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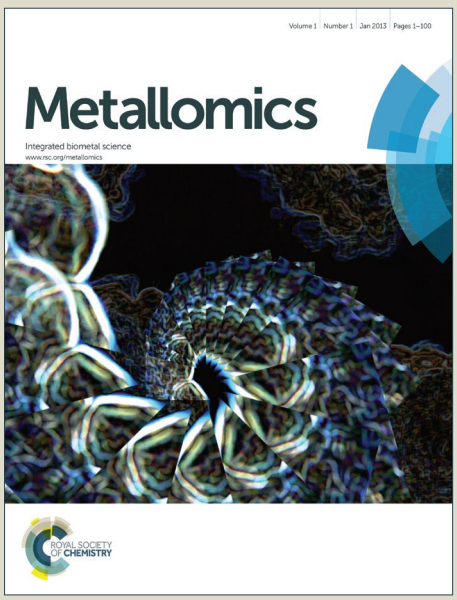

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Tyrosine Hydroxylase Regulation in Adult Rat Striatum following Short-term Neonatal Exposure to Manganese

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ABSTRACT

Manganese (Mn) is an essential trace element required for a range of physiological processes, but Mn can also be neurotoxic especially during development. Excess levels of Mn accumulate preferentially in the striatum and can induce a syndrome called manganism, characterized by an initial stage of psychiatric disorder followed by motor impairment. In the present study, we investigated the effects of Mn exposure on the developing dopaminergic system, specifically tyrosine hydroxylase (TH) protein and phosphorylation levels in the striatum of rats. Neonatal rats were exposed to Mn intraperitoneally (ip) from post-natal day 8 up to day 12 (PND8-12). Striatal tissue was analysed on PND14 or PND70, to detect either short-term or long-term effects induced by Mn exposure. There was a dose dependent increase in TH protein levels in the striatum at PND14, reaching significance at 20mg/kg Mn, and this correlated with an increase in TH phosphorylation at serines 40, 31 and 19. However, in the striatum at PND70, a time by which Mn levels were no longer elevated, there was a dose dependent decrease in TH protein levels, reaching significance at 20 mg/kg Mn, and this correlated with TH phosphorylation at Ser40 and Ser19. There was however a significant increase in phosphorylation of TH at serine 31 at 20 mg/kg Mn, which did not correlate with TH protein levels. Taken together our findings suggest that neonatal Mn exposure can have both short-term and long-term effects on the regulation of TH in the striatal dopaminergic system.

Keywords: Manganese, neurotoxicity, tyrosine hydroxylase, development, phosphorylation

Introduction

Manganese (Mn) is an essential trace element required for a range of physiological processes, but Mn can also be neurotoxic especially during development. Excess levels of Mn may be acquired via water, air, total parenteral nutrition (TPN), or exposure to some occupational settings. Since 1837 Mn has been recognized to induce a syndrome called manganism that is characterized by an initial stage of psychiatric disorder, followed by a motor impairment stage that has many similarities to Parkinson's disease [1, 2].

The immature central nervous system (CNS) is more sensitive to toxic insults and susceptible to developmental impairment, which can have lasting consequences on brain function. Furthermore, developmental insults may be unmasked at later life-stages [1, 3, 4]. Mn is present in TPN preparations and can therefore pose a risk for infants because the bioavailability of intravenous Mn in TPN is 100%, compared to only 5% absorption from enteral nutrition and because the hepatic mechanisms responsible for Mn elimination are not fully formed in infants; both these factors lead to Mn accumulation[5-7]. Fell and collaborators (1996) reported hypermanganesemia in eleven children receiving prolonged parenteral nutrition, one of which presented with a movement disorder. In spite of the recognition of an acute toxicity, the long-term consequences of this exposure were not reported [8].

It is estimated by the National Center for Health Statistics and reported by the American Society for Parenteral Nutrition (ASPEN) that patients received TPN on 360,000 hospital stays in 2009. About 33% of these patients were children or newborns. PN solutions are often supplemented with a multiple trace element supplement (TES) containing fixed ratios of zinc, copper, chromium and Mn, with or without selenium. It is estimated that the total Mn intake from TPN supplemented with TES is 100 times greater when compared to human milk. PN in the absence of TES contains 6.9 to 11.9 μg/L Mn and TES-containing PN contains 38.4 to 77.7 μg/L Mn. While human milk contains 3 to 10 μg/L Mn. Magnetic

resonance imaging (MRI) revealed that infants in neonatal intensive care unit (NICU) receiving standard-of-care PN show accumulation of Mn in basal ganglia [9].

Manganism and Parkinson's disease both involve brain areas rich in dopaminergic (DAergic) innervation and dopamine (DA) metabolism and signalling have been considered to be an important focus of the molecular action of Mn [10, 11]. The striatum expresses high levels of the divalent metal transporter-1 (DMT-1), which contributes to the transport of Mn and this may explain the accumulation of Mn and the prominent toxic effects of Mn that occur in this region [12]. However, Crossgrove and Yokel (2004) suggested that DMT-1 was not the main transporter for Mn across the BBB, using a model of rats with deficient DMT-1 expression (b/b Belgrade rat) [13]. Another factor contributing to the susceptibility of the striatum to Mn is the high DA content in this region, which contributes to the generation of oxidative stress in the presence of Mn [14, 15]. Rats exposed to Mn orally during development (PND1-21), had lower levels of the DA transporter (DAT) and efflux of [^{3}H] DA in the striatum and nucleus accumbens at PND90, indicating that Mn affects the nigrostriatal and mesolimbic DAergic pathways, and that these effects are long term [16]. The selectivity of Mn towards the basal ganglia is also confirmed by the motor damage that is observed in several models of Mn exposure *in vivo* [17-19].

An important aspect in regulating DA homeostasis is the rate of DA synthesis. Tyrosine hydroxylase (TH) is the rate-limiting enzyme in catecholamine synthesis and catalyses the first reaction of the biochemical synthetic pathway in which L-tyrosine is converted to L-3, 4-dihydroxyphenylalanine (L-DOPA). TH is regulated by phosphorylation of serine (Ser) residues (Ser40, Ser31 and Ser19) and all three sites can lead to TH activation either directly or indirectly [20, 21]. TH activity is also modulated by mRNA expression and protein synthesis [20]. Recently, it has been shown that DA deficiency also plays a role in TH modulation by inducing activation of TH via phosphorylation at Ser40 through D2-

autoreceptor and PKA-mediated pathways. This effect, in turn, gives a rise to TH degradation through an ubiquitin-proteasome pathway, resulting in a negative spiral of DA production when DA deficiency persists [22].

TH activity can be modulated by Mn *in situ* but the effects are dependent on exposure period and concentration of metal applied. Posser et al (2009) demonstrated that Mn (100 μM) stimulates phosphorylation of TH at Ser40 in undifferentiated rat pheochromocytoma 12 (PC12) DAergic cells with the first significant effects observed after 6 h and with subsequent increases up to 24 h; TH activity was increased at 24h without altering TH protein synthesis or cell viability *in situ* [23]. Zhang and colleagues (2011) demonstrated that 3 h treatment of differentiated N27 DAergic cells with sub-toxic concentrations of $MnCl_2$ (3 μM and 10 μM) induced an increase in TH activity as well as dose-dependent increase in the level of Ser40 phosphorylation *in situ*. However, when the period of Mn exposure was increased to 24 h, low Mn concentrations (0.1-1 μM) produced a decrease in TH activity without affecting the phosphorylation at Ser40 [24]. Moreover, striatal slices exposed to Mn (10-1000 μM) for 3-6 h did not display any alteration in TH content or Ser40 phosphorylation [25]. The variability in the responses to Mn *in vitro* raised the question of whether Mn would have any effect on TH regulation in the striatum *in vivo*.

Phosphorylation of TH at Ser40 causes the largest increase in TH activity due to a decrease in the feedback inhibition by DA [21, 26]. However, TH phosphorylation at Ser31 by ERK1/2 can also activate TH, but to a much smaller extent [21, 27]. TH phosphorylation at Ser19 does not directly activate TH, but it can lead to TH activation after binding to the 14:3:3 protein [21]. Protein kinase A (PKA), PKC and protein phosphatase 2A (PP2A) are involved in the phosphorylation and dephosphorylation of Ser40 *in situ* and their involvement has been demonstrated *in situ* in response to both acute and sustained stimuli [21, 28-32]. A functional interaction between PKCδ and TH has been recognized, in which PKCδ negatively

regulates TH activity and DA synthesis by enhancing PP2A activity in DAergic neurons [33].

In the present study, we investigated the dose dependent effects of neonatal Mn exposure on TH protein levels and TH phosphorylation at Ser40, Ser31 and Ser19 in the striatum. We employed a model of neonatal rats exposed to Mn intraperitoneally (ip) at PND8-12 to mimic TPN supplementation. Striatal tissue was analysed on PND14 in order to evaluate possible short-term effects, or PND70 to detect possible long-term effects of Mn exposure. We observed an increase in TH protein levels in the striatum of PND14, which was correlated with increased Ser40, Ser31 and Ser19 phosphorylation levels. In the striatum at PND70, TH protein levels were reduced, which suggested a loss of DAergic neurons and this effect was apparently compensated for by increased Ser31 phosphorylation.

Experimental procedures

Chemicals

Manganese chloride ($MnCl_2$) and protease inhibitor cocktail were obtained from Sigma (St. Louis, MO, USA). HEPES, Triton X-100, SDS, acrylamide, bis-acrylamide and Hybond ECL nitrocellulose were obtained from GE Healthcare Life Science (Piscataway, NJ, USA). Glycine, Tris, TEMED, and β-mercaptoethanol were obtained from Amresco Life Science (Solon, OH, USA). Avidin-biotin complex was obtained from Vector Laboratories (Burlingame, CA, USA). Mounting medium Entellan was obtained from Merck (Germany). All other reagents were of the highest analytical grade.

Animals

All animal studies were carried out in accordance with the ‘‘Principles of Laboratory Animal Care’’ (NIH publication, 8th edition, 2011) and approved by the local Ethical Committee for Animal Research. Litters containing 8 male Wistar rats at PND5 along with the dams were obtained from our own breeding colony at Universidade Federal de Santa Catarina (UFSC), Brazil. Rats were maintained in an air-conditioned room (21–23°C) on a 12-h light/dark cycle with water and food *ad libitum.* The food supplemented to the animals is produced by BioBase (Aguas Frias, SC, Brazil) and contains 70 mg/kg Mn.

On PND5, the litters were culled to 4 male pups. These pups were cross-fostered with male pups from another dam in order to keep litter size at 8 pups. The 2 sets of siblings were identified by different color atoxic ink and the pups were marked individually for each treatment. This procedure was carried out in order to maintain the same litter size that was used in our previous work [18]. We did not observe differences in maternal care towards the adopted pups as reported in our previous publication [34].

The pups were treated ip with saline (0.9% NaCl; controls) or $MnCl_2$ 5, 10, or 20 mg/kg for five consecutive days as previously described [18, 34]. After weaning (PND 21) and until PND70, each experimental group composed of 4 siblings was kept together in a cage (one control and three treated with the different doses of Mn). A total of seven groups were analyzed by western blotting on PND14 and seven groups on PND70. In addition, four groups were used for immunohistochemistry analysis on PND70 (for this experiment, only controls and 20 mg/kg Mn were analyzed). The litters used in the present study were not the same as the previous studies performed by our group, but were handled in the same way.

Western blotting

Western blot analysis of striatal samples was performed as previously described [35-37]. Briefly, the striatum was solubilized with 400 µl of lysis buffer (Tris 50 mM pH 7.0, EDTA 1 mM, NaF 100 mM, PMSF 0.1 mM, Na_3VO_4 2 mM, Triton X-100 1%, glycerol 10%, Sigma protease inhibitor cocktail) and then incubated for 10 min at 4°C. Lysates were centrifuged (10,000 x g for 10 min, at 4°C) to eliminate cellular debris. The supernatants were diluted 1/1 (v/v) in Tris 100 mM pH 6.8, EDTA 4 mM, SDS 8% and incubated for 5 min at 100°C. Next, sample dilution (40% glycerol, 100 mM Tris, bromophenol blue, pH 6.8) in the ratio 25:100 (v/v) and β-mercaptoethanol (final concentration 8%) were added. Protein concentrations were determined by the Peterson method [38] in aliquots collected before β-mercaptoethanol addition. Sixty µg of total protein were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels (miniVE Vertical Electrophoresis System™, GE Healthcare Life Sciences, Piscataway, NJ, USA), followed by transfer to nitrocellulose membranes using a semidry blotting apparatus (TE 70 SemiPhor™ Unit, GE Healthcare Life Sciences, Piscataway, NJ, USA) (1.2 mA/cm^2; 1 h 30 min) as described [39]. After blocking with 5% skim milk in Tris-buffered saline with Tween (TBST,

150 mM sodium chloride, 10 mM Tris, 0.075 % Tween-20, pH 7.5) for 1 hour, membranes were incubated overnight at 4ºC with homemade primary antibodies (total-TH, pSer40, pSer31 and pSer19) generated and tested for specificity as described [40, 41]. Subsequently, membranes were incubated for 1h at room temperature (RT) with anti-sheep-HRP (1:10,000) or anti-rabbit-HRP (1:7500) secondary antibodies. In between each incubation step, membranes were washed in TBST. Membranes were then re-probed for detection of anti-β-actin-HRP (1:50,000) from Sigma (St. Louis, MO, USA). For the analysis of MAPK, Akt, PKA and PKC we used commercially available antibodies, relevant information is presented in Table 1. Blots were developed by chemiluminescent reaction using detection reagent (Luminata Classico HRP substrate). The bands were quantified using the Scion Image™ software (Frederick, MD, USA). Total-TH was normalized to β-actin. pSer40, pSer31 and pSer19 were normalized to total-TH. The phospho kinases were normalized to their total protein levels. PKA and PKC substrates were normalized to β-actin. Data were expressed as a fold change of the mean ± SEM relative to the mean of control.

Immunohistochemistry

For immunohistochemical analysis of striatum and *substantia nigra pars compacta* (SNpc) rats (PND70) were anesthetized with 40% chloral hydrate and perfused through the left cardiac ventricle with saline (0.9% NaCl), followed by a fixing solution containing 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. After perfusion, the brains were removed and postfixed in the same fixing solution for 24 h at RT. Brains were cryoprotected by immersion in 30% sucrose in PBS at 4°C. Then, the brains were frozen by immersion in chilled isopentane and stored at -80°C until analysis.

Serial coronal sections (20 μm) of SNpc and striatum were obtained with a cryostat (Leica, Germany) at -21 °C. First, the sections were incubated with 0.3 % H_2O_2 in PBS

containing 0.15 % triton X-100 (PBS-T) for 30 minutes to inhibit endogenous peroxidase. Thereafter, blocking of nonspecific sites was carried out with 5% goat or rabbit serum in PBS-T for 1 h at RT. After three washes in PBS-T the sections were incubated overnight at 4°C with TH primary antibody (1:500; US Biological, MA, USA, catalogue number T9237-13). After three washes in PBS-T the tissue sections were incubated for 1h with the following secondary biotinylated antibody: anti-rabbit (1: 250, Vector Laboratories, CA, USA, catalogue number B-1000). Then, the samples were incubated for 1 h with avidin-biotin complex (1:125, Vectastain ABC kit, Vector Laboratories, CA, USA), followed by a developing reaction with 0.035% 3,3-diaminobenzidine chloride (DAB, St. Louis, MO, USA). The sections were mounted on gelatin-treated slides and covered with cover slip using mounting medium Entellan (Merck, Germany) for further observation using Zeiss Axio Observer CLEM (Correlative Light and Electron Microscopy). Images were analysed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical significance in western blotting data was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test using GraphPad Prism 6.0 (GraphPad Software Inc.). Linear regression and Pearson's correlation analysis was used to determine whether there were any significant relationships between $MnCl_2$ dose and tTH protein levels, and tTH protein levels and phospho-TH (pSer40, pSer31 and pSer19) levels. Immunohistochemistry data were analyzed by Student's t-test. The values were expressed as mean ± SEM and the relevant statistical information, such as p and F values, provided in brackets. Differences were considered to be significant when $p<0.05$.

Results

The effects of neonatal Mn exposure on TH regulation in the striatum at PND14

In the striatum of rats on PND14, the levels of total TH (tTH) normalized to β-actin were increased in rats exposed to 20 mg/kg $MnCl_2$ compared to the control (1.31 vs. 0.96 fold change; Figure 1 A) [F (3, 34) = 3.54; p <0.01]. The levels of phosphorylation at Ser40, Ser31 and Ser19 in relation to the total content of TH (pSer40/tTH, pSer31/tTH and pSer19/tTH) were not affected by treatment with Mn (Figure 1 B-D).

Because of the alterations in tTH levels, TH phosphorylation data was normalized to β-actin and analysed by linear regression and Pearson's correlation. On PND14 there is a positive correlation (r = 0.52; p < 0.01) between the levels of tTH and the dose of Mn (Figure 2 A). There was a positive correlation between the levels of tTH and pSer40 levels (r = 0.72; p < 0.001), pSer31 (r = 0.52; p < 0.01) and pSer19 (r = 0.63; p < 0.001) when they were normalized to β-actin (Figure 2 B-D).

The effects of neonatal manganese exposure on TH regulation in the striatum at PND70

On PND70, tTH levels normalized to β-actin are reduced in rats exposed to 20 mg/kg $MnCl_2$ compared to control (0.81 vs. 1.07 fold change; Figure 3A) [F(3, 24) = 5.69; p < 0.01]. On that postnatal period, the levels of pSer40/tTH and pSer19/tTH were not affected by treatment with Mn (Figure 3 B and D) and were indistinguishable from controls. However, levels of pSer31/tTH are increased in rats exposed to 20 mg/kg compared with the control (1.48 vs. 0.99 fold change; Figure 3 C) [F(3, 24) = 7.93; p < 0.001].

On PND 70 the correlation between tTH and the dose of Mn was negative (r = -0.60; p <0.001; Figure 4 A). There was a positive correlation between the levels of tTH and pSer40 (r = 0.44; p < 0.05) and pSer19 (r = 0.57; p < 0.01) when they were normalized to β-actin (Figure 4 B and D). However, there was no significant correlation between pSer31/β-actin vs tTH/β-actin (Figure 4 C).

Immunohistochemistry staining for TH in the striatum of rats on PND70 indicated a significant decrease in optical density in the 20mg/kg Mn group compared to controls (7.28% ± 0.51, $p<0.05$, Figure 5). In the SNpc no alterations in the number of TH positive neurons was observed (Supplemental Figure 1). Rats treated with 5 or 10 mg/kg Mn were not used for immunohistochemistry.

The levels of phosphorylation of the MAPK protein family (ERK1/2, JNK1 p54/p46 and $p38^{MAPK}$) and AKT underwent no significant change on PND70 (Supplemental Figure 2 A-D). Likewise, no significant changes were observed in the levels of phosphorylated substrates of PKA or PKC (Supplemental Figure 3 A-B).

Discussion

We investigated the effects of ip Mn exposure during development (PND8-12) on striatal TH protein and TH phosphorylation levels. TH activity is increased in the brain and adrenal glands in response to a range of stressors in order to make new catecholamines to replace those that are released from neurons and the adrenal medulla during the stress response. Two fundamental mechanisms lead to these increases in TH activity including increases in TH phosphorylation and increases in TH protein levels [20, 21, 41]. When TH is phosphorylated at Ser40 TH activity is increased because of the release of bound catecholamines which normally inhibit enzyme activity [20, 21]. When TH is phosphorylated at Ser31 TH activity is increased by decreasing the Km for the enzymes cofactor tetrahydrobiopterin (BH_4)[21], or decreasing the rate of TH degradation[42]. When TH is phosphorylated at Ser19 there is no direct increase in TH activity. However, Ser19 phosphorylation decreases TH degradation and subsequent binding of the 14:3:3 protein can increase TH activity[21, 42]. When TH protein levels are increased this increases TH activity, as long as no catecholamine becomes bound to the newly synthesised enzyme to inhibit it, and it also increases the TH available for phosphorylation which then would increase TH activity by the mechanisms described.

The increased expression of tTH in the striatum on PND14 is part of an initial response to the stress induced by elevated levels of Mn. Striatal tissue was collected 48 h after the end of Mn exposure, when the metal concentrations were increased in this structure, as previously reported by Cordova et al. (2012), with an equivalent model of Mn exposure. Mn levels in control striatum was approximately 0.05 μg Mn/g tissue and reached approximately 0.7, 4 and 10 μg/g tissue with exposure to 5, 10 and 20 mg/kg Mn, respectively[18]. On PND70, Mn concentration in striatum of controls and treated animals was not significantly different (approximately 0.3 μg/g tissue)[34]. It is known that Mn can substitute for calcium in causing catecholamine secretion[43] and as catecholamine levels are

maintained in the brain in response to stress, there would be a need to replace the secreted catecholamines by increasing its synthesis. There was a correlation between the dose of Mn and the level of TH protein, suggesting that as the levels of Mn increased there was either a concomitant increase in the capacity for synthesis of TH, or a decrease in the capacity for TH degradation. Whatever the mechanism the positive correlations between tTH and pSer40, pSer31 and pSer19 levels at PND14 indicated that an increase in TH activity occurred and this suggests an increased capacity for catecholamine synthesis, corroborating earlier studies in juvenile mice where Mn exposure increased the levels of striatal DA[44]. It is known that Mn can increase protein kinase activity directly leading to increased phosphorylation of TH *in vitro* as well as other proteins including the MARCKS protein (P80), which is a substrate for PKC [43]. It is most likely that the increase in TH protein in response to Mn is due to activation of protein kinases that act as transcription factors for TH synthesis and that activation of these or other kinases also modulates TH phosphorylation in parallel. Future studies should clarify the mechanism for Mn-induced alterations in TH levels and how it influences DA levels upon Mn exposure and also the functional consequences of this finding (e.g. behavioural alterations).

Treatment with the highest dose of $MnCl_2$ (20 mg/kg) during the neonatal period induced a long lasting reduction in TH levels in the striatum on PND70, as shown by western blotting and immunohistochemistry. No alterations were observed in DAergic neurons from the SNpc, whose axons project to the striatum. The levels of striatal Mn in this period in exposed rats are similar to control values [34]. Mn is known to induce a reduction in DA striatal levels in adult experimental models, consistent with basal ganglia dysfunction [44]. It is known that maintenance of high levels of TH activity can lead to neurotoxicity due to damage induced by the raised DA levels causing oxidative damage [45]. Hence, it is possible that the early increases in TH and TH Ser40 phosphorylation observed at PND14 caused neuronal

damage. A decrease in TH immunostaining in catecholaminergic neurons by Mn concomitant with functional (motor) alterations has been previously demonstrated in a zebrafish model, with reversal of these effects by DA supplementation [46]. However, Liu et al. (2006) demonstrated loss of striatal DA in the adult mice brain due to oral Mn exposure without loss of DAergic neurons [47]. Furthermore, in non-human primates receiving intravenous Mn there is a decrease in DA release without affecting DAergic terminal integrity [19]. Therefore, based on the conflicting reports published, the reduction in TH levels observed in our model does not necessarily indicate a loss of DAergic neurons in the striatum, but could demonstrate a decrease in TH protein expression.

Treatment with the highest dose of $MnCl_2$ (20 mg/kg) during the neonatal period induced a long lasting reduction in TH levels in the striatum on PND70 and this correlated with decreases in Ser40 and Ser19 phosphorylation, suggesting that TH activity would be decreased at this time. However, Ser31 phosphorylation was increased on PND70 despite the decreased levels of TH, suggesting a compensatory response to increase the synthesis of DA, considering that phosphorylation of Ser31 increases TH activity modestly *in vitro* and *in vivo* [21, 27, 48-52]. The striatum contains DAergic nerve terminals and not cell bodies and it is established that under basal conditions Ser31 phosphorylation is higher in nerve terminals in the striatum than either Ser19 or Ser40 phosphorylation [51, 53]. The reason(s) for this high level of Ser31 phosphorylation under basal conditions is not known. It is also not clear how Ser31 phosphorylation was maintained in response to Mn, since ERK1/2 the main kinase involved in the phosphorylation of this site was not modified at PND70. Moreover, the other members of the MAPK family, such as $p38^{MAPK}$ and JNK, as well as AKT, PKA and PKC activities were unaltered at PND70. It is important to note that the results of western blotting do not distinguish between TH-expressing and non-expressing cells in the striatum, therefore it is possible that kinase activity may be altered in TH containing neurons, but this couldn't be

detected by western blotting. Alternatively, PP2A acts on Ser31 dephosphorylation and its activity can also influence TH phosphorylation state [21, 32], but PP2A also dephosphorylates Ser40 and Ser19[21] and is unlikely to be responsible, especially as the major beta/beta prime subunit which facilitates PP2A activity in the brain against TH is not found in nerve terminals [54]. Cyclin-dependent kinase 5 also phosphorylates TH at Ser31 and regulates its stability [55, 56] and activation of this kinase, which is present in the striatum, may be involved. Therefore it remains to be determined what the mechanism is for the increased Ser31 phosphorylation in Mn exposed rats and how this may affect DA levels in the striatum of adult rats. Interestingly, O'Neal et al. (2014) reported increased DA levels in the striatum of adult mice exposed to Mn, via i.p., without alteration in TH protein levels. The authors point that the different outcomes upon Mn exposure may be related to different dosing regimens, routes of exposure and animal species [57].

Conclusion

Our results indicate that neonatal exposure to Mn differentially affects TH levels in the striatum of developing and adult rats. Initially there is an increase in TH protein and TH phosphorylation at Ser40, Ser31 and Ser19, which is likely to lead to higher levels of catecholamine synthesis to replace catecholamines that are lost during the initial responses to the stress of Mn exposure. Subsequently, there is a long term decrease in TH protein and TH phosphorylation at Ser40 and Ser19 most likely leading to lower levels of catecholamine synthesis. However, an increase in TH phosphorylation at Ser31 is likely to partially compensate for these lower levels of catecholamine synthesis.

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Figure 1: Levels of tyrosine hydroxylase (TH) in the striatum of PND14 rats exposed to $MnCl_2$ (5, 10 or 20 mg/kg/day, ip) during development (PND 8-12). Blots were developed by chemiluminescence. The bands were quantified by densitometry. (A) Total TH content (tTH) data were normalized to β-actin. Phosphorylated (p) Ser40 (pSer40) (B) pSer31 (C) and pSer19 (D) were normalized to tTH. The data represent changes compared to control and express the mean ± SEM of 7 experiments. ** $p < 0.01$ compared to control (one-way ANOVA followed by Dunett's test).

Figure 2: The content of tyrosine hydroxylase (tTH) was determined by western blotting in the striatum of rats on PND14 exposed to $MnCl_2$ (5, 10 or 20 mg/kg/day, ip) during development (PND 8-12). (A) Pearson correlation coefficient was calculated between $MnCl_2$ dose and tTH levels normalized to β-actin levels (tTH/β-actin). Pearson correlation analysis shows a significant positive correlation (Pearson $r = 0.52$, $p < 0.01$). (B) Pearson correlation analysis shows a significant positive correlation of tTH/β-actin versus pSer40/β-actin (Pearson $r = 0.72$, $p < 0.0001$), (C) pSer31/β-actin (Pearson $r = 0.52$, $p < 0.01$) and (D) pSer19/β-actin (Pearson $r = 0.63$, $p < 0.001$). The line represents linear regression. Statistical analysis was performed with two-tailed t test.

Figure 3: Levels of tyrosine hydroxylase (TH) in the striatum of PND70 rats exposed to $MnCl_2$ (5, 10 or 20 mg/kg/day, ip) during development (PND 8-12). Blots were developed by chemiluminescence. The bands were quantified by densitometry. (A) Total TH content (tTH) data were normalized to β-actin. Phosphorylated (p) Ser40 (pSer40) (B) pSer31 (C) and pSer19 (D) were normalized to tTH. The data represent changes compared to control and express the mean ± SEM of 7 experiments. ** $p < 0.01$, *** $p < 0.001$ compared to control (one-way ANOVA followed by Dunett's test).

Figure 4: The content of tyrosine hydroxylase (tTH) was determined by western blotting in the striatum of rats on PND70 exposed to $MnCl_2$ (5, 10 or 20 mg/kg/day, ip) during development (PND 8-12). (A) Pearson correlation coefficient was calculated between $MnCl_2$ dose and tTH levels normalized to β-actin levels (tTH/β-actin). Pearson correlation analysis shows a significant negative correlation (Pearson $r = -0.60$, $p < 0.001$). (B) Pearson correlation analysis shows a significant positive correlation of tTH/β-actin versus pSer40/β-actin (Pearson $r = 0.44$, $p < 0.05$), (C) no significant correlation in pSer31/β-actin (Pearson $r = 0.04$, $p > 0.05$) and (D) a significant positive correlation in pSer19/β-actin (Pearson $r = 0.57$, $p < 0.01$). The line represents linear regression. Statistical analysis was performed with two-tailed t test.

Figure 5: Representative photomicrographs of tyrosine hydroxylase (TH) immunostaining in the striatum of PND70 rats exposed to $MnCl_2$ (20 mg/kg/day, ip) during development (PND 8-12). The optical density of at least 7 different sections from both hemispheres at 400X magnification per animal were analysed. The graph shows the percentage relative to control mean of striatal TH optical density of 4 animals in each group. Data are expressed as mean ± SEM. *$p < 0.05$ (two tailed t test). Bars represent 1 mm.

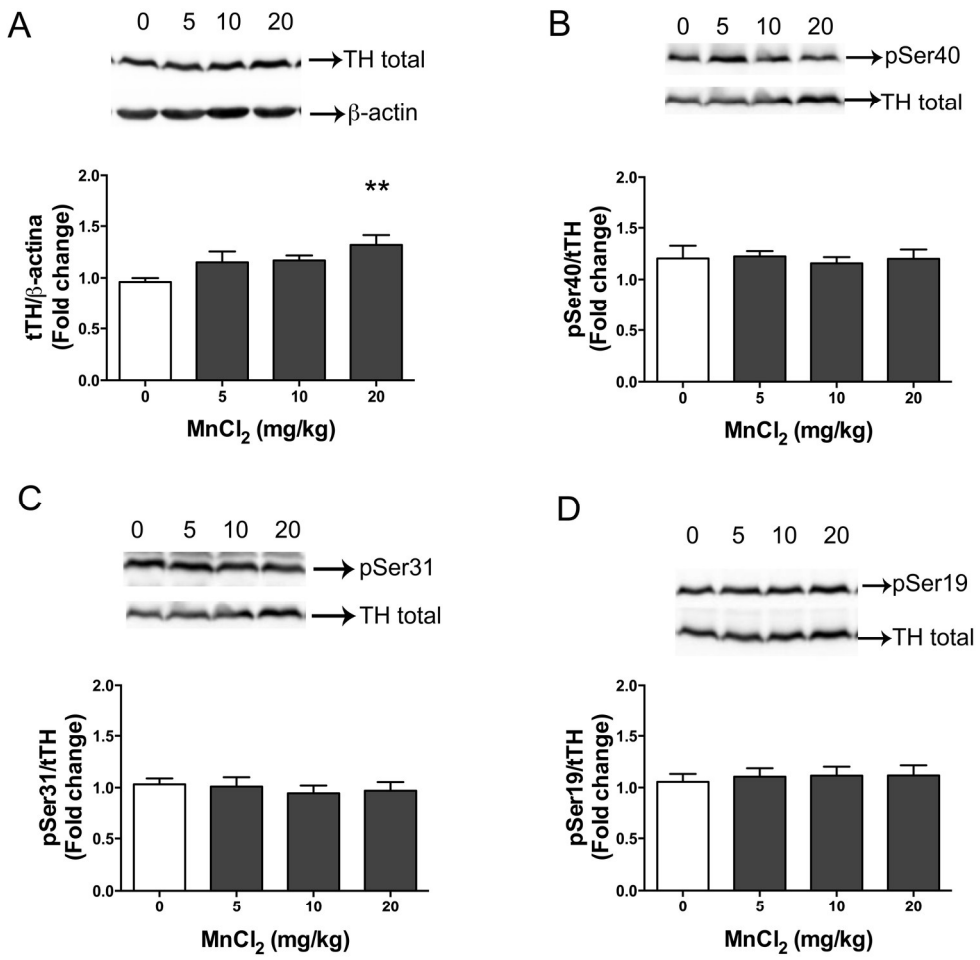

Fig 1

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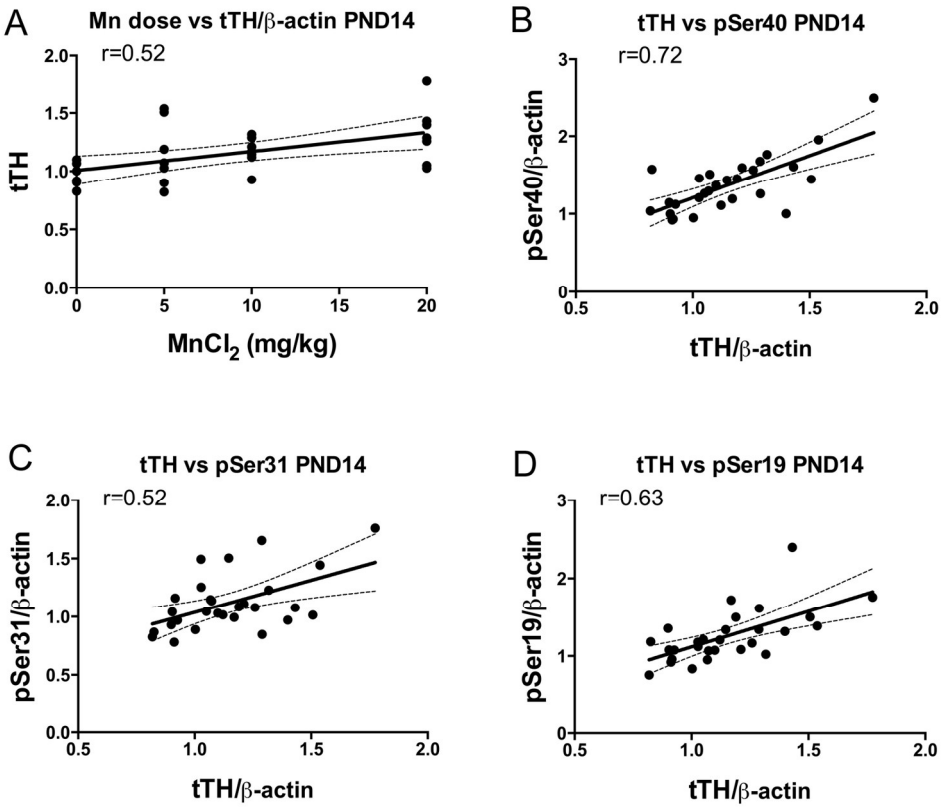

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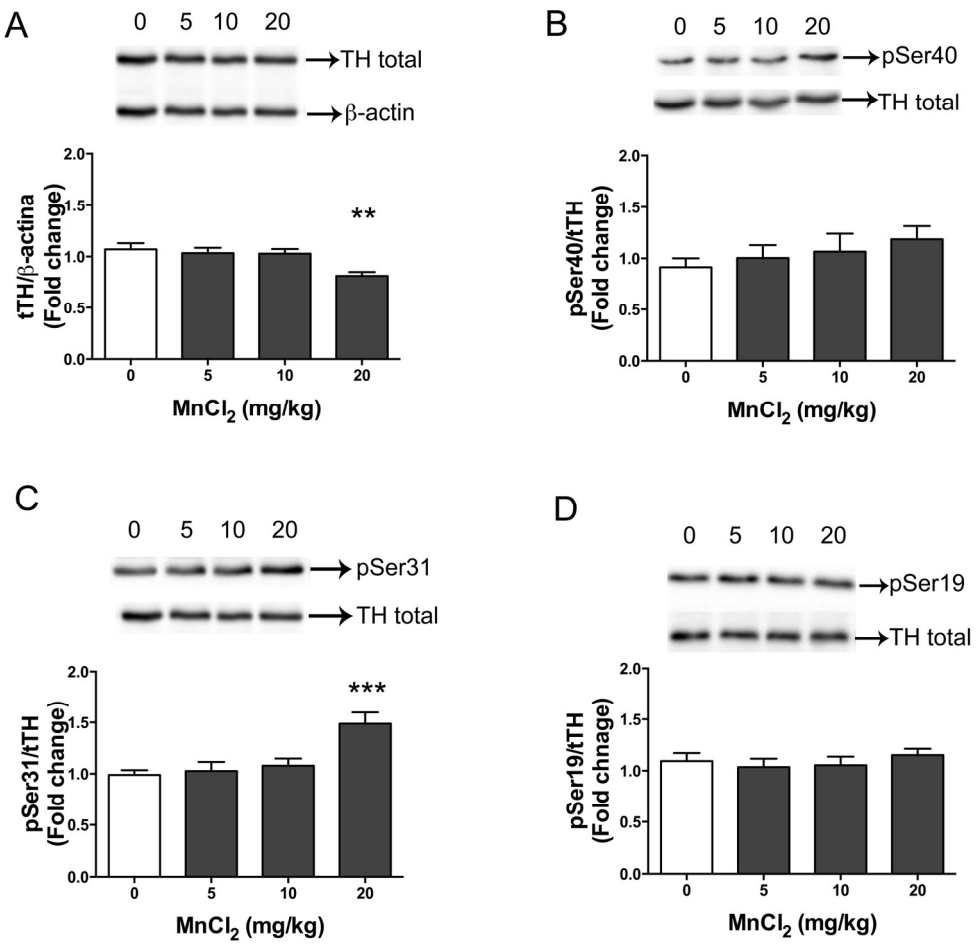

Fig 3

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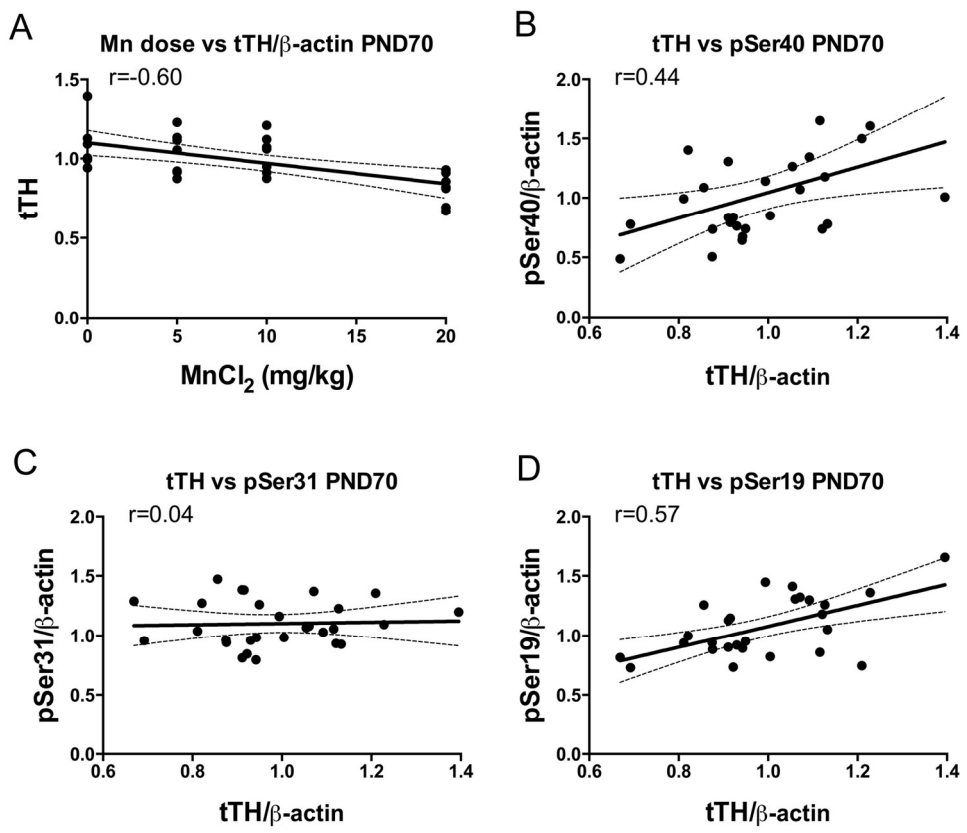

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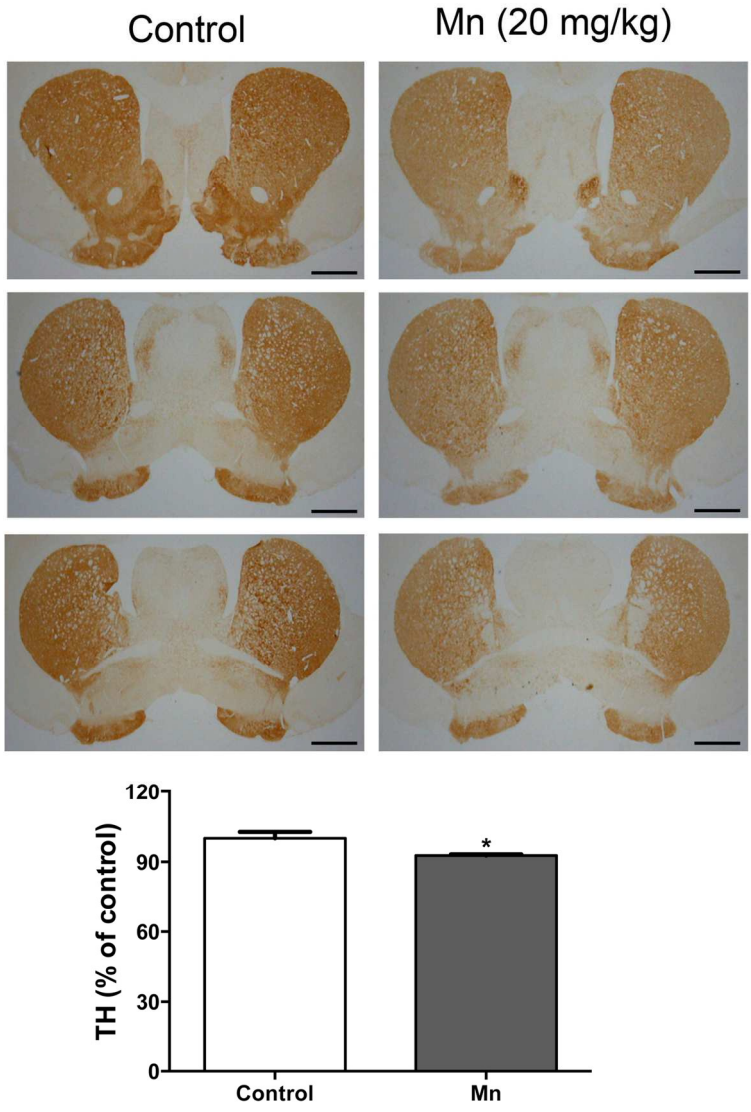

126x177mm (300 x 300 DPI)

Table 1: Information on antibodies used for western blotting.

Antibody	Molecular weight of target protein	Supplier (catalogue number)	Dilution
Phospho-p44/42 MAPK (Thr202/ Tyr204)	44/42 kDa	Cell Signaling Technology (#4370)	1:2000
Anti-Mitogen Activated protein kinase (ERK1 & ERK2)	44/42 kDa	Sigma (M5670)	1:40000
Anti-phospho-p38 ($pThr^{180}$/$pTyr^{182}$)	~38 kDa	Sigma (P1491)	1:10000
Anti-p38 MAP kinase	~38 kDa	Sigma (M0800)	1:10000
Phospho-SAPK/JNK (Thr183/Tyr185)	46/54 kDa	Cell Signalling Technology (#9251)	1:5000
Anti-c-Jun N-Terminal Kinase (p54/p46)	46/54 kDa	Sigma (J4500)	1:5000
Anti-phospho PKB (Ser473)	60 kDa	Sigma (P4112)	1:2000
Akt	60 kDa	Cell Signalling Technology (#9272)	1:1000
PKA phosphorylated substrates	30 to 80 kDa	Cell Signalling Technology (#9624)	1:2000
PKC phosphorylated substrates	30 to 80 kDa	Cell Signalling Technology (#2261)	1:2000
β-actin	43 kDa	Sigma (A5441)	1:50000
Goat anti-Mouse IgG, HRP Conjugated secondary antibody	-	Millipore (AP308P)	1:2500
Rabbit anti-Sheep IgG (H+L) Secondary Antibody, HRP conjugate	-	Thermo Fisher (61-8620)	1:10000
Rabbit IgG, HRP-linked whole Ab (from donkey)	-	GE Healthcare Life Sciences (NA934)	1:7500