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Production, regulation and role of nitric oxide in glial cells

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Introduction

The central nervous system (CNS) consists of two major cell types, neurones and glial cells. Neuronal communication with other neurones or glial cells is effected mainly through neuro-transmitters and peptides, while glial cells appear to use an abundant range of factors for the communication with either neurones or other glial cells. The communication between neurones can span large distances in the body, while glial cell communication is mainly local or paracrine. During the past decade it has become clear that the glial cells named after glue (= glia) have more functions besides acting as a "nerve-glue" to form the brain.[1,2] The glial cells appeared to be essential for neuronal protection, survival and outgrowth during development and for the neuronal degeneration and regeneration under pathological conditions. In this review we summarize glial cell functions and focus on the role, production and regulation of nitric oxide (NO), a molecule which has been shown to be involved in various neuroimmune processes.

Glial Cells

Glial cells can be divided into microglial cells and macroglial cells, the latter of which are subdivided in astroglial cells and oligodendrocytes. The oligodendrocytes are known for their myelin production that wraps the axons in the white matter of the brain, and are affected in diseases like multiple sclerosis (MS). Glial cell activation has been indicated in many neuropathological diseases like Alzheimer's disease,[3–7] Parkinson's disease,[5] multiple sclerosis,[8] acquired immune deficiency syndrome (AIDS) dementia complex (ADC)[9–11] and other disorders.

Microglial cells

Microglial cells, formerly named Hortegaglia or Mesoglia, have been described in detail for the first time in 1932 by Del Rio-Hortega[12] considered as the father of microglia. Microglial cells are ubiquitously distributed in the CNS, show heterogeneous morphology and comprise up to 20% of the total glial cell population in the brain.[13] It was Del Rio-Hortega who proposed that microglial cells occur in two morphologically distinct forms, the ameboid or macrophage-like form representing as active microglial cells seen in developing brain and at sites of injury. These cells convert into the highly branched ramified microglial cells[14–17] viewed as quiescent cells in the mature CNS[18] which eventually can transform into active macrophages (reactive microglial cells) (Fig. 1).[19,20] Del Rio-Hortega suggested that microglial cells served as macrophages or phagocytic cells, which has found substantial support.[21–23]

The name mesoglia was derived from their proposed mesodermal origin. Indeed more recent and elaborative studies support a bone-marrow origin[17,24] from the usual macrophage precursor. Blood monocytes or monocyte precursors invade the brain during development.[25,26] In studies using chimeric rats, support was found for the bone marrow origin hypothesis[27,28] but in other rat chimera experiments the contrary was found.[29,30] The prevailing concept is however that blood monocytes are the precursors of ameboid microglial cells. There is apparently no need

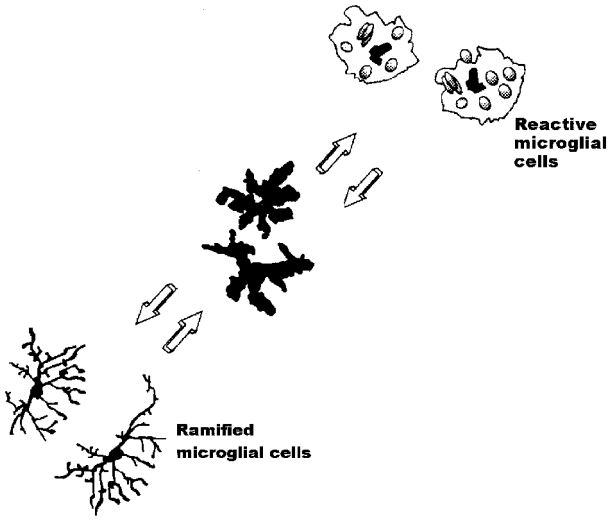

FIG. 1. Microglial cell activation cascade. Schematic drawing illustrating the transition of microglial cells from ramified microglial cells to the ameboid microglial cell, adapted from Ref. 371.

for the influx of a large number of monocytes into the brain parenchyma under normal conditions, since microglial cells have an extreme long life and have a low turnover rate.[31,32] Their numbers can be augmented by local proliferation[19,33–36] or by immigration of blood monocytes.[37] Under pathological conditions increased monocyte infiltration into the brain parenchyma is found[8,38,39] and the influx is suggested to be mediated by the microglial cell derived chemokine MCP-1[40] or adhesion molecules.

Microglial cell functions

The microglial cells serve as immunoregulatory cells, and are essential for resistance to inter- and intracellular pathogens. The microglial cells are present in a resting or a ramified form (Fig. 2) and are very important in immunosurveillance. After activation of the resting cells the microglial cells exhibit a highly potent phagocytic activity of foreign organisms and material, phagocytosis of injured or necrotic tissue, antimicrobial immunity, elimination of tumour cells and regulate inflammatory responses.[41] Microglial cells have been reported to possess Fc receptors,[18,42] CD4 antigen[43] and major histocompatibility complex (MHC) class I and II antigens[41,44–46] and thus are antigen presentating cells. Interferon-γ and interleukin-4 (IL-4) up-regulate MHC class II expression and induce microglial cell proliferation.[47] In addition, microglial cells show chemotaxic activity, like monocytes and macrophages, to several immunological factors such as complement factor C5a and to transforming growth factor β (TGFβ) suggesting that these cells can move to sites of injury and thereby participate in an inflammatory response.[12,42,48,49] These observations have led to a redefinition of the brain as immune privileged site a concept based on lack of inflammatory responses through the absence of T and B cells.

Microglial cells share many functional characteristics with cells of the monocytic lineage. Interleukin-1 (IL)-1 production by microglial cells has been demonstrated for the first time by Giulian,[50] and later by many others[41,51–54] and IL-1 is found to be present in injured brain tissue.[50,55] Other cytokines produced by microglial cells are TNF-α,[54,56,57] IL-6[57–59] and TGFβ[60–62] and they produce prostanoids such as prostaglandin E_2, (PGE_2), PGD_2, thromboxane[63,64] and leukotrienes, LTB_4, LTC_4 and 5-HETE.[65] IL-5 was found to be produced by microglial cells *in vitro* which may be involved in the interaction between glial cells and immune cells in the brain.[66] IL-10 and TGFβ both immunosuppressive and anti-inflammatory cytokines, have been demonstrated to be produced by human microglial cells and down-regulate microglial cell functions.[60,67–71] These properties confirm that microglial cells can initiate and regulate immune and inflammatory responses within the brain.

Astroglial cells

Astrocytes, oligodendrocytes and neurons are of ectodermal origin, and derive from the neuroepitelium of the primitive neural tube. Astroglial cells, unlike neurones, retain the ability to divide throughout life.[72] The multipotential stem cell develops into the bipotential progenitor cell and finally the glial lineage-restricted progenitor cell which can differentiate into the oligodendrocyte, the astrocyte type 1 and type 2.[73–76] The astrocytes outnumber neurones 10:1 in mammalian brain, and as their name imply, they have a star-shaped morphology. The astrocytes can be identified by using specific markers such as glial fibrillary acidic protein (GFAP), and glutamine synthase (GS), that are specific for both types of astroglial cells.[77–81]

Each cell forms processes that contact the blood vessels, where they form the so-called end-feet or sucker processes which also forms part of the blood–brain barrier (BBB) together with endothelial cells and the lamina basalis.[72] Astrocytes in the white matter are referred to as fibrous astrocytes, with numerous fibrils within their cytoplasm. In the grey matter, the astrocytes generally contain few fibrils and are called protoplasmic astrocytes. Interestingly, *in vitro* also two types of astrocytes can be identified, type 1 and type 2 astrocytes which are thought to be *in vitro* analogous of the protoplasmic and fibrous astrocytes respectively[82] (Fig. 2).

Astroglial cell functions

Initially the function of astroglial cells was thought to be a structural support within the CNS, with their processes having junctions with other astroglial cells, endothelial cells and neurones. In addi-

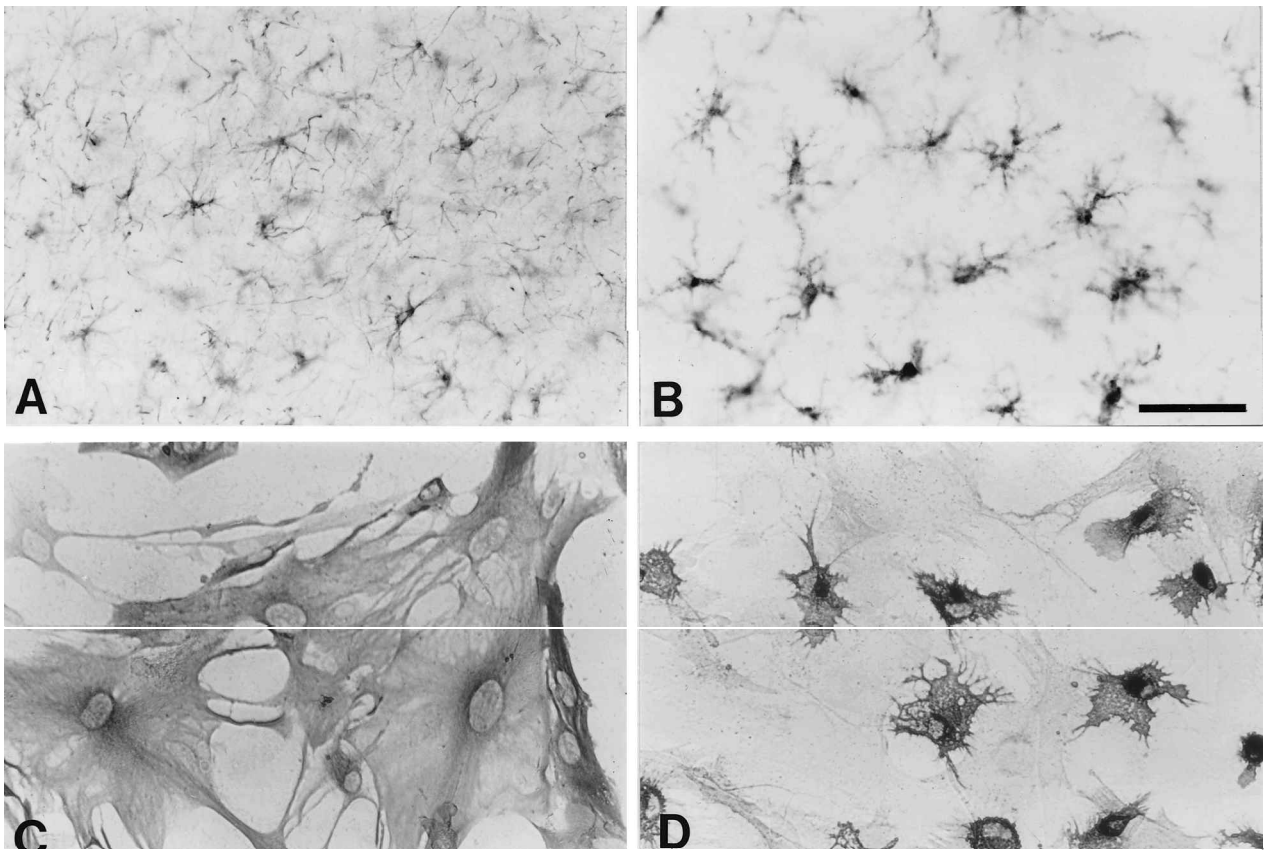

FIG. 2. Microglial cells and astroglial cells in rat brain ***in vivo*** and ***in vitro*** stained for GSA-I-B4-isolectin and GFAP respectively. (**A**) GFAP staining of astroglial cells in rat brain; (**B**) GSA-I-B4-isolectin staining ramified microglial cells in a rat brain; (**C**) GFAP ***in vitro*** in a purified astroglial cell culture; (**D**) GSA-I-B4-isolectin ***in vitro*** in a purified ameboid microglial cell culture. A, B, bar = 66 mm; C,D, bar = 25 mm.

tion in repair mechanisms the astroglial cells fill open spaces by proliferating and thereby forming a glial scar.[83–86]

It is now known that astroglial cells are very important cells in the outgrowth and survival of neurones during development and in neuropathology. Astroglial cells produce nerve growth factor (NGF)[87] *in vitro* and *in vivo* and the production of NGF is increased by IL-1,[88–92] TNFα,[93] IL-4 and IL-5.[94] Other neurotrophic factors produced by astroglial cells are ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factors (BDNF) and fibroblast growth factor (FGF).[95,96]

Astrocytes produce cytokines like IL-1,[41,97–100] IL-6,[101] IL-3,[102] TGFβ[60,62,103–105] and IL-15[106] and factors like prostaglandin E_2 (PGE_2),[97,99,107,108] granulocyte-macrophage colony-stimulating factor (GM-CSF)[109,110] and microglial mitogens (MM).[111] TNFα is also produced by astroglial cells *in vitro* after lipopolysaccharide (components of Gram-negative bacterial outer membranes; LPS)[56,112] and IL-1β stimulation[59,106] or mycoplasma infection.[108]

Astrocytes can be induced to express MHC I and II class molecules *in vitro* and are able to present antigen[113,114] and thus are important immune cells in the brain. However, in contrast to microglial cells they do not express significant levels of MHC class II molecules[115] *in vivo*.

Glia–glia interactions

Recent studies have shown various interactions between microglial cells and astroglial cells. IL-1 produced by microglial cells has been shown to stimulate astroglial cell proliferation *in vitro*.[116–119] Intracerebral injections of IL-1 or local production of IL-1 by microglial cells elicit astrogliosis which may result in scar formation[120,121] and thereby have a negative effect on axonal outgrowth and remyelination. In addition the microglial cells influence the production of NGF by astroglial cells, which is enhanced after IL-1 and IL-5 both produced by microglial cells.[66,94,122]

On the other hand, astroglial cells influence microglial cell functions. Interleukin-3 (IL-3) is mitogenic to microglial cells and has been suggested to be produced by astrocytes.[102,123] Mitotic activity of microglial cells can additionally be elevated by colony stimulating factor-1 (CSF-1), which production is increased by IL-1, TNFα,[124] granulocyte-macrophage

colony-stimulating factor (GM-CSF)[41,109,110,122,125–127] or microglial mitogen (MM).[111]

The microglial cells undergo morphological changes in response to factors released by or through cell–cell contact with astrocytes. These factors derived from astrocytes, induce microglial cells to become the ramified, functionally resting cells *in vitro*, while inflammatory mediators like interferon γ (IFNγ) and LPS induce microglial cells to become ameboid.[128,129] Also, blood monocytes and spleen macrophages differentiate into ramified microglial cells when cultured onto an astroglial cell monolayer.[130,131] This illustrates the capacity of astroglial cells to transform cells from monocytic origin into cells with microglial cell morphology.

In addition, astroglial cells have been shown to functionally regulate microglial cell activity. The endotoxin induced synthesis of iNOS and release of NO but not the production of IL-1β by microglial cells is inhibited in the presence of astroglial cells *in vitro*.[51]

Apparently both glial cells communicate through the production of various factors including cytokines and growth factors. In this way the glial cells appear to tightly regulate each others morphology, activity and secretion of products.

Glia–neurone interactions

The presence of glia-derived cytokines in the CNS and the function of these cytokines *in vitro* suggest that they are important for normal brain development and homeostasis.[132,133] However, excessive expression of these cytokines may be a factor in abnormal glial functions leading to neuropathological events.

In general astrocytes have been found to express neurotrophic factors such as ciliary neurotrophic factor (CNTF), neurotrophin-3, fibroblast growth factor (FGF) and NGF near the site of injury.[134,135] *In vitro* experiments show that astrocytes protect dopaminergic neurons against H_2O_2 toxicity[136] through actions of glutatione. Interestingly, survival of these dopaminergic neurones *in vitro* is enhanced by the presence of glial cells derived from striatal astroglia: the target-derived astroglial cells, illustrating astroglial cell heterogeneity.[137,138] The survival of dopaminergic neurones is promoted by glial cell-lined derived neurotrophic factor (GDNF) *in vivo*.[139] *In vitro* co-cultures of neurones and astrocytes induced an increased cell survival and neurite outgrowth[140–142] and axons might trigger glial differentiation.[143] The ability of astrocytes to augment neuronal survival was increased after treatment of the astrocytes with macrophage conditioned medium[144] indicating that factors produced by macrophages induce the production of neurotrophic factors.

Neurones are less sensitive to oxygen or glucose deprivation or treatment with glutamate when co-cultured with astrocytes.[145] In addition astrocytes increase neuronal survival under pathological conditions since they have an energy reserve stored as glycogen which becomes available for neurones under conditions of energy substrate limitations.[146] Astroglial cells are important in the metabolism of glutamate and GABA and other neurotransmitters,[147] and maintain the microenvironment by regulating the ionic composition of the extracellular space around the neurones.[148]

Neurotrophic effects i.e. neuronal survival and neurite extension have also been reported by microglial cell conditioned medium[149] and more specifically by the production of NGF[150] and thrombospondin.[151,152] In addition secretion of IL-6, IL-1, FGF, TGFβ, TNFα by microglial cells and astroglial cells may stimulate nerve growth factor production by astroglial cells for the regeneration of neurones.[93,134,153–158] These factors also directly improve the survival of neurones and/or have synergistic effects with NGF.[159,160]

While in general factors released by astroglial cells actually increase the survival of neurones,[161] microglial cells in contrast, can directly participate in neuronal cell death through the release of neurotoxins.[162] Microglial cells have been reported in the presence of degenerating neurones in various regions in the brain.[18,163–165] In these regions the microglial cells clearly contribute to the removal of pycnotic cell bodies. Prior to this scavenger role, the active participation of microglial cells in neurite amputation has been shown on electron microscopic pictures of microglial cells engulfing axon processes which display no obvious signs of degeneration.[166] It is therefore thought that interactions between neurones and microglial cells may not be restricted to cell debris scavenging but microglial cells may also induce neuronal cell death.

In vitro studies have demonstrated the production of many different neurotoxins by microglial cells. Activated microglial cells release several cytotoxic compounds i.e. reactive oxygen intermediates,[167,168] NO, proteases[51,169–172] and inflammatory cytokines i.e. IL-1, TNFγ or TNFα,[50,56,119,127,157,173,174] that play a role in neuronal damage in the CNS. Finally microglial cells produce large amounts of glutamate and aspartate *in vitro*.[175] The release of these excitatory amino acids points to a further role of microglial cells in NMDA receptor-mediated neuronal injury.[175] In addition, cultured microglial cell release large amounts of H_2O_2, which leads to neuronal cell death in neurone–microglial cell cocultures.[172,176,177]

Taken together, activated microglial cells display a broad repertoire of cytotoxic functions which could be involved in tissue damage during CNS injury. In addition, microglial cells activate astroglial cells in a way that benefits regeneration. Further, the effect of neurotoxic factors released by microglial cells can be

attenuated by proteins released from astroglial cells.[178,179] This illustrates the fascinating and delicate interactions that exist between glial cells and neurones, which are crucial in maintaining neural functioning and integrity. These studies have contributed towards a concept which considers reactive microglial cells as an opposing force to neurotrophic astroglia, the two glial cell populations rivaling in regulating survival of neurones.[180]

Nitric Oxide in the CNS

Various cell types in the CNS, i.e. neurones, endothelial cells, microglial cells and astroglial cells produce NO. Different isoforms of the nitric oxide synthase (NOS) are responsible for the production of NO by these cell types. NO in the brain is multipotent and is responsible for blood flow regulation, may act as a neurotransmitter or as a neurotoxic agent, depending on the cellular source, amount and production site.

Isoforms of nitric oxide synthase

NO, a free radical gas, was found to be responsible for the vasodilatation in arteries and at first named endothelium derived relaxing factor (EDRF).[181] Later the source of NO was elucidated revealing the enzymatically conversion of L-arginine to L-citrulline by NO synthase whereby NO is produced (Fig. 3).[182] Three isoforms of NOS, encoded by different genes[183] have been characterized, isolated and cloned to further study the physiologic and/or pathologic functions of NO.[184] All three isoforms, endothelial (eNOS, ecNOS or type III), constitutive (cNOS, nNOS, bNOS or type 1) found in astroglial cells and neurones and inducible (iNOS, mNOS, macNOS or type II), NOS are found in the CNS and play a role in certain physiological or pathological functions of the CNS (see following section).[185] Constitutive NOS is constitutively expressed in neurones, is Ca^{2+} and calmodulin dependent, and mediates the production of only small amounts of NO after stimulation. This neuron derived NO is very rapidly produced and released since cNOS is constitutively expressed and does not require mRNA synthesis, acts as a neurotransmitter with properties that differ from other neurotransmitters: (a) it is not stored in vesicles, (b) there are no specific release or uptake mechanisms and (c) its transmission is not synaptic. NO diffuses into the target cell and directly regulates enzymes systems, such as activation of guanylate cyclase, resulting in increased cGMP levels.[186,187] NO has a half life of a few seconds in contrast to the milliseconds of the neurotransmitters in classical synapses.[188,189] It is important in neurotransmission, and NO is considered a candidate for a memory-related process named long-term-potentiation (LTP).[190,191] Indeed, inhibitors of NOS can block LTP.[192,193] In areas such as the cerebral cortex, hippocampus, cerebellum and corpus striatum, cNOS expressing neurones compose 1–2% of all neuronal cells.[194] Activation of cNOS in astroglial cells was observed after challenge with calcium ionophores, bradykinin or glutamate.[185]

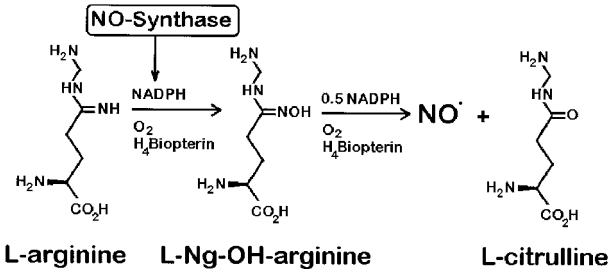

FIG. 3. Biosynthesis of NO through the so-called L-arginine-NO pathway, adapted from Ref. 372.

Endothelial NOS (eNOS) produced by endothelial cells is a constitutive Ca^{2+}-dependent enzyme that is essential for the control of vascular tone. NO transduces a signal from the endothelial cell to the vascular smooth muscle eventually leading to cGMP production and vasodilation. In the brain, eNOS-derived NO regulates cerebrovascular blood flow.[195]

Inducible NOS (iNOS) is a Ca^{2+} and calmodulin independent enzyme. It requires gene transcription, is slowly produced and is activated only under pathological situations where microglial cells and macrophages exert cytotoxic effects in response to cytokines.[196] The generation of NO by iNOS is long-lasting, in contrast to the cNOS and eNOS isoforms where NO is generated in short bursts.[197,198] The mechanism of iNOS induction involves transcription of mRNA and novel protein synthesis and it takes several hours before NO is generated after the initiating signal.[199] It induces a 100-fold higher local concentrations of NO than eNOS or cNOS and act as a antimicrobial defence mechanism of the immune system. iNOS is not expressed in normal brains but expression can be induced in astroglial and microglial cells through viral infection or trauma.[200,201] It is mainly expressed under inflammatory conditions, and after transient ischaemic periods.[202–205]

Nitric oxide in neuropathology

NO can be neurotoxic under different circumstances. The NO mediated neuronal cell death can be induced by overexpression of cNOS in neurones and astroglial cells or iNOS induction in glial cells, both pathways will be further discussed below (see Fig. 4).

cNOS induced NO mediated neurotoxicity

Glutamate binding to its NMDA receptor induces NO production by cNOS activation. Derangements of

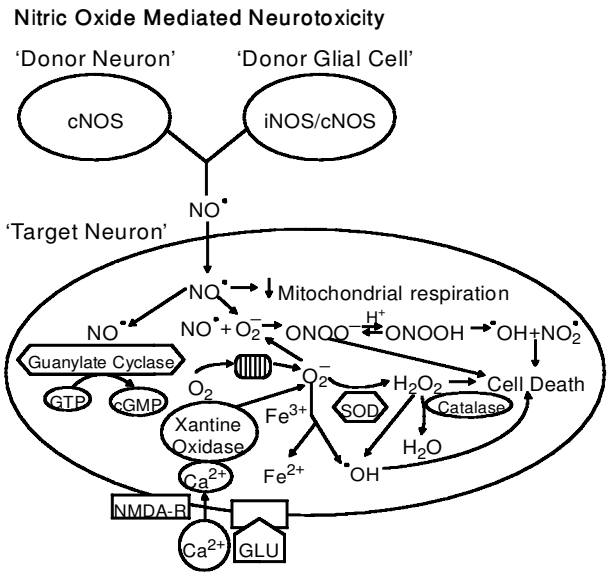

FIG. 4. NO as neurotoxin. Excessive NO is formed on sustained glutamate stimulation of NMDA receptors by cNOS in neurones or by activation of iNOS or cNOS in glial cells. NO freely diffuses to adjacent target neurones where it combines with the O_2^- to yield the peroxynitrite, $ONOO^-$, which is an extremely potent oxidant. Although NO can function as a toxin directly, the peroxynitrite pathway may be the major pathway of cell death, adapted from Ref. 303.

glutamate neurotransmission leading to neurotoxicity has been implicated in Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), epilepsy and stroke.[206,207] Glumate neurotoxicity is demonstrated to be mediated through NO production. After binding of glutamate with the NMDA subtype of glutamate receptors, Ca^{2+} enters the channel and binds to calmodulin, a cofactor for cNOS and stimulates NOS activity whereby NO is produced.[208] In addition, O_2^- is produced which results in the formation of $ONOO^-$ which subsequently leads to neuronal death.[209–211] Neurones obtained from cNOS null transgenic mice are markedly resistant to ischaemic conditions,[212] in which the primary mechanism of damage is mediated by activation of the NMDA receptor and subsequent formation of NO. This indicates that cNOS is capable of producing neurotoxic amounts of NO.[213]

iNOS induced NO mediated neurotoxicity

The iNOS-mediated release of NO by astrocytes and microglial cells in the brain may be important in antimicrobial or tumoricidal responses to inflammatory signals.[197] In acute CNS inflammatory conditions like rabies, herpes simplex, Borna, and lymphocytic choriomeningitis virus, iNOS is expressed[200,214–217] as well as in experimental pneumococcal meningitis and toxoplasmosis and in humans during encephalitis.[130,218–220] iNOS mediated NO is considered to mediate neuronal and oligodendrocyte degeneration under neuropathological conditions.[202,221,222] The role of iNOS in neuropathology in general is indirect, microglial cells and/or macrophages become activated either through direct infection with virus or other pathogens, or by local cytokine production and subsequently produce iNOS which eventually leads to damage. Excessive amounts of NO produced by glial cell are probably neurotoxic. iNOS-derived NO is one of the major sources of toxic free radicals in the brain, since its reaction with the superoxide anion (O_2^-) leads to the formation of peroxynitrite anion ($ONOO^-$) which is an extremely potent oxidizing agent.[223] Peroxynitrite generates DNA-single-strand breaks with subsequent activation of the DNA repair enzyme poly ADP ribosyltransferase (PARS).[224] Furthermore, NO and peroxynitrite have been shown to inhibit the mitochondrial respiratory chain and disrupt normal cellular iron homeostasis.[201,225–227] Peroxynitrite and/or NO can terminally damage neurones, leading to cell death.[228–232] Low levels of or sustained exposure to NO or peroxynitrite cause apoptosis, whereas sudden exposure to high concentrations of NO or peroxynitrite leads to necrosis.[233]

NO in Alzheimer's disease and MS

iNOS expression is found in Alzheimer's disease[234,235] and in experimentally infected brains of rats with various viral agents.[200] NO has been implicated in demyelination and destruction of oligodendrocytes and subsequent demyelination, the process found in MS[236] and in the primary animal model for MS, experimental allergic encephalomyelitis (EAE).[216,237] During EAE, iNOS mRNA is detectable before the onset of the clinical symptoms and the levels of protein correlate with the severity of the disease.[200] Further evidence supporting a role for iNOS in the pathogenesis of MS is the finding that human iNOS protein and mRNA is markedly elevated in the active lesions in brains of MS patients.[238,239] In MS lesions, macrophages appear to produce iNOS and are NADPH-diaphorase positive after histochemical staining.[240] In another study, NADPH-diaphorase activity in MS lesions was found in reactive astroglial cells[239] which was later shown to be cNOS.[240] The cellular source of iNOS mRNA expression in brains of patients with MS has been confirmed to be macrophages/microglial cells.[238] In EAE an increase was found in eNOS in blood vessels in the inflamed lesions and increase in iNOS in infiltrating inflammatory cells.[216,241] iNOS expression can be (further) induced by cytokines like TNFα, IFNγ and IL-1, that are all detected in brains of MS patients.[242–245]

NO in AIDS dementia complex

iNOS-mediated NO production has also been described to be involved in acquired immune deficiency syndrome (AIDS)-related neuropathology. iNOS protein and mRNA was found in the CNS of patients with AIDS dementia complex (ADC),[246,247] and is

expressed at higher levels than in brains of AIDS patients without neurological symptoms. The cell types expressing iNOS however, remain unknown. In these patients there is a clear correlation between the severity of the dementia and iNOS expression in the brain. In addition in brain tissue of simian immunodeficiency virus (SIV) infected monkeys iNOS mRNA was detected.[248] The microglial cells have been postulated to be involved in the pathogenesis of ADC because they are preferentially infected by the virus.[43,249–251] Neuropathological manifestations such as loss of cortical neurones, loss of synapses and neuronal apoptosis have therefore been suggested to be mediated indirectly by cytokines like TNFα[252,253] and IL-1[254–256] or by nitric oxide.[257–261] TNFα, IL-1 and iNOS have been demonstrated in brains of AIDS patients[247,256,262–265] as well as in ADC patients[259,266,267] (Vincent *et al.*, submitted). In cytomegalovirus (CMV) infected retinas from AIDS patients iNOS immunoreactivity and NADPH-diaphorase were found in (CMV)-infected cells identified as Müller cells and astrocytes.[268]

NO in parkinson's disease

In Parkinson's disease, which is primarily characterized by a loss of midbrain dopaminergic neurones, iNOS was present in glial cells[269] in the mesecephalon probably in activated macrophages.[269] In addition, NOS inhibitors attenuate malonate-induced degeneration by NMDA receptor activation of the nigrostriatal pathway in rats[270] suggesting a role for NO in neurodegeneration. The inhibition of tyrosine hydroxylase resulting in reduced dopamine synthesis is triggered by peroxynitrite, a reaction product of NO.

NO in brain activation

During ischaemic brain damage eNOS is induced in endothelial cells which then has beneficial effects by enhancing the vasodilation, further increasing blood flow in the peri-infarct area.[272,273] In addition iNOS is induced leading to NO production, which leads to neuronal death after cerebral ischaemia.[274] During postnatal brain development of rats, large numbers of NADPH-diaphorase positive neurones and NADPH-diaphorase positive cells with macrophage morphology were observed. The latter are possibly involved in developmental shaping of the brain, which includes cell death and fagocytosis of cellular debris.[275] High levels of NADPH-diaphorase were found in evenly distributed astroglial cells in areas surrounding a mechanical lesion in the brain. Within the lesion the NADPH-diaphorase positive cells most probably were macrophages.[276]

Thus, although iNOS and NADPH-diaphorase activity can be induced in astrocytes and microglial cells through viral infection or trauma most of the studies have revealed iNOS immunoreactivity and iNOS mRNA in the brain in infiltrating macrophages or microglial cells.[210] In general, exposure of brain cells to signals, such as microbial products, viruses, glutamate or yet unknown signals in diseases like MS, Alzheimer's and Parkinson's disease leads to the secretion of inflammatory cytokines that induce either *de novo* synthesis of iNOS by glial cells or cNOS in astroglial cells or neurons as has been demonstrated in *in vitro* studies. This NO may be neurotoxic and may subsequently lead to neuropathology.

Production of iNOS *in vitro*

To answer some more fundamental questions regarding iNOS production and regulation *in vitro* studies are widely used. Sources, induction mechanism and intervention of iNOS production are studied in detail in various glial cell cultures. In addition, a model has been developed that allows studies of the possible neurotoxic effects of glial NO, on cultured neurons. These *in vitro* studies have led to new insights in the functions of glial cells in normal brain and in neuropathology.

Several techniques have been described for isolation of rat murine or human brain microglial cells, astroglial cells and maintenance of these cells *in vitro*.[118,240,277–282] The microglial cells and astroglial cells are isolated from mixed glial cultures in most instances or from disrupted adult or neonatal tissue.[53,127,171,279,283–285] In tissue culture, the ameboid and ramified microglial cell can be identified, most probably corresponding to its morphological diversity in the adult brain.[286] The astroglial cell cultures can be contaminated with microglial cells, since the purification of astroglial cells is a rather delicate technique and microglial cells remain a significant contaminant.[56,161,287] Many studies showing production of, for example, cytokines like IL-1 or TNFα by astroglial cells *in vitro* have to be interpreted with care. *In vitro* studies also provide a very useful technique to study both the functions of glial cells and of neurones as well as the interactions between these cell types. Therefore techniques for selective isolation, co-culturing, labeling and stimulation of microglial cells and astroglial cells allow investigators to study some fundamental questions in neuro-immunology.

Glial cell-derived iNOS

iNOS expression by glial cells has been studied in *in vitro* systems of highly purified microglial cell and astroglial cell cultures and in mixed cultures containing both cell types from human or rodent brain. Following incubations with various stimuli, both microglial and astroglial cells have been demonstrated to produce nitrate, one of the end-products of nitric oxide oxidation.[203]

iNOS has been identified in rodent astrocytes and microglial cells in response to IL-1β, LPS or Gram-positive bacterial products.[185,259,288,289] IFNγ induces NO in microglial cells and macrophages but not in astroglial cells,[203,221,290–292] while synergism of IFNγ and IL-1β or TNFα induce significant levels of nitrite in rodent and human astroglial cells.[203,239,250,293,294] LPS induction of iNOS required CD14 expression on glial cells.[295] As yet it is not clear whether cytokines activate gene expression via one or multiple pathways. Experiments with phorbol esters, which induce iNOS, suggest that protein kinase C may be involved in the induction process in microglial cells and astrocytes.[296]

Agents like IFNγ and LPS are more effective inducers of iNOS in rodent than in human microglial cells,[198,250,288,297] which therefore appears to be species-dependent as described for NO production by retinal pigment epithelial cells.[298] Recently, some studies did however reveal NO and iNOS mRNA production in human ramified microglial cells upon LPS or TNFα stimulation.[267,299]

HIV or the HIV type I coat proteins gp120 or gp41 induce iNOS in cultured microglial cells, monocytes or macrophages.[9,246,257,258,266] In human fetal glial cells, comparable amounts of NO were induced by gp120, gp41 and the proinflammatory cytokines IFNγ and IL-1β.[9] HIV-infected brain mononuclear macrophage secrete NO and O_2^-,[266] especially after immune activation and TNFα further increases NO production. In MS and EAE, macrophages isolated from lesion areas produced significant amounts of nitrite and were shown to be iNOS positive without any further stimulation.[240,300]

β-amyloid, the major component of the senile plaques in Alzheimer's disease, causes a significant increase in NO by microglial cells and not in astroglial cells. The NO production induced by the β-amyloid was increased by IFNγ[301,302] or phorbol-myristate-acetate (PMA) challenge.[168]

Studies that have attempted to compare rat microglial cell and astrocyte NO production have concluded that microglial cells produce more NO on a per cell basis than astrocytes.[202,222,290] This has led to the suggestion that activated microglial cells rather than astrocytes are the principal source of reactive nitrogen intermediates in the CNS, and that the NO produced by astroglial cells might be beneficial, whereas microglial-derived NO might be involved in neurotoxicity.[185]

Nitric oxide mediated neurotoxicity in vitro

In vitro co-cultures of glial cells with neurones have proven to be a valuable tool in the identification of NO as a neurotoxin and the cellular sources of NO. Exogenous NO generated from NO donors have been shown to kill neurones *in vitro*.[224] Cytokine-activated murine microglial cells and astroglial cells apparently generate substantial amounts of NO that kill neurones *in vitro*,[202,222,259,288,303,304] since inhibition of endogenously formed NO by specific NOS inhibitors blocks this microglia- or astrocyte-mediated neurotoxicity.[202,260,305] For example, factors like LPS, gp41 and β-amyloid can indirectly kill neurones in mixed cultures with glial cells, which is abrogated by NOS-inhibitors.[246,301] LPS or cytokine activated glial cells stimulate the production of neurotoxins, e.g. NO, because LPS and cytokines do not directly influence the viability of purified neurones.[307] These studies clearly illustrate the indirect mechanism by which neurones are thought to be killed.[306]

The glial cells produce large amounts of NO by iNOS activity which can form peroxynitrite which is toxic to neurones as previously described. In addition, glutamate neurotoxicity is also mediated by NO in primary neuronal cultures.[303] Peroxynitrite, NO and NMDA can damage neurones *in vitro* leading to necrotic or apoptotic cell death pending on the concentration and duration of the exposure.[229] In addition, oligodendrocytes are also being killed by an NO dependent mechanism by ameboid microglial cells *in vitro*, suggesting that iNOS expression by invading and intrinsic brain cells play a role in lesion formation in multiple sclerosis.[221]

Although glial cells produce various neurotoxins i.e. TNFα, glutamate and PAF, production of NO appears to play a key role in different neurotoxic pathways since inhibition of iNOS spares the neurones.[260,305,308] These *in vitro* findings suggest an important role for glial iNOS derived NO in the pathophysiology of CNS diseases.

Regulation of NO Production

Since NO is shown to play an important role in neurotoxicity, inhibition of NO could be a possible route of intervention in the prevention of neuropathogenesis. Experimentally often used inhibitors of NOS production are synthetic arginine analogues like N^G-mono-methylarginine (NMMA), Nw-nitro-L-arginine methyl ester (L-NAME), aminoguanidine and N^G-nitroarginine.[239,258,309] They are often used in *in vitro* studies to certify the L-arginine dependent origin of NO.

Intervention of NO synthesis *in vivo* by using NOS specific inhibitors has been described in EAE using different arginine analogues e.g. aminoguanidine, L-NMMA and L-NAME.[241,300,310,311] In these experiments, the effects of these inhibitors on the clinical score of EAE were not conclusive. The aspecificity of these inhibitors for the subtypes of NOS, and thereby effects on eNOS and cNOS leading to e.g. vascular changes, may explain these different results. The search for pharmacological tools that selectively inhibit iNOS, eNOS or cNOS is currently getting much attention and thus far has yielded some agents. The

agent 7-nitroindazole is a selective cNOS inhibitor and N^G-nitro-L-arginine shows a preference for eNOS and cNOS over iNOS, whereas L-N^6-(1-iminoethyl)lysine is selective for iNOS over cNOS.[312]

Interestingly, NO can also act as a negative feedback signal on iNOS activity, by inhibiting the transcriptional induction of NOS, indicating a self-regulatory mechanism.[313,314] In cultures of rat astroglial cells norepinephrine and dexamethasone have been shown to suppress iNOS induction.[257,288,289,315] In addition, prostanoids are involved in the regulation of iNOS since prostaglandin E_2 but also cyclooxygenase inhibitors suppress iNOS expression in LPS activated rat microglial cells.[316] In addition several endogenously produced cytokines have been shown to inhibit NO production. TGFβ, IL-4 and IL-10, are known to inhibit NOS activity in monocytes[9,221,266,305,317–320] and synergistically suppress NO production.[70] TGFβ is an important modulator in the brain during development[321] and TGFβ has been demonstrated in brains of HIV patients, around brain tumours, in MS, Alzheimer's disease, brain ischaemia, peripheral nerve transections and in several experimental lesions in animals.[322–330]

Transforming growth factor β (TGFβ)

TGFβ is a 25 kDa homodimeric protein secreted by a variety of cells as a latent protein complex.[60] There are at least five distinct gene products that constitute the TGFβ family, TGFβ1 through TGFβ5 which show a high degree (70–80%) of amino acid sequence identity. The three highly homologous mammalian TGFβ isoforms are TGFβ1, TGFβ2 and TGFβ3 with relatively high sequence similarities but differences in receptor binding affinities.[331] Three different TGFβ receptors have been identified, type I to type III, which distributions are ubiquitous in various body tissues.

In a variety of studies of microglial cell cultures, TGFβ has been shown to be a potent immunosuppressive cytokine[332] and inhibits NO production by microglial cells.[333] TGFβ inhibits iNOS expression by decreasing iNOS mRNA stability and inhibiting its translation and increasing iNOS protein degradation,[334] resulting in reduced NO production.[317,335] Not only NO but also O_2^- is inhibited by TGFβ[71] and thereby the formation of the highly toxic peroxynitrite is prevented.

TGFβ is a chemotactic agent for monocytes and macrophages and is suggested to be important in the recruitment of circulating monocytes into brain tissue after damage.[336] TGFβ has protective effects in different experimental autoimmune diseases[337–339] whereas neutralizing antibodies to TGFβ1 worsen clinical severity.[340,341] During EAE, and after cerebral trauma or hypoxic-ischaemic damage, TGFβ expression is increased when neurological symptoms are severe, which might indicate an inflammation limiting of TGFβ in the recovery phase thereby controlling the inflammatory reaction.[342–345]

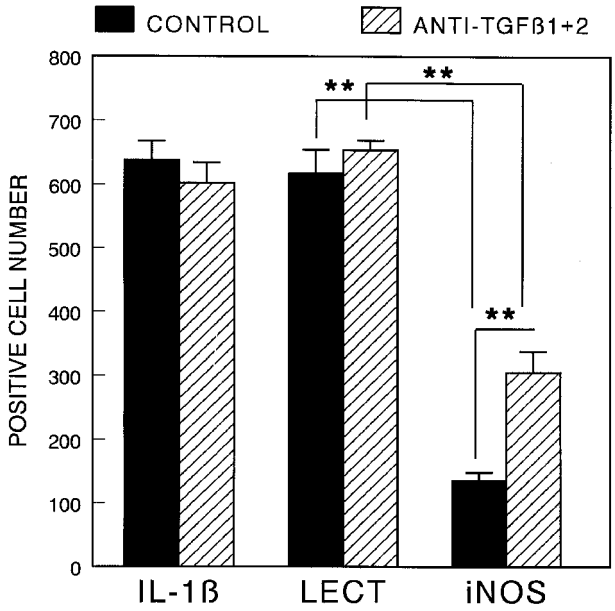

FIG. 5. Effect of immunoneutralizing of TGFβ1 and TGFβ2 on endotoxin induced expression of IL-1β and iNOS by confluent mixed glial cell cultures containing mainly astroglial cells and microglial cells. Numbers of immunopositive cells in 24 h endotoxin (1 μg/ml) stimulated mixed glial cell cultures. Different cultures were stained with the microglial cell marker GSA-I-B4-isolectin (LECT) or for IL-1β or iNOS. Data are expressed as the mean and SE (n = 3). $^{**}P < 0.005$.

Astroglial and microglial cells are known to constitutively produce TGFβ *in vivo*[61,324,325] and *in vitro*.[62,103–105,346,347] The isoforms TGFβ1, TGFβ2 and TGFβ3 are produced by astroglial cells *in vitro*, while microglial cells only produce the TGFβ1 isoform.[60] TGFβ production in microglial and astroglial cells is increased after TNFα[348] or IL-1[104,349] or TGFβ exposure itself.[350]

In co-cultures of astroglial cells and microglial cells bioactive TGFβ was found to inhibit iNOS expression and thereby NO production by endotoxin activated microglial cells. The presence of astroglial cells was shown to be essential for the activation of TGFβ in these co-cultures of astroglial and microglial cells[62] (see Fig. 5).

Regulation of TGFβ activity

TGFβ is produced by glial cells in a latent, inactive form[60] and forms a complex with the latency-associated protein (LAP).[351,352] Activation of latent TGFβ consists of releasing TGFβ from the LAP, which occurs after heat treatment, acidification, alkalization or proteolysis by plasmin.[351,353] Plasmin is generated by the plasminogen activator system. Plasminogen activators (PA)s are serine proteases consisting of a 50 kD urokinase-type plasminogen activator (u-PA)

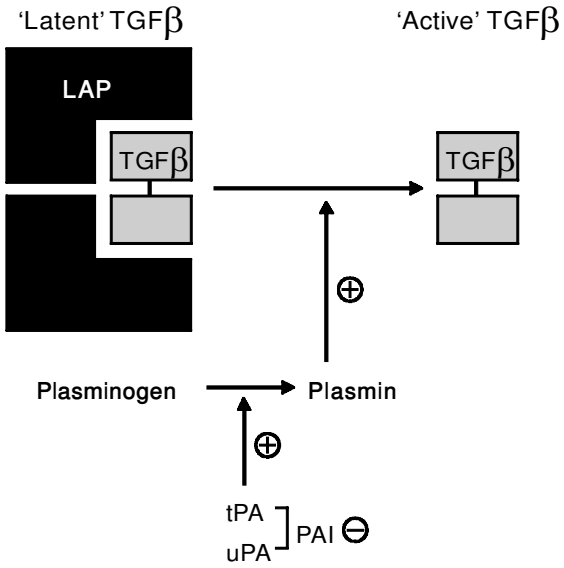

FIG. 6. Activation mechanism of latent TGFβ by plasmin. Plasmin releases TGFβ from its latency associated protein (LAP) whereby active TGFβ is generated. Plasminogen is cleaved into plasmin by plasminogen activators i.e. urokinase PA (uPA) or tissue-type PA (tPA) which are inhibited by plasminogen activators inhibitors (PAI).

and a 68 kD tissue-type plasminogen activator (tPA), secreted as inactive pro-forms, which major substrate is plasminogen. uPA and tPA can cleave plasminogen into plasmin which subsequently activates latent TGFβ[354,355] (Fig. 6). These PAs are specifically inhibited by the plasminogen activator inhibitors (PAI), PAI-1, PAI-2 and PAI-3 (Table 1),[356,357] by formation of a tight complex with PAs.

Plasmin has a broad range of substrates including fibrin, fibronectin, laminin and matrix metalloproteinases (MMP). The PAs and PAIs play an important role by fine regulating the proteolytic degradation of fibrin clots (fibrinolysis) in the circulation, mediated by plasmin[354,358] and therefore tPA is now used for the treatment of thrombotic stroke. Only recently functions for PA were found in the brain, and proteolysis of the extracellular matrix (ECM) by MMP activation, amplified by PAs, has been suggested. The breakdown of the ECM is thought to be involved in brain development and neurite outgrowth but also in neuropathology like growth and invasion of brain tumours, leukocyte infiltration in MS and EAE, breakdown of the BBB and nerve demyelination.[311]

Microglial cells secrete proteases such as elastase, uPA and secrete plasminogen[359,360] which have a direct neurotrophic effects on various types of neurones.[361] Astroglial cells can synthesize and secrete both tPA and uPA as well as PAI-1,[178,362–364] and tPA is involved in motor learning but also in Alzheimer's disease and neuronal degeneration.[365–367] PAs and PAIs have been demonstrated in cerebrospinal fluid of patients with neurological disease[368,369] and tPA expression was found[370] in MS lesions supporting a role of PA and PAI in neuropathological processes.

An important role of tPA and PAI and the regulation of TGFβ activity was shown in a glial cell coculture.[178] tPA and PAI-1 produced by astroglial cells regulated the bioactivity of TGFβ, and thereby indirectly the production of NO by microglial cells.[178] Therefore, we postulate that tPA-mediated activation of TGFβ plays an important role in neuroprotection.

Table 1. Plasminogen activators and their inhibitors (adapted from Ref. 311)

	Plasminogen activators (PAs)	
	Pro form molecular weight (kDa)	Active form molecular weight (kDa)
uPA	55	33.50
tPA	70	70
	Plasminogen activators inhibitors (PAIs)	
	Molecular weight (kDa)	Substrate specificity
PAI-1	46–54	uPA=tPA
PAI-2	47–60	uPA>tPA
PAI-3	50	uPA>tPA

Summary

In neuropathological conditions such as Alzheimer's disease, Parkinson's disease, AIDS dementia complex and multiple sclerosis, activation of microglial cells and astroglial cells is evident. Under these neuropathological conditions cellular damage in the brain is considered to arise indirectly from cytotoxic substances produced by activated glial cells. One of these toxins is NO which has been demonstrated to be produced during several neuropathological conditions. High NO levels are produced by glial cells and exert neurotoxic effects. Astroglial cells and microglial cells communicate in various ways to reduce NO production by microglial cells which is essential to maintain homeostasis in the brain. The production of TGFβ by glial cells and its activation by astrocyte-derived tPA represents one mechanism by which astroglia limit NO production in the brain.

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