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Manganese-Induced Downregulation of Astroglial Glutamine Transporter SNAT3 Involves Ubiquitin-Mediated Proteolytic System

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Abstract

SNAT3 is a major facilitator of glutamine (Gln) efflux from astrocytes' supplying Gln to neurons for neurotransmitter synthesis. Our previous investigations have shown that, in primary cortical astrocyte cultures' SNAT3 protein is degraded after exposure to manganese (Mn^{2+}). The present studies were performed to identify the processes responsible for this effect. One of the well-established mechanisms for protein-level regulation is posttranslational modification *via* ubiquitination' which leads to the rapid degradation of proteins by the 26S proteasome pathway. Here, we show that astrocytic SNAT3 directly interacts with the ubiquitin ligase, Nedd4–2 (neural precursor cells expressed developmentally downregulated 4–2), and that Mn^{2+} increases both Nedd4–2 mRNA and protein levels. Additionally, we have found that Mn^{2+} exposure elevates astrocytic ubiquitin B mRNA expression, free ubiquitin protein levels, and total protein ubiquitination. Furthermore, Mn^{2+} effectively decreases astrocytic mRNA expression and the phosphorylation of serum and glucocorticoid-inducible kinase, a regulatory protein, which, in the active phosphorylated form, is responsible for the phosphorylation and subsequent inactivation of Nedd4–2. Additional findings establish that Mn^{2+} increases astrocytic caspase-like proteolytic proteasome activity and that the Mn^{2+}-dependent degradation of SNAT3 protein is blocked by the proteasome inhibitors, *N*-acetyl-leu-leu-norleucinal and lactacystin. Combined, these results demonstrate that Mn^{2+}-induced SNAT3 protein degradation and the dysregulation of Gln homeostasis in primary astrocyte cultures proceeds through the ubiquitin-mediated proteolytic system.

Keywords

glutamine; SNAT3; Nedd4–2/SGK1 signaling; manganese; ubiquitination; proteasome

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INTRODUCTION

Manganese (Mn^{2+}) is essential for the proper functioning of various physiological processes and is required for normal human development (Prohaska, 1987; Takeda, 2003). However, despite its essentiality, chronic environmental exposure to high levels of Mn^{2+} is known to cause neurotoxicity and has been implicated as a risk factor for the neurodegenerative brain disorder referred to as manganism (Erikson and Aschner, 2003; Guilarte et al., 2008). It is characterized by chronic symptoms and morphological lesions similar to those seen in Parkinson's disease (Racette et al., 2001). Mn^{2+} has been identified as an occupational health hazard for miners, ferroalloy and battery manufacturers, and automotive repair workers (Roth and Garrick, 2003; Weiss, 2006).

It is well established that exposure of astrocytes to high levels of Mn^{2+} affects their ability to transport and metabolize glutamine (Gln) (Milatovic et al., 2007; Sidoryk-Wegrzynowicz et al., 2009). Gln transport in the central nervous system is essential for the glutamate (Glu)-Gln-γ-aminobutyric acid (GABA) cycle, which occurs between neurons and glia. Gln shuttling involves carriers showing specific neuronal and astrocytic localization (Bröer and Brookes, 2001). Adequate Gln supply is a key determinant of homeostasis for both Glu and GABA (Albrecht et al., 2007).

Mn^{2+} decreases the levels of Gln transporter proteins and inward and outward transport of Gln in astrocytes (Sidoryk-Wegrzynowicz et al., 2009). Our recent studies have established that system N transporter, SNAT3, which plays a prominent role in the uptake and release of Gln in astrocytes (Chaudhry et al., 1999; Deitmer et al., 2003), is the most sensitive to Mn^{2+} exposure among all of the Gln transporters. We have shown that Mn^{2+} impairs system N-dependent Gln transport and causes the complete degradation of SNAT3 transporter in these cells (Sidoryk-Wegrzynowicz et al., 2009).

A well-established mechanism for protein-level regulation is posttranslational modification *via* the covalent attachment of ubiquitin or polyubiquitin and the subsequent degradation of the protein by one of the ubiquitin-mediated proteolytic systems, namely the 26S proteasome pathway (Ciechanover, 1998; Hilt and Wolf, 1996; Pickart, 2000). This pathway has been implicated in a wide range of physiological functions and in various neuropathological states (Hegde and Upadhya, 2007). Ubiquitination involves several sequential actions of enzymes: an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3) (Hershko and Ciechanover, 1998). Ligases E3 activity and specificity are the key factors in substrate protein selection (Jentsch and Schlenker, 1995). Ubiquitin ligase, Nedd4–2 (neural precursor cells expressed developmentally downregulated 42), which belongs to the family of E3 ubiquitin ligases, is highly involved in the regulation of numerous receptor and transporter protein levels (Kabra et al., 2008; Kamynina and Staub, 2002; Zhou et al., 2007). A co-expression study has shown that Nedd4–2 inhibits the functional activity of SNAT3 and that this effect is blocked by serum and glucocorticoid-inducible kinase (SGK1), a known negative regulator of Nedd4–2 activity (Boehmer et al., 2003; Debonneville et al., 2001; Snyder et al., 2002).

In this study, we investigated mechanisms underlying the Mn^{2+}-mediated downregulation of SNAT3. Nedd4–2/SGK1 signaling, which is known to regulate SNAT3 activity, precedes the ubiquitination of various receptors and other transmembrane proteins, leading to the protein degradation. In this report, we present data supporting the hypothesis that Mn^{2+}-induced SNAT3 degradation in primary cultures of astrocytes involves Nedd4–2/SGK1 signaling and, consequently, the ubiqui-tin-mediated proteolytic system.

MATERIALS AND METHODS

Chemicals, Reagents, and Cell Culture Supplies

Manganese chloride ($MnCl_2$) and the proteasome inhibitors, *N*-acetyl-leu-leu-norleucinal (ALLN) and lac-tacystin (Lac) were purchased from Sigma Chemical Co. (St Louis, MO). Minimal essential medium with Earle's salts, heat-inactivated horse serum, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA).

Antibodies

Primary antibodies against the following proteins were used: SNAT3, SNAT2, ASCT2, and LAT2 (Santa Cruz Biotechnology, Santa Cruz, CA); Nedd4–2, conjugated ubiquitin, and free ubiquitin (Abcam, Cambridge, MA); phospho-SGK1 (Ser78) and SGK1 (Cell Signaling Technology, Beverly, MA); and β-actin (Sigma Chemical Co.). Secondary, horse radish peroxidase-conjugated antibodies against goat, mouse, and rabbit IgGs were purchased from Santa Cruz Biotechnology.

Primary Cultures of Astrocytes

Primary cultures of astrocytes were prepared according to previously established protocols (Aschner et al., 1992). One-day-old Sprague-Dawley rats were decapitated under halothane anesthesia, and the cerebral cortices were dissected out and digested with bacterial neutral protease (Dispase; Invitrogen, Eugene, OR). Astrocytes were then recovered by the repeated removal of dissociated cells and plated at a density of 1×10^5 cells/mL. Twenty-four hours after initial plating, the medium was changed to preserve the adhering astrocytes and to remove neurons and oligodendrocytes. The cultures were maintained at 37°C in a 95% air/5% CO_2 incubator for 3 weeks in minimal essential medium with Earle's salts supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The medium was replaced twice per week. The surface-adhering monolayer cultures were >95% positive for the astrocytic marker, glial fibrillary acidic protein. All experiments were performed 3 weeks after isolation.

Treatment

Where indicated, $MnCl_2$ was added to the culture medium. The chosen $MnCl_2$ concentrations were based on estimates from the literature. The physiological range (no symptoms) was 75–100 μ*M*, and clinical signs increased in frequency and severity above this level. Cells were treated with 100 μ*M* $MnCl_2$ (physiological range) and with 500 μ*M* or 1 m*M* $MnCl_2$, representing a range of Mn^{2+} concentrations considered to be toxic in cultured astrocytes (Milatovic et al., 2007) and in the *in vivo* brain (Suzuki et al.,1975). Details about concentrations and incubation times for each experiment are provided in the

Results Section. For assessment of proteasome involvement in Mn^{2+}-induced SNAT3 degradation, cells were treated 4 h with 10 μ*M* ALLN or 10 μ*M* Lac.

Western Blot Analysis

The astrocytes were washed twice with phosphate-buffered saline and then lysed with radio immunoprecipitation assay (RIPA buffer: 150 m*M* NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 50 m*M* Tris, pH 8.0) containing a protease inhibitor and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentrations in the astrocytic lysates were determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). An equal amount of protein (30 μg) was loaded and run on a 12% SDS-poly-acrylamide gel electrophoresis (PAGE) (for SNAT3, Nedd4–2, pSGK1, and total protein ubiquitination) or 15% SDS-PAGE gel (for free ubiquitin) and then transferred to a polyvinylidenefluoride membrane (Millipore, Bedford, MA). The membrane was blocked for 1 h in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 at room temperature. Incubations with primary antibodies were carried out in 5% milk in Tris-buffered saline with 0.1% Tween 20 at 4°C for 24 h at the following concentrations: anti-SNAT3 (1:400), anti-Nedd4–2 (1:1,000), anti-SGK1 (1:1,000), anti-pSGK1 (1:1,000), anti-free Ub (1:1,000), anti-conjugated Ub (1:1,000), and anti-beta actin (1:3,000). Secondary antibody incubations were performed at room temperature for 1.5 h using anti-rabbit IgG (1:1,000), anti-goat IgG (1:1,000), or anti-mouse IgG (1:2,000). Western blots were visualized with Thermo Scientific Pierce Supersignal West Dura Extended Duration Chemiluminescent Substrate (Pierce). Densitometry measurement of the band intensities was used for quantification (AlphaEaseFC Imaging System software; Alpha Innotech, San Leandro, CA). SNAT3, Nedd4–2, and Ub levels were normalized to p-actin, and phospho-SGK1 was normalized to total SGK1.

Co-Immunoprecipitation

Cells were washed twice with phosphate-buffered saline and then lysed with RIPA buffer containing protease and phosphatase inhibitors. Lysates containing 1,000 μg of protein were precleared by shaking with 40 μL of Pansorbin cells (Calbiochem, San Diego, CA) at 4°C for 1 h, centrifuged at 3,000g for 5 min at 4°C, and the supernatant was incubated overnight at 4°C with SNAT3, SNAT2, ASCT2, LAT2, or control rabbit IgG antibodies. Proteins were precipitated by incubating with 25 μL of protein-G agarose beads (Sigma-Aldrich) for 2 h at 4°C and subsequent centrifugation at 1,000*g* for 1 min at 4°C. Immunocomplexes were washed three times with RIPA buffer and once with 500 μL of wash buffer (100 m*M* NaCl, 50 m*M* Tris-HCl, pH 7.5, 0.5% Nonidet P-40) solubilized at 95°C for 5 min in 40 μL of SDS-PAGE loading buffer with 0.1*M* dithiothreitol and then subjected to Western blot analysis.

RNA Isolation and Real-Time Polymerase Chain Reaction

For real-time polymerase chain reaction (RT-PCR) analysis, total RNA was isolated from primary cultures of astrocytes with the RNeasy kit (Qiagen, Valencia, CA) followed by reverse transcription with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. RT-PCR analyses were carried out using the following TaqMan Gene Expression: glyceraldehyde 3-phosphate

dehydrogenase (GAPDH), Rn99999916_s1; Nedd4–2, Rn01530565_m1; SGK1, Rn00693143_g1; UbA52, Rn01785965_g1; ubiquitin B (UbB), Rn03062801_gH; and UbC, Rn01789812_g1 (Applied Biosystems). The reaction mixtures contained Taq-man Universal PCR Mastermix, TaqMan® Gene Expression, and 1 μL cDNA in a total volume of 5 μL. Reactions were performed in 384-well optical reaction plates using an Applied Biosystems 7500 Sequence Detection System. Expression values obtained from triplicate runs of each cDNA were calculated relative to the triplicate value for the GAPDH from the same cDNA preparation. Relative quantifications of mRNA in the samples were performed by the postrun data analysis software (SDS 2.3) provided by the Cycler system from Applied Biosystems.

Proteasomal Peptidase Activity Assay

Cells were grown in 96-well plates. After Mn^{2+} treatment, the astrocytes were incubated for 10 min in a buffer containing a specific luminogenic proteasome substrate according to the manufacturer's protocol (Promega Bioscience, Madison, WI). Cells treated with 10 μ*M* ALLN for 4, 8, and 24 h were used as the controls for the assay. The chymotrypsin-like, trypsin-like, and caspase-like proteasome activities were detected as the relative light unit generated from the cleaved substrate in the reagent. Luminescence generated from each reaction condition was detected with a Beckman Coulter DTX 880 multimode detector (Brea, CA). At the end of each experiment, cells were lysed in 150 μL of 1*M* NaOH, and the lysates were used for protein determination with the bicinchoninic acid protein assay kit. Proteasomal activity was expressed as relative light unit/mg protein.

Statistical Analysis

Results are expressed as the mean ± standard deviation (SD) from at least three independent astrocytic culture preparations of three or more samples per each. The statistical analysis was carried out with one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. All analyses were performed with GraphPad Prism 4.02 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Mn^{2+} Activates Nedd4–2/SGK1 Signaling

Studies by Boehmer et al., 2003 have shown that the SNAT3 transporter is regulated through Nedd4–2/SGK1 signaling. To test the hypothesis that this pathway may be activated in astrocytes in response to Mn^{2+} treatment, mRNA and protein levels of both Nedd4–2 and SGK1 were measured after exposure to 100 μ*M*, 500 μ*M*, or 1 m*M* Mn^{2+}. The quantitative RT-PCR analysis showed a significant increase in Nedd4–2 mRNA levels after the incubation of astrocytes with 1 m*M* Mn^{2+} for 8 h and with 500 μ*M* or 1 m*M* Mn^{2+} for 24 h (Fig. 1A). Treatment with 1 m*M* Mn^{2+} for 4 h or 500 μ*M* and 1 m*M* Mn^{2+} for 8 h significantly increased Nedd4–2 protein levels (Fig. 1B,C). Significant inhibition of SGK1 mRNA expression was observed after treatment with 1 m*M* Mn^{2+} for 8 h and with 500 μ*M* or 1 m*M* Mn^{2+} for 24 h (Fig. 2A). Exposing astrocytes to 1 m*M* Mn^{2+} for 4 h and 500 μ*M* or 1 m*M* Mn^{2+} for 8 h led to a significant decrease in pSGK1 protein levels (Fig. 2B,C). These data indicate that high Mn^{2+} levels enhance Nedd4–2/SGK1 signaling in astrocytes.

Nedd4–2 Associates with SNAT3 in Astrocytes

We used the co-immunoprecipitation method to examine possible SNAT3 and other Gln transporters conjuga-tion/complexation with the ubiquitin E3 ligase–Nedd4–2, in primary cultures of astrocytes. Lysates of cells were subjected to immunoprecipitation using antibodies against several Gln transporters: SNAT3, SNAT2, ASCT2, and LAT2. All these carriers are known to be downregulated at protein levels after Mn^{2+} exposure (Sidoryk-Wegrzynowicz et al., 2009). We found that Nedd4–2 was present only in SNAT3 immunoprecipi-tates, providing strong evidence for the interaction of these two proteins (Fig. 3). Notably, our observations are consistent with a previous report, suggesting a possible role of Nedd4–2 in the regulation of SNAT3 function (Boehmer et al., 2003).

Mn^{2+} Exposure Increases Astrocytic Ubiquitin Levels and Protein Ubiquitination

Next, we investigated Mn's effects on the expression of the following ubiquitin-related genes: UbB, UbC, and UbA_{52}. The quantitative RT-PCR analyses showed that treatment with 1 m*M* Mn^{2+} for 8 h and with 500 μ*M* or 1 m*M* Mn^{2+} for 24 h led to a significant increase in the expression of UbB mRNA (Fig. 4A), whereas the expression of mRNAs encoding UbC and UbA_{52} remained unaffected at all tested Mn^{2+} concentrations and exposure times (Fig. 4B,C). Western blot analyses revealed that treatment of astrocytes for 4 h and 8 h with 500 μ*M* or 1 m*M* Mn^{2+} significantly increased free ubiquitin levels (Fig. 4D,E). Additionally, we assessed overall levels of protein ubiquitination using an antibody that recognizes poly- and monoubiquitinated proteins. We found abundant and statistically significant protein hyperubiquiti-nation after 4 h and 8 h of exposure to 500 μ*M* and 1 m*M* Mn^{2+} (Fig. 4F).

Proteasome Activity Contributes to Mn^{2+}-Induced Decrease in SNAT3 Protein

Mn^{2+}-induced increases in protein ubiquitination and activation of the Nedd4–2/SGK1 signaling pathway (Figs. 1 and 2), as well as the association of Nedd4–2 with SNAT3 (Fig. 3), strongly suggest that Mn^{2+}-induced depletion of SNAT3 protein is mediated through the ubiquitin-mediated proteolytic systems. To test this hypothesis, we incubated the cells with 500 μ*M* Mn^{2+} for 4 h in the presence of the proteasome inhibitors, ALLN (10 μ*M*) or Lac (10 μ*M*), and measured astrocytic SNAT3 protein levels. We found that both inhibitors partially protect SNAT3 against Mn^{2+}-induced protein depletion. Levels of this transporter protein were significantly higher in cells co-incubated with Mn^{2+} and ALLN (86% ± 12% of control) or Mn^{2+} and Lac (61% ± 9% of control) than in cells incubated with Mn^{21} alone (32% ± 8% of control; Fig. 5). These data clearly indicate the prominent contribution of the proteasome in the astrocytic degradation of SNAT3 protein in response to Mn^{2+} treatment.

Mn^{2+} Exposure Affects Proteasome Proteolytic Activity

The 26S proteasome is composed of a 20S catalytic core and two 19S regulatory caps (Jentsch, 1992). The 20S core contains two pairs of different catalytic sites, each of which contains three active sites: chymotrypsin-like, trypsin-like, and caspase-like (Adams, 2003). To evaluate whether Mn^{2+} contributes to the proteasome activation, we used luminometric assays for the three different activities (see above). Treatment with 1 m*M* Mn^{2+} for 4 h or with 500 μ*M* and 1 m*M* Mn^{2+} for 8 h and 24 h led to an increase in caspase-like proteasomal

activity (Fig. 6A). No significant changes in the chymotrypsin-like or trypsin-like proteasomal activities were detected after Mn^{2+} exposure (Fig. 6B,C).

DISCUSSION

Our previous studies revealed that Mn^{2+} disrupts Gln transporter expression and function in astrocytes (Sidoryk-Wegrzynowicz et al., 2009). We showed that SNAT3, which, among all the Gln transporters is characterized by the highest affinity for this amino acid (Bode, 2001), was the most sensitive to Mn^{2+} exposure. Incubation of cells with 500 μ*M* or 1 m*M* Mn^{2+} for 4–8 h resulted in a significant loss of SNAT3 protein (Sidoryk-Wegrzynowicz et al., 2009). SNAT3 mediates the inward or outward transport of Gln, dependent on the amino acid or pH gradients (Chaudhry et al., 1999). Under physiological conditions, in astrocytes, where Gln concentrations are much higher intra- *vs.* extracellularly, the efflux of Gln significantly predominates over the uptake (Conti and Melone, 2006). SNAT3 is a major facilitator of Gln efflux from astrocytes, which constitutes its importance in the Gln-Glu cycle and in supplying neurons with precursors for Gln and GABA synthesis (Boulland et al., 2002). Despite this prominent role, little is known about SNAT3's activity and expression regulation mechanisms. Co-expression experiments in *Xenopus* oocytes have shown that Nedd4–2 inhibits the functional activity of SNAT3 (Boehmer et al., 2003). Our co-immunoprecipitation assay revealed an interaction of these two proteins in astrocytes cultures, significantly strengthening the hypothesis that Nedd4–2 regulates SNAT3 expression (Fig. 4A,B). The Nedd4–2/SGK1 signaling pathway is known to regulate several membrane channels as well as transporter expression and function (Boehmer et al., 2006, 2008; Debonneville et al., 2001; Lang et al., 2003). Interestingly, we found no interaction between other Gln carriers (ASCT2, SNAT2, and LAT2) with Nedd4–2, suggesting that Nedd4–2/SGK1 signaling is not a universal pathway of Gln transporter regulation, but is specific for SNAT3.

Nedd4–2 binds to and catalyzes ubiquitin attachment, but its phosphorylation by SGK1 blocks this interaction and decreases ubiquitination of the substrate proteins (Bhalla et al., 2005; Flores et al., 2003; Snyder et al., 2002). Taking into consideration our observation that Nedd4–2 interacts with SNAT3, we hypothesized that Mn^{2+}-induced SNAT3 loss is mediated by Nedd4–2/SGK1 signaling. Our data have indicated that Mn^{2+} exposure increases astrocytic Nedd4–2 mRNA and protein levels, and, at the same time, decreases expression of SGK1 mRNA and levels of its active, phosphorylated form (Figs. 1A–C and 2A–C). All of these processes are known to result in the decline of Nedd4–2 substrate protein levels, leading us to conclude that they are very likely responsible for SNAT3 protein degradation in astrocytes exposed to Mn^{2+}. To our knowledge, this is the first report to study the effect of Mn^{2+} on Nedd4–2. Nevertheless, gene expression profiling experiments in the frontal cortex of *Cynomolgous macaques* have previously shown that Mn^{2+} exposure increases the expression of genes associated with protein folding and turn over. Notably, a member of the E3 ubiquitin ligase family, namely RNF40, was found among them (Guilarte et al., 2008).

In addition to ubiquitination-promoting alterations in expression and the posttranslational modification of astrocytic Nedd4–2 and SGK1, we also found, in response, the Mn^{2+}

exposure-induced overexpression of UbB mRNA (Fig. 4A), the gene encoding three ubiquitin repeats (Hayashi et al., 1994), as well as an increase in free ubiquitin levels (Fig. 4D,E). These alterations undoubtedly contribute to the hyperubiquitination, which was inherent to Mn^{2+} treatment (Fig. 4F). The precise mechanisms of Mn^{2+}-induced increased levels of ubiquitin and ubiquitination remain unclear. Previous studies from our laboratory showed that Mn^{2+} also impairs the expression and function of other Gln transporters (LAT2, ASCT2, and SNAT2) (Sidoryk-Wegrzyno-wicz et al., 2009), as well as the Glu carrier, GLAST (Lee et al., 2009). In addition to their respective roles in Gln and Glu transport, each of these transporters is also responsible for the transport of many other amino acids. Accordingly, the Mn^{2+}-induced decrease in their expression levels and function likely leads to attenuated amino acid uptake in the astrocytes, a process which is known to activate the ubiquitin-mediated proteolytic system. For example, fasting experiments have shown that amino acid deprivation increases levels of mRNAs of all the genes encoding ubiquitin, as well as components of ubiquitin–protein ligase complexes (Jagoe et al., 2002).

Two very interesting findings from this study are that Mn^{2+} increases caspase-like activity of the proteasome (Fig. 6A) and that the Mn^{2+}-induced loss of SNAT3 is prevented by the proteasome inhibitors, ALLN and Lac (Fig. 5). These data strongly support the hypothesis that SNAT3 protein degradation due to Mn^{2+} exposure involves the 26S proteasome. Notably, our results show a stronger effect of ALLN, which, in addition to its proteasome inhibition, also inhibits cathepsins and calpains. Thus, ALLN's stronger effects likely involve lysosomal activity in combination with proteasome as opposed to the more limited effect of lactacytin, which is a selective proteasome inhibitor. Additionally, it is known that ubiquitination of cell surface proteins precedes not only proteasome-dependent, but also lysosome-dependent, proteolysis (Flores et al., 2003; Harris and Schaefer, 2009; Sheldon et al., 2008; Yang et al., 2008). Experiments aimed at evaluating the contribution of lysosomal-based pathways in Mn^{2+}-induced SNAT3 protein loss are currently under way in our laboratory.

In conclusion, this study significantly extends our previous investigations in defining the effect of Mn^{2+} on Gln transport regulation. Our results demonstrate a novel Mn^{2+}-mediated mechanism critical for the posttranslational regulation of SNAT3. We conclude that Mn^{2+}-induced SNAT3 degradation proceeds through the ubiquitin-proteasome system.

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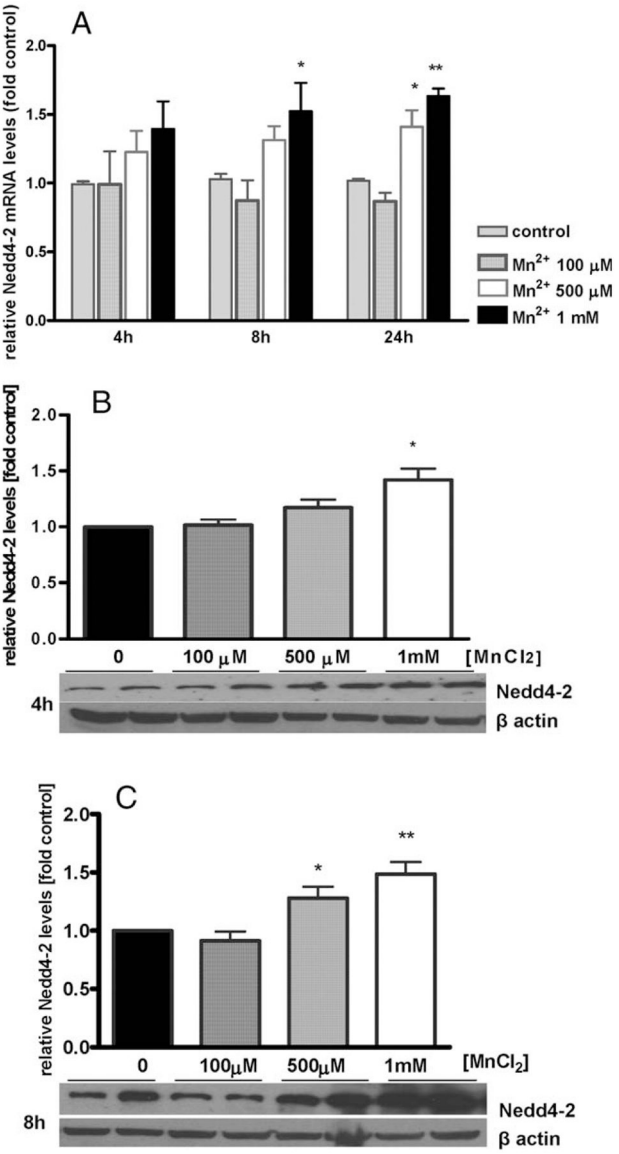

Fig. 1.

Mn^{2+} exposure increases astrocytic Nedd4–2 mRNA and protein levels. Levels of Nedd4–2 mRNA and protein were determined in primary cultures of astrocytes treated with 100 µ*M*, 500 µ*M*, or 1 m*M* Mn^{2+}. (A) Expression of Nedd4–2 mRNA after 4, 8, or 24 h exposure to Mn^{2+} was measured by quantitative RT-PCR and normalized to the levels of GAPDH m_1RNA. Levels of Nedd4–2 protein after (B) 4 h or (C) 8 h exposure to Mn^{21} were determined by Western blotting and normalized to the levels of b-actin protein. Data represent the mean ± SD (three independent sets of cultures, $n = 9$). *$P < 0.05$ and **$P < 0.01$ *vs.* control (ANOVA followed by Tukey's test).

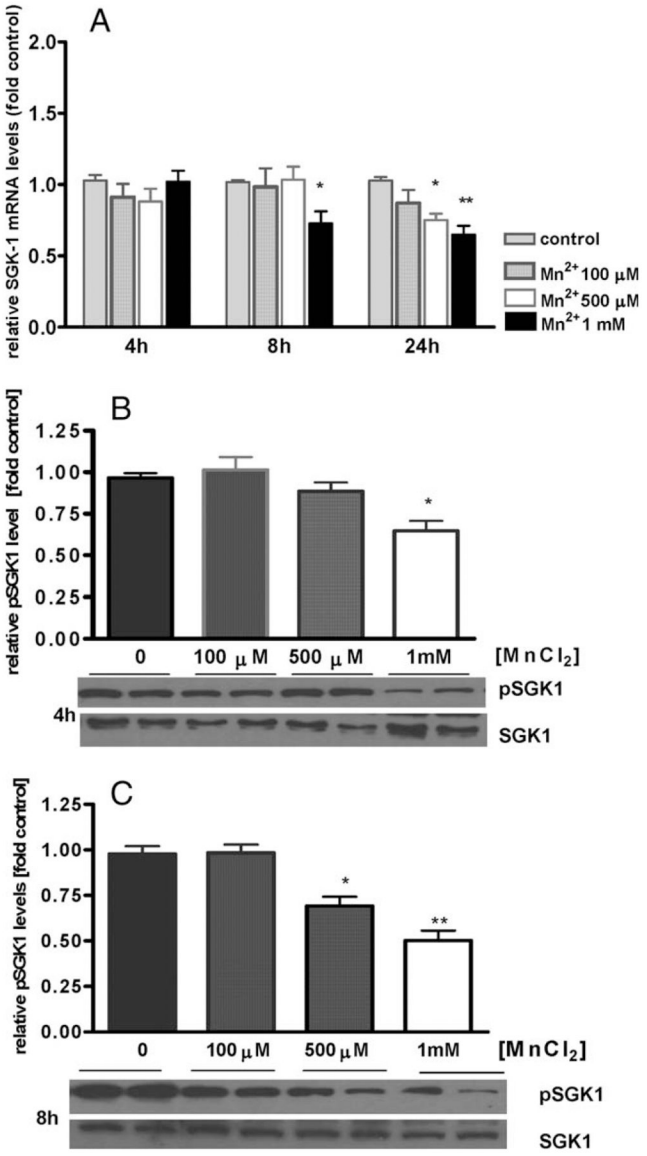

Fig. 2.

Mn^{2+} exposure decreases astrocytic SGK1 mRNA levels and protein phosphorylation. Levels of SGK1 mRNA and protein phosphorylation were determined in primary cultures of astrocytes treated with 100 µ*M*, 500 µ*M*, or 1 m*M* Mn^{2+}. (A) Expression of SGK1 mRNA after 4, 8, or 24 h exposure to Mn^{2+} was measured by quantitative RT-PCR and normalized to the levels of GAPDH mRNA. Levels of phos$_1$phoryl-ated SGK1 protein after (B) 4 h or (C) 8 h exposure to Mn^{21} were determined by Western blotting and normalized to the levels of total SGK1 protein. Data represent the mean ± SD (three independent sets of cultures, n = 9). *P < 0.05 and **P < 0.01 *vs.* control (ANOVA followed by Tukey's test).

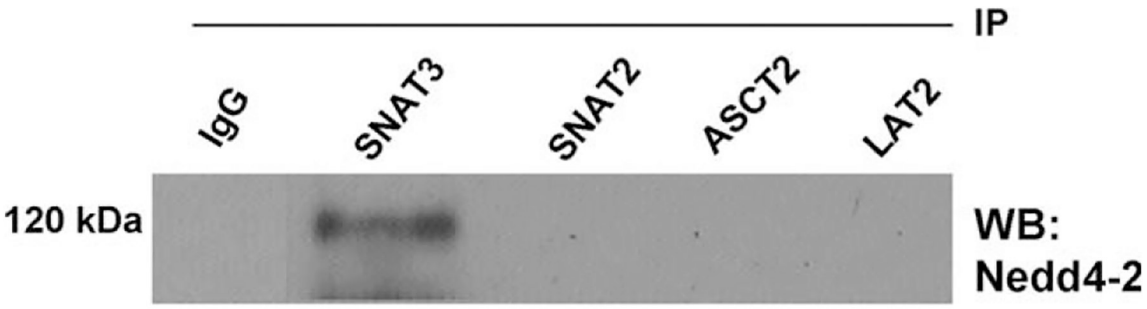

Fig. 3.

Astrocytic SNAT3 co-immunoprecipitates with Nedd4–2. Lysates of astrocytes were subjected to immunoprecipitation using an antibody against SNAT3, SNAT2, ASCT2, and LAT2 and probed for Nedd4–2. In each experiment, an antibody against control IgG was additionally used for immunoprecipitation as the control for nonspecific binding. Similar results were obtained in three to four independent sets of cultures.

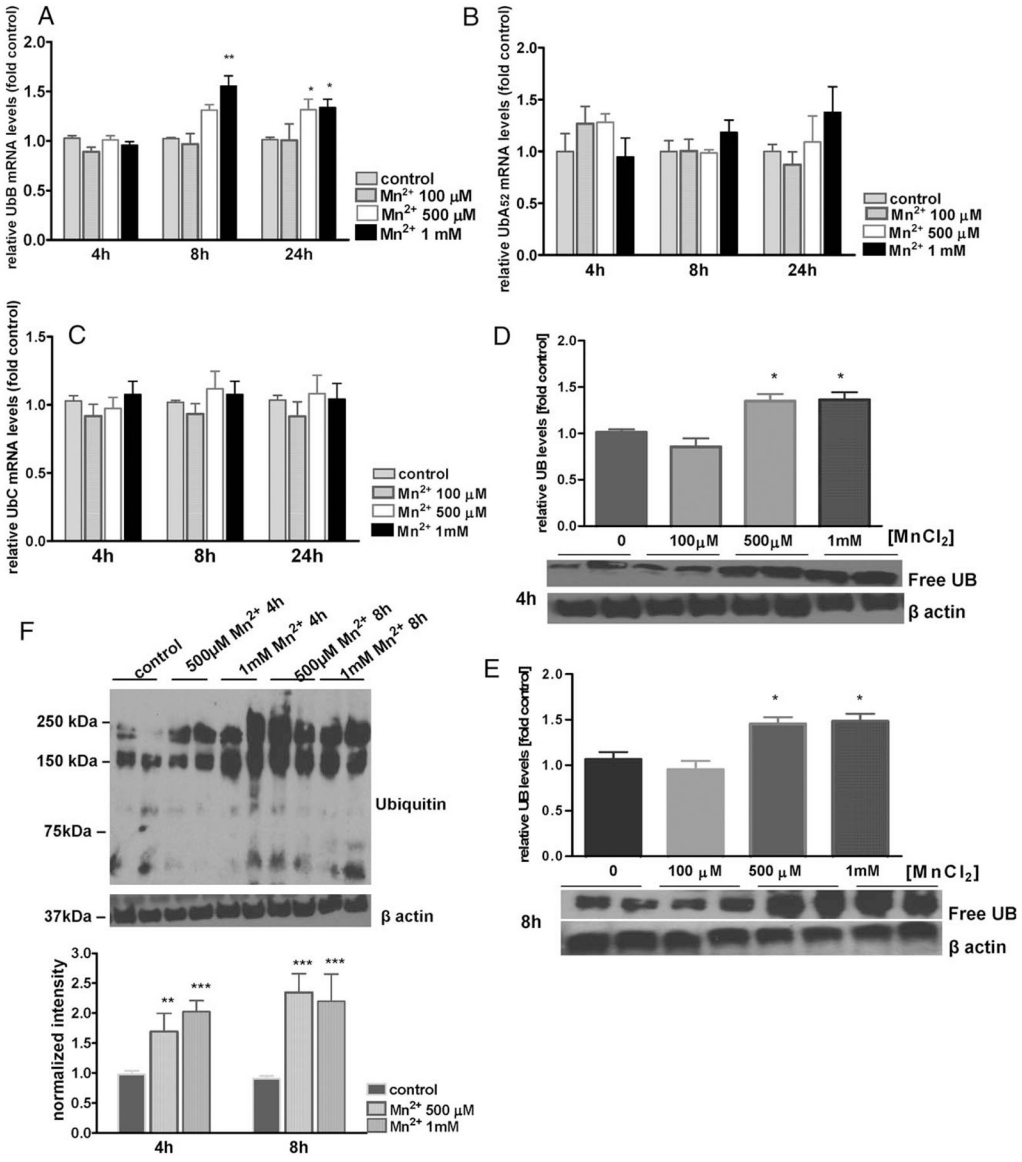

Fig. 4.

Mn^{2+} exposure increases astrocytic UbB mRNA expression, free ubiquitin protein levels, and protein ubiquitination. Levels of UbB, UbA_{52}, and UbC mRNA, free ubiquitin protein, and protein ubiquitination were determined in primary cultures of astrocytes treated with 100 µ*M*, 500 µ*M*, or 1 m*M* Mn^{2+}. Expression of (A) UbB, (B) UbA_{52}, and (C) UbC mRNAs after 4, 8, or 24 h exposure to Mn^{2+} was measured by quantitative RT-PCR and normalized to the levels of GAPDH mRNA. Levels of free ubiquitin protein after (D) 4 h or (E) 8 h exposure to Mn^{2+} were determined by Western blotting and normalized to the levels of b-actin protein. (F) Protein ubiquitination was assessed by Western blotting using an antibody that targets mono- and polyubiquitinated proteins. Quantification of ubiquitination was normalized to β-actin. Data represent the mean ± SD (three independent sets of cultures, n = 9–12). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ *vs.* control (ANOVA followed by Tukey's test).

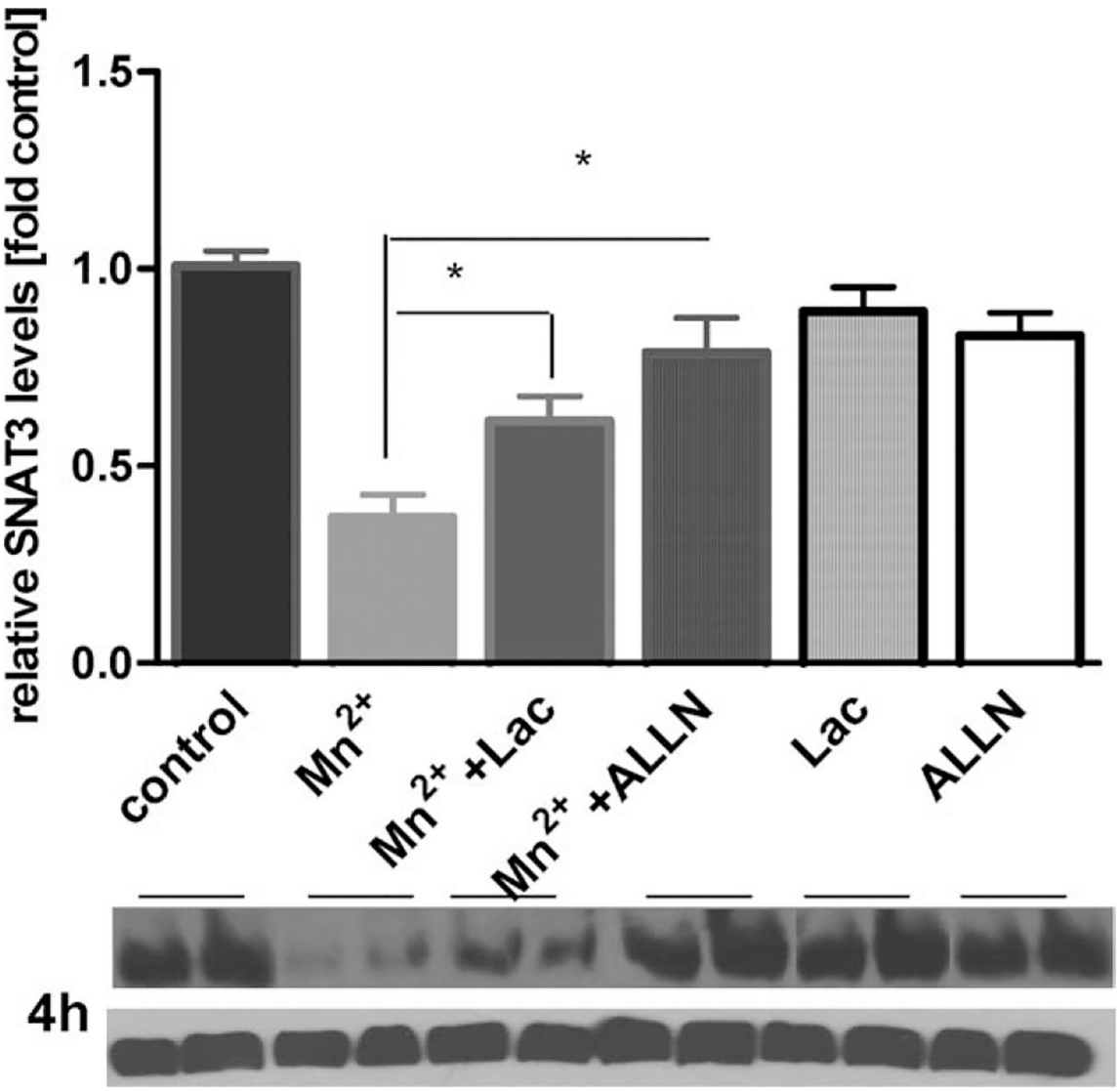

Fig. 5.

Proteasome inhibitors decrease Mn^{2+}-induced SNAT3 protein loss in astrocytes. Levels of SNAT3 protein in primary cultures of astrocytes treated for 4 h with 500 μ*M* Mn^{2+} in the presence of 10 μ*M* ALLN or 10 μ*M* Lac were determined by Western blotting and normalized to the levels of β-actin protein. Data represent the mean ± SD (three independent sets of cultures, n = 9). **P* < 0.05 *vs.* control (ANOVA followed by Tukey's test).

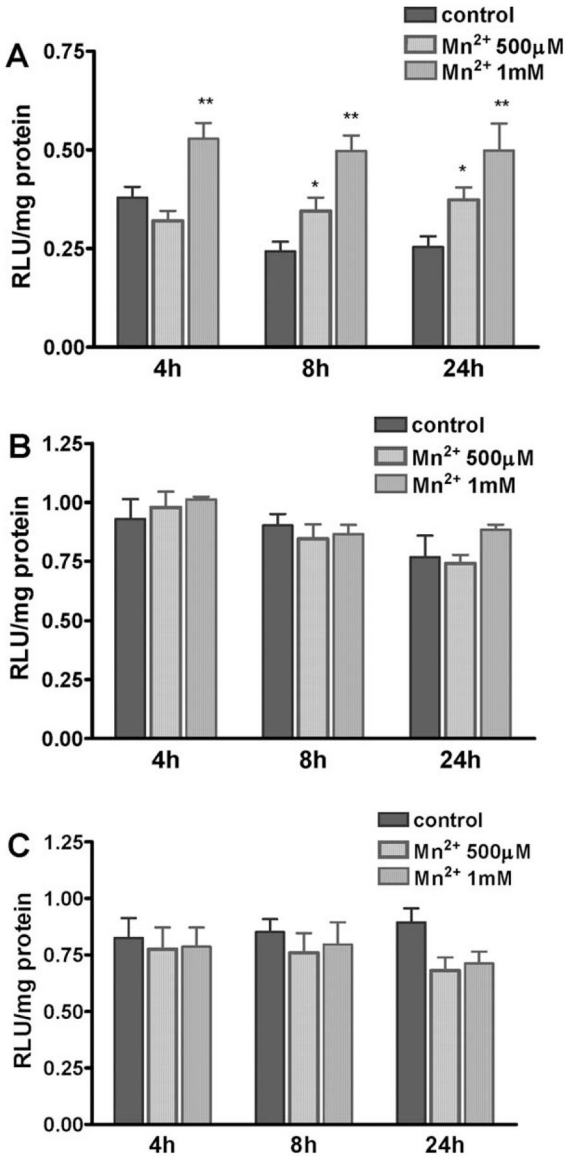

Fig. 6.

Mn^{2+} exposure increases caspase-like proteasomal activity in astrocytes. (A) Caspase-like, (B) chymotrypsin-like, and (C) trypsin-like proteasomal activities in primary cultures of astrocytes treated with 500 µ*M* or 1 m*M* Mn^{2+} for 4, 8, or 24 h were determined by luminometric assay and normalized to the protein content. Data represent the mean ± SD (three independent sets of cultures, $n = 9$). $*P < 0.05$ and $**P < 0.01$ *vs.* control (ANOVA followed by Tukey's test).