Chapter 24
Proteomic Approaches to Dissect Neuronal Signaling Pathways

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Abstract With an increasing awareness of mental health issues and neurological disorders, "understanding the brain" is one of the biggest current challenges in biological research. This has been recognized by both governments and funding agencies, and includes the need to understand connectivity of brain regions and coordinated network activity, as well as cellular and molecular mechanisms at play. In this chapter, we will describe how we have taken advantage of different proteomic techniques to unravel molecular mechanisms underlying two modulators of neuronal function: Neurotrophins and antipsychotics.

24.1 Introduction

Over the past years, proteomic studies have significantly advanced our understanding of the mechanisms of brain function. They have highlighted differences between normal and diseased brains (e.g. [1, 10]), identified the molecular composition of synapses [39], and in a landmark study, identified the entire and stoichiometric protein composition of a synaptic vesicle as the first ever fully characterized organelle [35]. It is well beyond the scope of this chapter to describe all the advances in neuroscience

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A.G. Woods and C.C. Darie (eds.), *Advancements of Mass Spectrometry in Biomedical Research*, Advances in Experimental Medicine and Biology 806,
DOI 10.1007/978-3-319-06068-2_24,

that were aided by proteomic approaches. Instead, here we focus on our recent studies that took advantage of proteomics to unravel molecular mechanisms of action, two distinct modulators of neuronal function: Neurotrophins and antipsychotics.

Neurotrophins are a family of growth factors comprising the first ever described growth factor, nerve growth factor (NGF) [8], as well as brain-derived neurotrophic factor (BDNF), and neurotrophins (NT) 3 and 4 [27]. Neurotrophins are best characterized for their role in mediating neuronal outgrowth, arborization, and survival, as well as synaptic plasticity. They signal through their cognate Trk receptors, the receptor tyrosine kinases (RTKs) TrkA, B, and C, and all neurotrophins also bind to and signal through the tumor necrosis family-type receptor, p75 pan-neurotrophin receptor [12, 13, 27, 37]. Similar to other secreted molecules, neurotrophins are initially synthesized as precursors, the proneurotrophins, which are then processed either intra- or extracellularly to generate the mature ligand [7]. However, proneurotrophins are not just precursors to the mature ligand, but can also act as active signaling molecules. They bind with high affinity to a complex of p75NTR and a sortilin family member, and interestingly, display largely opposing effects to their mature counterparts, inducing apoptosis and long-term depression of synapses and discouraging outgrowth [13, 22, 27, 36]. While the importance of neurotrophins for the formation, maintenance, and plasticity of the nervous system is undisputed, the intracellular mechanisms mediating their actions are not fully characterized. Below, we will describe our recent approaches to broaden our current knowledge of the intracellular pathways triggered by neurotrophins, and speculate how future approaches may further our understanding.

Given the significance of growth factors for the functioning of the central nervous system, it is not surprising, a large effort is put into finding small molecule mimetics to for example substitute for loss of BDNF in neurodegenerative conditions, and that drugs commonly used to treat mental health disorders interact with neurotrophic pathways. For example, the serotonin reuptake inhibitor fluoxetine, which is commonly used to treat major depression, also raises BDNF levels, and this BDNF increase is essential for the beneficial effects of the drug [2]. In this chapter we will discuss how proteomics have advanced our understanding of the intracellular mechanisms of neurotrophin action as well as of the trophic actions of antipsychotics.

24.2 Immediate Activation of Signaling Cascades: Isolating Tyrosine Phosphorylated Substrates

Neurotrophins signal through their cognate RTKs, and accordingly, many of the canonical pathways downstream of all RTKs, such as MEK-Erk1/2, PI3 kinase-Akt, and PLCγ signaling, are also activated by neurotrophins [12, 27, 37]. To broaden our understanding of neurotrophin signaling in primary neurons, we took an unbiased approach by isolating tyrosine-phosphorylated proteins following BDNF and NT-3 treatment, followed by mass spectrometry analysis. While many meaningful

studies have been completed through the addition of unlabelled samples to the mass spectrometer and their comparison following separate mass spectrometry analyses, the problem of natural technical variability persists. To address this technical variability that stems from comparing two groups in individual mass spectrometry analyses, [26] created stable isotope labeling of amino acids in culture (SILAC). In this method, cells in culture are fed amino acids (commonly Arginine and Lysine, as trypsin cleaves the C-terminal to these amino acids) that have different carbon or nitrogen weights (e.g. they have Carbon-13 instead of Carbon-12) that will in turn be incorporated into nascent proteins. Typically, cells are grown for ten divisions in SILAC media before being subjected to differential treatment and mass spectrometry. In order to adapt this technology to post-mitotic neurons, we analyzed the labeling efficiency over time and found that neurons grown in SILAC media for 10 days from the time of plating show >80 % labeling of their proteome [34]. We then used the SILAC approach to identify proteins that are tyrosine phosphorylated downstream of BDNF in primary cortical neurons. To this end, treated and untreated cells were lysed, lysates were combined and tyrosine phosphorylated proteins and their interactors were immunoprecipitated. Since the difference in weight will correspond to a specific higher or lower weight when measured by the mass spectrometer, the two treatment groups can be separated by the mass spectrometer and assigned to their original treatment groups, limiting experiment-by-experiment technical variability, including which proteins are measured in each group. This label (heavy or light) is reversed in subsequent experiments to avoid label bias in peptide detection or integration [34, 41]. Using this technique, we found a number of proteins that had not been identified as activated downstream of BDNF-TrkB signaling before, including a number of cytoskeleton-associated proteins. In addition, we identified and characterized the ESCRT machinery-associated protein, Hrs, as a novel regulator of TrkB turnover in cortical neurons [34].

While we observed significant labeling of the proteome after 10 days in culture, individual proteins of low turnover rate will show less label incorporation and thus may be assigned to the wrong experimental group. To avoid misinterpretation, label incorporation rates for individual proteins were measured and results were adjusted accordingly [34]. This labor-intensive correction step can be circumvented by using multiplex SILAC, i.e. labeling all treatment groups, one with “medium” and one with “heavy” amino acids and thus excluding “light” peptides from the analysis. Using this modified approach, we investigated which proteins are tyrosine phosphorylated downstream of NT-3 treatment in cortical neurons [40]. We expected to see a large overlap with the BDNF experiment since both neurotrophins activate canonical RTK signaling pathways. Interestingly, despite activating both TrkC and also TrkB robustly, many of the non-canonical downstream targets of NT-3 were quite different from those identified in response to BDNF. In fact, many of the candidates coming out of this screen were associated with synaptic vesicles and exocytosis [40]. This striking difference may be due to differential receptor engagement or receptor–ligand complex stability. It is well characterized that both signal localization and duration may critically influence the cellular outcome [14, 19, 25], which may in part underlie these observed differences.

24.3 Immediate Activation of Signaling Pathways: Characterizing Protein–Protein Interactions

In addition to phosphorylation events, signals are transduced through recruitment of adaptor proteins into effector complexes and formation of protein complexes. This is not only important downstream of RTK signaling, but also allows to dissect signaling events downstream of receptors lacking a kinase domain, such as p75NTR. One approach to isolate protein complexes is through affinity purification of the protein of interest and then submitting them to mass spec analysis, ideally from SILAC labelled cells [29]. These approaches have not only considerably broadened our understanding of cellular processes, but more recently have also highlighted how the cellular context may critically determine the composition of interaction networks [33]. We have used classical co-immunoprecipitation followed by mass spectrometry to identify a novel interactor of p75NTR, Trio. We further showed that Trio dissociates from p75NTR in response to proneurotrophin treatment and thus leads to an acute growth cone collapse response in primary neurons, discouraging neuronal growth [15]. This identified an intracellular pathway of proneurotrophin action on neuronal morphology, which opposes the well-established function of mature neurotrophins in promoting outgrowth.

Another unbiased approach to study protein complexes forming in response to a stimulus is through separating intact complexes from a lysate by blue native polyacrylamide gel electrophoresis, followed either directly by mass spectrometry of the entire complexes, or by a separation of the complexes into their subunits by SDS-PAGE. The resulting 2D gel may then be analyzed by Coomassie or silver staining to look for shifts between conditions, by mass spectrometry to identify components or by Western blot to probe for the presence of candidates. Using this technique, we isolated protein complexes containing tyrosine-phosphorylated proteins in response to ephrinB1 stimulation in NG108 cells. We detected many established and a number of new interactions, both leading to assembly or disassembly of complexes in response to ephrinB1 and implicating focal adhesion kinase and WAVE complexes in ephrinB1 signaling [11]. It will be of interest to use these approaches to further characterize differential p75NTR signaling in response to mature proneurotrophins, as well as to characterized to protein interaction networks downstream of Trk signaling in primary neurons.

24.4 Watching the Cell Change Its Proteome: Monitoring the Activation of Translation in Response to a Signal

Activation of signaling pathways often leads to translational activation and adjustment of the cellular makeup. Proteome changes have been identified by mass spectrometry following BDNF treatment of hippocampal neurons and radiolabeling of newly synthesized proteins [24], and in cortical synaptoneurosomes using MudPIT

(multidimensional protein identification technology) and relative quantification of all protein spectra [21]. Interestingly, both screens revealed that BDNF stimulation leads to upregulation of the translational machinery itself, thus increasing capability of the cells to synthesize new proteins. In addition, upregulation of a large variety of proteins including cytoskeletal and synaptic components were detected [21, 24].

Another way of measuring newly synthesized proteins is by labeling them with SILAC media. We have taken advantage of this approach to analyze proteomic changes in response to antipsychotics [4]. Previous studies have shown that the kinase Akt can be acutely activated after antipsychotic treatment [3], and that chronic antipsychotic treatment can lead to alterations in the proteomic content of neurons [6, 23]. We demonstrated that the antipsychotic haloperidol activated protein synthesis within 30 min of treatment in neurons in culture via the Akt—mammalian target of rapamycin complex 1 (mTORC1) signaling cascade that is known to influence both translation initiation and elongation in neurons [4, 31].

To measure which proteins were being synthesized in response to the antipsychotic haloperidol, we performed proteomic analysis at an early stage (5 h) and later stage (48 h) of treatment. Striatal neurons were grown in culture for 7 days. They were then treated with either an antipsychotic or vehicle for 48 h in the presence of SILAC Multiplex media (Medium: [$^{13}C_6$] Arginine [4,4,5,5-D_4] Lysine, Heavy: [$^{13}C_6$; $^{15}N_4$] Arginine [$^{13}C_6$; $^{15}N_2$] Lysine). This way we were able to detect nascent proteins that were synthesized and not degraded during the 48 h treatment period that were relevant to antipsychotic action. The advantage of this method is the ability to analyze nascent proteins in the mass spectrometry analysis. A potential caveat to this approach is that while it is very useful, SILAC label incorporation into primary neurons occurs at approximately 1 % per hour on average (based on [40]). This makes it difficult or impossible to quantify changes in total protein amounts by mass spectrometry after only a few hours of treatment without enrichment for newly synthesized proteins.

However, it is of keen interest to the translation community to study short timepoints to measure acute responses to stimuli. In order to achieve this, several new techniques were introduced. One such technique was developed by Schmidt et al. [32]—surface sensing of translation (SUnSET) (Fig. 24.1). In SUnSET, cells are fed a sublethal dose of the antibiotic puromycin, then lysed and analyzed with a monoclonal anti-puromycin antibody by Western blot. This allows identification of nascent proteins, as puromycin is structurally similar to tRNA, and the ribosome will stochastically incorporate it in place of a tRNA onto nascent peptide chains, forming a covalent bond. Puromycin differs from a tRNA in an oxygen-to-nitrogen substitution, which prevents the ribosome from hydrolyzing it as it would a tRNA and this causes premature truncation and release of the peptide from the ribosome. We combined SUnSET with Multiplex SILAC to allow for the labeling, isolation, and quantitation of nascent proteins following the short labeling period (5 h). Using this approach, we were able to enrich for bonafide proteins regulated by antipsychotic treatment in a relatively early time period of 5 h. SUnSEt allowed for the isolation of nascent proteins via an anti-puromycin immunoprecipitation, and SILAC enabled us to combine the lysates from the two treatment groups before

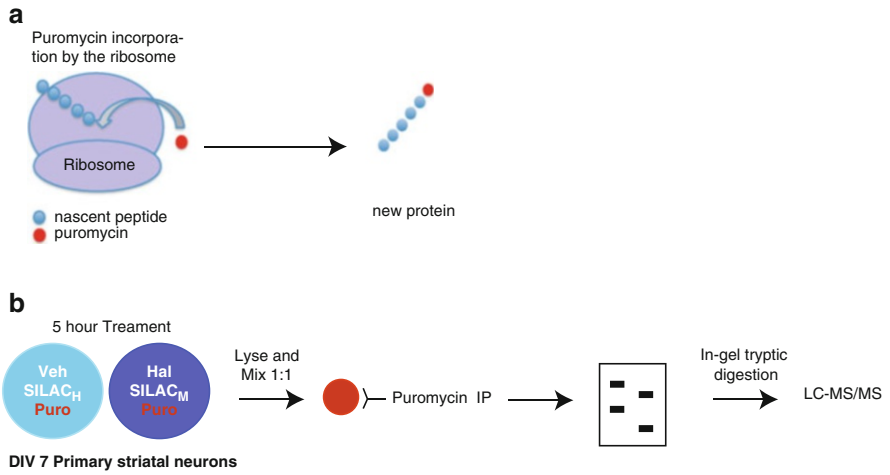

Fig. 24.1 Schematic of puromycin labelling and SUnSET/multiplex SILAC experiments. (**a**) Puromycin mimics a tRNA and is stochastically incorporated by the ribosome onto nascent peptide chains, causing release from the ribosome, and allowing for the labelling of nascent peptides. (**b**) Schematic of SUnSET/multiplex SILAC experiment. DIV7 primary striatal neurons were labelled for 5 h with puromycin and multiplex SILAC media upon treatment with vehicle or the antipsychotic, haloperidol. Following treatment, the neurons were lysed, mixed at a 1:1 ratio, and puromycin labelled proteins were immunoprecipitated, run on an acrylamide gel, isolated using in-gel tryptic digestion, and subjected to LC-MS/MS

immunoprecipitation and mass spectrometry analysis to reduce technical variability. With the enrichment of the nascent proteins with the puromycin immunoprecipitation, proteins could be measured and quantified by mass spectrometry, which was not possible without the enrichment. Using this method, we were able to identify several hundred proteins that were unique to antipsychotic treatment in the first 5 h that provided insight into the biological mechanisms of early antipsychotic response, specifically an upregulation of ribosomal proteins, including ribosomal protein S6 (Rps6). An increase in these proteins suggests "priming" for later stage changes in cellular protein content, such as the increase in specific cytoskeletal proteins identified after 48 h of treatment that were not detected at 5 h, which included ankyrin repeat-rich membrane-spanning protein (ARMS/Kidins220). Interestingly, there were several different types of proteins identified from ribosomal proteins, to mitochondrial proteins and cytoskeletal proteins, and nuclear proteins, suggesting that this technique is not limited to labeling specific subsets of only one type of protein. These data, especially the detection of ARMS/Kidins220, allowed us to predict an acute increase in morphological complexity, which was dependent on mTORC1 signaling effectors associated with protein synthesis [4].

Despite the fact that this method was helpful in identifying proteins synthesized in a short time period, there are drawbacks to its use. Though the cells are presented with a sublethal dose of puromycin, puromycin itself interrupts the normal function of the ribosome by causing premature truncation and release of nascent peptides. Over time, this disrupts cellular function—both by truncating important proteins

and by ultimately clogging the ribosome with puromycin instead of tRNAs. In our experiments, we analyzed striatal neurons treated with puromycin over time. While we measured SILAC incorporation at 5 h of treatment, we found that SILAC incorporation was markedly reduced in the presence of puromycin at 8, 12, and 24 h (unpublished observation).

An alternative approach to enrich newly synthesized proteins is BONCAT (biorthogonal non-canonical amino acid tagging), which is achieved by feeding cells a non-toxic amino acid analog (such as AHA or L-Azidohomoalanine) containing a heavily charged azide group or an alkyne group. Cells are amino acid starved for 30 min to encourage them to take up the tagged analog and other amino acids from the surrounding media. The tag is an amino acid analog, so it mimics an amino acid (methionine in the case of AHA) and is naturally incorporated by the ribosome onto newly synthesized proteins [17]. After labeling for the desired period of time, cells are lysed. Using an alkyne linked to biotin, a bead or fluorophore, a copper-catalyzed Huisgen-azide alkyne reaction is performed to form a covalent bond between the tag (biotin or fluorophore) and the nascent proteins [4, 17, #342], [38, #343]. Using Western blot or immunohistochemistry, general protein synthesis may be visualized. To target specific proteins, biotin can be removed using a filtration process and using the specific streptavidin-biotin interaction, nascent proteins can be precipitated from the lysate of the cell and examined by Western blot. Since this technique involves separating only the nascent proteins, it is possible to examine shorter timepoints to examine total proteomic changes, thus allowing to measure acute treatment responses. However, because there is no difference in label between treatment groups, separate mass spectrometry analyses must be performed and analyzed separately. Thus, while the new population can be analyzed under different conditions, the problem of technical variability persists.

To solve this problem, SILAC and BONCAT were combined to create QuaNCAT (quantitative noncanonical amino acid tagging). In QuaNCAT, cells are treated with the methionine analog AHA and SILAC media, and the BONCAT method is used to isolate nascent proteins while SILAC is used to measure the proteins by mass spectrometry with less variability so direct quantitative comparisons can be made. This method was pioneered by Howden et al. [20]. In their study, they plated freshly isolated human CD4+ T cells. These cells were starved of arginine, methionine, and lysine for 1 h then stimulated them for 2 h in methionine, arginine, and lysine-free media supplemented with AHA and Medium: [$^{13}C_6$] Arginine; [$^{2}H_4$] Lysine or Heavy: [$^{13}C_6$,$^{15}N_4$] Arginine; [$^{13}C_6$,$^{15}N_2$]-Lysine. Lysates from stimulated and unstimulated cells were combined and AHA-tagged proteins underwent cycloaddition via the Huisgen azide-Alkyne cycloaddition reaction, and were isolated by NeutrAvidin pull-down, digested with trypsin, and analyzed by LC-MS/MS. Since AHA has been shown to be non-toxic to cells [16], one could use this technique in place of the SUnSET/SILAC combination to label proteins made in a specific short timeframe and isolate them for quantitative analysis. This technique would allow for longer windows of time while analyzing only nascent proteins that respond to a given stimulus such as drug treatment. One possible application of this technique is in further drug studies, where one could examine changes in the 12–14 h treatment range that are still challenging with SILAC alone, but beyond the scope of the SUnSET technique.

24.5 Special Challenges Posed by Non-dividing Cells: Post-mitotic Systems and Data Analysis

Though there are many new and exciting methods to use in the field of neuroscience for examining proteomic changes, one problem that continues to plague the field is analyzing subtler proteomic changes. Cells undergoing mitosis, such as commonly used cell lines, have an overall higher rate of translation compared to quiescent cells (reviewed in [18, 30]). The need to identify significant smaller scale shifts is acute in quiescent and post mitotic systems such as neurons where few proteins have been reported to change above twofold or 200 % with various treatments [4, #336], [5], [34, #9]; however, changes as low as 20 % can be statistically significant in neurons and validated by Western blotting [5]. This begs the question of whether even smaller changes that are biologically relevant may be significant. Unfortunately, this point remains controversial in the field as the reliability and sensitivity of measuring proteomic changes can be variable, thus, any protein of interest in this range must be independently validated using a candidate approach. While there is no standard method of analyzing data in the proteomics field, many researchers agree that proteomic changes should be presented in ratio form (e.g. treated vs. untreated, transgenic vs. wildtype) and normalized to a peptide that falls in the middle of the calculated range of peptides in the dataset [9]. An alternative method suggested by the Yates lab aims to further refine this ratio ("fold change") calculation. Using an automated program called Census that they created, they remove statistical outliers from datasets by identifying and removing poor quality peptide measurements [5, 28]. They then use QuantCompare to generate protein ratios for peptides that meet the criterion and identify statistically significant changes in individual proteins. This new approach allows for a greater refinement of signal by purging outliers, which in turn facilitates the identification of significant shifts between treatment groups that are smaller. It will be exciting to see how this new analytical approach will aid to further advance our understanding of the molecular mechanisms underlying brain function.

Acknowledgment This work was supported by the Human Frontiers Science Program and the FNES start-up award (KD).

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