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**Title Page**

**The ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> stoichiometry of the nicotinic acetylcholine receptor predominates in the rat motor cortex**

Kristen E. DeDominicis, Niaz Sahibzada, Thao T. Olson, Yingxian Xiao, Barry B. Wolfe, Kenneth J. Kellar, and Robert P. Yasuda

Department of Pharmacology and Physiology, Georgetown University School of Medicine, Washington, DC 20057 (K.E.D, N.S., T.T.O., Y.X., B.B.W., K.J.K., R.P.Y.)

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**Running Title:** The ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChR predominates in the rat motor cortex

**Corresponding Authors:**

Robert P. Yasuda and Kenneth J. Kellar

Address: Department of Pharmacology and Physiology, Georgetown University Medical Center,  
3900 Reservoir Road NW, Washington, DC 20057

Tel.: 202-687-8189; Fax: 202-687-8825

E-mail: yasudar@georgetown.edu and kellark@georgetown.edu

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**Abbreviations:**

nAChR: nicotinic acetylcholine receptors

HS: high sensitivity ( $\alpha 4$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub> nAChR

LS: low sensitivity ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChR

DH $\beta$ E: dihydro- $\beta$ -erythroidine

Saz-A: Sazetidines-A

MLA: Methyllycaconitine

CARB: Carbachol.

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### Abstract

The  $\alpha 4\beta 2$  nicotinic acetylcholine receptor (nAChR) is important in CNS physiology and in mediating several of the pharmacological effects of nicotine on cognition, attention, and affective states. It is also the likely receptor that mediates nicotine addiction. This receptor assembles in two distinct stoichiometries:  $(\alpha 4)_2(\beta 2)_3$  and  $(\alpha 4)_3(\beta 2)_2$  referred to as high (HS) and low (LS) sensitivity nAChRs, respectively, based on a difference in the potency of acetylcholine to activate them. The physiological and pharmacological differences between these two receptor subtypes have been described in heterologous expression systems. However, the presence of each stoichiometry in native tissue remains unknown. In this study, different ratios of rat  $\alpha 4$  and  $\beta 2$  subunit cDNA were transfected into HEK293 cells to create a novel model system of HS and LS  $\alpha 4\beta 2$  nAChRs expressed in a mammalian cell line. The HS and LS nAChRs were characterized through pharmacological and biochemical methods. Isolation of surface proteins revealed higher amounts of  $\alpha 4$  or  $\beta 2$  subunits in the LS or HS nAChR populations, respectively. Additionally, sazetidine-A displayed different efficacies in activating these two receptor stoichiometries. Using this model system, a neurophysiological ‘two-concentration’ acetylcholine or carbachol paradigm was developed and validated to determine  $\alpha 4/\beta 2$  subunit stoichiometry. This paradigm was then utilized in layers I-IV of slices of rat motor cortex to determine the percent contribution of high and low sensitivity  $\alpha 4\beta 2$  receptors in this brain region. We report that the majority of  $\alpha 4\beta 2$  nAChRs in this brain region possess a stoichiometry of the  $(\alpha 4)_3(\beta 2)_2$  LS subtype.

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### Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated cation channels composed of different combinations of  $\alpha$  ( $\alpha 2$ -10) and  $\beta$  ( $\beta 2$ -4) subunits. Different combinations of subunits define nAChR receptor subtypes and confer distinguishing biophysical and pharmacological characteristics to the receptor. The  $\alpha 4\beta 2$  nAChRs are the predominant heteromeric nAChRs in the mammalian brain (Whiting and Lindstrom, 1987; Flores et al., 1992; Mao et al., 2008) and are thought to mediate important physiological actions of acetylcholine and pharmacological effects of nicotine related to attention and cognition (Picciotto et al., 1995; Guillem et al., 2011; Lozada et al., 2012; Paolone et al., 2013; Oda et al., 2014) and affective states (Mineur and Picciotto, 2010; Turner et al., 2010; Anderson and Brunzell, 2012; Hussmann et al., 2014; Anderson and Brunzell, 2015). In addition to their roles in normal CNS physiology, considerable evidence points to the involvement of these receptors in nicotine addiction and dependence (Marks et al., 1983; Schwartz and Kellar, 1983; Benwell et al., 1988; Flores et al., 1992; Flores et al., 1997; Perry et al., 1999; Staley et al., 2006; Wüllner et al., 2008; Marks et al., 2011). Because of the wide-ranging role these receptors play in the CNS, understanding their structure in native tissue is particularly important.

Although  $\alpha 4\beta 2$  nAChRs may include additional subunits, e.g.,  $\alpha 4\beta 2\alpha 5$  (Mao et al., 2008), the simpler receptor complex composed only of  $\alpha 4$  and  $\beta 2$  subunits appears to predominate in most rat brain regions (Perry et al., 2007; Mao et al., 2008). Early work with chick  $\alpha 4\beta 2$  nAChRs heterologously expressed in oocytes suggested that these receptors adopted a stoichiometry of  $(\alpha 4)_2(\beta 2)_3$  (Anand et al., 1991). However, subsequent experiments that altered the proportion of  $\alpha 4$  and  $\beta 2$  subunits expressed in oocytes by injecting different amounts of subunit cDNA demonstrated that in heterologous expression systems  $\alpha 4\beta 2$  nAChRs can form

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with two distinct stoichiometries (Zwart and Vijverberg, 1998; Nelson et al., 2003). The two stoichiometric isoforms,  $(\alpha 4)_2(\beta 2)_3$  and  $(\alpha 4)_3(\beta 2)_2$ , have been called high sensitivity (HS) and low sensitivity (LS), respectively, based on the potency of acetylcholine to activate these receptor subtypes (Moroni et al., 2006).

In addition to their different sensitivities to ACh, other important differences between HS and LS  $\alpha 4\beta 2$  nAChRs have been observed, including: 1) different potencies and efficacies of nicotine and sazetidine-A, (Moroni et al., 2006; Zwart et al., 2008; Carbone et al., 2009) and 2) differences in calcium conductance through the channel, with the LS passing more calcium than the HS receptor (Tapia et al., 2007). Furthermore, single channel recordings in heterologous cells indicate high and low conductance states of  $\alpha 4\beta 2$  nAChRs, which might be attributable to HS and LS receptors, although no consensus had been reached as to which stoichiometry corresponds to which conductance level (Buisson and Bertrand, 2001; Nelson et al., 2003). Indeed, a recent study revealed a low conductance channel (29 pS) corresponding to the HS receptor and a high conductance channel (44 pS) corresponding to the LS receptor (Carignano et al., 2016). Interestingly, in addition to the receptor's canonical orthosteric agonist binding site at the  $\alpha 4/\beta 2$  interface, it appears that activation of an  $\alpha 4/\alpha 4$  binding site, which is present only in the LS nAChR that possesses the  $(\alpha 4)_3(\beta 2)_2$  stoichiometry (Harpsøe et al., 2011; Mazzaferro et al., 2011; Wang et al., 2015), contributes to some of the differences between HS and LS receptor subtypes, including greater efficacy of some agonists (Harpsøe et al., 2011; Wang et al., 2015) and decreased desensitization (Benallegue et al., 2013; Eaton et al., 2014).

While both stoichiometries of the  $\alpha 4\beta 2$  nAChR assemble in heterologous expression systems, their presence in native tissue is not known with certainty. Some evidence suggests that both subtypes may assemble in the brain. For example, previous studies have demonstrated that

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mouse brain synaptosomes have a biphasic response to ACh with a high and low sensitivity component, each phase of which is substantially reduced upon knockout of the  $\alpha 4$  or  $\beta 2$  subunits (Marks et al., 1999; Marks et al., 2007).

We present here a model of non-linked, HS and LS rat  $\alpha 4\beta 2$  nAChRs expressed in mammalian cells. Using this model, we propose a method that exploits the differences in sensitivity between the two stoichiometric isoforms of the receptor to determine the contribution of HS or LS nAChR subtypes in rat motor cortex. We report that a majority of  $\alpha 4\beta 2$  nAChRs found in the rat motor cortex exhibits a response to ACh that is indicative of the LS  $(\alpha 4)_3(\beta 2)_2$  nAChRs.

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### **Materials and Methods**

#### ***Materials***

HEK293 cells, Dulbecco's Modified Eagle's Media (DMEM), Lipofectamine™ and Plus™ Reagent were purchased from Life Technologies, Grand Island, NY. Primary antibodies against the nAChR  $\alpha 4$  subunit (sc-1772) or the nAChR  $\beta 2$  subunit (sc-11372) were purchased from Santa Cruz Biotechnology, Dallas, TX. Polyclonal antibodies against the nAChR  $\alpha 3$  and  $\alpha 5$  subunits were produced as described previously (Yeh et al., 2001; Lomazzo et al., 2011). Carbachol (CARB, carbamylcholine chloride), atropine sulfate, acetylcholine chloride, dihydro- $\beta$ -erythroidine (DH $\beta$ E), Thesit, phosphatidylcholine, poly-D-lysine, and primary antibodies against  $\beta$ -actin (A5316), the nAChR  $\beta 2$  subunit (mAb290) and the nAChR  $\alpha 4$  (mAb299) were purchased from Sigma-Aldrich, St. Louis, MO. Secondary antibodies (800CW 926-32214 and 680LT 926-68023) and blocking buffer were purchased from LI-COR Biosciences, Lincoln, NE. NeutrAvidin Agarose Resin, Protein G Ultralink and EZ-Link Sulfo-NHS-LC-LC-Biotin were purchased from ThermoFisher Scientific, Pittsburgh, PA. Sazetidine-A (Saz-A) was obtained from RTI International, Research Triangle Park, NC. Methyllcaconitine (MLA) was supplied by NIDA, Bethesda, MD.

#### ***Cell Culture and Transfection***

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum. Cells were plated and transiently transfected with rat  $\alpha 4$  and  $\beta 2$  nAChR subunit cDNA using Lipofectamine™ and the Plus™ Reagent. Cells used for biotinylation experiments were plated in 100 mm dishes to ~90% confluency and transfected the following day with  $\alpha 4$  and  $\beta 2$  nAChR subunit cDNA in a molar ratio of 1:6 or 4:1  $\alpha 4$ : $\beta 2$  to generate high sensitivity (HS) or low sensitivity (LS)  $\alpha 4\beta 2$  nAChRs, respectively. The rat  $\alpha 4$  and  $\beta 2$  nAChR

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subunit plasmid cDNA construction has been previously described (Xiao and Kellar, 2004). Cells used for electrophysiology recording experiments were plated at low density on poly-D lysine coated coverslips using a flame-polished pipette. The next day, these cells were co-transfected with GFP and ( $\alpha 4 + \beta 2$ ) subunit cDNA in a molar ratio of 1:4 GFP:( $\alpha 4 + \beta 2$ ), in which the relative molar ratios of  $\alpha 4$  and  $\beta 2$  subunit cDNA were 1:6 (HS  $\alpha 4\beta 2$  nAChR) or 4:1 (LS  $\alpha 4\beta 2$  nAChR). To up-regulate receptors for both biotinylation and electrophysiology experiments, the HS  $\alpha 4\beta 2$  nAChRs were cultured in the presence of 1 mM carbachol + 10  $\mu$ M atropine while the LS  $\alpha 4\beta 2$  nAChRs were cultured in the presence of 1 mM carbachol.

### ***Cell Surface Labeling***

Surface proteins of transfected HEK293 cells were biotinylated 24 h after transfection. Cells were washed 3 times with PBS buffer (in mM: 1.1 potassium phosphate monobasic, 155 sodium chloride, 3 sodium phosphate dibasic; pH 8; 37°C) then incubated in Versene (37°C) and gently transferred to 15 mL conical tubes. Cells were spun at ~500g and gently resuspended in ice cold PBS then placed on ice. The biotinylating reagent (EZ-Link Sulfo-NHS-LC-LC-Biotin) at a final concentration of 1.7 mM was added to the cell suspension and incubated for 20 min on ice with gentle rotation. The reaction was quenched with three washes of 100 mM glycine. Cell membrane homogenates were isolated by sonication in TEE buffer (in mM: 10 Tris, 5 EDTA and 5 EGTA; pH 7.4) and centrifuged at 30,000g at 4°C for 15 min. The membrane pellet was resuspended in TEE buffer.

### ***Isolation of Biotin-Labeled Cell Surface Proteins and Immunoprecipitation***

Biotin-labeled HEK293 membrane homogenates were solubilized in TEE buffer containing 1% Thesit, 1.8 mM phosphatidylcholine, and 0.05% SDS for 4-6 h at 2-8°C with gentle rotation. Cortical brain tissue homogenates were solubilized in TEE buffer containing 2%



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Triton-X, 1.8 mM phosphatidylcholine, and 0.05% SDS for 4-6 h at 2-8°C with gentle rotation. Samples were then centrifuged at 30,000g for 30 min at 4°C to pellet the membrane fraction. Solubilized proteins in the supernatant were added to NeutrAvidin agarose resin or mAb299 ( $\alpha 4$  selective) or mAb290 ( $\beta 2$  selective) antibodies covalently coupled to Protein G resin as described previously (Husmann et al., 2014). Samples were then mixed, and incubated at 2-8°C with gentle rotation overnight. NeutrAvidin-biotin complexes and immunoprecipitations were pelleted at 1000g and washed 3 times with 1% Thesit in TEE buffer. The final pellet was suspended in Laemmli loading buffer (Laemmli, 1970) for subsequent analysis by Western blot.

### ***Western blots***

Denatured protein samples were separated by SDS-PAGE on 9% polyacrylamide gels. Proteins were transferred to PVDF-FL membranes and then blocked using the LI-COR Odyssey blocking buffer diluted 2x with PBS. Blocked membranes were probed with primary antibodies against the  $\alpha 4$  nAChR subunit (1  $\mu\text{g/ml}$ ),  $\beta 2$  nAChR subunit (1  $\mu\text{g/ml}$ ),  $\alpha 3$  nAChR subunit (1  $\mu\text{g/ml}$ ),  $\alpha 5$  nAChR subunit (1  $\mu\text{g/ml}$ ), or  $\beta$ -actin (1:5,000) and followed with secondary antibodies. All membranes were imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences). Bands for the  $\alpha 4$  and  $\beta 2$  subunits were imaged simultaneously from the same sample lane on a single membrane using dual color detection. Bands for  $\beta$ -actin were visualized on separate membranes. The  $\alpha 3$  and  $\alpha 5$  nAChR subunits were visualized on membranes containing controls with either stably transfected rat  $\alpha 4\beta 2$  nAChRs or rat  $\alpha 3\beta 4$  nAChRs, and transiently transfected rat  $\alpha 4\beta 2\alpha 5$  nAChRs in HEK cells.

### ***Preparation of Brain Slices***

Brain slices were obtained from P16 – P24 male Sprague Dawley rat pups. Animals were quickly decapitated and the brains were removed and transferred to ice cold artificial

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cerebrospinal fluid (ACSF) containing (in mM): 121 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 glucose, 5 HEPES and 3 *myo*-inositol which was continuously oxygenated with 95% O<sub>2</sub> + 5% CO<sub>2</sub>. Coronal brain slices (250 μm) were cut from the motor cortex region (~2.2 mm to bregma) using a vibratome (Leica VT1000S) and incubated for 30 min at 37°C in oxygenated ACSF followed by 30 min at room temperature (21°C) until used for recording. All procedures were performed according to the National Institutes of Health (U.S.A.) guidelines for the ethical use of animals in research and were approved by the Georgetown University Animal Care and Use Committee.

### *Preparation of HEK293 Cells for Electrophysiology Experiments*

Transiently transfected HEK293 cells expressing α4β2 nAChRs were used in experiments 24 h post-transfection. Coverslips were washed 6 times in fresh culture media to remove up-regulating agents and then incubated for at least 1 h at 37°C until use. Extracellular solution for recording consisted of (in mM): 130 NaCl; 5 KCl; 2 CaCl<sub>2</sub>; 2 MgCl<sub>2</sub>; 10 glucose; and 10 HEPES, pH 7.4.

### *Electrophysiology Experiments*

Coverslips containing transfected α4β2 HEK293 cells or brain slices from the motor cortex were transferred to a recording chamber (500 μL volume) attached to the stage of a Nikon E600-FN microscope where they were continuously perfused with the appropriate extracellular ACSF solutions listed above. The ACSF solutions were corrected for osmolarity to ~296 mOsm/l.

Recordings were made with patch electrodes (4-6 MΩ) pulled from borosilicate glass and filled with an internal recording solution containing (in mM): 145 K gluconate, 5 EGTA, 2.5 MgCl<sub>2</sub>, 10 HEPES, 5 ATP·Na, and 0.2 GTP·Na, pH 7.2, adjusted to 285 mOsm/l. Cells were

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identified visually by infrared-differential interference contrast (IR-DIC) and fluorescence optics via a CCD camera (Dage S-75). A 60X-water immersion objective (Nikon) was used to approach cells and neurons. Whole cell voltage clamp recordings of HEK293 cells and neurons ( $V_{\text{hold}} = -60$  mV) were obtained using a Multiclamp 700B (Molecular Devices). Signals, acquired at 20 kHz, were low passed filtered at 2 kHz, digitized (Digidata 1440A; Molecular Devices) and stored on a PC computer for subsequent analysis. Prior to recording agonist-induced whole-cell currents, pipette and cell capacitance was minimized and series resistance ( $<12 \text{ M}\Omega$ ) was compensated via the 'MultiClamp Commander' software (Axon instruments Inc.). Input and access resistances were monitored by a 5 mV hyperpolarization pulse throughout the recording session.

### *Drug Solutions and Application*

Ligands for application during electrophysiology experiments (ACh, CARB, Saz-A, DH $\beta$ E, MLA) were prepared in extracellular solutions (i.e., for HEK293 cells or brain slices). Atropine (1  $\mu\text{M}$ ) was added to all drug solutions to block possible long-term effects from activation of muscarinic receptors.

For ACh concentration-response curves and Saz-A experiments, agonists were applied by a 'y-tubing' positioned above the recording cell. For experiments examining the response to 10  $\mu\text{M}$  and 1000  $\mu\text{M}$  ACh or 32  $\mu\text{M}$  and 1000  $\mu\text{M}$  CARB, agonists were applied by two borosilicate glass pipettes located  $\sim 5\text{-}20 \mu\text{m}$  from the cell body using a picospritzer (one pipette for each concentration). In HEK293 cells at concentrations of ACh or CARB  $\leq 32 \mu\text{M}$ , an inter-stimulus interval (ISI) of at least 5 min was necessary to allow receptors to recover from desensitization, while an ISI of 10 min was required for ACh or CARB concentrations  $> 32 \mu\text{M}$ . In brain slice experiments, a 5 min ISI was sufficient to allow receptors to recover from desensitization between subsequent applications with ACh.

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Antagonists (MLA and DH $\beta$ E) were applied via bath application. The concentration of 10 nM MLA was chosen because it is routinely used in the literature to block responses from  $\alpha 7$  nAChRs (Christophe et al., 2002; Sahibzada et al., 2002; Xiao et al., 2009; Alves et al., 2010). In some experiments, MLA 10 nM was directly added to the ACSF that contained 1  $\mu$ M atropine. A concentration of 10  $\mu$ M DH $\beta$ E was used to inhibit both high and low sensitivity  $\alpha 4\beta 2$  nAChRs (Moroni et al., 2006).

### ***Data Analysis***

All data are presented as mean  $\pm$  standard error (SE). Quantification for electrophysiology experiments was based on peak amplitude values, which were obtained using Clampfit 10.2 software. Concentration-response curves were fit using the GraphPad Prism 5 equation: “log(agonist) vs. response -- Variable slope (four parameters)” and the reported EC<sub>50</sub> values were calculated from this equation. Differences between the HS and LS  $\alpha 4\beta 2$  nAChRs were compared statistically with an unpaired t-test. A value of  $p < 0.05$  was considered significant.

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### Results

#### *Altering transfection ratios of $\alpha 4$ and $\beta 2$ subunit cDNA into HEK293 cells favors incorporation of more $\alpha 4$ or $\beta 2$ subunit proteins at the cell surface*

Altering injection ratios of  $\alpha 4$  and  $\beta 2$  subunit cDNA or cRNA into *X. laevis* oocytes produces either HS or LS nAChRs (Moroni et al., 2006; Harpsøe et al., 2011). We hypothesized that altering transfection ratios of  $\alpha 4$  and  $\beta 2$  subunit cDNA into HEK293 cells would do the same. We initially biotinylated surface proteins to examine the relative proportion of  $\alpha 4$  and  $\beta 2$  subunit protein present at the cell surface after transfecting  $\alpha 4$  and  $\beta 2$  nAChR subunit cDNA at molar ratios of 1:6 or 4:1 to create the HS ( $\alpha 4$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub> or LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChR subtype, respectively. Isolating cell surface proteins is important because only receptors reaching the outside surface can mediate neurotransmitter signaling, thus allowing us to compare the subunit composition at the surface to function. As shown in Figure 1A, altering the ratio of  $\alpha 4$  and  $\beta 2$  subunit cDNA transfected into HEK293 cells results in a proportional shift of the  $\alpha 4$  and  $\beta 2$  subunit protein that reaches the cell surface. Figure 1B and 1C presents the quantification of the  $\alpha 4$  and  $\beta 2$  subunit band intensities, respectively, in the 1:6 and 4:1 transfection ratios of  $\alpha 4$  and  $\beta 2$  subunit cDNA. It is important to note that the intensity of the  $\alpha 4$  nAChR subunit protein and  $\beta 2$  nAChR subunit protein are not directly comparable since each antibody is unique and different secondary antibodies are used for each subunit. Therefore, it is inappropriate to draw quantitative conclusions regarding receptor composition from this data alone. Western blot bands for  $\beta$ -actin (an intracellular protein) were present in membrane homogenates prior to surface protein isolation but were negligible in the surface isolated fraction, which acts as an internal control for our experiment to confirm isolation of only surface labeled proteins. Please note that the load for the NeutrAvidin pull-down of the surface protein fraction is 16 times

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greater than the whole homogenate fraction; therefore, the trace of  $\beta$ -actin observed in the NeutrAvidin pull-down is nominal.

### *The transfection ratios yield receptors with different acetylcholine sensitivity*

As shown in Figure 1, transfection ratios that favor more  $\alpha 4$  or  $\beta 2$  subunit cDNA result in receptors with more  $\alpha 4$  or  $\beta 2$  subunits, respectively, being assembled and reaching the cell surface. Next, we sought to assess if these different  $\alpha 4$ : $\beta 2$  subunit ratios would functionally resemble HS or LS  $\alpha 4\beta 2$  nAChRs as reported by others. Whole cell voltage clamp ACh concentration-response curves were generated from rat  $\alpha 4\beta 2$  nAChRs expressed in HEK293 cells transfected with  $\alpha 4$ : $\beta 2$  subunit cDNA ratios of 1:6 or 4:1. When cells with a transfection ratio of 1:6  $\alpha 4$ : $\beta 2$  cDNA were used, the concentration-response relationship fit best to a single site which displayed a high sensitivity to ACh (Fig. 2A), with an  $EC_{50}$  of 5.2  $\mu$ M (95% CI 3.7 – 7.2  $\mu$ M) and a Hill slope of 0.97 (95% CI 0.74 – 1.19). When cells with a transfection ratio of 4:1 of  $\alpha 4$ : $\beta 2$  cDNA were used, the concentration-response relationship also fit best to a single site, but the sensitivity to ACh was 10-fold lower with an  $EC_{50}$  of 56  $\mu$ M (95% CI 26 – 119  $\mu$ M) and Hill slope of 0.61 (95% CI 0.41 – 0.81). Thus, the  $\alpha 4\beta 2$  nAChRs assembled from these different transfection ratios demonstrate a clear shift in sensitivity to activation by ACh. Moreover, these  $EC_{50}$  values are similar to others that have been reported previously for the ACh high- and low-affinity nAChR sites for human  $\alpha 4\beta 2$  stably expressed in HEK293 cells (Nelson et al. 2003) and for human HS ( $\alpha 4$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub> or LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChR subtypes expressed in oocytes (Harpsøe et al., 2011). Representative traces of ACh gated currents for the HS and LS nAChRs can be found in Figure 2B and 2C.

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***Characterization of the HS and LS  $\alpha 4\beta 2$  nAChR subtypes using Sazetidine-A***

Sazetidine-A (Saz-A), a high affinity ligand for  $\alpha 4\beta 2$  nAChRs (Xiao et al., 2006), is a nearly full agonist at human HS  $\alpha 4\beta 2$  nAChRs but a very weak partial agonist at human LS  $\alpha 4\beta 2$  nAChRs (Zwart et al., 2008; Eaton et al., 2014). Here, we compared the efficacy of 1  $\mu$ M Saz-A to 32  $\mu$ M ACh at rat nAChRs in cells expressing the HS or LS  $\alpha 4\beta 2$  nAChR subtype. We used a concentration of 1  $\mu$ M Saz-A because it elicits a maximal response at both the human HS and LS  $\alpha 4\beta 2$  nAChR subtypes, though the response at the LS receptors is only ~6% of that at the HS receptor (Zwart et al., 2008). A concentration of 32  $\mu$ M ACh was used for comparison because it provided robust responses at both forms of the receptor without the prolonged recovery time necessary after activation with higher concentrations of ACh. The mean peak amplitude of whole-cell responses elicited by 1  $\mu$ M Saz-A compared to 32  $\mu$ M ACh was significantly different at the HS and LS nAChRs (Figs. 3A and 3B, respectively). In cells that expressed the HS nAChR, Saz-A stimulated  $69 \pm 16\%$  of the peak amplitude response elicited by 32  $\mu$ M ACh, while in cells that expressed the LS nAChRs, it stimulated only  $25 \pm 6\%$  of the ACh response. By extrapolating the 32  $\mu$ M ACh to 1000  $\mu$ M ACh responses specific to the appropriate HS or LS subtype, Saz-A stimulated peak amplitude responses of  $56 \pm 13\%$  (HS) or  $10 \pm 2.4\%$  (LS) compared to 1000  $\mu$ M ACh (Fig. 3C). These results are consistent with the relative response elicited by Saz-A at HS and LS human  $\alpha 4\beta 2$  nAChRs (Zwart et al., 2008).

***High and low concentrations of ACh distinguish between the HS and LS nAChRs***

To test how well ACh distinguishes between this model of HS and LS nAChRs, we measured the responses to 10  $\mu$ M and 1000  $\mu$ M ACh in cells after transfection with our two different ratios of  $\alpha 4$  and  $\beta 2$  subunits (Fig. 4). We selected these two concentrations of ACh because, as shown in Figure 2A, 1000  $\mu$ M ACh maximally activates both HS and LS receptors,

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while 10  $\mu$ M maximizes the differences in responses to ACh observed in the HS and LS receptor subtypes.

Comparing the peak amplitude response of 10  $\mu$ M ACh against 1000  $\mu$ M ACh at each of our transfection ratios showed a statistically significant difference between the HS and LS models of nAChRs. In cells transfected to yield the HS  $\alpha 4\beta 2$  nAChR subtype, the peak amplitude whole-cell current response to 10  $\mu$ M ACh was  $50 \pm 10\%$  of the response to 1000  $\mu$ M ACh; while in cells transfected to yield the LS  $\alpha 4\beta 2$  nAChR subtype, the peak response to 10  $\mu$ M ACh was only  $13 \pm 2\%$  that of the 1000  $\mu$ M ACh response (Fig. 4). Thus, based on the responses to Saz-A and to the higher and lower concentrations of ACh, transfection of cells with these ratios of  $\alpha 4$  and  $\beta 2$  subunits can reliably produce the HS and LS nAChR subtypes.

### *Assessing the stoichiometry of $\alpha 4\beta 2$ nAChRs in rat brain*

Since normalized 10  $\mu$ M to 1000  $\mu$ M ACh responses display different relative efficacy at HS and LS receptors as evidenced by data from our model of HS and LS rat  $\alpha 4\beta 2$  nAChRs in transfected cells, we sought to use this paradigm to assess the stoichiometry of  $\alpha 4\beta 2$  nAChRs in a native rat brain tissue by determining the relative percentages of the HS or LS receptors in layers I-IV of the motor cortex in a rat brain slice preparation. We chose this brain region because: 1) the motor cortex contains a high percentage of neurons responsive to nAChR agonists (Porter et al., 1999; Christophe et al., 2002), 2) more than 90% of the heteromeric nAChRs in the rat cerebral cortex are an  $\alpha 4\beta 2$  nAChR subtype (Flores et al., 1992; Mao et al., 2008), although ~15-20% of these also contain the  $\alpha 3$  or  $\alpha 5$  nAChR subunit (Mao et al., 2008), and 3) the nAChR  $\alpha 5$  mRNA is restricted to cortical layer VI (Wada et al., 1990; Marks et al., 1992). Pharmacological investigation with galanthamine also supports the presence of  $\alpha 5$ -containing nAChRs exclusively



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in cortical layer VI (Kassam et al., 2008; Poorthuis et al., 2013). Galanthamine potentiates the  $\alpha 4\beta 2$  nAChR response when the  $\alpha 5$  nAChR subunit is present (Kuryatov et al., 2008).

To further determine if  $\alpha 3$  or  $\alpha 5$  nAChR subunits may assemble with the  $\alpha 4\beta 2$  nAChRs in the motor cortex, we performed immunoprecipitation experiments with solubilized motor cortex brain tissue with antibodies specific to the  $\alpha 4$  or  $\beta 2$  nAChR subunits followed by subsequent western blotting experiments to assess the presence of  $\alpha 3$  and  $\alpha 5$  in isolated  $\alpha 4$  and  $\beta 2$  nAChR complexes. The  $\alpha 3$  subunit was not detectable following immunoprecipitation with either  $\alpha 4$  or  $\beta 2$  nAChR antibodies (Supplemental Fig. 1A), indicating that the co-assembly of this subunit into  $\alpha 4\beta 2$  nAChRs in this region is negligible. We did observe the co-assembly of the  $\alpha 5$  nAChR subunit with  $\alpha 4\beta 2$  nAChRs (Supplemental Fig. 1B) but, as mentioned previously, the  $\alpha 5$  subunit has been demonstrated to be confined to layer VI (Kassam et al., 2008; Poorthuis et al., 2013). Thus, cortical layers I-IV of the motor cortex provide an excellent brain region in which to study responses of  $\alpha 4\beta 2$  nAChRs in isolation from other nAChR subunits.

To confirm this, we first assessed the nAChRs in layers I-IV of the motor cortex based on their responses to 1000  $\mu$ M ACh and the sensitivity of those responses to blockade by the  $\alpha 7$  antagonist MLA and the  $\alpha 4\beta 2$  antagonist DH $\beta$ E. While half of the neurons (18/36) were responsive to 1000  $\mu$ M ACh, an equal number were unresponsive. In 78% of the responsive neurons (n=14/18), 1000  $\mu$ M ACh elicited a whole-cell current response composed of two components, one that was blocked by a 5 min bath exposure to 10 nM MLA and a second component that was blocked upon a further 5 min bath exposure to 10  $\mu$ M DH $\beta$ E (Fig. 5A), implying that these neurons expressed both  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs. In 5% of the responsive neurons (n=1/18), a 5 min bath application of 10 nM MLA abolished whole-cell responses produced by 1000  $\mu$ M ACh (Fig. 5B), which indicates that a small percentage of neurons in layers I-IV express  $\alpha 7$  nAChRs alone. In

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17% of the neurons (n=3/18), whole-cell currents elicited by 1000  $\mu$ M ACh were completely inhibited by a 5 min bath exposure of 10  $\mu$ M DH $\beta$ E (Fig. 5C), suggesting that these neurons express only  $\alpha$ 4 $\beta$ 2 nAChRs.

The present data indicate that neurons in cortical layers I-IV contain nicotinic ACh sensitive neurons that express either a mixture of  $\alpha$ 4 $\beta$ 2 and  $\alpha$ 7 nAChRs,  $\alpha$ 4 $\beta$ 2 nAChRs alone, or  $\alpha$ 7 nAChRs alone. Furthermore, within this population, neurons expressing a mixture of  $\alpha$ 4 $\beta$ 2 and  $\alpha$ 7 nAChRs predominate. Thus, cortical layers I-IV of the motor cortex represent an appropriate region in which to measure responses from native  $\alpha$ 4 $\beta$ 2 nAChRs and to try to determine the stoichiometry of these receptors by utilizing our ‘two-concentration’ ACh (10 and 1000  $\mu$ M) whole-cell current activation paradigm.

### *Assessment of $\alpha$ 4 $\beta$ 2 stoichiometry by the ‘two-concentration’ ACh paradigm in the motor cortex*

Since the majority of ACh sensitive neurons in the motor cortex express a mixture of  $\alpha$ 4 $\beta$ 2 and  $\alpha$ 7 nAChRs, all recordings were performed in the presence of the  $\alpha$ 7 nAChR antagonist MLA (10 nM). This allowed us to assess the stoichiometry of  $\alpha$ 4 $\beta$ 2 nAChRs using the ‘two-concentration’ ACh paradigm without a contribution from  $\alpha$ 7 nAChRs. It should be noted that MLA did not decrease the peak amplitude of ACh currents elicited by the  $\alpha$ 4 $\beta$ 2 nAChRs expressed in the HEK293 cells. Additionally, following the ‘two-concentration’ ACh test paradigm, MLA resistant responses from each recorded neuron were confirmed to result from activation of  $\alpha$ 4 $\beta$ 2 nAChRs by subsequent bath application of 10  $\mu$ M DH $\beta$ E, thus supporting the pharmacological isolation of  $\alpha$ 4 $\beta$ 2 nAChRs in our experiments. In this recording paradigm, the peak amplitude currents elicited by 10  $\mu$ M ACh were  $22 \pm 3\%$  of the currents elicited by 1000  $\mu$ M ACh (Fig. 6). These results indicate that a majority of  $\alpha$ 4 $\beta$ 2 nAChRs in layers I-IV in the motor cortex have a LS subtype stoichiometry.

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### ***Assessment of $\alpha 4\beta 2$ stoichiometry by a ‘two-concentration’ carbachol paradigm in the motor cortex***

To confirm this finding with a second cholinergic agonist and to demonstrate that endogenous acetylcholinesterase activity does not affect the ACh results presented here, we also applied a ‘two-concentration’ carbachol (CARB) paradigm to our HEK HS (Fig. 7A) and LS (Fig. 7B) nAChRs and motor cortex recordings (Fig. 7C) using 32  $\mu$ M and 1000  $\mu$ M CARB. This data is quantified in Fig. 7D. CARB, a muscarinic and nicotinic acetylcholine receptor agonist, is insensitive to cholinesterase activity (Brown and Laiken, 2011). Since the HEK cells and brain slices are bathed in 1  $\mu$ M atropine in our experiments, CARB is only effective as a nAChR agonist under these conditions. These results look similar to those using a ‘two-concentration’ ACh paradigm, further corroborating the stoichiometry of  $\alpha 4\beta 2$  nAChRs in layers I-IV in the motor cortex is that of the LS subtype and indicating that cholinesterase activity does not affect the ACh paradigm presented here.

### ***Biochemical estimation of $\alpha 4\beta 2$ stoichiometry in the rat cortex***

To provide supporting biochemical evidence for the predominance of the LS  $\alpha 4\beta 2$  nAChR stoichiometry in the motor cortex as determined by our patch clamp electrophysiology recordings, we performed immunoprecipitation and western blotting experiments. In these experiments, rat cortex homogenates were first immunoprecipitated with mAb290, a  $\beta 2$  nAChR selective antibody which has previously been described as conformationally selective for fully assembled nAChRs (Sallette et al., 2005), then probed with both  $\alpha 4$  and  $\beta 2$  antibodies by western blot (Supplemental Fig. 2). Ratios of the  $\beta 2/\alpha 4$  band intensities were calculated and compared to ratios of the  $\beta 2/\alpha 4$  band intensities in our surface isolated LS and HS nAChRs in HEK cells presented in

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Supplemental Figure 2B. These data support the predominance of the LS subtype in cortical homogenates.

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### Discussion

This study reveals that the majority of  $\alpha 4\beta 2$  nAChRs examined in layers I-IV of the motor cortex are of the LS subtype. This was accomplished by normalizing responses of 10 to 1000  $\mu\text{M}$  ACh, a paradigm that was first validated in non-linked rat  $\alpha 4\beta 2$  nAChRs. Translating receptor pharmacology from heterologous systems to *ex-vivo* preparations involves inherent assumptions. Substantial evidence, including reporter mutations (Moroni et al., 2006),  $\alpha 4:\beta 2$  transfection ratios and concatemers (Harpsøe et al., 2011), supports the assumption that assembly of heterologously expressed  $\alpha 4\beta 2$  nAChRs is limited to  $(\alpha 4)_3(\beta 2)_2$  or  $(\alpha 4)_2(\beta 2)_3$ , rather than alternate conformations such as  $(\alpha 4)_4(\beta 2)_1$  or  $(\alpha 4)_1(\beta 2)_4$ . Whether native receptors are restricted to these conformations is uncertain but seems likely.

Our data demonstrate that the ratio of  $\alpha 4:\beta 2$  subunits in the HS subtype is opposite to that in the LS subtype (Figure 1), confirming previous studies (Nelson et al., 2003; Moroni et al., 2006; Harpsøe et al., 2011). Additionally, our functional results demonstrate a clear shift in sensitivity to activation by ACh. Moreover, the  $\text{EC}_{50}$  values (Fig. 2A) are similar to those for human HS and LS receptors (Buisson and Bertrand, 2001; Nelson et al., 2003; Moroni et al., 2006; Harpsøe et al., 2011; Mazzaferro et al., 2011; Nichols et al., 2014). The ACh concentration-response curves for both transfection ratios statistically fit best to one class of receptor. However, after transfection with the 4:1 ratio, the Hill slope was  $0.61 \pm 0.18$ . Conflicting modeling of ACh dose-response curves at LS receptors has been reported (Moroni et al., 2006; Carbone et al., 2009; Harpsøe et al., 2011; Mazzaferro et al., 2011; Eaton et al., 2014). The most likely explanation for the low value obtained here is that the ACh stimulation of these LS receptors has two components, consistent with previous reports of biphasic acetylcholine concentration-response curves (Nelson et al., 2003; Harpsøe et al., 2011; Mazzaferro et al.,

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2011). The low-sensitivity component of this biphasic ACh response has been attributed to an  $\alpha 4/\alpha 4$  agonist binding site while the high sensitivity component of this response has been attributed to the canonical  $\alpha 4/\beta 2$  binding site (Harpsøe et al., 2011).

The application of 1  $\mu$ M Saz-A elicited a response of 69% and 25% of the 32  $\mu$ M ACh response in the rat HS and LS nAChRs, consistent with findings demonstrating greater efficacy at human HS nAChRs (Zwart et al., 2008; Eaton et al., 2014). When the response to 32  $\mu$ M ACh is normalized to a calculated maximal response predicted by the ACh concentration-response curves, application of 1  $\mu$ M Saz-A stimulated a response of  $56 \pm 13\%$  of the maximum response at HS and  $9.9 \pm 2.4\%$  at LS nAChRs. The value for our rat HS receptors differs from reports that Saz-A is nearly a full agonist in oocytes expressing a presumably pure population of human HS concatemeric nAChRs (Zwart et al., 2008; Eaton et al., 2014). Possible explanations for this difference are that others have assessed the efficacy of Saz-A at human  $\alpha 4\beta 2$  nAChRs expressed in oocytes, whereas here we studied rat nAChRs expressed in mammalian cells. We studied the rat  $\alpha 4\beta 2$  nAChRs to more readily allow comparison to responses in *ex-vivo* rat brain slices. We decided to compare two doses of ACh in brain slices rather than the difference in efficacy of Saz-A to determine the presence of HS or LS receptors because Saz-A produces prolonged desensitization at  $\alpha 4\beta 2$  nAChRs (Xiao et al., 2006; Eaton et al., 2014) which would have precluded our pharmacological confirmation of  $\alpha 4\beta 2$  mediated responses using DH $\beta$ E.

We sought to establish normalized responses to 10  $\mu$ M and 1000  $\mu$ M ACh at HS and LS nAChRs (Fig. 4). In applying this paradigm to the rat motor cortex, we found that the normalized response of 10 to 1000  $\mu$ M ACh was 22% indicating a majority of the nAChRs are the LS nAChR subtype (Fig. 6). By utilizing our values for the same paradigm at the HS and LS  $\alpha 4\beta 2$  subtypes in our transfected cells and extrapolating a theoretical value based upon different

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percentages of HS and LS receptors, we estimate that in the rat motor cortex LS nAChRs contribute about 76% of the response and the HS nAChRs contributes around 24% (Fig. 8A). This assumes that the 10  $\mu$ M response as a percent of the 1000  $\mu$ M response increases in a linear fashion as more HS receptors are present in the overall  $\alpha 4\beta 2$  nAChR population.

To corroborate the two-dose ACh paradigm, we used the agonist carbachol (CARB). Since all of our experiments are done in the presence of 1  $\mu$ M atropine to block possible signaling resulting from activation of muscarinic receptors, we are only observing the nicotinic cholinergic agonist effects. Importantly, while endogenous cholinesterase activity in brain slices could alter the ACh responses in the motor cortex, CARB is not hydrolyzable by cholinesterases (Brown and Laiken, 2011). From our experiments with CARB (Fig 7), we estimate that LS nAChRs contribute approximately 70% of the response in the rat motor cortex (Fig. 8B); thus, confirming the findings obtained using our ACh paradigm and indicating that any endogenous acetylcholinesterase activity contributes little to our results.

An immunoprecipitation and western blot method to estimate the receptor stoichiometry also supports the conclusion of the the ‘two-concentration’ ACh and CARB paradigms (Supplemental Fig 2). This experiment utilizes whole cortical homogenates rather than the motor cortex region specifically. In these biochemical experiments, about 79% of the nAChRs in rat cortex homogenates are of the LS subtype (Supplemental Fig. 2C).

One concern with the slice recordings presented here is the differential access of agonists to nAChRs on HEK cells compared to neurons due to the extracellular matrix present in slice preparations which does not allow for instantaneous solution exchange and may impede our ability to capture accurate peak amplitude responses. This is exemplified by the delayed response and slower rise times in brain slices (Figs. 5 and 6) compared to the rapid rise times

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observed in HEK cells (Figs. 4 and 7). However, we were able to capture the rapid kinetics of ACh-induced  $\alpha 7$  nAChRs currents in brain slices using the same setup in both Figure 5 and a previous study (Sahibzada et al., 2002). This indicates that we are likely capturing the peak amplitude response from the much slower  $\alpha 4\beta 2$  nAChR responses. Since ACh was applied from pipettes located about ~5-20  $\mu\text{m}$  from the cell soma in both experiments, it is unlikely that these differential effects can be circumvented. However, in our experiments the critical interpretation is based upon the ratio of responses from low and high agonist concentrations. We assume that the ratio of these two responses is independent of the degree of differential access between HEK cells and brain slice recordings since the access in each recording scenario (i.e., HEK or slices) is similar for each pair of drug pipettes.

The possibility of channel block at higher concentrations of ACh is of concern as it may have influenced our estimate of the ratio. However, at the high dose (1000  $\mu\text{M}$ ), we did not observe evidence of channel block; particularly, as there was no evidence of ensuing 'hump currents' that are indicative of channel block with high doses of acetylcholine (Liu et al., 2008). Moreover, as seen in Figure 2A, there is a decline in the current at 3000  $\mu\text{M}$  ACh which could be due to channel block in the LS nAChRs, but this concentration is greater than the 1000  $\mu\text{M}$  ACh utilized in our 'two-concentration' paradigm.

Our data indicate that a majority of rat motor cortex  $\alpha 4\beta 2$  nAChRs have lower sensitivity to ACh ( $\text{EC}_{50} = 56$  vs 5  $\mu\text{M}$  for HS). The difference in sensitivity to ACh probably extends to nicotine as well, since Moroni et al. (2006) found a marked difference in sensitivity to nicotine at HS vs LS  $\alpha 4\beta 2$  nAChRs expressed in oocytes ( $\text{EC}_{50} = 1$   $\mu\text{M}$  for HS vs 34  $\mu\text{M}$  for LS). In addition to the different potencies of these principal nicotinic cholinergic ligands at the two  $\alpha 4\beta 2$  stoichiometries, some drugs such as Saz-A and A85380 as well as nicotine demonstrate different



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efficacies at HS and LS nAChRs (Moroni et al., 2006; Eaton et al., 2014). For example, measured as a percent of ACh response, nicotine has 60% efficacy at LS nAChR and only 30% efficacy at HS nAChRs (Moroni et al., 2006). The LS subtype is less sensitive to desensitization by nAChR agonists in comparison to the HS subtype, possibly because of the presence of the  $\alpha 4/\alpha 4$  binding site (Benallegue et al., 2013; Eaton et al., 2014). These pharmacological differences between the HS and LS  $\alpha 4\beta 2$  nAChRs highlight the importance of determining which of these  $\alpha 4\beta 2$  subtypes is present and in what proportions in native tissues. This may be especially important when considering the potential use of nicotinic receptor ligands as pharmacotherapies, since accurately predicting the *in vivo* pharmacology of a ligand will require knowledge of receptor stoichiometry. While the variable pharmacology and physiology between HS and LS nAChRs has been well-established in heterologous expression systems, we believe this is the first determination of the relative amounts of HS and LS subtypes in brain.

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### **Authorship Contributions**

Participated in research design: DeDominicis, Sahibzada, Xiao, Wolfe, Kellar, and Yasuda.

Conducted experiments: DeDominicis, Sahibzada, Olson, and Yasuda.

Contributed new reagents or analytic tools: Sahibzada, Xiao, and Kellar.

Performed data analysis: DeDominicis, Sahibzada, Olson, Kellar, and Yasuda.

Wrote or contributed to the writing of the manuscript: DeDominicis, Sahibzada, Wolfe, Kellar, and Yasuda.

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### **Footnotes**

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## Figure Legends

### **Figure 1. Western blots of the 1:6 and 4:1 $\alpha 4$ : $\beta 2$ transfection ratios in HEK293 cells**

#### **demonstrate a shift in the relative amount of surface $\alpha 4$ and $\beta 2$ subunit protein at the cell**

**surface.** Surface proteins were labeled by biotin and isolated with NeutrAvidin. Western blots

were used to determine the presence of the  $\alpha 4$  nAChR subunit, the  $\beta 2$  nAChR subunit, and  $\beta$ -

actin proteins at the outside cell surface. (A) Lanes 1 and 2 show the surface  $\alpha 4$  nAChR and  $\beta 2$

nAChR subunit proteins when transfected with  $\alpha 4$ : $\beta 2$  cDNA at 4:1 and 1:6 ratios, respectively.

$\beta$ -actin (Surface) is used to determine the integrity of the cell membrane.  $\beta$ -actin (Homogenate)

acts as an internal control to confirm isolation of surface proteins only. The banding pattern

shown here is representative of four different transfections and surface labeling experiments in

which both transfection ratios were assessed. Quantification of the  $\alpha 4$  nAChR subunit protein

(B) and the  $\beta 2$  nAChR subunit protein (C) in 1:6 (black) and 4:1 (white)  $\alpha 4$ : $\beta 2$  transfection ratios

is presented. Each bar represents 3 different transfections and surface labeling experiments in

which both transfection ratios were assessed. Please note that the number of samples for panels

B and C has dropped from four to three (as compared to panel A) because one experiment used a

different secondary antibody dilution and is therefore not included.

### **Figure 2. Alternate transfection ratios of rat $\alpha 4$ : $\beta 2$ cDNA into HEK293 cells favor assembly**

**of HS or LS  $\alpha 4$ : $\beta 2$  nAChRs.** Rat  $\alpha 4$  and  $\beta 2$  cDNA were transfected in a molar ratio of 1:6 (HS)

or 4:1 (LS)  $\alpha 4$ : $\beta 2$  cDNA. (A) Concentration-response curves to acetylcholine were obtained

using whole cell voltage clamp electrophysiology. Drugs were applied by y-tubing. The peak

amplitude response from each ACh concentration was normalized to the response from 32  $\mu$ M in

the same cell. Values were first normalized and plotted against the 32  $\mu$ M ACh response, then

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re-plotted here for clarity as a percentage of the maximal 1000  $\mu$ M response to highlight the difference in ACh sensitivity between the two ratios. Each data point represents values from 2-3 cells. No more than 3 ACh responses were obtained from each cell. 20 and 24 cells were recorded from 11 or 13 different transfections to obtain data for the transfection of 1:6 and 4:1 molar ratios, respectively. Data were fit using a non-linear regression model with variable slope.

(B) Representative traces of ACh responses in HS  $\alpha 4\beta 2$  nAChR from the same transfected cell.

(C) Representative traces of ACh responses in LS  $\alpha 4\beta 2$  nAChR from the same transfected cell.

The ACh currents are from the data used to generate the dose-response curves in Figure 2A. The arrows denote when the y-tubing application of ACh was started.

**Figure 3. Saz-A has greater potency at the HS ( $\alpha 4$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub> than the LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChR in HEK293 cells as determined by whole cell voltage clamp electrophysiology.** Representative trace overlays of 1  $\mu$ M Saz-A (red) and 32  $\mu$ M ACh (black) are shown for the  $\alpha 4:\beta 2$  cDNA transfections for the (A) HS ( $\alpha 4$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub> and the (B) LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub>. Drugs were applied using y-tubing. To avoid prolonged desensitization elicited by Saz-A in our experimental paradigm, 32  $\mu$ M ACh was applied first, followed by the application of 1  $\mu$ M Saz-A. (C) A compilation of the peak amplitude of the 1  $\mu$ M Saz-A response normalized to the 1000  $\mu$ M ACh response is shown for the  $\alpha 4:\beta 2$  cDNA transfections for the HS (black) and the LS (white). \* $p < 0.05$ , t-test,  $n = 4-6$  cells from 3-4 different transfection days.

**Figure 4. Normalized 10  $\mu$ M ACh responses demonstrate a stark difference in the HS ( $\alpha 4$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub> and LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChRs in HEK293 cells as determined by whole cell voltage clamp electrophysiology.** Representative trace overlays of 10  $\mu$ M ACh (red) and 1000  $\mu$ M ACh (black) are shown for the (A) HS ( $\alpha 4$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub> and (B) LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChRs. Drugs were applied by pressure (picospritzer). (C) A compilation of the peak amplitude of the 10  $\mu$ M ACh

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response normalized to the 1000  $\mu$ M ACh response is shown for both transfection ratios. \*\*  $p < 0.01$ , t-test,  $n = 9$  cells for each transfection ratio from 2 or 3 separate transfection days.

**Figure 5. Heterogeneity of nAChR responses in rat motor cortex.** Four different types of nAChR responses were elicited by 1000  $\mu$ M ACh in whole cell patch clamp electrophysiology recordings from neurons in layers I-IV of the motor cortex: non-responsive neurons (data not shown), (A) MLA sensitive and DH $\beta$ E sensitive neurons, (B) MLA sensitive neurons, and (C) MLA insensitive and DH $\beta$ E sensitive neurons. Responses to 1000  $\mu$ M ACh without blockers are shown in black. Responses elicited by 1000  $\mu$ M ACh following 5 min pre-incubation with 10 nM MLA are shown in red. Responses elicited by 1000  $\mu$ M ACh following 5 min pre-incubation with 10  $\mu$ M DHBE and 10 nM MLA are shown in green.

**Figure 6. Comparison of the responses to 10  $\mu$ M and 1000  $\mu$ M ACh in neurons of the motor cortex.** All recordings were performed after a 5 min bath application of 10 nM MLA to block  $\alpha 7$  nAChR responses. A) Representative trace overlays of 10  $\mu$ M (red) and 1000  $\mu$ M ACh (black). B) Compilation of normalized 10  $\mu$ M to 1000  $\mu$ M ACh responses in the motor cortex (stippled;  $n = 9$  neurons from 6 animals aged P18 – P24). The ACh response to HEK cells transfected with subunit ratios to produce HS ( $\alpha 4$ )<sub>2</sub>( $\beta 2$ )<sub>3</sub> nAChRs (black) or the LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChRs (white) are included for comparison. A 5 min inter-stimulus interval was used between exposures to ACh to mitigate any possible effects of desensitization.

**Figure 7. Comparison of the responses to 32  $\mu$ M and 1000  $\mu$ M carbachol in HS and LS HEK cells and neurons of the motor cortex.** Representative trace overlays of 32  $\mu$ M (red) and 1000  $\mu$ M CARB (black) in HS (A) and LS (B)  $\alpha 4\beta 2$  nAChR in transfected HEK cells ( $n = 4$  each transfection ratio). C) Representative trace overlays of 32  $\mu$ M (red) and 1000  $\mu$ M CARB (black) in the motor cortex neurons ( $n = 5$  neurons from 5 animals aged P18 – P24 ). D)

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Quantification of 32  $\mu$ M and 1000  $\mu$ M CARB in rat motor cortex neurons (stippled). The CARB response to HEK cells transfected with subunit ratios to produce HS (black) or LS nAChRs (white) are included for comparison. All recordings were performed after a 5 min bath application of 10 nM MLA to block  $\alpha 7$  nAChR responses (neurons only) and 1  $\mu$ M atropine to eliminate effects from activation of muscarinic receptors. A five min inter-stimulus interval was used between exposures to CARB to mitigate any possible effects of desensitization.

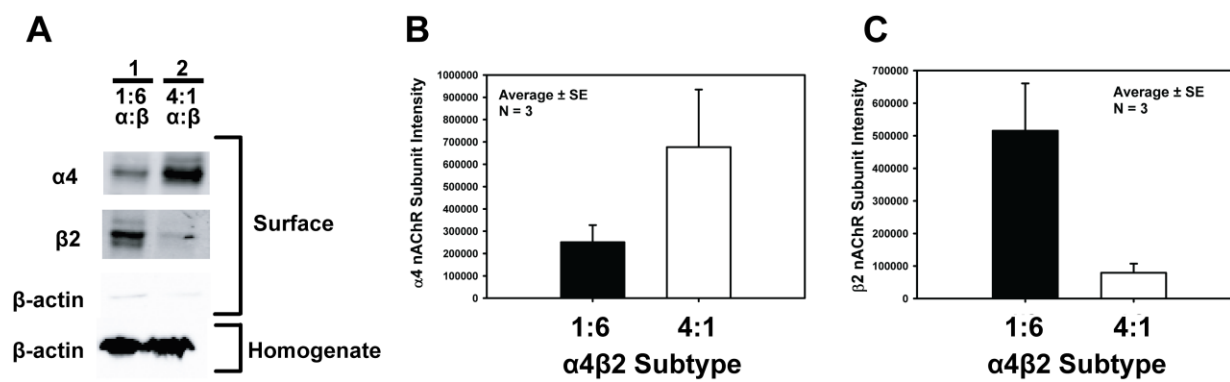
**Figure 8. Estimate of the percent of the LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChR in the rat motor cortex.** On the y-axis, normalized 10  $\mu$ M ACh (A) or 32  $\mu$ M CARB (B) responses from HS and LS HEK cells are plotted against the percent contribution of LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> nAChRs on the x-axis. Using the responses obtained from the motor cortex neuron recordings in presented in Figures 6 and 7, we estimate that the percent of the  $\alpha 4\beta 2$  nAChRs in the motor cortex that are of the LS ( $\alpha 4$ )<sub>3</sub>( $\beta 2$ )<sub>2</sub> subtype is ~76% as determined by ACh and ~70% as determined by CARB.



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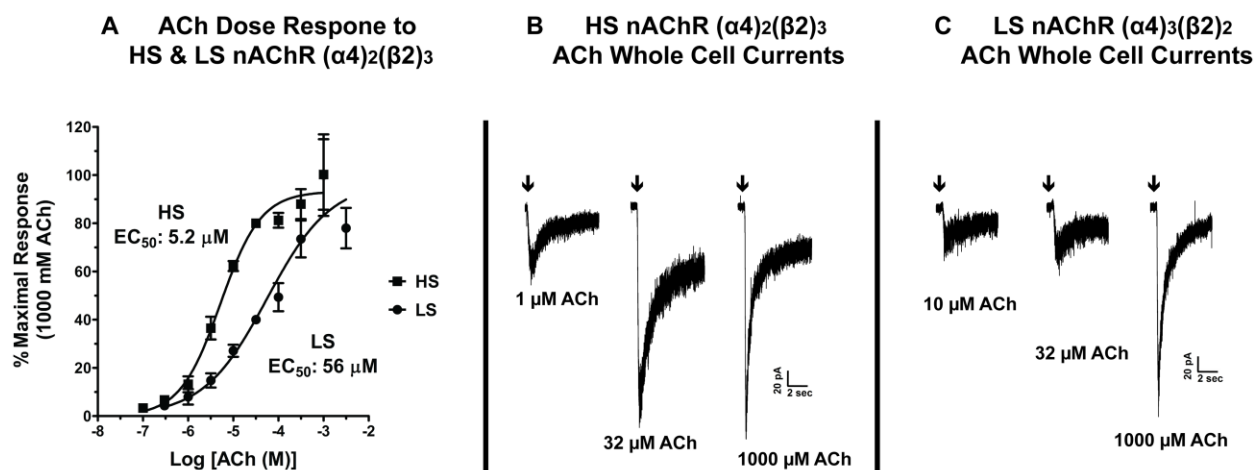
## Figures

Figure 1:



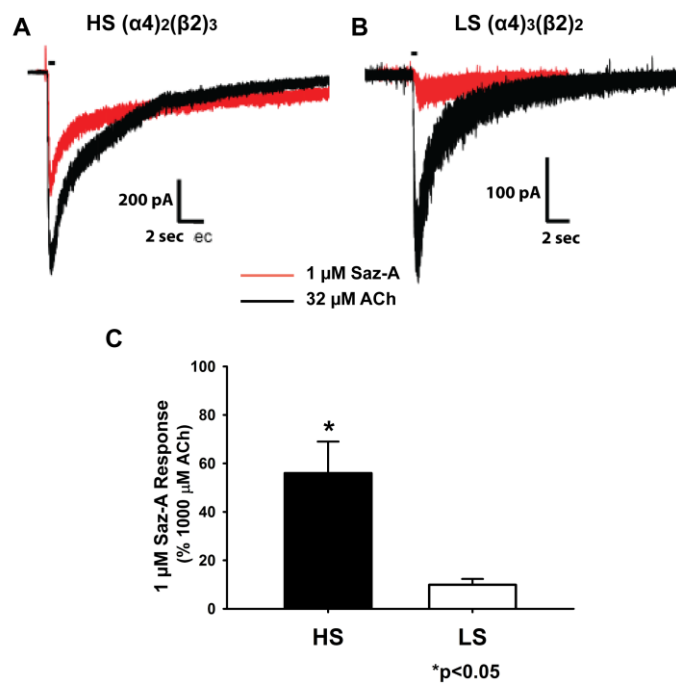
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Figure 2:



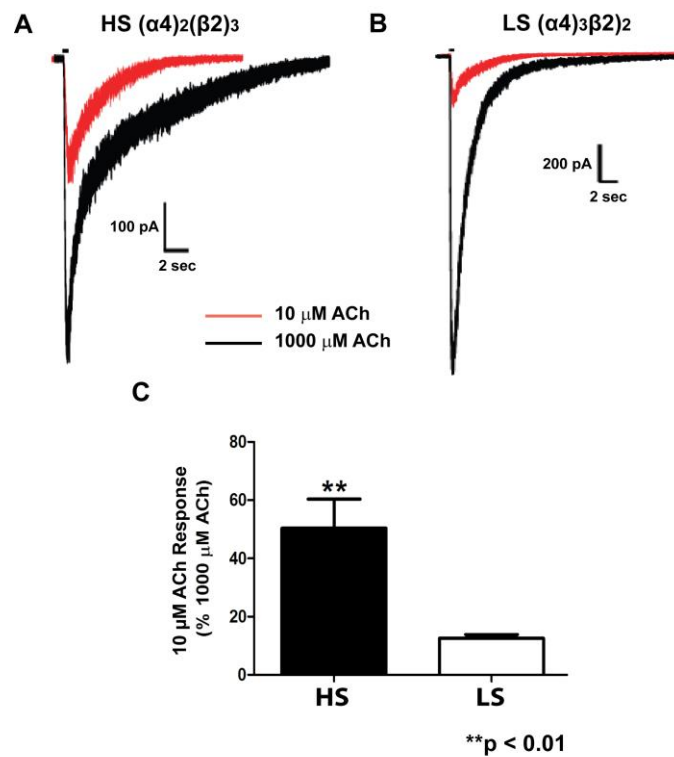
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Figure 3:



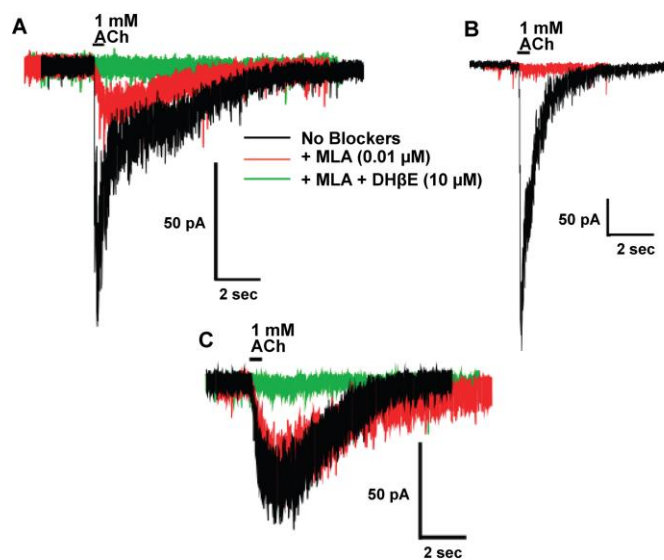
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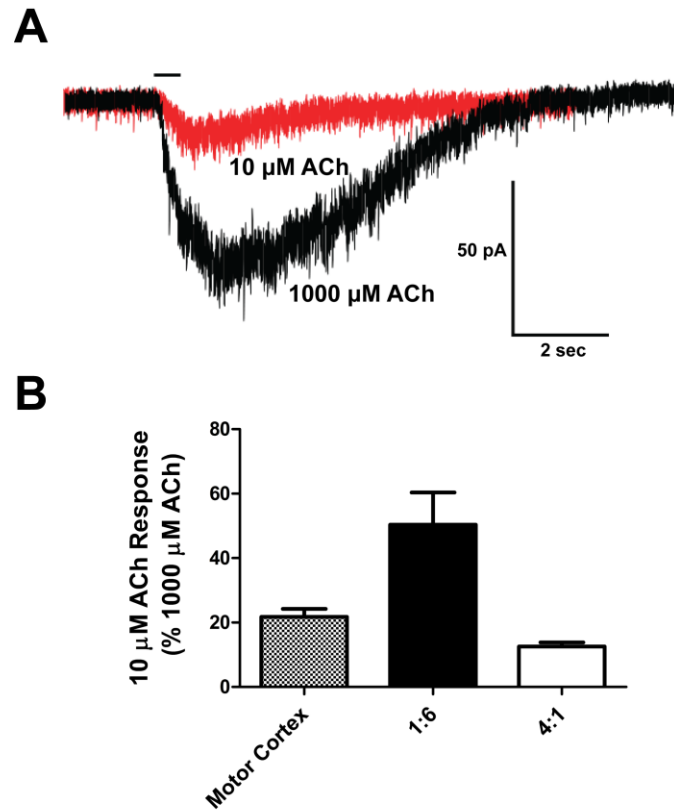
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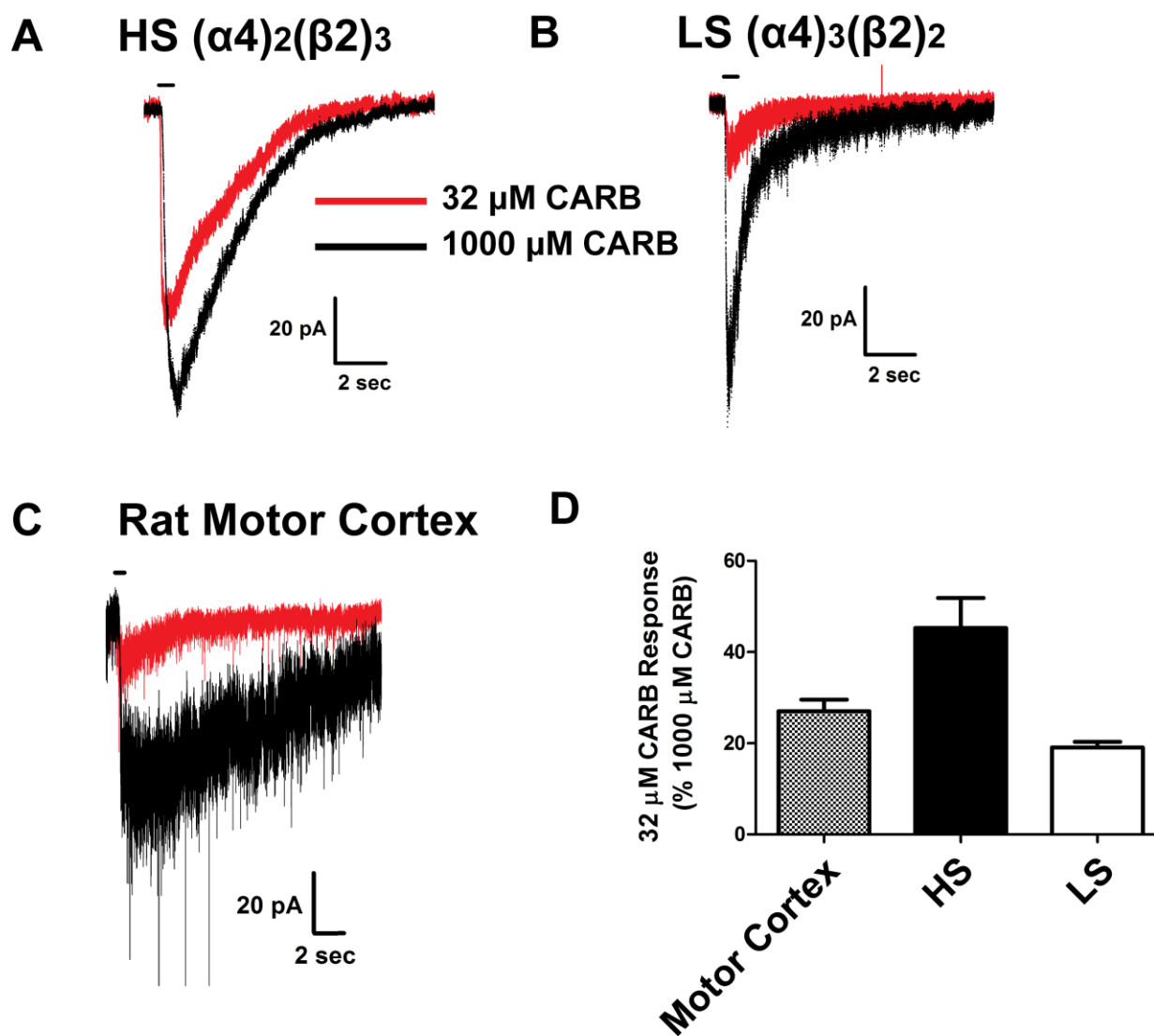
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Figure 6:



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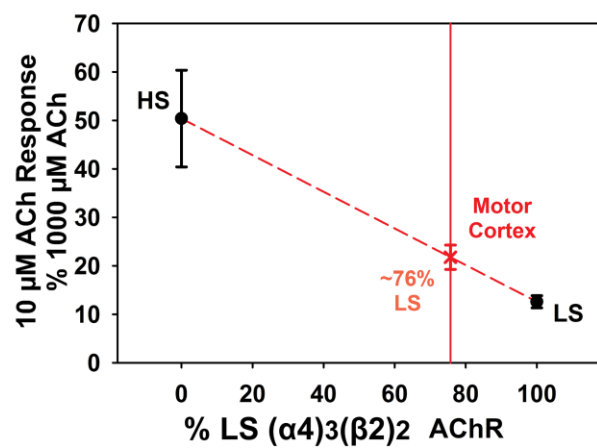
Figure 7:



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Figure 8:

**A**



**B**

