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Identification of ciliary neurotrophic factor receptor alpha as a mediator of neurotoxicity induced by α-synuclein

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Abstract

Accumulating evidence suggests that extracellular α-synuclein (eSNCA) plays an important role in the pathogenesis of Parkinson's disease (PD) or related synucleinopathies by inducing neurotoxicity directly or indirectly via microglial or astroglial activation. However, the mechanisms by which this occurs remain to be characterized. To explore these mechanisms, we combined three biochemical techniques - Stable Isotope Labeling of Amino acid in Cell cultures (SILAC); biotin labeling of plasma membrane proteins followed by affinity purification; and analysis of unique proteins binding to SNCA peptides on membrane arrays. The SILAC proteomic analysis identified 457 proteins, of which, 245 or 172 proteins belonged to membrane or membrane associated proteins, depending on the various bioinformatics tools used for interpretation. In dopamine neuronal cells treated with eSNCA, the levels of 86 membrane proteins were increased and 35 were decreased compared with untreated cells. In peptide array analysis, 127 proteins were identified as possibly interacting with eSNCA. Of those, seven proteins were overlapped with the membrane proteins that displayed alterations in relative abundance after eSNCA treatment. One was ciliary neurotrophic factor receptor alpha (CNTFR-α), which appeared to modulate eSNCA-mediated neurotoxicity via mechanisms related to JAK1/STAT3 signaling but independent of eSNCA endocytosis.

Keywords

Alpha-synuclein; Parkinson's disease; Proteomic

Introduction

Extracellular α-synuclein (eSNCA) has recently been a central focus of investigation into the cause of Parkinson's disease (PD) [1]. It is a potential biomarker in the cerebrospinal fluid

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(CSF) and/or plasma for PD diagnosis and for monitoring PD progression [2,3]. It has also been shown to induce neurotoxicity either directly [4,5] or indirectly by activating microglia [6] and astroglia [7]. Additionally, it appears that its aggregated forms are more potent than monomeric forms in activating glia or producing neural cell death [8]. The oligomers of SNCA has been shown to be cytotoxic by many investigators [9,10]. The mutated SNCA, overexpressed SNCA, or oxidatively damaged SNCA to form soluble oligomers or eventually insoluble fibrils in Lewy bodies that are refractory to degradation, resulting in cell death [10,11]. Finally, our previous experiments have demonstrated that aggregated eSNCA enter neurons much more effectively than monomers [5].

The mechanisms by which eSNCA mediates glial activation or neurotoxicity are not understood fully. Most proposed hypotheses are based on descriptions related to endogenous SNCA, e.g. inhibiting mitochondrial function, increasing oxidative stress [12], and interacting with other key intracellular proteins involved in cell death pathways. The key interacting proteins with endogenous SNCA include transcription factors like Elk-1 [13], the NF-κ signal transduction pathway [14], and apoptotic proteins like Bcl-xl and Bax [15]. Very little is known about the interactions between eSNCA and neuronal proteins currently, and it is expected that the interaction of eSNCA with neuronal cell surface proteins could play important roles in its regulation of cell function or eSNCA-mediated neurotoxicity. Furthermore, to study the membrane proteins interacting with eSNCA is also of potential importance since they might become targets for future therapeutic intervention.

One of the major problems associated with large-scale profiling of membrane proteins is the fact that proteins typically lose their structures or original cellular compartments upon cell lysis; therefore, traditional plasma membrane-enriched fractions by gradient or ultracentrifugation are heavily contaminated with other cellular components [16]. A major advance made recently to overcome this problem has been the development of the membrane-impermeable reagent biotin, which selectively labels plasma membrane proteins of intact cells. With affinity purification, it allows relatively homogeneous preparations of plasma membrane proteins from intact cells to be obtained [17].

In the current investigation, this technique was combined with SILAC (Stable Isotope Labeling of Amino acid in Cell cultures) and direct protein-protein binding with a peptide array to explore cell plasma membrane proteins that might be responsible for mediating eSNCA-induced neurotoxicity. The investigation identified a list of candidate proteins that might interact with eSNCA. Furthermore, we validated a membrane receptor, ciliary neurotrophic factor receptor alpha (CNTFR-α), in terms of its interaction with eSNCA and associated biological effects.

Finally, the investigation revealed that the JAK1/STAT3 pathway is a major downstream mechanism by which eSNCA produces neurotoxicity in dopaminergic (DAergic) cells.

Materials and methods

Reagents

Tissue culture media and supplements were obtained from Invitrogen (Carlsbad, CA, USA). All chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. $13C_6$ L-arginine (heavy) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Arginine-depleted medium was from JRH Biosciences (Lenexa, KS, USA). Purified human wild-type recombinant SNCA (MW: 14kDa; endotoxin <1.3 U/mg of peptide) was obtained from r-Peptide (Athens, GA, USA). Both anti-annexin II and anti-cytochrome C were from BD Biosciences (San Jose, CA, USA). The polyclonal anti-CNTFR-α antibody and anti-human SNCA antibody were from Imgenex (San Diego,

CA, USA) and Invitrogen. The anti-JAK1 and STAT3 antibodies were from Cell Signaling Technologies (Danvers, MA, USA). The polyclonal anti-microtubule associated protein 2 antibody (MAP-2) was from Millipore (Temecula, CA, USA).

Cell cultures

MES23.5 cells (MES), that possess many features of DAergic neurons of human substantia nigra, including synthesizing dopamine (DA), expression of DA transporter (DAT) and NMDA receptors, and commonly used in our lab as well as by many investigators [18–20], were cultured in Dulbecco's minimum essential medium F-12 (DMEM F-12) containing 1% N-2 supplement, 2% fetal bovine serum, and 50 units/ml penicillin and streptomycin at 37 °C in a 5% CO_2 humidified incubator.

Preparation of aggregated SNCA

Production of aggregated SNCA was performed following exactly the procedure described in our previous study [21]. It should be noted that the effects of eSNCA on cell biology, e.g. microglial activation, has been controversial in literature, largely due to the heterogeneity of the recombinant SNCA preparations added to the cell cultures. To this end, the nature of the aged SNCA in the current investigation has been characterized extensively by us with multiple methods, including size exclusion chromatograph and transmission electron microscopy, revealing that most species are indeed oligomers in the size of 3–5 nm[21].

MES cells SILAC labeling

SILAC labeling of MES cells was carried out using a method described by us previously with minor modifications [21]. MES cells were cultured using arginine depleted media and cells were incubated at 37°C for at least five generations in the presence of $^{12}C_6$ L-arginine (light) or $^{13}C_6$ L-arginine (heavy) before they were exposed to vehicle or aggregated eSNCA, respectively.

Cell plasma membrane biotin labeling

MES cells grown to 80% confluence on 150-mm tissue culture dishes were first incubated in serum-free medium for 1 hr, rinsed twice with ice-cold PBS, and then incubated with 1 mg/ml EZ-Link Sulfo-NHS-LC-biotin (Pierce Endogen Inc., IL, USA) in PBS for 20 min at 4°C with gentle agitation. After removal of the supernatant, residual Sulfo-NHS-LC-biotin was quenched with 100 mM glycine in PBS, and the cells were harvested using a plastic scraper.

Immunocytochemistry

Immunostaining was performed as described previously [21]. The 4% paraformaldehyde-fixed cells were blocked, and incubated overnight at 4°C with primary antibodies followed by fluorescent-labeled secondary antibodies, i.e. cross-absorbed goat anti-mouse (Alexa Fluor 488, Invitrogen), donkey anti-goat (Alexa Fluor 568, Invitrogen) and goat anti-rabbit (Alexa Fluor 568, Invitrogen) antisera or Alexa Fluor 488 conjugated avidin. Nuclei were stained by TO-PRO-3 (Invitrogen). Images were recorded using a laser scanning confocal microscope (Bio-Rad LS2000).

Neurite outgrowth assay

MES cells were seeded onto a poly-D-lysine coated 4-well Lab-Tek II chambered coverglass (Nagle Nunc). After incubation and treatment, cells were fixed in 4% formaldehyde, followed by staining with a rabbit anti-MAP2 antibody (1:1000) and a goat anti-rabbit IgG Alexa Fluor 568 secondary antibody (Invitrogen). Images of five randomly selected fields were then captured using a laser scanning confocal microscope. The lengths

of neurites on MAP2+ cells were quantified using Neurolucida software (version 8.0, MicroBrightField, Williston, VT, USA) by an observer blind to the experimental settings.

Sucrose gradient ultracentrifugation and magnetic streptavidin affinity purification of the biotinylated protein

The labeled MES cells were scraped into the lysis buffer (0.25 M Sucrose, 10 mM Hepes, 1 mM EDTA, protease inhibitors, pH 9) and homogenized. The nuclei and large cell debris were removed by centrifugation at 3,000 ×g for 10 min. The post-nuclear supernatant was loaded on 45% Sucrose-Hepes (10 mM pH 7.5) buffer and then centrifuged at 100,000 ×g for 17 hrs. A crude membrane fraction (containing biotinylated proteins) was then collected and diluted 4-fold with distilled water, followed by centrifugation at 120,000 ×g for 2 hrs. The resultant membrane pellet was solubilized in a hypotonic buffer (10 mM Hepes pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM NaF, 1mM Na_3VO_4 and protease inhibitors) with 0.5% NP40, and the protein concentration was measured using a bicinchoninic acid assay (BCA) protein assay kit (Pierce, Rockford, IL, USA). The membrane pellet was allowed to bind with Dynabeads M-280 streptavidin magnetic beads (Invitrogen) at 4°C on a shaker overnight. After binding, the magnetic beads were separated from the solution by holding the tube near a magnet; the unbound proteins (i.e., the flow-through fraction) were removed. The beads were washed three times with ice cold 1 M KCl and then three times with 0.1 M Na_2CO_3. To achieve efficient release, the mixture of purified membrane fraction and beads was fully dissociated in the presence of 8 M urea combined with 0.1% SDS. The eluted proteins were assayed by Western blotting or digested by mass spectrometry (MS) grade trypsin following a standard in-solution digestion protocol [21]. The trypsin digested sample was then further purified on a MCX column (Waters, MA) and stored at −80 °C before MS/MS analysis.

Detection and digestion of biotinylated protein bound to SNCA peptides on cellulose membranes

Preparation of human SNCA peptide membranes was as previously described [22]. SNCA peptides on cellulose membranes were rehydrated with methanol, and then washed using TBS–T buffer. The membranes were blocked with 5% skim milk overnight at 4 °C. After incubating the peptide membranes with the biotin labeled membrane protein fraction at 37 °C for 1 hr, followed by consecutive washing steps, the peptide membranes were incubated with Avidin-HRP (1:500). Then the membranes were extensively washed and incubated with ECL-chemiluminescence according to the manufacturer's instructions (Amersham Biosciences) to reveal biotin labeling of individual peptides on the membranes. The positive peptide array spots were excised from the membranes and washed three times with HPLC water. They were then incubated with 8 M urea buffer (combined with 0.1% SDS) for 1 hr at room temperature to release the bound proteins. Finally, the proteins were digested by MS grade trypsin following a standard in-solution digestion protocol.

Immunoprecipitation and Western blotting

Tosylated Dynabeads® (1×10^8 of Dynabeads®; Dynal Biotech, Invitrogen) were covalently conjugated to corresponding antibodies according to the manufacturer's instructions. Immunoprecipitation and Western blotting were performed as described previously [21]. Normal mouse and goat IgG were purchase from Santa Cruz Biotechnology (CA, USA).

CNTFR-α siRNA transfection in MES cells

MES cells were transfected with 5 nM of mouse CNTFR-α-specific siRNA [Mm_Cntfr_1 HP siRNA (Qiagen, Valencia, CA); target sequence CAGCTGTGACATGGAGGGTAA

(Gene accession number NM_016673)] or negative control siRNA (Qiagen) using the HiPerFect transfection reagent (Qiagen) according to the manufacturer's instructions. The negative control siRNA was purchased from Qiagen and the sequence is proprietary. It is claimed that the siRNA has no homology to any known mammalian gene and it has been validated using Affymetrix GeneChip arrays and a variety of cell-based assays and shown to ensure minimal nonspecific effects on gene expression and phenotype (https://www1.qiagen.com/products/genesilencing/allstarrnaicontrols/AllStarsNegativeControls.aspx).

Protein analysis by tandem mass spectrometry (MS/MS)

The purified labeled peptides were separated by a two dimensional microcapillary high performance liquid chromatography (μLC) system, which integrates a strong cation-exchange (SCX) column (100 mm in length × 0.32 mm inner diameter; particle size: 5 μm) with two alternating reverse phase (RP) C18 columns (100 mm in length × 0.18 mm inner diameter), followed by analysis of each peptide with MS/MS using an LCQ DECA XP PLUS ion trap (ThermoElectron, San Jose, CA). Settings for the μLC-MS/MS are as previously described [23]. Briefly, two or six fractions were eluted from the SCX, for peptide array or affinity purified samples, respectively, using a binary gradient of 2–90% solvent D (1.0 M ammonium chloride and 0.1% formic acid in 5% acetonitrile) versus solvent C (0.1% formic acid in 5% acetonitrile). Each fraction was injected onto an RP C18 column automatically with the peptides being resolved using a 120 min binary gradient of 5–80% solvent B (0.1% formic acid in acetonitrile) versus solvent A (0.1% formic acid in water). A flow rate of 160 μl/min with a split ratio of 1/80 was used. Peptides were eluted directly into the electrospray ionization (ESI) ion trap MS capable of data-dependent acquisition.

The intensities of eluting peptide pairs (light vs. heavy) were measured in the scanning mass spectrometer. Nonetheless, the entire peak area of each eluting peptide was reconstructed and used in the ratio calculation. To determine the amino acid sequence, the MS operated in a data-dependent mode (a full-scan mass spectrum followed by an MS/MS spectrum), where the precursor ion was selected “on the fly” from the previous scan. An m/z ratio for an ion that had been selected for fragmentation was placed in a list and dynamically excluded for 3 min from further fragmentation. Proteins from the mixture were later identified automatically using the computer program SEQUEST™, which searched MS/MS spectra against the International Protein Index (IPI) database (IPI, rat-mouse.fasta, V3.43). Search parameters for SEQUEST™ used in this study were the following: tryptic; +6 Da for ^{13}C isotopic-labeled arginine and +16 Da for oxidized methionine as differential options, and +57 Da for carbamidomethyl cysteine as a static option; mass tolerance ± 3 Da. Potential peptides and proteins were further analyzed with two software programs, PeptideProphet™ and ProteinProphet™ to enhance confidence of protein identification based on statistical models [24]. PeptideProphet™ uses various SEQUEST™ scores and a number of other parameters to calculate a probability score for each identified peptide. The peptides are then assigned a protein identification using ProteinProphet™. ProteinProphet™ allows filtering of large-scale data sets with assessment of predictable sensitivity and false-positive identification error rates. In our study, only proteins with a high probability of accuracy (< 5% error rate as determined by ProteinProphet™) were selected.

Relative protein abundance in experimental groups compared to controls was calculated as previously described using an algorithm termed the Automated Statistical Analysis of Protein abundance (ASAP) ratio [25]. The algorithm utilized for calculation of ASAP ratios of signals recorded for the different isotopic forms of peptides of identical sequence are based on numerical and statistical methods, such as Savitzky-Golay smoothing filters, statistics for weighted samples, and Dixon's test for outliers, to evaluate protein abundance

ratios and their associated errors. Information about these software tools and the software tools themselves can be found at: www.systemsbiology.org/Default.aspx?pagename_proteomicssoftware and can be downloaded freely.

Gene Ontology (GO) classification, TMHMM and SignalP search

All protein sequences were taken from IPI rat-mouse V3.43. The identified proteins were also classified using GO analysis as previously described [26]. To further increase confidence in the assignment of membrane proteins or secreted proteins, all identified proteins were also checked to meet the criteria set by TMHMM and SignalP predictions [27].

Statistical methods

Grouped data were expressed as a mean ± S.D. Changes between groups were analyzed by one-way analysis of variance (ANOVA), depending on the groups of comparison involved, using Graph Pad Prism 4.0 (San Diego, CA, USA).

Results

Purification of plasma membrane proteins of MES cells

Currently it is unknown about the interaction between eSNCA and the neuronal plasma membrane proteins, and it is expected that the interaction of eSNCA with plasma membrane proteins could play important roles in its regulation of cell function or eSNCA-mediated neurotoxicity. For further identifying some plasma membrane proteins interacting with eSNCA, we have utilized some proteomic approaches. A definitive proteomic study of the plasma requires preparation of samples that are highly enriched in membrane proteins. This goal was achieved by affinity purification of proteins biotinylated with sulfo-NHS-LC-biotin, which involves a reaction of primary amines in the cell membrane proteins with N-hydroxysuccinimide (NHS) on the biotinylation reagent [28]. The biotinylation reagent is water-soluble and membrane impermeable. Therefore, only plasma membrane proteins of intact cells, at least in theory, should be labeled by sulfo-NHS-LC-biotin. To examine the quality of the biotin labeling procedure, labeled MES cells were visualized (Fig 1A) with Alexa Fluor 488 conjugated-avidin, demonstrating that the avidin-biotin interaction was highly enriched on the external surface of the membrane compartment. A significant enrichment of membranous proteins after affinity purification was also validated by examining various cellular markers in the whole-cell lysate, the crude membrane preparation after sequential cellular fractionation, and the final affinity-purified membrane fraction. The markers used were annexin II (plasma membrane), cytochrome C (mitochondria), and β-actin (cytosol). The results, shown in Fig 1B, indicated that the affinity purified membranous fraction was very much enriched in annexin II with little, if any, cytochrome C and β-actin detected. Sample loading was compared using silver staining (Fig 1C). Simultaneously, The Streptavidin-HRP blot on plasma membrane fraction with or without biotin labeling was performed and shown that more proteins in plasma membrane fraction of biotinylated MES cells were enriched (**Supplemental Fig 2**).

Proteomic analysis of integral plasma membrane proteins of MES cells

Having confirmed the quality of the proteins isolated from the plasma membrane by affinity purification, we further investigated the membranous proteins that might be involved in the interaction with eSNCA in MES cells. One quantitative proteomic technique, SILAC was performed. Briefly, MES cells were pre-labeled with either $^{12}C_6$ L-arginine (light) or $^{13}C_6$ L-arginine (heavy) SILAC reagents. The cells were treated with vehicle or pre-aged SNCA

(eSNCA was used at 250 nM throughout the study), i.e. predominantly oligomers, not monomeric SNCA, respectively, for 1 hr before the samples were collected, when internalization of eSNCA was seen first by Western blotting (Fig 2A), and before overt neurotoxicity occurs [29]. Monomeric SNCA was not used because of a possibility of aggregation of these monomers during culture, thereby confounding the results. It should be noted also that most eSNCA entered cells based on our previous experiments (23). After the culture media was completely washed out, plasma membrane proteins were labeled with biotin, followed by affinity purification. They were then digested with trypsin, and the resulting peptides were analyzed by μLC–MS/MS for protein identification and quantification. After the results from two independent runs were combined, a total of 457 proteins were identified (Supplemental Table 2; all identification with $p>0.55$ [translating to a false positive rate of <0.05], as determined by PeptideProphet and ProteinProphet methods, and with ≥2 unique peptides). Among the identified proteins, 86 were up regulated and 35 down regulated, respectively, in MES cells treated with and without eSNCA. The list of the proteins that were altered in relative abundance (a minimum increase or decrease by 50%) after eSNCA treatment, i.e. with potential regulatory effects of protein-protein interactions or cellular functions, is highlighted in Supplemental Table 2. In addition, the identified proteins were cataloged for their biological processes as well as cellular components based on GO analysis, and the graphic presentation of protein groups can be found in Fig 2B. For the categories of biological processes, the proteins related to transport, signal, protein modification, metabolism, cell cycle, and cell migration are involved. Those belonging to one of the top-ranked GO biological process categories or most statistically overrepresented molecular functions of the identified proteins are shown in Table 1. GO analysis on cellular components revealed that 245 (53.6%) were annotated as membrane or membrane-associated proteins among the 457 proteins identified. Of the remainder, with subcellular annotation, 2.5% were annotated as mitochondrion, 2.9% as ER/Golgi, 0.6% as lysosome/proteasome, 9.3% as cytoplasmic and 16.0% as nuclear. As the transmembrane domain sequences are indicative of membrane proteins, the identified proteins were also analyzed by the software TMHMM and SignalP for these motifs in order to provide an additional criterion for the evaluation. TMHMM software is a Hidden Markov Model (HMM)-based transmembrane protein prediction algorithm that predicts protein topology and the number of transmembrane helices. Information from TMHMM and SignalP was combined to separate proteins into the following categories: a) cell surface proteins that contained predicted non-cleavable signal peptides and no predicted transmembrane segments; b) secreted proteins that contained predicted cleavable signal peptides and no predicted transmembrane segments; c) transmembrane proteins that contained predicted transmembrane segments and extracellular loops and/or intracellular loops; d) intracellular proteins that contained neither predicted signal peptides nor predicted transmembrane regions. The two softwares predicted 172 (37.6%) proteins to be transmembrane and/or extracellular proteins, of which, 128 had at least one predicted transmembrane domain and 46 were extracellular proteins. The results are also indicated in Supplemental Table 2.

Identification of membrane proteins interacting with eSNCA by peptide arrays

From the above results, though some plasma membrane proteins were identified by SILAC combing with neuronal membranous proteins biotin labeling, it is not impractical to confirm each protein one by one. To further narrow down the list of membranous proteins that directly interact with eSNCA (rather than secondary alterations due to changes in cellular function, which, though still important in terms of biology, are not the focus of the current investigation), an SNCA peptide array was synthesized on a cellulose membrane. Dodecamer peptides covering the whole SNCA sequence from N to C terminal, with each spot shifted by 2 amino acids, were synthesized robotically on the membrane. A total of 84 peptide spots were prepared, with the initial peptide being MDVFMKGLSKAK, and the

final peptide being TTVAEKTKEQVT. The peptides that code for each spot are listed in Supplemental Table 1.

The biotinylated enriched membranous proteins were incubated with a SNCA peptide array membrane, washed extensively with TBS-T, followed by incubation with avidin-HRP. The membrane was visualized with ECL reagents (Fig 3). The criteria to select positive spots for further protein identification were the following: 1) the spots must be homogenously stained at least in >50% of total area; 2) at least two consecutive spots must be stained. The following spots met these criteria: 1-b and 1-c; 2-a and 2-b; 3-a and 3-b; 4-c and 4-d; and 12-c and 12-d. These spots were excised and the bound proteins were eluted. The elutions from consecutive spots were combined before trypsin digestion and analysis of peptides by μLC–MS/MS. After two independent runs were combined, 127 proteins were identified on the basis of ≥2 unique peptides. The complete list of identified proteins is shown in Supplemental Table 3. The proteins that overlapped with those showing changes in relative abundance (Supplemental Table 2, p=1) after eSNCA treatment are shown in Table 2.

Validation of the interaction between SNCA and candidate proteins

As a cautionary note, proteins identified by proteomics, whether via direct profiling of affinity-purified proteins from SILAC labeled cells or those bound to an SNCA peptide array, might be incorrect due to the current incomplete protein database. Although proteins without quantitative changes could still be important in interacting with SNCA, proteins with quantitative changes of their plasma membrane levels when cells were treated with eSCNA might be more biological relevant, reflecting dynamic changes on plasma membrane when cells adapt to the external signal, e.g., receptor internalization or upregulation; as the first step towards validating novel proteins, we selected proteins displaying quantitative changes for validation with an independent means (Supplemental Fig 1). Due to the impracticability of verifying all candidate proteins, the following criteria were used in selecting candidate proteins for confirmation: a) the identification of proteins was based on multiple peptides; b) there was an overlap between the biotin plasma membrane enrichment and SNCA-associated proteins identification; c) there was an antibody commercially available; d) it belonged to a top-ranked biological process category as determined by GO analysis as well as being a membrane protein as determined by GO analysis and TMHMM software; and e) it demonstrated changes in relative abundance after eSNCA treatment. With these criteria in mind, CNTFR-α was selected as the prime candidate for confirming an association with eSNCA. Of note, though our focus in on the proteins with alterations in treated cells, proteins displaying quantitative changes in control cells could also be important. As shown in Fig 4A, spatial co-localization of eSNCA with CNTFR-α was apparent in the peri-membrane region. To further confirm the association of CNTFR-α with SNCA, co-immunoprecipitation was performed with CNTFR-α or SNCA antibody, followed by immunoblotting with anti-SNCA and anti-CNTFR-α respectively. The results, shown in Fig 4B, demonstrated that SNCA and CNTFR-α were indeed present whether CNTFR-α or SNCA antibody was used as the bait initially.

Biological roles of CNTFR-α in MES cells exposed to eSNCA

It is well known that a physical association of two proteins does not necessarily mean that two proteins are interacting biologically. As the first step to define the biological roles of CNTFR-α in aggregated SNCA-mediated neurotoxicity, we asked whether knocking down CNTFR-α could have any effect on the neurite out-growth, an early sign of neurotoxicity, which is also a more sensitive assay comparing to those determined by viability assays, e.g. MTT, in MES cells. The results (Fig 5A) indicated that siRNA of CNTFR-α significantly inhibited the expression of the CNTFR-α protein. In this part of the investigation, MES cells were pre-treated with a low level of rotenone (5 nM) briefly (6 hrs) to stress the system,

followed by treatment with 250nM pre-aged eSNCA for 1 hr. The results, shown in Fig 5B, revealed that rotenone or eSNCA alone did not cause significant loss of neurites in this treatment regimen. However, the average length of the neurites was decreased significantly when cells were treated with eSNCA in addition to rotenone, an effect significantly reversed by knocking down the CNTFR-α levels. It should be emphasized that eSNCA at 250 nM, alone, does cause appreciable neurotoxicity when assessed one day after exposure [5]. The image of neurite changes in MES cells has been shown in the Supplemental Fig 3.

CNTFR-α mediated neurotoxicity via JAK1/STAT3 signaling pathway

Aggregated eSNCA could mediate neurotoxicity either by entering neurons or by triggering a signal pathway critical to cell survival. To determine the mechanisms in which CNTFR-α is involved, we first determined the amount of internalized eSNCA in MES cells with vs. without suppression of CNTFR-α levels. The results demonstrated that manipulation of CNTFR-α levels had no effect on the internalization of aggregated eSNCA (Fig 6A). On the other hand, JAK1, a critical protein known to be modulated by CNTFR-α, demonstrated a significant induction in MES cells exposed to eSNCA, which was substantially attenuated by CNTFR-α siRNA (Fig 6B). It should be stressed that the changes in the signal pathway appeared to be more sensitive than neurite out-growth as an index, because the effect could be appreciated without rotenone pre-treatment. Additionally, of note was the observation that CNTFR-α siRNA seemed to block the translocation of STAT3, a down-stream effector of JAK1, from the cytosol to the nucleus (Fig 6C).

Discussion

The purpose of this investigation was to study the mechanisms mediating eSNCA-related neurotoxicity. By combining a few relatively new proteomic strategies, we identified a subset of proteins that appeared to interact with eSNCA and whose relative abundance was altered in a DAergic cell line after exposure to eSNCA. Among those proteins, we demonstrated that a membrane receptor, CNTFR-α, not only interacted with eSNCA but also mediated eSNCA-induced neurotoxicity, at least in part, via the JAK1/STAT3 pathway.

To increase the specificity of the study, we utilized the logical sequence of metabolic labeling (SILAC), tagging of plasma proteins with sulfo-NHS-LC-biotin, followed by biochemical fractionation and avidin affinity purification, and then identification and quantification of peptides by large scale 2D LC-MS/MS analysis. In the current investigation, a total of 245 or 172 (i.e. 53.6% or 37.6% of total proteins, depending on the bioinformatics tools used) were identified as membrane proteins (Supplemental Table 2). One might ask why there are so many non-specific proteins in the list despite the utilization of SILAC labeling, cellular fractionation and avidin-biotin affinity purification. The reasons might include: 1) since MS is an extremely sensitive technique, even minute contamination from other cellular compartments could produce false positive results (in reality, it is almost impossible to absolutely avoid cross-compartment contamination. However, this possibility has been diminished greatly by SILAC labeling, where minimal cellular manipulation is involved and the focus is placed on proteins with quantitative changes induced by aggregated -synuclein); 2) proteins traditionally considered as non-membranous could be identified in the membrane. A good example is SNCA itself, because SNCA was initially thought only to exist in the synapse but has now been demonstrated to be able to associate with membranes and present in multiple cellular compartments, including the extracellular space; and 3) intracellular proteins leaking out of unhealthy cells were labeled with the biotinylating reagent.

On the other hand, it should be emphasized that the biotinylating reagent does not label membrane proteins without an extracellular domain. Additionally, the efficiency diminishes

in proteins with few reactive lysine residues, small extracellular regions, or with many post-translational modifications (PTMs), such as glycosylation. In other words, the list of proteins shown in Supplemental Table 2 is by no means complete or even comprehensive. With that said, this analysis represents the first characterization of membrane proteins of DAergic cells that represent closely human nigral DAergic neurons that degenerate in PD, progressive nuclear palsy (PSP) and multiple system atrophy (MSA).

To reduce the dataset effectively, allowing for extensive investigations of candidate proteins, only those proteins demonstrating quantitative changes induced by aggregated eSNCA were focused on initially. Furthermore, the list of proteins was further refined by comparing SILAC profiling with those directly bound to an SNCA peptide arrays which include: 1) vesicle-associated membrane protein-associated protein a (IPI00125267.4), 2) Slc3a2 CD98 heavy chain (IPI00114641.2), and 3) heat shock protein 84b (IPI00229080.7). Remarkably, almost all of these proteins are associated with protein transportation, endocytosis, etc. Our previous study has indicated that clathrin interacted with SNCA and played an important role in the endocytosis of eSNCA in microglia [21]. Nonetheless, a detailed discussion on each of these proteins is not really meaningful until confirmational studies are performed, demonstrating that they are indeed involved in the process of eSNCA mediated neurotoxicity.

Although the functions of eSNCA remain to be defined, they appear to include the activation of glia as well as the production of neurotoxicity directly. We, as well as others, have demonstrated that oligomeric eSNCA can decrease the viability of neuronal cells over 1–3 days after treatment [5], and endocytosis of eSNCA appears to be at least partially involved. The goal of this study was to identify plasma membrane proteins that directly interact with eSNCA thereby mediating neurotoxicity, whether via endocytosis or not. The list of proteins was shown in Table 2, which are potentially interacting with SNCA. For further validation of interesting proteins, the criteria used in selecting candidate proteins included: 1) the identification and quantification of each candidate are based on multiple peptides; 2) there is an antibody available commercially; and 3) either the candidate has to be an important protein in signal transduction or endocytosis process, but its relationship to SNCA has not been studied yet. With these caveats in mind, we selected CNTFR-α, a protein normally localized to the neuronal plasma membrane and anchored by a glycosylphosphatidylinositol linkage that is sensitive to proteolysis and phospholipase-c-mediated cleavage following injury [30]. Although CNTFR-α was initially identified as a specific receptor of ciliary neurotrophic factor [31], it could interact with other proteins, as seen in more recent investigations [32]. In addition, CNTFR-α could be released into the extracellular space as a soluble component [33], and has been identified in proximity to injured tissues [34,35].

The role of CNTFR-α in eSNCA-mediated neurotoxicity has never been reported. One obvious possibility is that it serves as an adapter, linking eSNCA to endocytotic machinery, including clathrin [21], for entering neurons or glia. However, the fact that knocking down CNTFR-α expression did not affect the amount of internalized eSNCA seems to suggest that CNTFR-α is not vital to eSNCA endocytosis into the neuronal cells or endocytosis related neurotoxicity. To this end, it needs to be pointed out that our previous data suggest that the internalization of eSNCA is not necessarily required for eSNCA-mediated microglial activation [36]. As to how CNTFR-α might mediate eSNCA neurotoxicity, we explored two major independent pathways known to be important in CNTFR-α-mediating cell signaling: JAK1/STAT3 and Ras/MAPK, which is also mainly transmitted via the JAK1/STAT3 pathway [31]. JAK1/STAT3 signaling (with nuclear translocation) is uniquely susceptible to pro-oxidant compounds such as H_2O_2, nitric oxide, and rotenone, all of which are important to understanding PD pathogenesis [37]. It has also been implicated in the cellular effects

provoked by Aβ (a key protein involved in Alzheimer's disease) [38]. In addition, while STAT3 was activated, the neurons were in the course of cell death [39].

The results (Fig. 6) showed that eSNCA could regulate activation of the JAK1/STAT3 pathway. More importantly, manipulation of CNTFR-α not only partially restored changes in JAK1/STAT3 increase/activation induced by eSNCA but also significantly protected MES cells from eSNCA-related neurotoxicity. It should be stressed that the protection (after knocking down CNTFR-α) to eSNCA-mediated neurotoxicity was incomplete, which should not be surprising, for the reason that eSNCA can produce neurotoxicity via multiple pathways. For instance, in our recent investigation, it is clear that the recycling of internalized eSNCA also plays an important role in eSNCA-mediated neuronal dysfunctions [29]. Additionally, SNCA has been reported to interact with other proteins, including a transcription factor Elk-1 [40], mortalin [41] and DJ-1 (directly or indirectly) [42], all of which appear to be critical to PD pathogenesis (but under these circumstances, SNCA endocytosis-based mechanisms might become more important). Finally, because the JAK1/STAT3 signaling pathway is negatively regulated by receptor internalization to endocytic vesicles with subsequent receptor degradation [43], the effect of eSNCA through CNTFR-α on neuronal cell might be transient or minimized.

Concluding remarks

The current investigation has discovered that eSNCA could directly interact with a receptor, CNTFR-α, in neuronal cells. Additionally, JAK1/STAT3, a signal pathway known to be important in the function of neuronal cells, appeared to be involved in the process of eSNCA-mediated neurotoxicity. Further elucidation of the detailed mechanisms by which eSNCA mediates other cellular signal pathways will likely provide novel insight into neurodegenerative diseases with synucleinopathies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of Abbreviations

PD	Parkinson's disease
eSNCA	extracellular α-synuclein
SNCA	α-synuclein
CNTFR-α	ciliary neurotrophic factor receptor alpha
SILAC	stable isotope labeling of amino acid in cell cultures
MAP-2	microtubule associated protein 2
SDS	sodium dodecyl sulfate
GO	gene ontology
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

DMEM F-12	Dulbecco's modified eagle medium : Nutrient Mixture F-12
EDTA	ethylenediaminetetraacetic acid
PBS	phosphate buffered saline
TBS–T	Tris buffered saline

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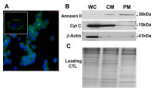

Fig. 1. Affinity enrichment of plasma membrane proteins

A) MES cells, grown in media at 37 °C, were washed with ice-cold PBS (pH 8.0) three times before incubating with sulfo-NHS-LC-biotin (0.5mg/ml in pH 8.0 PBS) for 20 min at 4°C. The unreacted sulfo-NHS-LC-biotin was quenched (at 37 °C for 10 min) by 100 mM glycine solution, followed by washing cells three times with ice-cold PBS. Biotinylated MES cells were visualized using avidin-linked fluorescent that highlights the plasma membrane (a magnified view shown as an insert at the right upper corner). Background staining could be due to leaking of biotin into the cells. Scale bar 27.5 μm. **B)** Biotinylated MES cells were fractionated with sucrose gradient ultracentrifugation before obtaining affinity-enriched membrane fraction (Dynabeads streptavidin at 4 °C for 1 hr). A total of 6 μg of whole–cell lysate (WC), crude membrane fraction (CM), or affinity-purified plasma membrane (PM) proteins were separated by 8–16% SDS-PAGE, transferred to a PVDF membrane, and blotted with an antibody against organelle-specific proteins: anti-annexin II for plasma membrane; anti-cytochrome C for mitochondrion; anti-β-actin for cytosol. **C)** Total amount of protein loaded for each sample was similar as revealed by silver staining.

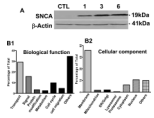

Fig. 2. Identification of plasma membrane proteins of MES cells

A) MES Cells were treated with human pre-aged eSNCA for 1, 3, or 6 hrs, washed extensively with PBS. The extent of internalization of human eSNCA was measured using Western blotting with β-actin as an internal control. Internalized eSNCA appeared as early as 1 hr after incubation. Notably, endogenous SNCA cannot be recognized with this antibody, i.e., rat-mouse SNCA level does not influence the level of internalized human eSNCA. **B)** GO analysis and annotation of total proteins identified by 2 or more peptides (proteins identified with high confidence). Proteins are classified according to their participation in specific biological functions (**B1**) and their cellular components (**B2**), respectively.

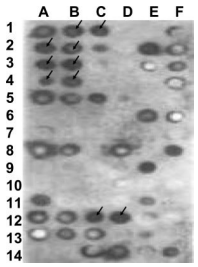

Fig. 3. Detection of biotinylated membrane proteins bound to SNCA peptide arrays

Biotinylated membrane proteins were enriched and the lysate was incubated with an SNCA peptide array membrane. After extensive washing to remove non-specific binding, the positive spots were detected using avidin-conjugated HRP, followed by ECL reagent development. Positive spots are marked with arrows.

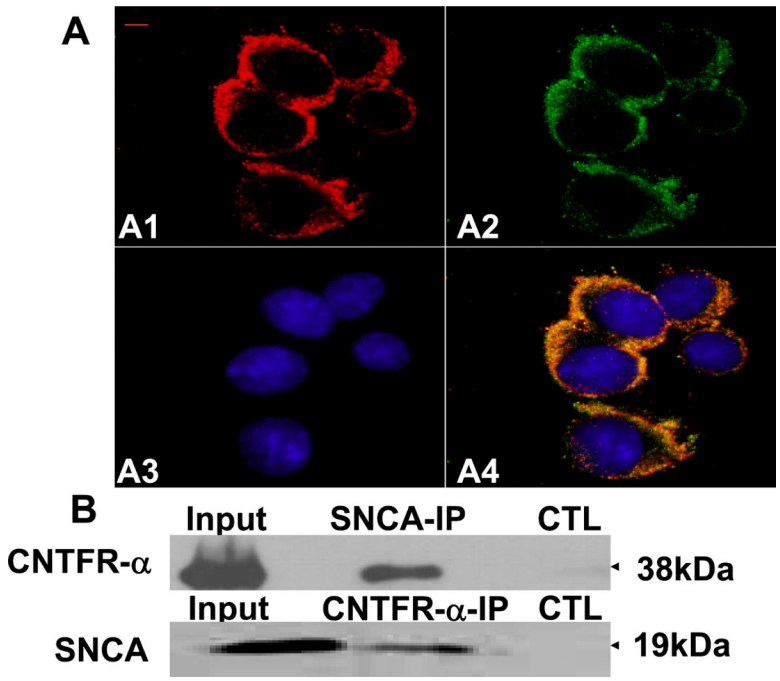

Fig. 4. Interaction between SNCA and CNTFR-α by immunostaining and immunoprecipitation

A) MES cells, seeded in a 4-well chamber at 0.05×10^6/well, were treated with eSNCA at 250 nM for 1 hr. Cells were stained with anti-CNTFR-α (red-A1) and anti-SNCA (green-A2) antibody, followed by examination with confocal microscopy. The nuclei are highlighted by DAPI (blue-A3). Note: SNCA was co-localized with CNTFR-α after 1 hr treatment (yellow color of the merged image-A4). Scale bar 5.4 μm. **B)** The protein complex of interest was isolated from MES cells homogenate after eSNCA treatment. Co-immunoprecipitation analysis using magnetic beads conjugated with either SNCA (top panel) or CNTFR-α (bottom panel) with subsequent pull-down revealed a noticeable protein-protein association between SNCA and CNTFR-α. Input represents original materials. CTL represents normal mouse or goat IgG control.

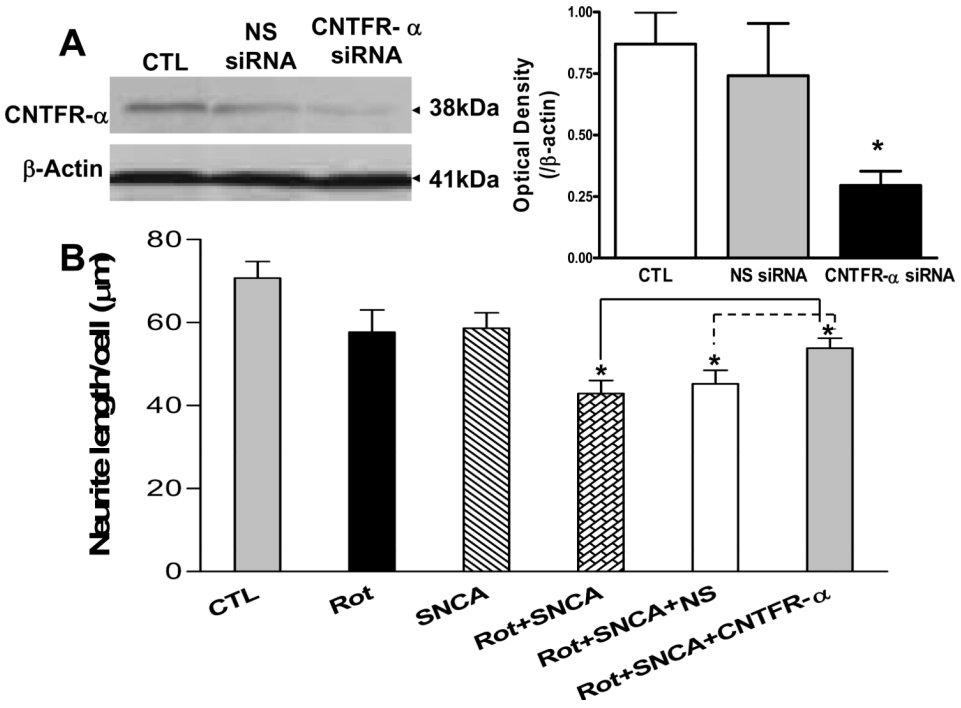

Fig. 5. Effect of knocking down CNTFR-α in MES cells on the loss of neurites

A) MES cells were transfected with CNTFR-α siRNA and expression levels of CNTFR-α were analyzed by Western blotting with an CNTFR-α antibody 72 hrs after gene manipulation, demonstrating that CNTFR-α siRNA effectively inhibited CNTFR-α expression (*$p<0.05$, compared to non-transfected control [CTL]/non-sense [NS] siRNA-transfected groups with β-actin as a loading control). **B)** Non-transfected MES cells or cells transfected with CNTFR-α siRNA or NS siRNA were seeded in 4-well chambers and incubated for 72 hrs. Cells were then treated with rotenone at 5 nM for 6 hrs, aggregated eSNCA for 1 hr, or a combination of rotenone and SNCA. After fixing and staining with an anti-MAP2 antibody, images of five randomly selected fields were then captured using a laser scanning confocal microscope and three wells were counted in each experimental condition. The length of neurites on MAP2+ cells and number of branching points of six cells in each field were quantified using Neurolucida software (version 8.0, MicroBrightField, Williston, VT, USA) by an observer blind to the experimental settings. Data are means ± SEM of at least three independent determinations (* $p<0.05$). Notably, cells transfected with NS siRNA or CNTFR siRNA and treated with rotenone or SNCA alone were also assessed, demonstrating no significant differences in terms of neurite out-growth when compared to non-transfected treated (Rot or SNCA) controls, or untreated (CNTFR siRNA-transfected or not) controls (data not shown). CTL represents non-transfected un-treated controls.

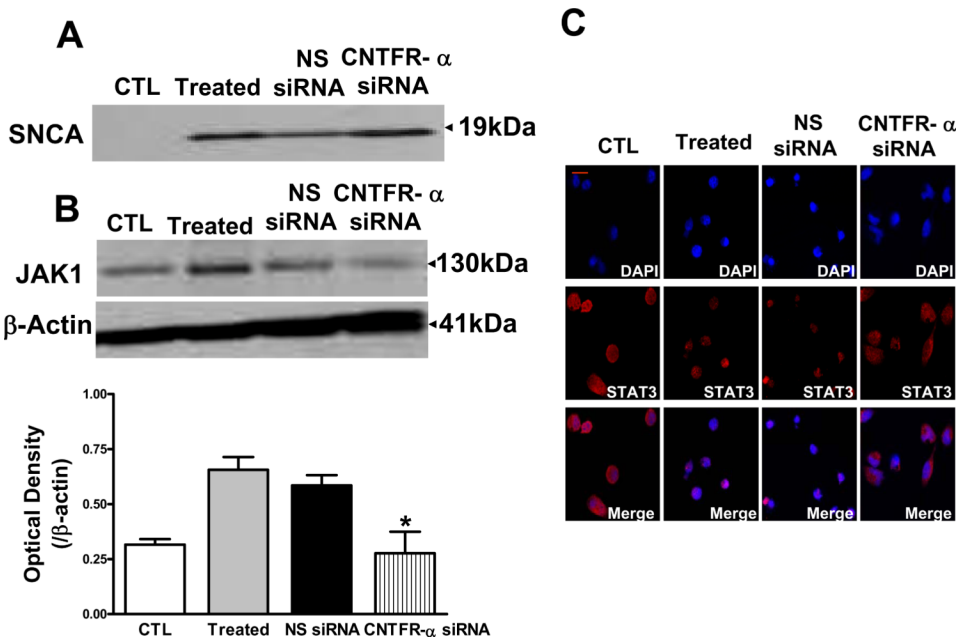

Fig. 6. JAK1/STAT3 signaling pathway and eSNCA-induced neurotoxicity via CNTFR-α

A) MES cells were transfected with CNTFR-α siRNA or non-sense [NS] siRNA before treatment with eSNCA. Untreated cells (CTL) and non-transfected treated cells were used as controls. The extent of internalization of human eSNCA in cells post-treatment 1 hr was measured by Western blotting analysis using an antibody specific to human α-synuclein. The internalization of human eSNCA was not blocked by knocking down CNTFR-α. **B)** MES cells were transfected with CNTFR-α siRNA or NS siRNA before treatment with eSNCA. The amount of JAK1 in cells was measured by Western blotting analysis. A significantly decreased JAK1 expression in cells was found after CNTFR-α knock-down (*$p<0.05$, densitometry values are represented as JAK1/β-actin ratio and means ± SD from three independent experiments, compared to treated non-transfected control [Treated] and non-sense [NS] siRNA-transfected groups; untreated NS or CNTFR siRNA-transfected groups were similar to the untreated control [CTL] group [data not shown]; β-actin was used as a loading control). **C)** The nuclear translocation of STAT3 in MES cells was visualized after eSNCA treatment with or without CNTFR-α siRNA transfection using immunostaining. While STAT3 was translocated to the nuclear compartment after eSNCA treatment, the knock-down of CNTFR-α appeared to inhibit the translocation. Scale bar, 9 μm.

Table 1

Top-Ranked Proteins (GO analysis) With Changes after eSNCA treatment

IPI No.	Common Name/ Function	GO Annotation Cellular Component	Ratio
IPI00113869.1	Isoform 2 of basigin precursor/ Plays pivotal roles in spermatogenesis, embryo implantation, neural network formation and tumor progression	Membrane	0.55±0.14
IPI00114641.2	CD98 heavy chain/Required for the function of light chain amino-acid transporters	Membrane	0.54±0.14
IPI00119618.1	Calnexin precursor/Calcium-binding protein that interacts with newly synthesized glycoproteins in the endoplasmic reticulum	Endoplasmic Reticulum	0.57±0.06
IPI00122559.3	Isoform 1 of Kinectin/Receptor for kinesin thus involved in kinesin-driven vesicle motility	Membrane	0.00±0.00
IPI00131693.2	Ciliary neurotrophic factor receptor alpha precursor/ Binds to CNTF and the alpha chain provides the receptor specificity	Membrane	0.63±0.08
IPI00129526.1	Endoplasmin precursor/Molecular chaperone that functions in the processing and transport of secreted proteins	Membrane associated	0.66±0.32
IPI00199571.1	Isoform 4 of Regulating synaptic membrane exocytosis protein 2/Rab effector involved in exocytosis	Membrane	0.00±0.00
IPI00204445.1	Tumor necrosis factor receptor superfamily member 16 precursor/Mediate cell survival as well as cell death of neural cells	Membrane	0.60±0.26
IPI00453847.2	Junctional adhesion molecule C precursor/Participate in cell-cell adhesion distinct from tight junctions	Membrane	0.56±0.40
IPI00476991.1	Neural cell adhesion molecule 1/A cell adhesion molecule involved in neuron-neuron adhesion, neurite fasciculation, outgrowth of neurites, etc	Membrane	0.49±0.44
IPI00331174.5	Cct7 T-complex protein 1 subunit eta/ Molecular chaperone; assist the folding of proteins upon ATP hydrolysis	Cytoplasm	0.60±0.15

Protein identifications are based on the IPI Rat-Mouse protein database (V3.43). Protein functions are from the UniProtKB/Swiss-Prot database. Ratio: Relative protein abundance in control groups compared to experimental groups (control/$^{12}C_6$ L-arginine-labeled *vs* eSNCA treatment/$^{13}C_6$L-arginine-labeled), calculated by an algorithm termed ASAP ratio. IPI, International protein index; GO, Gene Ontology; ER, Endoplasmic Reticulum; CNTF, Ciliary neurotrophic factor receptor alpha precursor; ATP, adenosine triphosphate.

Table 2

Proteins bound to SNCA peptide arrays and altered in relative abundance

P	Name(IPI)	Common Name	Top Rank	Membrane Protein
		Spot 2-a-b		
1	IPI00331174.5	Cct7 T-complex protein 1 subunit eta	+	-
		Spot 3-a-b		
1	IPI00114641.2	Slc3a2 CD98 heavy chain	+	+
1	IPI00125267.4	Vesicle-associated membrane protein-associated protein a	-	+
	IPI00209290.3	Vesicle-associated membrane protein-associated protein a		
1	IPI00131693.2	Ciliary neurotrophic factor receptor alpha precursor	+	+
	IPI00200440.3	Ciliary neurotrophic factor receptor alpha precursor		
	IPI00473582.1	Adult male testis cDNA, RIKEN full-length enriched library, clone:4930502A07 product:ciliary neurotrophic factor receptor, full insert sequence		
1	IPI00229080.7	Heat shock protein 84b	-	-
	IPI00471584.7	Heat shock protein HSP 90-beta		
	IPI00554929.2	Heat shock protein HSP 90-beta		
1	IPI00331361.2	Myb-binding protein 1a	-	-
1	IPI00195372.1	Elongation factor 1-alpha 1	-	-
	IPI00307837.6	Elongation factor 1-alpha 1		
	IPI00551729.1	50 kDa protein		

Protein identifications are based on the IPI Rat-Mouse protein database (V3.43). All proteins listed are with probability (p) =1 and identified by ≥ two peptides. Proteins listed in the same cell are either the same proteins with different IPI or proteins cannot be differentiated in the current database with the identified peptides.

+ Top rank protein is defined by GO analysis whereas membrane protein is defined by GO analysis and TMHMM software.

- Non-top rank or membrane protein. IPI: International protein index.