Increased thalamic neurodegeneration following ischaemic cortical stroke in osteopontin-deficient mice

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Inflammation aggravates brain injury caused by stroke and neurodegeneration. Osteopontin (OPN) is a cytokine-like glycoprotein that binds to various integrins and CD44 variants. OPN exerts proinflammatory effects in autoimmune conditions but also has cytoprotective properties and participates in wound healing. In this study, we addressed the role of OPN in ischaemic brain injury using OPN knock-out (KO) mice in models of cortical stroke. Compared with wild-type animals, OPN KO mice exhibited unaltered infarct development at the primary injury site but greatly increased retrograde degeneration of the ipsilateral thalamus. Thalamic neurodegeneration in OPN-deficient mice was associated with pronounced microglia activation and inflammatory gene expression and could be attenuated via pharmacological blockade of the inducible nitric oxide synthase (iNOS). Therefore, delayed neurodegeneration in OPN-deficient mice was at least partly due to an excessive release of nitric oxide via the iNOS pathway. Neuroprotective and anti-inflammatory effects of OPN may be relevant for a variety of neurological disease conditions.

Keywords: ischaemic stroke; inflammation; neurodegeneration; nitric oxide; microglia

Abbreviations: iNOS = inducible nitric oxide synthase; MCAO = middle cerebral artery occlusion; OPN = osteopontin; PCR = polymerase chain reaction; WT = wild-type

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Introduction

Brain inflammation holds promise as a therapeutic target in common neurological diseases such as stroke, Parkinson’s and Alzheimer’s disease (Perry et al., 1998; Allan and Rothwell, 2001). In ischaemic stroke, resident brain macrophages/microglia are activated within minutes after the insult (Stoll et al., 1998; Dirnagl et al., 1999; del Zoppo et al., 2000) involving the primary ischaemic focus as well as remote sites such as the ipsilateral thalamus and non-ipschaemic ipsilateral cortex (Myers et al., 1991; Schroeter et al., 1999; Pappata et al., 2000; Kury et al., 2004). Activated microglia produce a plethora of inflammatory mediators, which on the one hand exacerbate tissue damage (Banati et al., 1993; Barone et al., 1997; Rothwell et al., 1997) but can also protect the brain against ischaemic and excitotoxic injury (Mattson et al., 1997; Raivich et al., 1999; Hallenbeck, 2002). The inducible nitric oxide synthase (iNOS) acts as an important downstream mediator of inflammatory neurotoxicity in models of stroke and excitotoxic cell damage (Hewett et al., 1994; Iadecola et al., 1997). Thus, molecular pathways controlling the activity of iNOS may be critical in limiting toxic consequences of inflammation in stroke and neurodegeneration.

Osteopontin (OPN), an RGD-containing acidic glycoprotein, is among the most abundant secretory products of activated macrophages (Murry et al., 1994; Giachelli et al., 1998). In experimental stroke, OPN is expressed in a subacute stage of 3–6 days after the insult (Ellison et al., 1994; Wang et al., 1998; Ellison et al., 1999). OPN binds to a variety of integrin matrix receptors, such as the RGD-binding αvβ3 integrin (Liat et al., 1995), and the CD44 hyaluronate receptor (Weber et al., 1996). OPN exerts chemotactic effects on macrophages (Giachelli et al., 1998),
smooth muscle cells (Liaw et al., 1995) and astrocytes (Wang et al., 1998), and mediates proinflammatory effects in Th1-mediated immune responses (Ashkar et al., 2000; Chabas et al., 2001). On the other hand, OPN provides an autoregulatory feedback mechanism downregulating iNOS expression in various cell types (Hwang et al., 1994; Guo et al., 2001). Findings in experimental models of tissue injury suggest an important role of OPN in the maintenance of tissue homeostasis and the induction of wound healing (Denhardt et al., 2001).

In our present study, we addressed the functional role of OPN in brain ischaemia by using OPN knock-out (KO) mice in models of permanent cortical ischaemia. As the main result, OPN-deficient animals displayed greatly increased secondary neurodegeneration of the ipsilateral thalamus, which was associated with excessive thalamic microglia activation. Gene expression and pharmacological inhibition studies indicate that delayed neurodegeneration in OPN-deficient mice was at least partly due to an excessive release of nitric oxide via the iNOS pathway. Our data suggest protective effects of OPN, which may be relevant for a variety of neurological diseases.

Material and methods

Mice

Wild-type (WT; OPN+/+) and OPN KO (OPN−/−) mice on a 129SW background were obtained by crossing OPN+/− heterozygotes and identifying the WT and KO animals via PCR analysis of tail biopsies. For ischaemia experiments (see below), these OPN KO and WT mice were bred homozygously to obtain a total of 69 KO and 68 WT mice. To control for background gene effects (Banbury Conference on Genetic Background in Mice, 1997), additional OPN KO and WT mice obtained from the initial crossing were again crossed to obtain OPN +/+ F1 and subsequently OPN −/−, OPN +/- and OPN +/+ F2 littersmates. These heterozygously bred F2 littersmates were also subjected to focal ischaemia (n = 3 for each genotype). All animal procedures were approved by local authorities and were performed in accordance with international guidelines on handling laboratory animals.

Focal ischaemia

All experiments were performed on age-matched and gender-matched mice (20–25 g body weight, 10–14 weeks old) under anaesthesia with enflurane in a 2 : 1 N2O/O2 atmosphere. Permanent middle cerebral artery occlusion (MCAO) was induced by coagulation of the right MCA at the level of the inferior cerebral vein. The temporal muscle was dissected via a coronal skin incision, and the underlying skull exposed. Guided by the translucent MCA, a small burr hole was drilled just distal to the inferior cerebral vein, the dura opened with the tip of a 27G needle and the MCA coagulated with a bipolar coagulation forceps. For the induction of cortical photothrombosis (Schroeter et al., 2002), a fibre optic bundle coupled to a cold light source (Schott EL 1500, Mainz, Germany) was centred 2.5 mm posterior and 2.5 mm laterally from bregma. After intraperitoneal injection of 1 mg Rose Bengal (Sigma) the brain was illuminated through the intact skull for 15 min.

Pharmacological inhibition of iNOS

In a first experimental series, iNOS was blocked in OPN KO and WT mice using aminoguanidine as a specific inhibitor (Sugimoto and Iadecola, 2002). Between Day 7 and 13 after ischaemia, the animals were injected intraperitoneally twice daily with either aminoguanidine (200 mg/kg body weight; KO: n = 10, 6 male, 4 female; WT n = 9, 4 male, 5 female) in phosphate buffered saline (PBS) or identical volumes of PBS without AG (KO: n = 10, 5 male, 3 female; WT n = 8, 4 male, 4 female). Animals were sacrificed at Day 14 after photothrombosis. In an additional experiment, 1400 W (Alexis Corporation, Lausen, Switzerland) as a different iNOS inhibitor (Parmentier et al., 1999) was injected from Day 7 to 13 after photothrombosis (25 mg/kg i.p. twice daily, n = 4, all male; identical volume of sterile PBS as vehicle control, n = 4, all male), and animals were killed at Day 14 for histological analysis.

Histology, immunohistochemistry and image analysis

Adjacent serial coronal brain sections were cut from snap-frozen brain at 200 μm intervals and stained with (i) cresyl violet to depict histology and (ii) mAb M1/70 against the CD11b antigen (Serotec, Oxford, UK) to identify activated microglia/macrophages. In additional experiments, serial sections through degenerating thalamus (~AP −2.1 mm from bregma) from paraaffin-embedded brains were stained with either goat anti-mouse OPN antibody (R & D Systems, Minneapolis, MN), the microglia-specific rabbit antibody against the Iba1 antigen (1 : 2000, kindly provided by Y. Imai, Tokyo, Japan), mouse monoclonal antibody against the neuronal marker NeuN (1 : 1000, Chemicon International, Temecula, CA), rabbit polyclonal antibodies against iNOS (1 : 500, Upstate Biotechnology, Waltham, MA) or rabbit polyclonal anti-nitrotyrosine (1 : 500, Upstate Biotechnology). Prior to iNOS and nitrotyrosine immunostaining, deparaffinized sections were microwaved in 10 mmol/l sodium citrate buffer, pH 6.0, for 10 min.

For the analysis of neuron survival and infarct development over time, a total of 15 KO (11 male, 4 female) and 15 WT (11 male, 4 female) mice were used and sacrificed for quantitative analysis at 3, 7 and 15 days after photothrombosis (n = 5 per time point and genotype). In each animal, thalamic neurons were counted on five consecutive cresyl violet-stained brain sections taken at 200 μm intervals between AP −1 and −2.2 mm posterior from bregma. Identification of degenerating thalamic nuclei was guided by the occurrence of microglia activation on adjacent serial sections stained for CD11b. High-power fields of view (×40; 180 × 220 μm) from ipsilateral thalamus were acquired along with homotopic contralateral areas. Neuronal cells identified by the typical morphology of their nuclei were counted off-line on digitally stored high-power images by one investigator (M.S.) being unaware of the genotype or treatment of the animals. Neuronal cell counts in ipsilateral thalamus were expressed as the percentage of the contralateral thalamic neuron counts. Infarct volumes were determined planimetrically using cresyl violet-stained sections cut at 200 μm intervals throughout the photothrombotic infarct. On digitized microscope images infarct borders and ipsilateral as well as contralateral hemispheres were outlined employing tools of the AxioVision software (C. Zeiss, Mainz, Germany). Infarct volumes were calculated from the areas of the (i) total ipsilateral (Ii) and (ii) non-infarcted ipsilateral (In) hemisphere according to the equation: infarct volume (mm3) = \( \sum (I_i - I_n) \cdot mm^3 + 0.2mm^3 \).
Quantitative real-time PCR
A total of 24 WT (13 males, 11 females) and 24 KO (11 males, 13 females) mice was used. Total RNA was prepared from snap-frozen tissue specimens (n = 4 mice per group) using the Trizol reagent (Gibco BRL, Gaithersburg, MD) and reverse transcribed using oligo (dT) \(_{19} \) (G/A/C) primers and SuperscriptII reverse transcriptase (Gibco-BRL, Gaithersburg, MD). Quantitative determination of tumour necrosis factor (TNF)-\( \alpha \), interleukin (IL)-1\( \beta \) and iNOS gene expression levels was done on an ABI 5700 (Applied Biosystems, Weiterstadt, Germany) using the Sybr Green Universal Master Mix (Applied Biosystems) and gene-specific primer pairs described previously (Schroeter et al., 2003). OPN-specific primers [forward: 5'-GTCCCTCGATGTCATCCCTG-3'; reverse: 5'-TGATCAGAGGGCATGCTCAG-3'; amplifying nt 624–674 of mouse OPN cDNA (GenBank J04806)] were designed using the PrimerExpress 2.0 software (Applied Biosystems) and subsequently tested for the generation of specific amplicons. In all PCR analyses, glyceraldehyde 3-phosphate dehydrogenase [GAPDH (Schroeter et al., 2003)] was used as the reference gene since it exhibited constant expression levels under all conditions tested. Relative gene expression levels were determined according to the manufacturer’s \( \Delta \Delta C_{\text{t}} \) method.

Statistical analysis
Data were compared by ANOVA using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). \( P \) values < 0.05 were considered to indicate significant differences.

Results
OPN KO mice exhibit normal infarct development but increased thalamic neurodegeneration
To address the role of OPN in ischaemic stroke we first used a model of permanent middle cerebral artery occlusion, which causes predominantly cortical infarctions in the temporo-parietal cortex. Infarct development and microglial responses were compared between OPN KO and WT mice (Fig. 1). At Day 14 after ischaemia, cortical infarctions were evident as hypercellular areas on cresyl violet-stained brain sections (Fig. 1A and E), reflecting the massive activation of inflammatory cell populations occurring in the infarcted cortex at this stage. Accordingly, staining of serial sections for the
microglia/macrophage marker CD11b revealed a strong inflammatory response accentuated in the infarct periphery (Fig. 1C, D, G, H). No differences were evident between WT (Fig. 1A–D) and KO (Fig. 1E–H) mice with respect to the extent of cortical tissue damage and the degree of microglia/macrophage activation in the infarct area. However, relative to WT controls (Fig. 1D), KO mice (Fig. 1H) exhibited strongly increased induction of CD11b immunoreactivity in ipsilateral subcortical areas corresponding to the ventrolateral thalamic nucleus.

Thalamic microglia activation following cortical ischaemia has been demonstrated in numerous experimental and human studies (Myers et al., 1991; Pappata et al., 2000) and reflects secondary neurodegeneration of the respective thalamic nuclei. Thus, OPN deficiency may have caused excessive secondary neurodegeneration in the thalamus that is independent from direct effects of ischaemia. To address this possibility we performed additional experiments utilizing the photothrombosis model, which is characterized by circumscribed cortical infarctions that leave the ipsilateral thalamus completely non-ischaemic (Watson et al., 1985; Schroeter et al., 2002). Based on these features, a detailed spatiotemporal analysis of cellular responses and degenerative events was possible (Fig. 2). Microglia/macrophage responses were confined to the lesion and subcortical fibre tracts at Day 3 after lesion (Fig. 2A, B) and started to involve the ipsilateral thalamus at Day 7 (Fig. 2C, D). Up to this time point, the intensity and spatial extent of microglia activation were similar in WT and OPN KO mice. At Day 14, however, thalamic microglia responses were strongly exacerbated in KO animals, whereas they remained at low levels in WT mice (arrowheads, Fig. 2E, F).

On cresyl violet-stained serial sections, thalamic microglia activation in OPN KO was paralleled by an almost complete loss of neuronal cell profiles whereas numerous inflammatory cell nuclei were present (Fig. 3A, B). By contrast, in WT animals only mild chromatolysis of neurons was evident (Fig. 3C, D). Quantitative analysis over time confirmed a gradual neuron loss in the ipsilateral thalamus, which was not different between WT and KO mice until Day 7, but greatly exacerbated in the KO animals at Day 14 (Fig. 3G). Thus, excessive thalamic neurodegeneration in OPN-deficient mice occurred during the second week after injury. We additionally compared the infarct volume between WT and OPN KO mice and found no difference (Fig. 3H). Thalamic neurodegeneration was, therefore, not correlated with the extent of ischaemic tissue injury at the cortical insult site.

Neuron counts in control thalamus were not significantly different between WT and KO mice (28 ± 7 versus 26 ± 5 cells per high-power field, P = 0.8). Furthermore, baseline levels of CD11b expression on resident microglia were not visibly different between the genotypes.

To control for possible effects of background genes we performed additional experiments in heterozygously bred OPN +/+ and OPN +/− litters (see Material and methods). Quantitative analysis revealed a highly significant reduction of ipsilateral thalamic neuron counts at Day 14 in OPN −/− relative to both OPN +/+ and OPN +/− littermates (Fig. 4). These findings strongly supported a protective effect of the OPN gene.

**Fig. 2** Spatiotemporal pattern of microglia/macrophage responses following cortical photothrombosis in WT (A, C, E) and OPN KO (B, D, F) mice studied by CD11b immunohistochemistry. At Day 3 after ischaemia (A, B), mild upregulation of CD11b occurs at the cortical lesion site and in subcortical fibre tracts (arrows). Thalamic microglia responses (arrowheads in C–F) are first seen at day 7 (C, D). At this stage, no major differences are evident between WT (C) and OPN KO (D) mice. At Day 14, thalamic microglia activation remains at low levels in WT mice (E), whereas a striking increase is seen in the OPN KO mice (F).

**OPN is induced at sites of microglia activation in the ipsilateral thalamus**

In the ischaemic cortex of WT mice, quantitative PCR analysis showed massive induction of OPN mRNA within 4 days after
ischaemia (Fig. 5A), confirming previous findings in rat models of stroke (Wang et al., 1998). In the ipsilateral thalamus, OPN mRNA was expressed at ∼24-fold higher constitutive levels than in the cortex and exhibited more protracted upregulation between Days 4 and 10 after ischaemia (Fig. 5B). This time course was confirmed by immunohistochemistry showing peak levels of thalamic OPN protein expression during the second week after lesion induction (Fig. 5C). Serial sections were stained with an antibody against the microglia-specific antigen Iba1 (Ito et al., 1998) since the CD11b antibody did not work on the paraffin sections used for OPN immunostaining. OPN immunoreactivity in the thalamus throughout mirrored the distribution of activated Iba1-positive microglia on serial sections (Fig. 5D). At higher magnification, OPN expression (Fig. 5E) was frequently localized to Iba1-positive cell bodies (Fig. 5F, arrowheads denoting OPN/Iba1 double-positive cells in 5E and F). Furthermore, ubiquitous dot-like deposits of OPN immunoreactivity were present in the extracellular matrix (Fig. 5E).

Fig. 3 Increased microglia activation corresponds to thalamic neurodegeneration in OPN-deficient mice. Correlative analysis of CD11b+ microglia (A, C, E) and cresyl violet histology (B, D, F) on Day 14 after cortical photothrombosis reveals massive phagocyte transformation (A) and loss of neuronal cell profiles (B) in OPN KO mice, but only mild microglia activation (C) and slight alterations of neuronal morphology (D, arrows) in WT animals. Note that in B, cresyl violet staining reveals numerous inflammatory cells but almost no neuronal cell nuclei. Naïve WT mice lack CD11b expression (E) and exhibit normal neuronal morphology (F). Bar: 75 μm in A–F. G, H: Quantitative analysis of thalamic neuron counts (G) and infarct volumes (H) after cortical photothrombosis. Thalamic neuron counts (given as percentage of contralateral thalamus) in KO mice (light grey columns) are not different from WT mice (dark grey columns) until Day 7 after ischaemia but greatly decrease at Day 14 (G, ***P < 0.0001). At all time points studied infarct volume determined planimetrically is similar in KO and WT mice (H). Bars in G and H indicate mean ± SEM of n = 5 mice per group.

Fig. 4 Ipsilateral thalamic neuron counts at Day 14 following cortical photothrombosis in heterozygously bred OPN−/−, +/-, and +/- F2 littersmates (n = 3 per genotype; mean ± SD). Statistical analysis reveals significant reduction of neuron counts in OPN−/− mice relative to OPN +/- (**P = 0.04) and OPN +/-/+ (**P = 0.02) littersmates.
Fig. 5 OPN is induced at sites of microglia activation in the ipsilateral thalamus of WT mice. Expression of OPN mRNA in ischaemic cortex (A) and ipsilateral thalamus (B) after photothrombotic ischaemia in WT mice studied by quantitative real-time PCR. Total RNA samples from pooled tissue specimens ($n = 5$ mice per group) were analysed in triplicate. Bars indicate mean ± SEM. **$P < 0.05$, ***$P < 0.01$ versus control animals (ANOVA). Relative mRNA levels were obtained by normalization against normal control cortex, which was set at an expression level of 1. In ischaemic cortex, OPN mRNA is massively induced at day 4 after lesion (A). In the thalamus, constitutive OPN expression in control animals is ~6-fold higher than in the cortex and exhibits protracted upregulation at days 4 and 10. Immunohistochemistry reveals codistribution of OPN expression (C) and Iba1-positive microglia (D) in ipsilateral thalamus of WT mice at day 10 after ischaemia. At high power (E, F, nuclear counterstaining with Mayer’s haemalum), OPN (E) is partially colocalized with Iba1 immunoreactivity (F), suggesting OPN expression by microglia/macrophages. Arrowheads denote identical cells in E and F. Furthermore, significant OPN staining is present as ubiquitous dot-like deposits, presumably in the extracellular matrix. Bars: 100 µm (C, D), 25 µm (E, F).
Pharmacological inhibition of iNOS attenuates thalamic neurodegeneration in OPN-deficient mice

In vitro, OPN functions as a negative feedback regulator of nitric oxide synthesis via inhibition of iNOS expression (Hwang et al., 1994; Guo et al., 2001). TNF-α and IL-1β have been identified as key cytokines inducing iNOS in various cell types (Hewett et al., 1994; Skaper et al., 1995; Liu et al., 1996). To address potential mechanisms of thalamic neurodegeneration in OPN-deficient mice, we compared cytokine and iNOS gene expression in the cortex and thalamus of KO and WT mice (Fig. 6). In the cortical infarcts, we found identical expression levels of iNOS and TNF-α mRNA in KO and WT mice at all time points. IL-1β mRNA levels were moderately increased at Day 1 in KO versus WT mice whereas at later stages, no significant differences were found. Thus, we found overall only minor differences in the expression of inflammatory cytokines and iNOS in the ischaemic cortex of KO versus WT mice, which corresponded to the identical course of infarct development in both genotypes. In contrast, all genes exhibited consistent induction in the degenerating ipsilateral thalamus of KO mice. Upregulation of iNOS, IL-1β and TNF-α mRNA in KO versus WT mice was already present at day 1 after ischaemia and most pronounced at Day 10 after injury.

To corroborate disinhibition of the iNOS pathway in OPN-deficient mice, we performed an immunohistochemical analysis of iNOS protein expression and nitrotyrosine

![Figure 6](https://example.com/fig6.png)

**Fig. 6** OPN deficiency causes disinhibition of iNOS and cytokine gene expression in degenerating thalamus. Levels of iNOS, TNF-α, and IL-1β mRNA determined by real-time PCR analysis of total RNA from pooled tissue of n = 5 mice per group. Bars indicate mean ± SEM of triplicate analysis. *P < 0.05, **P < 0.01 versus WT animals (ANOVA). In ischaemic cortex, iNOS and TNF-α mRNA levels were similar in KO (light grey columns) and WT mice (dark grey columns) at all time points examined. IL-1β mRNA was moderately increased in the cortex of KO mice 1 day after ischaemia but not different at later stages. In contrast to the cortex, all three genes show consistent upregulation in the thalamus of KO mice with slight elevation already at Day 1 and peak levels reached at Day 10 after the insult.
immunoreactivity in the thalami of OPN KO and WT mice. These studies revealed upregulation of iNOS protein in degenerating thalamic nuclei of OPN-deficient mice at Day 10 after lesion (Fig. 7B), whereas no significant iNOS expression was found in WT animals (Fig. 7D). The distribution of iNOS immunoreactivity was throughout similar to that of activated microglia stained on serial sections (Fig. 7A, C). Nitrotyrosine immunoreactivity as a marker of NO-mediated cell damage was exclusively found in degenerating thalamic nuclei of OPN KO mice with peak levels reached at Day 14 after ischaemia (Fig. 7E–G).

To address the pathogenic relevance of increased iNOS expression in OPN-deficient mice, OPN KO mice were treated with the iNOS inhibitor aminoguanidine (Sugimoto and Iadecola, 2002). In line with the delayed time course of iNOS induction in the thalami of OPN-deficient mice, we administered aminoguanidine between Days 7 and 14 after ischaemia. This delayed treatment regime caused a significant attenuation of thalamic neurodegeneration in OPN KO mice with neuron counts at Day 14 almost doubled relative to sham-treated KO mice (Fig. 8A). However, even aminoguanidine-injected
KO mice still displayed enhanced thalamic neurodegeneration compared with WT animals. Infarct volumes at Day 14 were not different between vehicle-treated and aminoguanidine-treated KO mice (Fig. 8B). We additionally treated WT mice with AG between Days 7 and 14 after ischaemia and assessed thalamic neurodegeneration at Day 14. Thalamic neuron counts in WT mice treated with AG were not significantly different from that in WT mice treated with control saline (51.5 ± 3.6 versus 49.8 ± 3.7% of contralateral, P = 0.74). Thus, AG treatment did not influence thalamic neurodegeneration in WT mice. Finally, we performed experiments using 1400 W as an alternative iNOS inhibitor (Parmentier et al., 1999). 1400 W-treated OPN KO mice displayed significantly improved neuron survival in ipsilateral thalamus compared with sham-treated KO animals (36.6 ± 2.0 versus 22.1 ± 4.0% of contralateral, n = 4, P = 0.02), thereby confirming the protective effect of iNOS blockade in OPN KO mice.

Discussion

In this study, we addressed the role of the cytokine-like glycoprotein OPN for inflammatory responses and neurodegeneration in models of ischaemic cortical stroke. As the main finding, OPN KO mice exhibited greatly increased delayed neurodegeneration in thalamic nuclei that normally undergo only mild secondary cell death due to retrograde degeneration of thalamocortical projection fibres (Iizuka et al., 1990; Myers et al., 1991; Pappata et al., 2000). Thalamic neurodegeneration in OPN KO mice was associated with overexpression of iNOS and marked activation of microglia/macrophages. This was consistent with previous in vitro findings suggesting negative feedback regulation of iNOS transcription via OPN (Hwang et al., 1994; Guo et al., 2001). We therefore hypothesized that disinhibition of the iNOS pathway contributed to exaggerated neurodegeneration in OPN KO mice. Accordingly, delayed treatment of OPN KO mice with iNOS inhibitors such as aminoguanidine or 1400 W reduced thalamic neurodegeneration to a degree intermediate between sham-treated KO and WT mice. In contrast, iNOS inhibition did not influence thalamic neurodegeneration in WT mice. These data suggest that thalamic neurodegeneration in OPN-deficient animals was at least partly due to an excessive release of nitric oxide via the iNOS pathway.

Our findings add to previous evidence that RGD-containing ligands of integrin matrix receptors are regulators of neuroinflammatory responses in brain injury (Chabas et al., 2001; Milner and Campbell, 2003). Of interest is that vitronectin as an alternative ligand of the αvβ3 integrin seems to promote rather than inhibit microglia activation at least in vitro (Milner and Campbell, 2003) suggesting divergent actions of the various integrin ligands on CNS immunocompetent cells. The incomplete protection by iNOS inhibitors points to additional mechanisms involved in OPN-dependent neuroprotection. In vitro findings indicate that OPN signalling via integrins or CD44 exerts direct anti-apoptotic actions on various cell types (Denhardt et al., 2001; Khan et al., 2002; Meller et al., 2005). Osteopontin treatment of cortical neuron cultures caused an increase in Akt and p42/p44 MAPK phosphorylation suggesting OPN-induced neuroprotection via activation of these protein kinases (Meller et al., 2005).

This is in line with previous findings showing activation of PI3 kinase/Akt as a mechanism underlying OPN-induced cell migration (Zheng et al., 2000; Das et al., 2004; Rangaswami et al., 2004) and survival responses (Lin and Yang-Yen, 2001). Thus, direct neuroprotective effects may have contributed to the observed differences in thalamic neurodegeneration between OPN KO and WT mice.

In contrast to thalamic neurodegeneration, infarct development at the site of cortical ischaemia was similar in OPN KO and WT mice up to 14 days after the insult. This extends previous in vivo studies by Meller et al. (2005) who found neuroprotective effects of intracerebral OPN administration but unaltered infarct volumes in OPN-deficient mice at 24 h after transient MCAO. In addition, our study shows overall identical patterns of cytokine and iNOS expression as well as microglia activation in the cortical infarct zone, which is in
contrast to the consistently enhanced inflammatory responses in the thalami of OPN KO mice. Taken together, these findings suggest a context-dependent effect of OPN in ischaemic brain injury. As a possible explanation it is conceivable that protective effects of OPN are overridden by the hyperacute ischaemic injury in the cortex but sufficient to mitigate slowly evolving neurodegeneration in the thalamus.

In contrast to our present findings, previous studies suggested a proinflammatory, harmful role of OPN in multiple sclerosis and its animal model experimental autoimmune encephalomyelitis (Chabas et al., 2001; Jansson et al., 2002). This discrepancy may be largely explained by the different nature of the underlying disease processes, i.e. T cell-mediated autoaggression in multiple sclerosis versus T cell-independent neurodegeneration in our model. It is of note that similarly divergent effects, either harmful (Seun Riminton et al., 1998) or neuroprotective (Bruce et al., 1996) depending on the model studied, have been described for other inflammatory mediators such as TNF-α. Overall, the relationship between inflammation and neurodegeneration appears to be complex with reciprocal interactions between both processes, and additional studies into the mechanism of OPN-dependent neuroprotection are necessary.

Delayed thalamic neurodegeneration has been demonstrated in numerous studies of ischaemic stroke (Tamura et al., 1991; Pappata et al., 2000; Nakane et al., 2002; Herve et al., 2005). In a study combining neuroimaging with clinical scoring at various stages after hemiparetic stroke the degree of motor recovery was partly determined by the integrity of the thalamic circuitry (Binkofski et al., 1996). Secondary degeneration remote from the lesion, therefore, appears critical for the long-term functional prognosis of stroke patients. Beyond a role in stroke pathophysiology, increasing evidence implicates inflammatory microglia activation as a pathogenic factor in chronic neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease (McGeer et al., 2003; Mrak and Griffin, 2005; Ouchi et al., 2005). In the MPTP mouse model of Parkinson’s disease, iNOS expression by activated microglia aggravates dopaminergic neurodegeneration (Liberatore et al., 1999; Wu et al., 2002). Hence, OPN-mediated reduction of NO-induced cell damage as suggested by our present study may have broader implications for neurodegeneration in general. This view is supported by recent findings showing OPN induction in endotoxin-induced basal ganglia injury (Iczkiewicz et al., 2005) and in a transgenic model of Huntington’s disease (Karpuj et al., 2002). Of interest, the OPN gene is regulated by the orphan nuclear receptor Nur1 (Lammi et al., 2004), which has been implicated in dopaminergic cell survival at least during embryonic development (Perlmann and Wallen-Mackenzie, 2004). Thus, several lines of evidence link OPN with the maintenance of neuronal viability and neuroprotection. In vitro, inhibition of iNOS by OPN is dependent on RGD-binding integrins (Guo et al., 2001), which can be targeted by a variety of small-molecule ligands (Kerr et al., 2000). The known vitamin D3 response elements located in the OPN gene (Noda et al., 1990) offer additional possibilities for the modulation of OPN-dependent signalling. Taken together, OPN appears to be an important regulator of inflammation and cell death in CNS injury and may represent a new target for neuroprotective therapies in a variety of neurodegenerative disease conditions.

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