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A Method to Investigate Astrocyte and Microglial Morphological Changes in the Aging Brain of the Rhesus Macaque

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1 Introduction

Studies of alterations in brain structure associated with aging are ideally performed in nonhuman primates (NHP), due to the genetic and physiological similarities with humans. This is especially true when attempting to elucidate subtleties that develop over many years. Many of these processes are believed to be conserved across primate species, and thus, longitudinal assessments of NHPS provide invaluable data for eugenic or normal brain aging. We have utilized the rhesus monkey (*Macaca mulatta*), a “gold standard” system that has been in use since the 1960’s and has proven to have very high translational validity with respect to having a distinct adolescent period and cognitive assessments [1], combined with a true geriatric period [2]. A unique opportunity existed, therefore, to quantify glial morphometrics in primates with eugenic aging [3]. Crucially, rhesus macaques age approximately 4 times the rate of humans [4–6], providing a useful cohort of animals at Tulane National Primate Research Center.

Our previous study identified changes that occurred across the lifespan in healthy macaques [3]. Our data showed increased branching, and hence connectivity of astrocytes aged from juveniles through adolescence and into adulthood. In geriatric macaques, however, there was

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¹²Assuming you choose the same analyses every time, the sheets will stay constant. This is convenient for ensuring consistency especially if you do post-export batch sorting (see Note 15 on RDB Merge).

a sharp drop off in connectivity and branching of astrocytes, in combination with a decrease in total microglial arbor length, and increased numbers of microglia in geriatric macaques.

Our methodology utilized 6µm thick sections cut from formalin-fixed paraffin embedded frontal lobe sections collected in the past eight years and stored at the TNPRC tissue archive. The geriatric animals (over twenty years of age) were euthanized as a result of investigator-initiated control animals, trauma or natural causes. These protocols have been used to examine glial morphometrics in macaques using a number of infectious and behavioral pathologies [3, 7–14].

2 Materials

Most reagents are best prepared fresh, or for the xylene and graded ethanols, to be refreshed every few slide runs.

2.1 Immunohistochemistry

Xylene (histology grade)

Ethanol: 100%, 90%, 80% and 70% by volume with 18MΩ-cm ultrapure water.

Ultrapure 18MΩ water

Sodium Citrate Buffer (SSC at 2x strength made from 20x Roche 1666681)

Low pH Citrate based buffer for antigen retrieval (we use Dako S2369).

Blocking Solution (DAKO X0909)

Tris Buffered Saline – TBS (Fisher L-11690)

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Immunohistochemistry for GFAP (astrocytes) and/ or IBA1 (microglia)

Identify brain tissues for analysis and have 6µm paraffin sections cut.

1. Bake slides at 60°C overnight

Day 1

2.
 - Xylene 5 min (3X) (see Note 1)
 - 100% EtOH 3 min (2X). Note, each ethanol and water step is repeated.
 - 90% EtOH 3 min (2X)
 - 80% EtOH 3 min (2X)

¹·DON'T LET TISSUES DRY OUT AT ANY POINT!

- 70% EtOH 3 min (2X)
 - 18 MΩ Water 1 min (2X)
3. Rinse slides in SSC_{2x} (coplin jar) at room temperature for 30 minutes on a rocker.
 - -For antigen retrieval (breaks crosslinking)
 4. Steam slides for 20 min. (>95°C) in citrate-based buffer-pH6 in new coplin jar. (see Note 2)
 5. Allow slides to cool to room temperature in buffer for 20 minutes in chemical hood.
 6. Rinse slides in SSC_{2x} (coplin jar) at room temperature for 5 minutes on the rocker.
 7. Block for 1 hour (Blocking Soln. DAKO) at RT.
 8. Add primary antibody overnight at 4°C in blocking buffer.
- Day 2
9. Rinse 3 times for 5 minutes each with TBS.
 10. Add secondary antibody (1:1000) in blocking buffer for 1 hour at room temperature.
 11. Rinse 3 times for 5 minutes each with TBS.
 12. If using another antibody that is not pre-conjugated, complete blocking, primary antibody, and secondary antibody steps again.
 13. If other antibody is pre-conjugated, just block and add antibody,
 14. Rinse 3 × 5 mins TBS.
 15. Repeat for 3rd color / nuclear stain (e.g. DAPI).
 16. Coverslip.

3.1 Image capture and analyses.

Image slides using a fluorescence microscope pre-calibrated using a stage micrometer. For our studies, we used a 40X objective on a Nikon Eclipse TE2000-U microscope. Twenty non-overlapping fields were captured for microglia or astrocytes from each animal. (see Note 3)

²-Have buffer already heated before adding slides.

³-For these studies, as with previous studies by this group, only those cells with clear labeling (either GFAP or IBA1) were chosen. It was also important that clear cell bodies were apparent (DAPI staining), and that the processes did not extend beyond the edge of the field imaged. Cells were purposefully chosen far from the gray-white matter border to remain within designated cortical layers, as is routine [10, 11, 13, 14].

For astrocyte and/or microglia morphology, capture 10–14 cells each from gray (layers 2–6) and white matter at random. Captured cells are then analyzed using software designed to capture neuronal complexity [15–19].

3.2 Importing and tracing glia in Neurolucida software.

1. Ensure the correct magnification and microscope are selected before opening your captured image.
2. Drag and drop an image into Neurolucida's main window
3. Double check the correct magnification is selected (*see* Note 4)
4. Ensure your cell is reasonably clean and entirely visible. Cells with cut-off processes cannot be included in tracings. (Figure 1).
5. Select cell body from the pull down menu (*see* Note 5)
6. Click anywhere on the perimeter of the cell body to begin your tracing. Click along the perimeter to extend the tracing around the cell body (Figure 2). Once you've nearly returned to your starting point, right click and select 'Finish Cell Body' from the popup menu to finish the cell body. (*see* Note 6).
7. With 'Dendrite' selected, your cursor will become a cross inside a circle (Figure 3). The circle size can be adjusted using the scroll wheel on the mouse; adjust accordingly to match the width of the dendrite. Select a process and click once at the place where the dendrite meets the cell body.
8. Find a point further along the process, adjusting the size of the cursor to match the width of the dendrite. Click again to extend the process, similar to extending a cell body. This should trace your first dendritic segment: incidentally, this also creates a 'frustum' (*see* Note 7).
9. Arbor volume is found by revolving the two-dimensional process segments into three dimensional frusta, linking the series of frusta in each dendrite, and summing the combined volumes to estimate total process volume.
10. Once you reach a branching point, right click to bring up the popup menu, and select 'Bifurcating Node'. Continue tracing one segment as you normally would, and once you've reached the end, right click and select 'Ending' to end the segment. This is how you will end all dendritic trees (*see* Note 8). In the presence of a bifurcation, ending one segment will simply bring you back to the

⁴This is vitally important if you use more than one magnification for any analyses: it is not possible to correct this after analyses. Always check to make sure you've selected the right one before analyzing the data. Don't learn the hard way.

⁵For astrocytes or microglia, those will be the only two options you need. DO NOT mix them up. A cell body (Neurolucida calls these *contours*) cannot be a dendrite. You must have the icon to the right selected.

⁶You will have to use your best judgment to decide as to where the body extends or end. If you have 'Continuous Tracing' selected (Figure 2), you can trace the body by holding down the left click and dragging as opposed to clicking around the body. Experiment to see which method you are more comfortable with.

⁷Utilize the magnifier/dragging toolbar to manipulate the image to the optimum position for tracing. This might include zooming in on faint processes, and/or moving the cell around to focus on one particular aspect. The movement icon **directly** to the left of the magnifiers moves the image AND the tracing together. The icon to the left of that moves only the image, and may collapse the association between your existing tracing and the actual image.

bifurcating ‘node’, where you can begin tracing the other segment(s). For dendrites with several bifurcations, repeatedly bifurcating and ending will simply send you to the last bifurcating node, and then the last, and etc. Once you’ve ended the last segment, the tree will be complete (*see* Note 9).

11. In the upper left corner, open the dropdown menu ‘File’. From there, select ‘Save Data File...’, and navigate to your preferred save location in the resulting popup menu. To begin tracing a new image, hit the blank document icon in the upper right corner, and select ‘OK’ in the popup menu (*see* Note 10). Once this is done, follow the previous steps to trace more astrocytes.

3.3 Analyzing traces in Neurolucida Explorer

Having captured and traced the images, the Neurolucida Explorer application is used to analyze the tracings.

1. Open the dropdown menu ‘File’ and select ‘Open Data File...’. Navigate to where the tracings were saved and select one to bring it up.
2. In the top bar, navigate to the dropdown menu ‘Analysis’ and click on it. Our studies mostly used “Branched Structure Analysis” (Figure 4). The popup menu offers a variety of analyses you can do on your astrocyte (*see* Note 11). Figure 4b shows branches color coded by branch order, 4c shows an astrocyte within concentric red Sholl rings, and 4d demonstrates the same astrocyte color coded based on primary branches.
3. When the analyses are done, a popup window will appear for each individual analysis. To save these in a reasonably organized format, right click any of the data windows and click ‘Excel Export’. This will transfer the data to an Excel file where each separate analysis is contained in a separate sheet.
4. A Sholl analysis follows much the same format (Figure 5). Select the Sholl analysis to bring up a popup menu. Here, different analyses can be selected, and Sholl radii (routinely 10µm) can be adjusted (*see* Note 13).
5. A linear method Sholl analysis is performed on the data by placing concentric rings 10 µm apart around the cell centered at the centroid of the cell body and radiating outward. Intersections are determined as points where the astrocytic processes cross a concentric ring (Figure 6). Branching points, or nodes,

⁸If you accidentally traced a dendrite as a cell body, the ‘Select Objects’ icon can fix this. Click the icon, and then click on a tracing. Immediately, you will see dots which comprise the points that you clicked to make up the contour appear. This can be useful for manipulating the shape; these points can be dragged around to change the overall contour. If you’ve misclicked while tracing up a cell body but don’t want to retrace the whole thing, this tool will let you move the existing tracing to fit the proper shape. The previous steps also work for dendrites as well as contours. Not only can you manipulate the curvature, shape, and thickness, you can also add in bifurcation points you may have missed.

⁹Right click anywhere on the tree to bring up the popup menu and select ‘Insert Node into Selected Tree’. Click at the desired location to place a node there and use as you would a normal bifurcation point.

¹⁰Make sure you’ve checked both options for “close all images” and “new reference point” when you are tracing a new image.

¹¹For reference, the MacLean lab mainly uses the following: Cell Body Details, Neuron Summary, Dendrite Tree Totals, and Dendrite Terminal Distance. However, you can select ‘All Possible Analyses’.

¹³If you see red rings, you’ve done it right. As per branched structure analysis, right click any data window and select ‘Excel Export’ to export into an excel file. As above, if you use the same settings each time, this is very useful.

quantified during Sholl analysis are expressed as a quantity per concentric ring area. Sholl regression coefficient, obtained from the ImageJ Sholl Analysis plugin (v 1.0, Tiago Ferreira, 2012), is calculated on a normalized semi-log scale, with nodes per radial area versus cell radius plotted and a linear regression performed on the data. The resulting slope of this regression, or the Sholl regression coefficient [20], represents the rate of decay of branching as distance increases from the soma – the higher the coefficient, the greater the rate of decay, indicating lesser arbor complexity.

6. In the event that several astrocytes are saved in a single data file, you need to separate the cells. Select the pointer icon, and drag a box around a single astrocyte to select only that astrocyte. Any analyses done now will only be concerned with the single astrocyte you've selected.
7. If the astrocytes overlap each other, there is a solution to that too. Again, using the pointer tool, double click an 'interfering' process to select it. Right click to bring up the popup menu, and click 'Hide Selected Tree'. Once the other processes are gone, select the desired astrocyte and analyze as above (*see Note 14*).

3.4 Organizing Data

After saving all the excel data files from your analyses, you will end up with a folder with an excel data file for each of numerous cells. If all the setting were kept the same, each Excel file should be laid out the same.

1. On any open excel file, click on the 'Data' tab, and select the 'RDBMerge Add-In' icon (*see Note 15*). A pop-up window should appear. Under the 'Folder Location:' section, click the 'Browse' button. Navigate to the folder where all your excel files are stored. The program will proceed to consolidate all the data files in the selected folder into one master file. Thus, it is important that all the files in the selected folder are consistent (*see Note 16*).
2. Before merging, check the settings to make sure they are to your specifications. It is important to make sure in the 'Which range:' section that the 'First cell till last cell on Worksheet' is checked. Otherwise, the data will not be collected beyond the cell specified. Files will need to be re-merged for each individual sheet on the excel files. Under the 'Which worksheet(s):' section, select the 'Use the sheet index' option and manually select the appropriate sheet that needs to be consolidated. (Sheets are selected by switching between the tabs at the bottom of the window in an excel file.). At this point, all the data should be in a new Excel file, consolidated onto a single page.
3. Statistical analyses are routinely performed using GraphPad Prism (version 5, GraphPad Software, La Jolla, CA). Normality is assessed by Kolmogorov-

¹⁴. To restore the hidden cells, either right click and select 'Show last Hidden Object', or reopen the file again to restore all objects.

¹⁵. For a more in depth explanation of each setting, visit the website at : <http://www.rondebruin.nl/merge.htm>

¹⁶. We try to organize the data by animal condition, and then into grey and white matter folders to consolidate separately.

Smirnov test, and data that passes normality are analyzed by unpaired T-test. Data that are not distributed normally are assessed by Mann-Whitney test to determine significance between groups. For all analyses, significance is defined as $p < 0.05$. Data are usually presented as mean $\pm$ Standard Deviation. Branch structure data is assessed for significant changes between age groups using a one-way ANOVA with Bonferroni's Multiple Comparison Test.

1. Sholl data is assessed for significance using a one-way ANOVA with Bonferroni's Multiple Comparison Test.
2. Significant changes between sexes is assessed using an unpaired Student's t-test.

3.5 Cell Density Determination

1. At least five non-overlapping images are captured from both gray and white matter tissue from each stained brain section at 20X objective using the Nikon Eclipse TE2000-U microscope.
2. Cells are identified with either IBA1 or GFAP expression and clear cell bodies (DAPI). Each cell is then marked using the count feature in ImageJ, to ensure each cell is counted, but only once. Because most of the imaged gray matter fields include tissue borders, the images are first cropped and the pixel area contained within each field converted to μm^2 .
3. The number of each cell type within each tissue area are manually counted and calculated for cell density. Resulting data are analyzed with GraphPad Prism using a one-way ANOVA and Bonferroni's Multiple Comparison Test for significant values ($p < 0.05$).

References

1. Verrico CD, Liu S, Asafu-Adjei JK, Sampson AR, Bradberry CW, Lewis DA. Acquisition and baseline performance of working memory tasks by adolescent rhesus monkeys. *Brain Res* 2011; 1378: 91–104 [PubMed: 21215729]
2. Didier ES, MacLean AG, Mohan M, Didier PJ, Lackner AA, Kuroda MJ. Contributions of Nonhuman Primates to Research on Aging. *Vet Pathol* 2016; 53: 277–90 [PubMed: 26869153]
3. Robillard KN, Lee KM, Chiu KB, MacLean AG. Glial cell morphological and density changes through the lifespan of rhesus macaques. *Brain Behav Immun* 2016; 55: 60–9 [PubMed: 26851132]
4. Spear LP. The adolescent brain and age-related behavioral manifestations. *Neurosci Biobehav Rev* 2000; 24: 417–63 [PubMed: 10817843]
5. Colman RJ, Anderson RM. Nonhuman primate calorie restriction. *Antioxid Redox Signal* 2011; 14: 229–39 [PubMed: 20698791]
6. Kohama SG, Rosene DL, Sherman LS. Age-related changes in human and non-human primate white matter: from myelination disturbances to cognitive decline. *Age* 2012; 34: 1093–110 [PubMed: 22203458]
7. Lee KM, Chiu KB, Sansing HA, Didier PJ, Lackner AA, MacLean AG. The flavivirus dengue induces hypertrophy of white matter astrocytes. *J Neurovirol* 2016; 22: 831–39 [PubMed: 27273075]
8. Inglis FM, Lee KM, Chiu KB, Purcell OM, Didier PJ, Russell-Lodrigue K, Weaver SC, Roy CJ, MacLean AG. Neuropathogenesis of Chikungunya infection: astrogliosis and innate immune activation. *J Neurovirol* 2016; 22: 140–8 [PubMed: 26419894]

9. Lee KM, Chiu KB, Didier PJ, Baker KC, MacLean AG. Naltrexone treatment reverses astrocyte atrophy and immune dysfunction in self-harming macaques. *Brain Behav Immun* 2015; 50: 288–97 [PubMed: 26191654]
10. Snook ER, Fisher-Perkins JM, Sansing HA, Lee KM, Alvarez X, MacLean AG, Peterson KE, Lackner AA, Bunnell BA. Innate immune activation in the pathogenesis of a murine model of globoid cell leukodystrophy. *Am J Pathol* 2014; 184: 382–96 [PubMed: 24316110]
11. Lee KM, Chiu KB, Renner NA, Sansing HA, Didier PJ, MacLean AG. Form follows function: astrocyte morphology and immune dysfunction in SIV neuroAIDS. *J Neurovirol* 2014; 20: 474–84 [PubMed: 24970236]
12. Renner NA, Sansing HA, Inglis FM, Mehra S, Kaushal D, Lackner AA, Maclean AG. Transient acidification and subsequent proinflammatory cytokine stimulation of astrocytes induce distinct activation phenotypes. *J Cell Physiol* 2013; 228: 1284–94 [PubMed: 23154943]
13. Lee KM, Chiu KB, Sansing HA, Inglis FM, Baker KC, Maclean AG. Astrocyte atrophy and immune dysfunction in self-harming macaques. *PLoS One* 2013; 8: e69980 [PubMed: 23922882]
14. Lee KM, Chiu KB, Sansing HA, Didier PJ, Ficht TA, Arenas-Gamboa AM, Roy CJ, Maclean AG. Aerosol-induced brucellosis increases TLR-2 expression and increased complexity in the microanatomy of astroglia in rhesus macaques. *Frontiers in cellular and infection microbiology* 2013; 3: 86 [PubMed: 24350061]
15. Chen W, Prithviraj R, Mahnke AH, McGloin KE, Tan JW, Gooch AK, Inglis FM. AMPA glutamate receptor subunits 1 and 2 regulate dendrite complexity and spine motility in neurons of the developing neocortex. *Neuroscience* 2009; 159: 172–82 [PubMed: 19110036]
16. He H, Mahnke AH, Doyle S, Fan N, Wang CC, Hall BJ, Tang YP, Inglis FM, Chen C, Erickson JD. Neurodevelopmental role for VGLUT2 in pyramidal neuron plasticity, dendritic refinement, and in spatial learning. *J Neurosci* 2012; 32: 15886–901 [PubMed: 23136427]
17. Inglis FM, Crockett R, Korada S, Abraham WC, Hollmann M, Kalb RG. The AMPA receptor subunit GluR1 regulates dendritic architecture of motor neurons. *J Neurosci* 2002; 22: 8042–51 [PubMed: 12223558]
18. Prithviraj R, Kelly KM, Espinoza-Lewis R, Hexom T, Clark AB, Inglis FM. Differential regulation of dendrite complexity by AMPA receptor subunits GluR1 and GluR2 in motor neurons. *Dev Neurobiol* 2008; 68: 247–64 [PubMed: 18000827]
19. Thomas CC, Combe CL, Dyar KA, Inglis FM. Modest alterations in patterns of motor neuron dendrite morphology in the *Fmr1* knockout mouse model for fragile X. *Int J Dev Neurosci* 2008; 26: 805–11 [PubMed: 18638539]
20. Ferreira TA, Blackman AV, Oyler J, Jayabal S, Chung AJ, Watt AJ, Sjöström PJ, van Meyel DJ. Neuronal morphometry directly from bitmap images. *Nat Methods* 2014; 11: 982–4 [PubMed: 25264773]

Figure 1.
This cell cannot be used for tracing purposes, as one of the processes extends beyond the edge of the screen.

Figure 2.
If you've traced your cell body properly, it should look something like this!

Figure 3. Reconstructing astrocyte processes is a step-wise method. Once the crosshair is selected, and width adjusted (A), the segment is selected (B). If the process branches, select a “bifurcating node” from the drop down menu (C). Once you reach the end of this process, each branch is then traces in sequence until you complete an entire process (D).

Figure 4. Diagram of astrocyte reconstruction process. Fluorescent image of local field stained with GFAP was taken from 6-micron thick slides (a) and an astrocyte of interest is traced (b). Neurolucida software converts this tracing into morphological data (c), and creates a 3D reconstruction (d) by revolving individual process segments into frusta in order to estimate volume data.

Figure 5.
This is what your tracing looks like imported into Neurolucida Explorer. Note the red box surrounding the cell; this indicates that this particular cell has been selected.

Figure 6.

Morphometric analyses on a representative astrocyte. At left, an astrocyte lies in the center of a series of concentric rings spaced 10 microns apart as generated in a Sholl analysis. The analyzed soma area is highlighted in yellow, two example Sholl intersections are indicated by green circles, and an example branching point is highlighted by a blue arrow. At right, a representative dendrite from the astrocyte illustrates branching order; where primary processes emerging from the cell consist of the first order, the segments branching from the primary segment consist of the second order, and so on, ad infinitum. Total arbor length is the sum of the dendritic lengths, all branches included.