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ALTERED MICROGLIAL COPPER HOMEOSTASIS IN A MOUSE MODEL OF ALZHEIMER'S DISEASE

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Abstract

Alzheimer's disease (AD) is characterized by progressive neurodegeneration associated with the aggregation and deposition of β -amyloid ($A\beta_{40}$ and $A\beta_{42}$) peptide in senile plaques. Recent studies suggest that copper may play an important role in AD pathology. Copper concentrations are elevated in amyloid plaques and copper binds with high affinity to the $A\beta$ peptide and promotes $A\beta$ oligomerization and neurotoxicity. Despite this connection between copper and AD, it is unknown whether the expression of proteins involved in regulating copper homeostasis is altered in this disorder. In this study we demonstrate that the copper transporting P-type ATPase, ATP7A, is highly expressed in activated microglial cells that are specifically clustered around amyloid plaques in the TgCRND8 mouse model of AD. Using a cultured microglial cell line, ATP7A expression was found to be increased by the pro-inflammatory cytokine IFN- γ , but not by TNF α or IL-1 β . IFN- γ also elicited marked changes in copper homeostasis, including copper-dependent trafficking of ATP7A from the Golgi to cytoplasmic vesicles, increased copper uptake and elevated expression of the CTR1 copper importer. These findings suggest that pro-inflammatory conditions associated with AD cause marked changes in microglial copper trafficking, which may underlie the changes in copper homeostasis in AD. It is concluded that copper sequestration by microglia may provide a neuroprotective mechanism in AD.

Keywords

Alzheimer's disease; copper homeostasis; ATP7A; microglia; inflammation

INTRODUCTION

The propensity of the β -amyloid peptide ($A\beta$) to self-aggregate is thought to underlie its neurotoxic properties in Alzheimer's disease (Mattson et al. 1993; Pike et al. 1993).

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Although the precise mechanisms by which A β exerts its toxic effects on neurons remain to be fully elucidated, there is much evidence that reactive oxygen species generated by glial cells enriched at amyloid plaques contribute to disease pathology (Simonyi et al. 2010). The focal deposition of A β peptide in specific regions of the brain in AD patients is poorly understood, leading to postulation that certain modifiers of A β may promote its deposition. Recent attention has focused on extracellular transition metals (particularly copper) as potential modifiers of A β that drive AD pathology.

In vitro studies have demonstrated that A β aggregation can be initiated by low levels of copper ions (Atwood et al. 1998; Curtain et al. 2001; Harris 2002; Yoburn et al. 2003; Huang et al. 2004), which bind with apparent high affinity to histidine residues in the A β amino terminal region (Atwood et al. 2000; Dong et al. 2007). Several studies have demonstrated that copper binding influences the neurotoxicity of A β . For example, copper can potentiate the ability of A β to penetrate and disrupt membranes (Curtain et al. 2001; Curtain et al. 2003), inhibit mitochondrial cytochrome c oxidase (Crouch et al. 2005) and kill neurons (Huang et al. 1999c; Yoshiike et al. 2001; Giuffrida et al. 2007). In addition, copper coordinated by A β is able to catalytically generate neurotoxic H₂O₂ in the presence of reducing agents, such as ascorbate (Huang et al. 1999a; Barnham et al. 2004).

Studies of brain tissue suggest that copper-A β interactions occur *in vivo*. Copper concentrations are markedly elevated in cerebral amyloid plaques of AD patients and transgenic mice expressing human mutant forms of human β -amyloid precursor protein, APP (Lovell et al. 1998; Lee et al. 1999; Suh et al. 2000; Miller et al. 2006). Despite these localized increases in copper levels at sites of amyloidogenesis, brain tissues are copper deficient relative to age-matched controls from AD patients (Magaki et al. 2007) and APP-transgenic mice (Maynard et al. 2002; Bayer et al. 2003; Phinney et al. 2003). The mechanism(s) by which copper concentrations become elevated at sites of β -amyloid deposition is not fully understood, however, neurons are a likely source of this copper for two reasons. Firstly, copper is released from neurons in response to stimulation of the glutamatergic NMDA receptor (Schlief et al. 2005; Schlief et al. 2006; Bush 2008), and secondly, excessive NMDA receptor stimulation is thought to underlie excitotoxicity in AD (Chohan and Iqbal 2006; Bush 2008).

Chelators with high affinity for copper readily solubilize A β from post-mortem AD brain tissue, supporting the hypothesis that copper-A β interactions promote the aggregation and deposition of A β (Cherny et al. 1999; Dong et al. 2003). Metal binding compounds, such as clioquinol, that prevent or reverse copper-A β interactions reduce plaque burden in the brain of transgenic AD mouse models (Cherny et al. 2001; Lee et al. 2004), with accompanying marked improvements in cognitive performance (Adlard et al. 2008). Moreover, clinical trials with clioquinol or its derivative, PBT2, were found to improve cognitive scores and reduce A β levels in the cerebral spinal fluid of AD patients (Regland et al. 2001; Ritchie et al. 2003; Lannfelt et al. 2008). Collectively, these studies suggest a detrimental role for copper-A β interactions in AD pathogenesis, and highlight the importance of understanding the molecular basis for copper dyshomeostasis in AD.

In this study, we investigated whether the expression of a key transporter of copper, ATP7A, might be affected in a mouse model of AD. The ATP7A protein is a widely expressed copper pump whose intracellular location in the Golgi is required to transport cytoplasmic copper into secretory compartments, wherein the majority of copper-dependent enzymes obtain copper. ATP7A is also responsible for copper export from the cytoplasm via a process that involves copper-stimulated trafficking of ATP7A to post-Golgi vesicles or the plasma membrane (Petris et al. 1996; Yamaguchi et al. 1996). Results in the current study demonstrate that the ATP7A protein is over expressed in activated microglial cells

specifically clustered around β -amyloid plaques in the brains of TgCRND8 mice, a transgenic model of AD. Elevated expression of ATP7A was also observed in cultured BV-2 microglial cells stimulated with the pro-inflammatory agent IFN- γ , which also resulted in ATP7A trafficking into cytoplasmic vesicles. Interestingly, ATP7A trafficking into cytoplasmic vesicles was not associated with copper export; rather copper accumulation was observed in IFN- γ -stimulated microglial cells associated with increased copper uptake and expression of the copper importer, CTR1. These findings suggest that AD is characterized by altered copper homeostasis in activated microglial cells involving copper uptake via CTR1 and copper sequestration in vesicles via ATP7A. It is postulated that copper sequestration by activated microglia at sites of amyloidogenesis is a protective response in AD.

Materials and Methods

Reagents, antibodies and cell lines

All reagents were from Sigma unless otherwise indicated. Antibodies against CD68, NeuN and GFAP were from Serotec, Chemicon and BD Transduction LaboratoriesTM, respectively. Antibodies against ATP7A for immunofluorescence were provided by Dr. Betty Eipper (University of Connecticut Health Sciences Center). ATP7A antibodies used for Western analysis were raised against the last 20 amino acids of the C-terminus of the ATP7A protein (Bethyl Laboratories). WO2 monoclonal antibodies against the N-terminal region of the A β peptide were described previously (Ida *et al.* 1996). Antibodies against GM130 were from BD Transduction LaboratoriesTM. All cytokines were purchased from BD Transduction LaboratoriesTM. The mouse BV-2 microglia cell line was a kind gift from Professor Rosario Donato (University of Perugia, Italy) and has been well characterized (Bocchini *et al.* 1992). Cells were cultured using Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 4 mM glutamine, 100 U/ml penicillin, and 10 μ g/ml streptomycin in a humidified 5% CO₂ atmosphere at 37°C.

Animals

All mouse handling procedures were approved by the Animal Care and Use Committee of the University of Missouri. TgCRND8 mice on the C57BL/6 background and their wild type littermates were maintained on a 12-hour light/dark cycle and provided a normal chow diet and water *ad libitum*. Brain sections were obtained from mice between 7–9 months of age when there is extensive β -amyloid plaque formation in TgCRND8 mice (Janus *et al.* 2000; Chishti *et al.* 2001).

Immunofluorescence and immunoblot analysis

Whole brains were flash frozen by immersion in isopentane pre-chilled on dry ice. Coronal sections of 10 μ m thickness were prepared on glass slides using a cryostat, and then fixed in acetone at –20°C for 10 minutes. Slides were then blocked with 1% casein in PBS for 30 minutes and incubated with primary antibodies against A β , CD68, NeuN or GFAP, as indicated, for 1 hour at room temperature. Slides were washed 3 times in PBS and then incubated with the appropriate fluorescently labeled secondary antibody (Invitrogen), as indicated in the figure legends. After a further three washes in PBS, slides were mounted with coverslips using Mowiol medium and examined using a Leica DMRE fluorescence microscope with appropriate filters. Immunofluorescence microscopy of cultured BV-2 cells was performed as previously described (Mao *et al.* 2007).

For Western analysis, protein lysates were prepared by sonicating cell pellets in lysis buffer containing 62.5 mM Tris-HCl (pH 7.4), 2% SDS, 1% Triton X-100, 1 mM EDTA and CompleteTM protease inhibitor (Roche Applied Science). Protein concentrations were

determined using the *Dc* protein assay kit (Bio-Rad). Equal amounts of protein were fractionated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Primary and secondary antibodies (conjugated to horseradish peroxidase) were diluted in blocking buffer. Blots were developed using the SuperSignal West Pico Substrate according to the manufacturer's instructions (Pierce).

Copper uptake and accumulation assays

Radioactive ^{64}Cu transport assays were performed essentially as described previously (White *et al.* 2009). ^{64}Cu (specific activity ~ 40–200 mCi/ μg CuCl_2) was purchased from Washington University St. Louis. BV-2 cells were cultured in serum-free medium with or without 25 ng/ml IFN- γ for 16 hours. Cells were then incubated with 1 μM radioactive ^{64}Cu for 5 minutes (uptake) or 2 hours (accumulation), and washed three times with PBS to remove surface bound copper. Cell associated ^{64}Cu was then quantified using a gamma counter and normalized to the protein concentration.

Real time PCR

RNA was isolated using the RNeasy mini kit (Qiagen) from BV-2 cells treated for the indicated times with 25 ng/ml IFN- γ . Reverse transcription was performed using a kit from Roche. Real time PCR was performed using specific primers for murine CTR1 and GAPDH (Applied Biosystems) as part of the TaqMan Gene Expression Assay (Applied Biosystems). Results were analyzed using the Applied Biosystem 7500 Real Time PCR system.

RESULTS

We investigated whether the expression of the copper transporter ATP7A is altered in the brain of the transgenic mouse model of AD, TgCRND8, as compared to wild type mice. TgCRND8 mice harbor a double mutation of familial Alzheimer's disease within the human *APP* transgene (Swedish mutation, K670N/M671L, plus the Indiana mutation, V717F) and develop β -amyloid plaques and learning deficits by 12 weeks of age (Chishti *et al.* 2001). The expression of the *hAPP* transgene is under the control of the hamster prion protein gene promoter which directs neuronal expression (Chishti *et al.* 2001). Brain cryosections were prepared from 9 month-old TgCRND8 mice and their wild type control littermates, and the expression of ATP7A protein was investigated by immunofluorescence microscopy using affinity purified anti-ATP7A antibodies. In wild type mice, the ATP7A protein was uniformly expressed throughout the neocortex (Fig. 1A). However, in TgCRND8 mice, the expression of the ATP7A protein was enriched in cells clustered at distinct foci throughout the neocortex (Fig. 1A). These regions of elevated ATP7A protein were found to coincide with the presence of β -amyloid plaques identified with the WO2 monoclonal antibody directed against the N-terminal region of the A β peptide (Fig. 1A). Identical results were obtained with an alternative antibody to A β (6E10) (data not shown). Higher power microscopy demonstrated that the elevated expression of ATP7A in the TgCRND8 mouse brain occurred within small cells that clearly encircled the β -amyloid plaques (Fig. 1B). Despite this focal enrichment of ATP7A expression in the TgCRND8 mouse brain, Western blot analysis of total brain lysates did not reveal a demonstrable increase in ATP7A protein levels in TgCRND8 mice relative to wild type animals (Fig. 1C).

We then sought to identify the cell type in which ATP7A protein was upregulated in the brain of TgCRND8 mice using antibodies against marker proteins for the three major cell types in brain: NeuN for neurons, GFAP for astrocytes and CD68 for microglia. As expected, there was abundant labeling of CD68-positive microglia in close juxtaposition to β -amyloid plaques in the TgCRND8 mouse brain sections (Fig. 2A). This result was entirely consistent with the proliferation and activation of microglia observed around β -amyloid

plaques in AD brain (McGeer et al. 1987; Sasaki et al. 1997; Cagnin et al. 2001). Astrogliosis was also observed in brain sections from TgCRND8 mice, as indicated by the enrichment of GFAP-positive astrocytes in the vicinity of β -amyloid plaques (Fig. 2A). In double-labeling experiments using brain sections from TgCRND8 mice, we found that the expression of ATP7A was increased in CD68-positive microglia, as indicated by yellow labeling in the merged panels (Fig. 2B). Identical results were obtained with another marker of microglia, CD11b (data not shown). In contrast, the elevated expression of ATP7A was not evident in GFAP-positive astrocytes or in NeuN-positive neurons (Fig. 2B). These findings suggest that the ATP7A protein is specifically up regulated in clusters of activated microglia associated with β -amyloid deposits in TgCRND8 mouse brain.

The identification of microglia as a site for elevated expression of the ATP7A protein in TgCRND8 mice highlighted an unexpected and potentially important connection between inflammation and copper homeostasis in AD. To explore this further, we tested whether activation of cultured BV-2 microglial cells by pro-inflammatory agents could recapitulate the increased ATP7A expression seen in microglia of TgCRND8 mice. Several cytokines have been associated with inflammation in AD, most notably TNF- α , IL-1 β , and IFN- γ (Blasko et al. 1999; Tweedie et al. 2007; Yamamoto et al. 2007; Di Bona et al. 2008). Western blot analysis demonstrated a dose-dependent increase in ATP7A protein expression when BV-2 microglial cells were treated with a range of IFN- γ concentrations relative to untreated cells (Fig. 3A and B). In contrast, there was no effect of TNF- α or IL-1 β on ATP7A expression (Fig. 3A). The treatment of BV-2 cells with monomeric or aggregated forms of A β ₄₂ peptide (with or without copper) alone or in combination with cytokine treatment did not alter ATP7A expression levels, as compared to basal or cytokine-treated controls (data not shown). These findings demonstrate that ATP7A expression is up regulated by IFN- γ in BV-2 cells, and together with the findings in TgCRND8 mice, suggest that pro-inflammatory processes stimulate ATP7A expression in β -amyloid plaque-associated microglia.

Next, we investigated the intracellular localization of ATP7A and copper status in IFN- γ -treated BV-2 microglial cells. The intracellular distribution of ATP7A can provide a biological readout of copper status because ATP7A is restricted to the perinuclear region when copper levels are normal (or low), but traffics to post-Golgi compartments when copper levels are elevated to promote copper transport into vesicular compartments or across the plasma membrane (Petris et al. 1996; La Fontaine et al. 1998; Greenough et al. 2004). In untreated BV-2 cells, ATP7A protein was restricted to a perinuclear distribution typical of Golgi localization. Copper treatment (20 μ M) triggered trafficking of ATP7A to the cell periphery, consistent with previous observations (Fig. 4). Interestingly, treatment of cells with IFN- γ in the absence of added copper stimulated a shift in ATP7A location to a punctate vesicular distribution throughout the cytoplasm (Fig. 4). In contrast, TNF- α or IL-1 β treatment had no effect on ATP7A localization, as compared to untreated controls (Fig. 4). Other experiments demonstrated that the relocation of ATP7A to vesicles in response to IFN- γ was not due to a non-specific vesiculation of the Golgi, since distribution of the Golgi marker protein, GM130, which co-localized with ATP7A in untreated cells, was unaffected by IFN- γ (S.I. Fig. 1).

Because IFN- γ was able to elicit ATP7A trafficking to cytoplasmic vesicles, an event normally associated with copper stimulation, we tested whether this process might be dependent on copper by pre-treating cells with the membrane permeable copper chelator, tetrathiomolybdate (TTM). We found that ATP7A trafficking to cytoplasmic vesicles in response to IFN- γ was partially blocked by TTM, suggesting that this process requires copper (Fig. 5D). This finding prompted us to investigate whether copper uptake might be elevated in BV-2 cells following IFN- γ treatment. Copper transport studies using

radioactive ^{64}Cu demonstrated that the initial rate of copper uptake (5 min) was elevated in IFN- γ -treated cells relative to untreated controls (Fig. 6A). Moreover, there was a marked increase in steady-state ^{64}Cu accumulation after two hours in IFN- γ -treated cells relative to untreated controls (Fig. 6B). Copper export was not changed in IFN- γ -treated cells (data not shown) consistent with an increased copper accumulation at both early and late time points. We then examined whether this increased copper uptake might be associated with elevated expression of the CTR1 copper transporter. Although we could not detect CTR1 protein in BV-2 cells using available antibodies, real-time PCR analysis of mRNA expression in BV-2 cells treated with IFN- γ revealed a significant increase in the level of CTR1 mRNA, relative to untreated cells (Fig. 6C). Taken together, these findings indicate that IFN- γ treatment causes marked changes in copper homeostasis in BV-2 microglial cells, including elevated copper uptake and accumulation, increased expression of ATP7A protein and CTR1 mRNA, and copper-dependent trafficking of ATP7A to cytoplasmic vesicles.

DISCUSSION

A large body of evidence implicates a role for copper in the pathogenesis of AD. The most compelling of these data include: 1) high levels of copper (up to 500 μM) are locally deposited in senile plaques (Lovell et al. 1998; Lee et al. 1999; Suh et al. 2000; Miller et al. 2006); 2) copper potentiates the neurotoxicity of A β peptide (Huang et al. 1999b; Yoshiike et al. 2001; Giuffrida et al. 2007); and 3) copper chelation is an effective means of reducing A β plaque burden and preserving cognitive function in mouse models of AD (Cherny et al. 2001; Lee et al. 2004; Adlard et al. 2008). Clearly, to begin to elucidate the mechanisms by which copper functions in AD pathogenesis, it is important to understand how copper transporter expression and distribution might be altered in the brain of AD patients or mouse models of AD. In this study, we provide *in vivo* evidence that microglia specifically clustered around A β plaques in the brain of the TgCRND8 mouse model of AD are sites of increased ATP7A expression.

Since the local microenvironment of β -amyloid plaques is a site of inflammation in the AD brain, the elevated ATP7A expression in microglia surrounding A β deposits in TgCRND8 mice may be a response to pro-inflammatory mediators. Our finding that ATP7A expression was also increased in cultured BV-2 microglial cells treated with IFN- γ is consistent with this postulate. IFN- γ is a pro-inflammatory cytokine that is produced by microglia and astrocytes around β -amyloid deposits in transgenic AD mouse brain (Abbas *et al.* 2002), and IFN- γ has been shown to potentiate A β -induced encephalitis and neuronal death (Bate et al. 2006; Monsonego et al. 2006). In addition to stimulating ATP7A expression, IFN- γ evoked other responses that regulate copper homeostasis in BV-2 microglial cells, including the induction of CTR1 mRNA levels. Although the sensitivity of available CTR1 antibodies precluded detection of CTR1 protein via Western analysis, copper uptake and accumulation was increased in BV-2 cells in response to IFN- γ , consistent with an increase in copper uptake by CTR1 or other copper transporters. A related response in BV-2 cells to IFN- γ treatment is the copper-dependent redistribution of ATP7A from Golgi to cytoplasmic vesicles, a likely mechanism to sequester cytoplasmic copper. Interestingly, the pattern of ATP7A redistribution to cytoplasmic vesicles was more disperse with IFN- γ than with copper treatment alone, suggesting that inflammation alters ATP7A distribution in a different manner than that observed for copper. Whether ATP7A loads copper into these cytoplasmic vesicles in IFN- γ -treated microglial cells is not known. However, since ATP7A trafficking is known to be coupled with its catalytic activity in CHO cells and other cell types (Petris *et al.* 2002), it is probable that ATP7A trafficking in response to IFN- γ involves increased catalysis of the transporter. It is notable that the changes in copper homeostasis observed in BV-2 microglial cells are reminiscent of those we have recently documented in RAW264.7 macrophages stimulated with bacterial lipopolysaccharide in which the ATP7A

protein undergoes trafficking to vesicles and is also required for bacterial killing (White *et al.* 2009). Thus, marked changes in copper homeostasis, including increased copper uptake and ATP7A trafficking to cytoplasmic vesicles, may be an inflammatory response inherent to multiple cell types of the myeloid lineage.

Although our studies provide the first evidence of changes in microglial copper homeostasis in a model of the AD brain, deciphering whether such changes are protective or detrimental in disease pathogenesis will require further studies. Since copper is released from glutamatergic stimulation of neurons (Schlief *et al.* 2005), this may provide a source for neurotoxic A β -Cu interactions in the AD brain. We speculate that the uptake and sequestration of copper within activated microglia might confer protection in the AD brain by reducing copper-A β interactions in the extracellular milieu. This concept of a positive role of microglia in the pathogenesis of AD is not without precedent. Beneficial roles of microglia in mouse models of AD have been documented, including phagocytic clearance of A β peptides or A β -antibody complexes (Das *et al.* 2003; Lombardo *et al.* 2003; Wilcock *et al.* 2003). Our studies identify for the first time a pro-inflammatory pathway in microglia that links copper dyshomeostasis and Alzheimer's disease. Because ATP7A and CTR1 copper transporters are expressed throughout the CNS, future studies will require animal models in which these proteins are conditionally deleted in microglia in order to define their role in AD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations Used

AD	Alzheimer's disease
Aβ	β -amyloid peptide
IFN-γ	interferon-gamma

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Figure 1.

ATP7A protein expression is elevated in cell clusters associated with β -amyloid plaques. **A).** Expression of ATP7A (green) and β -amyloid (red) is shown in brain cortex of 9 month-old TgCRND8 transgenic mice and wild type littermates. Cryosections were probed with anti-ATP7A antibodies followed by anti-rabbit IgG antibodies conjugated to the Alexa 488 fluorophore (green). β -Amyloid plaques were detected using WO2 antibodies followed by anti-mouse IgG antibodies conjugated to the Alexa 596 fluorophore (red). Bar = 200 μ m. At least 10 mice of each genotype were investigated with the same findings as those shown. **B).** Same as A but at higher magnification. Bar = 20 μ m. Note the elevated expression of ATP7A in the TgCRND8 brain section occurs in small cells associated with β -amyloid plaque. **C).** Immunoblot analysis of ATP7A protein expression in total brain lysates from wild type (lanes 1 – 4) and TgCRND8 mice (lanes 5 – 8). Tubulin protein was detected as a loading control.

Figure 2.

A). Gliosis around β -amyloid plaques in TgCRND8 mouse brain. Brain cryosections from TgCRND8 mice were probed with WO2 antibodies to detect β -amyloid plaques and antibodies directed against either CD68 (microglia marker) or GFAP (astrocyte marker). WO2 antibodies were then detected with anti-mouse IgG antibodies conjugated to the Alexa 594 fluorophore (red), and anti-CD68 and anti-GFAP antibodies were detected with anti-rat IgG antibodies conjugated to the Alexa 488 fluorophore (green). Note the close juxtaposition of CD68- and GFAP-positive cells with β -amyloid plaques. Bar = 40 μ m **B).** Cell type specificity of elevated ATP7A over-expression in TgCRND8 mouse brain. Brain cryosections from TgCRND8 mice were probed with ATP7A antibodies and either anti-NeuN (neuron), anti-GFAP (astrocyte), or anti-CD68 (microglia) antibodies. ATP7A antibodies were detected with anti-rabbit IgG antibodies conjugated to the Alexa 488 fluorophore (green), and cell marker antibodies were detected with anti-rat IgG antibodies conjugated to the Alexa 596 fluorophore (red). Note the yellow labeling in the merged panels for ATP7A and the microglial marker, CD68. Bars = 40 μ m

Figure 3. IFN- γ increases ATP7A protein expression in cultured BV-2 microglial cells
A). BV-2 microglial cells were incubated overnight with or without 25 ng/ml pro-inflammatory cytokines, IFN- γ , TNF- α or IL-1 β . ATP7A protein levels were then detected by immunoblot analysis using anti-ATP7A antibodies. Tubulin protein was detected as a loading control. Relative band intensities vs tubulin are shown in parenthesis. **B).** IFN- γ dose-dependent ATP7A expression. BV-2 cells were incubated overnight with or without the indicated levels of IFN- γ and ATP7A protein levels were determined by immunoblot analysis. Relative band intensities vs tubulin are shown in parenthesis.

Figure 4.

IFN- γ stimulates the trafficking of ATP7A to vesicles in BV-2 microglial cells. Cells were incubated overnight with or without 20 μ M CuCl₂ or 25 ng/ml IFN- γ , TNF- α or IL-1 β . ATP7A protein (green) was then detected by immunofluorescence microscopy using anti-ATP7A antibodies followed by anti-rabbit IgG antibodies conjugated to the Alexa 488 fluorophore. Nuclei were labeled with DAPI (blue). Note the relocalization of ATP7A to cytoplasmic vesicles in cells treated with IFN- γ , but not with TNF- α or IL-1 β .

Figure 5.

The trafficking of ATP7A in IFN- γ stimulated cells is copper-dependent. BV-2 microglial cells were incubated overnight with or without IFN- γ (25 ng/ml), IFN- γ plus the copper chelator TTM (10 nM), or TTM alone. ATP7A protein was detected using anti-ATP7A antibodies followed by anti-rabbit IgG antibodies conjugated to the Alexa 488 fluorophore.

Figure 6.

IFN- γ stimulates copper uptake and accumulation in BV-2 microglial cells. **A**). ^{64}Cu uptake in BV-2 cells. Cells were incubated overnight with or without 25 ng/ml IFN- γ and copper uptake was determined the next day by incubation of the cells with 1 μM ^{64}Cu for 5 minutes. After extensive washing with PBS, cell associated ^{64}Cu was counted and normalized to total protein levels (mean $\pm$ SEM; * p <0.05). Each data point represents the mean of three experiments. **B**). Steady-state accumulation of ^{64}Cu in BV-2 microglial cells. Cells were incubated overnight with or without 25 ng/ml IFN- γ and the following day the steady-state accumulation of ^{64}Cu was determined over 2 hours (mean $\pm$ SEM; * p <0.05). Each data point represents the mean of three experiments. **C**). Real-time PCR analysis of

CTR1 mRNA expression in BV-2 microglial cells incubated overnight with or without 25 ng/ml IFN- γ for the indicated times. Total RNA was prepared and subjected to RT-PCR. Relative changes in CTR1 mRNA expression were determined in triplicate at each time point against the starting levels (mean $\pm$ SEM; n=3). Data were normalized to GAPDH mRNA expression.