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Manganese induced ROS and AChE variants alteration leads to SN56 basal forebrain cholinergic neuronal loss after acute and long-term treatment

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ABSTRACT

Manganese (Mn) induces cognitive disorders and basal forebrain (BF) cholinergic neuronal loss, involved on learning and memory regulation, which could be the cause of such cognitive disorders. However, the mechanisms through which it induces these effects are unknown. We hypothesized that Mn could induce BF cholinergic neuronal loss through oxidative stress generation, cholinergic transmission and AChE variants alteration that could explain Mn cognitive disorders. This study shows that Mn impaired cholinergic transmission in SN56 cholinergic neurons from BF through alteration of AChE and ChAT activity and CHT expression. Moreover, Mn induces, after acute and long-term exposure, AChE variants alteration and oxidative stress generation that leaded to lipid peroxidation and protein oxidation. Finally, Mn induces cell death on SN56 cholinergic neurons and this effect is independent of cholinergic transmission alteration, but was mediated partially by oxidative stress generation and AChE variants alteration. Our results provide new understanding of the mechanisms contributing to the harmful effects of Mn on cholinergic neurons and their possible involvement in cognitive disorders induced by Mn.

1. Introduction

Manganese (Mn) is a trace element used to produce fertilizers, gasoline additives, batteries, steel, ceramics, pigments, welding metals and pesticides (Adedara et al., 2017; Burton and Guilarte, 2009; Rivera-Mancía et al., 2011). It is also an essential element, playing an important role in normal growth, development, and cellular homeostasis in both animals and humans (Adedara et al., 2017; Rivera-Mancía et al., 2011). Exposure to excessive amounts of Mn happens through contaminated food and water intake; inhalation in occupational settings including mining, dyes, dry cell battery manufacturing and agriculture products (Adedara et al., 2017; Burton and Guilarte, 2009; Montes et al., 2008); or through environmental exposure to Mn-containing fuel additive, methylcyclopentadienyl manganese tricarbonyl (Burton and Guilarte, 2009).

Elevated brain Mn levels associated mainly with repeated exposure, but also acute exposure (Verhoeven et al., 2011; Montes et al., 2008) to excessive amounts of Mn results in development of a permanent neurodegenerative disorder, known as "manganism", which includes cognitive disorders among other toxic effects (Lebda et al., 2012; Aschner et al., 2007; Chang et al., 2010). In this regard, Mn significantly impairs learning and memory processes in exposed rats or non-human primates (Blecharz-Klin et al., 2012; Liang et al., 2015; Schneider et al., 2013). Moreover, epidemiological studies have reported that Mn also produces cognitive disorders, inducing learning and memory processes dysfunction in humans (Carvalho et al., 2014; Liang et al., 2015). However, the molecular mechanism of Mn-induced cognitive deficits remains unclear.

Abbreviations: Ach, acetylcholine; AChE, acetylcholine esterase; CHT, high-affinity choline transporter; ChAT, acetylcholine transferase; VAChT, vesicular acetylcholine transporter; DMSO, dimethylsulphoxide; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; BSA, bovine serum albumin; iso-OMPA, tetraisopropylpyrophosphoramide; PBS, phosphate-buffered saline; Mn, manganese; AD, Alzheimer's disease

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Mn could be accumulated in different brain regions, including basal forebrain (Tjälve et al., 1996). In this regard, Tjälve et al. (1996) reported that 1 day after intranasal injection of MnCl2 in male rats, the olfactory bulb contained 90% of the Mn administered, while the basal forebrain contained 6% of it. However, Mn concentrations of manganese in the basal forebrain increased to 21 and 28% of the total measured at 3 and 7 days post-dosing, respectively. One of the most important central cholinergic regions is basal forebrain region (Voytko, 1996), since cholinergic neurons project their axons throughout the hippocampal formation and the neocortex regulating learning and memory processes (Everitt and Robbins, 1997; Ward and Hagg, 2000). Li et al. (2015) described that Mn exposure induced learning and memory process alteration and basal forebrain cholinergic neuronal loss. Degeneration of cholinergic neurons, observed in Alzheimer's disease (AD) and other neurodegenerative diseases, results in memory deficits attributable to loss of cholinergic modulation of synaptic circuits (Scheiderer et al., 2006). In fact, the degree of cholinergic cell loss strongly correlates with severity of memory deficit (Bierer et al., 1995). In addition, cholinergic transmission displays an essential role in regulating vital functions, including learning and memory processes (Mesulam et al., 2002). Mn has been described to alter acetylcholinesterase (AChE) activity and disrupt cholinergic transmission (Babadi et al., 2014; Chtourou et al., 2012; Fernsebner et al., 2014; Lu et al., 2014). Thus, alteration of cholinergic transmission or cholinergic neuronal loss in this region could be related with Mn impairment of memory function among other actions (Andersson et al., 1997).

The mechanism through which Mn induces basal forebrain cholinergic cell death is unknown. In this regard, Mn induces reactive oxygen species (ROS) formation and lipid peroxidation, which can initiate apoptosis and/or necrosis in several tissues (Lebda et al., 2012; Orrenius et al., 2007). Acethylcholine (Ach) plays a role in cell survival through cholinergic receptor activation (Resende and Adhikari, 2009), and a reduction on its levels could increase cell death. In addition, it has also been proven that AChE overexpression, in particular AChE-S variant, results in increased apoptosis (Yang et al., 2002) and necrosis (Del Pino et al., 2016). Cells which otherwise do not express AChE, do so when entering apoptosis (Zhang et al., 2002). Mn has been reported to alter cholinergic neurotransmission and induce AChE overexpression (Lu et al., 2014), which could induce the cell death observed in basal forebrain cholinergic neurons.

According to all exposed, we hypothesized that Mn could induce cell death after acute and long-term exposure, on basal forebrain cholinergic neurons, through cholinergic transmission and AChE variants alteration and oxidative stress induction. The present work intends to study the Mn effect on basal forebrain cholinergic neuronal viability and the cholinergic mechanisms implicated on it, due to the importance of this effect to explain cognitive disorders induced by Mn. To reach this aim we have treated with different Mn concentrations and the antioxidant N-acetylcysteine (NAC), for 24 h or repeatedly for 14 days, wild type or transfected with siRNA for AChE SN56 cells from basal forebrain as an *in vitro* model of cholinergic neuronal cells from this region.

2. Materials and methods

2.1. Chemicals

We obtained manganaese chloride (99.99%), acetylcholine, tetraisopropylpyrophosphoramide (iso-OMPA), acetylthiocholine, dithionitrobenzoic acid, poly-L-lysine, dimethyl sulfoxide (DMSO), dibutyryl-cAMP, retinoic acid, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) from Sigma (Madrid, Spain). [^{14}C] acetyl-CoA was obtained from Perkin Elmer (Madrid, Spain). All other chemicals were reagent grade of the highest laboratory purity available.

2.2. Culture of SN56 cells

We used SN56 cells, a cholinergic murine neuroblastoma cell line derived from septal neurons (Hammond et al., 1990), as a model of cholinergic neurons from basal forebrain to evaluate Mn toxic effects on this specific type of neurons and the mechanisms through which they are induced. The cells were maintained at 37 °C and 5% CO2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, 2 mM L-glutamine (Sigma, Madrid, Spain), and 1 mM sodium pyruvate. Medium was changed every 48 h (Hudgens et al., 2009). We differentiated cells by culturing for 3 days with 1 mM dibutyryl-cAMP and 1 μM retinoic acid as previously described, which produces morphological maturation and 3–4-fold increase of acetylcholine transferase (ChAT) activity and acetylcholine level in the cells (Bielarczyk et al., 2003; Szutowicz et al., 2006). Differentiated cells have been reported to be more sensitives to neurotoxic compounds that affect cholinergic pathways (Bielarczyk et al., 2003; Szutowicz et al., 2006).

In order to determine the cellular Ach, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content, ChAT and AChE activities, AChE splice variants, high-affinity choline (CHT), ChAT and vesicular acetylcholine transporter (VAChT) gene expression, and AChE gene knockdown effects, cells were seeded in 6-well plates at a density of 10^6 cells/well. Cells were treated for 24 h or for 14 days with Mn in concentrations between 1 μM and 200 μM with or without ACh (10^{-8} to 10^{-4} M) and with or without NAC (1 mM). At least 3 replicate wells/treatment were used. A vehicle group was employed in parallel for each experiment as a control.

Previous studies have described that physiological concentration of Mn in the human brain are between 5.32 and 14.03 μg Mn/g protein (20.0–52.8 μM). However, pathophysiological threshold were estimated between 15.96 and 42.09 μg Mn/g protein (60.1–158.4 μM) (Bowman and Aschner, 2014). According to this information, we chose a range of concentration between physiological and toxicological threshold to study Mn cholinergic transmission disruption and cytotoxicity. Furthermore, we chose Mn 50 μM (MTT and caspases activity tests) and 100 μM (LDH test) concentrations, which were the lowest concentration observed to reduce cell viability in the present study after acute exposure, to study the mechanisms through which Mn decreases cell viability.

2.3. Measurement of cell viability (MTT assay)

SN56 cells viability was measured by MTT after 24 h and 14 days Mn treatment. The assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenase. Cells were incubated with 100 μL of yellow MTT solution (final concentration 0.5 mg/mL) for 4 h after treatment with Mn. After 4 h at 37 °C, the medium was removed and the formazan reaction product was dissolved in 250 μL DMSO. The formation of solubilized formazan product was measured spectrophotometrically at 570 nm (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, ThermoFisher Scientific, Madrid, Spain). Control cells treated with vehicle were taken as 100% viability.

2.4. Lactate dehydrogenase (LDH) assay

Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, Madrid, Spain), which measures the LDH released into the culture medium was used according to the manufacturer's instructions to assess the extent of cell death. Briefly, after removal, culture medium was pipetted into 96-well plates. The Master Mix reagent was added, and after 3 min colorimetric intensity was determined at 450 nm over every 5 min using a microplate spectrophotometer (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain).

2.5. Caspase activity analysis

After treatment with indicated concentrations of Mn or vehicle (solvent control), the presence of apoptotic SN56 cells was assessed by determining caspase activation using Caspase-Glo 3/7 luminescence assay kits (Promega, Madrid, Spain), according to the manufacturer's protocol. In brief, at the end of treatment, culture cells were washed with phosphate-buffered saline (PBS) and the cells were scraped and collected in a microfuge tube in dark. Equal volumes of reagent and cell lysis buffer were added to a white-walled 96-well plate and incubated at room temperature in dark for 1 h and the resultant luminescence was read in a Perkin Elmer LS50B plate-reading iluminometer (Perkin Elmer, Madrid, Spain). The luminescence of each sample was measured. The experiments were performed in triplicate.

2.6. Gene knockdown

SN56 cells were transfected with siRNAs in 6-well plates (1×10^6 cells/well) using HiPerfect Transfection reagent according to the manufacturer's instructions (Qiagen, Barcelona, Spain). Two sets of siRNA duplexes (Qiagen, Barcelona, Spain) homologous to mouse AChE sequences for each one were designed using the HiPerformance Design Algorithm (Norvatis AG) and were purchased from Qiagen (catalog numbers GS11423). As a transfection control, an All Stars Negative Control siRNA (Qiagen, Barcelona, Spain) was used. 48 h after transfection, the efficiency of siRNA-mediated AChE knockdown was determined by RT-PCR using primers specific for mouse AChE mRNA (Qiagen, Barcelona, Spain). The effects of AChE knockdown on cell injury were tested by MTT cell viability assay. After 24 h of incubation with the siRNAs, the cells were washed with PBS and incubated for a further 24 h or 14 days in culture medium with or without Mn.

2.7. Determination of AChE activity

The AChE activity was determined after 24 h and 14 days exposure to Mn, using standard Ellman's thiocholine technique (Ellman et al., 1961) with minor modifications (Hartl et al., 2011; Zimmermann et al., 2008) and normalized against total protein. Briefly, the supernatant cell lysate (10 μL) was pipetted into a 96-well microtiter plate which contained Ellman buffer as well as iso-OMPA, (final concentration 100 μM). The kinetic assay was initiated by addition of acetylthiocholine and dithionitrobenzoic acid (1 mM and 500 μM final concentrations, respectively). Absorbance was read using a plate reader (412 nm). All samples were run in triplicate. AChE activity was calculated as nmol/min/mg protein and presented as percent untreated control. Butyrylcholinesterase activity was inhibited by iso-OMPA.

2.8. Choline acetyltransferase activity measurement

ChAT catalyzes the transfer of an acetyl group from the coenzyme, acetyl-CoA, to choline yielding Ach. Control and treated cultures (from 6 well plates per sample) were washed with PBS, scraped in 100 μL cold homogenizing buffer (40 mM sodium phosphate buffer, pH 7.4, containing 200 Mm NaCl and 0.5% TritonX-100) and then homogenized by sonication (Zheng et al., 2002). ChAT activity was measured in the cell homogenates by a modification of the radio-enzyme assay of Fonnum, (1975), which involves the incorporation of [^{14}C] acetyl-CoA into Ach, as described before (Mennicken and Quirion, 1997; Zheng et al., 2002). Radioactivity corresponding to the reaction product ([^{14}C] Ach) was measured by organic liquid scintillation counting. All ChAT activity values, obtained in triplicate for each sample, were expressed as pmol Ach synthesized/h/mg protein.

2.9. Acetylcholine level measurement

A commercial colorimetric/fluorimetric kit (Abcam, Cambridge, UK) (Reale et al., 2012) was applied to detect the ACh release in culture medium. Briefly, culture medium was collected after 24 h and 14 days Mn treatment and spun at 800 × *g*. Subsequently, the supernatant was lyophilized, and reconstituted in 50 μL Choline Assay Buffer, and stored at −80ΊC until further analysis. 50 μL of the sample were mixed with 50 μL of reaction solution including choline assay buffer, choline probe, enzyme mix and AChE according to the instructions. Each sample was assayed in triplicate, and the whole experiment was carried out 3 independent times. The level of Ach (pmol/well) was calculated by plotting the fluorescence of each sample in relation to choline standard curve. The measurement of the fluorescence was determined at λ Ex/Em 535/587 nm.

2.10. Protein determination

At the end of the treatments, SN56 cells were washed with prechilled PBS, collected by scrapping, and lysed using RIPA buffer (Thermo Scientific, Madrid, Spain) with freshly added protease inhibitors cocktail (ThermoFisher Scientific, Madrid, Spain). After centrifugation at 10,000 × *g* for 10 min at 4 °C, cell lysate supernatant was collected. Protein concentration was assayed using a BCA kit (ThermoFisher Scientific, Madrid, Spain) and normalized.

2.11. Real-time PCR analysis

Total RNA was extracted using the Trizol Reagent method (Invitrogen, Madrid, Spain). The final RNA concentration was determined using a spectrophotometer Nanodrop 2000 (Thermo Fisher Scientific, Madrid, Spain) and the quality of total RNA samples was assessed using an Experion Lab Chip (Bio-Rad, Madrid, Spain) gel. First-strand cDNA was synthesized with 1000 ng of cRNA by using a PCR array first strand-synthesis kit (C-02; Super Array Bioscience, Madrid, Spain) following the manufacturer's instructions and including a genomic DNA elimination step and external RNA controls. After reverse transcription, we performed QPCR using prevalidated primer sets (SuperArray Bioscience) for mRNAs encoding VAChT (PPM04178), CHT (PPM31807A), AChE (PPM35356A), and ACTB (PPM02945B). Primers for both AChE isoforms R and S (Table 1) were taken from Shaltiel et al. (2013). We used ACTB as an internal control for normalization. Reactions were run on a CFX96 using Real-Time SYBR Green PCR master mix PA-012 (SuperArray Bioscience). The thermocycler parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 72 °C for 30 s. Relative changes in gene expression were calculated using the Ct (cycle threshold) method. The expression data are presented as actual change multiples (Livak and Schmittgen, 2001).

We chose ChAT, VAChT, CHT and AChE genes to determine the effect of Mn on cholinergic transmission, because Ach neurotransmitter is synthesized by the enzyme ChAT (Oda, 1999) and CHT mediates the presynaptic high-affinity choline uptake as the rate-limiting step in Ach synthesis (Bazalakova and Blakely, 2006). VAChT is responsible for the transportation of acetylcholine from the cytoplasm into the synaptic vesicles which is a limiting step in Ach release (Oda, 1999). Finally, AChE breaks down Ach, which terminates the neurotransmission process (Ballard et al., 2005).

Table 1
Primers used for quantitative real-time PCR analyses.

Abbreviation	Gene	Reference	Forward (F) and reverse (R) primers
AChE-S	Acetylcholinesterase	Shaltiel et al. (2013)	F-ctgaacctgaagcccttagagR-ccgcctcgtccagagtat
AChE-R	Acetylcholinesterase	Shaltiel et al. (2013)	F-gagcagggaatgcacaag R-ggggaggtaaagaagagag

2.12. Measurement of hydrogen peroxide levels

Hydrogen Peroxide (H_2O_2) is one of the reactive oxygen species produced under oxidative stress conditions. H_2O_2 content was measured using an using a hydrogen peroxide assay kit (Abcam, Cambridge, UK) according to the manufacturer's instruction. In brief, after 24 h and 14 days treatment with Mn with SN56 cells were harvested in H_2O_2 assay buffer and then centrifuged for 15 min at 1000 × *g*. A total of 50 μL of the supernatant was mixed with 50 μL of the reaction mix (assay buffer: 46 μL; OxiRed Probe: 2 μL; horse radish peroxidase (HRP): 2 μL) and then incubated at room temperature for 10 min. The optical density at 570 nm was read with a microplate reader (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain), and the H_2O_2 concentration was calculated according to a standard concentration curve. Hydrogen peroxide content in the samples was expressed in nanomole per milliliter and presented as percent untreated control.

2.13. Lipid peroxidation assay

Malondialdehyde (MDA) concentration was determined as an indicator of lipid peroxidation products. Intracellular MDA production was quantified after 24 h and 14 days exposure to Mn, using a Lipid Peroxidation MDA Assay Kit (Abcam, Cambridge, UK) following the manufacturer's protocol. Briefly, after treatment, 1×10^6 cells were collected and homogenized on ice in MDA lysis buffer (300 μL) with 3 μL butylated hydroxytoluene (BHT) (100X), centrifuged for 10 min at 13,000 × *g* to remove insoluble material. Sample (200 μL) or standard (200 μL of MDA) was mixed with 600 μL of thiobarbituric acid (TBA) solution, incubated at 95 °C for 50 min and cooled to room temperature in an ice bath for 10 min. Each sample and standard (200 μL) was loaded (duplicate) into a clear 96-well plate and the absorbance at 532 nm was recorded using a microplate reader (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain). Concentration of malondialdehyde determined as nmol/mg protein is presented as percent untreated control.

2.14. Protein oxidation assay

Carbonylation of protein was measured as an indicator of oxidative damage. The assessment of carbonyl formation was done based on the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) leading to the formation of stable dinitrophenyl (DNP) hydrazone adducts. Protein carbonyl contents was quantified after 24 h and 14 days exposure to Mn, using a Protein Carbonyl Content Assay Kit (Abcam, Cambridge, UK) following the manufacturer's procedures. Briefly, after treatment, 1×10^6 cells were collected and homogenized on ice in lysis buffer (100 μL), the lysates were derivatized with DNPH. Each sample and standard (100 μL) was loaded (duplicate) into a clear 96-well plate and the absorbance was recorded at 370 nm, using a microplate reader (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain). Protein carbonyl contents determined as nmol/mg protein is presented as percent untreated control.

2.15. Statistical analysis

At least three replicates for each experimental condition were performed, and the presented results were representative of these replicates. Data are represented as means ± standard deviation (SD). Comparisons between experimental and control groups were performed by Student's t (data analyzed in Fig. 2) and two-ways ANOVA analyses (gene manipulation vs treatment) or one-way ANOVA analyses (analysis of different Mn concentrations) followed by the Tukey post-hoc test. Statistical difference was accepted when $p \leq 0.05$. Statistical analysis of data was carried out by computer using GraphPad software.

3. Results

3.1. Effect of Mn on SN56 cell viability

We used the MTT test to evaluate cell survival after 24 h and 14 days exposure to Mn at increasing concentrations. MTT test after 24 h and long-term cell culture incubation with Mn showed, from 50 μM to 25 μM concentrations, respectively, a clear concentration-dependent reduction in cell viability compared with vehicle-treated cells (control negative) (Fig. 1A). After acute and long-term Mn exposure, the culture co-treatment with Ach (10^{-8}-10^{-4} M), did not induce a significant effect on cell death induced after Mn treatment alone (data not shown). However, after Mn treatment of SN56 AChE silenced cells or after antioxidant co-treatment of wild type or AChE silenced cells, an amelioration in the reduction in cell viability was observed (Fig. 1B). This reduction of cell viability decrease was higher after antioxidant co-treatment of AChE silenced cells. Transfection of cells with control siRNA or AChE siRNA showed no effect on cell viability (Fig. 2A). There was no significant difference between data of vehicle-treated cells and control cells.

The results obtained with the MTT assay were confirmed by the LDH determination. LDH is released from cells because of the loss of plasma membrane integrity and is indicative of necrotic cell death mechanism. An increased LDH release to the medium was observed in SN56 cells after 24 h and after long-term cell culture incubation with Mn, from 100 μM to 50 μM concentrations, respectively, compared with vehicle-treated cells (Fig. 3A). After Mn treatment of SN56 AChE silenced cells or after antioxidant co-treatment of wild type or AChE silenced cells, an amelioration in the LDH release was observed (Fig. 3B). This reduction of LDH release decrease was higher after antioxidant co-treatment of AChE silenced cells.

3.2. Effect on the activity of caspases 3/7

Mn activated the caspases in SN56 cells after 24 h and long-term treatment in a concentration-dependent manner from 50 μM to 25 μM concentrations, respectively, compared with control group (Fig. 4A). After 24 h and long-term treatment with Mn of AChE silenced cells, or after antioxidant co-treatment of wild type or AChE silenced cells, the activation of caspases 3/7 observed after Mn treatment alone was attenuated (Fig. 4B). This reduction of caspases 3/7 activation was higher after antioxidant co-treatment of AChE silenced cells.

3.3. Acetylcholine level measurement

Ach levels were assessed in culture cell supernatant after 24 h and long-term exposure to Mn at increasing concentrations. Ach levels were significantly decreased after 24 h and 14 days Mn exposure from 25 μM to 1 μM concentrations, respectively, compared with control group (Fig. 5A).

3.4. Determination of enzymatic activity

The AChE and ChAT activity after 24 h and long-term exposure Mn at increasing concentrations was assessed in SN56 cell lysates. A significant increase in AChE activity was observed from 25 μM to 1 μM after 24 h and 14 days exposure, respectively (Fig. 5B). Mn also induced a significant increase of ChAT activity after 24 h and 14 days treatment in a concentration-dependent manner from 200 μM to 1 μM concentrations, respectively, compared with control group (Fig. 5C). The Mn effect on ChAT activity could be masked by cell loss, but as the results were normalized with the concentration of proteins, the reduction of ChAT activity seems to be due to a direct effect of Mn on this enzyme.

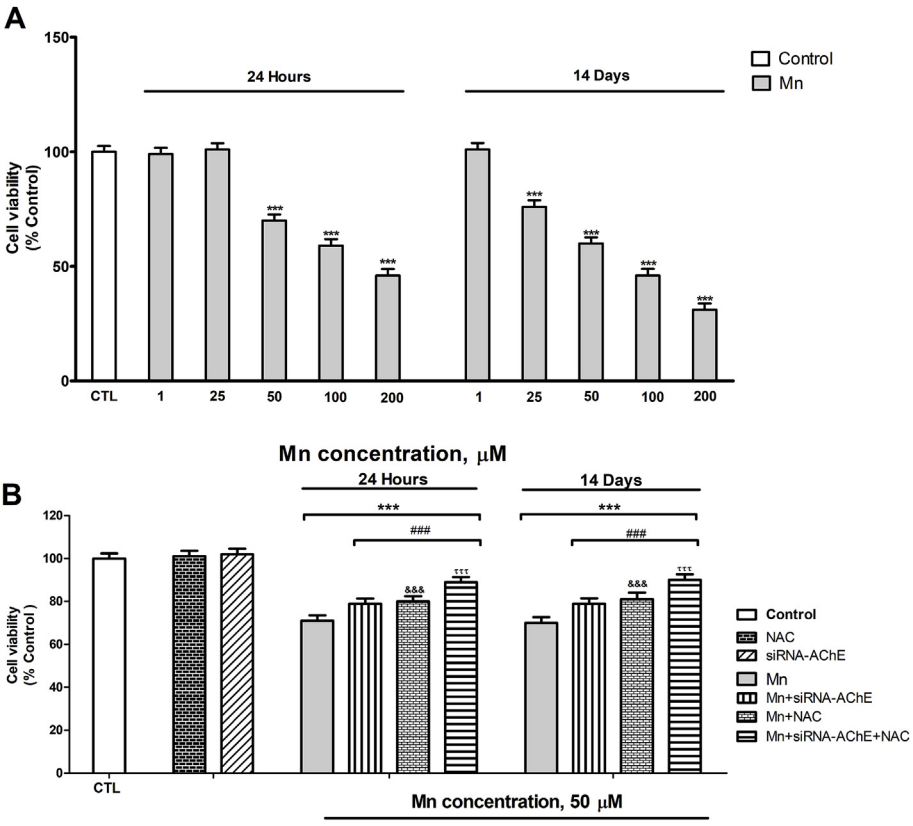

Fig. 1. Mn effect on cell viability of SN56 cells was determined by MTT assay. (A). Mn (1–200 μM) effect on cell viability. (B) Mn (50 μM) effect on cell viability of SN56 wild type or AChE silenced cells co-treated with or without NAC (1 mM). Cell viability was measured as MTT reduction (ordinate) and data were normalized as % control (CTL, white column). Data represents the mean ± SD of three independent experiments in triplicate. $^{***}p < 0.001$ compared to control. $^{\#\#\#}p \leq 0.001$ compared to Mn treatment. $^{\&\&\&}p \leq 0.001$ compared to AChE silenced cells treated with Mn. $^{\tau\tau\tau}p \leq 0.001$ compared to cells co-treated with NAC and Mn.

3.5. Real-time PCR analysis

After incubation for 24 h and long-term with different concentrations of Mn in SN56 cells, the gene expression of AChE-S and AChE-R variants were significantly induced in a concentration-dependent way (Fig. 6A and B). Moreover, the expression of CHT was reduced after 24 h and long-term exposure to Mn from 100 μM to 1 μM concentrations, respectively, (Fig. 6C). However, the expression of ChAT and VAChT were not affected at any concentration and time of exposure (data not shown). Transfection of cells with control siRNA showed no effect on AChE gene expression, but the AChE siRNA caused large reductions in AChE gene expression (Fig. 2B).

3.6. Measurement of hydrogen peroxide levels

H_2O_2 is a reactive oxygen metabolic byproduct that serves as a key regulator of a number of oxidative stress-related states. After 24 h and 14 days Mn treatment, the hydrogen peroxide content increased in a

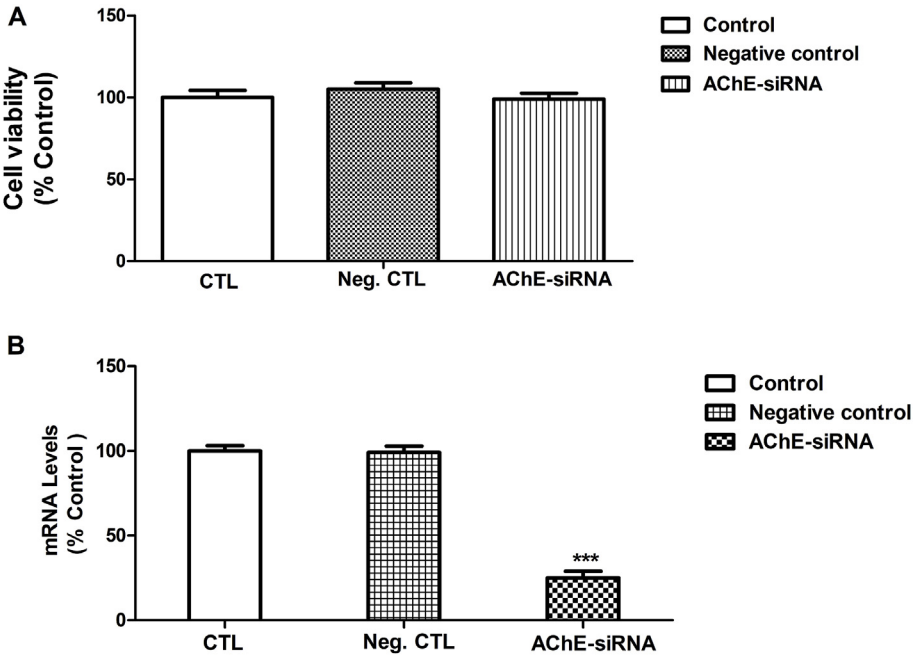

Fig. 2. Down regulation of AChE in SN56 cells and its impact on cell viability and gene expression was determined. Control: SN56 cells transfected without siRNA. Negative (Neg.) control: SN56 cells transfected with scrambled siRNA. AChE-siRNA: transfected with siRNA against AChE. (A) MTT test shows that AChE down regulation did not significantly induce cell damage after 48 h. (B) AChE down regulation could be detected by RT-PCR analysis 48 h after transfection. Values are given as mean ± SD of three separate experiments from cells of different cultures, each one performed in triplicate. $^{***}p \leq 0.001$ compared to control.

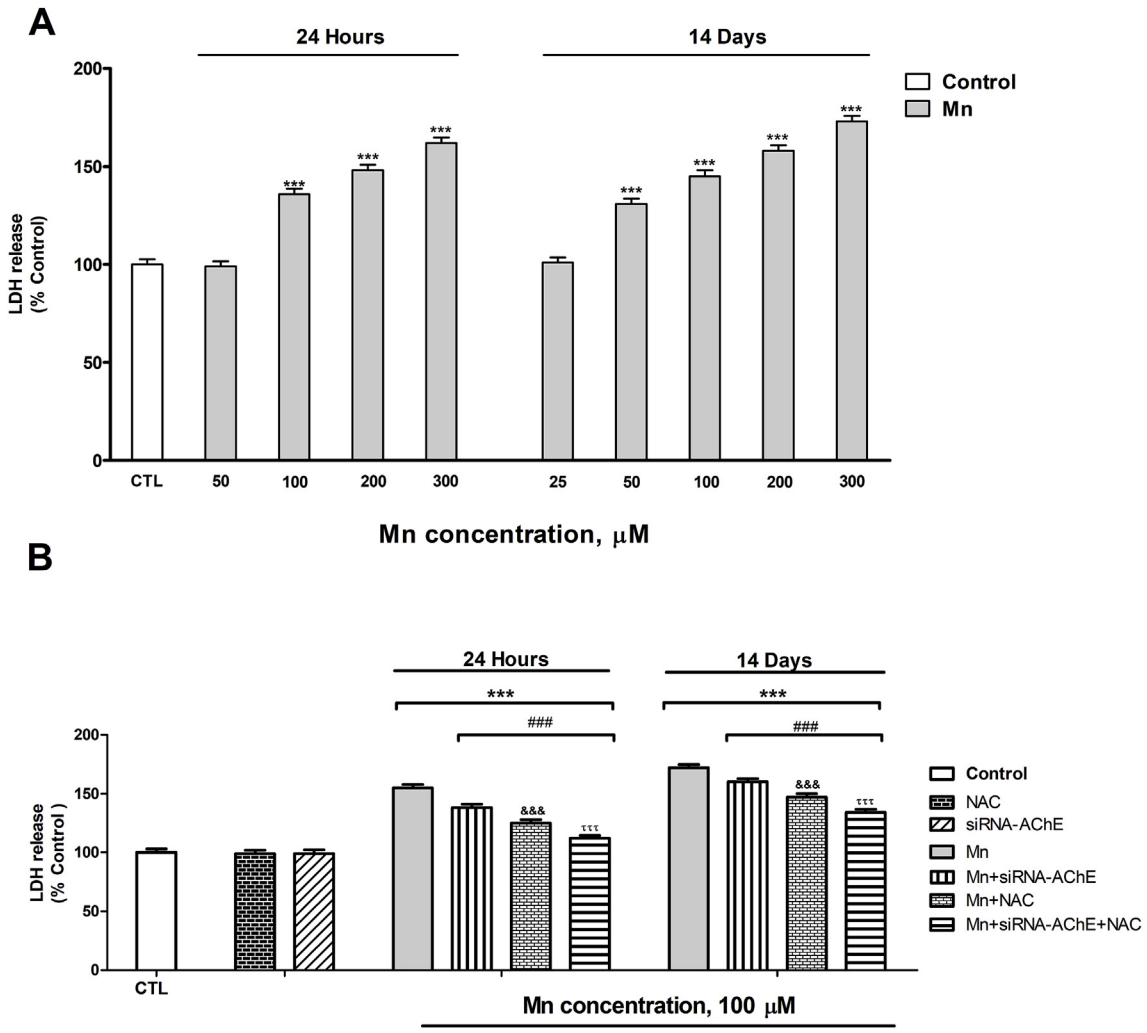

Fig. 3. Mn effect on LDH release in SN56 cells was determined by LDH assay. (A). Mn (1–200 μM) effect on LDH release. (B) Mn (50 μM) effect on LDH release in SN56 wild type or AChE silenced cells co-treated with or without NAC (1 mM). Results are expressed as percentages of LDH release after subtracting the control values. Data represents the mean ± SD of three independent experiments in triplicate. $^{***}p \leq 0.001$ compared to control. $^{\#\#\#}p \leq 0.001$ compared to Mn treatment. $^{\&\&\&}p \leq 0.001$ compared to AChE silenced cells treated with Mn. $^{\tau\tau\tau}p \leq 0.001$ compared to cells co-treated with NAC and Mn.

concentration-dependent manner from 50 μM to 25 μM concentrations, respectively, compared with control group (Fig. 7A). NAC treatment of SN56 cells did not induce an increase in H_2O_2 content and co-treatment with Mn completely attenuated the increase in H_2O_2 content observed after Mn treatment alone (Data not shown).

3.7. Lipid peroxidation and protein oxidation assay

Lipid peroxidation was measured in SN56 cells after 24 h and long-term exposure to Mn at increasing concentrations. Mn treatment induced an increase in MDA and protein carbonyl content in a concentration-dependent manner from 50 μM to 25 μM concentrations after 24 h and 14 days treatment, respectively, compared with control group, indicating the enhancement of lipid peroxidation (Fig. 7B) and protein oxidation (Fig. 7C). Otherwise, NAC treatment of SN56 cells did not did not induce an increase in the MDA and protein carbonyl content (Data not shown). Finally, short- and long-term co-treatment of SN56 cells with Mn and NAC produced a complete attenuation of the increased MDA and protein carbonyl content observed after Mn treatment alone (Data not shown).

4. Discussion

In the present work, we show that manganese induces, after 24 h and long-term exposure, a concentration-dependent reduction of Ach levels from 25 μM to 1 μM, respectively, confirming the cholinergic neurotransmission alteration in SN56 basal forebrain cholinergic neurons. Mn also induced an increase of AChE activity after 24 h (from 25 μM) and after long-term exposure (from 1 μM), which could explain the reduction in the Ach levels observed. In this regard, Mn has been described to increase AChE activity in rat brain after acute and long-term exposure (Babadi et al., 2014; Lu et al., 2014; Chtourou et al., 2012; Fernsebner et al., 2014; Lebda et al., 2012; Liapi et al., 2008; Nadeem et al., 2018), supporting our results. However, other studies has shown that Mn is able to also to inhibit AChE activity (Sitaramayya et al., 1974; Martinez and Bonilla, 1981; Santos et al., 2012). This contradictory effect has previously been suggested to be due to Mn effects in biological systems dependence on dose, route and period of exposure, age, environmental factors and nutritional state (Babadi et al., 2014; Fernsebner et al., 2014; Finkelstein et al., 2007; Peres et al., 2016). Besides, the differences with our results could be due to the species from which SN56 came from for the *in vitro* model used, the concentration used and frequency of exposure, being necessary further experiment to clarify this differences.

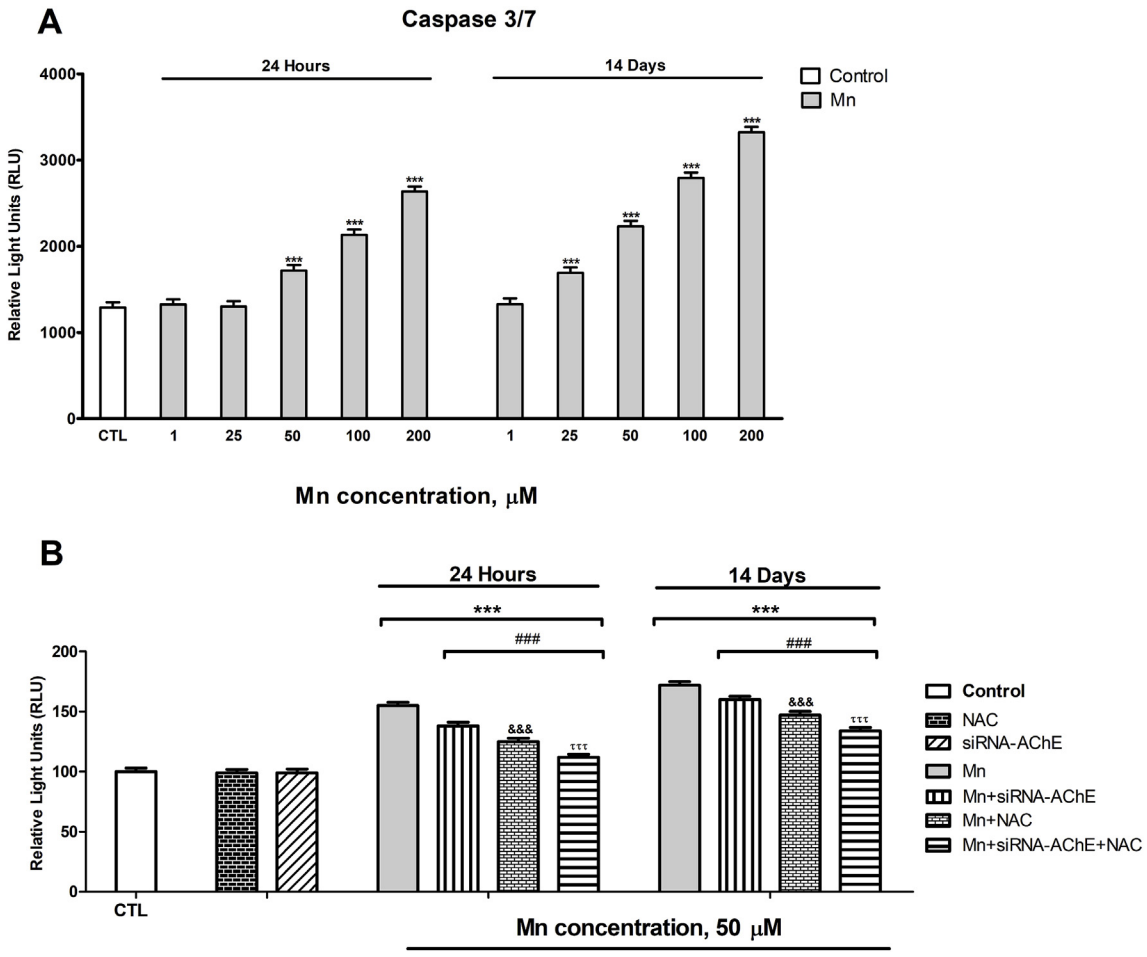

Fig. 4. Analysis of caspases 3/7 activity in SN56 cells after Mn treatment. (A) Analysis of caspases 3/7 activity after Mn (1–200 μM) treatment. Analysis of caspases 3/7 activity in SN56 wild type or AChE silenced cells after Mn (50 μM) treatment with or without NAC (1 mM). Values are expressed as mean ± SD. $^{***}p \leq 0.001$ compared to control. $^{\#\#\#}p \leq 0.001$ compared to Mn treatment. $^{\&\&\&}p \leq 0.001$ compared to AChE silenced cells treated with Mn. $^{\tau\tau\tau}p \leq 0.001$ compared to cells co-treated with NAC and Mn.

In addition, Mn induced a decrease in ChAT activity after 24 h (from 200 μM) and long-term (from 1 μM) exposure, effect that could also mediate the decrease in the Ach levels observed after long-term exposure and after 24 h exposure only from 200 μM concentration. This effect on ChAT activity was previously described in rats brain after long-term exposure (Lu et al., 2014), confirming our results. Moreover, VAChT expression was not affected by Mn exposure, but Mn induced after 24 h (from 100 μM) and after long-term (from 1 μM) exposure a reduction of CHT gene expression, which could also contribute to explain the reduction of Ach levels observed after long-term exposure and after 24 h exposure only from 100 μM concentration. Finally, we cannot rule out that there could also be a decrease in the Ach release that explains the effect observed on Ach levels. In this regard, previous studies have described that Mn presents an inhibitory effect on evoked Ach release in the presynaptic neurons (Finkelstein et al., 2007). Therefore, all these mechanism can induce the alteration of cholinergic transmission observed and could mediate the alteration on cognitive disorders described.

Acute and 14 days Mn exposure also induced, in a concentration-dependent way, ROS generation, lipid peroxidation and protein oxidation from 50 μM to 1 μM, respectively, on septal SN56 cholinergic basal forebrain neurons. In this regard, Mn has been reported to induce ROS generation, lipid peroxidation and protein oxidation after acute and repeated exposure in rat brain (Bahar et al., 2017; Bonke et al., 2016; Chtourou et al., 2012; El-Hady and Galal, 2018; Nadeem et al., 2018), which support our findings. Previous studies have described that the Mn effect on AChE and ChAT activity was mediated through the induction of oxidative stress and the antioxidant treatment reverses this effect (Adedara et al., 2017; Bahar et al., 2017; Lu et al., 2014; Chtourou et al., 2012; Liapi et al., 2008; Nadeem et al., 2018). The oxidative stress induced by Mn could mediate the decrease of ChAT activity through denaturalization of part of the enzymatic pool, but the increase of AChE activity could not be explain through this effect. In this regard, previous studies have shown that ROS could induce AChE activity through production of Aβ proteins (Melo et al., 2003) that have been reported to be produced after Mn exposure (Tong et al., 2014), which could explain this effect.

In addition, NAC co-treatment with Mn reversed completely the induction of oxidative stress. NAC, a potent antioxidant, has been shown to attenuate oxidative stress, recovering cellular redox status after Mn treatment (Wang et al., 2017; Zhu et al., 2016), which supports our results. NAC protective effect may be due to its action as a metal chelator (Jalilehvand et al., 2011), which could inhibit the Mn cellular uptake and its toxics effects. However, NAC co-treatment with Mn only completely reversed ROS generation, lipid peroxidation and protein oxidation induced by Mn treatment alone, while the cytotoxic effects were only partially reduced. Thus, NAC protection seems to be mediated by its antioxidant effect, although the possible reduction of Mn uptake cannot be excluded as a possible contributing mechanism. Otherwise, antioxidant co-treatment with Mn has been reported to reverse cognitive dysfunctions induced by Mn (Adedara et al., 2017; Lu et al., 2014), suggesting this mechanism could mediate the effect

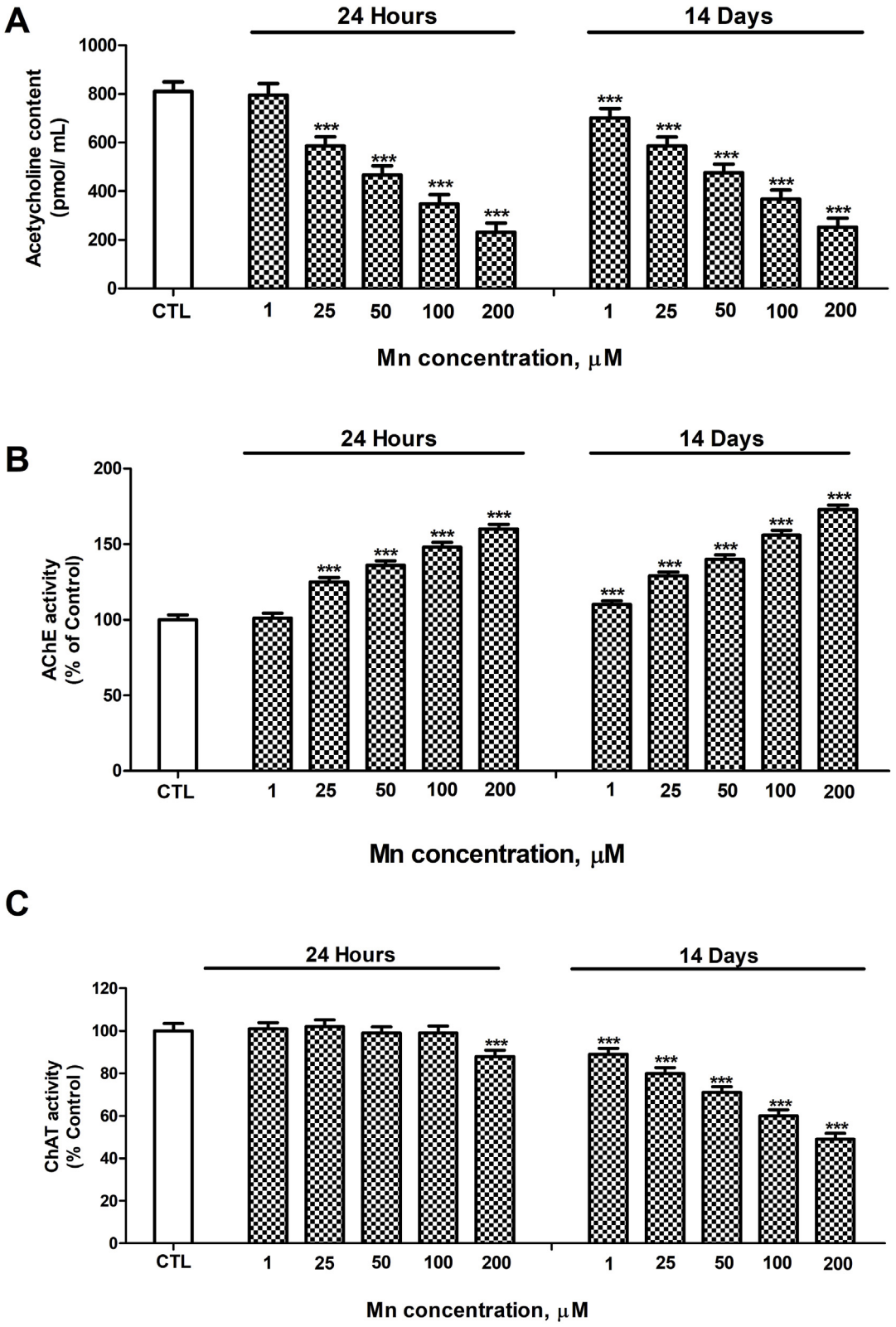

Fig. 5. Effects of different Mn concentrations treatment for 24 h or 14 days on (A) Ach content and (B) AChE and (C) ChAT activity was determined in SN56 cell homogenates. Data represents the mean ± SD of three independent experiments in triplicate. ***p < 0.001 compared to control.

observed after manganese treatment on memory and learning processes.

Otherwise, the present work shows that Mn induces after 24 h (from 50 μM) and 14 days (from 25 μM), concentration-dependent, cell death on SN56 basal forebrain cholinergic neurons. These results are similar to previous works which show that Mn induces cell death after acute and repeated exposure *in vivo* and *in vitro* (Bahar et al., 2017; Gandhi et al., 2018; Nadeem et al., 2018). Mn exposure also activates caspase 3/7 at the same range of concentrations suggesting that apoptosis takes place and confirms cell viability results. In addition, Mn increases LDH

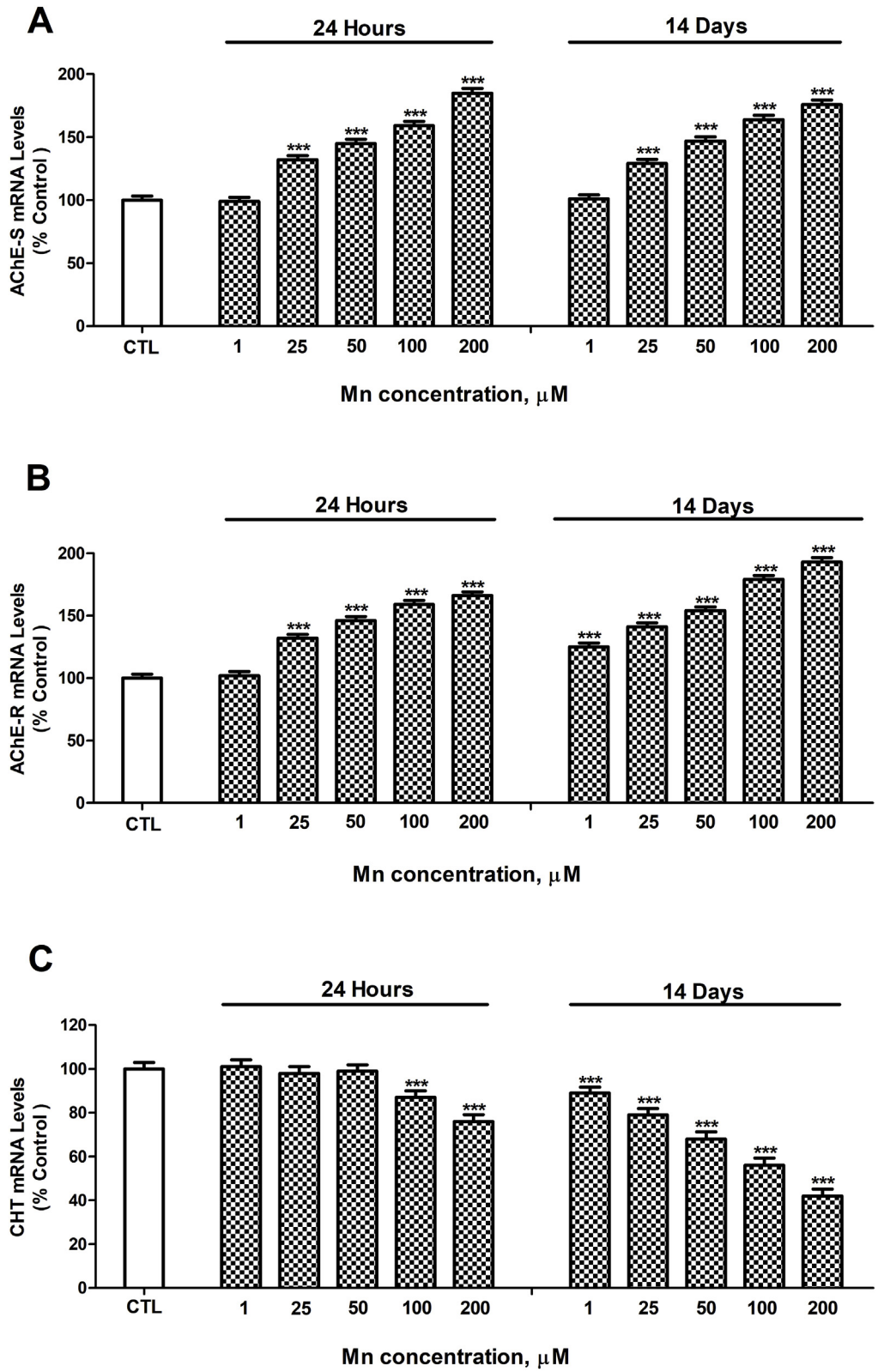

Fig. 6. Shows results from real-time PCR arrays targeting (A) AChE-S, (B) AChE-R and (C) CHT genes after 24 h and 14 days Mn treatment. AChE-S, AChE-R and CHT gene expression was compared with controls [cells treated with vehicle were the negative control]. Each bar represents mean ± SD of 6 samples. Levels were measured using QPCR. ACTB was used as an internal control. ***$p < 0.001$ significantly different from controls.

release, although it happens at higher concentrations, which could be due to necrosis taking place always at higher concentrations than apoptosis. Mn has been reported to induce cell death through apoptosis and necrosis (Bahar et al., 2017; Orrenius et al., 2007), which support our results. Previously, it has been described that Ach plays a role in cell survival through cholinergic receptor activation (Resende and Adhikari, 2009), and a reduction on its levels could increase cell death, being this a possible mechanism that could also mediate the cell death observed

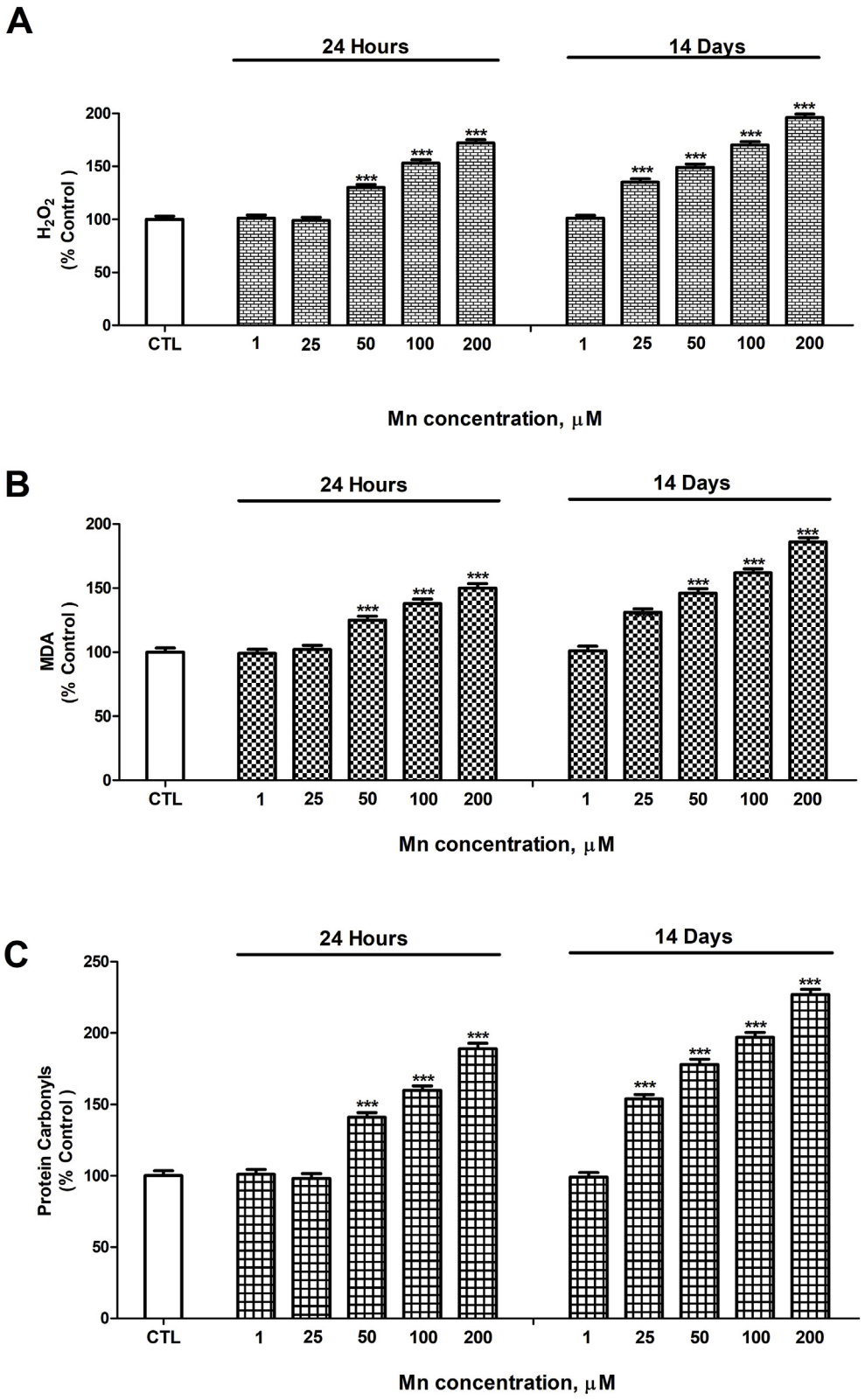

Fig. 7. Detection of ROS generation in SN56 cells was measures by hydrogen peroxide (H_2O_2) assay and H_2O_2 concentration is presented as percent untreated control (A). The lipid peroxidation level of SN56 cells was measured by MDA assay, and MDA concentration is presented as percent untreated control (B). H_2O_2 (A), MDA (B) and protein carbonyl (C) content generated after 24 h and 14 days Mn treatment. Values are given as mean ± SD of three separate experiments from cells of different cultures, each one performed in triplicate. $^{***}p < 0.001$ compared to control.

on cholinergic neurons. However, Ach co-incubation with Mn was not able to reduce cell death induced by Mn alone suggesting other mechanisms should be involved.

In addition, Mn increased the expression of the "synaptic" (S) and "readthrough" (R) variants that AChE present (Soreq and Seidman, 2001) after acute (from 25 μM in both variants) and 14 days (from 25 μM to 1 μM, respectively) exposure. AChE-R is induced under stress conditions, such as Mn exposure, with a neuroprotective and repair role (Adamec et al., 2008; Farchi et al., 2007). Conversely, AChE-S overexpression, either by itself or when up-regulated in conjunction with AChE-R, is linked to programmed and necrotic cell death depending on the stress stimulus (Greenberg et al., 2010; Zimmermann, 2013; Del Pino et al., 2016). Thus, the increase of AChE-S produced after 24 h and 14 days exposure could mediate the apoptotic and necrotic cell death observed, and the increase of AChE-R could be a neuroprotective mechanism against the possible hurtful effects induced by AChE-S overexpression. In this regard, AChE expression silencing partially reversed the induced cell death after 24 h and 14 days Mn exposure. AChE silencing has been shown to reduce activation of caspases and LDH release, reversing the apoptotic and necrotic cell death induction (Pegan et al., 2010; Del Pino et al., 2016). Therefore, these data support that AChE-S overexpression could be involved in the cell death observed in SN56 cholinergic neurons after 24 h and 14 days Mn exposure. Moreover, antioxidant NAC co-treatment with Mn attenuated the cell death induced after Mn treatment alone. Previous studies have shown that antioxidant treatment was able to reverse the effects on cell viability induced by Mn (Chtourou et al., 2012; Wang et al., 2017; Zhu et al., 2016), supporting our results. The antioxidant co-treatment with Mn of AChE silenced cell induced a higher reversion of the cell death observed, but was not completely suggesting that other mechanism are involved.

Mn has been reported to block nicotinic receptors (Ye and Kim, 2015), impairing cholinergic transmission, which could also mediate the effect observed on cholinergic neurons and cognitive deficits. In this regard, nicotinic receptor actions maintain basal forebrain cholinergic neurons and a loss of them exacerbates early-stage of cognitive decline and basal forebrain pathology (Hernandez et al., 2010). Besides, Mn has been described to increase Aβ protein production (Tong et al., 2014), tau and GSK-3β hyperphosphorylation (Cai et al., 2011), which have been related with induction of cell death in basal forebrain cholinergic neurons and AD (Kar et al., 2004), being also able to mediate this effect. Basal forebrain cholinergic neurons rely on brain-derived neurotrophic factor (BDNF), to maintain survival, differentiation, connectivity and function. BDNF, is decreased in AD, causing cholinergic dysfunction and synapse loss (Fahnestock et al., 2002). Chronic Mn exposure has been reported to decrease hippocampal BDNF levels (Liang et al., 2015), so this alteration could contribute to basal forebrain cholinergic neuronal loss. Further studies are needed to clarify the other mechanisms through which these effects are produced.

Taking all together, we can conclude that after acute and long-term exposure, Mn induces cell death on cholinergic neurons from basal forebrain, which could be mediated in part by AChE-S overexpression and oxidative stress generation, and is independent of cholinergic transmission alteration. These effects could explain cognitive alterations induced by Mn. Future studies should be developed to confirm, on *in vivo* studies, the effects observed and to determine the other mechanisms involved in the effects observed on cholinergic neurons and their implication in cognitive disorders. These results are of interest, since they provide new information on the mechanisms that mediate cholinergic transmission disruption and cell death induced by Mn, and should be taken into consideration in the risk assessment of this element.

Conflicts of interest

There are no conflicts of interest to declare.

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