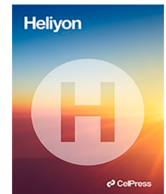
Heliyon 9 (2023) e21125

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Application of serum peptidomics for Parkinson's disease in SNCA-A30P mice

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ARTICLE INFO

Keywords:
Parkinson's disease
Peptidomics
Alpha-synuclein
SNCA-A30P
LC-MS/MS
Blood

ABSTRACT

Intraneuronal inclusions of alpha-synuclein (α-synuclein, α-syn) are commonly found in the brain of patients with Parkinson's disease (PD). The pathogenesis of the abundant α-syn protein in the blood has been extensively studied to understand its properties better. In recent years, peptidome analysis has received increasing attention. In this study, we identified and analyzed serum peptides from wild-type (WT) and the (Thy-1)-h[A30P] alpha-synuclein transgenic mice (SNCA-A30P mice) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). One thousand eight hundred fifty-six peptides from 771 proteins were analyzed. Among them, 151 peptides from 107 proteins were significantly differentially expressed. The glycoprotein VI platelet pathway (GP6) was the pathway's most significant differentially expressed signaling pathway. Cleavage sites of the differentially expressed peptides may reflect protease distribution and activity. We selected the most significantly differentially expressed peptide, VGGDPI, and found that it contained cathepsin K (Ctsk) and trypsin-1 cleavage sites, suggesting that Ctsk and trypsin-1 may be key peptidases in PD. α-syn is a protein associated with the pathogenesis of PD. mutations in several genes, including SNCA, which encodes α-syn, are associated with the development of PD. Bioinformatics analysis of the physiological pathways related to SNCA genes and apoptosis genes found the five most markedly up-regulated proteins: formin homology 2 domain-containing 1 (FHOD1), insulin receptor substrate 1(IRS1), TRPM8 channel-associated factor 1 (TCAF1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and interleukin-16 (IL-16). Therefore, the differentially expressed peptides in the five precursor protein domains may be potential bioactive

Abbreviations: ACN, acetonitrile; FHOD1, formin homology 2 domain containing 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GO, Gene Ontology; GP6, glycoprotein VI platelet; IL-16, interleukin-16; IPA, Ingenuity Pathway Analysis; IRS1, insulin receptor substrate 1; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MS/MS, tandem mass spectrometry; NDEVs, neuron-derived extracellular vesicles; PD, Parkinson's disease; TCAF1, TRPM8 channel-associated factor 1; TH, tyrosine hydroxylase; TRPM8, transient receptor potential melastatin-like 8; WT, wild-type; α-syn, α-synuclein; TTN, recombinant titin; DA, dopamine; CSF, cerebrospinal fluid; LBs, Lewy bodies; Ctsk, cathepsin K; MAP2, microtubule association protein-2; hDAT, human dopamine transporter; pI, isoelectric point; PVDV, polyvinylidene difluoride.

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https://doi.org/10.1016/j.heliyon.2023.e21125
Received 9 January 2023; Received in revised form 30 July 2023; Accepted 16 October 2023
Available online 14 November 2023
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peptides associated with α-syn and apoptosis. This study provides a validated peptidomics profile of SNCA-A30P mice and identifies potentially bioactive peptides linked to α-syn and apoptosis.

1. Introduction

PD is the second most common neurodegenerative disease worldwide [1]. Clinical motor symptoms include resting tremors, bradykinesia, myotonia, postural control deficits, and non-motor symptoms, including constipation, olfactory disorders, sleep disorders, autonomic dysfunction, and psychiatric and cognitive impairments [2]. It is estimated that 6.1 million people worldwide were affected by PD in 2016, with a higher incidence in people over 60 years. In addition, as the population ages, PD is becoming a significant societal burden [3,2]. The current treatment of PD is based primarily on dopamine (DA) therapy and surgery [4,5]. Although these approaches have been proven to control PD-related motor symptoms, the use of these approaches is often accompanied by severe adverse effects and does not effectively alter the pathological progression [5].

In recent years, it has been found that PD is classified as a synucleinopathy, and its leading pathological mechanism is the accumulation and transmission of pathological α-syn. Pathologically, the soluble monomer α-syn aggregates and misfolds into soluble oligomeric forms, insoluble fibrils, and then Lewy bodies (LBs) [6]. Recent studies have shown that α-syn oligomers are the leading cause of neurotoxicity and play a critical role in PD. Although α-syn has been tested extensively in cerebrospinal fluid (CSF), the relatively invasive procedure for collecting CSF is unsuitable in most clinical settings, leading to the investigation of plasma, blood, and saliva as alternatives. Evidence suggests potential diagnostic and prognostic value of blood biomarkers and bioactive compounds closely reflecting the pathophysiology of Parkinson's disease, such as α-syn species.A-syn has been identified as a characteristic biomarker of PD.Clinical studies have found that studies on α-syn levels in the peripheral blood of PD patients have been controversial. Total plasma α-syn levels in PD patients increased [7], decreased [8], or were similar to patients with control groups [9,10]. Contamination caused by the different degrees of hemolysis in plasma/serum (and CSF) could significantly affect α-syn levels. Consequently, some have suggested that red blood cells alone may be a potential biomarker for PD diagnosis and/or severity. For example, a phospholipid-ELISA assay of α-syn derived from packed red blood cells from twenty-one individuals with PD and 15 healthy controls showed a significantly lower ratio of total-to-proteinase K-resistant α-Syn levels was detected in PD patients versus in the healthy control group [11].

The A30P mutation in SNCA, the gene encoding α-syn, involves a change from guanine to cytosine at nucleotide position 88 (G88C), causing the replacement of alanine at amino acid position 30 by proline [12]. The study found that patients carrying the A30P mutation typically exhibit a similar age of onset and symptoms as sporadic PD [13]. Compared with wild-type (WT) mice and A53T mutants, the A30P mutants showed a faster oligomer formation [14,15]. Moreover, 9-month-old A30P mice exhibited S129-phosphorylated α-syn, abundant dystrophic neurites, some cell body inclusions, and many LBs-like structures [16]. It is worth noting that neuropathological analysis of SNCA-A30P mice at 8 and 11 months of age showed aggregation of oligomeric α-syn in neuronal cell bodies and decreased tyrosine hydroxylase (TH) levels [17]. We conducted a peptidomics analysis of the blood of WT and 10-month-old SNCA-A30P mice using liquid chromatography-tandem mass spectrometry (LC-MS/MS). We investigated the endogenous peptides involved in α-syn and apoptosis, which may contribute to our understanding of PD and have clinical implications for treatment.

Peptidomics, the comprehensive, qualitative, and quantitative analysis of all peptides in biological samples, is an emerging field related to proteomics and was enabled by advances in separation, analytical, and computational techniques [18]. Peptides are produced during metabolism or through non-specific proteolytic degradation of larger proteins by various peptidases [19]. Bioactive peptides are a group of protein fragments with several amino acid residues ranging from 2 to 20, and their molecular weight is 0.4–2 kDa. They have special physiological functions and potentially apply to human health and disease prevention. Compared with traditional drugs, bioactive peptides are highly selective, effective, versatile, safe, tolerant, and easier to metabolize in vivo. It is worth noting that bioactive peptides have powerful targeting effects and a magnificent ability to reach locations in the body. Therefore, it is considered to have great potential in treating diseases [20]. Studies have demonstrated a linear correlation between VGF peptide immunoreactivity, disease duration, levodopa equivalent dose, and olfactory dysfunction. As suggested by animal studies, reduced ghrelin excretion might increase the vulnerability of nigrostriatal dopaminergic neurons. Moreover, total tau and amyloid-β peptides (Aβ42, Aβ40) in serums of 22 PD patients and ten control subjects are correlated. The serum content of tau protein was inversely correlated with cognitive performances [21].

2. Materials and methods

2.1. Animals

In this experiment, SNCA-A30P-overexpressing [B6.Cg-Tg(THY1-SNCA*A30P)TS2Sud/J] mice (A30P, male, ten months, n = 3) were presented by Dr. Zhou Jiawei. Age-matched littermate WT mice were used as controls and were purchased from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China, n = 3). The mice were given standard chow and water ad libitum, housed at room temperature of 24 ± 2 °C, with 50–60 % humidity, adequate ventilation, and a 12-h/12-h light/dark cycle. All animals were acclimated to the environment for three days before the start of testing procedures. All animal procedures were carried out following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the related ethical regulations of

Nanjing Medical University.

2.2. Behavioral analysis

(1) For the rotarod test, three days before the test, the mice were trained for 5 min at a speed of 12–20 rpm every day with the rod-turning instrument (Jiliang, Shanghai, China). The retention time of the mice was recorded at a speed of 20 rpm during the formal test. For the pole test, during the experiment, the mouse was placed head up on the top of the pole and recorded two times: The time from the start of the movement to full head down (T-turn) and the total time the mouse was Climbing down to the bottom of the pole (T-turn). Training was conducted for three days before the experiment, twice a day, with an interval of 5 min. During the experiment, the minimum time was recorded. If the mice did not completely flip, drop or slip, it was 120s. For the open field test, mice were placed in an autonomous mobile box (20 cm × 20 cm × 15 cm) for 10 min of adaptation, and their crawling tracks and speed were observed and recorded within 5 min.

2.3. Western blotting analysis

Midbrain tissues were lysed in the RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 % NP-40(FNN0021, Thermo), 0.5 % sodium deoxycholate (D6750, Sigma), 0.1 % SDS (74255, Sigma) supplemented with phosphoprotease inhibitors (Roche, Shanghai, China). The amount of total extracted protein was determined by BCATM protein assay kit (Thermo, 23235). A 30 μg protein of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was electrophoretically transferred onto PVDF membranes (Millipore, Bedford, MA).The membranes were blocked and probed with the following primary antibodies overnight at 4 °C: Mouse anti-TH (1:1000, Sigma, Cat T1299), rabbit anti-P-alpha-syn (1:1000, abcam, ab51253), microtubule association protein-2 (MAP2) (1:1000, Santa Cruz, SC-32791), mouse anti-β-Actin (1:4000, abcam, ab179467).then added HRP labeled goat anti-rabbit (1:5000, Servicebio, GB23303) or HRP labeled goat anti-mouse (1: 5000, Servicebio, GB23301), incubated at room temperature for 1 h. Detection was performed using an ImageQuant LAS 4000 mini (Uppsala, Sweden) luminescent image analyzer.

2.4. Sample collection

Blood samples were collected from the retroorbital plexus of control and PD mice, thus allowing the fresh serum to be obtained without sacrificing the animals. Sodium citrate was used as an anticoagulant. The blood was centrifuged at 1000×*g* for 10 min at 4 °C to collect the supernatant. Aliquots of supernatants were mixed with protease inhibitor (Complete Mini EDTA-free, Roche Applied Science, Basel, Switzerland) and stored at −80 °C.

Before analysis, serum samples were thawed on ice and were collected and mixed thoroughly with 20 % acetonitrile (ACN) (v/v), and the mixture was incubated at room temperature for 20 min. Samples were filtered through a 10-kDa MW cut-off filter to remove highly abundant high-MW proteins. The filtrate was desalted and concentrated using C18 solid phase extraction and dried under a vacuum. Plasma protein concentrations were determined using a BCA assay (ThermoFisher Scientific, Waltham, MA, USA). Peptides in each sample were detected using tandem mass tags (ThermoFisher Scientific).

2.5. LC-MS/MS

The mass spectrometer was used to identify the labeled samples from the control or A30P groups. Peptides were loaded onto a trap column (Acclaim PepMap100C18, 75 μm × 20 mm, LC Packings) and separated with a C18 column (Acclaim PepMap C18, 75 μm × 150 mm, LC Packings) on an Ultimate 3000 nano-LC system (Dionex). The mobile phase consisted of (A) 2 % ACN and 0.5 % acetic acid and (B) 80 % ACN and 0.5 % acetic acid. Using a linear gradient, the eluted peptides were sprayed into the mass spectrometer at 1.8 V. Full scan analysis was performed at two spectra per second from m/z 600–5000. Compounds with a signal-to-noise ratio of 15 were selected for tandem mass spectrometry (MS/MS) analysis.

2.6. Bioinformatics analysis

Each peptide's isoelectric point (pI) and Molecular weight (MW) were calculated using an online pI/MW tool (http://web.expasy.org/compute_pi/). Search results were compiled into protein lists using ProteinScape (Bruker Daltonik GmbH, Bremen, Germany). Cellular components, biological processes, and molecular functions of peptides were determined by Gene Ontology (GO) analysis. Physiological and pathological functions of related proteins were analyzed using Ingenuity Pathway Analysis (IPA), which determined the signaling pathways involved and other molecular components associated with these pathways.

3. Results

3.1. Motor dysfunction in A30P transgenic mice

Tremor, bradykinesia, and postural instability are significant clinical manifestations of PD. To verify the success of SNCA-A30P mice PD model, the movement and behavior of mice were evaluated by the rotarod test, open field test, and pole test. As shown, compared with the control group, A30P transgenic mice have significantly shortened stick retention time (Fig. 1a), reduced the

distance and average speed of movement (Fig. 1 b, c), and reduced the turning and total time in the pole test (Fig. 1 d,e). These findings suggest that A30P transgenic mice have motor dysfunction.

3.2. Western blotting detects the expression levels of TH, MAP2, and α-syn in the midbrain of A30P-SNCA mutant mice

The main neuropathological characteristics of PD are LBs and progressive degeneration of dopaminergic neurons in the substantia nigra.LBs are the pathological hallmark of PD. More than 90 % of α-syn within LBs is phosphorylated at the serine-129 residue [pSer129 α-syn (p-α-syn)].TH was used to mark DA neurons, and MAP2 was used to mark neurons. Compared with control mice, TH and MAP2 protein levels in the midbrain of A30P transgenic mice were significantly decreased (Fig. 2 a, b, c), and the level of p-α-syn/α-syn significantly increased (Fig. 2 a, d). These results showed that neurons and DA neurons were lost, and endogenous p-α-syn was accumulated in 10-month-old A30P transgenic mice.

3.3. Identification of differentially expressed peptides in the blood of PD model mice

LC-MS/MS analysis of 1856 peptides from 771 proteins revealed that 151 peptides derived from 107 proteins were significantly differentially expressed (fold-change >1.5) between the A30P-SNCA mutant and WT mice. Of these, 78 peptides were up-regulated, and 73 were down-regulated in the mutant compared to the WT group. The peptide expression patterns between the two groups were

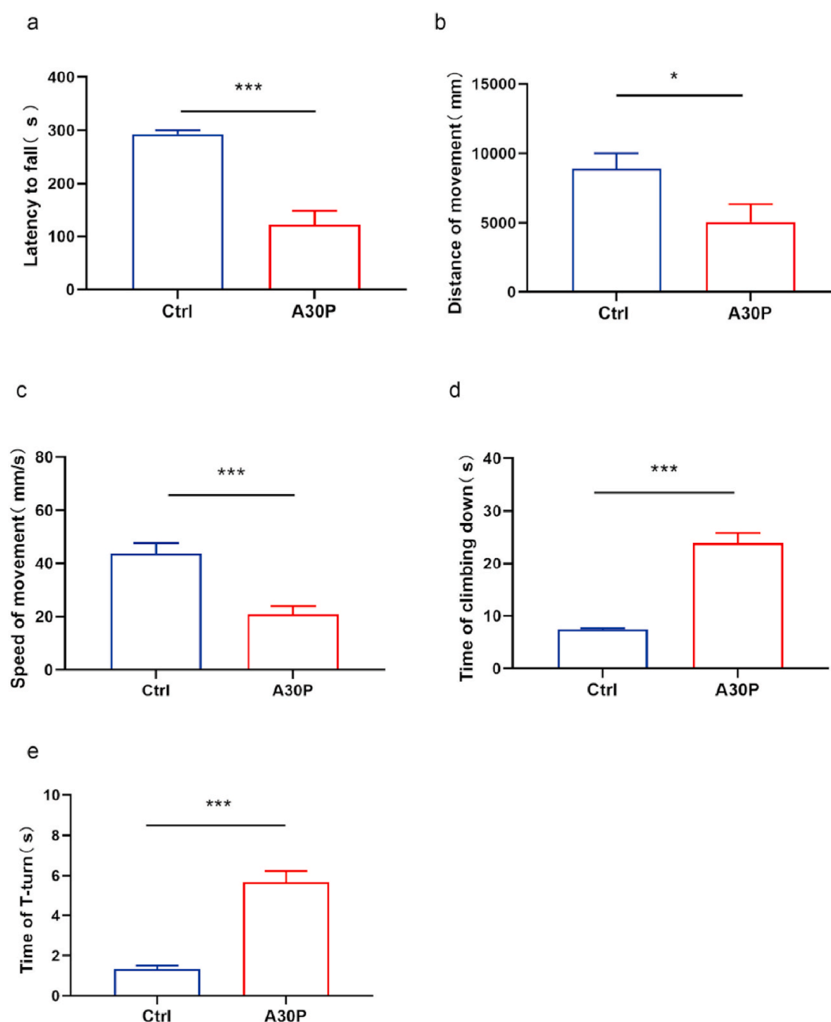

Fig. 1. Dysregulation of motor function in A30P transgenic mice. (a) Mice trained for three days on the rotarod until their motor performance was consistent. The duration of time sustained on the rod was calculated (in seconds). (b–c). Open field activities were simultaneously recorded for movement, distance, and speed by the Top View Animal Behavior Analyzing System produced in the USA. (d–e) The pole test: behavioral assays of climbing time represented as the time taken for mice to turn around (T-Turn) (d) and climb down a pole (e). Data were presented as mean ± SEM. (n = 9), *P < 0.05, **P < 0.01, ***P < 0.001vs control group by *t*-test.

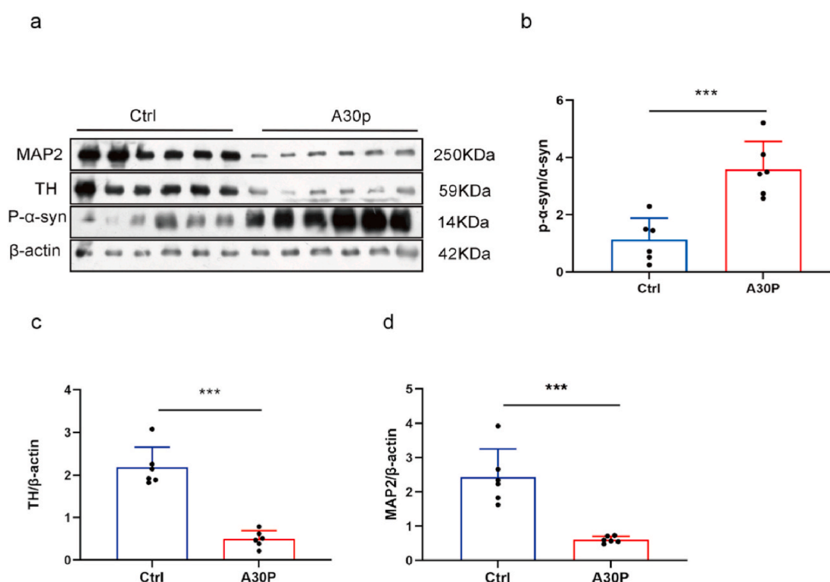

Fig. 2. TH, MAP2, p-α-syn protein expression in the midbrain of SNCA-A30P and WT mice. (a) Western-blot analysis of TH, MAP2, p-α-syn, α-syn protein expression in the midbrain and quantitative analysis (b),(c),(d). The data are expressed as the mean ± SEM and n = 6 per group.*P < 0.05, ***P < 0.01,****P < 0.001 vs. control group by *t*-test.

visualized using a heat map (Fig. 3).

3.4. Identification of peptides in the blood of SNCA-A30P mice

Fig. 4A and B shows MWs and pI of the significantly differentially expressed peptides. The MWs of most peptides ranged from 500 Da to 2000 Da. The pIs of up-regulated peptides were distributed in the 5–6 and 8–9 regions, whereas the pIs of down-regulated peptides were primarily distributed in the 5–7 and 8–10 regions. We also investigated the relative distribution of pIs to MWs (Fig. 4). The MWs of all identified peptides were under 3 KDa, confirming that the isolated peptides were of sufficient purity.

3.5. Cleavage-site patterns for identified endogenous peptides

LC-MS/MS was used in conjunction with bioinformatics analysis of the peptides' N- and C-terminal cleavage sites to evaluate their regulatory role through protease activity and distribution in the plasma of the A30P-SNCA mutant mice. Four cleavage sites were studied for each up-and-down-regulated peptide identified (Fig. 5). In the up-regulated group, the four most common amino acids associated with the four cleavage sites (N-terminal amino acid of the identified peptide, C-terminal amino acid of the identified peptide, C-terminal amino acid of the preceding peptide, and N-terminal amino acid of the subsequent peptide) were lysine (K), threonine (T), serine (S), and glycine (G). In contrast, the most common amino acids in the down-regulated group were glutamic acid (E), glycine (G), lysine (K), serine (S), proline (P), leucine (L), and aspartic acid (D).

3.6. Precursor annotation and canonical pathways of peptides

GO analysis was performed based on the interaction network of the identified peptides to examine the role of these peptides in biological processes, molecular functions, and cellular components. Regarding the cellular components of the precursor proteins: about 44 % of the precursor proteins were found to be derived from organelles, 42 % were derived from intracellular anatomical structures. Among the molecular functions of the precursor proteins, 73 % had binding functions, and 25 % had catalytic activity. About 74 % of the precursor proteins were involved in cellular processes, 64 % in biological regulation, and 36 % in developmental processes (Fig. 6). The precursor protein profiles of the differentially expressed peptides showed that the glycoprotein VI (GP6) signaling pathway was the most significant differential signaling pathway that may be closely associated with the development of PD.

3.7. Predicted changes in the development of PD

All differentially expressed peptide precursor proteins were analyzed by entering SNCA genes and apoptosis genes into the IPA software (Qiagen, Redwood City, CA, USA). to identify the molecular networks associated with the disease (Fig. 7) and listing all differentially up-regulated peptides in the network (Table 1). We screened for potential up-regulated protein roles in the network that showed the most marked differences in expression: formin homology 2 domain containing 1 (FHOD1), insulin receptor substrate 1

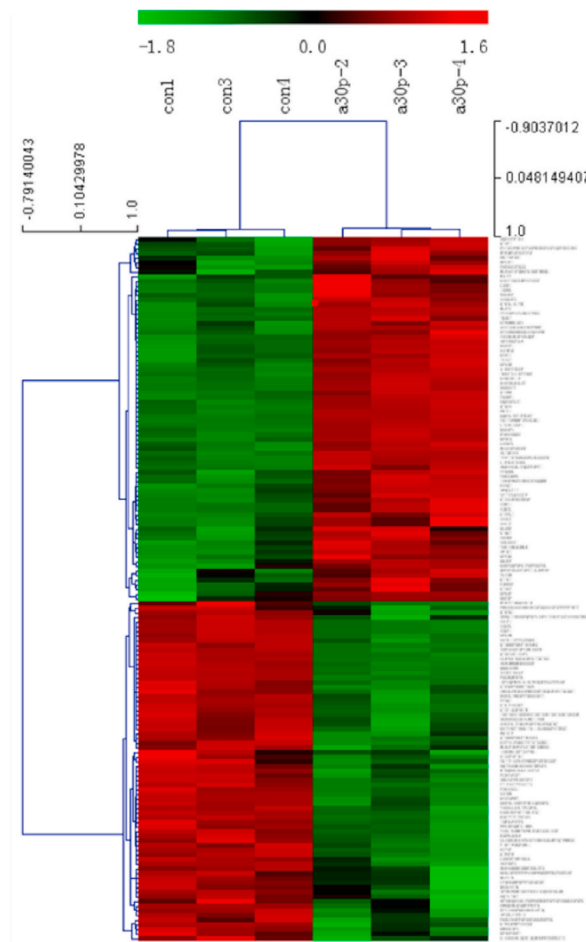

Fig. 3. Heat map showing the distribution of differentially expressed peptides between control and A30P-SNCA groups. The up-regulated peptides: red, the down-regulated peptides: green. The extent of peptide abundance is colored according to the heat map scale.

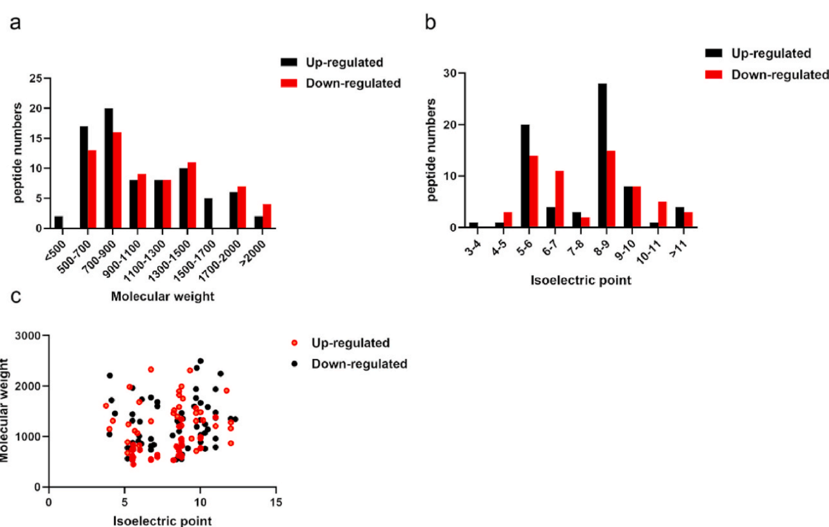

Fig. 4. Distribution of plasma peptides identified by liquid chromatography-tandem mass spectrometry. (a) Molecular weight of differential peptides. (b) Isoelectric points of differential peptides. (c) Scatter plots of molecular weight and relative isoelectric point distribution of differential peptides.The up-regulated peptides:red, the down-regulated peptides: black.

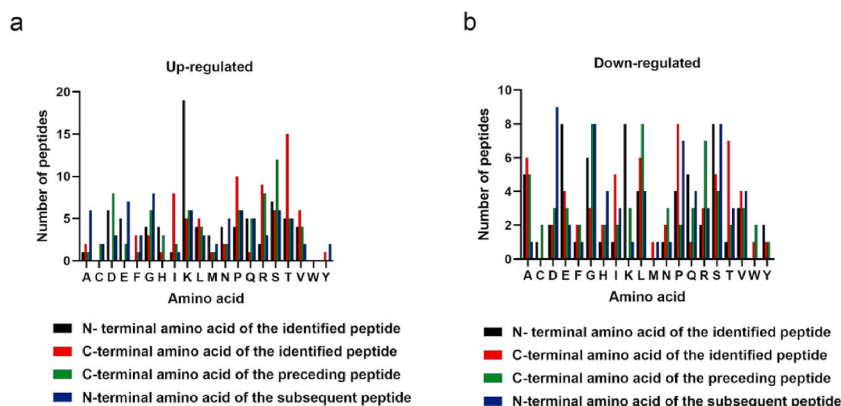

Fig. 5. Cleavage sites of differentially expressed peptides.(a) High expression group.(b) Low expression group.Black represents the N-terminal amino acid of the identified peptide.Red represents C-terminal amino acid of the identified peptide.Blue represents the C-terminal amino acid of the preceding peptide.Green represents the N-terminal amino acid of the subsequent peptide.

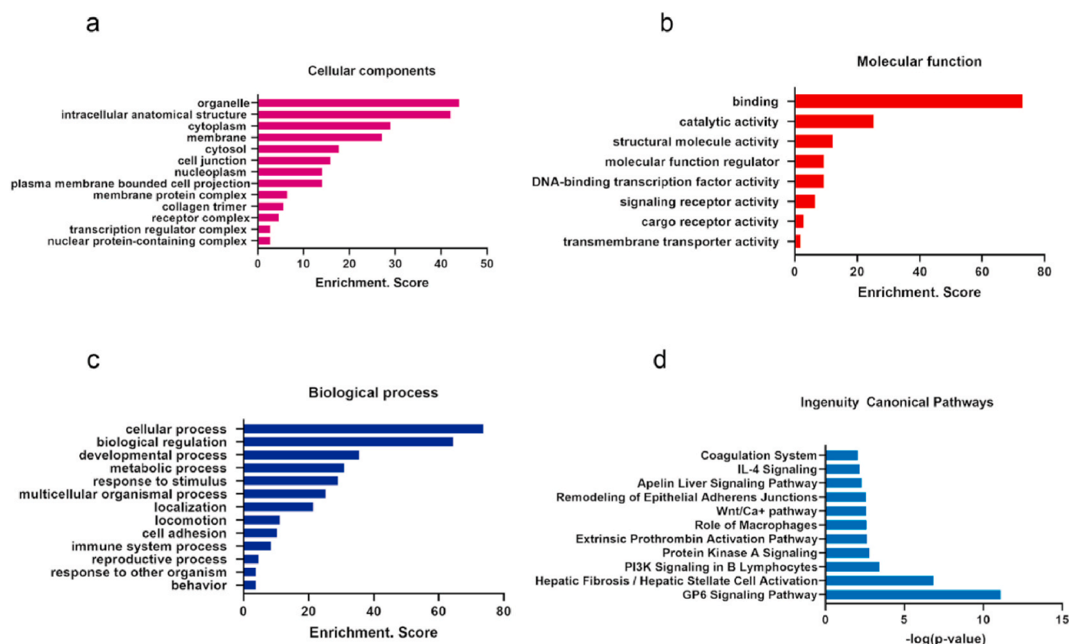

Fig. 6. GO analysis of precursor proteins associated with the identified peptides.(a) cellular components.(b) Molecular functional. (c) biological processes.(d) Ingenuity canonical pathways.

(IRS1), transient receptor potential melastatin-like 8 (TRPM8) channel associated factor 1 (TCAF1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), interleukin 16 (IL-16), and recombinant titin (TTN). We selected six up-regulated peptides corresponding to the six proteins as bioactive peptides.

3.8. Identification of candidate enzymes

Prediction of the cleavage sites of endogenous peptides revealed significant differences between amino acids, which warrants further investigation into the changes in peptidase functions. Peptide cleavage may be related to the presence of specific peptidases in serum. The types and numbers of these enzymes may alter during PD, reflected in the cleavage sites of the identified peptides.

We selected VGGDPI, the peptide showing the most marked expression change between the groups of the peptides screened. To find the key peptidases that cleave the VGGDPI peptide, we analyzed the peptides using the peptidase database MEROPS. Some candidate peptidases (Table 2) that may leave the start and end bonds may also cleave the Ile + Pro and Lys + Val-Gly sites of VGGDPI. The list of peptidases includes different peptidases and proteases (serine proteases, cysteine proteases, and metalloproteinases). According to this list, cathepsin K (Ctsk) and trypsin 1 can cleave VGGDPI at both cleavage sites, suggesting that an enzyme may regulate VGGDPI. In

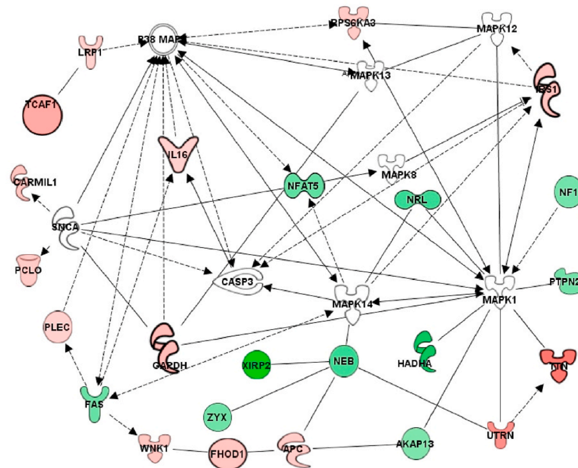

Fig. 7. Protein-protein interaction network of peptide precursors.

Table 1
Up-regulated peptides identified in structural domains of proteins found to be associated with PD.

Accession	Protein	Peptide	Unique	P-value	Fold-change
A2ASS6	TTN	VGGDPI	Y	0.000276168	4.29
Q8BNE1	TCAF1	SDNVDKMNLW	Y	0.000136782	2.82
A2ASS6	TTN	SLTAENSS	N	1.15964E-05	2.64
A2ASS6	TTN	EKTCI	Y	1.87306E-05	2.44
A0A0A0MQF6	GAPDH	VVSCDFNSNSHSSTFDA	Y	4.44484E-05	2.20
A2ASS6	TTN	SKDSSGALIVQEPPSFVT	Y	0.000938668	2.18
A2ASS6	TTN	KPGSR	Y	0.000608964	2.00
A0A0A6YWI6	DCLK2	SAKVL	N	0.005465207	1.99
P35569	IRS1	SAKVI	N	0.005465207	1.99
P35569	IRS1	QTKNLIGIYR	Y	0.000741585	1.95
D3Z030	CARMIL1	KVSSDKERDGQNSSQSSPR	Y	0.003536233	1.93
Q6P9Q4	FHOD1	RLLGAPLKLED	Y	0.000272527	1.92
P83741-5	WNK1	GQGSSVFTES	Y	0.006850851	1.91
A2ASS6	TTN	LSCKVL	N	0.000642422	1.89
A2ASS6	TTN	VGKAAKFL	Y	0.001945624	1.86
A2ASS6	TTN	TKEGTLSIKVVGKP	N	0.00023226	1.86
A2ASS6	TTN	VVQGDK	N	0.003443164	1.86
A2ASS6	TTN	DGGVPI	N	0.000400865	1.84
A2ASS6	TTN	QTQSS	N	6.50417E-05	1.79
A2ASS6	TTN	NGLSGSS	Y	0.008807609	1.78
B1AXN9	RPS6KA3	KLGMP	N	0.023192802	1.77
Q9QYX7-2	PCLO	KTTET	N	0.011080793	1.77
A2ASS6	TTN	KKAPP	N	0.003637478	1.70
A2ASS6	TTN	RVFARNAVGSVSNP	N	0.0035191	1.70
Q61315-3	APC	SSGQSPA	Y	0.000627952	1.66
A2ASS6	TTN	NVTTVLKSSATF	Y	0.004201939	1.66
A2ASS6	TTN	KTANCRVKVMDAP	N	0.001779386	1.63
A2ASS6	TTN	EVPVT	N	0.000407113	1.62
A2ASS6	TTN	TCSAT	Y	0.001634474	1.62
A2ASS6	TTN	KKPAP	N	0.040687554	1.60
A2ASS6	TTN	KPKAP	N	0.02903197	1.59
A2ASS6	TTN	ETVPLT	N	0.001515554	1.58
E9Q3W4	PLEC	HLPLEVAYQRGYLNKDTHDQL	N	0.023770377	1.55
A2ASS6	TTN	KPPAK	N	0.004686201	1.55
O54824	IL16	GATHND	Y	0.000683388	1.54
A2ASS6	TTN	KTQKT	N	0.006359466	1.54
A2ASS6	TTN	SFIKT	Y	0.001273731	1.54
A2ASS6	TTN	KTSEV	N	0.027154784	1.54
A2ASS6	TTN	DGGSPN	N	4.41978E-05	1.53
Q91ZX7	LRP1	HAIVV	N	0.036213134	1.52
A2ASS6	TTN	RFCAVI	N	0.016189181	1.50

Table 2
MEROPS prediction of VGGDPI peptidases.

Peptidase	Enzyme class
K-VG cleavage site	
Cathepsin S	Cysteine protease
Cathepsin K	Cysteine protease
Thermolysin	Metalloprotease
Immune inhibitor A peptidase	Metalloprotease
Chymotrypsin A (cattle-type)	Serine protease
Tryptase beta	Serine protease
Kallikrein-related peptidase 15	Serine protease
Granzyme A	Serine protease
Trypsin 1	Serine protease
Endopeptidase K	Serine protease
I–P cleavage site	
Cathepsin V	Cysteine protease
Cathepsin L	Cysteine protease
Cathepsin K	Cysteine protease
Calpain-1	Cysteine protease
Calpain-2	Cysteine protease
Legumain, animal-type	Cysteine protease
Matrix metallopeptidase-9	Metalloprotease
Matrix metallopeptidase-7	Metalloprotease
Matrix metallopeptidase-12	Metalloprotease
Xaa-Pro dipeptidase (bacteria-type)	Metalloprotease
Aminopeptidase P	Metalloprotease
Pro-Hyp dipeptidase	Metalloprotease
FtsH peptidase	Metalloprotease
Trypsin 1	Serine protease
Glutamyl endopeptidase I	Serine protease
HtrA peptidase	Serine protease

addition, previous studies have shown that Ctsk is a potent disaggregate of α-syn fibrils [21]. Taken together, this indicates that Ctsk may be a key peptidase in PD.

4. Discussion

Blood is a resource, rich in proteins secreted from organs, thus reflecting physiological and pathological conditions throughout the body [22]. Therefore, we identified differentially expressed peptides from WT and A30P-SNCA mutant mouse sera in the present study using LC-MS/MS. Specifically, we identified 1856 peptides derived from 771 proteins, of which 151 peptides from 107 were significantly differentially expressed between the A30P-SNCA mutant and control groups. GO analysis found that the GP6 pathway is the most significant differential signaling pathway. As a primary platelet receptor, its clustering or aggregation is crucial in promoting platelet activation, thrombus growth, and atherosclerosis by interacting with exposed collagen on injured vessel walls [23,24]. No direct studies have proven a link between PD and GP6. However, PD is associated with increased deep vein thrombosis [25]. It is suggested that the GP6 pathway may be closely related to the occurrence and development of PD.

Peptidase-catalyzed cleavage reactions can reflect peptidase specificity, and thus different cleavage sites may represent different peptidase activities [26]. This study analyzed the cleavage sites of the most markedly differentially expressed peptide VGGDPI between the WT and A30P mice groups. The proteases Ctsk and trypsin 1 cleaved TTN protein to VGGDPI at the Ile + Pro and Lys + Val-Gly sites. It suggests that Titin protein may be the substrate of Ctsk and trypsin 1.But it has not been reported that trypsin and cathepsin K can eliminate/Cleavage the titin protein in the PD model. However, Joumaa V et al. found that titin was degraded by 20 % because of the skinning protocol and conservation in titin-intact fibers. In trypsin-treated fibers, titin degradation was 68 % [27].The force of myocytes restoring was found to be depressed after titin had been degraded with trypsin [28]. Partial titin degradation with trypsin reduced substantially active and passive stresses [29]. Although the activity of cathepsin K and trypsin 1 in the PD model as a weakness was not experimentally validated in this paper, further studies will further validate these proteins and confirm their role.

In addition, substrates of Ctsk include fluorogenic peptides, two protein components of bone matrix, collagen, and osteonectin, and are inhibited by E−64 and leupeptin [30]. It's worth noting that Ctsk can break down α-syn amyloid fibrin, as well as mutant (A53T) and C-terminal truncated variants (1–122) in LBs to produce many small peptides [21]. Long-term memory impairments and the altered state of neurons and glial cells were found in the Ctsk^{-}/$^{-}$ mice [31]. These results indicate that Ctsk is closely related to PD. In addition, research findings that trypsin could increase the protein-protein interaction between α-synuclein and human dopamine transporter (hDAT), reverse the inhibition of hDAT by α-synuclein and A30P mutants, and affect the dopamine homeostasis produced by substantia nigra neurons [32]. These studies suggest that Ctsk and trypsin 1 may be the critical peptidases in PD.

Several studies have shown that overexpression of α-syn causes dopaminergic cell loss; however, the role of α-syn in apoptosis remains not fully known. Therefore, this study aims to identify bioactive peptides potentially related to α-syn and apoptosis. we screened for up-regulated peptides with unique sequences in functional and structural domains. Six up-regulated peptides with

significant fold changes were selected as potential bioactive peptides: FHOD1, IRS1, TCAF1, GAPDH, IL-16, and TTN. FHOD1 is a member of the diaphanous-related formin family and is considered to act as an actin nucleator and actin filament elongation factor. Previous studies have shown that FHOD1 is primarily expressed in mesenchymal lineage cells and is involved in maintaining cell shape, migration, formation of cellular protrusions [33], coordination of cytoskeletal organization, and stress fiber assembly [34,35]. However, no role for this protein has been identified in PD.

Prior studies in post-mortem tissue from patients with PD have identified elevated IRS-1 phosphorylation associated with attenuated insulin signaling [36]. Additionally, elevated IRS-1 p-S312 levels in blood neuron-derived extracellular vesicles (NDEVs) have been observed in PD patients. These findings suggest that p-IRS-1 in blood NDEVs could be a novel, potentially bioactive protein that can be assessed in the blood of PD patients [37].

TCAF1 is necessary for channel function as a partner protein for the cold sensor protein TRPM8. TCAF1 and TCAF2 bind to the TRPM8 channel and promote its trafficking to the cell surface [38]. Several studies have proposed that the transient receptor potential (TRP) channels are associated with neurological function and disorders, including pain perception, epilepsy, Alzheimer's disease, traumatic brain injury, and hypoxia. TRPM8 channels are expressed in neural tissues, such as the cerebral cortex, dorsal root ganglia, and hippocampal neurons. The SH-SY5Y neuronal cell line is frequently used as a cellular model of neurodegenerative diseases including Parkinson's disease. The differentiated SH-SY5Y cells have much neuronal structure, function and exaggerated neuronal marker expression.The study on the effect of neuron differentiation in Parkinson's disease model on TRPM8 channels found that it is involved in various neurological disorders and play critical roles in differentiating SH-SY5Y cells and MPTP-induced PD models [39].

IL-16 is a multifunctional inflammatory cytokine produced by $CD8^+$ T lymphocytes, mast cells, B lymphocytes, and cancer cells. Studies have demonstrated that IL-16 genetic variants are associated with sporadic PD [40]. IL-16 activates T cells and stimulates the production of proinflammatory factors associated with PD, such as TNF-a, IL-1β, IL-6, and IL-15 [41]. IL-16 might be used as a novel therapeutic target in the future.

GAPDH is a highly conserved protein that plays a vital role in glycolytic metabolism and is involved in autophagy and apoptosis [42,43]. Exogenous GAPDH has been shown to ameliorate neuronal death. Drugs such as selegiline have been shown to protect neurons by blocking the GAPDH death cascade [44,45]. GAPDH has been shown to play an essential role in the development of PD [46].

TTN is the largest known protein, with essential structural, developmental, mechanical, and regulatory functions in the heart and skeletal muscle [47]. TTN is reportedly involved in neuronal proliferation and cell migration in hippocampal graft tissue [48], which may be related to the pathogenic mechanism of PD.

Many recent studies have focused on PD-related bioactive peptides and biomarkers. However, few bioactive peptides and biomarkers have been successfully implemented in clinical practice. Bioactive peptides also have limitations, such as sensitivity to protease degradation, high clearance, high flexibility, low oral bioavailability, low membrane permeability, and low affinity.These problems can be solved by structural modification of peptides. Clinical studies have shown that exenatide has a neuroprotective effect, but there is a lack of contributing clinical data to support the impact. Moreover, although we found that six precursor proteins may be associated with PD, the biological role of peptides corresponding to the six upregulated proteins associated with SNCA remains largely unknown and requires further investigation.

The results of our combined analysis of plasma from A30P mutant mice can provide a reference for future exploration of the etiology and pathogenesis of PD. The peptidomics approach analyzes bioactive peptides, especially those identified in our study, by screening for signaling molecules that are closely related to the development and progression of PD. This study contributes to a deeper understanding of the etiology and pathogenesis of PD. It can be used to identify bioactive peptides for PD to reduce morbidity and mortality associated with the disease.

5. Conclusions

In summary, this study provides a validated blood peptidomics profile of SNCA-A30P mice, including differential peptides, signaling pathways, key peptidases, and potentially bioactive peptides linked to SNCA and apoptosis. We believe that the presented data will contribute to a deeper understanding of the etiology and pathogenesis of PD to reduce morbidity and mortality associated with the disease, particularly the A30P-*SNCA* mutation.

Data availability statement

The authors do not have permission to share data.

Funding statement

Huai'an Municipal Science and Technology Bureau(No: HAB201843); Natural Science Research Project of Jiangsu Universities (No:19KJD360001); 2021 Jiangsu Provincial Universities Qinglan Project.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Professor Jiawei Zhou for the supply of the A30P mice. Associate professor Guang Qiong Xu (Analysis and Testing Center of South Medical University) for creating peptide maps.

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