


Acute Manganese Exposure Modifies the Translation Machinery via PI3K/Akt Signaling in Glial Cells

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Abstract

Manganese (Mn) exerts serious neurotoxic effects, among which, the disruption of the glutamate/glutamine (Glu/Gln) cycle, leads to an excitotoxic insult. The molecular mechanisms mediating Mn-induced neurotoxicity, have not yet been fully understood. Glu, the major excitatory neurotransmitter in the nervous system, activates a variety of signal transduction cascades involved in protein synthesis regulation. Although protein translation is an exquisitely regulated process, translational dysregulation has been observed in many neurodegenerative disorders. Hence, we investigated the effect of a short-term Mn exposure in signaling pathways critically involved in protein synthesis, such as the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) cascade. To this end, we used the well-characterized chick cerebellar Bergmann glial cells (BGC) primary culture. Confluent BGC monolayers were exposed to different MnCl_2 concentrations (50–500 μM) for different time periods. The phosphorylation patterns of Akt, the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) as well as the adenosine monophosphate-dependent protein kinase (AMPK) were measured. A time and dose-dependent increase in the phosphorylation status of these proteins was found, thus the involvement of a Ca^{2+} /PI3K/mTOR pathway could be demonstrated. Accordingly, a modulation of [^{35}S]-methionine incorporation into newly synthesized polypeptides was found upon Mn acute exposure. These results demonstrate that Mn exerts triggers a change in the protein repertoire of glia cells that support their involvement in Mn neurotoxicity.

Summary Statement

We demonstrate herein that short-term exposure of radial glia cells to Manganese, a neurotoxic metal, induces an effect on protein synthesis, altering the protein repertoire of these cells.

Keywords

4E-BP1, AMPK, ERK 1/2, glial cells, manganese, PI3K/Akt

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Introduction

Mn is a transition metal naturally occurring in the earth's crust. More importantly, is an essential element critical for development, reproduction, antioxidant defense, energy metabolism, immune response, and regulation of brain function (Chen et al., 2018). Mn functions as a cofactor for a variety of enzymes, including arginase, glutamine synthetase (GS), pyruvate carboxylase, and Mn superoxide dismutase (Mn-SOD) (Bjørklund et al., 2020). Alterations in Mn homeostasis are associated with altered neuronal physiology and cognition in humans, and either overexposure or less likely insufficiency can cause neurological

dysfunctions (Balachandran et al., 2020). The main pathology due to Mn-overexposure is known as *Manganism*, which shares several pathological features of Parkinson's disease (PD)

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(Chen et al., 2018), and it is considered an important risk factor for the development of several neurodegenerative diseases. The proposed mechanisms of Mn neurotoxicity range from functional changes in neurotransmission, to mitochondrial damage, and oxidative stress (Aschner and Erikson, 2017). Regarding the effects on neurotransmitter regulation, numerous studies have documented that Mn induces neurotoxicity *via* Glu-mediated signaling (Escalante et al., 2019; Lee et al., 2017). Glu is the main excitatory neurotransmitter in the central nervous system, and its concentrations in the synaptic cleft are tightly regulated by a family of high-affinity transporters. The dysregulation of any of the main components of glutamatergic neurotransmission leads to excitotoxic cell death (Danbolt, 2001). Multiple studies implicate Glu signaling in changes in the protein synthesis machinery, influencing local translation in the axons (Hsu et al., 2015; Martínez-Lozada and Ortega, 2015).

Translation is one of the most energy-consuming cellular processes and several signaling pathways are involved in the regulation of its biochemical machinery (Moon et al., 2018). The dysregulation of these pathways results in anomalous protein synthesis which leads to the development of neurological diseases. The PI3K/Akt cascade engages with the mechanistic target of rapamycin (mTOR), known as the master regulator of protein synthesis (Roux and Topisirovic, 2018). mTOR serves an important role in neural plasticity (Cho et al., 2018). Canonically, growth factors activate receptor tyrosine kinases (RTKs) in the plasma membrane that become tyrosine phosphorylated and by these means serve as scaffold proteins for the recruiting of components of their signaling cascade that harbor the *so-called* SH2 (Src homology domain 2) domain. PI3K is one of these proteins, that once recruited to the growth factor receptor, phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) producing phosphatidylinositol-3,4,5-trisphosphate (PIP₃). PIP₃ recruits phosphoinositide-dependent kinase 1 (PDK1) and AKT to the plasma membrane where PDK1 phosphorylates and activates Akt (Thr³⁰⁸). mTORC2 increases Akt activity by phosphorylating its Ser⁴⁷³ residue (Ediriweera et al., 2019). Then, Akt phosphorylates the Tuberous sclerosis complex 2 (TSC2) and inhibits its GTPase activity, resulting in increased Rheb-GTP levels and the activation of the serine/threonine kinase mTORC1 which phosphorylates 4E-BPs and the 70-kDa ribosomal S6 kinases (S6Ks) 1 and 2, critically involved in mRNA translation regulation (Roux and Topisirovic, 2018). Furthermore, Mn exposure results in its accumulation in the mitochondria (Chen et al., 2018). Once inside, Mn impairs superoxide dismutase (SOD) generating ROS, which inhibits several metabolic enzymes, and disrupts the respiratory chain, particularly complexes I and II (Warren et al., 2020). All these biochemical reactions lead to a sustained energy deficit that shuts down most of the ATP-consuming functions in the cell. Alterations in cellular energetics are sensed by the AMP-activated protein kinase (AMPK). To ameliorate the ATP deficit, AMPK

downregulates anabolic processes, such as protein synthesis by inhibiting mTORC1 (Roux and Topisirovic, 2018).

It is broadly known that Mn accumulates preferentially in glial cells (Ke et al., 2019). BGC is a well-established model of radial glia that enwraps glutamatergic synapses, contributing to the recycling of Glu from the synaptic cleft (Glu/Gln shuttle) and the metabolic coupling with the surrounding neurons (astrocyte-neuron lactate shuttle) (Martínez-Lozada et al., 2013; Mendez-Flores et al., 2016). Moreover, Mn activates the PI3K/Akt pathway and promotes an energy deficit due to its interaction with the mitochondria (Peres et al., 2015; Warren et al., 2020). Currently, no studies have linked the effects of Mn on glutamatergic neurotransmission to translational control. Therefore, it is plausible that the translation process could be affected by Mn exposure, contributing to the neurotoxicity effects triggered by this metal. Hence, we decided to investigate herein the effect of Mn short-term exposure in signaling pathways involved in protein synthesis, such as the PI3K/Akt signaling cascade, in a model of chick cerebellar BGC.

Materials and Methods

Materials

Tissue culture reagents were obtained from GE Healthcare (Carlsbad, CA, USA). MnCl₂, D-Aspartic, and the MTT reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-Glutamate was obtained from Tocris Biosciences (Ellisville, IL, USA). L-[³⁵S]-Methionine (Cat# NEG009A, specific activity 1175 Ci/mmol) was purchased from PerkinElmer (Waltham MA, USA). Bradford and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were obtained from Bio-Rad (Hercules, CA, USA). Anti-pAkt (Cat# sc-514032, RRID: AB_2861344), anti-Akt (Cat# sc-81434, RRID:AB_1118808), anti-p4E-BP1 (Cat# sc-23768-R, RRID: AB_2095599), anti-4E-BP1 (Cat# sc-9977, RRID: AB_626621) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA); anti-pAMPK (Cat# 2535, RRID: AB_331250), anti-AMPK (Cat# 2532, RRID:AB_330331), anti-ERK1/2 (Cat# 9102, RRID:AB_330744) were purchased from Cell Signaling (Danvers, MA USA). Secondary antibodies were from Abcam. Horseradish peroxidase-linked secondary antibodies and the enhanced chemiluminescence reagent (ECL) were obtained from GE Healthcare (Carlsbad, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA).

Animals

Chicken embryos were kindly donated by Avimex, S.A de C.V. (Mexico City, Mexico) and maintained at 37°C until usage. All experiments were performed according to the International Guidelines on the Ethical Use of Animals in Research and were approved by the Cinvestav Animal

Ethics Committee. Every effort was made to reduce the number of embryos used and their suffering.

Bergmann Glial Cell Culture and Stimulation Protocol

Primary cultures of cerebellar BGC were prepared from 14-days-old chick embryos as previously described and characterized (Ortega et al., 1991). Chicken embryonic cerebella were dissected and dissociated by brief trituration and incubated in Puck's medium supplemented with trypsin (0.25 mg/mL) and DNase (0.08 mg/mL) for 15 min. The supernatant media was removed and, the sediment was resuspended in reduced-serum Minimal Essential Medium (Opti-MEM) containing 2.5% fetal bovine serum (FBS), 2 mM glutamine, and gentamicin (50 µg/mL) for mechanical dissociation. Cells were recovered by repeated removal of dissociated cells and seeded in plastic culture dishes at a density of 1×10^6 cells/mL. The cultures were maintained at 37°C and 95% air/5% CO₂ in a humidified incubator. For the experiments, cells were used 4–7 days post-isolation and serum-starved (DMEM with 0.5% FBS) for 12 h and then treated with different MnCl₂ concentrations at the indicated time periods. All inhibitors were added 30 min before the MnCl₂ treatments.

Cell Viability Assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were seeded in 96-well culture plates and treated with different MnCl₂ concentrations for 3, 6, 12, and 24 h. Then, 3 h before the end of each treatment, the cells were incubated with 5 mg/mL of MTT stock solution and maintained at 37°C. At the end of the treatments, the media was discarded and, 100 µL of dimethyl sulfoxide (DMSO) were added to each well to dissolve the formazan crystals formed in the MTT reaction. Absorbance was measured with a microplate reader (BioTek Instruments, VT, USA) at 570 nm. Experiments were performed in quadruplicates in three independent cultures. A 1% Triton X-100 was used as a positive control of cell death.

SDS-PAGE and Western Blots

Confluent monolayers were harvested with phosphate-buffered solution (10 mM K₂HPO₄/KH₂PO₄, 150 mM NaCl, pH 7.4) containing phosphatase inhibitors (10 mM NaF, 1 mM Na₂MoO₄, and 1 mM Na₃VO₄) and 1 mM of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF). The cells were lysed with RIPA buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1% NP-40, 0.25% sodium deoxycholate, 10 mM NaF, 1 mM Na₂MoO₄, and 1 mM Na₃VO₄, pH 7.4). Total cell lysates were denatured in Laemmli's sample buffer, and equal amounts of protein (approximately 50 µg of total protein as determined by the Bradford method) were resolved through 10% SDS-PAGE

slab gels and then electroblotted to nitrocellulose membranes. Blots were stained with Ponceau S stain to confirm equal protein content in all lanes. Membranes were soaked in PBS to remove the Ponceau S and incubated in TBS containing 5% dried skimmed milk and 0.1% Tween 20 for 2 h to block the excess of nonspecific protein binding sites. Then, the membranes were incubated overnight at 4°C with the primary antibodies indicated in each figure, followed by their respective secondary antibodies. The detection of the immunoreactive polypeptides was conducted using a MicroChem DNR Bio-Imaging System imager or a Li-COR Odyssey Imaging System and processed by the Image J software (NIH; Bethesda, Maryland, USA) or the Image Studio Lite (Li-COR, Lincoln, NE) to quantify the total intensity of the bands.

Metabolic Labelling and Assessment of Overall Protein Synthesis

Confluent BGC monolayers were labelled for 12 h with 1 µCi of L-[³⁵S] Methionine in methionine-free DMEM. The cells were extensively washed and treated for the indicated time points with 200 µM MnCl₂. The monolayers were washed twice with ice-cold PBS and lysed with cold RIPA buffer. An aliquot containing approximately 15 µg of protein was spotted onto GF/C microfiber filters (Whatmann). The filters were air-dried and washed for 10 min in ice-cold 10% trichloroacetic acid (TCA) followed by three 10 min washes in cold 5% TCA. After drying at room temperature, the filters were placed in scintillation vials with 3 ml of scintillation liquid containing 10 µl of glacial acetic acid. [³⁵S]-Methionine incorporation was determined *via* liquid scintillation counting in a PerkinElmer Tri-Carb 2810TR scintillation counter.

Statistical Analysis

Data are presented as the mean ± SD from at least three independent experiments. Data with a normal distribution (evaluated by the Shapiro-Wilk test) was analyzed by repeated-measures ANOVA followed by Dunnett's or Bonferroni's *post hoc* tests. Otherwise, non-parametric Friedman's test was used, and data were presented as median ± interquartile range. Differences with a $p \leq 0.05$ value were considered statistically significant. All statistical analyses were performed using GraphPad 9.0 software (GraphPad Software, La Jolla, California, USA).

Results

Mn Increases Akt Phosphorylation in BGC via PI3K

Within the cerebellum, BGCs actively participate in information processing, through the effective recycling of the major excitatory amino acid transmitter, glutamate. Interestingly, it has been documented that Mn significantly accumulates in the cerebellum (Pajarillo et al., 2020; Sepúlveda et al.,

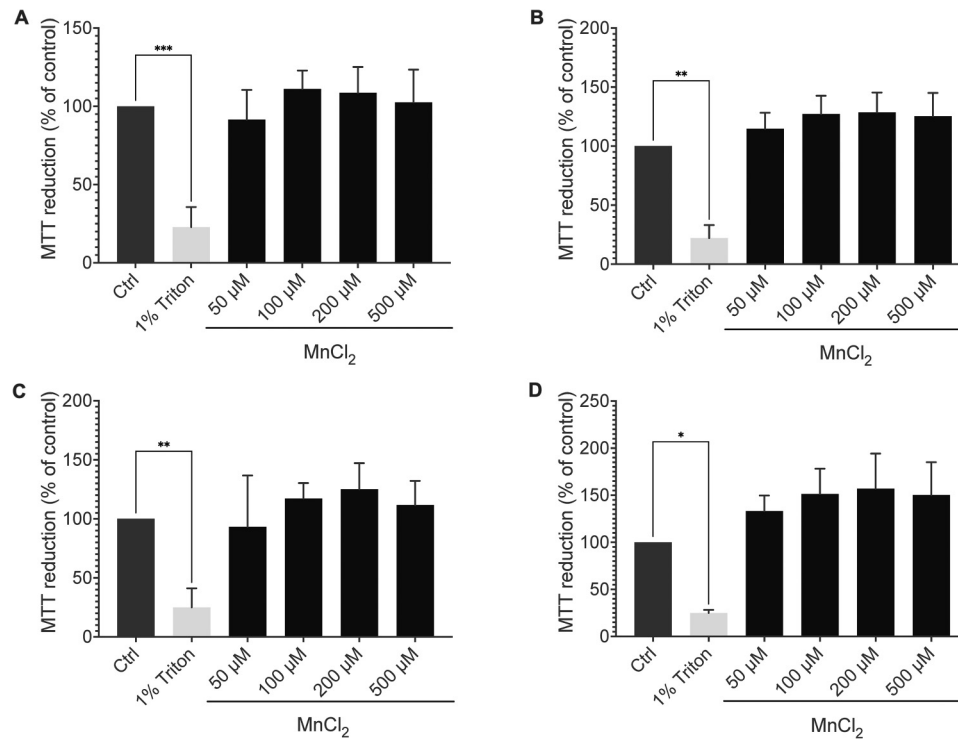


Figure 1. Mn effect on cell viability. Bergmann glial cells were treated with several concentrations of MnCl_2 (50, 100, 200, or 500 μM) for different time of exposure (A) 3 h ($n = 3$), (B) 6 h ($n = 4$), (C) 12 h ($n = 4$) and (D) 24 h ($n = 3$), and the reduction of MTT to formazan was evaluated. Control (Ctrl), 1% Triton X-100 was used as a control of cell death at each of the times. Data are expressed as the mean \pm SD of at least three independent experiments. A repeated-measures analysis of variance (ANOVA) and Dunnett's *post hoc* test were performed. Statistically significant differences are indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

2012; Ye and Kim, 2015). Several studies have indicated that astrocytes are more resistant to Mn insults when compared to neurons (Lee et al., 2009). To gain insight into the cytotoxic effect of MnCl_2 exposure on cultured Bergmann glia cells, we performed MTT assays. Confluent BGC monolayers were treated with MnCl_2 concentrations ranging from 50 to 500 μM exposed for 3, 6, 12, and 24 h. The results are presented in Figure 1. BGCs are resilient to the cytotoxic effects of Mn since there was no apparent cell death at any of the time points evaluated, even at the maximum concentration used (500 μM). As expected, the exposure to a 1% Triton X-100 solution reduced cell viability.

Previous findings from our group have demonstrated that MnCl_2 exposure increases the catalytic efficacy of the sodium-dependent glutamate/aspartate transporter (GLAST) (Escalante et al., 2019). Taking into consideration that we and others have demonstrated the signaling capabilities of this transporter (reviewed in (Martínez-Lozada and Ortega, 2015)), we decided to explore the signaling transactions activated by MnCl_2 . To evaluate the activation of the PI3K/PKB/Akt pathway we evaluated the Ser⁴⁷³ PKB/Akt phosphorylation patterns since it is established that this specific phosphorylation is a requirement for its kinase activity (Yu and Cui, 2016). A dose-dependent increase in PKB/Akt phosphorylation was found after 30 min of exposure to MnCl_2 (Figure 2A). The time-course

of this post-translational modification shows a rapid response to the exposure to 200 μM Mn (Figure 2B), reaching its peak just after 5 min of treatment and then decreasing to normal conditions at 60 min. Accordingly, the inhibition of PI3K with a 100 nM concentration of Wortmannin 30 min prevents the Mn effect (Figure 3). As control of these experiments, cells were exposed for 30 min to 1 mM glutamate (Glu) (Morales et al., 2006).

Mn-Induced Akt Phosphorylation Involves the Activation of NCX

To gain insight into the signaling mechanisms involved in the recorded Mn-mediated Akt phosphorylation and taking into account that this metal increases the catalytic efficacy of the sodium-dependent glutamate/aspartate transporter GLAST in our culture model (Escalante et al., 2019), we decided to explore the possibility that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) could be involved in Akt phosphorylation. To this end, we used the NCX inhibitor KBR7943. The results are presented in Figure 4. Preincubation with the exchanger blocker prevents the Mn-induced Akt phosphorylation, suggesting that manganese exposure augments $[\text{Ca}^{2+}]_i$, opening the possibility of the activation of several signal transduction cascades associated with the exposure to this metal.

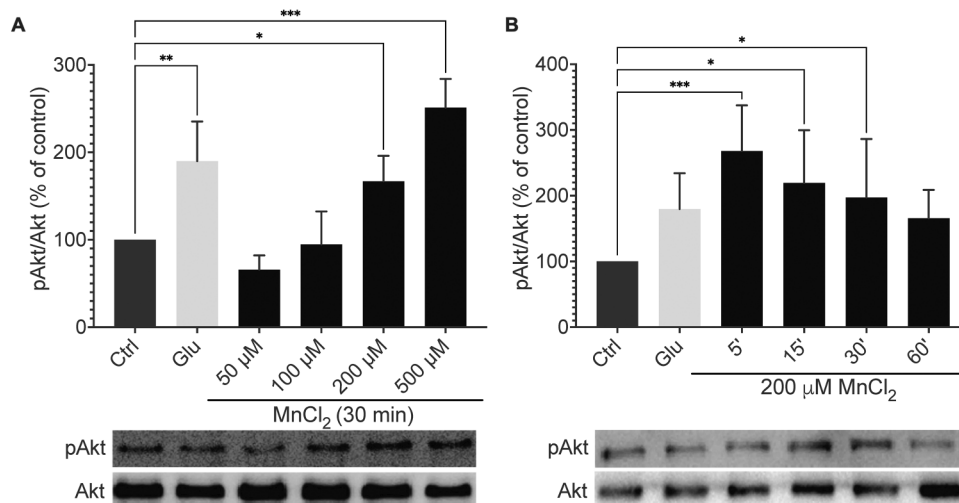


Figure 2. Mn treatment increases Ser⁴⁷³ Akt phosphorylation. (A) Dose-response curve of the effect of Mn on Akt phosphorylation in BGC. Bergmann glia was treated for 30 min with MnCl₂ (50, 100, 200, or 500 μM), n = 4. (B) Time course of the response of BGC to Mn in the phosphorylation of Akt. Bergman glia was treated with MnCl₂ 200 μM, for up to 60 min. Control (Ctrl), Glutamate (Glu) 1 mM (30 min) was used as a positive control, n = 3. A representative blot is presented below each graph. Data are expressed as the mean ± SD of at least three independent experiments. A repeated-measures analysis of variance (ANOVA) and Dunnett's *post hoc* test were performed. Statistically significant differences are indicated by *p < 0.05, **p < 0.01 and ***p < 0.001.

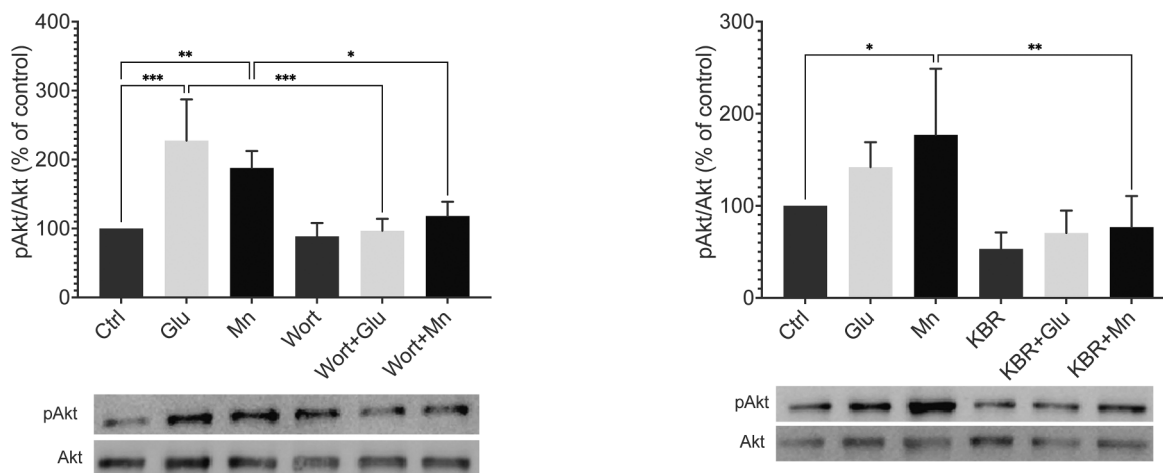


Figure 3. Phosphatidylinositol 3 kinase (PI3K) modulates the Mn effect on Akt phosphorylation (Ser⁴⁷³). Bergmann glial cells were treated with Wortmannin (Wort) 100 nM 30 min before the treatment with MnCl₂ 200 μM for 5 min. Control (Ctrl), Glutamate (Glu) 1 mM (30 min) was used as a positive control, n = 3. A representative blot is presented below each graph. Data are expressed as the mean ± SD of at least three independent experiments. A repeated-measures analysis of variance (ANOVA) and Bonferroni's *post hoc* test were performed. Statistically significant differences are indicated by *p < 0.05, **p < 0.01 and ***p < 0.001.

Figure 4. Inhibition of the Na⁺/Ca²⁺ exchanger (NCX) diminishes Mn-induced Ser⁴⁷³ Akt phosphorylation. 30 min before the treatment with MnCl₂ 200 μM (5 min), KBR7943 (KBR) was added to the Bergmann glial cells culture at a concentration of 15 μM. Glu 1 mM (15 min) was used as a control. Control (Ctrl), Glutamate (Glu) 1 mM (30 min) was used as a positive control, n = 4. A representative blot is presented below each graph. Data are expressed as the mean ± SD of at least three independent experiments. A repeated-measures analysis of variance (ANOVA) and Bonferroni's *post hoc* test were performed. Statistically significant differences are indicated by *p < 0.05 and **p < 0.01.

Mn Induces 4E-BP1 Phosphorylation via mTORC1 and Regulates de novo Protein Synthesis

An important downstream effector of the PI3K/Akt signaling pathway is the mechanistic target of rapamycin (mTOR) complex 1, the master regulator of protein synthesis (Roux

and Topisirovic, 2018). Moreover, mTOR activity promotes cap-dependent translation through 4E-BP1 phosphorylation (Santini and Klann, 2011). To explore the effect of Mn treatment on mTORC1 activity we measured phospho-4E-BP1 levels. Treatment with Aspartate 1 mM for 15 min was used

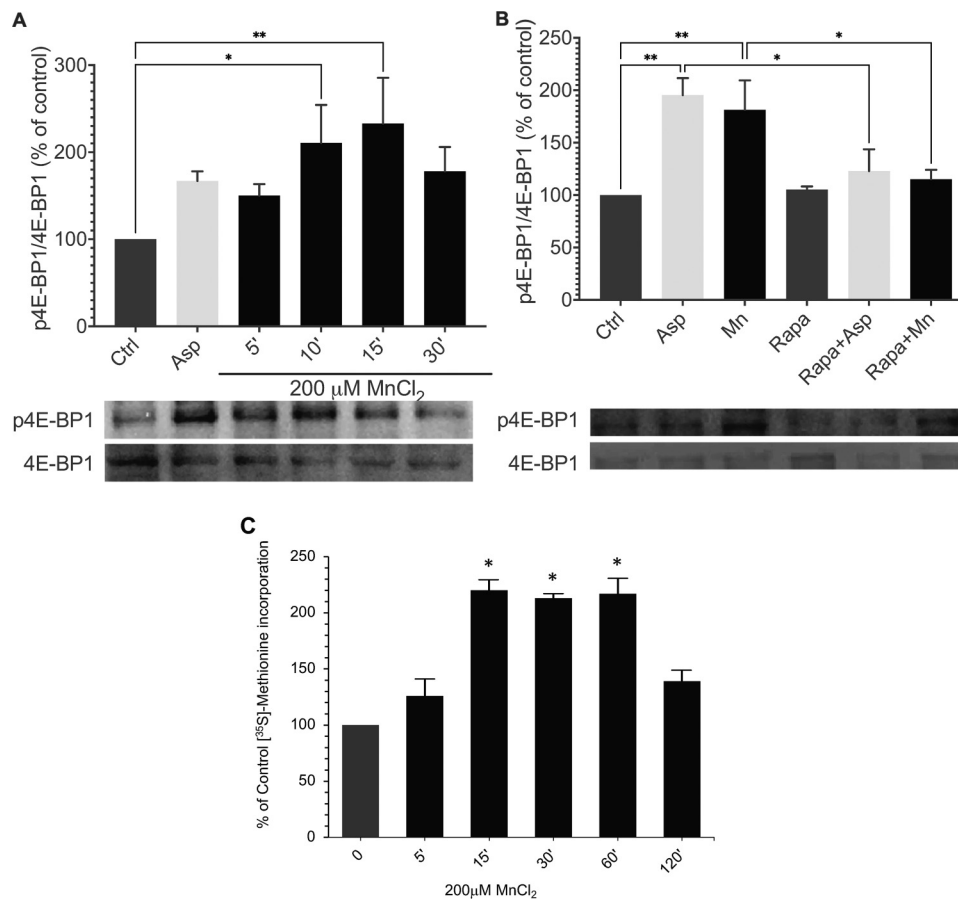


Figure 5. Mn treatment increases 4E-BP1 phosphorylation (Thr⁷⁰) through mTORC1 and regulates protein synthesis. (A) Time course of the response to Mn treatment on 4E-BP1 phosphorylation in BGC. Bergmann glial cells were treated with MnCl₂ 200 μM from 5 to 30 min of exposure. Friedman's test was performed, *n* = 3. (B) Participation of mTORC1 in the Mn-induced 4E-BP1 phosphorylation. 30 min before the treatment with MnCl₂ 200 μM (15 min), Rapamycin (Rapa) was added at 100 nM. (C) Time-dependent [³⁵S]-Methionine incorporation into TCA precipitable polypeptides after exposure to 200 μM. A repeated-measures analysis of variance (ANOVA) and Bonferroni's *post hoc* test was performed. Control (Ctrl), Aspartate (Asp) 1 mM (15 min) was used as a positive control, *n* = 4. A representative blot is presented below each graph. Data are expressed as the mean ± SD of at least three independent experiments. Statistically significant differences are indicated by **p* < 0.05 and ***p* < 0.01.

as a control for these experiments (Martínez-Lozada et al., 2011). A significant increase in the phosphorylation of 4E-BP1 in its Thr⁷⁰ residue was statistically significant after 15 min of MnCl₂ treatment (Figure 5A), after 30 min the effect is not significant. This effect is mediated by mTORC1 since the rapamycin abolished the Mn effect (Figure 5B). When phosphorylated, 4E-BP1 releases the eIF4E which is a cap-binding subunit of the eIF4F translation initiation complex that facilitates the recruitment of mRNAs to the ribosome (Qin et al., 2016). These results indicate that the initiation of cap-dependent translation is being up-regulated after a 15 min of exposure to Mn, from then the effect returns to its basal levels. In order to establish a functional consequence of this signaling pathway triggered by Mn, we decided to evaluate overall protein synthesis. To this end, we labeled confluent BGC cultures with 1 μCi of [³⁵S]-Methionine for 12 h in methionine-free medium. Cells were then exposed for different time periods to 200 μM

MnCl₂, and the polypeptides precipitated with TCA. An increase in [³⁵S]-Methionine incorporation into polypeptides was found after 15 min and lasted for up to 60 min of Mn exposure, returning to basal levels after 2 h (Figure 5, panel C). These results demonstrate a transient effect of this metal in the translation process.

Mn Induces AMPK Phosphorylation (Thr¹⁷²)

The well-established mitochondrial deleterious effects of Mn exposure in different model systems (Harischandra et al., 2019), the fact that Mn reduces glucose uptake in our model system (Escalante et al., 2019), and the return to basal translation levels after 120 min of Mn, prompted us to evaluate the ATP sensor, the adenosine monophosphate protein kinase (AMPK). It has been established that phosphorylation in its Thr¹⁷² residue is well-correlated with its enzymatic activity (Herzig and Shaw, 2018). Given the fact that the

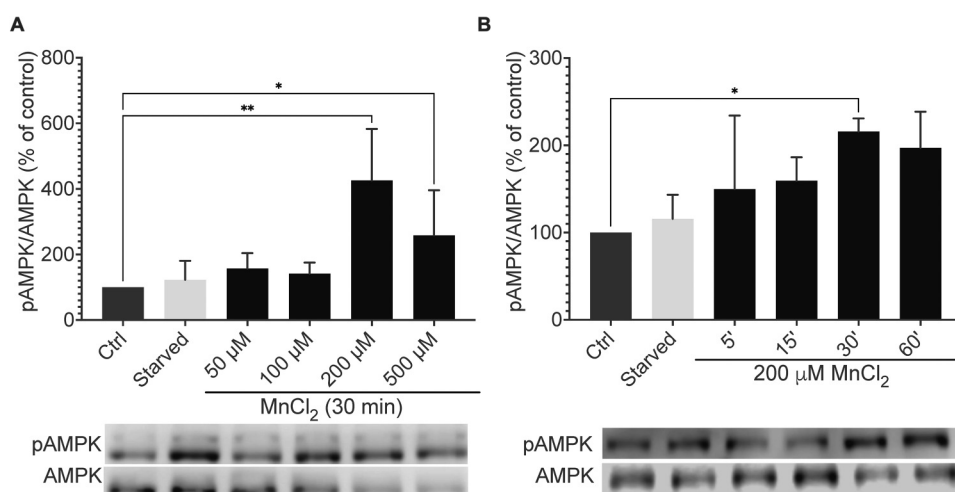


Figure 6. Mn treatment increases Thr¹⁷² AMPK phosphorylation. (A) Dose-response curve of the effect of Mn on AMPK phosphorylation in BGC. Bergmann glia was treated for 30 min with MnCl₂ (50, 100, 200, or 500 μM), n = 3. (B) Time course of the response of BGC to Mn treatment over the phosphorylation of AMPK. A repeated-measures analysis of variance (ANOVA) and Dunnett's *post hoc* test was performed. Control (Ctrl), Serum-starved cells were used as a control, n = 3. A representative blot is presented below each graph. Data are expressed as the mean ± SD of at least three independent experiments. Statistically significant differences are indicated by *p < 0.05 and **p < 0.01.

results described above demonstrate a Mn effect in protein synthesis, and that this is the most energy-consuming cellular process, we explored AMPK phosphorylation after different periods of Mn exposure. The results are presented in Figure 6, a 200 μM concentration of the metal increased the AMPK Thr¹⁷² phosphorylation levels after a 30 min of exposure (Figure 6A) that remain augmented after 60 min of Mn (Figure 6B). Moreover, these effects appear to be regulated by the PI3K/Akt pathway since PKB/Akt inhibition prevents AMPK phosphorylation at 30 min of exposure to Mn (Figure 7A), but after a 60 min exposure, an opposite effect is detected (Figure 7C) with no effects at shorter exposure times (5 and 15 min) (Figure 7B), suggesting a differential crosstalk between the AMPK and PI3K/Akt pathways as proposed for other models (El-Masry S. et al., 2015; Hawley et al., 2014). The Mn-induced increase of AMPK phosphorylation at 30 min and then a diminished AMPK phosphorylation after 60 min of Mn together with the kinetics of 4E-BP1 phosphorylation (Figure 5) correlate well with the recorded Mn effect in protein synthesis, in that its return to basal levels is most possibly linked to a reduction in ATP levels and the resulting change in the protein repertoire of these cells as shown in panel D of Figure 5.

MAPK ERK 1/2 Signaling Pathway Involvement in Mn-Mediated Effects in Bergmann glia

The mitogen-activated protein kinases (MAPKs) are Ser/Thr kinases also known for their involvement in translational control along with the PI3K/Akt pathway (Roux and Topisirovic, 2018). Therefore, we decided to explore the

phosphorylation status of the extracellular signal-regulated protein kinases 1/2 (ERK1/2) after the exposure to different MnCl₂ concentrations (50–500 μM) for different time periods (10–120 min). A non-monotonic effect in response to the treatment with increasing concentrations of MnCl₂ at 120 min of exposure was found, with an increase in ERK 1/2 phosphorylation up to 200 μM but at 500 μM such effect was not present (Figure 8A). On the other hand, the increase in ERK1/2 phosphorylation at 100 μM MnCl₂ was time-dependent (Figure 8B and C). These results confirm that the ERK1/2 pathway is activated in BGC treated after MnCl₂ exposure. Since ERK1/2 has several downstream effector proteins such as the p90 ribosomal S6 kinases (RSK) and the MAPK-interacting kinases (MNCs) (Roux and Topisirovic, 2018) these enzymes might be implicated in the regulation of mRNA translation. These and other possibilities will be addressed in our lab in the near future. In panel C, the treatment with 1 mM, Glu was used as a positive control (López-Colomé and Ortega, 1997).

Discussion

The duality of Mn as an essential trace element and a potent neurotoxic metal has been the object of discussion for the last few years. Since most of the *in vitro* studies have used Mn concentrations far from the ones physiologically relevant (Bowman and Aschner, 2014), in this work we focused on the use of Mn concentrations that result in acute neurotoxic effects. More importantly, Mn accumulates preferentially in glial cells in the CNS (Ke et al., 2019), and it can be found in the cerebellum (Blomlie et al., 2020). BGC is the most abundant non-neuronal cell type in the cerebellum which

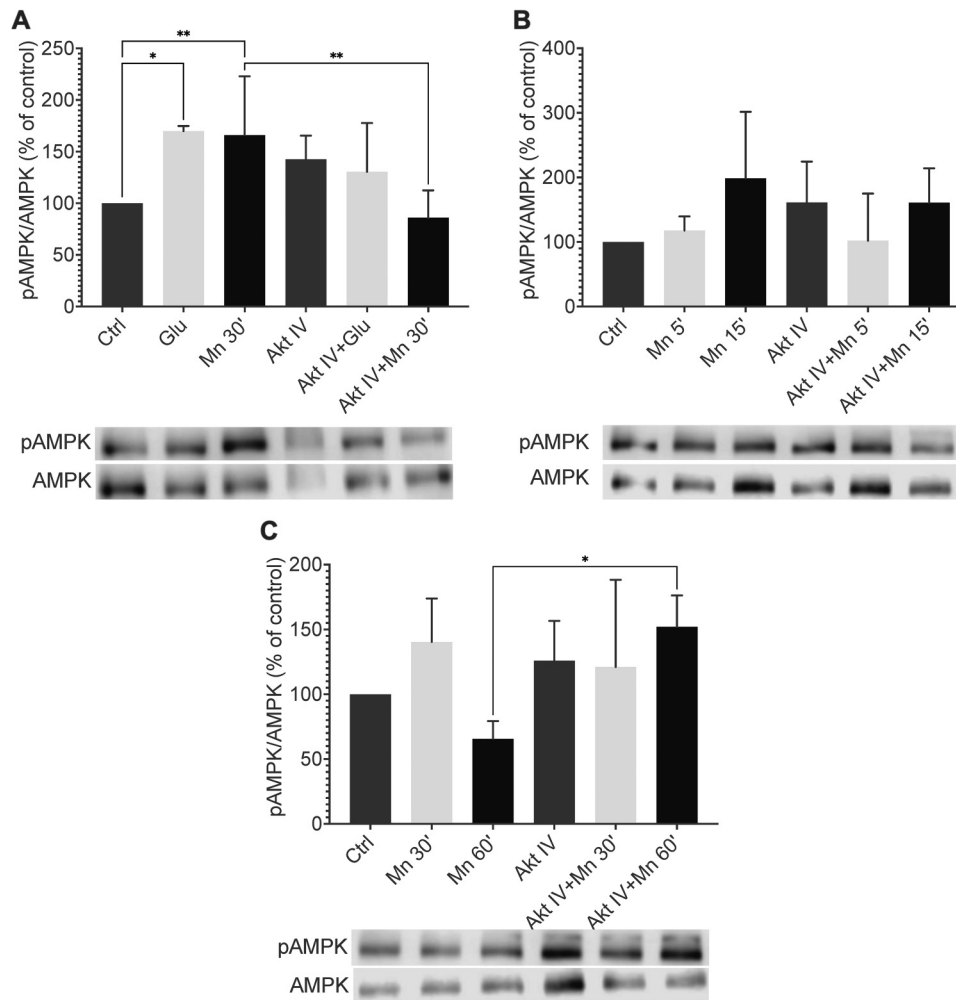


Figure 7. Inhibition of Akt downregulates Mn-induced AMPK phosphorylation (Thr¹⁷²). (A) 30 min before the treatment with MnCl₂ 200 μ M (30 min), Akt IV was added to the Bergmann glial cells culture at a concentration of 15 μ M. Glutamate (Glu) 1 mM (15 min) was used as a positive control (n = 4). After the 30 min of pretreatment with Akt IV BGC were also treated with MnCl₂ 200 μ M at (B) 5 and 15 min (n = 4), or (C) 30 and 60 min of exposure to assess different points of regulation (n = 4). Control (Ctrl). A representative blot is presented at the right of each graph. Data are expressed as the mean \pm SEM of at least three independent experiments. A repeated-measures analysis of variance (ANOVA) and Bonferroni's *post hoc* test was performed. Statistically significant differences are indicated by * $p < 0.05$ and ** $p < 0.01$.

makes these cells an excellent model to study neuron-glia interactions (Somogyi et al., 1990). BGC completely enwraps the parallel fiber-Purkinje cell synapses and takes an active part in the *so-called* Glu/Gln shuttle, ensuring sufficient neuronal neurotransmitter supply and thus, proper glutamatergic neurotransmission. Moreover, BGC are fundamental in preventing excitotoxicity insults linked to the activation of extra-synaptic receptors (Araujo et al., 2019). Several studies, mostly performed in cortical astrocytes, have established that Mn affects glutamate turnover through the impairment of the Glu/Gln cycle. A Mn dysregulation of the transcription of several of its components, like neutral amino acid transporters, has been reported (Sidoryk-Wegrzynowicz and Aschner, 2014). Other studies have demonstrated that Mn is capable to disturb anabolic metabolic pathways, such as protein synthesis (Bray et al., 2018; Korc, 1983; Zhang et al., 2002). The

control of the translation machinery provides an immediate mechanism of response to environmental signals (Browning and Bailey-Serres, 2015). We and others have described that Glu transporters can be regulated post-transcriptionally at the level of protein synthesis (Flores-Méndez et al., 2016; Tian et al., 2007). However, to our knowledge, no studies have linked Mn effects on glutamatergic neurotransmission resulting from the mentioned increase in GLAST catalytic activity, to translational control.

Mn exposure has been described to alter several signaling proteins, and this is not surprising since most kinases are either Mn or Mg-dependent (Ijomone et al., 2019; Kamada et al., 2020). ERK, Akt, mTOR, c-Jun N-terminal kinase (JNK), and more can be activated by Mn both *in vitro* and *in vivo* (Peres et al., 2015). Since Akt is known to play a major role in the control of cell metabolism, growth,

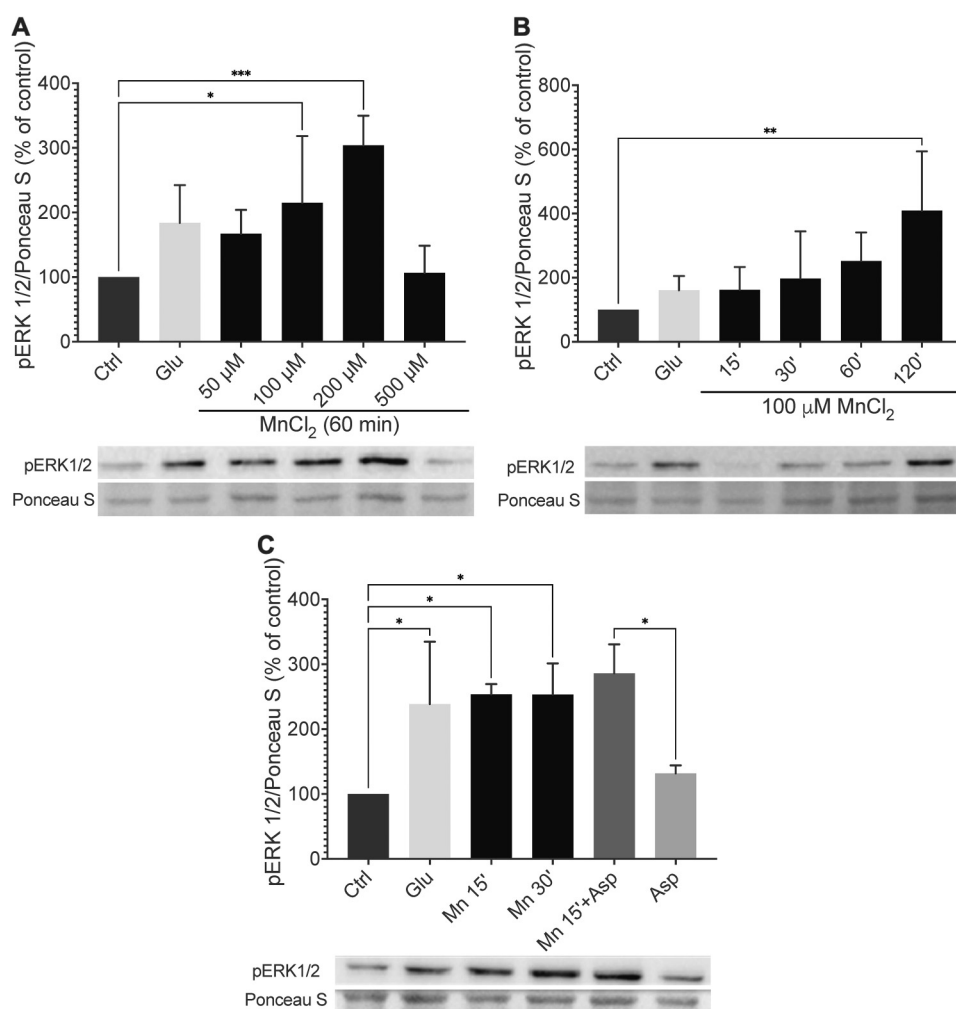


Figure 8. Mn treatment induces ERK 1/2 phosphorylation. (A) Dose-response curve of the effect of Mn on ERK 1/2 phosphorylation in BGC ($n = 4$). Bergmann glia was treated for 120 min with MnCl_2 (50, 100, 200, or 500 μM). (B) Time course of the response of BGC to Mn treatment over the phosphorylation of ERK 1/2. A repeated-measures analysis of variance (ANOVA) and Dunnett's *post hoc* test was performed ($n = 4$). (C) Glutamate transport and Mn mediated ERK 1/2 phosphorylation. Co-exposure of MnCl_2 200 μM and Aspartate 60 μM for 15 min. A repeated-measures analysis of variance (ANOVA) and Bonferroni's *post hoc* test was performed. Control (Ctrl), Glutamate (Glu) 1 mM (15 min), and Aspartate (Asp) 60 μM (15 min) were used as a positive control ($n = 3$). A representative blot is presented below each graph. Data are expressed as the mean \pm SD of at least three independent experiments. Statistically significant differences are indicated by n.s. not significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

proliferation, and survival, its phosphorylation is induced by Mn in different models (Bryan and Bowman, 2017; Peres et al., 2015), it was of our interest to elucidate the mechanisms triggered by Mn specifically in the PI3K/Akt pathway and its possible involvement in the translation process through the activation of effector molecules downstream of Akt. We demonstrate here that Mn treatment induces Ser⁴⁷³ Akt phosphorylation rapidly in BGC (Figure 2). The phosphorylation in the Ser⁴⁷³ residue is known as the regulatory site of Akt activation and this phosphorylation is carried out by mTORC2 (Sarbasov et al., 2005). Akt activation is controlled upstream by a multi-step process that involves PI3K activation, and we precisely observed that Mn-induced Akt activation involves PI3K activation (Figure 3).

The PI3K/Akt pathway is canonically activated downstream of plasma membrane tyrosine kinase receptors (i.e., growth factor, insulin receptors). Recent studies have shown that Mn exposure increases the Insulin-like growth factor 1 receptor (IGFR)/Insulin receptor (IR) phosphorylation and thus, the described signaling results from a direct Mn effect on these receptors (Bryan and Bowman, 2017). Another possibility is that Mn is indirectly affecting the tyrosine kinase receptors since it is known that the activation of certain membrane receptors is capable of transactivating IGFR/IR. This phenomenon has been largely described for GPCRs and neurotransmitter receptors (Schafer and Blaxall, 2017). Specifically, previous work from our lab has suggested that Glu receptors (α -amino-3-hydroxy-5-methyl-4-isoxazole

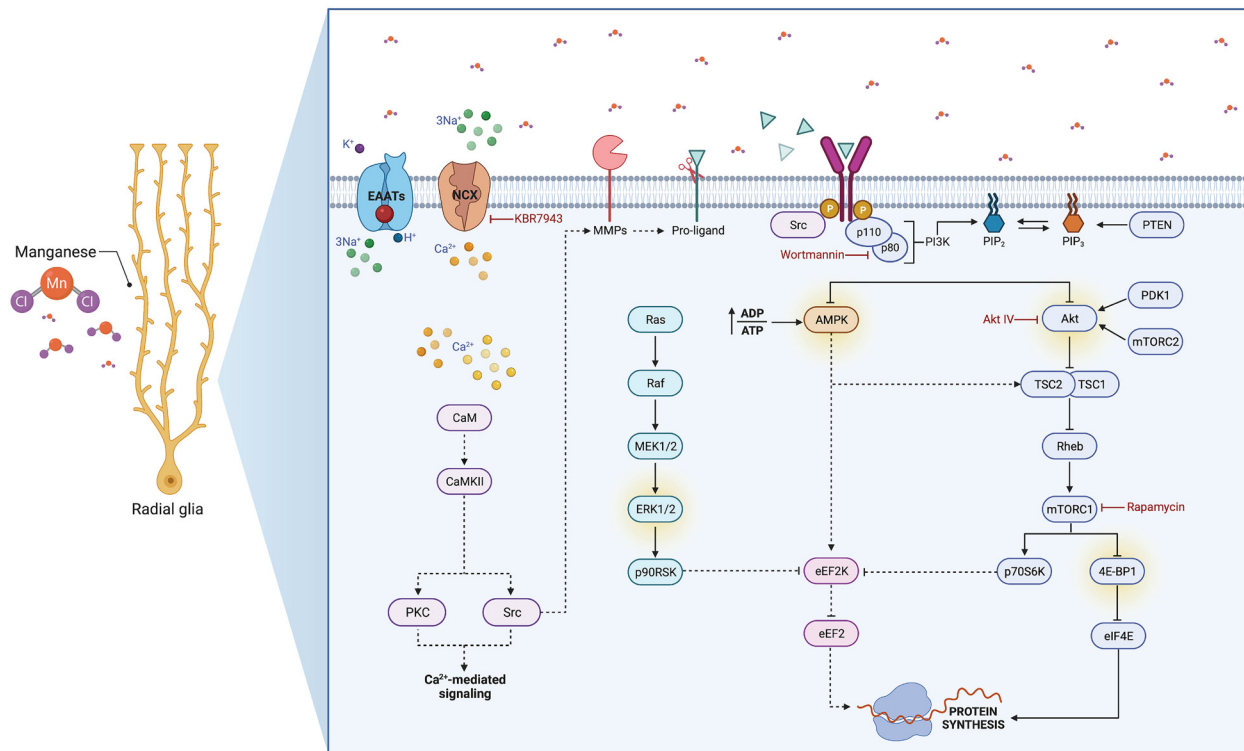


Figure 9. Proposed model for the effects of acute Mn exposure on PI3K/Akt/mTOR/4E-BP1 signaling pathway in BGC. For a detailed description please refer to the discussion section of this manuscript.

propionic acid: AMPA) transactivate IGFR (López-Bayghen et al., 2003). BGC AMPA receptors are Ca²⁺-permeable and are linked to the activation of the Ca²⁺/calmodulin-dependent multiprotein kinase II (CaMKII), the Ca²⁺/diacylglycerol-dependent protein kinase C (PKC), and the non-receptor tyrosine kinases (i.e., Src), that result in augmented tyrosine phosphorylation and RTK activation. On the other hand, the same Ca²⁺ influx may induce the activation of proteases such as matrix metalloproteases (MMPs) and/or A Disintegrin and Metalloprotease (ADAM), which cleave and release agonists that activate their respective receptors (Di Liberto et al., 2019). Src also plays a critical role in the activation of MMP and ADAM (Watson et al., 2016), which makes it an interesting protein to analyze due to its plausible involvement in Mn-mediated IGFR/IR transactivation.

We have previously demonstrated that the co-transport of Glu and Na⁺ leads to the activation of NCX (López-Colomé et al., 2012; Martínez-Lozada et al., 2011). The activation of this exchanger leads to Ca²⁺ influx driven by the Glu transport increased Na⁺ levels in BGC. The increase in intracellular Ca²⁺ is linked to the mTORC1 activation (López-Colomé et al., 2012; Martínez-Lozada et al., 2011). The present work demonstrates that NCX is involved in the Mn-induced Akt phosphorylation (Figure 4). Moreover, recent studies have indicated that Mn transport into the CNS cells may be influenced by Ca²⁺ transport and some of its downstream signaling proteins

(Ijomone et al., 2019). Taking into consideration that Mn exposure increases Ca²⁺ influx and knowing that the mitochondrial Ca²⁺ uniporter (MCU) is capable of Mn transport (Wettmarshausen et al., 2018), this metal may change the morphology and integrity of the mitochondrial plasma membrane disrupting the respiratory chain. Therefore, Mn-dependent mitochondrial damage could lead to impairment in protein synthesis because this process is the most energy-consuming cell function. In this context, the results presented in panel C of Figure 5, might appear to be contradictory at first sight, meaning that one would expect a sharp decrease in the translation process after Mn exposure, and in fact, an increase in [³⁵S]-Methionine polypeptide incorporation was found for up to 60 min. This response could be linked to a cellular effort to overcome the metal insult, and in fact matches with the time course of the Mn-triggered activation of the PI3K/Akt/mTORC1/4EBP1 cascade and the final AMPK phosphorylation. It is tempting to speculate that some of the proteins synthesized in this period represent the cell defense against MnCl₂ exposure, clearly, other experiments that are beyond the scope of this communication have to be done to gain insight into this paradox.

AMPK is an energy-sensing kinase that promotes catalytic processes that favor the replenishment of ATP stores in the energy-deprived cells while concomitantly shutting down anabolic processes, such as protein synthesis (Herzig and Shaw, 2018). We demonstrate here that short-term Mn exposure increases AMPK phosphorylation as early as 30 min of

exposure (Figures 6 and 7). The Thr¹⁷² AMPK phosphorylation is its hallmark of activation, that can disrupt translation by inhibiting the mTORC1 signaling (Ke et al., 2018). mTOR plays a pivotal role in the regulation of diverse aspects of cellular physiology such as energy metabolism, cell growth, and differentiation as well as protein synthesis. AMPK inhibits mTOR by activating TSC2, which is a signaling intermediate between Akt and mTOR. 4E-BP1 is a downstream target of mTOR, its Thr⁷⁰ phosphorylation prevents the binding of 4E-BP1 to eIF4E (Musa et al., 2016). In our model of BGC, we show that after the treatment with Mn there is an increase in 4E-BP1 phosphorylation (Figure 5A) that is dependent on the activation of mTOR (Figure 5B) that matches with an increase in [³⁵S]-Methionine incorporation into newly synthesized polypeptide (Figure 5C). The increase in 4E-BP1 phosphorylation enhances mRNA translation (Qin et al., 2016). Although phosphorylation of mTOR in the Ser²⁴⁴⁸ residue has been used as an indicator of its activation, the usefulness of this measurement has been a controversial topic since the mutation of Ser to Ala does not affect the mTOR-induced 4E-BP1 phosphorylation (Figueiredo et al., 2017). Interestingly, pharmacological activation of AMPK has shown to decrease phosphorylation of Akt, mTOR, S6K, and 4E-BP1 (Gwinn et al., 2008), which are indicative of suppressed protein translation. At this point is important to mention that the time frame for the activation of AMPK overlaps with the decrease in the phosphorylation of 4E-BP1 as well as Akt after the 20 min of treatment, although [³⁵S] methionine incorporation is still above control levels, nevertheless it is remarkable the inversely proportional nature of these signaling pathways upon Mn. This data highlights the transient effect in glial translational control in response to the exposure to this metal, as it has been shown for other neurotoxicants (Flores-Méndez et al., 2013, 2014; Rodríguez-Campuzano et al., 2020).

At this point, we show herein that Mn affects translation through a signal transduction cascade that changes the phosphorylation patterns of 4E-BP1 *via* the Ca²⁺/PI3K/Akt/mTOR pathway and AMPK, with the possible involvement of ERK 1/2 (Figure 8). Interestingly, these same signaling pathways have also an important role in the elongation phase of the protein synthesis (Johanns et al., 2017). The eukaryotic elongation factor 2 (eEF2) is a potential target of Mn exposure (Figure 9), eEF2 is indirectly regulated by mTOR through S6K and ERK 1/2 through RSK. It is the most energy-consuming step in the protein translation process. The phosphorylation of eEF2 at Thr⁵⁶ is carried out by the Eukaryotic Elongation Factor 2 Kinase (eEF2K) (Kenney et al., 2014). While S6K activates eEF2 by phosphorylating and inactivating eEF2K, AMPK induces the activation of eEF2K and the consequent eEF2 deactivation (Johanns et al., 2017). There is another important piece in the translation machinery that we need to take into consideration, the eukaryotic initiation factor 2 (eIF2), the phosphorylation of its alpha subunit (eIF2 α) in Ser⁵¹ prevents the nucleotide exchange of eIF2B and thus the formation of

the ternary complex of the initial translation step (Moon et al., 2018). There are four eIF2 α kinases: general control non-depressible 2 kinases (GCN2), heme-regulated inhibitor kinase (HRI), double-stranded RNA-dependent protein kinase (PKR), and PKR-like endoplasmic reticulum kinase (PERK) (Bond et al., 2020). Evidence has revealed that Mn exposure induces eIF2 α phosphorylation through PERK, activating the autophagic response (Liu et al., 2020). Work in progress in our lab is also aimed at this direction.

In summary, our findings suggest that an altered intracellular PI3K/Akt/mTOR signaling represent an early event in Mn toxicity mechanisms and that protein synthesis is altered by Mn exposure. These findings strengthen the idea of the critical role of glial cells in neurotoxicity processes. A summary of our findings is depicted in Figure 9.

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Author Contribution

JSV and AO conceptualized the experiments. JSV, JSP, and LHK performed the experiments, analyzed data, and prepared the figures. JSV wrote the first draft manuscript. AO supervised, obtained the funding, and wrote the final manuscript. All authors have approved the final manuscript.

Compliance with Ethical Standards

All experiments were performed according to the International Guidelines on the Ethical Use of Animals in Research and approved by the Cinvestav Animal Ethics Committee.



Declaration of Conflicting Interests

The authors declare that they do not have conflicts of interest regarding this article's research, authorship, and/or publication.

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- AMPK Adenosine monophosphate-dependent protein kinase
- BGC Bergmann glial cells
- Ca²⁺ Calcium
- CaMKII Ca²⁺/calmodulin-dependent multiprotein kinase II
- DMSO Dimethyl sulfoxide
- eEF2 Eukaryotic elongation factor 2
- eEF2K Eukaryotic Elongation Factor 2 Kinase
- eIF2 Eukaryotic initiation factor 2
- eIF4E Eukaryotic translation initiation factor 4E
- ERK 1/2 Extracellular signal-regulated protein kinases 1/2
- FBS Fetal bovine serum
- GCN2 General control non-depressible 2 kinases
- GLAST Glutamate/aspartate transporter
- Gln Glutamine
- Glu Glutamate
- GS Glutamine synthetase
- HRI Heme-regulated inhibitor kinase
- IGFR Insulin-like growth factor 1 receptor
- IR Insulin receptor
- JNK c-Jun N-terminal kinase
- MAPK Mitogen-activated protein kinase
- MCU Mitochondrial Ca²⁺ uniporter
- MMP Matrix metalloproteases
- Mn Manganese
- MNC MAPK-interacting kinases
- mTOR Mechanistic target of rapamycin
- MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- NCX Na⁺/Ca²⁺ exchanger
- Opti-MEM Reduced-serum Minimal Essential Medium
- PD Parkinson's disease
- PDK1 Phosphoinositide-dependent kinase 1
- PERK PKR-like endoplasmic reticulum kinase
- PI3K phosphatidylinositol 3 kinase
- PIP₂ Phosphatidylinositol 4,5-bisphosphate
- PIP₃ Phosphatidylinositol-3,4,5-trisphosphate
- PKC Ca²⁺/diacylglycerol-dependent protein kinase C
- PKR Double-stranded RNA-dependent protein kinase
- PMSF Phenylmethylsulphonyl fluoride
- RSK Ribosomal S6 kinases
- RTK Receptor tyrosine kinases
- S6K Ribosomal S6 kinase
- SH2 Src homology domain 2
- SOD Superoxide dismutase
- TSC2 Tuberosclerosis complex 2

List of Abbreviations

- 4E-BP1 Eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1
- ADAM A Disintegrin and Metalloprotease
- Akt Protein kinase B
- AMPA α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor