

Immunology of viral-vector-mediated gene transfer into the brain: an evolutionary and developmental perspective

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The immune system imposes limitations on gene transfer into the brain. Viral vectors injected into the brain's ventricular system elicit innate and adaptive immune responses. However, when injected directly into the brain parenchyma, they elicit only transient inflammation owing to the absence of dendritic cells, which transport antigen to lymph nodes and present it to naive T cells to initiate adaptive immune responses. This article explores the evolutionary and developmental basis of brain immune responses and their implications for viral-vector-mediated neurological gene therapy. Elucidating the cellular and molecular basis of these differential reactions is essential to the long-term success of neurological gene therapy.

'Continuous forms are the perfect ones. Usually, a distinction is created between supporting and supported elements, which is obviously wrong; there are those that both support and are supported. This distinction creates a degree of imperfection.'
(Antoni Gaudi, 1852–1926).

'Constraints upon evolutionary change may be ordered into at least two categories. All evolutionists are familiar with phyletic constraints... Developmental constraints, a subcategory of phyletic restrictions, may hold the most powerful rein of all over possible evolutionary pathways. In complex organisms, early stages of ontogeny are remarkably refractory to evolutionary change, presumably because the differentiation of organ systems and their integration into a functioning body is such a delicate process, so easily derailed by early errors with accumulating effects...' [1]

Gene therapy aims to use nucleic acids as drugs. Such an approach will be especially powerful for treating neurological diseases that, currently, remain untreatable. For example, disabling symptoms in many neurological diseases due to cell death could be remedied through the direct administration of neuronal growth and differentiation factors or, even,

specific transcription factors, such as glial-cell-line-derived neurotrophic factor (GDNF), Sonic hedgehog (Shh), bcl-2 or Gli-1, to the affected brain areas. However, to date, the widespread use of such therapeutic agents in classical pharmacological approaches has been precluded by the need for precise neuroanatomical delivery to restricted brain regions or nuclei, as well as the short half-lives of the peptides and their systemic toxicity. Therapeutic vectors derived from pathogenic viruses [e.g. adenovirus, adenovirus-associated virus (AAV), various lentiviruses and herpes simplex virus type 1 (HSV-1), among others [2]] allow high-level, cell-type-specific and anatomically restricted transgene expression in the brain. However, vector- and transgene-induced inflammation and immune responses limit both experimental and clinical gene therapy. Injection of vectors into the brain causes immune reactions, the mechanisms of which remain poorly understood [3–7]. A thorough understanding of the cellular and molecular mechanisms underlying viral-vector-induced inflammatory and immune reactions is essential for the long-term success of viral-vector-mediated gene therapy for brain diseases.

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The anatomy of brain immune responses

Immune responses, immune cells and immune-specific tissues are organ specific; examples of this include skin-associated lymphoid tissue (SALT) [8], nasal-mucosa-associated lymphoid tissue (NALT) [9], gut-associated lymphoid tissue (GALT) [10] and the eye-specific immune responses [11]. Regional immune responses result from the need to maintain effective immune surveillance while preserving organ function [12,13]. Although these local lymphoid tissues are certainly important in generating quick and efficient immune responses, their specific role in containing the response in any given anatomical location is less clear. In addition, even though few immune responses are organ-specific in a strict sense, memory lymphocytes are programmed to home preferentially either to skin, mucosa or lymphoid organs [14]. The brain lacks specific brain-associated lymphoid tissue, but benefits from brain-specific immune responses to antigens (Ags). These peculiar immune responses to infectious or particulate Ags injected into the central nervous system (CNS) have led to the brain being characterized as 'immune privileged' [12,13,15–17].

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Box 1. Characteristics of the two CNS immune compartments: the brain ventricular and parenchymal immune systems

The brain ventricular immune system

Anatomy:

- comprises the choroid plexus, ventricles, CSF and meninges
- characterized by the presence of all vascular and lymphatic channels, and immune cells found throughout the body

Physiology:

- elicits innate and adaptive immune responses to challenge with infectious particulate antigens and viral-vector-mediated gene transfer

The brain parenchymal immune system

Anatomy:

- comprises the CNS parenchyma proper
- lacks classical lymphatics, but outflow from CSF and brain parenchyma to cervical lymphatics has been described
- presence of blood–brain barrier
- lacks professional afferent APCs (i.e. DCs) able to prime naive lymphocytes by migrating from the

brain to secondary lymphoid tissue in the absence of inflammation

- myeloid-derived, but not lymphoid-derived, DCs are present in the brain only during infectious, autoimmune- or DTH-induced brain inflammation, or following expression of the DC growth factor Flt3L

- presence of monocyte-derived cells (e.g. macrophages and microglial cells)

- presence of all complement-activation pathways
- presence of proteasomes

Physiology:

- elicits only innate immune responses to challenge with infectious particulate infectious antigens and viral-vector-mediated gene transfer
- CTL responses are not primed following challenge with infectious particulate antigens or viral vectors
- neutralizing antibody responses are not primed following challenge with infectious particulate antigens
- slow recruitment of neutrophils

Infectious Ags and viral vectors injected anywhere in the body induce inflammation or activation of innate immune mechanisms and, at the same time, adaptive immune responses [12]. Similar reactions are elicited when viral vectors or infectious agents are introduced into the brain ventricular system (i.e. ventricles, choroid plexi or meninges) [17–20] (Box 1). However, if infectious particulate Ags (e.g. adenoviral or HSV vectors) are introduced carefully into the brain parenchyma proper [avoiding injections of either the systemic vasculature or the cerebrospinal fluid (CSF)], they elicit exclusively transient innate inflammatory immune responses (e.g. the release of cytokines, and the recruitment of monocytes and/or macrophages and neutrophils) [3–6,12,15,17–20], but no adaptive immunity.

However, if immunization is elicited by injecting the identical viral vector into a tissue outside of the

brain (e.g. skin), the adaptive immune system becomes primed, and antigenic epitopes anywhere within the brain (e.g. ventricles, meninges or, even, within the brain parenchyma) will be recognized as targets of either activated effector T cells [21] and B cells [22], or antibodies [23] (Box 1; Tables 1,2), irrespective of whether brain inflammation is present or not [3,5,12,17,19,21–23]. Complement components, and all pathways of complement activation present in non-nervous tissue, are present also in the brain and can become activated within the brain parenchyma or ventricles [24].

Historically, similar findings were obtained in the 1920s, when tissue grafts were shown to survive longer in the brain than elsewhere in the body [25,26]. Crucially, Medawar [27] demonstrated that extended survival of grafts in the brain was not sustained if

Table 1. Phylogeny of nervous, vascular and lymphoid cells and tissues^a

	CNS vessels	Peripheral vessels	CNS vessels	BBB	Peripheral lymphatics	CNS lymphatics	Choroid plexus	Meninges ^b	Lymphocytes	Macrophages	Afferent APCs ^c		
											Periphery	Ventricles	Brain proper
Hagfish	+	+	+	+	–	–	–?	–	?	+	–	–	–
Lamprey	+	+	+	+	–	–	+	–	+	+	–	–	–
Cartilaginous fishes	+	+	+	+	+	–	+	+	+	+	+?	+?	–
Bony fishes	+	+	+	+	+	–	+	+	+	+	+?	+?	–
Amphibians	+	+	+	+	+	–	+	+	+	+	+?	+?	–
Reptiles	+	+	+	+	+	–	+	++	+	+	+?	+?	–
Birds	+	+	+	+	+	–	+	+++	+	+	+	+?	–
Mammals	+	+	+	+	+	–	+	++++	+	+	+	+	–

^aAbbreviations: APC, antigen-presenting cell; BBB, blood–brain barrier; CNS, central nervous system.

^bThe increasing number of + signs for this structure represents its progressive growth and increasing complexity.

^c+? Indicates that the presence of these cells is inferred and suspected strongly, but has not been proven formally yet.

Table 2. Phylogeny of innate and adaptive immune responses in the brain and periphery^{a,b,c}

	Peripheral innate responses	Peripheral CTL responses	Peripheral neutralizing antibodies	CNS innate responses	CNS CTL responses	CNS neutralizing antibodies	Ventricular innate responses	Ventricular CTL responses	Ventricular neutralizing antibodies
Hagfish	+	?	–	+?	–	–	+?	–	–
Lamprey	+	–	–	+?	–	–	+?	–	–
Cartilaginous fishes	+	+	+	+	–	–	+	+	+
Bony fishes	+	+	+	+	–	–	+	+	+
Amphibians	+	+	+	+	–	–	+	+	+
Birds	+	+	+	+	–	–	+	+	+
Mammals	+	+	+	+	–	–	+	+	+

^aAbbreviations: CNS, central nervous system; CTL, cytotoxic T lymphocyte.
^bBased on information from Refs [32–34,39,41,57].
^c+? indicates that responses are inferred but remain to be proven formally.

animals were immunized against the same type of graft implanted into a non-CNS site. Medawar concluded that extended graft survival in the brain (and immune privilege) was a consequence of the absence of afferent priming of the immune response [thought, at the time, to be due to the presence of the blood–brain barrier (BBB) and the absence of brain lymphatics], but that an activated immune system could detect target Ags in the brain.

Why are adaptive immune responses not primed after parenchymal brain injections of viral vectors?

The lack of adaptive cellular immune responses to infectious particulate Ags injected into the naive brain can be most easily explained as a consequence of the absence of priming of naive T cells. This results from an anatomical or functional absence of afferent professional Ag-presenting cells (APCs) or dendritic cells (DCs) in the brain (Box 2). Lack of priming of naive T cells following the administration of infectious particulate Ags into the brain parenchyma

could be a consequence of: (1) the absence of DC precursors in the brain parenchyma; (2) an inability of DCs or their precursors to enter and colonize the brain; (3) a cytokine and biochemical environment that inhibits the differentiation of DC precursors into DCs; (4) a cytokine environment that is detrimental to DC survival in the brain; or (5) the anatomical impossibility for Ag-laden DCs to leave the brain parenchyma, owing to the absence of classical lymphatic exit channels. However, DCs are found within the choroid plexus and meninges [12,16,17,28–32], and this correlates with the induction of adaptive immune responses following injections of infectious agents or viral vectors into the brain ventricles.

Gene transfer into different brain compartments: an evolutionary and developmental perspective

The evolutionary appearance of the brain (in amphioxus and cyclostomes) pre-dates the appearance of the adaptive immune system in chondrichthyes by ~50–100 million years [33–35] (Fig. 1; Tables 1,2). Thus, during its early phylogeny, the brain co-evolved with the phylogenetically older innate immune system. Innate immune mechanisms are present in all animals preceding the cartilaginous fishes [33–35], as well as in insects and plants [36]. Thus, during the early stages of brain evolution, the brain acquired all innate immunity effectors, including cells of the innate immune system, as well as cytokines and chemokines.

To explain the peculiar brain immune responses to viral vectors, I propose that the brain was never 'colonized' by evolutionarily more-recent afferent APCs, such as DCs, which are the cell type responsible for initiating adaptive immune responses. Furthermore, it is proposed that the brain never became permissive to DC differentiation or evolved mechanisms to inhibit DC differentiation. This is supported by the lack of DCs in the naive brain parenchyma, as well as recent data demonstrating that brain microglial cells, which express c-kit and very low levels of Fms-like tyrosine kinase 3 (Flt3) and are not present in any other tissue outside the brain, are a very early precursor of both macrophages

Box 2. Classification of different types of antigen-presenting cells (APCs) according to their function [a–d]

Professional APCs

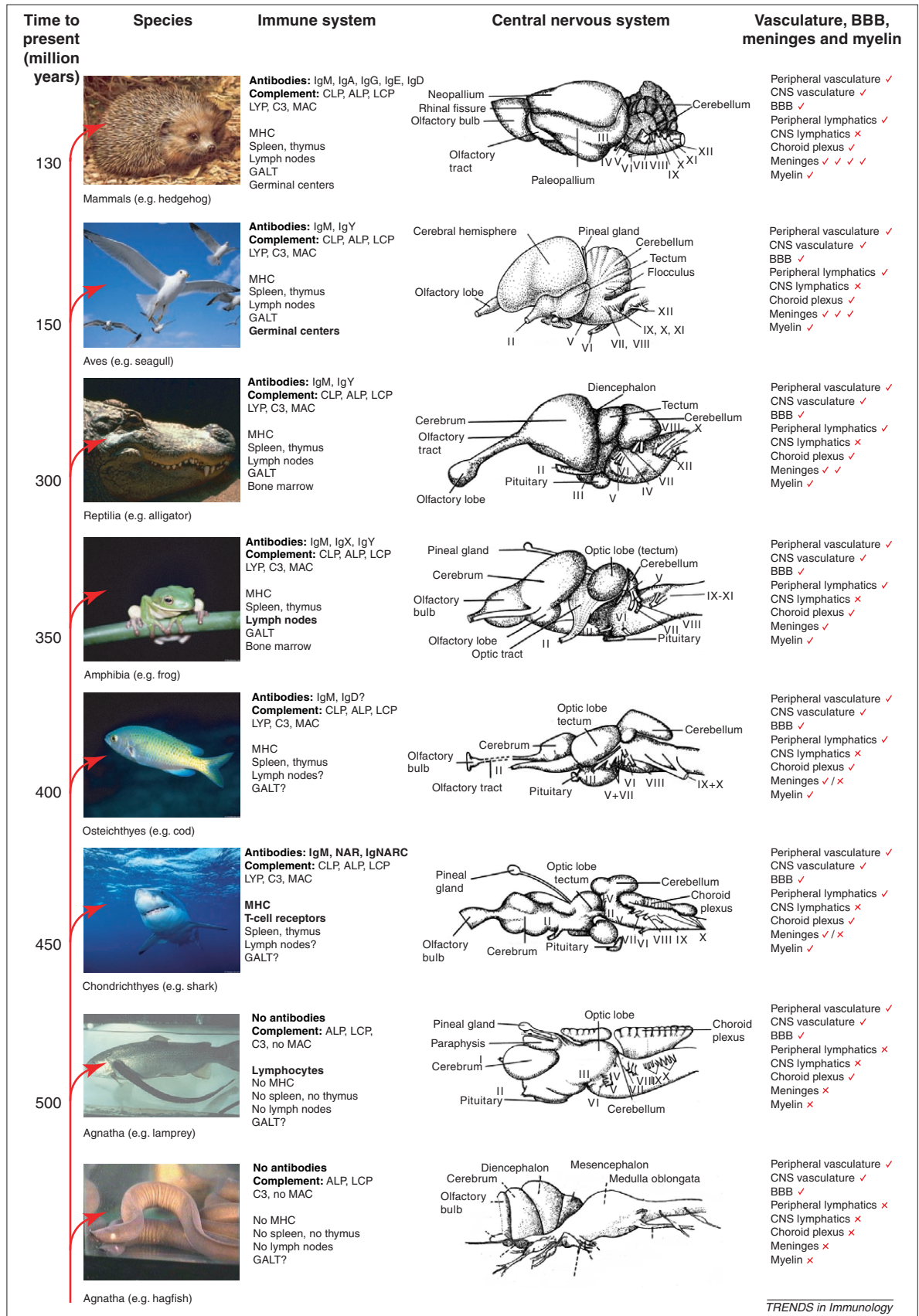
- **afferent APCs (dendritic cells):** cells that take up antigen, process it, become activated and migrate to secondary lymphoid organs, where they present antigenic epitopes on MHC class II molecules to naive T cells
- **effluent APCs (B cells and monocyte-derived cells):** cells that present antigenic epitopes on MHC molecules only to previously activated T cells

Nonprofessional APCs

- tissue cells that express MHC class I molecules and are capable of presenting antigenic epitopes to activated T cells

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TRENDS in Immunology

and DCs [37]. The evolutionarily late appearance or differentiation of the adaptive immune system should also be detected during development as the later

appearance of such cells and tissues during ontogeny, owing to constraints imposed by the developmental program [1] (Fig. 1; Tables 1–3).

Fig. 1. Comparative phylogeny of the brain and the immune system. This figure illustrates the co-evolution of molecules, cells and tissues of the innate and adaptive immune systems, as well as important anatomical and functional constituents of the central nervous system and the vasculature. The approximate time of origin of individual vertebrate groups is indicated by a red arrow. Individual vertebrate groups are illustrated by representative species. Central elements of the immune system are shown and the vertebrate group in which a particular cell type or tissue was identified for the first time is in bold. In particular, immunoglobulins described in each vertebrate group are indicated (a question mark indicates that the presence of a particular feature is disputed). Macrophages, which are present throughout all vertebrate groups, have not been indicated. A schematic view of the CNS of each species is shown also. The presence or absence of individual structures of the vasculature and nervous system are indicated by either a tick or cross, respectively; multiple ticks for the meninges during phylogeny indicate the progressive growth of this structure, which can be identified in sharks, but becomes progressively modified until adopting the classical trilaminar composition that can be identified in mammals. The figure illustrates the appearance of the adaptive immune response in chondrichthyes (appearance of MHC, antibodies, T-cell receptors, spleen and thymus), as well as the appearance for the first time of myelin in the same species. However, note that a BBB already exists in extant cyclostomes (e.g. hagfish and lampreys). Note also that lymph nodes are identified for the first time in amphibians; whereas germinal centers make their first appearance in aves. This figure is based on data taken from Refs [33–35,39,41]. Abbreviations: ALP, alternative pathway of complement activation; BBB, blood–brain barrier; C3, complement component 3; CLP, classical pathway of complement activation; CNS, central nervous system; GALT, gut-associated lymphoid tissue; LCP, lectin-dependent pathway of complement activation; LYP, lytic pathway of complement activation; MAC, membrane attack complex.

The brain ventricles and choroid plexi are anatomical structures already present in cyclostomes [33]. Lymphatic vessels, which appear together with the adaptive immune system in cartilaginous fish [33,35], colonized the choroid plexus and meninges, but never the brain parenchyma itself (Fig. 1; Tables 1,2). Thus, lymphocyte recirculation has been shown to occur through the CSF [38], but not the brain, and might provide the anatomical basis for the localization in the CSF of cells of the adaptive immune system, such as DCs. Therefore, DCs are able to enter, differentiate within and/or circulate through the developing choroid plexi and meninges, but not the brain parenchyma, which was never colonized by ingrowing lymphatic vessels and/or never became permissive to DC differentiation.

The BBB evolved to act as a selective barrier to the passage of solutes from the bloodstream to the brain and to keep the ionic concentrations of the internal milieu of the brain within a narrow physiological range [39]. Thus, the BBB only plays a limited role in regulating brain immune responses. A brain–lymph barrier is known to be present in insects [40], and the vertebrate BBB appeared early during vertebrate evolution, in cyclostomes (e.g. lampreys and hagfish), 50–100 million years before the appearance of adaptive immunity in cartilaginous fish [33–35] (Fig. 1; Tables 1,2). Lampreys and hagfish have an endothelial BBB, such as is found in vertebrates ranging from osteichthyes (i.e. bony fish) to mammals. By contrast, chondrichthyes (cartilaginous fish, such as sharks) are the only vertebrates with a glial BBB, which is only present in higher vertebrates in the hypothalamus and area postrema [39]. Thus, an evolutionary analysis fails to provide strong evidence either for a central role of the BBB in regulating brain–immune interactions or that it appeared in response to the adaptive immune system to protect the CNS. Whether or not certain specialized immunoregulatory features of the BBB did indeed co-evolve with the immune system remains to be determined.

An evolutionary analysis can also be used to elucidate the distribution of DCs throughout the meninges. Meninges are an even later phylogenetic acquisition than the brain ventricles and choroid plexi (Fig. 1; Tables 1,2). Primitive meninges are first recognized in cartilaginous fish (Fig. 1), but the three layers characteristic of mammals only become identifiable in amphibians [33,41]. Meninges, which co-evolved with the appearance and development of lymphatic vessels and the adaptive immune system, contain classical afferent lymphatic channels, and Ag exposure in the meninges leads to priming of naive T cells (Fig. 1; Tables 1,2). Accordingly, during development, meninges appear later, over the same time period that immune cells and tissues develop (Table 3).

Cells of the mononuclear lineages appear to have co-evolved with the brain, as suggested by the early evolutionary appearance of macrophages and recent evidence showing the existence of morphologically identifiable lymphocytes and lymphocyte-specific molecular markers [but not recombina-activating gene proteins 1 and 2 (RAG-1) and (RAG-2)] in lampreys [42]. Furthermore, no restrictions appear to have evolved for cells of the mononuclear lineages to enter and colonize the brain [43], whereas afferent APCs are never detected in the naive noninflamed brain.

'...the "immune privilege" of the brain is a result of the co-evolution of the brain and the immune system...'

Lymphocytes might also help to repair lesioned brain tissue. Recent data have demonstrated that specific lymphocytes reactive against myelin basic protein protect retinal neurons from axotomy-induced cell death. Also, activated macrophages promote spinal-cord regeneration, suggesting that potentially protective mechanisms of monocyte-derived cells and lymphocytes towards damaged brain tissue have been maintained during evolution [44,45].

Does the brain's capacity for Ag presentation change during inflammation?

During brain inflammation, cells displaying immunocytochemical, molecular or functional characteristics of DCs become detectable within the brain parenchyma. The appearance of DCs in the brain parenchyma, in experimental allergic encephalomyelitis (EAE), delayed-type hypersensitivity (DTH) or brain infection, or following the administration of the DC growth factor Flt3 ligand [17,30–32,46–48], suggests three possible mechanisms. These are: (1) DCs enter the brain after the induction of CNS inflammation (e.g. owing to

Table 3. Ontogeny of the brain, ventricles, choroid plexi, meninges, immune system and hemolymphoid system^{a,b}

	Brain ^c	Ventricles	Choroid plexus	Meninges	Immune system	Hemolymphoid system
Stage 12; day 8	Neural folds, pro-rhombomeres					
Stage 13; day 8.5	Rhombomeres 1–5					
Stage 14; day 9	Pros-, dien-, rhomb- and mesencephalic vesicles	IVth ventricle				
Stage 15; day 9.5	Telencephalon	IIIrd ventricle	Cavity (future IIIrd and IVth ventricle)			
Stage 16; day 10		Ventricular layer				
Stage 17; day 10.5						
Stage 18; day 11	Met- and myel-encephalon	Ependymal layer	Central canal and proto-ventricles become linked; ventricular system now filled with CSF			
Stage 19; day 11.5		Lateral ventricle	Lateral ventricle			
Stage 20; day 12	Hypothalamus		Choroid invagination	Primitive ectomeninx		
Stage 21; day 12.5	Thalamus, olfactory lobe and cortex		IVth and lateral ventricle choroid plexus	Arachnoid, dura- and pia-mater	Thymus primordium	B cells?
Stage 22; day 13.5	Ganglionic eminence	Pontine cistern and subarachnoid space		Falx cerebri, tentorium cerebelli and sub-arachnoid spaces	Spleen primordium	Lymph nodes?
Stage 23; day 14.5	Caudate-putamen		IIIrd ventricle choroid plexus			
Stage 24; day 15.5					Spleen and thymus	T cells? and Peyer's patches
Stage 25; day 16.5	Hippocampus					
Stage 26; day 17.5					Spleen: hilum and medulla; thymus: cortex and medulla	Thoracic duct ^d

^aThis table describes the detailed comparative ontogeny of individual structural components of the brain parenchyma, brain ventricles, choroid plexi, meninges and hemolymphoid and immune systems of the mouse.

^bBased on data from Refs [32–34,39,41,57].

^cIn all species, including the nonhuman and human primates, the nervous system is among the first structures to develop, and the choroid plexus, meninges and immune-system components appear later.

^dIn spite of the fact that the thoracic duct is only detected at a very late gestational stage in the mouse, lymphocyte circulation in the fetus has been shown to occur both in short-gestational species, such as mice, as well as in species with a long gestational period, such as sheep.

endothelial activation); (2) DC precursors enter the brain during inflammation and differentiate locally into DCs; or (3) inflammation induces the release of cytokines that induce the differentiation of very immature brain DC precursors into DCs [37].

The appearance of DCs in the brain during inflammation could provide the cellular basis underlying several different phenomena. They could explain epitope spreading during chronic demyelination induced by Theiler's virus [49], the capacity of bone-marrow cells to become perivascular Ag-presenting microglia [50] and, finally, the ability of mature, bone-marrow-derived DCs injected into the brain to recruit both CD4⁺ and CD8⁺ cells to the brain parenchyma and migrate to cervical lymph nodes [51]. Whether DCs recruited to the brain

during inflammation can migrate to local lymph nodes to present Ags remains to be demonstrated formally. However, available data suggest that, at least under some conditions, brain-infiltrating DCs could migrate from the brain to cervical lymph nodes during various pathological processes. However, brain inflammation *per se* might not be sufficient to recruit and induce APCs to exit from the brain. Brain inflammation occurs during stroke and Alzheimer's disease but these diseases are not associated with brain autoimmunity.

All markers that have been recognized on DCs within the brain parenchyma during inflammatory conditions are myeloid DC markers [17,30–32,46–48]. By contrast, DCs expressing lymphoid lineage markers have not been described in the brain

parenchyma proper, either in the absence or presence of inflammation. Therefore, a combination of the various mechanisms discussed above could account for the lack of lymphoid-derived DCs in the brain parenchyma. During brain inflammation, the local environment could allow the presence, differentiation and even, exit [51] of myeloid-derived DCs, but not lymphoid-derived DCs, which always remain excluded from the brain and are found only in the perivascular compartment (S. Ali *et al.*, unpublished).

Conclusion: clinical implications for the use of viruses as gene-therapy vectors for the treatment of brain diseases

This article proposes that the 'immune privilege' of the brain is a result of the co-evolution of the brain and the immune system (Fig. 1; Tables 1,2), maintained through phylogeny by constraints on the developmental programs (Table 3), as proposed by Gould and Lewontin [1]. This hypothesis is supported strongly by the parallel appearance of immune and nervous structures during ontogeny, following a time-course perfectly compatible with their evolutionary appearance.

The brain walks a tightrope between the evolutionary and developmental constraints, to build and maintain a working nervous system. Therefore, whereas macrophages and lymphocytes are required for the developmental remodeling of the brain and its protection from injury, they could potentially become APCs and thus, put the brain at risk of autoimmunity. The phagocytotic function of microglial cells during brain development precedes the recruitment of the macrophage and/or DC precursors [37] to the adaptive immune response. As a consequence, the brain is only permissive to the survival of the earliest myeloid precursors, and is nonpermissive for, or actively inhibits, the maturation of the endogenous, primitive myeloid precursors into DCs.

Now, viruses are being harnessed for neurological gene therapy as vectors of therapeutic nucleic acids. The evolutionary understanding of immune responses in the brain argues that, if viruses are delivered carefully into the brain parenchyma, and care is taken not to inject the ventricular compartment, viruses could avoid priming the adaptive immune response, such that both the gene therapy and the brain could remain unharmed. This is confirmed by experiments demonstrating the stability of adenovirus-mediated expression of transgenes, in the absence of peripheral priming of anti-adenoviral responses [5] or when using vectors deleted completely of any viral genes [5], even in the presence of pre-existing anti-adenoviral immunity [52]. However, it remains to be shown conclusively whether or not any lymphocytes primed to recognize adenoviral Ags could potentially cause bystander neurological damage in the brain. The hypothesis predicts that, if gene transfer is used to genetically engineer the brains of any species of vertebrate, including the cyclostomes, (in the absence of immune priming) transgene expression will be stable and long term. Also, it has implications for the treatment of brain tumors and brain autoimmune diseases using immunotherapy [53–56]. Furthermore, gene transfer into the brains of immunocompetent animals should be as stable as gene transfer into any other tissue of transgenic animals that lack the adaptive immune response. The absence of immune priming, even in the presence of damage to the brain and breakage of the BBB, suggests that, in the vertebrate brain, as opposed to other peripheral tissues, gene therapy using viruses might turn out to be safer than predicted. Whether this will also be true for chronically inflamed brains, such as those found in Alzheimer's or Parkinson's disease patients, remains to be assessed.

Acknowledgements

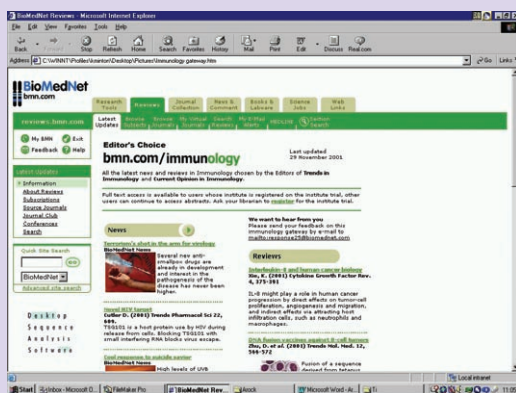
I wish to acknowledge the following for critical discussions of the ideas presented in this manuscript: M. Castro, C.A. Janeway, Jr, E. Bell, S. Ali, P. Walden and, especially, H. Lassmann, who has helped shape some of the ideas presented herein. I particularly wish to thank T. Southgate for his creative assistance in translating some of the essential ideas of this manuscript into its pictorial depiction in Figure 1. I was supported by a Research Fellowship from The Lister Institute of Preventive Medicine and was also funded by the Wellcome Trust, the Parkinson's Disease Society and the Cancer Research Campaign.

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