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Specificity of a rodent alpha(α)6 nicotinic acetylcholine receptor subunit antibody

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Abstract

Alpha(α)6-containing nicotinic acetylcholine receptors (nAChRs) have been implicated in nicotine reward and reinforcement. To date, a commercially available, validated α6 nAChR subunit antibody has not been reported. To evaluate a commercially available neuronal α6 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 α6 antibody from binding. Next, we tested the antibody in brain tissue of wild type and α6 knockout (KO) C57BL/6J mice. The α6 antibody was present at a higher than expected molecular weight (63 versus 57 kDa) and the control antigen blocked the α6 antibody, suggesting specificity. However, when we genetically validated the antibody, bands were present in both α6 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 α6 nAChR subunit antibody is non-specific.

Keywords

*CHRNA*6; 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 alpha(α)6 subunit (encoded

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Conflict of Interest: None.

by the *CHRNA*6 gene). α6-containing nAChRs have restricted expression patterns within the central nervous system and make up pentameric nAChR populations of varying compositions (α4α6β2*, α6β2β3* and α4α6β2β3*, *denotes possible other subunits)(Gotti et al. 2009). α6* 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; Champtiaux et al. 2002; Le Novere et al. 1996; Quik et al. 2011; Yang et al. 2009). Localization of the α6 nAChR subunit to the mesocorticolimibic and nigrostriatal pathways implicates a role of α6* 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 α6* 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. α6 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 α6 nAChR subunits would be a powerful tool to study receptor expression and function.

To date, a commercially available and validated α6 nAChR subunit antibody has not been reported. Previous studies assessing the specificity of α3, α4 and α7 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 α6 nAChR subunit antibody from Alomone Labs (cat. #: ANC-006, Jerusalem, Israel). In order to detect whether we can quantify α6 nAChR subunit protein expression in wild type (WT) Sprague Dawley rats and C57BL/6J mice we used quantitative western blot. The α6 nAChR subunit antibody used in this study is from a rabbit source, with rat and mouse reactivity, and was shown to bind to α6 nAChR subunit protein in rat PC12 pheochromocytoma cells as well as rodent brain lysates (Alomone). Although, a control antigen (Alomone) blocks the α6 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 α6 nAChR subunit protein expression with the α6 nAChR subunit antibody in wildtype versus α6 KO C57BL/6J mice.

As a first approach, we initially set out to develop a protocol to quantify α6 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 α6* nAChRs, quantifying protein expression of α6 nAChR subunits in the VTA is particularly challenging, given the α6 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 α6

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 α6 nAChR antibody reactivity in our quantitative western blots, illustrated by an absence of an observed band in the presence of the α6 nAChR subunit antibody suggesting that our antibody was detecting α6 nAChR subunits (Figure 1).

We then tested the α6 nAChR subunit antibody on brain tissue from α6 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 *Chrna*6 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 α6 nAChR antibody targets the extracellular N-terminus (amino acid residues 35–47) of the α6 nAChR subunit, we hypothesized that the α6 nAChR subunit band will be absent in α6 KO mice. Contrary to our prediction, we observed bands in α6 KO mice at the previously observed 63 kDa MW (Figure 2A). The control antigen blocked the α6 nAChR subunit antibody in α6 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 α6 KO mice (p=0.004) (Figure 2B).Taken together, our results support the conclusion that the commercially available α6 antibody in our study is nonspecific for α6 nAChR subunits.

The limited availability of highly specific antibodies for α6 nAChR subunit makes it necessary to test in the apporpriate tissue and validate them prior to conducting further experiments. By using a genetic control, we found that this antibody is nonspecific for α6 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 α6 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 α6 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 α6 nAChR subunit (Vailati et al. 1999). Ultimately, the development of validated α6 nAChR antibodies is necessary to assist in better understanding α6* nAChR disorders, such as nicotine/tobacco addiction and Parkinson’s disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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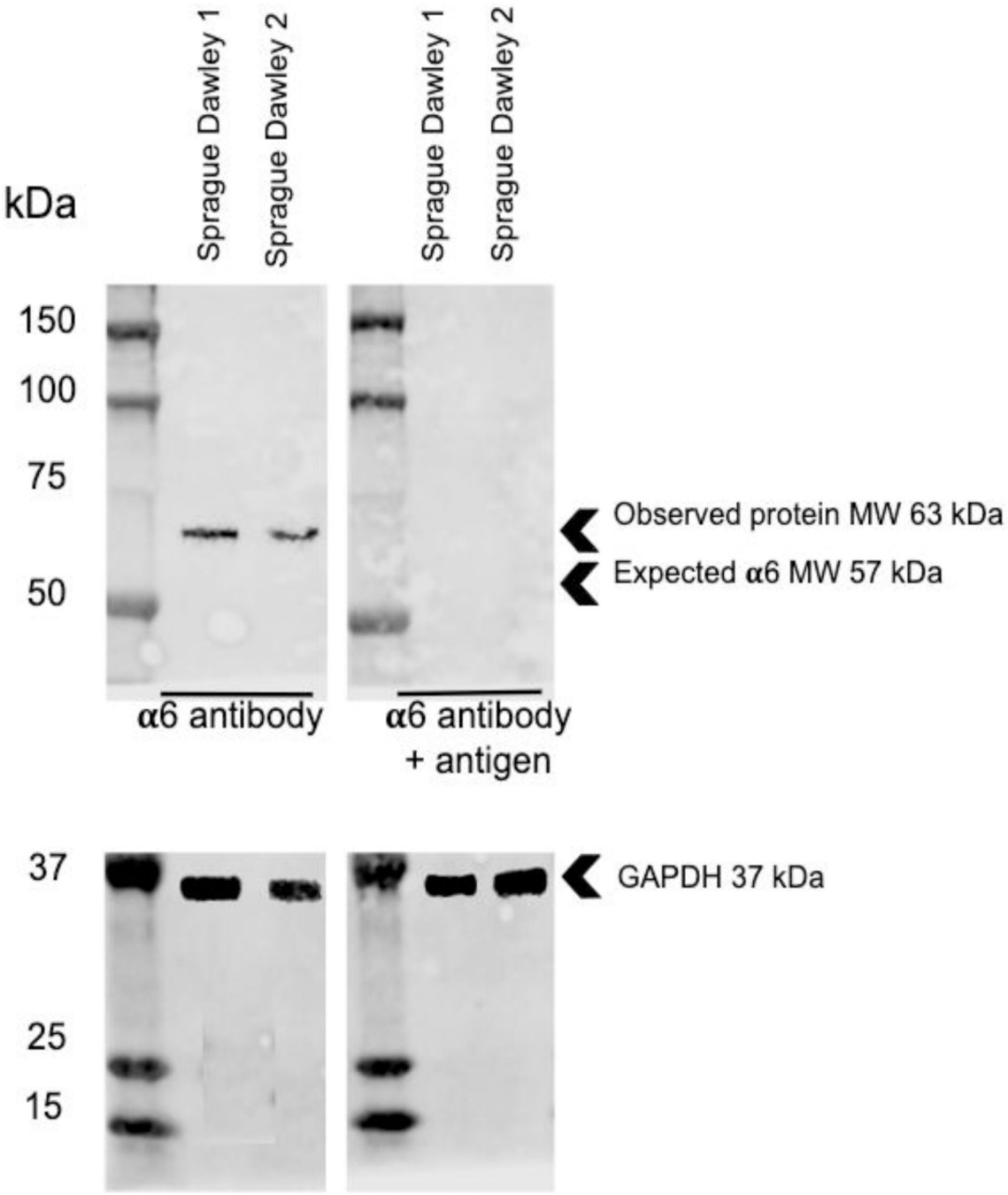

Figure 1:

Evaluation of an alpha(α)6 nicotinic acetylcholine receptor subunit antibody. A western blot of α6 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.

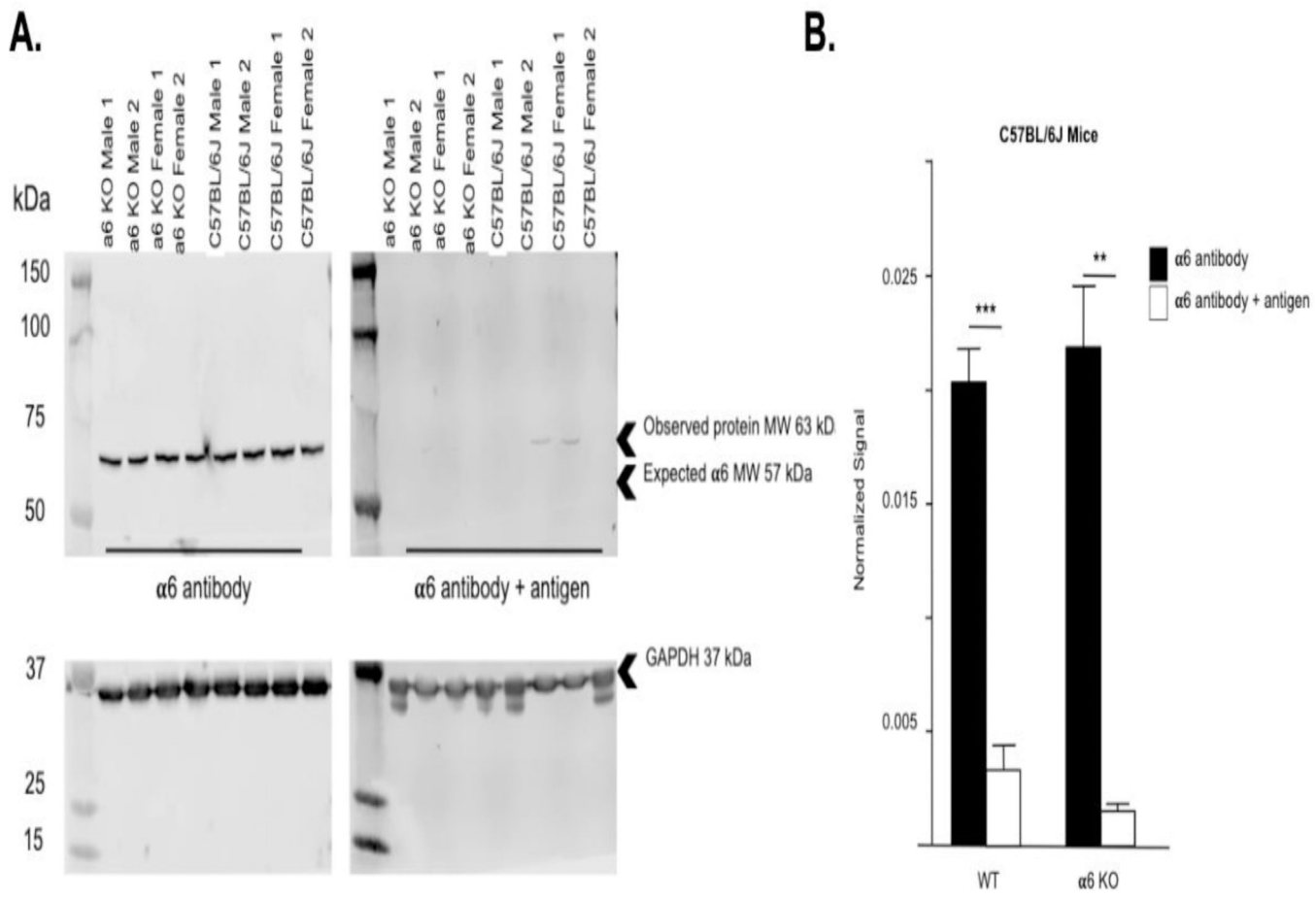

Figure 2:

Genetic validation of an alpha(α)6 nicotinic acetylcholine receptor subunit antibody. A.) Western blot and B.) relative band intensity quantification of α6 nAChR subunit expression and antigen block from whole brain tissue collected from male and female WT and α6 KO C57BL/6J mice, n=4 animals/genotype; **p< 0.01 and ***p< 0.001 α6 antibody vs. α6 antibody + antigen normalized signal. GAPDH is used as a loading control.