Published in final edited form as:

Brain Res. 2007 September 26; 1171: 18–29.

Brain mast cell relationship to neurovasculature during development

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Abstract

Mast cells, derived from the hematopoietic stem cell, are present in the brain from birth. During development, mast cells occur in two locations, namely the pia and the brain parenchyma. The current hypothesis regarding their origin states that brain mast cells (or their precursors) enter the pia and access the thalamus by traveling along the abluminal wall of penetrating blood vessels. The population in the pia reaches a maximum at postnatal (PN) day 11, and declines rapidly thereafter. Chromatin fragmentation suggests that this cell loss is due to apoptosis. In contrast, the thalamic population expands from PN8 to reach adult levels at PN30. Stereological analysis demonstrates that mast cells home to blood vessels. More than 96% of mast cells are inside the blood brain barrier, with ~ 90% contacting the blood vessel wall or its extracellular matrix. Mast cells express $\alpha 4$ integrins - a potential mechanism for adhesion to the vascular wall. Despite the steady increase in the volume of microvasculature, at all ages studied, mast cells are preferentially located on large diameter vessels ($>16\mu\text{m}$; possibly arteries), and contact only those maturing blood vessels that are ensheathed by astroglial processes. Mast cells not only home to large vessels but also maintain a preferential position at branch points, sites of vessel growth. This observation presents the possibility that mast cells participate in and/or regulate vasculature growth or differentiation. The biochemical and molecular signals that induce mast cell homing in the CNS is an area of active investigation.

Keywords

Astrocytes; blood-brain barrier; integrins; migration; endothelia; vasculature

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1. Introduction

Mast cells, derived from hematopoietic stem cells, populate the normal brain of many mammalian species including humans (Dropp, 1972; Dropp, 1976; Edvinsson et al., 1976; Ibrahim, 1974; Manning et al., 1994; Porzionato et al., 2004; Silverman et al., 1994; Turygin et al., 2005). Mast cells synthesize and store neuroactive and vasoactive substances within dense granules, along with unique serine proteases, cytokines, chemokines and growth factors. These mediators, as well as those made after stimulation, are secreted from mast cells upon receiving an appropriate signal (Galli et al., 2005). Mast cells are well known for their role in allergic inflammation and in host defense to immunologic stimuli in the periphery (Marshall, 2004). There is now increasing evidence that mast cells also function in normal physiology and in diseases of the CNS (Benoist and Mathis, 2002; Silver et al., 1996; Theoharides, 1996). In the normal adult brain, mast cell mediators influence CNS neuronal activity (Khalil et al., 2004) and vascular permeability (Esposito et al., 2001). In autoimmune demyelinating diseases, mast cells alter BBB permeability, resulting in inflammation and myelin degradation (Behi et al., 2005; Brosnan et al., 1990; Brown et al., 2002; Secor et al., 2000; Seeldrayers et al., 1989).

Mast cells lie at perivascular locations on the brain side of the BBB in apposition with astrocytic and neuronal processes (Florenzano and Bentivoglio, 2000; Manning et al., 1994; Silverman et al., 2000) and are concentrated in the thalamus (Asarian et al., 2002; Florenzano and Bentivoglio, M, 2000). The BBB is composed of endothelial cells that form elaborate tight junctions. Additional components of the microvascular wall include pericytes, endothelial cell basal lamina plus the astrocytic endfeet (Peters et al., 1976). Laminin and fibronectin (Tilling et al., 2002) are major constituent of the basal lamina and provide binding sites for a variety of cell surface receptors (Colognato et al., 2005). Pericytes provide structural support to vessels by extending long processes around capillary tubes, arterioles and venules (Gillard et al., 2003; Risau and Wolburg, 1990). Astrocytic processes terminate on the outer surface of the brain capillaries, and are necessary for the induction and maintenance of the BBB (Abbott, 2002).

The postnatal period is distinguished by significant changes in blood vessel density and organization of cells forming the BBB. Although brain angiogenesis and BBB formation begin during embryogenesis (Saunders and Mollgard, 1984), maximal capillary sprouting occurs in the second and third postnatal weeks in the rat cerebral cortex corresponding to the time of glial cell proliferation (Jacobson, 1991). The BBB matures gradually (Johanson, 1989; Risau and Wolburg, 1990), and in the rat, complete BBB closure occurs in the third postnatal (PN) week (Jacobson, 1991).

In the developing postnatal brain, there are rapid changes in mast cell size, mediator content and localization. Immature mast cells complete their maturation within their local microenvironment, a process characterized by changes in phenotype and an accumulation of granules containing neuroactive and vasoactive molecules, proteases, and proteoglycans (Dimitriadou et al., 1996; Lambracht-Hall et al., 1990; Zhuang et al., 1999). Changes in mast cell distribution during development have been documented in several mammalian and non-mammalian species (Gill and Rissman, 1998; Lambracht-Hall et al., 1990; Zhuang et al., 1999), but their localization relative to the cellular and molecular components of the BBB as well as the vascular changes coincident with mast cell entry into the brain remain incompletely understood. It has, however, been shown that mast cells gradually infiltrate the thalamus starting in the first week of postnatal development (Lambracht-Hall et al., 1990), in parallel with the developing vascular tree (Michaloudi et al., 2003).

These developmental changes in the spatial and temporal parameters of mast cell appearance at perivascular locations suggest that their infiltration may be controlled by the properties of the microvasculature. We, therefore, examined the location of mast cells relative to various molecular, cellular, and structural properties of blood vessels during development. To this end, we studied the localization of mast cells with respect to laminin, α -SMA, a label for pericytes and smooth muscle; glial fibrillary acidic protein, GFAP, a marker for astroglial processes; and RECA-1, a marker of endothelial cells. We also scored mast cell distribution and location on blood vessels according to blood vessel diameter and branch points. Our goals were: to determine quantitatively the time course and distribution of mast cell entry into the CNS; and to assess the biochemical and structural characteristics of blood vessels along which mast cells migrate. Our findings show that brain mast cells become integrated with the vasculature at a specific time in its development, and point to a role of mast cells in contributing to the regulation of the brain's circulation.

2. Results

Developmental changes in mast cell number and distribution

The size of the mast cell population was determined in both the thalamus and overlying pia during rat postnatal development ($n=5-8/\text{age}$; Fig. 1A, B). In the pia at PN0, mast cells numbered ~ 3500 and peaked at ~ 5000 at PN11. Mast cell number rapidly declined by about 74% to ~ 1500 at PN15, and reached adult levels of ~ 50 by PN30 (ANOVA; $F_6 = 58.6$; $p < 0.001$).

Thalamic mast cells were first observed at PN8 numbering ~ 140 and their numbers increased steadily between PN15 and 21, and reached adult values of ~ 1500 at PN30 (ANOVA, $F_6 = 56.3$, $p < 0.001$). This time period of increase coincided with the decline of the pial population.

Though their numbers declined between PN11-15, mast cells in the pia remained concentrated in the tissue overlying the anterior thalamic nuclei (Fig. 1C). In contrast, the anatomical concentration of thalamic mast cells shifted between PN21-30 from the anterior thalamus to the posterior thalamus, respectively (Fig. 1D). By PN30 they had reached their adult distribution (Asarian et al., 2002).

Mast cell apoptosis

Given the 74 % reduction in pial mast cells between PN11-15, we explored the possibility of ongoing apoptosis using DAPI to identify nuclear fragmentation (Fig. 2A-F). The majority of cells had a uniformly stained nucleus (Fig. 2 A-C). Cells with clumped chromatin and undergoing apoptosis (Fig. 2 D-F) represented 5% of the pial mast cell population at PN11.

Mast cell location relative to neurovascular elements

To determine their luminal or abluminal location, 282 mast cells divided equally among all age groups (PN8-30) were counted in RECA-avidin stained sections and scored as being on the brain side of the endothelial cell or in the lumen using confocal microscopy. This analysis demonstrated that 96.8% of mast cells lie on the brain side of blood vessels (Fig. 3A-C). Of these, approximately 10-15% were in the neuropil and not in contact with the wall of the blood vessel (data not shown). Finally, 3.2% of the mast cells were in the blood vessel lumen (Fig. 3DF).

To assess where mast cells are located relative to mural components of the blood vessels, tissue sections were stained with different combinations of anti-laminin, anti-smooth muscle actin, anti-GFAP and avidin (mast cells). Using the first two markers, thalamic mast cells were seen at blood vessels, which have (at a minimum) a SMA-positive pericyte in their walls (in addition

to the endothelial cell). For microvessels (<8 microns) mast cells lay just external to the endothelial cell and share the endothelial basal lamina (laminin) with the pericyte (data not shown). When associated with larger blood vessels, the mast cells were in physical contact with the smooth muscle cell or its basal lamina containing laminin as seen at PN11 (Fig. 4 A-D) and PN30 (Fig. 4 E-H; Fig. 6A-E, see below).

In addition to laminin, the extracellular matrix of blood vessels in the neonatal brain contained fibronectin (Krum et al., 1991; and data not shown). The integrin $\alpha_4\beta_1$, a ligand for fibronectin, is present on connective tissue mast cells (Gurish et al., 2001). To assess its presence in the developing brain, sections from PN11, PN21 and PN30 were examined using immunohistochemistry. The α_4 integrin chain is distributed as puncta on both the pial (Fig. 5AC) and thalamic mast cells (Fig. 5D-F). Interestingly, the majority of the mast cells in the pia express the integrin α chain, where as the expression of this protein by mast cells in the thalamus is less frequent.

Mast cell location: relationship to astroglial endfeet

From PN 0-8 the entire mast cell population is restricted to the pia and is surrounded by a mesh of astroglial process (Fig. 6A-1, 6A-2). During the time of entry of mast cells into the CNS, astroglia are proliferating and extending processes. Thalamic mast cells (>80%) were preferentially associated with those vascular elements ensheathed by astroglial endfeet (Fig. 6AC). Mast cells were often directly contacted by these endfeet (Fig. 6D, E).

This association between mast cells and astroglial endfeet was observed at PN8 when mast cells first entered the CNS and when astroglial processes (delineated by GFAP) were restricted to the lateral aspect of the thalamus, and also at PN30, when astroglia were distributed throughout the thalamus. Quantitative analysis further demonstrated that >90% of mast cells contact astroglia at all time points (Fig. 7).

Blood vessel density and size, and branch points

Blood vessel density increases between PN8 and 30. Stereological analysis demonstrated that this was due to increases in volume fraction of capillaries (<8 μ m) rather than medium and large blood vessels (Fig. 8A, B), with small vessels significantly increasing in density from P8 to P15 and P30 (Tukey Test, $P = 0.004$ for P8 vs. P15, and $P = 0.025$ for P8 vs. P30; $n = 3$ per age group). Despite the expansion in the microvasculature, 70-95% of thalamic mast cells were located preferentially on large blood vessels during this time period (Fig. 8C).

The brain's vasculature grows by the sprouting of branches from the original penetrating vessels. During the expansion of the vascular bed in development, mast cells were found nestled at these branch points (Fig. 9 A-C). To determine whether this was a preferential location, point counting stereology was used to calculate the total volume fraction of blood vessels and their branches (Fig. 9D). Blood vessels per se occupy a high proportion of the thalamic volume (over 50% of thalamic tissue at PN30) with branch points representing 2.4-6.9% of the total. Importantly, the proportion of mast cells located at branch points increases from 6.4 to 36.2 % from PN8 to PN30 (Fig. 9D). This preferential relationship is also evident in the computation of the ratio of mast cells at branch points (MC^{BP}) and the total number of branches where the ratio increases from ~1.7 at PN8 to 6.9 at PN15 and 5.7 at PN30 (Fig. 9E; $N = 3$ animals/group).

3. Discussion

The present results document for the first time the detailed anatomical relationship of mast cells to cellular and extracellular elements of the neonatal neurovascular unit. (This term refers

to the blood vessel endothelium, mural cells (pericytes and smooth muscle), astroglial endfeet and the various basal laminae associated with these cell types). The new observations indicate changes among the thalamic nuclei in the distribution of mast cells as they enter along the penetrating vessels. Mast cell association with the vascular bed persists throughout this post-natal period. They are part of the blood vessel wall and are surrounded by the laminin/fibronectin-containing basal lamina of the endothelia and/or smooth muscle. Mast cells become associated with blood vessels only when the vessels have been invested with astroglial processes. Quantitative stereological measures demonstrated that brain mast cells home to large, mature blood vessels and are preferentially associated with branch points, sites of angiogenesis.

Relationship to Neurovascular Unit

The results indicate that mast cells have a complex and intimate relationship to the cellular and extracellular components of the neurovascular unit (NVU) with the potential for multidirectional signaling. There are 3 characteristics of this preferential anatomical relationship of mast cells to the NVU: (1) Mast cells home to large diameter blood vessels with mural cells (pericytes and/or smooth muscle); these vessels have the appearance of arteries based on the circumferential arrangement of smooth muscle cells (Hughes and Chan-Ling 2004). This preferential association with arteries is found throughout development even as the density of the microvasculature increases. The potential functional consequences of mast cell activation on arteries are the possibility of regulating vasodilation and/or vasoconstriction (Filosa et al., 2004, Mulligan and MacVicar, 2004, Zonta et al., 2003). Mast cells are well known to induce vasodilation in the periphery by secreting vasoactive substances such as histamine and serotonin and can also mediate vasoconstriction in the CNS via adenosine receptors (Shepard et al., 1996). Thus mast cells may contribute to the regulation of blood flow in the brain (Owman et al., 1978).

(2) Based on stereological measurements, mast cells on blood vessels are predominately localized to branch points, the site of new blood vessel production. Angiogenesis in the CNS results from the growth of blood vessels on the leptomeningeal surface that invade the CNS and sprout new capillaries, extending the vascular network (Marin-Padilla, 1985). In the brain as elsewhere, blood vessel growth requires the recruitment and proliferation of endothelial cells and is regulated by angiogenic growth factors such as VEGF (Ruhrberg, 2002). The occurrence of mast cells and the vasculature wall, particularly their location at branch points might be explained by the fact that mast cells can also be recruited by angiogenic factors (Heissig et al., 2005) and can produce VEGF, fibroblast growth factor-2, transforming growth factor- β , tumor necrosis factor- α , interleukin-8 and tryptase (Ribatti et al., 2000). In addition, mast cell specific products such as tryptase can stimulate vascular tube formation (Blair et al., 1997) and its release could play a role in the expansion of the vascular bed.

(3) As might be expected from the prior statement, mast cell translocation from pia and their association with blood vessels is preceded by the expansion of astrocytic processes; those blood vessels that lack astrocytic endfeet (PN8-11) have no mast cells even when these vessels are located in thalamic nuclei that will contain mast cells later in development. Astrocyte production of VEGF regulates the growth and branching pattern of blood vessels in the CNS (hindbrain and retina, respectively) (Gerhardt et al., 2003; Ruhrberg, 2002) and induces the endothelial cells to develop barrier properties; it is possible that only then can mast cells home to a particular vessel (Abbott et al., 2006).

Mast cell dynamics in development

Not only do mast cells show a specific relationship to elements of the blood vessel but there are also age and sites specific changes in mast cell population size (also see Lambracht-Hall

et al. 1990; Michaloudi et al., 2003; Persinger, 1981). There are several possible mechanisms may underlie alterations in mast cell number.

Pia—The pial mast cell expands during the period from birth through PN11. The source of mast cells in the pia, as elsewhere, is likely to be from the circulating precursors derived from the bone marrow (Wendling et al., 1985). Committed mast cell progenitors circulate in the blood (Kitamura et al., 1979; Rodewald et al., 1996) and differentiate upon entering into tissues and organs (Kitamura et al., 1987). Mast cell maturation and survival are regulated by circulating or locally-produced cytokines and growth factors, with the most important being stem cell factor (SCF/c-kit ligand). This molecule that is also critical for mast cell homing; its absence is incompatible with mast cell survival or development (Zsebo et al., 1990). Systemic injections of SCF can increase mast cell number in many tissues indicating that circulating SCF is biologically active (Tsai et al., 1991; Galli et al., 1993). Neurons and glia produce SCF, at least under some conditions (Zhang and Fedoroff, 1997), providing a local supply within the brain. Mast cells in the pia express the c-kit receptor (Shanas et al., 1998) and would be responsive to its chemoattractive and mitogenic properties. It is also known that both fully differentiated mast cells as well as precursors continue to divide (Dvorak et al., 1976).

The mast cell population in the pia, however, declines precipitously between PN 11 to 15 with a rate of ~840 cells/day. Two possible mechanisms could account for this loss: apoptosis or emigration. The evidence suggests the former as 5% of the mast cells population had reached the stage of DNA fragmentation at PN11. This estimate of cell death is most likely to be an underestimate as apoptosis progresses rapidly and chromosome clumping is a late event (Ross and Pawlina, 2006). If mast cells do undergo cell death at approximately this rate between PN11 and 30, this would account in large part for the population decline. Although the trigger for apoptosis is unknown, mast cells do possess the biochemical machinery for at least one death pathway (Piliponsky and Levi-Schaffer, 2000). The sudden decline noted here *in vivo* is commensurate with cell loss in culture following withdrawal of survival factors. For example, *in vitro* mast cell apoptosis is initiated within 1-2 h after removal of SCF with 95% of the cell population being apoptotic after 30 h of growth factor deprivation (Yee et al., 1994). The apoptotic cascade can also be initiated by signals other than growth factor deprivation. For instance, incubation with TGF β 1 results in mast cell death via a mitochondrial/caspase 3-dependent mechanism (Norozi et al., 2006; Norizon, 2006). A potential source of TGF β 1 is the astrocytes with which the mast cell is intimately associated (Abbott et al., 2006). In the present experiments, analysis of additional time points during the period of cell loss and the use of methods to detect earlier steps in the apoptotic pathway such as caspase-3 activation would provide more detail on the mechanism of cell loss during this time period.

If, on the other hand, pial mast cells do not (all) undergo cell death it is possible that they emigrate to other tissues. The numbers of mast cells appearing in the thalamus during this time period does not account for the magnitude of loss. Another potential site of emigration is the leptomeninges; this population shows an increased abundance between postnatal week 2 and 4 in the rat (Dropp, 1976). This is an attractive hypothesis as cells in the leptomeninges could explain the capacity of the thalamic mast cell population to expand rapidly in response to psychological and physiological conditions (Asarian et al., 2002; Kovács and Larson, 2006) as well as pathological events (Jin et al., 2007).

Thalamus—In contrast to the pia, the mast cells population in the thalamus increases 10-fold from PN8 to 30 reaching adult levels at PN30 (Asaria et al., 2002). The current hypothesis is that the source of these mast cells is mast cells in the pia (also see (Lambracht-Hall et al., 1990; Michaloudi et al., 2003). However, one cannot rule out the prior establishment of a mast cell precursor pool and their subsequent differentiation within the neuropil. A large reservoir of mast cell precursors exists in other tissues, for example, in the intestine and their migration

into the gut is regulated by α chemokine receptor 2 and the integrin $\alpha 4\beta 7$ which is expressed on the precursors (Gurish and Boyce, 2006) (see below).

As the mast cell population expands, there is a time-dependent alteration in mast cell distribution in the thalamus. Initially the thalamic nuclei (and the blood vessels therein, see below) to which mast cells first “home” are in the more anterior aspect of the thalamus; over time the mast cell population “shifts” becoming concentrated in more posterior nuclei (and the blood vessels therein) reaching the adult distribution by PN30. One hypothesis for this shift could be alterations in the cell surface properties of cellular elements of the blood vessel wall or the composition of the extracellular matrix. Such phenomena would provide distinct “signatures” for blood vessels over developmental time. Mast cell progenitors respond to migration signals in an organ-specific manner and their trafficking includes both constitutive and inducible factors (Gurish and Boyce, 2006). The molecular mechanisms for populating the thalamus with mast cells are not known.

As stated above, mast cells enter the thalamus by accompanying the expanding vascular network. The vast majority of mast cells are located on the brain side of the blood brain barrier although confocal analysis identified a very small population of the thalamic population attached to the luminal side of the endothelia. For mast cells inside the brain, confocal analysis showed that mast cells are preferentially located in the perivascular extracellular matrix composed of fibronectin and laminin of the large blood vessels. It was already known that (activated, capable of migration) mast cells adhere to and move along these molecules (Thompson et al., 1993). In this study we found that cerebral mast cells express the $\alpha 4$ integrin to which fibronectin (and other matrix molecules) bind. It is known that α integrins are essential for recruitment and expansion of the mast cell (precursor) populations (Abonia et al., 2006).

In conclusion, mast cells are in position and armed with mediators that could participate in the growth and organization of the vascular bed of the thalamus. Future work will include more detailed analysis of angiogenic factors produced by mast cells during development of the CNS. Mast cells can be considered pluripotent in that their secretory armament reflects their prior history and their activation could alter the microenvironment of growing blood vessels.

4. Experimental Procedures

Animals and housing

Long-Evans rats (Charles River Laboratories Wilmington, MA) were bred in the laboratory. Pregnant dams were maintained two/cage on a 12:12 h light:dark cycle at a constant temperature (23°C). Pups remained with the dams after birth; only the males from these litters were used in the experiments. Animals aged PN 8, 11, 15, 21, 30 (PN 0, day of birth), and adult (16 weeks) were removed from their home cage and immediately anesthetized with an intraperitoneal injection of sodium pentobarbital (70 mg/kg, Henry Schein, Melville, NY). Experimental groups had 3-5 animals at each age. Food and water were provided *ad libitum*.

Tissue Preparation and processing

Rats were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.3. Brains were removed from the skull, post-fixed for 2 hours, and cryoprotected in 20% sucrose/PB/0.1% sodium azide at 4°C. Serial coronal sections of the thalamus were cut (50 μ m) on a cryostat (Zeiss Microm HM 500 OM, Walldorf, Germany) for analysis.

Microscopy

Sections were viewed on a conventional fluorescent microscope (Nikon Eclipse E800, Nikon, Inc., Melville, NJ) and images captured using a SPOT cooled CCD camera (Diagnostic Instruments, Inc., Sterling Heights, MI). In some experiments scanning confocal images were acquired using a fluorescence microscope with the LSM 510 capture program (Axiovert 200 LSM 510; 63X/W NA objective; lasers, Argon 488 and HeNe 543 and 633 nm; Zeiss, Inc., Thornwood, NJ).

Number and anatomical distribution

To assess MC numbers and distribution in the pia and thalamus (bregma -0.05 mm to -5.9 mm; Paxinos and Watson, 1986) alternate sections were stained with acidic toluidine blue (TB) (0.2% Toluidine Blue O (Sigma), pH 2.0) which is metachromatic for the sulfated proteoglycans in mast cell granules. Mast cells were counted only when cut through the plane of the nucleus. The Abercrombie correction factor was applied for cell counts (Abercrombie, 1946).

Programmed cell death

To assess mast cell apoptosis, selected sections of the pia at the level of the thalamus at PN11 were stained for 10 min with 10^{-5} M 4, 6 diamidino-2-phenylindole (DAPI; Sigma) and fluorophore conjugated avidin.

Mast cell relationship to neurovascular unit

Reagents: Methods used to characterize blood vessel components along with mast cells were single or double label immunohistochemistry combined with fluorophore- conjugated egg-white avidin (1:1000, 1 hr; Jackson ImmunoResearch Labs, West Grove, PA) which binds to heparin, a mast cell specific glycosaminoglycan (Lindahl and Kjellen, 1991). When double or triple labeling was performed cocktails of both primary and secondary antibodies as well as avidin were used. Antibodies used were: anti-glial fibrillary acidic protein (GFAP; rabbit polyclonal, 1:1000; Boehringer Mannheim, Indianapolis, IN), anti-smooth muscle actin (mouse anti- α SMA, monoclonal, 1:400, Sigma; St. Louis, Mo), anti-laminin (rabbit polyclonal, 1:500, Sigma), anti-rat endothelial cell surface antigen (RECA-1, mouse monoclonal, clone HIS52, 1:100; Serotec Ltd, Oxford, UK); anti- α 4 integrin (CD49d) (mouse anti-rat CD49d, clone TA-2, 1:100, Chemicon International, Inc., Temecula, CA,) and anti-fibronectin (rabbit anti-human fibronectin, 1:1000, Sigma, St. Louis, Mo). Sections were incubated overnight at 4°C with these primary antisera. Secondary antibodies were Cy2- Cy3- or Cy5-conjugated donkey immunoglobulins for the appropriate species (1:500, 1 hr at RT; Jackson ImmunoResearch Labs). Negative controls for immunoreactivity (ir) consisted of brain sections incubated in the absence of primary antibodies.

The physical and biochemical characteristics of the neurovascular unit including the mast cell were examined. Localization of mast cells vis à vis the blood vessel lumen required confocal microscopy and RECA-avidin staining. The basal lamina was visualized with immunocytochemistry for laminin or fibronectin and α SMA (mural cells) in sections from PN0, PN11, and PN30 animals.

Astrocyte ensheathment

Using confocal fluorescence microscopy, eight to twelve images were acquired per GFAP-stained section from 3 to 6 brains per age group PN8 to 30. Brain sections were stained for GFAP and heparin (avidin). A minimum of 5 confocal images were acquired from each brain with each image containing between 1 and 9 mast cells, totaling between 10 and 29 mast cells

per brain. The proportion of thalamic mast cells contacting GFAP positive processes was then calculated for each brain. SMA staining was used to define the blood vessel wall.

GFAP optical density

Using conventional fluorescence microscopy, the overall optical density (OD) of GFAP-IR in dorsal thalamic nuclei from animals PN8-30 was quantified using a method adapted from Simard et al. (2003). Eight to twelve images were acquired per GFAP-stained section from 3 to 6 brains per age group. The Photoshop magic wand tool was used to select regions above threshold staining in each image (tolerance value = 30) and NIH Image was used to calculate the area of the selected GFAP staining. The average GFAP OD was then calculated for each for each age.

Mast cell location and blood vessel size

Confocal microscopy was used to quantify the location of mast cells relative to blood vessel lumen diameter using immunocytochemistry for RECA-1 and avidin. Mast cell rich areas of the thalamus were identified at low power. Once mast cells were centered in the visual field, a z stack of 1-micron optical sections was collected through the cell(s). Mast cells were then scored as to their residence in the blood vessel wall vs. blood vessel lumen and relative to blood vessels size (small <8 μm , capillaries, medium 8-16 μm or large >16 μm) (see (Simard et al., 2003)).

Stereology

The volume fraction of blood vessels for each size category and of branch points was obtained using point-counting stereology (Russ, 1986). To this end, the frequency at which a blood vessel and a branch point occurred in each box in a 18 μm^2 grid in a 50 μm^2 section. Mast cells were scored as being at a branch point when they were in physical contact with both the main vessel and the branch.

Data analyses

Data are expressed as means $\pm$ SEM. One-way ANOVA followed by Tukey's all pair-wise multiple comparisons test was used to determine significant differences among groups using Sigmastat software (Jandel Scientific, San Rafael, CA). $P \leq 0.05$ was considered significant. Linear regression analysis was used to obtain the correlation coefficients of GFAP density and GFAP association with mast cells in development.

Acknowledgements

NIMH MH54088 (ajs)

NIMH 067782 (rs)

Fellowship: NIH 43035 (MK)

Training grant: NIH T32DK07328 (MK)

NSF DBI 320988 scanning confocal

Abbreviations

BBB, blood brain barrier; DAPI, 4, 6 diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; PN, post-natal; α SMA, alpha smooth muscle actin.

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Figure 1. Developmental changes in mast cell number

A. Pial mast cell population was 3558 ± 404 at P0, increased to 4958 ± 844 at P8, peaked at 5550 ± 764 at P11, then decreased by 74% to 1466 ± 220 at P15, and reached adult levels of 46 ± 14 at P30. B. Thalamic mast cells first appear at P8 (138 ± 38), increase rapidly between P15 and P21, and reach adult levels by P30 (1424 ± 267 at P30; 1566 ± 267 at P112). C, D. Distribution of mast cells in the pia and thalamus respectively, along the anterior - posterior axis with reference to bregma ($n = 5-8$ animals/age).

Figure 2. DAPI stained tissue to test for nuclear fragmentation in pial mast cells. (A-C) Pial mast cell with its characteristic pattern of eu- and heterochromatin. D-F. Apoptotic pial mast cell with fragmented chromatin (white arrows). Optical slices (z axis, 1 μ m). Scale bar: 5 μ m.

Figure 3.

Localization of mast cells in relation to the blood vessel wall. Mast cells are labeled with avidin (red) and endothelia are labeled with RECA-1 (white). (A-C) This is an example of a mast cell localized on the brain side of the endothelial cell. (D-F) This mast cell lies in the lumen of the blood vessel. (D-E) Composite image of 7 1- μ m serial optical slices. Scale bars; 10 μ m

Figure 4.

To determine the cellular localization of mast cells relative to the smooth muscle of the blood vessel and its basal lamina, triple-label immunocytochemistry was used. Mast cells are located between the smooth muscle actin positive cell (red) and the laminin-delineated basal lamina (white). (A-D) At PN11 thalamic mast cells (A) are associated with large blood vessels with smooth muscle containing α -SMA (B). Laminin (C) is present in the basal lamina of the smooth muscle and surrounds the mast cell. (D) Overlay of A-C. (E-G) A similar arrangement is shown for PN30 as in P11. Additionally, at this age, laminin is present in neuronal cell bodies (G). Images are composites of 4 (A-D) and 6 (E-H) optical slices (z axis, 1 μ m). Scale bars, 20 μ m.

Figure 5.

Mast cells in the thalamus seen express the integrin α_4 . (A-C) Mast cells in the P11 pia are labeled with avidin (A) contain α_4 integrin immunoreactivity (B). (C) Overlay. Image is a composite of 10 1-micron optical sections acquired using confocal microscopy. A similar observation was made in the PN30 thalamus (D-F). Scale bar 20 μm .

Figure 6.

Mast cells are associated with blood vessels which have acquired astrocytic endfeet. Triple label immunocytochemistry is used on tissue from P8, P15 and P30 to examine the physical interaction of mast cells and astrocytes. A1-A4 illustrate a large blood vessel (bv) just penetrating of the thalamus and a portion of the glial limitans and pia (indicated by the double headed yellow arrow in A2). Mast cells in the PN8 pia (yellow arrows, A1) are surrounded by GFAP positive processes (A2), These processes comprise much of the pia mater. The blood vessel, in addition to its ensheathment with glial endfeet, is surrounded by several layers of smooth muscle (A3). At P15 (B1-B4) and PN 30 (C1-C4) mast cells are again seen in the thalamus in association with glial endfeet. At higher magnification (Figure 6D and 6E) the GFAP processes cup the mast cell. Scale bars A-C= 20 μ m, D, E=10 μ m.

Figure 7.

The figure shows the frequency with which mast cells contact GFAP-positive processes from PN8-30. The percent mast cells contacted by GFAP-positive processes (circles) remains at about 80% at all ages. The OD of GFAP-positive processes (diamonds) increases with age.

Figure 8.

Stacked bar graphs reveal properties of vasculature in relation to localization of mast cells in development. A. The small blood vessels significantly increase in density from P8 to P15 and P30. B. The percent of blood vessels in each size category is shown over development. C. Mast cells are preferentially located on large blood vessels at all ages.

Figure 9.

Perivascular mast cells are preferentially located at blood vessel branch points. (A-C) (A) Mast cells at P15 are located at (B) endothelial (RECA-1, white) branch points (arrows). C. Overlay of (A) and (B). Images are composites of 13 optical slices (z axis, 1 μ m) acquired using confocal microscopy. Scale bar, 20 μ m. (D) Mast cells are preferentially located at blood vessel branch points at PN15 and PN30. Blood vessel density increases significantly from PN8 to PN15 and P30 (Tukey Test, $P = 0.008$ for PN8 vs. P15, and $P = 0.001$ for P8 vs P30; $n = 3$ per age group). Branch points compose between 2.4-6.9% of the blood vessel volume from PN8 to PN30, whereas the proportion of mast cells located at branch points increases from 6.4 at PN8 to 36.2% at PN30. E) The proportion of mast cells at branch points (MC^{BP}) vs. the proportion of branch points of all blood vessels indicates that mast cells are localized to branch points at P15 and P30. $N = 3$ animals/group. BP, branch point; MC, mast cells. Scale bar = 30 μ m