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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 subunit 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

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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 be associated with β2* nAChRs were identified previously by analyzing proteins isolated from the mouse brain tissue using a β2-selective monoclonal 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 [^{3}H]-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 [^{3}H]-epibatidine binding site capture. Depletion of [^{3}H]-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 (R^2 = 0.83; Figure S1B), but the calculated maximal binding site capture was not significantly different across the four mAb295-M270 bead ratios