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ORIGINAL

SODIUM BUTYRATE'S NEUROPROTECTIVE EFFECTS ON 6-OHDA-INDUCED DAMAGE IN PC12 CELLS: IMPLICATIONS FOR MENTAL HEALTH THROUGH BDNF/PROBDNF REGULATION

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ABSTRACT

Background: This study explores the neuroprotective role of sodium butyrate (NaB) in mitigating 6-hydroxydopamine (6-OHDA) induced damage in PC12 cells, with a focus on mental health implications via Brain-Derived Neurotrophic Factor (BDNF) and its precursor, proBDNF. **Methods:** We assessed cell viability using the Cell Counting Kit-8 and calculated the inhibitory concentration of 6-OHDA. Key protein expressions such as alpha-synuclein, tyrosine hydroxylase, BDNF, and proBDNF were analyzed through immunofluorescence and western blotting. We specifically examined the effects of NaB on cell viability and the BDNF/proBDNF ratio under 6-OHDA-induced stress. **Results:** 6-OHDA exposure led to a decrease in PC12 cell viability and alterations in key protein expressions relevant to neuronal health. Notably, NaB treatment countered these changes, particularly enhancing BDNF levels while not significantly affecting proBDNF. **Conclusion:** NaB demonstrates potential as a neuroprotective agent, particularly through modulating the BDNF/proBDNF ratio, underscoring its potential relevance in mental health contexts, specifically in conditions like Parkinson's disease where dopaminergic neuronal integrity is crucial. This suggests NaB's therapeutic prospects in neurodegenerative and mental health disorders.

KEYWORDS: Sodium butyrate, BDNF, proBDNF, Parkinson's disease, PC12 cells, mental health

1. INTRODUCTION

The intricate relationship between neuronal health and mental well-being is underscored in neurodegenerative diseases, with Parkinson's Disease (PD) serving as a prime example(Schulz-Schaeffer, 2010). The neuronal degeneration characteristic of PD, particularly in dopaminergic pathways, extends beyond physical motor impairments to encompass a range of mental health issues, including depression, anxiety, and cognitive decline(Mao, Qin, Zhang, & Ye, 2020). This multifaceted impact highlights the need for a comprehensive understanding of neuroprotective strategies (Fox et al., 2011).

Sodium butyrate, a histone deacetylase inhibitor, is increasingly recognized for its potential in neuroprotection, attributed to its epigenetic modulation capabilities(Verhagen Metman, Pal, & Slavin, 2016). Epigenetic modifications induced by NaB can alter gene expression patterns crucial for neuronal survival and plasticity(Verhagen Metman et al., 2016). This aspect is particularly vital in understanding and combating the neuronal damage induced by 6-hydroxydopamine (6-OHDA), a neurotoxin commonly used to model PD in research(Howells et al., 2000; Lee & Song, 2014; Mogi et al., 1999; Wang et al., 2019)

The neurotrophic factors, primarily BDNF and its precursor proBDNF, play a pivotal role in neuronal health(Chao, 2003). (Arancio & Chao, 2007). BDNF is essential for maintaining neuronal plasticity, which is crucial for cognitive function and mental health(Stahl, Mylonakou, Skare, Amiry-Moghaddam, & Torp, 2011; Sun et al., 2005). Dysregulation of BDNF and proBDNF is implicated in the pathophysiology of several mental health disorders(Taylor et al., 2012; Teng et al., 2005). The balance between BDNF and proBDNF is crucial, as it governs various aspects of neuron survival, differentiation, and synaptic connectivity(Baydyuk, Nguyen, & Xu, 2011; Studer et al., 1995).

In exploring the protective effects of NaB against 6-OHDA-induced neurotoxicity in PC12 cells, this study seeks to illuminate the pathways through which NaB modulates the BDNF/proBDNF ratio(Mowla et al., 2001). Understanding these mechanisms is key to developing therapeutic strategies for mental health issues associated with PD and other neurodegenerative diseases(Barichello et al., 2015). (Deng, Tao, Yu, & Jin, 2012). This research could pave the way for novel interventions that not only address the physical symptoms of such diseases but also the accompanying mental health challenges(Alquezar et al., 2015; KUNDU, KUMAR, TYAGI, & CHANDRA, 2019; Liu et al., 2017).

In this expanded introduction sets the stage for a detailed exploration of how NaB, through its influence on key neurotrophic factors, could be a beacon

of hope in the realm of mental health, particularly in the context of neurodegenerative diseases like PD (Davie, 2003).

1.1 Study design

It's basic study, doesn't involve human and animal studies. So Ethical approval and informed consent is not applicable in this study.

1.2 Cell culture

The PC12 cell line was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 U/mL penicillin and 100 μg/mL streptomycin (Procell, China) at 37°C in humidified air containing 5% CO_2.

1.3 Cell Counting Kit-8 (CCK-8) assay

PC12 cells were seeded in 96-well plates (1.0×10^5/mL, 100 μL/well) and incubated at 37°C for 24 hours. The cells were subsequently treated with 6-OHDA (Sigma-Aldrich, USA) at specific concentrations for 6, 12, or 24 hours. Cell viability was determined using the CCK-8 assay kit (Dojindo Molecular Technologies, USA) following manufacturer's instructions. The optical density at 450 nm was recorded on a BioTek ELx800 microplate reader (BioTek Instruments, Inc., USA). Cell viability was calculated using the following equation: Cell viability (%) = $100 \times (OD_{sample}-OD_{blank})/(OD_{control}-OD_{blank})$. To test the effects of NaB, the cells were first treated with NaB at specific concentrations for 1 hour. After that, 150 μM 6-OHDA was added, and the cells were incubated for another 24 hours. Cell viability was determined as above.

1.4 In situ cell immunofluorescence

PC12 cells were seeded in six-well plates (1.0×10^5/mL, 1 mL/well) and cultured till 80% confluence. The cells were stimulated with 150 μM 6-OHDA for 24 hours, washed, and fixed in 4% polyformaldehyde for 15 minutes. The fixed cells were washed and then permeabilized with 0.2% Triton X-100 (Sigma-Aldrich).

After blocking in 5% goat serum, the cells were incubated with rabbit anti-α-Syn antibody (1:400; Cell Signaling Technology, USA) over night at 4°C. After washing in PBS, the cells were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Abcam, USA) at room temperature for 2 hours. Cell nuclei were stained with DAPI (Thermo Fisher Scientific, USA). After embedding in resin, the cells were examined under a fluorescence microscope (Olympus, Japan).

To detect BDNF and tyrosine hydroxylase (TH), the cells were stimulated

with 150 μM 6-OHDA for 24 hours with or without 1-h pre-treatment with 100 μM NaB. Intracellular BDNF and TH were detected by cell immunofluorescence following identical procedures as above except the following: The primary antibodies used were a mixture of rabbit anti-BDNF (1:200; Abcam) and mouse anti-TH (1:500; Abcam), and the secondary antibodies used were a mixture of Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000; Abcam) and Alexa Fluor 647-conjugated goat anti-mouse IgG (1:1000; Abcam).

1.5 Western blot analysis

PC12 cells were cultured in T25 flasks till 85% confluence. The cells were stimulated with 150 μM 6-OHDA for 12 or 24 hours, and then lysed with RIPA lysis buffer (Beyotime Biotechnology, China) containing 1mM PMSF (Beyotime Biotechnology). Protein concentrations were determined using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific) following manufacturer's instructions. The proteins were separated by 15% SDS-PAGE (Sigma-Aldrich) and transferred to PVDF membranes (Millipore, USA).

After blocking in non-fat milk for 1 hour, the membranes were incubated with rabbit anti-BDNF antibody (1:1000; Abcam) or mouse anti β-actin antibody (1:5000; Sigma-Aldrich) overnight at 4°C. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG (1:2000; Cell signaling Technology) at room temperature for 1 hour. Protein bands were visualized with enhanced chemiluminescence (ECL) reagent (Thermo Fisher Scientific) and photographed on a Tanon 5500 imaging system (Tanon Science & Technology, China). To test the effects of NaB on the expression of BDNF and proBDNF, the cells were first treated with 100 μM NaB for 1 hour, and then co-treated with 150 μM 6-OHDA for another 24 hours. BDNF and proBDNF levels were determined by wester blotting following identical procedures as above. The primary antibody used to detect proBDNF was mouse anti-proBDNF (1:200; Santa Cruz Biotechnology, USA). All data were normalized to β-actin.

1.6 Statistical analysis

All data were analyzed with SPSS22.0 (IBM, USA) or GraphPad Prism 6 (GraphPad Software Inc., USA). All results are presented as mean ± SEM. Multigroup analysis was performed using one-way ANOVA. Multiple comparisons between groups were conducted with the Tukey's test. A P value of less than 0.05 was deemed statistically significant. Multi group analysis using one-way ANOVA (Analysis of Variance): This statistical test is used to determine whether there are any statistically significant differences between the means of three or more independent groups. Multiple comparisons between groups using Tukey's test: After finding a significant difference in the ANOVA, Tukey's test is employed to identify which specific groups are different from each other.

2. Results

2.1 In vitro PD model was established using 6-OHDA-stimulated PC12 cells

A cellular model of PD consisting of 6-OHDA-stimulated PC12 cells has been frequently used in PD-related studies (Ryu, Angelastro, & Greene, 2005). In this work, to establish an *in vitro* model of PD, we stimulated PC12 cells with 50, 100, 150, or 200 μM 6-OHDA for 6, 12, and 24 hours, respectively. As indicated in the CCK-8 assay, 6-OHDA reduced PC12 cell viability in a dose- and time-dependent manner, showing an IC_{50} value of 150 μM at 24-h treatment (Fig. 1A, Table 1). The accumulation of α-Syn aggregates is a histopathological hallmark of PD. *In situ* cell immunofluorescence imaging of PC12 cells after 24-h exposure to 150 μM 6-OHDA revealed the presence of α-Syn aggregates wrapping around the nucleus (Fig. 1B), indicating the successful establishment of an *in vitro* model of PD.

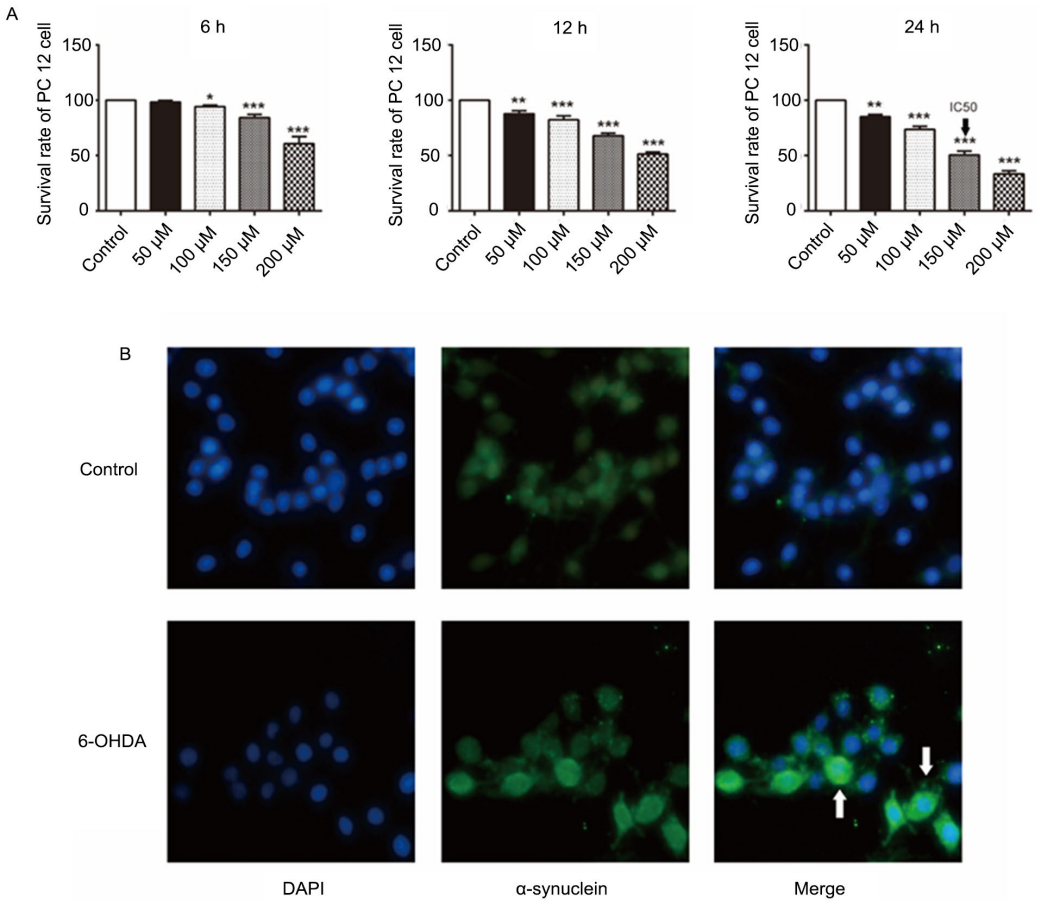

Figure 1: 6-OHDA reduced PC12 cell viability and induced α-Syn aggregation. A.PC12 cells were stimulated with 50, 100, 150, or 200 μM 6-OHDA for 6, 12, and 24 hours, respectively. Unstimulated cells were included as control. Cell viability was determined by the CCK-8 assay. n = 3, **P < 0.01, ***P < 0.001 vs. control. **B.** PC12 cells were stimulated with 150 μM 6-OHDA for 24 hours. Unstimulated cells were included as control. α-Syn was detected by cell immunofluorescence. The nuclei were stained with DAPI. Magnification, 400X.

Table 1: Viability of PC12 cells stimulated with 6-OHDA at different concentrations for various hours.

	CELL VIABILITY	MEAN ± SEM	P VALUE
6h	control	100.00±00.00	
	50μM	98.25±1.57	0.979
	100μM	94.34±0.78	0.043*
	150μM	84.31±2.92	0.00069**
	200μM	60.81±3.69	0.00025**
12h	control	100.00±00.00	
	50μM	87.79±2.81	0.0052**
	100μM	82.36±3.58	0.00012**
	150μM	67.98±2.38	0.00032**
	200μM	51.49±1.75	0.00041**
24h	control	100.00±00.00	
	50μM	85.17±1.87	0.0083**
	100μM	73.73±2.86	0.00032**
	150μM	50.51±3.64	0.00078**
	200μM	33.43±2.89	0.00049**

*Mean: mean of each control or 6-OHDA-treated group, n=3. SEM: standard error of the mean of each control or 6-OHDA-treated group. *: a difference significant at P<0.05. **: a difference significant at P<0.01.*

2.2 BDNF was downregulated in 6-OHDA-stimulated PC12 cells

To test the effects of 6-OHDA on BDNF expression, we used western blot analysis to assess BDNF levels in PC12 cells stimulated with 150 μM 6-OHDA for 12 or 24 hours. Compared with unstimulated control cells, 6-OHDA-stimulated cells exhibited significantly decreased BDNF levels, which were approximately 75% and 56% of control after 12 and 24-h exposure, respectively (Fig. 2A, B, Table 2).

Therefore, the time-dependent loss of cell viability induced by 6-OHDA stimulation was accompanied by time-dependent downregulation of BDNF. These findings indicate a clear time-dependent relationship between 6-OHDA exposure, BDNF expression, and cell viability in PC12 cells. The significant downregulation of BDNF after 6-OHDA stimulation suggests that this neurotoxin may be directly involved in suppressing BDNF production.

Additionally, the parallel decrease in cell viability highlights the potential role of reduced BDNF levels in contributing to the loss of cell viability induced by 6-OHDA. These results underscore the importance of further investigating

the molecular mechanisms underlying the regulation of BDNF expression in response to neurotoxic insults like 6-OHDA.

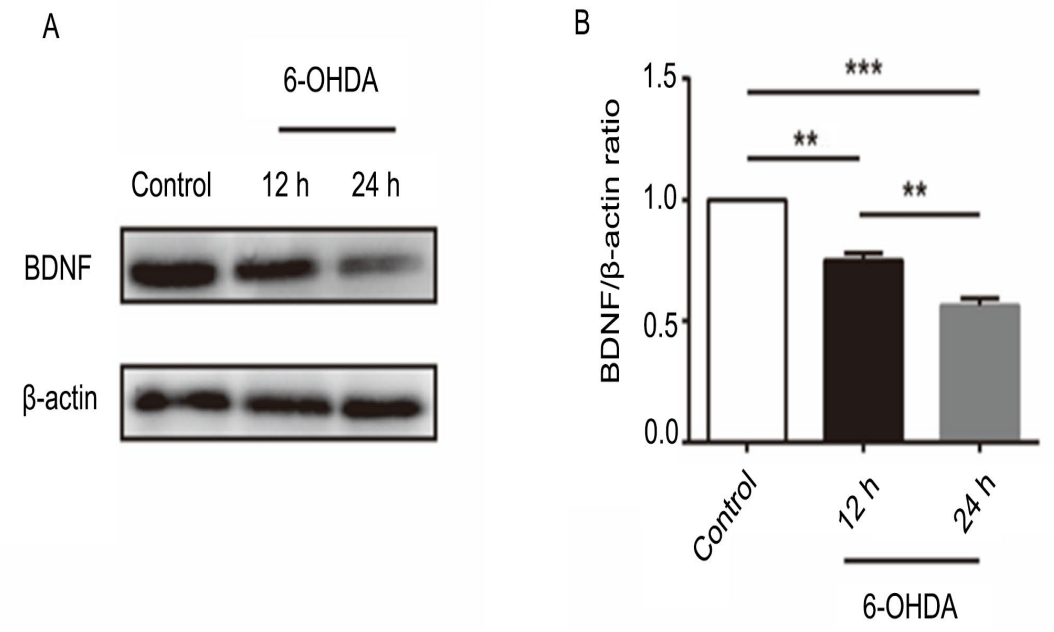

Figure 2: BNDF was downregulated in 6-OHDA-stimulated PC12 cells. **A** and **B.** PC12 cells were stimulated with 150 μM 6-OHDA for 12 or 24 hours. Unstimulated cells were included as control. BDNF levels were determined with western blot analysis. Representative gel images (**A**) and quantified BDNF levels (**B**) are shown. n = 3, **P < 0.01, ***P < 0.001.

Table 2: BDNF levels in PC12 cells stimulated with 150 μM 6-OHDA for 12 or 24 hours.

BDNF/B-ACTIN RATIO	MEAN ± SEM	P VALUE
CONTROL	1.00±0.00	
12h	0.75±0.28	0.0012**
24h	0.61±0.64	0.00034**

*Mean: mean of each control or treatment group, n=3. SEM: standard error of the mean of each control or treatment group, **: a difference significant at P<0.01 compared with the control*.

2.3 NaB protected PC12 cells from 6-OHDA-induced cell death

To test the potential cytoprotective effects of NaB, PC12 cells were pre-incubated with 10, 50, 100, 200, or 400 μM NaB for 1 hour before they were subjected to 24-h co-treatment with 150 μM 6-OHDA.

The CCK-8 assay revealed that NaB ameliorated 6-OHDA-induced cell death in a dose-dependent manner, with significant protective effects observed starting from 100 μM (P < 0.001; Fig. 2C, Table 3). Since 100μM NaB group resulted in ideal protective effects as a relative low concentration, we further selected this concentration for downstream experiments.

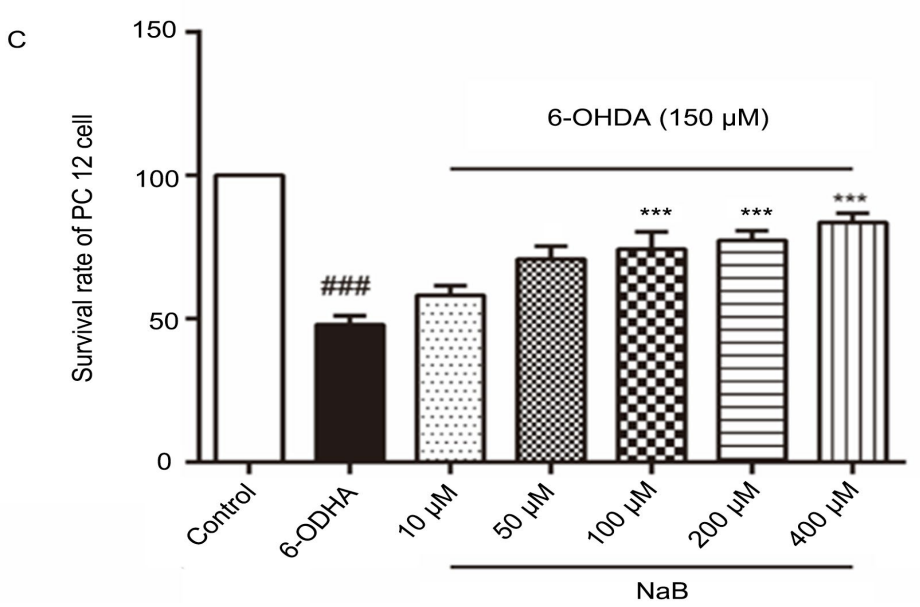

Figure 2: BNDF was downregulated in 6-OHDA-stimulated PC12 cells. **C.** PC12 cells were first treated with 10, 50, 100, 200, or 400 μM NaB for 1 hour, and then co-treated with 150 μM 6-OHDA for 24 hours. Untreated cells were included as control. Cell viability was determined with the CCK-8 assay. n = 3; *P < 0.5, **P < 0.01, ***P < 0.001 vs. control; $^{###}$P < 0.001 vs. control.

Table 3: Viability of 6-OHDA (150 μM) - stimulated or -unstimulated PC12 cells with or without pre-treatment with NaB at different concentrations.

CELL VIABILITY	MEAN ± SEM	P VALUE
CONTROL	100.00±00.00	
6-OHDA(150μM)	47.99±3.04	0.0019$^{##}$
10μM	58.25±3.30	0.86
50μM	70.85±4.45	0.45
100μM	74.29±6.03	0.0012**
200μM	77.30±3.36	0.0089**
400μM	83.60±3.18	0.0005**

*Mean: mean of each control or treatment group, n=3. SEM: standard error of the mean of each control or treatment group. **: a difference significant at P<0.01 compared with the control.*

2.4 NaB restored TH and BDNF expression downregulated by 6-OHDA

Tyrosine hydroxylase (TH) is a rate-limiting enzyme for the biosynthesis of catecholamines, and hence a marker for dopaminergic neurons. To explore the molecular mechanisms underlying the cytoprotective effects of NaB, we evaluated BDNF and TH expression in PC12 cells by *in situ* cell immunofluorescence. Compared with unstimulated control cells, 6-OHDA-stimulated cells showed significantly lower TH levels along with decreased

BDNF expression (Fig. 3A), suggesting that 6-OHDA caused injury in dopaminergic neurons in the PC12 cell population, at least partially, by downregulating BDNF. Treatment with NaB restored TH and BDNF expression downregulated by 6-OHDA (Fig. 3A), supporting that NaB protected dopaminergic neurons from 6-OHDA-induced cytotoxicity by upregulating BDNF.

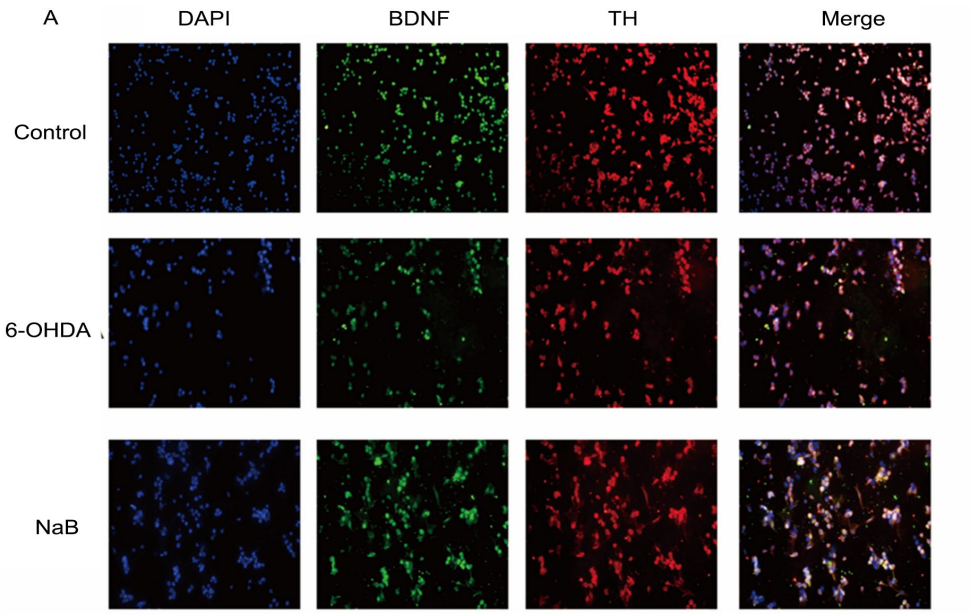

Figure 3: NaB restored BDNF and TH expression downregulated in 6-OHDA-stimulated PC12 cells. PC12 cells were first treated with 100 μM NaB for 1 hour, and then co-treated with 150 μM 6-OHDA for 24 hours. Untreated cells were included as control. **A.** Intracellular BDNF and TH were detected with *in situ* cell immunofluorescence. The nuclei were stained with DAPI. Magnification, 100X.

2.4 NaB restored the BDNF/proBDNF ratio downregulated by 6-OHDA

In contrast to the neuroprotective effects of BDNF, proBDNF appears to play an active role in neuronal cell death (Taylor et al., 2012; Teng et al., 2005). To investigate the balance between BDNF and proBDNF in PD, we evaluated BDNF and proBDNF levels in control and 6-OHDA-stimulated PC12 cells with western blot analysis. The results showed that 6-OHDA downregulated both BDNF and proBDNF; however, it had a more profound effect on BDNF (48% reduction) than on proBDNF (23% reduction) (Fig. 3B, Table 4), resulting in a lower BDNF/proBDNF ratio in 6-OHDA-stimulated cells compared with unstimulated control. NaB treatment restored the level of BDNF but not proBDNF downregulated by 6-OHDA (Fig. 3B), and as a result, the BDNF/proBDNF ratio in 6-OHDA-stimulated cells treated with NaB was higher than that in unstimulated, untreated control. Together, these data suggested that NaB protected PD dopaminergic neurons by upregulating BDNF and the BDNF/proBDNF ratio.

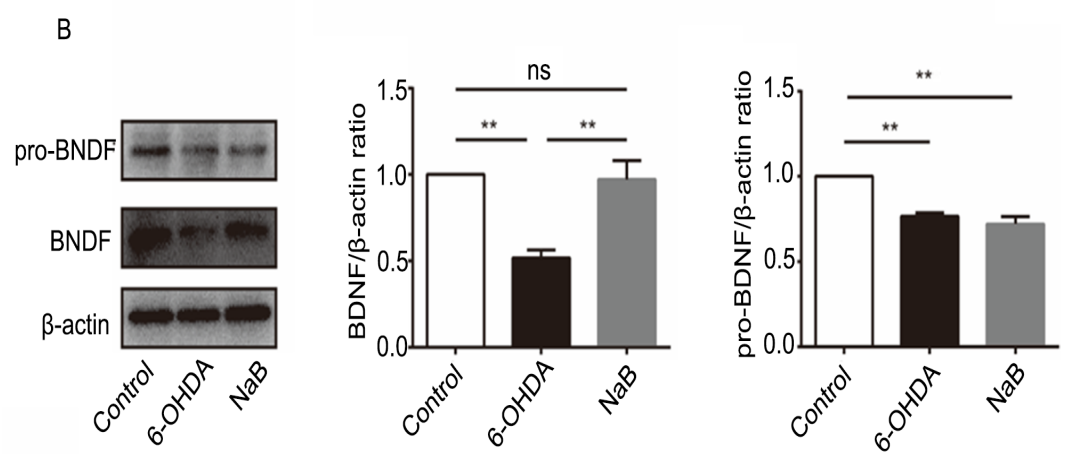

Figure 3: NaB restored BDNF and TH expression downregulated in 6-OHDA-stimulated PC12 cells. PC12 cells were first treated with 100 μM NaB for 1 hour, and then co-treated with 150 μM 6-OHDA for 24 hours. Untreated cells were included as control. **B.** The BDNF levels, pro-BDNF levels and BDNF/proBDNF ratios were determined with western blot analysis. n = 3, **P < 0.01, ***P < 0.001, ns = not significant.

Table 4: Analysis of BDNF and Pro-BDNF determined in western blot

	GROUP	MEAN ± SEM	P VALUE
BDNF/β-actin ratio	control	1.00±0.00	
	6-OHDA(150μM)	0.52±0.46	0.006**
	NaB(100Mm)	0.97±0.11	0.95
Pro-BDNF/β-actin ratio	control	1.00±0.00	
	6-OHDA(150μM)	0.77±0.02	0.0026**
	NaB(100μM)	0.72±0.44	0.0015**

3. Discussion

In this study, we found that BDNF and the BDNF/proBDNF ratio were downregulated in a common cellular PD model using 6-OHDA-stimulated PC12 cells. In addition, we found that NaB restored BDNF and the BDNF/proBDNF ratio downregulated by 6-OHDA and protected PC12 cells from 6-OHDA-induced cytotoxicity. In alignment with previous findings (Manouchehrabadi, Farhadi, Azizi, & Torkaman-Boutorabi, 2020; Nishiyama et al., 2019), 6-OHDA induced PC12 cell death in a dose- and time-dependent manner, showing an IC_{50} value of 150 μM at 24-h stimulation. The accumulation of α-Syn aggregates in dopaminergic neurons is a histopathological hallmark in various cellular and animal models of PD (Finkelstein et al., 2017). After 24-h stimulation with 150 μM 6-OHDA, our *in situ* cell immunofluorescence imaging revealed the presence of α-Syn aggregates as well as diminished level of the dopaminergic neuron marker TH. These results indicated that the cellular model used in this study presented histopathological characteristics of PD. BDNF plays a

significant role in PD, both for its involvement in the pathogenesis of the disease and for its neuroprotective effects as a pharmacotherapy (Palasz et al., 2020). The overexpression of α-Syn has been shown to downregulate BDNF (Yuan et al., 2010). In addition, α-Syn can directly bind to TrkB, thereby inhibiting the BDNF/TrkB signaling and triggering dopaminergic cell death (Kang et al., 2017). In this study, the *in situ* cell immunofluorescence imaging and western blot analysis showed decreased BDNF in 6-OHDA-stimulated PC12 cells, which could have resulted from the increased α-Syn expression and aggregation in this cellular model of PD. NaB, which is a known HDAC inhibitor (Davie, 2003), has shown neuroprotective effects in multiple *in vitro* and *in vivo* models of PD (Alquezar et al., 2015; Liu et al., 2017). In this study, NaB restored PC12 cell viability and TH expression reduced by 6-OHDA. These results were similar to the cytoprotective effects of NaB in another cellular model of PD using 6-OHDA-stimulated SH-SY5Y cells (Alquezar et al., 2015; Xicoy, Wieringa, & Martens, 2017). Valproate is another potent HDAC inhibitor (Gottlicher et al., 2001). Lai et al. have shown that valproate can mitigate 6-OHDA-induced loss of dopaminergic neurons in rodent midbrain through upregulating BDNF (Lai et al., 2019). Interestingly, NaB treatment in combination with nicorandil can upregulate BDNF in neural stem cells, leading to improved efficacy in stroke cell therapy (Hosseini et al., 2018). In this study, NaB restored BDNF expression in PC12 cells downregulated by 6-OHDA, suggesting that the cytoprotective effects of NaB in this cellular model of PD were mediated, at least partially, by BDNF upregulation.

The BDNF precursor protein proBDNF is considered to act against BDNF to promote neuronal cell death (Taylor et al., 2012; Teng et al., 2005). To further understand the mechanisms involved in the neuroprotective effects of NaB, we evaluated the level of proBDNF with western blot analysis. We found that proBDNF was also downregulated by 6-OHDA, but to a lesser extent than BDNF, leading to a lower BDNF/proBDNF ratio in 6-OHDA-stimulated PC12 cells than unstimulated control. NaB restored BDNF but not proBDNF downregulated by 6-OHDA, and consequently, 6-OHDA-stimulated cells treated with NaB exhibited a higher BDNF/proBDNF ratio than unstimulated, untreated cells. These results indicated that the BDNF/proBDNF ratio is downregulated in PD, and NaB can protect against dopaminergic neuronal loss through upregulating the BDNF/proBDNF ratio. The detailed molecular mechanisms are not clear, but may involve the regulation of enzymes responsible for the generation of BDNF from proBDNF, such as plasmin, matrix metalloproteinases, and furin, a proprotein convertase (Nagappan et al., 2009; Yang et al., 2009).

4. Conclusion

In conclusion, this study highlights the significant neuroprotective role of sodium butyrate (NaB) in counteracting 6-OHDA-induced damage in PC12

cells, specifically through the regulation of the BDNF/proBDNF ratio. Our findings indicate that NaB not only preserves cell viability but also modulates key neurotrophic factors crucial for neuronal health. These results underscore the potential of NaB as a therapeutic agent in mental health, particularly in the context of neurodegenerative diseases like Parkinson's Disease, where dopaminergic neuronal integrity is paramount. The study opens avenues for further research into NaB's application in mental health, providing a promising direction for future therapeutic strategies against neurodegeneration and associated mental health challenges.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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