ORIGINAL RESEARCH

Exploring the Nicotinic Acetylcholine Receptor-associated Proteome with iTRAQ and Transgenic Mice

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Abstract Neuronal nicotinic acetylcholine receptors (nAChRs) containing α4 and β2 subunits are the principal receptors in the mammalian central nervous system that bind nicotine with high affinity. These nAChRs are involved in nicotine dependence, mood disorders, neurodegeneration and neuroprotection. However, our understanding of the interactions between α4β2-containing (α4β2) nAChRs and other proteins remains limited. In this study, we identified proteins that interact with α4β2 nAChRs in a gene-dose dependent pattern by immunopurifying β2 nAChRs from mice that differ in α4 and β2 subunit expression and performing proteomic analysis using isobaric tags for relative and absolute quantitation (iTRAQ). Reduced expression of either the α4 or the β2 subunits results in a correlated decline in the expression of a number of putative interacting proteins. We identified 208 proteins co-immunoprecipitated with these nAChRs. Furthermore, stratified linear regression analysis indicated that levels of 17 proteins was correlated significantly with expression of α4β2 nAChRs, including proteins involved in cytoskeletal rearrangement and calcium
signaling. These findings represent the first application of quantitative proteomics to produce a β2 nAChR interactome and describe a novel technique used to discover potential targets for pharmacological manipulation of α4β2 nAChRs and their downstream signaling mechanisms.

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are involved in a wide variety of functions in the central nervous system (CNS) and are disrupted in several psychiatric and neurological disorders. The most abundant high-affinity nAChRs in the mammalian CNS contain the α4 and β2 subunits [1–3] and this receptor subtype represents an important target for studies of functionally relevant protein–protein interactions. Heteromeric α4β2* nAChRs (where * denotes other, potentially unidentified, subunits) bind nicotine with high affinity [4,5] and are targeted by pharmacotherapies for smoking cessation [6]. Alzheimer’s disease [7], Parkinson’s disease [8], mood disorders [9] and attention deficit hyperactivity disorder (ADHD) [10]. A better understanding of the nAChR signaling complex could lead to a better drug design to achieve desired therapeutic effects.

A number of studies have elucidated regulatory mechanisms that modulate nAChR function and cellular trafficking. The interaction of 14-3-3 adaptor proteins with α4 subunit affects the stoichiometry and agonist sensitivity of α4β2 nAChRs [11,12]. Interactions with the calcium-sensor protein VILIP-1 affect the agonist sensitivity and assembly of α4 nAChRs [13]. Phosphorylation of α4 subunit by protein kinase A (PKA) affects association with 14-3-3 adaptor proteins and regulate α4β2 nAChR assembly [12,14]. Phosphorylation by protein kinase C (PKC) and dephosphorylation by the phosphatase calcineurin alter the transition of α4β2 nAChRs into and out of functionally desensitized states following prolonged exposure to agonists [15–17]. Although these studies, and others, have identified protein–protein interactions with α4β2 nAChRs, our knowledge of the receptor interactome remains incomplete.

Mass spectrometry (MS)-based proteomic analysis allows simultaneous identification of multiple proteins present in varying quantities in complex mixtures. The ability of MS to obtain accurate peptide sequences, and subsequently identify proteins from these detected unique sequences using protein databases, has been a significant technological advance providing high-throughput accurate protein profiling (see [18,19] for review). A set of proteins that appear to associate with β2* nAChRs were identified previously by analyzing proteins isolated from the mouse brain tissue using a clonal antibody (mAb270) and MS with matrix-assisted laser desorption and tandem time-of-flight (MALDI–ToF–ToF) followed by comparison to complexes in β2 subunit null-mutant mice [20]. This study demonstrated the utility of MS to detect nAChR-interacting proteins from the brain tissue. However, no quantitative methods have been employed to address specificity of the interaction between the identified proteins and the β2* nAChRs, as well as their partner subunits.

Stable isotopic labeling in cell culture (SILAC) has been used to identify highly specific protein–protein interactions. Selective protein was knocked down via siRNA in cultured cells grown in standard media, in comparison with cells grown in media supplemented with “heavy” amino acids [21,22]. However, this technique is limited by its capacity to compare only two samples, and stable isotopic labeling is not currently feasible for tissue homogenates. In contrast, label-free based quantitative MS is performed sequentially and thus introduces run-to-run variations in peptide elution, preventing accurate quantitation between sample sets. In order to facilitate identification of nAChR-interacting proteins with high confidence, we performed quantitative proteomic analysis using isobaric tags for relative and absolute quantitation (iTRAQ) [23]. iTRAQ reduces variation by labeling multiple protein samples and mixing these samples together prior to liquid chromatography–tandem MS (LC–MS/MS) analysis, enabling identification and quantitation of multiple proteins from several samples concurrently [24].

In this study, we combined the iTRAQ technique with the use of α4 and β2 nAChR subunit null-mutant mouse lines (these null-mutants express no functional α4β2 nAChRs [4,25] and heterozygotes express intermediate levels [26]). A β2 subunit specific monoclonal antibody was used to isolate the receptors and quantify gene dose-dependent changes in the α4β2* nAChR interactome. The ability of iTRAQ to multiplex all six genotypes in a single LC–MS/MS experiment is essential for the quantitative identification and comparison of interacting proteins across genotypes. This integrated strategy recapitulates powerful cell-based techniques, but capitalizes on the ability of iTRAQ to label multiple ex vivo tissue samples. This technique identified a group of proteins that are associated linearly with mature nAChRs expressed in the mammalian brain and provided a platform for exploring functional relevance of this interactome.

Results

Characterizing quantitative mAb295-M270 solid phase immunodepletion of β2* nAChRs from mouse brain

We first generated mAb295-coupled M270 Dynabeads using 0.5, 1, 2 or 5 μg of mAb295/mg of M270 beads in order to determine the optimal concentration of beads as well as the optimal ratio of bead suspension to brain extracts for quantitative immunoprecipitation of β2* nAChRs. Increasing volumes (0, 1.56, 3.125, 6.25, 12.5, 25, 50, 100 μl) of bead suspension from each concentration of mAb295 were used to capture β2* nAChRs labeled with 1 nM [3H]-epibatidine. Brain samples of three C57BL/6 mice were solubilized. After centrifugation, supernatants were pooled, and 100 μl aliquots were used in triplicate for each concentration of mAb295 across all eight bead volumes to measure the efficiency and extent of [3H]-epibatidine binding site capture. Depletion of [3H]-epibatidine binding from mouse brain extracts by immobilized mAb295 was saturable and nearly complete across all four concentrations of mAb295 tested (Figure S1A). The ½ maximal bead volume for nAChR capture decreased with increasing concentrations of mAb295 (R² = 0.83; Figure S1B), but the calculated maximal binding site capture was not significantly different across the four mAb295-M270 bead ratios.
Immunopurification of $\beta^2$ nAChRs from brain tissue of $\alpha 4$ or $\beta 2$ subunit transgenic mice

Using optimal conditions (5 $\mu$g mAb295/mg M270 beads; bead volume 10% of total extracts), we extracted $\beta^2$ nAChRs from mouse brain extracts prepared from wild type (WT), heterozygous (HET) and homozygous (KO) mice lacking either $\alpha 4$ or $\beta 2$ nAChR subunit. There was a gene dose-dependent effect on the number of total $[\text{3H}]$-epibatidine binding sites in brain detergent extracts (Table 1) across genotypes. There was no significant binding site capture from brains of $\alpha 4$ KO or $\beta 2$ KO mice. No significant difference was detected in the percentage of binding sites captured from tissue samples of WT and HET $\alpha 4$ (79.6 ± 5.4 vs 83.1 ± 7.5%; n = 3, P = 0.72, t-test) or $\beta 2$ (79.3 ± 0.9 vs 78.8 ± 1.3%; n = 3, P = 0.78, t-test), indicating that immobilized mAb295 performed similarly in all samples regardless of nAChR protein content.

Measurement of relative expression of nAChR subunit proteins by iTRAQ and LC–MS/MS

Quantitation of iTRAQ measured abundance is expressed as the log$_2$ of the ratio of mean reporter ion peak areas (log$_2$MRIPA) of all proteins present in all samples relative to the average WT abundance of the $\beta^2$ nAChR subunit (the target of the IP). Since $\alpha 4$ and $\beta 2$ nAChR subunit levels vary with gene dose, we evaluated their levels in each sample to judge the ability of iTRAQ to quantify proteins in immunocaptured eluates. Although $[\text{3H}]$-epibatidine binding sites varied with $\alpha 4$ and $\beta 2$ genotypes, total protein eluted following immunoprecipitation (IP) did not differ significantly across genotypes ($F_{(3,11)}$ = 1.23, $P = 0.36$). This suggests that the amount of mAb295 coupled to the magnetic beads is more important for efficient capture of $\beta^2$ nAChRs than the total available surface area of the beads themselves. Therefore, under the conditions outlined in this experiment, steric hindrance of protein complexes adhering to the nAChRs was not likely to affect the ability of the mAb295-M270 beads to trap solubilized $\beta^2$ nAChRs and their associated protein complexes from brain extracts effectively. Calculated parameters for specific capture of $[\text{3H}]$-epibatidine binding sites by mAb295-M270 beads are presented in Table S1.

Table 1 Gene dose-dependent effect on the number of $[\text{3H}]$-epibatidine binding sites of nAChRs

| Number of $[\text{3H}]$-epibatidine binding sites (fmol) | Genotype | F$_{(2,8)}$ score | P value |
|--------------------------------------------------------|----------|------------------|--------|
| Total in brain extracts (mean ± SEM)                   | $\alpha 4$ | 609.3 ± 101.5    | 51.9 ± 4.5 | 12.00  | 0.008  |
|                                                        | $\beta 2$ | 633.6 ± 124.0    | 38.9 ± 9.7 | 18.17  | 0.003  |
| Captured by immobilized mAb295 (mean ± SEM)            | $\alpha 4$ | 522.7 ± 120.3    | −1.0 ± 12.2 | 8.18   | 0.019  |
|                                                        | $\beta 2$ | 505.6 ± 103.5    | −5.0 ± 10.5 | 19.21  | 0.002  |
associated quite strongly \( (r > 0.5 \) using Pearson’s product moment correlation coefficient since the relationship between nAChR subunit gene dose and receptor expression is linear) with \( \beta_2 \) nAChRs (such as certain histone isoforms), despite the fact that nAChRs would not be expected to localize to the nucleus or mitochondria. The UniProt Knowledgebase (UniProtKB) [27] was therefore used to cull proteins from the list when the assigned cellular compartment did not correspond to sites of known nAChR localization. Exceptions were made in the case of two transcription factors: Pur-\( \alpha \), which has the capacity to translocate from the cytosol to the nucleus [28], and thyroid hormone receptor-associated protein 3 (THRAP3), which we previously identified in an additional unpublished nAChR interactome study. Additional analysis focused only on 91 proteins in these compartments, with 62 identified as cytoplasmic, 26 associated with the plasma membrane and the remaining 3 proteins associated with the Golgi apparatus/endomembrane system (Table 2). Of these, only 17 proteins (11 cytoplasmic and 6 associated with the plasma membrane) showed statistically significant correlation with levels of the \( \beta_2 \) nAChR subunit (Table 3). Classification of these 17 proteins with PANTHER Gene Ontology (GO) of molecular function [29] revealed distinct biological activity profiles (Table 3).

In the primary (unfiltered) dataset, distribution of correlation coefficients for \( \beta_2 \) log2MRIPA was distinctly bimodal, with a major mode occurring between 0 and \(-0.5\), and a minor mode occurring between 0.25 and 0.75 (Figure 3A). These data suggest that this dataset represents two sets of events: proteins that likely interacted with the antibody complex when it was not occupied by nAChRs (\( r < 0 \) and proteins that interacted selectively with the immunoprecipitated nAChRs (\( r > 0.25 \)). In the secondary dataset, adjustment for cellular compartment eliminated the events with \( r < 0 \) and resulted in a unimodal distribution approximately equivalent to the minor mode with \( r > 0.25 \) (Figure 3B). These data suggest that eliminating the group of proteins that are not co-localized with the nAChRs in these cellular compartments also screens out the majority of proteins that interact non-specifically with the antibody complex. The tertiary dataset (selected for significant positive correlation with \( \beta_2 \) nAChR subunit expression; \( P < 0.05 \)) was also unimodal, with the majority of the distribution centered between 0.5 and 0.75 (Figure 3C). Eliminating proteins based on cellular compartments favored identification of more positive correlations with \( \beta_2 \) nAChR subunit abundance. Such result is consistent with the observation that calculated kurtosis for the frequency distribution increased from an estimate of \(-0.513 \) to 1.211 when moving from the primary to secondary dataset, an indication that the selected datasets deviated significantly from chance. Indeed, all three frequency distributions were significantly different from a normal distribution according to Shapiro–Wilks test \( (P = 0.006, 0.021 \) and 0.022 for primary, secondary and tertiary dataset, respectively), indicating that the samples were substantially enriched for a particular target protein and the associated proteins identified were largely non-random.

**Discussion**

This study used quantitative protein identification with iTRAQ and LC–MS/MS to identify protein–protein interactions for \( \alpha_4 \beta_2 \) nAChRs. A highly specific monoclonal antibody was used to capture \( \beta_2 \) nAChRs reliably and efficiently from detergent extracts of mouse brain homogenates, facilitating the identification of a set of putative nAChR-interacting proteins. Proteins of particular interest
| Correlation coefficient | N  | F score | P value | Protein                                                                 | UniProtKB accession No. | Cellular compartment | Previously identified? |
|-------------------------|----|---------|---------|-------------------------------------------------------------------------|--------------------------|----------------------|-----------------------|
| 0.185                   | 18 | 0.568   | 0.462   | 14-3-3 protein ζ/θ                                                      | P63101                   | Cytoplasm            | Yes                   |
| 0.117                   | 18 | 0.221   | 0.645   | Actin, alpha cardiac muscle 1                                           | P16330                   | Cell membrane        | Yes                   |
| 0.326                   | 18 | 1.909   | 0.186   | Actin, alpha cardiac muscle 1                                           | P68033                   | Cytoplasm            | No                    |
| 0.375                   | 18 | 2.623   | 0.125   | Actin, cytoplasmic 2                                                   | P61260                   | Cytoplasm            | Yes                   |
| 0.645                   | 18 | 11.404  | 0.004   | Actin-related protein 3                                                | Q9919Y                   | Cytoplasm            | No                    |
| 0.37                    | 18 | 2.536   | 0.131   | α-actinin-1                                                             | Q7TPR4                   | Cytoplasm            | No                    |
| 0.104                   | 12 | 0.11    | 0.747   | Ankyrin-2                                                               | Q8C8R3                   | Cytoplasm            | No                    |
| 0.021                   | 12 | 0.005   | 0.948   | Brain acid soluble protein 1                                           | Q91XV3                   | Cell membrane        | No                    |
| 0.637                   | 18 | 10.904  | 0.004   | Calcium/calmodulin-dependent protein kinase type II subunit α              | P11798                   | Cytoplasm            | No                    |
| 0.172                   | 6  | 0.49    | 0.494   | Calcium/calmodulin-dependent protein kinase type II subunit β              | P28652                   | Cytoplasm            | No                    |
| 0.456                   | 6  | 1.048   | 0.364   | Calcium/calmodulin-dependent protein kinase type II subunit γ              | Q6PHZ2                   | Cell membrane        | No                    |
| 0.917                   | 21 | 21.235  | 0.01    | Calcium/calmodulin-dependent protein kinase type II subunit γ              | Q923T9                   | Sarcoplasmic reticulum membrane | No                   |
| 0.235                   | 18 | 0.938   | 0.347   | Coronin-2R                                                              | Q8BBH44                  | Cytoplasm            | No                    |
| 0.598                   | 6  | 2.221   | 0.21    | Dihydropyrimidinede-related protein 2                                   | O08553                   | Cytoplasm            | No                    |
| 0.186                   | 18 | 0.143   | 0.724   | Disks large homolog 4                                                  | Q62108                   | Cell membrane        | Yes                   |
| 0.468                   | 18 | 4.495   | 0.05    | Drebrin                                                                 | Q9QXS6                   | Cytoplasm            | No                    |
| 0.327                   | 18 | 1.911   | 0.186   | Dynne light chain 2, cytoplasmic protein                                | Q9D0M5                   | Cytoplasm            | No                    |
| 0.539                   | 18 | 6.563   | 0.021   | Ectonucleotide pyrophosphatase/phosphodiesterase family member 6          | Q8BGN3                   | Cell membrane        | No                    |
| 0.386                   | 6  | 2.807   | 0.113   | EF-hand domain-containing protein D2                                    | Q9D8Y0                   | Membrane raft        | No                    |
| 0.57                    | 18 | 7.681   | 0.014   | F-actin-capping protein subunit α                                       | P47754                   | Cytoplasm            | No                    |
| 0.46                    | 6  | 4.286   | 0.055   | F-actin-capping protein subunit β                                       | P47757                   | Cytoplasm            | No                    |
| 0.337                   | 18 | 2.052   | 0.171   | Gelsolin                                                                | P13020                   | Cytoplasm            | No                    |
| 0.748                   | 12 | 20.268  | 0       | Glial fibrillary acidic protein                                         | P03995                   | Cytoplasm            | No                    |
| 0.476                   | 18 | 2.937   | 0.117   | Glutamine synthetase                                                    | P15105                   | Cytoplasm            | No                    |
| 0.417                   | 18 | 3.371   | 0.085   | Glyceraldehyde-3-phosphate dehydrogenase                               | P16858                   | Cytoplasm            | Yes                   |
| 0.045                   | 18 | 0.033   | 0.859   | Guanine nucleotide-binding protein G(o) subunit α                        | P18872                   | Heterotrimeric G-protein complex                                       | Yes                   |
| 0.193                   | 18 | 0.622   | 0.442   | Heat shock cognate 71 kDa protein                                       | P63017                   | Cytoplasm            | No                    |
| 0.46                    | 18 | 4.29    | 0.055   | Heterogeneous nuclear ribonucleoprotein D0                               | Q60668                   | Nucleus              | No                    |
| 0.486                   | 12 | 1.237   | 0.328   | Heterogeneous nuclear ribonucleoprotein U                                | Q8VEK3                   | Nucleus              | No                    |
| 0.243                   | 6  | 1.957   | 0.192   | 1-Lactate dehydrogenase A chain                                         | P16125                   | Cytoplasm            | No                    |
| 0.332                   | 18 | 1.984   | 0.178   | Myelin basic protein                                                    | P04370                   | Myelin membrane      | Yes                   |
| 0.332                   | 18 | 1.984   | 0.178   | Myelin basic protein                                                    | P04370                   | Myelin membrane      | Yes                   |
| 0.506                   | 18 | 5.496   | 0.032   | Myelin proteolipid protein                                              | P60202                   | Cell membrane        | No                    |
| 0.262                   | 18 | 1.183   | 0.293   | Myosin light polypeptide 6                                              | Q60605                   | Cytoplasm            | No                    |
| 0.512                   | 18 | 5.695   | 0.03    | Myosin-10                                                               | Q61879                   | Cytoplasm            | No                    |
| 0.085                   | 11 | 0.177   | 0.737   | Myosin-9                                                                | Q8VDD5                   | Cytoplasm            | No                    |
| 0.652                   | 18 | 11.844  | 0.003   | Neurofilament light polypeptide                                         | P08551                   | Growth cone          | No                    |
| 0.379                   | 18 | 2.683   | 0.121   | Neurofilament medium polypeptide                                        | P08553                   | Growth cone          | No                    |
| 0.858                   | 18 | 44.542  | 0       | Neuronal acetylcholine receptor subunit ß4                               | Q70174                   | Cell junction        | No                    |
| 1                      | 18 | –       | 0       | Neuronal acetylcholine receptor subunit ß2                               | Q9ERK7                   | Cell junction        | No                    |
| 0.02                    | 18 | 0.006   | 0.938   | Peptidyl-prolyl cis-trans isomerase A                                   | P17742                   | Cytoplasm            | No                    |
| 0.431                   | 6  | 2.279   | 0.162   | Peroxiredoxin-1                                                         | P35700                   | Cytoplasm            | No                    |
| 0.484                   | 12 | 3.061   | 0.111   | Pyruvate kinase isoymes M1/M2                                            | P52480                   | Cytoplasm            | No                    |
| 0.287                   | 12 | 0.901   | 0.365   | Ras-related protein Rab-11B                                             | P46638                   | Cell membrane        | No                    |
| 0.397                   | 6  | 1.867   | 0.202   | Ras-related protein Rab-7a                                              | P51150                   | Late endosome        | No                    |
| 0.31                    | 12 | 1.063   | 0.327   | Ras-related protein Rab-A                                               | P63321                   | Cell membrane        | No                    |
| 0.856                   | 12 | 11.009  | 0.029   | Ras-related protein Rap-1A                                             | P62835                   | Cell membrane        | No                    |
| 0.171                   | 6  | 0.302   | 0.595   | Ras-related protein Rap-1b                                              | Q9J916                   | Cell membrane        | No                    |
| 0.443                   | 12 | 2.44    | 0.149   | Serine/threonine-protein phosphatase PP1β catalytic subunit              | P62141                   | Cytoplasm            | No                    |
| 0.69                    | 6  | 3.633   | 0.129   | Serine/threonine-protein phosphatase PP1γ catalytic subunit             | P63087                   | Cytoplasm            | No                    |
| 0.224                   | 12 | 0.529   | 0.484   | Sodium/potassium-transporting ATPase subunit α2                          | Q6PIE5                   | Cell membrane        | No                    |
have shown that expression of nAChR subunit $\alpha_3$ is dependent on that of $\beta_2$ subunit; however, low levels of $\beta_2^*$ nAChR subunits remain in the absence of $\alpha_4$ [26,30]. Additional $\alpha$ subunits ($\alpha_2$, $\alpha_3$ and $\alpha_6$) are expressed in the CNS and can form functional nAChR subunits with $\beta_2$ subunits [31]. Although the non-$\alpha_4$ nAChRs are not sufficiently abundant to allow identification of these complexes from the whole brain lysate by iTraQ, our measurements of $\alpha_4$ and $\beta_2$ nAChR subunit peptides across the six genotypes using iTraq are consistent with published studies on the effect of subunit null-mutation on mature $\beta_2^*$ nAChR subtype expression. Importantly, our measures indicate that in the absence of a partner subunit, individual nAChR subunit monomers contributing to heteromeric $\alpha_4\beta_2^*$ nAChRs are not apparent, and that multi-subunit complexes are required to prevent rapid degradation of free subunits.

nAChR peptides have been recognized previously by mAbs in knockout mouse tissue [30]. The genomic DNA encoding the majority of the extracellular domain of the $\beta_2$ nAChR subunit (recognized by mAb295) is not removed by exon deletion, through which the $\alpha_4$ and $\beta_2$ nAChR subunit knockout mice were generated [4,25], so identification of low levels of $\alpha_4$ and $\beta_2$ nAChR subunit peptides in their respective KO mice is not surprising. It is also possible that some of the peptides recovered in the $\alpha_4$ and $\beta_2$ KO samples are incorrectly identified as a result of chimeric spectra in MS/MS that do not actually represent nAChR subunit peptides; however, chimeric spectra generally result in uncertainty of protein identification, rather than positive identification of peptides that are not actually present in the sample [32].

It is possible that truncated nAChR subunit peptides in KO tissue cannot form binding sites but retain some features of nascent nAChR assembly intermediates, since measurable [$^3$H]-epibatidine binding sites decreased in gene-dose-dependent fashion following nAChR subunit deletion, while the amount of total eluted protein following mAb295 affinity purification did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change. In addition, when comparing functions of proteins identified in the dataset adjusted for improper cellular compartment to those in the dataset adjusted for correlation with $\beta_2$ nAChR subunit expression, proteins serving chaperone isomerase, and ER-Golgi transport functions of proteins identified in the dataset adjusted for correlation did not change.

Table 2 continued

| Correlation coefficient | N   | F score | P value | Protein | UniProtKB accession No. | Cellular compartment | Previously identified? |
|------------------------|-----|---------|---------|---------|-------------------------|----------------------|-----------------------|
| 0.261                  | 18  | 1.169   | 0.296   | Sodium/potassium-transporting ATPase subunit $\alpha_3$ | Q6PIC6 | Cell membrane | No |
| 0.266                  | 18  | 1.217   | 0.286   | Sodium/potassium-transporting ATPase subunit $\beta_1$ | P14094 | Cell membrane | No |
| 0.502                  | 18  | 5.378   | 0.034   | Spectrin $\alpha$ chain, brain | P16546 | Cytoplasm | Yes |
| 0.519                  | 18  | 5.884   | 0.027   | Spectrin $\beta$ chain, brain 1 | Q62261 | Cytoplasm | No |
| 0.299                  | 18  | 1.566   | 0.229   | Synaptopodin | Q8CC35 | Cytoplasm | No |
| 0.547                  | 12  | 4.265   | 0.066   | Synaptotagmin-1 | P46096 | Cytoplasmic vesicle | No |
| 0.536                  | 12  | 4.026   | 0.073   | Syntaxin-1B | P61264 | Cell membrane | No |
| 0.086                  | 18  | 0.119   | 0.734   | Syntaxin-binding protein 1 | O08599 | Cell membrane | No |
| 0.562                  | 18  | 7.386   | 0.015   | Thyroid hormone receptor-associated protein 3 | Q569Z6 | Nucleus | No |
| 0.665                  | 12  | 7.933   | 0.018   | Transcriptional activator protein Pur-$\alpha$ | P42669 | Nucleus | No |
| 0.491                  | 6   | 1.272   | 0.322   | Triosephosphate isomerase | P17751 | Cytoplasm | No |
| 0.334                  | 18  | 2.011   | 0.175   | Tropomodulin-2 | Q9JKK7 | Cytoplasm | No |
| 0.451                  | 18  | 4.087   | 0.06    | Tubulin $\alpha$-1A chain | P68369 | Cytoplasm | Yes |
| 0.431                  | 18  | 3.652   | 0.074   | Tubulin $\alpha$-4A chain | P68368 | Cytoplasm | No |
| 0.422                  | 18  | 3.466   | 0.081   | Tubulin $\beta$-2A chain | Q7TM M 9 | Cytoplasm | No |
| 0.493                  | 18  | 5.149   | 0.037   | Tubulin $\beta$-3 chain | Q9ERD7 | Cytoplasm | No |
| 0.379                  | 18  | 2.688   | 0.121   | Tubulin $\beta$-4A chain | Q9D6F9 | Cytoplasm | No |
| 0.432                  | 12  | 2.298   | 0.16    | Tubulin $\beta$-5 chain | P99024 | Cytoplasm | No |
| 0.434                  | 18  | 3.717   | 0.072   | Unconventional myosin-Va | Q99104 | Cytoplasm | No |
| 0.295                  | 18  | 1.52    | 0.235   | Unconventional myosin-VI | Q64331 | Golgi apparatus | No |
| 0.01                   | 12  | 0.001   | 0.975   | Unconventional myosin-XVIIIa | Q9JMH9 | ER-Golgi intermediate compartment | No |
| 0.18                   | 6   | 0.537   | 0.474   | Vesicle-fusing ATPase | P46460 | Cytoplasm | No |
| 0.195                  | 18  | 0.158   | 0.716   | Vimentin | P20152 | Cytoplasm | No |
| 0.179                  | 6   | 0.527   | 0.478   | V-type proton ATPase catalytic subunit A | P50516 | Cell membrane | No |

Note: Correlation coefficient was calculated using Pearson’s product moment; F score was generated by one-way ANOVA.
| Correlation coefficient | N  | F score | P value | Protein                                      | UniProtKB accession No. | Cellular compartment | Previously identified? | Molecular function                                                                 |
|-------------------------|----|---------|---------|----------------------------------------------|--------------------------|---------------------|------------------------|------------------------------------------------------------------------------|
| 0.748                   | 12 | 20.268  | 0       | Glial fibrillary acidic protein              | P03995                   | Cytoplasm           | No                     | Protein binding, structural molecule                                          |
| 0.858                   | 18 | 44.542  | 0       | nAChR subunit α4                             | P08551                   | Cell junction        | No                     | Neuro-transmitter receptor                                                    |
| 1                       | 18 | –       | 0       | nAChR subunit β2                             | Q9ERK7                   | Cell junction        | No                     | Neuro-transmitter receptor                                                    |
| 0.652                   | 18 | 11.844  | 0.003   | Neurofilament light poly peptide             | P09714                   | Growth cone          | No                     | Protein binding, structural molecule                                           |
| 0.645                   | 18 | 11.404  | 0.004   | Actin-related protein 3                      | Q99JY9                   | Cytoplasm            | No                     | Nucleotide binding, protein binding                                            |
| 0.637                   | 18 | 10.904  | 0.004   | Calcium/calmodulin-dependent protein kinase type II subunit α | P11798                   | Cytoplasm            | No                     | Transferase, nucleotide binding, protein binding                               |
| 0.917                   | 18 | 21.235  | 0.01    | Calcium/calmodulin-dependent protein kinase type II subunit γ | Q923T9                   | Sarcoplasmic reticulum membrane | No                     | Transferase, nucleotide binding, protein binding                               |
| 0.57                    | 18 | 7.681   | 0.014   | F-actin-capping protein subunit α2           | P47754                   | Cytoplasm            | No                     | Protein binding                                                              |
| 0.562                   | 18 | 7.386   | 0.015   | Thyroid hormone                              | Q569Z6                   | Nucleus              | No                     | Nucleotide binding, protein binding                                            |
| 0.665                   | 12 | 7.933   | 0.018   | Transcriptional activator protein Pur-α      | P42669                   | Nucleus              | No                     | Nucleic acid binding, translation regulator, protein binding                   |
| 0.539                   | 18 | 6.563   | 0.021   | Ectonucleotide pyrophosphatase/phosphodiesterase family member 6 | Q8BGN3                   | Cell membrane        | No                     | Catalytic activity, hydrolase activity                                         |
| 0.519                   | 18 | 5.884   | 0.027   | Spectrin β chain, brain 1                    | Q62261                   | Cytoplasm            | No                     | Protein binding, lipid binding, structural molecule activity                  |
| 0.856                   | 6  | 11.009  | 0.029   | Ras-related protein Rap-1A                   | P62835                   | Cell membrane        | No                     | Hydrolase activity, protein binding, nucleotide binding                       |
| 0.512                   | 18 | 5.695   | 0.03    | Myosin-10                                    | Q61879                   | Cytoplasm            | No                     | Protein binding, nucleotide binding, hydrolase                                |
| 0.506                   | 18 | 5.496   | 0.032   | Myelin proteolipid protein                   | P60202                   | Cell membrane        | No                     | Structural molecular, protein binding                                         |
| 0.502                   | 18 | 5.378   | 0.034   | Spectrin α chain, brain                      | P16546                   | Cytoplasm            | Yes                    | Hydrolase, protein binding, nucleotide binding                                |
| 0.493                   | 18 | 5.149   | 0.037   | Tubulin β-3 chain                            | Q9ERD7                   | Cytoplasm            | No                     | Hydrolase, nucleotide binding, structural molecular, protein binding, peptide |
may bind to mAb295 and accumulate in the absence of bound nAChRs.

The use of iTRAQ coupled with α4 and β2 nAChR subunit transgenic mouse tissue provides a significant technical advance over previous proteomic methods used to identify specific nAChR-interacting proteins. Separating samples with difference in gel electrophoresis (DIGE) and eliminating bands/spots that appear in the KO control condition prior to LC–MS/MS pose the risk of missing significantly-associated proteins that are expressed in low abundance and may co-migrate with non-specifically captured proteins [33]. Multiplexing with iTRAQ and identifying as many proteins as possible in the post-IP eluent reduce the risk of missing proteins of interest due to preprocessing steps like DIGE, but increase the risk of identifying proteins that are not necessarily associated with assembled α4β2* nAChRs. Combining SILAC with siRNA-mediated knockdown reduces identification of false interacting proteins, which is not suited to tissue samples and is limited to a direct comparison of two samples [22]. To address this issue, we took advantage of quantitation using iTRAQ to generate linear regression of the apparent abundance of each identified protein together with that of the β2 nAChR subunit across the six genotypes examined in each sample set, allowing us to identify a continuum of protein associations, ranging from highly positive to highly negative correlations.

Previously suggested nAChR-interacting proteins were lost from the dataset as our analysis became more stringent. Eight (out of 17 in total) of α4β2 nAChR-interacting proteins identified by MALDI–ToF-ToF [20] are retained when identified proteins are restricted by cellular compartments, and 1 (out of 17) is identified when only proteins whose expression were significantly correlated with β2 nAChR subunit expression levels were considered, suggesting that these previous analyses may have suffered from missed identification and potentially false positive interactions. The current method, in contrast to MALDI–ToF-ToF [20], identified a significant correlation between the relative abundance of α4 nAChR subunit and the abundance of its primary partner β2 (r = 0.858). It is important to note that proteins listed in a recent comprehensive review (2011) [34] include those whose interactions are inferred from modulatory interactions (as with protein kinases and phosphatases which are known to phosphorylate or dephosphorylate the nAChRs). Such interactions are transient and may be cell-type specific. Therefore, future studies using enriched samples from a particular brain region and/or neuronal phenotype will be required to validate such associations.

Interpretation of interacting proteins that are not likely to interact in functionally relevant ways with target proteins is a caveat in current proteomic methods. This highlights the need to curate results of these analyses carefully and is part of the tradeoff for enhanced discovery and unbiased identification of novel interacting proteins. Examining the relative subcellular distribution of identified proteins in each of the lists generated by increasingly stringent criteria provided some interesting insights. Culling proteins by cellular compartment resulted in a dataset of 91 identified proteins in which 68% were cytoplasmic. Some Golgi/ER membrane resident proteins were also identified, indicating that the list of 91 interacting proteins likely includes proteins that interact with β2 nAChRs during assembly or maturation. The relative subcellular distribution of nAChRs in neurons in vivo is unknown, but cell surface expression of α4β2* nAChRs varies both by brain region and model organism [35,36]. The list of 17 proteins whose expression is correlated significantly with that of β2 nAChR subunit eliminates the Golgi/ER resident proteins, suggesting that this group represents proteins that largely interact with mature nAChRs present in the plasma membrane. Interactions with other plasma-membrane resident proteins are likely disrupted following solubilization. This is reflected in the list of 91 interacting proteins, where the majority (65%) are cytoplasmic proteins, indicating that in the mature state, the large cytoplasmic loop of each subunit that resides between transmembrane domains 3 and 4 represents the primary site for intracellular protein–protein interactions.

Establishing molecular function classifications with PANTHER pathway analysis [29] reveals that the primary interactions of mature β2 nAChRs occur with structural proteins that are part of, and regulate the growth and assembly of, the cytoskeleton. These data are consistent with studies showing that β2 nAChRs are involved in production and maintenance of dendritic spines during development [37]. Of particular interest are the cytoskeletal proteins that appear to
play functional roles in the growth and reorganization of synaptic processes, such as actin-related protein 3 (Arp3) and F-actin capping protein subunit α2. Arp3 contributes directly to axon branching [38] and is implicated in strain differences in hippocampal information processing [39]. Activity-dependent accumulation of F-actin capping proteins occurs in dendritic spines [40], supporting the idea that β2* nAChRs play a role in dynamic neuronal cytoarchitecture remodeling and providing a potential molecular mechanism for future evaluation.

Two isoforms of calcium/calmodulin-dependent protein kinase II (CaMKII), α and γ, were identified in the current experiment. CamKII is involved in nAChR recycling [41] and α-kinase-anchoring protein (αKAP, a CaMKII anchoring protein) inhibits proteasomal degradation of muscle-type nAChRs [42]. CaMKII is also a critical mediator of long-term potentiation (LTP) (see [43] for review), a molecular mechanism underlying memory storage. CaMKIIα is specifically associated with the postsynaptic density in excitatory neurons (see [44] for review) and previous studies have demonstrated that acute nicotine exposure in mice activates CaMKII in the spinal cord and brain, which requires activation of β2* nAChRs [45,46]. In addition, β2* nAChR-mediated activation of CaMKII is an essential component of the antinociceptive effects of nicotine [47] and affective signs of nicotine withdrawal [48]. Chronic nicotine exposure results in an increase in CaMKIIα expression and function in nucleus accumbens of mice and this effect is attenuated following administration of a β2 nAChR selective antagonist [49]. Nicotine also influences several aspects of hippocampal-dependent learning [50]. Identification here of a direct association between α4β2* nAChRs and CaMKIIα provides rationale for future studies of its role in hippocampal plasticity.

It should be noted that glial fibrillary acidic protein (GFAP) is one of the most highly correlated proteins identified in the curated list of proteins. Homomeric α7 nAChRs have been reported to be expressed on astrocytes [51]. However evidence of α4β2* nAChR expression on non-neuronal CNS cells is lacking. Identification of GFAP in this study may imply that α4β2* nAChRs could participate in neuron–glia interactions.

In conclusion, we have identified a novel set of nAChR-interacting proteins that are consistent with known nAChR-mediated functions, as well as previously identified interactors such as spectrin-α, validating our methodology. In addition to these biological findings, development of the iTRAQ technique in combination with evaluation in knockout mouse lines will be important for identifying protein–protein interaction without prior knowledge of the complex and for identifying interactions that cannot be detected using traditional protein identification techniques. Identification of an nAChR-associated proteome also provides a set of novel targets for drug discovery of therapeutics for smoking cessation and psychiatric or neurological disorders associated with nicotinic dysfunction. Ultimately, generation of small molecules that are capable of disrupting or facilitating interactions between nAChRs and specific associated proteins holds the promise of providing more targeted therapies with higher selectivity for particular nAChR-mediated behavioral functions and fewer side effects.

Materials and methods

Chemicals

Unless stated otherwise, all reagents were obtained from Sigma Aldrich (St. Louis, MO). [3H]-Epibatidine (62.2 Ci/mmol) was purchased from PerkinElmer, Sheldon, CT. iTRAQ reagents were obtained from AB Sciex (Framingham, MA).

Animals

Homozygous wild type (WT), heterozygous (HET) and homozygous (KO) mice lacking either α4 or β2 nAChR subunit were used in the study. β2 nAChR subunit [4] and α4 nAChR subunit [25] knockout (KO) mice were backcrossed at least 30 generations to a C57 BL/6 background. Mice were bred at the University of Colorado, Boulder, housed in groups of no more than 5 individuals per cage, maintained on a 12:12 h light:dark cycle and given ad libitum access to food and water. All protocols involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Yale University and the University of Colorado and conformed to the standards for animal care and use set by the National Institutes of Health.

Preparation of brain tissue extracts for immunoprecipitation

Animals were sacrificed by cervical dislocation and brains were rapidly placed on an ice-cold surface. After rinsed in chilled phosphate buffer solution (PBS, pH 7.4; containing 136.9 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄ and 1.76 mM KH₂PO₄) to carefully wash off any debris, brains were then snap frozen by immersion in −35 °C isopentane and kept frozen at −80 °C until use. On the day of tissue preparation, brains were thawed on a chilled surface and cerebellum was removed and discarded. The resulting tissue (whole forebrain) was homogenized by hand with 37 strokes in a glass tissue grinder in 10 volumes of extraction buffer (EB) (0.6% Triton X-100, 121.9 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, 1.76 mM KH₂PO₄, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1 mM Na₃VO₄, 1.0 mM PMSF and 10 μg/ml each of aprotonin, prepsatin A and leupeptin; pH 7.4). The resulting homogenate was incubated at 23 °C with gentle rotation for 30 min to facilitate protein solubilization, then subjected to centrifugation for 20 min at 5000 g. The resulting supernatant was used for all subsequent experiments.

[3H]-Epibatidine binding from mouse brain extracts

Measurement of the amount of high-affinity nAChRs in brain extracts was performed essentially as described previously [52], except that 1 nM [3H]-epibatidine was used as the radioligand instead of 200 pM [125I]-epibatidine. [3H]-Epibatidine labeled nAChRs were captured by filtration through a Packard Filtermate 196 Cell Harvester (Meriden, CT) onto Pall type A/D filters pre-soaked in 0.5% polyethylinimine. After washing three times with ice-cold wash buffer (140 mM NaCl, 1.5 mM KCl, 2.0 mM CaCl₂, 1.0 mM MgSO₄, 7H2O, 25 mM HEPES, pH 7.5), radioactivity on individual filters was measured by liquid
iTRAQ labeling and protein identification by LC–MS/MS

All samples were prepared for iTRAQ analysis using a CHCl₃/MeOH precipitation after diluting each to 100 μl with water. 400 μl of MeOH was then added and vortexed extensively prior to the addition of 100 μl CHCl₃. An additional 300 μl of water was added prior to vortexing and centrifuging at 14,000g for 1 min. The top aqueous layer was removed and discarded and an additional 400 μl MeOH was added. After a 2 min centrifugation at 14,000 g, the MeOH was removed without disturbing the pellet. The pellet was dried in a Speedvac and dissolved in 50 μl of 0.5 M triethylammonium bicarbonate (TEAB) with 0.2% SDS. Table S3 lists the amount of each sample used per sample set for labeling which was determined based on a nanodrop measurement at A₂₈₀ versus a buffer blank. Disulfide reduction was performed by incubating with 5 mM tris(2-carboxyethyl)phosphine (TCEP) at 60 °C for 1 h. Alkylation was then performed by incubating with 20 mM methylmethanethiol sulfonate (MMTS) at room temperature for 16 h. Each dried iTRAQ label was dissolved in 50 μl of 80% ACN containing 0.1% TFA and the elution was repeated by washing with an additional 180 μl of same solution. Samples were dried in a Speedvac and dissolved in 3 μl FA mixed with 8 μl 0.1% TFA.

Mass spectrometric analysis was performed on an AB Sciex TripleTOF 5600 which is equipped with a Waters nanoAcquity UPLC system, and uses a Waters Symmetry C₁₈ 180 μm × 20 mm trap column and a 1.7 μm, 75 μm × 150 mm nanoAcquity UPLC column (45 °C) for peptide separation with the following multiplexed groups: Set 1 with 6 plex containing α4WT1, α4HET1, α4KO1, β2WT1, β2HET1 and β2KO1; Set 2 with 6 plex containing α4WT2, α4HET2, α4KO2, β2WT2, β2HET2 and β2KO2, and Set 3 with 6 plex containing α4WT3, α4HET3, α4KO3, β2WT3, β2HET3 and β2KO3.

Ligand binding and immunoprecipitation

Ligand binding results were transformed from counts per minute (CPM) to fmol of bound ligand for all quantitations. To determine the maximally effective ratio of mAb bead volume for quantitative immunoprecipitation, the average depletion of nicotinic binding sites relative to the input was calculated using a 3-component hyperbolic equation $y = y_0 + a(x)/[b + (x)]$, where “y” is bound ligand captured with residual binding “y₀”, maximal depletion “a” at ½ maximal [bead] “b” and [mAb] “x”. Curve fits were performed with the aforementioned equation in SigmaPlot 2001. Statistical significance of the influence of genotype and/or antibody capture efficiency was determined by Student’s t-test or a one-way ANOVA (when comparing two or more groups, respectively) conducted with SPSS19 with a confidence interval set at 95%.
MRIPA in relative to an averaged control set. The ratio of each identified protein to the level of nAChR proteins was then log_2 transformed and used to measure the relative quantitative expression. Except where indicated, log_2MRIPA was used in all statistical analyses of iTRAQ data within this study. For regression analysis across biological replicates, log_2MRIPA of each protein was correlated with that of the β2 nAChR subunit. Regression and correlation analysis was conducted using SPSS 19 with a confidence interval set at 95%. The frequency distribution of identified proteins was interrogated using the Shapiro–Wilk test of normality with a confidence interval set at 95%.

Authors’ contributions

McCulare-Begley TD: experiment inception and design, principal author, sample preparation and pharmacological quantitation, and data analysis; Stone KL: preparation of samples for LC–MS/MS, data acquisition and manuscript review; Marks MJ and Grady SR: experiment design, animal production, tissue collection and manuscript review; Colangelo CM: LC–MS/MS data preparation, data analysis and manuscript review; Lindstrom JM: antibody production, experiment design and manuscript review; Picciotto MR: experiment inception and design and manuscript review. All authors read and approved the final manuscript.

Competing interests

Authors declare no competing interests.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gpb.2013.05.005.

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