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Valproate and sodium butyrate attenuate manganese-decreased locomotor activity and astrocytic glutamate transporters expression in mice

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Abstract

Manganese (Mn) is an essential trace element, but chronic overexposure to this metal, either environmentally or occupationally may cause manganism, a disease analogous to Parkinson's disease. Inhibitors of histone deacetylases, such as valproic acid (VPA) and sodium butyrate (NaB) exert neuroprotective effects in various animal models of neurological disorders. Thus, the present study investigated whether VPA or NaB prevent Mn-induced neurotoxicity by assessing locomotor activities and expression of astrocytic glutamate transporters, glutamate transporter 1 (GLT-1) and glutamate aspartate transporter (GLAST), in C57BL/6 mice. C57BL/6 mice were pretreated with VPA (200 mg/kg, i.p.) or NaB (1200 mg/kg, i.p.) prior to intranasal instillation of Mn (30 mg/kg) continually for 21 days, followed by open-field and rota-rod behavioral tests and analyses of astrocytic glutamate transporters GLT-1 and GLAST protein/mRNA levels. The results showed that Mn significantly decreased locomotor activity as determined by total distance travelled, stereotypic and ambulatory counts. Mn also significantly decreased rota-rod activity reflecting altered motor coordination. Pretreatment with VPA and NaB with Mn reversed the effects of Mn on the locomotor activity and motor coordination. VPA and NaB also attenuated the Mn-induced decrease in GLT-1 and GLAST mRNA and protein levels in the cerebral cortical and cerebellar regions of mice. These results suggest that VPA and NaB exert protective effects against Mn toxicity seem *in vitro* are also shown *in vivo*. VPA and NaB pretreatment in mice enhancing astrocytic glutamate transporter GLT-1 expression as well as locomotor activities. Future research endeavors are warranted to determine if the therapeutic potential of VPA and NaB is via common molecular mechanism, namely, inhibition of histone deacetylases.

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Conflicts of interest

There are no conflicts of interest.

Keywords

valproic acid; sodium butyrate; glutamate transporters; Manganese; behavior

1. Introduction

Abnormalities in behavioral and locomotor activities are considered as physiological indicators of neurological disorders, such as Parkinson's disease (PD), epilepsy, Alzheimer's disease (AD) and autism (Maski et al., 2011). Although the molecular mechanisms of these neurological disorders are still not completely understood, their etiology is based on genetic, environmental or gene \times environment interactions (Dauncey, 2012; Peres et al., 2016). Genetic abnormalities leading to neurological diseases are associated with rare gene mutations and irregular gene expression (Dauncey, 2012; Karki et al., 2015; Seifert et al., 2006).

Environmental and idiopathic factors also significantly influence the development and progression of neurodegenerative disorders (Peres et al., 2016; Robison et al., 2012). Previous studies have reported that environmental agents may induce toxicity, which can lead to serious brain injury and damage (Dauncey, 2012; Peres et al., 2016). One of the most studied metal toxicants is manganese (Mn), which is an essential trace element, but chronic high level exposures to this metal may cause a neurological disorder referred to as manganism, with similar clinical features to parkinsonism (Peres et al., 2016), characterized by locomotor dysfunction and behavioral abnormality (Peres et al., 2016; Robison et al., 2012). Upon overexposure, Mn accumulates preferentially in specific brain regions (i.e. globus pallidus), resulting in motor deficits and neurological disorders (Robison et al., 2012). Several therapeutic approaches have been reported for the treatment of manganism, such as levodopa, antioxidants, plant extracts, iron (Fe)-chelating agents and glutathione precursors (Peres et al., 2016).

Glutamate-mediated excitotoxicity is considered one of the critical mechanisms involved in Mn neurotoxicity (Brouillet et al., 1993). The astrocytic glutamate transporters, glutamate aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1) in rodents (excitatory amino acid transporter 1 (EAAT1) and excitatory amino acid transporter 2 (EAAT2) in humans, respectively) are the main transporters to uptake most of glutamate from synaptic clefts after binding to its postsynaptic receptors to transfer glutamate signals. Accordingly, dysfunction of these transporters is directly associated with glutamate excitotoxicity. In fact, several idiopathic neurodegenerative diseases including PD and manganism are associated with excitotoxicity along with a decrease in the function and expression of the glutamate transporter, GLT-1.

Targeting epigenetic regulation, in particular deacetylation of histone molecules using inhibitors of histone deacetylases (HDACs) for the development of neuroprotective and anti-cancer agents has shown significant progress in recent years. Valproic acid (2-n-propylpentanoic acid; VPA) is clinically used as an antiepileptic and anticonvulsant agent exhibiting increasing usage over the years for the treatment of neurological disorders. Interestingly, in addition to its beneficial effect against epilepsy, VPA exerts neuroprotective

effects in several animal models of neurodegenerative diseases (Monti and Contestabile, 2009). However, the exact molecular mechanism of its neuroprotective effect remains to be established.

VPA affects cell growth, differentiation and apoptosis, by regulating gene expression at the molecular level, through epigenetic mechanisms as an inhibitor of HDACs (Ximenes et al., 2013). In addition to inhibition of HDACs, VPA can also interfere with multiple regulatory mechanisms such as the glycogen synthase kinase (GSK)3- α and - β , protein kinase B (Akt), extracellular signal-regulated kinases (ERK) and phosphoinositol pathways, tricarboxylic acid (TCA) cycle, and γ -aminobutyric acid (GABA) system. VPA also showed beneficial effects in modulating blood brain barrier (BBB) disruption and brain edema in a rat model of cerebral ischemia (Wang et al., 2011). VPA also exerts anti-inflammatory and antioxidative properties.

Sodium butyrate (NaB), a short chain fatty acid) is an inhibitor of HDACs, and generally derived from the microbial fermentation product of dietary fiber in the colon and thus, inhibits intestinal pathogenic bacteria and maintains gastrointestinal homeostasis (Canani et al., 2011; Kim et al., 2012). NaB, thus, exerts multiple beneficial effects from the intestinal tract to the peripheral tissues (Canani et al., 2011). NaB has been shown to be effective in the prevention and treatment of multiple chronic disorders such as cancer, metabolic syndrome, cardiovascular diseases, and neurodegeneration. Inhibition of HDACs by NaB is related to its epigenetic regulatory effects on gene expression. Due to its modulation on epigenetic mechanisms, NaB has been considered as a specific and efficacious therapeutic strategy for treatment of variety of diseases, including neurodegenerative disorders. In rats, NaB exhibits neuroprotective effects against ischemic stroke by anti-inflammatory effects (Park and Sohrabji, 2016), and antidepressant-like effects (Yamawaki et al., 2012). NaB also exerts neuroprotective effects by anti-apoptotic, -oxidative and -inflammatory effects in a mouse model of cerebral ischemic injury (Sun et al., 2015). NaB improved memory deficits in a mouse model of traumatic brain injury, attributing to increased expression of tight junction-associated proteins such as occludin and ZO-1, and BBB permeability (Li et al., 2016).

Since VPA and NaB exert neuroprotective effects against numerous neurological disorders, not only by HDAC inhibition, but also many other molecular mechanisms, the current study investigated whether VPA and/or NaB attenuate Mn-induced neurotoxicity by assessing neurobehavioral deficits and expression of astrocytic glutamate transporters, GLT-1 and GLAST in mice.

2. Materials and Methods

2.1. Experimental animals

All animal protocols were reviewed and approved by the Meharry Medical College Institutional Animal Care and Use Committee (Nashville, TN). Adult male C57BL/6 mice (6-8 weeks old; weight 18-20 g) were purchased from the Jackson Laboratory and housed for one week prior to study (Bar Harbor, ME). The animals were randomly selected, group-housed (4 mice/cage) and maintained on a 12-h light/dark cycle at $25 \pm 2^\circ\text{C}$ and 60-70%

relative humidity with food, water and enrichment available *ad libitum* in the Animal Care Facility of Meharry Medical College (Nashville, TN).

2.2. Chemicals and reagents

Manganese chloride (MnCl_2 , 99% purity), valproic acid (VPA, 98% purity), and sodium butyrate (NaB, 98% purity) were obtained from Sigma-Aldrich (St. Louis, MO). EAAT1 (ab416) and EAAT2 (ab41621) antibodies were obtained from Abcam (Cambridge, MA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-14007) antibody was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit IgG-HRP-conjugated (W4018) secondary antibody was obtained from Promega (Madison, WI).

2.3. Experimental procedure

The number of animals needed for this study ($n=48$; 8 animals/group) was determined by power analysis. Mice were randomly separated into control, VPA, Mn, Mn plus VPA, NaB, Mn plus NaB groups, and initial body-weights were recorded. Male mice were used to alleviate any possible effects of female sex hormone estrogens on the modulation of astrocytic glutamate transporters (Dhandapani and Brann, 2007; Lee et al., 2009). Body-weights were recorded on a weekly basis. Mice were treated once daily for 21 consecutive days with 100 μl of intraperitoneal (i.p.) injection of VPA (200 mg/kg), NaB (1200 mg/kg), or saline (NaCl, 0.9%; control). VPA and NaB were diluted in 0.9% saline. After 30 min, Mn plus VPA, Mn plus NaB, and Mn groups received 2 μl of MnCl_2 (30 mg/kg) via intranasal instillation in the left nostril. Likewise, VPA, NaB and control groups received 2 μl of distilled water in the left nostril. Previously published protocols for Mn intranasal instillation (Kim et al., 2012), VPA (Loscher et al., 1993; Zaky et al., 2014) and NaB dosing and route (Ferrante et al., 2003; Langley et al., 2008) were implemented. During the instillation period the mice were placed under isoflurane-induced anesthesia for 3 min pre- and post-instillation to sedate and prevent expulsion of Mn from the nostril, respectively.

2.4. Open-field test

Behavioral data were collected on day 21 in Seamless Open-field Activity Arenas using Activity Monitor 5 software (Med Associates, Fairfax, VT). Each open-field arena was made of clear Plexiglas, measuring 27.3 cm \times 27.3 cm \times 20.3 cm, and was covered with a Plexiglas lid containing air holes. The following activity measures were calculated directly by the Activity Monitor 5 software: distance traveled, stereotypic counts, and ambulatory counts for each animal in each group. Each animal went through an acclimation period (1 trial for 30 min, 1 day before Mn and/or VPA or NaB treatment). On day 21, control, Mn, VPA, Mn plus VPA, NaB, and Mn plus NaB groups commenced open-field testing to monitor total distance traveled, ambulatory and stereotypic counts. Each mouse was placed in the center of the open-field for observation and the activity measures were recorded for each observed mouse within the 30-min period.

2.5. Rotarod test for motor performance and coordination

Rota-Rod system (Med Associates Inc., Fairfax, VT) was used to measure the time an animal maintains balance on a moving cylinder. Mice were trained for three consecutive

days, with one daily session consisting of 3 trials separated by 300 s resting periods. On days 1 and 21, treated mice were placed on the rod and trials were deemed to have started when rod begins start, with speed gradually increasing from 4 rpm to 40 rpm up to 10 min by 0.1 revolutions/sec. The fall latency, a time measurement of how fast a subject falls from the rotarod at various speeds was assessed. If the mouse persisted on the rod for the entire duration, the measurement value was recorded for 650 sec. The mean duration for each mouse was used for comparison.

2.6. Real time RT-PCR analysis

Tissue samples (3 samples/group) were extracted from cerebral cortical and cerebellar regions of C57BL/6 mice used in behavioral experiments. The mRNA levels of GLT-1 and GLAST from these regions were analyzed. Total RNA was extracted from mouse brain tissue using RNeasy® Mini Kit (Qiagen, Valencia, CA). The purified RNA was transcribed to cDNA with high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). For quantitative real-time PCR (qPCR), the following primers were used: for GLT-1, 5'-GCC AAT ACA ACC AAG GCA GTC-3' (forward) and 5'-TTC ATC CCG TCC TTG AAC TC-3' (reverse); for GLAST, 5'-GATCGGAAACATGAAGGAGC-3' (forward) and 5'-CAA GAA GAG GAT GCC CAG AG-3' (reverse) and for GAPDH, 5'-TCC CTC AAG ATT GTC AGC AA-3' (forward) and 5'-AGA TCC ACA ACG GAT ACA TT-3' (reverse). The total reaction volume (25 µl) contained 1µl of cDNA template of each sample 0.4 µM of primers and RT2 SYBR® Green qPCR Master Mix from SABiosciences/Qiagen (Frederick, MD). The qPCR parameters were set for one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min in CFX96 real-time PCR detection system from Bio-Rad (Hercules, CA). GAPDH was used to normalize all samples. Analysis of data was performed using the web-based RT2 PCR Array Profiler Data Analysis version 3.5 (SABiosciences/Qiagen).

2.7. Western blot analysis

For protein analysis, three mice from each treatment group were randomly selected and harvested brain regions (e.g. cerebral cortical and cerebellar regions) were used for protein extraction and further analysis. The protein concentration of homogenized tissue samples [3:1 ratio of radioimmuno precipitation assay (RIPA) buffer with protease inhibitor and tissue sample] was determined by bicinchoninic acid (BCA) assay. Equal amounts of proteins were run on 10% SDS-PAGE followed by western blot analysis. The primary antibody for GLT-1 or GLAST was at 1:5000 dilution, and HRP-conjugated secondary antibody was used at 1:5000 dilution. All blots were developed using a Pierce chemiluminescence detection kit (Rockford, IL), followed by blot imaging and quantification with the Molecular Imager VersaDoc™ MP 4000 System and Image Lab Software version 5.2.1 from Bio-Rad, respectively.

2.8. Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). Multiple comparisons analyses were performed using a one-way analysis of variance (ANOVA) or two-way ANOVA, followed by Tukey's *post-hoc* tests using the GraphPad Prism Software version 5.0 (San Diego, CA). F-statistics and degrees of freedom for all analysis were

performed, and the detailed F-statistics are described in supplementary tables. A p-value less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Effect of treatment of Mn, VPA, NaB, or Mn plus VPA or NaB on body weight of mice

Body weights were determined after treatment with saline, Mn, VPA, NaB, and Mn plus VPA or NaB for 21 days. Mn was administered into mice via intranasal instillation (daily, 30 mg/kg/day) (Gianutsos et al., 1997; Henriksson and Tjalve, 2000; Moberly et al., 2012). Saline, VPA (daily, i.p., 200 mg/kg/day) (Kimura et al., 2015; Qian et al., 2010), or NaB (daily, i.p., 1200 mg/kg/day) in respective treatment groups, was pre-treated 30 min before Mn instillation into mice (Kratsman et al., 2016; Malago and Sangu, 2015; Zhong et al., 2014). As shown in Table 1 using a two-way ANOVA, there was no significant change in body weights in any of the treated groups compared to the body weights on day 1. Table 1 shows the average weights of mice in grams (g) on days 1 and 21.

3.2. Pretreatment of VPA with Mn reversed the Mn-induced decrease in the locomotor activity in mice

As one of the main pathological effects of Mn toxicity or manganism is PD-like behavioral deficits, we assessed the locomotor activity altered by Mn exposure, and determined if VPA or NaB attenuates Mn effects. As shown in Fig. 1, on day 21 Mn induced a significant decrease in locomotor activities on several parameters such as total distance traveled (1A), ambulatory activity (1B) and stereotypic activity (1C) counts, compared to the control group. During the habituation period, there was no significant change between any animals in each treatment group (data not shown). The results illustrated that 21 days of continuous pretreatment with either VPA or NaB (30 min prior to Mn exposure) attenuated the Mn-induced behavioral deficits. Mn-induced reduction of total distance traveled were significantly reversed by both VPA ($F_{5,42} = 15.83$, $p < 0.01$) and NaB ($F_{5,42} = 15.83$, $p < 0.01$) (Fig. 1A). Additionally, ambulatory counts by VPA ($F_{5,42} = 17.64$, $p < 0.01$) and NaB ($F_{5,42} = 17.64$, $p < 0.05$) and stereotypic counts were reversed by VPA ($F_{5,42} = 6.545$, $p < 0.01$) and NaB ($F_{5,42} = 6.545$, $p < 0.05$) (Fig. 1B and 1C), respectively.

3.3. Effect of VPA and NaB on the Mn-induced decrease of rotarod performance

The rotarod performance test is especially useful to test the effect of experimental drugs or after traumatic brain injury (Mouzon et al., 2012). The length of time that a given animal stays on this rotating rod is a measure of their balance, coordination, physical condition and motor-planning. The Mn group exerted significantly reduced retention times on the bar compared to the control group on days 21 ($F_{5,42} = 7.566$, $p < 0.01$, Fig. 2), while pretreatment of VPA and NaB (Fig. 2) with Mn significantly attenuated motor coordination performance compared to the Mn alone group ($F_{5,42} = 7.566$, $p < 0.01$ for VPA; $F_{5,42} = 7.566$, $p < 0.05$ for NaBu). The VPA or NaB alone group did not change in rotarod performance compared to the control group.

3.4. VPA and NaB attenuated Mn-induced decrease of GLT-1 mRNA levels in the cerebral cortex and cerebellum of mouse brain

Mn induces glutamate excitotoxicity and dysregulates astrocytic glutamate transporters in the brain (Normandin and Hazell, 2002), and astrocytic glutamate transporters (GLT-1 and GLAST) are critically involved in glutamate toxicity (Rothstein, 1995; Sheldon and Robinson, 2007; Zhang et al., 2016). Since HDAC inhibitors including VPA and NaB have shown to reverse Mn-induced decrease in GLT-1 expression in astrocytes culture system, we investigated if VPA and NaB could reverse the Mn-induced effect. As shown in Fig. 3A and 3B, Mn significantly decreased GLT-1 mRNA levels in the cerebral cortex ($F_{3,8} = 179.5$, $p < 0.001$) and cerebellum ($F_{3,8} = 38.72$, $p < 0.001$) compared to the control group, whereas pretreatment of VPA with Mn significantly reversed GLT-1 mRNA levels compared to those of the Mn-treated group ($F_{3,8} = 179.5$, $p < 0.001$ for cortex and $F_{3,8} = 38.72$, $p < 0.01$ for cerebellum). Moreover, co-treatment of NaB with Mn also exerted a similar effect to those of VPA in attenuation of Mn-decreased GLT-1 mRNA levels in the cerebral cortex ($F_{3,8} = 47.63$, $p < 0.001$) and cerebellum ($F_{3,8} = 10.28$, $p < 0.01$) (Fig. 3C and 3D).

3.5. VPA and NaB attenuated Mn-induced decrease of GLT-1 protein levels in the cerebral cortex and cerebellum of mouse brain

For GLT-1 to be functional, it has to be in a form of protein, and thus, its protein expression levels are desirable to be tested. The results show a similar trend for GLT-1 protein expression to those of GLT-1 mRNA, as shown in Fig. 4. Mn decreased GLT-1 protein levels significantly in the cerebral cortex and cerebella regions of mice treated for 21 days, while VPA attenuated the Mn-decreased GLT-1 protein levels in cerebral cortex ($F_{3,8} = 10.22$, $p < 0.01$) (Fig. 4A) and cerebellum ($F_{3,8} = 40.38$, $p < 0.001$) (Fig. 4B). NaB also reversed Mn-induced reduction of GLT-1 protein levels in both cerebral cortex ($F_{3,8} = 31.72$, $p < 0.05$) (Fig. 4C) and cerebellum ($F_{3,8} = 14.81$, $p < 0.01$) (Fig. 4D). Neither VPA nor NaB alone altered GLT-1 protein levels significantly compared to the control groups.

3.6. VPA and NaB attenuated Mn-induced decrease of GLAST mRNA levels in the cerebral cortex and cerebellum

We also assessed the other main astrocytic glutamate transporter GLAST mRNA levels in cerebral cortex and cerebellum after 21 days of treatment with Mn, VPA, NaB, Mn plus VPA, or Mn plus NaB. While GLT-1 expression is more dominant in the cerebral cortex compared to cerebellum, GLAST is highly expressed in the cerebellar region compared to the cortex (Regan et al., 2007), indicating that these two transporters play a different role in different brain regions. As shown in Fig. 5, Mn significantly decreased GLAST mRNA levels in cerebral cortex ($F_{3,8} = 27.03$, $p < 0.001$) and cerebellum while co-treatment of VPA (30 min prior to Mn exposure) reversed Mn-decreased GLAST mRNA levels in both cerebral cortex ($F_{3,8} = 27.03$, $p < 0.01$) (Fig. 5A) and cerebellum ($F_{3,8} = 14.73$, $p < 0.05$) (Fig. 5B). NaB also reversed the Mn-induced reduction in GLAST mRNA levels in the cortex ($F_{3,8} = 106.3$, $p < 0.001$) (Fig. 5C) and cerebellum ($F_{3,8} = 19.01$, $p < 0.01$) (Fig. 5D).

3.7. VPA and NaB attenuated Mn-induced decrease of GLAST protein levels in the cerebral cortex and cerebellum

As shown in Fig. 6, Mn decreased GLAST protein levels significantly, where VPA attenuated Mn-decreased GLAST protein levels in both cerebral cortex ($F_{3,8} = 21.76$, $p < 0.01$) (Fig. 6A) and cerebellum ($F_{3,8} = 8.193$, $p < 0.01$) (Fig. 6B). NaB also reversed the Mn-induced decrease of GLAST protein expression levels in both cerebral cortex ($F_{3,8} = 10.18$, $p < 0.01$) (Fig. 6C) and cerebellum ($F_{3,8} = 8.193$, $p < 0.05$) (Fig. 6D) after 21 days of treatment.

4. Discussion

We demonstrate the therapeutic potential of VPA and NaB against Mn-induced behavioral deficits in mice. Moreover, VPA and NaB attenuated the Mn-induced dysregulation of astrocytic glutamate transporters by reversing the Mn-induced decrease in mRNA and protein levels of GLT-1 and GLAST in mouse cerebral cortex and cerebellum. These findings indicate that VPA and NaB may attenuate Mn-induced behavioral deficits, at least in part by reversing expression of GLT-1 and GLAST at the transcriptional level.

The molecular pharmacological commonality of VPA and NaB resides in their ability to inhibit HDACs via epigenetic mechanisms. We did not test the possible role of HDAC inhibition by these compounds in directly attenuating Mn toxicity. However, this attenuation is likely to involve HDAC inhibition, because HDACs regulate expression of GLT-1/GLAST as co-repressors of transcription factor yin yang 1 (YY1), and Mn enhances the interaction of YY1 with HDACs (Karki et al., 2015; Karki et al., 2014). HDAC inhibitors have been previously shown to reverse Mn-induced reduction in GLT-1 promoter activity as well as mRNA and protein levels (Karki et al., 2014). Additional studies on the precise mechanism/s by which VPA and NaB afford neuroprotection against Mn-induced toxicity should be advanced.

We tested motor performance and coordination by open-field and rotarod performance (Curzon et al., 2009). Since the symptoms of Mn toxicity or manganism are similar to those in PD, including tremor, gait, frequent falling and problems with posture and agility (Peres et al., 2016), analysis of Mn-induced reduction of locomotor activity and motor coordination are pertinent to test the effects of VPA and NaB in Mn-induced behavioral deficits. The cerebral cortex is a major component of the neural motor pathway in regulating body movements, and cerebellar regions play a critical role in motor coordination and balance. Therefore, studying these regions to examine any changes at the molecular levels are highly relevant for corroborating the efficacy of VPA and NaB in attenuating the Mn-induced behavioral deficits.

Ambulatory activity represents spontaneous physical activity, which comprises all activity other than formal exercise (Kotz et al., 2012). Animals with reduced motor muscle function have lower ambulatory activity, which is generally associated with decreased horizontal and vertical activities, total distance traveled and movement time (Nagaraju et al., 2000; Rayavarapu et al., 2010; Yu et al., 2013). It has been reported that PD patients showed a decrease in physical ambulatory activities due to motor deficits (Skidmore et al., 2008). The

stereotypic activity is believed to be correlated with signs of altered response selection by the basal ganglia (Garner and Mason, 2002). Assessing ambulatory activity associated with Mn toxicity in rodents is highly relevant, because in the initial stages of manganism, neurological symptoms consist of reduced response speed, irritability, mood changes and compulsive behaviors (Roth, 2006). In more advanced stages of the disease, Mn toxicity becomes more prominent in the basal ganglia, resembling the symptoms inherent to idiopathic PD. VPA is an anti-seizure agent, and has long been used clinically to treat epileptic patients with incomplete understanding of mechanism of action involved. VPA is known to modulate γ -aminobutyric acid (GABA) neurotransmission and also inhibit HDACs at the epigenetic levels, warranting further studies to understand molecular mechanisms by which VPA reversed Mn effects on behavioral deficits.

Although both VPA and NaB are well established HDAC inhibitors, they have also been shown to exert neuroprotective effects via other distinct cellular and molecular mechanisms (Chuang et al., 2009). The present study focused on their effects on modulation of astrocytic glutamate transporters GLT-1 and GLAST. Among the five subtypes of glutamate transporters identified in humans, two transporters, namely, EAAT1 (GLT-1 in rodents) and EAAT2 (GLAST in rodents), which are predominantly expressed in astrocytes, effectively remove the majority of glutamate from synaptic clefts. Accordingly, dysregulation of GLT-1 or GLAST leads to glutamate accumulation in the synaptic clefts, ultimately causing excitotoxic neuronal injury and death (Jayakumar and Norenberg, 2016; Karki et al., 2015; Leke and Schousboe, 2016). The results from the present study indicate that altered expression levels of GLT-1 and GLAST by Mn, and the reversing effects of VPA and NaB might be associated with dysregulation of glutamate signaling as well as excitotoxicity in the cerebral cortex and cerebellum, eventually leading to modulation of locomotor activity and motor coordination. Although the molecular mechanism underlying Mn-reduced GLT-1 and GLAST at the transcriptional level remains to be established, YY1 might be the key transcription factor for Mn-reduced expression of GLT-1/GLAST (Karki et al., 2014). Mn increased YY1 expression at the transcription level, and YY1 repressed GLT-1/GLAST with HDACs as co-repressors (Karki et al., 2014). These findings support the notion that inhibition of HDACs might represent a plausible mechanism of VPA/NaB-induced attenuation of Mn-induced motor deficits.

VPA, a conventional anti-epileptic drug, has been shown to improve seizure (Campos et al., 2016), primarily being used to treat epilepsy, bipolar disorder and migraine headaches. VPA-induced antiepileptic effects have been proposed to be due to modulation of levels of brain GABA by raising its levels in cerebral and cerebellar regions, possibly by inhibiting GABA degradative enzymes and transport of T-type calcium ions (Lason et al., 2011; Leke and Schousboe, 2016). However, a growing body of evidence indicates that VPA might be beneficial to treat many other neurologic and/or neurodegenerative diseases such as stroke, traumatic brain injury (TBI), Huntington's disease (HD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Fragile X Syndrome (FXS) via their diverse mechanisms of action (Monti et al., 2009). VPA modulates neurotrophic, angiogenic, and anti-apoptotic factors, survival signaling cascades, and oxidative stress pathways as well as HDAC epigenetic mechanisms (Ximenes et al., 2015).

At clinically relevant levels, VPA effectively inhibits HDACs (Gottlicher et al., 2001; Phiel et al., 2001), making it valuable for investigations into the therapeutic role of chromatin remodeling and epigenetic regulation in the CNS disorders. VPA significantly inhibits class I and, to a lesser extent, class II HDACs (Gottlicher et al., 2001). VPA also improves behavioral and cognitive performance, suppresses neurodegeneration and neuroinflammation, and prolongs cell survival in various animal models of CNS disorders such as stroke, PD, HD, AD and ALS (Chiu et al., 2013). Clinically, weight gain and increased appetite in obese patients have been reported as side effects of VPA treatment (Belcastro et al., 2013), but no significant changes in body weight or weight gain were observed in the current study. The protective effects of VPA against Mn-induced motor deficits are likely due to its efficacy in reversing the Mn-reduced effects on GLT-1/GLAST via inhibition of HDACs. VPA upregulates GLAST promoter activity and mRNA levels in chicken Bergmann glial cells (Aguirre et al., 2008), supporting the notion that VPA may exert neuroprotection against Mn-induced toxicity, at least in part, by modulation of histone acetylation and transcription of glutamate transporter activity. Nonetheless, further studies are warranted in order to elucidate the molecular mechanism by which VPA affords protection against Mn-toxicity.

Numerous studies have reported that NaB induces neuroprotection in various animal models of neurological disorders, suggesting a number of molecular mechanisms of action for NaB in its neuroprotection including anti-inflammatory effect (Park and Sohrabji, 2016), anti-oxidative effect, and anti-apoptotic properties (Sun et al., 2015). NaB has also been reported to modulate gene expression that is related to many behavior in the prefrontal cortex (Kratsman et al., 2016). NaB affects genes involved in neuronal excitation or inhibition, particularly promoting the transcription of genes on inhibitory pathways, but the exact mechanisms by which NaB regulates social behavior and neuronal activity on gene expression levels have yet to be established. At the epigenetic levels, NaB is a well-established HDAC inhibitor, suggesting that inhibition of these HDACs is likely involved in the molecular and behavioral effects. In general, it has been reported that HDAC inhibitors have potential to serve as therapeutics to treat learning and memory deficits (Takuma et al., 2014), depression (Wei et al., 2014) and cognition deficits (Graff and Tsai, 2013).

As histone hypoacetylation and transcriptional dysfunction are involved in numerous neurological disorders in both *in vivo* and *in vitro* models, particularly, class I and II HDAC inhibitors have shown to protect against neurodegeneration by modulating expression of multiple genes involved in neuroprotection and neurotrophicity (Chuang et al., 2009). Importantly, HDAC inhibitors modulate gene expression including neurotrophin not only in neurons but also in astrocytes. This suggests that astrocytes are also an important target for therapeutic intervention. In agreement with this previous reports, our findings that VPA and NaB attenuated Mn-induced behavioral deficits as well as a decreased expression of astrocytic glutamate transporters GLT-1 and GLAST which are predominantly expressed in astrocytes. Interestingly, VPA and NaB do not exert similar enhancing effects on GLT-1/GLAST between *in vivo* and *in vitro* conditions. We have reported that VPA and NaB increased promoter activity of GLT-1 (Karki et al., 2014), and increased the levels of mRNA and protein of GLAST in *in vitro* astrocytes (Karki et al., 2015). The present study shows that these HDAC inhibitors did not increase significantly expression of GLT-1 and GLAST

in mouse brain, while attenuating Mn-induced reduction of expression of these transporters. It is possible that in the *in vivo* condition, the mouse brain is compromised with the effects of HDAC inhibitors on GLAST/GLT-1 expression to maintain homeostasis. At this point, it is not conclusive whether these HDAC inhibitors attenuate Mn-induced neurotoxicity is solely via inhibition of HDACs or involving additional unknown mechanisms.

In conclusion, we demonstrate that VPA and NaB exert protective effects against Mn-induced decrease of locomotor activity as well as GLT-1/GLAST expression in mice. The VPA- and NaB-induced protective effects likely reflect VPA/NaB's propensity to increase glutamate transporter activity and glutamate clearance, thus inhibiting glutamate accumulation and excitotoxicity. Therefore, VPA and NaB have the potential to attenuate Mn toxicity as well as other neurodegenerative diseases driven by glutamate excitotoxicity. Further studies are required to establish the optimal doses of VPA and NaB, and identify the molecular targets of VPA and NaB which mediate their efficacy in attenuating the Mn-induced decrease in locomotor activity and GLT-1/GLAST expression in mouse brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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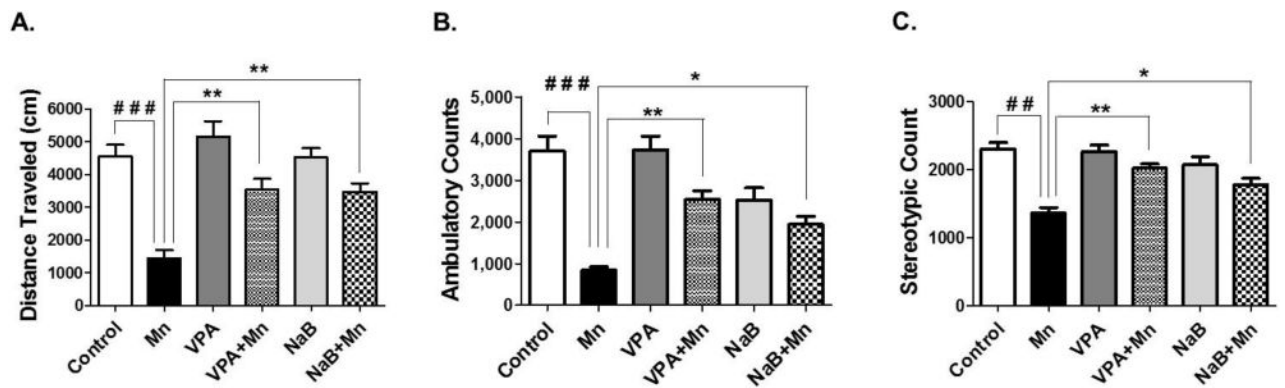


Fig. 1. Attenuating effect of VPA and NaB on Mn-induced decrease in locomotor activity in mice Total distance traveled (1A), ambulatory counts (1B) and stereotypic counts (1C) were measured in C57BL/6 mice. Mice were pretreated with VPA (100 μ l/200 mg/kg, i.p. daily) or with NaB (100 μ l/1200 mg/kg, i.p. daily) 30 min prior to Mn exposure (2 μ l/30mg/kg, intranasal instillation, daily) for 21 days as described in the Methods section. Saline (0.9% NaCl, i.p.) was used as vehicles for VPA and NaB; H₂O was used as vehicle for Mn. ###, $p < 0.001$; #, $p < 0.01$; * $p < 0.05$; ** $p < 0.01$ (ANOVA followed by Tukey's *post hoc* test; $n = 8$).

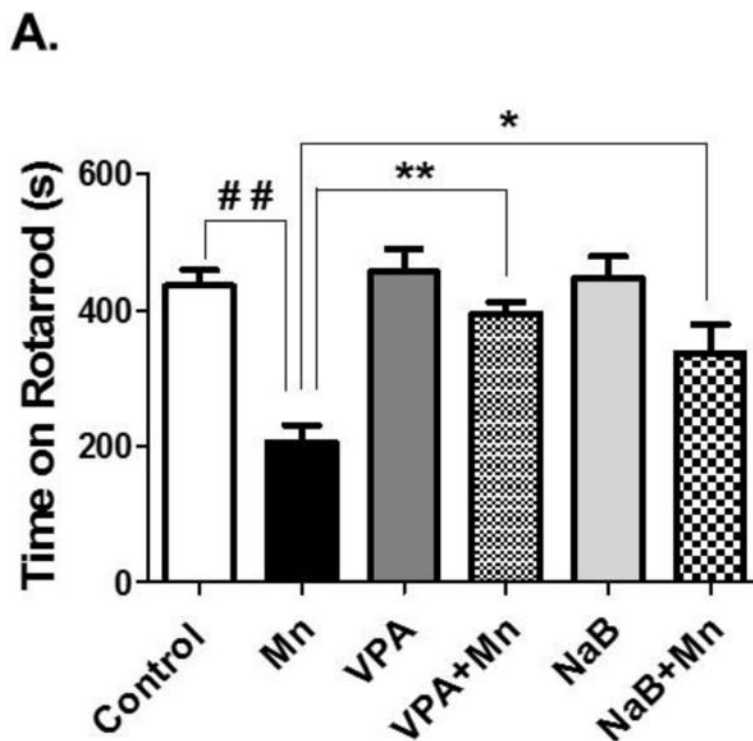


Fig. 2. Attenuating effect of VPA and NaB on Mn-induced decrease in motor coordination by Rota-Rod test in mice

Total time on rota-rod was measured after mice were pretreated with VPA (100 μ l/200 mg/kg, i.p. daily) or NaB (100 μ l/1200 mg/kg, i.p. daily) 30 min prior to Mn exposure (2 μ l/30mg/kg, intranasal instillation, daily) for 21 days as described in the Methods section. Saline (0.9% NaCl, i.p.) was used as a vehicle for VPA and NaB, and H₂O was used as a vehicle for Mn. ##, $p < 0.01$; *, $p < 0.05$; **, $p < 0.01$ (ANOVA followed by Tukey's *post hoc* test; n = 8).

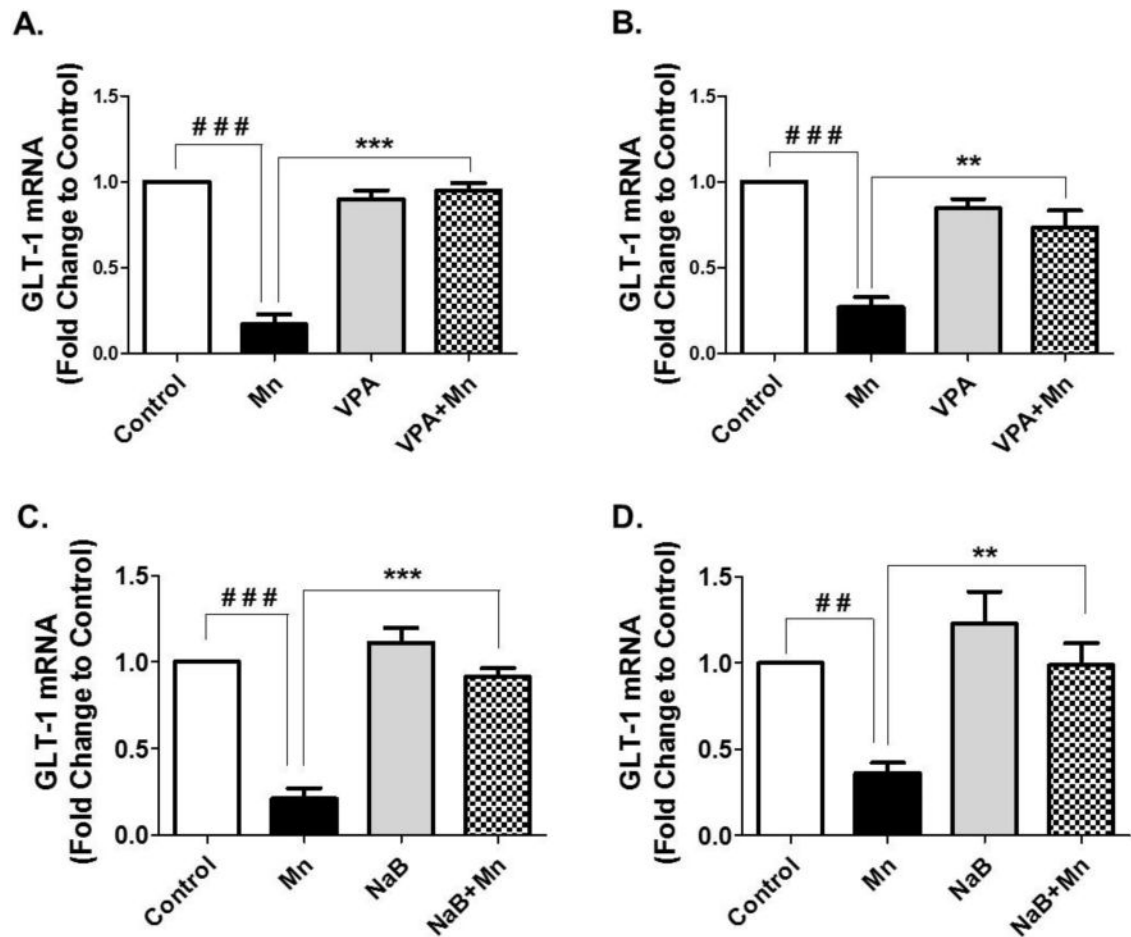


Fig. 3. Effects of VPA and NaB on Mn-induced decrease of GLT-1 mRNA levels in mouse cerebral cortical and cerebellar regions

At the end of 21 days of treatment with Mn, VPA, NaB as previously mentioned, tissues from cerebral cortex and cerebellum were prepared and measured for GLT-1 mRNA levels by qPCR. (A, B) GLT-1 mRNA levels in cerebral cortex (4A) and cerebellum (4B) from mouse brain treated with Mn, VPA or Mn plus VPA. (C, D) GLT-1 mRNA levels in cerebral cortex (4C) and cerebellum (4D) from mouse brain treated with Mn, NaB, or Mn plus NaB. ##, $p < 0.01$; ###, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.001$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

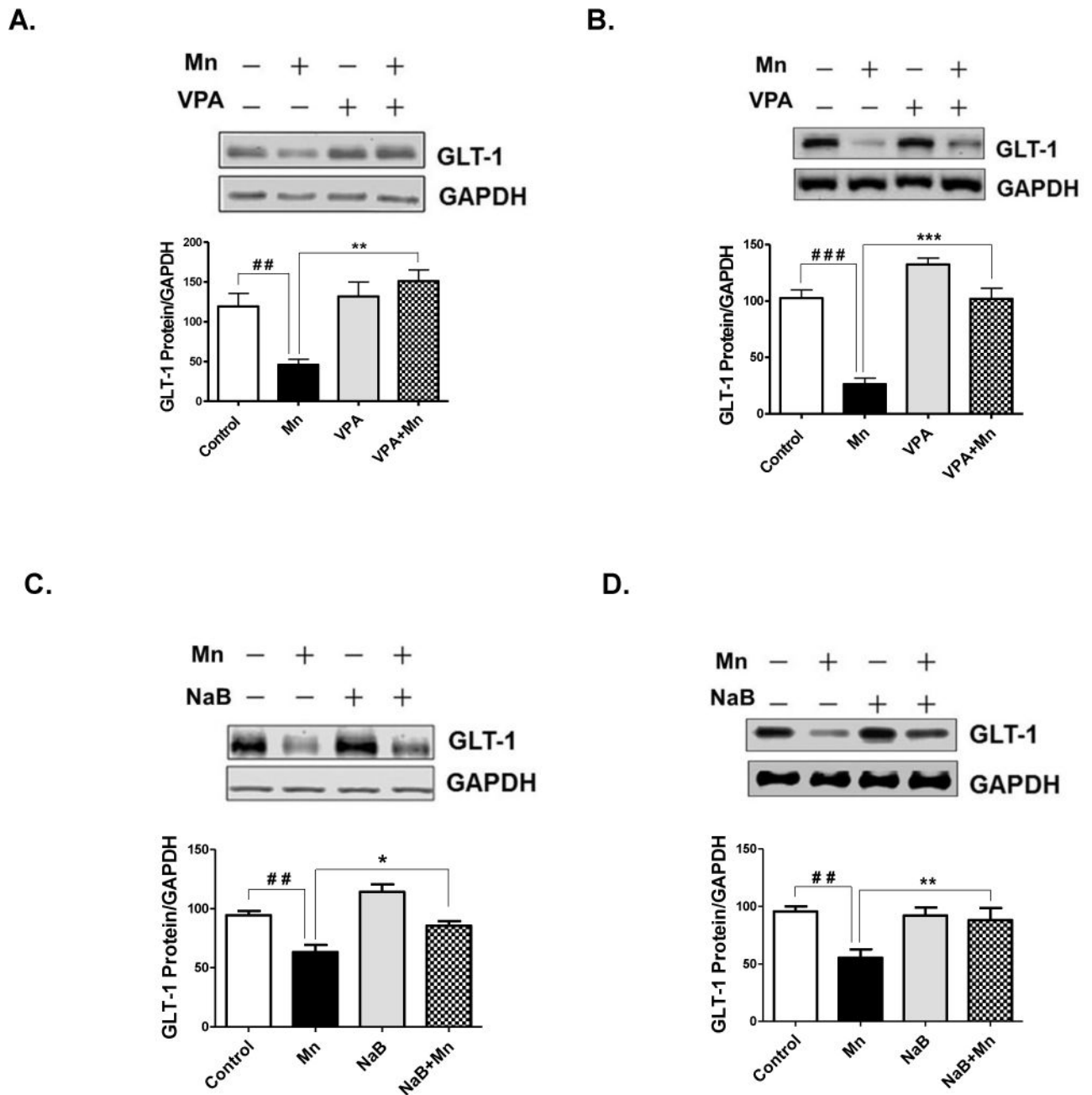


Fig. 4. Effects of VPA and NaB on Mn-induced decrease of GLT-1 protein levels in mouse cerebral cortical and cerebellar regions

At the end of 21 days of treatment with Mn, VPA, NaB as previously mentioned, tissues from cerebral cortex and cerebellum were prepared and measured for GLT-1 protein levels by western blot analysis. (A, B) GLT-1 protein levels in cerebral cortex (4A) and cerebellum (4B) from mouse brain treated with Mn, VPA or Mn plus VPA. (C, D) GLT-1 protein levels in cerebral cortex (4C) and cerebellum (4D) from mouse brain treated with Mn, NaB, or Mn plus NaB. ##, $p < 0.01$; ###, $p < 0.001$; *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

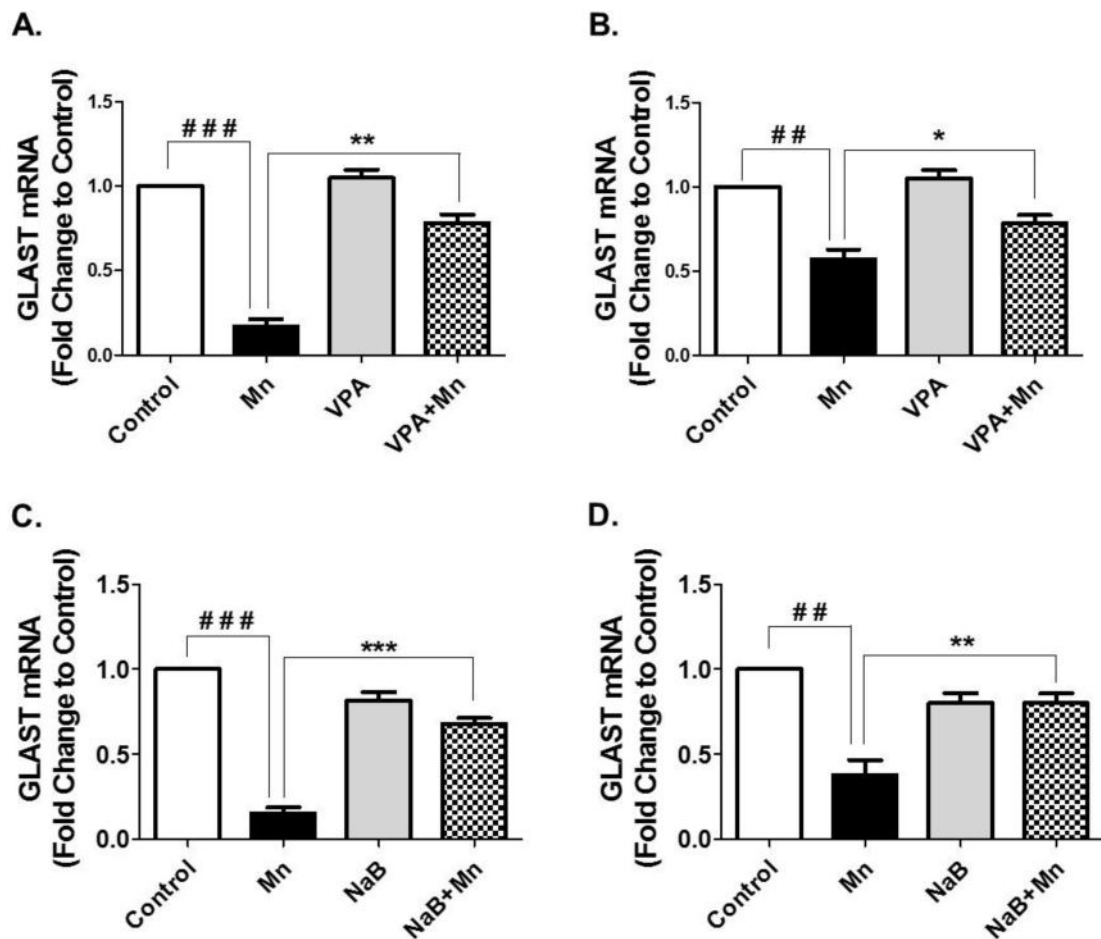
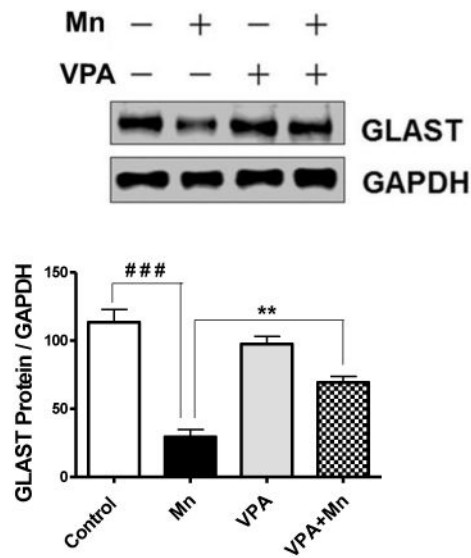


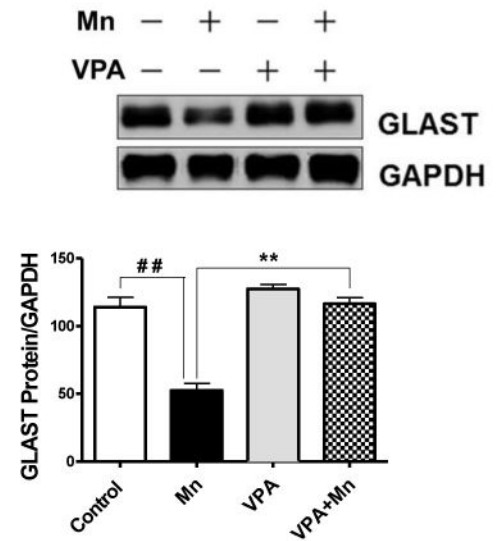
Fig. 5. Effects of VPA and NaB on Mn-induced decrease of GLAST mRNA levels in mouse cerebral cortical and cerebellar regions

At the end of 21 days of treatment with Mn, VPA, NaB as previously mentioned, tissues from cerebral cortex and cerebellum were prepared and measured for GLAST mRNA levels by qPCR. (A, B) GLAST mRNA levels in cerebral cortex (4A) and cerebellum (4B) from mouse brain treated with Mn, VPA or Mn plus VPA. (C, D) GLAST mRNA levels in cerebral cortex (4C) and cerebellum (4D) from mouse brain treated with Mn, NaB, or Mn plus NaB. ##, $p < 0.01$; ###, $p < 0.001$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

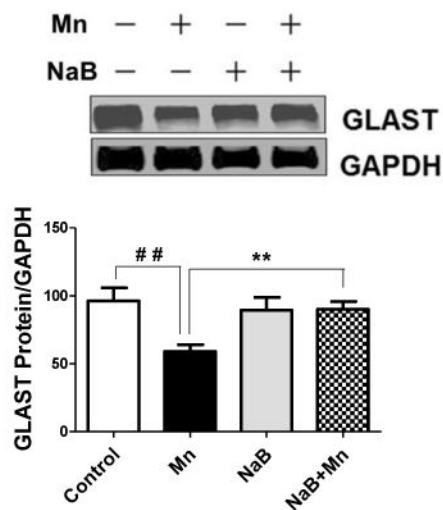
A.



B.



C.



D.

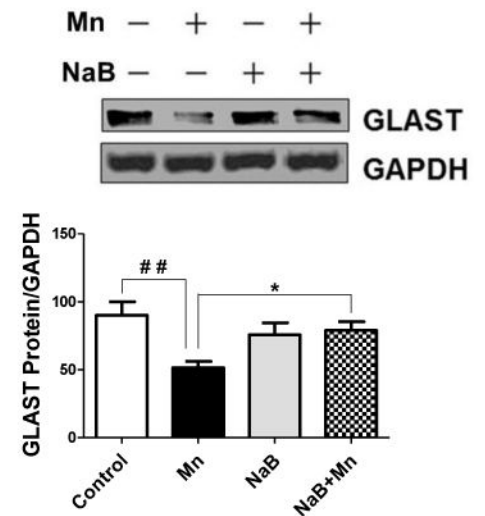


Fig. 6. Effects of VPA and NaB on Mn-induced decrease of GLAST protein levels in mouse cerebral cortical and cerebellar regions

At the end of 21 days of treatment with Mn, VPA, NaB as previously mentioned, tissues from cerebral cortex and cerebellum were prepared and measured for GLAST protein levels by western blot analysis. (A, B) GLAST protein levels in cerebral cortex (4A) and cerebellum (4B) from mouse brain treated with Mn, VPA or Mn plus VPA. (C, D) GLAST protein levels in cerebral cortex (4C) and cerebellum (4D) from mouse brain treated with Mn, NaB, or Mn plus NaB. ##, $p < 0.01$; ###, $p < 0.001$; *, $p < 0.05$; **, $p < 0.01$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

Table 1

Body weight changes in mice after treated with Mn, VPA, NaB, Mn plus VPA, or Mn plus NaB for 21 days

Treatment Groups	Day 1 Average Weight (g, n=8)	Day 21 Average Weight (g, n=8)	Weight Gain (+)/Loss (-) (g)
Control	23.04 ± 1.77	26.58 ± 1.74	+3.55 ± 0.94
Mn	19.54 ± 3.47	18.11 ± 2.06	-1.43 ± 2.02
VPA	22.58 ± 1.27	24.60 ± 1.82	+2.03 ± 1.16
VPA + Mn	21.99 ± 0.85	22.02 ± 0.99	+0.03 ± 0.43
NaB	22.48 ± 1.19	23.45 ± 1.65	+0.97 ± 1.37
NaB + Mn	21.90 ± 1.73	20.07 ± 1.41	-1.83 ± 0.44

Mice were pretreated with VPA (100 µl/200 mg/kg, i.p. daily) or with NaB (100 µl/1200 mg/kg, i.p. daily) 30 min prior to Mn exposure (2 µl/30mg/kg, intranasal instillation, daily) for consecutive 21 days. Body weights were measured throughout the experiment and weight gain recorded on day 1 and day 21. Results represent mean ± S.E.M derived from eight independent experiments and each of their weights are expressed in grams (g). Statistical analysis was performed by two-way ANOVA repeated measures, followed by Tukey's *post-hoc* test, (n=8).