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Binge Alcohol Alters Exercise-Driven Neuroplasticity

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Abstract

Exercise is increasingly being used as a treatment for alcohol use disorders (AUD), but the interactive effects of alcohol and exercise on the brain remain largely unexplored. Alcohol damages the brain, in part by altering glial functioning. In contrast, exercise promotes glial health and plasticity. In the present study, we investigated whether binge alcohol would attenuate the effects of subsequent exercise on glia. We focused on the medial prefrontal cortex (mPFC), an alcohol-vulnerable region that also undergoes neuroplastic changes in response to exercise. Adult female Long-Evans rats were gavaged with ethanol (25% w/v) every 8 hours for 4 days. Control animals received an isocaloric, non-alcohol diet. After 7 days of abstinence, rats remained sedentary or exercised for 4 weeks. Immunofluorescence was then used to label microglia, astrocytes, and neurons in serial tissue sections through the mPFC. Confocal microscope images were processed using FARSIGHT, a computational image analysis toolkit capable of automated analysis of cell number and morphology. We found that exercise increased the number of microglia in the mPFC in control animals. Binged animals that exercised, however, had significantly fewer microglia. Furthermore, computational arbor analytics revealed that the binged animals (regardless of exercise) had microglia with thicker, shorter arbors and significantly less branching, suggestive of partial activation. We found no changes in the number or morphology of mPFC astrocytes. We conclude that binge alcohol exerts a prolonged effect on morphology of mPFC microglia and limits the capacity of exercise to increase their numbers.

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Graphical abstract

Keywords

microglia; medial prefrontal cortex; binge alcohol consumption; exercise

Binge drinking damages corticolimbic brain regions important for memory, decision-making and behavioral control (Crews and Boettiger, 2009, Duka et al., 2011), and recent studies indicate that it results in detectable brain dysfunction (Maurage et al., 2012, Campanella et al., 2013). It also decreases hippocampal neurogenesis (Nixon and Crews, 2002, 2004, Nixon et al., 2008) and disrupts glial function (de la Monte and Kril, 2014). In contrast, exercise benefits neural health through a variety of mechanisms that include enhanced neurogenesis (van Praag et al., 1999), gliogenesis (Li et al., 2005b, Mandyam et al., 2007), angiogenesis (Black et al., 1990, Swain et al., 2003, Rhyu et al., 2010) and trophic factor upregulation (Gomez-Pinilla, 2001, Vaynman and Gomez-Pinilla, 2005). Exercise therefore has the potential to heal the alcohol-damaged brain and indeed has been shown to ameliorate the consequences of developmental alcohol exposure (Thomas et al., 2008, Helfer et al., 2009). However, the interactive effects of alcohol and exercise on the brain remain largely unexplored.

Exercise is increasingly being used as an adjunctive treatment for alcohol use disorders (AUD). Several recent reviews of a growing number of clinical trials indicate that exercise is feasible in those with AUD, and is effective at enhancing their cardiovascular health as well as treating co-morbid mental health problems, such as anxiety and depression (Giesen et al., 2015, Stoutenberg et al., 2016). The effect of exercise training on drinking behaviors is much less clear and there is a compelling need for carefully controlled trials (Stoutenberg et al., 2016). A better understanding of the interactive effects of alcohol and exercise on the brain will inform activity-based treatment strategies for AUD.

We have recently shown that exercise reverses binge-induced hippocampal damage in female rats (Maynard and Leasure, 2013), substantiating the idea that exercise can counter the damaging effects of binge alcohol. It remains unknown, however, whether alcohol influences the brain benefits of subsequent exercise. In the present report, we examined banked tissue from our prior study (Maynard and Leasure, 2013) in order to determine whether binge alcohol impacted exercise-induced cellular plasticity in the female brain. We focused on the medial prefrontal cortex (mPFC), a region that is both vulnerable to alcohol (Sullivan et al.,

2000, Kubota et al., 2001, Sullivan and Pfefferbaum, 2005) and responsive to exercise (Mandyam et al., 2007, Brockett et al., 2015). In addition, the mPFC has connections to the hippocampus (Warburton and Brown, 2010, Varela et al., 2014), which we and others have shown to be damaged by binge alcohol (Nixon and Crews, 2002, 2004, Nixon et al., 2008, Maynard and Leasure, 2013).

As neurogenesis does not occur in the mPFC, we focused on glial plasticity. As immunocompetent cells of the brain, microglia are constantly surveying the neural parenchyma, ready to respond to changes in their environment by taking on several stages of activation (Streit, 2002, Nimmerjahn et al., 2005). Previous research indicates that a single binge episode is able to prime microglia to respond to subsequent neuroimmune challenges (McClain et al., 2011, Marshall et al., 2016). This has been found in male rats, however, sex differences have been found in microglia priming in the mPFC in response to stress (Bollinger et al., 2016). Therefore, investigation into the effects of binge alcohol on microglia in the female brain is warranted.

We hypothesized that exercise would less effectively drive glial plasticity (including number and morphology of astrocytes and microglia) in the mPFC of binged rats, compared to controls. However, we anticipated that after 5 weeks of abstinence (including 4 weeks of exercise), binge effects on exercise-driven plasticity in the mPFC would be subtle. We therefore used computational image analysis, which can detect small morphological differences, and which generates a rich collection of quantitative measurements (Bjornsson et al., 2008, Al-Kofahi et al., 2010, Narayanaswamy et al., 2011), to analyze both number and morphology of mPFC astrocytes and microglia. These computational arbor analytics revealed that microglia in the binged animals continued to display an altered morphology following 5 weeks of abstinence. Additionally, the combination of binge exposure and exercise resulted in a drastic decrease in the number of surviving microglia. Our findings suggest that binge alcohol exerts a prolonged effect on microglia, suggestive of microglial priming, and alters the typical microglial response to exercise in the mPFC.

Experimental Procedures

Animals

In order to reduce the number of animals used, archived tissue from a previously published study (Maynard and Leasure, 2013) was used. The study had a 2×2 design, comparing Diet (ethanol versus isocaloric control) and Activity (exercise versus sedentary). Six animals per group were randomly chosen to be used in the current study. As previously described, adult female Long-Evans rats (170-200 g, purchased from Harlan) were given an ethanol diet (25% ethanol w/v in vanilla Ensure™; Abbot Laboratories, Columbus, OH), or an isocaloric control diet (dextrose w/ vanilla Ensure™) every eight hours for four days by intragastric gavage, using a paradigm modified from Majchrowicz (1975). The initial dose for each animal was 5g/kg; every additional dose was determined based on a 6-point scale of behavioral intoxication, such that the more intoxicated animals received less alcohol, and *vice versa*. Blood ethanol concentration (BEC) was determined from tail vein samples taken 90 minutes after the 7th dose. Seven days after the last dose of alcohol or isocaloric diet, rats in the exercise groups were given access to exercise wheels for approximately five hours

each day for four weeks. After 28 days of exercise (35 days from the last dose of alcohol or isocaloric diet), rats were killed with an anesthetic overdose and intracardially perfused with cold saline, followed by 4% paraformaldehyde until the upper body was stiff. Brains were then removed, post-fixed, and cut into 50 μm coronal sections on a microtome. Sections were stored in cryoprotectant in 96-well microtiter plates at -20°C . All experiments were carried out in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the Institutional Care and Use Committee of the University of Houston (protocol number 11-021).

Tissue Processing

To examine the mPFC, three 50 μm serial sections from each animal were processed using multi-channel immunofluorescence to label cell nuclei (4',6-diamidino-2-phenylindole; DAPI), microglia (Iba1), neurons (NeuN), and astrocyte fibrils (glial fibrillary acidic protein; GFAP). We used GFAP in lieu of the astrocytic protein S100 β , which is primarily expressed in nuclei (Donato, 1999, 2003), because we wanted to examine astrocyte fibrils. However, the presently available astrocytic markers that label fibrils (i.e., GFAP and vimentin) have also been found to label some stem cells. Thus, we used GFAP with the understanding that it is possible that this marker may also tag some cortical stem cells (Morrens et al., 2012). After 3 10-minute rinses in 0.1 M tris-buffered saline (TBS), the tissue sections were blocked in 3% normal donkey serum for 60 minutes (Sigma-Aldrich, MO, USA). The tissue was then incubated in the primary antibodies (rabbit anti-Iba1, Wako Chemicals USA, VA, USA, 1:10,000; guinea pig anti-NeuN, EMD Millipore, MA, 1:2,000; goat anti-GFAP, Santa Cruz Biotechnology Inc., CA, USA, 1:100) for 72 hours at 4°C . Following primary antibody incubation, and 2 15-minute rinses in TBS, the tissue was blocked in 3% normal donkey serum for 15 minutes. Following blocking, the sections were incubated in the secondary antibodies (donkey anti-rabbit Alexa 488, Life Technologies, NY, USA, 1:250; donkey anti-guinea pig Alexa 594, Jackson ImmunoResearch, PA, USA, 1:250; donkey anti-goat Alexa 633, Life Technologies, NY, USA, 1:250) at room temperature for 2 hours. Sections were then washed in TBS three times for 10 minutes each, and then incubated in DAPI (Life Technologies, NY, USA) for five minutes. Finally, the sections were washed 4 times in TBS, mounted onto SuperFrost Plus slides and coverslipped using ProlongGold as the mounting medium (Life Technologies, NY, USA).

Confocal Microscopy

A Leica SP8 confocal microscope was used to image eight fields of view (hereafter referred to as “tiles”) in a 2×4 rectangle (see Figure 1) covering a $775\ \mu\text{m} \times 1,550\ \mu\text{m}$ wide region for each tissue section, resulting in 24 tiles for each animal. The mPFC was identified using the 10x objective; images were acquired using the 40x oil objective. The tissue was imaged sequentially using the 405, 488, 594, and 633nm laser lines. Each tile encompassed an area of $387.50\ \mu\text{m} \times 387.50\ \mu\text{m}$ with a $1\ \mu\text{m}$ step size in the z-plane. The acquisition speed was 600 Hz, and the zoom factor was 0.75. The tiles were set to have 10% spatial overlap, and the z-stacks were collected encompassing the entire thickness of the tissue (section cut thickness was $50\ \mu\text{m}$). After image acquisition, each z-stack was separated by channel and saved in TIFF format.

Computational Image Analysis

For quantitative analysis of the confocal images, we used the FARSIGHT image analysis toolkit (www.farsight-toolkit.org). All images were smoothed using standard median filtering with a 2×2 pixel window, followed by illumination correction using the rolling-ball filter with a radius of 50 pixels (Sternberg, 1983, Narayanaswamy et al., 2011). The cell nuclei were then automatically identified and segmented (delineated) based on DAPI staining (see Figure 1C). Each DAPI+ nucleus was assigned a unique identifier and quantitative morphological measurements of each nucleus (number, volume, surface area, and orientation, see Table 1) were extracted (Bjornsson et al., 2008, Al-Kofahi et al., 2010). Because GFAP does not label astrocyte soma/nuclei as completely as cytoplasmic stains (Pekny et al., 2007), DAPI-tagged nuclei were not associated with astrocytes. An active machine-learning algorithm was then trained to identify the type of cell that each nucleus belongs to (Padmanabhan et al., 2014). Images from each experimental condition were randomly chosen and run through the algorithm and visually inspected for labeling errors. Once the algorithm produced labeling results with at least 95% accuracy, all images were run through the program and all nuclei were labeled.

Next, the cell arbors of the microglia were automatically traced starting from the identified microglia nuclei using an automated algorithm (Megjhani et al., 2015) and the reconstructions were inspected in the FARSIGHT Trace Editor program (Luisi et al., 2011, Narayanaswamy et al., 2011). A similar method was used for tracing and quantifying astrocyte fibrils. However, due to incomplete labeling of the soma by GFAP the nucleus could not be used at the starting point. Instead, traces of the GFAP fibrils began at the point of convergence for clusters of GFAP+ fibrils (Kulkarni et al., 2015). For both microglia and astrocytes, trace reconstructions from each experimental condition were sampled and visually examined by the user for tracing accuracy. When both cell types were traced with at least 95% accuracy in all sampled conditions, then the respective tracing algorithms were run on all images.

Group LASSO

The tracing program described above produces 130 measurements for each segmented cell (Scorcioni et al., 2008, Lu et al., 2015). To simplify analysis, these measurements were grouped into 16 new feature groupings, based on the nature of the measurement (see Table 2). For example, the arbor size-related measurements height, depth, and width were all combined to define a single group measurement named “bounding box”. To further streamline the data, a group LASSO (Least Absolute Shrinkage and Selection Operator) was used as a data mining technique to select a subset of the feature groupings that were sufficient to model and represent the cells typifying each of the experimental conditions (Bien et al., 2013). The group LASSO was run on the feature groupings with the experimental conditions as individual variables. Each feature group received a significance score that indicated its importance in differentiating between conditions (see Figure 2). The feature groups above the minimum square error threshold were determined to be the most salient feature groupings and were further examined using ANOVAs.

Statistical Analysis

All FARSIGHT measures (nuclear and arbor) of interest were analyzed using the statistical package R, using two-way factorial ANOVAs with the variables Activity, Diet, and the Activity by Diet interaction. Planned Tukey HSD *post hoc* comparisons were used when appropriate. The alpha level for all statistical analyses was set at 0.05.

Results

Number and size of neurons and glia in the medial prefrontal cortex

This study made use of banked tissue from a previous report, which details all binge and exercise-related data (Maynard & Leasure, 2013). As reported in Maynard and Leasure (2013), the average BEC of the animals used was 204.8 mg/dl $\pm$ 13.25. This is similar to what we have previously reported in females (Leasure and Nixon, 2010) as well as levels reported in male rats (Nixon and Crews, 2002). Computational image analysis with FARSIGHT yields data for the nucleus, soma, and arbors of each cell. Because each DAPI-tagged nucleus is segmented and analyzed, a vast amount of data is generated. The average number of nuclei segmented per tile was 1,339. Occasionally, a tile had to be discarded due to poor image quality, but this was uncommon - the average number of tiles analyzed per animal was 23.17.

Factorial analysis revealed a significant interaction of Diet and Activity on the average number of microglia per field of view ($F(1,19) = 21.948$, $p < .001$, see Figure 3). Post hoc analysis showed a significant increase in the number of microglia in exercised controls compared to their sedentary counterparts ($p = .05$), as well as compared to binged, exercised animals ($p < .001$). Post hoc analyses also showed a significant decrease in the number of microglia in exercised animals with prior binge exposure compared to sedentary binged animals ($p = .005$). Therefore, in the absence of binge alcohol, exercise resulted in a significant increase of microglia in the mPFC. However, binge exposure prior to exercise blocked this effect. There were no significant changes in the number of neurons or astrocytes. Additionally, there were no significant differences in nuclear size or texture for microglia or neurons.

Group LASSO Analysis of Glial Morphology

Of the 16 feature groups included in the group LASSO analysis, those best able to differentiate microglia between experimental groups were stems, bounding box, segment size, stem diameter, volume, branch, and leaf level (see Figure 2). Factorial analyses were performed on these feature groupings in order to determine whether there were differences between experimental conditions. Five weeks after binge exposure, there was a decrease in bounding box size of microglia in binged animals compared to animals given the control diet ($F(1,17) = 5.183$, $p = .03$). This means the microglia in binged animals were smaller and had arbors that did not extend as far as control animals. Binge exposure also decreased the leaf level, or number of branches, compared to the control diet ($F(1,17) = 4.691$, $p = .04$). Together these results show that microglia in the binged animals (regardless of whether or not they exercised) were smaller in size and had less extensive arbors (see Figure 4). Notably, these alterations were detectable after five weeks of abstinence from alcohol.

Additionally, there was a significant interaction of Diet and Exercise on the number of stems, or basal segments, of microglia arbors ($F(1,17) = 5.767, p = 0.03$). Post hoc analyses revealed that the microglia of binge exercisers had more stems compared to the other three groups ($p = .02$, see Figure 4). Binge exposure also increased the diameter of the microglia stems ($F(1,17) = 5.785, p = .03$, see Figure 4H) and the combined volume and surface area of all microglia arbor segments compared to animals given the control diet ($F(1,17) = 4.924, p = .04$). These results show that after five weeks, the microglia in binged animals had thicker arbors, and that binge exposure coupled with exercise resulted in more basal arbor segments.

For astrocytes, the group LASSO analysis revealed that the most salient feature groups were stem diameter, soma size, volume, branch, and bounding box (see Figure 2). However factorial analysis revealed no significant differences between groups for these measurements. This may be due to GFAP primarily labeling the fibrils of astrocytes. To observe subtle changes in astrocytic morphology a different method of visualizing astrocytes may be needed.

Discussion

In the current study, we found a prolonged effect of binge alcohol on microglia arbor morphology, present after 5 weeks of abstinence. Additionally, there was a significant decrease in microglia in the mPFC of animals that underwent both binge alcohol and exercise. Taken together, these data indicate that binge alcohol exerts a prolonged effect on the brain, and that the binged brain responds differently to exercise.

Microglia arbors were examined using quantitative analytics to detect changes in morphology due to binge alcohol and/or exercise. We found that binge alcohol independently exerted a protracted effect on microglia arbor morphology in the mPFC, decreasing their overall size (soma + arbors) and the level of branching, compared to control animals. Moreover, binge alcohol increased the thickness of microglial arbors, and when coupled with exercise, increased the number of basal segments. These results show that five weeks after binge alcohol, microglia are smaller in size and have thicker arbors with decreased branching and arbor extent. This morphology may indicate partial activation of microglia, which is consistent with other reports finding changes in microglia activation following alcohol exposure (McClain et al., 2011, Qin and Crews, 2012, Crews et al., 2015, Boschen et al., 2016, Kane and Drew, 2016, Marshall et al., 2016, Saito et al., 2016). Emerging evidence suggests that alcohol may act as a priming stimulus for microglia, causing them to mount an exaggerated response to subsequent challenges. The potential of alcohol to act as a priming stimulus is supported by increased microglial responses to LPS following alcohol exposure, as well as to a second binge exposure (Qin et al., 2008, Qin and Crews, 2012, Marshall et al., 2016). This priming of microglia does not appear to induce a full activation state, but instead results in a morphology and expression profile consistent with M2 activation (Perry and Holmes, 2014, Marshall et al., 2016). This alternative state of microglia activation is thought to be anti-inflammatory in comparison to the pro-inflammatory M1 activation state (Franco and Fernandez-Suarez, 2015, Tang and Le, 2016).

While speculative, it is possible that microglial priming may underlie why in the current study microglia in binged animals had a drastically different response to exercise. Consistent with prior findings (Ehninger and Kempermann, 2003), our results demonstrate that exercise increased cortical microglia. However, when binge alcohol preceded exercise, there was a significant decrease in their numbers. This reduction was an interactive effect of binge alcohol and exercise, as sedentary binged animals did not have a significant difference in microglia number compared to sedentary controls (see Figure 3). It may be that binge alcohol acted as a priming stimulus for the microglia, potentially explaining the drastic decrease in microglia number observed in animals exposed to both binge alcohol and subsequent exercise. Future research should address this possibility by examining markers of microglial priming, such as OX-42 and major histocompatibility complex (MHC) class II, following binge and exercise.

In the present study, we found no effects of binge exposure, exercise, or the combination of the two on astrocyte fibrils or on neurons. Prior studies using this binge model have shown degenerating cells in the olfactory bulb, frontal lobes, hippocampus and surrounding cortex several days post-binge (Obernier et al., 2002). Our results indicate that there is no significant loss of neurons in the mPFC, and that if astrocytes were affected early after binge, they are not showing changes 5 weeks later. Neurogenesis does not occur in the mPFC, so exercise was not expected to increase neuron numbers. However, an exercise-induced increase in GFAP expression has previously been reported in the frontoparietal cortex (Li et al., 2005a). We did not find any differences in GFAP-labeled arbors in the present study, but it should be noted that we examined a prefrontal region, and that exercise may have discrete effects on astrocyte populations in different regions of the cortex. Brockett et al. (2015) examined the effects of 12 days of exercise on astrocytes in the rat mPFC and found an increase in the cell body area of S100 β -labeled astrocyte cell bodies. We were not able to measure astrocyte cell body area due to GFAP not labeling the entire cell body. Additionally, this effect was only found in certain cortical areas, supporting the idea that astrocytes in discrete cortical regions respond differentially to exercise.

Taken together, our results indicate that binge alcohol exerts a prolonged effect on the morphology of microglia in the mPFC, and that binge-exposed microglia exhibit a strikingly altered response to exercise. To our knowledge, this is the first demonstration of binge effects on the capacity of microglia to react to subsequent behavioral experience. This reduction of glial plasticity in the mPFC due to alcohol fits nicely with previous research showing a reduction in dendritic plasticity in prefrontal regions following exposure to psychomotor-stimulant drugs (Robinson and Kolb, 1997, Kolb et al., 2003), in that it appears that alcohol and other drugs are capable of exerting prolonged changes on glial and neuronal plasticity in prefrontal regions. Furthermore, these findings offer specific insights into the interactive neural effects of binge alcohol and exercise. Exercise promotes brain health and is increasingly being used to treat AUDs, yet interactive effects of exercise and alcohol have not been fully examined. If exercise is to be incorporated successfully into AUD treatments, it is vital we have a better understanding of the interactive effects of alcohol and exercise on neuroplasticity.

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Abbreviations

mPFC	medial prefrontal cortex
DAPI	4',6-diamidino-2-phenylindole
GFAP	glial fibrillary acidic protein
LASSO	Least Absolute Shrinkage and Selection Operator
AUD	alcohol use disorder
TNF-α	tumor necrosis factor

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Highlights

- Binge alcohol exerts a prolonged influence on cortical microglia morphology
- Exercise increases cortical microglia
- Binge alcohol suppresses this exercise-driven increase in microglia

Figure 1. Imaging and segmentation of the medial prefrontal cortex (mPFC)

Panel A shows a complete montage of the mPFC labeled for cell nuclei (blue), microglia (green), neurons (magenta) and astrocyte fibrils (yellow). Each field of view (“tile”) covered an area of $387.50 \mu\text{m} \times 387.50 \mu\text{m}$. The step size used was $1 \mu\text{m}$, with a 10% overlap between tiles. The specific area imaged in the mPFC for each tissue section is depicted in (B). A single field of view from the complete montage is shown in Nucleus Editor (C).

Figure 2. Salient feature groups determined by LASSO analysis

The feature groups for microglia and astrocyte arbors that best distinguish between the experimental groups. The feature groups are on the X-axis and the significance weights are on the Y-axis. Feature groups above the minimum square error threshold (in red) were considered significant.

Figure 3. Binge alcohol suppressed the exercise-driven increase in microglia

Panel A depicts the average density of microglia per tile in the mPFC. In control animals, exercise increased the number of microglia present (C vs B). Note, however, that prior binge exposure in exercised animals (E) blocked this effect. * $p < 0.05$

Figure 4. Binge alcohol induced prolonged alterations of microglia arbor morphology

Panel A illustrates the microglia arbor measurements examined. Diet (control or binge) produced significant main effects on the bounding box (B), leaf level (C), segment size (D), and stem diameter (F) of microglia. Specifically, binge exposure decreased the bounding box (height, width, and depth) ($p = .036$) and leaf level (level of branching) ($p = .044$) of the microglia. Additionally, binge increased the diameter of the basal microglial arbors ($p = .028$) and the overall size (surface area and volume) of all microglia arbor segments ($p = .04$). Microglia from sedentary control (G) exercise control (H) animals have thinner processes coming off of the soma compared to microglia from sedentary binge (I) and exercise binge animals (J). Additionally, binge exercise animals had microglia with more basal segments (stems) compared to the other three groups ($p = .028$).

Table 1

Morphological measurements used for analysis of neuronal and microglial nuclei

Morphological Measurements	Description	Cell Type	
		Microglia	Neurons
Cell Number	Average number of specific cell type nuclei per tile.	$p = .00016^*$	NS
Volume	Number of voxels in specific nuclei.	NS	NS
Surface Area	Number of voxels on the surface of specific nuclei.	NS	NS
Orientation	Angle between the major axis of the best-fit hyper-ellipsoid and origin.	NS	NS

*
interaction of binge and exercise

Table 2

Feature groupings used for group LASSO analysis of microglia and astrocyte somas and arbors

Grouped Feature	Description
Bounding Box	Height (X), Width (Y), Depth (Z) of each cell
Total Volume	Number of pixels in arbors
Total Surface Area	Number of pixels on surface of arbors
Skewness	Skewness in the X, Y, and Z planes
Soma Size	Surface area and volume of soma
Leaf Nodes	Number of end tips of the arbors
Segments	Number of compartments in all arbors
Stems	Number of basal segments
Diameter	Diameter of stems
Segment Size	Surface area and volume of all segments
Stem Length	Length of stems
Segment Length	Length of all segments, including stems
Leaf Length	Length of the last segment ending in the tip
Leaf Level	Number of levels of branching
Bifurcations	Bifurcations
Branch	Number of stems with bifurcations