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Proteomic Analysis of an α7 Nicotinic Acetylcholine Receptor Interactome

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Abstract

The α7 nicotinic acetylcholine receptor (nAChR) is well established as the principal high-affinity α-bungarotoxin-binding protein in the mammalian brain. We isolated carbachol-sensitive α-bungarotoxin-binding complexes from total mouse brain tissue by affinity immobilization followed by selective elution, and these proteins were fractionated by SDS-PAGE. The proteins in subdivided gel lane segments were tryptically digested, and the resulting peptides were analyzed by standard mass spectrometry. We identified 55 proteins in wild-type samples that were not present in comparable brain samples from α7 nAChR knockout mice that had been processed in a parallel fashion. Many of these 55 proteins are novel proteomic candidates for interaction partners of the α7 nAChR, and many are associated with multiple signaling pathways that may be implicated in α7 function in the central nervous system. The newly identified potential protein interactions, together with the general methodology that we introduce for α-bungarotoxin-binding protein complexes, form a new platform for many interesting follow-up studies aimed at elucidating the physiological role of neuronal α7 nAChRs.

Keywords

α-bungarotoxin; α7 nAChR; membrane protein; mass spectrometry; nicotinic receptor

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) comprise a diverse family of ligand-gated ion channels (LGICs) that are expressed throughout the vertebrate central and peripheral nervous systems. nAChRs are composed of five subunits that assemble symmetrically around an axis perpendicular to the plasma membrane.1 Each subunit consists of a large N-terminal ligand-binding extracellular domain and four transmembrane helices, M1 through M4.2 Twelve neuronal nAChR subunits have been identified (α2–α10 and β2–β4). Subunit composition determines the localization and the physiological and pharmacological properties of each receptor subtype.3 Neuronal nAChR subtypes may be classified into two major subclasses based on sensitivity to the nicotinic antagonist α-bungarotoxin (α-bgtx). The neurotoxin, α-bgtx, has been used extensively to identify, localize, and purify neuronal α-bgtx-sensitive nicotinic acetylcholine receptors with α7, α8, α9, and α10 subunits, and the skeletal muscle-type nAChR.4

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The α7 nAChR is the only known endogenous receptor in the central nervous system (CNS) that binds α-bgtx with high affinity.[5] α7 subunit-containing receptors are abundant in the mammalian hippocampus and the cerebral cortex, brain regions associated with learning and memory.6 Located pre-and peri-synaptically, these receptors can modulate neurotransmitter release, while post-synaptic α7 nAChRs can mediate excitatory currents and are involved in complex signal transduction as a consequence of the intrinsic channel's high permeability to calcium.5 Similarly calcium entry through α7 nAChRs has been proposed to affect the initiation of secondary messenger systems during neuronal development.7 Although most abundantly expressed in neuronal tissue, recent evidence suggests that the α7 nAChR is found in mammalian macrophages,[8] microglia,[9, 10] keratinocytes,[11] smooth muscle cells,[12] astrocytes,[13] endothelial cells,[14, 15] and lymphocytes.[16, 17]

Additionally, the α7 nAChR is thought to interact with proteins involved in trafficking and targeting,[18–20] scaffolding,[21, 22] as well as with kinases and other signaling proteins.[23, 24] The intracellular region coupling the third and fourth transmembrane helices of the α7 nAChR, the M3-M4 linker, is potentially a major site for intracellular protein interaction. The bulk of the intracellular domain of nAChRs is comprised of the M3-M4 linker, which displays substantial variability in both sequence and length among nAChRs. The M3-M4 linker region has been implicated in receptor assembly,[25] folding,[5, 6] and trafficking.[22, 26–29] Several potential binding partners of the α7 nAChR have been reported in studies using a variety of methods including immunolabeling,[22] co-immunoprecipitations,[23, 24] yeast two-hybrid assays,[18] and Western blotting.[20, 21] However, our study is the first large-scale proteomics-based analysis of the α7 nAChR interactome.

As is well documented, mass spectrometry is a highly effective methodology for elucidating protein-protein interaction networks.30, 31 Following enzymatic digestion, peptides are fractionated and subjected to nano-electrospray ionization (nano-ESI). Mass measurement of the peptide ions followed by further fragmentation analysis allows for the determination of the primary sequences of the peptides, ultimately resulting in protein identification.32 We utilized mass spectrometry as a high-throughput method for identifying proteins that remain associated with the α7 nAChR following detergent solubilization from native neuronal tissue. Using α-bgtx-conjugated beads and specific competitive elution with carbachol (a low molecular weight cholinergic agonist of the α7 nAChR), we isolated protein complexes from murine brain membranes solubilized with the detergent, Triton X-100. Brain tissue from α7 nAChR knockout mice, lacking high-affinity binding sites, was used in our studies to control for proteins binding nonspecifically to the affinity bead matrix. The α7 nAChR knockout mice appear to be largely normal in development and in gross neurological function, although it has been observed that homozygotes may not breed reliably.33 Following SDS-PAGE fractionation and in-gel tryptic digestion of the selectively eluted binding proteins, the peptides were characterized by nano-ESI tandem mass spectrometry (MS/MS), and the SEQUEST algorithm was applied to the data analysis to determine the identity of the eluted proteins. Using these techniques, 55 potential members of the α7 nAChR interactome were identified reproducibly in at least two of the three independent mass spectrometric analyses performed as part of this study. Further validation by orthogonal methodologies of this preliminary list of proteins is necessary before these proteins can be deemed as *bona fide* interacting partners of the α7 nAChR. The α7 nAChR is thought to be involved in many cognitive and behavioral functions. Recent studies have shown a correlation between altered expression of the α7 nAChR and human cognitive disorders, such as epilepsy,[34, 35] schizophrenia,[36, 37] autism,[38, 39] and attention-deficit / hyperactivity disorder.[40, 41] Understanding the interplay of the α7 nAChR in various signaling pathways may have important implications in the diagnosis, treatment, and prevention of a variety of neuropsychiatric and neurodegenerative diseases.[42]

EXPERIMENTAL PROCEDURES

Materials

Trypsin Gold, mass spectrometry grade (V5280) was bought from Promega Corp. (Madison, WI). Triton X-100 (807426) was purchased from MP Biomedicals (Solon, OH). Complete, Mini protease inhibitor cocktail was purchased from Roche (Mannheim, Germany). Rabbit anti-α7 nAChR antibody (ab10096) was bought from Abcam (Cambridge, MA). Goat anti-α7 nAChR antibody (SC-1477) was from Santa Cruz Biotechnology (Santa Cruz, CA). CL-XPosure Film (34090) and Supersignal West Pico Chemiluminescence substrate (34080) were purchased from Pierce (Rockford, IL). BenchMark Pre-Stained (10748–010) and MagicMark XP (LC5602) protein standards were bought from Invitrogen (Carlsbad, CA). Strong cation exchange ZipTips (ZipTip$_{SCX}$, ZTSCXS096) were purchased from Millipore (Billerica, MA). Cyanogen bromide (CNBr) activated sepharose beads (C9142), Brilliant Blue G-Colloidal Coomassie stain (B2025), horse-radish peroxidase-conjugated goat anti-rabbit antibody (A9169), horse-radish peroxidase-conjugated rabbit anti-goat antibody (A5420), carbachol (C4382), α-bungarotoxin (T3019), and the remaining general chemicals used were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of ligand affinity beads

CNBr-activated sepharose 4B beads (1.5 g) were hydrated in 5 ml of 1 mM HCl for 30 minutes and washed on a coarse glass filter with 500 ml of 1 mM HCl. The beads were added to 7.5 ml of coupling buffer (0.25 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and centrifuged for 5 minutes at 1,500 × g. The beads were pelleted and resuspended in 15 ml of coupling buffer containing 3 mg of α-bgtx prior to gentle rotation overnight at 4°C. The beads were then pelleted, resuspended in 15 ml of 0.2 M glycine in 80% coupling buffer and gently rotated overnight at 4°C. This mixture was washed on a fritted coarse glass filter, first with 100 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0, next with 100 ml of 0.1 M $NaCH_3CO_2$, 0.5 M NaCl, pH 4.0, then again with 100 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0, followed by a wash in coupling buffer. The beads were finally washed twice with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.6), and resuspended in TBS supplemented with 0.1% Triton X-100 and 0.02% sodium azide.

Membrane protein solubilization

Frozen whole mouse brains of wild-type or α7 nAChR knockout mice (C57 strain genetic background) were thawed and homogenized in ice-cold TBSp (TBS supplemented with protease inhibitors) with 20–25 strokes of a Potter-Elvehjem glass homogenizer. One milliliter of buffer was added per 400 mg of mouse brain tissue. The homogenate was ultracentrifuged at 100,000 × g for 60 minutes at 4°C. The pellet (containing the cell membrane fraction) was reconstituted in an ice-cold TBSp plus 1% Triton X-100 solution. The resuspended pellet was homogenized with 20–25 strokes of a Potter-Elvehjem glass homogenizer and incubated on ice with gentle agitation for 2 hours. Detergent-solubilized proteins were separated from the insoluble proteins and cellular debris by ultracentrifugation at 100,000 × g for 60 minutes at 4°C.

α-Bgtx complex isolation

Detergent-solubilized proteins were incubated with 100 μl of α-bungarotoxin-conjugated sepharose bead slurry overnight at 4°C with gentle rotation. Following the incubation, the beads were centrifuged at 2,000 × g for 5 minutes and unbound proteins were removed. The beads were washed three times with 1.5 ml of ice-cold TBSp plus 1% Triton X-100 to reduce nonspecific binding. The α7 nAChR and associated proteins were selectively eluted by incubation with 50 μl of a 1 M carbamylcholine chloride (carbachol) solution, and

subsequently precipitated with acetone. In brief, four times the sample volume of cold (−20°C) 100% acetone was added to each sample, which was mixed by vortexing and then incubated for 60 minutes at −20°C. The samples were centrifuged for 10 minutes at 13,000 × g, after which the supernatants were aspirated and the pellets were air-dried.

Sample preparation, SDS-PAGE, and Western blotting

Acetone-precipitated samples were resuspended in 10 μl of SDS-loading buffer (100 mM Tris-HCl, pH 8, 2% SDS, 100 mM DTT, 20% glycerol, 0.002% bromophenol blue) and incubated at 55°C for 30 minutes. One microliter of 1 M iodoacetamide was added to the samples which were then incubated at room temperature in darkness for 30 minutes. Samples were loaded onto a 15% polyacrylamide gel and SDS-PAGE was performed at 125 V for approximately 45 minutes. Proteins were visualized by colloidal Coomassie staining following the manufacturer's protocol.

Western blotting confirmed that the α7 subunit was captured by the α-bgtx-conjugated sepharose bead procedure. Proteins were transferred from polyacrylamide gels onto a nitrocellulose membrane at 100 V for 90 minutes at 4°C in electroblotting buffer (25 mM Tris-HCl, 0.2 M glycine, 20% methanol). The membrane was blocked for 1 hour at room temperature with 5% nonfat dry milk dissolved in TBS-T (TBS supplemented with 0.1% Tween-20). The membrane was then incubated with 10 ml of a 1:500 dilution of anti-α7 nAChR antibody in TBS at room temperature for 2 hours. After two 5-minute washes in both TBS-T and TBS, a 1:20,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody in TBS was added to the membrane, which was incubated for 1 hour at room temperature. After another set of washes in TBS-T and TBS, the membrane was incubated with the chemiluminescence substrate for 5 minutes following the manufacturer's protocol. Finally, the membrane was exposed to X-ray film for 15–30 seconds and the film was developed.

In-gel digestion

Gel slices were excised from the Coomassie-stained polyacrylamide gel and washed three times with 200 μl of 50% acetonitrile, 50% 50 mM ammonium bicarbonate solution for 30 minutes at 37 °C. Gel slices were then dehydrated by adding 200 μl of neat acetonitrile and incubated at room temperature for 15 minutes. After removing the acetonitrile and drying for 30 minutes at 37 °C, the gel slices were rehydrated with the addition of 10 μl of 25 mM ammonium bicarbonate, pH 8.0 containing 100 ng of trypsin. Following a 30-minute room temperature incubation, an additional 50 μl of 25 mM ammonium bicarbonate was added to completely immerse the gel slices in buffer. After an overnight incubation at 37°C, the peptides were extracted with 40 μl of 0.1% TFA (trifluoroacetic acid) in increasing concentrations of acetonitrile (0%, 25%, 50%, and 75%). These fractions were combined in a single low-retention microcentrifuge tube and vacuum centrifuged to dryness. The peptides were desalted with a $ZipTip_{SCX}$ prior to analysis by mass spectrometry following the manufacturer's instructions. Briefly, the lyophilized peptides were resuspended in 10 μl of 0.1% TFA and the $ZipTip_{SCX}$ resin was equilibrated with 0.1% TFA. The peptides were bound to the ZipTip resin by trituration and washed in 0.1% TFA, 30% methanol. Finally, the peptides were eluted with 5 μl of 5% ammonium hydroxide, 30% methanol and the sample was vacuum centrifuged to dryness.

Mass spectrometry

One of the data sets (Data Set A) was obtained using an LTQ-Orbitrap at the Taplin Biological Mass Spectrometry Facility at Harvard Medical School (Boston, MA). The samples were reconstituted in 10 μl of HPLC solvent A (2.5% acetonitrile, 0.1% formic acid). A nano-scale reversed-phase HPLC capillary was prepared with 5 μm C_{18} spherical

silica beads packed into a fused silica capillary (100 μm inner diameter and approximately 12 cm in length) with a flame-drawn tip. After equilibrating the column, the sample was loaded via a Famos autosampler (LC Packings, San Francisco, CA). A gradient was formed and peptides were eluted with increasing concentrations of solvent B (97.5% acetonitrile, 0.1% formic acid). The eluted peptides were subjected to nano-ESI and the resulting ions were detected, isolated, and fragmented to produce MS/MS spectra of specific fragment ions using an LTQ Orbitrap mass spectrometer (ThermoFinnigan, San Jose, CA). The MS/MS spectra were automatically searched against the mouse NCBI non-redundant protein database (November 2007) using the SEQUEST algorithm.[43]

The other two data sets (Data Sets B and C) were obtained using an LTQ mass spectrometer at the NSF/EPSCoR Proteomics Facility at Brown University (Providence, RI). The peptides were reconstituted in 20 μl of 0.1 M acetic acid. A nano-scale reversed-phase HPLC capillary was prepared with 5 μm Monitor C_{18} spherical silica beads packed into a fused silica capillary (75 μm inner diameter and approximately 12 cm in length) with an integrated ESI emitter tip having a 4 μm opening and fritted with 3 μm silica particles (Bangs Labs, Fishers, IN). A gradient was formed and peptides were eluted with increasing concentrations of solvent B (90% acetonitrile, 0.1 M acetic acid). The eluted peptides were then scanned by an LTQ mass spectrometer (data-dependant scanning with 1 MS scan followed by 5 MS/MS scans). MS/MS spectra were generated and automatically searched against the mouse NCBI non-redundant protein database using the SEQUEST algorithm provided with BioWorks 3.2 SR (ThermoFinnigan, San Jose, CA). The list of predicted proteins was imported into a custom-made FileMaker Pro (FileMaker, Santa Clara, CA) relational database for data analysis.

Each data set (A through C) represents a different preparation of mouse brain tissue. The murine brain tissue samples were processed and in-gel digestions were preformed in parallel for Data Sets A and B. Data Set C samples were prepared several weeks later using identical protocols.

Data processing

Peak list files were created by the program “extract_msn.exe” that was installed with BioWorks version 3.2 SR using the following parameters: The mass must fall within the range of 600 to 4500 Daltons and the minimum total ion current for the scan must exceed 1000. The precursor tolerance for grouping was 1.5 Da, with no differing intermediate scans allowed and only a single scan required to create a peak file. The minimum signal-to-noise for a peak to be written to the peak file was 3, and 25 such peaks must be found for a peak file to be created. The program calculated charge states, however, in cases of ambiguity, peak files for both the +2 and +3 charge states were created. No smoothing or de-isotoping was performed. The precursor-ion tolerance was 2.0 Da and the fragment-ion tolerance was 0.8 Da. The modifications allowed were iodoacetamide alkylation of cysteine and oxidation of methionine. Enzymatic digestion was specified as trypsin, with up to 2 missed cleavages allowed. Searches were performed using the NCBI non-redundant mouse database (November, 2007). A list of reversed-sequences was created from these entries and appended to the original database for searching so that false positive rates could be estimated. Final false positive rates were estimated at approximately 1%. The mass tolerance parameter cut-offs were as follows: Peptides with charge of +1, +2 and +3 must have Xcorr values that are greater than 1.5, 2.0 and 2.5, respectively.

RESULTS

Affinity sedimentations, immunoblotting, and Coomassie staining

To achieve greater specificity, we used the nicotinic agonist, carbachol, to elute from α-bgtx-conjugated affinity beads those complexes bound in a manner consistent with the nicotinic pharmacology of the α7 nAChR. Carbachol eluants of α-bgtx bead isolations from wild-type and α7 knockout mouse brain tissue were analyzed on SDS-PAGE. Western blotting performed with antibodies against the α7 nAChR (Figure 1A) verified that the α7 receptor was successfully sedimented with the α-bgtx beads and released upon carbachol incubation. In these Western blots, no band was observed in the lane of samples originating from the α7 nAChR knockout mouse, confirming that the α7 nAChR is not present in the control mouse samples.

Affinity-isolated proteins were visualized on polyacrylamide gels stained with colloidal Coomassie blue. The protein staining showed multiple bands from the α-bgtx affinity sedimentations of wild-type samples (Figure 1B). These protein bands confirm the isolation of a diverse array of candidate interacting partners of the α7 nAChR. Protein bands in the sample lanes from the knockout mouse are most likely due to nonspecific binding, or could be novel non-α7 α-bgtx-binding proteins. Notably, protein bands from the wild-type mouse samples were significantly more intense (greater than two-fold) than those from the knockout mouse samples, although identical masses of brain tissue were homogenized in both cases. In our preliminary studies, we targeted specific protein bands for detailed MALDI MS/MS analysis which readily identified the α7 subunit at the correct molecular weight in the wild-type gel lane (Figure 1B, arrow). A similar MALDI analysis of the corresponding gel region in the α7 nAChR knockout mouse lane did not detect any α7 protein. In addition, we identified the most intensely stained band in the knockout sample lane as the highly abundant protein, tubulin.

Mass spectrometric analysis of α-bgtx affinity-isolated proteins

For a more thorough analysis of the proteins isolated by our methodology, SDS-PAGE gel lanes of α-bgtx isolations from wild-type and knockout mice were divided into 7 slices (Figure 1B) corresponding to the molecular weights: (1) 190–120 kDa, (2) 120–85 kDa, (3) 85–60 kDa, (4) 60–40 kDa, (5) 40–25 kDa, (6) 25–20 kDa, and (7) 20–15 kDa. Tryptic peptides prepared from each gel slice were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) prior to nano-ESI mass spectrometric analysis. Separation at the peptide level resulted in the identification of hundreds of proteins from a single gel slice. The use of $ZipTip_{SCX}$ sample preparation pipette tips also improved spectra quality by removing salts and residual detergent from the eluted peptides.

Proteins identified from wild-type mouse brain tissue, but absent in corresponding isolations from α7 nAChR knockout mice, and which also appear in at least two of the three independent data sets are listed in Table 1. These proteins were broadly categorized by their cellular functions by means of gene ontology queries.[44] Exclusively from wild-type mouse brain tissue, we identified 22 proteins that provide structural support and/or play a role in trafficking, 19 potentially interacting proteins involved in signal transduction, 7 proteins related to basic metabolism, 5 chaperone proteins, 2 proteins associated with proteolysis, for a total of 55 proteins. Supplementary Table 1 lists the identified peptides corresponding to the proteins listed in Table 1. An additional 153 proteins were identified in only one of the three acquired mass spectrometry data sets (Supplemental Table 2). In addition, a total of 129 proteins were identified by MS/MS as being represented in both the wild-type and the α7 nAChR knockout mouse samples (Supplemental Table 3). Proteins listed in Supplementary Table 2 and Table 3 were not included in our list of potential interacting

partners of the α7 nAChR. Further repetitions would be necessary to determine whether the proteins in Supplementary Table 2 are due to physiologically significant interactions with the network of proteins interacting with the α7 nAChR. Likewise, without further supporting data, the proteins listed in Supplemental Table 3 are most likely products of nonspecific binding, novel non-α7 nAChR α-bgtx-binding proteins, or false positive protein identifications resulting from the data analysis algorithm. Supplementary Table 4 lists additional information on the sub-cellular location and function of those candidate interacting proteins that are unique to the wild-type samples (i.e., those in Table 1).

DISCUSSION

The combination of affinity isolation and tandem mass spectrometry is a powerful method for revealing many potential interacting proteins associated directly or indirectly with a protein of interest. The isolation of a protein target is one of the major challenges of a proteomics experiment. Using antibodies coupled to a bead support for immunoprecipitations is a common technique for isolating proteins from their native environment. Antibodies, however, can lead to undesired sample complexity in mass spectrometry-based experiments. Anti-peptide antibodies against the α7 nAChR, for example, have been shown to cross-react with non-α7 nAChR proteins and are unreliable for use in many *in vivo* and *in situ* studies.[45, 46] In addition, antibodies binding to certain functionally relevant epitopes may perturb the conformation of the receptor and compromise important protein-protein interactions. Moreover, antibodies are not easily dissociated from their target, and an abundance of antibody protein in a sample may suppress the signal of peptides of interest.[47] The use of α-bgtx for affinity purification resolves many of these issues. In wild-type brain tissue, α-bgtx binds selectively to the α7 nAChR with high affinity, and brain sections from α7 receptor knockout mice show little evidence of α-bgtx binding to other sites.[33]

The interactions of various neuronal membrane receptors have been investigated via immunoprecipitations and/or affinity precipitations.48 These studies have both confirmed several previously suspected binding partners and yielded important information on novel interacting partners. Several such studies have investigated the post-synaptic density, which is a protein dense region at the post-synaptic plasma membrane of the neuronal synaptic cleft. Proteins in this region include glutamate receptors that are present at the major excitatory synapses in the mammalian CNS.[49–51] Recently, 698 proteins associated with post-synaptic terminals of the murine CNS have been identified via immunoprecipitations of the glutamate receptors, AMPAR (α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor) and NMDAR (N-methyl-D-aspartic acid receptor), as determined by LC-MS/MS.[52]

Our mass spectrometric analysis of proteins isolated by α-bgtx-affinity methods has identified several proteins that were among the 698 proteins listed by Collins, *et al.*[52] Twenty-six of the 55 proteins isolated using our α-bgtx binding strategy and identified only in the wild-type mouse sample are among the previously-described post-synaptic density proteins (listed in Table 2), including cell structure and trafficking proteins, molecular chaperones, and proteins involved in signal transduction. The α7 nAChR itself was not identified in the study by Collins, *et al.*, and, similarly, we also did not identify the NMDAR or the AMPAR subunits in our interactome study. These data suggest that α7 nAChRs are not directly complexed with glutamate receptors, although indirect interactions that are detergent sensitive remain a possibility.[53] It is certainly possible that the 26 proteins identified in common by both Collins, *et al.* and our study may interact with two different sets of receptor associated proteins, the α7 receptor interactome in one case, and the NMDAR/AMPAR post-synaptic density interactome in the other case. Further studies

would be needed to determine whether any of the overlapping 26 proteins are due to NMDAR/AMPAR protein complexes that include post-synaptic α7 nAChRs.

We also have identified potential α7 nAChR binding partners that may be associated with pre-synaptically localized α7 nAChRs, including the proteins, BASP1/NAP-22, tenascin-R, neuromodulin (GAP-43), and synaptogyrin-1. Of these identifications, BASP1/NAP-22 is particularly interesting as this protein binds cholesterol and has been associated with microdomains (lipid rafts) at axonal termini.[54, 55] Coincidently, several studies have suggested that α7 nAChRs may also be found in lipid rafts (as well as pre-synaptically), strengthening the possibility of an interaction between the two proteins.[23, 56, 57] The identification of both pre- and post-synaptic proteins in our proteomics study is not surprising considering that there exists much evidence that α7 nAChRs are associated with both presynaptic and postsynaptic membranes.[58, 59]

The intrinsic ion channel of the α7 nAChR is highly permeable to calcium ions, as has been established through electrophysiological recordings of heterologously-expressed receptors.[60, 61] In addition, the α7 nAChR has been implicated in calcium entry-dependent events, including the regulation of secondary messenger cascades and of neurotransmitter release.[13, 61] Phosphorylation of the receptor by protein kinase A (PKA) and/or protein kinase C (PKC), which play prominent roles in cellular regulation and signaling cascades, may also regulate and be regulated by α7 nAChR-mediated calcium modulation.[62–64] Interestingly, we have identified both PKA and PKC in our proteomics study as two of the 55 candidate interacting proteins present in samples prepared from wild-type mice, but absent in samples originating from α7 nAChR knockout mice (Table 1).

Calcium regulation by the α7 nAChR is thought to influence both neurite outgrowth and secondary messenger systems in the fetal and adult hippocampus.[65] The relatively high calcium permeability of the α7 nAChR may lead to the efficient co-localization of calcium-binding or calcium-dependent proteins in neurons. In the developing nervous system, an increase in calcium concentration in regions of neurite growth is directly associated with neurotransmitter release and neurite extension or retraction.[66] Terminal plasticity of neurites persists following synapse development.[67] In our interactome study, we identified several proteins that are known to be active in neurite outgrowth and maintenance, including alpha-catenin 2,[68] BASP1/NAP-22,[69] gelsolin,[70] MARCKS,[71] homer 1,[72] neuromodulin,[71] and tenascin C.[73] Many of the aforementioned proteins also interact with calmodulin, PKC, and protein phosphatase 2B, which have roles in the modulation of calcium-dependent processes and may indirectly affect the rate of elongation and branching of growing neurites. Tubulin, actin, and other cell structure proteins, such as fascin and tau, provide essential structural support to neurites.[74, 75] Moreover, actin has been shown, using confocal fluorescence microscopy and immunogold labeling in electron microscopic studies, to co-localize with the α7 nAChR.[22] In chick ciliary ganglion, the α7 nAChR has been shown to influence actin filament and/or microtubule assembly through calcium regulation.[76] Although actin and tubulin may interact with α7 nAChRs at some level in some tissues and regions, as has been suggested by the correlated presence of these proteins in neurite growth cones,[77] these two very abundant cytoskeletal proteins were eliminated from our list of potential interacting proteins because they were also found in samples originating from α7 nAChR knockout mice.

We have identified several proteins, as mentioned above, that are known to contribute to developmentally important regulatory mechanisms of the nervous system. Correspondingly, previous studies have shown that [^{125}I]-α-bgtx-binding is developmentally regulated in postnatal mice.[78] It would be a reasonable extension of our work to apply our α-bgtx-based isolation strategy to the identification of binding partners at various stages of postnatal

mouse development to search for novel developmental stage-specific α7 nAChR interactions. Of particular interest may be to further investigate the interaction of the α7 nAChR with chaperone proteins which we have identified as potential interacting partners. For example, 14-3-3η, a member of family of chaperone/ scaffolding/ adaptor proteins, has been linked with regulation of expression and subunit stabilization in the α4-subunit containing nAChR.[79] We have identified 14-3-3η, as well as14-3-3α/β and 14-3-3σ, exclusively in α-bgtx affinity isolations from adult wild-type mice (Table 1), but the characterization of their interaction with α7 nAChR in both a global and developmental context remains undefined. Another example of a protein that we identified that may also have a significant role in α7 nAChR-related nervous system development is protein disulfide-isomerase A3, which is a member of an enzyme family that catalyzes the rearrangement of disulfide bonds in proteins.[80] Although we present the first evidence that this enzyme may interact with the disulfide-bind containing α7 nAChR, there are reports in the literature of the function of protein disulfide-isomerases in developmental regulation.[81–83] These proteins, and others that we have identified, may have developmentally regulated roles for the localization and/or folding α7 nAChR. Further proteomic experiments at different mouse developmental stages could help clarify the roles and interactions of the α7 nAChR and its binding partners in the developing nervous system.

As is well known, the G protein (guanine nucleotide-binding protein) family functions in numerous important signal transduction and secondary messenger cascades. These signaling cascades influence embryonic development, learning and memory, as well as organismal homeostasis.[84] As is the case with α7 nAChRs, G proteins have been prominently implicated in neurite development 85 and in calcium-dependent signaling.86, 87 Several G proteins (Table 1) met the identification criteria of our mass spectrometric analysis of proteins enriched through α-bgtx affinity isolation. The likelihood of a physiologically important interaction between the α7 nAChR and G proteins is strengthened by the report of an association of the α7 nAChR with adenylyl cyclase. Adenylyl cyclase, an enzyme catalyzing the conversion of ATP to 3',5'-cyclic AMP (cAMP) and pyrophosphate, is known to be stimulated directly by G proteins.88 Similarly, immunoprecipitation and Western blotting data suggest that adenylyl cyclase interacts with the α7 nAChR23 Furthermore, although we did not identify the multi-transmembrane spanning protein adenylyl cyclase, we did identify, an adenylyl cyclase-associated protein, adenylyl cyclase-associated protein 2, as present only in those samples originating from wild-type mice. In addition, neuromodulin (GAP-43) is thought to bind G proteins and to stimulate binding of GTP to the G_o subunit.[89] In a related interaction, neuromodulin binds and inactivates calmodulin, which is then released in response to PKC activation.[90] Notably, neuromodulin, the G_o subunit, calmodulin, and PKC are all candidates for interacting partners with α7 nAChRs as they were identified only in samples originating from wild-type mice (Table 1).

Our results with respect to the G_o subunit differ from those of a previous study that failed to detect interaction between the α7 nAChR and the G_o subunit.[91] However, these researchers based their conclusions on Western blots of pull-downs performed using a GST-fusion protein of the α7 nAChR M3-M4 intracellular linker region. As a consequence of the prokaryotic expression of this GST-fusion protein, suboptimal folding, and/or deficiencies in post-translational modifications may not have permitted the association of the M3-M4 linker with all of its native binding partners in that study. Our data together with conclusions drawn from the available literature about common potential binding partners argue that associations between the α7 nAChR and G proteins are highly probable and clearly merit further investigation. For all proteins identified in our study, additional validation using independent methodologies is necessary to confirm that these proteins are *bona fide* interacting partners of the α7 nAChR *in vivo*.

CONCLUSION

We have successfully solubilized and affinity-isolated the α7 nAChR and associated proteins from murine brain tissue. Mass spectrometric analysis of in-gel, tryptically-digested peptides identified 55 proteins as members of the neuronal α7 nAChR interactome (Table 1). With the exception of tau, most of the proteins listed in Table 1 have not been previously identified as interaction partners of the α7 nAChR.[92] To our knowledge, of the proteins listed in Table 1, only PKA,[93, 94] PKC,[94] and 14-3-3η[93] have been shown previously to be associated with non-α7 nAChRs. The compiled list of proteins in the interaction network obtained from this initial study is not meant to be comprehensive, nor have we validated these identified proteins via orthogonal assays. Verification of the proteins that we identified as potential members of the α7 nAChR interactome will need to be performed via traditional biochemical methods, such as Western blotting and co-immunoprecipitations, before more concerted efforts are devoted to the detailed study of these interactions. Nevertheless, our work has laid a solid foundation for further exploration of the α7 nAChR interactome in both neuronal and non-neuronal tissues. Furthermore, the affinity purification with α-bgtx-conjugated beads together with the proteomic analysis procedure that we have developed could also be applied to the investigation of the interactomes of other receptors. The use of a pharmatope tag consisting of a short amino acid sequence to confer α-bgtx sensitivity to a target protein has been well described in cell culture[95] and oocyte studies.[96, 97] More specifically, such a tag may be inserted into a receptor for which suitable antibodies are not available and/or for which affinity-ligand beads are unavailable or impractical.[98] Coupling pharmatope-tagging with our methodology of α-bgtx isolation in an α7 nAChR null background may facilitate the proteomic analyses of other functionally important membrane proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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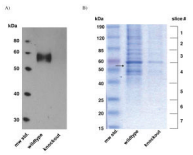

Figure 1.

Western blotting and SDS-PAGE analysis of mouse brain-derived samples following affinity isolation. A) Western blotting confirms that the α7 subunit was present in a lane loaded with the carbachol eluant from an α-bgtx affinity bead preparation of solubilized, wild-type mouse brain tissue. No corresponding band was present in the lane corresponding to a sample from the α7 nAChR knockout mouse. The nitrocellulose membrane was probed with anti-α7 nAChR antibody. B) Coomassie-stained SDS-PAGE gel illustrating the profile of proteins isolated by an α-bgtx affinity bead sedimentation from wild-type and α7 nAChR knockout mouse brain tissue. The approximate molecular weights of the protein standards and gel slice regions are denoted to the left and right of the gel, respectively.

Table 1

Proteins from wild-type mouse brain tissue identified by nano-ESI-MS/MS of peptides originating from in-gel digested proteins isolated by selective elution from α-bgtx-conjugated affinity beads

Only proteins that appear in wild-type samples and are absent in knockout samples are listed. This table lists proteins that were identified in at least two of three data sets from α-bgtx isolations of different mouse brain preparations. Supplemental Table 1 lists all those proteins from wild-type mouse brain tissue that appear in at least one of the three data sets associated with selective elution from α-bgtx-conjugated beads. Each Data Set (A through C) represents a separate preparation of the same amount of mouse brain tissue. The table lists the name of the protein, the Swiss-Prot accession number, protein mass in Daltons, as well as the total number of matched peptides per protein, and the gel slice from which these peptides were derived.

Protein	Swiss-Prot acc. no.	protein mass (Da)	Data Set A		Data Set B		Data Set C	
			matched peptides	slice no.	matched peptides	slice no.	matched peptides	slice no.
Basic metabolic proteins								
Adenylyl cyclase-associated protein 2	Q9CYT6	52,862	1	4	1	4	1	4
Aldehyde dehydrogenase, mitochondrial	P47738	56,538	2	4	1	4		
Glutathione S-transferase Mu 1	P10649	25,970	1	5			1	5
L-lactate dehydrogenase A chain	P06151	36,499	2	4	1	4		
Myristoylated alanine-rich C-kinase substrate	P26645	29,661	1	2	3	2		
Probable oxidoreductase KIAA1576	Q80TB8	45,817	1	4	1	4		
Vacuolar proton translocating ATPase A1	Q9Z1G4	96,501	1	1	2	2	2	2
Cell structure and protein trafficking								
Alpha-catenin 2	Q61301	105,286			1	1	1	1
Ankyrin 2, isoform 1	Q6PCN2	117,436	4	1	3	1	1	1
Ankyrin 2, isoform 2	Q8C8R3	131,202	2	1	2	1	1	1
AP-2 complex subunit beta-1	Q9DBG3	104,583			2	1	1	1
AP-3 complex subunit beta-2	Q9JME5	119,193	1	1	1	1		
Brain acid soluble protein 1; BASP1, NAP-22	Q91XV3	22,087	7	4	2	4		
Protein disulfide-isomerase A3	P27773	56,678	3	4			1	4
Endophilin-1	Q62420	39,877	2	4			1	4
Endophilin-2	Q62419	41,518	1	3			1	3
Fascin	Q61553	54,508	2	3	5	3		
Gamma-soluble NSF attachment protein	Q9CWZ7	34,732	1	4	1	4		
Homer protein homolog 1	Q9Z2Y3	41,413	1	4	1	4		
Kinesin-like protein KIF2	P28740	79,756			1	2	1	2
Microtubule-associated protein tau	P10637	76,243	2	4	1	4	5	4

Protein	Swiss-Prot acc. no.	protein mass (Da)	Data Set A		Data Set B		Data Set C	
			matched peptides	slice no.	matched peptides	slice no.	matched peptides	slice no.
Myosin-VI	Q64331	146,409	1	1	3	1		
Nck-associated protein 1	P28660	128,784	1	1	1	2		
Spectrin beta chain	P15508	245,250	1	1	2	1		
Synaptogyrin-1	O55100	25,653	1	5	1	5		
Synaptojanin-1	Q8CHC4	172,617	1	1	1	1		
Syntaxin-1 A	O35526	33,054	1	6	1	4		
Syntaxin-1 B	O35525	33,079	1	4	1	4		
Tropomodulin-2	Q9JKK7	39,511	1	4			1	4
Chaperones								
14-3-3 protein beta/alpha	Q9CQV8	28,086	1	6	2	6		
14-3-3 protein eta	P68510	28,212	1	5	1	5		
14-3-3 sigma	O70456	27,713			1	4	1	4
Gelsolin	P13020	85,942	1	2	3	2		
Heat shock protein HSP 90-beta	P11499	83,325	2	2	2	2		
Proteolytic pathway proteins								
Ubiquitin carboxyl-terminal hydrolase 5	P56399	95,833			1	1	2	2
Ubiquitin-activating enzyme E1	Q02053	117,809	2	1	2	1	3	1
Signal transduction								
Calmodulin	P62204	16,838	1	6	1	6	2	6
cAMP-dependent protein kinase, PKA	P68181	40,708	1	3	1	3		
Clathrin coat assembly protein AP180 (SNAP91)	Q61548	91,851	1	2			2	2
Contactin-1	P12960	113,388	1	1	3	1		
Guanine nucleotide-binding protein alpha-12	P27600	44,095	2	4			2	4
Guanine nucleotide-binding protein G(i), alpha-2	P08752	40,471	1	4	2	4		
Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta 3	Q61011	37,240	1	4			1	4
Guanine nucleotide-binding protein G(o) alpha 1	P18872	40,085	7	4	2	4	6	4
Guanine nucleotide-binding protein G(q) alpha	P21279	41,483	1	4	1	4		
Mitsugumin-23	Q3UBX0	26,306			1	6	1	6
Neuromodulin (GAP-43)	P06837	23,632	3	5			1	5
Phosphatase 2A inhibitor, TAF-1, SET	Q9EQU5	33,378			1	4	2	4

Protein	Swiss-Prot acc. no.	protein mass (Da)	Data Set A		Data Set B		Data Set C	
			matched peptides	slice no.	matched peptides	slice no.	matched peptides	slice no.
Protein kinase C gamma type	P63318	78,358	1	2			1	2
Protein phosphatase 1 regulatory subunit 7	Q3UM45	41,262	2	4			1	3
Ras-related protein Rab-2A	P53994	23,548	1	5			1	5
Serine/threonine-protein phosphatase 2B	P63328	58,644	2	3			1	3
Serine/threonine-protein phosphatase PP1-alpha	P62137	37,540	2	4	1	4		
Tenascin-R	Q8BYI9	149,563	1	1			1	1
Transgelin-3	Q9R1Q8	22,471	4	5	3	5		

Table 2

Proteins from Table 1 that have also been identified in the post-synaptic density proteome

These proteins are a subset of those in Table 1 that are among the 698 proteins identified in the study of the post-synaptic density proteome by Collins *et al.*[52]

Protein	Swiss-Prot acc. no.
Basic metabolic proteins	
L-lactate dehydrogenase A chain	P06151
Vacuolar proton translocating ATPase A isoform 1	Q9Z1G4
Cell structure and protein trafficking	
Alpha-catenin 2	Q61301
AP-2 complex subunit beta-1	Q9DBG3
Brain acid soluble protein 1; BASP1, NAP-22	Q91XV3
Fascin	Q61553
Gamma-soluble NSF attachment protein	Q9CWZ7
Homer protein homolog 1	Q9Z2Y3
Kinesin-like protein KIF2	P28740
Microtubule-associated protein tau	P10637
Myosin-VI	Q64331
Synaptogyrin-1	O55100
Syntaxin-1 A	O35526
Syntaxin-1 B	O35525
Tropomodulin-2	Q9JKK7
Chaperones	
14-3-3 protein beta/alpha	Q9CQV8
14-3-3 protein eta	P68510
Gelsolin; Actin-depolymerizing factor Brevin	P13020
Heat shock protein HSP 90-beta	P11499
Signal transduction	
cAMP-dependent protein kinase, PKA	P68181
Contactin-1	P12960
Guanine nucleotide-binding protein G(i), alpha-2 subunit	P08752
Guanine nucleotide-binding protein G(o) subunit alpha 1	P18872
Guanine nucleotide-binding protein G(q) subunit alpha	P21279
Protein kinase C gamma type	P63318
Ras-related protein Rab-2A	P53994