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DEVELOPMENT AND APPLICATIONS OF QUANTITATIVE MASS SPECTROMETRY TOOLS FOR THE INVESTIGATION OF THE SYNAPSE PROTEOME

Dissertation
for obtaining a doctorate degree
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Submitted by
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Declaration

This thesis is comprised of two published results that are compiled in a cumulative dissertation format. The author of this dissertation is the first author on both published articles. Additional publications that are appended at the end are theoretically or technically related field of research but are not included as the main part of the doctoral project. The work performed by the author was performed at the laboratory of Neuroproteomics Laboratory headed by Prof. Gert Lubec at Department of Pharmaceutical Chemistry, University of Vienna, Vienna, Austria and at the laboratory for Mass Spectrometry and Proteomics, headed by Dr. Keiryn L. Bennett, CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences in Vienna, Austria.

My contributions to the publications are described below:

Manuscript #1 was published under the title “Mass spectrometric analysis of synaptosomal membrane preparations for the determination of brain receptors, transporters and channels” in Proteomics, Reprinted with permission. co-authored by Fernando J. Sialana, Peter Gulyassy, Peter Majek, Evelina Sjostedt, Viktor Kis, Andre C. Muller, Elena L. Rudashevskaya, Jan Mulder, Keiryn L. Bennett and Gert Lubec. Proteomics, 2016, 16, 2911-2920,

FJS performed experiments, interpreted the data, prepared the figures and wrote the manuscript; PG performed the pilot synaptic preparations; PM provided technical supervision on the bioinformatics analyses; VK performed the electron microscopy experiments. AM provided technical supervision on LCMS analyses; ER provided preliminary methods for proteomic analyses; ES and JM provided preliminary immunohistochemistry data, KB analyzed the data, supervised the project and wrote the paper; GL analyzed the data, supervised the project and wrote the paper.

Manuscript #2 was published under the title “Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human DISC1 Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum” in Frontier of Molecular Neuroscience, Reprinted with permission. Co-authored by Fernando J. Sialana, An-Li Wang, Benedetta Fazari, Martina Kristofova, Roman Smidak, Svenja V. Trossbach, Carsten Korth, Joseph P. Huston, Maria A. de Souza Silva and Gert Lubec. Front. Mol. Neurosci. 11:26. doi: 10.3389/fnmol.2018.00026.

FJS performed experiments, interpreted the data, prepared the figures and wrote the manuscript, SVT designed and generated tgDISC1 rat animal model, A-LW and BF performed behavioural and brain dissections, RS provided assisted on the bioinformatics analyses, MK assisted on immunoblot-based validation, CK, JPH, MASS and GL contributed to the manuscript writing and provided a critical and detailed revision thereof.

In addition, publications arising not are listed in the “Appendix” (presented only by abstracts due to document size limitations)

Abstract

This cumulative doctoral thesis describes the implementation of a synaptic proteomics method via multiplexed quantitative analyses by tandem mass spectrometry and its application in studying animal models for disease and cognition.

The main aim of this thesis is to develop technological methods in mass spectrometry-based proteomics for the analyses of synaptic proteins. Paper 1 focuses on the identification of membrane proteins (i.e. receptors, transporters and channels) enriched from a detergent soluble and a detergent-resistant synaptosomal fraction of rat cortices. The initial step was to implement a tailored membrane-protein protocol to overcome the under-representation of this class of proteins in mass spectrometry-based proteomics. The approach generated a compendium of synaptic proteins, including receptors, transporters and channels that were underrepresented in databases. Furthermore, proteomics and bioinformatics analysis of the previously understudied detergent-soluble synaptosomal fractions showed enrichment of presynaptic proteins and proteins associated with diseases and disorders in the CNS.

The second study utilised the quantitative methods developed in the first paper to study the dynamics of synaptic proteome in animal disease models. Paper 2 explores the synaptic proteomic signatures of the tgDISC1 rat model (transgenic disrupted in schizophrenia 1 rat model) in dorsal striatum. This enables identification of novel proteins networks and signalling pathways upon overexpression of non-mutant human DISC1. The increase in DISC1 expression leads to changes in proteins and synaptic-associated processes associated with membrane trafficking, ion transport, synaptic organisation and neurodevelopment. These proteins are also associated with synaptic pathways, particularly axonal guidance and dopamine signalling

Keywords: synaptosome, DISC1, striatum, neuroproteomics, dopamine transporter

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List of Papers

This thesis is based on the following papers, which are referred to in the text by numbers.

1. **SIALANA, F. J.,** GULYASSY, P., MAJEK, P., SJOSTEDT, E., KIS, V., MULLER, A. C., RUDASHEVSKAYA, E. L., MULDER, J., BENNETT, K. L. & LUBEC, G. 2016. Mass spectrometric analysis of synaptosomal membrane preparations for the determination of brain receptors, transporters and channels. Proteomics, 16, 2911-2920.

2. **SIALANA, F. J.,** WANG, A.-L., FAZARI, B., KRISTOFOVA, M., SMIDAK, R., TROSSBACH, S. V., KORTH, C., HUSTON, J. P., DE SOUZA SILVA, M. A. & LUBEC, G. 2018. Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human DISC1 Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum. Frontiers in Molecular Neuroscience, 11.

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First Author Publications not Included in the Thesis

3. HAQ, N., * SCHMIDT-HIEBER, C., * **SIALANA, F. J. ***, CIANI, L., HELLER, J. P., STEWART, M., BENTLEY, L., WELLS, S., RODENBURG, R. J., NOLAN, P. M., FORSYTHE, E., WU, M. C., LUBEC, G., SALINAS, P., HAUSSER, M., BEALES, P. L. & CHRISTOU-SAVINA, S. 2019. Loss of Bardet-Biedl syndrome proteins causes synaptic aberrations in principal neurons. PLoS Biol, 17, e3000414. **equally shared first authorship*

4. DABA FEYISSA, D.,* **SIALANA, F. J.,*** KEIMPEMA, E.,* KALABA, P., PAUNKOV, A., ENGIDAWORK, E., HOGER, H., LUBEC, G. & KORZ, V. 2019. Dopamine type 1- and 2-like signaling in the modulation of spatial reference learning and memory. Behav Brain Res, 362, 173-180. *equally shared first authorship

5. **SIALANA, F. J**., RIBEIRODA SILVA MENEGASSO, A., SMIDAK, R., HUSSEIN, A. M., ZAVADIL, M., RATTEI, T., LUBEC, G., SERGIO PALMA, M. & LUBEC, J. 2019. Proteome Changes Paralleling the Olfactory Conditioning in the Forager Honey Bee and Provision of a Brain Proteomics Dataset. Proteomics, 19, e1900094.

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Other Publications not Included in the Thesis

8. DOS SANTOS-PINTO, J. R. A., ESTEVES, F. G., **SIALANA, F. J.,** FERRO, M., SMIDAK, R., RARES, L. C., NUSSBAUMER, T., RATTEI, T., BILBAN, M., BACCI JUNIOR, M., PALMA, M. S. & LUBEC, G. 2019. A proteotranscriptomic study of silk-producing glands from the orb-weaving spiders. Mol Omics, 15, 256-270.

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Table of contents

1 Summary Presentation of the Work

1.1 Introduction

1.1.1 Overview of Neuroproteomics

Neuroproteomics is a field of proteomics that studies the set of proteins in the nervous system in a specific cellular component or related to a specific condition such a disease or drug treatment. The characterisation of the spatial organisation of proteins and their interaction into functional networks in the nervous system, more importantly, the synapse is the aim of functional neuroproteomics (Bayes and Grant, 2009).

In designing a neuroproteomic analysis, the primary consideration is to focus on an individual brain region or subregion of biological interest. These sub-regions consist of several cell types, including different types of neurons, glia, and astrocytes that exhibit a diverse response to stimuli. The inherent complexity of the central nervous system (CNS) such as distinct brain regions, cell types and neuronal interconnection networks creates a high level of heterogeneity as each system can be described into different sets of proteomes (Sharma et al., 2015). Current proteomics technologies are not able to distinctly assess cell-type variation of the nervous system as compared to gene expression technologies (e.g. single-cell transcriptomics) (Cembrowski and Menon, 2018). The absence of amplification methods and their elaborate chemical nature makes the protein analysis of single-cell proteomes challenging (Budnik et al., 2018). Nevertheless, the complex and dynamic process of protein homeostasis provides essential information that complements genomic data. This qualitative and quantitative proteomic information, including expression levels (at subcellular levels), post-translational modifications and protein-protein interactions are determined in a non-aprioristic manner.

Proteomics is the large scale study of proteins. The proteome is the global set of proteins generated or modified by an organism or a system (Wasinger et al., 1995). The application includes the identification of proteins and their post-translational modifications, assembly into protein complexes and their sub-units, functional interactions and global protein measurements (Steen and Mann, 2004).

Quantitative protein mass spectrometry is an analytical chemistry technique for the separation, identification and quantitation of proteins in a sample.

Mass spectrometry is a vital method as it allows accurate determination of mass for the characterisation of peptides and

The chemical synapses or synaptic junctions represent the molecular substrate for synaptic plasticity. They consist of the pre-synapse that regulates the neurotransmitter release and the postsynaptic density (PSD) machinery for the detection, integration, and transduction of the transmitter signal. These neurotransmitters can diffuse, degrade or be removed by transporters. Receptors in the presynaptic terminal also detect these neurotransmitters and act as feedback to modulate synaptic transmission (Usiello et al., 2000). The well-designed assembly of these proteins in the synapse primary executes the synaptic function. By altering the composition, protein-protein interaction or the post-translational modification of the synaptic proteins, the synaptic activity can be modulated.

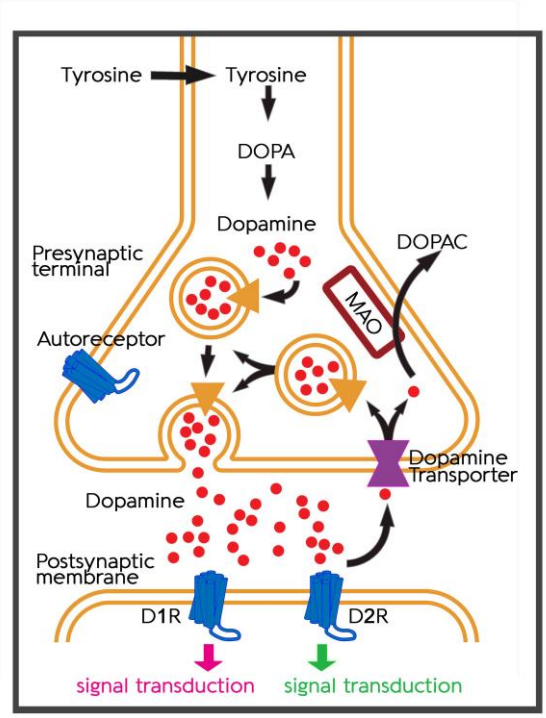

Figure 1 Graphical representation of a dopaminergic Synapse

Dopamine (DA) is a neurotransmitter involved in locomotor skills, motivation and reward, learning and memory and endocrine regulation.

Upon release from the presynaptic terminals, DA interacts with D1-like receptors (D1 and D5 receptors) that activates adenylyl cyclase and cAMP production, and the D2-like receptors (D2, D3, and D4 receptors) that inhibits adenylyl cyclase and cAMP production. Both types of receptors modulate Ca^{2+} dependent intracellular signaling processes that influence synaptic plasticity, neuronal activity, and behaviour. Presynaptic dopamine receptors regulate synthesis and release of DA as the major autoreceptor of the dopaminergic system.

After release, dopamine can be taken up again by dopamine transporter (DAT) or broken down by enzymes such as monoamine oxidases.

DOPA:3,4-dihydroxyphenylalanine, DOPAC: 3,4-dihydroxyphenylacetic acid, D1R: type 1 dopamine receptor, D2R: type 2 dopamine receptor, MAO: monoamine oxidase. Image adapted from http://www.nibb.ac.jp/annual_report/2004/img/240-01.jpg

Disturbance in synaptic transmission and functions are the basis for many neurodegenerative and psychiatric disorders. Diseases of the brain, spinal cord or peripheral nervous systems that are associated with synaptic dysfunction are referred collectively as synaptopathies (Lepeta et al., 2016). These neurological diseases can arise from gene mutations that alter synaptic protein expression or function that are involved in neurotransmission. Synaptic proteins are common drug targets as they play an integral role in psychiatric and neurodegenerative diseases. Small molecules drugs are designed to modulate protein targets in order to enhance or block activity at the synapse. In Table 1, are examples of drugs that alter dopamine or its metabolite levels in clinical disorders as reviewed by (Juarez Olguin et al., 2016)

Many proteins in the synaptic junctions are integral membrane proteins that act as receptors, transporters, channels, enzymes and anchors and are involved in signal transduction, cell regulation, cell to cell and cell to environment interactions (Fagerberg et al., 2010). They are attractive drug targets as they are more accessible than intracellular proteins. Of the currently approved drugs, 50% is directed towards integral membrane proteins (Vit and Petrak, 2017, Fagerberg et al., 2010).

Protein mass spectrometry has enabled the analysis of a large number of proteins from complex mixtures. Mass spectrometry-based proteomics of the synaptic proteome has reported more than 2000-6000 proteins in biochemical enrichments of the synapse (Pielot et al., 2012, Dieterich and Kreutz, 2016). Although a substantial number of proteins were identified from these synapse-enriched preparations, there is a notable under-representation of membrane proteins, particularly receptors, transporters and channels (Savas et al., 2011). Of particular interest are receptors and transporters of the dopaminergic system (Figure 1) that are elusive, nor quantified from proteomics studies (Pielot et al., 2012, Reig-Viader et al., 2018). A better understanding of these proteins is necessary, as perturbations of the dopaminergic system are involved in the pathophysiology of several mental disorders/diseases

(Juarez Olguin et al., 2016). This includes schizophrenia, autism, attention deficit hyperactivity disorders, drug abuse and other pathological disorders (Dahoun et al., 2017).

To unravel the protein-based molecular mechanisms of disease or drug action, the field of neuroproteomics has to shift from cataloguing to measuring protein levels. In order to understand the function of a protein and their role in the complex biological systems, it is necessary to measure the levels in different cellular compartments or to measure protein abundance changes relative to the state of the system. However, despite the advancement in quantitative proteomics in other fields, investigations in brain diseases and disorders, there are still limited. In a recent review of 87 synaptic proteomic studies on several mental disorders were included, only 6% of the proteins are replicated in subsequent experiments (Reig-Viader et al., 2018) due to high variability and a low number of biological replicates. As inherent high biological variability of tissue samples cannot be lowered, efforts to increase the reproducibility of quantitative proteomics measurements are essential.

Advancement in quantitative neuroproteomics requires the development of analytical methods in sample preparation, LCMS instruments and customised bioinformatics tools. Specific emphasis needs to be placed on the implementation of analytical methods for the determination of membrane proteins and the analysis of low abundance proteins, particularly receptors, transporters and channels, such as dopaminergic system.

Table 1 Studies of drugs that modulate levels of dopamine or its metabolites in clinical disorders.

Drug	Clinical disorder	Synaptic Changes	Reference
Risperidone/donepezil	Parkinsonian features	Dopamine transporter activity ↑	(Kang and Kim, 2013)
Cocaine, heroin, or methamphetamine	Addiction	Extracellular dopamine in CNS ↑	(Gaskill et al., 2013)
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	Parkinsonian features	Dopamine and TH ↓	(Lee et al., 2013)
PAOPA	Schizophrenia	Active site of the dopamine D(2) receptor ↓	(Tan et al., 2013)
Methylphenidate	Cocaine addiction	Dopamine transporter ↓	(Berglund et al., 2013)
Phenelzine	Depression and anxiety disorders	Dopamine levels in brain ↑	(Matveychuk et al., 2013)
Amphetamine	Attention deficit hyperactivity disorder	Extracellular dopamine ↑	(Daberkow et al., 2013)
L-DOPA	Parkinson disease	Brain dopamine levels ↑	(Szyrwiel et al., 2013)
3,4-Methylenedioxymethamphetamine	Addiction	Brain dopamine levels ↑	(Hondebrink et al., 2013)
Flupenthixol, perphenazine, and zotepine	Tauopathies	Dopamine D(2) receptor ↓	(McCormick et al., 2013)
Asenapine	Acute schizophrenia, manic episodes, bipolar I disorder	Brain dopamine levels ↑	(Tarazi and Neill, 2013)
Pramipexole	Depression	Dopamine receptor D(3) ↑	(Tokunaga et al., 2012)

1.1.2 Objective and definition of the research topic

This dissertation aims to develop technological methods in mass spectrometry-based proteomics for the analyses of synaptic proteins. This proteomics tools will allow investigations of the dynamic changes in animal models of disease and cognition in the field of neuroproteomics.

The first part of the dissertation addresses the optimisation of gel-free membrane proteomic methods for quantitative mass spectrometry (MS) to analyse the synaptic proteome. A large segment of the project was spent on the development of approaches enabling compatibility and in-depth analysis of membrane protein components by mass spectrometry. MS analyses of membrane preparations require strategies to address protein enrichment, solubility, detergent removal, proteolysis and dynamic range issues. Brain-specific informatics and annotation tools is required to interpret the large data sets generated and subsequent data analyses.

In the second part, the quantitative approach has been implemented for the proteomic profiling of tgDISC1 rat model. DISC1 is a gene for which chromosomal translocations or mutations have been linked to chronic mental illness including schizophrenia, bipolar disorder, autism and major depression. The mechanisms on how DISC1 gene dysfunction increases the susceptibility to brain disorders are still not well understood. The development of quantitative proteomics of the synapse is essential for the investigation of in-vivo protein alterations and the biological functions of DISC1. Identification of synaptic proteins and pathways altered by this protein is fundamental in understanding the DISC1-associated pathologies and development of novel therapies.

1.2 Methods in Synaptic Proteomics

1.2.1 Overview

The methods focus on the identification of membrane proteins (i.e., receptors, transporters and channels) in synapse-enriched preparations. The initial step was to implement a tailored membrane-protein protocol to overcome the under-representation of this class of proteins in mass spectrometry-based proteomics.

1.2.2 Enrichment of Synaptic Proteins by Subcellular Fractionation

In practice, dissected sub-regions are analysed as a total lysate or an in a subcellular compartment (e.g. synapse) to reduce the complexity of the sample and increase proteomic depth. The most common strategy to improve the detection of low abundance proteins is either by subcellular fractionation or by affinity purification using specific antibodies.

In this dissertation, synaptosomal preparations from tissue dissections are utilised for the majority of proteomic investigations. Synaptosomes are synapse-enriched biochemical fractions consisting of resealed nerve endings. These are formed from mild homogenization of tissues and enriched by differential and gradient ultracentrifugation. Several biochemical preparations were described to enrich synaptosomes (Bayes et al., 2011, Dunkley et al., 2008, Gray and Whittaker, 1962, Phillips et al., 2001). Enrichment of glutamatergic synaptosomes, such as fluorescence-activated synaptosome sorting, was also described (Biesemann et al., 2014).

In *Paper 1*, synaptosomal preparations were adapted from the protocols previously described (Hahn et al., 2009, Phillips et al., 2001). Rat cortices were utilised as the reported protocols required larger tissue quantities (~350mg) and express mid-level DRD1 and DRD2 and DAT1 relative to other brain regions. In *Paper 2*, a microscale sucrose gradient protocol for synaptosome preparations was developed for small tissue dissections such as dorsal striatum (~35mg tissue, ~140µg protein). This method enabled proteomic analysis from individual animals (10 biological replicates per group) for this study.

In addition, several biochemical preparations have been described to study subsynaptic proteins. This includes postsynaptic density, PSD (Collins et al., 2006, Peng et al., 2004, Yoshimura et al., 2004), presynaptic cytomatrix active zone (Boyken et al., 2013, Phillips et al., 2001, Weingarten et al., 2014), synaptic vesicles (Gronborg et al., 2010) and synaptic cleft (Loh et al., 2016).

In *Paper 1*, synaptosomes were further fractionated into the postsynaptic density preparations (PSD) and the detergent-soluble synaptosomal fractions (DSS). The aim was to characterise the DSS fraction as only a few proteomics studies were performed in these fractions. This fraction is the DSS fractions (PSD-depletion of synaptosomes) were predicted to be enriched with presynaptic proteins (Rice et al., 2019). However, the subsynaptic preparations that utilise non-ionic detergents (Triton-X100) are not readily compatible with mass spectrometry analysis.

1.2.3 Membrane protein-adapted sample preparation for mass spectrometry

To address the requirement for increased proteomic depth, a gel-free approach for membrane protein preparations solubilized in high concentrations of non-ionic detergents was optimized, as described in *Paper 1*. The membrane proteins-adapted (mem-FASP) approach consists of non-ionic detergent depletion by ethyl acetate extraction; followed by a sequential trypsin-chymotrypsin filter-aided digestion. The mem-FASP approach is tailored towards the increased identification of transmembrane proteins to address under-representation of membrane proteins. In Figure 2, the workflow is illustrated.

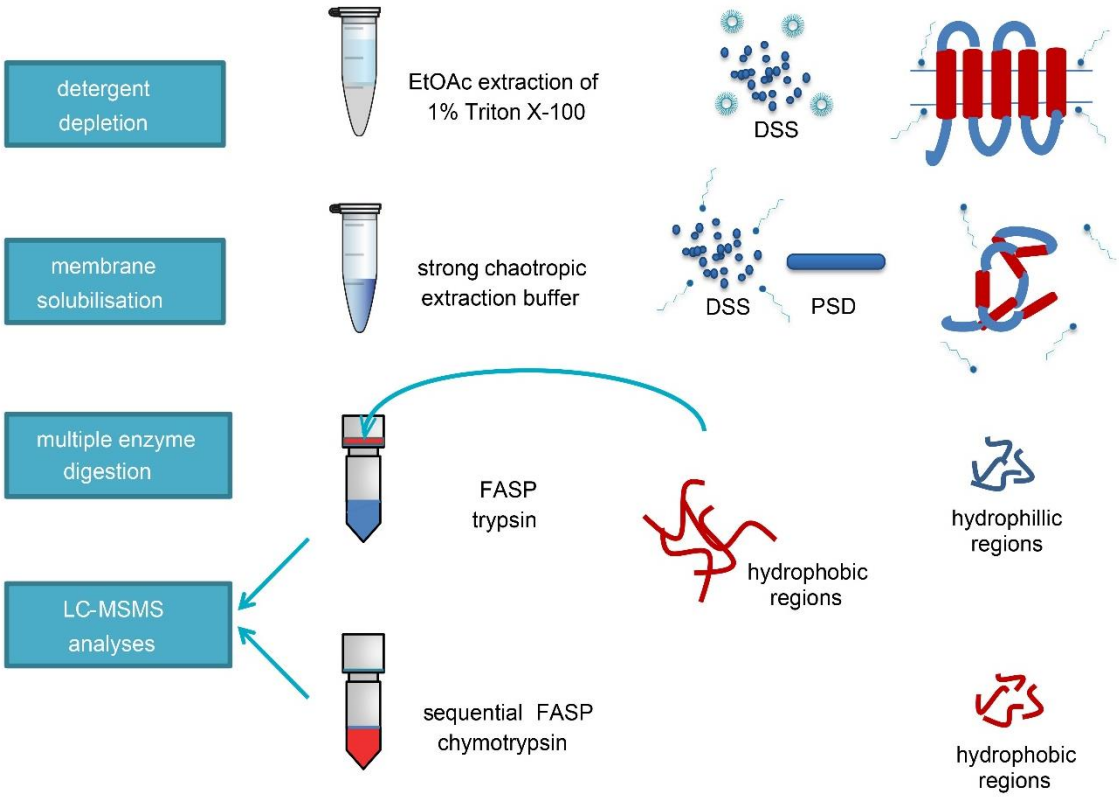

Figure 2 FASP protocol adapted for synaptic membrane proteins.

A membrane-tailored sample preparation protocol is tailored to address the under-representation of membrane proteins in proteomics. Following synaptosomal fractionation, the DSS samples were solubilised in 1% Triton X-100. Triton X-100 micelles were depleted by ethyl acetate solvent extraction to below the critical micelle concentration (CMC). Insoluble samples (PSD) were solubilised in a strong chaotropic extraction buffer. The gel-free digestion was accomplished using trypsin and FASP. To recover hydrophobic regions of membrane proteins, a sequential chymotryptic digest was performed on the sample that remained on the filter. *Reprinted with permission from John Wiley and Sons. © 2016 WILEY-VCH*

1.2.3.1 Detergents in membrane proteomics: the necessity and removal

Detergents are essential in membrane proteins preparation as they can be extracted and dispersed in aqueous solutions through disrupting the interactions of the lipid layer. Non-ionic detergents (*e.g.*, Triton X-100, DDM, NP-40 and Triton X-114) are so far the best choice for membrane enrichment, and affinity purifications as these enable retention of the native conformation of the membrane protein and protein-protein interactions or protein-antibodies in immuno-affinity experiments. A detergent solubility screen is often required to extract the desired transmembrane protein before affinity purifications. In *Paper 20*, optimum solubilisation of DAT1 was achieved using Triton X-100 and DDM detergent for dopamine receptors, DRD1 and DRD2 (Stojanovic et al., 2017).

Detergents such as these, however, are not compatible with gel-free liquid chromatography-mass spectrometry (LCMS) analyses as chromatographic co-elution with peptides occur, and prominent signals are observed in the mass spectra. These prominent detergent signals mask the signals from the peptides hampering their detection. Characteristic LCMS detergent peaks include adduct formation and shape distortion, signal suppression and mass-to-charge shifts of signals (Loo et al., 1994). LCMS detergent contamination is characterised by strong signal clusters of peaks with an interval of 44 m/z arising from repeating units of ethylene glycol subunits $[-CH_2CH\text{-}O-]_n$ of Triton derivatives (Figure 3).

Recent gel-free proteomic techniques use spin filters (filter-aided sample preparation, FASP) to free the samples from detergents and other small molecules (Manza et al., 2005, Wisniewski et al., 2009). However, for cellular membrane samples, which are solubilised in non-ionic detergents, removal by spin filters are not sufficient. These non-ionic detergents form 90 kDa micellar structures that cannot be fully removed with 30 kDa molecular weight cut-off (MWCO) FASP filters at detergent concentrations above the critical micelle concentrations (CMC).

In order to address these problems, a detergent removal protocol was developed in *Paper 1,* a phase transfer using ethyl acetate extraction was employed to deplete the non-ionic detergents concentration. This was performed in the protein level rather than at the peptide level as compared previously described (Yeung and Stanley, 2010). By introducing detergent depletion step before FASP, detergents exist as molecules rather than as micelles and the remaining detergents pass-through the 30 kDa cut-off filter. After filter aided digestion of samples, no traces of the Triton X-100 and Triton X-114 on both peptide samples were detected upon LCMS analyses.

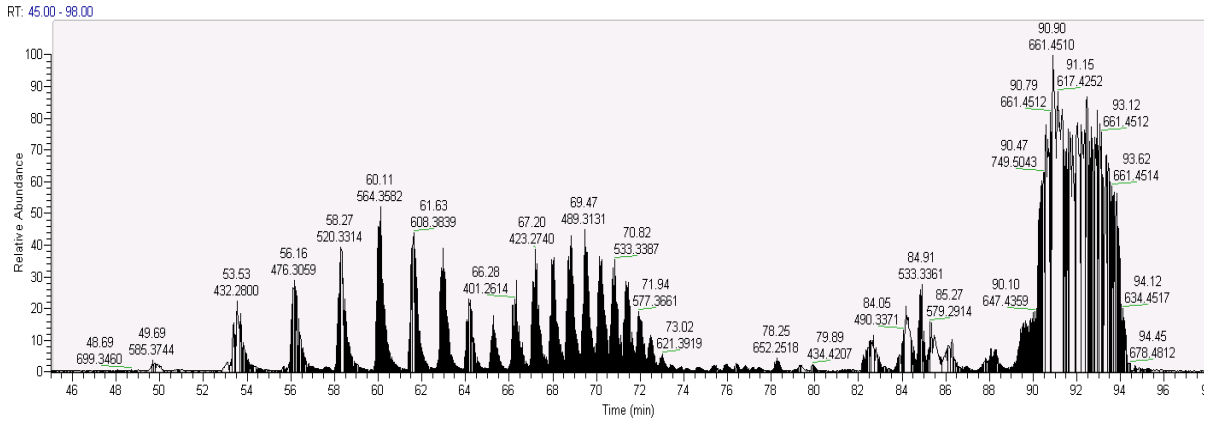

Figure 3 Characteristic LCMS detergent contamination peaks

Characteristic LC-MS detergent contamination characterised by clusters of peaks with an interval of 44 m/z arising from repeating units of ethylene glycol subunits $[-CH_2CH\text{-}O-]n$ of from Triton detergent derivatives in ESI positive mode.

This workflow was further utilised for analyses of hydrophobic protein enrichment preparations from cell cultures for breast cancer (Karabacak and Sialana, manuscript in preparation) and glioblastoma (Erhart et al., 2019a), *Paper 9*. In these studies, a Triton-X114 detergent phase separation (Bordier, 1981) was implemented to enrich the integral membrane proteins and to minimise the aqueous-soluble proteins associated with the cultivation media with serum (*e.g.,* albumin) used. The previous studies rely on acetone precipitation to remove detergents and concentrate samples where the pellets formed are challenging to resolubilize (Donoghue et al., 2008, Chapel et al., 2013, Abul-Husn et al., 2011, Doucette et al., 2014).

The lack of solubility of integral membrane proteins could prevent their complete digestion. Solubilization of membrane material, therefore, plays a significant role in membrane proteomics. In our hands, protein aggregation occurs on integral membrane proteins are solubilised in SDS with heating, particularly with the PSD preparations when original FASP workflows were implemented (Wisniewski et al., 2009). Strong chaotropic extraction buffer sample (7M urea, 2M thiourea, 4%CHAPS, 50mM TEAB, 100mM DTT) were used to solubilise the membrane proteins at room temperature was used instead. The non-ionic detergent (CHAPS) reduce the interaction of the proteins to the lipid bilayer while urea and thiourea reduce the hydrogen bonding and ionic interactions between proteins. Following protein solubilisation, the use of filters enables removal of the remaining detergent via washing step with urea. Reduction of disulphide bonds and carbamidomethylation of cysteine carried out on the same sample filter. (Wisniewski and Mann, 2012).

In our recent studies, we modified solubilisation of membrane protein samples in SDS buffer (2% SDS, 150mM NaCl, 100mM DTT and 50mM TEAB) at 37^{o}C as it was comparable the strong chaotropic extraction buffer. The advantage is that the remaining lysates can be used for immunoblotting techniques.

1.2.3.2 Proteolytic digestion on membrane proteins

In bottom-up proteomics workflow, proteolytic digestion is employed to generate peptides before MS analyses. Trypsin is by far the preferred enzyme due to its high cleavage specificity on the carboxy-terminal of basic amino acid residues (lysine and arginine) protein sequences. This generates at least doubly positively charged peptides that increase ionisation efficiency (Steen and Mann, 2004).

Integral membrane proteins have transmembrane domains that span the lipid bilayer and some domains exposed in both intracellular and extracellular portions. The membrane-spanning parts are typically alpha-helices consisting of 20 to 25 hydrophobic amino acids with limited tryptic cleavage sites (Vit and Petrak, 2017).

In *Paper 1,* a sequential chymotrypsin digest of the large, tryptically-digested peptides that remained on the filter was performed to generate peptides from the hydrophobic protein regions. Sequential chymotrypsin digestion generates peptides from the transmembrane domain (TMD) regions as it cleaves preferentially at the carboxyl side of tyrosine, phenylalanine, and tryptophan.

In the label-free analyses of DSS samples in *Paper 1*, in addition to tryptic digestion, a 36% were identified from sequential chymotrypsin digestion of the same samples of which 46% were predicted by TMHMM (Sonnhammer et al., 1998) to have transmembrane domains. It includes the identification of the DAT, DRD1 and DRD2 transmembrane proteins on samples without prior peptide pre-fractionation.

Although using chymotrypsin increases the protein sequence coverage and identification, the low-cleavage specificity of chymotrypsin makes it less desirable for protein quantification. Relative quantification using the combination of spectral counts from tryptic and sequential tryptic/chymotryptic peptides was proposed in *Paper 1*. Only the tryptic digests were used in *Paper 1* for actual quantitative measurements.

In *Paper 2*, a mixture of LysC (cleaves the carboxy-terminal of lysine residues) and trypsin was used to minimise proteolytic miscleavage leading to higher reproducibility of generated peptides (Wisniewski and Mann, 2012).

1.2.3.3 Separating complex peptide digests

The proteolytic digestion from complex protein samples generates a large number of peptides. Although the current mass spectrometer technologies are reasonably able to separate the different ions introduced, the current proteomics methods rely on peptide separation techniques to reduce the complexity before mass spectrometric analysis. Typically, the nano flow high-performance liquid chromatography (nano-HPLC) is directly coupled to the MS via an ESI source.

In this dissertation, an ion pair reverse-phase chromatographic system was used for separation of peptides. This consists of a hydrophobic C18 separating column (octadodecyl carbon chain bound to silica) and a hydrophilic aqueous-acetonitrile mobile phase. Peptides are sequentially eluted by increasing the composition of acetonitrile during the chromatographic run (gradient elution). A C18 pre-column is used before the separating column for peptide enrichment and as a protective cleaning to increase the column lifespan. In *Paper 2,* The length of the column was upgraded (13 cm to 50cm) to increase peak capacities at the expense of high column backpressures. Column back pressures are reduced by increasing the column oven temperatures to 55^{o}C.

The retention of highly charged amphiprotic peptides is inherently weak in reverse phase chromatography. The addition of weak acid modifiers (e.g. formic acid and trifluoroacetic acid) protonates the peptides while the formate anions form ion pair with basic moieties on the peptide whereby cloaking the charge (Apffel et al., 1995). Ion pairing improves the retention of peptide to the hydrophobic surface of the stationary phase and promotes better separation.

Efficient peptide separation increases the likelihood of the detection of low-abundance species that are usually masked by the high-abundance ones.

In in-depth proteomics experiments, an additional offline peptide pre-fractionation is employed before the LCMS analyses of the samples. This step reduces sample complexity and maximizes proteomic depth. The high-pH reversed-phase chromatography, anion or cation exchange chromatography, and hydrophilic interaction liquid chromatography are the most popular peptide pre-fractionation techniques used in proteomics.

In this dissertation, high-pH reversed-phase chromatography was used as the mobile phase modifiers are readily removed by evaporation (Gilar et al., 2005). This allows further reduces the complexity of the peptide samples prior to LCMS analysis. In the basic environment, (ammonium formate buffer, pH 10), the retention of deprotonated species is orthogonal to that in the acidic environment.

1.2.3.4 Sample Ionisation

The eluted peptides from the chromatographic separation are first ionised before mass spectrometry. In shotgun proteomics, the two popular ionisation source include electrospray ionisation, ESI (Fenn et al., 1989) and matrix-assisted laser desorption ionisation (MALDI) (Karas et al., 2002).

In this dissertation, applied potential in ESI source generates a fine spray of charged droplets from the eluted peptides and travel towards the MS inlet (Figure 4). Once the droplets are heated, solvent evaporation occurs, leading to the reduction of droplet size. The increase of charge density and electrostatic repulsion generates a coulombic explosion of the droplets (Ho et al., 2003). This generates smaller and more stable de-solvated protonated ions (Steen and Mann, 2004) that accelerate into the high vacuum inlet of the mass spectrometer. These ions are guided through voltage and radio frequency-focused openings (Pitt, 2009). Subsequently, a mass analyser determines the mass to charge ratio (m/z, Thompsons) of the ions and corresponding signal intensities.

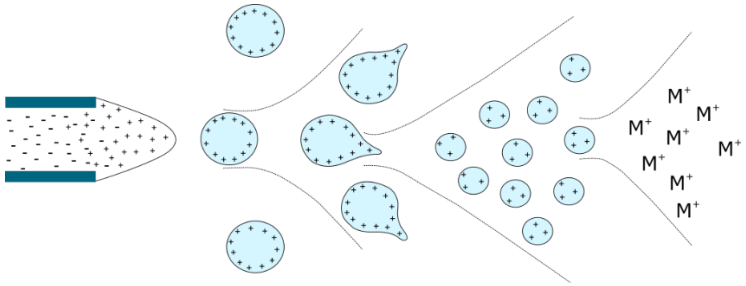

Figure 4 Generation of ions by electrospray ionisation

Droplets are heated, and the solvent evaporation leads to droplet size reduction. This increases the charge density and electrostatic repulsion generating a coulombic explosion of the ions.

1.2.3.5 Mass analysis

Mass analysers are the essential components of a mass spectrometer. In principle, it takes the ions, separates them based on charge to mass ratio (m/z) and conveys them to a detector (Graves and Haystead, 2002). The desolated ions are controlled and measured in response to the electromagnetic field (Savaryn et al., 2016). This principle generally applies to mass analysers such as Ion trap, Time-of-flight, Quadrupole mass filter and Orbitraps.

Linear ion traps utilise quadruples to trap ions radially while an electrical potential is used to trap the ions axially. In an Orbitrap mass analyser (Figure 5), the ions are trapped in an electrostatic field between an inner (spindle-like, coaxial) and outer electrode (barrel-like) in an orbital motion around the inner electrode (Makarov, 2000). As the ions rotate, they oscillate around the axis at a frequency characteristic of their mass to charge (m/z). The axial oscillations are detected as they induce image current on the outer electrodes. The outer electrode is split into two detectors that is connected to a differential amplifier. As the ions are introduced into the Orbitrap, a concurrent detection of the oscillatory motion of each ion population is measured. The Fourier transformation of generated oscillation signals (time-domain oscillation) are converted into frequencies and intensities. The frequencies are further converted into m/z generate the mass spectra.

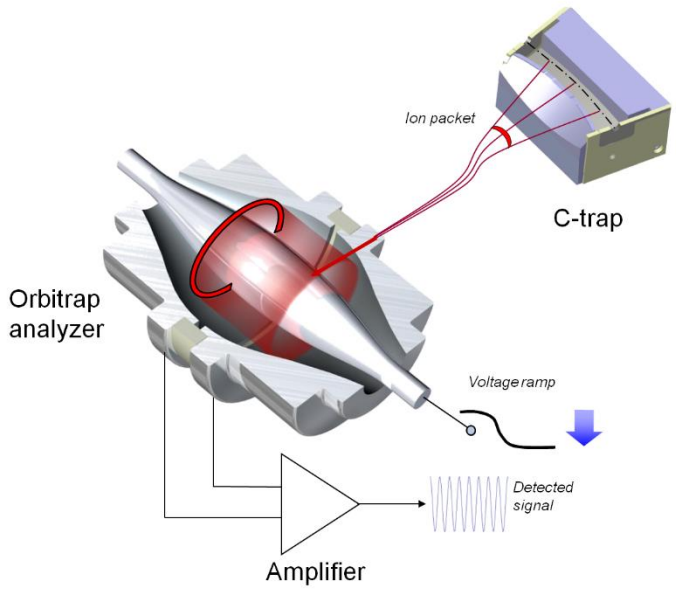

Figure 5 Injection of ions into the Orbitrap mass analyser

Artwork by Thermo Fisher Scientific CC BY-SA 3.0

1.2.3.6 Tandem Mass Spectrometry of Peptides

The method to determine the amino acid sequence of the peptides utilize tandem mass spectrometry (Hunt et al., 1986). Modern instruments used in proteomics utilise hybrid mass spectrometers that combine several mass analysers in one instrument to perform tandem mass spectrometry.

In the course of this dissertation, a linear trap quadrupole (LTQ) Orbitrap Velos and the Q-Exactive hybrid quadruple-Orbitrap tandem mass spectrometers were employed (Figure 6).

In the first step of tandem mass spectrometry, ionised peptides are filtered by the linear ion trap typically at a range of 350-1800m/z. As the ions are introduced, the Orbitrap generates a mass spectrum of intensity vs. m/z of the isolated ions designated as MS (MS1).

In the acquisition of tandem mass spectra, a data-dependent acquisition (DDA) mode was employed. Using DDA mode, the mass spectrometer isolates the most intense peptide ions in the first stage (MS1). These peptides are fragmented and analysed in the second step of the tandem mass spectrometry to generate an MS/MS (MS2) peptide fragment spectra (Michalski et al., 2011).

The fragmentation generates ions from the cleavage of the peptide backbone into a/x, b/y and c/z type of ions depending on the technique used (Figure 6).

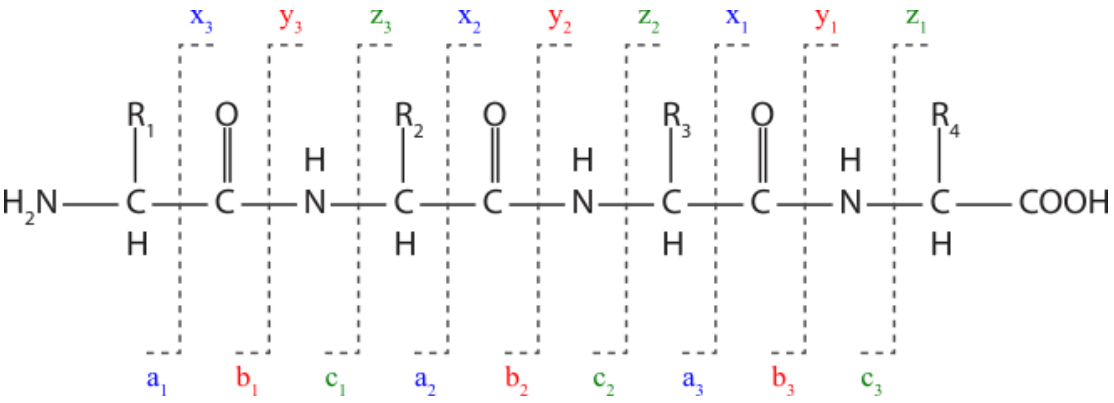

Figure 6 Peptide fragmentation sites and ion series generated

The most common peptide fragmentation techniques are collision-induced dissociation (CID), high collision dissociation (HCD) and electron transfer dissociation (ETD). Different fragmentation techniques affect the generated ion series in MS/MS.

CID is performed in the ion trap of the LTQ Orbitrap Velos. The isolated ions are exposed to radiofrequency excitation, whereby increasing kinetic energy. The fragmentation occurs as the excited peptides collide in the cloud of helium gas. This technique results in generation b and y type ions as CID favourably fragments peptides in the peptide bond (amide) position. Ion traps typically have a low mass resolution, and low mass cut-off (about 1/3 of the selected m/z) as higher radiofrequency energies are required (Kocher et al., 2009).

HCD fragmentation was introduced to overcome the limitation of CID. The peptides are fragmented in octopole HCD collision cell (Figure 7) while using Orbitrap as a detector (high resolution). In HCD fragmentation, the ions from the LTQ, pass through the C-trap towards the HCD cell (see Figure 7). In the HCD cell, peptide fragmentation occurs due to the varied direct current offset in a shroud of nitrogen gas (Olsen et al., 2007). These fragmented ions are returned and collected in the C-trap before injection to the Orbitrap analyzer.

In ETD, an anion reagent (e.g. fluoranthene) induce fragmentation of the cationic peptides to generate c and z ion series (Swaney et al., 2007). This incredibly useful in the detection of post-translational modifications as they are preserved on the fragment ions.

In the majority of this dissertation, HCD fragmentation is used for the analysis of TMT-labeled peptides. The HCD fragmentation generates ions from 100-150m/z range to detect reporter ions for quantitation and yields b- and y-type ions series for peptide sequencing.

In *Paper 2*, TMT-10plex quantification relies on high resolution and accuracy measurements as mass differences between reporter ions are small (0.00632 Da). The modern hybrid instruments used combine quadrupole precursor selection with the Orbitrap Fourier transform-based mass analyser that can achieve high resolution (> 100,000) and high mass accuracy (≤ 5ppm) (Makarov, 2000). Using a lock-mass scheme can lower mass errors to < 2ppm (Olsen et al., 2005). Internal calibration utilises protonated polycyclodimethylsiloxane from the atmosphere as lock mass and external calibration that consist of caffeine, MRFA and Ultramark 1621 mixture (Cox et al., 2011).

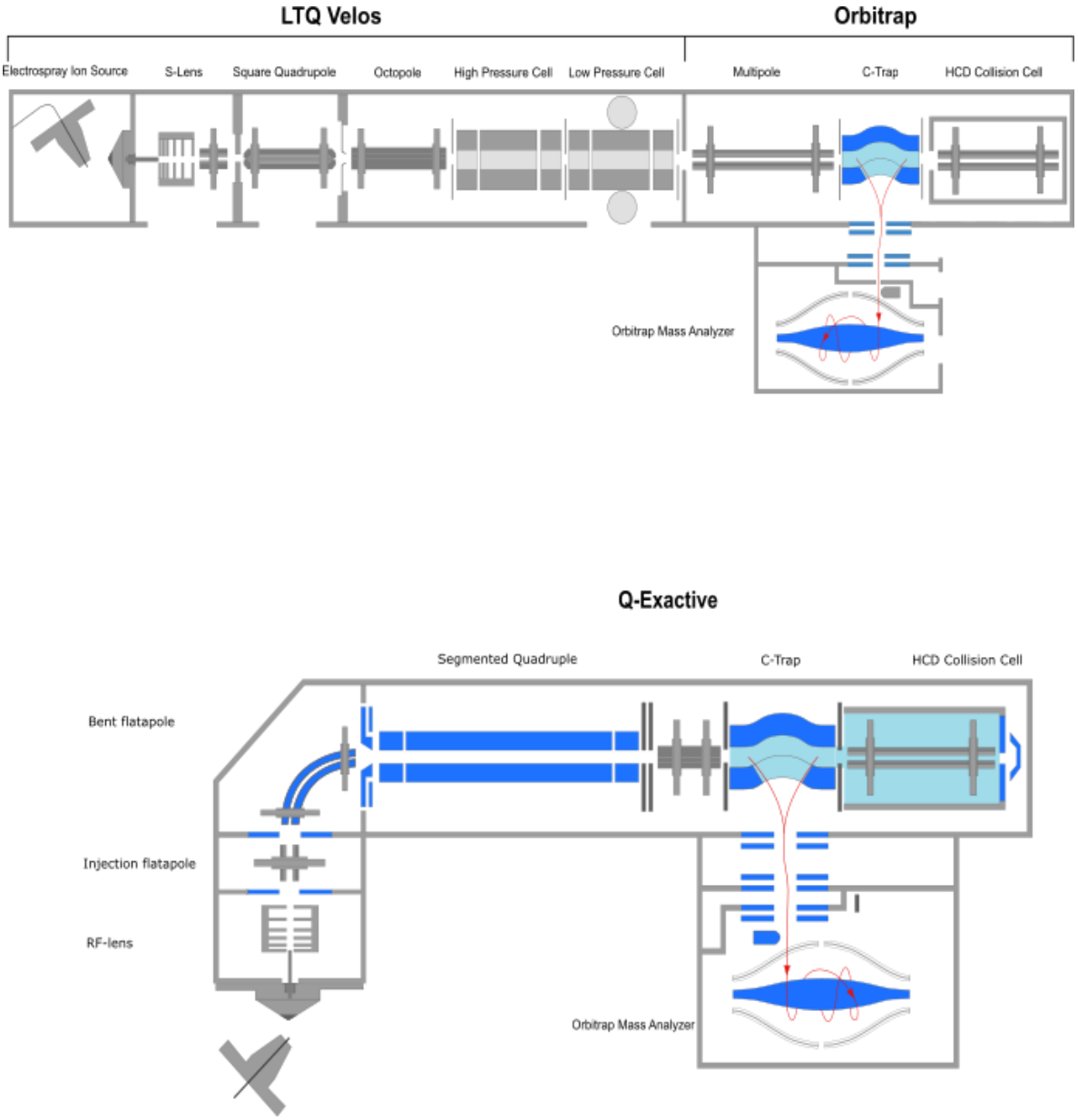

Figure 7 Schematic Diagram of LTQ Orbitrap Velos and Q-Exactive

1.2.4 Quantitative proteomics

To understand the function of individual proteins and their role in the complex biological systems, it is often necessary to measure the levels in different cellular compartments or to measure protein abundance changes relative to the state of the system.

In this dissertation, a discovery-based relative quantification was the analytical approach used to reveal relative protein abundance across different groups of samples simultaneously. Several quantitative proteomic technologies have been described including TMT (Thompson et al., 2003), iTRAQ (Wiese et al., 2007), SILAC (Ong et al., 2002), SILAM (McClatchy and Yates, 2014), and label-free quantification (Wiener et al., 2004) approaches.

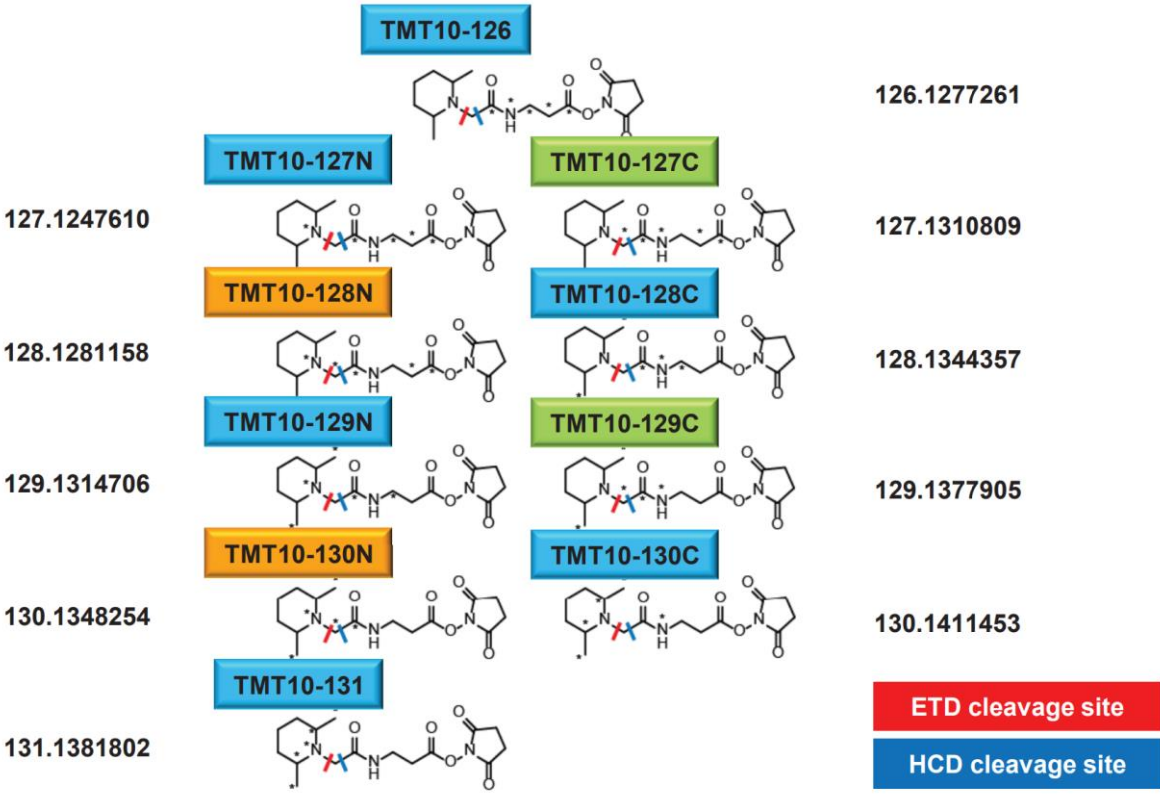

Figure 8 Chemical Structures of Tandem Mass Tags (TMT)

TMT10plex structures and reporter ion mass for TMT6plex tags (blue), TMT8plex (green) and TMT10plex (orange). Artwork by Rosa Viener, Thermo Scientific.

For relative quantitation in this dissertation, isobaric labelling using tandem mass tags (TMT) using TMT-6plex and TMT-10plex was utilised (Figure 8). Recently, this multiplexing technique (TMTpro) allows simultaneous analysis of up sixteen (16) samples. (Thompson et al., 2019). The TMTs tags for peptide bind covalently to the lysine or N-terminus of peptide residues (Thompson et al., 2003).

In an in-depth proteomics analysis, two chromatographic separations are used prior to MS analysis results in variable peptide separation. In addition, ionisation efficiency in separate LCMS runs could result in different charges and precursor intensities. In TMT multiplex experiment (Figure 9), each sample (peptides) are covalently labelled with a different isotopic variant of a TMT isobaric mass tag and then pooled before chromatography. Since the isobaric tags have the same chemical properties, the derivatised peptides from the different TMT

labelled peptides co-migrate as a single entity during LC separations and ionisation. This result as a single peak in MS scans leading to higher reproducibility of measurements.

The TMT labelled peptides made up of reporter-, balance-, and amine-reactive –regions (Rauniyar and Yates, 2014) which exhibit unique, intense, low-mass MS/MS reporter ions. Peptide identification is achieved by matching the fragment ion peaks to the sequence databases. By comparing the intensities of reporter ions, relative peptide/protein quantitation is determined (Figure 9).

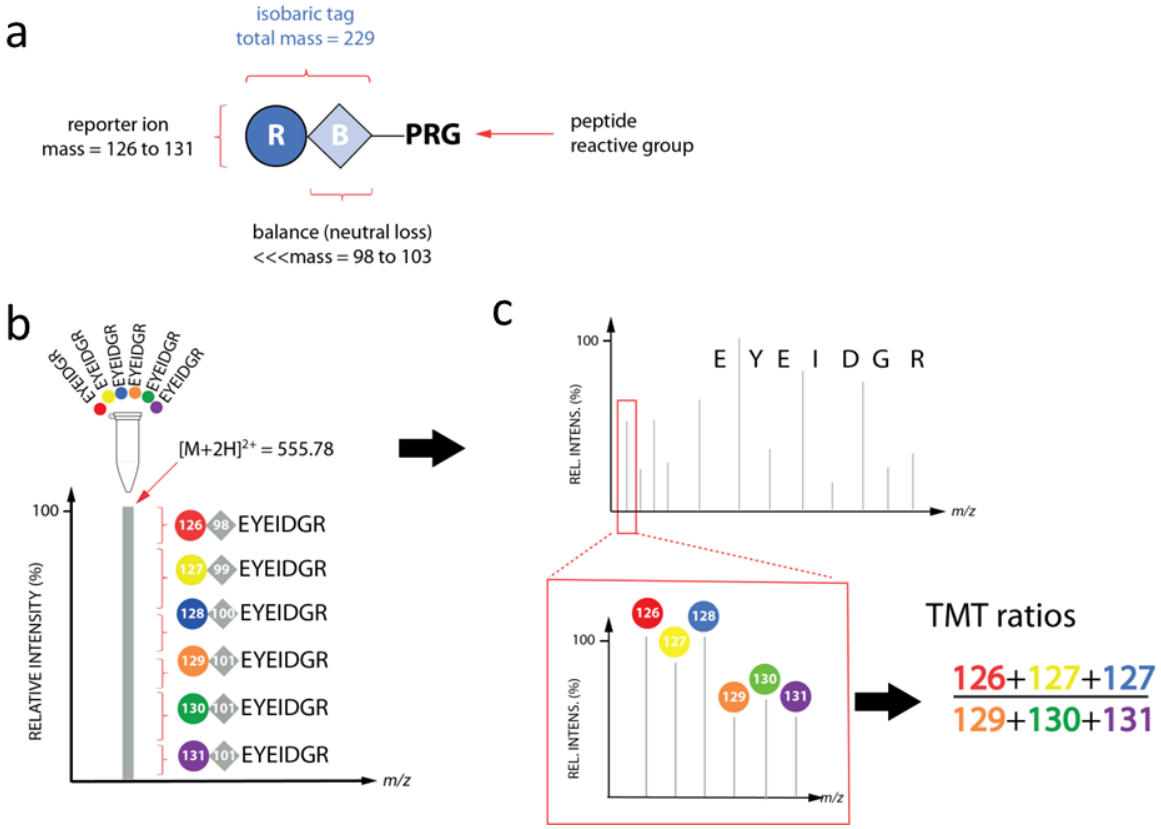

Figure 9 Multiplexing with Tandem Mass Tags

The use of isobaric tags warranted multiplexing of six quantitative proteomic experiments. Isobarically TMT labeled tryptic peptides (a) from different samples co-elute from liquid chromatography and are detected by MS^1 scans as an additive signal (b). Isobaric labeling ensures that even low abundance peptides are fragmented (together with other samples) that may be excluded by abundance-based data-dependent methods using label-free methods. Upon MS^2 fragmentation, specific reporter ions at *m/z* 126 to 131 are generated together with peptide sequence information (c). Relative quantitation of the peptide is determined by calculating the relative ratios of the reporter ion intensities. Relative quantitation of 4,218 synaptic proteins was simultaneously determined with high reproducibility between animal triplicates.

1.2.5 Data analysis and bioinformatics

1.2.5.1 Peptide and Protein Identification

The process of separating the peptides by liquid chromatography, detection in tandem mass spectrometer, isolation and fragmentation and recording of the spectra of generated fragments that is collected over several hours will produce thousands to millions of tandem mass spectra. High-throughput identification of peptides is automated using search engines such as MASCOT, SEQUEST and PHENYX (Colinge et al., 2004, Eng et al., 1994, Perkins et al., 1999) was utilised in this dissertation

The search engines match the observed spectra (experimental) with the reference peptide sequences (theoretical) and assign a score reflecting the probability of matching. The reference database consist of the predicted sequences from *in silico* protease (e.g. trypsin or chymotrypsin) cleavage of a protein database (e.g. human, mouse, rat). This protein sequence databases are generated from completely sequenced genomes of organisms (Consortium, 2014). In *Paper 8* (Dos Santos-Pinto et al., 2019), where spider genome sequences are unavailable, we generated the protein database from assembled transcriptome sequences.

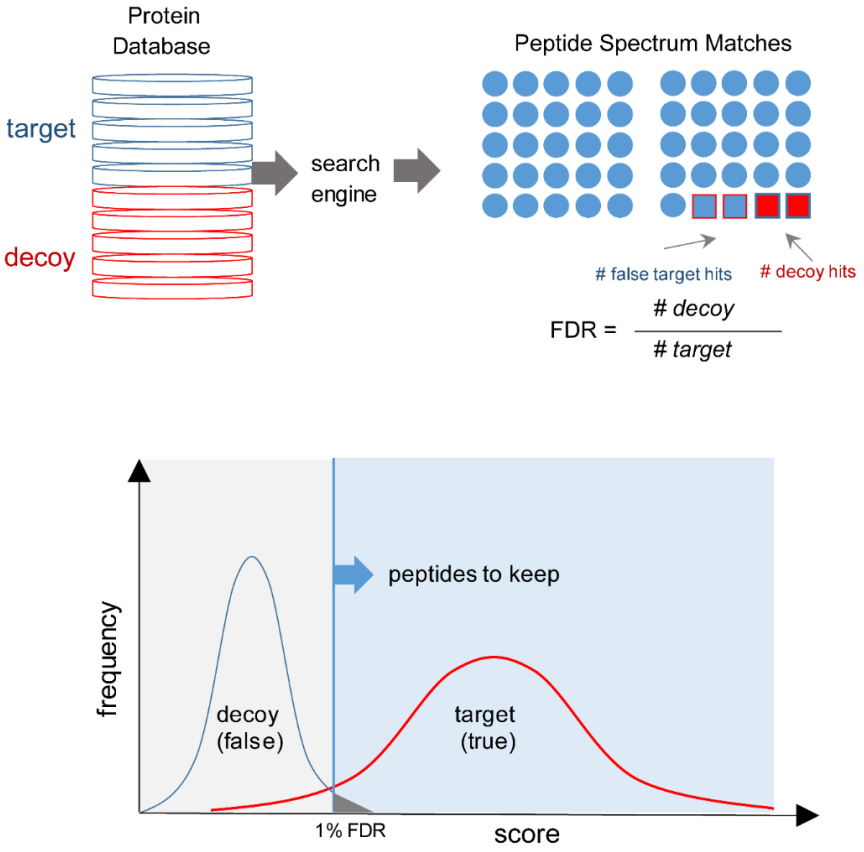

Figure 10 Peptide identification through target-decoy search strategy

Search engine software uses a scoring algorithm to differentiate true and false identifications. False discovery rate is the fraction of false positives identified from the decoy peptides. A score threshold is usually set at 1% FDR where peptides spectral matches above this score is reported.

To simplify data analysis of mass spectra, the complex multi-isotopic peak lists (m/z) are first grouped into a simple monoisotopic mass list by a process of deconvolution. (Mujezinovic et al., 2006, Liu et al., 2010). These mass lists are used to determine a set of candidate reference peptides that is near the observed experimental peptide precursor ion in a mass range.

For each candidate peptide, the search algorithm matches the theoretical spectra to the observed tandem mass spectrum by assigning a probability match score (probability that the observed match is a random event). The highest scoring peptide sequence that match to the spectrum is reported as the best identification and is usually referred to as a peptide-spectrum match (PSM).

In addition, the experimental spectra are also matched to a decoy database. This is usually generated from the reversed sequences of the target protein database hence is assumed to be absent in the sample (Figure 10). The distribution of scores from the spectra that match the decoy peptide sequences are used to estimate the false discovery rate. The commonly accepted FDR is 1% on the identification level, where an accepted small proportion of peptide identifications (1%) will likely be false identification.

To improve discrimination of correct and incorrect peptide spectrum identifications, a semi-supervised machine-learning algorithm is used in combination with the database search (Kall et al., 2007). The subset of the high scoring target peptides is used as the positive subset while the peptides matched to the decoy database are assigned as the negative subset. The Percolator algorithm trains the support vector machine (SVM) to discriminate the positive and negative subsets by assigning weight to PSM features that may include score, precursor and fragment mass errors. The weighted features allow re-ranking of matches from all the data and assigns false discovery rate (q-value) and a posterior error probability (PEP) that indicates that the individual match is a chance event (number of hits from a decoy database per number of hits at a given score). In the Mascot search engine, the -10log (PEP) is reported as the mascot score.

In this dissertation, for large scale analysis q-value cut-off was used to estimate the misclassification in a large set of PSMs (Kall et al., 2008). For identifying specific peptide or protein, PEP is used in combination with manual inspection of raw spectra for low scoring PSMs to assign score thresholds.

1.2.5.2 Functional Annotation of Proteins

Interpreting high-throughput proteomics data into biologically meaningful information relies on in-depth bioinformatics analysis. In discovery proteomics, a differential analysis experiment aims to reveal a set of biomarkers or clues of a relevant pathway or process. The assumption is that proteins showing similar experiment profiles share biological mechanisms that could provide hints to molecular processes or pathways.

However, in the course of this dissertation, bioinformatics workflows and tools specialised for neuro-proteomics studies are limited. In our hands, annotations that are experimentally observed from tissues and cells from the nervous system pathways and processes terms was used to provide relevant information.

Using the proteomic methods used in this dissertation, a differential proteomics experiment could identify and quantify 4000 to 7000 proteins. About 200 proteins (~ 5% of the data) are reported as differentially expressed using two-sided T-test. These list of differentially expressed proteins are annotated with biological terms from curated databases. Fisher enrichment analysis is used to identify terms that are over-represented within the experimental protein list that could describe an underlying or shared biological insight.

Several annotation terms were used in this study: Gene Ontology (Ashburner et al., 2000) for biological process, cellular components molecular functions, pathways using (Ingenuity Pathway analysis, www.ingenuity.com and KEGG (Kanehisa et al., 2002)), protein classes and domains (PANTHER (Mi et al., 2013), IUPHAR/BPS (Pawson et al., 2014)), drug targets

(IUPHAR and DGIdb (Griffith et al., 2013, Pawson et al., 2014), and brain disease and disorders (Comparative Toxigenomics database (Davis et al., 2013).

As the heterogeneity of results were reported depending on the software and database used (Mooney and Wilmot, 2015), several software and programs were also used to confirm results including DAVID (Huang et al., 2007), Reactome Pathway Database (Croft et al., 2014), and NCI Pathway Interaction Database (Schaefer et al., 2009)

Further, to reduce the redundancy of terms, platforms such as ClueGO via the Cytoscape platform (Bindea et al., 2009, Huntley et al., 2015) was used. This allows grouping and fusion of functionally related terms are into functional networks, and only the most significant terms per group are reported.

2 Selected Scientific Publications

2.1 Article 1: Mass spectrometric analysis of synaptosomal membrane preparations for the determination of brain receptors, transporters and channels.

Sialana FJ, Gulyassy P, Májek P, Sjöstedt E, Kis V, Müller AC, Rudashevskaya EL, Mulder J, Bennett KL, Lubec G.

Proteomics. 2016 Nov;16(22):2911-2920. doi: 10.1002/pmic.201600234.

The manuscript focuses on the identification of membrane proteins (i.e., receptors, transporters and channels) enriched from a detergent soluble and a detergent-resistant synaptosomal fraction of rat cortices. The initial step was to implement a tailored membrane-protein protocol to overcome the under-representation of this class of proteins in mass spectrometry-based proteomics.

In total, 4,784 synaptic proteins were identified including 274 receptors, 394 transporters/channels and 1,377 transmembrane proteins. Of these, 1,781 are potential drug targets and 834 are linked to brain disorders. The so far largest datasets on synaptic proteins *i.e. SynProt* contains only a third of the receptors, transporters and channels identified herein.

The vast majority of pharmacological targets are represented by transmembrane proteins and CNS disorders/diseases are also often linked to such proteins. Thus, this data is an unprecedented resource of synaptic membrane proteins that will be of interest to a broad community focused on basic and clinical neurosciences.

RESEARCH ARTICLE

Mass spectrometric analysis of synaptosomal membrane preparations for the determination of brain receptors, transporters and channels

Fernando J. Sialana[1,2], Peter Gulyassy[3], Peter Májek[2], Evelina Sjöstedt[6,7], Viktor Kis[4], André C. Müller[2], Elena L. Rudashevskaya[5**], Jan Mulder[8], Keiryn L. Bennett[2***] and Gert Lubec[1]*

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Accepted: October 17, 2016

The molecular composition of synaptic signal transduction machineries shapes synaptic neurotransmission. The repertoire of receptors, transporters and channels (RTCs) comprises major signaling events in the brain. RTCs are conventionally studied by candidate immunohistochemistry and biochemistry, which are low throughput with resolution greatly affected by available immunoreagents and membrane interference. Therefore, a comprehensive resource of synaptic brain RTCs is still lacking. In particular, studies on the detergent-soluble synaptosomal fraction, known to contain transporters and channels, are limited. We, therefore, performed sub-synaptosomal fractionation of rat cerebral cortex, followed by trypsin/chymotrypsin sequential digestion of a detergent-soluble synaptosomal fraction and a postsynaptic density preparation, stable-isotope tryptic peptide labeling and liquid chromatography mass spectrometry. Based on the current study, a total of 4784 synaptic proteins were submitted to the ProteomExchange database (PXD001948), including 274 receptors, 394 transporters/channels and 1377 transmembrane proteins. Function-based classification assigned 1781 proteins as probable drug targets with 834 directly linked to brain disorders. The analytical approach identified 499 RTCs that are not listed in the largest, curated database for synaptosomal proteins (SynProt). This is a threefold RTC increase over all other data collected to date. Taken together, we present a protein discovery resource that can serve as a benchmark for future molecular interrogation of synaptic connectivity.

Keywords:
Animal proteomics / Channels / Membrane / Receptors / Transporters

Additional supporting information may be found in the online version of this article at the publisher's web-site

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The authors would like to dedicate this paper to Dr. Michael J. Dunn for his many editorial accomplishments, energy and tremendous contribution to the proteomics community as Editor-in-Chief of Proteomics.

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Colour Online: See the article online to view Figs. 1–4 in colour.

Significance of the study

Brain membrane receptors, transporters and channels (RTCs) are key elements of synaptic functions. The identification, quantitation and localization of RTCs are fundamental to a basic understanding of the molecular mechanisms of information processing, cognition, pathologies and also possible therapeutic interventions. The vast majority of pharmacological targets are represented by transmembrane proteins and CNS disorders/diseases are also mainly linked to such proteins. The characterization of the previously understudied detergent-soluble synaptosomal fractions showed enrichment of transporters and receptors. Proteins enriched in this fraction showed strong association with dementia, Alzheimer's disease, substance withdrawal syndromes and Parkinson´s disease. Thus, this data is an unprecedented resource of synaptic membrane proteins that will be of interest to a broad community focused on basic and clinical neurosciences.

1 Introduction

A series of attempts have been made to analyze, characterize and quantitate receptors, transporters, channels (RTCs) and other synaptic membrane proteins [1]. Although there is more data available on postsynaptic density preparations (PSD) [1], the number and characterization of RTCs of the detergent-soluble synaptosomal fraction is limited [2–4].

Biochemical methods were developed to enrich the synaptic junctions that include synaptosomal preparations. Synaptosomes are the resealed nerve endings formed after mild homogenization of nerve tissues and enriched through differential and gradient centrifugation. Widely used PSD preparations are the detergent-resistant synaptosomal fractions showing enrichment of the morphologically defined PSD structures [1]. Large-scale proteomic studies of this detergent-resistant synaptic junction fraction have allowed characterization of proteins localized to the postsynaptic membrane [1].

For the detergent-soluble synaptosomal fractions that has been depleted of PSD, however, only few proteomic studies have been performed [4–6]. This fraction should contain equally important presynaptic constituents involved in neurotransmitter release and reuptake mechanisms. Hydrophobic membrane proteins have remained under-represented in proteomics due to inherent technical difficulties in solubilization, detergent removal, limited tryptic cleavage sites and low abundance.

The aim of the study was to perform a thorough and detailed analysis of synaptic membrane proteins to generate a comprehensive resource database for neuroscientists. To achieve this objective, a tailored membrane protein sample preparation protocol including detergent removal and filter aided sample preparation with subsequent trypsin-chymotrypsin digestion, was coupled to gel-free LC-MS to identify and quantitate proteins. Herewith, we report a proteomic approach to systematically study synaptic membrane RTCs. The combined methodology from this study has generated a compendium of synaptic proteins with enrichment in detergent-soluble and PSD fractions. The dataset is an unprecedented resource that provides a wealth of information on receptors, transporters/channels and other proteins for studies in neurobiology, neuropathology, neuropharmacology and related research.

2 Materials and methods

2.1 Enriched synaptosomal preparations

The synaptosomal preparations were adapted from two previously published protocols [7, 8]. Briefly, cortices from three male Wistar rats were dissected and stored at –80°C. Rat cortices (about 350 mg, wet weight) were homogenized with a Dounce-type homogenizer. Sucrose gradient ultracentrifugation was performed at 100 000 × *g*, the band between 1 M and 1.25 M sucrose was collected, pelleted and designated as synaptosomal preparation. Synaptosomal preparations were reconstituted with 1% Triton X-100 at pH 6, shaken and centrifuged at 35 000 × *g*. The synaptic membrane (SM) pellet produced was reconstituted with 1% Triton X-100 at pH 8 and centrifuged at 140 000 × *g*. The supernatant was designated as the detergent-soluble synaptic membrane fraction (DSS) that was collected and concentrated tenfold using a 3 kDa centrifugal filter. The pellet was designated as the postsynaptic density (PSD) (Supporting Information Methods).

2.2 Immunoblotting

The following antibodies were used according to the instructions supplied by the manufacturer: mouse anti-PSD95 (124011, Synaptic Systems), mouse anti-SYP (sc-55507,

Abbreviations: DSS, detergent soluble synaptic membrane fraction; **PSD**, postsynaptic density; **RTCs**, receptors, transporters and channels; **TMT**, tandem mass tag; **TMHMM**, transmembrane helices hidden markov models

Santa Cruz Biotechnology), mouse anti-NMDAR1 (ab32915, Abcam), mouse anti VGLUT1 (135311, Synaptic Systems), mouse anti-VDAC1 (ab14734, Abcam) and rabbit anti-GAPDH (ab9485, Abcam). Detailed immonublotting conditions and antibody dilutions are provided in the Supporting Information Methods.

2.3 Proteolytic digestion of the synaptic membranes

The concentration of the Triton X-100 detergent was reduced to below the critical micellar concentration by a modified ethyl acetate extraction approach [9]. Briefly, ethyl acetate (water-saturated) was added to the samples in a 1:1 v/v ratio, gently swirled for 10 min and then centrifuged for 10 min at 13 000 rpm. The ethyl acetate layer was siphoned and the extraction was repeated twice. Membrane samples were reconstituted in urea buffer and sonicated. Protein amounts were estimated with the Pierce 660 protein assay.

Fifty micrograms of samples (2 × 50 μg each) were digested with trypsin (1:100 w/w) using the filter-aided sample preparation (FASP) as previously described with minor modifications [10, 11]. Tryptic peptides were recovered and the remaining material on the FASP filter was washed with 50 μL 50 mM TEAB buffer. This material was further digested with chymotrypsin (enzyme to protein ratio of 1:200 w/w based on the initial protein amount) for 2 h at 25°C with constant shaking at 300 rpm Doubly-digested tryptic-chymotryptic peptides were recovered separately as described for trypsin. All digests for label free analyses were desalted and concentrated with customized reversed-phase C18 tips. Lyophilized peptides were reconstituted in 5% formic acid and analyzed by LC-MS/MS (Supporting Information Methods).

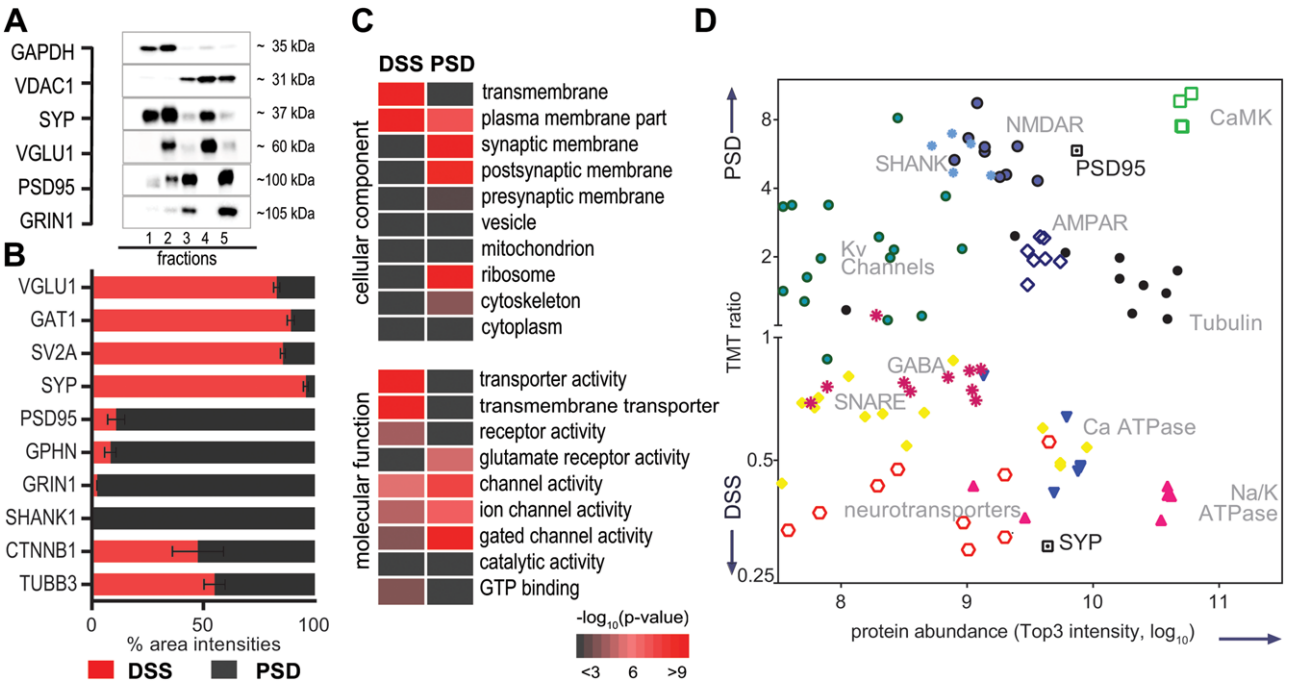

Figure 1. Proteomic profile of the synaptosomal fractions. (A) Immunoblot of representative proteins for the following biochemical fractionation stages: (1) whole cortex, (2) synaptosomes (3) synaptic membrane preparation, (4) detergent-soluble synaptic membrane preparation, DSS (5) postsynaptic density preparation, PSD. The cytosolic protein GAPDH is decreased in the fractionated stages. Presynaptic (VGLU1, SYP) and postsynaptic (GRIN1 and PSD95) protein markers are enriched in DSS and PSD preparations, respectively. Mitochondrial protein VDAC1 that is commonly co-fractionated in the enrichment steps. Postsynaptic markers GRIN1 and PSD. (B) Protein levels of representative synaptic markers were estimated from the area of the sum of the three most intense tryptic peptides (Top3) from label-free LC-MS analyses of the DSS and POST fractions. Enrichment of the presynaptic (VGLU1, GAT1, SV2A, SYP) and postsynaptic markers (PSD95, GPHN, GRIN1, SHANK1) are shown. (C) GO annotation of the highly enriched proteins illustrated as a heat map. Integral membrane components are enriched in both the DSS and PSD. Weak annotation enrichment for cellular components of the DSS fraction as these proteins are poorly annotated in available databases. However, annotation for molecular function of the DSS fraction showed enrichment of transporters that are associated with the presynaptic membrane region. The enriched PSD fractions are populated with postsynaptic membrane and ribosomal components. (D) Synaptosomal DSS and PSD peptides were labeled with stable-isotope TMT 6-plex reagents (129–131 and 126–128, respectively). Relative quantitation of 4177 proteins in the DSS and PSD fractions was obtained from the intensities of the TMT reporter ions. When the DSS to PSD ratio (TMT reporter ion intensities) was plotted against the relative protein abundance (Top3 peptide area intensities), major protein complexes (e.g., AMPAR and NMDAR) co-clustered.

2.4 Tandem mass tag derivatization and reversed-phase peptide fractionation

One-hundred micrograms of the tryptically digested samples were purified with reversed-phase C18 and labeled with TMT 6-plex according to the instructions supplied by the manufacturer. For the three biological replicates, the PSD and DSS preparations were labeled with the 126, 127, 128 channels and the 129, 130, 131 channels, respectively. All six samples were pooled, the peptides were separated by basic reversed-phase liquid chromatography [12] and 50 collected fractions were analyzed by LC-MS. Details of the procedure are essentially as described previously [13] and in the Supporting Information Methods.

2.5 Liquid chromatography tandem mass spectrometry

Peptides were analyzed by nano-LC-MS/MS as previously described with minor alterations [13]. Briefly, samples were analyzed on a linear trap quadrupole (LTQ) Orbitrap Velos coupled to an Agilent 1200 HPLC nanoflow system. HPLC solvent A consisted of 0.4% formic acid in water and solvent B consisted of 0.4% formic acid in 70% methanol and 20% isopropanol. The peptide mixture was loaded using 0.1% TFA onto a pre-column at a flow rate of 45 μL/min. After washing, the peptides were eluted by back-flushing onto an analytical column (16 cm x 50 μm i.d. and packed with a 3 μm C18 material). The peptides were eluted from the analytical column with the following solvent B gradient: 3 to 30% for 25 min, 30 to 70% for 25 min and 70 to 100% for 7 min solvent B at a constant flow rate of 100 nL/min.

The analyses were performed in a top15 CID and top10 HCD data-dependent acquisition mode for unlabeled and TMT-labeled peptides, respectively. The 10 or 15 most intense ions were isolated and fragmented by CID for peptide fragmentation alone; or HCD for peptide identification and relative quantitation of reporter ions for analyses of TMT-labeled peptides. Dynamic exclusion for selected ions was 60 s and a single lock mass at m/z 445.120024 was used for internal mass calibration [14]. The maximum ion accumulation time was set to 500 and 50 ms for MS^1 and MS^2 scans, respectively. Overfilling of the ion traps was prevented by automatic gain control set to 10^6 ions for a full Fourier transform MS scan and 5×10^4 ions for MS^2 HCD scans. Intact peptides were detected in the Orbitrap mass analyzer at a resolution of 60 000 with a signal threshold of 5000 counts for triggering an MS^2 event. HCD MS^2 spectra were acquired at a resolution of 7500 (Supporting Information Methods).

2.6 Protein identification

All $MSMS^2$ spectra were searched against UniProtKB/Swiss-Prot rat with MASCOT [15] and Phenyx [16] using mass tolerances of ± 10 ppm and ± 0.1 Da for precursor and fragment ions, respectively. One missed tryptic cleavage site and five for tryptic/chymotryptic digested samples were allowed. Oxidation of methionine was set as variable modification, whilst carbamidomethylation of cysteine residues was set as fixed modification. Additionally, TMT 6-plex labeling of peptide N-termini and lysine residues were added as fixed modifications for the labeled analyses. Thresholds were determined via the target-decoy approach [17] using a reversed protein database as the decoy by imposing 1% false discovery rate (FDR). The

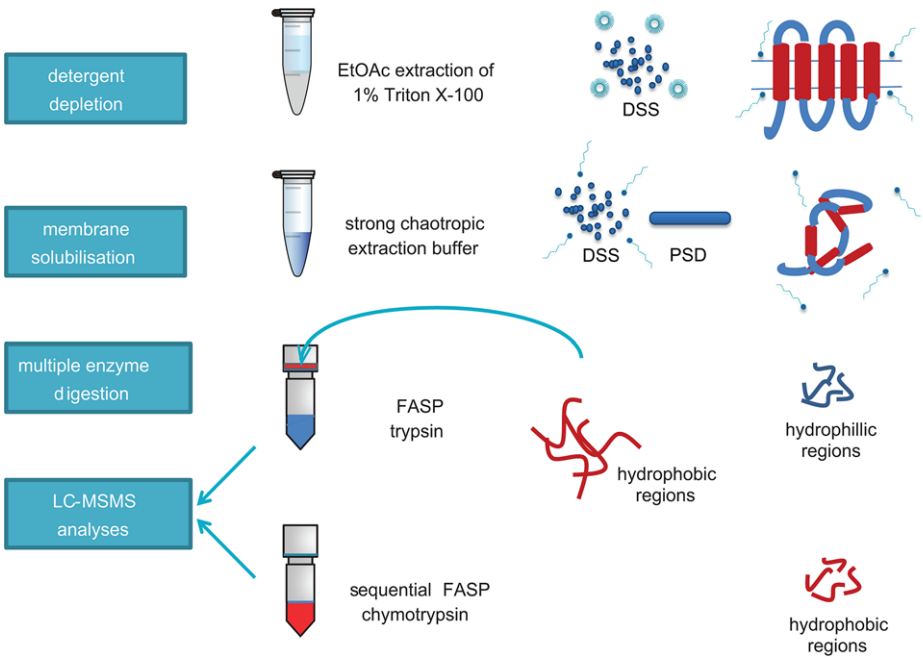

Figure 2. FASP protocol adapted for synaptic membrane proteins. A membrane-tailored sample preparation protocol for gel-free LC-MS analyses was implemented. Following synaptosomal fractionation, the DSS samples were solubilized in 1% Triton X-100. Triton X-100 micelles were depleted by ethyl acetate solvent extraction to below the critical micelle concentration (CMC). Insoluble samples (PSD) were solubilized in a strong chaotropic extraction buffer. The gel-free digestion was accomplished using trypsin and FASP. To recover hydrophobic regions of membrane proteins, a sequential chymotryptic digest was performed on the sample that remained on the filter.

validated proteins retrieved by the two search engines were merged, any spectral conflicts discarded and grouped according to shared peptides.

The mass spectrometry proteomic data have been deposited into the ProteomeXchange Consortium [18] via the PRIDE partner repository with the dataset identifier PXD001948.

2.7 Quantitative data analysis

For TMT 6-plex labeled samples, relative abundances of proteins were determined from the TMT reporter ions using Isobar [19]. For each identified protein a ratio of abundance between the DSS and PSD together with the corresponding *P*-values were calculated. Protein abundances were denoted as significantly different between DSS and PSD when both the technical and biological *P*-values were <0.05. Protein abundance ratios were calculated based on unique peptides only.

For label-free analyses, protein abundances were determined using the Top3 algorithm [20] as implemented in Skyline (v 3.1) [21] for peptide area calculation. The protein abundance is the sum of area intensities of the three most abundant peptides identified per protein (Supporting Information Methods).

2.8 Functional data analysis

Protein classes were annotated using PANTHER [22] and IUPHAR/BPS [23]. Enriched GO annotations were determined using Gorilla [24]. Significant GO terms were reported as determined by Fisher's exact test (*P*-value ≤ 0.05). Drug target terms were annotated using IUPHAR and DGIdb [23,25]. Curated gene-disease annotations were obtained from Comparative Toxigenomics database [26].

3 Results and discussion

3.1 DSS and PSD preparations

Enriched synaptosomal preparations from rat cortical material were prepared by differential sucrose gradient ultracentrifugation. The synaptosomes contained the presynaptic terminal enclosing the synaptic vesicles and mitochondria, the postsynaptic membrane and the postsynaptic density (PSD). The PSD preparation was characterized by the presence of the marker protein GRIN1 and DLG4/PSD95 (Fig. 1A). The DSS preparation was characterized by the presence of vesicular glutamate transporter 1 (VGLU1) and synaptophysin, (SYP), a marker protein that has not been shown to be present in the PSD fraction but in the synaptic junction.

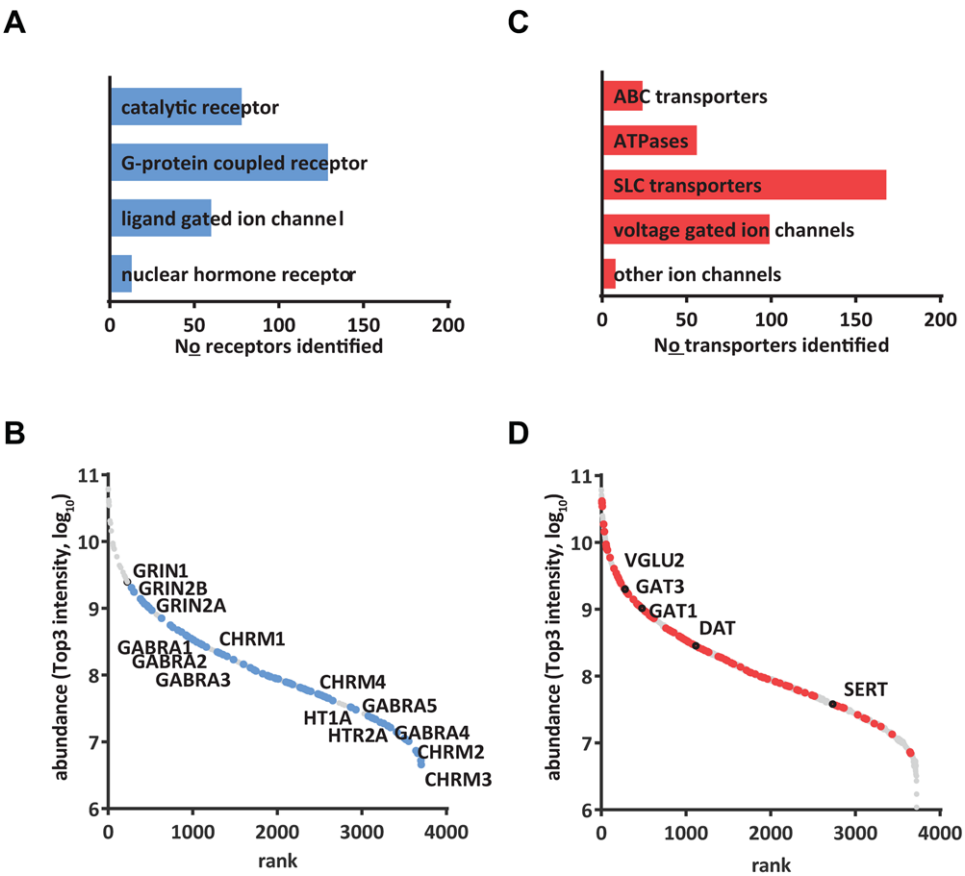

Figure 3. Synaptic proteome. LC-MS analyses of the synaptic membrane resulted in the identification of 4784 proteins. From the analyses, 274 receptors and 394 transporters/channels from major protein classes were identified (A and C). G-protein coupled receptors and solute carrier (SLC) transporters are well represented in the analyses. The range of protein abundance in the synaptic membrane of important neurotransmitter receptors and transporters in the synaptic membrane are illustrated (B and D).

Table 1. Receptors, transporters and channels not previously identified by mass spectrometry (not reported in the SynProt database)

	Protein	Accession	DSS/PSD	*P*-value	Description
(A) Detergent soluble synaptosomal fraction, DSS					
Receptors	ADCYAP1R1-5	P32215-5	2.14	2.2E–09	Pituitary adenylate cyclase-activating polypeptide type I receptor
	ADORA1	P25099	1.54	2.3E–03	Adenosine receptor A1
	ADRA1B	P15823	1.81	2.0E–02	Alpha-1B adrenergic receptor
	CHRM2	P10980	1.15	2.0E–02	Muscarinic acetylcholine receptor M2
	CNR1	P20272	1.44	8.0E–05	Cannabinoid receptor 1
	ERBB2	P06494	1.45	1.3E–03	Receptor tyrosine-protein kinase erbB-2
	GABRA5	P19969	1.52	3.8E–07	Gamma-aminobutyric acid receptor subunit alpha-5
	GABRD	P18506	1.36	1.1E–02	Gamma-aminobutyric acid receptor subunit delta
	PPARG-1	O88275-1	1.54	3.5E–05	Peroxisome proliferator-activated receptor gamma
	TAS1R2	Q9Z0R7	1.51	3.4E–02	Taste receptor type 1 member 2
	SLC10A4	Q5PT56	3.97	1.7E–06	Sodium/bile acid cotransporter 4
	SLC12A4	Q63632	1.46	2.3E–02	Solute carrier family 12 member 4
	SLC13A5	Q8CJ44	1.56	1.5E–02	Solute carrier family 13 member 5
	SLC15A1-1	P51574-1	1.45	3.9E–02	Solute carrier family 15 member 1
	SLC20A2	Q63488	1.61	1.8E–04	Sodium-dependent phosphate transporter 2
	SLC22A17	Q9P290	1.54	3.8E–02	Solute carrier family 22 member 17
	SLC22A23	Q9QZG1	2.14	1.4E–06	Solute carrier family 22 member 23
	SLC22A4	Q9R141	1.96	2.0E–07	Solute carrier family 22 member 4
	SLC29A3	Q80WK7	2.52	3.1E–05	Equilibrative nucleoside transporter 3
	SLC2A1	P11167	2.07	1.4E–07	Solute carrier family 2, facilitated glucose transporter member 1
	SLC31A1	Q9JK41	1.64	4.8E–17	High affinity copper uptake protein 1
	SLC35F6	Q5RKH7	2.33	4.3E–21	Solute carrier family 35 member F6
	SLC38A3	Q9JHZ9	1.57	4.7E–02	Sodium-coupled neutral amino acid transporter 3
	SLC6A4	P31652	2.71	4.2E–12	Sodium-dependent serotonin transporter
	SLC6A6	P31643	1.78	9.8E–05	Sodium- and chloride-dependent taurine transporter
	SLC7A10	P63116	1.72	9.8E–05	Asc-type amino acid transporter 1
	SLC7A5	Q63016	1.71	4.5E–03	Large neutral amino acids transporter small subunit 1
	SLC7A8	Q9WVR6	1.90	3.0E–06	Large neutral amino acids transporter small subunit 2
	SLCO3A1	Q99N02	1.61	3.9E–02	Solute carrier organic anion transporter family member 3A1
Channels	CACFD1	D4A9I3	1.98	3.1E–03	Calcium channel flower homolog
	CLCN3-1	P51792-1	1.57	4.8E–06	H(+)/Cl(-) exchange transporter 3
	CLCN5	P51796	1.46	7.1E–06	H(+)/Cl(-) exchange transporter 5
	CLIC6	Q811Q2	1.70	1.5E–06	Chloride intracellular channel protein 6
	CNGA1	Q62927	1.49	8.1E–04	cGMP-gated cation channel alpha-1
	KCNC4	Q63734	2.83	7.0E–03	Potassium voltage-gated channel subfamily C member 4
	KCNK18	Q6Q1P3	1.73	9.8E–12	Potassium channel subfamily K member 18
	KCNMB4	Q9ESK8	1.50	1.1E–02	Calcium-activated potassium channel subunit beta-4
	KCNN3	P70605	1.67	2.1E–06	Small conductance calcium-activated potassium channel protein 3
	KCNQ4	Q9JK96	1.55	3.4E–04	Potassium voltage-gated channel subfamily KQT member 4
	ORAI1	Q5M848	1.71	1.1E–02	Calcium release-activated calcium channel protein 1
	SCN1B	Q00954	2.82	2.3E–17	Sodium channel subunit beta-1
	SCN2B	P54900	1.37	2.1E–02	Sodium channel subunit beta-2
	SCN3B	Q9JK00	1.95	1.8E–04	Sodium channel subunit beta-3
	SCN4B	Q7M730	1.77	7.3E–06	Sodium channel subunit beta-4
	SCN8A-1	O88420-1	1.61	1.1E–03	Sodium channel protein type 8 subunit alpha
	Protein	Accession	PSD/DSS	*P*-value	Description
(B) Detergent resistant synaptosomal fraction, PSD					
Receptors	ADRB3	P26255	2.00	1.3E–02	Beta-3 adrenergic receptor
	CHRNA4-1	P09483-1	1.37	3.4E–02	Neuronal acetylcholine receptor subunit alpha-4
	EPHA6	P54758	1.65	6.2E–03	Ephrin type-A receptor 6

Table 1. Continued

	Protein	Accession	PSD/DSS	*P*-value	Description
	EPHA8	P29321	1.58	1.3E–06	Ephrin type-A receptor 8
	GLRA2-1	P22771-1	1.38	5.6E–03	Glycine receptor subunit alpha-2
	GPR176	Q64017	1.28	4.0E–02	Probable G-protein coupled receptor 176
	GRIK3-2	P42264-2	1.69	1.1E–02	Glutamate receptor ionotropic, kainate 3
	GRM4	P31423	1.48	1.9E–02	Metabotropic glutamate receptor 4
	ITGA7-3	Q63258-3	1.69	7.0E–07	Integrin alpha-7
	LPHN2-7	O88923-7	1.46	2.6E–02	Latrophilin-2
	NR1D2-1	Q63504-1	1.40	2.9E–02	Nuclear receptor subfamily 1 group D member 2
	TAAR5	Q5QD23	1.86	2.6E–02	Trace amine-associated receptor 5
	TAS1R3	Q923K1	1.64	8.0E–03	Taste receptor type 1 member 3
Transporters	SLC14A1-1	P97689-1	1.89	1.3E–06	Urea transporter 1
	SLC27A1	P97849	1.37	2.7E–03	Long-chain fatty acid transport protein 1
	SLC6A9-1	P28572-1	3.98	6.0E–17	Sodium- and chloride-dependent glycine transporter 1
	SLC7A2-1	B5D5N9-1	1.34	3.4E–02	Low affinity cationic amino acid transporter 2
	SLC8A1-7	Q01728-7	8.52	3.7E–05	Sodium/calcium exchanger 1
	SLC8A1-6	Q01728-6	2.06	2.0E–06	Sodium/calcium exchanger 1
Channels	ASIC2-2	Q62962-2	1.79	8.8E–07	Acid-sensing ion channel 2
	CACNA1I-1	Q9Z0Y8-1	2.34	6.1E–19	Voltage-dependent T-type calcium channel subunit alpha-1I
	KCNA3	P15384	1.72	1.8E–03	Potassium voltage-gated channel subfamily A member 3
	KCNA4	P15385	1.42	2.2E–02	Potassium voltage-gated channel subfamily A member 4
	KCNAB1	P63144	1.50	6.8E–04	Voltage-gated potassium channel subunit beta-1
	KCNB2	Q63099	1.67	5.6E–03	Potassium voltage-gated channel subfamily B member 2
	KCNG3-1	Q8R523-1	1.19	4.0E–02	Potassium voltage-gated channel subfamily G member 3
	KCNH2	O08962	1.83	8.7E–03	Potassium voltage-gated channel subfamily H member 2
	KCNJ9	Q63511	3.50	1.8E–03	G protein-activated inward rectifier potassium channel 3
	SCLT1-2	Q8CJ99-2	1.27	1.7E–02	Sodium channel and clathrin linker 1
	SCN3A	P08104	1.34	2.0E–02	Sodium channel protein type 3 subunit alpha

Only proteins that are significantly different (*P*-value ≤ 0.05) in DSS c.f. PSD are shown.

3.2 Membrane proteomics

A membrane-tailored sample preparation coupled to a gel-free liquid chromatography mass spectrometry was employed for the analyses of synaptosomal membrane protein preparations (Fig. 2, Supporting Information Fig. S1). Triton X-100 detergent-removal was coupled to filter-aided sample preparation (FASP) to ensure compatibility of the DSS samples with LC-MS. Solubilization of the detergent-insoluble PSD samples was achieved with a strong chaotropic extraction buffer.

Following tryptic digestion, DSS and PSD ($n = 3$ individual rat cortices) were analyzed by LC-MS and 2182 proteins were identified. To generate peptides from the hydrophobic protein regions, a sequential chymotryptic digest of the large, tryptically-digested peptides that remained on the filter was implemented. The principle is based on previously-described methods for the consecutive digestion on spin filters and the ability of chymotrypsin to generate peptides from the transmembrane domain (TMD) regions [27, 28]. Additional 966 proteins (Supporting Information Fig. S2A) with 44% predicted by TMHMM [29] to have transmembrane domains were identified (Supporting Information Fig. S2B, S2C, S2D).

3.3 Synaptosomal membrane proteome

Analysis of low-abundance synaptosomal proteins and relative quantitation of the proteins in the DSS and PSD fractions was achieved by multiplexing six samples and orthogonal peptide fractionation [30]. Analysis of the combined samples by two-dimensional gel-free LC-MS resulted in the identification of four proteins and the relative quantitation of a wide dynamic range of proteins (five orders of magnitude; $n = 4177$) (Supporting Information Table 2).

Combining the data from the TMT 6-plex labeling experiments with the unlabeled tryptic and sequential tryptic/chymotryptic data resulted in the identification of 4784 proteins (Supporting Information Table 4A). Relative to the whole rat genome, enrichment of integral transmembrane proteins (1245 proteins), receptors (297 proteins) and transporters (487 proteins) were 2.07-, 1.73- and 3.74-fold, respectively. Note, however, that there is a caveat with the chosen sample preparation in that proteins are co-fractionated that does not originate from the synapse. These include mitochondrial membrane proteins ($P = 3.21 \times 10^{-4}$) and axonal ($P = 2.29 \times 10^{-4}$) components. The ten most high-ranking proteins in the synaptosomal preparations are scaffolding proteins (ACTB, TUBB4), Ca^{2+}/calmodulin complex and the

sodium/potassium ATPases which are typically associated with synaptic membrane.

All data were also searched against a reference rat proteome database (27 815 entries) and 6365 proteins were identified (Supporting Information Table 4B). Most of the entries in this database are unreviewed, thus curated only SwissProt proteins (9626 entries) are described in the subsequent sections.

From the largest curated database for synaptosomal proteins (Synprot, Supporting Information Fig. S3) [1], a total of 158 RTCs and 291 transmembrane proteins have been previously identified in synaptosomal preparations. In contrast, our approach identified 657 RTCs and 1343 transmembrane proteins. This is about a four-fold increase in RTCs compared to all other data collected to date.

Here, 129 G-protein coupled receptors, 60 ligand-gated ion channels, 13 nuclear hormone receptors and 78 catalytic receptors were identified (Fig. 3A). To illustrate the dynamic range of neurotransmitter receptors, abundance was ranked based upon protein intensity areas (Fig. 3B). The neurotransmitter receptors, glutamate AMPA (GRIA2, rank 51) and NMDA (GRIN1, rank 387) are the most abundant; whereas serotonin (HTR2A, rank 3953) and muscarinic (CHRM4, rank 3,145) receptors are of lower abundance. The characterization of 274 receptors by LC-MS is a major advancement in neurobiological studies. In particular, neurotransmitter receptors as serotonergic, dopaminergic, cholinergic (neuronal and muscarinic), histaminergic, adrenergic, opioid, cannabinoid and trace-amine receptors were identified. As expected, the majority of the neurotransmitter receptors and other receptor types were observed in the PSD fraction.

From the in-depth characterization of the synaptosomal fractions, 394 transporters were identified. In particular, the membrane transporters of the solute carrier group (SLC transporters; 158 proteins) and the voltage gated ion channels (VGIC; 99 proteins) were observed. Other membrane transporters included 24 ATP-binding cassette (ABC) transporters, 56 ATPases, and 8 other ion channels (Fig. 3C). Of these, 65% had not been previously identified by LC-MS (Supporting Information Fig. S3A and S3B). Of considerable pharmacological interest are the 158 SLC and 24 ABC transporters, as these have important roles in the absorption, distribution, metabolism and elimination of diverse substrates [32]. Only 31 SLC and 5 ABC transporters were previously reported from high-throughput MS studies [1]. Further, neurotransmitter transporters such as vesicular glutamate transporters (VGLU1-3), excitatory amino acid transporters (EAAT 1–4), GABA transporters (GAT 1–3, BGT1, VGAT), glycine transporters (GlyT 1–2), monoamine transporters (DAT, SERT, VMAT2), adenosine transporters (ENT 2–3) and the acetylcholine transporter (VACHT) were identified.

Using an expertly curated resource for established/potential human drug targets [20, 22], a large number of the synaptic proteins ($n = 1781$) were classified as drug targets.

3.4 Detergent-soluble proteome of the synaptosome

As only a few proteomic studies have been performed on DSS (PSD-depleted, detergent solubilized samples), we performed a quantitative multiplexing experiment to investigate proteins distinct from the PSD (detergent-resistant synaptosomal preparations). The detergent-resistant synaptosomal fractions are the widely-used PSD preparations which showed proteins localized to the postsynaptic membrane as verified by immunogold electron microscopy [1]. Tryptic peptides from the three PSD and three DSS samples were labeled with stable-isotope tandem mass tag (TMT) reagents 126–128 and 129–131, respectively. The coefficient of variation between the animal triplicates was 8.1% (SD = 6.9%) and 8.5% (SD = 7.8%) for the DSS and PSD preparations, respectively (Supporting Information Fig. S4).

The proteomic profile of synaptosomal fractions is given in Fig. 1B. Enrichment of presynaptic markers was observed in the DSS while of postsynaptic markers was observed in the PSD. In agreement, immunoblotting (Fig. 1A) showed the presence of the presynaptic marker proteins (SYP and VGLU1) in the DSS fraction and postsynaptic marker proteins (PSD95 and GRIN1) in the PSD fraction.

From the quantitative experiment, 802 detergent soluble proteins were significantly different from that of the PSD preparations. Receptors, transporters and channels which were not previously identified by mass spectrometry and reported in SynProt Database are listed in Table 1.

The top five GO terms enriched in the DSS were substrate-specific/transmembrane transporter activity ($P = 2.99 \times 10^{-13}$, $P = 8.06 \times 10^{-13}$ respectively), vesicular components ($P = 1.82 \times 10^{-28}$), and transport/establishment of localization processes ($P = 4.24 \times 10^{-15}$, $P = 1.22 \times 10^{-13}$). To illustrate the distribution and dynamic range of cortical proteins in the synaptic membrane, protein abundance (Top3 intensity, $\log_{10}$ scale) was plotted against the relative ratio of the PSD c.f. DSS fractions in Fig. 1D. Interestingly, what we observed was that the PSD-to-DSS ratios and overall protein abundance for subunits of some known complexes (e.g., NMDAR, AMPAR) had the tendency to cluster together. This suggests that the fractionation conditions employed retain protein complexes. In this study, 163 transmembrane transporters (17 of which are neurotransmitter transporters) were identified DSS alone. In our and previous studies, the enrichment protocol by Philips and coworkers [4–6, 8] was used. While this method is not able to discriminate presynaptic from postsynaptic localization, our data indicates that the DSS fraction represents many constituents of the presynaptic region and this was particularly evident with the neurotransmitter transporters. Previous proteomic studies on the DSS fraction suggested that under-representation of receptors was due to an overall low abundance [3]. We propose, however, that this is not the case and the analytical proteomic approach used here is the crucial factor for the increase in the identification of receptors. For the first time, our study revealed that 50

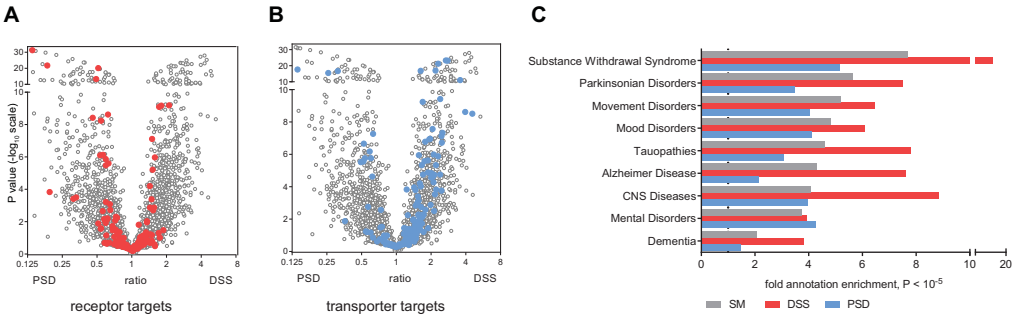

Figure 4. Function-based classification of the synaptic proteins. Identified proteins were annotated using the curated IUPHAR/BPS database for human drug targets. From the proteins identified, 37% (1781) are potential drug targets. Proteins identified in the DSS- and PSD-enriched fractions by TMT 6-plex were annotated using the curated IUPHAR/BPS database as human drug targets. Volcano plots were generated from the *P*-values ($p \leq 0.05$, $-\log_{10}$ scale) versus the DSS to PSD ratio ($\log_2$ scale) for transporters and receptor targets. Whilst the transporters are primarily higher in the DSS fractions, receptor targets were observed in both the DSS and the PSD. Representative human diseases were associated with synaptic membrane (SM) proteins using the disease annotation database DGIdb. Significant fold annotation enrichment for representative brain diseases for the SM, DSS and PSD fractions are shown in (B). The majority of the proteins linked to brain disorders and diseases are associated with the DSS fraction. To date, these proteins are poorly studied.

neurotransmitter receptors were identified in this fraction without prior immunoprecipitation.

Of the identified synaptic membrane proteins, 685 proteins were associated with brain disorders and/or brain diseases as assigned by MeSH descriptors [31]. Disease-gene associations were based on genomic, transcriptomic and proteomic studies on the sequence variation and expression changes associated with brain diseases and disorders [31]. Mutations of genes of identified PSD cortical proteins were previously linked to 133 diseases of the nervous system [33]. To complement the proteins from the PSD data, the current study showed that 156 of genes/proteins enriched in the DSS preparations were linked to diseases/disorders (Fig. 4C). Notable annotation enrichment relative to the DSS proteins includes dementia, schizophrenia, substance withdrawal syndromes, plus tauopathies including Alzheimer's disease.

4 Concluding remarks

The large number of membrane proteins identified herein, highlights the necessity of implementing membrane-tailored proteomic protocols to overcome the underrepresentation of RTCs in proteomics. The feasibility of our combined sample preparation and proteomic methodology is clearly shown by the high number of identified membrane proteins.

This resource and the analytical approach used to collect, analyze and define the data can be used by the neuroscience community to concomitantly identify and quantitate a large series of RTCs and other transmembrane proteins from the synaptosomal preparation. The quantitative techniques that were utilized (label-free or multiplexing) were highly amenable to sensitive and specific RTC analyses in a high-throughput fashion.

Last but not least, the proteomic data generated from this current study are highly-relevant for interpretation of previous studies and the design of future investigations on RTCs in particular of the so far poorly-studied DSS proteins in diseases and disorders of the CNS.

We thank Katja Parapatics, Dijana Vitko, and Juliane Weißer for their excellent technical assistance and experimental advice. We thank Dr. Rainer Pielot for his invaluable assistance in accessing the SynProt database. We thank Prof. Tibor Harkany and Dr. Volker Korz for their critical comments on the manuscript. Research in the Bennett laboratory is supported by the Austrian Academy of Sciences.

The authors declare that there is no conflict of interest.

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PROTEOMICS

Supporting Information for Proteomics

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André C. Müller, Elena L. Rudashevskaya, Jan Mulder, Keiryn L. Bennett, Gert Lubec

Mass spectrometric analysis of synaptosomal membrane preparations for the determination of brain receptors, transporters and channels

Supplementary

Mass spectrometric analysis of synaptosomal membrane preparations for the determination of brain receptors, transporters and channels

Table of Contents

Figure S1

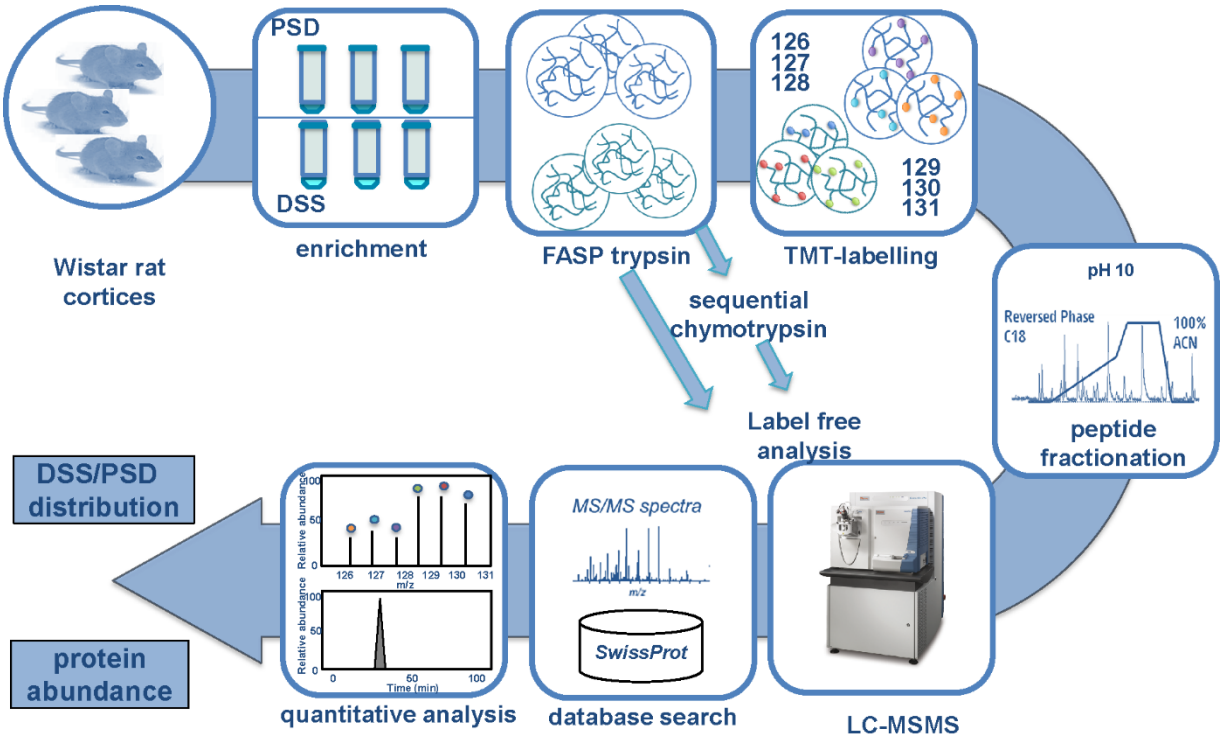

Figure S1. *Experimental Workflow.* Synaptosomes from the cortices of three Wistar rats were separated into detergent soluble synaptosomal (DSS) and postsynaptic density (PSD) fractions. A FASP protocol adapted for synaptic membrane proteins is coupled to a gel-free LCMS to allow analyses of synaptosomal fractions. The tryptic peptides from the DSS and PSD were labeled with TMT 6-plex reagents and fractionated by ion-pair reversed-phase chromatography at pH 10. Fractions were analysed by liquid chromatography tandem mass spectrometry using an LTQ Orbitrap Velos with high-collision dissociation fragmentation. Database searching was performed with search engines against the rat SwissProt protein database. Quantitative information were determined using the software tools, Proteome Discoverer and Isobar.

Figure S2

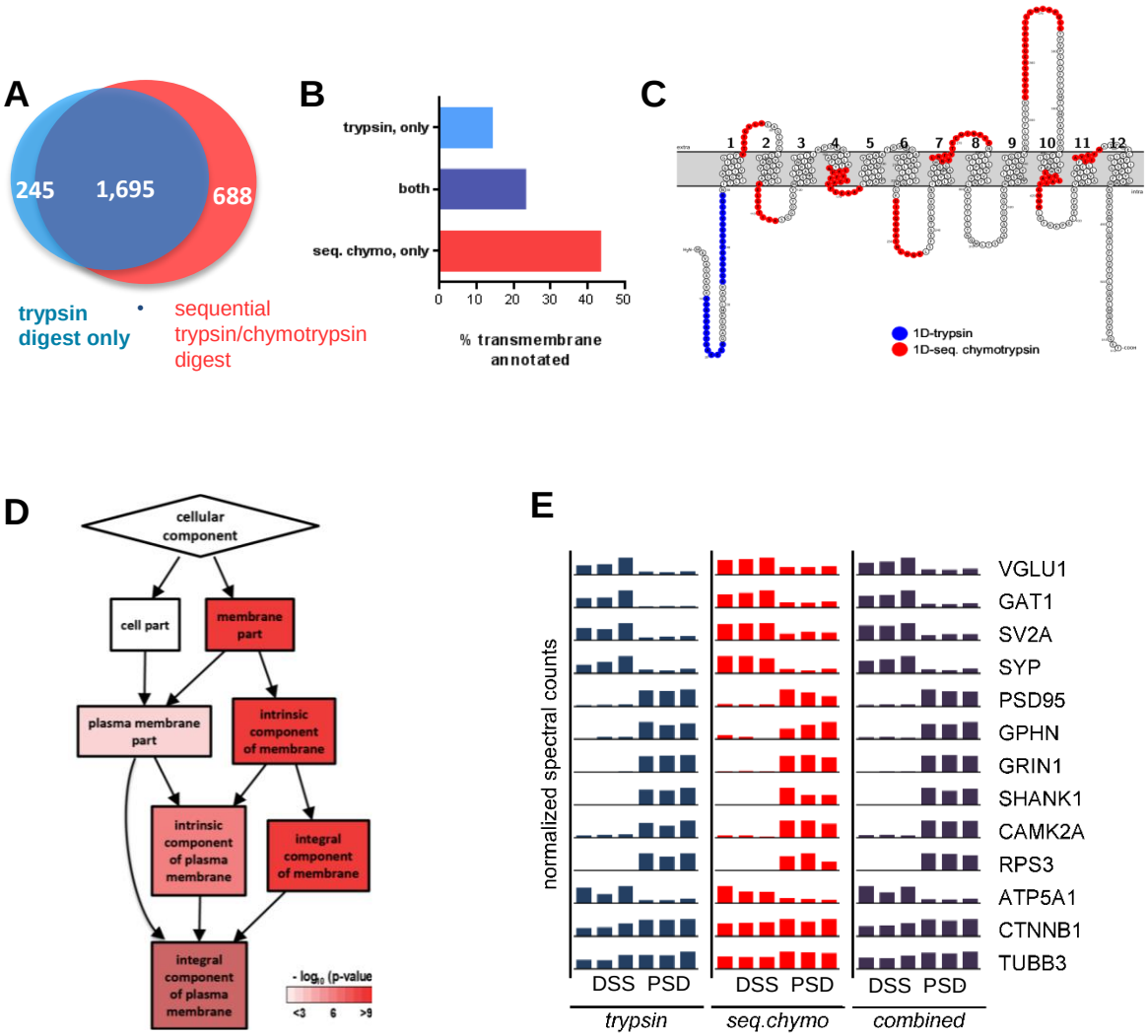

Figure S2 *Enhancement of Transmembrane Recovery with Sequential Chymotrypsin Digestion.* Chymotryptic digestion of the material that remained on the FASP filter following tryptic digestion resulted in the identification of an additional 688 proteins (A). Of these, 43% were annotated as transmembrane proteins (B). The marked increase in sequence coverage was more evident with integral membrane proteins (C). With this two-enzyme protocol, peptides are generated in both the hydrophilic (trypsin = blue) and transmembrane domain regions (chymotrypsin = red) of the proteins (*e.g.,* LAT1) (D). Proposed relative quantitation by combining the spectral counts from the label-free LCMS analyses of the tryptic and sequential tryptic/chymotryptic digests of the synaptosomal fractions. The relative protein levels of presynaptic (VGLU1, GAT1, SV2A, SYP), postsynaptic (PSD95, GPHN, GRIN1, SHANK1), ribosomal (RPS3), mitochondrial (ATPA1), cytosolic (CAMK2A, SOD1), synaptic junction (CTNNB1) and housekeeping (TUBB3) protein markers are shown for the DSS and PSD.

Figure S3

A

	Proteins	Receptor	Transporter	Channel	Transmembrane Proteins
Synaptosomal Fraction					
DSS, label free	2,072	123	163	82	397
PSD, label free	1,968	128	131	88	627
DSS/PSD, multiplex	4,218	194	190	140	1108
Total	4,784	274	237	149	1377

B

	Proteins	Receptor	Transporter	Channel	Transmembrane Proteins
SynProt					
DSS	540	5	56	9	153
PSD	1122	49	55	29	207
TOTAL	1342	52	76	30	291

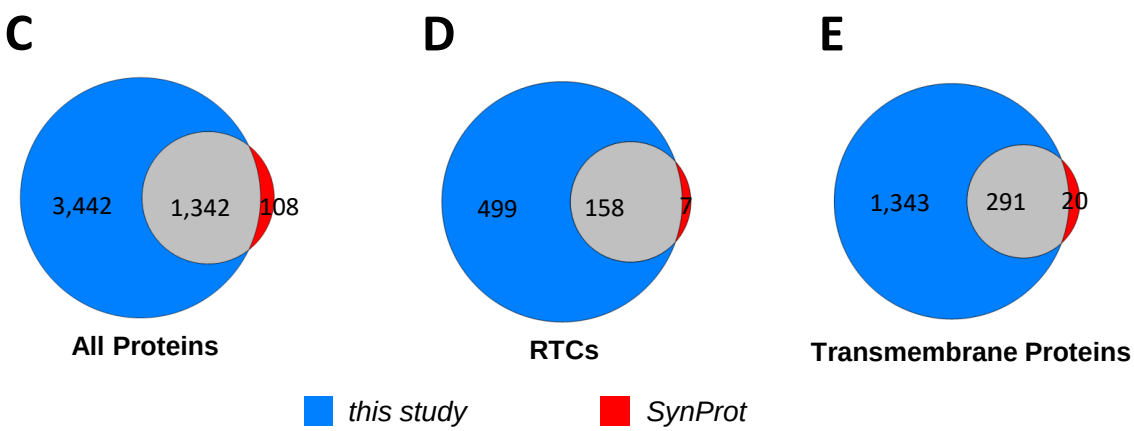

Figure S3. *Receptors, Transporters and Channels Protein Identifications.* (A) Proteins identified from the LCMS analyses of the DSS and PSD in both the label-free and DSS/PSD multiplexing experiments. Annotations for receptors, transporters, channels and transmembrane classes are also provided. For comparison, previously identified proteins collated in the SynProt database (v20150515) for the LCMS analyses of DSS (vesicles/active zone, 3 publications) and PSD (PSD, 14 publications) fractions (B). Venn diagrams show the overlap of the proteins identified in this study with the SynProt database for all proteins, RTCs (receptors, transporters and channels) and transmembrane proteins (C-E).

Figure S4

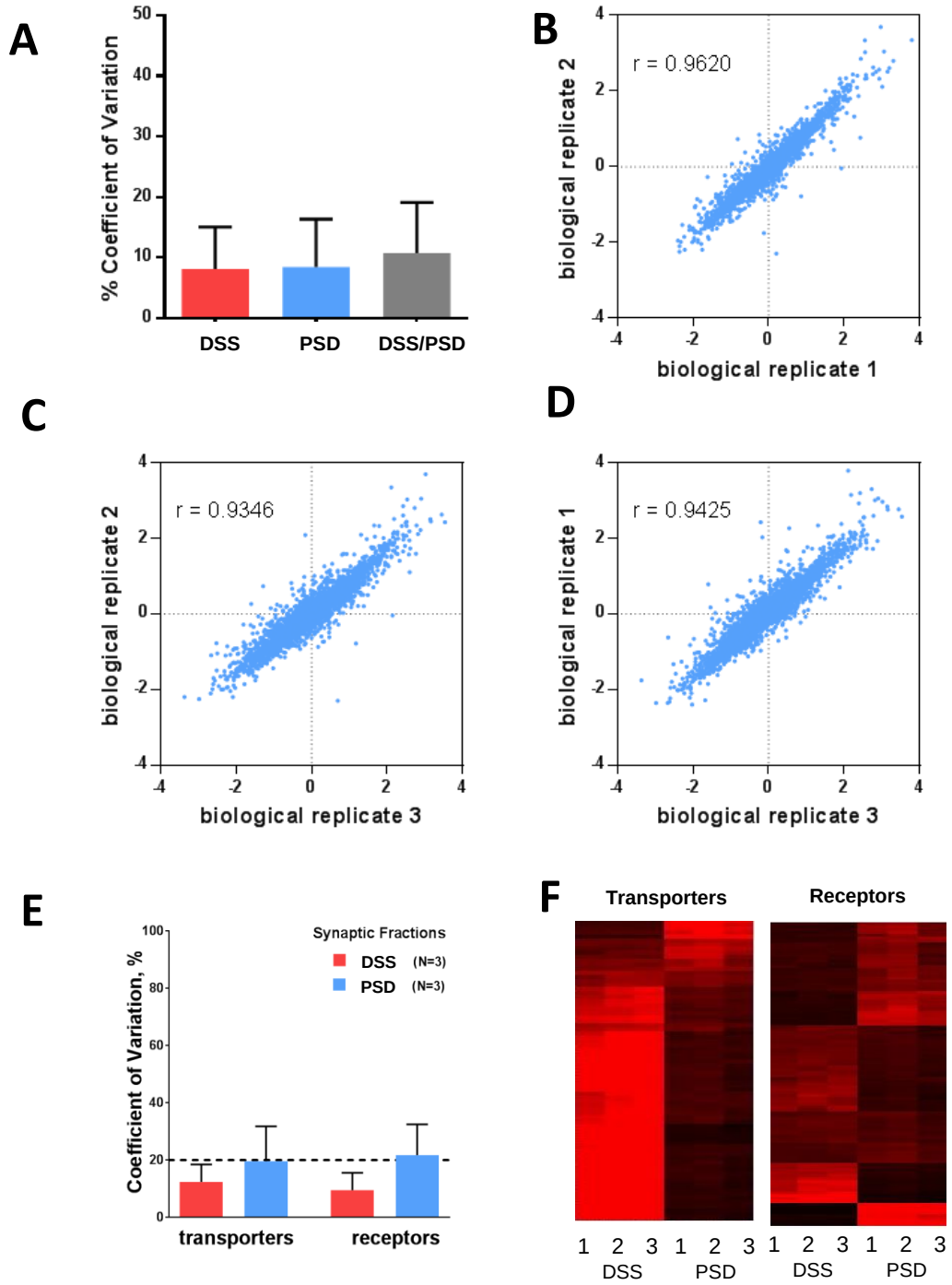

Figure S4. *Reproducibility of Biological Replicates in TMT Multiplexing Data.* The variability of biological triplicates in DSS, and PSD preparations and as DSS/PSD ratios illustrated as coefficient of variation (CV) (A). Biological and technical variability together is below 10%. Comparisons of the DSS/PSD ratios between animals shows good correlation (B-D). For the analyses of receptors and transporters, variability is shown as %CV (E) and visualized as heat map in (F).

2.2 Article 2: Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human DISC1 Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum.

Sialana FJ, Wang AL, Fazari B, Kristofova M, Smidak R, Trossbach SV, Korth C, Huston JP, de Souza Silva MA, Lubec G.
Front Mol Neurosci. 2018 Feb 6;11:26. doi: 10.3389/fnmol.2018.00026

Paper 2 demonstrates the use of quantitative proteomics to study the dynamics of the synaptic proteome in animal models of disease. Specifically, methods developed in *Paper 1* were used to investigate synaptic perturbations in the tgDISC1 rat model. This model is a transgenic rat overexpressing wild type human DISC1, modeling aberrant proteostasis of the DISC1 protein.

The paper focuses on the proteomic signatures of the tgDISC1 rat model to identify novel proteins networks and signaling pathways regulated by an increase of non-mutant DISC1 expression. We have previously reported evidence for a focal role of the DISC1 protein in striatal functions, and particularly on dopamine homeostasis in relation to behavioral changes using this rat model. In this study, we performed quantitative analyses (LCMS-based) of the proteins from the synapse-enriched membrane (synaptosomes) in the dorsal striatum of the tgDISC1 and wildtype.

The increase in DISC1 expression leads to changes in proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment is observed. Furthermore, dysregulation of DISC1 potentially modulates pathways brain specific pathways, particularly axonal guidance and dopamine signalling. DISC1-regulated proteins are also highly associated with neurodevelopmental and mental disorders. Our analysis demonstrates that the effects of a relatively small overexpression of non-mutant DISC1 can lead to profound changes in protein networks relevant in behavioral and neural processes and disorders.

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
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Quantitative Proteomics of Synaptosomal Fractions in a Rat Overexpressing Human DISC1 Gene Indicates Profound Synaptic Dysregulation in the Dorsal Striatum

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Disrupted-in-schizophrenia 1 (DISC1) is a key protein involved in behavioral processes and various mental disorders, including schizophrenia and major depression. A transgenic rat overexpressing non-mutant human DISC1, modeling aberrant proteostasis of the DISC1 protein, displays behavioral, biochemical and anatomical deficits consistent with aspects of mental disorders, including changes in the dorsal striatum, an anatomical region critical in the development of behavioral disorders. Herein, dorsal striatum of 10 transgenic DISC1 (tgDISC1) and 10 wild type (WT) littermate control rats was used for synaptosomal preparations and for performing liquid chromatography-tandem mass spectrometry (LC-MS)-based quantitative proteomics, using isobaric labeling (TMT10plex). Functional enrichment analysis was generated from proteins with level changes. The increase in DISC1 expression leads to changes in proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment. Canonical pathway analysis assigned proteins with level changes to actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling. DISC1-regulated proteins proposed in the current study are also highly associated with neurodevelopmental and mental disorders. Bioinformatics analyses from the current study predicted that the following biological processes may be activated by overexpression of DISC1, i.e., regulation of cell quantities, neuronal and axonal extension and long term potentiation. Our findings demonstrate that the effects of overexpression of non-mutant DISC1 or its misassembly has profound consequences on protein networks essential for behavioral control. These results are also relevant for the interpretation of previous as well as for the design of future studies on DISC1.

Keywords: DISC1, proteomics, synapses, animal model, dopaminergic system, axon guidance, striatum

INTRODUCTION

Disrupted-in-schizophrenia 1 (DISC1) is a gene originally identified as a translocation mutation in an extended Scottish pedigree where carriers suffered from diverse mental disorders comprising schizophrenia and affective disorders (Millar et al., 2000). Similarly, the DISC1 haplotype was associated with schizophrenia in a Finnish cohort (Hennah et al., 2003). A second family was later identified with a missense mutation and associated diverse clinical phenotypes (Sachs et al., 2005), and genetic association studies have supported association of DISC1 with mental disorders (Chubb et al., 2008). A role of the DISC1 gene for adaptive behavior was also suggested by various animal studies (Brandon and Sawa, 2011; Dahoun et al., 2017).

The DISC1 protein has features of a scaffold protein (Yerabham et al., 2013) and several subdomains have an intrinsic tendency to form high molecular multimers (Yerabham et al., 2017). Insoluble DISC1 protein has been identified in human post mortem brains with mental disorders (Leliveld et al., 2008), indicating that the DISC1 protein can be subject to aberrant proteostasis *in vivo*. For modeling the effects of aberrant proteostasis *in vivo*, a transgenic rat model overexpressing (approximately 11-fold) the full length, non-mutant human DISC1 gene (transgenic DISC1, tgDISC1 rat) was generated that exhibited perinuclear aggregates throughout the brain, accentuated in dopamine-rich regions such as in the striatum (Trossbach et al., 2016). The tgDISC1 rat exhibited phenotypes such as amphetamine supersensitivity, an increase in D2Rhigh receptors, and dopamine transporter mislocalization and dysfunction consistent with phenotypes observed in schizophrenia (Trossbach et al., 2016). Also, at the neuroanatomical level fewer dopaminergic neurons and projections into the dorsal striatum, as well as aberrant interneuron positioning was observed indicating subtle neurodevelopmental disturbance (Hamburg et al., 2016).

These findings, induced by aberrant proteostasis of the DISC1 protein, leading to its misassembly and perinuclear deposition, suggest an important role of the DISC1 protein and its correct assembly for protein networks involved in adaptive behavior. Such protein networks have been described both, at the protein and the genetic level. At the genetic level, Teng et al. (2017) carried out targeted sequencing of 59 DISC1 interactome genes and 154 regulome genes in psychiatric patients, identifying altered regulation of schizophrenia candidate genes by DISC1. In an attempt to dissect DISC1 function through protein-protein interactions based upon a yeast two-hybrid system along with bioinformatic methods, a comprehensive network around DISC1 was generated (Camargo et al., 2007). Using this iterative yeast two-hybrid system, a framework was provided to explore the function of DISC1, and interrogation of the proposed interactome has shown DISC1 to have protein-protein interactions consistent with that of an essential synaptic protein (Camargo et al., 2007). Current evidence suggests that DISC1 functions as a neuronal intracellular trafficking regulator that includes transport of neurotransmitter receptors, vesicles, mitochondria and mRNA, rendering synaptic regulation vulnerable to DISC1 dysfunction (Devine et al., 2016).

The objective of this study was to identify the proteomic signatures of the tgDISC1 rat model vs. its littermate wild type (WT) control to gain insights onto the DISC1-regulated proteins and downstream synaptic processes and to identify molecular circuitry regulated by relatively modest changes in expression level leading to DISC1 misassembly. Identification of changes in protein networks relevant for behavioral processes would raise the possibility for the DISC1 protein to represent a non-genetic interface with exogenous influences for mental disorders.

There is mounting evidence for a focal role of the DISC1 protein in striatal functions, and particularly on dopamine homeostasis in relation to behavioral changes (Trossbach et al., 2016; Wang et al., 2017). Therefore we chose to select proteins from the synapse-enriched membrane fractions (synaptosomes) from the dorsal striatum for this study. Differential proteomics by isobaric labeling (TMT10plex) enable multiplexed protein identification and quantitative analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). This allows the unbiased analyses of approximately 6000 proteins and targets synaptic proteins including receptors, transporters and channels that have been implicated in psychiatric disorders. Combining proteomics and bioinformatics approaches enabled a comprehensive view on the *in vivo* protein changes and the biological functions of DISC1.

MATERIALS AND METHODS

Animals

Previously described tgDISC1 Sprague-Dawley rats and WTs were used in this study (Trossbach et al., 2016). Briefly, full-length, non-mutant human DISC1 as transgene with the polymorphisms F607 and C704 were integrated into the pronuclei of Sprague Dawley rats. Ten male tgDISC1 rats and 10 male WT littermate control rats, aged 14–15 months (ZETT, Heinrich Heine University, Düsseldorf, Germany) were used. One WT rat and one tgDISC1 rat were derived from each pair of parents. The study was carried out in accordance with the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985), and the German Law on the Protection of Animals. It was approved by the Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV) NRW.

Preparation of Synaptosomal Fractions

Dorsal striata from fresh brains were dissected and stored at −80°C. Synaptosomal fractions from bilateral regions were prepared for individual animals (for tgDISC1 and WT; $n = 10$ each), using a microscale discontinuous sucrose gradient modified from previous protocols (Hahn et al., 2009; Sialana et al., 2016). Collected synaptosomes from 1.25/1.0 M sucrose interface were diluted with 10 mM HEPES, divided into two and pelleted at 15,000× *g* for 30 min. Pelleted synaptosomal samples were reconstituted in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 50 mM TEAB supplemented with protease inhibitors) for LCMS analyses and SDS buffer

(1.5% SDS, 100 mM NaCl, 20 mM Tris supplemented with protease inhibitors) for WB analyses and were sonicated for 1 h. Protein amounts were estimated using the Pierce 660 protein assay or BCA protein assay (ThermoFisher Scientific).

Proteolytic Digestion and Isobaric Labeling

Fifty micrograms of samples were digested with a Trypsin-LysC enzyme mixture (1:100 w/w, Promega) using the filter-aided sample preparation (FASP), as previously described, with minor modifications (Wisniewski et al., 2009). The resulting peptide samples were purified with reversed-phase C18 and labeled with TMT 10-plex according to the instructions supplied by the manufacturer. Two TMT-10plex experiments were performed, with each experiment consisting of five tgDISC1 and five WT animals (n = 10 biological replicates per group). For each TMT experiment, ten isobarically labeled peptide samples were pooled, the peptides separated by high pH reversed-phase LC into 100 time-based fractions and pooled into 25 samples (Gilar et al., 2005). The peptides were vacuum concentrated and reconstituted in 5% formic acid. Details of the procedure are essentially as described previously (Sialana et al., 2016) and in the Supplementary Figure S1.

Liquid Chromatography and Tandem Mass Spectrometry

Samples were injected onto a Dionex Ultimate 3000 system (ThermoFisher Scientific) coupled to a Q-Exactive Plus mass spectrometer (ThermoFisher Scientific, Schwerte, Germany). Software versions used for the data acquisition and operation of the Q-Exactive were Tune 2.8.1.2806 and Xcalibur 4. HPLC solvents were as follows: solvent A consisted of 0.1% formic acid in water and solvent B consisted of 0.1% formic acid in 80% acetonitrile. From a thermostated autosampler, 10 μL that correspond to 1 μg of the peptide mixture were automatically loaded onto a trap column (PM100-C18 3 μm, 75 μm × 20 mm, ThermoFisher Scientific, Austria) with a binary pump at a flow rate of 5 μL/min using 2% acetonitrile in 0.1% TFA for loading and washing the pre-column. After washing, the peptides were eluted by forward-flushing onto a 50 cm analytical column with an inner diameter of 75 μm packed with 2 μm-C18 reversed phase material (PepMap-C18 2 μm, 75 μm × 500 mm, ThermoFisher Scientific, Austria). For label free quantification (LFQ), the LCMS analyses was performed using a single-shot LCMS approach with 4-h gradient with LCMS parameters as described previously (Stojanovic et al., 2017).

The fractionated TMT10plex labeled peptides were eluted from the analytical column with a 120 min gradient ranging from 5% to 37.5% solvent B, followed by a 10 min gradient from 37.5% to 50% solvent B and finally, to 90% solvent B for 5 min before re-equilibration to 5% solvent B at a constant flow rate of 300 nL/min. The LTQ Velos ESI positive ion calibration solution (Pierce, IL, USA) was used to externally calibrate the instrument prior to sample analysis and an internal calibration was performed on the polysiloxane ion signal at m/z 445.120024 from ambient air. MS^1 scans were performed from m/z 375–1400 at a resolution of 70,000. Using a data-dependent acquisition mode, the 15 most intense precursor ions of all precursor ions with +2 to +7 charge were isolated within a 1.2 m/z window and fragmented to obtain the corresponding MS/MS spectra. The fragment ions were generated in a higher-energy collisional dissociation (HCD) cell at 32% normalized collision energy with a fixed first mass at 100 m/z and detected in an Orbitrap mass analyzer at a resolution of 35,000. The dynamic exclusion for the selected ions was 30 s. Maximal ion accumulation time allowed in MS and MS^2 mode was 50 and 100 ms, respectively. Automatic gain control was used to prevent overfilling of the ion trap and was set to 3×10^6 ions and 1×10^5 ions for a full Fourier transform MS and MS^2 scan, respectively.

Protein Identification and Quantification

All MS-MS^2 spectra were searched against UniProtKB/Swiss-Prot rat protein database version v 2016.04.14 (27,815 sequences, including isoforms). In addition, sequences of the human DISC1 protein and 11 isoforms produced by alternative splicing with the polymorphisms F607 and C704 were appended to the rat database. All spectra files were processed in Proteome Discoverer 2.1 (Thermo Scientific, Germany) platform with Mascot using mass tolerances of ±10 ppm and ±0.02 Da for precursor and fragment ions. One missed tryptic cleavage site was allowed. Oxidation of methionine was set as variable modification, whilst carbamidomethylation of cysteine residues, TMT 10-plex labeling of peptide N-termini and lysine residues were set as fixed modification. Thresholds were determined via the target-decoy approach using a reversed protein database as the decoy by imposing 1% false discovery rate (FDR). Label-free quantitation was implemented using the Minora feature of Proteome Discoverer 2.2. The following parameters are used: maximum retention time alignment of 10 min with minimum of S/N of 5 for feature linking mapping. Abundance were based precursor/peptide area intensities. Normalization was performed such that the total sum of the abundance is the same for all sample channels. Imputation was performed by replacing the missing values with random values from the lower 5% of the detected values. For TMT 10-plex labeled samples, relative abundances of proteins were determined from the TMT reporter ions without imputation. Protein abundance ratios were calculated based on unique and razor peptides. Relative protein levels were determined from the sum of the reporter ion intensities per quantitative channel that correspond to each biological animal replicate.

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2014) partner repository with the dataset identifier PXD008123.

Bioinformatics

Quantitative data were analyzed using Perseus statistical package (version 1.5.1.6; Tyanova et al., 2016). Statistical significance of differences in protein levels between the groups were evaluated using a two-sided T-test with $P < 0.05$ (either Student's or Welch's as required). Enrichment of GO annotations were

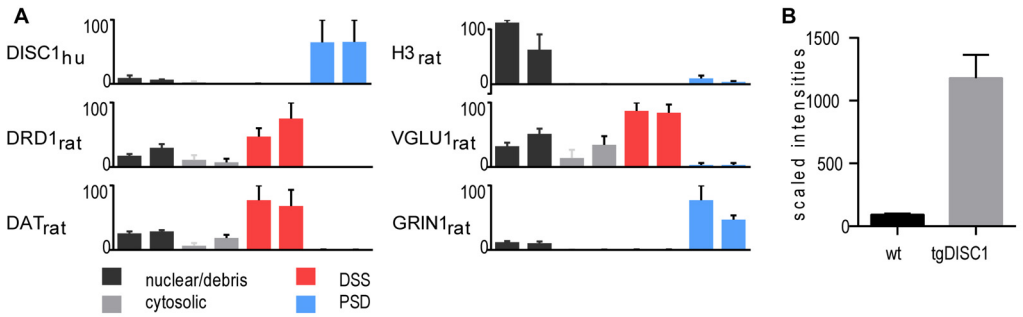

FIGURE 1 | Proteomic profile of the transgenic DISC1 (tgDISC1) fractions. **(A)** Protein levels of representative proteins for the following biochemical fractions of the dorsal striatum: nuclear/debris, cytosolic, detergent-soluble synaptosomal preparation (DSS), postsynaptic density (PSD) preparation. Protein levels of representative synaptic markers were estimated from label-free LCMS analyses. Protein levels of the nuclear (H3), presynaptic (VGLU1) and postsynaptic (GRIN1) protein markers are enriched in nuclear/debris, DSS and PSD preparations, respectively. The majority of the human Disrupted-in-schizophrenia 1 (DISC1) protein was enriched in the PSD preparations. The level of overexpression is approximately 10-fold higher than the endogenous DISC1 protein in the whole synaptosomes **(B)**.

performed on the significant proteins using GOA database (v30.08.2017) using the ClueGO via the Cytoscape platform (Bindea et al., 2009; Huntley et al., 2015). To reduce redundancy of GO terms the fusion option was selected. Enriched GO terms (Benjamini–Hochberg P-value < 0.05) are functionally grouped into networks linked by their kappa score level ($\geq$0.40). Functionally related groups partially overlap and only the most significant terms per group are labeled. Pathway analyses on the significant proteins were performed through the use of IPA (Ingenuity® Systems[1]). The differentially expressed genes were categorized to related canonical pathways. Only those experimentally observed or highly predicted molecules and/or relationships from tissues and cells from the nervous system were considered. The top enriched categories of canonical pathways with a P-value $< 10^{-3}$ as well as representative differentially expressed proteins in each canonical pathway is reported. Curated gene-disease annotations were obtained from Comparative Toxigenomics database (Davis et al., 2015). The IPA regulation z-score algorithm was used to predict biological functions that are expected to be activated (z-score ≥ 2; $P \leq 0.05$). The z-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset.

Immunoblotting

The following antibodies were used according to the instructions supplied by the manufacturer: mouse anti-PSD95 (124011, Synaptic Systems), mouse anti-SYP (sc-55507, Santa Cruz Biotechnology), rabbit anti-NMDAR1 (ab32915, Abcam), mouse anti-VGLUT1 (135311, Synaptic Systems), rabbit anti-GAPDH (ab9485, Abcam), rabbit anti-DAT1 (ab111468, Abcam) and mouse anti-huDISC1 (3D4, Korth lab; Ottis et al., 2011). Immunoblot data were normalized to corresponding whole-lane densitometric volumes of protein-stained membranes (Welinder and Ekblad, 2011). Immunoblotting conditions were as previously described (Sialana et al., 2016) and antibody dilutions are provided in the Supplementary Table S1.

[1]www.ingenuity.com

RESULTS

In the current study, a high-throughput proteomic approach was employed to generate a comprehensive view of the *in vivo* protein changes in striatal synaptosomes of the tgDISC1 rat model (experimental workflow, Supplementary Figure S1). Methodologically, tissue fractionation was initially performed on the dorsal striatum of tgDISC1 rats to determine the subcellular expression of tgDISC1 and which enrichment steps would be employed in this study (Phillips et al., 2001; Sialana et al., 2016). Dorsal striata of tgDISC1 rats were fractionated into nuclear/debris, cytosolic, detergent soluble synaptosome (DSS) and postsynaptic density (PSD) preparations. LCMS-based proteomic analyses of the biochemical fractions resulted in the identification and LFQ of 5002 protein groups (Supplementary Data 1). Distribution of the nuclear (H3), presynaptic (VGLU1) and postsynaptic (GRIN1) protein markers enriched in nuclear/debris, DSS and PSD preparations is given in **Figure 1A**. Although DISC1 was observed in all preparations, the majority of the human DISC1 protein was enriched in the Triton-X100-resistant PSD fractions. This is in agreement with previous immunoblotting studies of DISC1 in adult rats (Hayashi-Takagi et al., 2010). We have previously shown that dopaminergic pathways are modulated in the striatum of the tgDISC1 rat (Trossbach et al., 2016). Taking into account that dopamine receptor 1 and the dopamine transporter were highly enriched in the DSS preparations (**Figure 1A**), it was decided to study the *whole synaptosome* for quantitative proteomics experiments. Immunoblots of postsynaptic (GRIN1 and PSD95) and presynaptic (VGLU1 and SYP) proteins show enrichment of synaptosomal proteins on the biochemical fraction (Supplementary Figure S2). The level of overexpression is approximately 10-fold higher than

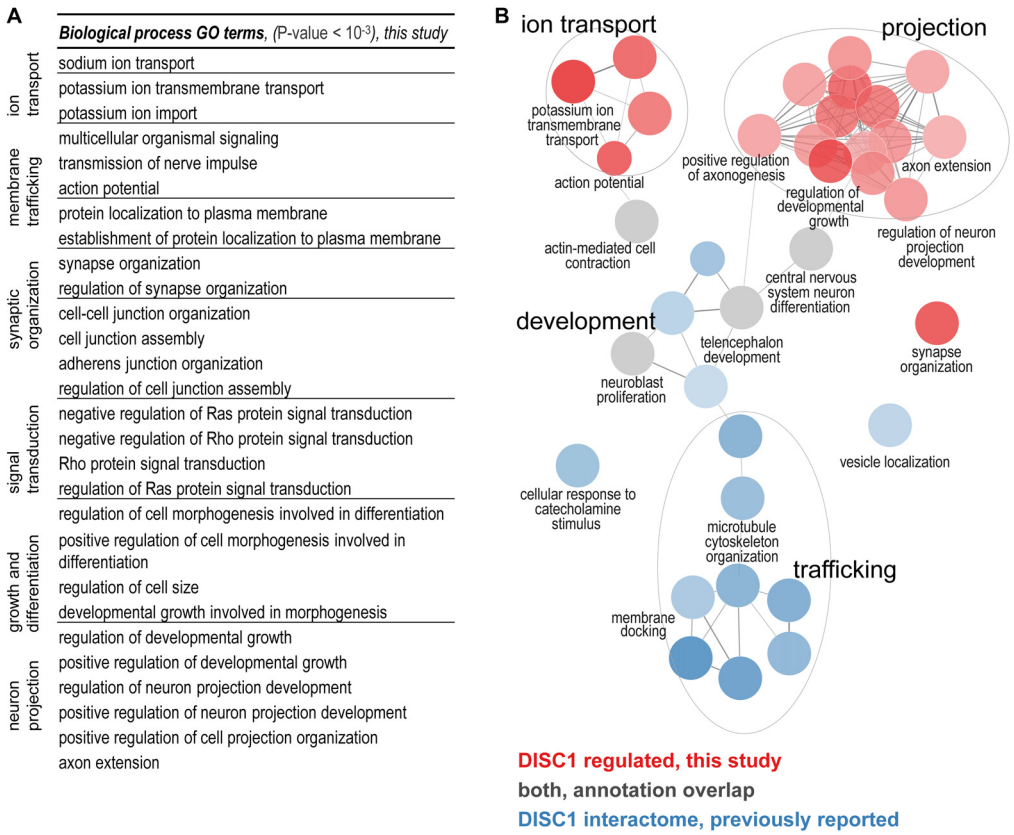

FIGURE 2 | (A) Biological processes regulated by DISC1. Enriched GO terms (*P*-value < 10^{-3}) are functionally grouped. The synaptic components terms as well as membrane trafficking, ion transport, synaptic organization and neurodevelopment processes are well represented. **(B)** Comparison of the DISC1-regulated proteins and the previously reported interacting proteins. In the current study ion transport, projections and synaptic organization were novel findings. Developmental processes from previous studies were confirmed (gray GO enrichment analyses was performed using ClueGO. Enriched GO terms (Benjamini–Hochberg *P*-value < 10^{-3}) are functionally grouped into networks linked by their kappa score level (≥0.40). Functionally related groups partially overlap and only the most significant terms per group are labeled.

the endogenous DISC1 protein in the whole synaptosomes (**Figure 1B**).

DISC1 Regulated Proteins—Proteomic Profiling of Striatal Synaptosomes

An expression proteomics experiment was performed to identify the proteins potentially regulated by DISC1. Synaptosomal fractions of bilateral dorsal striata of 10 wt and 10 tgDISC1 rats using TMT10plex were analyzed in two separate 10-plex experiments (5 tgDISC1 and 5 WT). In total, 7227 protein groups were identified (Supplementary Data 2) including 252 receptors and 672 transporters/channels. Out of the 6153 quantifiable protein groups, 213 proteins were statistically different between the tgDISC1 and WT rats (Supplementary Table S2, Supplementary Data 3). Protein levels were considered statistically different between groups when $P \leq 0.05$ using a two-sided *T*-test (either Student's or Welch's as required). Given the large number of comparisons made and the possibility of Type 1 error, the *p* values given cannot be interpreted in terms of "significance", but rather as "measures of effect".

As we used a good number of biological replicates for TMT-based proteomics (10 animals per group), we opted to use *T*-test that performs "individual proteins-based" hypotheses test (*T*-test) rather than a background "all-proteins-based" hypothesis test (FDR). TMT-based proteomics experiments are sensitive and precise but quantification is known to undergo ratio compression (Ow et al., 2011). The values from FDR corrections depend on effect size; smaller differences yield higher P-corrected (q-values); thus only two proteins passed the corrected thresholds. An additional filter is applied when enrichment analyses (GO annotation, IPA) is employed. Slight differences in the levels of multiple proteins should cluster relevant processes and the proteins from the top enriched processes/pathways are of higher emphasis (Pascovici et al., 2016).

Immunoblotting analyses of DAT1, GRIN1 and DISC1 of WT and tgDISC1 indicated that the direction of fold differences measured by TMT-proteomics and western blotting (Supplementary Figure S3) was consistent.

A

Canonical Pathways	P-value	Proteins
Signaling by Rho Family GTPases	4.57E-06	MYL6, ARHGEF4, RAF1, ROCK1, GNAZ, FGFR3, DES, PIK3CA, ARPC1B, CDH20, GNAS, ITGA3
Axonal Guidance Signaling	1.07E-04	MYL6, LINGO1, RAF1, ABLIM2, EPHA6, ROBO2, ROCK1, GNAZ, FGFR3, SEMA7A, PIK3CA, ARPC1B, GNAS, ITGA3
RhoGDI Signaling	2.57E-04	MYL6, ARHGEF4, ROCK1, GNAZ, ARPC1B, CDH20, GNAS, ITGA3
Ephrin Receptor Signaling	2.69E-04	RAF1, EPHA6, ROCK1, GNAZ, ARPC1B, GNAS, ITGA3, GRIN1
Actin Cytoskeleton Signaling	3.47E-04	MYL6, ARHGEF4, RAF1, ROCK1, CSK, FGFR3, PIK3CA, ARPC1B, ITGA3
Gαq Signaling	8.71E-04	RAF1, ADRA1A, ROCK1, CSK, FGFR3, PIK3CA, GNAS
Dopamine-DARPP32 Feedback in cAMP Signaling	9.33E-04	CACNA1A, ADCY3, KCNJ4, KCNJ11, GNAS, KCNJ12, GRIN1

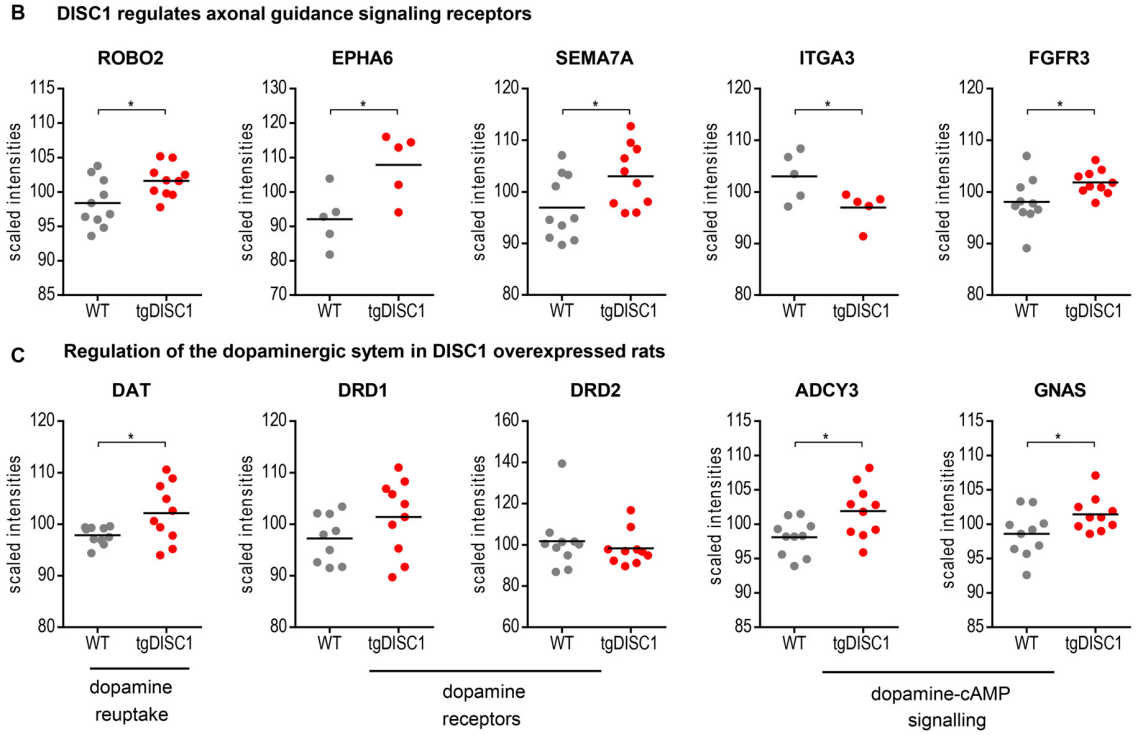

FIGURE 3 | Pathways regulated by DISC1. Significantly enriched canonical pathways (Fishers' exact test, $P < 10^{-3}$, IPA) of the proteins altered in by tgDISC1 rats in the dorsal striatum **(A)**. Representative proteins from the dopaminergic **(B)** and axonal guidance signaling pathway **(C)** are shown. Values represent $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared using two-sided *T*-tests.

Functional Classification of Proteins Modulated in tgDISC Rats

The biological functions of the 213 proteins with highly different protein level changes between wildtype and tgDISC1 rats were explored using GO enrichment analyses. Enrichment of synaptic components such as axons, dendritic spines, membrane rafts, neuron projection membrane, and the ion channel complex were revealed (Supplementary Table S3, Supplementary Figure S4). The voltage gated ion channels were the major protein classes represented (Supplementary Table S4). The results suggest that the modest overexpression of the full-length human DISC1 alters proteins linked to synaptic processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment (**Figure 2A**).

Functional Comparison of the DISC1 Regulated Proteins to Known Interacting Proteins

To determine the biological functions unique to DISC1 regulated proteins, we performed enrichment analyses for the

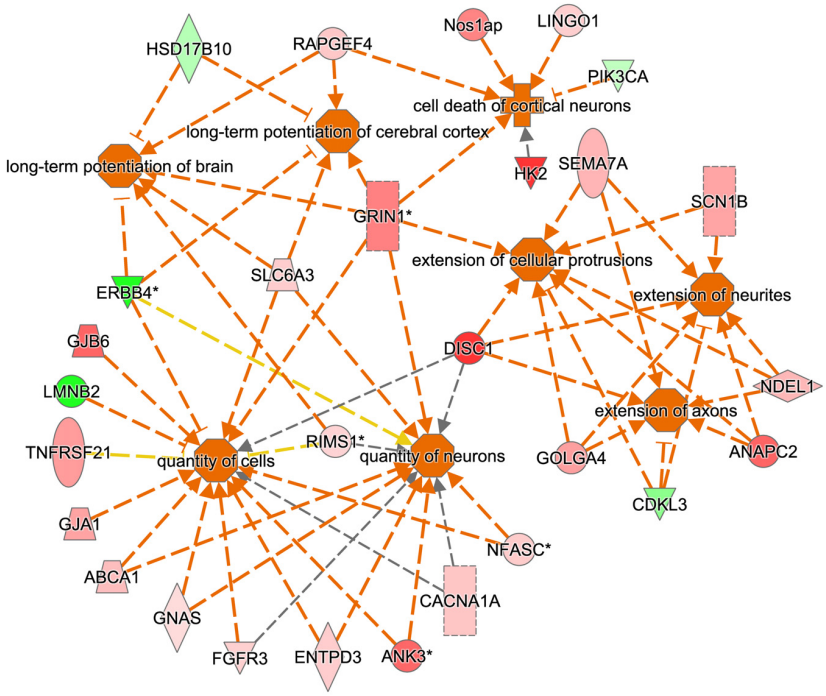

FIGURE 4 | Predicted biological functions of the tgDISC1 regulated proteins as evaluated by IPA. The IPA regulation *z*-score algorithm was used to predict the activation of biological functions in tgDISC1 rats relative to wild type (WT) according to our proteomics data. The network displays functional interactions between proteins (z-score ≥ 2 and p-value ≤ 0.05). Dashed lines indicate direct or indirect interactions. Proteins up-regulated in tgDISC1 rats are colored in shades of red; proteins down/regulated are colored in green.

DISC1 regulated proteins in comparison to previously reported interacting proteins (Camargo et al., 2007; Boxall et al., 2011; Bradshaw and Porteous, 2012; Thomson et al., 2013) as compiled by a recent study (Teng et al., 2017). Using ClueGO, 36 biological processes with strong enrichment ($P < 10^{-6}$) were revealed (**Figure 2B**; Supplementary Figure S5). The clusters of biological processes exclusive to the proteins regulated by DISC1 include: "regulation of neuron projection development", "positive regulation of axonogenesis", "action potential/potassium ion transport and synapse organization". Terms associated with microtubule development and neuronal transport were highly represented in the DISC1-interacting proteins. Biological processes such as "CNS differentiation" and "telencephalon development" were enriched in both, DISC1 regulated and interacting protein data sets.

Prediction of Canonical Pathways and Biological Function

To investigate the molecular mechanisms modulated by DISC1, data were analyzed through the use of Ingenuity Pathway analysis (IPA; Ingenuity® Systems[2]). The differentially expressed proteins were categorized to related canonical pathways. Canonical pathway analysis assigned proteins with level changes to actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI-, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling (Fisher's exact test, $P < 10^{-3}$, **Figure 3A**, Supplementary Figure S6). Only robustly predicted or experimentally observed molecules and/or relationships from tissues and cells from the nervous system were considered. Receptors from the axonal guidance signaling and the dopamine-DARPP32 feedback from the cAMP signaling canonical pathway are illustrated in **Figures 3B,C**).

The IPA regulation *z*-score algorithm was used to predict biological functions that are expected to be activated in tgDISC1 rats rather than in wildtype (positive *z*-score) according to own proteomics data (z-score ≥ 2; $P \leq 0.05$). The z-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset. From the expression data of the regulated proteins, the following processes are predicted to be activated: "activation regulation of cell quantities", "neuronal and axonal extension", "long term potentiation" and "apoptosis" (**Figure 4**, Supplementary Table S5).

Annotation of the DISC1 altered protein levels revealed that 54 proteins are associated with mental disorders and/or nervous system diseases as implemented by the Comparative Toxicogenomics Database (CTD; Davis et al., 2015). Disease-gene associations were based on genomic, transcriptomic and proteomic studies on the sequence variation

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and expression changes associated with brain diseases and disorders. Over-represented disease-protein associations (Fishers' exact test, $P < 0.05$) include: neurodevelopmental disorders, autistic disorders, schizophrenia spectrum, anxiety disorders, substance-related disorders (e.g., cocaine) and intellectual disability (**Table 1**). In particular, the schizophrenia-associated proteins including dopamine transporter 1 (SLC6A3), receptor tyrosine-protein kinase erbB-4 (ERBB4), glutamate ionotropic receptor NMDA type subunit 1 (GRIN1), membrane associated guanylate kinase WW and PDZ domain containing 2 (MAGI2) and regulator of G-protein signaling 12 (RGS12) were also regulated by DISC1 (Mateos et al., 2006; Silberberg et al., 2006; Xu et al., 2011; Koide et al., 2012; Guipponi et al., 2014; Jaros et al., 2015; Zhang et al., 2015; Li et al., 2017).

DISCUSSION

By the use of quantitative proteomics of synapse-enriched membrane (synaptosome) fractions of the dorsal striatum of the tgDISC1 rat, we have identified novel protein networks and signaling pathways regulated by an increase of non-mutant DISC1 expression or DISC1 misassembly. These results suggest that the DISC1 protein and its disturbed proteostasis can have an effect on mental disorder-relevant protein networks independent of genetic mutations. Likely, multiple exogenous or endogenous factors other than overexpression could lead to a failure of DISC1 proteostasis, such as exposure to high dosages of dopamine or other oxidants, making DISC1 protein an oxidation "sensor" (Atkin et al., 2012; Trossbach et al., 2016).

In the tgDISC1 rat, an about 11-fold overexpression, leading to DISC1 misassembly, changed proteins and synaptic-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment is observed. Furthermore, dysregulation of DISC1 potentially modulates pathways including actin cytoskeleton, Gαq, Rho family GTPase and Rho GDI-, axonal guidance, ephrin receptor and dopamine-DARPP32 feedback in cAMP signaling associated with the synaptic pathologies. DISC1-regulated proteins are also highly associated with neurodevelopmental disorders, autistic disorder, schizophrenia spectrum, anxiety disorders, substance-related disorders and intellectual disability (**Figure 5**).

Previously known DISC1-protein interactors have been reported to modulate synaptic processes. The current study revealed that DISC1 regulates an array of synaptic proteins and processes that complements previous protein interaction results (Supplementary Figure S7). Proteins that were previously reported to interact with DISC1 (Millar et al., 2003; Camargo et al., 2007) were also modified in the current study in the tgDISC1 rat. These include microtubule proteins pericentrin (PCNT), GRIP1 associated protein 1 (GRIPAP1), microtubule associated protein 1A (MAP1A), nudE neurodevelopment protein 1 (NDEL1) and microtubule-actin crosslinking factor 1 (MACF1) that are involved in neuronal cytoskeleton organization and membrane transport processes.

Dysregulation of DISC1 was reported to modulate glutamatergic and dopaminergic systems as previously reviewed (Hayashi-Takagi et al., 2010; Ramsey et al., 2011; Dahoun et al., 2017). Own results herein show that NMDAR1 is increased in the striatum of the tgDISC1 rat. A relationship between NMDAR1 and DISC1 has been shown, as knockdown and antagonists of NMDAR1 reduced numbers of synapses and synaptic DISC1 mainly in the striatum (Ramsey et al., 2011). Further, the DISC1 interactor GRIPAP1 is increased in the tgDISC1 rat. GRIPAP1 controls the AMPA receptors/GRIP-complex transport to the synapse by NMDA receptor activation (Ye et al., 2000).

As shown by MS, dopamine transporter levels were highly increased in the tgDISC1 rats, consistent with own previous studies by immunoblotting (Trossbach et al., 2016). Whereas levels of dopamine receptors 1 and 2 were not significantly altered, pathway enrichment analyses (**Figure 3C**) suggest that proteins (e.g., ADCY3, GNAS) from the dopamine-DARPP32 feedback of the cAMP signaling canonical pathway, may be involved in modulation of the known dopaminergic deficits in tgDISC1. Adenylate cyclase ADCY3 as a downstream effector of dopaminergic pathways catalyzes the formation of cAMP in response to G-protein signaling.

TABLE 1 | Disease-protein association of the DISC1 regulated proteins.

Disease name	*P*-value	Proteins
Neurodevelopmental disorders	1.02E-07	ANK3, ASIC2, CADM1, CTTNBP2, DISC1, GJA1, GNAS, GRIN1, KCNA2, KCNMA1, RIMS1, ROBO2, SCN2A, SLC4A4, SLC6A3, STAMBP, TCN2
Mental disorders	5.11E-07	ANK3, ASIC2, CADM1, CTTNBP2, DISC1, GC, GJA1, GNAS, GRIN1, KCNA2, KCNMA1, KLHL5, LINGO2, MAGI2, RGS12, RIMS1, ROBO2, SCN2A, SLC4A4, SLC6A3, STAMBP, TCN2
Autistic disorder	3.40E-05	ASIC2, CADM1, DISC1, GJA1, KCNMA1, RIMS1, ROBO2, TCN2
Schizophrenia spectrum and other psychotic disorders	4.10E-04	DISC1, GC, GRIN1, MAGI2, RGS12, SLC6A3
Anxiety disorders	2.53E-02	MAGI2, SLC6A3
Cocaine-related disorders	1.74E-02	GRIN1, KLHL5, SLC6A3
Intellectual disability	2.52E-02	ANK3, DISC1, GNAS, GRIN1, KCNA2, SLC4A4
Psychotic disorders	1.13E-02	GRIN1, SLC6A3
Schizophrenia	1.62E-03	DISC1, GC, MAGI2, RGS12, SLC6A3
Substance-related disorders	3.98E-02	GNAS, GRIN1, KLHL5, LINGO2, SLC6A3

Gene-disease associations on the DISC1 regulated proteins were implemented in the Comparative Toxicogenomics Database, CTD. Fifty-four DISC1-regulated proteins are associated with mental disorders and/or nervous system disease disorders. Over-represented disease-protein associations (Fishers' exact test, $P < 0.05$) are illustrated.

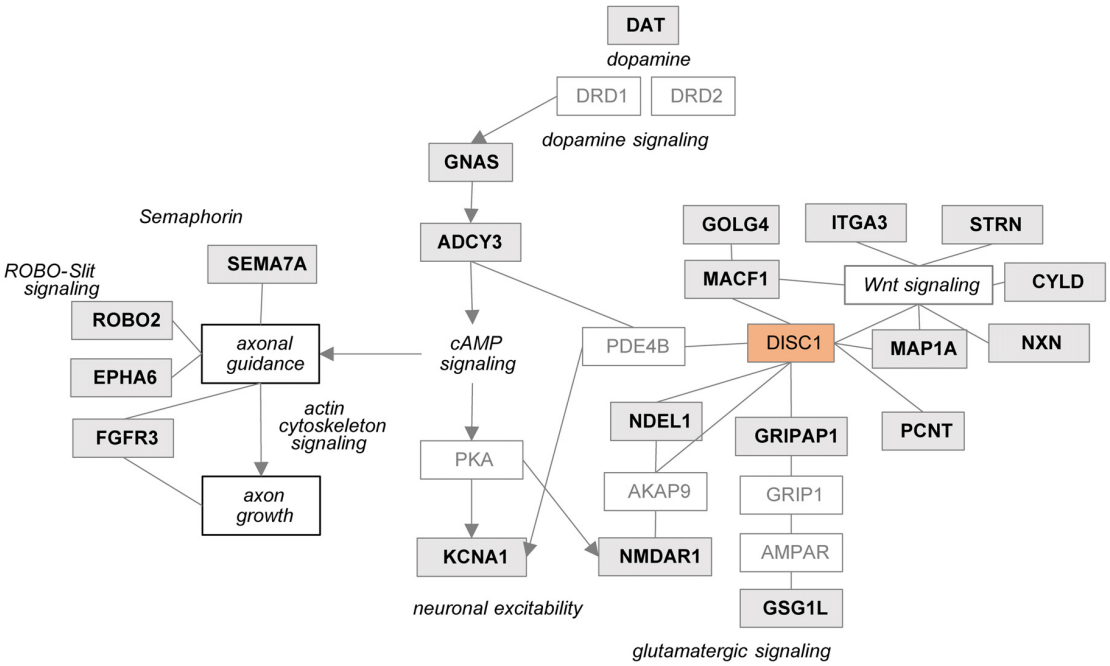

FIGURE 5 | Potential relationship between the DISC1 regulated proteins and synaptic processes. Proteins regulated in tgDISC1 rats from this study are marked in gray boxes.

The protein level changes of this enzyme along with the corresponding G-protein GNAS observed herein supports previous studies proposing dysregulation of cAMP signaling by DISC1 (Millar et al., 2005; Kvajo et al., 2011; Crabtree et al., 2017).

In a mouse Disc1 mutant model, functional reduction of Kv1.1/KCNA1 was proposed to contribute to alterations in neuronal excitability and short-term plasticity. Reduction of this channel was accompanied by reduced phosphodiesterase 4 activity and elevated cAMP levels in the PFC of *Disc1* mutant mice (Crabtree et al., 2017). Interestingly, in our DISC1 overexpressing transgenic model, we found an increase of this and several proteins in the voltage-gated potassium channel complex suggesting potential dyregulation of electrophysiological synaptic functions (Supplementary Figure S8).

Current data also revealed that proteins associated with axonal guidance pathways were altered by DISC1 overexpression: the axonal guidance receptors semaphorin 7A (SEMA7A), EPH receptor A6 (EPHA6), roundabout receptor 2 (ROBO2), fibroblast growth factor receptor 3 (FGFR3) and integrin subunit alpha 3/very late activation protein 3 receptor, alpha-3 subunit (ITGA3) were shown to be modulated by DISC1 (**Figure 3B**). The leading edge of the axons contains receptors that sense guidance cues and aid in the navigation and migration of axons. The attraction or repulsion of cues promotes or decreases active actin polymerization, resulting in axonal extension or retraction by triggering the actin cytoskeleton signaling and Rho-GTPase pathways, as also proposed in the current pathway enrichment analysis (reviewed in Dent et al., 2011; Spillane and Gallo, 2014; Van Battum et al., 2015). The receptor SEMA7A stimulates axonal growth through integrins and MAPK signaling (Pasterkamp et al., 2003). The roundabout receptor 2, ROBO2 is the main receptor from the Slit-Robo pathway, that is involved in axon guidance and which is also associated with DISC1-interacting proteins SRGAP2 and 3 (Camargo et al., 2007). The Ephrin receptor signaling pathway, predicted to be regulated by DISC1, is critical for embryonic development and known as a mediator of axon guidance (Kvajo et al., 2011).

In perspective, alterations of these developmental pathways and processes could explain the subtle neurodevelopmental phenotypes in the tgDISC1, where the substantia nigra (SN) contains fewer dopaminergic neurons (DA), fewer projections into dorsal striatum, and a shift in the parvalbumin-positive interneurons (Hamburg et al., 2016). DA homeostasis deficiency and the proposed disturbed dopaminergic signaling could explain the observed decrease of DA neurons in the SN. The disturbed axonal guidance signaling could lead to the reduction of the projections into the dorsal striatum and the shift of the parvalbumin-positive interneurons. As protein profiles were obtained from adult tgDISC1 rats, it would be interesting to follow up by studying the profiles in the developing brain to reveal the etiopathology effects of DISC1 which exceeds the scope of this study.

Bioinformatics analyses from the current study predicted that the following biological processes were activated by overexpression of DISC1, i.e., regulation of cell quantities, neuronal and axonal extension and long term potentiation

(**Figure 4**). These results may be relevant for interpretation of previous as well as for the design of future studies on DISC1.

CONCLUSION

Our results suggest that overexpression and/or aberrant DISC1 proteostasis can lead to profound changes in protein networks relevant for mental disorders or endophenotypes and may signify a role for the DISC1 protein alone—in the absence of mutations—in behavioral and neural processes and disorders. DISC1 expression levels likely have to be controlled in a narrow expression window in order to execute adaptive behavior. These findings make the DISC1 protein and its posttranslational modifications a molecular convergence point or sensor for environmental interactions such as oxidative stress. The findings also strongly support the earlier literature indicating involvement of the dopaminergic systems, particularly in the dorsal striatum in functional properties of the DISC1 protein.

AUTHOR CONTRIBUTIONS

FJS, SVT, CK, JPH, MASS and GL conceived and designed the experiments. FJS, A-LW, BF and MK performed experiments. FJS and RS collected data and processed them. FJS, CK, JPH, MASS and GL interpreted the results. FJS, CK, JPH, MASS and GL wrote the article. CK, JPH, MASS and GL revised the intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2018.00026/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Quantitative proteomics of synaptosomal fractions in a rat overexpressing human DISC1 gene indicates profound synaptic dysregulation in the dorsal striatum

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1 Supplementary Data (xlsx files)

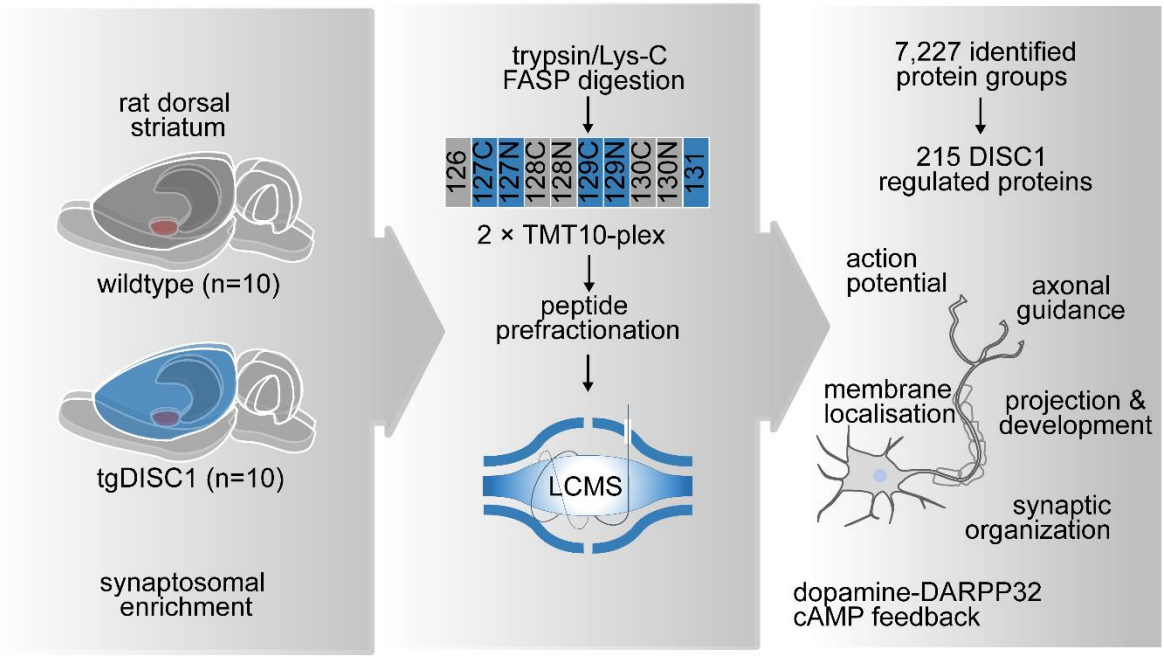
Supplementary Data 1. Label free quantification of the biochemical fractions of tgDISC1 rats.

Supplementary Data 2. Protein and peptide identifications data for TMT10plex.

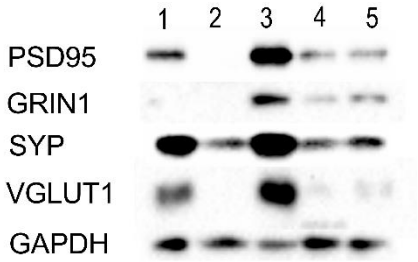
Supplementary Data 3. Quantitative proteomics data of synaptosomal fractions of tgDISC1 and wild type rats in the dorsal striatum by TMT10plex.

2 Supplementary Figures and Tables

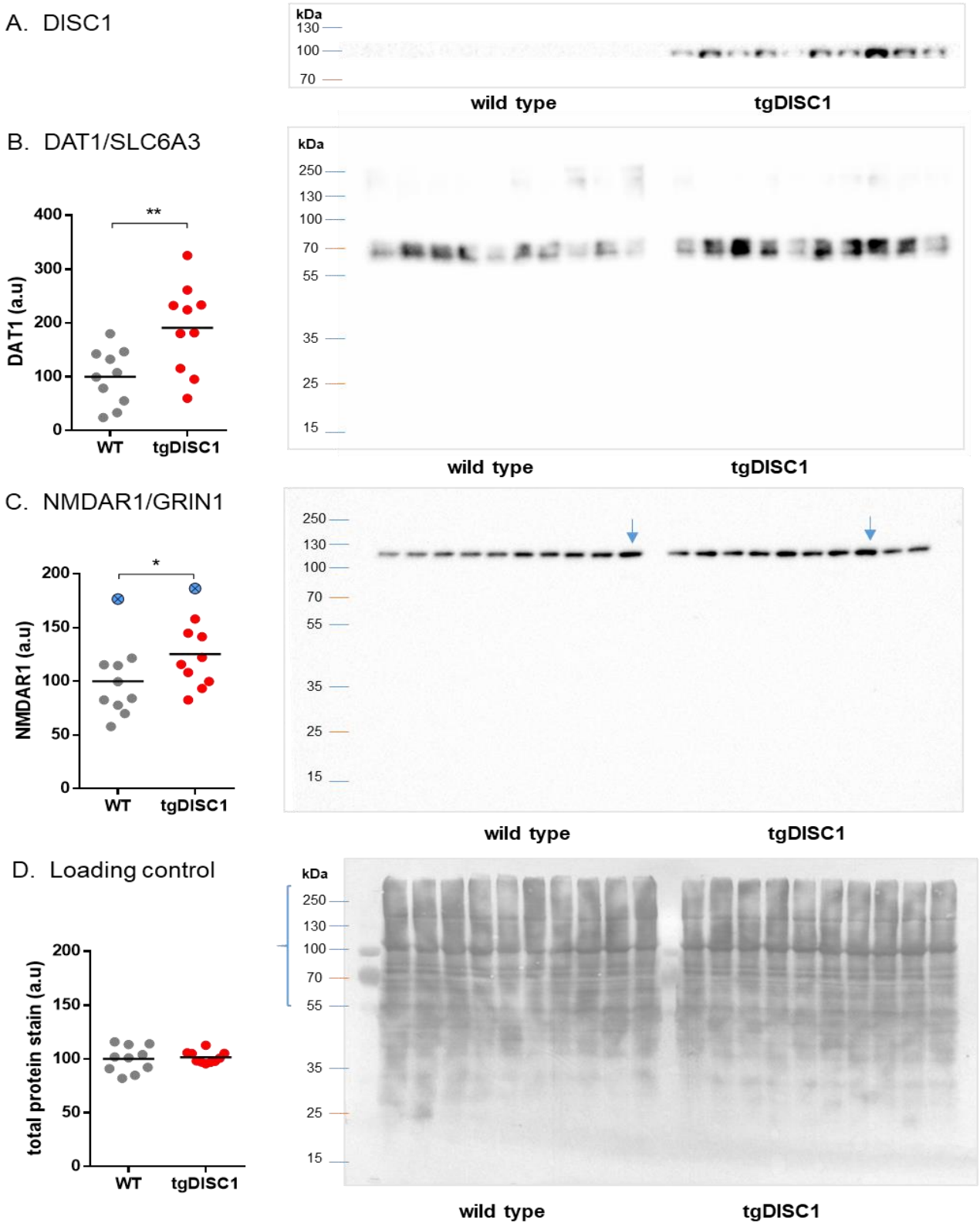
Supplementary Figure S1. Experimental Workflow. Synaptosomal fractions from bilateral regions were prepared for individual animals (for tgDISC1 and wild type; n=10 each). Synaptosomes were digested with a Trypsin-LysC enzyme mixture using the filter-aided sample preparation (FASP). The peptides were labeled with TMT 10-plex isobaric labels and fractionated by ion-pair reversed-phase chromatography at pH 10. Fractions were analysed by liquid chromatography tandem mass spectrometry using a Q-Exactive Plus. Database searching was performed with search engines against the rat SwissProt protein database. Functional enrichment analyses were performed using ClueGO and Inguinuity Pathaway analyses (IPA).



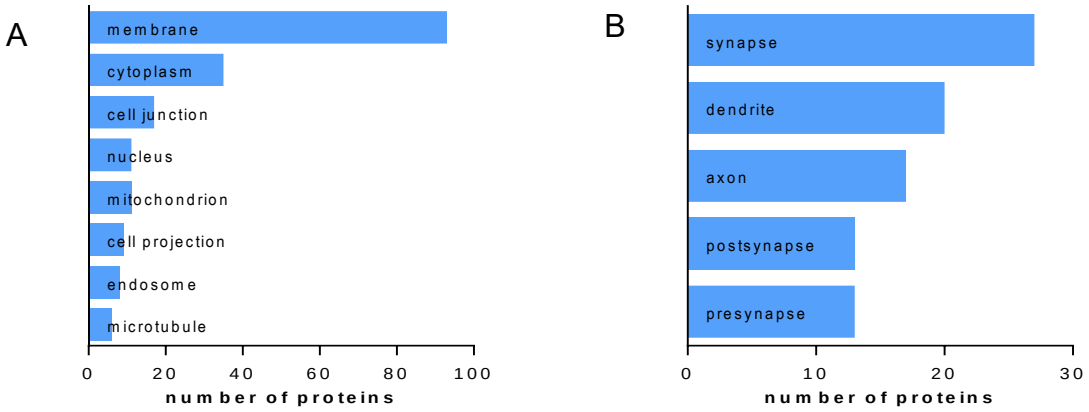
Supplementary Figure S2. Proteomic profile of the striatal synaptosomal fractions. Synaptosomal fractions were prepared using a microscale discontinuous sucrose gradient and collected at the 1.25/1.0 M sucrose interface. Immunoblot of representative proteins for the following biochemical fractionation stages: (1) whole dorsal striatum, (2) cytosolic (3) synaptosomes at 1.25/1.0M sucrose interface (4) below 1.25M sucrose (5) above 1.0M sucrose. The presynaptic (VGLU1, SYP) and postsynaptic (GRIN1 and PSD95) protein markers are enriched in the synaptosomal fractions. The cytosolic protein GAPDH is depleted in the synaptosomal fraction.



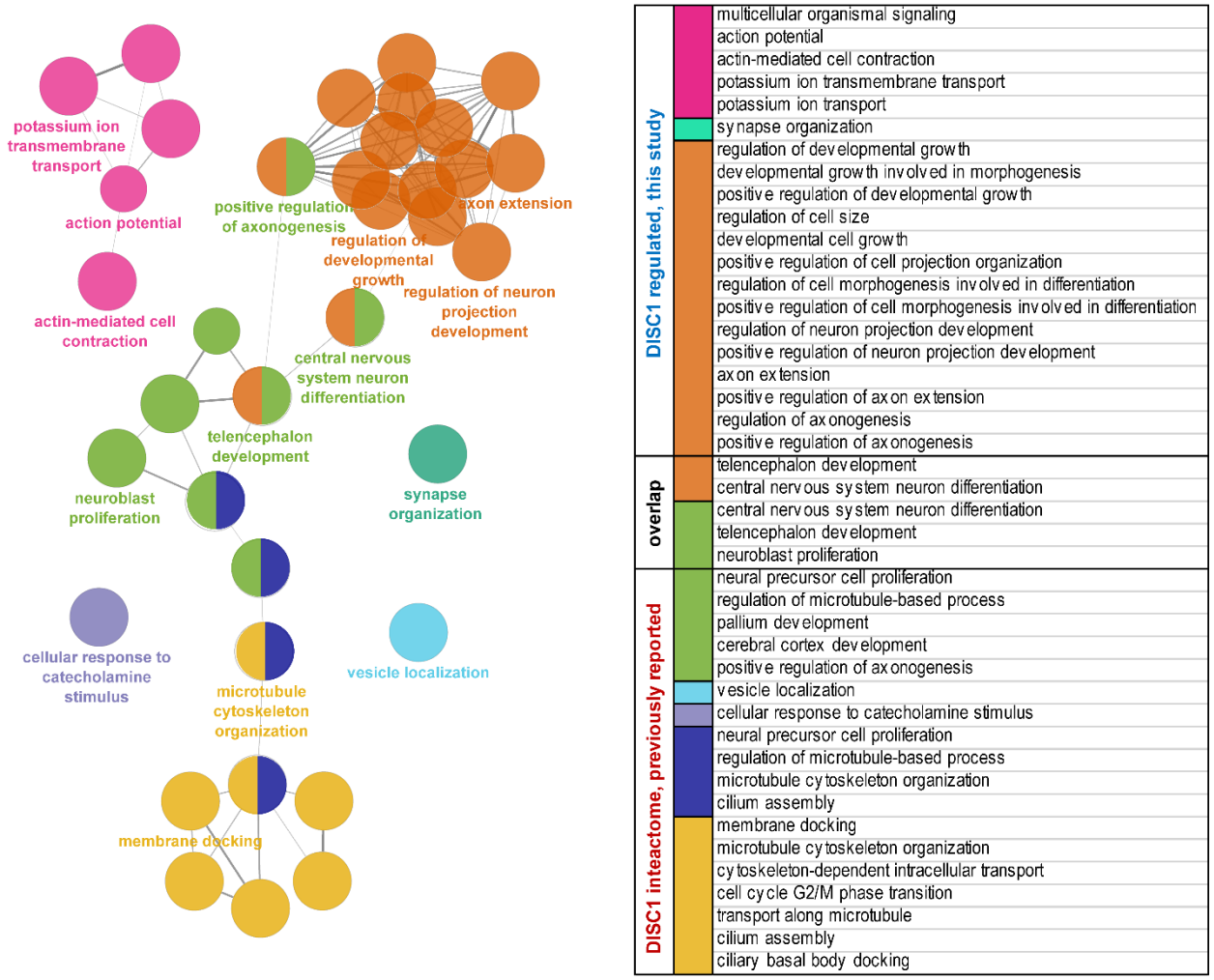
Supplementary Figure S3. Immunoblot of DISC1, DAT1 and NMDAR1 proteins (A-C) of the synaptosomal fractions of tgDISC1 and wild type rat. Total protein stain was used as loading control (D). Values represent *p < .05, **p < .01, ***p < .001 compared using two-sided *T*-tests. Outliers (blue arrows) are identified using Grubb's test.



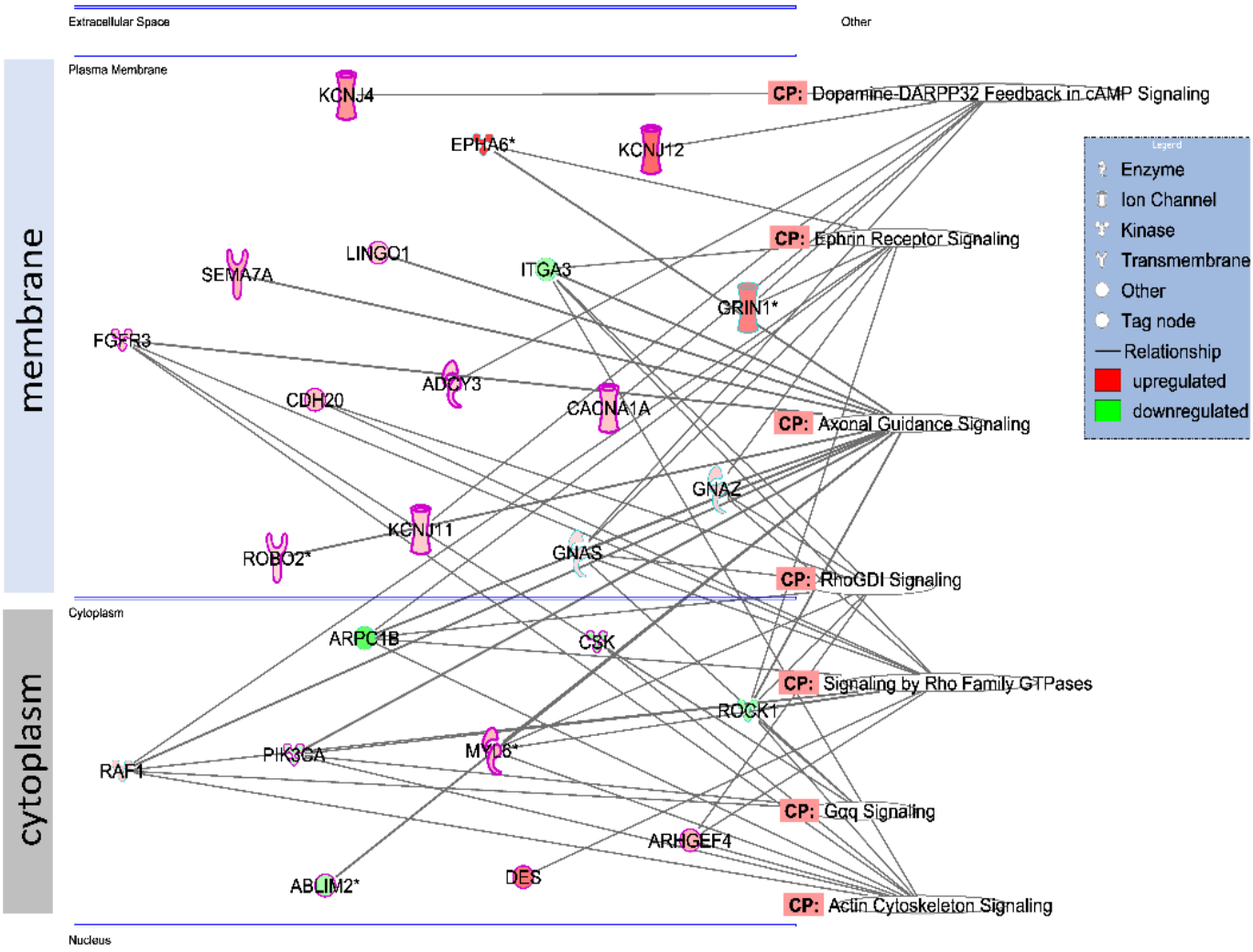
Supplementary Figure S4. Distribution of cellular (A) and synaptic components (B) of the DISC1-regulated proteins



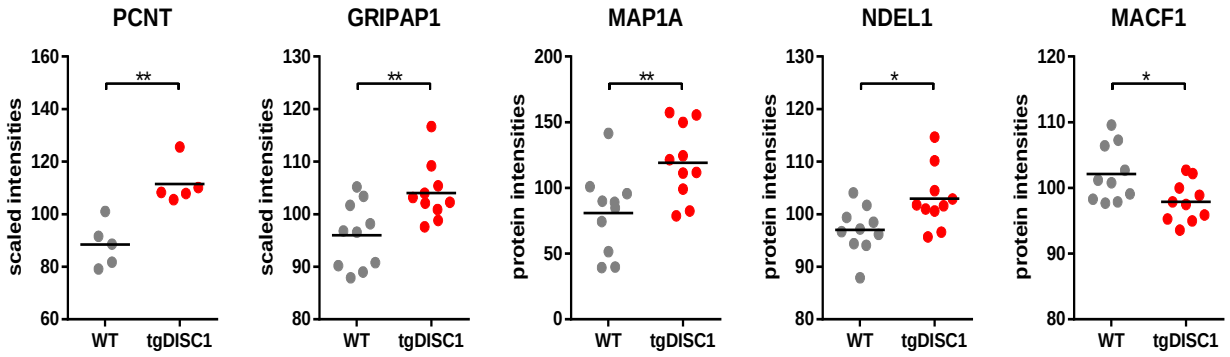
Supplementary Figure S5. Graphical representation of the biological processes of DISC1 regulated proteins (this study) and previously reported DISC1 interactors. Functionally related processes are clustered into networks using ClueGO and illustrated in different colors.



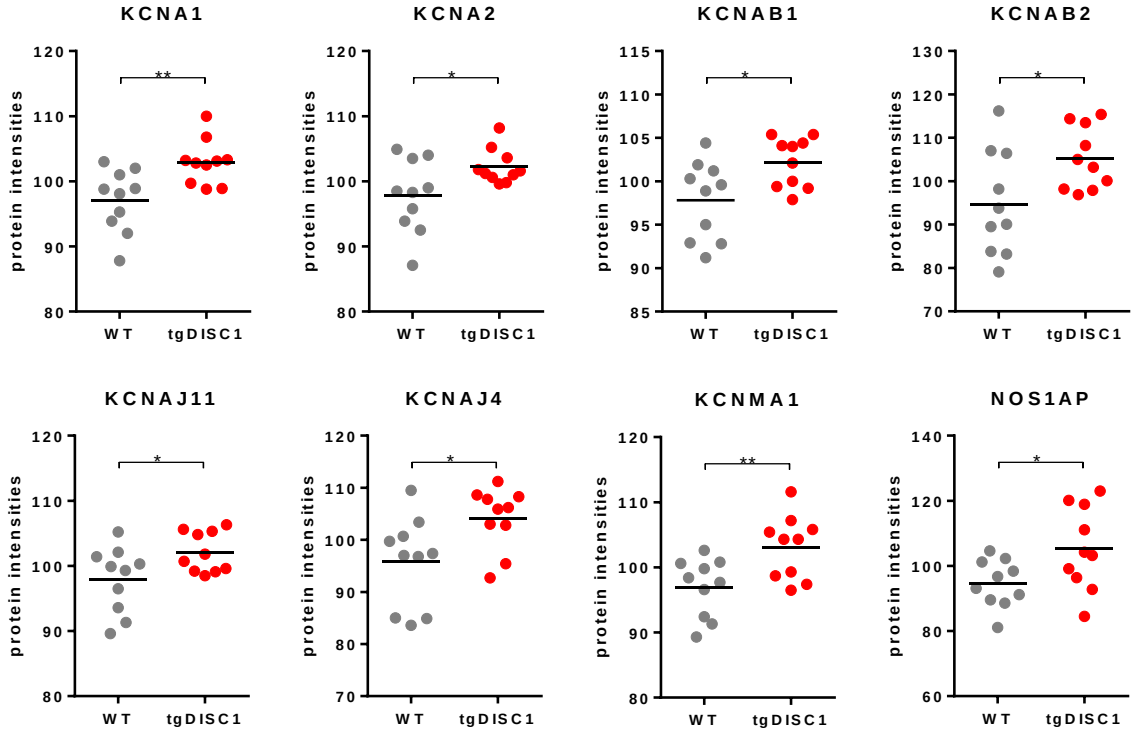
Supplementary Figure S6. Integrated canonical pathways of the DISC1-regulated proteins. Significantly enriched canonical pathways ($P < 10^{-3}$) in tgDISC1 in the dorsal striatum using Ingenuity Pathway Analyses. The network in the compartments "membrane" and "cytoplasm" is illustrated. Proteins up-regulated in tgDISC1 rats are colored in shades of red; proteins down-regulated are colored in green.



Supplementary Figure S7. DISC1–interactors altered in tgDISC1 rat as compared to the wild type animals. Values represent *p < .05, **p < .01, ***p < .001 compared using two-sided *T*-tests.



Supplementary Figure S8. Voltage-gated potassium channel complex proteins altered in tgDISC1 rat as compared to wild type animals. Values represent *p < .05, **p < .01, ***p < .001 compared using two-sided *T*-tests



2.1 Supplementary Tables

Supplementary Table S1. Immunoblotting materials and conditions.

Protein	Sample	Primary Antibodies		Secondary Antibodies	
	(µg)	Supplier, Cat #	Dilution	Supplier, Cat #	Dilution
PSD95	5	Synaptic Systems , 124011	1:1000	Abcam, ab6728	1:20000
SYP	5	Santa Cruz, sc-55507	1:5000	Abcam, ab6728	1:40000
NMDAR1	10	Abcam, ab32915	1:1000	Abcam, ab6728	1:10000
VGUT1	10	Synaptic Systems, 135311	1:1000	Abcam, ab6728	1:10000
GAPDH	10	Abcam, ab9485	1:2000	Abcam, ab191866	1:10000
DAT1	10	Abcam, ab111468	1:1000	Abcam, ab191866	1:10000
DISC1	10	Korth lab, 3D4	1:1000	Abcam, ab6728	1:10000

Supplementary Table S2. Proteins statistically different between the tgDISC1 and wild type rats (Protein levels were considered statistically different between groups when $P \leq 0.05$ using a two-sided T-test.

Accession	Protein	P-value	
actin cytoskeleton organization			
F1LMV9	coronin 2B (CORO2B)	1.4E-02	*
P62024	phosphatase and actin regulator 1 (PHACTR1)	2.8E-02	*
GPCR signaling			
P43140	adrenoceptor alpha 1A (ADRA1A)	3.1E-02	*
B4F7C1	G protein-coupled receptor 37 like 1 (GPR37L1)	3.7E-02	*
axonal guidance signaling			
A0A0G2JTB5	actin binding LIM protein family member 2 (ABLIM2)	2.0E-02	*
O88656	actin related protein 2/3 complex subunit 1B (ARPC1B)	4.5E-02	*
F1LQK6	EPH receptor A6 (EPHA6)	2.2E-02	*
A0A0G2K210	fibroblast growth factor receptor 3 (FGFR3)	3.7E-02	*
P19627	G protein subunit alpha z (GNAZ)	3.3E-02	*
D3ZHA7	myosin light chain 6 (MYL6)	2.8E-03	**
A0A0G2K344	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)	4.0E-02	*
P11345	Raf-1 proto-oncogene, serine/threonine kinase (RAF1)	1.9E-02	*
D4A5S0	Rho associated coiled-coil containing protein kinase 1 (ROCK1)	3.8E-02	*
brain development			
A0A0G2JXE5	ankyrin repeat and LEM domain containing 2 (ANKLE2)	5.9E-02	*
Q99J86	attractin (ATRN)	3.0E-02	*
Q2IBD4-2	cortactin binding protein 2 (CTTNBP2)	1.6E-02	*
D3Z9H2	hyaluronan and proteoglycan link protein 4 (HAPLN4)	1.7E-03	**
cell adhesion			
G3V7W5	cadherin 20 (CDH20)	4.1E-02	*
Q2KN99	sperm antigen with calponin homology and coiled-coil domains 1 like (SPECC1L)	2.1E-02	*
B4F773	tweety family member 1 (TTYH1)	4.4E-02	*
cell junction			
F7EYW4	cell adhesion molecule 1 (CADM1)	1.9E-02	*
P32577	c-src tyrosine kinase (CSK)	3.2E-02	*
Q5U302	catenin alpha 1 (CTNNA1)	4.5E-02	*
P08050	gap junction protein alpha 1 (GJA1)	6.9E-04	***
E9PSV8	glycoprotein m6b (GPM6B)	3.9E-03	**
P34901	syndecan 4 (SDC4)	7.2E-03	**

G3V6L8	striatin (STRN)	4.6E-02	*
A0A0G2K2P5	tight junction protein 1 (TJP1)	4.7E-02	*
B2RYG8	elongator acetyltransferase complex subunit 6 (ELP6)	9.7E-03	**
differentiation			
F1M7X4	erb-b2 receptor tyrosine kinase 4 (ERBB4)	3.3E-02	*
B1WC24	CTD small phosphatase 1 (CTDSP1)	2.5E-02	*
Q9Z0G8	WAS/WASL interacting protein family member 3 (WIPF3)	9.2E-03	**
A0A0G2JUX5	purine rich element binding protein B (PURB)	1.7E-02	**
F1M987	signal peptide, CUB domain and EGF like domain containing 1 (SCUBE1)	3.2E-02	*
dopamine response			
G3V6I2	adenylate cyclase 3 (ADCY3)	1.8E-02	*
A0A0G2JXK1	calcium voltage-gated channel subunit alpha1 A (CACNA1A)	1.6E-02	*
P63095	GNAS complex locus (GNAS)	4.6E-02	*
Q62648	glutamate ionotropic receptor NMDA type subunit 1 (GRIN1)	4.6E-02	*
P63142	potassium voltage-gated channel subfamily A member 2 (KCNA2)	4.0E-02	*
P70673	potassium voltage-gated channel subfamily J member 11 (KCNJ11)	3.8E-02	*
P52188	potassium voltage-gated channel subfamily J member 12 (KCNJ12)	4.2E-02	*
G3V9M7	potassium voltage-gated channel subfamily J member 4 (KCNJ4)	2.1E-02	*
P23977	solute carrier family 6 member 3 (SLC6A3)	4.6E-02	*
A0A0G2JYY3	tyrosine kinase non receptor 2 (TNK2)	3.6E-02	*
ER to Golgi vesicle-mediated transport			
Q5XHZ8	component of oligomeric golgi complex 3 (COG3)	4.7E-02	*
Q99M64	phosphatidylinositol 4-kinase type 2 alpha (PI4K2A)	3.6E-02	*
Q9Z158	syntaxin 17 (STX17)	7.0E-03	**
Q6AZ42	gap junction protein beta 6 (GJB6)	2.8E-02	*
D3ZD84	leucine rich repeats and immunoglobulin like domains 1 (LRIG1)	3.8E-02	*
ion transport, other			
P60571	pannexin 2 (PANX2)	3.2E-02	*
A0A0H2UHA7	solute carrier family 15 member 2 (SLC15A2)	4.3E-02	*
D4A517	solute carrier family 39 member 10 (SLC39A10)	2.1E-02	*
D3ZZM0	solute carrier family 39 member 14 (SLC39A14)	4.0E-02	*
lipid-related processes			
M0RA83	ATP binding cassette subfamily A member 5 (ABCA5)	2.3E-02	*
Q5PQS4	GULP, engulfment adaptor PTB domain containing 1 (GULP1)	4.9E-02	*
Q5BK77	retinoic acid receptor responder 2 (RARRES2)	4.3E-02	*
Q99MS0	SEC14 like lipid binding 2 (SEC14L2)	8.1E-04	***
P58405-2	striatin 3 (STRN3)	7.7E-02	*
F1LNL3	ATP binding cassette subfamily A member 1 (ABCA1)	2.9E-04	***
B0BMW2	hydroxysteroid 17-beta dehydrogenase 10 (HSD17B10)	3.9E-02	*
P04276	GC, vitamin D binding protein (GC)	1.4E-02	*
localization to plasma membrane			
P97846	contactin associated protein 1 (CNTNAP1)	5.1E-02	*
F1LTW9	EFR3 homolog B (EFR3B)	5.4E-02	*
F1M8X9	golgi brefeldin A resistant guanine nucleotide exchange factor 1 (GBF1)	4.6E-02	*
A0A0A0MY13	G protein-coupled receptor 158 (GPR158)	1.9E-02	*
D3ZII1	raftlin family member 2 (RFTN2)	4.2E-03	**
Q99PV2	syntaxin binding protein 3 (STXBP3)	2.8E-02	*
metabolism			
G3V7H6	arginase 2 (ARG2)	2.8E-02	*
Q5U2N0	CTP synthase 2 (CTPS2)	1.7E-02	*
P15429	enolase 3 (ENO3)	4.7E-02	*
P84039	ectonucleotide pyrophosphatase/phosphodiesterase 5 (putative) (ENPP5)	4.0E-02	*
Q5PPI4	lysosomal associated membrane protein family member 5 (LAMP5)	4.9E-02	*
D4A197	methylmalonyl-CoA epimerase (MCEE)	3.8E-02	*
F1M5N4	malic enzyme 3 (ME3)	1.7E-02	*

D3ZKY0	N-terminal EF-hand calcium binding protein 3 (NECAB3)	4.3E-02	*
Q99PW5	neuraminidase 3 (NEU3)	1.1E-02	*
F7F588	nicotinamide nucleotide adenylyltransferase 3 (NMNAT3)	5.9E-03	**
Q66H59	N-acetylneuraminate pyruvate lyase (NPL)	3.7E-03	**
E9PTV9	similar to glyceraldehyde-3-phosphate dehydrogenase (RGD1562758)	6.4E-03	**
F1M0M3	UEV and lactate/malate dehyrogenase domains (UEVLD)	4.9E-02	*
D4A7Z0	WD and tetratricopeptide repeats 1 (WDTC1)	3.6E-02	*
microtubule organization			
Q78P75	dynein light chain LC8-type 2 (DYNLL2)	1.8E-02	*
P34926	microtubule associated protein 1A (MAP1A)	9.8E-03	*
Q4QQR9	mediator of cell motility 1 (MEMO1)	5.3E-02	*
A0A0G2K4U7	microtubule associated tumor suppressor candidate 2 (MTUS2)	2.8E-02	*
D3ZMY8	pericentrin (PCNT)	2.4E-03	**
D3Z7Z5	RAN binding protein 10 (RANBP10)	3.5E-02	*
migration			
Q66HC5	nucleoporin 93 (NUP93)	4.5E-02	*
mitochondrial processes			
Q68FT1	coenzyme Q9 (COQ9)	4.4E-02	*
M0R4L6	glutamyl-tRNA amidotransferase subunit B (GATB)	4.9E-02	*
P27881	hexokinase 2 (HK2)	1.9E-02	*
M0RDC8	mitochondrial translation release factor 1 (MTRF1)	2.7E-03	**
Q5M7W7	prolyl-tRNA synthetase 2, mitochondrial (putative) (PARS2)	3.1E-02	*
G3V7I0	peroxiredoxin 3 (PRDX3)	2.7E-02	*
Q6TUF2	succinate dehydrogenase complex assembly factor 3 (SDHAF3)	2.7E-02	*
Q9R0E0	UDP-glucose ceramide glucosyltransferase (UGCG)	5.1E-02	*
Q6AY19	coenzyme Q8B (COQ8B)	3.9E-02	*
neuron projection development			
B2RYJ1	anaphase promoting complex subunit 2 (ANAPC2)	3.1E-02	*
A0A0G2K5J4	cyclin dependent kinase like 3 (CDKL3)	4.8E-03	**
Q9NRI5	disrupted in schizophrenia 1 (DISC1)	7.9E-13	****
A0A0G2JUE4	golgin A4 (GOLGA4)	4.2E-02	*
D3ZQM3	integrin subunit alpha 3 (ITGA3)	4.6E-02	*
G3V881	leucine rich repeat and Ig domain containing 1 (LINGO1)	5.2E-03	**
A0A0G2JWA8	microtubule-actin crosslinking factor 1 (MACF1)	2.1E-02	*
O88382	membrane associated guanylate kinase, WW and PDZ domain containing 2 (MAGI2)	4.0E-02	*
Q78PB6	nudE neurodevelopment protein 1 like 1 (NDEL1)	1.9E-02	*
A0A0G2JTW1	RAP2A, member of RAS oncogene family (RAP2A)	2.3E-02	*
A0A0G2JZA1	roundabout guidance receptor 2 (ROBO2)	2.7E-02	*
B2GUZ6	reticulon 4 interacting protein 1 (RTN4IP1)	4.4E-02	*
Q00954	sodium voltage-gated channel beta subunit 1 (SCN1B)	2.0E-02	*
D3ZQP6	semaphorin 7A (SEMA7A)	4.0E-02	*
F1LXV3	serine/threonine kinase 26 (STK26)	2.1E-02	*
A0A0G2JSR2	synaptotagmin 3 (SYT3)	1.2E-02	*
neurotransmitter metabolism, secretion and signaling			
D3Z890	diacylglycerol lipase beta (DAGLB)	4.6E-04	***
F1MAB7	diacylglycerol kinase iota (DGKI)	5.1E-02	*
P63170	dynein light chain LC8-type 1 (DYNLL1)	3.3E-02	*
P09606	glutamate-ammonia ligase (GLUL)	1.8E-02	*
Q9JHZ4	GRIP1 associated protein 1 (GRIPAP1)	7.0E-03	**
D3ZK93	GSG1 like (GSG1L)	7.9E-03	**
O54960	nitric oxide synthase 1 (neuronal) adaptor protein (NOS1AP)	3.4E-02	*
Q9JIR4	regulating synaptic membrane exocytosis 1 (RIMS1)	3.2E-02	*
D3ZWS6	N(alpha)-acetyltransferase 30, NatC catalytic subunit (NAA30)	3.0E-02	*
Q5XIS7	ubiquitin associated protein 1 (UBAP1)	2.9E-02	*
potassium ion transmembrane transport			
P10499	potassium voltage-gated channel subfamily A member 1 (KCNA1)	6.0E-03	**

P63144	potassium voltage-gated channel subfamily A member regulatory beta subunit 1 (KCNAB1)	1.8E-02	*
P62483	potassium voltage-gated channel subfamily A regulatory beta subunit 2 (KCNAB2)	2.9E-02	*
A0A0G2K104	potassium calcium-activated channel subfamily M alpha 1 (KCNMA1)	9.4E-03	**
G3V6N7	solute carrier family 12 member 6 (SLC12A6)	1.1E-02	*
signal transduction			
F1M4N6	dedicator of cytokinesis 3 (DOCK3)	1.3E-02	*
F1LYG2	signal-induced proliferation-associated 1 like 3 (SIPA1L3)	3.1E-02	*
A0A0G2K2B9	ankyrin 3 (ANK3)	5.1E-02	*
D3ZGL1	Rho GTPase activating protein 25 (ARHGAP25)	4.4E-02	*
D3ZKB4	Rho guanine nucleotide exchange factor 4 (ARHGEF4)	1.1E-02	*
Q64542-2	ATPase plasma membrane Ca2+ transporting 4 (ATP2B4)	4.6E-02	*
D4A758	leucine rich repeat containing 8 family member B (LRRC8B)	4.9E-02	*
Q6AYR2	NDRG family member 3 (NDRG3)	5.2E-02	*
F1LQ26	Rap guanine nucleotide exchange factor 4 (RAPGEF4)	2.0E-02	*
A0A0G2JTA7	RAS protein activator like 2 (RASAL2)	3.8E-02	*
D4AB55	regulator of G-protein signaling 12 (RGS12)	1.7E-02	*
F1M3P6	suppressor of cancer cell invasion (SCAI)	4.1E-02	*
Q8R424	STAM binding protein (STAMBP)	4.7E-03	**
sodium ion transport			
Q62962	acid sensing ion channel subunit 2 (ASIC2)	2.5E-02	*
P04775	sodium voltage-gated channel alpha subunit 2 (SCN2A)	1.7E-02	*
Q9JJP0	solute carrier family 20 member 1 (SLC20A1)	3.8E-03	**
Q9JHE5	solute carrier family 38 member 2 (SLC38A2)	1.7E-02	*
P23347	solute carrier family 4 member 2 (SLC4A2)	2.5E-02	*
G3V8P8	solute carrier family 4 member 3 (SLC4A3)	4.9E-04	***
Q9JI66-3	solute carrier family 4 member 4 (SLC4A4)	3.2E-02	*
D3ZEE4	plexin domain containing 1 (PLXDC1)	2.5E-02	*
synaptic organization			
M0R752	leucine rich repeat and Ig domain containing 2 (LINGO2)	2.1E-02	*
D3ZLC1	lamin B2 (LMNB2)	3.7E-02	*
Q45R42	leucine rich repeat containing 4 (LRRC4)	3.4E-02	*
D3ZNW5	neurofascin (NFASC)	9.1E-03	**
F1M4R7	chondroitin sulfate proteoglycan 5 (CSPG5)	2.1E-02	*
D4A2L1	RIMS binding protein 2 (RIMBP2)	3.3E-02	*
transciption			
D3Z941	methionyl-tRNA synthetase (MARS)	4.7E-02	*
A0A0G2K1A1	zinc finger protein 706 (ZFP706)	2.6E-02	*
translation			
A9CMB7	aspartyl-tRNA synthetase (DARS)	3.9E-02	*
Q68G14	DEAD-box helicase 25 (DDX25)	2.2E-04	***
F7EPZ4	DIS3 homolog, exosome endoribonuclease and 3'-5' exoribonuclease (DIS3)	4.7E-02	*
Q4G061	eukaryotic translation initiation factor 3 subunit B (EIF3B)	1.2E-02	*
Q5RKI9	mitochondrial ribosome recycling factor (MRRF)	2.6E-02	*
D3ZQL8	pumilio RNA binding family member 2 (PUM2)	3.1E-02	*
P38983	ribosomal protein SA (RPSA)	4.9E-03	**
Q09167	serine/arginine-rich splicing factor 5 (SRSF5)	9.7E-03	**
F1M8H2	tryptophanyl tRNA synthetase 2, mitochondrial (WARS2)	3.9E-02	*
Q4V8I7	leucine rich repeat containing 8 family member A (LRRC8A)	2.2E-03	**
D3ZGN0	TBC1 domain family member 4 (TBC1D4)	6.9E-03	**
D3ZE59	transmembrane protein 115 (TMEM115)	9.7E-04	***
G3V6K1	transcobalamin 2 (TCN2)	1.0E-02	**
Q66H62	CYLD lysine 63 deubiquitinase (CYLD)	3.3E-02	*
D4A0M2	nucleoredoxin (NXN)	2.1E-02	*
B5DEL1	potassium channel tetramerization domain containing 5 (KCTD5)	2.6E-02	*
F1LQN1	polypeptide N-acetylgalactosaminyltransferase 16 (GALNT16)	2.5E-02	*

apoptosis			
D4AA14	apoptosis inducing factor, mitochondria associated 2 (AIFM2)	3.9E-02	*
A0A0G2K427	influenza virus NS1A binding protein (IVNS1ABP)	3.0E-02	*
Q91XT9	N-acylsphingosine amidohydrolase 2 (ASAH2)	3.9E-02	*
Q5FWU3	autophagy related 9A (ATG9A)	1.6E-05	****
others			
Q5XIJ5	abhydrolase domain containing 17A (ABHD17A)	2.1E-02	*
Q6AY72	chromosome 19 open reading frame 25 (C19ORF25)	1.2E-02	*
D3ZV63	chromosome 1 open reading frame 27 (C1ORF27)	9.6E-03	**
Q498D0	coiled-coil domain containing 28A (CCDC28A)	3.1E-02	*
G3V674	claudin domain containing 1 (CLDND1)	5.1E-03	**
M0RAD5	caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP)	3.9E-02	*
D4A1C0	cyclin and CBS domain divalent metal cation transport mediator 1 (CNNM1)	3.2E-02	*
Q499V0	COMM domain containing 7 (COMMD7)	3.4E-02	*
P48675	desmin (DES)	3.9E-02	*
B2RYA8	DnaJ heat shock protein family (Hsp40) member B2 (DNAJB2)	2.8E-02	*
G3V7G9	eukaryotic translation initiation factor 3, subunit L (EIF3L)	1.2E-02	*
Q80Z26	ectonucleoside triphosphate diphosphohydrolase 3 (ENTPD3)	4.7E-02	*
M0RAC2	family with sequence similarity 102 member A (FAM102A)	3.5E-02	*
D4A1T7	family with sequence similarity 173 member A (FAM173A)	4.3E-03	**
M0RDY2	family with sequence similarity 185 member A (FAM185A)	2.1E-02	*
Q7TP54	family with sequence similarity 65 member B (FAM65B)	4.0E-03	**
B2RYH5	interleukin 1 receptor associated kinase 1 (IRAK1)	3.3E-02	*
Q1EG90	kelch like family member 5 (KLHL5)	3.7E-02	*
Q561R9	lactamase beta 2 (LACTB2)	1.6E-02	*
D3ZW19	leucine rich repeat and Ig domain containing 3 (LINGO3)	2.1E-02	*
P05544	Serine protease inhibitor (LOC299282)	5.7E-02	*
A0A096MK47	muscular LMNA interacting protein (MLIP)	2.7E-02	*
B1H218	NIMA related kinase 6 (NEK6)	3.9E-02	*
A0A0G2JUP0	NIMA related kinase 7 (NEK7)	2.2E-02	*
D4A478	nucleoside-triphosphatase, cancer-related (NTPCR)	2.0E-02	*
G3V834	proline rich coiled-coil 1 (PRRC1)	2.7E-02	*
D4A9R4	proline rich transmembrane protein 4 (PRRT4)	4.2E-02	*
Q66H61	glutaminyl-tRNA synthetase (QARS)	1.5E-02	*
B5DFB2	RB binding protein 4, chromatin remodeling factor (RBBP4)	1.5E-02	*
D4AAW6	rhomboid like 3 (RHBDL3)	4.6E-02	*
D3ZAB6	ring finger protein 219 (RNF219)	1.1E-02	*
M0RCV5	RUN and SH3 domain containing 2 (RUSC2)	1.9E-02	*
A0A0G2JSK1	serine (or cysteine) proteinase inhibitor, clade A, member 3C (SERPINA3C)	4.1E-02	*
Q499T3	signal regulatory protein alpha (SIRPA)	2.8E-02	*
G3V702	DNA replication regulator and spliceosomal factor (SMU1)	3.2E-02	*
D4A9H7	spermatogenesis associated 2 like (SPATA2L)	1.3E-02	*
D3ZF92	TNF receptor superfamily member 21 (TNFRSF21)	2.7E-02	*

Supplementary Table S3. Cellular components of the proteins altered by DISC1 overexpression. Enrichment of GO annotations were performed on the significant proteins using GOA database (v30.08.2017) using the ClueGO via the Cytoscape platform (Benjamini–Hochberg P-value $<10^{-3}$)

GO ID	GOTerm	P-value	Associated Proteins Found
GO:0043197	dendritic spine	3.70E-03	ASIC2, CACNA1A, CTTNBP2, GRIN1, LRRC4, STRN
GO:0045121	membrane raft	3.80E-04	ABCA1, ATP2B4, CSK, CTNNA1, DYNLL1, GJA1, GPM6B, KCNMA1, NOS1AP, PI4K2A, RFTN2, SDC4, SLC6A3
GO:0005901	caveola	1.50E-03	ATP2B4, CTNNA1, DYNLL1, KCNMA1, NOS1AP, SLC6A3
GO:0009898	cytoplasmic side of plasma membrane	2.50E-03	CSK, CYLD, GNAS, GNAZ, KCNAB1, KCNAB2, RASAL2, TNK2
GO:0019897	extrinsic component of plasma membrane	1.70E-04	ANAPC2, CSK, CTNNA1, CYLD, GNAS, GNAZ, KCNAB1, KCNAB2, SCUBE1, TNK2
GO:0014704	intercalated disc	6.00E-05	CTNNA1, DES, GJA1, KCNJ11, SCN1B, SCN2A, TJP1
GO:0042383	sarcolemma	4.30E-04	ADRA1A, ATP2B4, DES, KCNJ11, NOS1AP, SCN1B, SCN2A, SLC38A2
GO:0030315	T-tubule	1.50E-04	ADRA1A, ATP2B4, KCNJ11, NOS1AP, SCN1B, SCN2A
GO:0031252	cell leading edge	8.00E-05	ANAPC2, ARHGEF4, CACNA1A, CTNNA1, DYNLL1, GBF1, GNAS, KCNA2, KCNJ11, LAMP5, MACF1, NDEL1, PIK3CA, ROBO2, ROCK1, SLC39A14
GO:0031256	leading edge membrane	4.00E-04	ANAPC2, ARHGEF4, CACNA1A, DYNLL1, KCNA2, KCNJ11, LAMP5, MACF1, ROBO2
GO:0032589	neuron projection membrane	1.80E-03	CACNA1A, KCNJ11, LAMP5, ROBO2
GO:0033267	axon part	1.60E-04	GRIN1, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, NDEL1, NFASC, ROBO2, SCN1B, SCN2A
GO:0034702	ion channel complex	1.10E-05	CACNA1A, GRIN1, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ4, KCNMA1, LRRC8A, LRRC8B, NOS1AP, SCN1B, SCN2A, TTYH1
GO:0034703	cation channel complex	5.30E-05	CACNA1A, GRIN1, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ4, KCNMA1, NOS1AP, SCN1B, SCN2A
GO:0044304	main axon	1.00E-06	KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, NFASC, ROBO2, SCN1B, SCN2A
GO:0008076	voltage-gated potassium channel complex	8.30E-05	KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ4, KCNMA1, NOS1AP
GO:0033268	node of Ranvier	2.00E-03	NFASC, SCN1B, SCN2A
GO:0033270	paranode region of axon	1.20E-03	KCNA1, NFASC, SCN2A
GO:0044224	juxtaparanode region of axon	8.70E-05	KCNA1, KCNA2, KCNAB1, KCNAB2

Supplementary Table S4. Molecular Function of the proteins altered by DISC1 overexpression. Enrichment of GO annotations were performed on the significant proteins using GOA database (v30.08.2017) using the ClueGO via the Cytoscape platform (Benjamini–Hochberg P-value $<10^{-3}$)

GO ID	GO Term	*P*-Value	Associated Genes Found
GO:0004812	aminoacyl-tRNA ligase activity	7.80E-05	DARS, MARS, PARS2, QARS, SRSF5, WARS2
GO:0015293	symporter activity	9.70E-05	SLC12A6, SLC15A2, SLC20A1, SLC38A2, SLC4A10, SLC4A2, SLC4A3, SLC4A4, SLC6A3
GO:0005452	inorganic anion exchanger activity	2.30E-04	SLC4A10, SLC4A2, SLC4A3, SLC4A4
GO:0015301	anion:anion antiporter activity	6.80E-04	SLC4A10, SLC4A2, SLC4A3, SLC4A4
GO:0015077	monovalent inorganic cation transmembrane transporter activity	2.40E-06	ASIC2, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ12, KCNJ4, KCNMA1, SCN1B, SCN2A, SLC12A6, SLC20A1, SLC4A10, SLC4A2, SLC4A3, SLC4A4, SLC6A3
GO:0008510	sodium:bicarbonate symporter activity	1.10E-05	SLC4A10, SLC4A2, SLC4A3, SLC4A4
GO:0015081	sodium ion transmembrane transporter activity	1.60E-04	ASIC2, SCN1B, SCN2A, SLC20A1, SLC4A10, SLC4A2, SLC4A3, SLC4A4, SLC6A3
GO:0005244	voltage-gated ion channel activity	1.20E-05	CACNA1A, GRIN1, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ12, KCNJ4, KCNMA1, SCN1B, SCN2A
GO:0005261	cation channel activity	1.40E-04	ASIC2, CACNA1A, GRIN1, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ12, KCNJ4, KCNMA1, SCN1B, SCN2A
GO:0015077	monovalent inorganic cation transmembrane transporter activity	2.40E-06	ASIC2, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ12, KCNJ4, KCNMA1, SCN1B, SCN2A, SLC12A6, SLC20A1, SLC4A10, SLC4A2, SLC4A3, SLC4A4, SLC6A3
GO:0046873	metal ion transmembrane transporter activity	3.80E-09	ASIC2, ATP2B4, CACNA1A, GRIN1, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ12, KCNJ4, KCNMA1, SCN1B, SCN2A, SLC12A6, SLC20A1, SLC39A10, SLC39A14, SLC4A10, SLC4A2, SLC4A3, SLC4A4, SLC6A3, TTYH1
GO:0022843	voltage-gated cation channel activity	6.80E-05	CACNA1A, KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ12, KCNJ4, KCNMA1
GO:0015079	potassium ion transmembrane transporter activity	1.60E-04	KCNA1, KCNA2, KCNAB1, KCNAB2, KCNJ11, KCNJ12, KCNJ4, KCNMA1, SLC12A6
GO:0015081	sodium ion transmembrane transporter activity	1.60E-04	ASIC2, SCN1B, SCN2A, SLC20A1, SLC4A10, SLC4A2, SLC4A3, SLC4A4, SLC6A3

Supplementary Table S5. Predicted biological functions of the tgDISC1 regulated proteins as evaluated by IPA. The IPA regulation z-score algorithm was used to predict biological functions that are expected to be activated in tgDISC1 rats rather than in wildtype (positive z-score) according to own proteomics data (z-score ≥ 2; $P \leq 0.05$). The z-scores take into account the directional effect of one protein on a process and the direction of change of molecules in the dataset.

Functions	Diseases or Functions Annotation	*P*-value	Activation z-score	Proteins
Cell death	cell death of cortical neurons	4.83E-02	2.21	GRIN1,HK2,LINGO1,Nos1ap,PIK3CA,RAPGEF4
Extension	extension of cellular protrusions	1.85E-03	2.79	ANAPC2,CDKL3,DISC1,GOLGA4,GRIN1,NDEL1,SCN1B,SEMA7A
Extension	extension of neurites	5.64E-03	2.61	ANAPC2,CDKL3,DISC1,GOLGA4,NDEL1,SCN1B,SEMA7A
Extension	extension of axons	3.78E-03	2.43	ANAPC2,CDKL3,DISC1,GOLGA4,NDEL1,SEMA7A
Long-term potentiation	long-term potentiation of brain	1.37E-02	2.40	ERBB4,GRIN1,HSD17B10,RAPGEF4,RIMS1,SLC6A3
Long-term potentiation	long-term potentiation of cerebral cortex	3.08E-02	2.19	ERBB4,GRIN1,HSD17B10,RAPGEF4,SLC6A3
Quantity	quantity of cells	9.54E-03	2.67	ABCA1,ANK3,CACNA1A,DISC1,ENTPD3,ERBB4,FGFR3,GJA1,GJB6,GNAS,GRIN1,LMNB2,NFASC,RIMS1,SLC6A3,TNFRSF21
Quantity	quantity of neurons	1.62E-02	2.43	ABCA1,ANK3,CACNA1A,DISC1,ENTPD3,ERBB4,FGFR3,GNAS,GRIN1,NFASC,RIMS1,SLC6A3

3 Further discussion and concluding remarks

Development of proteomic methods for analysis of transmembrane proteins

The methods developed in the project forming part of this cumulative thesis allowed for the identification of a large number of membrane proteins from brain tissue. To a large degree, these proteins were not identified by the conventionally applied proteomics technologies. Therefore, these data highlights the necessity of implementing membrane-tailored proteomic protocols to overcome the underrepresentation of receptors, transporters and channels in standard synaptic proteome analyses.

The protocols developed in *Paper 1* include removal of non-ionic detergents and sequential proteolytic cleavage of transmembrane proteins prior to standard peptide pre-fractionation. These techniques were used for in-depth characterisation of the brain proteomes of other model organisms including Drosophila *(Paper 11)*, Zebrafish *(Paper 18)* and Honeybee (*Paper 5*) (Aradska et al., 2015a, Smidak et al., 2016b, Sialana et al., 2019), further highlighting applicability of this workflow.

The feasibility of the developed methodologies that combined sample preparation, mass spectrometry and bioinformatics is clearly shown by the identification of large number of synaptic proteins from different brain regions and different model systems.

(Sub)synaptic proteomics from biochemical enrichments: limitations and applications

The synaptosomal enrichment and further separation into subfractions are classical biochemical preparations described in several studies. The (sub)synaptic fractionation prior to protein analysis allowed reduction of the complexity of the samples and provided a comprehensive inventory of the proteome. The quantitation experiment between the PSD and the DSS fractions to determine the proteins enriched in these (sub) synaptic fractions revealed presynaptic proteins in the DSS fractions.

However, our in-depth proteomic analysis have revealed limitations of these classical enrichment protocols. Although the synaptosomal preparations are enriched in known synaptic proteins, the caveat of this preparation is that there are co-fractionated proteins that do not originate from the synapse. These include mitochondrial membrane proteins and axonal components. The purity of synaptosomes prepared from classical differential sucrose gradient typically reaches up to ~60 to 80% at most.

Further, although the results showed the enrichment of known presynaptic components in the DSS preparations. The biochemical fractionation is not sufficiently pure such that it cannot be used to assign localisation of a protein (e.g. presynaptic or postsynaptic). Therefore, the proteomic data by itself should not serve as an authoritative inventory of pre- and postsynaptic proteins. It is expected that the extraction efficiency of membrane proteins in Triton-X100, primarily depends on whether they are free in the membrane, form larger complexes with other membrane proteins ("rafts", i.e. detergent resistant membranes), or are present in large aggregates or anchored to an insoluble matrix (e.g PSD95). Thus, extraction largely depends on physical properties including the specifics of the large Triton micelles rather than the source of the membrane. The most prominent insoluble matrix includes the postsynaptic density (PSD95) to which some receptors and transporters are anchored. In contrast, the membranes of trafficking organelles, such as synaptic vesicles are not stably anchored to scaffolds, resulting in an almost complete extraction of synaptic vesicle proteins - hence to a certain extent, this classical protocol enriches the pre- and postsynaptic compartments.

For this reason, PSD fractions and detergent-soluble synaptosomal (DSS) fractions were the more appropriate terms to describe these biochemical fractions that were characterised in *Paper 1*.

Herein, two inherent limitations in *Paper 1* that needs to be addressed in future experiments include: (1) the purity of the starting synaptosome material and (2) the separation of the pre- and postsynaptic components from the synaptosomes.

Nevertheless, as demonstrated in *Paper 3* and *Paper 12,* the (sub)synaptic data from Paper 1 has a predictive value for localisation of candidate synaptic proteins. These candidates however has to be validated with imaging techniques such as immunohistochemistry or electron microscopy.

For instance, in collaboration with Beales group (University of College London, UK) we predicted the synaptic localization of the BBS proteins based on the proteomic data from Paper 1. These were further validated by immunofluorescence techniques on tissue or neuronal cultures. In Paper 3, (Haq et al., 2019) our collaborators generated knockout mouse lines for Bardet-Biedl syndrome Bbs4 and Bbs5 genes to understand the molecular mechanisms of cognitive impairment phenotype observed in BBS patients. Results from our collaborators revealed that the loss of the BBS proteins was related to synaptic dysfunction including a reduction in dendritic spine density, aberrant synaptic insulin-like growth factor (IGF) receptor signalling and altered neurotransmitter receptor levels (NMDA and AMPA). Data mining of our previous results in (Sialana et al., 2016, Sialana et al., 2018b, Smidak et al., 2017b) (Paper 1, 2, 19) revealed the presence of the BBS1, BBS2, BBS4, BBS5, BBS7, and BBS10 proteins in synaptosomal fractions from different rat brain regions. Additional proteomic revealed the presence of BBS1, BBS2, BBS4, BBS5, and BBS9 mainly in the PSD fractions while BBS7 was mostly present in the cytosolic fractions. The dendritic synaptic localisation of BBS4 and BBS5 was confirmed in mouse hippocampal neurons by immunofluorescence indicating the synaptic localisation of BBS proteins.

In addition, in *Paper 12 (Bitencourt et al., 2015a)*, our collaborators (Köfalvi group, University of Coimbra, Portugal) asked whether the subsynaptic localisation of the glucocorticoid receptor (GcR) and the cannabinoid receptor (CB1) would allow for a direct interaction between the proteins in the pre-frontal cortex in rodents. In *Paper 1*, both GcR and CB1 were identified in the synaptosomal preparations. Quantitative proteomics data from *Paper 1* revealed that CB1Rs were predominantly DSS (presynaptic-enriched) and in contrast, GcRs were primarily enriched in the PSD fractions. These data were confirmed by immunohistochemical analysis performed by our collaborators indicating that the lack of interaction between the two receptors.

This two studies demonstrates that the qualitative and quantitative data in *Paper 1* is valuable resource for molecular interrogation of the synapse in combination with imaging techniques as demonstrated by these two studies.

Quantitative proteomics of the synapse

The quantitative proteomics workflow for synaptosomal preparations from small brain sub-regions (~35mg) is highly amenable to studies in a high-throughput fashion (20 individual animals), as demonstrated in the tgDISC1 rat study *(Paper 2)*.

As discussed in the introduction, brain tissue samples from different donors are inherently heterogeneous. As biological variability of tissue samples cannot be lowered, high reproducibility of quantitative proteomics measurements described are essential. By using TMT-multiplexing, which allows simultaneous analysis of multiple samples (up to 16) whereby decreasing technical variability between sample runs. The advantages of TMT-multiplexing include increased proteomic depth, reduced machine time and increased reproducibility, while overcoming the issue of missing values characteristic for label free analysis.

In the future, this can used for the proteomic profiling of post-mortem samples of patients with chronic mental disorders where biological markers or sub-groups are not defined. Previous studies from post-mortem of brains with chronic mental disorder, CMD revealed an increased DISC1 aggregation is associated with only around 20% of the cases (Leliveld et al., 2008). The higher proteomic depth could reveal additional disease or disorder subsets from proteomic profiles using this method.

Synaptic profiles of the tgDISC1 rat in the dorsal striatum

The application of quantitative proteomics methods developed in this thesis was essential in revealing that aberrant DISC1 protein homeostasis can lead to synaptic changes relevant to traits associated with mental disorders. The modest increase of DISC1 modulates the proteins involved in synapse-associated processes including membrane trafficking, ion transport, synaptic organization and neurodevelopment, which were previously not reported. These proteins are also involved in brain specific pathways, particularly axonal guidance and dopamine signalling.

In particular, an increase in the synaptic dopamine transporter levels was revealed by quantitative proteomics and validated by immunochemistry. This was consistent with our previous observation on the increased clearance rate of extracellular dopamine (DA) (Trossbach et al., 2016) and the reduction of total dopamine content from post-mortem neurochemistry *(Paper 23)*. Subsequently, in *(Paper 23)* we were able to show that intranasal administration of dopamine reversed the cognitive deficits of the tgDISC1 model (Wang et al., 2017). Our results also supports the involvement of the dopaminergic system in the function of DISC1 protein. A summary of molecular and anatomical phenotypes of the tgDISC1 rat model is summarized in Table 2.

Table 2 Molecular and anatomical/behavioural phenotypes of tgDISC1 rat model

Molecular	**Anatomical/Behavioral**
↑ dopamine transporter ↓ dopamine ~ DRD receptor feedback	↓ dopaminergic neurons (SN) cognitive deficits reversed with dopamine (intranasal)
axonal guidance receptor alterations	↓ projections to the dorsal striatum aberrant interneuron positioning (cortex)

The functional enrichment analysis of the significantly changed proteins indicate that modest increase of DISC1 modulates the proteins involved in synapse-associated processes including membrane trafficking, ion transport, synaptic organisation and neurodevelopment, which were previously not reported. These proteins are also involved in brain specific pathways, particularly axonal guidance and dopamine signalling. These proteomic data could explain the biochemical, behavioural and anatomical deficits of the tgDISC1 rat model. Further, the DISC1 altered protein levels also highly associated with other mental disorders and/or nervous system diseases (Figure 11). These data suggest that alterations of the DISC1 protein levels can lead to changes in protein networks that are relevant to the brain disorders and diseases.

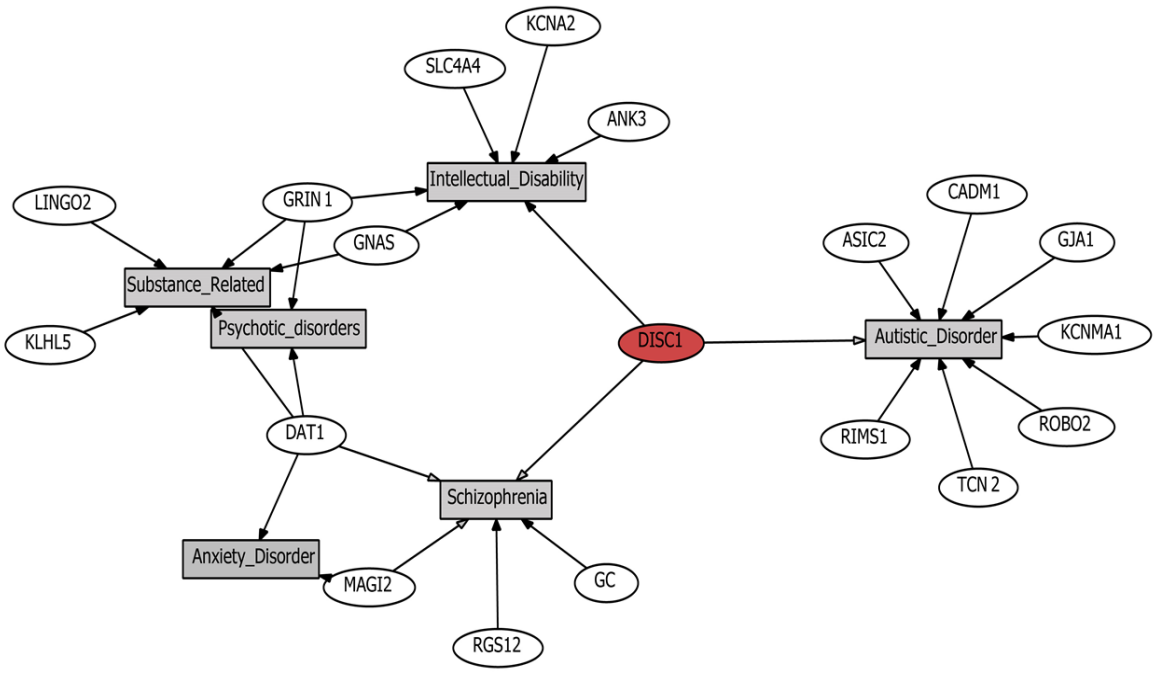


Figure 11 Disease-gene associations of DISC1 regulated proteins

Molecular tools for the investigation of dopaminergic systems.

In these dissertation, we have demonstrated the applicability of our methods the quantitation of the low abundance proteins of the dopaminergic system. Further, we have reported DRD1, DRD2 (*Paper 20*) and DAT1 (*data unpublished*) specific antibodies as by both receptor deficient mice and immunoprecipitation followed by mass spectrometry. Moreover, we have characterized molecular tools to study the dopamine transporter and receptors (DRD1 and DRD2). These tools include pharmacological drugs such as dopamine transporter reuptake inhibitor *(Paper 15)* and dopamine receptor agonists/antagonists *(Paper 4)*.These molecular tools are essential for further interrogation of the dopaminergic system in the tgDISC1 and other models.

4 Zusammenfassung (German)

Diese kumulative Doktorarbeit beschreibt die Implementierung einer Methode der synaptischen Proteomik mittels multiplexierter quantitativer Analysen in der Tandem-Massenspektrometrie und demonstriert ihre erfolgreiche Anwendung in Tiermodellen für Krankheit und Kognition. Das ursprüngliche Ziel dieser Arbeit ist es, technologische Methoden in der massenspektrometrischen Proteomik zur Analyse von synaptischen Proteinen zu entwickeln. Die erste Publikation befasst sich mit der Identifizierung von Membranproteinen (d.h. Rezeptoren, Transporter und Kanäle), die in Detergenzien löslichen und Detergenzien unlöslichen synaptosomalen Fraktionen von Rattenhirnrinden angereichert werden können. Der erste Schritt bestand darin, ein maßgeschneidertes Membran-Protein-Protokoll zu implementieren, um der Unterrepräsentation dieser Klasse von Proteinen in der massenspektrometrischen Proteomik entgegenzuwirken. Dies führte zur Beschreibung eines Kompendiums von synaptischen Proteinen, einschließlich Rezeptoren, Transportern und Kanälen, die zuvor nicht in Datenbanken für synaptische Proteine verzeichnet wurden. Darüber hinaus zeigten proteomische und bioinformatische Analysen der untersuchten in Detergenzien löslichen synaptosomalen Fraktionen eine Anreicherung von präsynaptischen Proteinen und von Proteinen, die mit Krankheiten und Störungen des Zentralennervensystems assoziiert sind.

In der zweiten Publikation wurde die entwickelte quantitative Proteomikmethode angewandt, um die Dynamik des synaptischen Proteoms in Tierkrankheitsmodellen zu untersuchen. Die proteomischen Signaturen des tgDISC1-Rattenmodells (transgenic disrupted in schizophrenia 1 rat model) wurden untersucht, um neue Proteinnetzwerke und Signalwege zu identifizieren, die durch eine Erhöhung der nichtmutanten DISC1-Expression reguliert werden. Die Zunahme der DISC1-Expression führt zu Veränderungen in Proteinen und in synaptischen Prozessen wie z.B. Membran- und Ionentransport, synaptische Organisation und Neuroentwicklung. Die identifizierten Proteine sind außerdem an Signalwegen beteiligt, insbesondere im Zusammenhang der axonalen Steuerung und des Dopaminsignalwegs.

Keywords: synaptosome, DISC1, striatum, neuroproteomics, dopamine transporter

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8 Appendix

8.1 Loss of Bardet-Biedl syndrome proteins causes synaptic aberrations in principal neurons (Haq et al., 2019).

Haq N, Schmidt-Hieber C, Sialana FJ, Ciani L, Heller JP, Stewart M, Bentley L, Wells S, Rodenburg RJ, Nolan PM, Forsythe E, Wu MC, Lubec G, Salinas P, Häusser M, Beales PL, Christou-Savina S.

PLoS Biol. 2019 Sep 3;17(9):e3000414. doi: 10.1371/journal.pbio.3000414. eCollection 2019 Sep. Erratum in: PLoS Biol. 2019 Oct 8;17(10):e3000520.

PMID:31479441

Abstract

Bardet-Biedl syndrome (BBS), a ciliopathy, is a rare genetic condition characterised by retinal degeneration, obesity, kidney failure, and cognitive impairment. In spite of progress made in our general understanding of BBS aetiology, the molecular and cellular mechanisms underlying cognitive impairment in BBS remain elusive. Here, we report that the loss of BBS proteins causes synaptic dysfunction in principal neurons, providing a possible explanation for the cognitive impairment phenotype observed in BBS patients. Using synaptosomal proteomics and immunocytochemistry, we demonstrate the presence of Bbs proteins in the postsynaptic density (PSD) of hippocampal neurons. Loss of Bbs results in a significant reduction of dendritic spines in principal neurons of Bbs mouse models. Furthermore, we show that spine deficiency correlates with events that destabilise spine architecture, such as impaired spine membrane receptor signalling, known to be involved in the maintenance of dendritic spines. Our findings suggest a role for BBS proteins in dendritic spine homeostasis that may be linked to the cognitive phenotype observed in BBS.

8.2 A proteotranscriptomic study of silk-producing glands from the orb-weaving spiders (Dos Santos-Pinto et al., 2019).

Dos Santos-Pinto JRA, Esteves FG, **Sialana FJ,** Ferro M, Smidak R, Rares LC, Nussbaumer T, Rattei T, Bilban M, Bacci Júnior M, Palma MS, Lübec G.

Mol Omics. 2019 Aug 5;15(4):256-270. doi: 10.1039/c9mo00087a.

PMID:31268449

Abstract

Orb-weaving spiders can produce different silk fibers, which constitute outstanding materials characterized by their high strength and elasticity. Researchers have tried to reproduce the fibers of these proteins synthetically and/or by using recombinant DNA technology, but only a few of the natural physicochemical and biophysical properties have been obtained to date. Female orb-web-spiders present seven silk-glands, which synthesize the spidroins and a series of other proteins, which interact with the spidroins, resulting in silk fibers with notable physicochemical properties. Despite the recognized importance of the silk-glands for understanding how the fibers are produced and processed, the investigation of these glands is at a nascent stage. In the current study we present the assembled transcriptome of silk-producing glands from the orb-weaving spider Nephila clavipes, as well as develop a large-scale proteomic approach for in-depth analyses of silk-producing glands. The present investigation revealed an extensive repertoire of hitherto undescribed proteins involved in silk secretion and processing, such as prevention of degradation during the silk spinning process, transportation, protection against proteolytic autolysis and against oxidative stress, molecular folding and stabilization, and post-translational modifications. Comparative phylogenomic-level evolutionary analyses revealed orthologous genes among three groups of silk-producing organisms - (i) Araneomorphae spiders, (ii) Mygalomorphae spiders, and (iii) silk-producing insects. A common orthologous gene, which was annotated as silk gland factor-3 is present among all species analysed. This protein belongs to a transcription factor family, that is important and related to the development of the silk apparatus synthesis in the silk glands of silk-producing arthropods.

8.3 Proteome Changes Paralleling the Olfactory Conditioning in the Forager Honey Bee and Provision of a Brain Proteomics Dataset.(Sialana et al., 2019)

Sialana FJ, Ribeiroda Silva Menegasso A, Smidak R, Hussein AM, Zavadil M, Rattei T, Lubec G, Sergio Palma M, Lubec J.

Proteomics. 2019 Jul;19(13):e1900094. doi: 10.1002/pmic.201900094. Epub 2019 Jun 24.

PMID:31115157

Abstract

The olfactory conditioning of the bee proboscis extension reflex (PER) is extensively used as a paradigm in associative learning of invertebrates but with limited molecular investigations. To investigate which protein changes are linked to olfactory conditioning, a non-sophisticated conditioning model is applied using the PER in the honeybee (Apis mellifera). Foraging honeybees are assigned into three groups based on the reflex behavior and training: conditioned using 2-octanone (PER-conditioned), and sucrose and water controls. Thereafter, the brain synaptosomal proteins are isolated and analyzed by quantitative proteomics using stable isotope labeling (TMT). Additionally, the complex proteome dataset of the bee brain is generated with a total number of 5411 protein groups, including key players in neurotransmitter signaling. The most significant categories affected during olfactory conditioning are associated with "SNARE interactions in vesicular transport" (BET1 and VAMP7), ABC transporters, and fatty acid degradation pathways.

8.4 Spheroid glioblastoma culture conditions as antigen source for dendritic cell-based immunotherapy: spheroid proteins are survival-relevant targets but can impair immunogenic interferon γ production.(Erhart et al., 2019b)

Erhart F, Weiss T, Klingenbrunner S, Fischhuber K, Reitermaier R, Halfmann A, Blauensteiner B, Lötsch D, Spiegl-Kreinecker S, Berger W**, Sialana FJ,** Lubec G, Felzmann T, Dohnal A, Visus C.

Cytotherapy. 2019 Jun;21(6):643-658. doi: 10.1016/j.jcyt.2019.03.002. Epub 2019 Apr 8.

PMID:30975602

Abstract

BACKGROUND: Glioblastoma is the most aggressive type of brain cancer. Dendritic cell (DC)-based immunotherapy against glioblastoma depends on the effectiveness of loaded antigens. Sphere-inducing culture conditions are being studied by many as a potential antigen source. Here, we investigated two different in vitro conditions (spheroid culture versus adherent culture) in relation to DC immunotherapy: (1) We studied the specific spheroid-culture proteome and assessed the clinical importance of spheroid proteins. (2) We evaluated the immunogenicity of spheroid lysate - both compared to adherent conditions.

METHODS: We used seven spheroid culture systems, three of them patient-derived. Stemness-related markers were studied in those three via immunofluorescence. Spheroid-specific protein expression was measured via quantitative proteomics. The Cancer Genome Atlas (TCGA) survival data was used to investigate the clinical impact of spheroid proteins. Immunogenicity of spheroid versus adherent cell lysate was explored in autologous ELISPOT systems (DCs and T cells from the three patients).

RESULTS: (1) The differential proteome of spheroid versus adherent glioblastoma culture conditions could successfully be established. The top 10 identified spheroid-specific proteins were associated with significantly decreased overall survival (TCGA MIT/Harvard cohort; $n = 350$, $P = 0.014$). (2) In exploratory experiments, immunogenicity of spheroid lysate vis-á-vis interferon (IFN)γ production was lower than that of adherent cell lysate (IFNγ ELISPOT; $P = 0.034$).

CONCLUSIONS: Spheroid culture proteins seem to represent survival-relevant targets, supporting the use of spheroid culture conditions as an antigen source for DC immunotherapy. However, immunogenicity enhancement should be considered for future research. Transferability of our findings in terms of clinical impact and regarding different spheroid-generation techniques needs further validation.

8.5 Dopamine type 1- and 2-like signaling in the modulation of spatial reference learning and memory.(Daba Feyissa et al., 2019)

Daba Feyissa D, **Sialana FJ**, Keimpema E, Kalaba P, Paunkov A, Engidawork E, Höger H, Lubec G, Korz V.

Behav Brain Res. 2019 Apr 19;362:173-180. doi: 10.1016/j.bbr.2019.01.028. Epub 2019 Jan 16.

PMID:30659847

Abstract

Spatial reference memory is known to be modulated by the dopaminergic system involving different brain regions. Here, we sought to identify the contribution of D1 (D1R) and D2 (D2R)-like dopamine receptor signaling on learning and memory in a food rewarded hole-board task by intracerebroventricular infusing D1R- and D2R- like receptor agonists (SKF-81297 and Sumanirole) and antagonists (SCH 23390 and Remoxipride) once 30 min prior to daily training sessions. D1R agonism induced persistent enhancement of performance, whereas D1R antagonism impaired reference memory formation. D2R agonist and antagonist exerted no effects. Phase specific comparisons revealed an enhancement of spatial acquisition in the presence of the D1R but not D2R agonism on acquisition, but not during retention. Since task difficulty might skew dopamine-induced improvements in learning and memory, we tested the D1R agonist in the hole-board task with increased difficulty. Drug treated animals performed significantly better during all training phases, with results better resolved than in the easy task. Additionally, proteomic analysis of the prefrontal cortex revealed ninety six proteins to be regulated by D1R agonism, from which 35 were correlated with behavioral performance. Obtained targets were grouped by function, showing synaptic transmission, synaptic remodeling, and dendritic spine morphology as the major functional classes affected. In sum, we find that activation of D1R signaling during spatial acquisition and retention improved reference memory index, depended on the task difficulty, and altered the proteome landscape of the prefrontal cortex indicative of massive organizational synaptic restructuring.

8.6 A novel role for NUPR1 in the keratinocyte stress response to UV oxidized phospholipids.(Narzt et al., 2018)

Narzt MS, Nagelreiter IM, Oskolkova O, Bochkov VN, Latreille J, Fedorova M, Ni Z, Sialana FJ, Lubec G, Filzwieser M, Laggner M, Bilban M, Mildner M, Tschachler E, Grillari J, Gruber F.

Redox Biol. 2019 Jan;20:467-482. doi: 10.1016/j.redox.2018.11.006. Epub 2018 Nov 13.

PMID:30466060

Ultraviolet light is the dominant environmental oxidative skin stressor and a major skin aging factor. We studied which oxidized phospholipid (OxPL) mediators would be generated in primary human keratinocytes (KC) upon exposure to ultraviolet A light (UVA) and investigated the contribution of OxPL to UVA responses. Mass spectrometric analysis immediately or 24 h post UV stress revealed significant changes in abundance of 173 and 84 lipid species, respectively. We identified known and novel lipid species including known bioactive and also potentially reactive carbonyl containing species. We found indication for selective metabolism and degradation of selected reactive lipids. Exposure to both UVA and to in vitro UVA - oxidized phospholipids activated, on transcriptome and proteome level, NRF2/antioxidant response signaling, lipid metabolizing enzyme expression and unfolded protein response (UPR) signaling. We identified NUPR1 as an upstream regulator of UVA/OxPL transcriptional stress responses and found this protein to be expressed in the epidermis. Silencing of NUPR1 resulted in augmented expression of antioxidant and lipid detoxification genes and disturbed the cell cycle, making it a potential key factor in skin reactive oxygen species (ROS) responses intimately involved in aging and pathology.

8.7 Proteomic Studies on the Swim Bladder of the European Eel (Anguilla anguilla) (Sialana et al., 2018a).

Sialana FJ, Schneebauer G, Paunkov A, Pelster B, Lubec G.

Proteomics. 2018 Apr;18(8):e1700445. doi: 10.1002/pmic.201700445. Epub 2018 Mar 30.

PMID:29469228

Abstract

The swim bladder of a fish is a vital organ that with gas gland cells in the swim bladder wall enables key physiological functions including buoyancy regulation in the face of different hydrostatic pressures. Specific gas gland cells produce and secrete acidic metabolites into the blood in order to reduce the physical solubility of gases and blood gas transport capacity for regulating the volume of the swim bladder. Transcriptomic analyses have provided evidence at the RNA level but no specific studies at the protein level have been carried out so far. Herein, it was the aim of the study to show swim bladder proteins of the yellow stage European eel by label-free LCMS (Q-Exactive Plus) that resulted in the identification of 6223 protein groups. Neurotransmitter receptors and transporters were enriched in the membrane fraction and enzymes for acid production were observed. The list of identified proteins may represent a useful tool for further proteomics experiments on this organ. All MS proteomics data are available at the PRIDE repository with the dataset identifier PXD007850.

8.8 Early Presymptomatic Changes in the Proteome of Mitochondria-Associated Membrane in the APP/PS1 Mouse Model of Alzheimer's Disease. (Volgyi et al., 2018)

Völgyi K, Badics K, **Sialana FJ**, Gulyássy P, Udvari EB, Kis V, Drahos L, Lubec G, Kékesi KA, Juhász G. Mol Neurobiol. 2018 Oct;55(10):7839-7857. doi: 10.1007/s12035-018-0955-6. Epub 2018 Feb 22. PMID:29468564

Abstract: Intracellular β-amyloid (Aβ) accumulation is an early event in Alzheimer's disease (AD) progression. Recently, it has been uncovered that presenilins (PSs), the key components of the amyloid precursor protein (APP) processing and the β-amyloid producing γ-secretase complex, are highly enriched in a special sub-compartment of the endoplasmic reticulum (ER) functionally connected to mitochondria, called mitochondria-associated ER membrane (MAM). A current hypothesis of pathogenesis of Alzheimer's diseases (AD) suggests that MAM is involved in the initial phase of AD. Since MAM supplies mitochondria with essential proteins, the increasing level of PSs and β-amyloid could lead to metabolic dysfunction because of the impairment of ER-mitochondrion crosstalk. To reveal the early molecular changes of this subcellular compartment in AD development MAM fraction was isolated from the cerebral cortex of 3 months old APP/PS1 mouse model of AD and age-matched C57BL/6 control mice, then mass spectrometry-based quantitative proteome analysis was performed. The enrichment and purity of MAM preparations were validated with EM, LC-MS/MS and protein enrichment analysis. Label-free LC-MS/MS was used to reveal the differences between the proteome of the transgenic and control mice. We obtained 77 increased and 49 decreased protein level changes in the range of - 6.365 to + 2.988, which have mitochondrial, ER or ribosomal localization according to Gene Ontology database. The highest degree of difference between the two groups was shown by the ATP-binding cassette G1 (Abcg1) which plays a crucial role in cholesterol metabolism and suppresses Aβ accumulation. Most of the other protein changes were associated with increased protein synthesis, endoplasmic-reticulum-associated protein degradation (ERAD), oxidative stress response, decreased mitochondrial protein transport and ATP production. The interaction network analysis revealed a strong relationship between the detected MAM protein changes and AD. Moreover, it explored several MAM proteins with hub position suggesting their importance in Aβ induced early MAM dysregulation. Our identified MAM protein changes precede the onset of dementia-like symptoms in the APP/PS1 model, suggesting their importance in the development of AD.

8.9 **Reduced Levels of the Synaptic Functional Regulator FMRP in Dentate Gyrus of the Aging Sprague-Dawley Rat.** (Smidak et al., 2017a)

Smidak R, **Sialana FJ**, Kristofova M, Stojanovic T, Rajcic D, Malikovic J, Feyissa DD, Korz V, Hoeger H, Wackerlig J, Mechtcheriakova D, Lubec G.

Front Aging Neurosci. 2017 Nov 23;9:384. doi: 10.3389/fnagi.2017.00384. eCollection 2017.
PMID:29218006

Abstract

Fragile X mental retardation protein (FMRP) encoded by Fragile X mental retardation 1 (*FMR1*) gene is a RNA-binding regulator of mRNA translation, transport and stability with multiple targets responsible for proper synaptic function. Epigenetic silencing of *FMR1* gene expression leads to the development of Fragile X syndrome (FXS) that is characterized by intellectual disability and other behavioral problems including autism. In the rat FXS model, the lack of FMRP caused a deficit in hippocampal-dependent memory. However, the hippocampal changes of FMRP in aging rats are not fully elucidated. The current study addresses the changes in FMRP levels in dentate gyrus (DG) from young (17 weeks) and aging (22 months) Sprague - Dawley rats. The aging animal group showed significant decline in spatial reference memory. Protein samples from five rats per each group were analyzed by quantitative proteomic analysis resulting in 153 significantly changed proteins. FMRP showed significant reduction in aging animals which was confirmed by immunoblotting and immunofluorescence microscopy. Furthermore, bioinformatic analysis of the differential protein dataset revealed several functionally related protein groups with individual interactions with FMRP. These include high representation of the RNA translation and processing machinery connected to FMRP and other RNA-binding regulators including CAPRIN1, the members of Pumilio (PUM) and CUG-BP, Elav-like (CELF) family, and YTH N(6)-methyladenosine RNA-binding proteins (YTHDF). The results of the current study point to the important role of FMRP and regulation of RNA processing in the rat DG and memory decline during the aging process.

8.10 Intra-nasal dopamine alleviates cognitive deficits in tgDISC1 rats which overexpress the human DISC1 gene (Wang et al., 2017).

Wang AL, Fazari B, Chao OY, Nikolaus S, Trossbach SV, Korth C, **Sialana FJ**, Lubec G, Huston JP, Mattern C, de Souza Silva MA.

Neurobiol Learn Mem. 2017 Dec;146:12-20. doi: 10.1016/j.nlm.2017.10.015. Epub 2017 Oct 28.

PMID:29107702

Abstract

The Disrupted-in-Schizophrenia 1 (DISC1) gene has been associated with mental illnesses such as major depression and schizophrenia. The transgenic DISC1 (tgDISC1) rat, which overexpresses the human DISC1 gene, is known to exhibit deficient dopamine (DA) homeostasis. To ascertain whether the DISC1 gene also impacts cognitive functions, 14-15 months old male tgDISC1 rats and wild-type controls were subjected to the novel object preference (NOP) test and the object-based attention test (OBAT) in order to assess short-term memory (1 h), long-term memory (24 h), and attention.

RESULTS:

The tgDISC1 group exhibited intact short-term memory, but deficient long-term-memory in the NOP test and deficient attention-related behavior in the OBAT. In a different group of tgDISC1 rats, 3 mg/kg intranasally applied dopamine (IN-DA) or its vehicle was applied prior to the NOP or the OBAT test. IN-DA reversed cognitive deficits in both the NOP and OBAT tests. In a further cohort of tgDISC1 rats, post-mortem levels of DA, noradrenaline, serotonin and acetylcholine were determined in a variety of brain regions. The tgDISC1 group had less DA in the neostriatum, hippocampus and amygdala, less acetylcholine in neostriatum, nucleus accumbens, hippocampus, and amygdala, more serotonin in the nucleus accumbens, and less serotonin and noradrenaline in the amygdala.

CONCLUSIONS:

Our findings show that DISC1 overexpression and misassembly is associated with deficits in long-term memory and attention-related behavior. Since behavioral impairments in tgDISC1 rats were reversed by IN-DA, DA deficiency may be a major cause for the behavioral deficits expressed in this model.

8.11 Validation of dopamine receptor DRD1 and DRD2 antibodies using receptor deficient mice.(Stojanovic et al., 2017)

Stojanovic T, Orlova M, **Sialana FJ**, Höger H, Stuchlik S, Milenkovic I, Aradska J, Lubec G.

Amino Acids. 2017 Jun;49(6):1101-1109. doi: 10.1007/s00726-017-2408-3. Epub 2017 Mar 18.

PMID:28316027

Abstract

Dopamine receptors 1 and 2 (DRD1, DRD2) are essential for signaling in the brain for a multitude of brain functions. Previous work using several antibodies against these receptors is abundant but only the minority of antibodies used have been validated and, therefore, the results of these studies remain uncertain. Herein, antibodies against DRD1 (Merck Millipore AB1765P, Santa Cruz Biotechnology sc-14001, Sigma Aldrich D2944, Alomone Labs ADR-001) and DRD2 (Abcam ab21218, Merck Millipore AB5084P, Santa Cruz Biotechnology sc-5303) have been tested using western blotting and immunohistochemistry on mouse striatum (wild type and corresponding knock-out mice) and when specific, they were further evaluated on rat and human striatum. Moreover, a DRD1 antibody and a DRD2 antibody that were found specific in our tests were used for immunoprecipitation with subsequent mass spectrometrical identification of the immunoprecipitate. Two out of nine antibodies (anti DRD1 Sigma Aldrich D2944 and anti DRD2 Merck Millipore AB5084P) against the abovementioned dopamine receptors were specific for DRD1 and DRD2 as evaluated by western blotting and immunohistochemistry and the immunoprecipitate indeed contained DRD1 and DRD2 as revealed by mass spectrometry. The observed findings may question the use of so far non-validated antibodies against the abovementioned dopamine receptors. Own observations may be valuable for the interpretation of previous results and the design of future studies using dopamine receptors DRD1 or DRD2.

8.12 A detailed proteomic profiling of plasma membrane from zebrafish brain. (Smidak et al., 2016a)

Smidak R, Aradska J, Kirchberger S, Distel M, **Sialana FJ**, Wackerlig J, Mechtcheriakova D, Lubec G.

Proteomics Clin Appl. 2016 Dec;10(12):1264-1268. doi: 10.1002/prca.201600081. Epub 2016 Aug 12.

PMID:27459904

Zebrafish (Danio rerio) is a well-established model organism in developmental biology and disease modeling. In recent years, an increasing amount of studies used zebrafish to analyze the genetic changes underlying various neurological disorders. The brain plasma membrane proteome represents the major subsets of signaling proteins and promising drug targets, but is often understudied due to traditional experimental difficulties including problems with solubility, detergent removal, or low abundance. Here, we report a comprehensive dataset of the proteins identified in the enriched plasma membrane of the zebrafish brain by applying sequential trypsin/chymotrypsin digestion with multidimensional LC-MS/MS. A total number of 97 017 peptide groups corresponding to 9201 proteins were identified. These were annotated in various molecular functions or neurological disorders. The dataset of the current study provides a useful data source for further utilizing zebrafish in basic and clinical neuroscience.

8.13 Transcriptomic and Proteomic Analysis of Arion vulgaris--Proteins for Probably Successful Survival Strategies?(Bulat et al., 2016)

Bulat T, Smidak R, **Sialana FJ**, Jung G, Rattei T, Bilban M, Sattmann H, Lubec G, Aradska J. PLoS One. 2016 Mar 17;11(3):e0150614. doi: 10.1371/journal.pone.0150614. eCollection 2016.

PMID:26986963

Abstract

The Spanish slug, Arion vulgaris, is considered one of the hundred most invasive species in Central Europe. The immense and very successful adaptation and spreading of A. vulgaris suggest that it developed highly effective mechanisms to deal with infections and natural predators. Current transcriptomic and proteomic studies on gastropods have been restricted mainly to marine and freshwater gastropods. No transcriptomic or proteomic study on A. vulgaris has been carried out so far, and in the current study, the first transcriptomic database from adult specimen of A. vulgaris is reported. To facilitate and enable proteomics in this non-model organism, a mRNA-derived protein database was constructed for protein identification. A gel-based proteomic approach was used to obtain the first generation of a comprehensive slug mantle proteome. A total of 2128 proteins were unambiguously identified; 48 proteins represent novel proteins with no significant homology in NCBI non-redundant database. Combined transcriptomic and proteomic analysis revealed an extensive repertoire of novel proteins with a role in innate immunity including many associated pattern recognition, effector proteins and cytokine-like proteins. The number and diversity in gene families encoding lectins point to a complex defense system, probably as a result of adaptation to a pathogen-rich environment. These results are providing a fundamental and important resource for subsequent studies on molluscs as well as for putative antimicrobial compounds for drug discovery and biomedical applications.

8.14 Gel-free mass spectrometry analysis of Drosophila melanogaster heads (Aradska et al., 2015b).

Aradska J, Bulat T, **Sialana FJ**, Birner-Gruenberger R, Erich B, Lubec G.
Proteomics. 2015 Oct;15(19):3356-60. doi: 10.1002/pmic.201500092. Epub 2015 Sep 10.
PMID:26201256

Abstract
Membrane proteins play key roles in several fundamental biological processes such as cell signalling, energy metabolism and transport. Despite the significance, these still remain an under-represented group in proteomics datasets. Herein, a bottom-up approach to analyse an enriched membrane fraction from Drosophila melanogaster heads using multidimensional liquid chromatography (LC) coupled with tandem-mass spectrometry (MS/MS) that relies on complete solubilisation and digestion of proteins, is reported. An enriched membrane fraction was prepared using equilibrium density centrifugation on a discontinuous sucrose gradient, followed by solubilisation using the filter-aided sample preparation (FASP), tryptic and sequential chymotryptic digestion of proteins. Peptides were separated by reversed-phase (RP) LC at high pH in the first dimension and acidic RP-LC in the second dimension coupled directly to an Orbitrap Velos Pro mass spectrometer. A total number of 4812 proteins from 114 865 redundant and 38 179 distinct peptides corresponding to 4559 genes were identified in the enriched membrane fraction from fly heads. These included brain receptors, transporters and channels that are most important elements as drug targets or are linked to disease. Data are available via ProteomeXchange with identifier PXD001712.

8.15 Lack of presynaptic interaction between glucocorticoid and CB1 cannabinoid receptors in GABA- and glutamatergic terminals in the frontal cortex of laboratory rodents (Bitencourt et al., 2015b).

Bitencourt RM, Alpár A, Cinquina V, Ferreira SG, Pinheiro BS, Lemos C, Ledent C, Takahashi RN, **Sialana FJ**, Lubec G, Cunha RA, Harkany T, Köfalvi A.

Neurochem Int. 2015 Nov;90:72-84. doi: 10.1016/j.neuint.2015.07.014. Epub 2015 Jul 18.

PMID:26196379

Abstract

Corticosteroid and endocannabinoid actions converge on prefrontocortical circuits associated with neuropsychiatric illnesses. Corticosteroids can also modulate forebrain synapses by using endocannabinoid effector systems. Here, we determined whether corticosteroids can modulate transmitter release directly in the frontal cortex and, in doing so, whether they affect presynaptic CB1 cannabinoid receptor- (CB1R) mediated neuromodulation. By Western blotting of purified subcellular fractions of the rat frontal cortex, we found glucocorticoid receptors (GcRs) and CB1Rs enriched in isolated frontocortical nerve terminals (synaptosomes). CB1Rs were predominantly presynaptically located while GcRs showed preference for the post-synaptic fraction. Additional confocal microscopy analysis of cortical and hippocampal regions revealed vesicular GABA transporter-positive and vesicular glutamate transporter 1-positive nerve terminals endowed with CB1R immunoreactivity, apposing GcR-positive post-synaptic compartments. In functional transmitter release assay, corticosteroids, corticosterone (0.1-10 microM) and dexamethasone (0.1-10 microM) did not significantly affect the evoked release of [(3)H]GABA and [(14)C]glutamate in superfused synaptosomes, isolated from both rats and mice. In contrast, the synthetic cannabinoid, WIN55212-2 (1 microM) diminished the release of both [(3)H]GABA and [(14)C]glutamate, evoked with various depolarization paradigms. This effect of WIN55212-2 was abolished by the CB1R neutral antagonist, O-2050 (1 microM), and was absent in the CB1R KO mice. CB2R-selective agonists did not affect the release of either neurotransmitter. The lack of robust presynaptic neuromodulation by corticosteroids was unchanged upon either CB1R activation or genetic inactivation. Altogether, corticosteroids are unlikely to exert direct non-genomic presynaptic neuromodulation in the frontal cortex, but they may do so indirectly, via the stimulation of trans-synaptic endocannabinoid signaling.

8.16 Frontal cortex and hippocampus neurotransmitter receptor complex level parallels spatial memory performance in the radial arm maze

(Shanmugasundaram et al., 2015).

Shanmugasundaram B, Sase A, Miklosi AG, **Sialana FJ**, Subramaniyan S, Aher YD, Gröger M, Höger H, Bennett KL, Lubec G.

Behav Brain Res. 2015 Aug 1;289:157-68. doi: 10.1016/j.bbr.2015.04.043. Epub 2015 Apr 28.

PMID:25930220

Abstract

Several neurotransmitter receptors have been proposed to be involved in memory formation. However, information on receptor complexes (RCs) in the radial arm maze (RAM) is missing. It was therefore the aim of this study to determine major neurotransmitter RCs levels that are modulated by RAM training because receptors are known to work in homo-or heteromeric assemblies. Immediate early gene Arc expression was determined by immunohistochemistry to show if prefrontal cortices (PFC) and hippocampi were activated following RAM training as these regions are known to be mainly implicated in spatial memory. Twelve rats per group, trained and untrained in the twelve arm RAM were used, frontal cortices and hippocampi were taken, RCs in membrane protein were quantified by blue-native PAGE immunoblotting. RCs components were characterised by co-immunoprecipitation followed by mass spectrometrical analysis and by the use of the proximity ligation assay. Arc expression was significantly higher in PFC of trained as compared to untrained rats whereas it was comparable in hippocampi. Frontal cortical levels of RCs containing AMPA receptors GluA1, GluA2, NMDA receptors GluN1 and GluN2A, dopamine receptor D1, acetylcholine nicotinic receptor alpha 7 (nAChR-α7) and hippocampal levels of RCs containing D1, GluN1, GluN2B and nAChR-α7 were increased in the trained group; phosphorylated dopamine transporter levels were decreased in the trained group. D1 and GluN1 receptors were shown to be in the same complex. Taken together, distinct RCs were paralleling performance in the RAM which is relevant for interpretation of previous and design of future work on RCs in memory studies.

8.17 Individual phases of contextual fear conditioning differentially modulate dorsal and ventral hippocampal GluA1-3, GluN1-containing receptor complexes and subunits. (Sase et al., 2015).

Sase S, Sase A, **Sialana FJ** Jr, Gröger M, Bennett KL, Stork O, Lubec G, Li L.
Hippocampus. 2015 Dec;25(12):1501-16. doi: 10.1002/hipo.22470. Epub 2015 Jul 14.
PMID:25914080

Abstract

In contextual fear conditioning (CFC), the use of pharmacological and lesion approaches has helped to understand that there are differential roles for the dorsal hippocampus (DH) and the ventral hippocampus (VH) in the acquisition, consolidation and retrieval phases. Concomitant analysis of the DH and the VH in individual phases with respect to α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptors and N-methyl-D-aspartate receptor subtype N1 (GluN1)-containing complexes (RCC) and subunits has not been reported so far. Herein, CFC was performed in mice that were euthanized at different time points. DH and VH samples were taken for the determination of RCC and subunit levels using BN- and SDS-PAGE, respectively, with subsequent Western blotting. Evaluation of spine densities, morphology, and immunohistochemistry of GluA1 and GluA2 was performed. In the acquisition phase levels of GluA1-RCC and subunits in VH were increased. In the consolidation phase GluA1- and GluA2-RCC levels were increased in DH and VH, while both receptor subunit levels were increased in the VH only. In the retrieval phase GluA1-RCC, subunits thereof and GluA2-RCC were increased in DH and VH, whereas GluA2 subunits were increased in the VH only. GluN1-RCC levels were increased in acquisition and consolidation phase, while subunit levels in the acquisition phase were increased only in the DH. The immunohistochemical studies in the individual phases in subareas of hippocampus supported immunochemical changes of GluA1 and GluA2 RCC's. Dendritic spine densities and the prevalence of thin spines in the acquisition phase of VH and mushroom spines in the retrieval phase of the VH and DH were increased. The findings from the current study suggest different receptor and receptor complex patterns in the individual phases in CFC and in DH and VH. The results propose that different RCCs are formed in the individual phases and that VH and DH may be involved in CFC.

8.18 Drebrin depletion alters neurotransmitter receptor levels in protein complexes, dendritic spine morphogenesis and memory-related synaptic plasticity in the mouse hippocampus (Jung et al., 2015).

Jung G, Kim EJ, Cicvaric A, Sase S, Gröger M, Höger H, **Sialana FJ**, Berger J, Monje FJ, Lubec G.

J Neurochem. 2015 Jul;134(2):327-39. doi: 10.1111/jnc.13119. Epub 2015 Apr 29.

PMID:25865831

Abstract

Drebrin an actin-bundling key regulator of dendritic spine genesis and morphology, has been recently proposed as a regulator of hippocampal glutamatergic activity which is critical for memory formation and maintenance. Here, we examined the effects of genetic deletion of drebrin on dendritic spine and on the level of complexes containing major brain receptors. To this end, homozygous and heterozygous drebrin knockout mice generated in our laboratory and related wild-type control animals were studied. Level of protein complexes containing dopamine receptor D1/dopamine receptor D2, 5-hydroxytryptamine receptor 1A (5-HT1(A)R), and 5-hydroxytryptamine receptor 7 (5-HT7R) were significantly reduced in hippocampus of drebrin knockout mice whereas no significant changes were detected for GluR1, 2, and 3 and NR1 as examined by native gel-based immunoblotting. Drebrin depletion also altered dendritic spine formation, morphology, and reduced levels of dopamine receptor D1 in dendritic spines as evaluated using immunohistochemistry/confocal microscopy. Electrophysiological studies further showed significant reduction in memory-related hippocampal synaptic plasticity upon drebrin depletion. These findings provide unprecedented experimental support for a role of drebrin in the regulation of memory-related synaptic plasticity and neurotransmitter receptor signaling, offer relevant information regarding the interpretation of previous studies and help in the design of future studies on dendritic spines.

8.19 Hippocampal receptor complexes paralleling LTP reinforcement in the spatial memory holeboard test in the rat (Subramaniyan et al., 2015).

Subramaniyan S, Hajali V, Scherf T, Sase SJ, **Sialana FJ**, Gröger M, Bennett KL, Pollak A, Li L, Korz V, Lubec G.

Behav Brain Res. 2015 Apr 15;283:162-74. doi: 10.1016/j.bbr.2015.01.036. Epub 2015 Jan 30.

PMID:25639541

Abstract

The current study was designed to examine learning-induced transformation of early-LTP into late-LTP. Recording electrodes were implanted into the dentate gyrus of the hippocampus in male rats and early-LTP was induced by weak tetanic stimulation of the medial perforant path. Dorsal right hippocampi were removed, membrane proteins were extracted, separated by blue-native gel electrophoresis with subsequent immunoblotting using brain receptor antibodies. Spatial training resulted into reinforcement of LTP and the reinforced LTP was persistent for 6h. Receptor complex levels containing GluN1 and GluN2A of NMDARs, GluA1 and GluA2 of AMPARs, nAchα7R and the D(1A) dopamine receptor were significantly-elevated in rat hippocampi of animals underwent spatial learning, whilst levels of GluA3 and 5-HT1A receptor containing complexes were significantly reduced. Evidence for complex formation between GluN1 and D(1A) dopamine receptor was provided by antibody shift assay, co-immunoprecipitation and mass spectrometric analysis. Thus our results propose that behavioural stimuli like spatial learning reinforce early LTP into late LTP and this reinforced LTP is accompanied by changes in certain receptor levels in the membrane fraction of the rat hippocampus.

8.20 Modafinil improves performance in the multiple T-Maze and modifies GluR1, GluR2, D2 and NR1 receptor complex levels in the C57BL/6J mouse (Sase et al., 2012).

Sase S, Khan D, **Sialana F**, Höger H, Russo-Schlaff N, Lubec G.
Amino Acids. 2012 Dec;43(6):2285-92. doi: 10.1007/s00726-012-1306-y. Epub 2012 May 22.
PMID: 22614872

Abstract

Modafinil has been shown to modify behavioural and cognitive functions and to effect several brain receptors. Effects, however, were not observed at the receptor protein complex level and it was therefore the aim of the study to train mice in the multiple T-Maze (MTM) as a paradigm for spatial memory and to determine paralleling brain receptor complex levels. Sixty C57BL/6J mice were used in the study and divided into four groups (trained drug injected; trained vehicle injected; yoked drug injected; yoked vehicle injected). Animals obtained training for 4 days and were killed 6 h following the last training session on day 4. Hippocampi were dissected from the brain, membrane fractions were prepared by ultracentrifugation and were run on blue-native gels and immunoblotted with antibodies against major brain receptors. Modafinil treatment led to decreased latency and increased average speed, but not to changes in pathlength and number of correct decisions in the MTM. Drug effects were modifying receptor complexes of GluR1, GluR2, D2 and NR1. Training effects on receptor complex levels were observed for GluR3, D1 and nicotinic acetylcholine receptor alpha 7 (Nic7). GluR1 levels were correlating with GluR2 and D1 levels were correlating with D2 and NR1. Involvement of the glutamatergic, NMDA, dopaminergic and nicotinergic system in modafinil and memory training were herein described for the first time. A brain receptor complex pattern was revealed showing the concerted action following modafinil treatment.

8.21 Proteomic and Clinical Analysis of a Fine-Needle Aspirate Biopsy from a Single Cold Thyroid Nodule: A Case Study (Vitko and Sialana, 2016)

Vitko D, Sialana FJ, Parapatics K, Koperek O, Pötzi C, et al. (2016) Proteomic and Clinical Analysis of a Fine-Needle Aspirate Biopsy from a Single Cold Thyroid Nodule: A Case Study

J Clin Case Rep 6:766. doi:10.4172/2165-7920.1000766

Abstract

Background: For cases where clinical and cytological data from cold thyroid nodules are ambiguous; presurgical proteomic profiling of fine-needle aspirate biopsies of cold thyroid nodules in situ can provide additional diagnostics to avoid invasive surgical intervention and thyroidectomy of benign or non-cancerous tissue. Methods: The fine-needle aspirate biopsy lysate was digested with trypsin, and analysed by liquid chromatography mass spectrometry on a linear trap quadrupole Orbitrap Velos. Remaining peptides were separated by reversedphase chromatography and fractions analysed as technical duplicates. Identified proteins were analysed by Gene Ontology and protein abundance were calculated using the Top3 label-free method. The proteomic data was complemented with ultrasonography and scintigraphy of the thyroid gland; and cytology of the cold thyroid nodule fine-needle aspirate biopsy. Results: Sixty seven and 2,595 non-redundant protein groups (≥2 unique peptides) were identified from unfractionated and fractionated cold thyroid nodule fine-needle aspirate biopsy, respectively. Label-free protein abundance ranged over 6 orders of magnitude from the most abundant proteins, haemoglobin and thyroglobulin; to the low-abundance protein SON. Many previously-reported markers of thyroid cancer were in the top 23% of the identified proteins. Gene Ontology analysis revealed high-enrichment for cytoplasmic and membrane-bound organelle (cellular component); single-organism and small molecular processes (biological processes); and poly(A) ribonucleic acid, ribonucleic acid and protein-binding (molecular function). Conclusions: The cold thyroid nodule was clinically-classified as benign. Proteomic data from fine-needle aspirate biopsies can provide additional diagnostic candidates indicative of a benign or cancerous cold thyroid nodule without the need for invasive surgical intervention.

9 Table of abbreviations

AMPA/ AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BBS	Bardet-Biedl syndrome
CB1	cannabinoid receptor 1,
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DISC1	Disrupted in schizophrenia 1
DRD1	dopamine receptor 1
DRD2	dopamine receptor 2
DA	dopamine
DAT1	dopamine transporter 1
DGIdb	Drug Gene Interaction Database
DSS	detergent soluble synaptosome
DTT	Dithiothreitol
DDM	n-Dodecyl β-D-maltoside
ESI	electrospray ionisation
FASP	filter-aided sample preparation
FDR	false discovery rate
HPLC	high performance liquid chromatography
iTRAQ	Isobaric tags for relative and absolute quantitation
IUPHAR	International Union of Basic and Clinical Pharmacology
GcR	glucocorticoid receptor
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCMS	Liquid chromatography–mass spectrometry
MALDI	Matrix Assisted Laser Desorption/Ionization
NMDA/NMDAR	N-methyl-D-aspartate receptor
PEP	posterior error probability
ppm	parts per million
PSD	postsynaptic density
PSM	peptide spectral matches
SDS	sodium dodecyl sufate
SILAC	Stable Isotope Labeling with Amino Acids in Cell Culture
SVM	Support vector machine
TEAB	trimethylamine bicarbonate
TMD	transmembrane domain
TMHMM	transmembrane hidden Markov model
TMT	Tandem mass tag

10 Curriculum Vitae

Personal data

Name	Fernando Jayson Sialana, Jr
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Education

2020	Dr.-Studium der Ingenieurwissenschaften; Lebensmittel- und Biotechnologie
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Professional and Research experience

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2013-2015	Ph.D. Student (shared), CeMM Center for Molecular Medicine of the Austrian Academy of Science, Vienna, Austria, Dr. Keiryn L. Bennett (Mass spectrometry and Proteomics)
2004-2011	Full Instructor, Chemistry Department, University of San Carlos, Philippines
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2001-2003	Plant Supervisor/HACCP Accreditation Leader, RGE Agridev Corporation, Philippines
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