

# Proteomics in postgenomic neuroscience: the end of the beginning

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**Proteomics is complementary to genomic approaches anchored in DNA and RNA. Global characterization of proteins is providing new insights into general biological structures as well as synapses, receptor complexes and other neuronal and glial features. Current challenges for proteomics of the nervous system include problems relating to sample preparation, brain complexity, limited databases and informatics tools. The combination of proteomics with other global functional genomic approaches at the levels of genome and transcriptome, together with network biology, will provide important bridges between genes, physiology and pathology.**

The proteome is the genome complement of proteins, and proteomics is the study of proteomes. Proteomics originally focused on identifying proteins and the differences between pairs of samples derived from different tissues or conditions, but has since extended to define structural and functional features of proteins on a large scale. The analysis of proteomes is significantly more challenging than that of genomes. In particular, there is greater diversity in proteins at the amino acid composition level; the proteome is dynamic, both spatially and temporally; and a wide range of variation of protein concentrations exists within cells. Moreover, proteomic analysis is substrate limited, because methods for protein amplification are not available.

Two main areas of this field are 'profiling' and 'functional' proteomics. Profiling proteomics encompasses the description of the whole proteome of an organism (by analogy with the genome) and includes organelle mapping and differential measurement of expression levels between cells or conditions. Functional proteomics characterizes protein activity, interactions and the presence of post-translational modifications. Here, we provide an overview of current proteomic technologies in both these areas along with their implications for neuroscience (for a more thorough review of proteomic technologies generally, see refs. 1–4).

Global protein analysis, although advanced in yeast, is in its infancy in multicellular organisms and neuroscience. In yeast, systematic proteomic scale investigation of protein interactions and the organization of complexes, subcellular localization and post-translational modifications have been reported. In contrast, the proteome of a neuron or glia is only partially characterized. Studies have focused on subcellular structures, including neurotransmitter and adhesion protein com-

plexes, synaptic preparations and axo-glial junctions (Table 1). Programs are now underway to map the proteome of the human brain (loosely coordinated by the Human Brain Proteome Project, www.hbpp.org). Global proteomic datasets are vital to systems biology, which seeks to describe and simulate biological systems through the integration of comprehensive data from diverse sources, using computation and mathematical tools<sup>5</sup>. Proteomics has also been used to study neurological and psychiatric disorders, but this is currently limited by access to brain and nerve tissue from living subjects.

## Core proteomic methodologies

Because of protein diversity, a range of proteomic technologies has emerged, which rely on integration of biological, chemical and analytical methods. In particular, methodologies for production of recombinant protein, cellular fractionation and biochemical purification are proving extremely valuable in contemporary proteomics. The principal technologies that currently underlie most proteomic platforms can be classified as mass spectrometry (MS) coupled with protein separation<sup>2</sup> (Fig. 1) and methods based on protein microarrays<sup>1</sup>. These approaches are complementary and are increasingly used in combination.

MS is a highly sensitive and versatile technique for studying proteins. It can be used to derive sequences *de novo* and determine structural information (in particular post-translational modifications) as well as to quantify relative and absolute amounts of proteins (Fig. 1). In proteomics, the most common approaches used are peptide mass fingerprinting and tandem mass MS sequencing<sup>2</sup>. Technical improvements and the availability of genome sequences have established the use of these MS methods as a powerful tool for rapidly identifying proteins from very complex biological samples.

The combination of MS protein identification with the unique resolving power of two-dimensional gel electrophoresis (2DE) is the most mature platform for large-scale analysis of gene products directly from biological samples<sup>6</sup>. This conventional proteomics platform has been widely used in neuroscience for profiling and differential protein expression analysis (Table 1). However, 2DE remains subject to technical and analytical limitations, the most significant of these being that certain key classes of proteins, such as membrane proteins, are not efficiently represented on 2DE.

Several alternative and complementary methods for proteomic analysis have emerged, in particular those using high-efficiency capillary separation<sup>7</sup>. Liquid chromatography (LC) is a complementary technique for separating proteins and is also effective for resolving peptides. Moreover, reverse-phase chromatography can be directly interfaced to MS. The implementation of nanoscale LC on-line with MS (referred to as LC-MS and LC-MS/MS) offers automated, high-

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sensitivity MS analysis. An extension of this approach involves adding another dimension of chromatography, for example including a cation-exchange phase. The combination of multidimensional chromatography with MS provides a gel-free approach to analyzing very complex protein samples; examples of its application include profiling of the total yeast<sup>8</sup> and malaria<sup>9</sup> proteomes. An alternative gel-free strategy that has been particularly effective for differential proteomic profiling uses a combination of affinity capture and isotope labeling<sup>10</sup>—the former component reducing sample complexity while the latter provides the capability for quantification by MS. The isotope-coded affinity tag (ICAT) strategy is becoming increasingly popular in the field of neuroproteomics<sup>11</sup>.

Despite these technological advances, comprehensive proteome analyses that include low-abundance proteins or in-depth information of primary structure remain challenging. Fractionation methods that reduce complexity or affinity purifications for selective enrichment are commonly used to enhance proteomic analysis<sup>12</sup>. For example affinity methods for selecting post-translational modification groups have been combined with MS and applied to proteome-scale characterization of phosphorylation, glycosylation, ubiquitylation and N-terminal processing<sup>13</sup>. Methods for the identification of two further classes of post-translational modifications, O-GlcNAc<sup>14</sup> and carbonylation<sup>15</sup>, have been optimized with brain samples.

Protein and peptide microarrays involve the spotting of proteins and peptides at high density on surfaces (such as glass slides) and can be used for both profiling and functional proteomics<sup>1</sup>. Profiling of protein levels, which is typically done by western blotting or enzyme-linked immunosorbent assays (ELISA), is being superseded by antibody microarrays, which allow large-scale measurements with small sample volumes<sup>16</sup>. Functional protein arrays allow for testing of activities and interactions with lipids, nucleic acids and small molecules as well as other proteins<sup>17</sup>. These strategies are based on genome sequence data, from which open reading frames (ORFs) are identified; they are then amplified and expressed in recombinant systems and printed on arrays. Whole-proteome ORFs have been compiled in expression vectors for yeast<sup>18</sup> and are underway in *Caenorhabditis elegans*<sup>19</sup>, *Drosophila melanogaster*<sup>20</sup> and several mammalian species. Specialist arrays consisting of protein families (such as kinases) have also been developed. ORFs can also be tagged with fluorescent proteins for studies of subcellular localization, labeled with affinity tags for complex isolation, and used in yeast two-hybrid screens for mapping protein interactions<sup>1</sup>.

Synthetic peptides can be generated at high throughput and readily printed on arrays, where they can be used as substrates for studies of

phosphorylation<sup>21</sup> and protein interactions<sup>22</sup>. In addition, proteins captured on arrays (by interaction with immobilized peptides, proteins or other ligands) can be directly analyzed by MS<sup>23</sup>. Proteomic microarrays, however, have yet to make inroads into neuroscience.

### Profiling proteomics

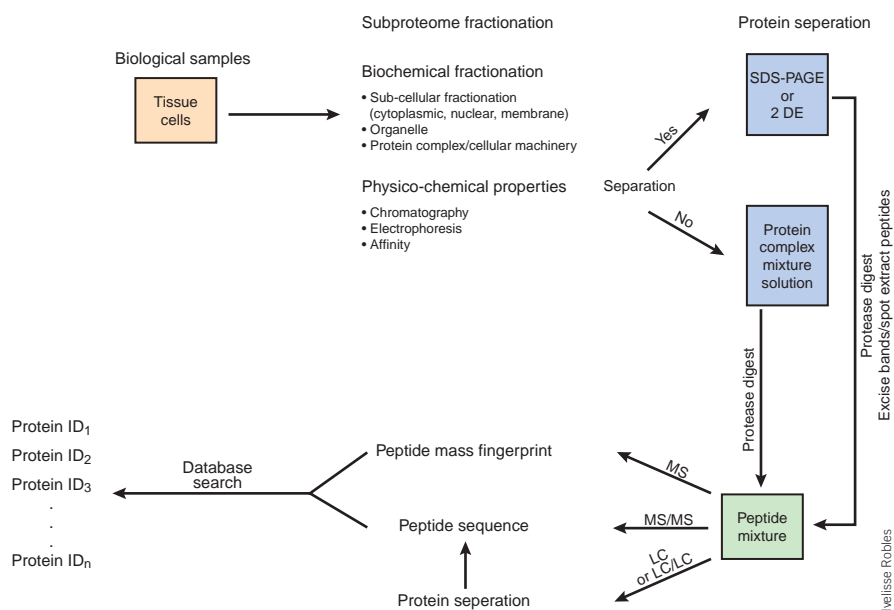
Key examples of proteome catalogs in neuroscience are now emerging (Table 1). One detailed analysis of the mouse brain proteome<sup>24</sup> established a protein index of over 8,500 proteins by 2DE, with MS identification of ~500. Another profile of human fetal brain identified 1,700

**Table 1 Summary of representative proteomic profiling studies in neuroscience**

Profiling	Samples	Species	Subjects	Methods
General	Brain, fetal brain	Human, rodent	Whole lysate, regions, specific enzyme class	2DE MS
	Neuronal cell line	Human	Cortical neuronal cell line, neuroblastoma, hippocampal neural stem cells	2DE MS
	Neuronal fluids	Human, rodent	Astrocyte secretions, cerebrospinal fluid	2DE MS
	Larval CNS	<i>D. melanogaster</i>	Peptides	LC-MS
Organelle	Brain	Rodent	Cytosol, mitochondria and microsomes, plasma membrane, clathrin-coated vesicles	2DE MS, isotope tag LC-MS
	Forebrain	Rodent	Presynaptic region	1DE MS
	Forebrain	Rodent	Postsynaptic density	2DE MS, ICAT, LC/LC-MS/MS, MS
	Optic nerve	Rodent	Oligodendrocytes and associated proteins	2DE MS
	Optic lobes	Squid	Synaptosomes	2DE MS, radiolabeling
Differential expression	Brain	Human, rodent	Age-related change, development	2DE MS
Treatment	Brain	Rodent	Chemical treatments	2DE MS
	Cultured cortical neurons, endothelial cells, neuronal cell line	Human, rodent	Chemical treatments (e.g., camptothecin), nerve growth factor, tumor necrosis factor- $\alpha$	ICAT, MS
Genetic	Brain	Rodent	Genetic knockout, mutant	2DE MS, DIGE, MS
Disease	Adult brain, fetal brain, body fluids	Human	Down syndrome, Alzheimer disease, Pick disease, schizophrenia, bipolar disorder and major depressive disorder	2DE MS
	Brain, body fluids	Rodent	Cerebral ischemia in stroke-prone, stroke-prone spontaneously hypertensive, traumatic brain injury, controlled cortical impact	2DE MS
	Cerebral microdialysate	Human	Stroke	2DE MS
	Primary muscle cultures, neuronal cell line (NSC34)	Human	Type II spinal muscular atrophy, amyotrophic lateral sclerosis, SOD mutant cell lines	2DE MS, antibody array
	Glioblastoma cell lines and tumors	Human	Gliomas, microdissected tumors	2DE MS
	Cerebrospinal fluid	Human	Primary brain tumors	2DE MS

A summary of representative papers illustrating the spectrum of applications and technology. For a complete version of this table including references, see **Supplementary Table 1** online.

**Figure 1** Strategies for mass spectrometry (MS)-based proteomic analysis. Proteins from biological samples (tissues or cell lines) can be fractionated by one or more steps at the cellular level, the protein level or both. Samples for protein identification are usually subject to separation at the protein level with gel electrophoresis, and/or separation after proteolysis at the peptide level by chromatography. Proteins are identified by searching MS data corresponding to peptide mass fingerprints or sequence against a protein sequence database.



proteins corresponding to 437 genes<sup>25</sup>. Proteins from all functional classes have been identified by such analyses. In addition, cerebrospinal fluid and various cultured neuronal cell lines and brain regions have been profiled (Table 1). Although protein detection has been predominantly based on staining, immunodetection and radiolabeling, more recently chemical probes for profiling functional classes have emerged<sup>26</sup>.

Strategies that reduce proteome complexity and increase the dynamic range of protein detection, particularly cellular fractionation methods and separations based on the proteins' chemical and physical properties, have been used to extend proteomic profiling of neuronal samples (Table 1). Organelle mapping has expanded the brain proteome catalog by the addition of information from general cellular structures such as mitochondria, microsomes, cytosolic proteins and plasma membranes<sup>12</sup>.

Because of its accessibility and biological interest, the synapse (synaptosomes, synaptosomal membranes, presynaptic vesicles and postsynaptic densities; Table 1) has been a focus of profiling studies. Recent analyses based on current MS and gel-free methods have detected hundreds of proteins in these samples. Many of these proteins were known synaptic proteins, but new components (hypothetical proteins or expressed sequence tags) were also found<sup>27</sup>.

Differential protein expression analysis based on 2DE separation and visualization methods have been used to compare the anatomy of different brain regions and to profile molecular changes associated with physiological states and development (Table 1). Comparative proteome analysis has also been used to study pathology associated with neurodegeneration, psychiatry, trauma, stroke and nervous system tumors. Expression proteomics of cerebrospinal fluids, astrocyte secretions and microdialysates of brain are under investigation to identify biomarkers for diagnostics and prognostics. In addition, profiling studies in transgenic mouse models examine the effects of genetic mutations or overexpression at the protein level. One noteworthy method is difference gel electrophoresis (DIGE), in which separate protein samples are independently fluorescently labeled before 2DE separation<sup>28</sup>. This approach has been used to identify age-related proteins from the striate cortex of cats and stress-related proteins in genetically altered mice.

Gel-free methods of protein profiling and differential expression analysis are increasingly becoming more routine. The combination of LC-MS with ICAT has been used in several neuroproteomic studies as an alternative to 2DE (Table 1). This approach has been particularly effective in the differential analysis of plasma membrane proteins from fore- and hindbrain<sup>11</sup> and in the study of differences in neu-

ropeptides from pituitary extract between wild-type and mutant mice<sup>29</sup>, because such samples are difficult to profile by 2DE.

Collectively, the proteome catalogs that have been assembled could be used to provide information about the anatomy and physiology of brain regions. Furthermore, processes may be discovered that are either commonly or specifically associated with neurological conditions and some unique markers of disease pathologies. Thus far, however, profiling has been used mainly to develop suitable methods for proteome analysis. Perhaps the more useful biological application for this technology has been in identifying previously unassigned ORFs<sup>30,31</sup>. Analyses of cellular substructures are likely to provide functionally relevant insights in the near future.

**Functional proteomics**

Proteins exist in complexes ranging from a few subunits to more than 100 components that are tethered by binary and ternary interactions. Native protein interactions can be characterized by the analysis of isolated protein complexes. Two studies in yeast have shown that generic affinity-capture methods can be used to isolate protein complexes in a systematic, proteome-wide manner<sup>32,33</sup>. The approach is based on genetically modifying endogenous proteins to express a fusion product with an affinity tag that enables isolation of the complexes by standardized protocols followed by MS analysis. These large-scale studies have shown that a representative of the majority multiprotein complexes can be isolated using this approach. Systematic analysis of such protein assemblies suggests that a single large network of interactions exists between proteins. Proteins of similar function cluster together and are separated by no more than two other proteins. Based on the 'guilt-by-association' concept, this allows predictions of protein function.

Focused characterization of protein complexes also provides information about molecular organization<sup>34</sup> as well as cellular pathways<sup>35</sup>. The retrieval of complexes based on the protein tagging strategy has recently been adapted for *Escherichia coli*<sup>36</sup>, cultured *D. melanogaster*<sup>37</sup>, mammalian cell lines<sup>38</sup> and neuronal cell lines (J.C. and Cellzome Inc., unpublished data). The recombinant affinity tag strategy is also suitable for transgenic mice, although it may be important to express the tagged protein at natural levels,

**Table 2 Integrating proteomics into the systems biology of the nervous system**

Level	Biological feature	Cellular complexity	Analytic throughput
1	Genome	Single	High
2	Transcriptome	Single	High
3	Proteome	Single	High
4	Organelles and subcellular structures	Single	Medium
5	Synapse	Single	Low
6	Cells (neuron, glia)	Single	Low
7	Circuits and regions	Multiple	Low
8	Brain	Multiple	Low
9	Organism, behavior	Multiple	Low

An ascending hierarchy of nine biological systems between genome and behavior. At each level of the hierarchy, there are methods of analysis with different rates of throughput. At higher levels in the hierarchy, there is increasing cellular complexity, which is inversely correlated with the throughput of relevant analytical methods. The first four levels are generic to all cells, and the top five levels are specific to neuroscience.

using homologous recombination into bacterial artificial chromosomes or insertion into the endogenous locus.

In neuroscience, the more common strategy for isolating protein complexes relies on affinity methods in which the natural proteins, antibodies, ligands or peptides are used to capture the complex<sup>39</sup>. Immunoblotting with specific antibodies has been used for sensitive, quantitative detection of components; however, this approach is limited by prior knowledge of likely constituents and by reagent availability. The use of MS to identify subunits of protein complexes is becoming an increasing widespread and complementary approach<sup>39–41</sup>. An example of the value of proteomic analysis of complexes in neuroscience is the study of the complex between PSD-95 and the *N*-methyl-D-aspartate (NMDA) receptor<sup>39</sup>. The NMDA receptor is an important subtype of glutamate receptor involved in modulating many downstream signaling events in neurons. Biochemical isolation of the PSD-95–NMDA receptor complex followed by large-scale immunoblotting and MS revealed 77 associated proteins, many of which are regulators of these downstream processes and involved with behavioral functions in rodents and humans. Almost all the interactions were new and were subsequently supported by yeast two-hybrid screening results.

An alternative strategy for mapping binary protein interactions is yeast two-hybrid screening, which is used extensively in neuroscience, although not usually on a global scale. Large-scale interaction maps based on such screening have been constructed for bacteria, yeast, *C. elegans*<sup>42</sup> and *D. melanogaster*<sup>43</sup>. Analysis of well-characterized interactions indicate that the method is very sensitive and should be capable of detecting the weak or transient interactions that are of particular interest in regulatory processes. However, many of the detected interactions may not be valid in a cellular environment, consistent with the high rate of false positives in such studies<sup>44</sup>. Moreover, the yeast two-hybrid approach detects direct interactions but not interactions based on cooperativity, which is often found in protein complexes. It is now recognized that the quality of interaction data is enhanced by combining multiple complementary approaches. The value of these large-scale ‘interactome’ maps is increased by broad coverage: this will allow orthologous maps to be derived between species, which could in turn facilitate the mapping of networks in the mammalian nervous system.

Post-translational modifications are features of proteins that affect functional activity, localization and stability. Phosphorylation is the

most widely studied post-translational modification in neurobiology, where it is critical for synaptic plasticity and development. Earlier methods for characterizing phosphorylation used radiolabeling and 2DE separation methods, or complementary immunological detection approaches (such as phospho-specific antibodies)<sup>45,46</sup>. Methods based on affinity enrichment and MS analysis have now been introduced for the large-scale, proteome-wide identification and quantification of phosphorylation sites<sup>47</sup>. We have developed methods for purifying soluble phosphoproteins and have used established MS phosphopeptide methods to study synaptic proteins (M.O. Collins, L. Yu, H. Husi, J.C. and S.G.N.G., unpublished data). In our analysis of mouse brain, we have so far characterized approximately 250 phosphoproteins (around 50% of them previously identified) and over 100 defined phosphorylation sites. Another group used *in vitro* phosphorylation of postsynaptic preparations to identify 28 substrates of CaM kinase II by MS<sup>48</sup>. MS-based methods for quantitative monitoring of phosphorylation levels have also become available<sup>49</sup>. Changes in protein interactions as a result of phosphorylation can also be investigated<sup>50</sup>.

Proteomic-scale methods for profiling oxidative modifications have been developed and used to investigate molecular changes associated with neuropathological events. Approaches based on 2DE profiling, coupled with immunological detection of protein carbonylation and tyrosine nitration events followed by MS analysis, have been used to identify proteins oxidized in Alzheimer disease<sup>51,52</sup>. An alternative approach, based on affinity capture and LC-MS, has been implemented for large-scale profiling of carbonylation in samples from aged mouse brains<sup>15</sup>.

### Proteomic problems in neuroscience

The most pressing problems are those involving sample preparation. First, the brain is highly complex and heterogeneous, and whole cellular proteomes cannot be profiled without contaminants. In an ideal situation, a single, defined cell type is used as starting material: examples are clonal neuronal cell lines<sup>53</sup> and primary cultures of neurons<sup>54</sup> and astrocytes<sup>55</sup>. Laser-capture microdissection of brain<sup>56</sup> has been used to isolate small groups of cells. Comprehensive proteome analyses that are able to include low-level regulatory components are not yet routine, proteomics is substrate limited, and protein amplification methods are not available. Thus, the challenge will be to extend analytical sensitivity, with concurrent increase in throughput. This is particularly an issue if proteomics is to be applicable in clinical settings, where tissue samples are limited. An additional complication in working with human brain material is that post-mortem tissues are subject to protein degradation and generation of artifacts. It should be noted that these problems challenge most tissue systems research in multicellular organisms and are not restricted to neuroscience.

A major problem is handling the substantial volume of data and developing approaches that effectively use its content. Generating lists alone provides limited biological insight, whereas complementing these lists with useful bioinformatics approaches or protein interactions results in leads to an understanding of function and molecular organization. The larger proteomic data sets are becoming harder to handle, and there is a growing need to collate data from the increasing number of studies. Thus there is a need for repositories where data can be obtained and shared between laboratories. In our view, these data sets should be made freely available, as was achieved with genome sequences by the public sequencing consortium. Such an undertaking could help in assessing fractionation protocols and validating data. It should include batch processing of protein lists with informatic and annotation tools that identify com-

mon features, protein interactions, subcellular localization, roles in disease and overlap with other datasets<sup>57</sup>. An example of a tool that helps assemble a list of mammalian neuronal proteins into networks and pathways is the Protein-Protein Interaction Database ([www.ppid.org](http://www.ppid.org))<sup>58</sup>. The addition of neurobiological information as annotations to genome and proteome data will add value to this data; such information could include histological expression profiles in the nervous system and phenotypes for mutations in mouse, human and other model organisms.

### Neuroproteomics meets neurosystems biology

Extracting insights into physiological and pathological mechanisms from large, complex sets of neuroproteomic experiments is a fascinating challenge. Systems biology, which aims to explain the function of sets of molecules and biological structures (tissues, cells, organelles, complexes), has been reinvigorated by the large-scale data sets now available<sup>5</sup> and is poised to tackle neurobiological questions<sup>59</sup>. In yeast, a combination of genetic, mRNA microarray and quantitative proteomics data on metabolic pathways revealed global regulatory mechanisms<sup>60</sup>. The mathematical analysis of these large datasets now includes network studies, which in yeast show that the proteome is organized as a 'small world' scale-free network<sup>61</sup>. We have found that the mammalian synaptic proteome and the NMDA receptor complex are also organized as a scale-free network (J.D. Armstrong, M. Cumiskey, J.C. & S.G.N.G., unpublished data). Notably, the network properties are predictive of the robustness to perturbation of individual genes in the physiological and behavioral systems. It is likely that experimental design and interpretation of results will increasingly involve collaboration between scientists with expertise in modeling and statistical methods.

A general neurosystems biology strategy, which integrates proteomics and other functional genomics approaches, is outlined in Table 2 and discussed in detail elsewhere<sup>59</sup>. Here behavior and brain are broken down into a hierarchy with genome, transcriptome and proteome studies at the base and electrophysiological, anatomical and behavioral analysis at higher levels. The general goal is to obtain large-scale data from multiple levels of analysis and then integrate and model it<sup>62</sup>. Although this sounds overwhelming in scale, it is possible to tackle it with a specific focus—such as the NMDA receptor and the synapse proteome. There are now draft proteomes for the NMDA receptor complex<sup>39</sup>, and large-scale mutation studies with vertically integrated analysis are in progress (under the auspices of the Genes to Cognition research program, [www.genes2cognition.org](http://www.genes2cognition.org)). It is likely that these strategies can be extended to study the synapse proteome and ultimately all the proteins expressed in the brain. The scale of data collection and the diversity of expertise required make these endeavors well suited to multicenter collaborations involving both small, specialized and large, high-throughput laboratories.

Proteomics technologies are progressing rapidly and contributing to crucial biological findings in areas that are not amenable to genomic studies. The enhanced protein information offered by proteomics has superior functional value and can generate context-based understanding of cellular protein networks. Consequently, proteomics has been gaining wider acceptance as a functional high-throughput approach. Integration of proteomics with other experimental disciplines, particularly biochemistry, cell biology, molecular genetics and chemistry, will continue to extend its application. Finally, in addition to addressing many basic neurobiological issues and questions relating to disease, brain proteomics is beginning to aid understanding of the evolution of human behavior. The proteome seems to have diverged more rapidly in the brain than in other tissues<sup>63</sup>, and specific post-

translational modifications occur in human brains as a result of recent mutations in modifying enzymes<sup>64</sup>.

*Note: Supplementary information is available on the Nature Neuroscience website.*

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### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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