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Specialized Roles of Neurofilament Proteins in Synapses: Relevance to Neuropsychiatric Disorders

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Abstract

Neurofilaments are uniquely complex among classes of intermediate filaments in being composed of four subunits (NFL, NFM, NFH and alpha-internexin in the CNS) that differ in structure, regulation, and function. Although neurofilaments have been traditionally viewed as axonal structural components, recent evidence has revealed that distinctive assemblies of neurofilament subunits are integral components of synapses, especially at postsynaptic sites. Within the synaptic compartment, the individual subunits differentially modulate neurotransmission and behavior through interactions with specific neurotransmitter receptors. These newly uncovered functions suggest that alterations of neurofilament proteins not only underlie axonopathy in various neurological disorders but also may play vital roles in cognition and neuropsychiatric diseases. Here, we review evidence that synaptic neurofilament proteins are a sizable population in the CNS and we advance the concept that changes in the levels or post-translational modification of individual NF subunits contribute to synaptic and behavioral dysfunction in certain neuropsychiatric conditions.

Keywords

Neurofilament subunit; synapse; dendritic spine; neuropsychiatric disease

1. Introduction

Neurofilaments (NFs), the intermediate filaments of mature neurons, are among the most abundant proteins in brain. Unlike the intermediate filaments of other cell types, which are usually homopolymers, NFs in the CNS are hetero-polymers composed of NFL, NFM, NFH and alpha-internexin subunits [187]. Although structurally distinctive, these four NF subunits share a basic tripartite domain structure consisting of a conserved central α-helical

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rod region, a short variable head domain at the amino-terminal end and a tail of highly variable length at the C-terminal end. The short head domain is rich in serine and threonine residues and contains consensus sites for O-linked glycosylation and phosphorylation [188]. The central rod domain, which is relatively conserved among intermediate filament family members, contains long stretches of hydrophobic heptad repeats favoring formation of α-helical coiled-coil dimers. The C-terminal domains contain glutamic- and lysine-rich stretches of varying length that mainly establish the size range (58–200 kDa on SDS gels) of the four NF subunits. Although all intermediate filament types serve roles as structural scaffolds, NF subunit heterogeneity also confers specialized structural properties to NF, in axons where the filaments are extremely long and are often arranged in parallel with uniform spacing conferred by the long C-terminal tails of NFM and NFH extending perpendicularly from the filament core [141, 142]. These unique space filling properties of NF facilitate their well-established role in caliber expansion of large-diameter myelinated axons of peripheral nerves, which is critical for effective nerve conduction [198]. NFs also extensively cross-link with other cytoskeletal elements along axons to form a large metabolically stable stationary NF network [126, 185, 190] that is critical to axon caliber expansion [66, 84, 129] and organelle distribution along axons [143].

Besides these unique structural and functional features, NF subunits are distinguished from other intermediate filament proteins by the complex regulation of their head and tail domains by phosphorylation, especially those of NFM and NFH, which involves actions of multiple protein kinases and phosphatases at many polypeptide sites [133]. Although certain phosphorylation events are known to control tail extension and subunit assembly and slow turnover, the purpose of such complex and dynamically changing phosphate topography on NF subunits [49, 127, 133] has remained puzzling, given the mainly static structural support roles ascribed to NF. Both the complex hetero-polymeric structure and dynamically changing phosphate topography of NF proteins suggests that individual NF proteins might serve additional biological roles although there has been relatively little exploration of this issue until recently.

NF gene mutations are well recognized as causes of several neurological disorders mainly involving degeneration of peripheral nerve fibers in accordance with the prominent function of NF in supporting large-diameter myelinated axons [26]. Notably, however, NF proteins are abundant in grey matter CNS regions as well as white matter [33] but influence caliber expansion much less dramatically in most populations of CNS axons [55]. These observations and evidence that NF subunits can be axonally transported in various minimally assembled forms (including as heterodimers) suggest a broader distribution and range of assembly forms of NF subunits within CNS neurons, including substantial populations in synapses. In light of these findings, alterations of a particular NF subunit as seen in specific brain regions in psychiatric and neuropsychiatric disorders [30, 40, 69] may reflect an alteration within synapses which influences the clinical phenotype.

Psychiatric diseases, affecting an estimated 54 million Americans yearly, cause mild to severe disturbances in thought or behavior usually in the absence of known changes in axonal integrity. Nevertheless, changes in levels and phosphorylation of NF subunits have consistently been noted in certain psychiatric disorders although the location of these

changes within neurons is poorly understood. Psychiatric diseases prominently involve alterations of synaptic transmission. The highly specialized composition of synapses includes not only the well characterized vesicular and protein receptor machinery supporting neurotransmission but also a specialized cytoskeleton important for delivering, inserting, and recycling synaptic components. Like other domains in a neuron, the cytoskeleton in synapses is composed of microtubules, actin filaments, the spectrin-rich membrane skeleton, and as recently shown, NF assemblies [191, 193]. Evidence is also emerging that the cytoskeleton, and especially the NF scaffold, acts as a docking platform to organize the topography of organelles within different neuronal compartments. Rearrangements of this topography are dynamically coordinated at least in part by cellular signals regulating the phosphorylation state of the binding partners [137, 143, 163, 193]. Such evidence suggests a range of possibilities for understanding the newly recognized roles of individual NF subunits in modulating synaptic function. In this review, we consider the properties of synaptic NF assemblies in the CNS and, in this context, raise the possibility that certain changes in levels or phosphorylation of NF subunits reported in neuropsychiatric disorders (Table 1) disrupt synaptic signaling or, in some cases, reflect adaptive or maladaptive responses to these synaptic disruptions.

2. NF proteins in synapses

2.1 Distinctive assemblies of NF subunits in synapses

Synapses have long been considered to be degradative sites for NF reaching terminals by axonal transport [145]. When NF proteins have been detected in synaptic fractions and bound to synaptic proteins *in vitro*, they have previously been viewed as contaminating axonal NF proteins [112] and their possible role in synapses has rarely been entertained. Using multiple independent approaches, however, Yuan et al. recently provided definitive evidence that all four CNS NF subunits are integral resident proteins of synapses and have distinct roles in synaptic function and behavior (Figure 1)[191, 193]. The NF assemblies isolated from synapses are distinctive as compared to those in other parts of the neuron in terms of their morphology and in having higher proportions of alpha-internexin, a lowered phosphorylation state of NFM, and a higher NFH phosphorylations state. This unique population of synaptic NF proteins is more abundant in the postsynaptic area than in adjacent dendritic areas or presynaptic terminals and exhibits a different response to subunit perturbation than the axonal population. For example, when NFM is deleted from mice, NFH phosphorylation and the ratio of NFH to NFL subunit increase significantly in the synaptic pool in comparison to the total NF pool [193]. Because NF subunit monomers are inefficiently transported along axons [186, 187, 193], NF proteins in synapses are at least oligomeric in form and can be transported as such in axons, providing one possible basis for their synaptic location. It is also possible that some NF protein in postsynaptic boutons is synthesized locally because mRNAs of NFL [45, 134] and alpha-internexin [180] are reported to be present in dendrites and polyribosomes are preferentially localized under the base of dendritic spines [161]. In this regard, evidence suggests that NF proteins identified in nerve terminals isolated from squid brain are produced by local protein synthesis [46, 110].In light of these findings, aforementioned reports noting the presence of NF in synaptic

fractions and interactions of NF proteins with known synaptic proteins can be viewed as further support for a substantial synaptic pool of NF proteins in the brain.

Remarkably, previous proteomic analyses of synaptic fractions have consistently identified NF proteins but these findings were not followed up with additional proof of a synaptic localization or function. During the study of activity-regulated proteins in postsynaptic density fractions by two-dimensional gel electrophoresis and mass spectrometry, Satoh et al. identified a total of 90 spots containing 47 different protein species in the PSD fractions isolated from mouse forebrain [148]. Among these spots, alpha-internexin and NFM accounted for 8 and 4 spots, respectively. With an improved proteomic method involving nanoflow HPL coupled to electrospray tandem mass spectrometry (LC-MS/MS), Jordan et al. identified all four CNS NF proteins (NFL, NFM, NFH and alpha-internexin) as abundant components of biochemically purified PSD fractions from rat or mouse brain[92]. A subsequent phospho-proteomic analysis of PSD proteins, identified 42 phosphoproteins in PSD preparations from mouse brains [172]. Out of 90 phosphorylated peptides derived from these 42 phosphoproteins, NF proteins accounted for 23 (NFL 1, NFM 5 and NFH 17), indicating the considerable abundance of NF proteins in the PSD. NF proteins are also glycosylated and in a glyco-proteomic analysis of PSD preparations from mouse brain, NFL, NFM and alpha-internexin were among 18 identified GlcNAc-modified proteins [181]. A limited idea of how NF proteins are organized within the PSD has been achieved using a novel solid phase and chemical crosslinking approach to distinguish proteins that reside either at the surface of the PSD or in its interior [108]. The analysis suggested that NFM, NFH and alpha-internexin together with tubulin subunits reside in the interior of the PSD while actin resides primarily on the surface. Recently, Moczulska et al. performed precise quantification of the mouse synaptosomal proteome during postnatal maturation (from 3 – 8 weeks of age) using peptide-based iTRAQ labeling and high-resolution two-dimensional peptide fractionation [119]. Among the 3422 identified proteins with complete quantifications, NFL, NFM, NFH and alpha-internexin were established to be components of the synaptosomes and their levels increased during brain postnatal maturation, a period when synapse formation sharply increases [2]. Alpha-internexin had long been believed to form a separate filament system from NF triplet proteins until it was shown to be the fourth subunit of NFs in the mature CNS [187] although somewhat over-represented among the other NF subunits present in synapses [193]. Benson et al. detected alpha-internexin immunoreactivity in dendrites extending into dendritic spines of cultured hippocampal neurons [17]. This immunoreactivity receded from spines and remained at the base of dendritic protrusions when cells were treated with latrunculin A to disrupt actin filaments [195].

Besides their association with the PSD, NF subunits have been noted to interact with other synaptic proteins. The synapse-specific PSD95-associated protein SAPAP interacts via its N-terminal region with all 4 NF subunits, co-immunoprecipitates with NF proteins from rat brain, and co-localizes with NFs in transfected COS cells [80]. Spinophilin, a synaptic protein highly enriched in dendritic spines [3], regulates the formation and function of the spines [59]. Using a shotgun proteomic approach, Baucum et al. showed that spinophilin interacts via its N-terminal domain with NFL, NFM, and alpha-internexin in mouse striatum [13, 14, 79]. Moreover, the association increased significantly during mouse brain

maturation [14] during the period of synaptogenesis [2] and was dependent on the phosphorylation state of either spinophilin or NF protein [79]. The NFL subunit also directly binds to tau [118], a protein initially considered to localize in dendritic spines only under pathological conditions [86], but is not believed to have a physiological role in dendritic functioning [65, 90]. Knockdown of tau using specific shRNA significantly decreased the spine density of cultured hippocampal neurons [36] and deletion of tau in mice abolished long-term depression [95]. Thus the interaction between NFL and tau in dendritic spine could be important for normal synaptic transmission.

NF polymers are highly abundant in myelinated axons as estimated by conventional electron microscopy but 10nm filaments have also been noted in the perikarya and dendrites of neurons, including ones lacking a myelinated axon [55, 81, 98, 196]. In 1977, Blomberg et al. reported 10 nm filaments as major constituents of the partially broken up PSD isolated from dog cerebral cortex and assumed that they were composed of NF proteins [21]. Using a rapid-freezing technique, Landis and Reese described infrequent 9–10nm filaments in dendritic spines of mouse brain but interpreted these structures as actin filaments due to their periodicity [104]. Yuan et al. also identified in some synapses that short 10 nm filaments were decorated by immunogold antibodies to different NF subunits indicating that at least some synaptic NF subunits exist in polymerized form (Yuan et al. 2015b)[193]. Although NF proteins are present in both pre- and postsynaptic areas, 10nm NF polymers are not abundant in synaptic areas compared to myelinated axons, estimated by conventional electron microscopy. The infrequency of conventional long intermediate-sized polymers in synapses indicates that most NF subunits in synapses are probably in the form of oligomeric structures (protofilaments or protofibrils). Consistent with this interpretation, transport of NFM in non-filamentous, but oligomeric form, has been reported [166, 184, 186]. The organization of oligomeric NF assemblies in synapses, quite different from those parallel arrays with spacing controlled by C-terminal extensions of NFH/MFM in axons, might be closely associated with the synaptic actin network since disruption of such actin filaments also remarkably affects the distribution of NF assemblies [195]. The relative abundance of NF proteins in synapses is synapse type-dependent, e.g., NF proteins are not present at the postsynaptic sites of neuromuscular junctions. Instead, another intermediate filament protein desmin is highly concentrated near the AChR-rich crests of the junctional folds at the neuromuscular junctions [154].

2.2 NF subunit-specific modulation of synaptic functions and behavior

N-methyl-D-aspartate (NMDA) receptors are excitatory neurotransmitter receptors critical for synaptic plasticity and neuronal development in the mammalian brains [107]. These receptors are highly concentrated in postsynaptic membranes of glutamatergic synapses. Yeast-two-hybrid screening revealed that NFL interacts through its rod domain directly with the cytoplasmic C-terminal domain of NR1 [56] suggesting that NFs may anchor or localize NMDA receptors within the neuronal plasma membrane. Co-expression of NFL with either NR1 or NR2B subunits in HEK293 cells did not increase surface expression of these subunits whereas expression of all three components increased surface abundance of NR1 by 20% and concomitantly increased NMDA-mediated cytotoxicity [144]. The NR1 subunit is ubiquitinated in HEK293 cells and the co-expression with NFL antagonizes this

ubiquitination process, suggesting another way that the interaction of NFL with NR1 may stabilize the NMDA receptor. NFL also binds to protein phosphatase-I (PP1), a protein / serine /threonine phosphatase in the PSD [168]. NFL-bound PP1 could regulate the phosphorylation states of NF subunits and NMDA NR1 receptors the cellular distribution of which is regulated by the phosphorylation of specific serines in the C1 exon cassette [57]. On the one hand, NFL may modulate synaptic plasticity through interactions with NR1, PP1 or 14-3-3 [56, 115, 168]. On the other hand, the phosphorylation state of NFL is regulated by synaptic plasticity events such as long-term potentiation (LTP) [78] and long-term potentiation depression (LTD) [77]. NFL selectively influences dendritic spine and glutamate receptor function since NFL-null mice not only have abnormal spines but also dysfunction of hippocampal-dependent spatial memory, NMDA receptor protein expression and synaptic plasticity (Yuan, Veeranna et al. unpublished data). LTP is a persistent increase in synaptic strength following high-frequency stimulation of a chemical synapse and is generally considered one of the major cellular mechanisms that underlie learning and memory [20]. Yuan et al. recently determined LTP in the Schaffer collateral pathway of the hippocampal slices prepared from wild-type control and IHL-TKO mice lacking alpha-internexin, NFH and NFL subunits [192]. The tetanic stimulation evoked a typical LTP of fEPSP in slices from wild-type mice. These responses were stable for 120 min. However, tetanic stimulation evoked a significantly reduced fEPSP slopes in IHL-TKO mice, indicating NF proteins are, therefore, required for synaptic plasticity. Because LTP in the Schaffer collateral pathway of the hippocampus is NMDA receptor-dependent, altered LTP may be associated with reduced levels of NMDA-NR1 receptor (Yuan, Veeranna et al. unpublished data). Yuan et al. also conducted a 5-trial social memory assay to determine if IHL-TKO mice have a social memory defect. In this test, the subject mouse was given four brief exposures (trials 1–4) in its home cage to the same stimulus mouse (intruder). In the 5^{th} trial, the subject mouse encountered an entirely novel stimulus mouse (novel intruder). Wild-type control mice displayed normal social memory, as demonstrated by a marked habituation (decreased exploration) during the first 4 trials and a striking dishabituation (increased exploration) on the presentation of a novel animal on the 5^{th} trial. In contrast, IHL-TKO mice showed no significant habituation during the 4 exposures to the stimulus mouse or dishabituation to the novel stimulus mouse. Consistent with reduced LTP, IHL-TKO showed social interaction deficits, indicating that mice lacking NF proteins failed to develop normal social memory.

Dopamine receptors are G-protein-coupled receptors that mediate functions of dopamine ranging from voluntary movement and reward to hormonal regulation and hypertension [72]. These receptors are highly concentrated in the postsynaptic membrane of dopaminergic synapses. Yeast-two-hybrid screening has shown that the C-terminal tail of NFM directly interacts with the cytoplasmic loop of dopamine D1receptor [94] and that their co-expression in HEK-293 cells resulted in more than 50% reduction of receptor binding. Recently, the relevance of this interaction at synapses was established in genetically modified mice. Deletion of NFM, but not deletion of any of the other NF subunits, greatly amplified dopamine D1-receptor-mediated motor responses to cocaine while redistributing postsynaptic D1-receptors from endosomes to the plasma membrane (Figure 2)[193]. In wild-type mice, NFM colocalized with the D1 receptor in synaptic boutons by immunogold

electron microscopy. Deletion of NFM also significantly increased D1R–stimulated hippocampal LTP further indicating an *in vivo* interaction between NFM and D1R [193]. Depressed hippocampal LTP induction is NF subunit-selective: maintenance of LTP is deficient in NFH-null mice while basal neurotransmission and induction of LTP are normal in NFM-null mice. Eliminating all NF proteins from the CNS profoundly disrupted synaptic plasticity and social memory without altering the structural integrity of synapses [193].

Peripherin, a subunit of peripheral nerve NFs, is almost exclusively expressed in the neurons of PNS and in the CNS only in defined subsets of neurons directly projecting to the periphery [25, 189]. However, brain injuries such as stab lesions and focal ischemia can trigger peripherin expression in CNS neurons normally silent for this gene [15]. Although definitive evidence for the presence of peripherin in synapses in the CNS is lacking, electrophysiological studies revealed synaptic plasticity was markedly altered in transgenic mice over-expressing the normal peripherin gene under its own promoter [101]. LTP was 50% greater in CA3 and 50% diminished in CA1 of hippocampus of the mutant mice than in wild-type controls. These data indicate a role for peripherin as a mediator of plasticity of hippocampal synapses potentially relevant to cognitive diseases. Peripherin was recently shown to be a strong binding partner of Rab-7A mutations of which are capable of altering the ratio of soluble and insoluble peripherin in vitro and are causative of CMT2B [42], indicating that peripherin may also have a role in vesicular transport. More recent studies involving yeast two-hybrid screens established interactions of peripherin and SNAP25-interacting protein SIP30 through coiled-coil domains [71] further suggesting a potential important role of peripherin in regulating vesicular trafficking at the synapses.

3. Alterations of NF proteins in psychiatric disorders

3.1. Schizophrenia and bipolar disorder

Schizophrenia, a severe chronic brain disorder affecting ~1% of the population [136], is characterized by abnormal interpretation of reality and social behavior. The behavioral syndrome reflects a combination of positive symptoms (eg. hallucinations, delusions, and disordered thinking and behavior), negative symptoms (eg. reduced expression, feelings, and speech production), and cognitive deficits (eg. poor executive functioning, working memory, and attention). The causes of schizophrenia are unknown. Various alterations of neuronal and glial functions have been reported although loss of neurons or neurodegenerative change is not considered fundamental to disease development. Postmortem brains from individuals with schizophrenia are usually characterized by reduced dendritic spine density [70, 73, 165].

Pharmacological and biochemical evidence supports an important role of NMDA receptor hypofunction in the pathophysiology of schizophrenia [11, 44]. Many genetic risk factors for schizophrenia have been reported, including inherited common single nucleotide polymorphisms, copy number variants, rare single nucleotide variants and rare de novo variants. *De novo* genomic copy number variants known to substantially increase susceptibility to schizophrenia are enriched for members of the NMDA receptor postsynaptic signaling complex and are significantly more frequent in individuals with schizophrenia than in controls [96]. Fromer et al. confirmed these findings and further

implicated abnormalities of synaptic cytoskeleton regulation and glutamatergic neurotransmission [67]. The fact that NFL, NFM and NFH genes map to chromosomal regions (8p21, 8p22 and 22q12, respectively) that are strongly implicated in schizophrenia suggests the involvement of NF proteins in this disease [8, 106]. As earlier noted, NFL in synapses [193] binds the NR1 receptor and may stabilize its presence in the cell membrane by preventing its ubiquitination and subsequent degradation [56, 144]. Besides binding NR1 to influence NMDA receptor function, NFL has also been shown to interact in a phosphorylation-dependent manner with 14-3-3 gamma [115], another protein implicated in schizophrenia [117, 171].

Of further potential relevance to NMDA receptor dysfunction in schizophrenia is a consistent body of evidence that points to selectively reduced levels of NFL subunits in brain regions essential for the cognitive and behavior functions affected in schizophrenia [100]. In dorsolateral prefrontal cortex (DLPFC), NFL transcript expression is significantly increased across all cortical isodense layers in DLPFC from elderly individuals with schizophrenia (mean patient age 80y) while levels of NFL protein are decreased about 50%, collectively suggesting possibly that turnover of NFL is abnormally high. Proteomic analysis of the DLPFC in schizophrenia revealed synaptic and metabolic abnormalities, including significantly reduced NFL (1.5-fold) and NFM levels (1.2-fold) without changes in NFH and alpha-internexin proteins [135]. Reduced NFL and NFM levels were also found in study of DLPFC deep white matter in schizophrenia (English et al.) and in the ACC (anterior cingulate cortex) white matter [39] where a 3-fold increase ($p<0.05$) in alpha-internexin was also measured. Similar changes in NFL and alpha-internexin levels were seen in another proteomic analysis of schizophrenic corpus callosum [157]. Although NFL transcription was significantly decreased in the thalamus of younger adult schizophrenics (mean age 43y), it was increased 25%-30% relative to controls, along with NFM levels, in elderly schizophrenics (mean age 70y) [41]. Collectively, these results implicate regional abnormalities of NFL transcription and /or accelerated NFL degradation in schizophrenia. NFL protein has been shown to be more vulnerable than NFM and NFH subunits to calcium-dependent proteases [199]. The changes observed in NFs may be part of the dynamic cytoskeletal remodeling in the pathogenesis of schizophrenia. Dramatic changes in the levels of NF proteins were also observed in axotomized motor and sensory neurons [84, 169, 183]. In light of evidence linking NFL to the functional activity of the NMDA receptor, the lowered levels of NFL could be a potential contributor to the NMDA hypofunction proposed to underlie various phenotypic features of schizophrenia.

Bipolar disorder is a brain disease that causes extreme mood swings that include emotional highs and lows. The exact cause of bipolar is unknown. Imaging and cellular studies have identified dysfunction of the dorsolateral prefrontal cortex in bipolar disease [54, 140, 162]. NFL transcripts are reported significantly reduced, along with NFM and alpha-internexin proteins [135], in adults with bipolar disorder (mean age 42y), but not in major depressive disorder [41]. Examining the differential protein expression in the deep white matter from DLPFC from individuals with bipolar disorder and controls, English et al. identified differences in 15% of proteins classified as cytoskeletal proteins which included increased NFM (1.66 fold, $p= 0.03$)[58].

Given that most studies of psychiatric disorders focusing on cytoskeletal changes analyzed brain areas enriched in white matter axons and the alterations of NF proteins may well reflect disturbed function of the axonal compartment. Given the ubiquity and abundance of synapses in CNS, it is also possible that even white matter analyses capture alterations of the significant synaptic NF protein pool in neuropsychiatric disorders. It is also reasonable to expect that future deeper analyses that include terminal regions of these same vulnerable brain regions and target additional relevant grey matter regions enriched with synaptic boutons, may identify dysfunction of the synaptic cytoskeletal network, including NF subunit interactions, more sensitively.

3.2. Drug addiction

Drug addiction is a chronic brain disorder that causes compulsive drug seeking and use despite adverse consequences. The mesolimbic pathway, which connects the ventral tegmental area (VTA) to the nucleus accumbens, has been implicated in common mechanisms of drug addition [139]. The mesolimbic pathway releases dopamine into the nucleus accumbens, where it affects motivation for rewarding stimuli. Drugs abused by humans such as morphine, cocaine, ethanol and nicotine have very different chemical structures but all preferentially increase synaptic dopamine concentrations in the mesolimbic system [53]. This is believed to be the neural substrate mediating the reinforcing properties of drugs of abuse. Given their diverse primary sites of action on cell surface receptors, these addictive drugs have to act indirectly on intracellular proteins as a convergence point to exert similar effects on mesolimbic dopamine function. Beitner-Johnson et al. first reported that NF proteins NFL, NFM and NFH were decreased 15–50% in the VTA by chronic administration of morphine or cocaine in rats [16]. The lowering of NF protein levels by these drugs was selective since the levels of 8 other major cytoskeletal or cytoskeletal-associated proteins did not change. Chronic exposure to psychotropic drugs lacking reinforcing properties did not alter NF levels. In separate studies, morphine administration caused a decrease of NFL immunoreactivity in rat cerebral cortex, which was antagonized completely by concurrent administration of naloxone [22]. Similar antagonism was achieved in three separate knockout mice of opioid receptors (mu or delta or kappa) [68], implicating all opioid receptor subtypes in these effects of morphine. Chronic morphine and cocaine also increased phosphorylation of NFH and NFM tail domains, an effect ablated by knockout of cortical mu-opioid receptors [68]. Interestingly, chronic cocaine, but not chronic morphine, lowered alpha-internexin levels in the VTA, hinting at somewhat different mechanisms at play for these two drugs, which are known to act at different receptors [16].

Rats inbred for alcohol preference expressed lower levels of NF subunits, suggesting that NF proteins in the VTA may influence preference for drugs [74, 75]. Examining its influence on NF proteins, GFAP and NMDA receptors, Ortiz et al. reported that chronic ethanol treatment significantly decreased NF protein levels in VTA (by 14%-37%) while increasing GFAP (by 40%) and NMDA NR1 subunit (by 27%) [131]. Similar to other drugs of abuse, nicotine activates the mesolimbic pathway by increasing cell firing of dopaminergic neurons in the VTA via nicotinic receptors [113]. Nicotine microinfusions into the VTA which resulted in sensitization of dopamine release [10], also reduced NFL (by 34%, $p<0.05$), NFM (by 42%, $p<0.01$), and NFH levels (by 38%, $p<0.05$) in the VTA but not in the substantia nigra in rats

[28, 150] without altering neuron numbers. The similar reductions of NF proteins in the VTA by the chronic treatment of different drugs of abuse with reinforcing properties suggest that common mechanisms underlie these addictive states.

Other brain targets, such as frontal cortex [29, 156], also show lowered NFL levels (47%, $p < 0.001$) in brains of addicts dying from opiate overdose [69]. Similar NFL reductions (49%, $p<0.001$) are also seen in the frontal cortex of rats after chronic morphine treatment. Narayana et al. reported immunohistochemical evidence for a significant decrease in NFH expression in the fimbria and internal capsule of rats after chronic cocaine administration [123]. Analysis of NF phosphorylation in the prefrontal cortex of human opioid addicts uncovered significantly reduced nonphosphorylated forms of NFH, NFM, and NFL and corresponding increases in phosphorylated forms [61, 62]. Similar changes are seen in brain regions of rats chronically treated with cocaine [109]. Acute morphine exposure also induces a marked increase in phosphorylated NFH whereas chronic morphine administration followed by opiate withdrawal results in a time-dependent decline in phosphorylated NFH [32, 91]. A recent study found chronic morphine causes persistent nitration of NFL in cortex and subcortex in mice even during abstinence [132], although its significance remains unclear.

Since PP2A has been shown to be the most effective in the nervous system in dephosphorylating the sites in NFH known to be phosphorylated by cdk5 [177], this phosphatase together with cdk5 were also examined. Surprisingly, the levels of PP2A were found unchanged while the levels of cdk5 and its neuron-specific activator p35 in the prefrontal cortex of human opioid addicts were decreased by 18% and 26–44%, respectively [62]. In these brains, phosphorylated NFH significantly correlated with p35 but not with cdk5. Further studies showed that acute treatment of rats with morphine increased the density of cdk5 by 35% in the cerebral cortex. In contrast to the acute effects, chronic morphine induced a marked decrease in cdk5 by 40% and p35 by 47% in rat brain [62]. It therefore appears that, despite the possible stimulating effect of the deadly opiate overdose on cdk5, the reduced expression of the cdk5/p35 complex in brains of opioid addicts is the net result of a chronic opiate effect. These results indicate that opioid addiction is associated with down regulation of cdk5/p35 levels in the brain and the hyperphosphorylation of NFH is not the result of a reduced dephosphorylation process. Although the abundance of cdk5 was decreased in the brains from chronic opioid abusers, Narita et al. showed that the level of phosphorylated cdk5 (serine-159) immunoreactivity in the frontal cortex was significantly increased by chronic morphine treatment [124] and phosphorylation of cdk5 at serine-159 dramatically increases cdk5 activation [155]. They also demonstrated that the treatment of selective cdk5 inhibitor, roscovitine and a half deletion of the cdk5 gene caused a significant inhibition of the rewarding effect of morphine. In addition, chronic cocaine or morphine administration also activates extracellular signal-regulated kinase (ERK), a known NF protein kinase [176], by promoting its phosphorylation [18, 174]. Altogether, these data indicate the hyperphosphorylation of NF proteins under these conditions may be related to the up-regulation of phosphorylated cdk5 and increased ERK activity.

D1R and NMDA NR1 receptors are assembled as an oligomeric complex and are functionally interdependent [63, 105]. Both receptors bind to NF proteins [56, 94] and are

also involved in drug addiction [43, 173]. Cocaine has been shown to reduce the D1R-NR1 physical interaction and may influence intracellular signaling [164]. On the one hand, D1R is a primary target of stimulant drugs and chronic exposure to drugs of abuse lower levels of NF proteins and alter their phosphorylation state [16]. On the other hand, NMDA receptor inhibition increases the phosphorylation state and the solubility of NFM protein [64]. Glutamate has been shown to inhibit NF transport, which can be reversed by NMDA receptor blocker MK-801 [1]. Over-activation of primarily NMDA receptors by glutamate leads to rapid disruption of NF proteins in cultured neurons [37]. The basis for these NF changes and their relationship to synaptic function remain elusive. As discussed above, our recent studies demonstrated that deletion of NFM in mice amplifies D1R–mediated motor responses to cocaine while redistributing postsynaptic D1R from endosomes to the plasma membrane, indicating a role of this NF subunit in drug addiction [193].

4. Alterations of NF proteins in Alzheimer's disease

Alzheimer's disease is an irreversible, degenerative brain disorder that progressively destroys memory and other mental functions. Early striking loss of synaptic connections within brain regions involved in memory and thinking skills is followed by loss of neurons of many types. Accompanying neurodegenerative changes is the development of defining neuropathological hallmarks of AD, neuritic plaques, and neurofibrillary tangles, along with extracellular deposition of β-amyloid. The causes of the most common late-onset forms of AD (more than 95% of all cases) are not fully understood: the much less common early-onset familial forms (less than 5% of all cases) are caused by mutations of either of three genes, amyloid precursor protein (APP) and presenilins 1 and 2(PSEN1 and PSEN2) [76, 175]. These causative genes and other risk factors influence the production and clearance of APP metabolites that mediate APP signaling cascades and may also have cytotoxic properties.

An important feature of AD is the disruption of the neuronal cytoskeleton leading to formation of neurofibrillary tangles (NFTs) [27, 102] numbers of which correlate well with neurodegeneration and severity of cognitive decline in AD [5, 19, 23]. The main constituents of tangles are paired helical filaments (PHF), initially believed to be composed of NF proteins [4, 47, 88] and later shown to be mainly fibrillar aggregates of tau protein [99, 103, 128]. Although NF proteins were somewhat ignored as tangle constituents, data has amply confirmed that NF proteins are integral components of the NFTs [138]. Most recently, mass spectrometry analysis by Pant and colleagues confirmed the presence of NFM and NFH in purified NFTs from AD brain independently of antibody cross-reaction between NF protein and tau [147]. The NFTs are far more complex than that of tau - PHFs. They also contain abundant NF proteins, vimentin, phosphorylated MAP1, phosphorylated MAP2, nonphosphorylated MAP1B, nonphosphorylated MAP2, nonphosphorylated MAP4 and nonphosphorylated MAP6. The question of whether the formation of tau-PHF is the initiating event in NFT formation has not been thoroughly addressed and the possibility that another NFT constituent, like NF protein, may initiate the process has not been excluded. In this regard, Morrison and colleagues reported that, certain populations of cortico-cortical pyramidal neurons that contain abundant perikaryal NF in relatively low states of phosphorylation are highly vulnerable to neurodegeneration through NFT formation [82, 83,

121, 178, 179]. More recent studies have provided independent evidence for loss of SMI32 immunopositive neurons in temporal cortical areas of AD brain [170]. The loss of SMI32 immunoreactivity on cortical regions of AD brain is often paralleled by increase in NFTs and AT8-positive tau immunoreactivity in neurons [121], indicating nonphosphorylated NF proteins may play a protective role in preventing the formation of NFTs. In fact, it is suggested that the development of neurofibrillary pathology may begin with abnormal NF accumulations in damaged distal processes followed by reactive perikaryal cytoskeletal changes that ultimately lead to tau hyperphosphorylation and NFT formation [178]. The co-localization of caspase-3 cleaved spectrin with nonphosphorylated NF-immunoreactive neurons in AD suggests that caspase-3 activation may be a pathological event responsible for the loss of these vulnerable neurons [6].

NFL mRNA is significantly reduced in AD cortex [38, 114] [97] and CA1 and CA2 regions of hippocampus [159]. Like NFL, NFM mRNA was also significantly decreased while NFH mRNA was unaltered in AD temporal cortex as compared to controls [97]. Also significantly increased in AD are NFH (by 1.7-fold), NFM (by 1.5-fold) and NFL proteins (by 1.6-fold) and the degree of phosphorylation of NFH (by 1.6–2.7-fold) and NFM (by 1.3–1.9-fold) as compared to Huntington disease [182]. Proteomic studies revealed increases of alpha-internexin and NFM proteins in the postsynaptic densities isolated from AD cortex as compared to controls with the increase of alpha-internexin being validated by Western blots [197]. Using quantitative phosphoproteomic methodology, Pant and colleagues recently reported 13 hyperphosphorylated sites in the C-terminal domain of NFM and 10 hyperphosphorylated sites of that of NFH in the frontal cortex of AD brain and all of these sites are in a higher state of phosphorylation in AD (4–8-fold higher) compared with control brains [146]. Surprisingly, the abundance and number of KSP repeat hyperphosphorylation of NFM are significantly higher compared with NFH, even though potential phosphorylation sites in NFH are more numerous. Based on the NF stoichiometry [4:2:2:1 (NFL:alpha-internexin:NFM:NFH)] [187], there are 2-fold more KSP repeat sites hyperphosphorylated in NFM compared with NFH, indicating that NFM might contribute more to aberrant NF phosphorylation in AD compared with NFH.

Additional abnormalities of NF have also been observed in AD. Significantly decreased O-GlcNAcylation of NFM was observed in AD cortex as compared to controls [51] while carbonyl-related posttranslational modification of NFH protein was also present in the AD NFTs [158]. Chapman et al. showed that sera of AD patients contain antibodies that bind specifically to NFH of Torpedo cholinergic neurons [35]. AD patients also have elevated intrathecal synthesis of anti-NFH antibody [12]. Behavioral tests revealed that rats immunized with cholinergic NFH for 12 month performed significantly worse than controls in in T-maze alternation test [34]. This prolonged immunization of rats with NFH results in cognitive impairment, IgG accumulation in the septum, hippocampus and entorhinal cortex with marked loss of neurons in the septum [130]. Interestingly, this cognitive impairment can be reversed by the acetylcholinesterase inhibitor physostigmine administered 30 min prior to testing [116]. These findings suggest that NFH may play a role in the pathogenesis of AD neurons.

Further support for NF protein involvement in AD pathogenesis comes from genetic studies. Deletion of NFL or NFH subunits in T44 mice overexpressing htau resulted in a dramatic decrease in the total number of tau-positive spheroids in the spinal cord and brainstem, indicating a role of NF proteins in the pathogenesis of neurofibrillary tau pathology [89]. Deletion of NFL in APP/PS1 transgenic mice, unexpectedly, increased neocortical β-amyloid deposition, synapse vulnerability and microgliosis surrounding β-amyloid plaques, suggesting a protective role of this NF subunit in AD [60].

The abundance of NFs in axons and the frequency of axonal injury in neurodegenerative disease has led to the use of NF protein measurements as a clinical index of brain injury in various neurological disorders. Higher than normal levels of NFL and NFH in CSF have been reported in AD [24, 87] and correlate with disease severity in a recent study of frontotemporal dementia [153]. CSF NFL concentration is increased by the early clinical stage of AD, correlates with cognitive deterioration and structural changes over time [194] and may be a useful marker of disease progression in AD [7].

Although amyloid plaques and tau tangles are two hallmarks of Alzheimer's disease, synaptic loss is more robustly correlated than these pathological lesions with cognitive deficits [50, 167]. Synaptic loss is more pronounced in the hippocampus [152] than in temporal and frontal cortex [48] and disturbance of synaptic integrity occurs very early on in the process of AD [111]. Ultrastructural stereological studies on rapid postmortem autopsy samples showed an 18% synapse loss in the hippocampal CA1 region of MCI patients that progressed to a 55% synapse loss in mild AD [151]. Since oligomeric NF proteins are present in synapses where they interact with synaptic proteins, loss of synapses in early AD could affect the level of this synaptic NF pool and may thus contribute to the increased NF protein levels in CSF and blood [7]. Alterations of NF proteins in early AD could also reflect synaptic reorganization while changes of NF subunits in late AD may suggest reorganization of both synaptic and axonal pools.

5. Conclusion

The etiology of major neuropsychiatric disorders critically involves dysfunction of synapses and, in late-onset dementias, the ultimate loss of synapses early in the disease. Relatively little attention, however, has been paid to the dynamics of cytoskeletal proteins at synaptic terminals and particularly the possible synaptic involvement of NF proteins. Until recently, the functioning of NF protein assemblies within synapses has been unappreciated and the focus has been on their axonal roles (eg. radial growth) and disruption in axonopathies involving mainly neurons with long myelinated axons that contain abundant NFs. In the CNS, however, even axons in major fiber tracts like the corpus callosum change minimally in caliber in NF triple knockout mice nearly completely lacking all 4 CNS NF subunits. This finding underscores growing evidence that NF proteins in the CNS have important functions beyond caliber expansion. Emerging evidence shows that NF proteins are present and functional in synapses. The synaptic population of NF proteins is distinctive in phosphorylation state, likely present in unconventional stoichiometries and assembly forms including oligomeric structures, and possibly more plastic in exchanging subunits and altering form in response to signals. Further evidence that individual subunits differentially

modulate neurotransmission and behavior through interactions with specific neurotransmitter receptors heightens expectations that alterations of NF proteins could influence synaptic function in psychiatric disorders and dementias. Despite the very frequent occurrence of NF protein changes in multiple major psychiatric states, most of the analyses have been on white matter tracts and the changes seen, often involving levels of only one or two subunit, have been difficult to interpret mechanistically from a perspective of changes in the axonal NF cytoskeleton. The newly uncovered sizable population of synaptic NF proteins should encourage more directed attention to regional analyses that interrogate this synaptic NF protein pool and will potentially yield valuable new insights into the significance of NF subunits in neuropsychiatric disease.

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Highlights

- Distinctive assemblies of neurofilament subunits are integral components of synapses
- Synaptic neurofilament proteins are a sizable population in the CNS
- NF subunits modulate neurotransmission and behavior via interactions with receptors
- Alterations of NF subunits may contribute to neuropsychiatric dysfunction

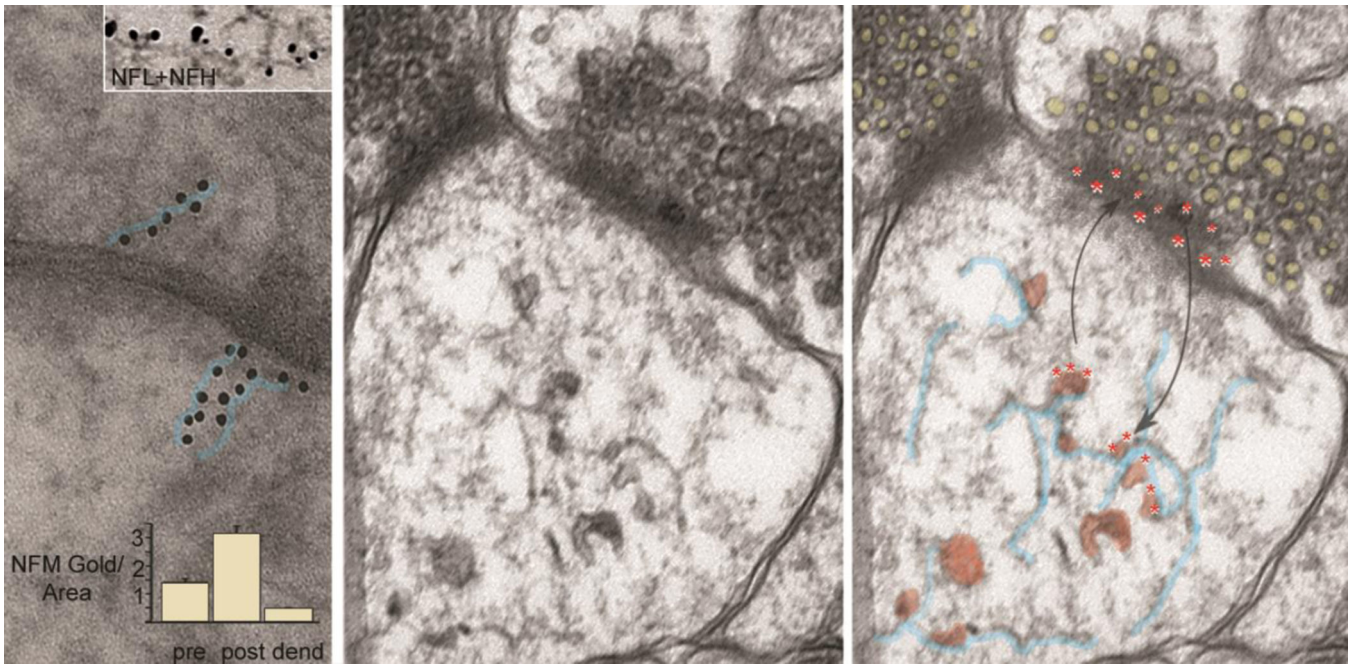

Figure 1. Functional NF subunit assemblies in synapses

Left panel: Immunogold labeled antibodies against the NFM subunit decorating synaptic structures in a linear pattern (immunogold particles outlined in blue) suggesting the presence of short NFs and protofilament /protofibril or unit length filament assemblies. In the upper inset, a filament within a postsynaptic bouton is decorated by immunogold antibodies to both NFL (large gold dots) and NFH (small gold dots). Morphometric analysis indicates a higher density of immunogold labeling in postsynaptic boutons than in preterminal dendrites or presynaptic terminals (graph inset). Middle panel: Ultrastructural image of a human synapse depicts membranous vesicles, many of which appear to be associated with a loose network of short 10nm filaments in the post-synaptic region. Right panel: Evidence supports a biological mechanism whereby D1 dopamine receptors internalized on endosomes from the postsynaptic surface (red asterisks) dock on synaptic NF subunit assemblies (outlined in blue) where they are readily available to recycle on endosomes to the surface in response to ligand stimulation (adapted from Yuan et al. Mol Psychiatry 2015; 20:915 with permission).

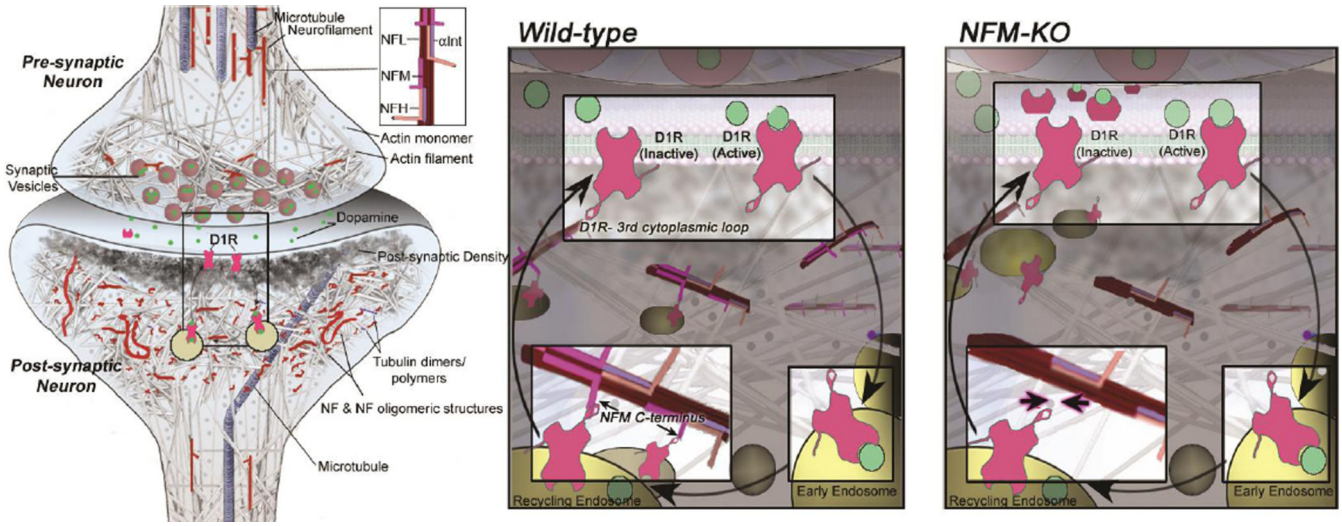

Figure 2. Model of D1R–containing endosomes anchored on NFM-containing cytoskeletal assemblies

Based on collective findings on NF scaffolding functions and our D1R data on NF subunit null mice, we propose a model by which NFM acts in synaptic terminals to anchor D1R–containing endosomes formed after agonist-induced internalization of membrane D1R. Retention of D1R in a readily available internal pool within the synapse would favor desensitization to D1R stimulation: in the absence of NFM, the greater recycling back to the plasma membrane surface would favor hypersensitivity to D1R agonists, as observed in our in *vivo* studies (adapted from Yuan et al. Mol Psychiatry 2016; 20:986–994 with permission).

Table 1

Neurofilament Subunit Expression in Human and Rodent Models of Psychiatric Disease

Disease	NF subunit (gene symbol)	Model system	Regulation				
			Protein level	Phosphorylation	Brain region	Method	Reference
Schizophrenia	NEFL	Human	Decrease		DLPFC	WB	[100]
		Human	Decrease		ACC white matter	Proteomic	[39]
		Human	Decrease		CC	Proteomic	[157]
		Human	Decrease		DLPFC	Proteomic	[135]
		Human	Decrease		DLPFC white matter	Proteomic	[58]
	NEFM	Human	Decrease		CC	Proteomic	[157]
	NEFH	Human	Decrease		DLPFC white matter	Proteomic	[58]
	INA	Human	Increase		ACC white matter	Proteomic	[39]
		Human	Increase		CC	Proteomic	[157]
Bipolar disorder	NEFL	Human	Decrease		DLPFC	Prpteomic	[135]
		Human	Decrease		DLPFC white matter	Proteomic	[58]
	NEFM	Human	Increase		DLPFC	Proteomic	[135]
		Human	Increase		DLPFC white matter		[58]
	NEFH	Human	Decrease		DLPFC white matter	Proteomic	[58]
	INA	Human	Decrease		DLPFC	Proteomic	[135]
Drug addiction	NEFL	Rat	Decrease		VTA	WB	[16]

Disease	NF subunit (gene symbol)	Model system	Regulation		Brain region	Method	Reference
			Protein level	Phosphorylation			
		Rat	Decrease		VTA	IHC	[28]
		Human	Decrease		Frontal cortex	WB	[69]
		Neuronal culture	Decrease		Hippocampus	IHC	[149]
	NEFM	Rat	Decrease	Increase	VTA	WB	[16]
		Rat	Decrease		VTA	IHC	[28]
		Neuronal culture	Decrease		Hippocampus	IHC	[149]
	NEFH	Rat	Decrease	Increase	VTA	WB	[16]
		Rat		Decrease	VTA	IHC	[28]
		Neuronal culture	Decrease		Hippocampus	IHC	[149]
Alzheimer disease	NEFL	Human	Decrease		Occipital cortex	WB	[9]
	NEFM	Human	Decrease	Decrease	Sciatic nerve	WB	[85]
		Human		Increase	Hippocampus	IHC	[160]
		Human		Increase	Hippocampus	WB	[179]
		Human		Increase	Frontal cortex	WB, IHC & iTRAQ	[146]
	NEFH	Human	Decrease	Decrease	Sciatic nerve	WB	[85]
		Human		Increase	Hippocampus	IHC	[160]
		Human		Increase	Hippocampus	WB	[179]
		Human		Increase	Frontal cortex	WB, IHC & iTRAQ	[146]
		Human		Increase	Temporal cortex	IHC	[170]
	INA	Human	Increase		Cortex	Proteomic & WB	[197]

Note: DLPFC, dorsolateral prefrontal cortex; ACC, anterior cingulate cortex; CC, corpus callosum; WB, Western blotting; IHC, immunohistochemistry; iTRAQ, isobaric peptide tags for relative and absolute quantification;