Neuroproteomics Approaches to Decipher Neuronal Regeneration and Degeneration*

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Given the complexity of brain and nerve tissues, systematic approaches are essential to understand normal physiological conditions and functional alterations in neurological diseases. Mass spectrometry-based proteomics is increasingly used in neurosciences to determine both basic and clinical differential protein expression, protein-protein interactions, and post-translational modifications. Proteomics approaches are especially useful to understand the mechanisms of nerve regeneration and degeneration because changes in axons following injury or in disease states often occur without the contribution of transcriptional events in the cell body. Indeed, the current understanding of axonal function in health and disease emphasizes the role of proteolysis, local axonal protein synthesis, and a broad range of post-translational modifications. Deciphering how axons regenerate and degenerate has thus become a postgenomics problem, which depends in part on proteomics approaches. This review focuses on recent proteomics approaches designed to uncover the mechanisms and molecules involved in neuronal regeneration and degeneration. It emerges that the principal degenerative mechanisms converge to oxidative stress, dysfunctions of axonal transport, mitochondria, chaperones, and the ubiquitin-proteasome systems. The mechanisms regulating nerve regeneration also impinge on axonal transport, cytoskeleton, and chaperones in addition to changes in signaling pathways. We also discuss the major challenges to proteomics work in the nervous system given the complex organization of the brain and nerve tissue at the anatomical, cellular, and subcellular levels. Molecular & Cellular Proteomics 9:963–975, 2010.

Neurons are extremely polarized cells that rely on intracellular signaling pathways for development and function. The length of axons often exceeds the dimension of the neuronal cell body by several orders of magnitude with length reaching up to 1 m in humans. Anterograde and retrograde axonal transport coupling the distantly located synaptic terminals with the cell soma is essential for neuronal differentiation, survival, and function. The importance of these trafficking routes is underscored by the findings that disruption of axonal transport is an early and perhaps causative event in many neurodegenerative diseases (1, 2). In addition, retrograde axonal transport has recently emerged as a key factor in nerve repair, mediating transfer of information from the axonal injury site back to the cell body in peripheral neurons (3, 4). This transport system is critical to allow peripheral neurons to repair themselves after injury and may be absent or deficient in neurons within the central nervous system (CNS). Failure to repair or protect CNS axons has remained a recalcitrant problem despite a century of research and continues to be one of the biggest challenges in neuroscience. Changes in axons after injury or in disease states often occur without the contribution of transcriptional events in the cell body in part because of the distance separating the injury site from the nucleus. Indeed, the current understanding of axonal function in health and disease emphasizes the role of proteolysis, local axonal protein synthesis, and a broad range of post-translational modifications. Deciphering how axons regenerate and degenerate has thus become a postgenomics problem, which depends in part on proteomics approaches. In this review, we describe the current understanding in neuronal regeneration and degeneration and discuss recent studies designed to uncover the mechanisms and molecules involved with an emphasis, when available, on those linking axonal transport to regeneration and disease. We also discuss advantages and limitations of proteomics approaches to study the nervous system.

NEURONAL REGENERATION

Axonal Injury Signals—Primary sensory neurons with cell bodies in the dorsal root ganglion (DRG) provide a useful

1 The abbreviations used are: CNS, central nervous system; DRG, dorsal root ganglia; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal kinase; PNS, peripheral nervous system; CRMP-2, collapsin response mediator protein-2; OEC, olfactory ensheathing cell; GAP-43, growth-associated protein 43; RGC, retinal ganglion cell; NGF, nerve growth factor; OMgp, oligodendrocyte myelin glycoprotein; aFGF, acidic fibroblast growth factor; SCI, spinal cord injury; Uch-L1, ubiquitin COOH-terminal hydrolase isozyme L1; Wld(s), Wallerian Degeneration Slow; AD, Alzheimer disease; PD, Parkinson disease; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; SILAC, stable isotope labeling with amino acids in cell culture; SOD1, copper-zinc superoxide dismutase; iTRAQ, isobaric tag for relative and absolute quantitation; crybb2, crystallin β b2; GDI, guanine nucleotide dissociation inhibitor; gad, gracile axonal dystrophy; Aβ, amyloid-β peptide; CREB, cAMP-response element-binding protein; ERK, extracellular signal-regulated kinase.

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In recent years, studies by several groups have emphasized the role of axonal protein synthesis in axon regrowth and repair (for reviews, see Refs. 9 and 10). Proteomics approaches on purified axons have enabled the identification of injury signals and their downstream signaling cascades. To identify protein synthesized in axons upon injury, Willis et al. (11) used metabolic labeling of axons purified from cultures of injury-conditioned adult DRGs followed by proteomics and validation by reverse transcription. This elegant approach led to the identification of 40 locally synthesized proteins. This complex set of proteins included cytoskeletal, heat shock, antioxidant, metabolic, and resident endoplasmic reticulum proteins and proteins associated with neurodegenerative diseases. However, the total number of locally synthesized proteins could have been underestimated because this study used gel-based proteomics assays. In another study, Perison et al. (12) performed differential proteomics on axoplasm purified from injured and uninjured molluscan Lymnaea nerve to identify signaling molecules retrogradely transported following injury. Among 40 identified proteins, the authors characterized a proteolytic fragment of the retrograde protein of 51 kDa (RGP51), a homolog of the mammalian intermediate filament protein vimentin. Inhibition of RGP51/vimentin function by RNA interference blocked regenerative growth of Lymnaea neurons in culture (12). The same group then showed that vimentin is locally translated following injury in rat sciatic nerve, and its proteolytic cleavage promotes interaction with the retrograde motor dynein following injury (13). Interestingly, the authors found that vimentin also interacts with the MAPK ERK1 upon injury and protects it from dephosphorylation (14), thereby providing a mechanism to prevent deactivation of the injury signal during the long journey to the cell body. The importance of vimentin for regeneration was confirmed because neurite outgrowth and recovery of sensory responses after lesion of the sciatic nerve are reduced in animals lacking vimentin (13). The same group then showed that in addition to vimentin components of the nuclear import machinery are also synthesized locally upon axonal injury and link MAPK injury signals to dynein for retrograde transport (15, 16). These studies reveal that axons have the potential to respond to injury by synthesizing a complex population of proteins and led to the identification of a new nuclear import machinery-based retrograde injury signal mechanism.

Another adaptor protein linking MAPK signaling molecules to the dynein-dynactin-dependent retrograde transport after nerve injury is the JNK scaffolding protein Sunday Driver (also known as syd/JIP3/JSAP1). Syd and JNK are present on vesicular structures in axons and are transported in both anterograde and retrograde directions (17). Following nerve injury, activated JNK and syd are primarily transported retrogradely, likely because of an enhanced interaction between syd and the dynein-dynactin complex (17). To further examine whether syd-associated vesicles function as a mobile injury signaling platform, Abe et al. (18) identified the molecular anatomy of syd-associated vesicles using an immunoiolation strategy followed by electron microscopy and mass spectrometry analysis. This study revealed that syd associates in part with endosomes in axons and points to a role for syd endosomes in the transport of signals along the axon. This study also suggests that syd may also play a role in axonal growth and guidance through its interaction with another class of small anterograde vesicles. Another type of axonal endosome recently characterized by magnetic isolation and proteomics transports neurotrophins and their receptors retrogradely along axons (19). Axonal injury may limit the amount of neurotrophin signaling reaching the cell body, and this specific set of endosomes may therefore represent a negative injury signal. Different organelles, endosomes, and protein complexes involved in axonal transport may thus collaborate to promote nerve regeneration in peripheral neurons (20).

Given the importance of axonal transport in the response of axons to injury, Michaelievski et al. (21) sought to characterize the protein ensemble associated with anterograde and retrograde axonal transport in injured axons. The authors used an isobaric tag for relative and absolute quantitation (iTRAQ) proteomics approach on purified axoplasm from lesioned rat nerves. This study revealed extensive changes in the proteins undergoing anterograde and retrograde axonal transport. In addition, this study further emphasizes the important role of local protein synthesis in the response of axons to injury.

The lack of regenerative growth capacity of CNS axons can be attributed in part to their inability to activate or transport injury signals. The conditioning injury paradigm emphasizes that the protein composition may be different in DRG central and peripheral branches, thereby underscoring their differential response to injury. Proteomics approaches, aimed at comparing peripheral nervous system (PNS) and CNS axonal responses to injury, may shed light on the poor regenerative capacity of central neurons. Recently, Katano et al. (22) used a proteomics approach to define the differential protein composition of DRG central and peripheral branches. Whole ly-
sates from peripheral and central branches of DRG neurons were used, and gel-based proteomics led to the identification of over 800 proteins. However, the authors only reported the characterization of one candidate present solely in the peripheral fraction, a novel isoform of the collapsin response mediator protein-2 (CRMP-2) called periCRMP-2. They report that the level of periCRMP-2 is decreased upon nerve injury, but the true functional role of periCRMP-2 awaits future work (23). This study emphasizes that broad conclusions are difficult to draw without proper enrichment methods. Nevertheless, the authors demonstrate the power of proteomics for understanding the mechanisms by which injury can be differentially sensed in central and peripheral axons. The observations that CRMP-2 interacts with both kinesin (24) and dynein (25) combined with the findings that CRMP-2 undergoes proteolytic cleavage in degenerating neurites (26) and that its levels are down-regulated with age (27) suggest that CRMP-2-dependent transport is important for axon outgrowth and regeneration. These studies in peripheral nerves emphasize that coordination between several injury signaling pathways may be necessary to specify the nature and the location of injury to allow neurons to mount the appropriate cell body response (for further reading on injury signals, see Refs. 3 and 28).

Schwann Cell and Glial Response to Injury—Injury to peripheral neurons initiates a sequence of events in the nerve segment distal to the injury site termed “Wallnerian degeneration.” The distal portion of the axon degenerates, and the associated Schwann cells dedifferentiate to a non-myelinating state and produce a number of neurotrophic factors that support the survival of injured neurons. This is followed by a wave of Schwann cell proliferation, which provides the permissive environment at the injury site to guide injured axon toward their original target. These Schwann cells then ensheath and remyelinate the regenerating axons to allow functional recovery of the injured nerve. At the molecular level, the reaction of Schwann cells to injury and their role in axonal regeneration have been well defined (29). However, many questions remain unanswered. In particular, the exact similarity between remyelination after injury and during development remains to be determined. Furthermore, the pathways that trigger dedifferentiation and proliferation of Schwann cells after injury are still unclear.

In a comparative study, Jiménez et al. (30) used gel-based proteomics to determine the temporal changes in protein expression in rat sciatic nerve distal to a crush injury. Whole lysates of sciatic nerve segments distal to the injury site were prepared at different time points ranging from 5 to 35 days postinjury. Cluster analysis of 121 proteins that changed at one or more time points grouped proteins by cellular origin in addition to different functional roles, revealing the difficulty of proteomics data interpretation when whole nervous tissue lysate is used. However, in addition to the stress response- and cytoskeleton-related proteins, several proteins not previously implicated in nerve regeneration were identified, including components of protein synthesis, maturation, and degradation machinery. One such protein, annexin A9/31, belongs to a novel subfamily of annexins and is up-regulated in sciatic nerve after injury. Annexin I in macrophages of PNS was suggested to function as an endogenous regulator of inflammation during nerve repair (31), and annexin III is up-regulated in stimulated microglia surrounding injured motor neurons (32), suggesting a role in morphological changes of microglia following injury. Up-regulation of several members of the annexin family may thus exert immunoregulatory functions following peripheral nerve injury. These experiments by Jiménez et al. (30) did not detect changes in low abundance proteins such as receptors, cytokines, and growth factors, which are known to be induced in Schwann cells upon nerve injury, indicating that gel-based approaches may lack the necessary sensitivity to identify low abundance proteins regulated by sciatic nerve crush in vivo.

Although Schwann cells contribute to a large extent to the successful regeneration of peripheral neurons, impaired myelination following CNS trauma represents a major hurdle to neurological recovery. Recently, transplantation of olfactory ensheathing cells (OECs) in addition to the use of Schwann cell transplants has been tested in clinical trials to ameliorate the functional deficits following CNS injuries. Once transplanted, OECs transform into Schwann cell-like cells that myelinate axons and support new growth. It is important to accurately identify grafted OECs and distinguish them from endogenous Schwann cells that can invade the spinal cord after injury to accurately estimate the benefit of OEC transplantation. A recent gel-based proteomics study identified calponin, an actin-binding protein, as a phenotypic marker that distinguishes between OECs and Schwann cells (33). Future proteomics studies of OECs and remyelinating Schwann cells may enable manipulation of these cells to promote remyelination and facilitate functional recovery.

Neurite and Axon Outgrowth—Although much of the axonal elongation process following injury may be reminiscent of the developmental stages, the distances that regrowing axons have to cover to reconnect with their target tissue is several orders of magnitude larger than during their embryonic development. The damaged axon needs to form a dynamically extending growth cone, which is achieved by calcium-dependent remodeling of the cytoskeleton (34). This initial phase also requires local protein synthesis in the axon, occurring without contribution of transcriptional events in the cell soma (35, 36). The growth-associated protein 43 (GAP-43) is considered to be a crucial component of axonal outgrowth, representing a major factor enhancing sprouting in adult neurons (37, 38). However, expression of GAP-43 alone appears insufficient to stimulate regeneration of DRG central branches into the spinal cord (6). Retinal ganglion cells (RGCs) are often used as a CNS neuronal model because they possess only a limited ability for regrowth after birth when compared with DRG neurons. In these cells, GAP-43 is developmentally reg-
ulated such as that it is associated with axonal growth only within a 2-week time window of postnatal life, from day 14 to day 30, and reappears only following injury (39, 40). To assess which factors may regulate GAP-43 expression, Schröer et al. (40) used a gel-based proteomics profiling of retinal explants and identified translin-associated factor X, a DNA-binding factor that appears to regulate GAP-43 transcription and regeneration-promoting effects during the postnatal maturation period of RGCs. Translin-associated factor X expression correlated with the developmental time window in which RGCs regenerate in vitro and appears to regulate the switch to the no-growth stage reached at day 30. Such proteomics studies may be valuable in identifying new potential therapeutic targets for optic nerve and spinal cord injuries.

Similarly to the conditioning injury in DRG neurons, optic nerve crush or lens injury stimulates the growth capacity of RGCs, a property that permitted the identification of growth-promoting factor secreted by activated macrophage in the retina, such as oncomodulin (41). Two studies recently took advantage of this feature to reveal a role for the small heat shock protein crystallin in RGC regeneration. Liedtke et al. (42) used a gel-based proteomics approach to analyze the supernatant of cultured retinal explants that did or did not receive a conditioning injury. They found that crystallin β2 (crybb2) is up-regulated in the regenerating retina and that overexpression of crybb2 in RGCs and hippocampal neurons increases axonogenesis. This result implies that crybb2, unlike oncomodulin, which is produced by macrophages, may function in an autocrine manner to stimulate axonal growth. To determine whether aging affects the regenerative ability of retinal explants, Rose et al. (43) used organotypically cultured monkey retina between the day of birth and adulthood for gel-based proteomics profiling. Although regeneration of RGCs axons occurred throughout all stages, the numbers of axons decreased with age (43). Among the proteins strongly expressed in the neonatal retina, they found α-crystallin and ribosomal subunits involved in protein synthesis. This latter finding is consistent with the recent finding that reactivation of protein synthesis promotes RGC regeneration (44). Together with the observation that α-crystallin is locally synthesized in injury-conditioned DRG axons (11), these studies emphasize the role of molecular chaperones and axonal protein synthesis in axon outgrowth and regeneration.

Another important class of molecules that promote neurite outgrowth and neuronal survival are neurotrophins such as nerve growth factor (NGF). Acting synergistically with neurotrophins to stimulate nerve regeneration are immunophilins, a family of proteins that also serve as receptors for immunosuppressant drugs (45). One such immunosuppressant, rapamycin, has a potential clinical use to promote neurological recovery following peripheral nerve and spinal cord injuries (46). To understand how immunophilin ligands cooperate with neurotrophic factors in promoting neurite outgrowth, Liu et al. (47) performed an iTRAQ-based proteomics approach to determine the differential neurite outgrowth patterns brought about by treatment of DRG neurons with NGF or the neurophilin ligand JNJ460. They report that DRG neurite outgrowth following NGF treatment appears to rely on proteins possessing biosynthesis function and ribosomal localization, whereas neurite outgrowth following JNJ460 treatment relies on proteins promoting organogenesis and mediating signal transduction. This study thus points to a possible mechanism for the observed synergistic actions of neurotrophic factors and immunophilin ligands, but further studies are needed to examine the precise nature of these interactions.

To gain insight into the spatial organization of signaling networks that enable neuritogenesis, Pertz et al. (48) performed a proteome profiling of the soma and neurites of neuroblastoma cells. Neuroblastoma cells were grown on a microporous filter system, allowing neurites and soma to be purified. Although this study used neuroblastoma cells rather than primary cultured neurons, the elegant design allowed the authors to determine the spatial compartmentalization of signaling networks involved in neurite outgrowth. The authors found an asymmetry in the signaling pathways present in both subcellular domains. Notably, a highly compartmentalized and complex set of potential Cdc42 guanine nucleotide exchange factors and GTPase-activating proteins are present in neurites, suggesting that these regulators precisely control neurite extension in time and space.

Another set of proteins controlling neurite outgrowth originates from the myelin. It is well established that CNS myelin proteins have the capacity to inhibit neurite outgrowth and that this represents a major factor limiting regeneration in the CNS. In contrast, PNS myelin provides a permissive substrate for neurite outgrowth. This permissive environment allows axonal sprouting from neighboring non-damaged axons at nodes of Ranvier, which represents one of the mechanisms contributing to the reestablishment of functional connections in the injured PNS. This collateral sprouting mechanism is lacking in CNS nodes despite the absence of myelin at the node. To determine whether non-myelin-derived factors present in the CNS nodal vicinity possess sprouting inhibitory activity, Huang et al. (49) purified the CNS nodal axoglial apparatus and determined its protein composition. More than 300 proteins were identified, including proteins known to be localized at the nodes as well as novel node constituents, such as CRMP-2 and the oligodendrocyte myelin glycoprotein (OMgp), which is generally believed to be enriched in myelin membranes. Surprisingly, Huang et al. (49) found that OMgp is enriched at the nodal region, encircling the CNS axon. The nodal region in PNS also contained OMgp but in a more diffuse and less intense pattern. OMgp-null mice exhibited collateral sprouting, suggesting that OMgp might be an important factor preventing collateral axon outgrowth during development and also restraining regeneration after CNS injury. This study illustrates that enrichment methods and rigorous validation experiments combined with proteomics are...
extremely helpful in discovering new pathways regulating nerve regeneration.

Pain—Although peripheral nerves have the ability to regenerate following injury, neurological recovery is rarely complete and can often be associated with the development of severe neuropathic pain. Neuropathic pain results from synaptic changes in both the peripheral and central nervous systems and leads to long term mechanical sensitivity. Following spinal nerve ligation, a widely used model of neuropathic pain, the number of DRG neurons decreases by 30%, and neuronal degeneration affects neighboring intact neurons as well as non-neuronal cells in their surrounding. Komori et al. (50) sought to determine the changes in DRG cell bodies following spinal nerve ligation that underlie neuronal cell death and potentially contribute to the development of chronic pain. DRG whole lysates from injured or uninjured spinal nerves were analyzed by gel-based proteomics, and the expression levels of 67 proteins were found to be tightly regulated 1 week after ligation. Most proteins identified are related to stress response, cellular defense, and antioxidation mechanisms and may originate from the circulation, invading macrophages or Schwann cells. Similarly to the sciatic nerve lysate preparation used by Jiménez et al. (30), DRG includes several types of neurons and glial cells. Therefore, despite the different injury model used, the proteomics data obtained by Komori et al. (50) are very similar to those obtained by Jiménez et al. (30), emphasizing that without enrichment strategies abundant proteins related to glia and circulation overwhelm the low abundance neuronal proteins. The few differences may reflect different regulatory processes elicited by injury proximal or distal from the cell body. Most of the proteins identified also display similar regulatory patterns in other types of peripheral nerve injury, including HSP27, peroxiredoxin 6, annexins, and vimentin. Therefore, a careful choice of model system and enrichment strategy is needed to make proteomics approaches useful to better understand the molecular determinants of increased sensitivity and pain following peripheral nerve injury. Nonetheless, Komori et al. (50) report the interesting observation that ligation of the L5 spinal nerve leads to the proteomic changes in the L4 DRG whose spinal nerve was left intact, suggesting that neighboring cells are affected by injury, possibly due to the fact that both uninjured L4 and injured L5 axons commingle in the sciatic nerve. This phenomenon may contribute to the development of chronic pain following nerve injury.

Neuropathic pain is also believed to rely on changes to cellular signals and altered synaptic transmission at the level of the spinal cord. To understand the mechanisms underlying this central sensitization, Singh et al. (51) sought to determine the global expression changes of synaptosome-associated proteins in the dorsal horn after spinal nerve injury. Using a gel-based approach, 27 spots showing at least a 2-fold change between injured nerves and control were identified. In addition to the metabolic and oxidative stress-related proteins, this study revealed that spinal nerve injury increased the expression of synaptosome-associated proteins that are involved in synaptic transmission and modulation of noxious information in the dorsal horn, such as glutamate dehydrogenase 1 and glutamine synthetase. Because this study used synaptosome preparation as an enrichment method, it also enabled the detection of changes in proteins involved in plasma membrane preparation, such as GRB2-like protein 1 and septin 5. When compared with total protein sample form dorsal horn, some identified proteins did not reveal any significant changes, indicating that translocation of these proteins into the synaptosomal fraction rather than the global expression level might be regulated following nerve injury and thereby contribute to the development of chronic pain. This study thus emphasizes the power of enrichment procedures to elucidate new molecular and cellular mechanisms underlying the development of neuropathic pain.

Neuroprotection—Microglia are pivotal cells of the immune system that influence the progression of neurodegenerative diseases but can also lead to neuroprotective activities via the release of neurotrophic factors (52). Thus, elucidation of destructive and protective microglial functions has significant implications in the understanding of both the pathogenesis and treatment of nervous system disorders. Toward this goal, Glanzer et al. (53) used genomics and proteomics approaches to explore the molecular signature of microglia. To reflect the immune consequences of nervous system injury, optic and sciatic nerve fragments were used to stimulate cultured microglia. Both cytosolic and secreted proteins were included in the experiment. The genomics approach indicated that nerve-stimulated microglia show fluctuations in the levels of neurotrophins, lysosomal enzymes, antioxidants, and NFκB, distinguishing their resting and activated states. Proteomics analyses identified lysosomal, antioxidant, and cytoskeletal proteins as being up-regulated in nerve-stimulated microglia. The up-regulation of cathepsin and other lysosomal enzymes is critical for microglial clearance and for the mobilization of adaptive immunity. Increased secretion of ferritin upon nerve stimulation may be important to control the levels of reactive oxygen species (54). The combination of genomics and proteomics approaches in this study thus supports a role for microglia in debris clearance and promotion of neural repair after injury.

Another mechanism that protects axons and synapses following injury involves the Wallerian Degeneration Slow Wld(s) gene. Wld(s) is a spontaneous mutation leading to expression of a fusion protein that combines a fragment of the ubiquitination assembly factor (Ube4b/Uf2a) with the nicotinamide mononucleotide adenylyltransferase 1 (Nmnat-1). To determine the mechanisms underlying resistance to degeneration, Wishart et al. (55) used differential proteomics analysis to identify proteins whose expression levels are altered by the presence of Wld(s). The authors took advantage of the well-established method for synaptosome preparation to isolate
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...synaptic preparations from the striatum, an area of the CNS in which synapses are known to be protected from neurodegeneration in Wld(s) mice. Indeed, of the 16 proteins identified, half serve as regulators of mitochondrial stability, whereas the other half is related to both ubiquitination and NAD-dependent pathways. This study thus supports the idea that nicotinamide mononucleotide adenylyltransferase activity is responsible for axon protection (56) and that the Ube4b domain is important for localization of the Wld(s) fusion protein (57, 58).

Acidic fibroblast growth factor (aFGF) is known to promote survival and sprouting in various neural systems (59, 60). Tsai et al. (61) found that administration of aFGF induces functional recovery of locomotion following spinal cord injury (SCI) in rats and that functional recovery is accompanied by proteomic changes. Extracts from total spinal cord segments that were treated or not with aFGF were prepared, and following a gel-based approach, 51 proteins that had significant differential expression were identified. In particular, proteins involved in the process of secondary injury, such as the glial fibrillary acidic protein, S100B, and the keratan sulfate proteoglycan lumican were up-regulated after SCI and down-regulated following aFGF treatment. HSP27 was found to be up-regulated by SCI and down-regulated by aFGF treatment, whereas annexin A3 was down-regulated by SCI and only minimally affected by aFGF treatment. The expression of the antioxidant proteins peroxiredoxin 2 and peroxiredoxin 6 is down-regulated by SCI, and levels are partially recovered by aFGF treatment, indicative of a reduced protection to oxidative stress upon injury. Peroxiredoxin itself is oxidized in the gracile axonal dystrophy (gad) mouse model of axonal degeneration (62), indicating that axonal integrity is related to control of oxidative stress. Functional recovery achieved by aFGF was also parallel with elevated levels of GAP-43 and stathmin, two important regulators of neurite outgrowth, similar to studies in which growth inhibitors such as Nogo were neutralized (63). However, differential expression of GAP-43 and stathmin was established by Western blot analysis and not by the gel-based proteomics method, revealing the limitation of the latter approach. Interestingly, another gel-based proteomics study aimed at identifying molecular changes due to aging showed that stathmin levels are reduced in old sciatic nerve (27), underscoring the role of stathmin in axonal regenerative ability.

The mechanisms of neuroprotection provided by Wld(s) and aFGF may share common pathways. Indeed, both Wld(s) and aFGF treatment following SCI reduces the levels of RabGDI, a regulator of RabGTPase association with membrane compartments (55, 61). Wld(s) also partially rescues the axonal degeneration in gad mice (64), a mouse model of axonal degeneration in which oxidized RabGDI is detected (62). The gad mouse lacks functional ubiquitin COOH-terminal hydrolase isozyme L1 (Uch-L1), a deubiquitinating enzyme, indicating that RabGDI function may be altered as a result of modifications in the ubiquitin-proteasome system in both Wld(s) and gad mice. Further studies are needed to examine the precise role of RabGDI in axonal degeneration. However, its role in controlling the association of RabGTPases with subcellular organelles suggests that vesicular transport may play a role in axon protection.

The importance of the ubiquitin-proteasome system in protecting axons and neurons is evidenced by the findings that the ubiquitin-like modifier-activating enzyme 1 (UBA1) is up-regulated in Wld(s) mice (55), Uch-L1 is down-regulated following SCI (61), and Uch-L1 function is lost in gad mice. In addition to function as a deubiquitinating enzyme, Uch-L1 also binds and stabilizes monoubiquitin (65). Consequently, the level of monoubiquitin is decreased in gad mouse sciatic nerve, suggesting that target proteins of the ubiquitin-proteasome system are not sufficiently ubiquitinated and thus not sufficiently degraded in the gad mouse (65). Goto et al. (65) used a gel-based proteomics approach to identify proteins that accumulate in the sciatic nerve of gad mice. Among several proteins identified, the authors validate the axonal accumulation of glyceraldehyde-3-phosphate dehydrogenase. Together with the observation that glyceraldehyde-3-phosphate dehydrogenase localizes to extracellular plaques in neurodegenerative disorders, this study delineates a role for glyceraldehyde-3-phosphate dehydrogenase in the axonal degeneration in gad mice and possibly other neurodegenerative diseases.

NEURODEGENERATION

Neurodegenerative disorders such as Alzheimer disease (AD), Parkinson disease (PD), or amyotrophic lateral sclerosis (ALS) may be caused by genetic mutations but also occur in sporadic forms. Although these diseases have different causes and affect different regions of the nervous system, the underlying degenerative mechanisms have much in common and include axonal pathologies. Abnormal accumulation of protein aggregates and organelles along the axon due to disruption of axonal transport is thought to be an early pathogenic event leading to the demise of neurons (1, 2). We discuss below several studies that have used proteomics approaches to define the molecular components associated with pathogenic protein aggregates and the mechanisms contributing to the pathogenesis of neurodegenerative diseases.

Alzheimer Disease—Alzheimer disease is the leading cause of dementia and the most common neurodegenerative disease. The disease is largely sporadic with a small percentage of cases being inheritable. AD characteristic pathologies include neurofibrillary tangles, areas containing aggregated microtubule-associated protein tau, and amyloid plaques, areas containing amyloid-β peptide (Aβ). Gene mutations in the amyloid precursor protein (APP) and presenilins, which are involved in APP processing, are linked to some familial cases of AD. Both extra- and intracellular Aβ resulting from aberrant APP proteolysis are implicated in the initiation of axonal transport defects and axonal degeneration (66, 67). Aβ appears to
initiate multiple pathways, including oxidative stress and signaling cascades, leading to defective axonal transport (66, 67). To identify proteins that may favor Aβ deposition, Liao et al. (68) used laser capture microdissection for proteomic profiling of amyloid plaques from post-mortem AD brain tissues. Using immunoprecipitation of APP, this study identified 26 proteins enriched in amyloid plaques as compared with non-plaque areas (68). Surprisingly, among these, only a few known proteins (Fe65 and α-crystallin) and mostly novel APP-interacting proteins such as the retrograde motor dynein were found (69). Dynein was also among the list of 21 proteins found to be associated with APP in brain samples of patients with Alzheimer disease (69). The association of dynein with senile plaques and APP underscores the role of axonal transport in the disease. To characterize neurofilibrillary tangles from patients with AD, Wang et al. (70) performed proteomic profiling also using laser capture microdissection. This approach allowed the identification of 72 proteins, which function in diverse biological processes including energy and metabolism, intracellular trafficking, signaling, stress response, and cytoskeletal or extracellular matrix components (70). Although these studies demonstrate the power of enrichment strategies such as laser capture microdissection to the comprehensive analysis of neuropathological structures, further studies are needed to determine the causal or consequential effect of specific proteins associated with plaques.

Accumulating evidence suggests that oxidative stress contributes to AD pathogenesis. Protein oxidation may lead to loss or reduction of protein function during oxidative stress. Redox proteomics approaches are thus useful to determine which proteins undergo oxidative modifications in AD. Several studies on human AD brain tissue report the oxidation of multiple proteins, including Uch-L1, CRMP-2, α-enolase, HSC71, α-crystallin, and glyceraldehyde-3-phosphate dehydrogenase (71–77). In addition to human tissue, proteomics studies on human AD brain tissue report the oxidation of multiple proteins, including Uch-L1, CRMP-2, and cytoskeletal or extracellular matrix components (70). Although these studies demonstrate the power of enrichment strategies such as laser capture microdissection to the comprehensive analysis of neuropathological structures, further studies are needed to determine the causal or consequential effect of specific proteins associated with plaques.

To reveal proteins linked to PD disease, Basso et al. (83) used a gel-based approach to compare the proteomic profile of human PD and control substantia nigra. Among 44 proteins identified, nine displayed significant alteration in their relative abundance. The elevation of peroxiredoxin 2, mitochondrial complex III, and ATP synthase (83) and the decrease of mortalin (84) are consistent with the role of mitochondrial dysfunction and oxidative stress in PD pathogenesis. Using the stable isotope labeling with amino acids in cell culture (SILAC) approach, Jin et al. (85) report that mortalin interacts with α-synuclein in dopaminergic mouse embryonic stem cells. Further characterization of this interaction as well as of other α-synuclein interactors (85) is needed to understand their role in PD progression. Oxidation of dopamine and dopamine metabolites has also been linked to the pathology of PD (86). The substantial loss of mortalin observed in rats exposed to an oxidized product of dopamine (87) and the observed decrease of mortalin in PD brains (84) may reflect the particular susceptibility of mortalin to dopamine oxidation. In light of the critical role of mortalin in mitochondrial function (88), future work is needed to establish the role of mortalin in the context of dopamine neurodegeneration.

Another mechanism that may affect dopaminergic neurons in PD is rearrangement of the cytoskeleton. Proteomics studies revealed that neurofilament chains are less abundant in human substantia nigra from PD patients (83), and deregulation of actin cytoskeleton is observed in a Drosophila PD model (89) and in a Caenorhabditis elegans PD model (90). Both α-synuclein and parkin interact with the actin cytoskeleton (91, 92), and α-synuclein also interacts with the micro-
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...tubule protein tau (92), reflecting their potential roles in regulating the dynamics of actin filaments and microtubules during dopaminergic degeneration (93). Continued efforts to elucidate the cytoskeletal and mitochondrial changes prior to the onset of PD may thus offer a plethora of new therapeutic targets.

Lewy bodies, a hallmark of PD disease, are abnormal intracellular protein aggregates made up mostly of α-synuclein fibrils. As mentioned, laser capture microdissection represents a powerful tool to characterize human cortical Lewy bodies. Using this approach, Leverenz et al. (94) identified 296 proteins and validated a novel component of Lewy bodies, the heat shock protein HSC70/HSP73. Further investigation will reveal novel mechanisms by which Lewy bodies form and lead to neurodegeneration. Interestingly, other heat shock proteins may be involved in dopaminergic neurons protection. Using a gel-based proteomics approach combined with phosphoprotein enrichment, Hong et al. (95) found that HSP27 phosphorylation is increased in PC12 cells treated with the neurotrophic factor glial cell-derived neurotrophic factor. Moreover, overexpression of HSP70 protects dopaminergic neurons from neurotoxicity and suppresses α-synuclein aggregation (96). Proteomic profiling of Parkin knock-out mice, which reproduce some of the symptomatic aspects of Parkinson disease in the absence of neuronal degeneration, points to energy metabolism, stress response chaperones, and the ubiquitin-proteasome pathway as possible protective mechanisms (97). Similar studies may be valuable to identify additional factors that contribute to dopaminergic neuron protection.

Microglial activation following the initial neurodegeneration may subsequently enhance the degenerative process. Bioactive substances released by degenerating dopaminergic neurons (98), including aggregated α-synuclein (99), are known to activate microglia (98). Proteomic profiling of microglia exposed to nitroated and aggregated α-synuclein identified inflammatory proteins, indicative of the important role of secondary neuroinflammation in the progression of nigral degeneration and PD (100).

Amyotrophic Lateral Sclerosis—ALS, also known as Lou Gehrig disease, is a fatal, neurodegenerative disease caused by the progressive death of motor neurons. Mutations in the copper-zinc superoxide dismutase (SOD1) as well as defects in the retrograde motor machinery have been linked to ALS disease (1). Neurotrophic signaling from the target tissue to the neuronal cell body critically depends on the retrograde transport machinery. The lack of neurotrophic support may thus result in part underlie motor neuron degeneration (1). To examine further the contributions of the reduced axonal transport to the pathogenesis of neuronal degeneration, Perlson et al. (101) determined the dynein-associated proteins in wild-type and mutant SOD1 mice, a widely used ALS mouse model. The authors used a protein microarray analysis, which is based on a microarray chip containing ∼600 antibodies against signal...

...ing molecules, allowing one to track the differential binding of dye-labeled proteins prepared from nerve axoplasm. They report that in ALS the retrograde signal is changed such that dynein-associated cargoes switch from survival-promoting in wild-type mice to death-promoting signaling pathways in mutant SOD1 mice. In particular, they observed decreased retrograde transport of phospho-Trk and phospho-ERK1/2 and increased retrograde stress factor signaling, including phosphorylated JNK, caspase-8, and p75 neurotrophin receptor p75NTR cleavage fragment in the SOD1 mouse model. Interestingly, no appreciable differences were observed in the overall levels of proteins such as phospho-JNK and phospho-ERK, although significant changes in their association with dynein were found. The finding that JNK signaling may be associated with regeneration (3, 102, 103) and degeneration (101, 104) illustrates the complexity of the signaling pathways involved. The ability of the study by Perlson et al. (101) to quantify changes in the retrograde signaling in the ALS mouse model rather than total proteomic changes relied on the preparation of axoplasm-enriched fraction. This elegant work thus indicates that neurodegeneration may depend on a change in the balance of survival and stress signaling along the axon. In addition, an aberrant interaction between dynein and mutant SOD1 (101, 105, 106) may compromise the efficiency of the retrograde transport. Indeed, the lower retrograde transport velocities observed in mutant SOD1 mice (101, 105) may be due to a differential regulation of the molecular motors, leading to an overall decrease in the efficiency of transport.

Mitochondrial dysfunction also plays a critical role in etiology of familial ALS. In particular, recruitment of mutant SOD1 to spinal mitochondria was proposed as the basis for their selective toxicity in ALS (107). To identify mitochondrial proteins that are altered in the presence of mutant SOD1, Fukada et al. (108) determined the mitochondrial proteome in a motor neuron-like cell line (NSC34 cells) expressing wild-type or mutant SOD1. Purified mitochondria and a gel-based proteomics approach were used to determine 45 spots displaying modified protein abundance, including proteins involved in mitochondrial membrane transport, apoptosis, and the respiratory chain and molecular chaperones. More than half of the protein spots that are altered in the mitochondria of the mutant SOD1-expressing cells contain post-translational modifications. For example, modifications of the mitochondrial outer membrane protein VDAC2, which was shown to inhibit the mitochondrial apoptotic pathway (109), could be involved in regulating apoptosis in ALS. Protein oxidation, as described above for AD, may also underlie axonal degeneration in ALS. Indeed, similarly to AD, the proteins CRMP-2, HSP70, and possibly α-enolase are oxidized in the spinal cord of mutant SOD1 transgenic mice (110). The antioxidant protein peroxiredoxin 3 localizes within mitochondria in the spinal cord and is down-regulated in mutant SOD1 mice (111), representing a possible deficit in mitochondrial antioxidant defense. Together, these studies emphasize that protein oxidation can...
lead to the breakdown of mitochondrial energetics, contributing to axonal loss in ALS and possibly other neurodegenerative diseases.

CONCLUSIONS

Although peripheral neurons have a remarkable ability to repair themselves after injury, neurons within the central nervous system do not spontaneously regenerate and are more vulnerable to degeneration induced by genetic and environmental factors. Initiation of axonal regeneration and degeneration does not require transcription in the cell body and depends mostly on post-transcriptional processes. It emerges that the principal degenerative mechanisms converge to oxidative stress, dysfunction of axonal transport, mitochondria, chaperones, and the ubiquitin-proteasome system. The mechanisms regulating nerve regeneration also impinge on axonal transport, the cytoskeleton, and chaperones in addition to changes in signaling pathways (Fig. 1). The recent technical advances in quantitative proteomics are emerging as a powerful tool to elucidate mechanisms involved in the life and death of neurons. However, the extreme complexity of brain and nerve tissue at the anatomical, cellular, and subcellular levels presents major challenges to proteomics work in the nervous system. Unless enrichment strategies for sample preparation are used, results may often be difficult to interpret and biased toward identification of abundant cytoskeletal and glia proteins. Furthermore, gel-based proteomics approaches are biased toward the identification of abundant proteins. Therefore, experimental designs need to involve enrichment strategies such as immunoprecipitation of organelle or protein complexes of interest or laser microdissection to reduce sample complexity and increase sensitivity of detection (Fig. 2).
The development of non-gel-based approaches for quantitative proteomics together with advances made to detect post-translational modification will guide progress toward delineating the mechanisms involved in nerve regeneration and degeneration dysfunctions. Such neuroproteomics approaches will lay the foundation for further detailed functional studies and may thus bring our understanding of axonal biology to an unforeseen level.

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**Fig. 2. Flowchart outlining overall strategies for neuroproteomics studies.** Black arrows, tissue or cells are first fractionated to enrich for organelle, protein complex, or structure of interest. The protein mixture is then digested by a protease, typically trypsin, to produce peptides. The resulting peptides are then separated by LC and analyzed by a first mass spectrometer (MS), which determines the mass of a given peptide. Peptides are further analyzed by fragmentation in a second mass spectrometer, which determines the peptide sequence and thus the protein identity by simultaneously searching against a protein database. Characterization of complex protein mixtures using LC/MS/MS is also called *shotgun proteomics* (see Ref. 112 for more details on mass-spectrometry based proteomics). Gray arrows, two-dimensional (2D) PAGE offers another approach to protein identification. The protein mixture is first analyzed by 2D-PAGE, and then protein spots of interest are excised, digested, and analyzed by MS/MS. Orange arrows, metabolic and chemical labeling or fluorescence labeling are commonly used for quantification of proteins by mass spectrometry (quantitative proteomics). In the metabolic and chemical labeling, different isotopes are incorporated into each sample to be compared. Pairs of peptides with different isotope composition are identified, and their peak intensities provide a quantitative measurement of their relative abundance. Metabolic labeling with SILAC allows mixing samples early in the analysis process, decreasing variation due to the numerous steps in sample preparation. Chemical labeling strategies of protein mixture such as ICAT and iTRAQ offer the ability to perform quantitative proteomics on samples prepared from different tissue sources and control for some possible errors due to sample preparation. "Semi-quantitative" mass spectrometry can be performed without sample labeling: the peak intensity (or area) or the peptide counts are correlated with the amount of protein in the sample. However, because samples are never mixed, errors due to sample preparation can be relatively high. When using fluorescence labeling (two-dimensional DIGE), the “spot maps” generated from the two different dyes discern the pattern of the two samples being compared. Although two-dimensional DIGE is a sensitive technique, it is limited by the two-dimensional gel electrophoresis step, which limits the type and amount of proteins being studied. Blue arrows, in addition to protein identification and quantification, mass spectrometry allows determination of post-translational modifications. For example, single phosphorylation of a peptide results in a known mass gain that allows searching for peptides with such a mass shift. However, the stoichiometry of modified versus unmodified protein species can be low, and enrichment strategies can be used before MS/MS analysis. Current strategies used to enrich for phosphoproteins include affinity purification using IMAC or titanium dioxide chromatography. Other post-translational modifications such as ubiquitination can be identified and/or quantified using affinity chromatography (AC) prior to mass spectrometry. Protein oxidation can also be studied using derivatization of oxidized proteins followed by immunodetection on two-dimensional PAGE (oxyblot). Biological networks can also be identified when proteomics approaches are combined with powerful statistical and cluster analysis.
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