ARL-TR-8989 ● JULY 2020

DEVCOM ARMY RESEARCH LABORATORY

Co-Cultures of Neurons and Astrocytes: Alterations to Morphology and Cell-Type Distribution

by Erika A Matheis, Amy J Wegener, Meaghan Richardson, and Ann Mae DiLeonardi

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED (From - To)
July 2020	Technical Report	November 2017–August 2019

4. TITLE AND SUBTITLE
Co-Cultures of Neurons and Astrocytes: Alterations to Morphology and Cell-Type Distribution

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Erika A Matheis, Amy J Wegener, Meaghan Richardson, and Ann Mae DiLeonardi

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
CCDC Army Research Laboratory
ATTN: FCDD-RLW-PB
Aberdeen Proving Ground, MD 21005

8. PERFORMING ORGANIZATION REPORT NUMBER
ARL-TR-8989

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES
ORCID ID(s): Ann Mae DiLeonardi, 0000-0002-1476-7726; Erika A Matheis, 0000-0002-5122-1503

14. ABSTRACT

The brain is possibly the most complicated organ in the body, making understanding its function in normal or pathological states difficult. In vitro models using brain cells reduce some complexity and offer a high degree of experimental control compared with in vivo experiments. Many in vitro models include only neurons in culture, but neurons are surrounded by glial cells in the brain. Including other cell types in co-culture can dramatically change the response of those cells and is more representative of in vivo conditions but increases culture complexity. The astrocyte-to-neuron ratio can be manipulated via different culturing techniques but is not well characterized. Primary pyramidal neurons from rat hippocampi were cultured to obtain three configurations: confluent astrocyte, sporadic astrocyte, and astrocyte free. Qualitatively, neurons appeared healthier in astrocyte-containing cultures, as indicated by thicker neurites and no evidence of free-floating debris or neurite beading, compared with astrocyte free. Each condition was characterized via astrocyte-to-neuron ratio, neurite length, and branching analysis with significant differences among all groups. The confluent-astrocyte group had the largest astrocyte-to-neuron ratio and the lowest values for neurite length and branching. This study characterized cell cultures under different media conditions to help inform future work.

15. SUBJECT TERMS
cell culture, media characterization, glia, astrocyte, primary neurons

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Ann Mae DiLeonardi
Unclassified	Unclassified	Unclassified	UU	23	19b. TELEPHONE NUMBER (Include area code) (410) 306-0689

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

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1. Introduction

The brain is possibly the most complicated organ in the body, making understanding its function in normal or pathological states difficult. In vitro models of brain cells provide a means to reduce the complexity of the full brain by observing how brain cells respond to various stimuli as well as offer a high degree of experimental control compared with in vivo experiments. Many in vitro models include only neurons in culture, but neurons are surrounded by glial cells in vivo, and in some areas of the brain, glial cells can outnumber neurons 10:1.[1,2] The concentration of glial cells is not uniform throughout the brain,[3] therefore building an in vitro model where the concentration of glial cells can be controlled is important.

There are four types of glial cells present in the central nervous system (CNS): oligodendrocytes (myelin sheath), microglia (immune response), ependymocytes (cerebrospinal fluid production), and astrocytes (homeostasis). Astrocytes are the most abundant type of glial cell and provide homeostasis to the neurons by regulating extracellular concentrations of ions and neurotransmitters available, among many other functions (reviewed by Araque and Navarrete[4]). Astrocytes offer support to neurons via release of trophic factors, important for neuronal growth and repair, and molecules to shape the extracellular matrix, the 3-D network that provides structural and biochemical support to the cells.[5] In culture, the quality and attachment of neurons improve when astrocytes are present compared with a glass coverslip alone.[6,7] Following mechanical trauma, astrocytes proliferate and serve as chemical "buffers" to potentially reduce the load on injured neurons.[8–11] Including other cell types in co-culture can dramatically change the response of neurons following mechanical trauma.[12]

Astrocytes can greatly affect the health of neurons; however, astrocytes can be cultured in various ways to support neurons and model the physiological environment. Astrocytes can be grown in culture as a distinct layer to offer support to neurons grown separately.[13–18] This method is particularly advantageous for low-density neuronal cultures.[17] The astrocyte layer is suspended over neuron cultures to release trophic substances into the medium that enhance the development of neuronal processes, accelerate the presence of electrophysiological activity, and improve neuron viability.[13–17] Distinct astrocyte and neuron layers are useful in culture to monitor responses of the neurons alone because the two layers are separate. Alternatively, astrocytes and neurons can be co-cultured to better model the physiological environment.[19] This is achieved by adding astrocytes directly to neuron cultures or by culturing the neurons in astrocyte-conditioned

medium. Astrocytes in co-culture promote greater synaptic density, increased electrophysiological response, and better neuron survival.[14]

Considering the importance of astrocytes in neuronal health and injury response, investigating the effects of a simplified neuronal co-culture with a single glial cell type (i.e., astrocytes) on the morphological development of pyramidal neurons is beneficial for selecting the most appropriate in vitro model for future neuronal injury studies. Using two methods of in vitro astrocyte inclusion is explored in this study to determine the effect of each configuration on neuronal health and provide recommendations for which configuration is most appropriate for specific applications.

2. Methods

2.1 Cell Culture

Two days prior to culturing neurons, 22- × 22-mm coverslips (Corning, catalog [cat.] no. 2845-22) were placed in a glass dish on gauze pads and covered with aluminum foil. The coverslips were sterilized in an autoclave at 138 °C for 30 min. Once the coverslips cooled, the dish was moved to the biosafety cabinet (BSC) and a single coverslip was placed in each well of sterile Costar 6 well plates (Corning, cat. no. 3736). Poly-L-lysine (PLL; Sigma, cat. no. P1524) was diluted to 0.01 mg/mL by adding 1 mL of stock PLL (1 mg/mL) to 99 mL of sterile water. Working PLL was filter-sterilized by passing the solution through a 0.22-µM filter, and 3 mL of PLL was added to each well, then left in the BSC with the blower off for 20–24 h. The following day, PLL was removed from the plates using vacuum suction and coverslips were washed with sterile water twice for 10 min. After the second wash, additional sterile water was put on the coverslips and plates were moved to the incubator at 37 °C and 5% CO_2 overnight until culturing the following day.

On the day of the cultures, about 20–24 h after the sterile water washes, the water was removed from the wells using vacuum suction and 1.5 mL of filter-sterilized feeding media (Neurobasal media, Gibco, cat. no. 21103049; 2% B-27, Invitrogen, cat. no. 17504044; and 0.4 mM GlutaMAX, Invitrogen, cat. no. 35050061) was placed in the wells. Plates were returned to the incubator.

2.2 Neuron Isolation and Dissociations

Embryonic day-18 (E18) rat hippocampi (cat. no. SDEHP) were purchased from BrainBits, LLC (Springfield, Illinois). Hippocampi arrived in BrainBits Hibernate

media and were stored at 4 °C for 24 h prior to culturing. When ready to culture, the hippocampi were incubated in 5 mL of feeding media containing trypsin (0.3 mg/mL, Sigma-Aldrich, cat. no. T9935-100mg) and DNase I (0.2 mg/mL, Sigma-Aldrich, cat. no. 10104159001) for 20 min in the incubator at 37 °C. Hippocampi were carefully removed and placed into a clean 15-mL conical tube using a glass Pasteur pipette, and the remaining feeding media with trypsin/DNase I was removed. Next, 3 mL of plating media (feeding media containing 5% heat-inactivated fetal bovine serum; Sigma-Aldrich, cat. no. F4135) was added to the hippocampi.

Hippocampi were dissociated by repeatedly pipetting them up and down in progressively smaller pipettes: 10-mL stereological pipette for 10 reps, then a regular Pasteur pipette for 10 reps and finished with a flame-polished Pasteur pipette (BrainBits cat. no. FPP). Cells were counted on a hemocytometer to determine density. Cells were then plated on the glass coverslips and returned to the incubator. Filter-sterilized, warmed feeding media was replaced 4 days after the initial plating, then 1–2 times per week. Cells were maintained at 37 °C, 5% CO_2 for up to 12 days in vitro (DIV). If a well became infected, it was cleared and excluded from the study.

2.3 Media Manipulation

Three different neurobasal media (NBM) configurations were chosen to provide a range of astrocyte-to-neuron ratio conditions for evaluation:

- Condition 1 (n = 5 wells): Astrocyte-free, NBM–B27–GlutaMAX (ThermoFisher Scientific, Grand Island, New York), where the anti-mitotic agent Cytosine β-D-arabinofuranoside (Ara-C, 1 μM; Sigma, cat. no. C1768) was added 24 h post-plating. Ara-C inhibits mitosis, resulting in little to no astrocyte being present in the culture.
- Condition 2 (n = 6 wells): Sporadic Astrocyte, NbActiv1 (BrainBits), produced neuronal cultures with sporadic astrocyte growth intermingled among neurons.
- Condition 3 (n = 6 wells): Confluent Astrocyte, co-culture media (BrainBits), produced cultures with a bed of confluent astrocytes where neurons grow on top of the confluent astrocytes.

2.4 Fixation and Immunocytochemistry

At DIV 12, cells were incubated with 4% paraformaldehyde for 15 min at room temperature, then washed with cold (4 °C) 1× phosphate-buffered saline (PBS)

three times. For immunocytochemistry, cells were permeabilized with 0.25% Triton X-100 for 10 min and blocked with 10% bovine serum albumin for 30 min. Cultures were incubated with the 1° antibodies (glial fibrillary acid protein [GFAP], a marker for glia and Beta-III Tubulin, a marker for neurites) for 1 h, followed by a 1-h incubation with the 2° antibodies. Cultures were then mounted to slides using Prolong Diamond with 4′,6-diamidino-2-phenylindole (DAPI), for visualizing the nuclei.

2.5 Imaging and Cell Quantification

Using a Nikon Eclipse Ti-U inverted microscope (Nikon Instruments Inc, Melville, New York), phase-contrast images (20×) were taken to capture images at DIV 12 and used to determine the ratio of neurons to astrocyte in each of the three conditions. Eight images were captured per well and each image needed a minimum of 10 cells to be included in the analysis (astrocyte free: n = 41 images; confluent astrocyte: n = 24 images; and sporadic astrocyte: n = 41 images). Additionally, for the sporadic and confluent conditions, a minimum of two of each cell type was required to be included in the analysis. Cells were manually counted by an experienced, condition-blinded scientist with 6 years of cell-culture microscopy experience. Cells with phase-bright cell bodies were counted as neurons, and cells with granulated cell bodies were counted as astrocytes. This was qualitatively confirmed by visualization with immunocytochemical markers. Following completion of immunocytochemistry, fluorescent images (20×) were acquired from the neurons fixed at DIV 12. Astrocytes were identified with a GFAP-specific antibody and visualized with a fluorescein-isothiocyanate or FITC filter (green). Neurites were identified with a Beta-III Tubulin-specific antibody visualized with a tetramethylrhodamine-isothiocyanate or TRITC filter (red). DAPI was used to stain nuclei of both cell types and visualized with the DAPI filter (blue).

2.6 Image Processing

Image processing was performed using the FIJI[20] plug-in, Simple Neurite Tracer,[21] to characterize neuronal features such as neuronal bodies, or somas, dendrites, and branching for additional analysis. The program identified and outlined the neuronal dendrites from the microscope images by their tube-like structures, demarcated by changes in brightness and contrast within the image (Fig. 1A). The program allowed the user to confirm the accuracy of the automatic tracing; if not accurate, pathways were manually edited. Once the initial dendrite pathways were captured originating from the soma, the secondary and tertiary branches were selected and categorized.

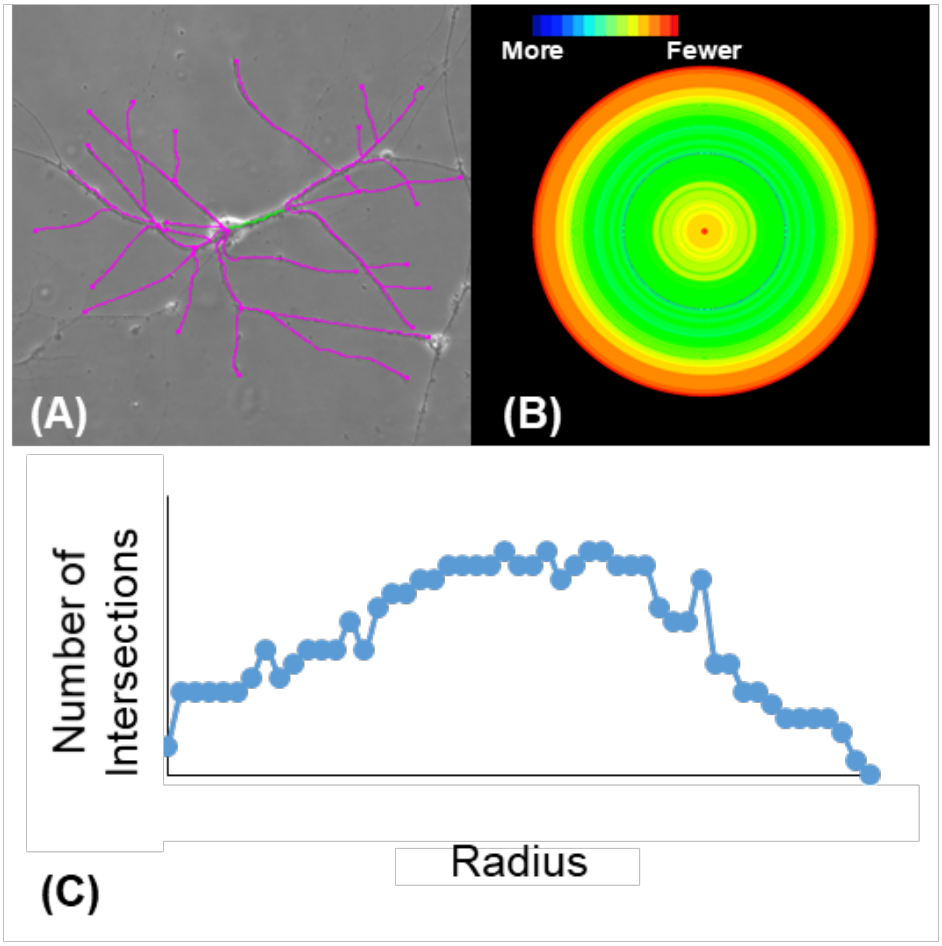

Fig. 1 Sholl analysis using FIGI plug-in Simple Neurite Tracer: A) single neuron was selected from each image and traced, path zero selected (green) and analysis performed; B) Sholl image, a density map of number of intersections as function of radius; and C) Sholl profile, the number of intersections as function of radius

After the somas, dendrites, and branches were identified, additional metrics to describe the neuronal morphology were performed. Neurite length was defined as the length of the longest branch traced from a soma in the initial Simple Neurite Tracer analysis. Branching of neurites was described using the Sholl analysis. The Sholl analysis measures branch intersections, span, and amount of branching through a method of concentric circles starting from the center of the soma and extending outward (Figs. 1A and 1B).[22–24] Within Simple Neurite Tracer, the image was converted to an 8-bit luminance format and the center of a single-cell soma was selected from each image. All branches were traced from this starting point using the path feature. The number of branches was plotted as a function of distance from the soma (Fig. 1C). The Simple Neurite Tracer function to fit a polynomial of any degree, up to 8, used a heuristic algorithm to guess a polynomial best-fit.

Other reported values from the Sholl analysis include maximum number of intersections at a concentric circle, maximum intersection radius (radius at which the maximum number of intersections occurs), critical value (the local maximum of intersections in the polynomial fit), and critical radius (distance at which the critical value occurs).

2.7 Data Analysis

Statistical analysis was performed using JMP 14 (SAS Institute Inc., Cary, North Carolina) for cell count, neurite length, and Sholl-analysis data. Averages and standard deviations were calculated for all measurements taken. A one-way analysis of variance (ANOVA) was performed between media conditions. If significance was found at a p-value of less than 0.05 (95% confidence interval) a Tukey HSD* post-hoc analysis was completed to determine the significance between groups.

3. Results

3.1 Morphology

In phase-contrast images, regardless of astrocyte condition, neurons had phase-bright, nongranulated somas (Fig. 2A's arrows) with neurites extending out from the soma. Neurites in astrocyte-free cultures were contiguous; however, they had a punctate appearance and were not smooth (Fig. 2A's arrowheads). Immunoreactivity for Beta-III Tubulin was observed, indicating the presence of neurites, while there was no evidence of GFAP immunoreactivity, demonstrating the lack of astrocytes in these cultures (Fig. 2B). In the confluent astrocyte condition, the astrocytes, confirmed by immunoreactivity for GFAP, cover the entire substrate with phase-bright neurons growing on top (Fig. 2C). The growth of neurons on top of the astrocytes was verified by positive staining for Beta-III tubulin (Fig. 2D). Neurites in this condition were contiguous and lacked the punctated appearance of neurons from the astrocyte-free condition. The final condition resulted in a sporadic distribution of neurons and astrocytes. Neurons can still be identified by their phase-bright somas with contiguous neurites (Fig. 2E's arrows), while somas of astrocytes are granulated and not phase bright (Fig. 2E's arrowheads). Immunoreactivity for GFAP- and Beta-III tubulin-labeled astrocytes and neurons, respectively (Fig. 2E), allowing for the appreciation of the different cell-type morphologies. For example, neurites appeared longer in comparison with

* Honestly Significant Difference

astrocytic branches. Additionally, neurites tapered at their terminals, whereas astrocyte processes branched.

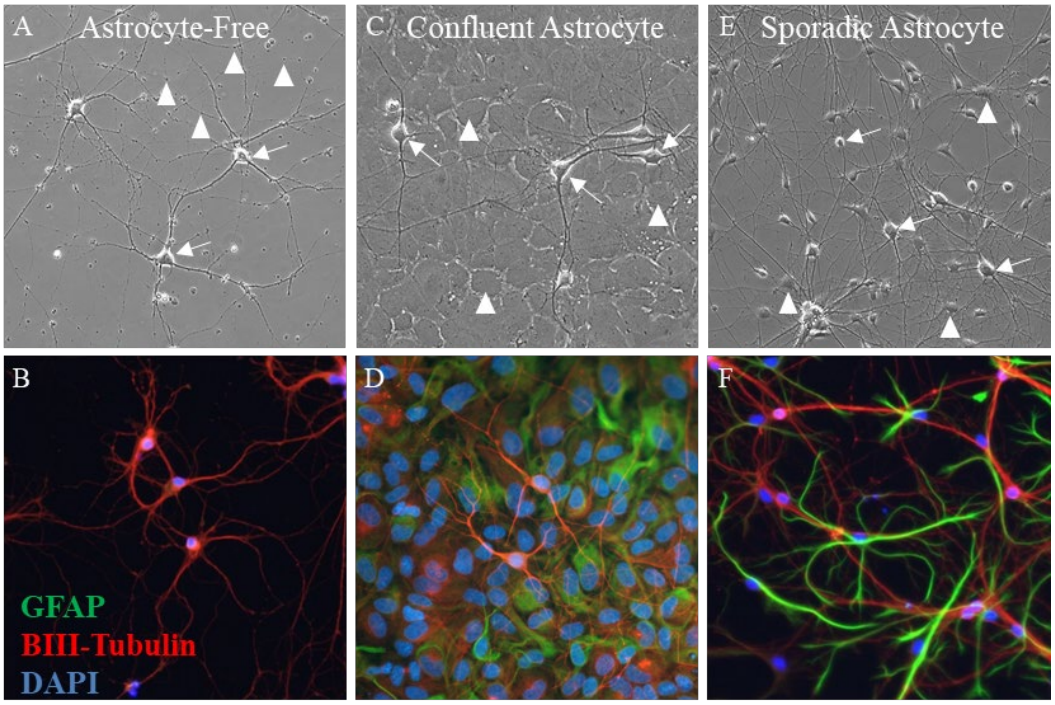

Fig. 2 Representative 20× images of three conditions: A) and B) astrocyte free, arrows are neuronal cell bodies, arrowheads are punctate; C) and D) confluent astrocyte, arrows are neuronal cell bodies, arrowheads are astrocytes; and E) and F) sporadic astrocyte, arrows are neuronal cell bodies, arrowheads are astrocytes.

3.2 Cell-Type Ratio

The numbers of neurons and astrocytes were manually counted to compare the relative amount of each cell type among the different conditions. As expected, the astrocyte-free condition had a ratio of astrocytes-to-neurons of 1:96, with 98.97% ± 3.94 neurons and 1.03% ± 3.94 astrocyte. In the confluent astrocyte condition, the astrocyte-to-neuron ratio was 10:1, with 8.7% ± 5.15 neurons and 91.30% ± 5.15 astrocyte population. The sporadic astrocyte condition had a 1:1 ratio, 45.85% ± 23.32% of neurons and 54.15% ± 23.32% of astrocytes. The average percent of neurons and astrocytes comprising the cultures was significantly different among all groups (Fig. 3; $p < 0.01$).

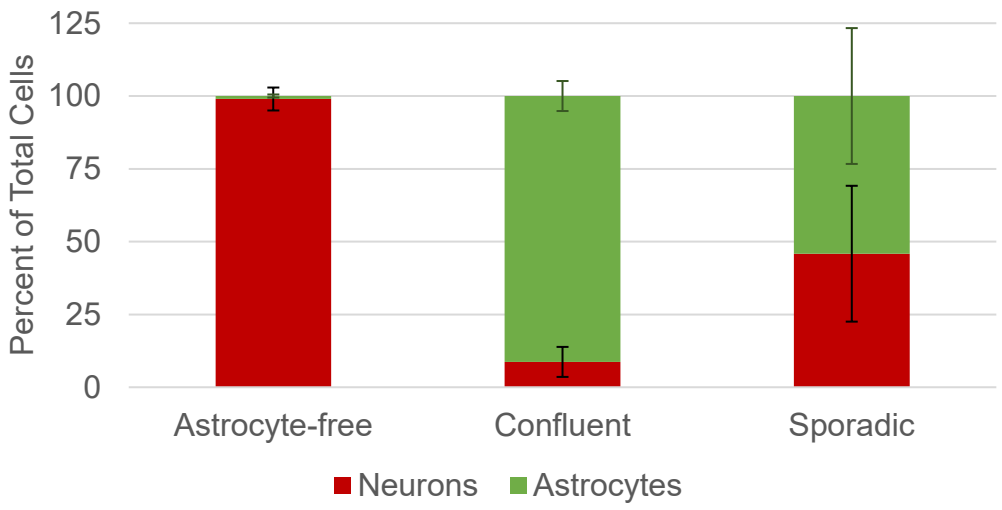

Fig. 3 All astrocyte conditions were significantly different from each other in astrocyte-to-neuron ratio ($p < 0.01$) where confluent astrocyte had the largest percentage of astrocytes; astrocyte free had the largest percentage of neurons and sporadic astrocyte was close to a 1:1 ratio of astrocyte-to-neuron.

3.3 Sholl Analysis

The average Sholl profiles for each astrocyte condition are graphed as the number of intersections plotted as a function of distance from the center of the cell body (Fig. 3). Qualitatively, the profiles show there is a decrease in intersections for the confluent astrocyte condition, suggesting there is less branching of neurites in this condition. The parameters selected for quantitative comparison included neurite length, mean number of intersections, maximum number of intersections, the maximum intersecting radius, the critical value, and the critical radius (Table 1).

Table 1 Quantitative measurements from neurite tracings for the different astrocyte conditions

Measurement	Astrocyte condition		
	Astrocyte free	Sporadic	Confluent
Neurite length (µm)	224.74 ± 78.15	202.26 ± 76.76	189.29 ± 80.23
Mean intersections (N)	8.94 ± 3.00	8.31 ± 2.94	5.18 ± 2.27[a]
Max intersections (N)	20.56 ± 6.34	20.67 ± 9.63	13.55 ± 6.39[a]
Max intersecting radius (µm)	68.47 ± 51.32	58.15 ± 42.07	53.17 ± 52.63
Critical value	12.56 ± 4.29	11.67 ± 4.19	7.56 ± 3.58[a]
Critical radius (µm)	65.32 ± 34.13	72.58 ± 45.64	52.75 ± 50.39

[a] $p < 0.01$

A one-way ANOVA, grouped by astrocyte condition, found there was a significant decrease in both mean ($p < 0.01$) and maximum ($p < 0.01$) number of intersections and critical value ($p < 0.01$) for the confluent astrocyte condition compared with

both the sporadic astrocyte and astrocyte-free conditions (Table 1), suggesting neurons grown on a confluent bed of astrocytes branch less than those cultured in the absence of or intermingled with astrocytes. For the full Sholl profiles, there was a significant decrease in intersections between 48 and 195 µm from the center of the cell body of the confluent astrocyte condition compared with the astrocyte free or sporadic astrocyte (Fig. 4). At points both more distal and more proximal to the cell body, there is no significant difference among the different conditions. This suggests the neurons in different astrocyte conditions differ mostly in the number of secondary and tertiary neurites rather than primary. As Table 1 shows, there was no significant difference among the three groups for neurite length, maximum intersecting radius, or critical radius.

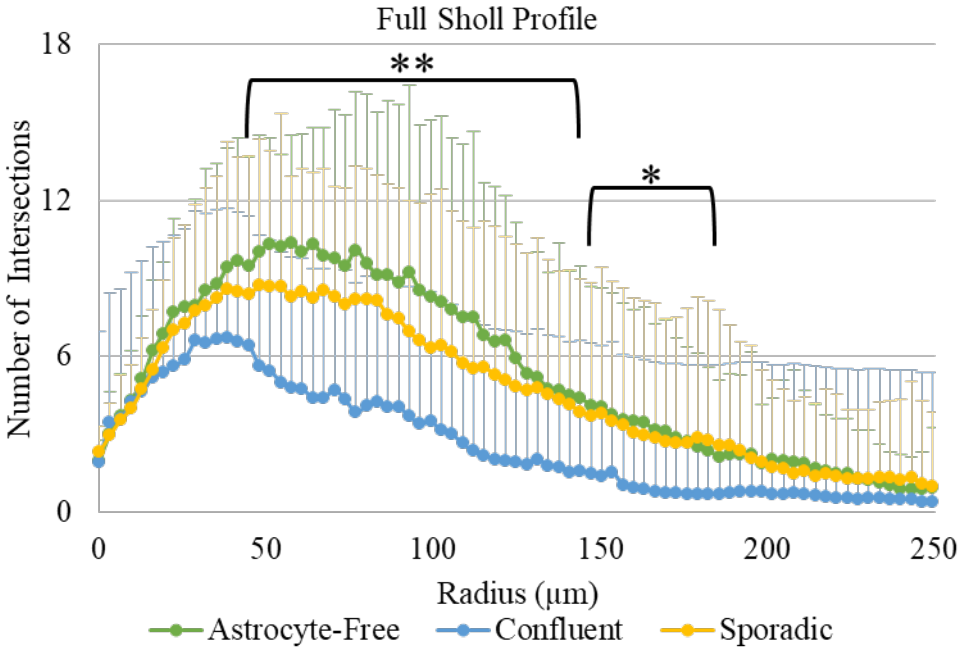

Fig. 4 Full Sholl profile graphing average number of intersections as function of distance from cell body for each astrocyte condition; neurons grown on confluent astrocytes had significantly less branching at 48–195 µm (48–145 µm, **p < 0.01; 145–195 µm, *p < 0.05)

4. Discussion

Three different ratios of astrocytes-to-neurons were successfully cultured from embryonic rat hippocampi. The cultures were characterized at DIV 12 to describe their cellular composition, cellular morphology, and neurite outgrowth. As expected, astrocyte-free cultures contained neurons but few to no astrocytes. In contrast, the confluent astrocyte cultures had a high astrocyte-to-neuron ratio, and sporadic astrocyte cultures had a relatively equal number of neurons and astrocytes intermingled together. Neurites from astrocyte free cultures did not appear as

healthy as their astrocyte-containing counterparts; the neurites appeared thinner with a punctate appearance along their lengths. While not quantified in this study, neurite thickness appeared to increase with the amount of astrocytes present in the culture: astrocyte free < sporadic astrocyte < confluent astrocyte. Furthermore, astrocyte free cultures were more likely to produce free-floating debris when compared with cultures containing astrocytes.

Astrocyte morphology was different among the two astrocyte-containing groups: the confluent astrocyte cultures presented as a fully connected layer of polygonal-shaped astrocytes similar to what others have described as the classical morphology.[25,26] It is most likely the addition of serum in the confluent condition that is responsible for these differences. The astrocytes in the sporadic culture had defined processes extending outward from the cell body. Others have reported astrocytes of this morphology were indistinguishable from classical morphology in regard to functional and metabolic characteristics.[26]

The healthy appearance of the neurons and neurites in the cultures containing astrocytes in this study is not surprising since astrocytes provide both functional and structural support to neurons. Functionally, astrocytes communicate with neurons to provide network regulation and neuroprotection,[27–30] which is important for neuronal health. Even in astrocyte-free cultures, adding media or serum that mimics an astrocytic environment improves the health of neurons in culture.[15,31,32] Structurally, astrocytes provide a more compliant environment on which neurons prefer to attach both in vivo and in vitro; having the astrocytes present in the culture will increase the attachment of neurons and quality of the culture compared with a glass cover slip alone.[6,7]

While the inclusion of astrocytes is more representative of in vivo conditions, the actual ratio of astrocytes to neurons in vivo is still ambiguous.[3,27,33,34] The ratio is influenced by the size of the brain, region examined, and the functional capacity of the animal species.[3] In human brains, early studies suggested a 10:1 glia-to-neuron ratio, but 1:1 has been proposed more recently.[3,27,34] In this study, the confluent (10:1) and sporadic (1:1) astrocyte conditions represent the published ratios for human brains, and the culture conditions can be selected based on the experimental needs. Although an astrocyte free condition does not represent any in vivo condition, an astrocyte free culture may be used to evaluate isolated neuronal responses without the influence of other cell types.[35]

The Sholl analysis demonstrated a reduction in neurite branching as the density of astrocytes increased. Neuronal growth patterns in culture can be affected by neighboring cells, media additives, and the incubation environment.[36–41] For example, mature astrocytes have been shown to inhibit neurite outgrowth compared

with immature astrocytes.[42] This may explain the smallest amount of branching in the confluent astrocyte condition witnessed in this study. Interestingly, the number of intersections among all groups were indistinguishable from one another until about 50 μm, suggesting all neurons regardless of astrocyte presence had similar numbers of primary branches. The confluent astrocyte condition had significantly fewer branches from about 50 to 200 μm, suggesting there were fewer secondary and tertiary branches. This is possibly the result of increased communication/ support between the neurons and surrounding astrocytes. In contrast, the astrocyte free condition had a trend toward an increased number of secondary and tertiary branches that may be influenced by the neurons seeking to increase the chances of receiving trophic support from more remote neighboring cells, or by the lack of astrocyte-associated inhibition.

Depending on the type of experiment and analysis required, each media condition has benefits and limitations. A bed of confluent astrocytes provides a supportive structure for neurons to grow; however, because of the large ratio of astrocytes to neurons, it is difficult to discern subtle fluctuations in neuron morphology due to obstruction in visualization.[43] Furthermore, the specific cause of any neuronal changes, either structural or functional, is confounded by the presence of astrocytes. Astrocyte free conditions may allow for single neuronal cell analysis without the complication of other cells being present; however, this does not mimic the in vivo milieu. Even without an insult or treatment, neurons from these cultures do not survive as long as neurons cultured with astrocytes. To overcome these challenges, some laboratories add astrocyte-conditioned media or a feeding layer of astrocytes suspended above the neuronal culture.[15,31,32,44]

5. Conclusion

In vitro models using brain cells reduce some of the complexity and offer a high degree of experimental control compared with in vivo experiments. Increased complexity better mimics the in vivo environment, but it is more difficult to resolve subtle effects. In this study, primary pyramidal neurons from hippocampi were cultured to obtain three configurations of varying complexity: confluent astrocyte—a layer of confluent astrocytes with neurons grown on top; sporadic astrocyte—neurons and astrocytes intermingled; and astrocyte free—neurons with no astrocytes present. This study describes the methodology to obtain these different neuronal-culture configurations and describes their neuronal environment, thereby providing a resource for researchers to choose the best neuron–astrocyte configuration for their intended research model.

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List of Symbols, Abbreviations, and Acronyms

2-D	2-dimensional
3-D	3-dimensional
ANOVA	analysis of variance
ARL	Army Research Laboratory
BSC	biosafety cabinet
CCDC	US Army Combat Capabilities Development Command
CNS	central nervous system
DAPI	4',6-diamidino-2-phenylindole
DIV	days in vitro
FBS	fetal bovine serum
GFAP	glial fibrillary acidic protein
HEB	Hibernate EB
NBM	neurobasal media
PBS	phosphate-buffered saline
PLL	poly-L-Lysine

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