Manganese Accumulation in Membrane Fractions of Primary Astrocytes is Associated with Decreased γ-Aminobutyric Acid (GABA) Uptake, and is Exacerbated by Oleic Acid and Palmitate

By: Steve C. Fordahl, Keith M. Erikson

Fordahl, S.C., & Erikson, K.M. (2014). Manganese accumulation in membrane fractions of primary astrocytes is associated with decreased γ-aminobutyric acid (GABA) uptake, and is exacerbated by oleic acid and palmitate. *Environmental Toxicology and Pharmacology, 37(3),* 1148-1156.doi: 10.1016/j.etap.2014.03.016

Made available courtesy of Elsevier: http://www.dx.doi.org/10.1016/j.etap.2014.03.016

Abstract:

Manganese (Mn) exposure interferes with GABA uptake; however, the effects of Mn on GABA transport proteins (GATs) have not been identified. We sought to characterize how Mn impairs GAT function in primary rat astrocytes. Astrocytes exposed to Mn (500 μM) had significantly reduced ^{3}H-GABA uptake despite no change in membrane or cytosolic GAT3 protein levels. Co-treatment with 100 μM oleic or palmitic acids (both known to be elevated in Mn neurotoxicity), exacerbated the Mn-induced decline in ^{3}H-GABA uptake. Mn accumulation in the membrane fraction of astrocytes was enhanced with fatty acid administration, and was negatively correlated with ^{3}H-GABA uptake. Furthermore, control cells exposed to Mn only during the experimental uptake had significantly reduced ^{3}H-GABA uptake, and the addition of GABA (50 μM) blunted cytosolic Mn accumulation. These data indicate that reduced GAT function in astrocytes is influenced by Mn and fatty acids accumulating at or interacting with the plasma membrane.

Abbreviations: Mn, manganese; GABA, γ-aminobutyric acid; GAT, GABA transporter; PKC, protein kinase C; pPKC,phosphorylated PKC; PMA, phorbol 12-myristate 13-acetate; Slc, solute carrier family; D-MEM,Dulbecco's minimal essential

medium; ISO, isorhamnetin; PBS, phosphate buffered saline; RIPA,radioimmunoprecipitation assay; BIS II, bisindolylmaleimide II; DFO, desferrioxamine; DAT, dopamine transporter

Keywords: Manganese | Neurotoxicity | GABA | Astrocyte | Fatty acid

Article:

1. Introduction

Manganese (Mn) neurotoxicity is associated with distinct neurochemical changes that contribute to extrapyramidal symptoms similar to Parkinson's disease. While most of the phenotypic changes resulting from prolonged Mn exposure are associated with changes in the dopamine system, evidence shows that Mn induced changes in the GABA neurotransmission exist prior to dopaminergic dysfunction (Gwiazda et al., 2002). GABA is the main inhibitory neurotransmitter in the brain that is responsible for modulating excitatory signals within the basal ganglia to help coordinate smooth motor function. Motor control issues observed with Mn exposure (e.g. bradykinesia) and other artifacts of Mn accumulation, such as glutamate excitotoxicity, may result from disrupted GABA signaling (Erikson and Aschner, 2003). Specific mechanisms as to how Mn alters GABA are unclear, but Mn appears to target GABA uptake leading to a greater than 3-fold increase in extracellular GABA (Fordahl et al., 2010). GABA clearance is mediated by pre- and extra-synaptic GABA transport proteins GAT1 and GAT3, respectively (Kersanté et al., 2012). Astrocytes primarily express the GAT3 isoform and are integral to clear GABA overflow from the synapse. The relatively high Km for GAT3 compared to neuronal GAT1 (33 μM versus 7 μM, respectively) suggests that GAT3 plays a critical role in clearing GABA spillover from the synaptic cleft during Mn neurotoxicity. Under these conditions, GAT3 reduces the duration and intensity of inhibitory neurotransmission. Astrocytes also sequester Mn and other metals that pose a threat to more vulnerable neurons (Aschner et al., 1992); however, as Mn concentrations increase, the protective function of astrocytes and their role in GABA clearance may be jeopardized.

Several studies have shown that Mn exposure disrupts GABA levels in tissue and extracellular space of the striatum (Bonilla, 1978, Gianutsos and Murray, 1982, Gwiazda et al., 2002, Takeda et al., 2002, Takeda et al., 2003, Anderson et al., 2008 and Fordahl et al., 2010). The direct effect of Mn on GABA transporters, however, has only been measured in striatal synaptosomes using ^{3}H-GABA (Anderson et al., 2007a) and with in vivo microdialysis in Mn exposed rats after delivery of the GAT inhibitor nipecotic acid (Fordahl et al., 2010). These studies reveal a marked decline in GABA uptake, increased extracellular GABA, and impaired GAT protein function. Coupled with evidence that Mn exposure does not alter GAT protein or mRNA levels in Mn exposed rodents (Anderson et al., 2008), these findings suggest a functional decline in GABA transport proteins due to Mn.

We hypothesize the decline in GABA transport is due to Mn altering cellular regulation of the GATs presumably through protein kinase C (PKC) signaling. PKC activation has been reported

in dopaminergic N27 cells exposed to Mn (Latchoumycandane et al., 2005). Additionally, internalization of GATs from the plasma membrane to cytosolic vesicles is dependent on PKC phosphorylation of GATs (Gadea and Lopez-Colome, 2001 and Quick et al., 2004), but can be blocked by PKC inhibitors (Sato et al., 1995). Moreover, other solute carrier family (Slc) transporters, like the glutamine transporter SNAT3, are impaired by Mn induced activation of PKC (Sidoryk-Wegrzynowicz et al., 2011). Furthermore, recent data from our lab showed profound increases in oleic acid (12-fold), palmitic acid (15-fold) and cholesterol (4-fold) levels in brains of Mn exposed rats (Fordahl et al., 2012) and both oleic and palmitic acids are known modulators of PKC activity (Troeger et al., 1984, Khan et al., 1992 and Ragheb et al., 2009). Thus, the goals of our study are to: (1) specifically examine GABA uptake and GAT3 protein levels in primary astrocytes after Mn exposure, (2) investigate the role of PKC signaling in GAT3 regulation with the use of a PKC inhibitor quercetin, and (3) characterize the effect of oleic acid and palmitate on GAT3 protein levels and function. We hypothesized that Mn directly regulates GAT3 proteins through PKC signaling leading to transporter internalization, similar to other transport proteins in the solute carrier family. Additionally, we hypothesized that oleic acid and palmitate would exacerbate Mn induced GAT3 dysfunction. Lastly, we wanted to quantify Mn accumulation in cytosolic and membrane fractions of astrocytes to identify if Mn distribution within these cellular fractions is associated with GAT3 localization, PKC signaling, and/or GABA uptake.

2. Methods

2.1. Cell isolation and culture

Cortical astrocytes were isolated from Sprague Dawley rat pups PND 1–3 (Harlan Laboratories) following the methods described by Allen et al. (2001) with slight modifications. All procedures were approved by the University of North Carolina Animal Care and Use Committee. Briefly, the pups were retrieved, cleaned using the antiseptic microbiocide Betadine, and swiftly decapitated. Using dissecting scissors and forceps the skull cap was detached and the brain was removed after carefully dissecting away any intact meninges to reduce fibroblast contamination. Once removed, well-defined cortices were carefully dissected apart from the rest of the brain and placed into serum free Dulbecco's minimal essential medium (D-MEM) (Sigma-Aldrich). The isolated tissue was minced by titration using a Pasteur pipette treated with Sigmacote®(Sigma-Aldrich) to prevent cell lysing. Astrocytes were dissociated using a 1:5 dilution of Trypsin 0.05% in serum free D-MEM. Dissociated cells were removed and placed in D-MEM containing 10% heat inactivated horse serum (Sigma-Aldrich) to neutralize the Trypsin. Cells were plated in 100 mm dishes at a density of 7.5×10^5 or 6-well plates at a density of 1.0×10^5 and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. Media were changed twice a week until cultures were 90% confluent. Culture purity was verified using immunocytochemistry where >95% stained positive for the astroglial marker GFAP (Invitrogen). Experimental treatments were as follows: 500 μM Mn in the form of $MnCl_2$ was used for all

Mn exposures unless otherwise indicated. This concentration of Mn is above physiologic levels, but has been demonstrated to be neurotoxic in astrocytes (Milatovic et al., 2007 and Milatovic et al., 2009) and falls in the symptomatic range of Mn neurotoxicity, 300 μM-1000 μM, as measured in the brains of non-human primates (Suzuki et al., 1975). Clinical data of Mn exposed humans show a greater than 3-fold accumulation in brain Mn compared to controls (Tracqui et al., 1995). 10 μM isorhamnetin (ISO) (Sigma-Aldrich), the methylated metabolite of quercetin and protein kinase C (PKC) inhibitor, was used as a pre- and co-treatment with Mn for uptake, western blot, and metal analyses. Isorhamnetin concentrations in rat plasma and brain have been reported at 15 μM and 200 nM, respectively (de Boer et al., 2005). Total GABA concentrations used in experiments were 50 μM representing physiologically relevant synaptic concentrations of GABA during inhibitory neurotransmission (Grabauskas, 2005). Logarithmic concentrations (10, 100, and 1000 μM) of albumin bound Oleic acid and palmitic acid (Sigma-Aldrich) were used for individual and co-treatment with Mn for all fatty acid experiments; however, decreased cell viability was observed during co-treatment of 1000 μM fatty acid and Mn so these data were not reported.

2.2. ^{3}H-GABA uptake

Uptake of tritiated GABA (^{3}H-GABA) was measured as described by Erikson and Aschner (2002). Astrocytes (90% confluent in 6-well plates) were incubated for 1, 6, or 24 h at 37 °C with treatment media containing 0 or 500 μM Mn in the form of $MnCl_2$. An additional group of control astrocytes received 500 μM Mn (CnMn) in the experimental buffer only for the duration of the uptake experiment. The CnMn group simulates Mn in the extracellular space, and was used to observe the interaction of Mn with extracellular GABA and GABA transport proteins. Cells were washed 3× with HEPES buffer [122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, and 25 mM N-2-hydroxy-ethylpiperanzine N'-2-ethansulfonic acid, pH 7.4] and incubated for 1, 2, or 4 min with HEPES buffer containing 0.5 μCi ^{3}H-GABA (PerkinElmer) and cold GABA (Sigma-Aldrich) resulting in a final concentration of 50 μM total GABA. The reaction was stopped by aspirating the tritiated HEPES and washing the cells 4× with cold (4 °C) 290 mM mannitol buffer containing 0.5 mM calcium nitrate to maintain cell adhesion to the substrate. Cells were solubilized in 500 μL NaOH (1 N) and 400 μL aliquots were neutralized with 33.3 μL HCl (12 N) then used for β-counting with a Perkin Elmer liquid scintillation analyzer (PerkinElmer). The remaining 100 μL was used for protein determination using the bicinchoninic assay (BCA, Pierce Chemicals).

2.3. Cell fractionation

Astrocyte cytosolic and membrane fractions were obtained through differential centrifugation. Astrocytes from two 100 mm plates were pooled into 1 mL phosphate buffered saline (PBS),

centrifuged (1000 × *g*) to pellet, then rinsed with PBS and re-pelleted twice. The PBS was then aspirated and 100 μL cold 0.32 M sucrose pH 7.4 containing a protease inhibitor cocktail (Calbiochem) and phosphatase inhibitors sodium orthovanadate, sodium fluoride, and β-glycerolphosphate (Sigma–Aldrich) was added to the final pellet to maintain membrane integrity. The pellet was sonicated on ice using ten one-second bursts. The remaining homogenate was centrifuged at 750 × *g* for 5 min to pellet nuclear material and any unbroken cells. The supernatant was decanted and centrifuged at 41,000 × *g* for 30 min at 4 °C. The resulting supernatant and pellet were separated and represent the cytosol and crude membrane fractions, respectively. The crude membrane fraction was solubilized in 100 μL radioimmunoprecipitation assay (RIPA) lysis buffer (99 mL 1× PBS, 1 mL Nonidet 40, 0.1 g sodium dodecyl sulfate, 0.5 g sodium deoxycholate, pH 7.4). Cellular fractions of astrocytes were obtained from least three independent culture dates and used for western blot and metal analyses.

2.4. Western blot analysis

Protein concentrations of cytosolic and plasma membrane samples were determined using BCA analysis. For western blots, 20 μg of protein from each sample was loaded into NuPAGE® Bis-Tris precast gels, run with MES running buffer for 40 min using NuPAGE® preset conditions on the PowerEase® 500 power supply system, and transferred to a Immobilon® PVDF membrane (Millipore) for 1 h using the XCell II™ blot module in transfer buffer (all from Invitrogen). The following antibodies and dilutions were used for protein detection: GAT3 1:500 (Abcam), phospho-PKC (pan, βII Ser 660) 1:1000 (Cell Signaling), PKC (pan, Thr 495) 1:1000 (Novus Biologicals), β-Actin 1:1000–1:5000 (Cell Signaling), and anti-rabbit IgG HRP-linked secondary (Cell Signaling). Immunoblotting occurred overnight at 4 °C after membranes were blocked with Tris-buffered saline containing tween (TBST, 2.42 g Tris, 8 g NaCl, 1 L deionized water, 500 μL Tween 20, pH 7.6) and 5% instant milk. The membrane was then rinsed 4× with TBST and blocked again with 5% milk prior to being exposed to the secondary antibody for 2 h at room temperature. Protein detection was acquired using Western Lighting Chemiluminescence (PerkinElmer) on a BioRad Chemidoc imaging system, and the band signal intensity was assessed using QuantityOne software (BioRad).

2.5. Metal analysis

Mn concentrations were measured with graphite furnace atomic absorption spectrometry (Varian AA240, Varian, Inc., USA). Aliquots of astrocyte cytosolic and plasma membrane homogenates (30 μL) were digested in ultra-pure nitric acid (1:2 v/v dilution) for 24–48 h in a sand bath (60 °C). Each sample was further diluted with a 2% nitric acid solution as needed for analysis. A bovine liver (NBS Standard Reference Material, USDC, Washington, DC) (10 μg Mn/g) was digested in ultrapure nitric acid and used as an internal standard for analysis (final concentration 5 μg Mn/L). Metal data are expressed as μM/mg protein.

2.6. Statistical analysis

^{3}H-GABA uptake and metal data were analyzed using SPSS v20 for Windows. Data were examined for the presence of outliers by boxplot analysis. Analysis of variance was conducted to identify mean differences between treatment groups for uptake and metal analyses with a significance threshold set at $p < 0.05$. Tukey's post hoc tests were conducted when a significant difference in means was detected to identify significant variations among individual treatments within the statistical model. Pearson's correlational analyses were then performed to examine relationships between metal concentrations and ^{3}H-GABA uptake. The threshold of significance for all tests was set at $p < 0.05$.

3. Results

3.1. Mn decreases ^{3}H-GABA uptake in a PKC independent manner

A time dependent decrease in ^{3}H-GABA uptake was observed in astrocytes exposed to 500 μM Mn (Fig. 1A). Mn significantly reduced GABA uptake after 24 h of exposure ($p < 0.001$) and in astrocytes that were only exposed to Mn (CN Mn) during experimental conditions ($p < 0.05$), compared to control. The CN Mn group was added to identify the effect of extracellular Mn on GABA uptake. A time dependent increase in PKC phosphorylation (pPKC) was also observed with Mn exposure (Fig. 1B); however, no appreciable changes in plasma membrane or cytosolic GAT3 protein levels resulted due to elevated pPKC, contrary to our hypothesis. To further investigate the involvement of PKC in Mn reduced GABA transport we repeated the uptake experiments using the PKC inhibitors BIS II and isorhamnetin (ISO). Pretreatment with 10 μM ISO failed to restore Mn-impaired GABA uptake (Fig. 1C), despite decreasing pPKC in the plasma membrane fraction (Fig. 1D); furthermore, ISO reduced GABA uptake when Mn was present in the extracellular space (Fig. 1C). Slight reductions in membrane bound GAT3 were observed with ISO treatment corroborating decreased uptake. ISO driven changes in membrane GAT3 is not likely due to PKC signaling because 24 h Mn exposure did not yield similar results although pPKC was abundant. Moreover, treatment with the broad PKC inhibitor BIS II (10 μM) did not restore Mn-impaired GABA uptake (data not shown) further supporting a diminished role of PKC phosphorylation in Mn reduced GABA uptake.

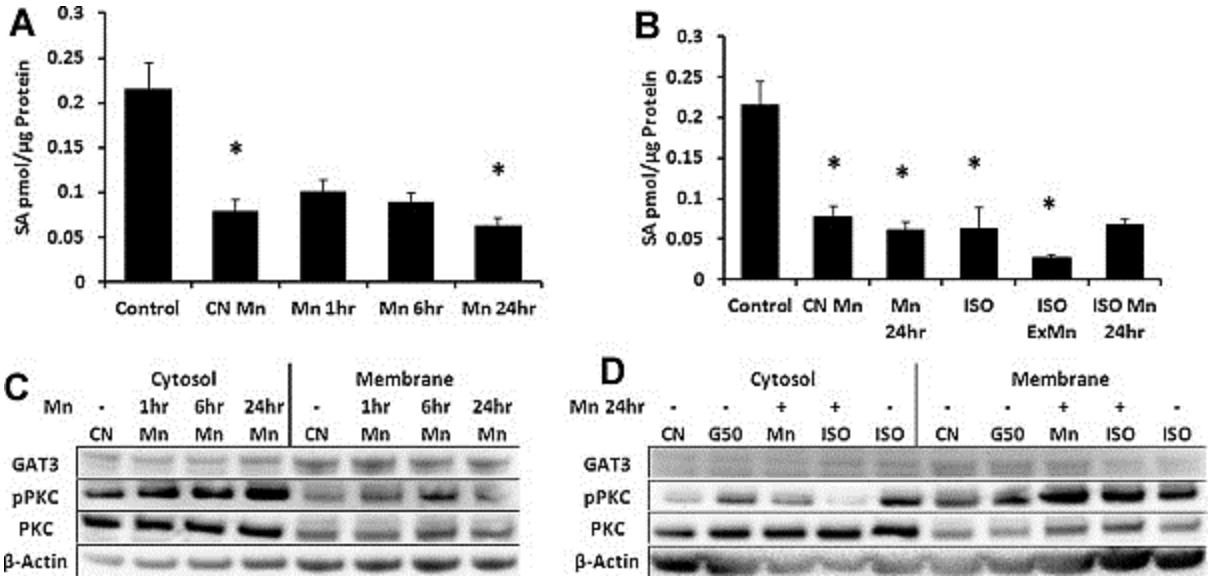

Fig. 1. ^{3}H-GABA uptake and GAT3 protein levels in Mn exposed astrocytes. Mn and isorhamnetin (ISO) significantly reduce ^{3}H-GABA uptake independent of PKC phosphorylation. (A) ^{3}H-GABA uptake, reported as specific activity (SA), of astrocytes exposed for 2 min to an experimental buffer containing 0.5 μ Ci ^{3}H-GABA (total GABA concentration, 50 μ M). Prior to uptake, astrocytes were exposed to 500 μ M Mn for 0 (control), 1, 6, or 24 h. An additional group of control astrocytes were exposed to experimental buffer containing ^{3}H-GABA and 500 μ M Mn (CN Mn) to simulate extracellular Mn. (C) 2 min uptake of 3H-GABA in astrocytes pretreated for 72 h with 10 μ M ISO, ISO plus extracellular Mn (ISO ExMn), and 24 h Mn exposure after ISO pretreatment (ISO Mn 24 h). All uptake data represent three sample replicates from three independent culture dates (n = 9) and are expressed as mean specific activity normalized to protein $\pm$ SEM. (B and D) Western blot analysis on cytosolic and plasma membrane fractions of cells exposed to 500 μ M Mn for 0, 1, 6, or 24 h (B), or 24 h exposure to Mn after pretreatment with ISO (D). An additional group of cells were treated with 50 μ M GABA (G50) (D) to control for protein expression changes due to the uptake experimental conditions. Analysis of variance was performed on uptake data, and when applicable, Tukey's post hoc analysis was conducted to determine significant differences between treatment groups, (*) $p \leq 0.05$.

3.2. Membrane GAT3 may be influenced by cellular Mn localization

Metal analysis of membrane and cytosolic fractions provide novel data that suggest Mn and ISO induced changes in GABA uptake may be associated with cellular Mn localization. Astrocytes exposed to 500 μ M Mn rapidly accumulate cytosolic Mn by 600-fold within 1 h of exposure, followed by drastic Mn efflux resulting in a net retention of cytosolic Mn that is ~100-fold greater compared to control (Fig. 2A). Interestingly, ISO pretreatment blunted cytosolic Mn accumulation by 60% after 1 and 6 h of Mn exposure, but cytosolic Mn normalized after 24 h (Fig. 2A and B). Cytosolic and membrane Mn levels were significantly elevated from controls after 24 h of Mn exposure ($p < 0.05$), corresponding with significantly reduced GABA uptake but stable membrane GAT3 levels. Decreased Mn uptake in the ISO treatment group, and a near

significant (p = 0.06) reduction in membrane Mn levels compared to the Mn alone treatment, may allow for proper GAT3 membrane recycling.

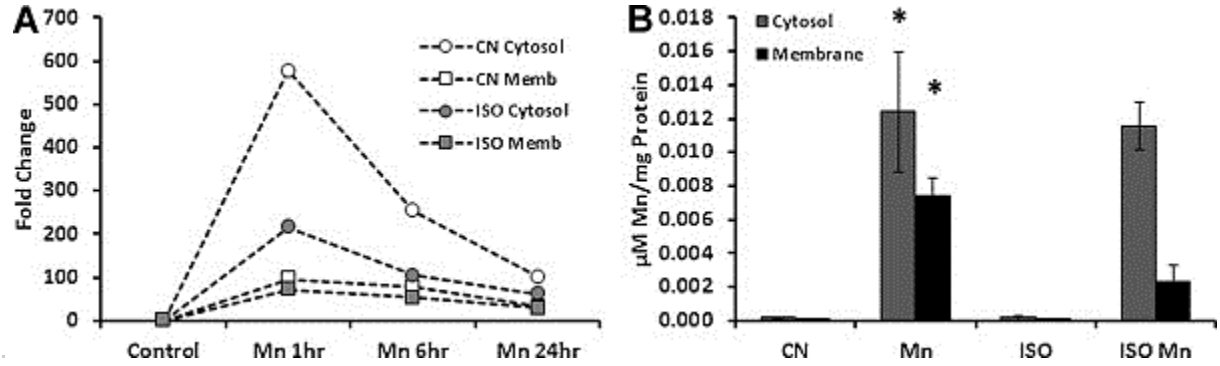

Fig. 2. Astrocyte Mn accumulation after Mn exposure and ISO pretreatment. (A) Astrocytes exposed to 500 μ M Mn responded with rapid accumulation of cytosolic Mn after 1 h of exposure but retained less cytosolic Mn with longer term exposure. The Mn content in plasma membrane fractions of astrocytes responded similarly over time with less magnitude of change. After 24 h of Mn exposure, cytosolic and membrane Mn concentrations were significantly elevated compared to control (B). Pretreatment with ISO blunted the initial cytosolic accumulation of Mn (A), and caused a near significant (p = 0.06) reduction in plasma membrane Mn content after 24 h of Mn exposure (B). Data from control and 24 h time points were collected from three independent culture dates (n = 9), 1 h and 6 h Mn time points were pooled samples from three separate 100 mm plates. Analysis of variance was performed on metal analysis data, and when applicable, Tukey's post hoc analysis was conducted to determine significant differences between treatment groups, (*) $p \leq 0.05$.

3.3. Oleic acid and palmitic acid exacerbate Mn accumulation resulting in decreased GABA uptake

Administration of oleic acid and palmitic acid to astrocyte cultures resulted in concentration dependent changes in GABA uptake (Fig. 3A). Low concentrations (10 μ M) of oleic acid (O10) or palmitic acid (P10) had little effect on GABA uptake but a logarithmic increase in fatty acid dose (O100 or P100) reduced uptake. Mn exposure compounded this effect significantly impairing GABA uptake with 10 or 100 μ M treatment of either fatty acid (Fig. 3A). Decreased uptake did not correspond with changes in GAT3 protein levels (Fig. 3B and C). The application of fatty acids did, however, enhance Mn accumulation in cytosolic and membrane fractions of astrocytes (Fig. 4A and B). Co-application of oleic acid and Mn significantly increased membrane Mn content (O10 Mn, $p < 0.001$; O100 Mn, $p < 0.05$) over Mn treatments alone (Fig. 4B). Similarly, combined palmitic acid and Mn applications significantly elevated membrane Mn levels over Mn treatments alone (P10 Mn and P100 Mn, $p < 0.001$), but palmitic acid also exacerbated Mn uptake leading to significantly greater cytosolic Mn concentrations than Mn treatment alone ($p < 0.001$) (Fig. 4D). Correlational analysis revealed significant inverse relationships between GABA uptake and both cytosolic (Fig. 4C, $p < 0.05$) and membrane (Fig.

4D, $p < 0.05$) Mn concentrations. Decreased uptake and augmented Mn accumulation with oleic acid and palmitic acid were not associated with changes in GAT3 protein levels (Fig. 3B and C).

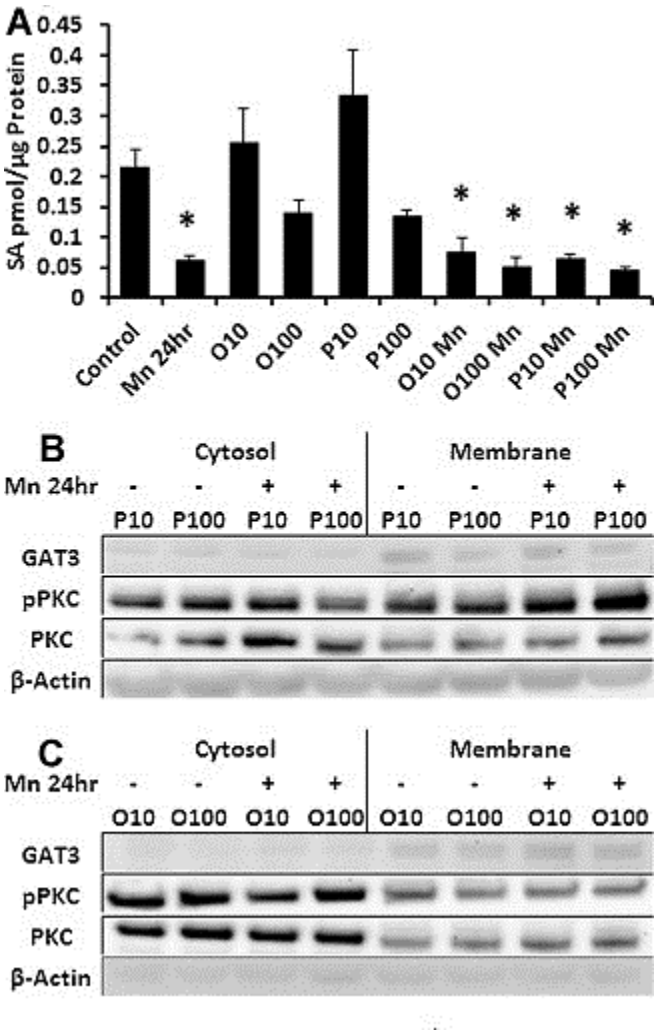

Fig. 3. ^{3}H-GABA uptake and western blot analysis of astrocytes exposed to oleic acid and palmitate. Astrocytes were exposed to oleic acid or palmitate (10 μ M (O10 and P10) or 100 μ M (O100 and P100)) for 24 h with or without 500 μ M Mn. Uptake and western blot analyses were performed on astrocytes from three independent culture dates. Uptake samples (n = 9) and western blots are representative of protein changes from the three culture dates. (A) Oleic acid and palmitate significantly decrease ^{3}H-GABA uptake when combined with Mn exposure, but not independently. (B) Changes in ^{3}H-GABA uptake did not correspond with changes in plasma membrane GAT3 content or PKC phosphorylation. Analysis of variance was performed on uptake data, and when applicable, Tukey's post hoc analysis was conducted to determine significant differences between treatment groups, $p \leq 0.05$.

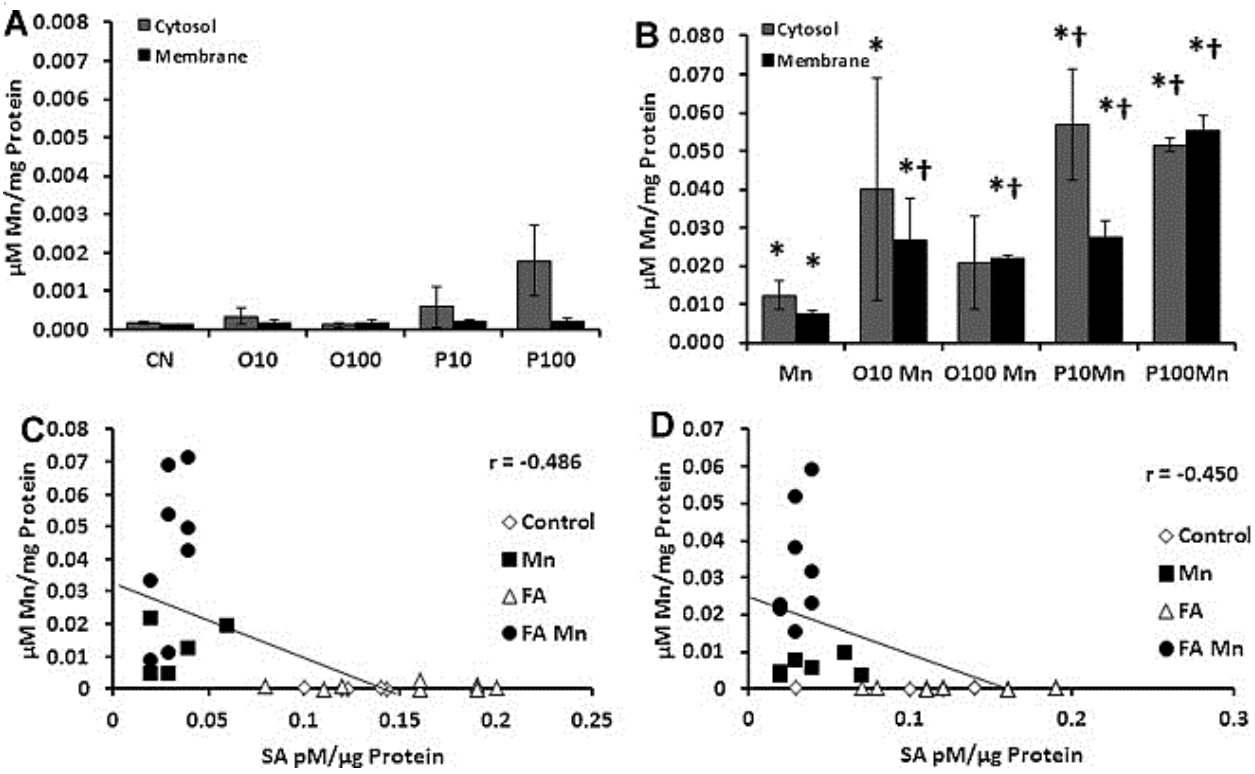

Fig. 4. Cytosolic and plasma membrane metal content of astrocytes exposed to oleic acid and palmitate. Exposure to oleic acid and palmitate exacerbate Mn accumulation due to 500 μ M Mn exposure in cytosolic and membrane fractions, and was negatively correlated with ^{3}H-GABA uptake. Data represent three sample replicates from two independent culture dates (n = 6) and are expressed as μ M Mn normalized to sample protein $\pm$ SEM. (A) Mn concentrations of cytosolic and membrane fractions of cells exposed to 10 or 100 μ M fatty acid. (B) Co-treatment with 500 μ M Mn and fatty acids enhance Mn accumulation in both cell fractions compared to control (*) and Mn exposure (†) alone. (C) Cytosolic Mn concentrations of Mn and fatty acid (FA) exposures had a significant negative correlation with ^{3}H-GABA uptake. (D) A significant negative correlation was also observed between plasma membrane Mn content and ^{3}H-GABA uptake of Mn and FA exposed astrocytes. Analysis of variance with Tukey's post hoc analysis was used to determine significant differences in Mn content between treatment groups, and Pearson's correlational analysis was conducted to elucidate the relationship between ^{3}H-GABA uptake and Mn accumulation, $p \leq 0.05$.

3.4. Extracellular Mn and GABA interact reducing their transport into cells

Astrocytes exposed to Mn only during the uptake experiment (CN Mn) had reduced GABA uptake similar to astrocytes that were exposed to Mn for 24 h (Fig. 1A). Additionally, extracellular Mn reduced GABA uptake in the ISO treatment group versus the ISO treatment without Mn in the experimental buffer (Fig. 1C). These data show that extracellular Mn decreases GABA uptake. Next we tested whether increasing concentrations of GABA had the same effect on Mn uptake. Astrocytes exposed to 10, 100, and 1000 μ M Mn had significantly

elevated cytosolic Mn compared to control (Fig. 5); however concomitant administration of 50 μ M GABA with 1000 μ M Mn decreased cytosolic Mn concentrations compared with 1000 μ M Mn alone ($p < 0.05$) (Fig. 5).

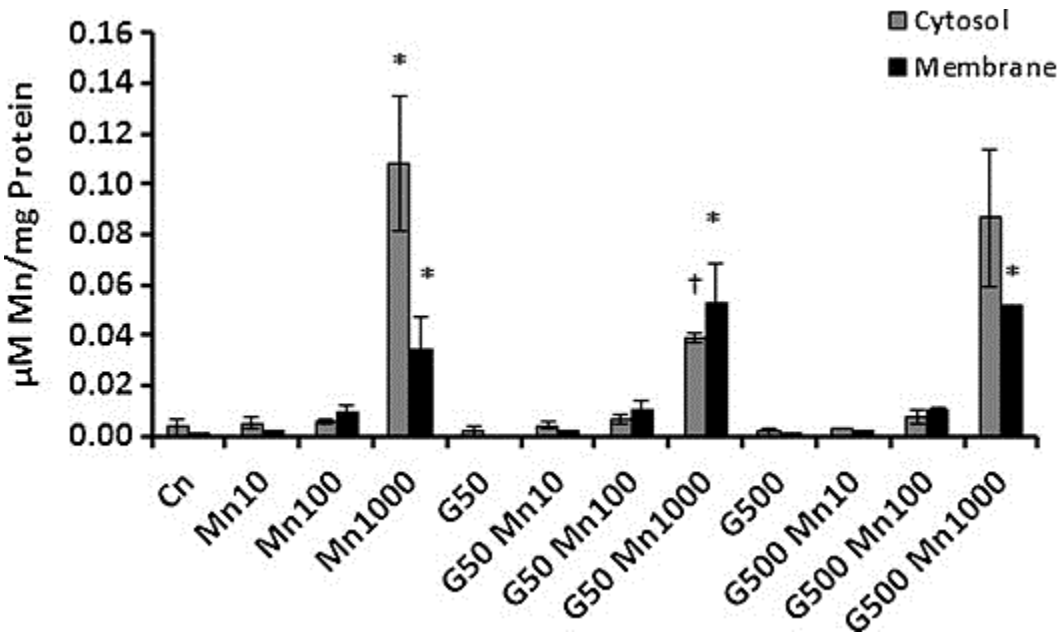

Fig. 5. Dose response: cytosolic and membrane Mn concentrations with increasing Mn and GABA. These data represent cytosolic and membrane Mn concentrations of astrocytes exposed to logarithmic increases in Mn (10, 100, and 1000 μ M) and GABA (50 and 500 μ M) independent or in combination for 24 h. These data represent three pooled culture dishes from two culture dates. ($n = 4$). Analysis of variance with Tukey's post hoc analysis, were applicable, was used for statistical analysis. (*) represents a significant change from control and (†) represents a significant change from Mn 1000, $p \leq 0.05$.

4. Discussion

The purpose of this study was to characterize how Mn decreases GABA uptake in primary astrocytes. Because astrocytes maintain the extracellular milieu around synaptic terminals, understanding how Mn and indirect consequences of Mn exposure (i.e. increased fatty acids) alter astrocyte GABA uptake is valuable to understand neurochemical changes associated with Mn neurotoxicity. These data corroborate previous findings of impaired GABA uptake extending to primary astrocytes and novel evidence that mechanisms driving this impairment involve Mn interacting with the plasma membrane. Additionally, our results show that increases in brain fatty acid levels associated with Mn neurotoxicity exacerbate the effect of Mn on GABA uptake by enhancing Mn accumulation in the membrane fraction of astrocytes.

We hypothesized that Mn would decrease GABA uptake in astrocytes via PKC phosphorylation and internalization of GAT3. This assumption was based on data indicating that Mn activates PKC (Latchoumycandane et al., 2005 and Kitazawa et al., 2005), PKC mediates membrane

recycling of GAT1 (Wang and Quick, 2005), and that Mn induced PKC activation led to internalization of other transporters in the Slc6 family (Sidoryk-Wegrzynowicz et al., 2011). We observed a time dependent increase in PKC phosphorylation with Mn exposure; however, this increase did not correspond with internalization of GAT3 protein (Fig. 1B). There is abundant evidence in the literature supporting the role of PKC in the regulation of GAT1 (Whitworth and Quick, 2001, Quick et al., 2004 and Wang and Quick, 2005), but a paucity of experimental studies examining GAT3 regulation. We measured both GAT1 (data not shown) and GAT3 in our primary astrocytes, but only detected the presence of GAT3. It is possible that GAT3 regulation functions through different mechanisms, or that GAT3 does not recycle as dynamically as GAT1 to and from the plasma membrane. Alternatively, increased pPKC in astrocytes promotes SNARE protein interactions with GAT1 (and likely GAT3), which has been shown to stabilize GAT1 on the membrane, but due to conformational changes, decreases GAT function (Quick, 2006 and Wang et al., 2003). However, because the PKC inhibitors Bis II and ISO did not restore GABA uptake in our study, and the addition of Mn to the experimental buffer decreased uptake in control astrocytes, we postulate that Mn interacts directly with the plasma membrane and possibly the GAT proteins. Mn binding GATs could reduce GABA transport similar to decreased dopamine transporter (DAT) function upon zinc binding to extracellular loops of DATs (Norregaard et al., 1998).

Mn is chaperoned by intra- and extra-cellular transport proteins, but once the need for Mn in metalloenzymes is met and storage mechanisms become overwhelmed, excess Mn can impair cellular respiration, induce lipid peroxidation, and initiate apoptosis (Malecki, 2001, Yiin et al., 1996 and Yoon et al., 2011). The unbound species of Mn (Mn^{3+}) is highly reactive and will quickly bind to sulfhydryl moieties of amino acid residues (Fisher and Jones, 1981) or scavenge electrons initiating oxidative damage. The production of F_2-isoprostanes and increase of prostaglandin E_2 due to Mn exposure demonstrates this reactivity (Milatovic et al., 2009), but also provides evidence that Mn interacts with the plasma membrane. Our data confirm that Mn content in the membrane fraction of astrocytes is significantly elevated with Mn exposure (Fig. 5), and was also associated with decreased GABA uptake (Fig. 1A). Sulfhydryl containing cysteine residues on the short extracellular loop four of the GABA transporters are fundamental to sodium and GABA ligand binding for translocation to the cytosol (Zomot and Kanner, 2003). We hypothesize that interactions between Mn and these sulfhydryl residues are a putative mechanism for decreased GABA uptake in primary astrocytes. Additionally, the DAT (of the same, largely conserved, Slc6 transporter family as GAT isoforms) was identified to play a role in Mn transport in striatal synaptosomes and the globus pallidus of Mn exposed rats (Anderson et al., 2007b). It is possible that Mn has a similar affinity for GAT isoforms as DAT, accounting for a decline in GAT function. We observed that adding physiological concentrations of GABA (50 μ M) to astrocyte cultures decreased cytosolic Mn accumulation (Fig. 5), and speculate that Mn interacts with extracellular GABA or competes as a ligand for GATs. To identify direct binding of Mn to GATs would require further studies using structural modeling to predict metal binding sites, similar to the elucidation of the zinc/DAT binding site, in conjunction with

immunoprecipitation of GATs from Mn exposed cells followed by metal analysis of the eluted protein to identify Mn/GAT binding.

Recently, data were published by our lab showing profound changes in brain lipid metabolism of Mn exposed rats (Fordahl et al., 2012). Significant elevations of oleic acid (12-fold), palmitic acid (15-fold), and cholesterol (4-fold) were observed. These fatty acids are primary constituents of plasma membrane and membrane raft composition (Schumann et al., 2011), and oleic and palmitic acid have been independently associated with decreased GABA uptake and increased PKC activity (Troeger et al., 1984, Khan et al., 1992 and Ragheb et al., 2009). Drastic changes in these fatty acids may contribute to GABA related dysfunction observed in Mn neurotoxicity; however to our knowledge this relationship has not been investigated.

The effect of fatty acid treatment on GABA uptake observed in our study was less than previously reported (Troeger et al., 1984 and Rhoads et al., 1982). A significant decline in GABA uptake was only achieved when oleic acid or palmitate were co-administered with Mn. Again, PKC associated internalization of GAT3 did not occur with either fatty acid alone or combined fatty acid/Mn treatments (Fig. 3B and C). Changes in the amount of these fatty acids, specifically the saturated fatty acid palmitate, may compromise the membrane fluidity and impair protein-mediated transport (Hulbert et al., 2005). Oleic acid is one of the most abundant fatty acids in the plasma membrane, and oleic and palmitic acids are the predominant fatty acids found in membrane lipid rafts (Schumann et al., 2011). Disrupting lipid composition in membrane rafts resulted in a 50% decrease in transport rate by GATs (North and Fleischer, 1982 and Allen et al., 2007). Since GATs are associated with lipid raft micro-domains (Allen et al., 2007) the debilitating effect of fatty acids and Mn on GABA uptake observed in our study could be influenced by changes in lipid raft composition. Moreover, oleic and palmitic acids significantly elevated Mn accumulation in membrane fractions over Mn exposure alone. It is unclear whether decreased GABA uptake is primarily affected by Mn directly or Mn induced changes in fatty acids, but our data show a synergistic relationship between the two.

While direct binding of Mn to GATs needs to be confirmed, we provide evidence that an interaction between Mn and these transmembrane proteins may exist. Furthermore, it appears that Mn exposure does not profoundly alter cellular localization of GAT3 in primary astrocytes, specifically via PKC signaling. It is possible that endoplasmic reticular stress compromises post translational modification of neurotransmitter transporters, but results from this and previous studies from our lab suggest that Mn exposure alters membrane structure/function as evidenced by the oleic acid, palmitic acid, and cholesterol changes in vivo with Mn exposure (Fordahl et al., 2012) and their influence on in vitro GABA uptake. These changes may influence membrane dynamics, and our data suggest that elevations of these fatty acids augment Mn aggregation at the membrane where Mn is available to interact with transmembrane proteins or instigate oxidative damage. Further characterizing the effects of Mn accumulation on membrane integrity will provide valuable information on how Mn neurotoxicity affects GABA and other neurotransmitter systems within the basal ganglia.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Allen, J.W., Mutkus, L.A., Aschner, M., 2001. Isolation of neonatal rat cortical astrocytes for primary cultures. Curr. Protoc. Toxicol. 4, 12.4.1–12.4.15.

Allen, J.A., Halverson-Tamboli, R.A., Rasenick, M.M., 2007. Lipid raft microdomains and neurotransmitter signaling. Nat. Rev. Neurosci. 8 (2), 128–140.

Anderson, J.G., Cooney, P.T., Erikson, K.M., 2007a. Inhibition of DAT function attenuates manganese accumulation in the globus pallidus. Environ. Toxicol. Pharmacol. 23, 179–184.

Anderson, J.G., Cooney, P.T., Erikson, K.M., 2007b. Brain manganese accumulation is inversely related to –amino butyric acid uptake in male and female rats. Toxicol. Sci. 95(1), 188–195.

Anderson, J.G., Fordahl, S.C., Cooney, P.T., Weaver, T.L., Colyer, C.L., Erikson, K.M., 2008. Manganese exposure alters extracellular GABA, GABA receptor and transporter protein and mRNA levels in the developing rat brain. Neurotoxicology 29, 1044–1053.

Aschner, M., Gannon, M., Kimelberg, H.K., 1992. Manganese uptake and efflux in cultured rat astrocytes. J. Neurochem. 58 (2), 730–735.

Bonilla, E., 1978. Increased GABA content in caudate nucleus of rats after chronic manganese chloride administration. J. Neurochem. 31, 551–552.

de Boer, V.C.J., Dihal, A.A., van der Woude, H., Arts, I.C.W., Wolffram, S., Alink, G.M., Rietjens, I.M.C.M., Keijer, J., Hollman, P.C.H., 2005. Tissue distribution of quercetin in rats and pigs. J. Nutr. 135, 1617–1618.

Erikson, K.M., Aschner, M., 2003. Manganese neurotoxicity and glutamate-GABA interaction. Neurochem. Int. 43 (4–5), 475–480.

Erikson, K.M., Aschner, M., 2002. Manganese causes differential regulation of glutamate transporter (GLAST) taurine transporter and metallothionein in cultured rat astrocytes. Neurotoxicology 23, 595–602.

Fisher, N.S., Jones, G.J., 1981. Heavy metals and marine phytoplankton: correlation of toxicity and sulfhydryl-binding. J. Phycol. 17, 108–111.

Fordahl, S.C., Anderson, J.G., Cooney, P.T., Weaver, T.L., Colyer, C.L., Erikson, K.M., 2010. Manganese exposure inhibits the clearance of extracellular GABA and influenced taurine homeostasis in the striatum of developing rats. Neurotoxicology 31, 639–646.

Fordahl, S., Cooney, P., Qiu, Y., Xie, G., Jia, W., Erikson, K.M., 2012. Waterborne manganese exposure alters plasma, brain, and liver metabolites accompanied by changes in stereotypic behaviors. Neurotoxicol. Teratol. 34, 27–36.

Gadea, A., Lopez-Colome, A.M., 2001. Glial transporters for glutamate, glycine, and GABA: II GABA transporters. J. Neurosci. Res. 63 (6), 461–468.

Gianutsos, G., Murray, M.T., 1982. Alterations in brain dopamine and GABA following inorganic or organic manganese administration. Neurotoxicology 3 (3), 75–81.

Grabauskas, G., 2005. Time course of GABA in the synaptic clefts of inhibitory synapses in the rostral nucleus of the solitary tract. Neurosci. Lett. 373, 10–15.

Gwiazda, R.H., Lee, D., Sheridan, J., Smith, D.R., 2002. Low cumulative manganese exposure affects striatal GABA but not dopamine. Neurotoxicology 23, 69–76.

Hulbert, A.J., Turner, N., Storlein, L.H., Else, P., 2005. Dietary fats and membrane function: implications for metabolism and disease. Biol. Rev. 80 (1), 155–169.

Kersanté, F., Rowley, S.C., Pavlov, I., Gutièrrez-Macinas, M., Semyanov, A., Reul, J.M.H.M., Walker, M.C., Linthorst, A.C.E., 2012. A functional role for both GABA transporter-1 and GABA transporter-3 in the modulation of extracellular GABA and GABAergic tonic conductances in the rat hippocampus. J. Physiol. 591 (10), 2429–2441.

Khan, W.A., Blobe, G.C., Hannun, Y.A., 1992. Activation of protein kinase C by oleic acid. J. Biol. Chem. 267 (6), 3605–3612.

Kitazawa, M., Anantharam, V., Yang, Y., Hirata, Y., Kanthasamy, A., Kanthasamy, A.G., 2005. Activation of protein kinase C by proteolytic cleavage contributes to manganese-induced apoptosis in dopaminergic cells: protective role of Bcl-2. Biochem. Pharmacol. 69, 133–146.

Latchoumycandane, C., Anantharam, V., Kitazawa, M., Yang, Y., Kanthasamy, A., Kanthasamy, A.G., 2005. Protein kinase C is a key downstream mediator of manganese-induced apoptosis in dopaminergic neuronal cells. J. Pharmacol. Exp. Ther. 313(1), 46–55.

Malecki, E.A., 2001. Manganese toxicity is associated with mitochondrial dysfunction and DNA fragmentation in rat primary striatal neurons. Brain Res. Bull. 55 (2), 225–228.

Milatovic, D., Yin, Z., Gupta, R.C., Sidoryk, S., Albrecht, J., Aschner, J.L., Aschner, M., 2007. Manganese induces oxidative impairment in cultured rat astrocytes. Toxicol. Sci. 98 (1),198–205.

Milatovic, D., Zaja-Milatovic, S., Gupta, R.C., Yu, Y., Aschner, M., 2009. Oxidative damage and neurodegeneration in manganese-induced neurotoxicity. Toxicol. Appl. Pharmacol. 240, 219–225.

Norregaard, L., Frederiksen, D., Nielsen, E.Ø., Gether, U., 1998. Delineation of an endogenous zinc-binding site in the human dopamine transporter. EMBO J. 17 (15), 4266–4273.

North, P., Fleischer, S., 1982. Alteration of synaptic membrane cholesterol/phospholipid ratio using a lipid transfer protein. J. Biol. Chem. 258 (2), 1242–1253.

Quick, M.W., Hu, J., Wang, D., Zhang, H.Y., 2004. Regulation of a-aminobutyric acid transporter by reciprocal tyrosine and serine phosphorylation. J. Biol. Chem. 279 (16), 15961–15967.

Quick, M.W., 2006. The role of SNARE proteins in trafficking and function of neurotransmitter transporters. Handb. Exp. Pharmacol. 175, 181–196.

Ragheb, R., Shanab, G.M.L., Medhat, A.M., Seoudi, D.M., Adeli, D., Fantus, I.G., 2009. Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: evidence for PKC activation and oxidative stress-activated signaling pathways. Biochem. Biophys. Res. Commun. 389, 211–216.

Rhoads, D.E., Kaplan, M.A., Peterson, N.A., Raghupathy, E., 1982. Effects of free fatty acids on synaptosomal amino acid uptake systems. J. Neurochem. 38, 1255–1260.

Sato, K., Betz, H., Schloss, P., 1995. The recombinant GABA transporter GAT1 is downregulated upon activation of protein kinase C. FEBS Lett. 375, 99–102.

Schumann, J., Leichtle, A., Thiery, J., Fuhrmann, H., 2011. Fatty acid and peptide profiles in plasma membrane and membrane rafts of PUFA supplemented RAW264.7 macrophages. PLoS ONE 6 (8), e24066.

Sidoryk-Wegrzynowicz, M., Lee, E., Mingwei, N., Aschner, M., 2011. Disruption of astrocytic glutamine turnover by manganese is mediated by the protein kinase C pathway. Glia 59, 1732–1743.

Suzuki, Y., Mouri, T., Suzuki, Y., Nishiyama, K., Fujii, N., 1975. Study of subacute toxicity of manganese dioxide in monkeys. Tokushima J. Exp. Med. 22, 5–10.

Takeda, A., Sotogaku, N., Oku, N., 2002. Manganese influences the levels of neurotransmitters in synapses in rat brain. Neuroscience 114, 669–674.

Takeda, A., Sotogaku, N., Oku, N., 2003. Influence of manganese on the release of neurotransmitters in rat striatum. Brain Res. 965 (1–2), 279–282.

Tracqui, A., Tayot, J., Kintz, P., Alves, G., Bosque, M.A., Mangin, P., 1995. Determination of manganese in human brain samples. Forensic Sci. Int. 76, 199–203.

Troeger, M.B., Rafalowska, U., Erecinska, ´ M., 1984. Effect of oleate on neurotransmitter transport and other plasma membrane functions in rat brain synaptosomes. J. Neurochem. 42, 1735–1742.

Wang, D., Deken, S.L., Whitworth, T.L., Quick, M.W., 2003. Syntaxin 1A inhibits GABA flux, efflux, and exchange mediated by the rat brain GABA transporter GAT1. Mol. Pharmacol. 64 (4), 905–913.

Wang, D., Quick, M.W., 2005. Trafficking of the plasma membrane GABA transporter GAT1: size and rates of an acutely recycling pool. J. Biol. Chem. 280 (19), 18703–18709.

Whitworth, T.L., Quick, M.W., 2001. Substrait-induced regulation of -aminobutyric acid transporter Trafficking requires tyrosine phosphorylation. J. Biol. Chem. 276 (46), 42932–42937.

Yiin, S.J., Lin, T.H., Shih, T.S., 1996. Lipid peroxidation in workers exposed to manganese. Scand. J. Work. Environ. Health 22 (5), 381–386.

Yoon, H., Kim, D.S., Lee, G.H., Kim, K.W., Kim, H.R., Chae, H.J., 2011. Apoptosis induced by manganese on neuronal SK-N-MC cell line: endoplasmic reticulum (ER) stress and mitochondria dysfunction. Eviron. Health Toxicol. 26, e2011017.

Zomot, E., Kanner, B.I., 2003. The Interaction of the-aminobutyric acid transporter GAT-1 with the neurotransmitter is selectively impaired by sulfhydryl modification of a conformationally sensitive cysteine residue engineered into extracellular loop IV. J. Biol. Chem. 278, 42950–42958.