Published in final edited form as:

Psychopharmacology (Berl). 2020 January ; 237(1): 283–285. doi:10.1007/s00213-019-05413-x.

Specificity of a rodent α 6 nicotinic acetylcholine receptor subunit antibody

Anjelica Cardenas¹, Mina Elabd², Shahrdad Lotfipour^{1,3}

¹Department of Pharmaceutical Sciences, College of Health Sciences, University of California, Irvine, Irvine CA, USA

²Biological Sciences, University of California, Irvine, Irvine CA, USA

³Department of Emergency Medicine, School of Medicine, University of California, Irvine, Irvine, CA, USA

Abstract

Alpha(α)6-containing nicotinic acetylcholine receptors (nAChRs) have been implicated in nicotine reward and reinforcement. To date, a commercially available, validated α 6 nAChR subunit antibody has not been reported. To evaluate a commercially available neuronal α 6 nAChR subunit antibody we performed quantitative western blots on protein from the ventral tegmental area of wild type Sprague Dawley rats. As a first approach to determine the specificity of the antibody, we used a control antigen to block the α 6 antibody from binding. Next, we tested the antibody in brain tissue of wild type and α 6 knockout (KO) C57BL/6J mice. The α 6 antibody was present at a higher than expected molecular weight (63 versus 57 kDa) and the control antigen blocked the α 6 antibody, suggesting specificity. However, when we genetically validated the antibody, bands were present in both α 6 KO mice and C57BL/6J samples. Taken together, our study highlights the necessity to genetically validate antibodies when possible and we report that a commercially available α 6 nAChR subunit antibody is non-specific.

Keywords

CHRNA6; Reward; Nicotine; Addiction; Tobacco; Dopamine; Cigarettes; Ventral Tegmental Area; Nucleus Accumbens; Reinforcement

Neuronal nicotinic acetylcholine receptors (nAChRs) interact with the brain's endogenous ligand, acetylcholine, and the exogenous ligand, nicotine. Neuronal nAChRs regulate mood, memory, cognition, and reward. A subset of nAChRs contain the α 6 subunit (encoded

Correspondence should be addressed to: Shahrdad Lotfipour, Ph.D., Assistant Professor, Principal Investigator, 303 Med Surge II, Irvine, CA. 92697-4625, shahrdad@uci.edu.

Author Contributions: AC and SL conceived the study design and supervised data collection. All authors conducted data analyses, contributed to manuscript writing, conducted a literature review, and contributed to the critical revision of data analysis. Authors approved the final version of the manuscript.

Conflict of Interest: None.

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by the *CHRNA6* gene). $\alpha 6$ -containing nAChRs have restricted expression patterns within the central nervous system and make up pentameric nAChR populations of varying compositions ($\alpha 4\alpha 6\beta 2^*$, $\alpha 6\beta 2\beta 3^*$ and $\alpha 4\alpha 6\beta 2\beta 3^*$, *denotes possible other subunits)(Gotti et al. 2009). $\alpha 6^*$ nicotinic receptors are expressed on the terminals of dopaminergic neurons in the striatum, and reach peak mRNA expression in dopaminergic cell bodies in the ventral tegmental area (VTA) and substantia nigra during adolescence (Azam et al. 2007; Champitiaux et al. 2002; Le Novère et al. 1996; Quik et al. 2011; Yang et al. 2009). Localization of the $\alpha 6$ nAChR subunit to the mesocorticolimbic and nigrostriatal pathways implicates a role of $\alpha 6^*$ nAChRs role in nicotine reinforcement and reward as well as motor control (Bruijnzeel and Markou 2004; Exley et al. 2008; Gotti et al. 2010; Jackson et al. 2009; Laviolette and van der Kooy 2003; Pons et al. 2008). As research begins to further elucidate $\alpha 6^*$ nAChR pharmacology, function, and its behavioral role in learning, memory, and addiction, we are limited in our tools to study the receptor complexes at a protein level. $\alpha 6$ nAChR expression has previously been detected via *in situ* hybridization (mRNA), genetic approaches or by labeled selective antagonists, α -conotoxinMII (α -CtxMII) and PIA (Drenan et al., 2008; Yang et al. 2009). However, a general, validated antibody, selective for $\alpha 6$ nAChR subunits would be a powerful tool to study receptor expression and function.

To date, a commercially available and validated $\alpha 6$ nAChR subunit antibody has not been reported. Previous studies assessing the specificity of $\alpha 3$, $\alpha 4$ and $\alpha 7$ nAChR subunit antibodies have challenged the specificity of these antibodies. Data highlights that the immunoreactivity for the antibody binding of these nAChR subunits is equivalent in wild-type and nAChR subunit knock-out (KO) animals (Moser et al. 2007). Thus, the purpose of this study is to validate the specificity of the commercially available polyclonal $\alpha 6$ nAChR subunit antibody from Alomone Labs (cat. #: ANC-006, Jerusalem, Israel). In order to detect whether we can quantify $\alpha 6$ nAChR subunit protein expression in wild type (WT) Sprague Dawley rats and C57BL/6J mice we used quantitative western blot. The $\alpha 6$ nAChR subunit antibody used in this study is from a rabbit source, with rat and mouse reactivity, and was shown to bind to $\alpha 6$ nAChR subunit protein in rat PC12 pheochromocytoma cells as well as rodent brain lysates (Alomone). Although, a control antigen (Alomone) blocks the $\alpha 6$ nAChR subunit antibody from binding in the PC12 pheochromocytoma cells, a more standard form of validation is necessary (Uhlen et al. 2016). Thus, the aim of our current studies is to use a genetic approach, to assess $\alpha 6$ nAChR subunit protein expression with the $\alpha 6$ nAChR subunit antibody in wildtype versus $\alpha 6$ KO C57BL/6J mice.

As a first approach, we initially set out to develop a protocol to quantify $\alpha 6$ nAChR subunits within the VTA of male Sprague Dawley rats. The VTA is an important structure within the mesolimbic pathway that plays a role in mediating reward, motivation, and attention (Spanagel and Weiss 1999). This pathway is composed of dopaminergic neurons that originate in the VTA and innervate the limbic system including the nucleus accumbens (Di Chiara and Imperato 1988). Although the VTA is rich in $\alpha 6^*$ nAChRs, quantifying protein expression of $\alpha 6$ nAChR subunits in the VTA is particularly challenging, given the $\alpha 6$ nAChR subunit is expressed in very low quantities in the brain. Despite lower levels of expression, we observed protein expression at 63 kDa, a higher molecular weight (MW) than the expected 57 kDa (Consortium 2018)(Figure 1A). The slightly higher observed MW (63 versus 57 kDa) found in our studies may be due to post-translational modifications. The $\alpha 6$

nAChR subunit has multiple sites for post-transcriptional modifications such as glycosylation (Asparagine-55) and phosphorylation (Serine-401), which mediate subunit folding, assembly and trafficking (Alexander et al. 2010; Consortium 2018). We also found that the control antigen blocked the $\alpha 6$ nAChR antibody reactivity in our quantitative western blots, illustrated by an absence of an observed band in the presence of the $\alpha 6$ nAChR subunit antibody suggesting that our antibody was detecting $\alpha 6$ nAChR subunits (Figure 1).

We then tested the $\alpha 6$ nAChR subunit antibody on brain tissue from $\alpha 6$ KO mice on a C57BL/6J background (Champtiaux et al. 2002). These mice were generated by the deletion of the first two exons of the *Chrna6* gene which encode the ATG initiator codon, the signal peptide, and the N-terminal extracellular domain of the subunit (Champtiaux et al. 2002). Since the $\alpha 6$ nAChR antibody targets the extracellular N-terminus (amino acid residues 35–47) of the $\alpha 6$ nAChR subunit, we hypothesized that the $\alpha 6$ nAChR subunit band will be absent in $\alpha 6$ KO mice. Contrary to our prediction, we observed bands in $\alpha 6$ KO mice at the previously observed 63 kDa MW (Figure 2A). The control antigen blocked the $\alpha 6$ nAChR subunit antibody in $\alpha 6$ KO and C57BL/5J mice (Figure 2A–B). No between main effect was observed for genotype. We observed a within effect for antigen ($F_{1,6}=172.34$; $p<0.0001$). Post-hoc comparisons illustrate a significant decrease in protein expression in the presence of the antigen for WT ($p=0.0007$) and $\alpha 6$ KO mice ($p=0.004$) (Figure 2B). Taken together, our results support the conclusion that the commercially available $\alpha 6$ antibody in our study is nonspecific for $\alpha 6$ nAChR subunits.

The limited availability of highly specific antibodies for $\alpha 6$ nAChR subunit makes it necessary to test in the appropriate tissue and validate them prior to conducting further experiments. By using a genetic control, we found that this antibody is nonspecific for $\alpha 6$ nAChR subunits. These issues are likely persistent across vendors and with other commercially available nAChR subunit antibodies (Moser et al. 2007). A review of available rodent $\alpha 6$ nAChR antibodies (Supplementary Table 1) suggest that a large majority target the N-terminus or an unspecified portion of the human CHRNA6 protein. Commercially available $\alpha 6$ nAChR subunit- and species-specific antibodies may need to be developed to target alternative regions such as the COOH terminal or the CYT loop of the $\alpha 6$ nAChR subunit (Vailati et al. 1999). Ultimately, the development of validated $\alpha 6$ nAChR antibodies is necessary to assist in better understanding $\alpha 6^*$ nAChR disorders, such as nicotine/tobacco addiction and Parkinson's disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We would like to thank Professor M. Imad Damaj and Bryan McKiver for their assistance in providing us with the $\alpha 6$ nAChR knockout mice brain tissue used in our current studies.

Funding: This work was supported by the Institute for Clinical and Translational Science Pilot Studies Program, National Center for the Advancement of Clinical Science (SL); UCI School of Medicine start up fund (SL); and by

the 2018–2019 Undergraduate Research Opportunities Program Fellowship from the University of California, Irvine [96052s1] (ME, SL). AC is supported by the Ford Foundation Predoctoral Fellowship.

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Figure 1:

Evaluation of an alpha(α)6 nicotinic acetylcholine receptor subunit antibody. A western blot of $\alpha 6$ nAChR subunit expression and antigen block in bilateral ventral tegmental tissue punches collected from male Sprague Dawley rats. n=2 animals total. GAPDH is used as a loading control.

Figure 2:

Genetic validation of an alpha(α)6 nicotinic acetylcholine receptor subunit antibody. A.) Western blot and B.) relative band intensity quantification of $\alpha 6$ nAChR subunit expression and antigen block from whole brain tissue collected from male and female WT and $\alpha 6$ KO C57BL/6J mice, n=4 animals/genotype; **p< 0.01 and ***p< 0.001 $\alpha 6$ antibody vs. $\alpha 6$ antibody + antigen normalized signal. GAPDH is used as a loading control.