

## Mass spectrometry-based proteomic characterization of the middle-aged mouse brain for animal model research of neuromuscular diseases

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### Abstract

Neuromuscular diseases with primary muscle wasting symptoms may also display multi-systemic changes in the body and exhibit secondary pathophysiological alterations in various non-muscle tissues. In some cases, this includes proteome-wide alterations and/or adaptations in the central nervous system. Thus, in order to provide an improved bioanalytical basis for the comprehensive evaluation of animal models that are routinely used in muscle research, this report describes the mass spectrometry-based proteomic characterization of the mouse brain. Crude tissue extracts were examined by bottom-up proteomics and detected 4558 distinct protein species. The detailed analysis of the brain proteome revealed the presence of abundant cellular proteoforms in the neuronal cytoskeleton, as well as various brain region enriched proteins, including markers of the cerebral cortex, cerebellum, hippocampus and the olfactory bulb. Neuroproteomic markers of specific cell types in the brain were identified in association with various types of neurons and glia cells. Markers of subcellular structures were established for the plasmalemma, nucleus, endoplasmic reticulum, mitochondria and other crucial organelles, as well as synaptic components that are involved in presynaptic vesicle docking, neurotransmitter release and synapse remodelling.

**Key Words:** mass spectrometry; mouse brain; neuromuscular disease; neuroproteomics.

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The mouse is one of the most widely used animal models in neuroscience,<sup>1</sup> including studies of neuromuscular disorders such as muscular dystrophy,<sup>2</sup> or motor neuron disease.<sup>3</sup> Many genetic or acquired muscle diseases exhibit besides characteristic symptoms of fiber degeneration and/or contractile dysfunction also body-wide changes that are illustrated by pathophysiological alterations in non-muscular tissues. For example, the highly heterogeneous group of motor neuron diseases displays severe dysfunction of the motor system, but also a variety of extra-motor abnormalities. The most frequently observed adult-onset form of motor neuron disease is amyotrophic lateral sclerosis, which is characterized by progressive muscular weakness.<sup>4</sup> Primary abnormalities in an extremely large number of

genes have been associated with this disorder. Established animal models of amyotrophic lateral sclerosis include the SOD1 mouse and the wobbler mouse.<sup>3</sup> In both patients and animal models of amyotrophic lateral sclerosis, abnormalities are observed in the flow of excitatory signals from neurons in the cortex, brain stem, spinal cord and the neuromuscular junction.<sup>5</sup> Thus, in order to better understand the pathobiochemical mechanisms that underlie muscular atrophy due to the degeneration of lower and upper motor neurons, a comprehensive analysis of the peripheral and central nervous system is essential. Proteomics suggests itself as the methodological approach of choice for such a large-scale and high-throughput analysis due to its swift, unbiased and technology-driven nature.<sup>6</sup> However,

the mass spectrometric survey of the mouse model brain and its pathophysiological involvement in motor neuron disease is ideally based on previously established catalogues of the normal brain proteome.<sup>7</sup> This should preferably include the biochemical detection and characterization of brain region enriched markers, cell type specific proteins and the identification of subcellular markers with a distinct function in key organelles of the diverse types of neurons and glial cells.

Another example of a neuromuscular disorder with complex body-wide alterations including abnormal brain functions is Duchenne muscular dystrophy (DMD).<sup>8</sup> This X-linked inherited disorder can be clearly defined as a primary muscle wasting disorder but can also be categorized as a multi-systemic disease.<sup>9</sup> Dystrophinopathies are due to mutations in the *DMD* gene that results in the almost complete loss of the membrane cytoskeletal protein dystrophin and a reduced expression of its associated glycoprotein complex in voluntary contractile fibers. This weakens the muscle fiber periphery and renders the sarcolemma more susceptible to contraction-induced rupturing of the surface membrane. Hence, the main pathophysiological hallmarks of X-linked muscular dystrophy are muscle membrane leakage and calcium-related myonecrosis, combined with fat substitution, reactive myofibrosis and chronic inflammation of skeletal muscles.<sup>10</sup> Many of these symptoms are also seen in the *mdx*-type mouse models of dystrophinopathy.<sup>2</sup> However, since the extremely large *DMD* gene has several promoter regions that produce 8 different tissue-specific dystrophin isoforms, mutation-specific alterations in combination with secondary changes can occur in many organ systems besides the skeletal musculature. This may include the liver, kidney, spleen, gastrointestinal tract and the nervous system.<sup>11-13</sup> In this context, it is encouraging that the proteomic survey of the dystrophin-deficient brain of the *mdx* model of Duchenne muscular dystrophy has identified a large number of altered proteins involved in the maintenance of intermediate filaments, membrane repair mechanisms and calcium handling.<sup>14</sup>

In order to establish the scientific basis for comparative proteomic surveys of the central nervous system in relation to mouse model research of neuromuscular diseases, this report outlines the mass spectrometry-based proteomic characterization of the normal middle-aged mouse brain. The proteomic data of markers of brain regions such as the olfactory bulb, hippocampus, cerebellum, hypothalamus and cerebral cortex, various brain cell types and subcellular structures found in neurons, synapses and glia cells should be helpful as a reference guide for the comprehensive evaluation of the central nervous system in genetic mouse models that are routinely used in basic and applied myology research.

## Materials and Methods

For the proteomic profiling of the mouse brain, general materials and analytical grade reagents were obtained

from GE Healthcare (Little Chalfont, Buckinghamshire, UK), Sigma Chemical Company (Dorset, UK) and Bio-Rad Laboratories (Hemel-Hempstead, Hertfordshire, UK). Controlled protein digestion was carried out with sequencing grade-modified trypsin and the proteolytic enzyme Lys-C, in combination with Protease Max Surfactant Trypsin Enhancer, from Promega (Madison, WI, USA). Vivacon 500 (Product number: VN0H22) filter units from Sartorius (Göttingen, Germany) were used for filter-aided sample preparation. Buffers for tissue extraction were supplemented with a protease inhibitor cocktail from Roche Diagnostics (Mannheim, Germany). The protein concentration of extracted samples was determined with the Pierce 660-nm Protein Assay from ThermoFisher Scientific (Dublin, Ireland).

## Ethical approval, animal license and animal maintenance

Male wild type C57/BL6 mice were obtained from the Bioresource Unit of the University of Bonn.<sup>13</sup> All procedures adhered to German legislation on the use of animals in experimental research (Amt für Umwelt, Verbraucherschutz und Lokale Agenda der Stadt Bonn, North Rhine-Westphalia, Germany). Mice were kept under standard conditions.<sup>15</sup> For tissue dissection, protein extraction and subsequent proteomic analyses, 12-months old mice were used. Freshly dissected brain tissue specimens were quick-frozen in liquid nitrogen and transported to Maynooth University in accordance with the Department of Agriculture (animal by-product register number 2016/16 to the Department of Biology, National University of Ireland, Maynooth) on dry ice and stored at -80°C prior to proteomic analysis.<sup>14</sup>

## Preparation of middle-aged mouse brain protein extracts for mass spectrometric analysis

This study was carried out with 12-month old tissue specimens since this represents the fully matured and middle-aged central nervous system.<sup>16</sup> Importantly, at this age the proteomic data are suitable for comparative studies of late-onset neuromuscular changes, such as reactive myofibrosis.<sup>17</sup> In general, mice of 3-6, 10-15 and 18-24 months of age are considered 'mature adult', 'middle aged' and 'old', respectively. The middle-aged group represents a phase in the life of mice during which early changes due to the natural aging process can be detected. Thus, the murine middle-aged group can be conveniently used in comparative aging studies to evaluate whether an age-associated effect is of an early onset and progressive nature or only seen at much older age.<sup>18</sup> Whole brain specimens from wild type (n=6) mice were lysed by homogenization using 4% (w/v) sodium dodecyl sulfate, 0.1M dithiothreitol, 100mM Tris-Cl pH 7.6; supplemented with a protease inhibitor cocktail.<sup>19</sup> Following incubation at 95°C for 3 minutes, sonication for 30 seconds and centrifugation at 16,000xg for 5 minutes, the resulting tissue extracts were analyzed by mass spectrometry-based proteomics.<sup>20</sup> Peptides were

generated by digestion with the proteolytic enzymes LysC and trypsin and then processed using standardized filter-aided sample preparation (FASP).<sup>21</sup>

### **Mass spectrometry-based proteomic profiling of the mouse brain**

All preparative steps and analytical procedures using label-free liquid chromatography mass spectrometry and data-dependent acquisition methodology were recently described in detail.<sup>20</sup> For mass spectrometry, a Thermo UltiMate 3000 nano system was used for reverse-phased capillary high-pressure liquid chromatography, which was directly coupled in-line with a Thermo Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The generated mass spectrometric files were qualitatively analyzed using the UniProtKB-SwissProt database (*Mus musculus*) with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and Percolator.<sup>19</sup> The following search parameters were employed for protein identification: (i) an allowance of up to two missed cleavages, (ii) MS/MS mass tolerance set to 0.02 Da, (iii) (i) peptide mass tolerance set to 10 ppm, (iv) methionine oxidation set as a variable modification, and (v) carbamido-methylation set as a fixed modification.<sup>15</sup> Individual peptides were filtered using a minimum XCorr score of 1.5 for 1, 2.0 for 2, 2.25 for 3 and 2.5 for 4 charge states, with peptide probability set to high confidence. Progenesis QI for Proteomics (version 2.0; Nonlinear Dynamics, a Waters company, Newcastle upon Tyne, UK) was used for quantitative label-free data analysis. Protein identification was carried out with Proteome Discoverer 2.2 using Sequest HT (Thermo Fisher Scientific) and Percolator. Data sets were then imported into Progenesis QI software for further analysis.<sup>19</sup> The .raw MS files and the multi-consensus data of this mass spectrometric study have been deposited to the Open Science Framework (OSF) under the title “Proteomic profiling of mouse brain” (<https://osf.io/345w8/>) with the file name ‘345w8’. Bioinformatic PANTHER analysis of protein families was performed with a publicly available search engine (<https://pantherdb.org>).<sup>22</sup>

## **Results**

### **Mass spectrometric analysis of crude extracts from middle-aged mouse brain**

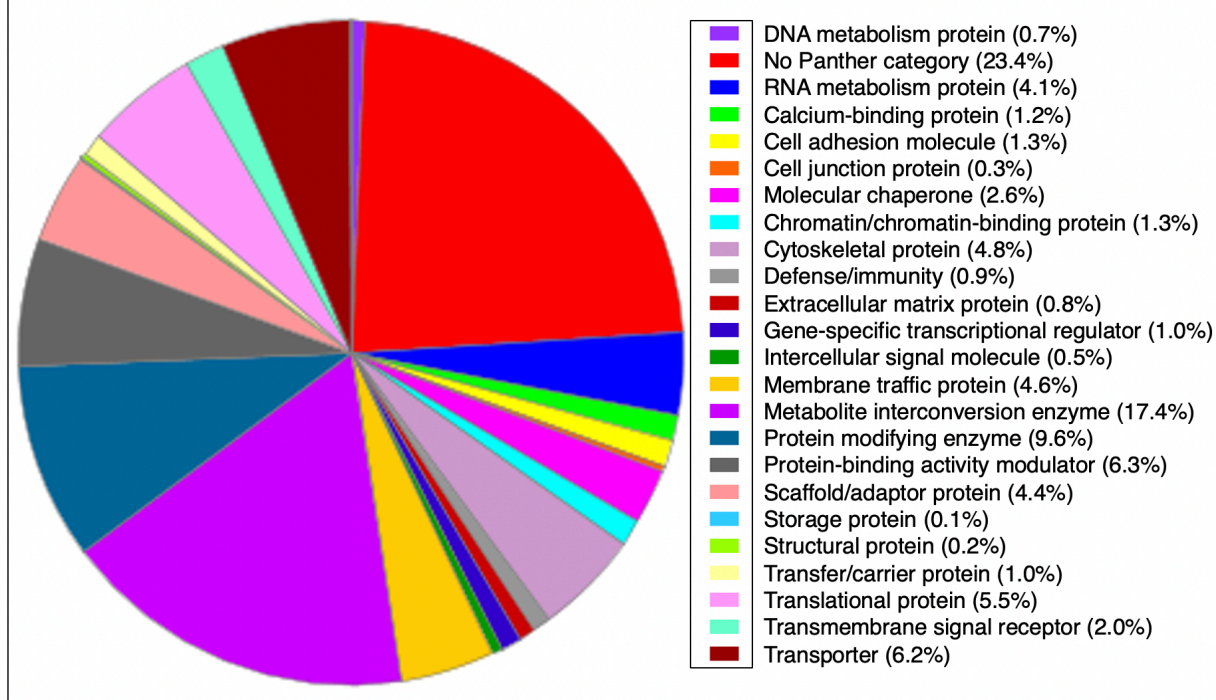
The proteomic analysis of total brain extracts detected 4,558 distinct protein species. Proteins identified with greater than 100 peptides were found to be predominately cytoskeletal proteins. The most abundant brain proteins were identified as the alpha and beta chains of spectrin (*Sptan1* gene, *Sptbn1* gene), the heavy chain of cytoplasmic dynein-1 (*Dync1h1* gene), plectin (*Plec* gene), ankyrin-2 (*Ank2* gene), microtubule-associated proteins 1A and 1B (*Map1a* gene, *Map1b* gene) and protein bassoon (*Bsn* gene). The bioinformatic PANTHER analysis of protein classes in total extracts of mouse brain are presented in Figure 1. Major protein

classes include components involved in RNA metabolism, calcium-binding, cell adhesion, cell junction formation, the cellular stress response, chromatin binding, cytoskeletal maintenance, membrane trafficking, enzymatic conversion of metabolites, protein modification, modulation of protein-binding activity, scaffolding and adaptations, translation, transmembrane signaling and transportation.<sup>22</sup> The section ‘No Panther category’ consists of protein species without class annotation. These proteins may have unknown functions, have not yet being curated, or no protein class terms describes their function(s).<sup>22</sup> A variety of brain region enriched/specific proteins were detected in crude tissue extracts, including generally accepted markers of the cerebral cortex, cerebellum, hippocampus and the olfactory bulb.<sup>7,23-25</sup> As a marker of the cerebral cortex, Septin-7 (*Sept7* gene) was detected by 24 peptides (63.3% sequence coverage, accession number O55131, molecular mass 101.9 kDa). Protein markers of the cerebellum were established as peripherin (*Prph* gene) by 6 peptides (13.5% sequence coverage, accession number P15331-3, molecular mass 52.7 kDa) and the metabotropic glutamate receptor 1 (*Grm1* gene) by 5 peptides (6.2% sequence coverage, accession number P97772-2, molecular mass 101.6 kDa), as well as cerebellin-1 (*Cbln1* gene) by 2 peptides (6.7% sequence coverage, accession number Q9R171, 21.1 kDa molecular mass). The protein fraction derived from the hippocampus was recognized by the presence of neuronal pentraxin-1 (*Nptx1* gene) using 10 peptides (27.3% sequence coverage, accession number Q62443, molecular mass 47.1 kDa) and the neuron-specific calcium-binding and calcium-sensing protein hippocalcin (*Hpca* gene) using 11 peptides (55.4%, accession number P84075, molecular mass 22.4 kDa). An excellent marker of the olfactory bulb is presented by the olfactory marker protein (*Omp* gene). This protein was detected by 2 peptides (21.5% coverage, accession number Q64288, molecular mass 18.9 kDa).

### **Proteomic identification of cell type specific proteins in middle-aged mouse brain**

The proteomic identification of select mouse brain proteins that are associated with specific cell types using label-free mass spectrometry is listed in Supplemental Table 1. This included the brain/neuron-specific glycolytic enzyme  $\gamma$ -enolase ENO2 and the neuronal beta-3 isoform of tubulin named TUBB3, two well established marker proteins of neurons.<sup>7,23-25</sup> Additional neuronal marker were identified as the NeuN/Fox-3 RNA binding protein fox-1 homolog 3, the brain isoform of glycogen phosphorylase, hippocalcin, septin-3, protein kinase C and casein kinase substrate in neurons protein pacsin-1 and calbindin-2. Specialized markers of somato-dendritic neurons, dendritic spines in neocortical pyramidal neurons and cerebellar granule cells were detected in the form of the microtubule-associated protein MAP2, the synaptic Ras GTPase activating

## PANTHER analysis of protein classes in total extracts of mouse brain



**Fig 1.** Bioinformatic analysis of the distribution of protein classes in total extracts of mouse brain as determined by mass spectrometric analysis. The pie chart was generated with the help of the PANTHER analysis tool.<sup>22</sup>

protein 1 and the calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1A, respectively.<sup>7,23-25</sup> As a general marker of glia cells, protein S100-B was determined by proteomic analysis. More cell-specific glia cell markers included the glial fibrillary acidic protein GFAP for astrocytes. Oligodendrocyte-enriched proteins were identified as the myelin-oligodendrocyte glycoprotein MOG, the myelin-associated oligodendrocyte basic protein MOBP and oligodendrocyte-myelin glycoprotein OMG. Myelin sheath and Nodes of Ranvier were recognized by the presence of myelin basic protein MBP and neurofascin, respectively.<sup>7,23-25</sup> The mass spectrometric analysis also detected the proteins contactin-1 and contactin-2 as integral parts of neuron-glia interactions zones, and the enzyme gamma-glutamyl-transferase GGT7 as a marker of cerebral capillaries at the blood-brain barrier.

#### **Proteomic identification of subcellular protein markers in middle-aged mouse brain**

The proteomic identification of mouse brain associated proteins that are located in distinct subcellular structures and/or are involved in specific cellular processes using label-free mass spectrometry is listed in Supplemental Table 2. The proteomic survey identified markers of the plasma membrane, neuronal cell adhesion processes, the neuronal cytoskeleton, the nucleus, the endoplasmic reticulum, the Golgi apparatus, mitochondria, ribosomes,

lysosomes, peroxisome, proteasome, the cytosol, extracellular space and the extracellular matrix. Surface membrane markers included the Na<sup>+</sup>/K<sup>+</sup>-ATPase, the plasma membrane Ca<sup>2+</sup>-ATPase and the neuronal cell adhesion molecules NCAM1, NCAM2, NRCAM, NEGR1, L1CAM AND CADM3.<sup>7,23-25</sup> Surface membrane markers involved in glial-guided migration, neurite outgrowth and synaptic plasticity were identified as astrotactin ASTN3, neurotrimin and neuroplastin.

The detection of abundant cytoskeletal proteins that are involved in organizing the cytomatrix included the protein named bassoon, the presynaptic scaffolding protein piccolo PCLO, the major membrane cytoskeletal component spectrin and the cytoskeletal network organizer plectin. In contrast, the membrane cytoskeletal protein dystrophin appears to be of much lower abundance in the brain as compared to skeletal muscles.<sup>9</sup> The mass spectrometric survey presented here also covered the main intermediate filament proteins that structurally support the neuronal cytoskeleton of axons, i.e. the light NEFL, medium NEFM and heavy NEFH polypeptides of the neurofilament triplet protein assembly.<sup>7,23-25</sup> Microtubules were recognized by the neuronal TUBB3 isoform of tubulin, and the microtubule-associated protein MAP1A and MAP1B. Standard organellar markers included emerin, lamin LMNB2 and histone H4 for the nucleus, the protein

ERP29, the Ca<sup>2+</sup>-ATPase isoform SERCA2 and the RYR2 isoform of the ryanodine receptor Ca<sup>2+</sup>-release channel for the endoplasmic reticulum, the protein ERGIC1 for the endoplasmic reticulum-Golgi intermediate compartment, and neurocalcin and the general vesicular transport factor USO1 for the Golgi apparatus. Mitochondria were recognized by the organelle-specific metalloproteinase neurolysin, and the various subfractions of mitochondria by the voltage-dependent anion-selective channel protein mt-VDAC1 (outer membrane), NADH dehydrogenase (inner membrane complex I), succinate dehydrogenase (inner membrane complex II), cytochrome b-c1 (inner membrane complex III), cytochrome c oxidase (inner membrane complex IV), ATP synthase (inner membrane complex V) and isocitrate dehydrogenase (mitochondrial matrix).

The mass spectrometric analysis identified 40S ribosomal protein RPSA, lysosome-associated membrane glycoprotein LAMP1, catalase and ubiquitin-conjugating enzyme E2 as markers of ribosomes, lysosomes, peroxisomes and proteasomes, respectively. Cytosolic markers were identified in the context of the glycolytic pathway (phosphofructokinase), metabolite transportation (brain isoform FABP7 of the fatty acid-binding protein), the cellular stress response (the small heat shock protein alpha-B crystallin) and signal transduction (neurochondrin).

Proteins of the extracellular region were covered by the mass spectrometric detection of laminin of the basal lamina, collagen isoform COL-IV of the extracellular matrix, the basement membrane-specific heparan sulfate proteoglycan core protein and neurocan core protein of the proteoglycan matrix, and serum albumin in the extracellular space.

#### ***Proteomic identification of synaptic markers in middle-aged mouse brain***

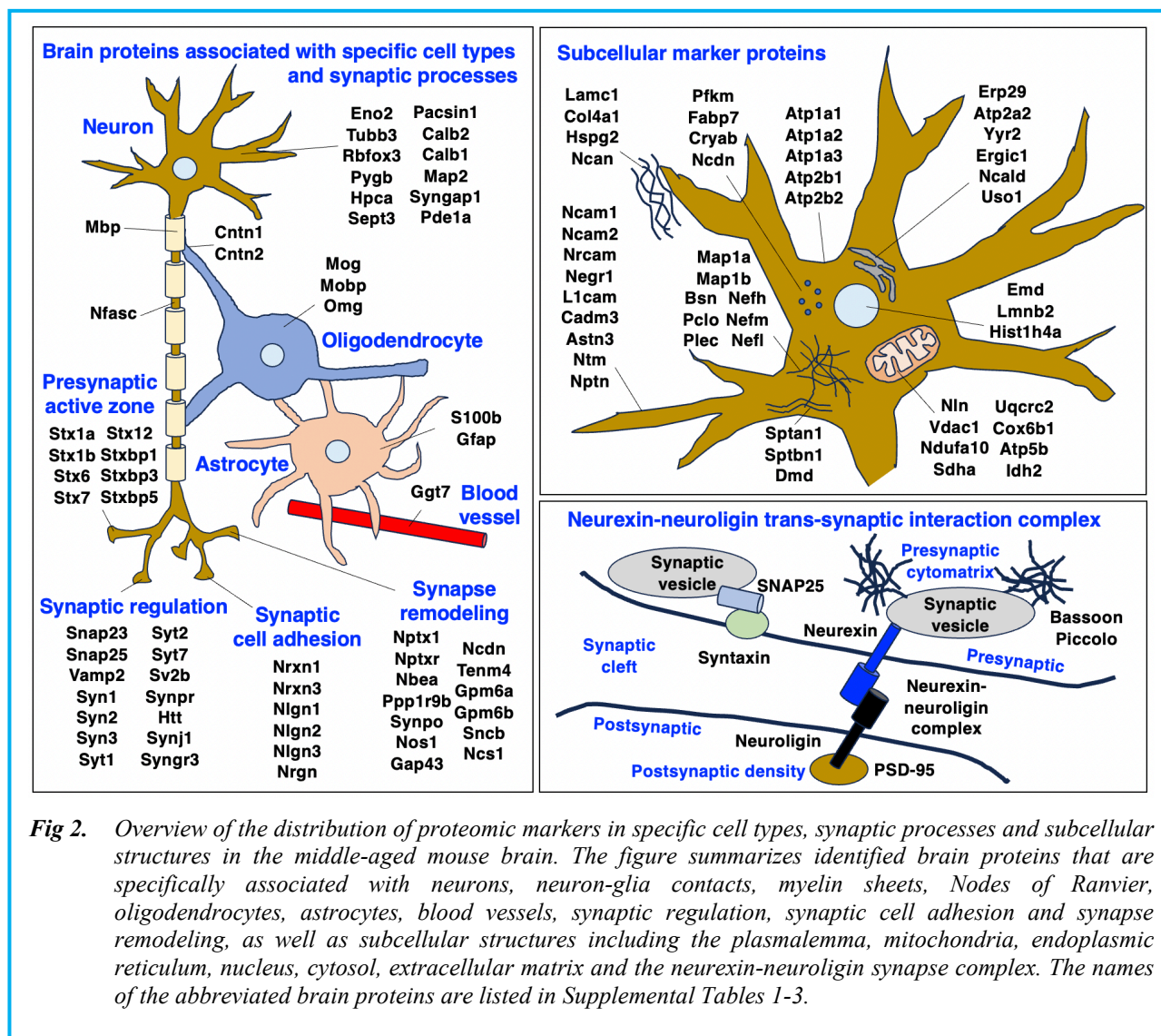
The proteomic identification of mouse brain proteins that are associated with the formation, function and maintenance of synapses using label-free mass spectrometry is listed in Supplemental Table 3. This included marker proteins of the presynaptic active zone that are involved in docking of synaptic vesicles at presynaptic active zones, synaptic cell adhesion, formation of synaptic contacts for efficient neurotransmission, synaptic vesicles maintenance, neurotransmitter release, neuronal modulation and synapse remodelling. Mass spectrometry detected neuronal markers that are involved in (i) docking of synaptic vesicles at presynaptic active zones and neurotransmitter exocytosis (syntaxin isoforms STX-1A/1B/6/7/12, and syntaxin-binding protein isoforms STXBP-1/3/5), (ii) synaptic cell adhesion and the formation of synaptic contacts for efficient neurotransmission (neurexin-1/3, neuroligin-1/2/3, neurogranin, and the postsynaptic scaffolding protein PSD-95), (iii) the regulation of synaptic vesicles and

neurotransmitter release (synaptosomal-associated proteins SNAP-23 and SNAP-25, vesicle-associated membrane protein VAMP2, synapsin-1/2/3, synaptotagmin-1/2/7, synaptic vesicle glycoprotein 2B, synaptoporin, huntingtin, synaptotagmin-1, and synaptogyrin-3), and (iv) neuronal modulation and synapse remodelling (neuronal pentraxin-1, neuronal pentraxin receptor, neurobeachin, neurabin-2, synaptopodin, brain nitric oxide synthase, neuromodulin, neurochondrin, teneurin-4, neuronal membrane glycoproteins M6-a/M6-b, beta-synuclein, and neuronal calcium sensor NCS1).

#### **Discussion**

The major aim of this study was to establish useful neuroproteomic markers of the middle-aged mouse brain, which is frequently used in basic and applied studies in neurosciences as a model system. The mass spectrometric survey was carried out with crude brain extracts and identified brain region markers of the cerebellum, hippocampus, the olfactory bulb and the cerebral cortex. In addition, a large number of cell type markers of the brain were detected for the differential analysis of neurons versus glia cells. The proteomic identification of subcellular markers included brain proteins located in the nucleus, endoplasmic reticulum, plasma membrane, mitochondria and other organelles. Importantly, a large number of components that are involved in presynaptic vesicle docking, neurotransmitter release and synapse remodelling were clearly detected by mass spectrometry. Building on the results of previous cataloguing studies of mouse brain parts and neuronal tissue specimens to establish a brain region and cell type resolved murine brain proteome,<sup>7,23-36</sup> this investigation focused on the refined and comprehensive detection of robust proteomic markers of whole mouse brain preparations. Thus, the main objective of this proteomic survey was to establish a proteomic reference map of the middle-aged murine brain using crude protein extracts from small tissue specimens. The biochemical analysis described here employs a minimum of preparative steps for a streamlined proteomic analysis platform that eliminates excessive artefacts for the routine detection of brain proteoforms. The graphical presentations of Figure 2 summarize the distribution of key proteomic markers in specific cell types of the mouse brain and subcellular structures, as well as synaptic processes.

Of special interest is the mass spectrometric identification of isoforms of the neurexin,<sup>37</sup> and neuroligin,<sup>38</sup> families and the large neurexin-neuroligin complex with its presynaptic linkage to neurotransmitter vesicles and its associations with postsynaptic scaffolds.<sup>39</sup> This involves an indirect connection to the postsynaptic density protein PSD-95 and a linkage to the scaffolding protein piccolo PCLO and the cytoskeletal component bassoon on the presynaptic side. The cytomatrix-organising protein bassoon is one of the most



**Fig 2.** Overview of the distribution of proteomic markers in specific cell types, synaptic processes and subcellular structures in the middle-aged mouse brain. The figure summarizes identified brain proteins that are specifically associated with neurons, neuron-glia contacts, myelin sheets, Nodes of Ranvier, oligodendrocytes, astrocytes, blood vessels, synaptic regulation, synaptic cell adhesion and synapse remodeling, as well as subcellular structures including the plasmalemma, mitochondria, endoplasmic reticulum, nucleus, cytosol, extracellular matrix and the neurexin-neuroigin synapse complex. The names of the abbreviated brain proteins are listed in Supplemental Tables 1-3.

abundant brain proteins that was identified by mass spectrometry in this study. Bassoon is intrinsically involved in the regulation of neurotransmitter release at the nerve terminal active zone.<sup>40</sup> It has previously been shown by proteomics to be changed in abundance in the brain of the *mdx* mouse model of dystrophinopathy.<sup>14</sup> Previous gel-based and top-down approaches, as well as gel-free and bottom-up proteomic studies, produced large data sets of proteins that are associated with the mouse brain.<sup>7,23-36</sup> This included the proteomic establishment of general markers of the olfactory bulb, medulla, midbrain, hypothalamus, hippocampus, cerebellum and cortex using two-dimensional gel electrophoresis,<sup>23,27,28</sup> and liquid chromatography,<sup>7,25</sup> based protein separation prior to mass spectrometric analysis. A variety of mass spectrometric labelling methods were also used to study the mouse brain proteome.<sup>26,29,35,36</sup> Focused cataloguing surveys centered on brain development,<sup>27,30</sup> cell type-resolved proteomes,<sup>7,35</sup> the composition of the synaptome,<sup>32</sup> and regional differences in neuronal versus

astrocyte cell populations.<sup>36</sup> The application of top-down proteomics, for the detailed identification and characterization of proteoforms<sup>41,42</sup> resulted in the establishment of the Mouse Brain Proteoform Atlas.<sup>33,34</sup> This report has confirmed many of the detected cellular and subcellular brain markers, and has extended the list of suitable proteoforms that are associated with specific regions, cell types, synaptic processes and subcellular structures in the middle-aged murine brain. The expression of previously established cell specific markers was confirmed in this study, including proteomic markers of neurons such as the light NEFL, medium NEFM and heavy NEFH polypeptides of the neurofilament triplet, as well as the synaptosomal-associated protein SNAP-25.<sup>7,25,32</sup> The detection of established synaptic markers included the postsynaptic density protein PSD-95.<sup>32</sup> Well-established glia cell markers were confirmed, such as the glial fibrillary acidic protein GFAP for astrocytes, and the myelin-associated proteins MBP and MOG for oligodendrocytes.<sup>7,25</sup>

Crucial brain region enriched markers detected in this study included (i) septin of the cerebral cortex, (ii) peripherin, the metabotropic glutamate receptor 1 and cerebellin-1 of the cerebellum, (iii) neuronal pentraxin-1 and the neuron-specific calcium sensor hippocalcin of the hippocampus, and (iv) olfactory marker protein OMP for the olfactory bulb region in the brain.<sup>7,23-25</sup> Septin-7 is a cytoskeletal component of the postsynaptic density and exhibits high expression levels in the cerebral cortex. It acts as a GTP-binding protein which polymerizes into heteromeric filaments and interacts with actin and microtubules.<sup>43</sup>

Crucial cerebellum markers were identified, including peripherin, the metabotropic glutamate receptor 1 and cerebellin-1, which is involved in synapse formation, axon growth and guidance. Peripherin is a major cytoskeletal component and belongs to the desmin-like type III intermediate filament class of proteins that are located in neurons. The peripherin isoform in the cerebellum, which is different from the one present in the photoreceptor outer segments, represents an excellent marker of cerebellar climbing fibers. Mutations in the *PRPH* gene confer susceptibility to motor neuron disease.<sup>44</sup>

The metabotropic glutamate receptor is found in dendrites, the soma region, axons and synapses of neurons with a high expression level in the cerebellum. Neuronal pentraxin-1 is found in axons and synapses and shows high expression levels in the hippocampus, as well as the cerebral cortex. Pentraxin-1 is probably involved in synaptic remodeling mechanisms and was shown to affect the numbers of excitatory synapses via a modulating process of neuronal excitability.<sup>45</sup> The olfactory marker protein OMP is a cytoplasmic marker of olfactory receptor neurons in the mature olfactory bulb that plays a key role in odor detection and cellular signaling. OMP sharpens both the response profile to odorants and accelerates the response kinetics to odorants, which is clearly supported by the characterization of OMP-KO mice that have lost the ability to properly respond to distinct odor stimuli.<sup>46</sup> The proteomic identification of cell type specific proteins in mouse brain confirmed the presence of general neuronal markers, including the brain/neuron-specific glycolytic enzyme  $\gamma$ -enolase (ENO2) that exhibits neurotrophic and neuroprotective properties, and the neuronal tubulin beta-3 isoform TUBB3/TUJ1 that supports axon guidance and neuron differentiation.<sup>7,23-36</sup> The gamma-glutamyl-transferase GGT7 was clearly detected and represents as an established marker of cerebral capillaries at the blood-brain barrier.<sup>47</sup>

Important proteins that mediate neuron-glia interactions and the organization of axonal domains at Nodes of Ranvier were identified as contactin-1, contactin-2 and neurofascin.<sup>7,23-36</sup> Interactions between amyloid precursor protein and contactin are implicated in the pathogenesis of Alzheimer's dementia.<sup>48</sup> The presence of established glia markers was confirmed by the mass

spectrometric coverage of glial fibrillary acidic protein GFAP, glia-derived nexin, vimentin and the myelin-associated proteins MBP, PLP1, MOG, OMG, MAG and MOBP.<sup>7,23-36</sup> Altered GFAP expression levels are widely used as a marker of astrogliosis, but also in the general context of traumatic brain injury, certain psychiatric disorders, neurodegenerative processes and neuroinflammation.<sup>49</sup>

The field of neuroproteomics, which represents an important sub-discipline of neurochemistry, focuses on the mass spectrometric identification and biochemical characterization of protein species that are critical for the development, maturation and adult functioning of the nervous system.

Proteomic surveys of the central and peripheral nervous system also involve studying the molecular and cellular basis of neurological and neuromuscular disorders. The mass spectrometry-based proteomic profiling of the normal middle-aged mouse brain presented here has identified a large number of brain-associated marker proteins. Since murine brain tissues are frequently used as model systems to study neurological abnormalities in neuromuscular disorders and test novel therapeutic approaches, the identified neuronal and glial proteoforms can now be employed as highly useful biomarkers of the middle-aged central nervous system, brain-specific regions, specific brain-associated cell types and subcellular structures. This includes various protein species that are highly specific for their expression levels in neurons and glia cells in distinct brain regions, such as the cerebral cortex, cerebellum, hippocampus and the olfactory bulb. For the evaluation of cellular interactions, subcellular structures and synaptic systems, this proteomic study has provided detailed information on the identification of key organellar markers and proteins that are located in synaptic vesicles and the neurotransmitter release apparatus.

In conclusion, this report provides the field of experimental myology with an improved set of bioanalytical tools for the detailed characterization of genetic mouse models.

#### **List of acronyms**

ASTN3 - astrotactin 3  
CADM3 - cell adhesion molecule 3  
COL-IV - collagen isoform IV  
DMD - Duchenne muscular dystrophy  
ENO2 - brain/neuron-specific  $\gamma$ -enolase  
ERGIC1 - endoplasmic reticulum-Golgi intermediate compartment protein 1  
ERP29 - endoplasmic reticulum resident protein 29  
FABP7 - fatty acid binding protein 7  
GFAP - glial fibrillary acidic protein  
GGT7 - gamma-glutamyl-transferase 7  
LICAM - neural cell adhesion molecule L1  
LAMP1 - lysosome-associated membrane glycoprotein 1  
LMNB2 - lamin B2

Lys-C - endoprotease that cleaves on the C-terminal side of lysine residues  
 MAP2 - microtubule-associated protein 2  
 MOBP - myelin-associated oligodendrocyte basic protein  
 MOG - myelin-oligodendrocyte glycoprotein  
 NCAM 1/2 - neural cell adhesion molecule 1/2  
 NCS1 - neuronal calcium sensor 1  
 NEFH - neurofilament, heavy polypeptide  
 NEFL - neurofilament, light polypeptide  
 NEFM - neurofilament, medium polypeptide  
 NEGR1 - neuronal growth regulator 1  
 NRCAM - neuronal cell adhesion molecule  
 OMG - oligodendrocyte-myelin glycoprotein  
 OMP - olfactory marker protein  
 PCLO - protein piccolo  
 RPSA - 40S ribosomal protein SA  
 RYR2 - ryanodine receptor calcium-release channel 2  
 SERCA2 - endoplasmic reticulum calcium ATPase 2  
 SNAP-23/25 - synaptosomal-associated proteins 23/25  
 STX-1A/1B/6/7/12 - syntaxin isoforms 1A/1B/6/7/12  
 STXBP-1/3/5 - syntaxin-binding protein isoforms 1/3/5  
 SOD1 - superoxide dismutase 1  
 TUBB3 - Tubulin isoform beta-3, neuronal  
 VAMP - vesicle-associated membrane protein  
 VDAC1 - voltage-dependent anion-selective channel protein 1

### Contributions of Authors

PD, HS, DS, and KO were involved in the conceptualization and initiation of this project, as well as the design of the research strategy. PD performed the biochemical experiments and analyzed the data. MZ was involved in the preparation of brain tissues. MH and PM performed the mass spectrometric and bioinformatic analysis. All authors were involved in the writing and final editing of the manuscript. All authors approved the final edited Early Release.

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### Conflict of Interest

The authors declare no competing interests.

### Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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**Proteomics of mouse brain for neuromuscular diseases**

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**Supplemental Table 1.** Proteomic identification of select mouse brain proteins that are associated with specific cell types using label-free mass spectrometry

Brain cell type	Marker proteins	Accession number	Gene name	Coverage (%)	Peptides	kDa
Neurons	Gamma-enolase, ENO2 isoform, neuronal	P17183	Eno2	69.4	19	47.3
Neurons	Tubulin, beta-3, neuronal	Q9ERD7	Tubb3	78.9	25	50.4
Neurons	NeuN/Fox-3 RNA binding protein fox-1 homolog 3	Q8BIF2-4	Rbfox3	8.0	2	34.0
Neurons	Glycogen phosphorylase, brain isoform	Q8C194	Pygb	53.7	36	96.7
Neurons	Hippocalcin, neuron-specific	P84075	Hpca	55.4	11	22.4
Neurons	Septin-3, neuronal-specific	Q9Z1S5-2	Sept3	44.2	13	38.7
Neurons	Protein kinase C and casein kinase substrate in neurons protein Pacsin1	Q61644	Pacsin1	57.6	24	50.5
Neurons	Calbindin-2 (Calretinin)	Q08331	Calb2	54.6	16	31.4
Neurons (GABAergic)	Calbindin-1 (Calbindin-D28k)	P12658	Calb1	66.3	16	47.5
Neurons (somato-dendritic)	Microtubule-associated protein 2	P20357	Map2	59.5	85	199.0
Neurons (pyramidal, dendritic spines in neocortex)	Synaptic Ras GTPase activating protein 1	F6SEU4	Syngap1	39.1	37	148.1
Neurons (cerebellar granule cells)	Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1A	Q61481-2	Pde1a	40.6	14	60.6
Glia cells	Protein S100-B	P50114	S100b	23.5	2	11.2
Glia cells (astrocytes)	Glial fibrillary acidic protein	P03995	Gfap	57.2	23	49.9
Glia cells (oligodendrocytes)	Myelin-oligodendrocyte glycoprotein	Q61885	Mog	38.21	10	28.3
Glia cells (oligodendrocytes)	Myelin-associated oligodendrocyte basic protein	Q9D2P8	Mobp	34.1	6	19.2
Glia cells (oligodendrocytes)	Oligodendrocyte-myelin glycoprotein	Q63912	Omg	17.3	6	49.3
Myelin sheath	Myelin basic protein	P04370	Mbp	39.20	11	27.2
Nodes of Ranvier	Neurofascin	Q810U3	Nfasc	35.4	33	137.9
Neuron-glia interactions	Contactin-1	P12960	Cntn1	57.0	42	113.3
Neuron-glia interactions	Contactin-2	Q61330	Cntn2	20.96	15	113.1
Cerebral capillaries (blood-brain barrier)	Gamma-glutamyl-transferase 7	Q99JP7	Ggt7	12.2	6	70.2

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**Supplemental Table 2.** Proteomic identification of mouse brain associated proteins that are located in distinct subcellular structures and/or are involved in specific cellular processes using label-free mass spectrometry

Subcellular structure / process	Marker proteins	Accession number	Gene name	Coverage (%)	Peptides	kDa
<b>(i) Plasma membrane</b>						
Plasmalemma, membrane potential	Na <sup>+</sup> /K <sup>+</sup> -ATPase, alpha-1	Q8VDN2	Atp1a1	44.8	43	112.9
Plasmalemma, membrane potential	Na <sup>+</sup> /K <sup>+</sup> -ATPase, alpha-2	Q6PIE5	Atp1a2	54.4	54	112.1
Plasmalemma, membrane potential	Na <sup>+</sup> /K <sup>+</sup> -ATPase, alpha-3	Q6PIC6	Atp1a3	56.3	52	111.6
Plasmalemma, calcium homeostasis	Plasma membrane calcium-transporting ATPase 1	G5E829	Atp2b1	35.7	34	134.7
Plasmalemma, calcium homeostasis	Plasma membrane calcium-transporting ATPase 2	Q9R0K7	Atp2b2	40.0	37	132.5
<b>(ii) Neuronal cell adhesion</b>						
Surface adhesion, neurons, glia cells	Neural cell adhesion molecule 1	P13595	Ncam1	44.9	33	119.4
Surface adhesion, neurons, glia cells	Neural cell adhesion molecule 2	O35136	Ncam2	31.2	19	93.1
Surface adhesion, neurons, glia cells	Neuronal cell adhesion molecule, isoform 2	Q810U4-2	Nrcam	25.1	16	131.2
Neural cell adhesion	Neuronal growth regulator 1	Q80Z24	Negr1	31.9	8	37.9
Neurite outgrowth, cell migration	Neural cell adhesion molecule L1	P11627	L1cam	15.6	14	140.9
Cell-cell adhesion	Cell adhesion molecule 3	Q99N28	Cadm3	35.9	10	42.9
Glial-guided migration	Astrotactin-1	Q61137-2	Astn3	13.8	10	143.9
Neurite outgrowth	Neurotrimin	Q99PJ0	Ntm	26.7	8	38.0
Synaptic plasticity	Neuroplastin	P97300	Nptn	32.2	10	44.3
<b>(iii) Neuronal cytoskeleton</b>						
Cytomatrix organization	Protein bassoon	O88737	Bsn	41.8	100	418.6
Presynaptic scaffolding	Protein piccolo, isoform 2	Q9QYX7-2	Pclo	24.1	75	527.9
Membrane cytoskeleton	Spectrin, alpha chain, non-erythrocytic 1	P16546-2	Sptan1	71.7	163	282.2
Membrane cytoskeleton	Spectrin, beta chain, non-erythrocytic 1	Q62261	Sptbn1	66.5	133	274.1
Membrane cytoskeleton	Dystrophin	P11531	Dmd	1.4	4	425.6
Cytoskeletal organization	Plectin	Q9QXS1-6	Plec	39.7	144	519.4
Microtubules	Microtubule-associated protein 1A	Q9QYR6	Map1a	52.7	106	300.0
Microtubules	Microtubule-associated protein 1B	P14873	Map1b	50.9	91	270.1
Neurofilament	Neurofilament, heavy polypeptide	P19246	Nefh	36.3	38	116.9
Neurofilament	Neurofilament, medium polypeptide	P08553	Nefm	64.0	46	95.9

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Neurofilament	Neurofilament, light polypeptide	P08551	Nefl	56.4	35	61.5
<b>(iv) Nucleus</b>						
Inner nuclear membrane	Emerin	O08579	Emd	29.0	5	29.4
Nuclear lamina	Lamin-B2	P21619	Lmnb2	52.4	28	67.3
Nucleus	Histone H4	P62806	Hist1h4a	45.3	7	11.4
<b>(v) Endoplasmic reticulum and Golgi apparatus</b>						
Endoplasmic reticulum	Endoplasmic reticulum resident protein 29	P57759	Erp29	30.9	7	28.8
Endoplasmic reticulum, membrane	Endoplasmic reticulum calcium ATPase SERCA2	O55143	Atp2a2	47.5	41	114.8
Intracellular calcium handling	Ryanodine receptor calcium-release channel 2	E9Q401	Ryr2	12.3	39	564.5
Endoplasmic reticulum-Golgi intermediate compartment	Endoplasmic reticulum-Golgi intermediate compartment protein 1	Q9DC16	Ergic1	23.8	5	32.5
Perinuclear trans-Golgi network	Neurocalcin, delta	Q91X97	Ncald	50.8	10	22.2
Golgi apparatus	General vesicular transport factor p115	Q9Z1Z0	Uso1	24.8	15	106.9
<b>(vi) Mitochondria</b>						
Mitochondria (general)	Neurolysin, mitochondrial metallopeptidase	Q91YP2	Nln	6.8	3	80.4
Mitochondrium, outer membrane	Voltage-dependent anion-selective channel protein, isoform mt-VDAC1	Q60932-2	Vdac1	63.3	14	30.7
Mitochondrium, inner membrane complex I	NADH dehydrogenase, mitochondrial	Q99LC3	Ndufa10	56.3	18	40.6
Mitochondrium, inner membrane complex II	Succinate dehydrogenase, mitochondrial	Q8K2B3	Sdha	56.6	28	72.5
Mitochondrium, inner membrane complex III	Cytochrome b-c1, mitochondrial	Q9DB77	Uqcrc2	54.3	17	48.2
Mitochondrium, inner membrane complex IV	Cytochrome c oxidase	P56391	Cox6b1	61.6	5	10.1
Mitochondrium, inner membrane complex V	ATP synthase, subunit beta, mitochondrial	P56480	Atp5b	78.5	26	56.3
Mitochondrium, matrix	Isocitrate dehydrogenase, mitochondrial	P54071	Idh2	37.8	14	50.9
<b>(vii) General organelle markers</b>						
Ribosome	40S ribosomal protein SA	P14206	Rpsa	36.6	8	32.8

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Lysosome	Lysosome-associated membrane glycoprotein 1	P11438	Lamp1	8.4	3	43.8
Peroxisome	Catalase	P24270	Cat	29.6	10	59.8
Proteasome	Ubiquitin-conjugating enzyme E2	P61089	Ube2n	60.5	6	17.1
<b>(viii) Cytosol</b>						
Cytosol, glycolytic pathway	Phosphofructo-kinase	P47857	Pfkm	59.4	32	85.2
Cytosol, metabolite transportation	Fatty acid-binding protein, brain, FABP7	P51880	Fabp7	28.8	2	14.9
Cytosol, cellular stress response	Alpha-crystallin B	P23927	Cryab	61.7	9	20.1
Cytosol, signal transduction	Neurochondrin	Q9Z0E0	Ncdn	48.6	23	78.8
<b>(ix) Extracellular region</b>						
Basal lamina	Laminin, subunit gamma-1	P02468	Lamc1	22.1	24	177.2
Extracellular matrix	Collagen IV, alpha-1 chain	P02463	Col4a1	1.7	2	160.6
Proteoglycan matrix	Basement membrane-specific heparan sulfate proteoglycan core protein	Q05793	Hspg2	9.9	21	398.0
Proteoglycan matrix, modulation of cell adhesion	Neurocan core protein	P55066	Ncan	20.0	19	137.1
Extracellular space	Serum albumin	P07724	Alb	73.4	44	68.6

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**Supplemental Table 3.** Proteomic identification of mouse brain proteins that are associated with the formation, function and maintenance of synapses using label-free mass spectrometry

Synaptic structure / process	Marker proteins	Accession number	Gene name	Coverage (%)	Peptides	kDa
<b>(i) Presynaptic active zone (docking of synaptic vesicles at presynaptic active zones)</b>						
Neurotransmitter exocytosis	Syntaxin-1A	O35526	Stx1a	56.9	18	33.0
Neurotransmitter exocytosis	Syntaxin-1B	P61264	Stx1b	61.1	19	32.2
Neurotransmitter exocytosis	Syntaxin-6	Q9JJK1	Stx6	25.5	4	29.0
Neurotransmitter exocytosis	Syntaxin-7	O70439	Stx7	40.2	8	29.8
Neurotransmitter exocytosis	Syntaxin-12	Q9ER00	Stx12	27.0	5	31.2
Neurotransmitter exocytosis	Syntaxin-binding protein 1	O08599	Stxbp1	72.6	40	67.5
Neurotransmitter exocytosis	Syntaxin-binding protein 3	Q60770	Stxbp3	16.4	7	67.9
Neurotransmitter exocytosis	Syntaxin-binding protein, isoform 5	Q8K400	Stxbp5	23.8	17	127.6
<b>(ii) Synaptic cell adhesion (formation of synaptic contacts for efficient neurotransmission)</b>						
Presynaptic neuroligin-binding receptor	Neurexin-1	Q9CS84-4	Nrxn1	23.9	24	164.3
Presynaptic neuroligin-binding receptor	Neurexin-3	Q6P9K9	Nrxn3	21.3	24	173.3
Postsynaptic cell adhesion protein	Neuroligin-1	Q99K10	Nlgn1	5.1	3	94.1
Postsynaptic cell adhesion protein	Neuroligin-2	Q69ZK9	Nlgn2	20.5	9	90.9
Postsynaptic cell adhesion protein	Neuroligin-3	Q8BYM5	Nlgn3	19.4	10	91.1
Postsynaptic scaffolding	Postsynaptic density protein PSD-95	Q62108-3	Dlg4	43.1	24	80.1
Post-synaptic calmodulin-binding protein	Neurogranin	P60761	Nrgn	60.3	3	7.5
<b>(iii) Regulation of synaptic vesicles and neurotransmitter release</b>						
Regulation of transport vesicle docking and fusion	Synaptosomal-associated protein SNAP-23	O09044	Snap23	12.9	2	23.2
Synaptic vesicle trafficking and docking	Synaptosomal-associated protein SNAP-25	P60879	Snap25	62.6	14	23.3
Synaptic vesicle trafficking and docking	Vesicle-associated membrane protein VAMP2	P63044	Vamp2	34.5	3	12.7
Regulation of neurotransmitter release	Synapsin-1	O88935	Syn1	64.6	27	74.1
Regulation of neurotransmitter release	Synapsin-2	Q64332	Syn2	59.4	24	63.3



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Regulation of neurotransmitter release	Synapsin-3	Q8JZP2	Syn3	30.1	11	63.3
Trafficking of synaptic vesicles at the active zone	Synaptotagmin-1	P46096	Syt1	49.2	22	47.4
Trafficking of synaptic vesicles at the active zone	Synaptotagmin-2	P46097	Syt2	46.2	16	47.4
Trafficking of synaptic vesicles at the active zone	Synaptotagmin-7	Q9R0N7-4	Syt7	18.5	7	63.1
Control of regulated secretion	Synaptic vesicle glycoprotein 2B	Q8BG39	Sv2b	23.9	11	77.4
Membrane integrity of small neurosecretory vesicles	Synaptoporin	Q8BGN8-2	Synpr	13.3	3	31.3
Synaptic neurotransmission	Huntingtin	P42859	Htt	9.0	18	344.5
Synaptic vesicle un-coating and recycling	Synaptojanin-1	Q8CHC4	Synj1	37.8	42	172.5
Neurotransmitter recycling from the synaptic cleft towards the presynaptic terminal	Synaptogyrin-3	Q8R191	Syngr3	24.0	5	24.5
<b>(iv) Neuronal modulation and synapse remodelling</b>						
Synapse remodelling and clustering	Neuronal pentraxin-1	Q62443	Nptx1	37.3	10	47.1
Synapse remodelling and clustering	Neuronal pentraxin receptor	Q99J85	Nptxr	33.1	10	52.3
Modulation of signal transduction and vesicular trafficking	Neurobeachin	Q9EPN1-3	Nbea	17.1	32	323.0
Modulation of excitatory synaptic transmission	Neurabin-2	Q6R891	Ppp1r9b	37.1	22	89.5
Postsynaptic density integrity and synaptic plasticity	Synaptopodin	Q8CC35-3	Synpo	44.1	17	74.0
Maintenance of synaptic plasticity	Nitric oxide synthase, brain isoform N-NOS-2	Q9Z0J4-2	Nos1	16.0	15	148.3
Nerve growth and migration	Neuromodulin	P06837	Gap43	71.37	16	23.6
Nerve growth and migration	Neurochondrin	Q9Z0E0	Ncdn	48.56	23	78.8
Nerve growth and migration	Teneurin-4	Q3UHK6	Tenm4	2.60	5	308.2
Neuronal plasticity	Neuronal membrane glycoprotein M6-a	P35802	Gpm6a	31.3	10	31.1
Neuronal plasticity	Neuronal membrane glycoprotein M6-b	P35803-8	Gpm6b	30.2	9	31.6
Neuronal plasticity	Beta-synuclein	Q91ZZ3	Sncb	52.6	7	14.0
Dynamic regulation of nerve terminal growth	Neuronal calcium sensor NCS1	Q8BNY6	Ncs1	32.6	5	21.9