Published in final edited form as:

Neuroscience. 2019 February 21; 400: 98–109. doi:10.1016/j.neuroscience.2018.12.044.

Region Specific Differences in Morphometric Features and Synaptic Colocalization of Astrocytes During Development

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Abstract

It is well established that astrocytes play pivotal roles in neuronal synapse formation and maturation as well as in the modulation of synaptic transmission. Despite their general importance for brain function, relatively little is known about the maturation of astrocytes during normal postnatal development, especially during adolescence, and how that maturation may influence astroglial-synaptic contact. The medial prefrontal cortex (mPFC) and dorsal hippocampus (dHipp) are critical for executive function, memory, and their effective integration. Further, both regions undergo significant functional changes during adolescence and early adulthood that are believed to mediate these functions. However, it is unclear the extent to which astrocytes change during these late developmental periods, nor is it clear whether their association with functional synapses shifts as adolescent and young adult maturation proceeds. Here we utilize an astrocyte-specific viral labeling approach paired with high resolution single cell astrocyte imaging and threedimensional reconstruction to determine whether mPFC and dHipp astrocytes have temporally distinct maturation trajectories. mPFC astrocytes, in particular, continue to mature well into emerging adulthood (postnatal day 70). Moreover, this ongoing maturation is accompanied by a substantial increase in colocalization of astrocytes with the postsynaptic neuronal marker, PSD-95. Taken

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AUTHOR CONTRIBUTIONS

M-LR, HSS, KJR and AT designed the experiment, M-LR, HGS, MA, SH, and KD performed the surgeries and processed the tissue, AT acquired and processed the images, analyzed the data and prepared the figures. The manuscript was written by M-LR and AT, edited by M-LR, AT, HGS, HSS and KJR and reviewed by all parties.

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AUTHORS REPORT NO CONFLICTS OF INTEREST (COI)

together, these data provide novel insight into region-specific astrocyte-synapse interactions in late CNS development and into adulthood, thus raising implications for the mechanism of post-adolescent development of the mPFC.

Graphical Abstract

Keywords

astrocyte; development; adolescence; Lck-GFP; postsynaptic density protein 95; three-dimensional reconstruction

INTRODUCTION

The ability of astrocytes to ensheath synapses and communicate with multiple neurons places them in the ideal position to regulate the initiation, formation, maturation, and maintenance of synapses throughout life. These regulatory processes occur through the secretion of a variety of astrocyte signaling factors and via contact-mediated signals (Garrett & Weiner 2009, Risher & Eroglu 2012), both of which depend on physical proximity to the synaptic region. Extracellular matrix proteins, including the thrombospondin (TSP) family, are astrocyte-secreted factors localized at pre- and postsynaptic neuronal processes that promote their structural formation and maturation (Christopherson et al 2005, Eroglu et al 2009, Kucukdereli et al 2011). While TSPs are involved in the initiation of synaptogenesis, other secreted and contact-mediated factors are also critical for various stages of this process. For example, the emergence of synapse function requires signaling via astrocyte-secreted glypicans (Allen et al 2012, Allen & Eroglu 2017, Allen & Lyons 2018, Farhy-Tselnicker & Allen 2018, Farhy-Tselnicker et al 2017), while neuroligins (cell adhesion proteins) have been shown to not only modulate astrocyte morphology via contact-mediated signaling but also to influence neuronal excitatory-inhibitory balance (Allen & Eroglu 2017, Stogsdill & Eroglu 2017, Stogsdill et al 2017).

While the mechanisms by which astrocytes extend their fine processes toward synaptic regions, thereby enabling these highly controlled events to occur, is beginning to be elucidated, the developmental trajectories of the relationship between astrocytes and synaptic regions throughout adolescence and into adulthood are less clear (Molofsky & Deneen 2015). Investigation into this trajectory is fundamental to understanding brain development throughout adolescence, adulthood, and the aging brain. Adolescence, despite

being a period of critical maturation for many brain regions, has historically been overlooked but is now the subject of intensive investigation due to the unique sensitivity to stress and neurotoxic effects of illicit drugs and alcohol (Lockhart et al 2018, Spear 2018).

Importantly, the prefrontal cortex circuits, known to be involved in executive function (e.g., forethought and inhibitory control), continue to mature throughout adolescence and into early adulthood in rodents and humans (Colonnese et al 2008, Tian & Ma 2017) and the hippocampal formation shows marked differences in excitability and drug responsiveness in adolescence compared to adulthood (Spear & Swartzwelder 2014). Moreover, this is a period in which psychiatric disorders and indications of future substance abuse begin to emerge (Lockhart et al 2018). Accordingly, we sought to delineate the time course of astrocyte morphometric change and synaptic colocalization to astrocytes in two brain regions which undergo critical developmental changes throughout adolescence.

Previous studies investigating the relationship between astrocytes and neurons have relied heavily on labor-intensive electron microscopy or immunohistochemical techniques using antibodies against the traditional astrocytic marker, glial fibrillary acidic protein (GFAP). These approaches come with a critical caveat as GFAP labeling only allows for visualization of primary, secondary, and occasionally tertiary branches of the astrocyte, thus failing to capture the processes that actually ensheath synapses (Bushong et al 2002). This incomplete labeling provides incomplete structural information, as approximately 50% of the mature astrocyte volume and 80% of the total surface area (Chao et al 2002, Wolff 1970) are composed of finer processes that are not captured using typical antibody staining techniques.

With new technological advances in microscopy and cell labeling techniques (Scofield et al 2016, Shigetomi et al 2011, Silver & Stryker 2000, Testen et al 2018), we are now able to systematically assess detailed astrocyte structural features within the rodent brain, improving our understanding of how astrocytes elaborate and refine their processes to influence local synaptic activity during development and into adulthood. Here we combine targeted adeno-associated virus (AAV) labeling techniques with immunohistochemistry to reconstruct individual astrocytes in their entirety at multiple time points throughout periadolescent development and into adulthood (PND24, 30, 44-47, and 70) within the medial prefrontal cortex (mPFC) and dorsal hippocampus (dHipp). Using this approach, we can observe that mPFC and dHipp astrocytes have temporally unique maturation trajectories in which astrocytes continue to mature well into adulthood (PND70) within the mPFC but not in the dHipp. This ongoing maturation is accompanied by a substantial increase in astrocyte-synaptic colocalization, indicative of increased interactions between the two. This process appears to be locally controlled and coincides with observations of continued cortical development in sexually mature animals and humans (Crews et al 2007, Semple et al 2013, Tian & Ma 2017). These data provide novel insight into how astrocyte morphology is regionally and temporally controlled, and it begins to elucidate the developmental regulation of astrocytes' interaction with synapses during the critical transition from early life into adulthood.

METHODS

All of the procedures used in this study were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and the National Research Council's Guide for Care and Use of Laboratory Animals and were approved by the Durham VA Medical Center.

Animals and Surgical Procedures.

Male Sprague Dawley rats of various ages were purchased from Charles River (Indianapolis, IN). They were double-housed in a normal light cycle (9AM-9PM) with *ad libitum* access to food and water. After approximately one week of vivarium habituation, animals were moved to the surgery room and administered mannitol (0.3ml/kg, 25% w/v, i.p.) (Fu et al., 2003, Carty et al., 2010). Animals were anaesthetized with a cocktail of ketamine (30 mg/kg), xylazine (2.5 mg/kg), and acepromazine (0.5 mg/kg), and secured in the stereotax. For visualization of astrocytes we used a green fluorescent protein (GFP) with the lymphocyte protein tyrosine kinase (Lck) tag. Lck is a short amino acid sequence within a Src family of protein tyrosine kinases that leads to the membrane insertion of the GFP and subsequent enhanced visualization of finer astrocyte processes over soluble protein reporters (Benediktsson et al 2005, Shigetomi et al 2013, Zlatkine et al 1997). Lck-GFP was expressed under the control of the astrocyte-specific GfaABCID promoter using adeno-associated virus type 5 (Scofield et al 2016, Shigetomi et al 2013, Testen et al 2018). The viral construct was unilaterally microinjected with 26-gauge injectors (1.0 μ L per side, 7.3×10^{12} particles/mL at 0.1 μ L/min) with a dwell time of 15 minutes at the following coordinates (minor adjustments were made based on age): +3.3mm anterior/posterior, +/- 0.6mm medial/lateral, -3.5mm dorsal/ventral for mPFC, and -3.2mm anterior/posterior, +/- 2.6mm medial/lateral, +3.0mm dorsal/ventral for dHipp. Animals that received surgery at the two earliest time points (PND10 for the PND 24 group and PND 16 for the PND 30 group) were still at a pre-weaning stage of development. They were thus removed from the litter, received surgery, and placed back into their original litter. The older groups were double housed prior to surgery and single housed following surgery until tissue collection. The duration of maternal care prior to weaning was constant for all four groups.

Immunohistochemistry.

Fourteen days post-surgery (at PND 24, 30, 45-48, 70), animals were anesthetized with isoflurane, perfused with phosphate buffered saline (PBS, 138mM NaCl, 2.7mM KCl, pH7.4) for 5 minutes followed by 4% PFA for 25 minutes (20mL/min). Brains were harvested and post-fixed in 4% PFA at 4°C overnight followed by 30% sucrose in PBS at 4°C for 1-2 days. Brains were placed in 2:1 of 30% sucrose to OCT freezing compound (TissueTek, Electron Microscopy Sciences) and stored at -80°C. 80 μ m slices were collected from the cortical and hippocampal regions in their entirety. Sections underwent antigen retrieval (as described in Jiao et al., 1999). Briefly, free-floating sections were rinsed three times (5 minutes each) in 0.1M PB (3.1g/L NaH₂PO₄, 10.9g/L Na₂HPO₄, pH 7.4) then transferred to 10mM sodium citrate buffer preheated to 80°C for 30 minutes. Sections were cooled and washed 3 times (5 minutes each) in 0.1M PB (pH 7.4). Sections were blocked with 5% normal goat serum (NGS, Jackson Immunolabs, cat#005-000-121) in PBST (2%

triton 100-X, Roche, cat#13134900) for one hour and probed at 4°C for four days in anti-PSD-95 (1:450 Thermo, cat# MA1-045) and rabbit anti-GFAP (1:500 cat#DAKO Z0334) in PBST (2% triton, 5% NGS). Sections were washed three times in PBST (0.2% triton) and incubated for 8 hours at room temperature in Alexa Fluor goat anti-mouse 555 (Thermofisher, cat#A21424) and Alexa Fluor goat anti-rabbit 647 (Thermofisher, cat#A21245) (1:200) in PBST (2% triton X-100, 5% NGS). Sections were washed three times in PBST (0.2% triton) and once in PBS. Slices were mounted with Vectorshield (Vector Laboratories, cat#H-1200) and coverslips were sealed with nail varnish.

Data acquisition and processing.

A Zeiss LSM 800 laser-scanning confocal microscope with 405, 488, 561 and 640 diode lasers, Gallium Arsenide Phosphid (GaAsP) detectors, 63× oil-immersive objective (Zeiss, Oberkochen, DE) and ZEN software suite (ZEN 2.3-Blue edition, Zeiss) was used for image acquisition. Acquisition parameters were set at 1024×1024 pixels frame size, 16-bit depths, 4× averaging, 1 µm z-step size. Lck-GFP expression pattern is diffused to a great extent thus aiding in the acquisition of single, isolated astrocytes. A limited amount of signal saturation was observed in dHipp (Figure 1B-I) likely due to the isolated nature of the hippocampus (which is flanked by alveus and fimbria of the hippocampus, respectively), preventing the diffusion of the virus beyond the surrounding white matter tracks. Based on a previous report suggesting that astrocyte volumes from different hippocampal subregions (CA1, CA3 and DG) do not vary significantly (Bindocci et al 2017), hippocampal astrocytes were merged into one group and analyzed collectively.

AutoQuant software (v. X3.0.4, MediaCybernetics) was used to deconvolve raw images before the reconstruction. Algorithm for blind deconvolution with 10 iterations was run on each z-stack. Output file was directly imported to the Imaris software (v. 8.4.1, Bitplane, Zurich, CH) for 3-dimensional reconstruction. Using Imaris, each individual astrocyte was first isolated from a Lck-GFP background signal using a surface building feature. Surface rendering enables extraction of the surface area and volume values, and generates a new Lck-GFP channel devoid of any background, encompassing only a signal from a single isolated astrocyte. Close attention was paid to verify collected Lck-GFP signal indeed accounted for a single astrocyte, collected in its entirety. To this end, colocalization of the Lck-GFP and GFAP as well as Lck-GFP and DAPI signals were performed and analyzed. Lck-GFP and DAPI colocalization can readily return multiple nuclei since astrocytes connect to neuronal somata as they do endothelial cells of the vasculature. At the same time colocalization of Lck-GFP and GFAP can produce a complex yet uninformative branching pattern. Only results of both colocalizations together produce a reliable validation of the single astrocyte acquisition (Supplemental video SV1). The isolated Lck-GFP channel (surface mask) was used, in conjunction with the PSD-95 channel, to perform colocalization analysis as a proxy for proximity between the astrocytes and post-synaptic neuronal terminals. It should be noted the colocalization analysis is not a substitute for detection of direct molecular interactions, and indicates one or multiple fluorescent signals within a given voxel. Voxel dimensions were 0.099 µm × 0.099 µm × 1.000 µm for x, y and z, respectively. Before the colocalization analysis the threshold for a PSD-95 signal was manually determined by measuring fluorescent intensity of unambiguous PSD-95 positive

puncta on multiple optical planes. An average of these measurements was used as a final PSD-95 signal threshold value. Colocalization is reported as percentage of region of interest (ROI, being a new masked Lck-GFP channel) colocalized with the PSD-95 channel.

PSD-95 expression was evaluated for each cell separately. A 50×50×50 µm box was built around the isolated astrocyte, and PSD-95 positive puncta above the previously determined threshold level were automatically counted within the box's volume. Throughout the experiment, the experimenter was blinded to the groups, tissue samples and computer files to avoid potential bias during the analysis.

Statistical analysis:

Data were grouped using Excel 365 (Microsoft, Redmond, WA) and analyzed using Prism (v. 8.0.0, GraphPad, La Jolla, CA) and SAS (v. 9.4, SAS Institute, Cary, NC). Nested ANOVA analysis was performed on all groups (Heimer-McGinn et al 2013, Risher et al 2014) with a Tukey-Kramer post hoc test. $p < 0.05$ was considered significant.

RESULTS

Post-adolescent increase in astrocyte surface area and volume in mPFC but not in the dHipp.

Membrane-tagged Lck-GFP expressed under the control of the GfaABC1D promoter allowed visualization and quantification of morphometric features of astrocytes at different developmental timepoints (Figure 1A). Fluorescent signal in the dHipp (Figure 1B I) as well as in mPFC (Figure 1B II) showed a robust and sparsely diffused expression pattern, enabling unobstructed single cell imaging of GFAP-positive astrocytes in the dHipp (Figure 1C) and mPFC (Figure 1D). A surface building function was used on isolated astrocytes (Figure 2A, 3A) to enable extraction of the morphometric data. A significant increase in mPFC astrocyte surface area was observed at PND70 compared to all other time-points (Figure 2B-I; PND24: $29,074 \pm 2,356 \mu\text{m}^2$; PND30: $26,895 \pm 2,619 \mu\text{m}^2$; PND44-47: $32,185 \pm 2,391 \mu\text{m}^2$; PND70: $44,212 \pm 2,629 \mu\text{m}^2$; $p\text{-value} = 0.008$, $F\text{-value} = 8.83$; significant post-hoc test (Tukey-Kramer): PND24:PND70 – adjusted $p\text{-value} 0.002$; PND30:PND70 – adjusted $p\text{-value} 0.001$; PND44-47:PND70 – adjusted $p\text{-value} 0.016$). In contrast, this developmental difference was not observed in astrocytes from dHipp (Figure 3B-I; PND24: $34,154 \pm 3,256 \mu\text{m}^2$; PND30: $32,882 \pm 2,799 \mu\text{m}^2$; PND44-47: $36,488 \pm 2,973 \mu\text{m}^2$; PND70: $41,063 \pm 2,983 \mu\text{m}^2$; $p\text{-value} = 0.243$, $F\text{-value} = 1.49$). The increase in surface area of mPFC astrocytes was accompanied by a statistically significant increase in astrocyte volume at PND70 (Figure 2B-II; PND24: $14,696 \pm 1,088 \mu\text{m}^3$; PND30: $13,594 \pm 1,226 \mu\text{m}^3$; PND44-47: $16,035 \pm 1,119 \mu\text{m}^3$; PND70: $19,203 \pm 1,234 \mu\text{m}^3$; $p\text{-value} = 0.025$, $F\text{-value} = 3.97$; significant post-hoc test (Tukey-Kramer): PND30:PND70 – adjusted $p\text{-value} 0.022$; PND24:PND70 – adjusted $p\text{-value} 0.059$ (trending)). Consistent with the astrocyte area measurements, this developmental increase in astrocyte volume was also limited to mPFC, with no statistically significant changes in astrocyte volume observed in the dHipp (Figure 3B-II; PND24: $17,487 \pm 1,565 \mu\text{m}^3$; PND30: $18,237 \pm 1,345 \mu\text{m}^3$; PND44-47: $18,787 \pm 1,425 \mu\text{m}^3$; PND70: $19,758 \pm 1,431 \mu\text{m}^3$; $p\text{-value} = 0.743$, $F\text{-value} = 0.42$).

Post-adolescent increase in astrocyte synaptic contact in the mPFC.

In order to assess developmental effects on synaptic colocalization of astrocytes the degree of colocalization between astrocytic Lck-GFP signal and IHC signal from PSD-95 neuronal postsynaptic marker was measured (Figure 4). There was a statistically significant increase in the degree of synaptic colocalization of mPFC astrocytes at PND70 compared to two earlier time points (Figure 5A-I; PND24: $4.1064 \pm 0.4553\%$; PND30: $2.4007 \pm 0.5027\%$; PND44-47: $3.3206 \pm 0.4589\%$; PND70: $5.7903 \pm 0.5037\%$; p-value = 0.001, F-value = 8.26; significant post-hoc test (Tukey-Kramer): PND30:PND70 – adjusted p-value 0.001; PND44-47:PND70 – adjusted p-value 0.009). This developmental increase in colocalization was absent in dHipp, where no statistically significant change in synaptic contact by astrocytes was observed in the post-adolescent period compared to earlier time points (Figure 5A-II; PND24: $5.6239 \pm 0.7077\%$; PND30: $3.9517 \pm 0.6102\%$; PND44-47: $4.2963 \pm 0.6506\%$; PND70: $4.6217 \pm 0.6217\%$; p-value = 0.352, F-value = 1.14). The analysis also shows PSD-95 expression remained stable with no statistically significant changes observed in either mPFC (Figure 5B-I; PND24: $5,259.28 \pm 432.48$; PND30: $3,975.10 \pm 471.66$; PND44-47: $4,391.56 \pm 432.46$; PND70: $5,445.69 \pm 472.75$; p-value = 0.111, F-value = 2.31) or dHipp (Figure 5B-II; PND24: $4,899.28 \pm 607.67$; PND30: $5,554.22 \pm 523.21$; PND44-47: $5,677.89 \pm 558.26$; PND70: $5,776.88 \pm 559.38$; p-value = 0.723, F-value = 0.44) across the observed timepoints.

DISCUSSION

Results from the current study reveal two fundamental principles regarding maturation of mammalian astrocytes. Specifically, (1) astrocyte growth (assessed by measuring total surface area and volume), and increased astrocyte-synaptic colocalization, continue well into adulthood in the rat mPFC, and (2) astrocyte maturation was observed during adolescence in mPFC but not in dHipp. These findings bolster the emerging literature related to the critical roles of astrocytes with regard to synaptogenesis and neural circuit formation and function (Baldwin & Eroglu 2017, Miller & Gauthier 2007) and carry significant implications regarding the regional specificity of astrocyte-synapse interactions in the late-maturing CNS. Importantly, they also address the previously unexplored question of astrocyte maturation during adolescent and young adult development. As such, these findings can serve to guide future investigations of astrocyte development, synaptogenesis, and experience-dependent effects on astrocyte maturation and consequently neural function across adolescence and early adulthood.

The currently appreciated developmental trajectory of astrocytes includes astrogliogenesis starting in the late embryonic stage into the first few weeks postnatally, with finer processes continuing to emerge and develop for several more weeks thereafter (Molofsky & Deneen 2015, Sauvageot & Stiles 2002, Tien et al 2012, Yang et al 2013). For example, in EAAT2 tdT reporter mice cortical astrocyte arborization and synaptic coverage increases from PND14-26 (Morel et al 2014) following initial neurogenic and gliogenic periods. A recent paper by Stogsdill and colleagues (2017) demonstrates a role for contact-mediated processes, via astrocytic neuroligins and neuronal neuroligins, in establishing astrocyte complexity in cortical layers using Ald1L1-EGFP BAC-transgenic mice during early

postnatal development (Stogsdill et al 2017). The current findings indicate that ongoing maturation of astrocytes in the mPFC continues well into late adolescence and early adulthood while, interestingly, being absent in the hippocampus.

Increased surface area is functionally significant because approximately 80% of the astrocyte total surface area consists of fine processes (Wolff 1970) and accordingly, astrocyte coverage of synapses accounts for ~50-90% of synapses, depending on brain region and type of synapse (Verkhratsky & Nedergaard 2014). Among the functional significance of these interactions, synapses require astrocytic contact to mediate cell adhesion molecule engagement and for the generation of appropriate stable excitatory and inhibitory synapses, while astrocytes require contact-mediated interactions to develop appropriate complexity (Stogsdill et al 2017). Further, alterations in astrocyte-secreted contact and signaling factors have been implicated in neuronal morphology and synaptic changes seen in many neurodegenerative disorders (Blanco-Suarez et al 2017).

Colocalization analysis in the current study indicates a nonsignificant trend toward a decrease in colocalization of astrocytes with PSD-95 following PND 24 in both brain regions (Figure 5A-I, II). Reports show up to 50% of all synapses within specific circuits are pruned during adolescence (Rakic et al 1994, Spear 2013); thus, circuit pruning might contribute toward this apparent decrease. Of relevance, we did not observe a significant decrease in PSD-95 expression at this time point following PND 24, consistent with findings from other laboratories (Pinto et al 2013, Shapiro et al 2017). Also, it has been shown that astrocyte processes do not colocalize with all synapses, and that coverage varies greatly depending on the brain region (see review by (Bernardinelli et al 2014)), however nuances across development and particularly into adolescence remain unexplored. We can speculate that if astrocytes are only being colocalized with a portion of synapses/PSD-95, and those synapses are preferentially eliminated via astrocyte-mediated phagocytosis (Chung et al 2013), this may explain why we see such a trending, yet not statistically significant, decline in colocalization between PND 24 and PND 30 in both brain regions. The quantitative impact of these events could be enhanced due to astrocytes preferentially targeting a subpopulation of synapses that have been 'tagged' for elimination. However, if this elimination accounts for a small portion of synapses overall, assessing total PSD-95 expression may not be a sufficiently sensitive measure to capture the nuances of these pruning events. More work will be required to investigate these events in the context of synaptic pruning. Future experiments will undoubtedly be important for understanding the role of astrocytes in developmental synaptic refinement.

For efficient and localized communication and signaling to occur, astrocytic processes and synapses must be in close proximity. Electron microscopy and super-resolution studies reliably show astrocyte processes within tens of nanometers of neuronal terminals (Heller et al 2017, Heller & Rusakov 2015, Panatier et al 2014) although functionally relevant distances might very well be greater. For example, it was recently shown that striatal astrocyte-neuron colocalization analysis at distances of up to 600 nm can report changes in interactions in response to oxygen-glucose deprivation and genetic mutations in a Huntington's Disease mouse model (Oceau et al 2018). Moreover, serial block face electron microscopy indicates that the average distance between excitatory synaptic terminals and

striatal astrocyte processes is approximately 50 nm, with 47% of astrocyte-synaptic pairs being between 10 nm and 400 nm apart, while the average distance between striatal astrocytes and TH-positive synaptic elements is 250 nm, with 89% being between 10 nm and 1,100 nm apart (Octeau et al 2018). These findings collectively indicate that our colocalization analysis, reporting changes within a ~200 nm window, likely reflects physiologically meaningful changes.

Regional and circuit specificity of post-adolescent astrocyte maturation

While differences among astrocytes, such as GFAP expression, have long been appreciated, more systematic analyses of region-specific heterogeneity of astrocytes have only recently been performed. Early work demonstrated region-dependent variations in astrocyte-specific genes from microarray datasets both *in vitro* (Bachoo et al 2004) and *in vivo* (Yeh et al 2009). More recently, a very comprehensive assessment of striatal and hippocampal astrocyte characteristics was conducted (Chai et al 2017). However, only one time point (PND 59-64) was used to compare these brain regions, and more focus was placed on the relationship between spine type and proximity rather than any developmental/temporal changes that may have been occurring (Chai et al 2017).

The present results also suggest that any attempts to understand how and when the proximity events that drive astrocyte-synapse interactions occur will likely be complicated by their brain region-, circuit-, and temporal-dependency. A recent study using adult (PND 70) BAC *aldh111* TRAP mice showed that in addition to the regional- and temporal-dependent events that control astrocyte development, there may also be a circuit-dependent component that follows a dorsal to ventral pattern (Morel et al 2017). Though these findings are exciting with regard to how astrocytes may shape circuit connectivity, much more work is required to fully understand how these changes relate to the development of astrocyte-synapse colocalization across development and into adulthood.

Implications for Human Health

Our findings indicate that developmental astrocyte-dependent synaptic modulation in the mPFC continues beyond adolescence (~PND 30-50) and into emerging/young adulthood (~PND 65), whereas dHipp astrocyte maturation occurs much earlier. This timeline is consistent with mPFC-specific cortical synaptic development and maturation that can continue well into young adulthood (~PND 55) (Crews et al 2007, Semple et al 2013, Tian & Ma 2017). The prolonged development of the mPFC has been associated with delayed development of executive function, which continues throughout the late teens and early twenties in humans as forebrain areas continue to mature and refine local circuits. This trajectory is consistent across humans and rodents (Schubert et al 2015). Accordingly, the present data demonstrate a likely and important role of astrocytes in the emergence of frontally-mediated cognitive functioning, and the integration of cognitive with affective decision-making processes, which are associated with post-adolescent maturation and the development of executive function.

As noted above, adolescence is a time of dynamic physiological and behavioral change. It is a period in which many psychiatric disorders emerge, when the brain is particularly sensitive

to stress and to the neurotoxic effects of illicit drugs and alcohol. Understanding the effects of these distinct stressors on astrocyte maturation during adolescence and young adulthood, will markedly further our understanding of both the acute and enduring effects of stress and exposure to such drugs during adolescence, and may also provide insight into the etiology of substance use disorders and other diseases that begin to manifest during this period (Casey & Jones 2010, Gomes & Grace 2017, Scofield & Kalivas 2014). Thus, the present findings of changes in astrocyte morphology and astrocyte-synapse proximity across the transition from adolescence to adulthood will provide an important backdrop for such studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This research was supported by NIH/NIAAA U01-AA019925 NADIA (HSS), VA Career Development Award 11K2BX002505 (M-LR) and NARSAD Young Investigator Award 25432 (M-LR), and R01DA041455 (KJR).

REFERENCES

- Allen NJ, Bennett ML, Foo LC, Wang GX, Chakraborty C, et al. 2012 Astrocyte glypicans 4 and 6 promote formation of excitatory synapses via GluA1 AMPA receptors. *Nature* 486: 410–4 [PubMed: 22722203]
- Allen NJ, Eroglu C. 2017 Cell Biology of Astrocyte-Synapse Interactions. *Neuron* 96: 697–708 [PubMed: 29096081]
- Allen NJ, Lyons DA. 2018 Glia as architects of central nervous system formation and function. *Science* 362: 181–85 [PubMed: 30309945]
- Bachoo RM, Kim RS, Ligon KL, Maher EA, Brennan C, et al. 2004 Molecular diversity of astrocytes with implications for neurological disorders. *Proc Natl Acad Sci U S A* 101: 8384–9 [PubMed: 15155908]
- Baldwin KT, Eroglu C. 2017 Molecular mechanisms of astrocyte-induced synaptogenesis. *Curr Opin Neurobiol* 45: 113–20 [PubMed: 28570864]
- Benediktsson AM, Schachtele SJ, Green SH, Dailey ME. 2005 Ballistic labeling and dynamic imaging of astrocytes in organotypic hippocampal slice cultures. *J Neurosci Methods* 141: 41–53 [PubMed: 15585287]
- Bernardinelli Y, Muller D, Nikonenko I. 2014 Astrocyte-synapse structural plasticity. *Neural Plast* 2014: 232105 [PubMed: 24511394]
- Bindocci E, Savtchouk I, Liaudet N, Becker D, Carrierio G, Volterra A. 2017 Three-dimensional Ca(2+) imaging advances understanding of astrocyte biology. *Science* 356
- Blanco-Suarez E, Caldwell AL, Allen NJ. 2017 Role of astrocyte-synapse interactions in CNS disorders. *J Physiol* 595: 1903–16 [PubMed: 27381164]
- Bushong EA, Martone ME, Jones YZ, Ellisman MH. 2002 Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J Neurosci* 22: 183–92 [PubMed: 11756501]
- Casey BJ, Jones RM. 2010 Neurobiology of the adolescent brain and behavior: implications for substance use disorders. *J Am Acad Child Adolesc Psychiatry* 49: 1189–201; quiz 285 [PubMed: 21093769]
- Chai H, Diaz-Castro B, Shigetomi E, Monte E, Oceau JC, et al. 2017 Neural Circuit-Specialized Astrocytes: Transcriptomic, Proteomic, Morphological, and Functional Evidence. *Neuron* 95: 531–49 e9 [PubMed: 28712653]

- Chao TI, Rickman M, Wolff JR. 2002 The synapse-astrocyte boundary: an anatomical basis for an integrative role of glia in synaptic transmission In *Tripartite Synapse: Glia in Synaptic Transmission*, ed. Volterra PJMA, and Haydon PG, pp. 3–23. Oxford, UK: Oxford University Press
- Christopherson KS, Ullian EM, Stokes CC, Mallowney CE, Hell JW, et al. 2005 Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120: 421–33 [PubMed: 15707899]
- Chung WS, Clarke LE, Wang GX, Stafford BK, Sher A, et al. 2013 Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. *Nature* 504: 394–400 [PubMed: 24270812]
- Colonnese MT, Phillips MA, Constantine-Paton M, Kaila K, Jasanoff A. 2008 Development of hemodynamic responses and functional connectivity in rat somatosensory cortex. *Nat Neurosci* 11: 72–9 [PubMed: 18037883]
- Crews F, He J, Hodge C. 2007 Adolescent cortical development: a critical period of vulnerability for addiction. *Pharmacol Biochem Behav* 86: 189–99 [PubMed: 17222895]
- Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, et al. 2009 Gabapentin receptor $\alpha 2\delta$ -1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. *Cell* 139: 380–92 [PubMed: 19818485]
- Farhy-Tselnick I, Allen NJ. 2018 Astrocytes, neurons, synapses: a tripartite view on cortical circuit development. *Neural Dev* 13: 7 [PubMed: 29712572]
- Farhy-Tselnick I, van Casteren ACM, Lee A, Chang VT, Aricescu AR, Allen NJ. 2017 Astrocyte-Secreted Glypican 4 Regulates Release of Neuronal Pentraxin 1 from Axons to Induce Functional Synapse Formation. *Neuron* 96: 428–45 e13 [PubMed: 29024665]
- Garrett AM, Weiner JA. 2009 Control of CNS synapse development by $\{\gamma\}$ -protocadherin-mediated astrocyte-neuron contact. *J Neurosci* 29: 11723–31 [PubMed: 19776259]
- Gomes FV, Grace AA. 2017 Adolescent Stress as a Driving Factor for Schizophrenia Development-A Basic Science Perspective. *Schizophr Bull* 43: 486–89 [PubMed: 28419390]
- Heimer-McGinn V, Murphy AC, Kim JC, Dymecki SM, Young PW. 2013 Decreased dendritic spine density as a consequence of tetanus toxin light chain expression in single neurons in vivo. *Neurosci Lett* 555: 36–41 [PubMed: 24035894]
- Heller JP, Michaluk P, Sugao K, Rusakov DA. 2017 Probing nano-organization of astroglia with multicolor super-resolution microscopy. *J Neurosci Res* 95: 2159–71 [PubMed: 28151556]
- Heller JP, Rusakov DA. 2015 Morphological plasticity of astroglia: Understanding synaptic microenvironment. *Glia* 63: 2133–51 [PubMed: 25782611]
- Kucukdereli H, Allen NJ, Lee AT, Feng A, Ozlu MI, et al. 2011 Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins Hevin and SPARC. *Proc Natl Acad Sci U S A* 108: E440–9 [PubMed: 21788491]
- Lockhart S, Sawa A, Niwa M. 2018 Developmental trajectories of brain maturation and behavior: Relevance to major mental illnesses. *J Pharmacol Sci*
- Miller FD, Gauthier AS. 2007 Timing is everything: making neurons versus glia in the developing. *Neuron* 54: 357–69 [PubMed: 17481390]
- Molofsky AV, Deneen B. 2015 Astrocyte development: A Guide for the Perplexed. *Glia* 63: 1320–9 [PubMed: 25963996]
- Morel L, Chiang MSR, Higashimori H, Shoneye T, Iyer LK, et al. 2017 Molecular and Functional Properties of Regional Astrocytes in the Adult Brain. *J Neurosci* 37: 8706–17 [PubMed: 28821665]
- Morel L, Higashimori H, Tolman M, Yang Y. 2014 VGluT1+ neuronal glutamatergic signaling regulates postnatal developmental maturation of cortical protoplasmic astroglia. *J Neurosci* 34: 10950–62 [PubMed: 25122895]
- Octeau JC, Chai H, Jiang R, Bonanno SL, Martin KC, Khakh BS. 2018 An Optical Neuron-Astrocyte Proximity Assay at Synaptic Distance Scales. *Neuron* 98: 49–66 e9 [PubMed: 29621490]
- Panatier A, Arizono M, Nagerl UV. 2014 Dissecting tripartite synapses with STED microscopy. *Philos Trans R Soc Lond B Biol Sci* 369: 20130597 [PubMed: 25225091]
- Pinto JG, Jones DG, Murphy KM. 2013 Comparing development of synaptic proteins in rat visual, somatosensory, and frontal cortex. *Front Neural Circuits* 7: 97 [PubMed: 23754984]

- Rakic P, Bourgeois JP, Goldman-Rakic PS. 1994 Synaptic development of the cerebral cortex: implications for learning, memory, and mental illness. *Prog Brain Res* 102: 227–43 [PubMed: 7800815]
- Risher WC, Eroglu C. 2012 Thrombospondins as key regulators of synaptogenesis in the central nervous system. *Matrix Biol* 31: 170–7 [PubMed: 22285841]
- Risher WC, Patel S, Kim IH, Uezu A, Bhagat S, et al. 2014 Astrocytes refine cortical connectivity at dendritic spines. *Elife* 3
- Sauvageot CM, Stiles CD. 2002 Molecular mechanisms controlling cortical gliogenesis. *Curr Opin Neurobiol* 12: 244–9 [PubMed: 12049929]
- Schubert D, Martens GJM, Kolk SM. 2015 Molecular underpinnings of prefrontal cortex development in rodents provide insights into the etiology of neurodevelopmental disorders. *Molecular Psychiatry* 20: 795–809 [PubMed: 25450230]
- Scofield MD, Kalivas PW. 2014 Astrocytic dysfunction and addiction: consequences of impaired glutamate homeostasis. *Neuroscientist* 20: 610–22 [PubMed: 24496610]
- Scofield MD, Li H, Siemsen BM, Healey KL, Tran PK, et al. 2016 Cocaine Self-Administration and Extinction Leads to Reduced Glial Fibrillary Acidic Protein Expression and Morphometric Features of Astrocytes in the Nucleus Accumbens Core. *Biol Psychiatry* 80: 207–15 [PubMed: 26946381]
- Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. 2013 Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol* 106-107: 1–16 [PubMed: 23583307]
- Shapiro LP, Parsons RG, Koleske AJ, Gourley SL. 2017 Differential expression of cytoskeletal regulatory factors in the adolescent prefrontal cortex: Implications for cortical development. *J Neurosci Res* 95: 1123–43 [PubMed: 27735056]
- Shigetomi E, Bushong EA, Hausteiner MD, Tong X, Jackson-Weaver O, et al. 2013 Imaging calcium microdomains within entire astrocyte territories and endfeet with GCaMPs expressed using adeno-associated viruses. *J Gen Physiol* 141: 633–47 [PubMed: 23589582]
- Shigetomi E, Tong X, Kwan KY, Corey DP, Khakh BS. 2011 TRPA1 channels regulate astrocyte resting calcium and inhibitory synapse efficacy through GAT-3. *Nat Neurosci* 15: 70–80 [PubMed: 22158513]
- Silver MA, Stryker MP. 2000 A method for measuring colocalization of presynaptic markers with anatomically labeled axons using double label immunofluorescence and confocal microscopy. *J Neurosci Methods* 94: 205–15 [PubMed: 10661840]
- Spear LP. 2013 Adolescent neurodevelopment. *J Adolesc Health* 52: S7–13 [PubMed: 23332574]
- Spear LP. 2018 Effects of adolescent alcohol consumption on the brain and behaviour. *Nat Rev Neurosci* 19: 197–214 [PubMed: 29467469]
- Spear LP, Swartzwelder HS. 2014 Adolescent alcohol exposure and persistence of adolescent-typical phenotypes into adulthood: a mini-review. *Neurosci Biobehav Rev* 45: 1–8 [PubMed: 24813805]
- Stogsdill JA, Eroglu C. 2017 The interplay between neurons and glia in synapse development and plasticity. *Curr Opin Neurobiol* 42: 1–8 [PubMed: 27788368]
- Stogsdill JA, Ramirez J, Liu D, Kim YH, Baldwin KT, et al. 2017 Astrocytic neuroligins control astrocyte morphogenesis and synaptogenesis. *Nature* 551: 192–97 [PubMed: 29120426]
- Testen A, Sepulveda-Orengo MT, Gaines CH, Reissner KJ. 2018 Region-Specific Reductions in Morphometric Properties and Synaptic Colocalization of Astrocytes Following Cocaine Self-Administration and Extinction. *Front Cell Neurosci* 12: 246 [PubMed: 30147645]
- Tian L, Ma L. 2017 Microstructural Changes of the Human Brain from Early to Mid-Adulthood. *Front Hum Neurosci* 11: 393 [PubMed: 28824398]
- Tien AC, Tsai HH, Molofsky AV, McMahon M, Foo LC, et al. 2012 Regulated temporal-spatial astrocyte precursor cell proliferation involves BRAF signalling in mammalian spinal cord. *Development* 139: 2477–87 [PubMed: 22675209]
- Verkhratsky A, Nedergaard M. 2014 Astroglial cradle in the life of the synapse. *Philos Trans R Soc Lond B Biol Sci* 369: 20130595 [PubMed: 25225089]
- Wolff JR. 1970 The astrocyte as link between capillary and nerve cell. *Triangle* 9: 153–64 [PubMed: 5521752]

- Yang Y, Higashimori H, Morel L. 2013 Developmental maturation of astrocytes and pathogenesis of neurodevelopmental disorders. *J Neurodev Disord* 5: 22 [PubMed: 23988237]
- Yeh TH, Lee DY, Gianino SM, Gutmann DH. 2009 Microarray analyses reveal regional astrocyte heterogeneity with implications for neurofibromatosis type 1 (NF1)-regulated glial proliferation. *Glia* 57: 1239–49 [PubMed: 19191334]
- Zlatkine P, Mehul B, Magee AI. 1997 Retargeting of cytosolic proteins to the plasma membrane by the Lck protein tyrosine kinase dual acylation motif. *J Cell Sci* 110 (Pt 5): 673–9 [PubMed: 9092949]

Highlights:

- There is continued, region-specific maturation of astrocytes present through adolescence and into adulthood
- Cortical astrocytes continue to increase their volume and surface area into adulthood
- Increased astrocyte-synapse colocalization is also observed into adulthood

Figure 1:

Experimental timeline and specificity validation of Lck-GFP expression. (A) The analysis was performed three weeks after animal arrival at the PND 24, 30, 44-47 and 70 time points. (B) Tiled scans of brain regions analyzed, showing robust Lck-GFP expression in the dorsal hippocampus (dHipp, I) and prelimbic region of prefrontal cortex (mPFC, II). Dashed rectangle (dHipp) and dashed square (mPFC) represent the general regions of interest where the astrocytes were imaged, while the smaller solid line squares demark the area later represented in panels C (I-IV) and D (I-IV), respectively. Scale bar 1 mm. (C) 10×

magnification of the medial part of dHipp showing sparsely labeled Lck-GFP expressing astrocytes (II) as well nuclear stain DAPI (I), IHC for astrocyte-specific GFAP (III) and a merger of all channels (IV). Scale bar 100 μm . Area within the dashed squares magnified below every image (V-VIII) showing in more detail individually labeled Lck-GFP astrocytes (VI), undisturbed GFAP network (VII) and colocalization of the Lck-GFP and GFAP channels (white), confirming astrocyte specificity of Lck-GFP (VIII). Scale bar 50 μm . (D) Same as above, only showing Lck-GFP and GFAP expression in the mPFC. Scale bar (I-IV) 100 μm , (V-VIII) 50 μm .

Figure 2: Single cell imaging and measurements indicate post-adolescent increase in astrocyte surface area and volume in mPFC. (A) Panel composed of representative images of cortical astrocytes, showing isolated, Lck-GFP expressing cells at each of the selected developmental time points. Below them are their respective 3-D surface renderings. The surface renderings were used to extract measurements of surface area and volume for each individual astrocyte. Scale bar 8 μm . (B) Scatter plots showing measurements extracted from above depicted surface renderings. Surface area (I) (p-value = 0.008 as reported by nested

ANOVA, F-value = 8.83; significant post-hoc test (Tukey-Kramer): PND24:PND70 – adjusted p-value 0.002; PND30:PND70 – adjusted p-value 0.001; PND44-47:PND70 – adjusted p-value 0.016) and volume (II) (p-value = 0.025 as reported by nested ANOVA, F-value = 3.97; significant post-hoc test (Tukey-Kramer): PND30:PND70 – adjusted p-value 0.022; PND24:PND70 – adjusted p-value 0.059 (trending)) of mPFC astrocytes were significantly increased at the PND70 time point compared to other time points. Error bars represent SEM, bar inserts represent the number of animals used and the number of cells analyzed (in brackets). * = ($p < 0.05$)

Figure 3: Single cell imaging and measurements indicate a lack of post-adolescent increase in astrocyte surface area and volume in dHipp. (A) Panel composed of representative images of hippocampal astrocytes, showing isolated, Lck-GFP expressing cells at each of the selected developmental time points. Below them are their respective 3-D surface renderings. The surface renderings were used to extract measurements of surface area and volume for each individual astrocyte. Scale bar 8 μm . (B) Scatter plots showing measurements extracted from above depicted surface renderings. There was no statistically significant changes in either

surface area (I) (p-value = 0.243 (nested ANOVA), F-value = 1.49) or volume (II) (p-value = 0.743 (nested ANOVA), F-value = 0.42) in the area of dHipp at any of the four developmental time points. Error bars represent SEM, bar inserts represent the number of animals used and the number of cells analyzed (in brackets). * = ($p < 0.05$)

Figure 4:

Astrocyte-PSD-95 colocalization analysis in the mPFC. Displayed are previously shown isolated mPFC astrocytes expressing Lck-GFP (I) at all four developmental time points as well as IHC for post-synaptic neuronal protein PSD-95 (II). These two channels are then merged (III) and voxels colocalized are shown in white (IV). Scale bar 8 μ m.

Figure 5:

Post-adolescent increase in astrocyte synaptic colocalization observed in mPFC but not in dHipp. (A) Scatter plot representing measurements of the Lck-GFP and PSD-95 IHC colocalization. A statistically significant increase in colocalization is observed at PND70 compared to two previous time points in mPFC (I) (p-value = 0.001 (nested ANOVA, F-value = 8.26; significant post-hoc test (Tukey-Kramer): PND30:PND70 – adjusted p-value 0.001; PND44-47:PND70 – adjusted p-value 0.009). No change in colocalization was observed in dHipp between any of the developmental time points (II) (p-value = 0.352

(nested ANOVA), F-value = 1.14). (B) PSD-95 expression remains stable with no statistically significant changes observed in either mPFC (I) (p-value = 0.111 (nested ANOVA), F-value = 2.31) or dHipp (II) (p-value = 0.723 (nested ANOVA), F-value = 0.44). Error bars represent SEM, bar inserts represent the number of animals used and the number of cells analyzed (in brackets). * = ($p < 0.05$).