Basal Forebrain Deep Brain Stimulation Impacts the Regulation of Extracellular Vesicle Related Proteins in the Rat Brain

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Abbreviations

AD, Alzheimer disease;

ALS, amyotrophic lateral sclerosis;

BF, basal forebrain;

CID, collision induced disassociation;

CMT, Charcot-Marie-tooth;

CNS, central nervous system;

CSF, cerebrospinal fluid;

DBS, deep brain stimulation;

EV, extracellular vesicle;

FDA, federal drug & administrative

FDR, false discovery rate;

GABA, gamma-aminobutyric acid;

GPi, globus pallidus;

MCH, melanin-concentrating hormone;

MDD, major depressive disorder;

NBM, nucleus basalis magnocellularis;

NFH, neurofilament protein heavy peptide;

NFL, neurofilament protein light peptide;

NFM, neurofilament protein medium peptide;

NIFID, neuronal intermediate filament inclusion disease;

OCD, obsessive-compulsive disorder;

PD, Parkinson’s disease;

PFC, prefrontal cortex;

PTM, post-transnational modification;

SNpr, substantia nigra pars reticulata;

STN, subthalamic nucleus;

TEAB, triethylammonium bicarbonate;

TEM, transmission electromicropy;

VC, visual cortex;

Abstract

Extracellular vesicle (EV) signaling has attracted considerable attention in recent years because EVs play a key role in long distance cellular communication functions. EV studies have begun to reveal aspects of physiological and physiopathological regulation in numerous applications, although many areas remain to date largely unexplored. Deep brain stimulation (DBS) has shown remarkable therapeutic benefits of patients with neuropsychiatric disorders, but despite of the long and successful history of use, the mechanisms of action on neural ensemble activity are not yet fully understood. Here we explore how DBS of the basal forebrain impacts EV signaling in the rat brain. We employed differential centrifugations to isolate the EVs prefrontal cortex (PFC), hippocampus and striatum. We then performed quantitative analysis of EV-associated proteins using an MS-based proteomics method. We identified a considerable number of EV-associated proteins are modulated by DBS in three brain regions, some of which have been previously linked with central nervous system disorders. Particularly, neurofilament proteins NFL and NFM were both significantly changed in EVs of PFC, hippocampus and striatum after DBS stimulation compared with controls. The SOD1 protein, associated previously with neurodegenerative diseases, was significantly increased only in PFC. Our study is the first, to our knowledge, to use EV protein analysis to examine DBS effects on brain physiological regulation. Our findings open an entirely new perspective on brain area specific DBS effects.

Introduction

Extracellular vesicles (EVs), which are most prominently involved in shuttling reciprocal signals between myelinating glia and neurons, contribute to many aspects of central nervous system (CNS) development and function, including regulation of synaptic communication, synaptic strength, and synapse assembly and plasticity (1-3). EVs can selectively shuttle cytosolic and membrane proteins, as well as RNA and lipids, across cells to achieve an exchange of signaling molecules (4, 5). For example, cultured neurons from embryonic and mature mammalian neural tissue release exosome-like EVs after stimulation with depolarization or alternative means to induce excitation, such as application of Ca^{2+} ionophores, GABA receptor blockers or Glutamate receptor agonists α-amino-3-hydroxy-5-methyl-4-isoxazole- propionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA) (6, 7). EVs have also been identified as important factors in the progression of neurodegenerative diseases (2), such that EVs have been implicated in the regulation of amyloid-ß protein (8-11), tau protein (12-14), alpha-synuclein (15-18), TAR DNA-binding protein 43 (TDP-43) (19-21) and leucine-rich repeat serine/threonine kinase 2 (LRRK2) (22, 23). Some of these proteins are considered to be potential biomarkers for neurodegenerative diseases such as Alzheimer disease (AD), Parkinson disease and amyotrophic lateral sclerosis (ALS). For example, alpha-synuclein, which is packaged into EVs for extracellular transport and released into extracellular space, has been closely linked to the pathogenesis of PD (16). Additional evidence suggests that EVs play a role in the sequestration of toxic oligomers and in the release of oligomers from protein aggregates (24-26). The loss of functional aggregated proteins can interference with axonal transport and lead to proteasomal inhibition, synaptic toxicity and endoplasmic reticulum stress (27-32). While the mechanisms that contribute to neurodegenerative diseases are diverse, protein aggregates are widely considered to have a central role (2), and EVs are thus likely candidates as vehicles of propagation of neurodegenerative disease and the cell- or region-specific cargos that EVs carry give them the potential to harbor disease-specific molecular signature.

The modulation of brain activity by way of direct electrical stimulation has a long history, with early studies more than a century ago showing that motor cortex stimulation in dogs can elicit limb movements (33). In recent years, deep brain stimulation (DBS) in specific brain regions has provided therapeutic benefits for otherwise treatment-resistant disorders such as chronic pain (34, 35), Parkinson's disease (36, 37), essential tremor (38, 39), dystonia (40, 41), major depressive disorder (MDD) (42), obsessive-compulsive disorder (OCD) (43), Tourette's syndrome (44-46), addiction (47) and obesity (48). Furthermore, for a number of these disorders DBS has been approved for clinical therapy. Advantages of DBS include the direct and spatially specific modulation of brain activity as well as the controlled manner of stimulation and reversibility of effects. Despite the long history of DBS, its underlying principles and mechanisms remain incompletely understood (49-51). A large number of studies have reported the release of neurotransmitters induced by DBS in brain areas that are relevant for movement disorders. Initial studies on rats showed that the subthalamic nucleus (STN) stimulation increase extracellular glutamate levels in the STN, Globus pallidus (GPi) and substantia nigra pars reticulata (SNpr) (52). In anaesthetized 6-OHDA-lesioned rats, STN stimulation caused GABA increasing in the SNpr (53), while another study showed the STN stimulation may differentially regulate both glutamate and GABA release (54). Parkinson's disease patients showed increase of cyclic GMP in the GPi instead of increasing of extracellular glutamate (55). Based on this evidence, we hypothesized that DBS might regulate EVs containing signaling molecules in a brain region specific manner.

The basal forebrain (BF) is considered to be a major cholinergic hub of the central nervous system. It consists of several structures that send cholinergic projections to the cortex, hippocampus and other brain regions where they play an important role in various cognitive functions including learning, memory, formation and attention. The Nucleus basalis magnocellularis (NBM) is part of this structure, projecting mainly to the neocortex, amygdala, and thalamus (56, 57). Previous studies showed that the NBM has important role in the neural activity in learning and memory (58-62), such

that for example NBM lesions affect the acquisition of spatial memory and produce a severe selective disturbance in recent memories (63, 64) and electrical NBM stimulation improves cognitive functions of patients suffering from dementia (65). Effects of NBM electrical stimulation have been demonstrated also at the level of neural activation patterns in cortex, such that for example BF activation robustly enhances contrast sensitivity in visual cortex (VC), an effect that likely involves cholinergic modulation but also BF GABAergic projections to VC (66). The effects of DBS at the level of physiological signaling cascades has been much less investigated.

Here we hypothesize that BF simulation may also exert a profound influence on signaling pathways within the BF and at BF projection brain regions. We employed differential centrifugations for isolation of EVs from PFC, hippocampus and striatum brain regions after unilateral electrical stimulation in the NBM of the rat brain, with the other non-stimulated brain hemisphere serving as control. Such an internal control allows us to reduce data variability across animals and improves the accuracy of results. In addition, we employed the dimethyl label method for relative quantification on levels of proteins and peptides. We observed a large number of significantly modulated proteins in brain regions of the stimulated hemisphere compared with the control hemisphere. Using functional analysis with the DAVID algorithm, we linked these identified EV- proteins to oxidative stress, DNA damage, nervous system development and signaling processes.

EXPERIMENTAL SECTION

Materials

LC-MS grade acetonitrile and formic acid were purchased from Fisher and Fluka, respectively. Acetic acid was purchased from Fluka (Buchs, Switzerland). Pure water was prepared by GenPure system (TKA, Niederelbert, Germany). Siliconized micro centrifuge tubes were purchased from Eppendorf (Hamburg, Germany). Formaldehyde (CH_2O, 37%, v/v, cat. no. 252549), sodium cyanoborohydride ($NaBH_3CN$, cat. no. 156159) and triethylammonium bicarbonate buffer (TEAB, cat. No. T7408) were purchased from Sigma. Sodium cyanoborohydride ($NaBD_3CN$, 96%

D, cat. no. 190020) and formaldehyde ($^{13}CD_2O$, 20%, 99% ^{13}C, 98% D, cat. no. 596388) was purchased from Cambridge Isotope Laboratory, Inc (Tewksbury, UK).

DBS animals

DBS is known to have an excitatory or inhibitory effect depending on stimulation frequency stimulation, although results tend to vary as a function of stimulation parameters and brain region under study (67-69), and biphasic cathode-first stimulation tends to be more effective in eliciting functional responses (70-72). In the present study, we tested different stimulation parameter combinations and selected biphasic pulse trains with 20 Hz frequency, ±8 V amplitude, 100µs pulse duration, 500 ms pulse train duration and 10s pulse trains intervals. This stimulation protocol was applied during two consecutive days with three stimulation sessions per day lasting 1h each with a 1h pause between sessions. BF electrodes were implanted bilaterally, but only one hemisphere was stimulated in each animal with the other hemisphere serving as a control. A total of n=6 animals were used in this study.

EVs Isolation and Sample Preparation

The EVs isolation protocol was modified according to a previous report (73). In order to obtain enough analyte for the procedures, samples from two animals were pooled together as one biological replicate. Brain tissue was dissected and treated with 20 units/ml papain with Hibernate A (Gibco) for 15 min at 37 ^{o}C. It was then gently washed with another two volumes cold Hibernate A and then sequentially centrifuged 3000 g for 20 min and then filtered through a 40 µm mesh filter (BD Bioscience) and a 1 µm syringe filter (Acrodisc, Sigma). EVs isolation centrifuge steps were modified as described previously (74). Briefly, the filtrate was sequentially centrifuged at 5000g for 20 min at 4 ^{o}C to discard small debris. The supernatant was centrifuged at 20,000g for 1 h at 4 ^{o}C. The pellets were washed with cold PBS twice collecting pellets as microvesicles, and supernatants were further performed ultracentrifuge 200,000 g for 2 h at 4 ^{o}C. The pellets were washed with PBS twice collected as exosomes. The isolated EVs were incubated with 100 µL 8 M urea (pH=7.4)

containing Cocktail proteinase inhibitors for 30 min in the ice after vortex properly. 50 mM DTT and IAA were first used for disulfide bond reduction and alkylation as described, respectively. Urea concentration was diluted to below 1 M for trypsin (Roche) digestion at 37 ^{o}C for overnight. The dimethyl labeling was performed after desalting with C18 StageTip (75). The dried samples were resolved with 100 mM TEAB first and subsequently, add 5 µL of 4% (v/v) CH_2O and $^{13}CD_2O$, 5 µL of 0.6 M $NaBH_3CN$ and $NaBD_3CN$ to the samples for "light" and "heavy" dimethyl labeling, respectively. The labeling reaction was put in a working fume hood for 1 hat room temperature. Quench the labeling reaction by adding 10 µL of 1% (v/v) ammonia solution and then acidify the sample with 20 µL 0.2 % formic acid. The differential labeled samples were mixed first and desalted with C18 StageTips (75) again, which pre-washed with 200 µL 0.2 % acetonitrile and 200 µL 0.2% formic acid in water twice, respectively. Finally, 200 µL of 80% acetonitrile containing 0.2% formic acid was used for eluting peptides and then directly analysis by nanoLC-MS with three replicates. For each peptide sample, three technique replicates were performed.

Transmission Electromicroscopy (TEM)

Microvesicle and exosome fractions were enriched with above centrifugation steps and then fixed in 2% paraformaldehyde/ 2.5% glutaraldehyde in 0.1M cacodylate buffer solution for 30 minutes, washed and embedded in 2% AGAR/water. Specimens were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 minutes, washed, dehydrated and epoxy resin embedded. 70 nm ultrathin sections were contrasted with uranyl acetate and lead citrate and examined by TEM (Philips CM100).

LC-MS Data Acquisition

Samples were measured on Orbitrap Discovery (Thermo Fisher Scientific) coupled with UltiMate^{TM} 3000 RSLCnano System (Thermo Fisher Scientific). Analytical column tips with 75 µm inner diameter (New Objective) were self-packed with 1.9 µm Reprosil-Pur C18 AQ (100Å, Dr. Maisch GmbH) to a length of 20 cm. The

precolumn (µ-precolumn, C18 Pepmap, 300 µm ID, Thermo Scientific) was used for sample loading with 3 µL/min 100% A (0.2% formic acid in water) in loading pump and then switch to analytical column after 5 min. A gradient of A and B (0.2% formic acid in 80% ACN in water) with increasing organic proportion was used for peptide separation (separation ramp: from 2-30% B within 170 min). The separation flow rate was 300 nL/min and totally 190 min running for each sample. The mass spectrometry was operated in the data-dependent mode and switched automatically between MS (Orbitrap analyzer, resolution 30,000, AGC $1e^6$) and MS/MS (Ion trap analyzer, AGC $1e^4$) with maximum top ten. The fragmentation was performed in CID mode with normalized collision energy 35%. Parent ions with a charge state of z =1 and unassigned charge state were excluded for fragmentation. The mass range of MS was from 400 to 1800 m/z. The other mass spectrometry parameters were as follows: spay voltage 2.5 kV; no sheath and auxiliary gas flow; and ion-transfer tube temperature 200 ^{o}C.

Data analysis and Statistics

All the raw MS data files were processed on MaxQuant software (version 1.6.1.0) (76) for proteins and peptides identification and quantification, and trypsin was specified as digestion for Uniprot rat database (January 2017) searching with a false discovery rate (FDR)<0.01 at level of proteins, peptides, and modifications. DimethLys0 and DimethNter0 were specified as “Light Labels”; DimethLys8 and DimethNter8 were specific as “Heavy Labels”, and 3 maximum labeling for each peptide. Acetylation (protein N-term), phosphorylation (S/T/Y), and oxidation on methionine were selected for the variable PTMs with 3 maximum modifications for each peptide, and carbamidomethyl as fixed modification. The minimum peptide length is seven amino acids, and “match between runs” enabled with a matching time window of 1 min. Proteins and peptides were identified using the Andromeda search engine integrated into the MaxQuant environment (77). Bioinformatics analysis was performed on Perseus(78) and significant A with p-value (FDR 0.05) was used for proteins significantly analysis. A site localization probability of 0.75 was used as cutoff for

localization of phosphopeptides sites. The phosphorylation motifs were determined by pLogo program (79). The proteins network was analyzed with STRING(80) and visualized by Cytoscape (81). We used Perseus significance A (78) to determine differential expression of EV proteins in the PFC, hippocampus and striatum of stimulated samples compared with controls.

Results

EVs, including microvesicles and exosomes, were isolated from rat brain tissue PFC, hippocampus and striatum regions with differential high speed centrifugations and ultrahigh speed centrifugations after incubation with papain and series filtration steps (see methods, Figure 1 and Figure S1) (82). TEM was employed to confirm that excellent EV purity was achieved using this procedure (Figure 1C). After lysis of EVs, dimethyl reagents were used for peptide level stable isotope labeling (83), and mixed peptides were desalted with StageTips (75) and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on a high-speed, high-resolution mass spectrometer.

Our strategy allowed us to identify 1037, 634 and 713 EV proteins from PFC, hippocampus and striatum brain regions, which were derived from 1292, 744 and 861 genes, respectively. Gene ontology analysis of the identified EVs proteins indicated overall similar cellular components and biological functions in PFC, hippocampus and striatum (Figure 2A and 2B). In Figure 2C, we illustrate the gene overlap of identified EV proteins from PFC, hippocampus and striatum. Note that the identification ratio tended to be higher for the PFC compared to the other two regions; an effect we attribute to the relatively large tissue sample for PFC. Common EV biomarkers were identified in different brain regions (Figure 2D), such that the EV marker proteins CD9, CD81, CD82 and HSC70 were in fact present in all three brain areas (PFC, hippocampus and striatum), emphasizing the high purity of our EV isolation procedure from tissue samples.

Based on quantitative statistical analysis, 23, 20 and 24 significantly changed EVs proteins were identified in PFC, hippocampus and striatum respectively. These

proteins were derived from 39, 32 and 35 genes respectively. A complete list of all identified and significantly DBS modulated proteins are shown at Supporting Information Table S1. Approximately fifth percentage biological replicate correlation in PFC, hippocampus and striatum has been shown at Figure 3A-C. Since EVs have important communication roles in the CNS, we focused analyses on identified phosphopeptides that result from post-translational modifications of signaling molecules. Note that these analyses were possible despite the fact that no phosphopeptides enrichment was employed in this work. We identified 549, 88 and 552 phosphopeptides respectively in PFC, hippocampus and striatum. The phosphorylation site localization probability was 0.75 and pS/pT/pY site and sequence motif distributions are shown at Figures 3D and Figure S2-5. We observe that the hippocampus exhibits less phosphopeptides identification than other regions, and pY EVs are more common in PFC than hippocampus or striatum. In addition, the hippocampal pT site proportion was significantly increased compared to the other regions. The complete list of identified phosphopeptides were shown at Supporting Information Table S2. All of these disparities may bear relevance to differential brain regions in pathological processes.

To better understand the biological roles of differentially expressed EV proteins, we examined the relationship between EV proteins and specific CNS related diseases, using STRING to identify enriched gene ontology categories and signaling networks. In PFC, these significant changed proteins were classified into protein biosynthetic process, oxidative stress, signaling, cytoskeleton and nervous system development processes. For the hippocampus, DNA damage, oxidative stress/nervous system development and signaling related EVs proteins were found to be significantly modulated. The striatum had similar EV protein function classification as hippocampus, with exception of an additional role in protein biosynthetic processes. It is interesting to note that the oxidative stress plays a central role in stimulation caused EV proteins differential expression compared with controls, which is consistent with the previous studies that oxidative stress may be involved in many neurodegenerative diseases(84). Network visualizations of these effects are shown in Figure 4. Focusing

on specific proteins, we found that the two NF proteins NFL and NFM were significantly modulated in all three brain regions, but in different directions. NFL and NFM were both downregulated average -5.6 times (-2.0 ~ -14.1) and -10.8 times (-4.2 ~ -23.5) in PFC EVs in stimulated samples compared with control; whereas upregulation both average 2.2 times (1.2 ~ 3.1) and 2.1 times (1.5 ~ 2.7) was observed in hippocampus and striatum (Figure 5A), respectively. Protein NFH was also identified but levels for this protein did not modulate significantly excepting in PFC EVs (reduced average -8.4 times (-3.6 ~ -21.3)). These findings are consistent with previous studies suggesting that NF proteins may be involved in neurodegeneration (85). We proceeded to summarize NF proteins subunits that have different regulation in different brain regions in neurodegenerative diseases and others CNS related disease studies, such as Schizophrenia and MDD (86-88) as illustrated in Figure 5B and 5C. These data support the idea that the EVs proteome is a sensitive indicator for physiological regulation and that quantitative analysis of EVs proteome can be disease specific.

Besides, alpha-internexin was also found significantly downregulated average -3.9 times (-1.2 ~ -7.9) in the EVs of the PFC brain region. Alpha-internexin, is another neuronal intermediate filament protein, and three neurofilament subunits (NFL, NFM, MFH) have recently been identified as the pathological hallmarks of neuronal intermediate filament inclusion disease (NIFID) (89), a neurological disease of early onset with a variable clinical phenotype including frontotemporal dementia, pyramidal and extrapyramidal signs (90). The granin family protein secretogranin-2 (Scg2), which is the precursor for biologically active peptides such as neuropeptides, also exhibited significant differences (reduced average -4.5 times (-4.4 ~ -4.7)) in EVs of PFC between animal groups in our study. Indeed, other studies have shown that Scg2 is strongly modulated in cerebrospinal fluid (CSF) of AD patients, using a parallel reaction-monitoring mass spectrometric method (91). Scg2 was also identified and significantly changed in the CSF of multiple sclerosis patients (92). Additional neuropeptide precursor proteins ProSAAS (reduced average -5.5 times (-3.1 ~ -8.5)) and protachykinin-1 (Tac1, reduced average -3.7 times (-2.3 ~ -5.0))

also were identified significantly changed in the EVs of PFC. Previous immunoreactivity study has shown a novel function for ProSAAS protein as amyloid anti-aggregant in AD, which may play a role in AD pathology. Tac1 can be cleaved to 11 amino acids neuropeptide substance P, the first identified neuropeptide in the history, and corresponding receptor antagonist (aprepitant) has been developed approved by FDA for treatment of chemotherapy-induced emesis; it also has demonstrated clinical efficacy in the treatment of major depression (93). Furthermore, other neuropeptides precursors also identified in the EVs of brain regions such as Pro-MCH, vasopressin-neurophysin 2-copeptin (Avp), chromogranin-A, neuroendocrine protein 7B2.

Discussion

This study employs a novel analytical approach to monitor EV associated proteins, in order to investigate the DBS regulation on different rat brain regions. This method has merits on high specificity, with the potential to uncover molecules linked to brain pathology. We discovered over a dozen of significantly changed proteins in PFC, hippocampus and striatum in DBS treatment animals compared with controls, some of which had been previously reported and linked to neurodegenerative diseases.

Neurofilaments (NF) are intermediate filament in neurons composed of three subunits, neurofilament light polypeptide (NFL), neurofilament medium polypeptide (NFM), and neurofilament heavy polypeptide (NFH). NF proteins are major components of large myelinated axons. Abnormal accumulation of NF proteins is a primary finding in several human neurodegenerative diseases, such as ALS, PD, AD and Charcot-Marie-tooth (CMT) (85). Brettschneider and colleagues reported that NFH protein increased 10 folds in CSF of AD patients compared with controls, using ELISA and antibodies (94). NFL is generally considered to be a biomarker of large-caliber myelinated axon injury (95) and in CSF as a biomarker of neurocytoskeleton impairment in AD (96). One paper has systematically reviewed that CSF NFL elevated average 2.35 (1.90 ~2.91) times in AD patients compared with controls (97). It suggested that the NEF was not only strongly associated with AD but

also strongly associated with mild cognitive impairment due to AD. Besides, it also found that the NFL mRNA is selectively reduced by up to 70% in degenerating neurons of AD and ALS (98, 99). The proteomic analysis of substantia nigra (SN) showed reduced protein level of NFL and NFM in PD patients (100). NFL mRNA is also decreased and correlated with the severity of PD (101). In contrast, Abdo and colleagues (102) have reported significant increased CSF level of NFL and NFH in multiple system atrophy predominated by Parkinsonism. In our study, we found significant changed NFL and NFM in all of PFC, hippocampus and striatum in stimulated animals compared with controls, while NFH was not significantly modulated. The result shows that DBS brain stimulation can profoundly impact biomarker proteins that are sensitive indicators of neurodegenerative diseases.

Other CNS related diseases have also been linked to modulations of neurofilament proteins such as Schizophrenia (87), MDD (88) and suicide (86). We can conclude from the present study that EV associated NF proteins exhibit brain region specific regulation, and EV monitoring may thus provide a more sensitive opportunity to study the pathogenesis of related brain disorders.

We document that in all three brain regions, oxidative stress related proteins were found significantly changed in DBS stimulated animals compared to controls. High levels of oxidative stress have been implicated in many common neurodegenerative diseases. Neuron cells are particularly vulnerable to oxidative damage due to high polyunsaturated fatty acid content in membranes, high oxygen consumption, and weak antioxidant defense (84, 103). Neurodegenerative diseases are characterized by progressive damage in neural cells and neuronal loss, which lead to compromised motor or cognitive function. The overproduction of reactive oxygen species may have complex roles in promoting neurodegenerative diseases according to previous researches (104, 105). The exact molecular pathogenesis of this disturbance of redox balance remains unclear. Our findings certainly suggest that DBS brain stimulation might modulate or even reverse oxidative stress caused damage in CNS.

The protein SOD1 is thought to be an important cell-signaling molecule with neuromodulatory properties. Mutant SOD1 can contribute to the progression of

familial ALS through the dysregulation of signal transduction pathways in motor neurons and in the activity of supportive glial cells (106, 107). Studies in vitro, CSF and in transgenic mouse models show that SOD1 is secreted via the microvesilcular secretory pathway(108-110). In our study, SOD1 was significant decreased average -2.3 times (-1.6 ~ -3.0) in stimulated PFC EVs related to control, demonstrating a strong association between DBS and CNS related diseases although the precise mechanism remains unclear. DNA damage process related proteins were also found frequently in our study through the functional analysis. Particularly, mitochondria dysfunction could be linked with sustained oxidative stress in neurodegenerative disorders, which would be have strongly relevant with DNA damage process.

Conclusions

Brain stimulation profoundly impacts the regulation and transmission of signaling molecules in different brain regions. Here, we evaluate DBS effects on brain regions by MS-based EVs proteins quantitative analysis. Last, we got an amount of significant changed EVs proteins in PFC, hippocampus and striatum after stimulation on rat brain BF position. Through functional analysis, these proteins were involved in oxidative stress, nervous system development, DNA damage and protein biosynthetic processes, and some of them are strongly associated with CNS related disorders. Particularly, the NF proteins that were reported as potential biomarkers in many CNS related diseases. In our study, the NF subunits NFL and NFM were both significantly changed in EVs of PFC, hippocampus and striatum in stimulated samples compared with control. The strongly related to neurodegenerative disease SOD1 protein was also found significantly changed in PFC. All of this founding shown that the brain BF stimulation can impact the EVs proteins differential expression in different regions, which would be helping to explain the stimulation certainly potential effects on benefits of patients in the future.

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References

1. Budnik, V., Ruiz-Canada, C., and Wendler, F. (2016) Extracellular vesicles round off communication in the nervous system. *Nature reviews. Neuroscience* 17, 160-172
2. Thompson, A. G., Gray, E., Heman-Ackah, S. M., Mager, I., Talbot, K., Andaloussi, S. E., Wood, M. J., and Turner, M. R. (2016) Extracellular vesicles in neurodegenerative disease - pathogenesis to biomarkers. *Nature reviews. Neurology* 12, 346-357
3. Zappulli, V., Friis, K. P., Fitzpatrick, Z., Maguire, C. A., and Breakefield, X. O. (2016) Extracellular vesicles and intercellular communication within the nervous system. *The Journal of clinical investigation* 126, 1198-1207
4. Babst, M. (2011) MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between. *Current opinion in cell biology* 23, 452-457
5. Raposo, G., and Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *The Journal of cell biology* 200, 373-383
6. Faure, J., Lachenal, G., Court, M., Hirrlinger, J., Chatellard-Causse, C., Blot, B., Grange, J., Schoehn, G., Goldberg, Y., Boyer, V., Kirchhoff, F., Raposo, G., Garin, J., and Sadoul, R. (2006) Exosomes are released by cultured cortical neurones.

Molecular and cellular neurosciences 31, 642-648
7. Lachenal, G., Pernet-Gallay, K., Chivet, M., Hemming, F. J., Belly, A., Bodon, G., Blot, B., Haase, G., Goldberg, Y., and Sadoul, R. (2011) Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Molecular and cellular neurosciences* 46, 409-418
8. Yuyama, K., Sun, H., Sakai, S., Mitsutake, S., Okada, M., Tahara, H., Furukawa, J.-i., Fujitani, N., Shinohara, Y., and Igarashi, Y. (2014) Decreased Amyloid-β Pathologies by Intracerebral Loading of Glycosphingolipid-enriched Exosomes in Alzheimer Model Mice. *Journal of Biological Chemistry* 289, 24488-24498
9. Yuyama, K., Sun, H., Mitsutake, S., and Igarashi, Y. (2012) Sphingolipid-modulated exosome secretion promotes clearance of amyloid-beta by microglia. *The Journal of biological chemistry* 287, 10977-10989
10. An, K., Klyubin, I., Kim, Y., Jung, J. H., Mably, A. J., O'Dowd, S. T., Lynch, T., Kanmert, D., Lemere, C. A., Finan, G. M., Park, J. W., Kim, T. W., Walsh, D. M., Rowan, M. J., and Kim, J. H. (2013) Exosomes neutralize synaptic-plasticity-disrupting activity of Abeta assemblies in vivo. *Molecular brain* 6, 47
11. Dinkins, M. B., Dasgupta, S., Wang, G., Zhu, G., and Bieberich, E. (2014) Exosome reduction in vivo is associated with lower amyloid plaque load in the 5XFAD mouse model of Alzheimer's disease. *Neurobiol Aging* 35, 1792-1800
12. Saman, S., Kim, W., Raya, M., Visnick, Y., Miro, S., Saman, S., Jackson, B., McKee, A. C., Alvarez, V. E., Lee, N. C., and Hall, G. F. (2012) Exosome-associated tau is secreted in tauopathy models and is selectively phosphorylated in cerebrospinal fluid in early Alzheimer disease. *The Journal of biological chemistry* 287, 3842-3849
13. Santa-Maria, I., Varghese, M., Ksiezak-Reding, H., Dzhun, A., Wang, J., and Pasinetti, G. M. (2012) Paired helical filaments from Alzheimer disease brain induce intracellular accumulation of Tau protein in aggresomes. *The Journal of biological chemistry* 287, 20522-20533
14. Asai, H., Ikezu, S., Tsunoda, S., Medalla, M., Luebke, J., Haydar, T., Wolozin, B., Butovsky, O., Kugler, S., and Ikezu, T. (2015) Depletion of microglia and inhibition of exosome synthesis halt tau propagation. *Nature neuroscience* 18, 1584-1593
15. Lee, H. J., Patel, S., and Lee, S. J. (2005) Intravesicular localization and exocytosis of alpha-synuclein and its aggregates. *J Neurosci* 25, 6016-6024
16. Lee, H. J., Suk, J. E., Bae, E. J., and Lee, S. J. (2008) Clearance and deposition of extracellular alpha-synuclein aggregates in microglia. *Biochemical and biophysical research communications* 372, 423-428
17. Emmanouilidou, E. (2010) Cell-produced [alpha]-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. *J. Neurosci.* 30, 6838-6851
18. Alvarez-Erviti, L. (2011) Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission. *Neurobiol. Dis.* 42, 360-367
19. Nonaka, T. (2013) Prion-like properties of pathological TDP-43 aggregates from diseased brains. *Cell Rep.* 4, 124-134
20. Feneberg, E. (2014) Limited role of free TDP-43 as a diagnostic tool in

neurodegenerative diseases. *Amyotroph. Lateral Scler. Frontotemporal Degener.* 15, 351-356
21. Feiler, M. S. (2015) TDP-43 is intercellularly transmitted across axon terminals. *J. Cell Biol.* 211, 897-911
22. Fraser, K. B. (2013) LRRK2 secretion in exosomes is regulated by 14-3-3. *Hum. Mol. Genet.* 22, 4988-5000
23. Martin, I., Kim, J. W., Dawson, V. L., and Dawson, T. M. (2014) LRRK2 pathobiology in Parkinson's disease. *J. Neurochem.* 131, 554-565
24. Leitman, J., Ulrich Hartl, F., and Lederkremer, G. Z. (2013) Soluble forms of polyQ-expanded huntingtin rather than large aggregates cause endoplasmic reticulum stress. *Nature communications* 4, 2753
25. Joshi, P., Turola, E., Ruiz, A., Bergami, A., Libera, D. D., Benussi, L., Giussani, P., Magnani, G., Comi, G., Legname, G., Ghidoni, R., Furlan, R., Matteoli, M., and Verderio, C. (2014) Microglia convert aggregated amyloid-beta into neurotoxic forms through the shedding of microvesicles. *Cell death and differentiation* 21, 582-593
26. Yuyama, K., Sun, H., Usuki, S., Sakai, S., Hanamatsu, H., Mioka, T., Kimura, N., Okada, M., Tahara, H., Furukawa, J., Fujitani, N., Shinohara, Y., and Igarashi, Y. (2015) A potential function for neuronal exosomes: sequestering intracerebral amyloid-beta peptide. *FEBS Lett* 589, 84-88
27. Terwel, D., Dewachter, I., and Van Leuven, F. (2002) Axonal transport, tau protein, and neurodegeneration in Alzheimer's disease. *Neuromolecular medicine* 2, 151-165
28. McNaught, K. S., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C. W. (2003) Altered proteasomal function in sporadic Parkinson's disease. *Exp Neurol* 179, 38-46
29. Katayama, T., Imaizumi, K., Manabe, T., Hitomi, J., Kudo, T., and Tohyama, M. (2004) Induction of neuronal death by ER stress in Alzheimer's disease. *Journal of chemical neuroanatomy* 28, 67-78
30. Ilieva, H., Polymenidou, M., and Cleveland, D. W. (2009) Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *The Journal of cell biology* 187, 761-772
31. Winklhofer, K. F., and Haass, C. (2010) Mitochondrial dysfunction in Parkinson's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1802, 29-44
32. Vanden Broeck, L., Callaerts, P., and Dermaut, B. (2014) TDP-43-mediated neurodegeneration: towards a loss-of-function hypothesis? *Trends in molecular medicine* 20, 66-71
33. Kringelbach, M. L., Jenkinson, N., Owen, S. L. F., and Aziz, T. Z. (2007) Translational principles of deep brain stimulation. *Nature reviews. Neuroscience* 8, 623-635
34. Owen, S. L., Green, A. L., Stein, J. F., and Aziz, T. Z. (2006) Deep brain stimulation for the alleviation of post-stroke neuropathic pain. *Pain* 120, 202-206
35. Marchand, S., Kupers, R. C., Bushnell, M. C., and Duncan, G. H. (2003)

Analgesic and placebo effects of thalamic stimulation. *Pain* 105, 481-488
36. Bittar, R. G., Burn, S. C., Bain, P. G., Owen, S. L., Joint, C., Shlugman, D., and Aziz, T. Z. (2005) Deep brain stimulation for movement disorders and pain. *Journal of Clinical Neuroscience* 12, 457-463
37. Krack, P., Batir, A., Van Blercom, N., Chabardes, S., Fraix, V., Ardouin, C., Koudsie, A., Limousin, P. D., Benazzouz, A., LeBas, J. F., Benabid, A. L., and Pollak, P. (2003) Five-year follow-up of bilateral stimulation of the subthalamic nucleus in advanced Parkinson's disease. *The New England journal of medicine* 349, 1925-1934
38. Koller, W. C., Lyons, K. E., Wilkinson, S. B., and Pahwa, R. (1999) Efficacy of unilateral deep brain stimulation of the VIM nucleus of the thalamus for essential head tremor. *Movement disorders : official journal of the Movement Disorder Society* 14, 847-850
39. Rehncrona, S., Johnels, B., Widner, H., Tornqvist, A. L., Hariz, M., and Sydow, O. (2003) Long-term efficacy of thalamic deep brain stimulation for tremor: double-blind assessments. *Movement disorders : official journal of the Movement Disorder Society* 18, 163-170
40. Vidailhet, M., Vercueil, L., Houeto, J. L., Krystkowiak, P., Benabid, A. L., Cornu, P., Lagrange, C., Tezenas du Montcel, S., Dormont, D., Grand, S., Blond, S., Detante, O., Pillon, B., Ardouin, C., Agid, Y., Destee, A., and Pollak, P. (2005) Bilateral deep-brain stimulation of the globus pallidus in primary generalized dystonia. *The New England journal of medicine* 352, 459-467
41. Hu, W., and Stead, M. (2014) Deep brain stimulation for dystonia. *Translational Neurodegeneration* 3, 2-2
42. Delaloye, S., and Holtzheimer, P. E. (2014) Deep brain stimulation in the treatment of depression. *Dialogues in Clinical Neuroscience* 16, 83-91
43. Denys, D., and Mantione, M. (2009) Deep brain stimulation in obsessive-compulsive disorder. *Prog Brain Res* 175, 419-427
44. Kuhn, J., Lenartz, D., Mai, J. K., Huff, W., Lee, S. H., Koulousakis, A., Klosterkoetter, J., and Sturm, V. (2007) Deep brain stimulation of the nucleus accumbens and the internal capsule in therapeutically refractory Tourette-syndrome [8]. *Journal of Neurology* 254, 963-965
45. Servello, D., Sassi, M., Brambilla, A., Porta, M., Haq, I., Foote, K. D., and Okun, M. S. (2009) De novo and rescue DBS leads for refractory Tourette syndrome patients with severe comorbid OCD: A multiple case report. *Journal of Neurology* 256, 1533-1539
46. Servello, D., Porta, M., Sassi, M., Brambilla, A., and Robertson, M. M. (2008) Deep brain stimulation in 18 patients with severe Gilles de la Tourette syndrome refractory to treatment: The surgery and stimulation. *Journal of Neurology, Neurosurgery and Psychiatry* 79, 136-142
47. Kuhn, J., Möller, M., Treppmann, J. F., Bartsch, C., Lenartz, D., Gruendler, T. O. J., Maarouf, M., Brosig, A., Barnikol, U. B., Klosterkötter, J., and Sturm, V. (2014) Deep brain stimulation of the nucleus accumbens and its usefulness in severe opioid addiction. *Molecular psychiatry* 19, 145-146

48. Nangunoori, R. K., Tomycz, N. D., Oh, M. Y., and Whiting, D. M. (2016) *Deep Brain Stimulation for Obesity: From a Theoretical Framework to Practical Application*
49. Gildenberg, P. L. (2005) Evolution of Neuromodulation. *Stereotactic and Functional Neurosurgery* 83, 71-79
50. Hammond, C., Ammari, R., Bioulac, B., and Garcia, L. (2008) Latest view on the mechanism of action of deep brain stimulation. *Movement disorders : official journal of the Movement Disorder Society* 23, 2111-2121
51. Garcia, M. R., Pearlmutter, B. A., Wellstead, P. E., and Middleton, R. H. (2013) A slow axon antidromic blockade hypothesis for tremor reduction via deep brain stimulation. *PloS one* 8, e73456
52. Windels, F., Bruet, N., Poupard, A., Feuerstein, C., Bertrand, A., and Savasta, M. (2003) Influence of the frequency parameter on extracellular glutamate and γ-aminobutyric acid in substantia nigra and globus pallidus during electrical stimulation of subthalamic nucleus in rats. *Journal of neuroscience research* 72, 259-267
53. Windels, F., Carcenac, C., Poupard, A., and Savasta, M. (2005) Pallidal origin of GABA release within the substantia nigra pars reticulata during high-frequency stimulation of the subthalamic nucleus. *Journal of Neuroscience* 25, 5079-5086
54. Boulet, S., Lacombe, E., Carcenac, C., Feuerstein, C., Sgambato-Faure, V., Poupard, A., and Savasta, M. (2006) Subthalamic stimulation-induced forelimb dyskinesias are linked to an increase in glutamate levels in the substantia nigra pars reticulata. *Journal of Neuroscience* 26, 10768-10776
55. Stefani, A., Fedele, E., Galati, S., Pepicelli, O., Frasca, S., Pierantozzi, M., Peppe, A., Brusa, L., Orlacchio, A., Hainsworth, A. H., Gattoni, G., Stanzione, P., Bernardi, G., Raiteri, M., and Mazzone, P. (2005) Subthalamic stimulation activates internal pallidus: Evidence from cGMP microdialysis in PD patients. *Annals of Neurology* 57, 448-452
56. Detari, L., Rasmusson, D. D., and Semba, K. (1999) The role of basal forebrain neurons in tonic and phasic activation of the cerebral cortex. *Progress in neurobiology* 58, 249-277
57. Vale-Martinez, A., Guillazo-Blanch, G., Marti-Nicolovius, M., Nadal, R., Arevalo-Garcia, R., and Morgado-Bernal, I. (2002) Electrolytic and ibotenic acid lesions of the nucleus basalis magnocellularis interrupt long-term retention, but not acquisition of two-way active avoidance, in rats. *Experimental brain research* 142, 52-66
58. Wenk, G. L. (1997) The Nucleus Basalis Magnocellularis Cholinergic System: One Hundred Years of Progress. *Neurobiology of Learning and Memory* 67, 85-95
59. Wrenn, C. C., and Wiley, R. G. (1998) The behavioral functions of the cholinergic basalforebrain : lessons from 192 IgG-SAPORIN. *International Journal of Developmental Neuroscience* 16, 595-602
60. Lehmann, J., Nagy, J. I., Atmadja, S., and Fibiger, H. C. (1980) The nucleus basalis magnocellularis: The origin of a cholinergic projection to the neocortex of

the rat. *Neuroscience* 5, 1161-1174
61. Baxter, M. G., and Chiba, A. A. (1999) Cognitive functions of the basal forebrain. *Current opinion in neurobiology* 9, 178-183
62. Hasselmo, M. E., and Sarter, M. (2011) Modes and Models of Forebrain Cholinergic Neuromodulation of Cognition. *Neuropsychopharmacology* 36, 52-73
63. Murray, C. L., and Fibiger, H. C. (1985) Learning and memory deficits after lesions of the nucleus basalis magnocellularis: reversal by physostigmine. *Neuroscience* 14, 1025-1032
64. Bartus, R. T., Flicker, C., Dean, R. L., Pontecorvo, M., Figueiredo, J. C., and Fisher, S. K. (1985) Selective memory loss following nucleus basalis lesions: long term behavioral recovery despite persistent cholinergic deficiencies. *Pharmacol Biochem Behav* 23, 125-135
65. Freund, H. J., Kuhn, J., Lenartz, D., Mai, J. K., Schnell, T., Klosterkoetter, J., and Sturm, V. (2009) Cognitive functions in a patient with Parkinson-dementia syndrome undergoing deep brain stimulation. *Archives of neurology* 66, 781-785
66. Bhattacharyya, A., Veit, J., Kretz, R., Bondar, I., and Rainer, G. (2013) Basal forebrain activation controls contrast sensitivity in primary visual cortex. *BMC Neuroscience* 14, 55-55
67. Holtzheimer, P. E., and Mayberg, H. S. (2011) Stuck in a rut: rethinking depression and its treatment. *Trends in neurosciences* 34, 1-9
68. Kringelbach, M. L., Jenkinson, N., Owen, S. L., and Aziz, T. Z. (2007) Translational principles of deep brain stimulation. *Nature reviews. Neuroscience* 8, 623-635
69. Sankar, J., Das, R. R., Jain, A., Dewangan, S., Khilnani, P., Yadav, D., and Dubey, N. (2014) Prevalence and outcome of diastolic dysfunction in children with fluid refractory septic shock--a prospective observational study. *Pediatric critical care medicine : a journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies* 15, e370-378
70. Gong, C. S., Lai, H. Y., Huang, S. H., Lo, Y. C., Lee, N., Chen, P. Y., Tu, P. H., Yang, C. Y., Lin, J. C., and Chen, Y. Y. (2015) A programmable high-voltage compliance neural stimulator for deep brain stimulation in vivo. *Sensors (Basel, Switzerland)* 15, 12700-12719
71. Hofmann, L., Ebert, M., Tass, P., and Hauptmann, C. (2011) Modified Pulse Shapes for Effective Neural Stimulation. *Frontiers in Neuroengineering* 4
72. Macherey, O., van Wieringen, A., Carlyon, R. P., Deeks, J. M., and Wouters, J. (2006) Asymmetric pulses in cochlear implants: effects of pulse shape, polarity, and rate. *Journal of the Association for Research in Otolaryngology : JARO* 7, 253-266
73. Perez-Gonzalez, R., Gauthier, S. A., Kumar, A., and Levy, E. (2012) The exosome secretory pathway transports amyloid precursor protein carboxyl-terminal fragments from the cell into the brain extracellular space. *The Journal of biological chemistry* 287, 43108-43115
74. Chen, I. H., Xue, L., Hsu, C. C., Paez, J. S., Pan, L., Andaluz, H., Wendt, M. K., Iliuk, A. B., Zhu, J. K., and Tao, W. A. (2017) Phosphoproteins in extracellular vesicles as

candidate markers for breast cancer. *Proceedings of the National Academy of Sciences of the United States of America*
75. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature protocols* 2, 1896-1906
76. Tyanova, S., Temu, T., and Cox, J. (2016) The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nature protocols* 11, 2301-2319
77. Cox, J., Neuhauser, N., Michalski, A., Scheltema, R. A., Olsen, J. V., and Mann, M. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 10, 1794-1805
78. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat Methods* 13, 731-740
79. Schwartz, D., and Gygi, S. P. (2005) An iterative statistical approach to the identification of protein phosphorylation motifs from large-scale data sets. *Nature biotechnology* 23, 1391-1398
80. Snel, B., Lehmann, G., Bork, P., and Huynen, M. A. (2000) STRING: a web-server to retrieve and display the repeatedly occurring neighbourhood of a gene. *Nucleic acids research* 28, 3442-3444
81. Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research* 13, 2498-2504
82. Vella, L. J., Scicluna, B. J., Cheng, L., Bawden, E. G., Masters, C. L., Ang, C. S., Willamson, N., McLean, C., Barnham, K. J., and Hill, A. F. (2017) A rigorous method to enrich for exosomes from brain tissue. *Journal of extracellular vesicles* 6, 1348885
83. Boersema, P. J., Raijmakers, R., Lemeer, S., Mohammed, S., and Heck, A. J. (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nature protocols* 4, 484-494
84. Liu, Z., Zhou, T., Ziegler, A. C., Dimitrion, P., and Zuo, L. (2017) Oxidative Stress in Neurodegenerative Diseases: From Molecular Mechanisms to Clinical Applications. *Oxidative medicine and cellular longevity* 2017, 2525967
85. Liu, Q., Xie, F., Alvarado-Diaz, A., Smith, M. A., Moreira, P. I., Zhu, X., and Perry, G. (2011) Neurofilamentopathy in neurodegenerative diseases. *The open neurology journal* 5, 58-62
86. Kekesi, K. A., Juhasz, G., Simor, A., Gulyassy, P., Szego, E. M., Hunyadi-Gulyas, E., Darula, Z., Medzihradszky, K. F., Palkovits, M., Penke, B., and Czurko, A. (2012) Altered functional protein networks in the prefrontal cortex and amygdala of victims of suicide. *PloS one* 7, e50532
87. Davalieva, K., Maleva Kostovska, I., and Dwork, A. J. (2016) Proteomics Research in Schizophrenia. *Front Cell Neurosci* 10, 18
88. Ditzen, C., Tang, N., Jastorff, A. M., Teplytska, L., Yassouridis, A., Maccarrone, G.,

Uhr, M., Bronisch, T., Miller, C. A., Holsboer, F., and Turck, C. W. (2012) Cerebrospinal fluid biomarkers for major depression confirm relevance of associated pathophysiology. *Neuropsychopharmacology* 37, 1013-1025
89. Cairns, N. J., Uryu, K., Bigio, E. H., Mackenzie, I. R., Gearing, M., Duyckaerts, C., Yokoo, H., Nakazato, Y., Jaros, E., Perry, R. H., Arnold, S. E., Lee, V. M., and Trojanowski, J. Q. (2004) alpha-Internexin aggregates are abundant in neuronal intermediate filament inclusion disease (NIFID) but rare in other neurodegenerative diseases. *Acta neuropathologica* 108, 213-223
90. Cairns, N. J. (2011) Neuronal Intermediate Filament Inclusion Disease. *Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders*, pp. 404-411, Wiley-Blackwell
91. Brinkmalm, G., Sjodin, S., Simonsen, A. H., Hasselbalch, S. G., Zetterberg, H., Brinkmalm, A., and Blennow, K. (2017) A Parallel Reaction Monitoring Mass Spectrometric Method for Analysis of Potential CSF Biomarkers for Alzheimer's Disease. *Proteomics. Clinical applications*
92. Mattsson, N., Ruetschi, U., Podust, V. N., Stridsberg, M., Li, S., Andersen, O., Haghighi, S., Blennow, K., and Zetterberg, H. (2007) Cerebrospinal fluid concentrations of peptides derived from chromogranin B and secretogranin II are decreased in multiple sclerosis. *Journal of neurochemistry* 103, 1932-1939
93. Hokfelt, T., Bartfai, T., and Bloom, F. (2003) Neuropeptides: opportunities for drug discovery. *Lancet Neurol* 2, 463-472
94. Brettschneider, J., Petzold, A., Sussmuth, S. D., Ludolph, A. C., and Tumani, H. (2006) Axonal damage markers in cerebrospinal fluid are increased in ALS. *Neurology* 66, 852-856
95. Mattsson, N. (2011) CSF biomarkers in neurodegenerative diseases. *Clinical chemistry and laboratory medicine* 49, 345-352
96. Fialova, L., Bartos, A., and Svarcova, J. (2017) Neurofilaments and tau proteins in cerebrospinal fluid and serum in dementias and neuroinflammation. *Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia*
97. Olsson, B., Lautner, R., Andreasson, U., Ohrfelt, A., Portelius, E., Bjerke, M., Holtta, M., Rosen, C., Olsson, C., Strobel, G., Wu, E., Dakin, K., Petzold, M., Blennow, K., and Zetterberg, H. (2016) CSF and blood biomarkers for the diagnosis of Alzheimer's disease: a systematic review and meta-analysis. *Lancet Neurol* 15, 673-684
98. Wong, N. K., He, B. P., and Strong, M. J. (2000) Characterization of neuronal intermediate filament protein expression in cervical spinal motor neurons in sporadic amyotrophic lateral sclerosis (ALS). *Journal of neuropathology and experimental neurology* 59, 972-982
99. Menzies, F. M., Grierson, A. J., Cookson, M. R., Heath, P. R., Tomkins, J., Figlewicz, D. A., Ince, P. G., and Shaw, P. J. (2002) Selective loss of neurofilament expression in Cu/Zn superoxide dismutase (SOD1) linked amyotrophic lateral sclerosis. *Journal of neurochemistry* 82, 1118-1128
100. Basso, M., Giraudo, S., Corpillo, D., Bergamasco, B., Lopiano, L., and

Fasano, M. (2004) Proteome analysis of human substantia nigra in Parkinson's disease. *Proteomics* 4, 3943-3952
101. Hill, W. D., Arai, M., Cohen, J. A., and Trojanowski, J. Q. (1993) Neurofilament mRNA is reduced in Parkinson's disease substantia nigra pars compacta neurons. *The Journal of comparative neurology* 329, 328-336
102. Abdo, W. F., Bloem, B. R., Van Geel, W. J., Esselink, R. A., and Verbeek, M. M. (2007) CSF neurofilament light chain and tau differentiate multiple system atrophy from Parkinson's disease. *Neurobiol Aging* 28, 742-747
103. Rego, A. C., and Oliveira, C. R. (2003) Mitochondrial Dysfunction and Reactive Oxygen Species in Excitotoxicity and Apoptosis: Implications for the Pathogenesis of Neurodegenerative Diseases. *Neurochemical Research* 28, 1563-1574
104. Albers, D. S., and Beal, M. F. (2000) Mitochondrial dysfunction and oxidative stress in aging and neurodegenerative disease. *Journal of neural transmission. Supplementum* 59, 133-154
105. Dias, V., Junn, E., and Mouradian, M. M. (2013) The role of oxidative stress in Parkinson's disease. *Journal of Parkinson's disease* 3, 461-491
106. Lee, J., Hyeon, S. J., Im, H., Ryu, H., Kim, Y., and Ryu, H. (2016) Astrocytes and Microglia as Non-cell Autonomous Players in the Pathogenesis of ALS. *Experimental neurobiology* 25, 233-240
107. Li, Q., Spencer, N. Y., Pantazis, N. J., and Engelhardt, J. F. (2011) Alsin and SOD1(G93A) proteins regulate endosomal reactive oxygen species production by glial cells and proinflammatory pathways responsible for neurotoxicity. *The Journal of biological chemistry* 286, 40151-40162
108. Mondola, P., Ruggiero, G., Seru, R., Damiano, S., Grimaldi, S., Garbi, C., Monda, M., Greco, D., and Santillo, M. (2003) The Cu,Zn superoxide dismutase in neuroblastoma SK-N-BE cells is exported by a microvesicles dependent pathway. *Brain research. Molecular brain research* 110, 45-51
109. Mondola, P., Damiano, S., Sasso, A., and Santillo, M. (2016) The Cu, Zn Superoxide Dismutase: Not Only a Dismutase Enzyme. *Frontiers in physiology* 7, 594
110. Basso, M., and Bonetto, V. (2016) Extracellular Vesicles and a Novel Form of Communication in the Brain. *Frontiers in neuroscience* 10, 127

Legends

Figure 1 The scheme of stimulation procedure in rat. A, the deep brain stimulation related parameters; B, the workflow of animal experiment; C, the transmission electromicroscopy picture for EVs after isolation, left, exosome, right, microvesicles.

Figure 2 The EV proteins identification from PFC, hippocampus and striatum. A, the cellular component distribution of EV proteins identified from three brain regions; B, the biological function distribution of EV proteins identified from three brain regions; C, the genes overlap of identified EV protein from the three brain regions; D the comparison of EV marker proteins identified from different brain regions.

Figure 3 The biological Pearson's correlation of EV proteins for PFC (A), hippocampus (B) and striatum (C) brain regions. D, distribution of S/T/Y phosphopeptides from EVs in three brain regions.

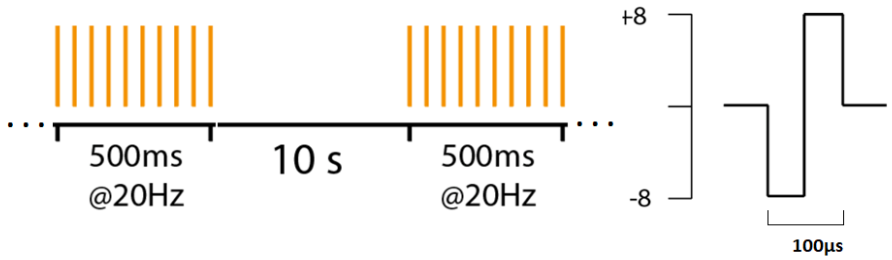
Figure 4 The network analysis for significant changed EV proteins from PFC (A), hippocampus (B) and striatum (C).

Figure 5 The neurofilament (NF) proteins regulation direction comparison with literatures. A, the different alterations of NF proteins in PFC, hippocampus and striatum with the brain stimulation; B, the NFL, NFM, NFH proteins different regulation in neurodegenerative diseases according to literatures. C, the NFL, NFM, NFH proteins different regulation in Schizophrenia, MDD and suicide. NFL, neurofilament light polypeptide; NFM, neurofilament medium polypeptide; NFH,

neurofilament heavy polypeptide; PFC, prefrontal cortex; DPFC, dorsal prefrontal cortex; CC, corpus callosum; TL, temporal lobe; Hip, hippocampus; MT, mediodorsal thalamus; CSF, cerebrospinal fluid; SN, substantia nigra; LVN, lateral vestibular nucleus; MDD, major depressive disorder.

Figure 1

A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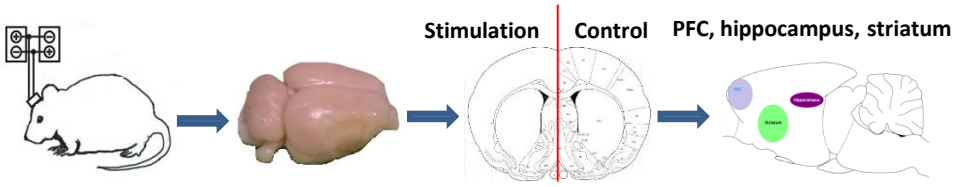
B


Stimulation
Control
PFC, hippocampus, striatum

C

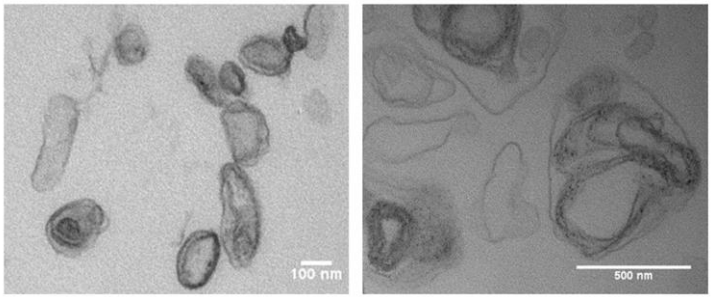

100 nm
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Figure 2

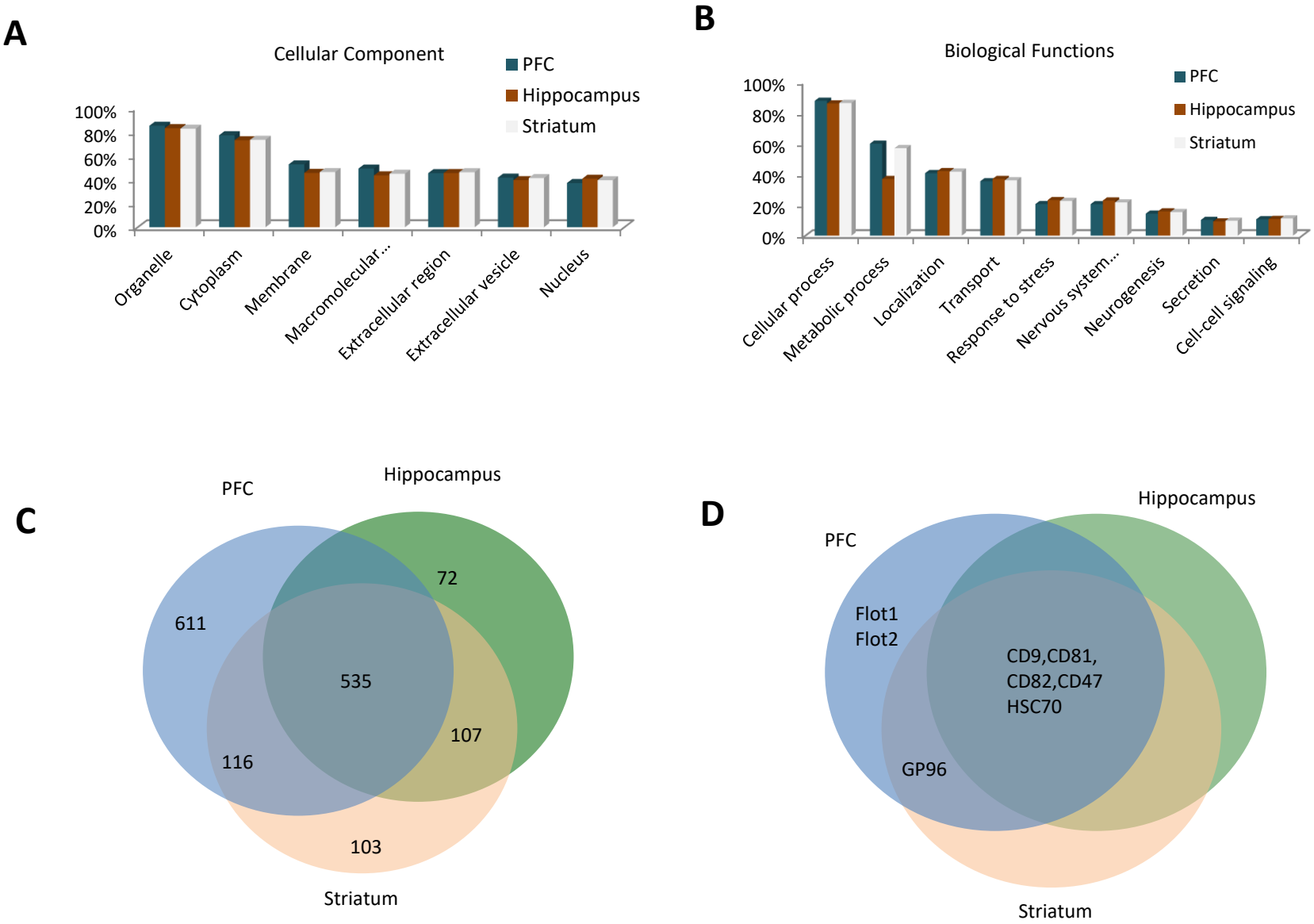
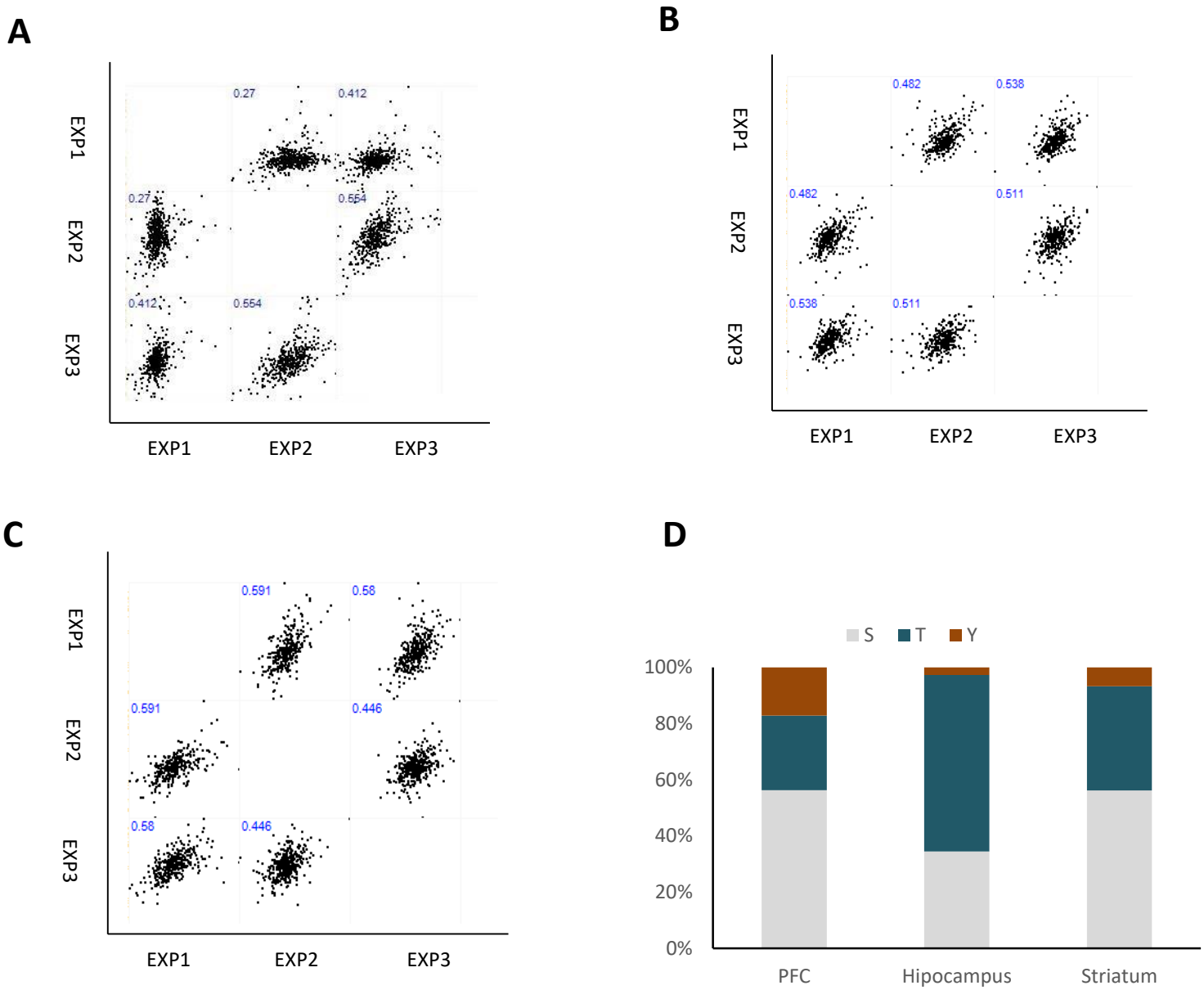

A
Cellular Component
PFC
Hippocampus
Striatum
100%
80%
60%
40%
20%
0%
Organelle
Cytoplasm
Membrane
Macromolecular...
Extracellular region
Extracellular vesicle
Nucleus
B
Biological Functions
PFC
Hippocampus
Striatum
100%
80%
60%
40%
20%
0%
Cellular process
Metabolic process
Localization
Transport
Response to stress
Nervous system...
Neurogenesis
Secretion
Cell-cell signaling
C
PFC
Hippocampus
Striatum
611
72
535
107
116
103
D
PFC
Hippocampus
Striatum
Flot1
Flot2
CD9,CD81,
CD82,CD47
HSC70
GP96

Figure 3


A
0.27
0.412
0.27
0.554
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0.554
EXP1
EXP2
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B
0.482
0.538
0.482
0.511
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C
0.591
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100%
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PFC
Hipocampus
Striatum

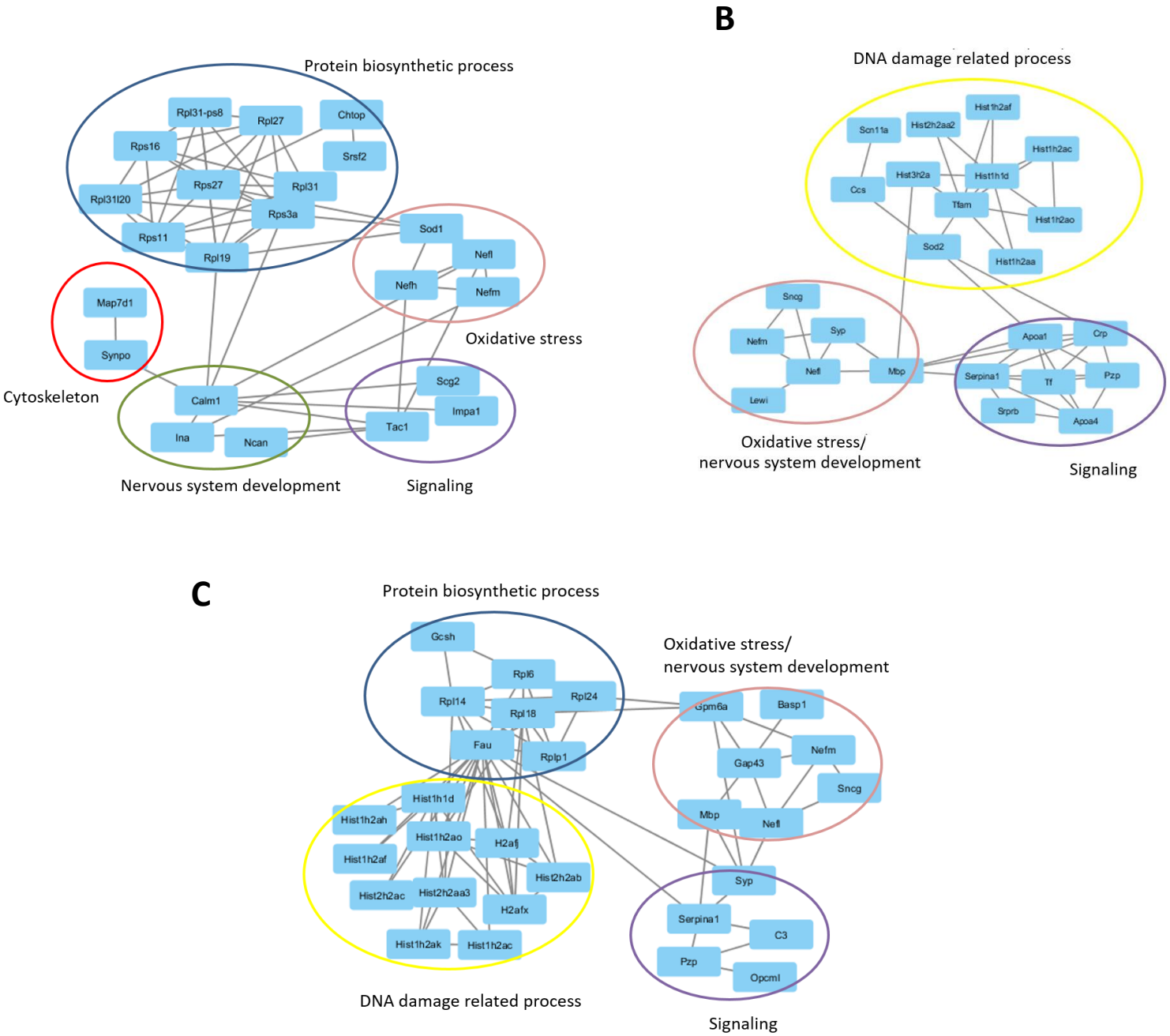
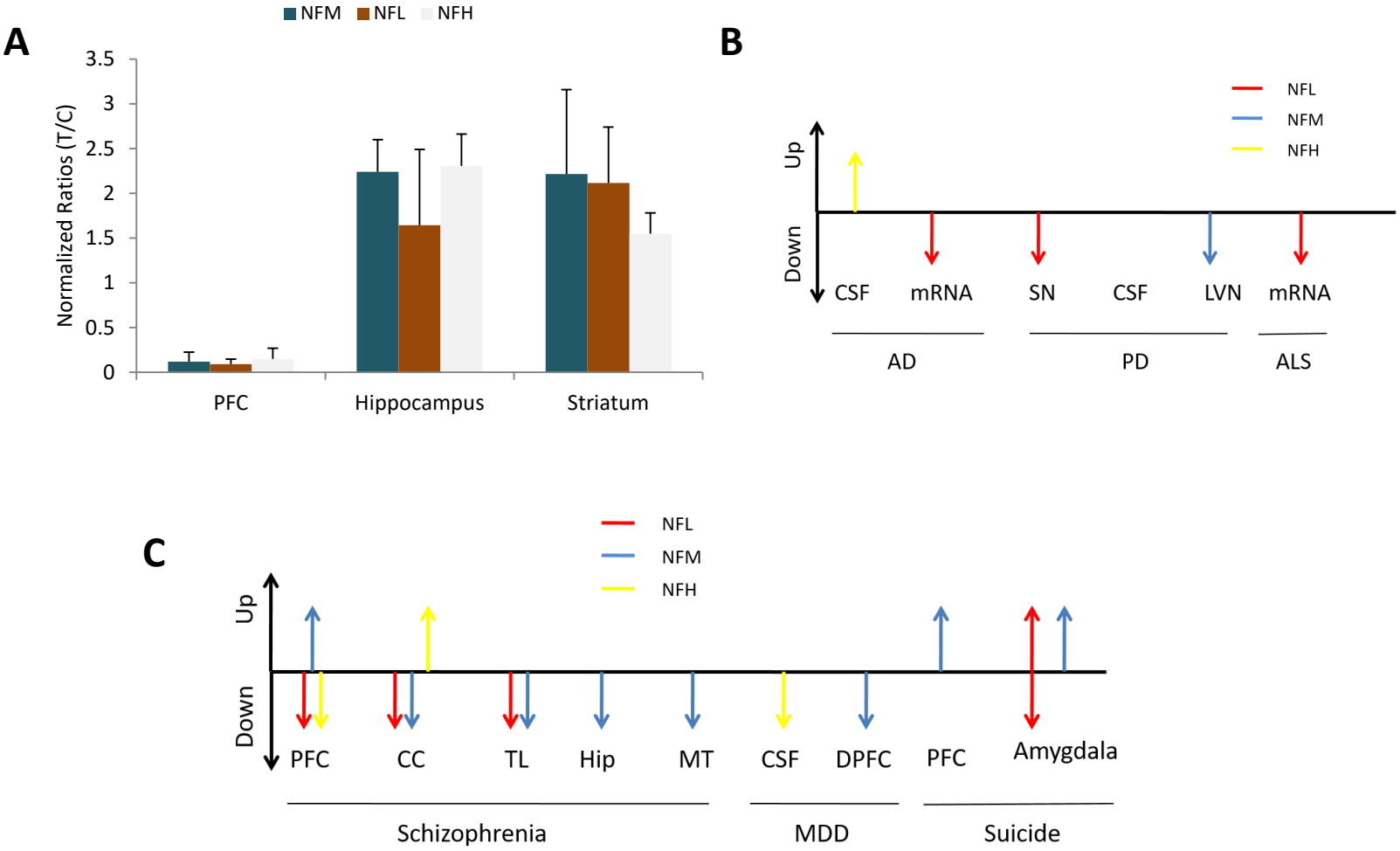

A
Protein biosynthetic process
Rpl31-ps8
Rpl27
Chtop
Rps16
Srsf2
Rps27
Rpl31
Rpl31l20
Rps3a
Rps11
Rpl19
Sod1
Nefl
Nefh
Nefm
Oxidative stress
Map7d1
Synpo
Cytoskeleton
Calm1
Scg2
Impa1
Tac1
Ina
Ncan
Nervous system development
Signaling
B
DNA damage related process
Scn11a
Hist2h2aa2
Hist1h2af
Hist1h2ac
Ccs
Hist3h2a
Hist1h1d
Tfam
Hist1h2ao
Sod2
Hist1h2aa
Sncg
Nefm
Syp
Nefl
Mbp
Lewi
Apoa1
Crp
Serpina1
Tf
Pzp
Srprb
Apoa4
Oxidative stress/
nervous system development
Signaling
C
Protein biosynthetic process
Gcsh
Rpl6
Rpl14
Rpl24
Rpl18
Fau
Rplp1
Oxidative stress/
nervous system development
Gpm6a
Basp1
Gap43
Nefm
Sncg
Mbp
Nefl
Hist1h1d
Hist1h2ah
Hist1h2ao
H2afj
Hist1h2af
Hist2h2ab
Hist2h2ac
Hist2h2aa3
H2afx
Hist1h2ak
Hist1h2ac
Syp
Serpina1
C3
Pzp
Opcml
DNA damage related process
Signaling

Figure 4

Figure 5


A
NFM
NFL
NFH
Normalized Ratios (T/C)
3.5
3
2.5
2
1.5
1
0.5
0
PFC
Hippocampus
Striatum
B
NFL
NFM
NFH
Up
Down
CSF
mRNA
SN
CSF
LVN
mRNA
AD
PD
ALS
C
NFL
NFM
NFH
Up
Down
PFC
CC
TL
Hip
MT
CSF
DPFC
PFC
Amygdala
Schizophrenia
MDD
Suicide