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Isolation and sequencing of salsolinol synthase, an enzyme catalyzing salsolinol biosynthesis

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Abstract: Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline), a derivate of dopamine, is suspected the most probable neurotoxin in the degeneration of dopaminergic neurons. Numerous hypotheses regarding its pathophysiological roles have been raised, especially related to Parkinson’s disease and alcohol addiction. In the mammalian brain, salsolinol may be enzymatically synthesized by salsolinol synthase from dopamine and acetaldehyde. However, the direct evidence of its biosynthesis was still missing. In this study, we purified salsolinol synthase from rat brain by a systematical procedure involving acid precipitation, ultrafiltration, and hydrophilic interaction chromatography. The molecular weight of salsolinol synthase determined by MALDI-TOF MS is 8622.29 Da, comprising 77 amino acids (MQIFVKTLLT KTITLEVEPS DTIKNKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYI IQKKSLTHLV LRLRVDY). Homology analysis showed that the enzyme is a ubiquitin-like protein, with four amino acids difference, which suggests it
is a novel protein. After it was over-expressed in eukaryotic cells, the production of salsolinol was significantly increased as compared with control, confirming the catalytic function of this enzyme. To our knowledge, it’s the first systematic purification and sequencing of salsolinol synthase. Together, this work reveals a formerly anonymous protein, and urges to explore its possible prognostic value and implications in Parkinson’s disease and other related disorders.

**Keywords:** salsolinol, Sal synthase, biosynthesis, purification, amino acid sequence, Parkinson’s disease.

**Introduction**

Parkinson’s disease (PD) is the second most common chronic, progressive neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain. The catechol isoquinolines derived from dopamine (DA) are compounds widely present in the mammalian brain and are also detected in the urine of patients suffering from PD who have been treated with L-DOPA (1). The metabolic precursor of catechol isoquinolines, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol, Sal), has recently invited much attention due to its similar structure with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which could cause the symptoms of PD (2). As reported, Sal is a prominently detected catechol isoquinoline in the postmortem of PD patient’s brain (3). Compared with healthy subjects, it is significantly present in the urine and cerebrospinal fluid of PD patients (4). Previous
studies on Sal revealed its involvement in the progression of disease characterized by
dysfunction of dopaminergic neurons (5). Moreover, as the oxidative stress level
increases, the lipid peroxidation-induced formation of acetaldehyde (AcH) can
promote the synthesis of Sal as well. The increased concentrations of Sal induce the
mitochondrial toxicity, which could also cause PD (6-8). These results implicated that
Sal is one of the candidate neurotoxins in PD pathogenicity.

In addition, Sal has also been implicated in the development of alcoholism and in the
release of the hormone prolactin (PRL). Many reports have shown that chronic
administration of ethanol to animals leads to increase in brain Sal (9, 10). Furthermore,
the increase of Sal was found in the brain of alcoholics intoxicated at the time of death
as well (11). It suggested that Sal can be used as a potential biomarker for the
detection of alcoholism. In ruminants, Sal was demonstrated to stimulate the secretion
of prolactin PRL (12, 13); however, the precise mechanism of it is not yet known.

Many reports revealed that Sal was unable to cross the blood-brain barrier (BBB)
(14-16), so its existence in the brain indicates endogenous synthesis. Furthermore,
Quintanilla et al. reported that systemically administered Sal is able to cross the BBB
(10). The truth about BBB penetration of Sal still needs to be explored. Sal has an
asymmetric center at C-1 and exists as (R)- and (S)-enantiomers (17). In the human
brain, reports showed that the concentration of (R)-Sal was much higher than (S)-Sal
(18, 19), and the extra (R)-Sal may be due to the presence of a putative enzyme. Until
now, Sal is proposed to be formed in three pathways (Figure 1). The first is the
non-enzymatic Pictet-Spengler (P-S) reaction to yield the racemic (R/S)-Sal from DA
and AcH (20). In the second pathway, an enzyme which names salsolinol synthase (Sal synthase), is recognized to catalyze the formation of (R)-Sal from DA and AcH (21, 22). The third one showed that an unknown enzyme is involved in the conversion of DA and pyruvic acid into (R)-Sal via formation of an intermediate metabolite (salsolinol-1-carboxylic acid) (23).

For a long time, the existence of Sal synthase is doubtful because neither has it been isolated nor has its amino acid sequence been determined (24). In the previous study, we have confirmed the presence of Sal synthase in rat brain (25), but it is still not fully understood. In 1996, Naoi et al had reported the preliminary isolation of the enzyme without identification (21). From then on, Sal synthase has not been reported extensively, and some researchers also suspected its existence. Consequently, our work was to develop a viable procedure for isolation and purification of Sal synthase from rat brain, and to identify its amino acid sequence. This study is the first systematic attempt for purification, sequencing and functional analysis of Sal synthase.
Figure 1. Synthesis pathways of salsolinol (? - unknown enzyme). (A) Pictet-Spengler reaction to yield the racemic (R/S)-Sal from DA and AcH. (B) Sal synthase catalyzes the formation of (R)-Sal from DA and AcH. (C) An unknown enzyme is involved in the conversion of DA and pyruvic acid into (R)-Sal via formation of an intermediate metabolite (salsolinol-1-carboxylic acid).

Results

1. Activity and distribution of Sal synthase in rat brain

In our study, crude enzyme extracts prepared from rat brain were used to isolate and purify Sal synthase. To begin with, we detected the activity of Sal synthase in whole rat brain. As shown in Figure 2A, DA and AcH can produce Sal by the Pictet-Spengler reaction, and a relatively low level of endogenous Sal also exists in the crude enzyme extract, which is in accordance with previous reports (26-28). Attractively, the incubation of the two substrates (DA and AcH) with crude enzyme fraction produced a significantly larger amount of Sal. To calculate the true quantity of Sal produced by
the catalytic reaction of enzyme, we subtracted the amount of Sal already present in
the crude extract and that produced by the Pictet-Spengler reaction from the Sal
quantity obtained in DA+AcH+E reaction. We found that increase in the catalytically
produced amount of Sal is positively correlated with the amount of crude enzyme
fractions (Figure 2B). These results clearly suggested the existence of Sal synthase
activity in the rat brain that catalyzes the condensation of DA and AcH into Sal.

We also detected the distribution of Sal synthase in different regions of rat brain, and
found its activity exist in almost all regions with the highest in substantia nigra
(Figure 2C). Musshoff et al reported that significant amount of Sal was only found
in the dopamine-rich areas such as striatum, substantia nigra and hypothalamus,
whereas in the other regions no alkaloids were detected (29). Our finding of the
higher Sal synthase activity in substantia nigra and striatum can be positively
correlated with the higher Sal production in these regions. These results suggest that
the concentration of the substrate dopamine and the activity of Sal synthase may
determine the Sal level in vivo.
Figure 2. Activity and distribution of Sal synthase in rat brain. (A) Comparison among the endogenous Sal in crude enzyme extracts, amount of Sal produced by Pictet-Spengler reaction and by the reaction incubated with crude enzyme extracts (E = crude enzyme extract). (B) Incubated with DA and AcH in the same condition, the production of Sal were increased and positively correlated with the amount of the crude enzyme extracts. (C) Activity distribution of Sal synthase among the different regions of rat brain.

2. Isolation and purification of Sal synthase

In our research, the whole rat brains were used to isolate Sal synthase, and the process of purification and identification was shown in Figure 3. After obtaining the crude enzyme fractions, a systematical procedure comprising acid precipitation,
ultrafiltration, and hydrophilic interaction chromatography was developed for purification. Finally, the molecular weight and the amino acid sequence of Sal synthase were determined by MALDI-TOF MS.

Figure 3. Schematic diagram of the purification and identification processes of Sal synthase.

2.1 Acid precipitation

During our studies, it was surprising to find that Sal synthase couldn’t be removed by strong acid, although it showed the highest activity in neutral environment (Supplementary Figure S1). Therefore, we used perchloric acid (PCA) to get rid of the great majority protein in the crude enzyme extract and then the supernatant containing the target enzyme was neutralized with K$_2$CO$_3$. Nearly 98% of Sal synthase activity was observed in the neutralized supernatant, whereas the activity was seriously lost.
without neutralization (Figure 4A). However, it is difficult to determine whether the activity is inhibited by PCA itself or acidity surroundings. Hydrochloric acid (HCl) was then used as control, and both HCl and PCA were neutralized by NaOH (Figure 4B). We observed the same phenomenon that the activity of Sal synthase was lost in acid environment and can be recovered after base neutralization. These results confirmed that Sal synthase could not be precipitated by acids, as its activity is lost temporarily which can be regained by neutralization.

Figure 4. Comparison of Sal synthase activity after acid precipitation and base neutralization (R= DA+ AcH+ Enzyme). (A) PCA was used to remove the great majority protein in the crude enzyme extract and then the supernatant containing the enzyme was neutralized with K$_2$CO$_3$. (B) PCA and HCl were used for acid precipitation of proteins and both were neutralized with NaOH.

2.2 Ultrafiltration and dialysis

The supernatant obtained from acid precipitation was then isolated by ultrafiltration using YM-100 and YM-3 membrane filters with molecular weight cut-off (MWCO) around 100 kDa and 3 kDa respectively. After centrifugation, three fractions, P1
(MWCO > 100 kDa), P2 (3 kDa < MWCO < 100 kDa) and P3 (MWCO < 3 kDa) were obtained according to molecular mass. The result of activity detection showed that P3 has the highest activity, and it is closer to the total activity of the crude extract. It suggests that most of Sal synthase exists in the P3 fraction (Figure 5). Then, we used dialysis to remove the small-molecular compounds of this fraction through a selective diffusion of dissolved particles by 0.5 kDa semipermeable membranes. Figure 5 showed that almost 75% of the Sal synthase activity was retained after dialysis, precluding the query that Sal synthase maybe one kind of small-molecular compound in our previous report (25). Within that paper, we also suspected that the molecular mass of Sal synthase maybe less than 3 kDa, as most of its activity exists in P3 fraction. However, this presumption is not accurate enough in current view, since the ultrafiltration always has three times effect, which means that YM-3 membrane filter could cut off proteins with nearly 10 kDa molecular weight. Based on this, we supposed that the molecular mass of Sal synthase is in the range of 0.5 to 10 kDa.

![Graph showing Sal synthase activity](image-url)
Figure 5. Comparison of Sal synthase activity obtained from different parts of ultrafiltration. P1 (MWCO >100 kDa), P2 (3 kDa < MWCO < 100 kDa) and P3 (MWCO < 3 kDa). D represents the Sal synthase activity after dialysis from P3 fraction.

2.3 Hydrophilic interaction chromatography (HILIC)

As Sal synthase could not retain on reversed phase chromatography (RP), which is the most powerful HPLC separation mode (as shown in Supplementary Figure S2), we used Hydrophilic interaction chromatography (HILIC) for further purification. At present, the HILIC mode is widely used for the separation of highly polar substances including biologically active compounds such as pharmaceutical drugs, neurotransmitters, peptides, amino acids and nucleosides. HILIC with low aqueous/high organic mobile phase was emerging as a valuable alternative to the reversed-phase LC-MS/MS (30). Hence, the enzyme samples after ultrafiltration and dialysis were further isolated by HILIC with two kinds of columns. From the larger HILIC column, the active fraction was eluted at specifically 51-52 min with 55.5-61% ACN (Figure 6). This active eluate was further isolated using a smaller HILIC column, getting the active fraction at 39-40 min with 64-67% ACN (Figure 7).
Figure 6. Elution profile of Sal synthase activity from the larger HILIC column. The enzyme samples were first eluted with 95% ACN, then with a linear ACN gradient (...). Every fraction per minute was collected and analyzed for their absorbance at 280 nm (—) and their Sal synthase activity (♦—♦).

Figure 7. Elution profile of Sal synthase activity from the smaller HILIC column. The fractions which contained the activity of Sal synthase from the larger HILIC column were collected and separated on a smaller HILIC column in the same condition.
2.4 Recovery of purification

The purification procedure and activity recovery of Sal synthase are summarized in Table 1. Initially, the total activity was 625.96 pmol/min, and it was reduced to about 226.24 pmol/min after purification. The final recovery was about 36.14%. This enzyme was relatively stable and could be stored at -20°C for a period of up to one year without appreciable loss of catalytic activity.

<table>
<thead>
<tr>
<th>Purification procedure</th>
<th>Total activity (pmol/min)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme extract</td>
<td>625.96±153.41</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant of acid precipitation</td>
<td>592.87±80.20</td>
<td>94.71</td>
</tr>
<tr>
<td>Ultrafiltration (P3)</td>
<td>496.60±51.89</td>
<td>79.33</td>
</tr>
<tr>
<td>Dialysis</td>
<td>372.45±45.33</td>
<td>59.50</td>
</tr>
<tr>
<td>1st HILIC (larger) column chromatography</td>
<td>272.35±26.23</td>
<td>43.51</td>
</tr>
<tr>
<td>2nd HILIC (smaller) column chromatography</td>
<td>226.24±33.35</td>
<td>36.14</td>
</tr>
</tbody>
</table>

3. Identification and sequencing of Sal synthase

3.1 Tris-Tricine SDS-PAGE of purified Sal synthase

The purity of enzyme obtained from the smaller HILIC column was then determined by Tris-Tricine SDS-PAGE using silver staining. Presence of only one band of nearly 9 kDa proved the purity of enzyme (Figure 8A). As shown in Figure 8B, the protein band recovered from SDS-PAGE gels still contained high catalytic activity, confirmed that the purified protein is Sal synthase.
Figure 8. Silver staining analysis of purified Sal synthase on SDS-PAGE gels. (A) The active fractions isolated by HILIC system were resolved on Tris-Tricine SDS gels. Silver-stained gel indicates that the purified protein contained only one band with a small molecular weight. (B) The protein band from the gel was recovered and the activity was detected by HPLC-ECD.

3.2 Analysis by MALDI-TOF MS

The purified product recovered from Tris-Tricine SDS-PAGE gel was digested by trypsin and then analyzed with MALDI-TOF. In the linear mode, two clusters of peaks with different mass-to-charge ratio (m/z) were observed (Figure 9). One cluster was observed with the highest peak at 8622.29 m/z, whereas there is also a weak peak at about 8463.43 m/z. The other cluster was at 4314.48 m/z, and it is the doubly charged ion of 8622.29 m/z. In general, intensities of individual peptides in spectra for sample replicates somewhat varied, and occasionally weak peaks were not observed between replicate samples. Therefore, the observed weak peak of 8463.43 m/z seems
to be the same peak of 8622.29 m/z missing one amino acid. Mass spectrometric analysis revealed that 8622.29 m/z peak is the purified enzyme, which is in agreement with the result of SDS-PAGE, demonstrating the molecular mass of Sal synthase is about 8622.29.

Table 2 shows the representative peptides of purified Sal synthase observed from MALDI-TOF MS spectra. Combined with MS/MS sequence data, the major peptide ions were tabulated and compared with the theoretical peptides obtained from NCBI database by Mascot searching. It routinely provided 100% confidence in identification of Sal synthase. The ion masses, amino acid sequences and ions score of the four representative peptides were summarized in Table 2. These peptides were matched with theoretical peptides with the highest ions score of 90. Detailed MS/MS analysis of major peptide ions was used to assess peptide signatures in Sal synthase. Computer-assisted interpretation of MS/MS spectral data provided the de novo amino acid sequence assignments. Representative MS/MS spectra and amino acid sequence results are presented in Figure 10.
Table 2. Peptides observed in spectra from Sal synthase by MALDI-TOF MS.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Calculated m/z</th>
<th>Database Sequence</th>
<th>Ions score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12-27</td>
<td>1787.9305</td>
<td>K.TITLEVEPSDTIENVK.A</td>
<td>26</td>
</tr>
<tr>
<td>30-42</td>
<td>1523.8191</td>
<td>K.IQDKEGIPPDQQR.L</td>
<td>90</td>
</tr>
<tr>
<td>34-42</td>
<td>1039.5829</td>
<td>K.EGIPPDQQR.L</td>
<td>51</td>
</tr>
<tr>
<td>64-72</td>
<td>1067.6832</td>
<td>K.KSTLHLVLR.L</td>
<td>67</td>
</tr>
</tbody>
</table>

![Mass spectrum of IQDKEGIPPDQQR peptide](image)
Figure 10. Representative MS/MS spectra and novo sequence of peptide from purified Sal synthase: (A) Peptide ion m/z 1523.8191. (B) Peptide ion m/z 1067.6832.

From Mascot searching of the NCBI database, we found the theoretical amino acid sequence of Sal synthase contains 77 amino acids (MQIFVKTLTG KTITLEVEPS DTIKNVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN IQKKSTLHLV LRLRVDY). The NCBI reference of the sequence is XP_006221493.1. It is an unnamed ubiquitin-like protein which was yet unknown. We compared it with the ubiquitin amino acid sequence of rattus norvegicus, and found that four amino acids are different between them. The twenty-fourth and the sixty-fourth amino acids of the N-terminal of the ubiquitin is glutamic acid (E), and the corresponding position of the unknown protein is occupied by lysine (K). In the C-terminal, two glycines (G) of the ubiquitin are replaced by valine (V) and aspartic Acid (D) in unnamed protein (Figure 11). As glutamic acid is an acidic amino acid and lysine is a basic amino acid, it is presumed that this purified enzyme is alkaline. This result is consistent with the result
that Sal synthase has strong retention ability on weak anion exchange column during purification (data not shown). The comparison of this purified enzyme with ubiquitin should require further characterization.

Figure 11. Full amino acid sequence alignment of Sal synthase and ubiquitin from *Rattus norvegicus*, showing amino acid replacements (highlighted in red).

4. Activity confirmation of the amino acid of Sal synthase

The cDNA sequence of unnamed protein obtained from the NCBI database was synthesized. pcDNA3.1(+) vector was used to construct recombinant plasmid [pcDNA3.1(+)-Sal] of unnamed protein, and the plasmid was overexpressed in PC12 cells. The level of Sal synthase was determined by Western Blot which showed high level of the enzyme after transfection (antibody of the unnamed protein was prepared by Huada Beijing Protein Innovation, China). Relatively, the endogenous Sal synthase was difficult to detect in control which was transfected with pcDNA3.1(+) empty vector (Figure 12A). After transfection, the level of Sal produced in cells was detected
by HPLC-MS/MS to evaluate the catalytic function of Sal synthase. As shown in Figure 12B, there is a significant increase of Sal compared to control (*P<0.05), indicating that the overexpressed protein could catalyze the production of Sal and display Sal synthase activity. This result indicates that the unnamed protein in NCBI database can be called Sal synthase, although need further investigation.

Figure 12. Overexpression of Sal synthase in PC12 cells and its catalytic function was detected. (A) Western-blot assay of the Sal synthase. (B) Salsolinol synthase induced the increasing level of Sal in transfected PC12 cells. PC12 cells transfected with pcDNA3.1(+) empty vector was used as control.

Discussion

Sal synthase is one kind of Pictet-Spenglerases that catalyze the first step of the indole alkaloids pathways (31). Up till now, three kinds of Pictet-Spenglerases have been found in plants (32-34), which are strictosidine synthase, norcoclaurine synthase and deacetylisoipecoside synthase. According to our latest knowledge, it is the first study of its kind which identified and confirmed the presence of a Pictet-Spenglerase, Sal synthase, in animals.
In this study, we have isolated and identified Sal synthase from the rat brain which catalyzes the condensation of dopamine and acetaldehyde to Sal. We established a simple procedure involving acid precipitation, ultrafiltration and HILIC for purification of Sal synthase. Precipitation by PCA could preserve more than 98% of Sal synthase activity and remove most of unrelated proteins from the crude extract. HILIC with low aqueous/high organic mobile phase is free from the risks of ion adulteration. This resulted in the purification enzyme with high recovery. The molecular mass of purified Sal synthase confirmed by MALDI-TOF MS is approximately 8.6 kDa which suggested that this enzyme is a small active protein. Our results are different from those reported by Naoi in 1996(21), who estimated the molecular weight was $34.3 \pm 8.3$ kDa. It may due to the difference in the origin of Sal synthase, since Naoi purified this enzyme from human brain whereas we isolated it from rat brain. It’s difficult to make a detailed comparison between them, as neither Naoi nor the others published any further reports about the Sal synthase since 1996.

After purification, we matched from the NCBI database that the purified enzyme consists of 77 amino acids. Moreover, the database suggests that it is one kind of a novel protein, and predicted that it is an ubiquitin-like protein with four amino acids difference (Figure 11). To confirm the function of identified amino acid sequence, we overexpressed its cDNA in PC12 cells, and found that it displays Sal catalytic activity. Interestingly, we also detected the activity of ubiquitin from rattus norvegicus, and didn’t find any catalytic activity. Overall, we suspect this unnamed protein in NCBI database is Sal synthase.
In the human brain, reports showed that only (R)-Sal was detected, and Naoi found Sal synthase enantio-selectively synthesizes (R)-Sal as well (18, 21). However, the enantiomeric ratio of (R)-/(S)-Sal was approximately 2 in rat brain (20), indicating that the dissimilar production of Sal between different species. Likewise, our previously researches in rat brain also found that (R)-Sal exhibits an approximately 1.49-fold increase in enzymatic reaction compared to the production of (S)-Sal(35).

Based on these results, we presume that both Pictet-Spengler reaction and enzymatic reaction take place in rat brain. Pictet-Spengler reaction produces the racemic (R/S) Sal at the ratio of 1: 1, and the enzymatic reaction produces only (R)-Sal catalyzed by Sal synthase. Studies showed that (R)-Sal could be metabolized by N-methyltransferase to N-methyl-(R)-salsolinol which subsequently converted into 1,2-dimethyl-6,7- dihydroxyisoquinolinium ion (DMDHQ⁺) by amine oxidase (36, 37). The up-regulation of Sal and N-methyl-salsolinol (NMSal) in the cerebrospinal fluid of PD patients suggests that these endogenous catechol isoquinolines may be involved in the pathogenesis of PD (20). In addition, our results showed that the activity of Sal synthase was much higher in substantia nigra compared with the other regions which is consistent with the distribution of Sal and its metabolites (29, 38).

Altogether, we suggested that Sal synthase is the key enzyme in the metabolism of tetralydroisoquinoline, and maybe play a role underlying the etiopathogenesis of PD. As acetaldehyde may react with DA either spontaneously or enzymatically, many investigations have highlighted that Sal may lead to alcohol addiction and regulate hormone release of the neuroendocrine system, which indicated that it is a potential
regulator of dopaminergic neurons (10, 39). Quintanilla et al indicated that (R)-Sal stereospecifically induces motivational effects, behavioral sensitization and increases ethanol intake (40). Although much works have identified the role of acetaldehyde and Sal in the central effects of ethanol, the pharmacologically significant, the clinical application, and the potentially responsible for alcohol use disorders (AUDs), are still need further disclosed.

As the neuromodulatory role of Sal is still poorly understood, extensive research should be carried out to reveal the metabolic pathway of Sal and its metabolites in vivo. Both in rodents and humans, Sal concentration tends to be higher in dopamine-rich brain areas (29) and our study witnessed higher activity of Sal synthase at these regions. It can be proposed that the production and distribution of Sal in brain should not solely depend on dopamine but also on the activity of the synthesizing enzyme. Moreover, Maruyama et al reported that the N-methyltransferase activity was higher in the rat nigro-striatum than in other brain regions (41), suggesting that Sal could also cause the accumulation of NMSal. In the metabolic process of these tetralydroisoquinolines, Sal synthase is the key enzyme and may play prognostic role in the whole metabolism pathway. It can affect the production of series neurotoxins, which may be related to PD, alcohol addiction and other related diseases. Based on the amino acid sequence of Sal synthase, extensive studies are warranted to explore its biological functions. In addition, characterization of Sal synthase should be further revealed.
Materials and methods

Reagents

Dopamine (DA), acetaldehyde (AcH), 1-methyl-6,7-dihydroxy-1,2,3,4
tetrahydroisoquinoline (salsolinol), sodium 1-heptanesulfonate (SHS), HPLC-grade
methanol (Me) and acetonitrile (ACN) were purchased from Fisher Scientific Canada
(Edmonton, Canada). 50 mM Tris-HCl (pH 7.4), 1 M perchloric acid (PCA)
containing 1 mM ethylenediamine tetra-acetic acid (EDTA) and sodium metabisulfite,
citric acid and pentobarbital sodium were purchased from Sigma (Sigma-Aldrich,
Oakville, ON, Canada). Disodium ethylenediamine tetra-acetic acid (Na$_2$EDTA) was
purchased from Sino-American Biotechnology Company. Tris-Glycine and
Tris-Tricine were purchased from Bio-Rad Laboratories (Mississauga, Canada). A
pre-stained low molecular weight protein marker was purchased from Sigma. All
other chemicals were obtained from the Beijing Chemical reagent Company in China.
All of the chemicals used were of analytical grade and organic solvents were of
high-performance liquid chromatography (HPLC) grade. A Milli-Q water purifying
system was utilized to generate 18.2 MΩ deionized water.

Animal Experiments

Animals and Treatment

Male Sprague-Dawley rats (Central Animal House of Chinese Academy of Medical
Sciences), weighing 200 ± 20 g (45 days old), were given a commercial standard rat
cube diet and water ad libitum. They were housed in an animal facility under
controlled environmental conditions (ambient temperature, 24 ± 1°C; relative
humidity, 50 ± 5%; and a 12 h light/12 h dark cycle) for 7 days before experimental conditions. The experimental protocols were carried out following guidelines on the Care and Use of Animals developed by the National Advisory Committee for Laboratory Animal Research and in accordance with ARRIVE guideline. All animals received humane care and their use was approved by the Animal Ethics Committee of China Pharmaceutical University (No. CPU-PCPK-B1210071).

**Crude enzyme extraction**

Crude enzyme fractions were extracted from whole brains or different brain regions of Sprague-Dawley rats. Rats were anesthetized with pentobarbital (64.8 mg/mL, 50 mg/kg) and decapitated (according to institutional protocols). Brain, cortex, striatum, substantia nigra, and cerebellum were quickly dissected on a cold plate at 4°C. The tissues were immediately homogenized with a pestle using 2 mL extraction buffer (50 mM Tris-HCl, pH 7.4) per gram wet weight. The homogenate was centrifuged at 100,000 g for 1 h at 4°C and the supernatant was collected. In total, 30-35 frozen Sprague-Dawley rat brains were thawed and homogenized during the whole purification processes. The animal studies have been conducted in accordance with an Institutional Animal Care and Use Committee of Institute of Biophysics, Chinese Academy of Sciences.

**Reaction Condition**

Enzyme fractions (0.1 mL homogenate supernatant) were incubated in 50 mM Tris-HCl (pH 7.4) with 1 mM dopamine and 1 mM acetaldehyde in a total volume of 1 mL (21, 23). After an incubation period of 40 min at 37°C, 20 μL of PCA (1 M) was
added to terminate the reaction. After mixing and centrifugation at 17,000 g for 15 min, the supernatant was diluted 25 fold and filtered through a 0.22 µm cellulose membranes, then stored at -80°C until detection. The blank control was treated at the same reaction conditions without enzyme.

**Sal synthase activity assay by HPLC-ECD**

The assay system for determining the catalytic activity of Sal synthase was developed based on the enzymatic product. Enzyme activity was calculated by subtracting the background Sal in control reactions, such as endogenous Sal and non-enzymatic Pictet-Spengler reaction. The product Sal was measured by HPLC-electrochemical detection (ECD) (ESA, Chelmsford, MA, USA) (35). The oxidation voltage of a model 5020 guard cell was 400 mV, 350 mV, 100 mV and -50 mV. Samples were separated on a reverse phased inertosil ODS-C18 column (4.6 (i.d.) × 150 mm), with a mobile phase (pH 4.0) of citric acid (40 mmol/L), Na<sub>2</sub>HPO<sub>4</sub> (20 mmol/L), Na<sub>2</sub>EDTA (0.3 mmol/L), 5% methanol, and 1.0 mM heptane sulphonic acid at a flow rate of 1.0 mL/min. The quantity of Sal was calculated by means of a standard curve generated by standard chemicals. The specific activity of Sal synthase was estimated with the increase of Sal per min per mg of tissue at 37°C, represented as pmol/min/mg tissue.

**Purification and identification of salsolinol synthase**

**Acid precipitation**

The crude enzyme fraction of whole rat brain was transferred to a 1.5 mL microfuge tube and 200 µL of ice-cold perchloric acid (PCA, 1 M) was added per milliliter. The
acidic supernatant was centrifuged at 17,000 g and 4°C for 10 min. The supernatant was neutralized with 100 µL of 1 M K₂CO₃ (42), kept on ice for 10 min, and then at -80°C for 1 h, to promote precipitation of the perchlorate. In addition, hydrochloric acid (HCl, 1 M) was also used to precipitate proteins and 1 M NaOH was used to neutralize acidic supernatant induced both by PCA and HCl as controls. Finally, the mixture was centrifuged as described above. The supernatant referred as the crude enzyme extract was stored at -80°C until further purification.

**Ultrafiltration and dialysis**

Ultrafiltration (UF) is a kind of membrane filtration in which hydrostatic pressure forces a liquid against a semipermeable membrane. Suspended solids and solutes of high molecular weight are retained, while water and low molecular weight solutes pass through the membrane. In this paper, two different types of Microcon ultrafiltration tubes, 15 mL YM-3 and YM-100 were used to segregate the crude enzyme extract by molecular weight. First, enzyme fractions were transferred to the ultrafiltration tube YM-100 and centrifuged at 12,000 g for 20 min at 4°C. The filtrate was transferred to YM-3 tube and centrifuged under similar conditions. Finally, from these two kinds of ultrafiltration tubes, the three fractions (MWCO > 100 kDa, 3 kDa < MWCO < 100 kDa, MWCO < 3 kDa) were collected to detect the activity of Sal synthase by HPLC-ECD. After ultrafiltration, the fractions which contained enzyme activity were further purified by dialysis using a 0.5 kDa semipermeable membrane for selective diffusion of dissolved particles. The fractions were dialyzed for 24 h in 250 mL of deionized water as solvent. The analytes inside semipermeable membrane
were then collected to detect the activity of Sal synthase after freeze-drying.

**Hydrophilic interaction chromatography (HILIC)**

The dialyzed fraction which contains the enzyme activity was separated by two kinds of HILIC column. First, on a larger venusil HILIC column (21.5×150 mm; particle size, 5 µm; pore size, 100 Å) of HPLC/DAD system containing a gradient pump and a UV detector. The mobile phase consisting of solvent A (10 mM ammonium formate in water) and solvent B (acetonitrile) was delivered at a flow rate 5 mL/min. The injection volume was 2 mL. The stepwise elution was as follows: 0.0 min [A/B: 5/95], 10.0 min [A/B: 5/95], 30.0 min [A/B: 15/85], 45.0 min [A/B: 50/50], 50.0 min [A/B: 60/40], and 60.0 min [A/B: 5/95]. The chromatogram was recorded at a wavelength of 280 nm. In total, 60 fractions were collected in 60 minutes. Identical fraction numbers from each run were combined, dried via nitrogen and detected for the Sal synthase activity by HPLC-ECD. Second, the fractions which contained the activity of Sal synthase were then separated on a smaller venusil HILIC column (4.6×150 mm; particle size, 5 µm; pore size, 100 Å) with an Agilent 1200 series (Agilent, Palo Alto, CA) having a quaternary pump. The mobile phase and stepwise elution conditions were same as in the first separation step. Then, the activity was detected.

**Tris-Tricine SDS-PAGE**

Tris-Tricine SDS polyacrylamide gels were run using a Mini-Protean II slab gel electrophoresis unit (Bio-Rad) according to the method of Schagger (43). The gel contained two parts: stacking gel and separating gel. The purified samples were
lyophilized and dissolved in loading buffer. Gels were run at 50 V until the tracking dye passed stacking gel (T=5%, C=3.3%), and then, a constant voltage of 200 V was applied until the dye reached the bottom of the separating gel (T=18%, C=5%). Silver staining of SDS gels was performed using the Silver Staining kit from Bio-Rad Laboratories.

**Amino acid sequencing**

The purified Sal synthase was subjected to trypsin and chymotrypsin digestion. Peptides were separated using HPLC with Symmetry C18 reverse phase column (4.6 (i.d.) × 150 mm). Two solvents used were Solvent A (Water containing 0.1% TFA) and Solvent B (Acetonitrile containing 0.1% TFA). The peptide was eluted with a 60 min linear gradient to reach from 100% A to 100% B. Flow rate was maintained at 1 mL/min. Analysis of the sample was performed by MALDI-TOF MS (Bruker Daltonics, Germany). The sequence obtained from N-terminal sequencer was matched with the database of NCBI (National Center for Biological Information) using BLAST (Basic Local Alignment Search Tool).

**Cell Experiments**

**Recombinant plasmid construction**

The cDNA sequence of Sal synthase which was obtained from the NCBI database was synthesized (Sangon Biotech, China). Primer 1 (5’-GACACGGATCC (Bamh1) gccac-cATGCAAATCTTCGTGAAGACCCT-3’ (Forward) and 5’-GACACCTCGAG (Xho-1) TTAATAGTCACCCTCAGGC-3’ (Reverse)) and pcDNA3.1(+) vector were used to construct the Sal synthase recombinant plasmid, named
pcDNA3.1(+)-Sal. pcDNA3.1(+) empty vector was used as control.

**Cell culture and transfection**

Rat pheochromocytoma PC12 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium (Gibco, USA) with 10% fetal bovine serum (Wisent, Canada) and 5% horse serum (Gibco, USA) with 5% CO$_2$ and maximal humidity at 37°C. Differentiated PC12 cells were grown to ~80% and transfected with pcDNA3.1(+) empty vector and pcDNA3.1(+)-Sal using Lipofectamine 3000 (Invitrogen, USA). In this research all the experiments were performed after transfection for 48 h.

**Western-blot Assay**

Cells were lysed in RIPA buffer plus a cocktail protease inhibitor (Solarbio, China). Lysates were centrifuged and the concentration of protein was measured by BSA (Sigma, USA) method. The samples were diluted with the protein loading buffer and boiled for 5 min. After centrifugation at 10,000 g for 5 min, aliquots of cell lysates (30 µg total protein/sample) were separated on 12% SDS-PAGE, and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, CA, USA). The blocking buffer was 5% non-fat milk in TBS-Tween buffer. The antibody of β-actin was obtained from Sigma. The antibody of Sal synthase was prepared by Huada Beijing Protein Innovation, China. The bands were visualized with enhanced chemiluminescence reagents (Thermo scientific) and exposed to X-ray film (Kodak, USA). The quantitative analysis was performed by Bio-Rad imaging system and signals were normalized for β-actin probed on the same blots as loading controls.
**Analysis of Sal by HPLC-MS/MS**

After transfection, PC12 cells were harvested and sonicated in 50 mM Tris-HCl (pH 7.4) containing protease inhibitor after transfection for 48h. The concentration of protein was measured and then the proteins were removed by adding 1 M perchloric acid (PCA) solution (1 M HClO₄, 10 mM Na₂S₂O₅, 10 mM EDTA) at the rate of 5:1 (v/v). The suspensions were centrifuged and the supernatants were filtered through a 0.22 µm membrane. The quantitative analysis of Sal was done by HPLC-MS/MS using a Discovery® HS F5-5 (15cm × 2.1mm, 5µm) column. Isoproterenol (ISOP) was added as the internal standard. The mobile phase consisting of methanol-water (25/75, v/v) with 10 mM ammonium formate (pH 3.5), was delivered at flow rate of 0.2 mL/min. The injection volume was 5 µL. The instrument was operated according to methods described previously (44).

**Statistics analysis**

The results were presented as the mean ± SEM 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.

**Abbreviations**

AcH, acetaldehyde; BBB, blood-brain barrier; DA, dopamine; DMDHIQ⁺, 1,2-dimethyl-6,7- dihydroxyisoquinolinium ion; EDTA, ethylenediamine tetra-acetic acid; HILIC, Hydrophilic interaction chromatography; HPLC- ECD,
high-performance liquid chromatography-electrochemical detection; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MWCO, molecular weight cut-off; Na₂EDTA, Disodium ethylenediamine tetra-acetic acid; NMSal, N-methyl-salsolinol; PCA, perchloric acid; PD, Parkinson’s disease; Sal, Salsolinol, 1-methyl-6,7-dihydroxy- 1,2,3,4-tetrahydroisoquinoline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SHS, sodium 1-heptanesulfonnate; SNpc, substantia nigra pars compacta.

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**Author Contributions**

X.C. is the first author, did almost all experiments and wrote this manuscript. X.Z. and Z.C. collaborated to conduct the cell transfections and Western-blot assays; M.G. and Y.Z. helped to collect samples and do LC/MS detection; S.A. helped to revise the manuscript in language editing; R.Z. and H.Q. helped to guide experiments; Y.D., as the corresponding author, guided and organized all experiments and revised this manuscript.

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**Supporting Information**

The pH-activity profile of Sal synthase and the chromatogram of P3 fraction separated by reversed-phase chromatography.

**References**


Isolation and sequencing of salsolinol synthase, an enzyme catalyzing salsolinol biosynthesis

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