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Low-Level Manganese Exposure Alters Glutamate Metabolism in GABAergic AF5 Cells

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Abstract

Recent studies have suggested that the globus pallidus may be a particularly sensitive target of manganese (Mn), however, *in vitro* studies of the effects of Mn on GABAergic neurons have been restricted by the lack of a cell model expressing GABAergic properties. Here we investigated the effects of low-level Mn treatment on cellular GABA and glutamate metabolism using the newly characterized AF5 rat neural-derived cell line, which displays GABAergic properties during culture *in vitro*. Intracellular GABA and glutamate levels were measured along with measurement of the release of GABA and glutamate into the culture medium, glutamine uptake from the culture medium, and the specific effects of Mn on the enzymes directly responsible for the synthesis and degradation of GABA, glutamate decarboxylase (GAD) and GABA transaminase (GABA-T). Our results demonstrate that Mn had no effect on the activities of GAD or GABA-T. Similarly, low-level Mn treatment of AF5 cultures had only a small effect on intracellular GABA levels (114% of control) and no effect on the release of GABA. In contrast, intracellular and extracellular glutamate levels were enhanced to 170% and 198% of control during Mn treatment, respectively, while extracellular glutamine decreased to 73% of controls. Together, these results suggest that glutamate homeostasis may be preferentially affected over GABA in AF5 cells during low-level Mn treatment, suggesting a novel mechanism by which Mn-induced excitotoxicity might arise.

Keywords

GABA; glutamate; manganese; rat; glutamic acid decarboxylase

Introduction

Manganese (Mn) is essential for biological function, though elevated exposures to Mn are well known to produce neurotoxicity. Previous studies have indicated that the motor dysfunction and behavioral deficits seen in individuals suffering from Mn toxicity arise at least in part from disruptions in the basal ganglia (Mergler and Baldwin, 1997; Pal *et al.*, 1999). More recently, several recent pediatric epidemiological studies have shown that elevated exposures to environmental Mn, including contaminated well water, are associated with neurocognitive deficits in children (Wasserman *et al.*, 2006; Wright *et al.*, 2006), substantiating the hypothesis

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that important behavioral deficits may arise from environmental exposures before manifestation of the motor deficits characteristic of occupational exposures.

Decreased striatal dopamine has historically been considered a hallmark neurochemical outcome of Mn neurotoxicity (Cotzias *et al.*, 1976), based in part on the similarities in motor symptoms between Manganism and Parkinson's disease. However, recent studies have indicated that GABAergic nuclei of the basal ganglia such as the globus pallidus and the striatum may be early, more sensitive targets of Mn (Wolters *et al.*, 1989; Spadoni *et al.*, 2000; Gwiazda *et al.*, 2002; Kim *et al.*, 2005; Liu *et al.*, 2006). These findings are consistent with a recent review of animal model studies suggesting that Mn produces an effect on striatal GABA levels over a wide range of cumulative doses, while decreased striatal dopamine is observed only at very high exposures (Gwiazda *et al.*, 2006). The neurochemical basis for the effects of Mn on GABAergic nuclei in these brain regions, however, remains elusive.

Several mechanisms of Mn neurotoxicity have been proposed, including disruption of mitochondrial metabolism (Gavin *et al.*, 1992; Brouillet *et al.*, 1993; Zwingmann *et al.*, 2003), induction of oxidative stress (Olanow and Arendash, 1994; Ali *et al.*, 1995), and alteration of iron homeostasis (Lai *et al.*, 1999; Zheng *et al.*, 1999; Ellingsen *et al.*, 2003; Kwik-Urbe *et al.*, 2003; Kwik-Urbe and Smith, 2006). The heightened sensitivity of mitochondria appears to be due at least in part to their propensity to accumulate Mn to relatively high levels (Gavin *et al.*, 1990). Moreover, Mn has been shown to cause inhibition of several mitochondrial enzymes including Complex I (Chen *et al.*, 2001), Complex II (Tomas-Camardiel *et al.*, 2002), and mitochondrial (m-) aconitase (Villafranca and Mildvan, 1971; Zheng *et al.*, 1998). In line with these observations, Zwingmann *et al.* observed significant alteration of mitochondrial energy metabolism and amino acid homeostasis in cultured neurons and astrocytes (Zwingmann *et al.*, 2003), and in the brains of rats (Zwingmann *et al.*, 2004) following treatment with Mn.

Interestingly, Zwingmann *et al.* also reported increased intracellular synthesis of glutamate in Mn-treated astrocyte/neuron co-cultures (Zwingmann *et al.*, 2003). This finding is consistent with other studies reporting that Mn alters the metabolism of cellular glutamate in several other cell types (Hazell and Norenberg, 1997; Chen and Liao, 2002; Erikson and Aschner, 2002; Normandin and Hazell, 2002; Mutkus *et al.*, 2005). Collectively, these observations suggest that increased extracellular glutamate brought on by decreased astrocytic glutamate uptake, or by enhanced neuronal release, might lead to excitotoxicity in post-synaptic neurons expressing glutamate receptors (Fitsanakis *et al.*, 2006).

The number of studies specifically investigating the effects of Mn on GABA metabolism has been limited, in part due to the lack of a suitable *in vitro* tissue culture model. Here we investigated the effects of Mn on cellular GABA and glutamate homeostasis in the recently characterized AF5 rat neural-derived cell line (Truckenmiller *et al.*, 2002). AF5 cells possess several GABAergic properties including the expression of GAD65, GAD67, and GAT-1, and the ability to synthesize and release GABA (Sanchez *et al.*, 2006). Our results demonstrate that glutamate metabolism is preferentially impacted by Mn as compared to GABA, leading to significant increases in intracellular glutamate accompanied by increased levels of glutamate in the extracellular medium. These results substantiate prior suggestions that Mn exposure may contribute to glutamate excitotoxicity through altered glutamate metabolism in GABAergic nuclei.

Materials and Methods

Cell Culture

The immortalized mesencephalic-derived AF5 cell line was a generous gift of Dr. W.J. Freed of NIDA/NIH. For all experiments utilizing the AF5 cell line, cells were seeded at ~70%

confluence and allowed to grow to 4 days post-confluence on either 24-well plates or T-75 flasks (B D Falcon) in Neurobasal-A medium with B27 growth supplement (Invitrogen) and 10% FBS (NB27 medium) in a 37°C humidified environment maintained at 5% CO₂, as previously described (Sanchez *et al.*, 2006). For Mn treatments culture medium was removed and replaced with culture medium spiked with a concentrated MnCl₂ solution for final Mn concentrations ranging from 25µM to 1.8 mM. Following incubation for either 24 or 48 h, cells were harvested by trypsinization unless otherwise noted; after centrifugation, cells were washed once with phosphate buffered saline (PBS), and cell pellets were frozen at -70°C until further analyses.

Lactate dehydrogenase assay

AF5 cells were cultured on 24-well plates and treated with Mn for 24 h. Following Mn treatment, the culture medium was removed and centrifuged at 200 x g for 5 min, and the supernatants were assayed for lactate dehydrogenase (LDH) activity by monitoring the disappearance of NADH at 340 nm in the presence of excess pyruvate as previously described (Wroblewski and Ladue, 1955).

Measurement of intracellular Mn concentration

Cellular manganese levels were measured using trace metal clean methods as previously described (Smith *et al.*, 2000;Kwik-Urbe *et al.*, 2003). Briefly, AF5 cells were harvested by trypsinization, and the pellets were washed once with PBS supplemented with 10 mM EDTA, followed by a second wash with PBS alone to remove surface-associated Mn from the cells. Cell pellets were lysed by sonication in de-ionized water, and aliquots of total cell lysates were digested with 16N HNO₃ (Optima grade, Fisher Scientific) and re-dissolved in 1N HNO₃ for spectroscopic analyses. Manganese levels were determined using Zeeman graphite furnace atomic absorption spectrometry (GFAAS; Perkin-Elmer 4100ZL), with external standardization using certified SPEX standards. NIST 1577 (bovine liver) was used to evaluate procedural accuracy. The analytical detection limit for Mn analyses was 0.10 ng/ml.

Effects of Mn on intracellular and extracellular amino acid levels in AF5 cells

AF5 cells were cultured on 24-well plates and treated with Mn for 24 or 48 h. Following Mn treatment, the culture medium was centrifuged at 200 x g for 5 min, and the supernatants were frozen and stored for HPLC analyses. AF5 cell monolayers from these plates were washed twice with PBS before addition of hypotonic lysis buffer consisting of 1% Triton X-100 and 30mM HEPES (pH 7.4). Lysates were centrifuged at 10,000 x g for 5 min, an aliquot from each sample was assayed for total protein concentration using the BCA assay (Pierce), and the remainder was stored for HPLC analysis.

Cell lysate and culture medium samples were prepared for HPLC analysis by perchlorate precipitation and o-phthalaldehyde derivitization, and analyzed by HPLC as described previously (Gwiazda *et al.*, 2002), except that 25 mM EDTA was included in the derivitization buffer. Amino acid concentrations measured in treatment medium incubated for 24 h without cells were subtracted from concentrations in treatment medium incubated with cells for 24 h to obtain the total amount released (Glu, GABA), or consumed (Gln) by the cells. Intracellular and extracellular amino acid levels were normalized to the amount of total cellular protein measured in each well using the BCA assay.

Glutamate Decarboxylase Assay

GAD activity was measured in rat brain homogenate by HPLC. Adult (350 g) female Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA) and maintained on rat

chow and water ad libitum for one week until sacrifice. All animal protocols were approved by the University's IACUC and conformed to NIH guidelines.

Rats were sacrificed by decapitation without anesthesia, and whole intact brains rapidly removed and minced on ice. A portion of minced brain tissue was weighed and added to ten volumes of ice cold homogenization buffer consisting of 30 mM HEPES, 0.1% Triton X-100, and 5% glycerol, and homogenized in a Teflon tissue grinder. The resulting homogenate was centrifuged at 10,000 x g for 5 minutes; two aliquots of the supernatant were transferred to separate test tubes for GAD assay, and an additional aliquot was taken for measurement of total protein. The assay aliquots were then spiked with MnCl_2 solutions or water to achieve final Mn concentrations of 0, 0.7, or 2.5 mM Mn and incubated on ice for 20 min. Next, GAD assay reaction mixture was added to the tubes for a final concentration of 100 μM pyridoxal-5-phosphate, 5 mM glutamic acid, and 100 μM gabaculine, and reactions were either stopped immediately or incubated at 37° C with constant shaking for 30 min. Reactions were stopped by addition of perchlorate buffer to give a final concentration of 0.5 M acetic acid, 0.5 M sodium acetate, and 0.4 M sodium perchlorate. The reaction mixtures were then centrifuged at 15,000 x g for 5 min, and an aliquot of the resulting supernatant was analyzed by HPLC using the method described above. The rate of GABA synthesis was calculated by subtracting the GABA concentration measured in reactions stopped immediately from concentrations measured in reactions incubated for 30 min. GAD activities were normalized to total protein concentrations in the lysates.

GABA Transaminase Assay

GABA transaminase (GABA-T) activity in fresh rat whole brain mitochondrial lysates was determined using a coupled enzyme assay with modifications to a published method (De Boer and Bruinvels, 1977). Rats ($n=3$) were sacrificed as above, and aliquots of minced fresh rat brain tissue were homogenized in buffer consisting of 3 mM HEPES, 225 mM sucrose, 20 mM KCl, 5 mM MgCl_2 , and 500 μM EDTA, pH 7.5 using a Teflon tissue grinder. The brain homogenate was centrifuged at 500 x g for 10 min, and the supernatant was collected and centrifuged at 25,000 x g for 20 min. The resulting enriched mitochondrial pellet was then re-suspended in lysis buffer consisting of 30 mM HEPES (pH 7.5), 0.1% Triton X-100, 10 % (v/v) glycerol, and 5 mM beta-mercaptoethanol, and aliquots taken for assay of GABA-T activity and total protein measurement.

The GABA-T assay aliquots were spiked with MnCl_2 solutions or water to achieve final Mn concentrations of 0, 0.7, or 2.5 mM Mn and incubated on ice for 20 min. Separate sample aliquots were spiked with CaCl_2 (5 mM final concentration) as a cation-chloride control. Inherent GABA-T activity was assayed in a reaction mixture containing 100 mM pyrophosphate buffer (pH 8.4), 3.5 mM BME, 5 mM $\alpha\text{-KG}$, 1 mM NAD^+ , and 18 mM GABA. Reaction mixture components (ice cold) were added to samples tubes on ice and incubated for 30 min. GABA was then added to start the reaction, and the change in absorbance at 340 nm was measured for 20 min in quartz cuvettes at 30°C. The GABA-T inhibitor gabaculine (500 μM) was included in separate control reactions to verify specificity of the enzyme assay.

Statistical Analysis

Treatment comparisons were made using analysis of variance (ANOVA) and Tukey's post-hoc tests. P values of less than 0.05 were considered significant. All analyses were conducted using SYSTAT (SPSS Inc., 10th edition, 2000).

Results

Cytotoxicity of Mn

In order to determine the Mn treatment levels that lead to overt cytotoxicity in AF5 cells, lactate dehydrogenase (LDH) activity was measured in the culture medium supernatants of AF5 cultures treated with increasing concentrations of MnCl₂. Following a 24 h treatment period, only cells treated with the highest (1800μM) Mn exposure showed measurably increased levels of LDH in the culture medium (839% of control) indicative of overt cytotoxicity (Figure 1). All lower Mn exposures (25 – 750μM) did not produce overt cytotoxicity based on culture medium LDH levels. Mn exposures in subsequent experiments were restricted to concentrations of 300μM Mn or lower to ensure that Mn treatment levels were well below those that cause overt cytotoxicity.

Intracellular accumulation of Mn in AF5 cells

Mn levels were measured in AF5 lysates by Zeeman graphite furnace atomic absorption spectroscopy to facilitate the comparison of the effects observed here with other published studies based on intracellular Mn levels, and not simply on exposure concentrations. Intracellular Mn levels increased in a dose-dependant manner from 0.15 nmol Mn/mg protein in control cultures up to 1.19 nmol/mg protein, or nearly 800% of control levels, following 300μM Mn treatment (Figure 2).

Mn increases intracellular glutamate and GABA levels in AF5 cells

To investigate the effects of Mn on glutamate and GABA metabolism in AF5 cells, the intracellular concentrations of these neurotransmitters were measured following 24 h Mn treatment. Manganese treatment produced a significant and robust increase in intracellular glutamate levels following 24 h exposure to levels of ~170% of control at the highest (300μM Mn) exposure level (Figure 3). Intracellular GABA levels were also significantly increased in Mn-treated AF5 cells, reaching a peak of approximately 114% of control following 300μM Mn treatment. To determine whether these significant increases in intracellular glutamate and GABA persisted beyond 24 h, we exposed AF5 cells to Mn for 48 h and found that glutamate levels in Mn-treated AF5 cells remained elevated at ~124% of control with 100μM Mn treatment, while intracellular GABA levels were no longer measurably increased as compared to controls (Figure 3, inset). None of these 48h Mn treatments caused overt cytotoxicity, based on absence of a significant increase in culture medium LDH activity (data not shown).

Mn does not directly alter the activity of GAD or GABA-T

Cellular metabolism of glutamate and GABA in neurons is directly linked, suggesting that the above changes observed in intracellular GABA following Mn treatment might arise from a direct interaction between Mn and the enzymes responsible for the synthesis and degradation of GABA, namely glutamate decarboxylase (GAD) and GABA transaminase (GABA-T). A direct effect of Mn on these enzymes might also help explain the changes in GABA levels observed in the globus pallidus and striatum following chronic Mn exposure reported by others (Bonilla, 1978; Gianutsos and Murray, 1982; Brouillet *et al.*, 1993; Gwiazda *et al.*, 2002). To explore this hypothesis, we established optimized *in vitro* activity assays for both GAD and GABA-T in rat brain extracts, and then evaluated the effect of exogenously added Mn on the activity of each enzyme. Manganese had no measurable effect on the rate of either GAD or GABA-T when added to the assay reactions at final concentrations of 0.7 or 2.5 mM (n=3 for each assay; *p*>0.9 for both assays at both treatment levels). These data strongly suggest that elevated Mn does not directly interfere with the activity of either of these enzymes in the brain.

Mn increases extracellular glutamate and reduces extracellular glutamine in AF5 cultures

To determine whether the altered intracellular metabolism of glutamate and GABA in low-level Mn-treated AF5 cells reported above also produced changes in the concentrations of these metabolites in the extracellular space, we measured the concentrations of glutamate, GABA, and glutamine in the culture medium of control and Mn-treated AF5 cultures. Manganese treatment produced significant increases in extracellular glutamate levels, as well as a significant decrease in the extracellular concentration of glutamine over the 24 h exposure period (Table 1, Figure 4). When the initial concentrations of these amino acids in cell-free medium are considered (Table 1), AF5 cells exposed to 150 μ M Mn increased the concentration of glutamate in the culture medium by 1.49 μ mol/mg cellular protein, or 198% of control (Figure 4A). In addition, Mn treatment significantly reduced extracellular glutamine concentrations by 3.5 μ mol/mg cellular protein as compared to controls (Figure 4C). Remarkably, despite the significant Mn-induced alterations in extracellular glutamate and glutamine that were observed here, no change in extracellular GABA was observed in AF5 cells treated with either 50 μ M or 150 μ M Mn (Figure 4B).

Discussion

To investigate a possible mechanism underlying Mn neurotoxicity in GABAergic nuclei of the basal ganglia, AF5 cells expressing a GABAergic phenotype (Truckenmiller *et al.*, 2002; Sanchez *et al.*, 2006) were treated with concentrations of Mn well below dosage levels that cause overt cytotoxicity to determine whether Mn disrupts GABA and/or glutamate metabolism. Our results demonstrate that Mn treatment produced significant (up to ~170% of control) and sustained (i.e., at 24 and 48 h) increases in intracellular glutamate concentrations, as well as significantly increased extracellular glutamate concentrations of up to ~198% of control. A significant and substantial reduction in culture medium glutamine levels was also observed, suggesting that Mn increased the cellular consumption of glutamine and/or increased the capacity for transport of glutamine into the cells from the extracellular space. Manganese treatment also produced significant but smaller increases (~114% of control) in intracellular GABA levels, though no detectable changes in extracellular GABA were observed. Moreover, as the activities of the enzymes responsible for synthesis and degradation of GABA (GAD and GABA-T) were unaffected by the addition of Mn *in vitro*, it can be inferred that the effects of Mn on glutamate homeostasis observed here occur via a pathway independent of the GABA shunt in AF5 cells.

The substantial ~170% increase in intracellular glutamate levels with 300 μ M Mn treatment, accompanied by a ~198% increase in extracellular glutamate following 150 μ M Mn treatment demonstrates significant Mn-induced alteration of glutamate homeostasis in AF5 cells (Figure 4A, Table 1). Our observation of a Mn-induced increase in intracellular glutamate is consistent with results reported by Zwingmann *et al.*, who observed a 70–80% increase in the intracellular abundance of glutamate in primary astrocyte and astrocyte/cortical neuron co-cultures treated with 100 μ M Mn for 5 days (Zwingmann *et al.*, 2003). The significant increases in extracellular glutamate in Mn-treated AF5 cultures might be a consequence of increased glutamate efflux from plasma membrane glutamate transporters caused by the significant increases in intracellular glutamate levels. Alternatively, the increased extracellular glutamate levels might also reflect reduced glutamate (re)uptake via high affinity glutamate transporters, as observed by others in Mn-treated astrocytes (Hazell and Norenberg, 1997; Chen and Liao, 2002; Erikson and Aschner, 2002; Erikson *et al.*, 2002; Mutkus *et al.*, 2005). Our results also substantiate prior suggestions that Mn exposure may contribute to glutamate excitotoxicity through altered glutamate metabolism in the basal ganglia, possibly potentiating the effects of increased cortical excitatory glutamatergic input (Brouillet *et al.*, 1993), enhanced sensitivity of pallidal post-synaptic neurons to glutamatergic input (Spadoni *et al.*, 2000), or decreased astrocytic

glutamate uptake (Hazell and Norenberg, 1997; Chen and Liao, 2002; Erikson and Aschner, 2002; Erikson *et al.*, 2002). Finally, the findings presented here underscore the need for further research into the nature of glutamate and GABA release in AF5 cells, as it is currently not known whether these neurotransmitters are released from AF5 cells via carrier mediated or vesicular mechanisms.

AF5 cells treated with Mn for 24 h showed a significant but small 114% increase in intracellular GABA (Figure 3), which is consistent with the magnitude of striatal and pallidal GABA increases reported in Mn exposed rodents (Lipe *et al.*, 1999; Gwiazda *et al.*, 2002; Reaney *et al.*, 2006). Though the increased intracellular GABA observed here cannot be attributed to a direct effect of Mn on the activities of GAD or GABA-T, we cannot rule out possible indirect effects of Mn on the activity of these enzymes in the brain, which could arise from altered expression or distribution of these enzymes in GABAergic neurons. For instance, one study reported decreased expression of GAD mRNA levels in pallidal neurons of rats treated sub-chronically with Mn (Tomas-Camardiel *et al.*, 2002). Alternatively, this GABA effect may have arisen from the much larger increase in intracellular glutamate, the substrate for GAD and precursor of GABA.

Notably, AF5 cells accumulated intracellular Mn to levels far lower than those observed in PC12 cells treated with similar doses of Mn. Several other studies reported that 24 h treatment of undifferentiated PC12 cells with 50–100 μ M MnCl₂ resulted in intracellular accumulation of Mn to amounts ranging from approximately 10–18 nmol/mg protein, levels that were more than ten-fold higher than those observed here (Kwik-Urbe *et al.*, 2003; Gunter *et al.*, 2005b; Reaney and Smith, 2005). Similarly, Gunter *et al.* reported that treatment of cultured primary astrocytes with 100–300 μ M Mn resulted in accumulation of intracellular Mn to levels more than ten-fold higher than those observed in 300 μ M Mn-treated AF5 cultures (Gunter *et al.*, 2005a). These differences might be attributed to the fact that AF5 cells were largely in the stationary phase of growth during Mn exposure, as all experiments here were performed with cultures grown to four days post-confluence at which point AF5 cells exhibit contact inhibition and growth arrest. The stationary phase of growth in these cultures was also reflected in the large Mn dose (i.e. 1800 μ M) required to induce overt cytotoxicity as measured by LDH release following 24 h treatment.

In summary, we have reported the effects of Mn on GABA and glutamate metabolism in AF5 cells. Together, these results give insight into the neurochemical alterations that take place in GABAergic cells during elevated Mn exposure. While the moderate Mn-induced increase in intracellular GABA observed here is consistent with striatal and pallidal GABA increases reported in Mn exposed rodents, the more dramatic increases in intracellular and extracellular glutamate levels observed in Mn-treated AF5 cells suggest that diminished inhibitory tone and excitotoxicity might be important toxic outcomes in Mn-affected GABAergic brain nuclei.

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Figure 1. Cytotoxicity in Mn-treated AF5 cells. Mean culture medium LDH activity following 24 h Mn exposure, displayed as percent of control values. Error bars represent standard deviation ($n=4$). Data were analyzed by ANOVA with Tukey's post hoc test; letters denote significant differences between experimental groups ($p < 0.05$).

Figure 2.

Intracellular Mn concentrations in AF5 cells increased in a stepwise manner following Mn treatment. AF5 cultures were treated for 24 h with increasing concentrations of Mn, and intracellular Mn levels were measured by GFAAS as described in Materials and Methods. Letters denote significant differences between treatment groups ($p < 0.05$).

Figure 3.

Mn exposure increases intracellular glutamate and GABA in AF5 cells. Glutamate (filled bars) and GABA (unfilled bars) concentrations were measured in AF5 cell lysates following 24 and 48 h (inset) Mn exposure. Data are displayed as percent of control concentrations. Error bars represent standard deviation (n=4). Data were analyzed by ANOVA with Tukey's post hoc test; asterisks denote significant difference from respective controls (* $p < 0.05$, ** $p < 0.01$). Intracellular glutamate and GABA concentrations in control cells after 24 h incubation were 156 and 17.9 nmol/mg protein, respectively.

Figure 4.

Mn exposure alters the extracellular concentrations of glutamate and glutamine, but not GABA, in AF5 cultures. The average net change in concentration of (A) glutamate, (B) GABA, and (C) glutamine in the culture medium of control and Mn-treated AF5 cultures was measured following 24 h treatment. Data represent the 24h change in culture medium amino acid abundance normalized to total cellular protein. Error bars represent standard deviation (n=3–4). Data were analyzed by ANOVA with LSD post hoc test; letters denote significant differences between experimental groups ($p < 0.05$).

Table 1

Concentrations of glutamate, GABA and glutamine in fresh culture medium and in the culture medium of control and Mn-treated AF5 cells following 24 h incubation. Data are the average of 3–4 replicates.

	Time	Concentration, μ M		
		Glu	GABA	Gln
<i>fresh medium</i>	<i>0h</i>	96.2	0.00	2380
control	24h	145	18.7	1770
50 μ M Mn	24h	155	17.4	1730
150 μ M Mn	24h	193	17.6	1540