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*Short Communication*

# Sal synthase induced cytotoxicity of PC12 cells through production of the dopamine metabolites salsolinol and N-methyl-salsolinol

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**Abstract**

As a catechol isoquinoline, salsolinol (Sal) is widely distributed in mammalian brains, and is increased in the cerebrospinal fluid (CSF) and urine of Parkinsonian patients. Sal can be metabolized to N-methyl-salsolinol (NM-Sal), an $MPP^+$-like neurotoxin, and impairs the function of dopaminergic neurons, causing the clinical symptoms of Parkinson's disease (PD). Sal synthase, which catalyzes the production of Sal from dopamine and acetaldehyde, may be the important enzyme in the metabolism of catechol isoquinolines (CTIQs). Previously, our work demonstrated the existence of Sal synthase in rat brain and identified its amino acid sequence. However, the biological function of Sal synthase has not been thoroughly explored, especially its role in dopaminergic neuronal degeneration. In this study, we tried to clarify the catalytic role of Sal synthase in the formation of CTIQs which are endogenous neurotoxins in the mammalian brain. Furthermore, the cytotoxicity of Sal synthase was also observed in dopaminergic PC12 cells. The results demonstrated that Sal synthase overexpression can increase the level of Sal and NM-Sal, and ultimately cause mitochondria damage and apoptosis.

**Keywords:** Sal synthase; Parkinson's disease; PC12 cells; Salsolinol; N-methyl-salsolinol; Mitochondrial membrane potential; Apoptosis

## 1. Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder with a multitude of causes, including aging, genetic and environmental factors. Environmental toxin exposures may be one of the most significant risk factors for PD [1,2]. For example, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a very selective neurotoxin that cause a clinical syndrome similar to PD [3]. Since MPTP is an exogenous compound, catechol isoquinolines (CTIQs) are of interest as possible endogenous neurotoxins because they share a similar chemical structure with MPTP and its active metabolite, $MPP^+$(1-methyl-4-phenylpyridinium) [4]. Among CTIQs, salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4- tetrahydroisoquinoline, Sal) level is increased significantly in the cerebrospinal fluid (CSF) and urine of PD patients [5]. Moreover, Sal can be methylated to produce N-methyl-salsolinol (1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, NM-Sal) by N-methyltransferase [6]. Research indicates that Sal and NM-Sal are neurotoxic to dopaminergic neurons, and may play a key role in the pathogenesis of PD [7–9].

Sal formation in brain has been studied for almost three decades. Two pathways for Sal synthesis from dopamine (DA) and acetaldehyde (AcH) have been identified. The first pathway is the non-enzymatic Pictet-Spengler reaction, which produces racemic (R/S)-Sal. The second pathway is catalyzed by salsolinol synthase (Sal synthase), which can synthesize (R)-Sal stereoselectively [10]. Sal synthase was first isolated and characterized by Naoi M, who isolated it from the human brain and indicated that it may be a key enzyme in the metabolism of endogenous neurotoxins [11]. Our group previously demonstrated the existence of Sal synthase in rat brain [12], and also established a systematical procedure to purify Sal synthase and identified its amino acid sequence [13]. However, the catalytic function of Sal synthase in the formation of CTIQs requires further verification, especially its role in the pathogenesis of PD.

In this study, Sal synthase was overexpressed in PC12 cells to identify its function in the production of the dopamine metabolites Sal and NM-Sal. Furthermore, the neurotoxicity of Sal synthase was also investigated to evaluate the potential biological mechanism of Sal synthase in the degeneration of dopaminergic neurons to provide novel insight on the development of PD.

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## 2. Material and methods

### 2.1 Sal synthase recombinant plasmid

Sal synthase cDNA was synthesized by Sangon Biotech, China. The pcDNA3.1(+), pEGFP N2 vectors were chosen to construct the Sal synthase recombinant plasmids, and empty vectors were used as the control group, respectively. Primers 1 [5'-CCGCTCGAG (Xho1) ATGCAAATCTTCGTG (Forward) and 5'-CGCGGATCC (BamH1) ATAGTCAACCCT-3' (Reverse)] were used for the plasmid construction of pcDNA3.1(+)-Sal synthase. Primer 2 [5'-CCGCTCGAG (Xho1) ATGCAAATCTTCGTG-3' (Forward) and 5'-CGCGGATCC-(BamH1) ATAGTCAACCCT-3' (Reverse)] was used for pEGFP N2-Sal synthase construction.

### 2.2 Cell culture and transfection

The rat pheochromocytoma cell line (PC12) was obtained from cell center of the Chinese Academy of Sciences (Shanghai, China). PC12 cells were cultured in 1640 medium (Gibco, USA) with 5% fetal bovine serum (Wisent, Canada) and 10% horse serum (Gibco, USA), and were incubated with 5% $CO_2$ and maximal humidity at 37 °C. PC12 cells were grown to about 80% confluent for 48 h transfection, which was performed using Lipofectamine 3000 according to the manufacture's direction (Invitrogen, USA).

### 2.3 HPLC-MS/MS analysis of Sal and NM-Sal

The transfected cells were ultra-sonicated for 6 min in 50 mM Tris-HCl (pH 7.4). After quantification, proteins were removed using 1 M perchloric acid (PCA) solution (1 M $HClO_4$, 10 mM $Na_2S_2O_5$, 10 mM EDTA) [13]. The supernatants were collected and filtered through Millipore 0.22 $\mu$m filters. Afterwards, quantitative analysis of Sal and NM-Sal was completed on a Discovery®HS F5-5 column by HPLC-MS/MS. The mobile phase was delivered at flow rate of 0.2 mL/min, which consisted of methanol-water (25/75, v/v) with 10 mM ammonium formate (pH 3.5). The injection volume of each sample was 5 $\mu$L. Isoproterenol (ISOP) was used as an internal standard. The quantitative analysis of Sal and NM-Sal was detected according to methods described previously [14].

### 2.4 Activity detection of Sal synthase

The transfected cells were lysed in RIPA buffer, and then centrifuged at 4 °C at 12,000 g for 10 min and the supernatants were used as the crude enzyme of Sal synthase. The crude enzyme was incubated with 1 mM DA and 1 mM AcH in Tris-HCl (50 mM, pH 7.4) for 40 min at 37 °C. PCA was added to stop the reaction. After centrifugation, the supernatants were filtered with 0.22 $\mu$m filters, and the content of Sal was measured by HPLC-MS/MS. Sal synthase's catalytic activity was estimated from the increase of Sal (per mg protein per minute, $ng \cdot mg^{-1} \cdot min^{-1}$). The Sal produced by the Pictet-Spengler reaction was deducted from the enzyme-activity calculation.

### 2.5 Mitochondrial membrane potential and apoptosis

After a 48 h transfection, Sal synthase overexpression cells and control group cells were collected for the detection of mitochondrial membrane potential (MMP) and apoptosis. The fluorescent probe JC-1 (Beyotime, China) and an Annexin V-FITC/PI Apoptosis Detection Kit (DOJINDO, Japan) were utilized as described previously [15], and the final detection was determined with flow cytometry (Becton-Dickinson, USA).

### 2.6 Western blot assay

Protein samples obtained from cell lysates were mixed with 5× protein loading buffer and boiled at 100 °C for 5 min. The samples were separated on 12% SDS-PAGE, and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Germany). The membranes were blocked by 5% non-fat milk in TBS-Tween buffer. The primary antibodies of Bax, Bcl-2 and GAPDH were acquired from Beyotime of China, while $\beta$-actin was from Sigma. The Sal synthase antibody was prepared by Huada Beijing Protein Innovation of China. Primary antibodies were diluted in TBST or primary antibody dilution buffer at 4 °C overnight and the HRP-conjugated secondary antibody at RT for 2 h. The bands were visualized using ECL (enhanced chemiluminescence) reagents (Millipore, Germany) and an autoradiographic film (Tanon, China). The quantitative analysis was performed by Image J, and signals were normalized for $\beta$-actin or GAPDH as loading controls.

### 2.7 Statistics analysis

Data were presented as mean ± standard deviation (SD) values from at least three independent experiments. Statistical significance was evaluated using unpaired *t*-test with Welch's correction. $p$-value $< 0.05$ (*) or $<0.01$ (**) were considered as statistically significant.

## 3. Results

### 3.1 Overexpression and subcellular localization of Sal synthase

PC12 is a rat adrenal pheochromaocytoma-derived cell line that produces and releases dopamine [16], and has been commonly used for studies of cell toxicology, neuronal development, and neurodegenerative diseases. In our research, it was used to investigate the function of Sal synthase. The amino acid sequence of Sal synthase was reverse transcripted to cDNA to construct the recombinant plasmid [pcDNA3.1(+)-Sal synthase], which then transfects PC12 cells for Sal synthase overexpression (Fig. 1A). After a 48 h transfection, the Sal synthase protein level was determined. The results indicated that Sal synthase was successfully overexpressed in PC12 cells at a high level compared with the control which used empty vectors (Fig. 1B). Consistent with our previous study, endogenous Sal synthase in control

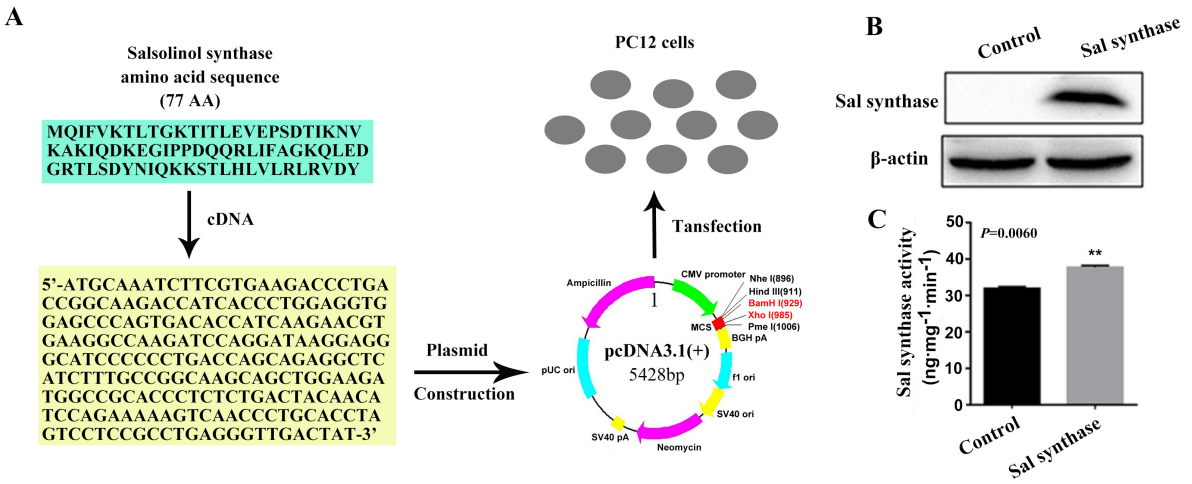


**Fig. 1. Overexpression and the activity detection of Sal synthase in PC12 cells**. (A) Recombinant plasmid was constructed based on the amino acid sequence of Sal synthase and transfected in PC12 cells for 48 h. (B) Quantification of overexpressed Sal synthase by western blot. (C) The activity of Sal synthase was increased in overexpressed PC12 cells. Error bar indicates means ± SD (n = 3), $^{**}p < 0.01$.

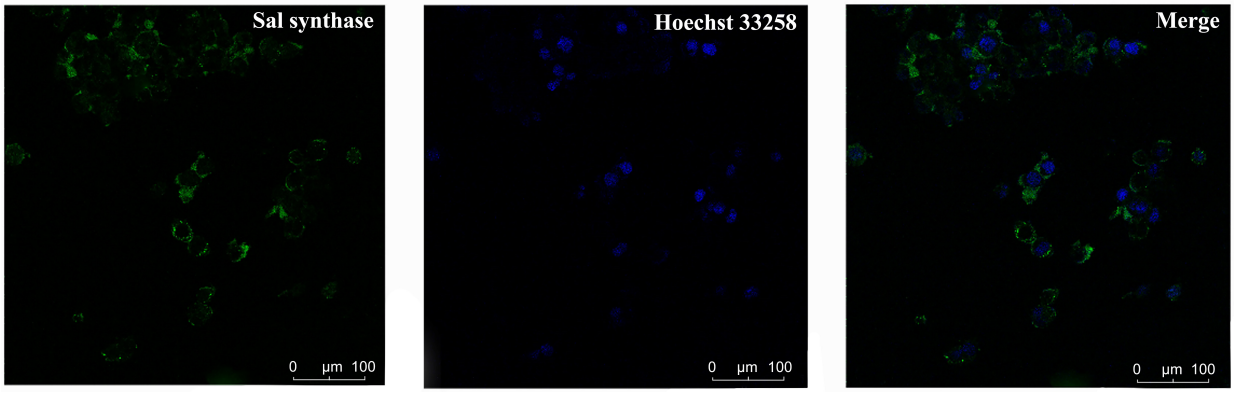


**Fig. 2. Subcellular localization of Sal synthase in PC12 cells**. After transfection for 48 h, a laser scanning confocal microscope was used to observe the subcellular distribution of Sal synthase. Green fluorescence indicated Sal synthase and the blue fluorescence represented cell nucleus.

PC12 cells was difficult to detect with western blot [13]. In addition, the soluble whole proteins of PC12 cells were extracted and evaluated the catalytic activity of Sal synthase by HPLC-MS/MS. Compared with control, Sal synthase's activity increased by approximately 20% in overexpressed PC12 cells ($p = 0.0060$, Fig. 1C). This result confirmed that the overexpressed Sal synthase maintains catalytic activity.

In order to detect the subcellular distribution of Sal synthase, co-expression plasmids containing both Sal synthase and Enhanced Green Fluorescent Protein (EGFP) were used to determine its subcellular localization. The EGFP representing the fusion protein of Sal synthase and Hoechst 33258 was used for nuclear staining. As shown in Fig. 2, Sal synthase-EGFP fusion protein was found within the cytoplasm of PC12 cells, suggesting it is one type of cytoplasmic protein and has enzymatic functions.

### *3.2 Sal synthase increased the production of Sal and NM-Sal*

To investigate the biocatalytic function of Sal synthase, the level of Sal was detected by HPLC-MS/MS using ISOP as an internal standard. In our previous study, (R)-Sal exhibits an approximately 1.49-fold increase compared to the production of (S)-Sal in enzymatic reaction [12]. This indicated that the (R)-Sal in excess was biosynthesized by Sal synthase and two pathways (the other one being the Pictet-Spengler reaction) of Sal production may co-existed. As it is difficult to completely prevent the Pictet-Spengler reaction inside PC12 cells, we measured the total Sal instead of (R)-Sal in this study. Sal may be further metabolized by N-methyltransferase to NM-Sal, so we also determined the content of NM-Sal in PC12 cells.

Typical MRM ion chromatograms of ISOP, Sal and NM-Sal are shown in Fig. 3A. The retention time of Sal

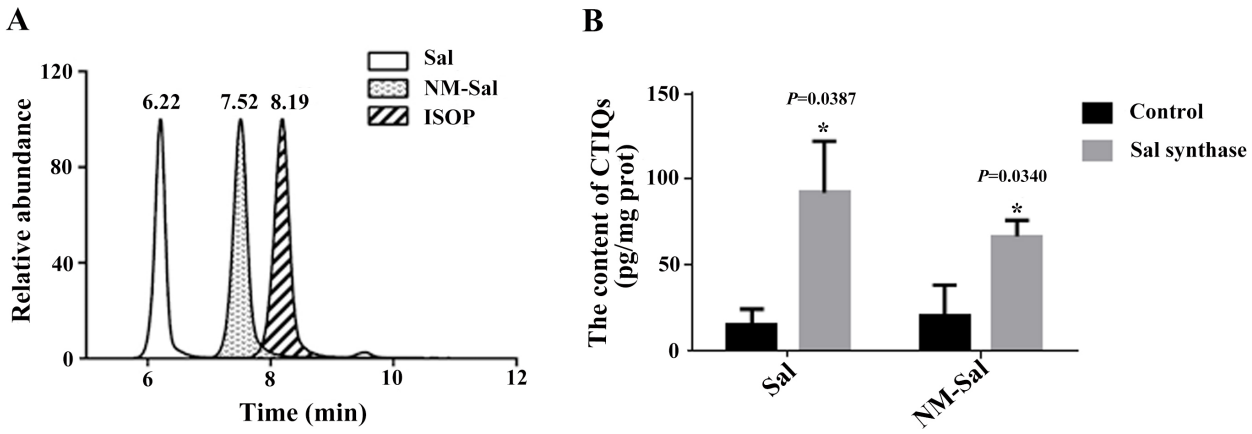


**Fig. 3. Sal synthase improved the production of Sal and NM-Sal**. (A) Typical MRM ion chromatograms of ISOP, Sal and NM-Sal detected by HPLC-MS/MS. (B) Quantification of Sal and NM-Sal in PC12 cells. Error bars indicate means ± SD (n = 3), *$p < 0.05$.

(m/z 180.1/117.1) was 6.22 min and NM-Sal (m/z 194/145) was 7.52 min. The levels of Sal and NM-Sal are presented as pg per mg protein (pg/mg protein). Results indicate that both Sal and NM-Sal were increased in Sal synthase overexpressed PC12 cells (Sal, $p = 0.0387$; NM-Sal, $p = 0.0340$) (Fig. 3B). This result indicated that Sal synthase could induce the production of Sal, and the accumulation of Sal could be further metabolized to NM-Sal.

### *3.3 Sal synthase induced mitochondria damage and apoptosis*

Previous studies suggest that the excessive formation of Sal and NM-Sal can induce oxidative stress and neurotoxicity in numerous cells [17,18], and Sal can also cause apoptosis in neural stem cells [19]. In order to evaluate the potential mechanism of Sal synthase, mitochondria damage and apoptosis were evaluated in Sal synthase overexpressed PC12 cells. For mitochondria damage detection, we estimated the MMP by loading the JC-1 probe. In normal mitochondria, JC-1 assembled in the matrix and the polymer emitted red fluorescence. However, when mitochondria were damaged and MMP decreased, the JC-1 monomer dissociated in the cytoplasm and displayed green fluorescence. Therefore, the ratio of green and red fluorescence intensity reflected the change of MMP. As shown in Fig. 4A,B, Sal synthase significantly increased the ratio of green/red fluorescence ($p = 0.0140$), which indicated mitochondrial damage.

We next investigated the apoptotic rate using an Annexin V-FITC/PI Apoptosis Detection Kit (Fig. 4C). The apoptosis rate was significantly increased (twelve-fold) compared with the negative control ($p = 0.0081$), indicating the substantial neurotoxicity of Sal synthase. Additionally, the expression of the anti-apoptotic protein Bcl-2 and the correlated pro-apoptotic protein Bax were detected. The results illustrate a remarkable decrease of Bcl-2 in overexpressed group (Bcl-2, $p = 0.0132$), along with no significant change in Bax (Fig. 4D). The significant decrease in the Bcl-2/Bax ratio caused by Sal synthase was in accordance with the Sal exposure [19], which demonstrated that the cytotoxicity of Sal synthase may be caused by the toxicity of Sal.

## 4. Discussion

Sal was first detected in the urine of Parkinsonian patients on L-DOPA medication in 1973 [20]. Further studies have characterized levels of this catechol isoquinoline and its metabolites in different visceral organs and brain regions in greater detail [21,22]. The identification of Sal bio-synthesis has led to more interest in the function of Sal synthase. Since its preliminary identification by Naoi M and purification by Deng's group, there has been limited study of its biological function. In the present study, we attempted to characterize the catalytic role of Sal synthase and its cytotoxicity in dopaminergic PC12 cells.

Our work revealed that the catalytic activity of Sal synthase was increased in overexpressed PC12 cells and promoted the production of Sal. Previous reports indicate that Sal can be N-methylated to NM-Sal, which possesses more potent neurotoxicity toward dopaminergic neurons [4]. Based on this observation, we optimized the chromatographic conditions to increase the detection limit for NM-Sal. Sal synthase overexpression can also increase the content of NM-Sal, indicating that Sal accumulation induces the production of NM-Sal. There is another neutral N-methyltransferase which contributes to the synthesis of NM-Sal, and its activity is significant higher in lymphocytes from PD patients [23]. Since Sal synthase increases the production of Sal and NM-Sal, we assumed that the metabolic pathway of NM-Sal also exists in PC12 cells, which could be verified by the knocking-down of N-methyltransferase. Unfortunately, the N-methyltransferase gene and its amino acid sequence have not been identified and purified until recently, and future research on this target is warranted.

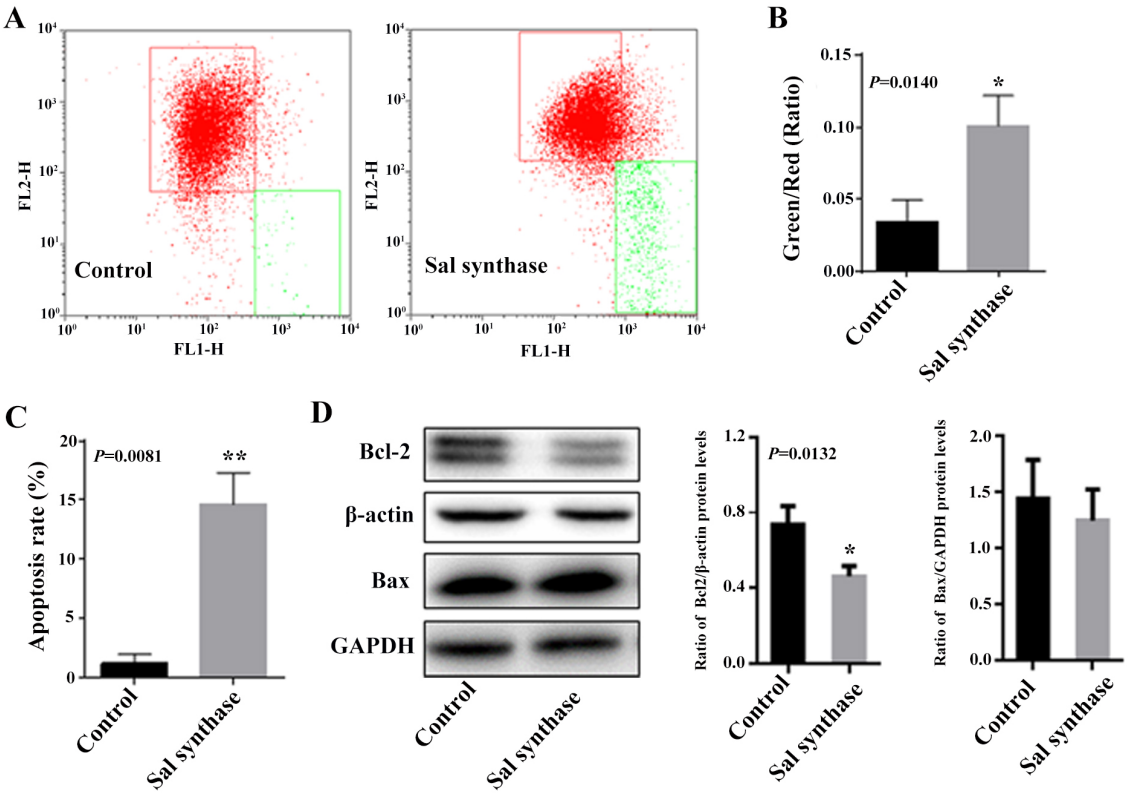


**Fig. 4. The effect of Sal synthase on MMP levels and apoptosis**. (A,B) MMP levels were estimated by flow cytometry using JC-1 probe, and the ratio of green and red fluorescence intensity reflected the change of MMP. (C) After transfection, PC12 cells were dually stained with Annexin V/PI (Propidium iodide, PI) to quantify the apoptotic cells. (D) Detection and quantification of Bcl-2 (26 kD, the upper band; the band below is non-specific band as using the polyclonal antibody) and Bax by western blot. Error bars indicate means ± SD (n = 3), *$p < 0.05$, **$p < 0.01$.

The neurotoxic mechanisms of both Sal and NM-Sal are still unclear. Storch *et al*. [24] reported that Sal exhibited higher potency and selective toxicity than NM-Sal in expression systems of the dopamine transporter (DAT) in Neuro-2A cells, indicating that the toxicity of isoquinoline derivatives is closely related to DAT. Moreover, the production of Sal may reduce the content of dopamine, which is in accordance with the loss of dopamine in substantia nigra of PD patients [25]. As postulated by Deng Y [25–27], the increased level of Sal and NM-Sal may increase oxidative stress by affecting the activity of Complex I, causing mitochondrial dysfunction. To confirm this hypothesis, we also determined mitochondrial function. Results reveal that MMP was substantially decreased in Sal synthase overexpressed cells compared with control, confirming that the impairment of mitochondria is caused by the increased levels of Sal and NM-Sal produced by Sal synthase.

In previous studies, researchers have often used Sal and NM-Sal to induce PD in cellular models. The toxicity of these CTIQs resulted in apoptosis and the death of neuronal cells [28–30]. After the Sal synthase is overexpressed in PC12 cells, more endogenous neurotoxins are produced, and the rate of apoptosis is also increased. It is hypothesized that the increasing neurotoxin could lead to the damage of mitochondria, leading to apoptosis. Therefore, the expression of apoptosis-related proteins including Bcl-2, Bax, Bad, p53 and caspase-3, were investigated in the present study. The results indicate that there was a significant decrease only in Bcl-2. Consistent with previous reports, Sal exposure could also induce a significant decrease in the expression of Bcl-2 and decrease the Bcl-2/Bax ratio, also leading to apoptosis [19]. Nevertheless, it is unclear how and by which pathways the Sal synthase worked. Notably, our results illustrate that endogenous Sal synthase is difficult to detect by western blot but exhibits high catalytic activity, indicating that the purified Sal synthase maybe just one subunit or requires the presence of other molecules. As the amino acid sequences of Sal synthase and ubiquitin are similar, with only four amino acids difference [13], it is difficult to knock-down endogenous Sal synthase or detect its mRNA level without ubiquitin interference. Consequently, further research should involve eliminating ubiquitin interference and screening for the interacting proteins of Sal synthase. This will be critical for elucidating the roles of Sal synthase in PD.

## Abbreviations

AcH, acetaldehyde; CSF, cerebrospinal fluid; CTIQs, catechol isoquinolines; DA, dopamine; DAT, dopamine transporter; ISOP, isoproterenol; MMP, mitochondrial membrane potential; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NM-Sal, N-methyl-salsolinol, 1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; PD, Parkinson's disease; Sal, salsolinol, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; Sal synthase, salsolinol synthase.

## Author contributions

QX and XZ performed the experiments, XZ and JiW analyzed the datas, XC wrote the paper and supervised the overall project. ZC, YD, RZ, and JuW contributed to revise the manuscript.

## Ethics approval and consent to participate

Not applicable.


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## Conflict of interest

The authors declare no conflict of interest.

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