

N-acetylcysteine ameliorates repetitive/stereotypic behavior due to its antioxidant properties without activation of the canonical Wnt pathway in a valproic acid-induced rat model of autism

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Abstract. N-acetylcysteine (NAC) is widely used as an antioxidant, and previous studies have suggested that it may have potential as an alternative therapeutic strategy for the treatment of patients with autism. However, the exact effects of NAC administration on the development of autism, as well as the molecular mechanisms underlying its actions, have yet to be fully elucidated. The present study aimed to investigate the effects of NAC on the oxidative status of rats in a valproic acid (VPA)-induced model of autism, and to examine the involvement of the canonical Wnt signaling pathway in the actions of NAC. Rats exposed to VPA were monitored for behavioral changes, and oxidative stress indicators and key molecules of the canonical Wnt pathway were investigated using colorimetric and western blot analysis, respectively. The present results demonstrated that NAC ameliorated repetitive and stereotypic activity in autism model rats. Furthermore, NAC was revealed to relieve oxidative stress, as demonstrated by the increased glutathione and reduced malondialdehyde levels compared with VPA-treated rats. However, NAC did not appear to affect the activity of the canonical Wnt signaling pathway. The present findings suggested that the beneficial effects of NAC in autism may be associated with its antioxidative properties, and may not be mediated by the canonical Wnt pathway. However, it may be hypothesized that the canonical Wnt pathway can be indirectly regulated by NAC through the activation of other signaling pathways or upstream factors.

Taken together, the present study has contributed to the elucidation of the molecular mechanisms that underlie the actions of NAC in autism, suggesting its potential for the development of novel therapeutic strategies for the treatment of patients with autism.

Introduction

Autism is a complex neurodevelopmental disorder characterized by three central features: Social interaction deficits, language retardation and aberrant stereotypic behavior (1,2). In 2016, the Centers for Disease Control and Prevention reported that 1 in 68 children is diagnosed with autism spectrum disorder in the United States (3). Currently, no effective therapeutic strategies are available to ameliorate the key deficits associated with autism. Therefore, the need for the development of novel targeted treatments is urgent for the management of autism, and for improvement of the patients' quality of life.

Previous studies have suggested that oxidative stress may be involved in the development of autism (4,5). N-acetylcysteine (NAC) is a source of the amino acid L-cysteine, and is a precursor in the formation of glutathione (GSH) (6), which is a major intracellular antioxidant within the central nervous system (CNS) (7). In addition to providing cysteine for GSH production, NAC has been revealed to directly scavenge oxygen free radicals through its thiol-reducing group, and has been suggested as a promising neuroprotectant in the CNS (8,9). NAC has been reported to cross the blood brain barrier (10,11), decrease oxidative stress and inflammatory cytokine production, replenish GSH levels, and thus ameliorate autism-associated irritability, including self-injurious behavior (12). Previous placebo-controlled human trials demonstrated that, following administration of NAC, autism-associated irritability was attenuated in pediatric patients with autism (12,13). In addition, two clinical trials demonstrated that the combination of NAC with risperidone significantly alleviated irritability in young patients with autism (14,15). Furthermore, NAC has been suggested to ameliorate additional features of autism, as it was demonstrated

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to improve social interaction (7,16) and reduce anxiety-like behaviors (16). However, to the best of our knowledge, the effects of NAC on repetitive/stereotypic activity and oxidative stress in autism models, and the molecular mechanisms underlying the effects of NAC in the treatment of autism, have yet to be elucidated.

The canonical Wnt pathway includes the major effector β -catenin and glycogen synthase kinase (GSK)-3 β . In the absence of Wnt ligands, β -catenin associates with a cytoplasmic degradation protein complex, leading to proteasomal degradation (17). With secreted Wnt ligands, the canonical Wnt pathway is activated, and the cytoplasmic degradation protein complex is prevented from forming. As a result, β -catenin accumulates in cytoplasm and translocates to the nucleus (18). GSK-3 β destabilizes β -catenin by phosphorylating its inhibitory sites, such as Ser33, Ser37, and Thr41 (19). Previous studies have reported that oxidative stress activated the canonical Wnt intracellular signaling pathway during the pathogenesis of various diseases, including preeclampsia (20), diabetic nephropathy (21) and gastric cancer (22). The canonical Wnt pathway has also been reported to be involved in the development of autism (23-25). Therefore, elucidating the molecular mechanisms that are involved in the modulation of oxidative processes, and the roles of the Wnt pathway in the pathogenesis of autism, is of critical importance.

The present study aimed to investigate the effects of NAC on autism-like behavioral phenotypes, and to examine the molecular mechanisms underlying its actions in a valproic acid (VPA)-induced animal model of autism. NAC was demonstrated to reverse behavioral abnormalities, including stereotypic behaviors, and its therapeutic effects were associated with a reduction in oxidative stress. However, the molecular mechanisms involved in the actions of NAC did not appear to be associated with the canonical Wnt signaling pathway. The Wnt/ β -catenin pathway may be indirectly regulated by NAC through the activation of alternative signaling pathways or upstream factors, and further studies are required to elucidate the mechanisms that may be involved in its actions.

Materials and methods

Animals and experimental groups. Experimental procedures were approved by the Laboratory Animal Care and Use Committee of Xinxiang Medical University, and were in accordance with the guidelines for animal experimentation of the Institutes of Health of Xinxiang Medical University (Xinxiang, China). A total of 60 Sprague-Dawley rats (age, 12 weeks; 40 female, 220-250 g; 20 male, 350-390 g) were purchased from Vital River Laboratories Co., Ltd. (Beijing, China) and were allowed to breed. The rats were kept in a cage with 40-80% relative humidity and a controlled temperature of 24 \pm 1 $^{\circ}$ C on a 12-h light/dark cycle (lights off at 19:00). Rats had free access to food and water. The presence of vaginal plugs was used to indicate gestation. For the establishment of the autism model (26,27), female rats were intraperitoneally injected with 600 mg/kg VPA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) on gestation day 12.5, vehicle-treated rats received physiological saline. The pups were weaned on postnatal day 23 and housed separately. Offspring (23 days old) of VPA-treated mothers were randomly assigned into the following 2 groups:

VPA and VPA + NAC (Sigma-Aldrich; Merck KGaA) groups. The 23-day-old-offspring of saline-treated mothers were randomly divided into the following 2 groups: Control and NAC groups. NAC (150 mg/kg) was intraperitoneally administered to rats in the NAC and VPA + NAC groups once daily for 4 weeks. Subsequently, male offspring were used for repetitive/stereotypic-like behavioral testing and then sacrificed by decapitation. Prefrontal cortex (PFC) and hippocampus (HC) samples were isolated and frozen at -80 $^{\circ}$ C for further analysis.

Behavioral testing. Behavioral testing was conducted using an open field test, as previously described (28). Briefly, rats were placed individually in a plywood apparatus (100x100x40 cm). Testing was performed between 9:00 am and 3:00 pm. Two rats were observed simultaneously, with one animal per chamber. The animals were individually placed in the field and allowed to explore it freely for 60 min. Movements were recorded using a digital video camera linked to a computer (Ethovision 3.0; Noldus Information Technology BV, Wageningen, The Netherlands); the mean activity during the 60-min testing session, divided into 6 time bins (10 min each), was then analyzed (EthoVision 3.0; Noldus). Repetitive/stereotypic-like movements (for example run and rotate, nose picking, lip biting, repeated sucking) were measured and analyzed by an observer blind to the treatments.

Oxidative stress assays

Malondialdehyde (MDA) assay. As a marker of lipid peroxidation, the MDA contents were evaluated in brain tissue samples isolated from rats using a commercially available MDA assay kit (cat no. S0131; Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. Briefly, brain tissue sections were homogenized in 0.15 ml thiobarbituric acid (TBA) diluent, 0.05 ml TBA and 3 μ l antioxidant then heated and boiled for 15 min. Following centrifugation at 1,000 x g for 10 min at room temperature, the supernatants were collected, and the absorbance of the samples was measured at 532 nm using a microplate reader. MDA contents were expressed as μ mol/mg protein.

GSH assay. 5,5'-Dithiobis-(2-nitrobenzoic acid) was used to detect intracellular GSH levels with a Total GSH assay kit (cat no. S0052; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Briefly, brain tissue sections were homogenized with glass homogenizer and incubated with the assay solution for 5 min at 25 $^{\circ}$ C. The concentration of GSH was measured spectrophotometrically at 412 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GSH contents were expressed as μ mol/mg protein.

Western blot analysis. Cytoplasmic and nuclear proteins were isolated from brain tissue samples using a Nuclear and Cytoplasmic Protein Extraction kit (cat no. P0028; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Tissue samples (60 mg) were lysed in a 200 μ l solution containing cytoplasmic protein extraction agent A and B (20:1) supplemented with phenylmethylsulfonyl fluoride (PMSF). Following tissue homogenization, the samples were incubated for 15 min on ice. Then the lysates were centrifuged for 5 min at 1,500 x g at 4 $^{\circ}$ C, and the supernatants, consisting

of the cytoplasmic fraction, were immediately frozen at -80°C for subsequent experiments. The pellets were resuspended in nuclear protein extraction agent B supplemented with PMSF. Following vortexing for 5 sec, the mixture was incubated on ice for 1 min and centrifuged for 10 min at $12,000 \times g$ at 4°C to obtain supernatants containing nuclear proteins. Proteins were quantified with a bicinchoninic acid assay kit (cat no. P0010; Beyotime Institute of Biotechnology). Equal amounts of extracted protein samples ($20\text{--}50 \mu\text{g}$) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin (cat no. A7030; Sigma-Aldrich; Merck KGaA) or fat-free milk in TBS containing 0.1% Tween-20 for 1 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C . Following incubation with horseradish peroxidase-conjugated goat anti-rabbit antibodies (cat no. 111035003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at a 1:5,000 dilution for 1 h at room temperature, the protein bands were visualized using an enhanced chemiluminescence kit (EMD Millipore). Blots were semi-quantified using densitometric analysis with ImageJ software (version 1.44p; National Institutes of Health, Bethesda, MD, USA). GAPDH (1:5,000; cat no. HRP-60004; ProteinTech Group, Inc., Chicago, IL, USA) or histone H3 (1:1,000; cat no. AF0009; Beyotime Institute of Biotechnology,) were used as the internal control for cytoplasmic and nuclear proteins, respectively. The following primary antibodies were used in the present study: Anti-glycogen synthase kinase (GSK)- 3β (1:1,000; cat no. 9315), anti-phosphorylated (p)-GSK- 3β (Ser-9; 1:500; cat no. 9336) (both from Cell Signaling Technology, Inc., Danvers, MA, USA), anti- β -catenin (1:1,000; cat no. sc-7199; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-p- β -catenin (Ser-33/37/Thr41; 1:500; cat no. 9561; Cell Signaling Technology, Inc.).

Statistical analysis. Data are expressed as the mean \pm standard error of the mean, for 5 repeated experiments. The statistical significance of the differences between groups was assessed using a one-way analysis of variance (ANOVA) followed by Fisher's least-significant difference post hoc correction for multiple comparisons. The statistical significance of the differences between correlated samples was assessed using a repeated measures two-way ANOVA followed by Fisher's least-significant difference post hoc test for multiple comparisons. Statistical analyses were performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of NAC administration on repetitive/stereotypic behavior in VPA-exposed rats. Previous studies have reported that treatment with NAC ameliorated behavioral disorders in children with autism (13,15,29,30). Therefore, the present study investigated the effects of NAC on autism-associated behavioral disorders in a VPA-induced rat model of autism.

An open field test was used to assess repetitive/stereotypic behavior in VPA-exposed male rats. The present results demonstrated that the distance traveled by rats in the VPA

group was significantly increased compared with the control group between 0 and 40 min of testing (Fig. 1A). The distance traveled by rats in the NAC and NAC + VPA groups was comparable to that of rats in the control group, and was significantly decreased compared with the VPA group across the 0-20, 30-40 and 50-60 min testing durations (Fig. 1A). In addition, VPA-treated rats spent significantly more time engaged in repetitive/stereotypic activities, and their number of repetitive/stereotypic movements was increased compared with the control group. VPA-treated rats were overactive between 0 and 40 min, and between 50 and 60 min of testing (Fig. 1B), and they spent more time occupied in repetitive/stereotypic movements between 0 and 40 min of testing compared with the control rats (Fig. 1C). Conversely, rats in the VPA + NAC group were not overactive and spent significantly less time occupied in repetitive/stereotypic movements between 0 and 40 min of testing (Fig. 1C). Furthermore, they exhibited significantly fewer repetitive/stereotypic movements throughout the duration of the experiment compared with rats in the VPA group (Fig. 1B). VPA + NAC-treated rats exhibited a similar behavior to rats in the control group throughout the 60-min duration of the experiment.

Effects of NAC on oxidative stress in VPA-exposed rats. NAC has previously been demonstrated to ameliorate autism-associated social interaction deficits and anxiety-related behaviors (16), and the present results suggested it could also attenuate repetitive/stereotypic movements. Therefore, the molecular mechanisms underlying the effects of NAC on autism-like phenotypes were further investigated, and its restorative actions on abnormal oxidative homeostasis were revealed. MDA and GSH contents were measured in brain tissue samples isolated from VPA-exposed rats, in order to examine the effects of NAC on lipid peroxidation and total antioxidative capacity in the brain. As demonstrated in Fig. 2, MDA levels were significantly increased, whereas GSH levels were significantly decreased, in the prefrontal cortex and hippocampus of VPA-treated rats compared with controls. Notably, treatment with NAC was revealed to significantly downregulate cortical and hippocampal MDA levels, and upregulate GSH levels compared with VPA-exposed rats (Fig. 2). No significant differences were detected in MDA and GSH levels between rats in the VPA + NAC and control groups (Fig. 2).

Effects of NAC on the canonical Wnt signaling pathway. Oxidative stress has been suggested to be involved in the pathogenesis of autism (4,5), and the canonical Wnt signaling pathway has been implicated in oxidative processes (20-22). Therefore, the present study investigated the involvement of the Wnt pathway in the beneficial effects of NAC in VPA-exposed rats.

β -catenin is a key signaling molecule involved in the canonical Wnt pathway, whereas GSK- 3β negatively regulates β -catenin via phosphorylation of its inhibitory sites, including Ser-33, Ser-37 and Thr-41 (19). The present results demonstrated that GSK- 3β Ser-9 phosphorylation was enhanced in brain tissue samples isolated from VPA-exposed rats compared with control rats (Fig. 3A and B), thus suggesting that GSK- 3β activity may be suppressed in VPA-induced

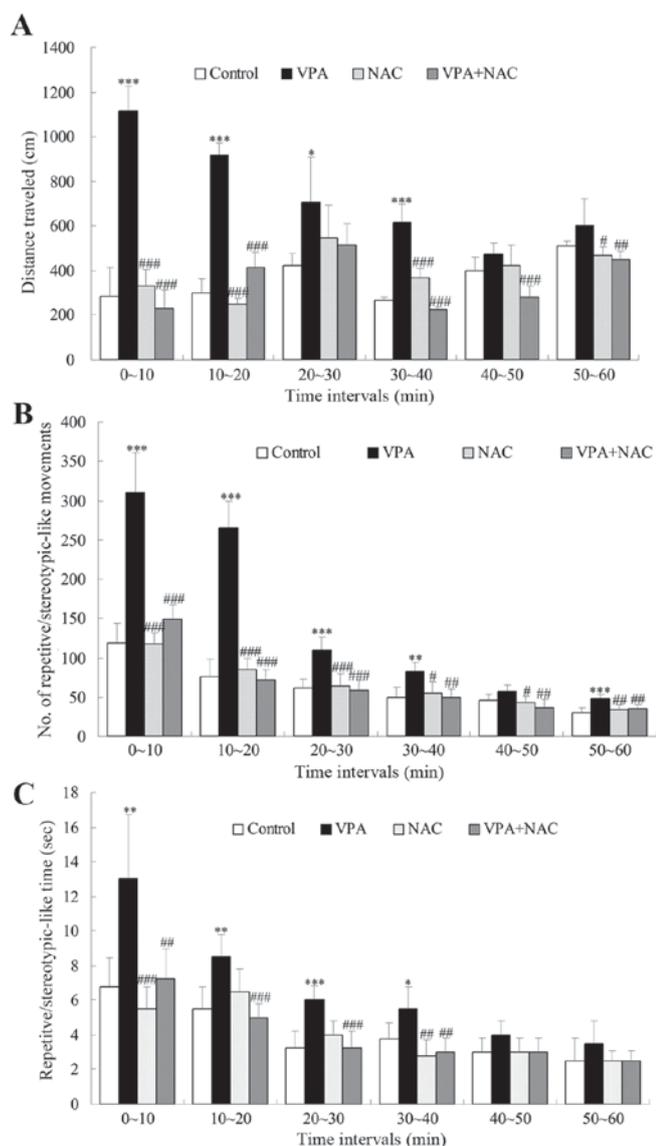


Figure 1. Effects of NAC on repetitive/sterotypic behaviors in VPA-exposed rats during an open field test. (A) The distance traveled, (B) the number of repetitive/sterotypic movements and (C) the time spent engaged in repetitive/sterotypic behaviors were detected for 60 min. Control, rats received no treatment; VPA, rats were prenatally exposed to VPA for the establishment of an autism model; NAC, rats received daily treatment with NAC for 4 weeks; VPA + NAC, rats were prenatally exposed to VPA for the establishment of an autism model, and subsequently received daily treatment with NAC for 4 weeks. Data are expressed as the mean \pm standard error. Control, $n=8$; VPA group, $n=10$; NAC group, $n=7$; VPA + NAC group, $n=12$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. VPA. NAC, N-acetylcysteine; VPA, valproic acid.

autism. In addition, VPA-exposed rats exhibited significantly decreased β -catenin phosphorylation levels (Ser-33/37/Thr-41) compared with the controls (Fig. 3C and D), thus suggesting that VPA may inhibit GSK-3 β to stabilize β -catenin. However, no statistically significant differences were detected in GSK-3 β and β -catenin phosphorylation levels between rats in the VPA + NAC and VPA groups (Fig. 3).

Active β -catenin is translocated into the nucleus to activate the transcription of target genes of the Wnt/ β -catenin pathway (31). Therefore, the protein expression levels of β -catenin were further investigated in the cytoplasm and

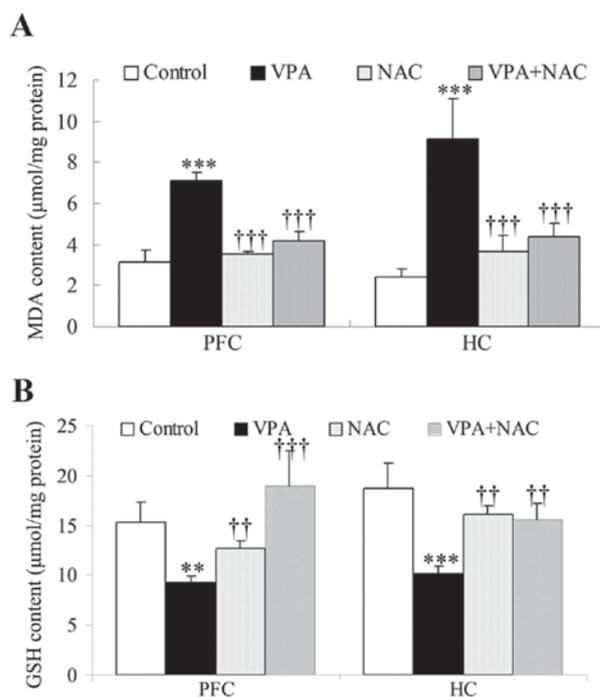


Figure 2. Effects of NAC on MDA and GSH levels in rat brain tissue samples. Intracellular total (A) MDA and (B) GSH levels were measured using commercially available kits in PFC and HC tissue samples. Control, rats received no treatment; VPA, rats were prenatally exposed to VPA for the establishment of an autism model; NAC, rats received daily treatment with NAC for 4 weeks; VPA + NAC, rats were prenatally exposed to VPA for the establishment of an autism model, and subsequently received daily treatment with NAC for 4 weeks. Data are expressed as the mean \pm standard error ($n=5$). ** $P<0.01$, *** $P<0.001$ vs. control; †† $P<0.01$, ††† $P<0.001$ vs. VPA. NAC, N-acetylcysteine; MDA, malondialdehyde; GSH, glutathione; PFC, prefrontal cortex; HC, hippocampus; VPA, valproic acid.

nucleus. VPA-exposed rats exhibited significantly increased nuclear and cytoplasmic protein expression levels of β -catenin compared with rats in the control group (Fig. 4). Compared with the VPA group, NAC alone decreased the protein levels of β -catenin in the cytoplasm and nucleus (Fig. 4). However, no statistically significant differences were detected in nuclear and cytoplasmic β -catenin levels between rats in the VPA + NAC and VPA groups (Fig. 4).

Discussion

The present study demonstrated that NAC exerted beneficial effects in a VPA-induced rat model of autism. Treatment with NAC was demonstrated to reverse abnormal locomotor behaviors, including repetitive/sterotypic activity in rats prenatally exposed to VPA, compared with untreated VPA-exposed offspring. The beneficial effects of NAC in autism-induced deficits were associated with its antioxidative properties, resulting in the restoration of redox homeostasis in the brain. However, its actions did not appear to be associated with the canonical Wnt signaling pathway, as demonstrated by the unaltered GSK-3 β and β -catenin phosphorylation levels, and the β -catenin protein levels in the cytoplasm and nucleus. These results suggested that the Wnt/ β -catenin signaling pathway may be indirectly regulated by NAC through the activation of alternative signaling pathways or upstream factors. NAC alters glutathione

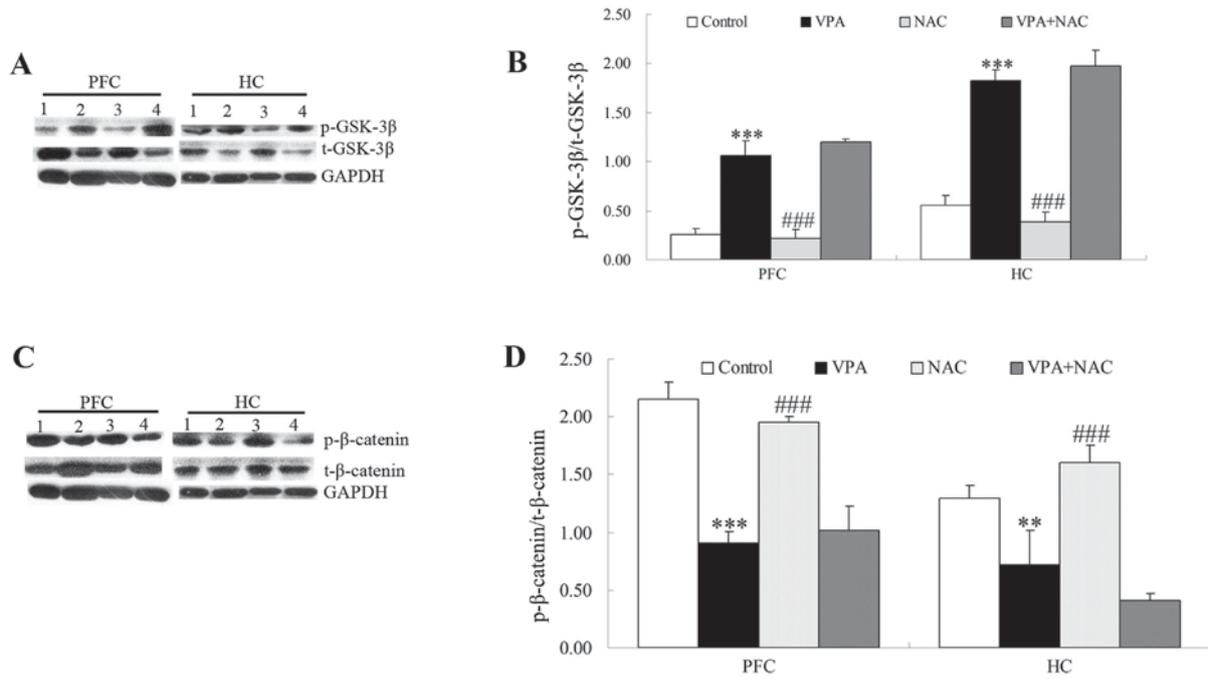


Figure 3. Effects of NAC on the Wnt/ β -catenin signaling pathway. Protein expression levels of (A and B) GSK-3 β and p-GSK-3 β , and (C and D) β -catenin and p- β -catenin were assessed using western blot analysis in PFC and HC tissue samples. GAPDH was used as an internal control. 1, Control; 2, VPA; 3, NAC; 4, VPA + NAC groups. Control, rats received no treatment; VPA, rats were prenatally exposed to VPA for the establishment of an autism model; NAC, rats received daily treatment with NAC for 4 weeks; VPA + NAC, rats were prenatally exposed to VPA for the establishment of an autism model, and subsequently received daily treatment with NAC for 4 weeks. Data are expressed as the mean \pm standard error (n=5). **P<0.01, ***P<0.001 vs. control; ###P<0.001 vs. VPA. NAC, N-acetylcysteine; GSK, glycogen synthase kinase; p-, phosphorylated; PFC, prefrontal cortex; HC, hippocampus; VPA, valproic acid.

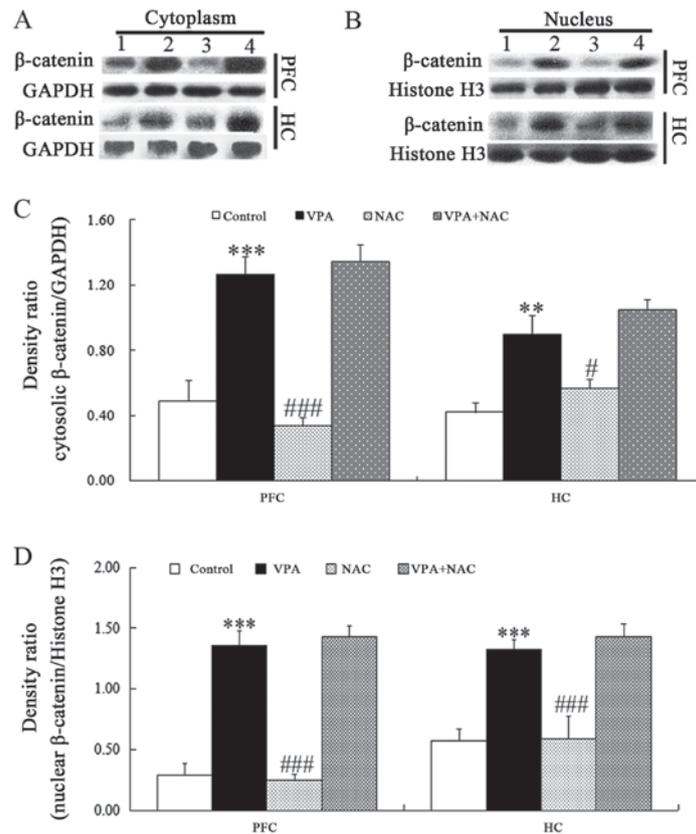


Figure 4. Effects of NAC on β -catenin protein expression levels in the cytoplasm and nucleus of rat brain tissue samples. Western blot analysis was used to assess β -catenin protein expression levels in the (A and C) cytoplasm and (B and D) nucleus in PFC and HC tissue samples. GAPDH and histone H3 were used as internal controls. 1, Control; 2, VPA; 3, NAC; 4, VPA + NAC. Control, rats received no treatment; VPA, rats were prenatally exposed to VPA for the establishment of an autism model; NAC, rats received daily treatment with NAC for 4 weeks; VPA + NAC, rats were prenatally exposed to VPA for the establishment of an autism model, and subsequently received daily treatment with NAC for 4 weeks. Data are expressed as the mean \pm standard error (n=5). ***P<0.001 vs. control; **P<0.01, ###P<0.001 vs. VPA. NAC, N-acetylcysteine; PFC, prefrontal cortex; HC, hippocampus; VPA, valproic acid.

metabolism and improves mitochondrial dysfunction in a subset of autistic lymphoblastoid cell lines (32). In accordance with these results, NAC has been reported to ameliorate autism phenotypes, associated with altered GSH levels (7,30).

Autism is a complex neurodevelopmental disorder, and its etiology has yet to be fully elucidated. Previous studies have suggested that oxidative stress may be involved in the pathogenesis of autism (30,33-36). NAC is a potent antioxidant that is widely used for the treatment of an acetaminophen overdose (37), as a renal protectant in contrast-induced nephropathy (38), and as a preventive agent for atrial fibrillation (39). NAC is a membrane-permeable L-cysteine precursor, and is intracellularly reduced to L-cysteine, a precursor of the endogenous antioxidant, GSH (40). In the brain, the oxidation of NAC-derived L-cysteine to cystine has been reported, which can subsequently modulate extracellular glutamate levels (41). The regulatory effects of NAC on extracellular glutamate levels, and its restorative actions on intracellular GSH levels, in addition to its well-established safety profile, suggest a potential for NAC as an alternative therapeutic strategy for the treatment of patients with autism. Clinical studies have reported that NAC alone, or in combination with risperidone improved some of the core symptoms of autism, including irritability or aggressive behavior (7,12,14,15), self-injurious behavior (13), social interaction impairments (7,16), and anxiety-like behavior (16). In the present study, NAC was demonstrated to ameliorate stereotypic/repetitive locomotor activity in a VPA-induced rat model of autism.

During open field behavioral testing, NAC was revealed to attenuate repetitive/stereotypic activity in VPA-exposed rats, possibly through a decrease in hyper-reactivity to stress. A previous study from our laboratory demonstrated that VPA-treated rats have reduced sensitivity to stress and exhibited repetitive/stereotypic activity (28). Conversely, when stress sensitivity is restored to physiological levels, repetitive/stereotypic activities are abolished (28). Therefore, it may be hypothesized that the frequent repetitive/stereotypic movements in VPA-exposed rats are a result of hyper-reactivity to stress. However, a previous study reported contradictory results, demonstrating that the distance traveled in the open field was similar between VPA + NAC-treated and VPA-treated rats (16). This discrepancy may be due to the different doses of VPA used and the variation in NAC treatment duration. However, further studies are required in order to examine the complex interactions between neuropeptides and neuroendocrine factors that modulate stress reactions, and to determine their effects on movement impairments in VPA-treated rats.

The present study investigated the involvement of the antioxidative properties of NAC in the amelioration of autism-like behavioral abnormalities. In order to assess the antioxidative effects of NAC, the levels of MDA, a marker of lipid peroxidation, and thus of oxidative stress, were assessed in rat brain tissue samples. GSH is an intracellular antioxidant that participates in the endogenous mechanisms of defense against reactive oxygen species (42). The present results demonstrated that prenatal exposure to VPA induced oxidative stress in rats, as demonstrated by the increased MDA and decreased GSH levels in brain tissue samples. Notably, following treatment with NAC, MDA levels were suppressed, whereas GSH levels were enhanced in VPA-exposed rats compared with the

untreated VPA group. These findings are in accordance with a previous study reporting that NAC significantly enhanced GSH levels in diabetic rats (43).

Further studies are required to elucidate the molecular mechanisms underlying the effects of NAC in the prevention of abnormal locomotor behaviors in autism. NAC is a GSH precursor and augments intracellular GSH levels. Wnt and its associated signaling proteins have been identified as targets for GSH (44), whereas NAC has been demonstrated to inhibit the activation of the canonical Wnt pathway in the retinas of diabetic rats (43). Furthermore, previous studies have reported a critical role for the Wnt pathway in the pathogenesis of autism (23,28,45). In a previous study by the present authors, VPA was revealed to inhibit the phosphorylation, and thus the inactivation, of β -catenin, which is a key effector during canonical Wnt signaling, possibly through the upregulation of *WNT1* and *WNT2* expression, thereby activating the canonical Wnt pathway (46). Notably, following treatment with the specific Wnt pathway inhibitor sulindac, Wnt/ β -catenin signaling was suppressed and autism-like behavioral abnormalities in a VPA-induced rat model of autism were attenuated (28). These results suggested that increased Wnt-mediated signaling may contribute to the susceptibility to autism.

Oxidative stress and the Wnt signaling pathway have been implicated in numerous pathological conditions (21,47,48); however, further studies are required to elucidate the modulatory mechanisms that affect oxidative processes and implicate the Wnt pathway in the pathogenesis of autism. As a critical effector of Wnt signaling, β -catenin can activate the transcription of various target genes, following its cytoplasmic accumulation and subsequent nuclear translocation (18,49). In the present study, VPA was revealed to induce oxidative stress, as demonstrated by the increased levels of the oxidative stress marker, MDA, and the downregulation in the levels of the endogenous antioxidant, GSH, in VPA-exposed rats. Furthermore, prenatal exposure to VPA enhanced β -catenin levels in the cytoplasm and nucleus, whereas it was revealed to inhibit the phosphorylation of β -catenin, and promote the phosphorylation of GSK-3 β . These results indicated that VPA activated the canonical Wnt signaling pathway in the rat autism model. However, treatment with NAC did not appear to affect the nuclear and cytoplasmic β -catenin levels, or the phosphorylation of β -catenin and GSK-3 β , thus suggesting that the Wnt pathway may not be involved in NAC-mediated amelioration of autism-like behavior in VPA-treated rats. These results are in accordance with our previous *in vitro* study, which demonstrated that NAC reduced oxidative stress in primary neuronal cultures following pre-treatment with VPA without affecting the activity of the Wnt pathway (45). These findings suggested that the unaltered activity of the canonical Wnt pathway may be an indirect consequence, of reduced oxidative stress in VPA-exposed offspring followed by NAC administration.

The association between the Wnt signaling pathway and oxidative stress under various pathological conditions remains controversial. A previous study from our group reported that treatment with sulindac, a small-molecule inhibitor of the canonical Wnt pathway, reduced the levels of 4-hydroxynonenal, a marker of lipid peroxidation, in a VPA-induced rat model of autism, thus suggesting that oxidative stress may be involved in the regulation of Wnt signaling (28). However,

it may be hypothesized that sulindac directly suppressed oxidative stress due to its antioxidative properties, and these effects may not be mediated by the Wnt pathway. Additionally, sulindac, a nonsteroidal anti-inflammatory drug, which has also been examined in cancer, neurodegenerative disease, and age-related macular degeneration, is able to reduce the levels of lipid peroxidation in an N-nitrosamine-induced mouse model of lung tumorigenesis (50), to reduce the generation of superoxide anions in Alzheimer's disease (51), and to lower ROS levels in a retinal pigmented epithelial cell line (52). VPA and sulindac reduce the activity of the canonical Wnt signaling pathway, compared with VPA exposure alone (28), however, sulindac has anti-inflammatory properties. Therefore, VPA may induce inflammatory status by possibly, though not exclusively, affecting the Wnt pathway. Further studies are required to investigate the association between oxidative stress and inflammation in the pathogenesis of autism. The molecular mechanisms underlying the direct and indirect implication of the canonical Wnt pathway in oxidative stress during the pathogenesis of autism require further investigation.

In conclusion, the results of the present study demonstrated that NAC reversed abnormal locomotor behaviors, including repetitive/stereotypic activity, in a VPA-induced rat model of autism, possibly due to its antioxidative properties. Notably, the beneficial effects of NAC in autism-like behavior did not appear to be associated with the canonical Wnt signaling pathway, suggesting that Wnt/ β -catenin signaling may be indirectly regulated by NAC through alternative signaling pathways or upstream mediators. However, the results of the present study should be interpreted with caution, as autism is a complex and highly heterogeneous group of pathologies, and a VPA-induced rat model is not representative of the wide spectrum of disorders that autism encompasses.

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