








Metallothionein Treatment Attenuates Microglial Activation and Expression of Neurotoxic Quinolinic Acid Following Traumatic Brain Injury

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Abstract The kynurenine pathway has been implicated in brain injury and neurodegeneration. We found that rapid microglial activation and migration to the site of neurotoxic kynurenine pathway intermediate quinolinic acid (QUIN) is rapidly expressed, within 24 h, by reactive component of the neuroinflammatory response to traumatic microglia following traumatic injury to the rodent neocortex. Furthermore, administration of the astrocytic cytokines and neurotoxic compounds. There is growing recognition that an important component of the microglial response by reducing microglial activation (by approximately 30%) and QUIN expression. The suppressive effect of the kynurenine pathway (KP) of MT was confirmed upon cultured cortical microglia. Within the CNS, more than 95% of tryptophan is catabolized by the KP (Ruddick et al. 2006). This pathway induced activation of microglia and QUIN appears to be a key regulator of neuroimmune response, and is directly implicated in neurotoxicity associated with modulatory properties of MT, which may have therapeutic applications for the treatment of traumatic brain injury.

Keywords Traumatic brain injury · Neuroinflammation · Neuron–glia interactions

Introduction

Recent studies have demonstrated that resident microglia within the cortex rapidly respond to traumatic injury in the

Alzheimer's disease (Heyes 1996; Guillemin et al. 2005; Hartai et al. 2005). There are several reports of elevated KP activity in patients with chronic brain injury (Christopoulos et al. 2006; Mackay et al. 2006), although this has not yet been investigated fully in animal models of traumatic brain injury. The KP produces several neuroactive intermediates, including the endogenous neuroprotective agents kynurenic acid and picolinic acid (Foster et al. 1984; Jhamandas et al. 2000). It is the expression of a third KP intermediate, quinolinic acid (QUIN), an endogenous NMDA receptor agonist and neurotoxin, which appears to be a major driver of neurotoxicity associated with neuroinflammation. QUIN is synthesized predominantly by activated macrophage and microglial cells (Guillemin et al. 2003). It is not produced by astrocytes (Guillemin et al. 2001) and neurons (Guillemin et al. 2007) instead these two cell types are able to catabolize some QUIN. Recent reports have demonstrated that inhibiting KP activity (and subsequently QUIN production) attenuates neuroinflammation and significantly prolongs survival in an experimental murine model of

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multiple sclerosis (Platten et al. 2005), indicating that administered into the injury site (1 ml of a 1 mg/ml solution of QUIN represents a promising therapeutic target for the injured or diseased brain.

It is well established that the astrocytic protein metallothionein (MT) has essential protective roles in wound healing following cortical brain injury (recently reviewed by Chung et al. 2008). Of particular interest within the context of the injured brain are the MT-I and -II isoforms (hereafter referred to as MT), expressed primarily by reactive astrocytes (see review by Hidalgo et al. 2001). In particular, the presence of MT is associated with reduced neuroinflammation and microglial activation. This is apparent in transgenic animal studies where MT knockout mice have significantly increased microglial activation following cortical cryolesion injury (Penkowa et al. 1999). Notably, elevated numbers of reactive microglia was associated with enhanced neuronal apoptosis, suggesting that inflammation has a direct impact upon neuronal survival following brain injury. Conversely, MT-overexpressing mice exhibit less microglial activation and subsequently less neuronal death than wildtype mice following traumatic brain injury (Giralt et al. 2002; Penkowa et al. 2002). Importantly, exogenous administration of MT also able to attenuate microglial inflammation and provide neuroprotection following traumatic brain injury (Giralt et al. 2002; Chung et al. 2003). Hence, there is substantial circumstantial evidence that the protective effect of MT within the injured brain involves a direct suppressive action upon neuroinflammation.

The goal of this study was to investigate the activation of microglia within the injured brain. We also investigated whether MT directly attenuates inflammatory microglia, which might provide a mechanism to explain the neuroprotective actions of MT following brain injury.

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Materials and Methods

Focal Cortical Injury to the Adult Rat Neocortex

Focal injuries were made to the Par 1 somatosensory region of the neocortex of adult Hooded Wistar rat as reported previously (Chung et al. 2003). Briefly, a 25-gauge Hamilton syringe was inserted to a depth of 1.5 mm, and held in place for 10 min. The Hamilton syringe was then retracted and a small piece of absorbant gelfoam was placed into the skull cavity and the skin was sutured over the skull. Rats were kept warm at 37°C until they awoke, and then housed as normal. In some cases, at the time of injury, sterile saline counts were also made from the two neighbouring tissue or purified rabbit MT-IIA (Bestenbalt LLC, Estonia) was

Counting Activated Microglia and Neurons Within the Injured Neocortex

For direct counting of reactive microglia, the section representing the middle of the lesion was identified, and the number of ferritin-immunoreactive microglia within a 1 mm² grid (with the lesion tract within the middle of the grid) were counted manually from a digital image captured at 100× magnification (Leica DMIRB). Microglial counts were also made from the two neighbouring tissue sections. At least 500 positive-cells were counted per

section from vehicle-treated animals. The average number of reactive microglia within and surrounding the injury site was calculated from four animals per treatment group. SMI-32 was used to identify neocortical neurons within the same tissue sections, and labelled neurons were counted in the same 1 mm² grids that were used for microglial counting.

Biochemical Determination of Quinolinic Acid Levels in the Injured Neocortex

In a separate experiment, an area of tissue encompassing the injury site (approximately 5 × 5 × 5 mm in volume; 30–50 mg total tissue weight) was collected from saline- and MT-treated rats at 2 DPI (three animals per group). The tissue sample was snap frozen and stored at -80 °C. The levels of QUIN were measured using gas chromatography mass spectrometry (GCMS), a method that has been previously described (Smythe et al 2002). Briefly, 500 µl of 10% trichloroacetic acid (Sigma) was added to each tissue sample and the mixtures were sonicated to form a homogenized suspension. The samples were then centrifuged at 1000 rpm for 5 min. Following that, 100 µl of sample solution was transferred into a glass tissue culture vial with 10 µl of [²H₃]QUIN (Le Research Inc., St. Paul, MN), as internal standard, and evaporated to dryness (Savant SpeedVac). A total of 60 µl each of trifluoroacetic anhydride and hexafluoroisopropanol (both from Sigma) were then added to the residues and the glass vials were capped and heated at 60 °C for 30 min. The hexafluoroisopropyl ester product formed was then dissolved in 880 µl toluene and transferred into a glass vial. The vials were then placed onto an autosampler (Agilent 7683), whereby 1 µl of sample was injected into a gas chromatography (Agilent 6890) interfaced to a mass selective detector (Agilent 5973) via the autosampler (Agilent Technologies, NSW, Australia).

Cortical Microglia Cultures

We obtained highly purified cultures of cortical microglia (>95%) using previously established glial culturing protocols (Chung et al 2004). Briefly, cerebral cortices were dissected from postnatal day 13 Hooded Wistar rats and collected in HBSS medium (Sigma). An equal volume of trypsin (0.25% final concentration; Sigma) was added, and the tissue suspension was incubated at 37 °C for 25 min. Medium was replaced with 2 ml DMEM-10% foetal calf serum (DMEM-10S; both from Invitrogen) and the tissue was gently triturated with a 1 ml pipette. The cell suspension was filtered through an 80 µm gauze filter to remove any undigested tissue or aggregated cells. The cell suspension was made up to 10 ml with DMEM-10S and

centrifuged for 10 min at 500g, 4 °C. The supernatant was removed and the cell pellet was gently resuspended in 1 ml of fresh DMEM-10S. The entire cell suspension was added to a poly-L-lysine (Sigma) coated (1:25 dilution of a 0.01% solution) 75 cm² flask containing 9 ml of pre-warmed DMEM-10S. Cells were maintained in an incubator in 5% CO₂ at 37 °C. After 24 h the media was replaced with fresh pre-warmed DMEM-10S, and thereafter was replaced for every 72 h.

After approximately 8–10 days a confluent mixed glial culture was established, containing an underlying monolayer of astrocytes, supporting a top layer of microglia and oligodendrocyte-precursor cells (Chung et al 2007). To remove the microglia, the flask was shaken at 250 rpm, 37 °C for 30 min. The medium (containing microglia) was collected and microglia was plated directly into 24-well plates onto glass coverslips (treated overnight with 0.01% poly-L-lysine) at a density of approximately 5 × 10⁴ cells per well. After 24 h the medium was gently replaced with DMEM, and experiments were performed around 3–4 days later when at least 70% of the microglia had a non-reactive phenotype.

IFN-γ Stimulation and Classifying Microglial Reactivity

Cultured microglia were stimulated into a reactive phenotype by treatment with 20 ng/ml recombinant IFN-γ (Biosource) for 24 h. In some cases, microglia were pre-treated with HPLC-purified rabbit MT-IIA (Bestenbalt LLC) 30 min prior to IFN-γ stimulation. Cells were fixed with 4% paraformaldehyde for 15 min, and immunocytochemistry performed using a polyclonal rabbit anti-ferritin antibody (1:500; Abcam). Microglial reactivity was determined using a similar classification method to that recently reported by Kauppinen et al (2008). Unreactive microglia were defined as stellate-shaped cells bearing one or more processes, while reactive microglia exhibited a round morphology with prominent and thick lamellae surrounding the cell body. For direct cell counting, three digital images were captured per coverslip (a minimum of three coverslips per treatment group) at 20× magnification on a Leica DMIRB microscope. At least 200 cells were counted per treatment group.

Quinolinic Acid Quantitation from Microglia-Conditioned Media

Conditioned media was collected from cultured microglia 24 h after IFN-γ stimulation, snap frozen and stored at -80 °C. QUIN levels were determined by GCMS as described above.

Results

Microglial Activation, QUIN Production and MT-Expression Following Traumatic Injury to the Adult rodent Brain

A focal cortical injury was performed to the neocortical tract as was observed at 1 DPI. Interestingly, reduced QUIN region of the adult rat brain by inserting a 28-gauge expression occurs when the expression of MT was significantly elevated by reactive astrocytes bordering the injury tract (Fig. 1a). At 1 day post-injury (DPI), there was a high degree of microglial activation (Fig. 1a), surrounding and within the core of the injury tract (Fig. 2e).

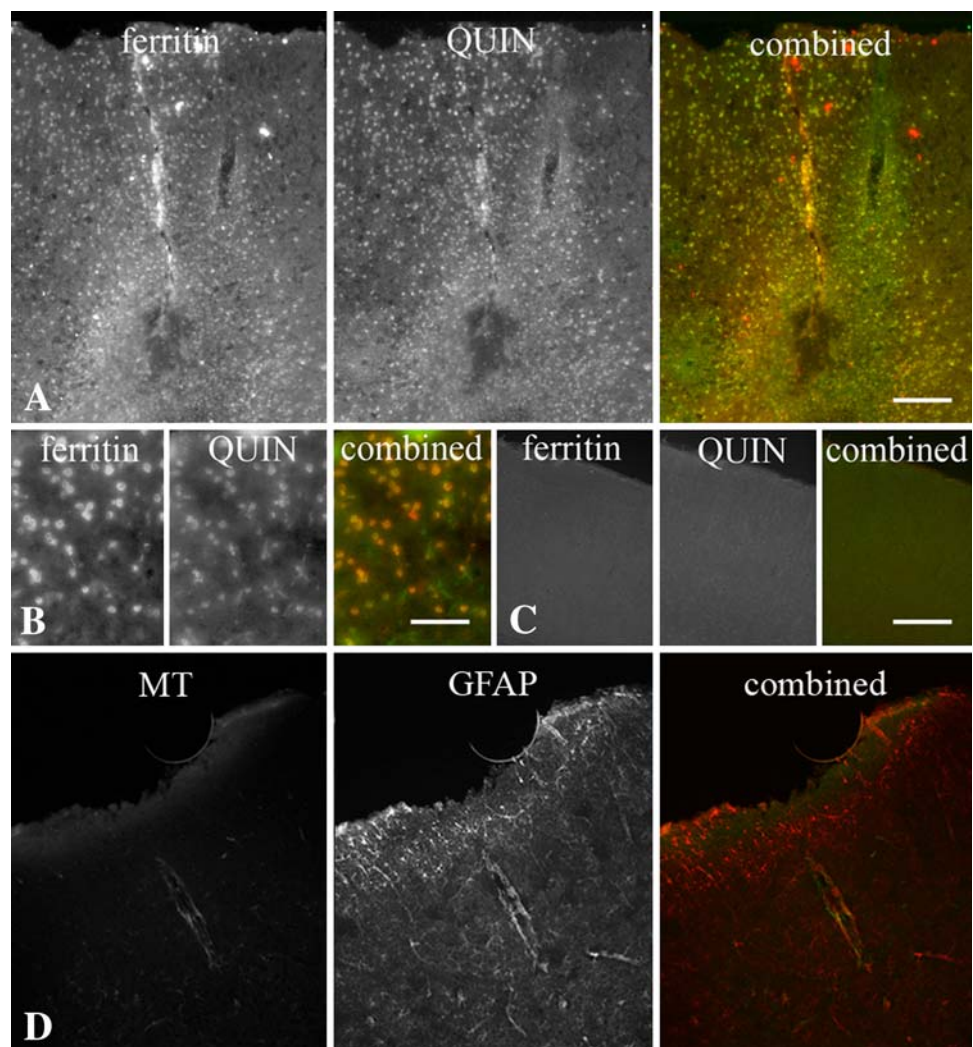
All ferritin-immunoreactive microglia were found to highly express QUIN (Fig. 1a, b). No immunoreactivity was observed in the ipsilateral, non-injured cortex (Fig. 1c). There was no MT-expression present at this time after injury (Fig. 1d), as we have reported previously (Chung et al. 2004).

At 4 DPI, the injury tract had enlarged greatly, filled with reduced microglial activation at 1 DPI, concomitant with reactive microglia that exhibit strong immunolabelling for reduced QUIN expression (Fig. 3a, b). Direct counting

ferritin (Fig. 2a). However, QUIN immunoreactivity appeared much lower at this time point. The bright, distinct expression of QUIN observed at 1 DPI was replaced by low, diffuse immunostaining (Fig. 2b). QUIN expression was also limited to microglia directly within the lesion tract (Fig. 2b), but not in microglia along the border of the injury

The correlation between microglial activation, reduced levels of QUIN, and elevated MT-expression following traumatic brain injury suggests that MT might be involved in modulating the neuroinflammatory response. To test this hypothesis, MT was administered directly into the lesion site at the time of injury. Treatment with MT resulted in

Fig. 1 At 1 day post-injury (DPI), there was a high degree of microglial activation (ferritin; red) and QUIN expression (green) surrounding and within the core of the injury tract (a). All ferritin-immunoreactive microglia were found to highly express QUIN (b). No ferritin or QUIN immunoreactivity was observed in the ipsilateral, uninjured cortex (c). There was no MT-expression (green) present at this time after injury (d), either within or bordering the injury site. Scale bars = 300 μ m (a, d), 75 μ m (b, c)



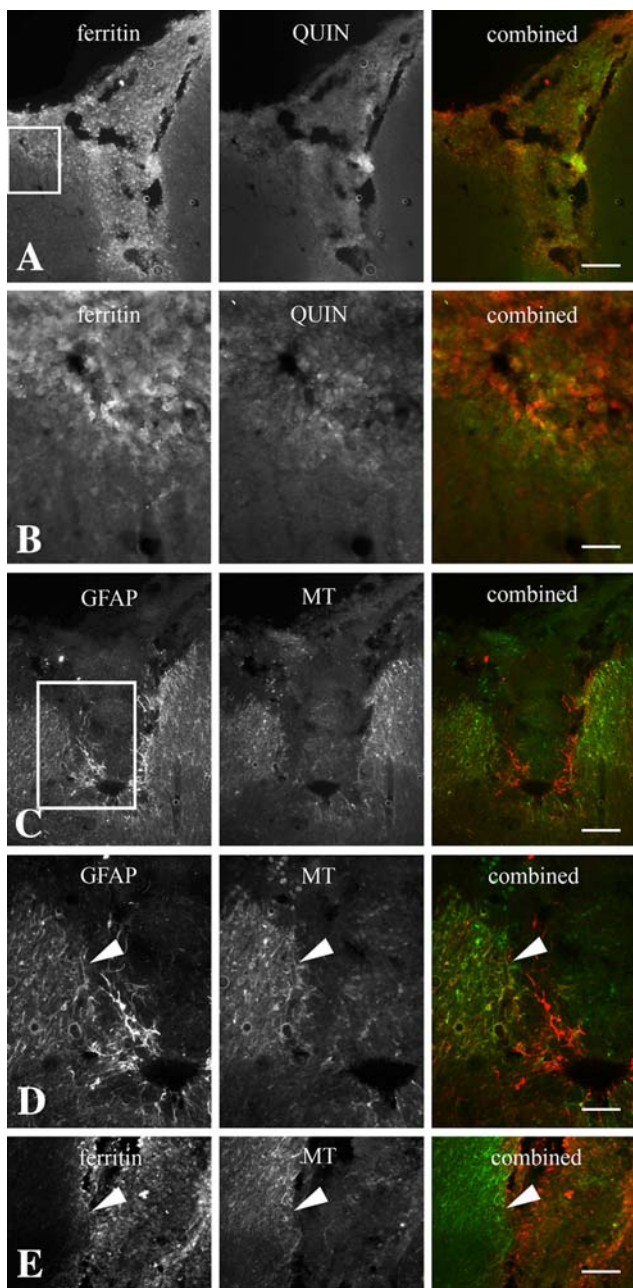


Fig. 2 At 4 DPI, ferritin-immunoreactive microglia persisted within the injury site (a). However, QUN immunoreactivity was relatively low and diffuse (a, inset magnified in panel b). QUN expression was limited to microglia directly within the lesion tract (b), but not in those microglia along the border of the injury tract. MT-expression was significantly elevated by reactive astrocytes bordering the injury tract, but not those further away (inset magnified in panel d). MT-expressing astrocytes formed a border surrounding the injury tract (indicated by arrowheads) in direct contact with reactive microglia filling the injury tract (Fig. 2e). Scale bars = 300 μm (a, c), 150 μm (d, e) and 75 μm (b)

revealed an approximate 30% decrease in the number of QUN-immunoreactive microglia following MT treatment by approximately 80% following treatment with MT (Fig. 5a). MT treatment also resulted in significant

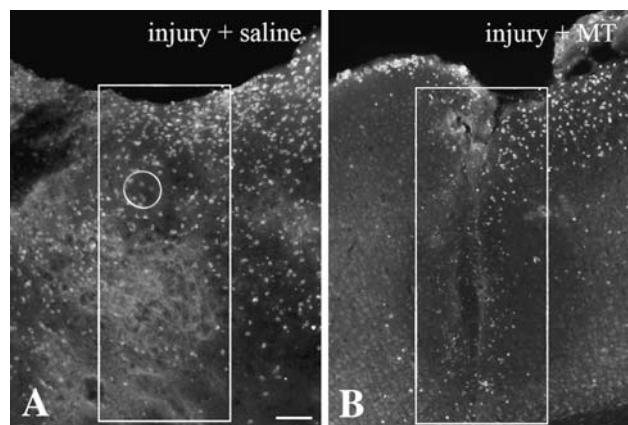


Fig. 3 Treatment with MT (b) reduced the number of QUN-expressing microglia within the injury site at 1 DPI compared to saline-treatment (a). Insets are representative of the area in which direct cell counts were performed. Scale bar = 50 μm

neuroprotection (Fig. 4); with the number of SMI-32-immunoreactive neurons in the cortex in MT-treated animals almost threefold greater than the number in vehicle-treated animals (Fig. 5b). Treatment with the zinc chelator CaEDTA significantly reduced microglial activation to a similar degree as MT treatment (Fig. 5a). CaEDTA treatment also improved neuronal survival following brain injury, but was significantly less neuroprotective than MT (Fig. 5b).

In parallel experiments, we measured levels of QUN within the injury site at 1 DPI by GCMS. Cortical injury resulted in a significant threefold elevation in QUN levels compared to uninjured cortex (Fig. 6a). In a separate experiment, we found that treatment with MT prevented elevation in QUN levels, with QUN levels in the injury site of MT-treated animals comparable to levels in uninjured cortex (Fig. 6b).

MT Attenuates Microglial Activation and QUN Production In Vitro

To directly test the ability of MT to modulate microglial activation and QUN production, a model of cortical microglia activation was established. QUN expression in unreactive microglia was relatively low (Fig. 7a). Treatment with IFN-γ induced reactivity in the cultured microglia (Fig. 7b), evidenced by a distinct change in morphology from stellate to round with large prominent lamellipodia surrounding the cell body. IFN-γ also induced QUN expression in reactive microglia (Fig. 7c). Pre-treatment with MT blocked IFN-γ-induced microglial activation (Fig. 7a) and QUN expression (Fig. 7b). Direct counting confirmed the attenuation of microglial activation (Fig. 7c). In parallel experiments, we also determined

Fig. 4 At 1 DPI, there was a high degree of neuroPlament (SMI-32) fragmentation within the injury tract of saline-treated animals (a), higher magnification in panel (b), indicating neuronal degeneration. Several SMI-32 labelled neurons (indicated by arrows) were observed along the edge of the injury tract at this time (b). Treatment with MT resulted in less neuronal fragmentation within the injury site (injury on left-side of image), and proportionately more SMI-32 labelled neurons (arrows) along the injury border (c). For comparison, SMI-32 labelling in uninjured neocortex demonstrated the presence of a number of neuronal cell bodies present in the layer III region (indicated by arrowhead), which was dramatically reduced following focal injury. Scale bars= 150 μ m (a, d) and 100 μ m (b, c)

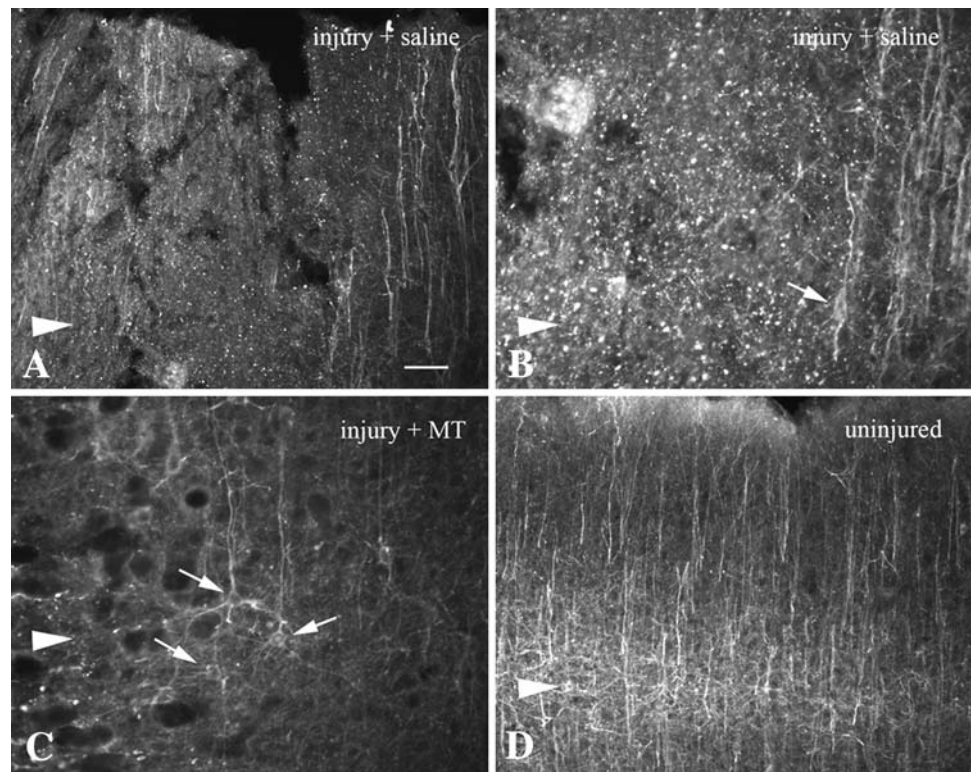
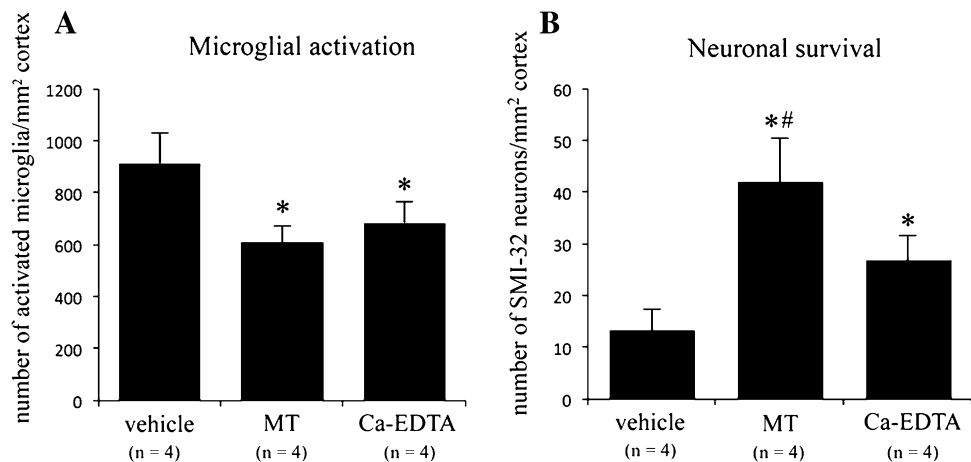


Fig. 5 Direct cell counting revealed that microglial activation was significantly reduced by treatment with MT and Ca-EDTA (b). Both treatments also significantly improved neuronal survival. However, MT treatment was significantly more neuroprotective than Ca-EDTA (b). * $P < 0.05$ versus vehicle-treated animals; ANOVA. # $P < 0.05$ versus Ca-EDTA-treated animals; ANOVA. Error bars represent standard error of the mean values ($n = 4$ animals per experimental group)



levels of QUIN secretion by microglia in microglia-conditioned media 24 h after IFN- γ stimulation. IFN- γ treatment led to an approximately 30% increase in media levels of QUIN, which was prevented by pre-treatment with MT (Table 1).

Discussion

We demonstrate that QUIN is rapidly expressed by reactive microglia following traumatic injury to the rodent neocortex, and that administration of MT attenuates this neuroinflammatory response. The suppressive effect of MT on QUIN expression was confirmed upon cultured cortical microglia, indicating the direct action of MT upon these cells. These results demonstrate the neuroimmunomodulatory properties of MT, which may have therapeutic applications for the treatment of traumatic brain injury.

We report for the first time that the neurotoxic compound QUIN is highly expressed by reactive microglia following traumatic brain injury. This observation is in accordance with the previous reports that QUIN is expressed by reactive microglia in neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease (Heyes 1996; Guillemín et al. 2005; Hartai et al. 2005).

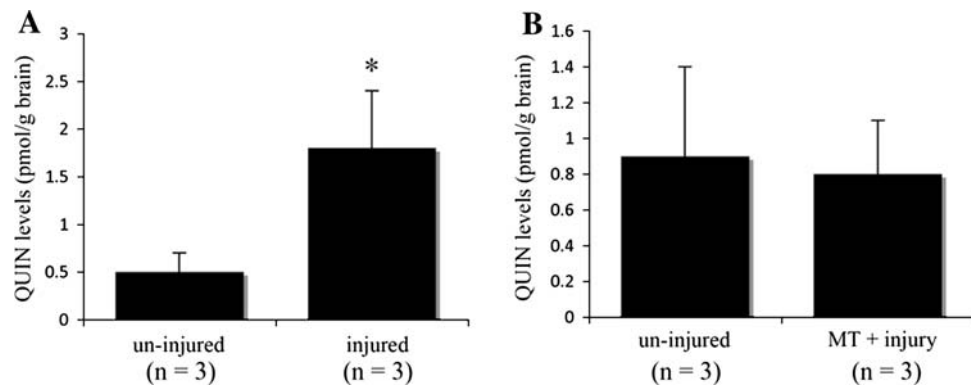
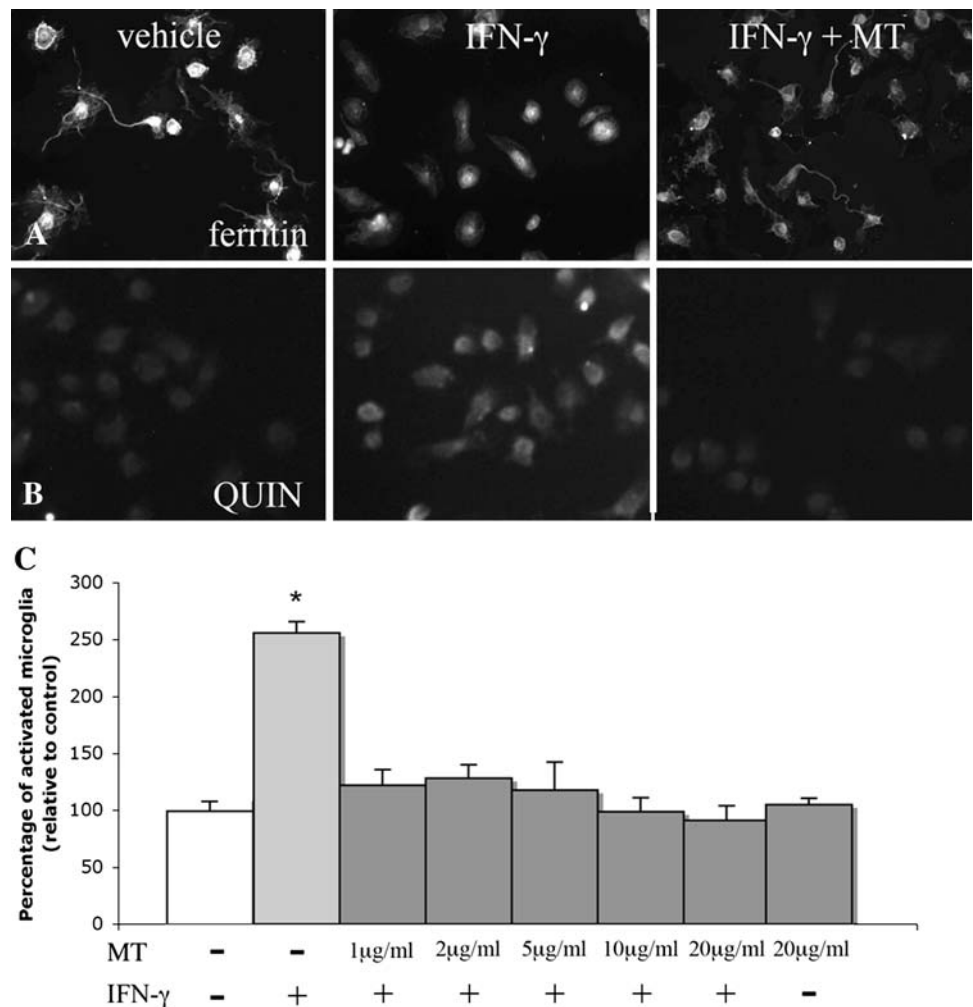


Fig. 6 Levels of QUIN within the injury site were measured by animals not statistically different to levels in uninjured cortex GCMS. At 1 day after cortical injury, there was a significant threefold ($P = 0.1625$; ANOVA). * $P < 0.05$ versus vehicle-treated animals; elevation in QUIN levels compared to un-injured cortex (ANOVA). Error bars represent standard error of the mean values Treatment with MT prevented elevation in QUIN levels in response ($n = 3$ animals per experimental group) to brain injury (b), with QUIN levels in the injury site of MT-treated

Fig. 7 QUIN expression in unreactive cultured cortical microglia was relatively low (a). Treatment with IFN γ induced a reactive microglial morphology with large prominent lamellaepodia surrounding the cell body, and also induced QUIN expression within reactive microglia (b). Pre-treating microglia with MT blocked IFN γ induced QUIN expression by microglia (c). Direct counting confirmed the attenuation of microglial activation by treatment with MT (c). * $P < 0.05$ (1-way ANOVA). Error bars represent standard error of the mean values (three different experiments)



4 DPI, concomitant with a significant increase in the MT significantly attenuates microglial activation and expression of MT. This suggests that MT might be directly QUIN expression following cortical injury. This was involved in modulating the neuroinflammatory response to observed both by direct counting of activated microglia, brain injury. To test this, we found that administration of and also by measuring levels of QUIN in injured

Table 1 Levels of QUIN in microglia (MG) conditioned-media 24 h after IFN- γ stimulation

	QUIN (nM)
Vehicle-treated MG	4.8 \pm 0.2
IFN- γ treated MG	6.6 \pm 0.6
IFN- γ + MT treated	5.0 \pm 0.4
MT treated MG	5.0 \pm 0.1

Data are the mean \pm SEM. Measurements were performed as described in the methods section. * represents $p < 0.05$ compared to all treatment groups, two-way ANOVA with Bonferroni post-hoc test. No other treatment groups were significantly different within the statistical analyses

neocortical tissue. These observations were replicated in vitro. We also found significantly less neuronal death following traumatic brain injury (Choi and Koh 1998). MT-treated animals versus saline-treated animals. Given that MT is one of the major zinc-binding proteins that there are a number of examples demonstrating a direct correlation between the presence of MT (either endogenous and is involved in maintaining metal homeostasis, it is MT in the case of transgenic mice or exogenous MT administration) and reduced neurotoxicity following traumatic brain injury (Penkowa et al. 1999, Giralt et al. 2002, Penkowa et al. 2002, Chung et al. 2003), we propose that this neuroprotective action of MT might be mediated in part by reduction of QUIN expression by inflammatory treatment. Interestingly, while CaEDTA was able to microglia. Our data also suggest that pharmacological targeting of QUIN represents a novel therapeutic target for the treatment of traumatic brain injury.

While there is substantial circumstantial evidence, primarily from transgenic animal studies, suggesting that MT can directly modulate neuroinflammatory responses within the injured brain, we provide the first evidence for this direct action of MT. Our observations are in accordance with work from the Lynes group on the function of 401 g/g brain tissue (Erickson et al. 1994). While it is not MT within the peripheral immune system. They have reported that exogenous MT directly affects leukocyte migration (Yin et al. 2005), suppresses T-lymphocyte actions of macrophages (Youn et al. 1995). Intriguingly, they have drawn analogies between the structure of MT and the immunomodulatory chemokine family of molecules, and have suggested that MT may act as chemokine under certain situations (Yin et al. 2005). Our observations would directly support this hypothesis. Significantly, we have recently reported the secretion of MT, which is elevated within the injured rodent neocortex or by reactive astrocytes in culture (Chung et al. 2008b), providing a clear explanation for how this astrocytic protein might mediate microglial inflammation within the injured brain. The mechanisms underlying the direct action of MT upon microglia are currently unclear, although analogies can be drawn from recent insightful studies in neuronal models. Ambjörnsson et al. (2008) have reported that exogenous MT interacts with neurons via the low-density lipoprotein family of receptors (LRP), activating a MAPK-dependent signalling pathway that promotes neurite outgrowth and blocks neuronal apoptosis. Microglia are known to express some of the same LRP receptors (Laporte et al. 2004), which may provide a mechanism facilitating the action of MT upon microglia. Indeed it is well established that beta-amyloid is internalised by microglia via interaction with LRP receptors (Laporte et al. 2004), and that beta-amyloid induces reactive microglial phenotypes (Floden and Combs 2006, Garica et al. 2006). Alternatively, Kauppinen et al. (2008) have unexpectedly reported that zinc can directly induce microglial reactivity, and it is well established that extracellular levels of zinc are increased following traumatic brain injury (Choi and Koh 1998). Given that MT is one of the major zinc-binding proteins within the brain (MT binds seven zinc ions per molecule) and is involved in maintaining metal homeostasis, it is possible that MT might attenuate microglial activation in vivo following brain injury by chelating extracellular zinc. To support this hypothesis, we observed that the zinc-chelating compound CaEDTA was also able to reduce microglial activation, to a similar degree as MT. Interestingly, while CaEDTA was able to significantly improve neuronal survival following cortical injury, it was only half as neuroprotective as MT. These results suggest that exogenous administration of MT has dual beneficial actions within the injured brain; firstly by reducing microglial activation (by modulating zinc) and acting directly upon neurons to promote their survival. It is important to note that the endogenous level of MT within the uninjured adult brain is approximately 401 g/g brain tissue (Erickson et al. 1994). While it is not known whether MT levels are upregulated in clinical cases of traumatic brain injury, levels of MT are significantly elevated in rodent models of traumatic brain injury (see review by West et al. 2004). Notably, levels of MT are elevated in the serum of patients who have suffered a traumatic brain injury (Kukacka et al. 2006). This suggests that it is conceivable that the level of MT that we have administered in our studies (11 of a 1 mg/ml solution directly into the site of injury) may resemble the physiological level found in the extracellular environment of the injured brain. In summary, we demonstrate that MT is able to directly modulate microglial activation, and in particular attenuates injury-induced expression of the neurotoxic compound QUIN. This may explain why the presence or absence of inflammation within the injured brain. The mechanisms underlying the direct action of MT upon microglia are reactive microgliosis and neuronal apoptosis, and indicates a potential therapeutic strategy for traumatic brain injury based upon MT administration.

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