile scissors [**Figure 1 (1)**].
3. Perform a midline incision from posterior to anterior skull with small scissors along the scalp to expose the brain [**Figure 1 (2-3)**].
4. Cut the cranium carefully from the neck to the nose with small scissors. Then cut cranium anterior to the olfactory bulbs and inferior to the cerebellum to disconnect the cranium from the skull base.
5. Make a small incision at the base of the skull and cut along the midline. Use sterile flat-tip forceps to peel away the skull. Remove the brain stem with the help of a curved forceps. Remove brain from the cranium and place into the first dissecting dish [**Figure 1 (4-9)**][21].
6. Using a curved forceps, flip the brain so that the ventral surface of the brain is facing up and then separate both hemispheres with a sharp sterile surgical blade [**Figure 1 (10,11)**]
7. Peel back the cerebral hemispheres and carefully remove the bulging midbrain and thalamic tissue with a sterile flat curved forceps to reveal the hippocampus, a small, seahorse-shaped structure in the medial temporal lobe [**Figure 1 (12-14)**].
8. Remove both the hippocampal lobes [**Figure 1 (15,16)**] and carefully dissect the meninges from the lobes by pulling with the fine forceps. This step avoids contamination of the final astrocyte culture by meningeal cells and fibroblasts.
9. Transfer the prepared hippocampal lobes into the second dish filled with HBSS and return it onto ice [**Figure 1 (17,18)**]. Pool both hippocampal lobes from a single mouse (P0 - P2) for preparation of hippocampal astrocyte cultures. This gives the astrocyte proper density. Mince hippocampal lobes using a sharp sterile surgical blade (approximately 4 times).
10. Aspirate HBSS from dish using a sterile tip attached to a 1 mL pipette and add 3 mL of 0.25% trypsin, mix, transfer to a 15 mL sterile conical tube and incubate the tissue at 37 °C for 20 min with gentle shaking.
11. Centrifuge tube at 300 x g for 5 min. Aspirate supernatant carefully using a sterile glass pipette attached to a vacuum line. Add 10 mL astrocyte plating medium and triturate (20 - 30 times) using a fire polished glass pipette until tissue pieces homogenize.
12. Centrifuge tube at 300 x g for 5 min. Aspirate supernatant and add 2 mL of fresh prewarmed astrocyte plating media. Pass cells through a 70 µm mesh filter (cell strainer) into a new 50 mL conical tube.
13. Plate entire cell suspension on a Poly-D-Lysine/Laminin coated sterile T25 culture flask containing 3 mL of astrocyte plating media, then incubate the flask at 37 °C in a 5% CO_2 incubator.
14. Aspirate entire media at day-*in-vitro* (DIV) 1, DIV 3 and DIV 7, and replace with 2 mL of fresh astrocyte plating media. Observe the progression and astrocytic confluence under light microscope each time while replacing the astrocyte plating media.
    NOTE: At DIV 1, all the viable astrocytes are attached to the surface of the culture flask and the dead or dying cells that include neurons are floating in the supernatant. At DIV 3, the attached cells start to divide to form astrocytic cell layer. In the absence of the supportive conditions, culture is almost devoid of any neuronal growth. At DIV 7, astrocyte layer is about 80 - 90% confluent and a few microglia as well as oligodendrocyte precursor cells are present on top of the astrocytic layer.
15. Aspirate the plating medium from the flask, add 2 mL of fresh prewarmed astrocyte plating medium, rinse and remove again at DIV 11, when astrocytes are confluent and ready for sub culturing.
16. Add 2 mL of 0.25% trypsin, gently rotate the flask a few times and aspirate trypsin using a sterile glass pipet.
17. Add 2 mL of 0.25% trypsin and let the flask sit in the tissue culture hood for 4 - 5 min at RT.
18. Remove the trypsin and keep the flask at 37 °C in a 5% CO_2 incubator for 10 min. Add 5 mL prewarmed astrocyte plating medium and detach the astrocytic layer by tapping the flask against the palm of your hand (3 - 4 times) followed by gentle trituration to achieve complete detachment and collect the astrocytes in a 15 mL conical tube.
19. Centrifuge tube at 300 x g for 5 min, aspirate the supernatant, and add 1 mL fresh astrocyte plating medium.
20. Add 10 µL of the cell suspension to a hemocytometer and count the cells in the large central gridded area (1 mm^2) using an inverted phase contrast microscope (10X objective). Multiply by 10^4 to estimate the number of cells per mL. One T25 flask will yield ~1 x 10^6 total dissociated cells. Seed ~1 x 10^5 cells in 2 mL astrocyte plating medium on a 12 mm diameter Poly-D-Lysine precoated glass coverslip in the well of a 24-well culture dish.
21. Incubate at 37 °C in a 5% CO_2 incubator. Perform OGD/REOX at DIV 12 - 14 (section 2).
22. Treat the astrocyte cultures at DIV 3 with 5 mM L-leucine Methyl Ester (LME) hydrochloride until DIV 11 if hippocampal cultures devoid of microglia are desired. After LME incubation, subject cultures to shaking (300 rpm for 1 h) to remove contaminating microglia.
    NOTE: LME is a microglial cytotoxic agent that has been used extensively as a method to eliminate proliferating microglia[22].

2. OGD/REOX Treatment

1. Aspirate media from coverslip containing adherent astrocyte culture (DIV 12 - 14) and rinse 3 times by gently rotating the 24-well culture dish holding the coverslip a couple times with 1 mL isotonic OGD solution (pH 7.4) containing (in mM): 0 glucose, 21 $NaHCO_3$, 120 NaCl, 5.36 KCl, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 1.27 $CaCl_2$, and 0.81 $MgSO_4$.
2. Add 0.2 mL OGD solution to well to cover the coverslip and incubate for 2 h in a hypoxic incubator containing 94% N_2, 1% O_2, and 5% CO_2. Gently mix with an orbital shaker (50 rpm) for the first 30 min of hypoxia to facilitate gas exchange.
3. Incubate normoxic control cells for 2 h in 5% CO_2 and atmospheric air in a buffer identical to the OGD solution except for the addition of 5.5 mM glucose.
4. For REOX, aspirate OGD solution and add 2 mL of astrocyte plating medium. Incubate in 5% CO_2 and atmospheric air at 37°C for 5 h.

3. Immunocytochemical Staining

1. Aspirate the culture media using a sterile glass pipette attached to a vacuum line and quickly rinse coverslip once by adding 1mL of 0.1 M Tris Buffered Saline (TBS) (154 mM NaCl, 16 mM Trizma Base, 84 mM Tris-HCl, pH 7.4) to the well. Aspirate and add 2 mL of 4% paraformaldehyde (PFA) in 1x Phosphate Buffered Saline (PBS). Incubate for 15 min at RT.
2. Aspirate and add 1 mL of 0.1 M TBS, then place on a rocking shaker for 2 min. Repeat 3 times. Add 1 mL blocking solution (10% goat serum, 10 mg/mL BSA, 0.025% Triton X-100 in 0.1 M TBS) and incubate for 30 min at 37 °C on a rocking shaker.
3. Aspirate blocking solution and add primary mouse monoclonal anti-GFAP antibody (0.2 mL of a 1:500 dilution in blocking solution) and rabbit polyclonal anti-S100B (1:500) or rabbit polyclonal anti-HIF1α (1:200) for 60 min at 37 °C on a rocking shaker.
4. Alternately, some astrocytes are incubated with rabbit polyclonal anti-IBA1 (1:200) and mouse monoclonal anti-MAP2 to access culture purity.
5. Aspirate primary antibody and rinse coverslip by adding 1 mL of 0.1 M TBS and placing on a rocking shaker for 2 min and then aspirate. Repeat twice.
6. Add 0.2 mL of a 1:200 dilution of the secondary goat anti-rabbit 488-conjugated and anti-mouse 568-conjugated antibodies in blocking solution for 60 min at 37 °C on a rocking shaker.
7. Aspirate secondary antibody and rinse coverslip by adding 1 mL of 0.1 M TBS and place on a rocking shaker for 2 min and then aspirate. Repeat twice.
8. Remove coverslip from well and dry by placing on a slide positioned on a slide-drier. Mount coverslip on a new slide by inverting on a single drop of VECTASHIELD hardset mounting medium with DAPI (1.5 ng/µL).
9. Image coverslips on a confocal microscope using either 20X dry or 60X oil objective. Acquire images (512 x 512) for DAPI (405 nm ex / 450 nm em) and fluorochrome 488 tagged GFAP antibody (488 nm ex / 515 nm em). Keep acquisition parameters constant within the 20X and 60X groups.

4. Sex Determination Using PCR

1. Heat pup toe or finger clippings at 95 °C for 45 min in 50 mM NaOH and neutralize with equal volume of 1 M Tris, pH 6.8.
2. Add 1 µL of the extracted DNA solution to 19 µL of the following mixture: 5 pmoles of primers for the Myog and Sry genes, 1x reaction buffer, 0.2 mM each deoxynucleotide and 8 U Taq polymerase.
3. Use primers sequences; Sry 5'TCATGAGACTGCCAACCACAG3', 5'CATGACCACCACCACCACCAA3' and Myog 5'TTACGTCCATCGTGGACAGC3', 5'TGGGCTGGGTGTTAGTCTTA3'[23].
4. Perform the following PCR protocol: 95 °C for 3 min then 30 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and elongation at 72 °C for 1 min. Following this 30 cycles, the reaction concludes with a final 72 °C elongation for 1 min.
5. Separate PCR products electrophoretically on an ethidium bromide-containing 2% agarose gel and visualize under UV illumination. (**Figure 2A**.) **CAUTION!** Ethidium bromide is a potential carcinogen and must be handled carefully and disposed of properly as per institution's regulations.

Representative Results

Understanding the roles of sexed astrocyte functions under physiologic or pathophysiological conditions have been immensely elucidated by culturing these cells under *in vitro* conditions. The important aspect of performing sexed culturing is to determine the sex of the mouse pup prior to its use. We determined the sex of the mouse genetically by PCR and by visual assessment (**Figure 2**)[16]. The methodology of sex determination using PCR was adopted with modifications from McClive and Sinclair, is quick, simple, and highly reproducible method[23]. **Figure 2A** shows the representative results of sex determination using PCR. PCR products were generated with DNA extracted from two representative P1 male and female tail snips. The reaction includes multiplexed primer pairs for *Sry* and *Myog* genes that generate male specific bands of 441 bp and female specific bands of 245 bp, respectively. Visual determination of neonatal (P 1 - 3) mouse sex with naked eye was established by looking for the presence of small darkened spot between anogenital openings only in males (**Figure 2B**)[16,24].

Although, culturing of astrocytes is convenient and relatively easy to establish under laboratory set up, this advantage can be hindered by its contamination primarily with microglia or neurons to a lesser extent. Contamination of the astrocytes can be easily determined by immunolabeling of the cultured cells with cell specific markers for either microglia (IBA1) or neurons (MAP2). In the present study, hippocampal astrocytes growing in primary monolayer cultures were observed to have ~6% of contaminating microglia at DIV 14, as suggested by a few cells that expressed IBA1 (**Figure 3**). On the contrary, none of the cells were found to express MAP2 suggesting the culture was totally devoid of any neuronal contamination (**Figure 3**). The minor microglial contamination observed in our cultures is in accordance with other reports, wherein the authors reported a 5% microglial contamination in their primary cortical astrocyte cultures derived from 1 - 3-day-old mouse pups[25]. Astrocytes possess a specific cytoarchitecture that allow them to respond to changes in their microenvironment including hypoxia. In order to determine any sex specific astrocytic response to *in-vitro* ischemia, we subjected sexed hippocampal astrocytes to OGD/REOX. Successful induction of OGD/REOX was determined by the increased nuclear expression of hypoxia inducible factor 1 alpha (HIF1α observed in $GFAP^+$ astrocytes subjected to 2 h OGD followed by 5 h REOX as compared to the HIF1α expressions in cells under normoxic conditions (**Figure 4**).

Immunocytochemical staining of sexed hippocampal cultured astrocytes with GFAP or S100B (mature astrocyte marker) resulted in a highly selective and reproducible staining pattern (**Figure 5**). GFAP and S100B were colocalized in the cytoplasm and cell bodies of astrocytes, characterizing a matured astrocytic developmental stage where these cells tend to lose their Neural Stem Cell (NSC) potential [26]. The morphology and density of GFAP or S100B immunoreactivities were similar in both male and female astrocyte cultures as observed in their respective normoxic and OGD/REOX treatment groups. Under normoxic conditions, hippocampal astrocytes presented a polygonal to fusiform and flat morphology [**Figure 5A, B** (normoxia 60X; arrow)], as evaluated using confocal microscopy. These cells were able to divide until confluent for approximately 2 weeks prior to the induction of OGD/REOX. After 2 h OGD and 5 h REOX, astrocytes exhibited classical reactive astrocyte morphology displaying retracted primary processes, hypertrophy of the soma and processes, and increased expression of GFAP or S100B [**Figure 5A, B** (OGD/REOX, 60X; arrowhead)]. Following the OGD/REOX, the GFAP/S100B staining also exhibited an extensive meshwork extending across the cytoplasm in both the male and female hippocampal astrocytes. Lack of GFAP staining observed in the cultured hippocampal astrocytes from GFAP null mice served as a negative control (**Figure 5B**, inset). The above results provide direct evidence that the morphology of GFAP/S100B-immunoreactive astrocytes undergoes significant changes when subjected to OGD-REOX.

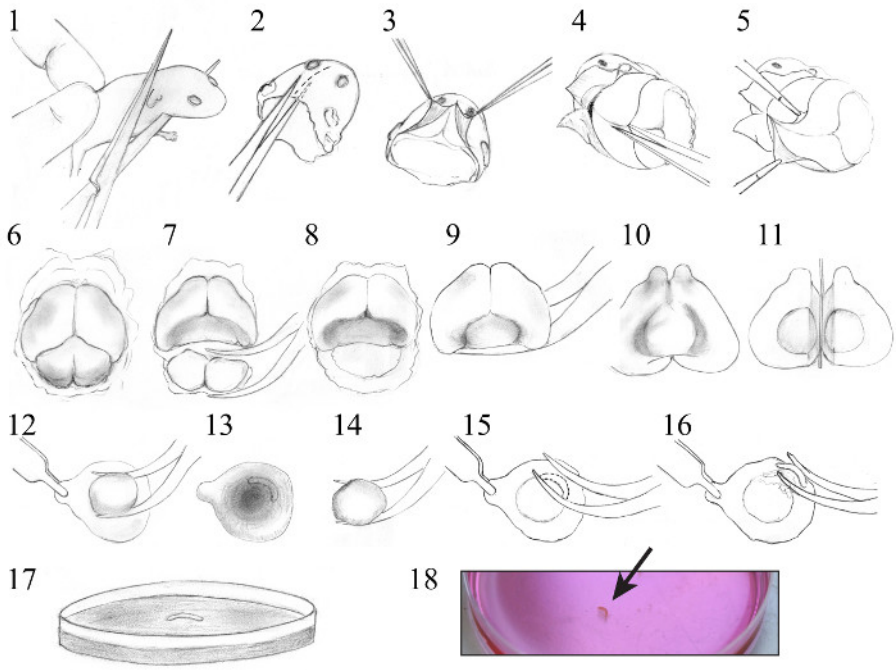

Figure 1. Illustrations of the Hippocampal Removal Technique from P1 Mice. 1. Decapitate pup to remove the head. **2, 3.** Using fine scissors, make a midline incision of the skin, posterior to anterior, and peel back the skin with the help of a flat-tip forceps. **4, 5, 6**. Make a small incision at the base of the skull. Cut the skull along the midline and peel away two halves to expose the brain. **7, 8**. Remove cerebellum with the help of a curved forceps. **9**. Carefully remove brain from the cranium and place into a sterile Petri dish. **10, 11**. Flip the brain so that the ventral surface of the brain is facing up using a curved forceps, then separate both hemispheres with a sharp sterile surgical blade. **12, 13, 14**. Remove the bulging midbrain and thalamic tissue leaving the intact underlying hemisphere and revealing the hippocampus. **15, 16**. Remove the hippocampus (small C-shaped structure). **17, 18.** Immediately place the hippocampal lobes obtained from both of the hemispheres in a separate Petri dish containing sterile HBSS. Please click here to view a larger version of this figure.

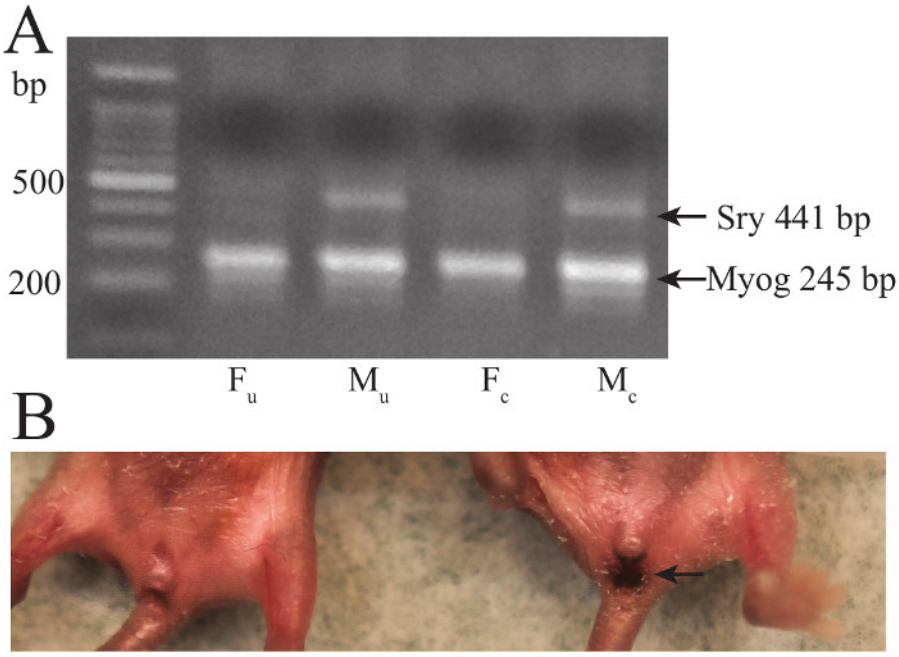

Figure 2. Sex Determination of Neonatal Mice. A. Mouse sex was genetically determined by PCR with primers specific to the Myog (X chromosome) and Sry (Y chromosome) genes using DNA extracted from finger or toe clippings (F_1; M_1). The bands of PCR were visualized on a 2% agarose gel with Ethidium Bromide under UV illumination. F_2 and M_2 served as positive controls for female and male, respectively. **B**. Visual determination of male from female was established by identifying a darkened spot between the anogenital openings (arrow). Please click here to view a larger version of this figure.

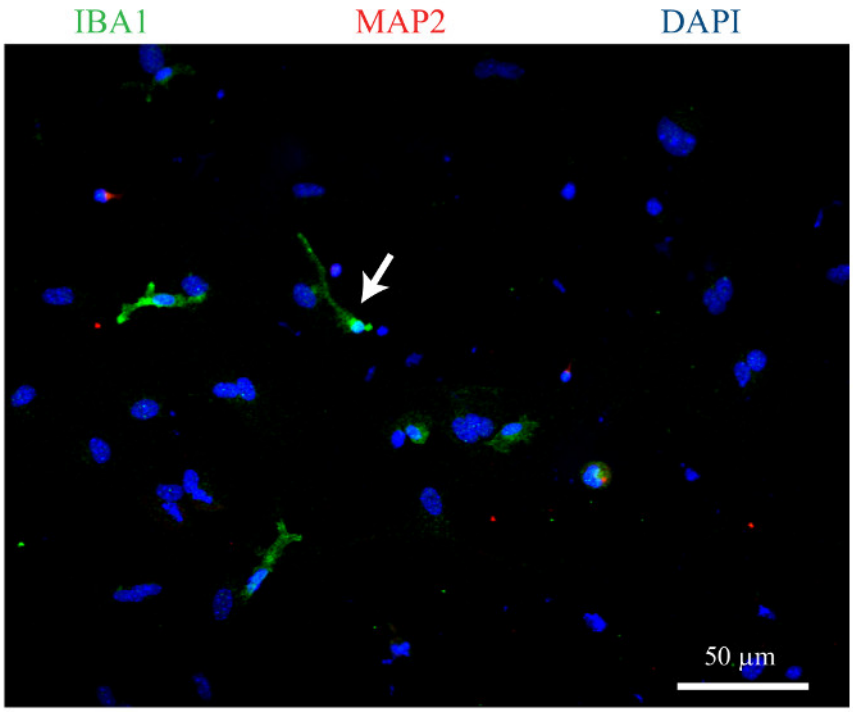

Figure 3. Purity of the Primary Mouse Hippocampal Astrocyte Culture. Immunostaining of astrocyte culture with neuronal marker MAP2 (red) and microglia marker IBA1 (green). Nuclei are stained with 4',6'-diamidino-2-phenylindole (DAPI) (blue). Arrow indicates microglia contamination. Scale bar: 50 µm. Please click here to view a larger version of this figure.

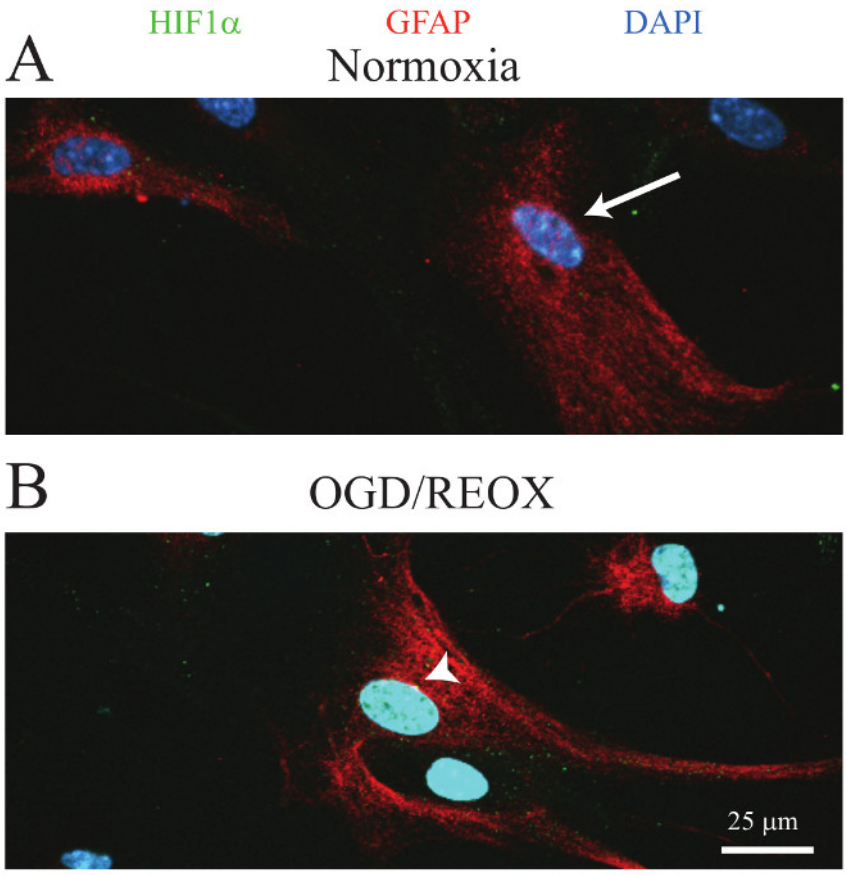

Figure 4. HIF1α Expression Was Increased in Cultured Hippocampal Astrocytes Exposed to OGD/REOX. Representative immunofluorescent images showing GFAP (red) and HIF1α (green) labeling in astrocytes subjected to (**A**) normoxia or (**B**) OGD (2 h)/REOX (5 h). Nuclei are stained with 4',6'-diamidino-2-phenylindole (DAPI) (blue). Arrow, low HIF1α expression; arrowhead, elevated HIF1α nuclear expression. Scale bar: 25 μm. Please click here to view a larger version of this figure.

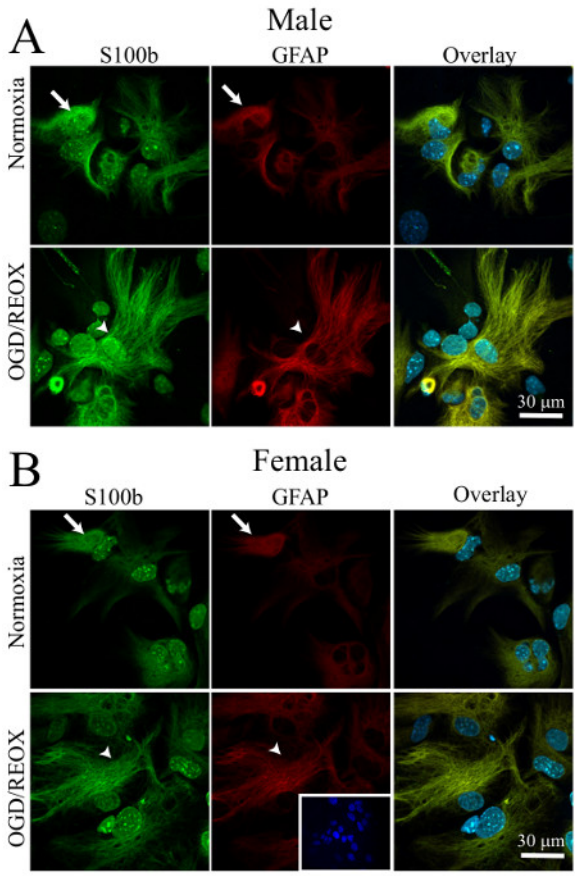

Figure 5. Up-regulation of S100B or GFAP Expression in Sexed Hippocampal Astrocyte Cultures following OGD-REOX. Sexed cultured hippocampal astrocytes were stained for S110b or GFAP expression under normoxic conditions or after 2 h OGD followed by 5 h of REOX (OGD/REOX). Inset: 60X image from hippocampal astrocytes cultured from GFAP null mice. Scale bar: 30 µm.

Discussion

In order to study the sex differences in the properties and function of astrocytes under physiological and pathological conditions, preparation of sexed primary astrocytes in cell culture is an important tool to utilize. In the present study we report a highly efficient and a reproducible method to culture a highly enriched homogeneous population of sexed hippocampal astrocytes from newborn (P0-P2) C57Bl/6 (wild type) or K19F (GFAP null) mouse pups *in-vitro*. Establishing this methodology helps the investigators understand the physiological and pathological functions of sexed hippocampal astrocytes after *in-vitro* ischemia.

There are two critical steps in establishing this methodology including the maintenance of sterility and adequate digestion of hippocampal lobes during trypsinization followed by trituration of the digested tissue in order to achieve the single cell suspension. Prevention of primary astrocytes from getting contaminated is one of the biggest challenges throughout the culture process. Bacterial and/or fungal are two common modes of contamination that can render the cultured cells useless if proper care is not adopted while performing the technique. Hence, in order to ensure experimental success, it is imperative to perform the process under a sterile work setting that includes, disinfecting the work place prior to use, using sterile surgical equipment and materials, avoid contact of sterile materials with nonsterile surfaces, using a laminar flow hood and avoid taking uncapped culture flasks out of the laminar hood. In addition, during enzymatic dissociation, longer incubation of hippocampal tissue with trypsin followed by extensive physical trituration can lead to its over-digestion resulting in poor cell viability and inefficient attachment of dissociated cells to culture flasks.

On the contrary, underdigestion of the brain tissue provides insufficient dissociation of glia cells thereby resulting in poor yield and seeding of cells as large clumps. Therefore, in order to obtain healthy astrocyte cultures, it is necessary to optimize trypsin concentration, incubation time and trituration to achieve optimum digestion of the hippocampal tissue. In our hands, incubation of hippocampal lobes with the working concentration of 0.25% trypsin for 20 min at RT followed by gentle trituration for 20 - 30 times yielded strong adherent, healthy and highly reproducible astrocyte cultures. Also, repeated freezing and thawing of reagents prior to use every time may reduce their effectiveness. Therefore, it is very important to aliquot reagents in small desirable quantities and freeze. For example, store trypsin, penicillin/streptomycin as 5 mL aliquots and fetal bovine serum as 50 mL aliquots in numerous sterile conical tubes at -20 °C until use. We advise not to use antibiotics beyond the expiration date.

GFAP is a well-characterized marker of reactive fibrous astrocytes. Following OGD/REOX, upregulation of GFAP expression in response to reactive astrogliosis has been reported[20]. Although, it has been proposed that GFAP overexpression could be used as specific target for neuronal repair strategies[27] its role in hippocampal damage after HI in neonates is still unclear. Immunohistochemical staining of astrocytes

with GFAP enables us to identify and characterize these cells under the physiological and pathological conditions. GFAP staining like any other marker has some limitations that need to be recognized. One of the limitations of the GFAP staining of astrocytes is that not all the astrocytes express GFAP under physiological conditions. Especially the immature astrocyte expression rate of GFAP does not exceed 40% under physiological conditions[28]. Depending on the age- and type-specific use of astrocytes, other markers of choice can be considered such as GLAST, ALDH1L1, BLBP, Aquaporin-4 and S100B[29].

Moreover, presence of NSCs disguised as astrocytes in the primary cultures cannot be ruled out. These NSCs have been shown to present phenotypical and ultrastructural characteristics similar to mature astrocytes including the expression of GFAP. Therefore, identification of molecular markers that are specific for fully differentiated and matured astrocytes is a must. Astrocytes were reported to expressed S100B at their later fully differentiated developmental stages. Recently, it has been reported that neonatal cortical $GFAP^+$ astrocytes lose their progenitor stem cell potential with the onset of S100B expression that happen around DIV 15 [26]. Similarly, in our culture conditions at DIV 14, 91% of GFAP expressing cells were found to coexpress S100B, which suggest that the cultures consist of primarily terminally differentiated matured hippocampal astrocytes.

It is almost impossible to obtain pure primary hippocampal astrocyte cultures totally devoid of contaminating microglial cells that reside above and below the astrocyte monolayer. Therefore, it is important to know the proportion of contaminating microglia in astroglial cultures and also to keep this proportion as low as possible. In the present study we stained astrocyte cultures with microglia specific marker IBA1 or neuronal marker MAP2 in order to determine the presence of any microglia or neurons as potential contaminants. The proportion of microglia in rodent astrocyte culture may vary from 1% to 30% depending upon several factors that include animal age, species, region, culture medium, subculturing method and shaking[30]. In our cultures we observed approximately 6% of microglial contamination which is comparable with other reports[25]. If the intended use of the astrocyte culture is to study the role of hippocampal astrocytes in inflammation it is imperative to treat the cultures with 5 mM LME for 10 d to eliminate possible microglial contamination in the astrocyte cultures starting from DIV 3. LME is a microglial cytotoxic agent that has been used extensively as a method to remove proliferating microglia[20,22]. In addition, LME-treated cultures should be subjected to a shaking protocol (300 rpm for 1 h).

Another technical challenge in performing OGD/REOX is to determine if a sufficient degree of hypoxia has been achieved to induce astrogliosis. Markers for hypoxia in astrocytes include the up-regulation of GFAP and the induction of HIF-1α. Thus, in this study hypoxia was confirmed by the increase in GFAP immunoreactivity (data not shown) and the upregulation of HIF1-α staining (**Figure 4**).

In summary, the results obtained from sexed neonatal hippocampal astrocyte cultures showed successful cell attachment with healthy homogeneous astrocyte layer displaying typical morphology on coverslips and the expression of the major reactive astrocytic markers GFAP and S100B upon induction of *in-vitro* ischemia. We believe that the protocol adopted here has the potential to answer important mechanistic and translational questions pertaining to the role of astrocytes in developing male and female brains. The entire culturing procedure as opposed to the majority of existing techniques generates mature and confluent sexed hippocampal astrocytes in two weeks, granting a rapid turnaround of experiments and the simplicity of the technique will facilitate the use of astrocytic culturing method as a tool to study the sex specific role of glia cells in developing brain thereby allowing its wide dissemination in brain research.

Disclosures

None of the authors have competing or conflicting interests.

Acknowledgements

Clinical and Translational Science Award program of NCATS UL1 TR0000427 and KL2 TR000428 (Cengiz P), UL1TR000427 to the UW ICTR from NIH/NCATS and funds from Waisman Center (Cengiz P), K08 NS088563-01A1 from NINDS (Cengiz P) and NIH P30 HD03352 (Waisman Center), NIH/NINDS 1K08NS078113 (Ferrazzano P). We would like to thank Albee Messing, PhD, for providing us the GFAP knockout mice.

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