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Astrocyte Fatty Acid Binding Protein-7 is a Marker for Neurogenic Niches in the Rat Hippocampus

John K. Young¹, Thomas Heinbockel¹, and Marjorie C. Gondré-Lewis¹

¹Dept. of Anatomy, Howard University College of Medicine, 520 W St., NW, Washington DC 20059

Abstract

Recent research has determined that newborn neurons in the dentate gyrus of the hippocampus of the macaque are frequently adjacent to astrocytes immunoreactive for fatty acid binding protein-7 (FABP7). To investigate if a similar relationship between FABP7-positive (FABP7+) astrocytes and proliferating cells exists in the rodent brain, sections of brains from juvenile rats were stained by immunohistochemistry to demonstrate newborn cells (antibody to Ki67 protein) and FABP7+ astrocytes. In rat brains, FABP7+ astrocytes were particularly abundant in the dentate gyrus of the hippocampus and were frequently close to dividing cells immunoreactive for Ki67 protein. FABP7+ astrocytes were also present in the olfactory bulbs, arcuate nucleus of the hypothalamus, and in the dorsal medulla subjacent to the area postrema, sites where more modest numbers of newborn neurons can also be found.

These data suggest that regional accumulations of FABP7+ astrocytes may represent reservoirs of cells having the potential for neurogenesis. Since FABP7+ astrocytes are particularly abundant in the hippocampus, and since the gene for FABP7 has been linked to Alzheimer's disease, age-related changes in FABP7+ astrocytes (mitochondrial degeneration) may be relevant to age-associated disorders of the hippocampus.

Keywords

glia; neurogenesis; hippocampus; hypothalamus; Alzheimer's

Introduction

Research performed during the early 1990's confirmed earlier observations that large numbers of neurons are born and differentiate within a few restricted regions of the juvenile and adult mammalian brains. Neurogenesis in the postnatal brain takes place mainly in the subventricular zone and within the dentate gyrus of the hippocampus. Newly divided cells initially express astrocyte proteins such as glial fibrillary acidic protein (GFAP); 60–70% of such cells mature further to acquire a neuronal phenotype (Cameron, et al., 1993; Ming and Song, 2011). Considerably more modest numbers of newborn neurons can also be detected in other brain regions such as the olfactory bulbs, arcuate nucleus of the hypothalamus, the medial habenula, and in the dorsal medulla subjacent to the area postrema (Cifuentes, 2011; Pierce and Xu, 2010). Ischemia or deafferentation of these brain areas, or exposure of these areas to growth factors such as epidermal growth factor, can substantially increase postnatal neurogenesis (Cifuentes, 2011; Pecchi, et al., 2007; Pierce and Xu, 2010).

One unresolved aspect of postnatal neurogenesis is the question of how these restricted neurogenic niches promote the birth of new neurons. The presence of specialized astrocytes within these niches and their interaction with neural progenitors may be one such mechanism. During the early 1990's, around the time that adult neurogenesis was widely recognized to occur, it was determined that a family of fatty acid binding proteins existed in the brain and showed changes in expression during brain development (Sellner, et al., 1995). Subsequently, it was found that specialized cells immunoreactive for fatty acid binding protein-7 (FABP7) existed mainly within neurogenic niches of the rodent brain (Kurtz, et al., 1994; Young, et al., 1996). FABP7+ cells stain positively for astrocyte markers such as glial fibrillary acidic protein and for the astrocyte-specific glutamate transporter GLAST (Anthony, et al., 2004; Kurtz, et al., 1994). More recently, a study of the postischemic adult hippocampus of macaques has shown that astrocytes immunoreactive for FABP7 are frequently close to newly divided cells (Boneva, et al., 2011). The possible relationship between FABP7+ astrocytes and newly divided cells in the rodent brain has not thus far been explored. A nuclear protein called Ki67 is a marker for newly divided cells (Kee, et al., 2002). The main goal of this study was to determine if the anatomical relationship between astrocytes immunoreactive for FABP7 and newly divided cells in the hippocampus in the macaque also exists in the rodent brain. Other regions of coronal brain sections containing the hippocampus were also examined for FABP7, along with parasagittal sections of the olfactory bulbs, which are known to be enriched in FABP7.

Materials and Methods

Animals

Experimental protocols were approved by the Howard University Institutional Animal Care and Use Committee. All experiments utilized young, juvenile (35–45 days old) male Sprague-Dawley rats (Harlan, Indianapolis, IN) according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This age range was selected to maximize the possibility of detecting proliferating cells. Neurogenesis declines by 50% between the ages of 45 and 90 postnatal days in rats and differentiation into neurons also shows an age-related decline (Ho, et al., 2012; Lichtenwalner, et al., 2001). At 1400–1600 hrs. animals were deeply anesthetized (Nembutal, 60 mg/kg) and wash-perfused with saline through the left ventricle followed by 10% formalin in 0.2 M phosphate buffer. Entire brains were immersed in 20% sucrose-phosphate buffer for cryoprotection. Parasagittal frozen sections (35 μ thick) in the olfactory bulb region, or coronal sections, in the hippocampus and medullary regions, were prepared using a sliding microtome.

For each brain, every fifth section was retained for immunostaining. Coronal sectioning of brain tissue containing the hippocampus was begun just caudal to the level of the anterior commissure, where the dentate gyrus first makes its appearance; sectioning continued to the level of the posterior hypothalamus, where the hippocampus begins to enter the temporal lobe. Mostly the midportion of the hippocampus, the adjacent habenula, and the midportion of the hypothalamic arcuate nucleus were examined.

Staining

To stain for FABP7, sections were first all washed for 30 min. in phosphate buffered saline (PBS) containing 0.3% Triton X and 5% normal goat serum. Sections from a total of 10 rat brains were added to buffer-filled large diameter tissue culture plates (6 wells/plate, Thermo Fisher Scientific Co., Pittsburgh, PA) (sections from 3 brains were used for each staining session). Sections were then incubated overnight at 4 degrees C with a rabbit antibody to FABP7 (diluted 1:3000) (Millipore, Temecula, CA) or with non-immune rabbit serum. Following this, sections were washed in PBS-0.1% Triton-X, incubated for 30 min. in

biotinylated goat-anti rabbit IgG (Vectastain ABC kit, Vector Laboratories, Burlingame, Ca.) and were washed once more in PBS-0.1% Triton. To nullify endogenous sources of pseudoperoxidase, sections were then incubated for 30 min. in PBS-0.3% hydrogen peroxide, followed by washing in PBS and a 60 min. incubation in avidin-biotin-peroxidase complex (ABC). Immunoreactivity was then localized via the production of a brown precipitate of diaminobenzidine (DAB) at the sites of peroxidase-labeled antibody by exposing the sections to a 0.05% solution of DAB plus 0.03% hydrogen peroxide for 1–2 min. (Young et al. 1996). Brain sections were dried down onto subbed slides, dehydrated in an ascending series of alcohols and xylene, and coverslipped using Permount. To stain for dividing cells, rabbit or mouse antibodies to Ki67 protein (Novacastra laboratories, Leica Microsystems, Newcastle upon Tyne, UK), a marker for proliferating cells, were utilized at a dilution of 1:1500 (rabbit antibody) or 1:300 (mouse antibody) and the same procedure described above was followed (Kee, et al., 2002). For double-staining studies, the staining procedure for Ki-67 was modified by adding 600 µl of 1% nickel sulfate to 20 ml of the DAB solution, so that Ki-67 immunoreactive (Ki-67 ir) nuclei stained dark black instead of brown. Subsequently, staining for FABP7 was performed on the same sections without the addition of nickel, so that cells stained brown.

Results

Immunoreactivity for FABP7

In rat brains, immunoreactivity for FABP7 was intense in astrocytes of the dentate gyrus of the hippocampus, arcuate nucleus of the hypothalamus, the habenula, and the margins of the area postrema of the medulla, consistent with previous reports of the distribution of FABP7 (Figs. 1–5) (Young, et al., 1996). No staining was present in sections incubated with normal rabbit serum alone (data not shown). In sections of rat hippocampus (Fig. 1), FABP7 immunoreactivity was found in the cell nucleus and in fine processes of astrocytes that penetrated into the granule cell layer of the dentate gyrus. Since these astrocytes were distributed in a linear fashion along the dentate gyrus and rarely overlapped each other, it was feasible to roughly estimate total numbers of astrocytes per section. About 35–45 FABP7+ astrocytes could be detected per hippocampal section (n = 10 brain sections from 10 different rat brains). The purpose of these rough estimates was not to produce a rigorously accurate count of cells in the hippocampus, but was to simply provide for a qualitative sense of how abundant these FABP+ astrocytes were in comparison with Ki67-ir cells in adjacent sections of the hippocampus. In the hypothalamus and habenula, FABP7+ astrocytes were present in denser concentrations and no attempt was made to estimate their numbers (Fig. 2–3). FABP7 immunoreactivity was particularly intense in olfactory ensheathing glial cells of the olfactory nerve, but was also present in astrocytes scattered between olfactory glomeruli and in lower layers of the olfactory bulb (external plexiform layer, internal plexiform layer, and granule cell layer) (Fig. 5).

Immunoreactivity for Ki67

In the rat dentate gyrus, roughly 10–15 clusters of Ki67-ir cells per hippocampal cross-section were detectable. Each cluster of newly divided cells contained between 2–6 cells; boundaries of overlapping cells generally were unclear due to intense staining and/or to section thickness, so it was difficult to determine the precise number of cells per cluster. Clusters tended to be more abundant at the medial border of the dentate gyrus, but could be found throughout this structure (Fig. 1). Much smaller numbers of Ki67-ir cells were observed within the rat arcuate nucleus, habenula, the area postrema and subjacent regions, and the olfactory bulb (Figs. 2–5). Occasionally, Ki67 immunoreactivity could be found in an ependymal cell of the third ventricle (Fig. 2). This is consistent with reports that ependyma as well as astrocytes may develop into neuroblasts (Coskun, et al., 2008).

Simultaneous staining for FABP7 and Ki67

In 20 sections of rat hippocampus (2 from each rat) double-stained for both antigens, clusters of Ki67-ir cells (Fig. 6, black stain due to nickel toning of brown diaminobenzidine (DAB) reaction product) were frequently adjacent to FABP7+ astrocytes (Fig. 6, brown stained diaminobenzidine reaction product). Black-stained nuclei immunoreactive for Ki67 protein generally did not seem to be present in cells bearing brown-stained glial cell projections immunoreactive for FABP7, so, within the limits of the procedures employed, the Ki67-ir cells did not appear to contain FABP7 (Fig. 6).

Numbers of FABP7+ astrocytes in the dentate gyrus outnumbered clusters of newly divided cells and many FABP7+ astrocytes were located 50–100 micrometers away from Ki67-ir cells. In other brain regions such as the habenula and arcuate nucleus, numbers of Ki67-ir cells were too small to realistically assess the proportions of such cells adjacent to FABP7+ astrocytes, but occasionally, the two cell types could be seen to be very close to each other (Fig. 6).

Discussion

This study of the rat brain confirms the proximity between FABP7+ astrocytes and newly divided cells that was first reported by Boneva, et al. in the macaque hippocampus (Boneva, et al., 2011). A further finding of this study was that FABP7+ cells are also abundant in brain areas showing more modest amounts of neurogenesis. Two main explanations for this association between the two cell types in the hippocampus seem likely.

The first explanation for the association between FABP7+ astrocytes and newly dividing cells originates from well-known features of embryological neurogenesis. In the developing neural tube, radial glia serve as progenitors for neurons in the CNS. These relatively undifferentiated radial glia are immunoreactive for FABP7 (also known as brain lipid binding protein) (Anthony, et al., 2004). Daughter cells of these FABP7+ glia differentiate into neurons, move short distances away from the radial glia, and lose the ability to synthesize FABP7 (Gerstner, et al., 2008; Kurtz, et al., 1994; Matsumata, et al., 2012). Thus, the anatomical proximity of the two cell types may result from the differentiation of the FABP7+ astrocytes into neurons that remain nearby. FABP7+ astrocytes may represent a reservoir of immature cells contained within specific brain regions that have a capacity to divide and differentiate into neurons.

A second and not mutually exclusive explanation for the association between the two cell types could be that FABP7+ astrocytes influence the division and differentiation of neuroblasts. This influence could be mediated via cell-cell contact or via secretion of neuroactive substances by astrocytes.

Confirmation of actual contact between the cell membranes of the two cell types at the ultrastructural level would require electron microscopy. If the two cell types in question were different types of neurons, such an ultrastructural study would be fruitful, because it could confirm the existence of functional synapses between the two cell types. However, since there is no analogous morphological signature of a functional relationship between neurons and astrocytes, which do not produce synapses or synaptic vesicles, even an ultrastructural study could not be helpful in establishing a functional relationship between FABP7+ astrocytes and newly divided cells. Indeed, recent research on astrocytes has shown that close contact between neuronal and astrocytic cell membranes is not necessary for an influence of astrocytes upon neurons.

Secretion of soluble, diffusible factors like glutamine, lactate, and thrombospondin into the extracellular environment by astrocytes is now known to influence neuronal function and synapse formation (Ransom and Ransom, 2012). More specifically, secretion of Wnt protein into the environment of astrocytes has recently been shown to have a powerful influence upon neurogenesis (Miranda, et al., 2012). The proximity of FABP7⁺ astrocytes to newly divided cells shown in the present study shows that diffusion of such signaling molecules between these two cells types is possible.

More substantial evidence for the general principal that FABP7 may influence neurogenesis was provided by a recent report that in mice genetically engineered to lack FABP7, hippocampal neurogenesis is reduced by about 20% (Matsumata, et al., 2012). The signaling pathways responsible for this influence of FABP7 are yet unclear. It is known that upstream modulators of FABP7 expression include transcription factors like Notch and Pax6; downstream mediation of effects of FABP7 may involve the ability of fatty acid binding proteins to enhance the cellular uptake of retinoic acid and guide this lipid molecule to nuclear receptors that modulate gene transcription (Matsumata, et al., 2012). Retinoic acid is known to influence neurogenesis in both the hippocampus and hypothalamus (Shearer, et al., 2012). The proposal that FABP7⁺ cells may influence neurogenesis is in accord with other observations showing that astrocyte-derived molecules modulate neurogenesis (Barkho, et al., 2006; Bozoyan, et al., 2012; Lie, et al., 2005).

The association between newly born cells and FABP7⁺ astrocytes, shown here, does not exclude the likelihood of interactions between newly born cells and other conventional or mature types of astrocytes. Indeed, quantitative studies have shown that glial cells and neurons are both abundant in the dentate gyrus and are closely intermixed in a ratio of about 1.5 neurons for every glial cell (Roy, et al., 2005). Staining of the dentate gyrus for other types of glial markers would likely also show an association between newborn neurons and glia. The novelty of this study arises from the fact that FABP⁺ astrocytes are not widespread in the brain and are present only in sites of neurogenesis.

Inspection of sections double-labeled for both proteins (black, nickel-toned DAB for Ki67, brown DAB for FABP7) suggested that most Ki67-ir nuclei did not overlap with astrocytic projections ir for FABP7 (Fig. 6). This may signify that newly divided cells no longer produce FABP7. Our observation is in conflict with the report by Boneva, et al. that FABP7 immunoreactivity could sometimes be detected in newly divided cells (Boneva, et al., 2011). The reasons for this difference are uncertain.

The staining procedure we employed would seem to be a reliable method of determining if nuclear immunoreactivity for Ki67 accompanies cytoplasmic immunoreactivity for FABP7 in the same cell. Nickel toning of the DAB chromogen has been widely used in neural tissues to distinguish between nuclear and cytoplasmic antigens, for example, to determine if nuclear FOS immunoreactivity is accompanied by cytoplasmic immunoreactivity for tyrosine hydroxylase in the same cell (Briski, 1998). We know of no reason why this procedure could not identify cells immunoreactive for both Ki67 and FABP7.

One possible explanation for the difference between our results and those of Boneva, et al. is that the use of fluorescently labeled antibodies may be a more sensitive procedure and could detect smaller levels of FABP7 than the procedure we utilized. We attempted to use secondary antibodies labeled with an orange-red fluorescent dye (Alexafluor 555) or a green fluorescent dye (Alexafluor 488, Life Technologies, Inc.) following incubation in the primary antibodies as described above. This approach, utilizing various concentrations of primary antibodies, did not yield a sufficient signal-to-noise ratio in our hands and was not further pursued.

Another possible explanation for the difference between our results and those of Boneva, et al. is that FABP7 expression may persist in newly born neurons for a longer time in the macaque than in the rat. One thing that seems certain is that FABP7 immunoreactivity in newly born neurons must be a relatively transient phenomenon in rats, since our previous inspection of FABP7 immunoreactivity in the rat hypothalamus, where newly born and immature neurons are scarce, showed no instances of FABP7 immunoreactivity in mature neurons (Young, 2002).

This study did not attempt to determine the neural or glial fate of the newly divided cells immunoreactive for Ki-67 that were studied here. Other investigators have found that newly divided cells retain astrocytic markers (eg., S100 protein) for 1–3 days following division and then only later (14 days) acquire a neuronal phenotype (Namba, et al., 2005). Since, in young rats, about 80% of newly divided cells eventually mature into neurons, it is likely that most of the Ki-67 ir cells studied here were destined to become neurons (Lichtenwalner, et al., 2001).

In other brain regions, like the arcuate nucleus and the area postrema, the abundance of newly divided cells was smaller than in the hippocampus, so the relationship between the two cell types was less easily understood. It seems clear that the presence of FABP7+ cells alone is not a sufficient cue for robust neurogenesis, since neurogenesis is much less intense in the arcuate nucleus and habenula than in the hippocampus, in spite of the presence of numerous FABP7+ cells in these regions.

In the arcuate nucleus, many newly divided neural progenitors differentiate into neurons responsive to the adipocyte hormone, leptin (Pierce and Xu, 2010). A previous study of ours demonstrated frequent associations between leptin-sensitive neurons in the arcuate nucleus and FABP7+ astrocytes (Young, 2002). Also, in the region subjacent to the area postrema, numerous atypical, immature astrocytes, immunoreactive for vimentin and nestin, can be found in an area that precisely matches the distribution of FABP7+ astrocytes seen in this study (compare results of Pecchi, et al., 2007 to Fig. 4). When the normally sparse neurogenesis in this region is stimulated by epidermal growth factor or by lesions of the vagus nerve, the majority of newly born neurons are in close proximity to these immature astrocytes (Pecchi, et al., 2007). An association between newly divided neural progenitor cells and immature astrocytes in these additional neurogenic niches of the rodent brain has thus been documented both by this and other studies.

The control of lipids in the environment of neural progenitor cells may be a particularly important aspect of astrocyte function. A high-fat diet has recently been shown to have an important influence upon hypothalamic neurogenesis (McKay, et al., 2012). A high-fat diet also promotes obesity and resistance to effects of leptin in the arcuate nucleus that is not seen in other leptin-responsive brain regions (Münzberg, et al., 2004). These effects of high-fat diets may be due to effects of palmitic acid upon neurons (Benoit, et al., 2009). Mice genetically engineered to lack FABP7 have elevated levels of palmitic acid in the brain. This suggests that FABP7+ astrocytes adjacent to leptin-sensitive neural progenitor cells could be modulating the exposure of these cells to lipids and their progression through neurogenesis (Owada, et al., 2006).

Finally, the synthesis of FABP7 by astrocytes in neurogenic niches may have implications for aging and neurogenesis. Hippocampal neurogenesis is substantially reduced in aged rats (Kuhn, et al., 1996). Many factors could contribute to this aging effect, but age-related changes in astrocyte signaling to neural progenitor cells has recently been identified as a major contributor to effects of aging on neurogenesis (Miranda, et al., 2012). The possession

of FABP7 by astrocytes in neurogenic niches may contribute to this aging-related change in astrocyte function.

In the aging rat and human brain, many FABP7+ astrocytes develop prominent cytoplasmic inclusions. These inclusions, stainable with Gomori's aldehyde fuchsin stain, gave rise to the term of "Gomori-positive astrocytes" to describe the phenotype of these cells. It is now known that these iron- and copper-rich inclusions are derived from mitochondria that have been damaged due to oxidative stress (Justino, et al., 1997; Young, 1996). However, the source of this oxidative stress in astrocyte mitochondria has been unknown, and it is not clear why mitochondria of nearby neurons are spared. It now appears that mitochondria of astrocytes, but not neurons, have the capacity to oxidize fatty acids, and that this oxidative process can damage astrocyte mitochondria (Schmidt, et al., 2010). Thus, the possession of FABP7 by astrocytes in neurogenic niches may indicate an enhanced metabolism of fatty acids that provokes an aged-linked degeneration of astrocyte mitochondria. This degenerative process may be related to abnormalities in hippocampal function and neurogenesis.

In the context of aging and the hippocampus, a recent analysis of genes upregulated in the hippocampus of Alzheimer's disease (AD) patients may be relevant to this study. Out of a total of 26,000 genes analyzed via microarray methods, only 24 genes were found to be overexpressed in AD. Two of these genes--those for amyloid precursor protein and the astrocytic lipid binding protein apolipoprotein E--have well recognized roles in the AD disease process. The other 21 genes have less obvious roles that merit further study. One of these genes codes for FABP7 (Guttala, et al., 2012).

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Fig. 1.
Top: Distribution of FABP7 immunoreactive (FABP+) astrocytes (arrow) within the rat hippocampus. Bottom: Distribution of newly divided cells immunoreactive (ir) for Ki67 (arrow) within the rat hippocampus. Magnification bar = 100 μ m.

Fig. 2.
Top: Distribution of FABP7+ astrocytes (arrow) within the rat arcuate nucleus. Bottom:
Distribution of newly divided cells ir for Ki67 (arrow) within the rat arcuate nucleus. Rarely,
immunoreactivity could also be detected in isolated ependymal cells lining the third
ventricle (arrow). Magnification bar = 100 μ m.

Fig. 3.
Top: Distribution of FABP7+ astrocytes (arrow) within the rat habenula. Bottom:
Distribution of newly divided cells ir for Ki67 (arrow) within the rat habenula.
Magnification bar = 100 μ m.

Fig. 4.

Top: Distribution of FABP7+ astrocytes (arrow) in the rat dorsal medulla. Bottom: Distribution of newly divided cells ir for Ki67 (arrow) within the rat dorsal medulla. Magnification bar = 100 μ m.

Fig. 5.

Top: Distribution of FABP7+ astrocytes (arrow) within the olfactory nerve and glomerular layer of the rat olfactory bulb. Bottom: Newly divided cells immunoreactive for Ki67 in the rat olfactory bulb adjacent to olfactory glomeruli and in the internal plexiform layer. Magnification bars = 100 μ m.

Fig. 6.
A and B: FABP7+ astrocytes (brown) were frequently associated with newly divided cells ir for Ki67 (black) in the dentate gyrus of the hippocampus. C and D: FABP7+ astrocytes (brown) were adjacent to sparse, newly divided cells ir for Ki67 (black) in the habenula. Magnification bars = 100 μ m.