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## ***Chrna4* A529 knockin mice exhibit altered nicotine sensitivity**

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### **Abstract**

The reasons why people smoke are varied, but research has demonstrated that genetic influences on various aspects of nicotine addiction are a major factor. There also is a strong genetic influence on measures of nicotine sensitivity in mice. Despite the established contribution of genetics to nicotine sensitivity in mice and humans, no naturally occurring genetic variation has been identified that demonstrably alters sensitivity to nicotine in either species. However, one genetic variant has been implicated in altering nicotine sensitivity in mice is a T529A polymorphism in *Chrna4*, the gene that encodes the nicotinic receptor (nAChR)  $\alpha 4$  subunit. The *Chrna4* T529A polymorphism leads to a threonine to alanine substitution at position 529 of the  $\alpha 4$  subunit. To more definitively address whether the *Chrna4* T529A polymorphism does, in fact, influence sensitivity to nicotine, knockin mice were generated in which the threonine codon at position 529 was mutated to an alanine codon. Compared to *Chrna4* T529 littermate controls, the *Chrna4* A529 knockin mice exhibited greater sensitivity to the hypothermic effects of nicotine, reduced oral nicotine consumption and did not develop conditioned place preference to nicotine. The *Chrna4* A529 knockin mice also differed from T529 littermates for two parameters of acetylcholine-stimulated <sup>86</sup>Rb+ efflux in midbrain: maximal efflux and the percentage of  $\alpha 4\beta 2^*$  receptors with high sensitivity to activation by agonists. Results indicate that the polymorphism affects the function of midbrain  $\alpha 4\beta 2^*$  nAChRs and contributes to individual differences in several behavioral and physiological responses to nicotine thought to be modulated by midbrain  $\alpha 4\beta 2^*$  nAChRs.

### **Keywords**

Nicotinic receptor; genetics; polymorphism; conditioned place preference; oral consumption; addiction

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Cigarette smoking is the single most preventable cause of premature death in the United States. It has been estimated that over 440,000 people died annually between the years 1998–2007 from smoking related illnesses [1]. In fact, the number of deaths related to tobacco is more than all deaths related to human immunodeficiency virus (HIV), illegal drug use, motor vehicle injuries, suicides, and murders combined. Despite these morbid statistics, 20% of adults in the United States continue to smoke [1].

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Although the specific reasons why people smoke are varied, it is known that genetics plays a vital role. Twin studies, beginning with those of Fisher [2,3] demonstrated that smoking behavior is heritable with a mean estimate of heritability of 0.53 [4]. Likewise, genetic factors influence the behavioral and physiological effects of nicotine in mice [5]. For example, an examination of 19 inbred mouse strains demonstrated that there is a 2 to 6-fold difference in sensitivity to nicotine across a battery of tests [6,7]. Moreover, genetic influences on the development of tolerance to nicotine [8–10], nicotine oral consumption [11–13] conditioned place preference [14,15] and conditioned taste aversion [16] have been reported in mice.

Due to the fact that there are clear genetic influences on nicotine dependence in humans and nicotine sensitivity in rodents, a number of attempts have been made to identify the genetic variants in both species that lead to individual differences in nicotine dependence/sensitivity [17,18]. Not surprisingly, these studies have uncovered several chromosomal regions and specific polymorphisms that may contribute to variable sensitivity to nicotine. Nonetheless, none of these naturally-occurring polymorphisms have been experimentally demonstrated to contribute to individual differences in nicotine dependence and/or sensitivity. Moreover, it remains to be determined whether any single naturally-occurring polymorphism is actually sufficient to alter individual responsiveness to nicotine in any measurable way.

One genetic variant that has been implicated in altering nicotine sensitivity in mice is the naturally occurring T529A polymorphism in *Chrna4*, the gene that encodes the nicotinic receptor (nAChR)  $\alpha 4$  subunit. Several studies have shown that this polymorphism is associated with individual differences in a variety of measures of nicotine sensitivity in mice [12,19–21]. The *Chrna4* T529A polymorphism leads to a threonine for alanine substitution at amino acid residue 529 of the  $\alpha 4$  subunit and is located within a region of the large cytoplasmic loop that is conserved across mammalian species. In mammals, the  $\alpha 4$  subunit combines with the nAChR  $\beta 2$  subunit to form the  $\alpha 4\beta 2^*$  nAChR (\* indicates the possible inclusion of other nAChR subunits) [22]. The  $\alpha 4\beta 2^*$  nAChR is not only the most abundant nicotinic receptor expressed in the brain [23,24] but also is critical for self-administration of and positive reinforcement to nicotine [25–28]. Heterologous expression of  $\alpha 4\beta 2$  nAChRs possessing the variant forms of the  $\alpha 4$  subunit demonstrated that the T529A polymorphism affect the function of  $\alpha 4\beta 2$  nAChRs in vitro [29]. Moreover, the *Chrna4* T529A polymorphism has been shown to be associated with differences in the function of  $\alpha 4\beta 2^*$  nAChRs in mouse brain synaptosomes [30,31]. Because the *Chrna4* T529A polymorphism is associated with differences in nicotine sensitivity,  $\alpha 4\beta 2^*$  nAChR function, and is located within a gene known to be critical for nicotine reinforcement, it is a strong candidate for a single naturally occurring polymorphism that contributes to individual variability in sensitivity to nicotine. However, all previous studies that have implicated the *Chrna4* T529A polymorphism in nicotine sensitivity and nicotinic receptor function in mice were either association or linkage studies. Consequently, it remains to be determined if the *Chrna4* T529A polymorphism or a polymorphism that is linked to it is responsible for individual differences in nicotine sensitivity and/or nAChR function. The studies described in this report utilized a knockin mouse for the *Chrna4* T529A polymorphism in order to provide a more definitive assessment of the role of the polymorphism in influencing nicotine sensitivity and nAChR function.

## Materials and Methods

### Animals

Mice were housed in a colony room that was on a 12 h light and 12 h dark cycle (lights on at 0700) and maintained at  $21^{\circ}\text{C} \pm 1$ . Mice were weaned between 21 and 25 days of age. All studies used male and female adult mice between 60 and 120 days of age. The animals were

provided with food (Teklad Rodent Diet (Harlan, Madison, WI)) and water *ad libitum*. All procedures were approved by the University of Colorado or University of Pittsburgh Institutional Animal Care and Use Committees.

### Generation of *Chrna4* T529A knockin mice

The DNA construct used for homologous recombination was generously provided by Dr. Henry Lester (CalTech) and has been described previously [32]. The mutation introduced by the Lester laboratory [32] was reverted and a new mutation changing the threonine codon to an alanine codon at amino acid position 529 was introduced using site directed mutagenesis. Reversion of the mutation introduced by the Lester laboratory and introduction of the threonine to alanine codon change at residue 529 were verified by sequencing. Sequencing also confirmed that there were no unwanted mutations introduced during the mutagenesis. The construct was introduced into the Strain 129SvJ Go Germline ES cell line (Genome Systems, Inc., St. Louis, MO) and homologous recombinants were identified by Southern blot using the exact same restriction digest and probes as described in Labarca *et al.* [32]. ES cells containing the targeted construct were introduced into C57BL/6J blastocysts and one chimeric mouse was identified as carrying the mutation germline. An intronic neomycin cassette was removed from the knockin allele of *Chrna4* by breeding the *Chrna4* T529A knockin progenitor mice with C57BL/6J-TgN(Zp3-Cre)<sup>93Kw</sup> mice (Jackson Laboratories, Bar Harbor, ME). The ZP3-Cre transgene was subsequently removed by backcrossing the *Chrna4* T529A knockin mice with C57BL/6J mice. *Chrna4* T529A knockin mice were then backcrossed to C57BL/6J mice for at least 8–9 generations prior to testing.

All animals used in the experiments were produced by matings between mice heterozygous for the T529A polymorphism. For all experiments, *Chrna4* A529 knockin mice refer to mice that possess an alanine at amino acid position 529 where as T529 control mice refer to mice that have a threonine at amino acid position 529. Genotyping of the animals was done exactly as described previously by Dobelis *et al.* [31].

### Materials

All materials were purchased (unless otherwise noted) from Sigma Aldrich (St. Louis, MO). Nicotine used for all experiments was free base.

### Y-maze and Body Temperature

Using a counterbalanced design, animals received either a 0.5 mg/kg nicotine or saline injection (i.p.) on day one. Three minutes following injection, the animals were transferred to a darkened Y-maze where the number of horizontal and vertical beam breaks was recorded for 3 minutes. Fifteen minutes after the nicotine injection, body temperature was recorded by a rectal thermometer. On the following day, animals were tested under the exact same conditions but received the opposite injection.. Approximately half of the animals per genotype received nicotine on day one and the other half received nicotine on day two.

### Nicotine Oral Consumption

A two bottle free choice paradigm was used as previously described [13]. The day before the experiment started, the mice were weighed, singly housed, and given free access to food and water. At the start of the experiment, mice were given two bottles: one containing tap water and the other containing 25 µg/ml nicotine in tap water. Both bottles were made from glass culture tubes fitted with standard stainless steel drinking spouts. The bottles were rotated each day to account for a side bias. After four days, the nicotine concentration was increased to 50 µg/ml and animals received fresh water. Following the four days of 50 µg/ml nicotine solution, the nicotine concentration was increased to 100 µg/ml for four days and animals

received fresh water. Bottles were weighed at the start and finish of each new concentration and the volume consumed per bottle was recorded. Animals were reweighed on the final day in order to determine the average weight of each animal throughout the study. In addition, cages without mice were fitted with two bottles to determine volume lost due to spillage and evaporation during the experiment.

### **Non-biased Nicotine Induced Conditioned Place Preference (CPP)**

The place preference apparatus consisted of 3 distinct compartments, two conditioning chambers and a smaller central chamber. Preliminary experiments were done to establish chamber conditions that prevented a bias towards one of the two conditioning chambers. As a result of these preliminary experiments, one conditioning compartments had vertical black and white striped walls with a mesh floor and the other conditioning compartment had black and white checkered walls with a rod floor. The middle chamber consisted of smooth black and white walls with smooth flooring. CPP was conducted in three phases: preconditioning, conditioning, and testing. In the preconditioning phase, mice were allowed to freely roam between the three chambers for 15 minutes. The time spent in each chamber was recorded and then animals were separated into counterbalanced groups. On day two, conditioning began with an injection (i.p.) of 0.09 mg/kg nicotine or saline and immediate isolation in one of the conditioning compartments for 30 minutes. Five hours later the same animal was injected with the opposite drug condition and confined to the other conditioning chamber for 30 minutes. Conditioning was continued for two more days with animals being paired in the same conditioning chamber with the same drug treatment. The experiment was counterbalanced in order to assure that some animals were drug paired in the checkered compartment and some in the striped compartment. On day five, animals were placed in the middle chamber with access to all three chambers for 15 minutes. The preference score for each compartment was determined by subtracting the preconditioning score from the test day score (in seconds).

### **<sup>86</sup>Rb<sup>+</sup> Efflux**

Crude synaptosomes were prepared from hippocampus, striatum, thalamus, and midbrain and loaded with <sup>86</sup>Rb<sup>+</sup> purchased from Perkin-Elmer Life and Analytical Sciences, Inc. (Waltham, MA, USA) using a technique that has been previously described [33]. Perfusion of synaptosomes with buffer (135 mM NaCl, 5 mM CsCl, 1.5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 20 mM glucose, 50 nM tetrodotoxin, 1 μM atropine, 25 mM HEPES hemisodium, 0.1% bovine serum albumin, pH 7.5) occurred for 5 minutes prior to the initiation of data collection. Data were then collected for 90 seconds to determine basal efflux and immediately thereafter samples were stimulated with acetylcholine for 5 seconds followed by 2 minutes of buffer perfusion. Data were collected on a β-RAM Radioactivity HPLC detector (IN/US Systems, Inc., Tampa, FL). The magnitude of efflux due to stimulation was determined relative to the basal efflux before and after the agonist peak. An exponential decay function was used to fit the basal efflux and subtracted from the efflux resulting from agonist stimulation as previously described [33]. Data was analyzed by determining the magnitude of agonist stimulated <sup>86</sup>Rb<sup>+</sup> release as the counts per minutes (cpm) exceeding basal efflux during exposure to the agonist. A nonlinear curve fitting algorithm in SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA) was used to fit the data. Michaelis-Menten equations were used to generate concentration-response curves.

### **<sup>125</sup>I-Epipatidine Binding**

Measurement of <sup>125</sup>I-epibatidine binding in synaptosomes was quantified as previously described [34]. Briefly, incubations were completed in 96 well polystyrene plates in a final volume of 30 μl in 1X binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 20 mM HEPES, pH = 7.5) at 22°C for 2 h in the presence of 200 pM <sup>125</sup>I-

epibatidine purchased from Perkin-Elmer Life and Analytical Sciences, Inc. (Waltham, MA, USA). Total and non-specific binding were measured in the presence of binding buffer or 100  $\mu\text{M}$  cytosine, respectively. Samples were counted on a Packard Cobra counter. Specific binding was determined by subtracting the non-specific binding from the total binding. Protein content was determined by the method of Lowry [35].

### Statistical Analyses

Most comparisons between genotypes (*Chrna4* A529 and *Chrna4* T529) were analyzed using the Student's t-test. All data are reported as an average  $\pm$  SEM. Oral nicotine consumption was calculated by determining the  $\mu\text{g}$  of nicotine consumed divided by the weight of the mouse (g)  $\pm$  SEM. Student's t-test was used to determine genotypic differences for average nicotine consumption and a two-way ANOVA was used to calculate an effect of genotype and nicotine concentration on nicotine consumption. The ACh elicited  $^{86}\text{Rb}^+$  efflux was calculated using a nonlinear two site regression with constrained  $\text{EC}_{50}$  values in GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A two site regression analysis of the concentration response curves was used to establish  $\text{EC}_{50}$  and  $E_{\text{max}}$  (maximal efflux) for agonist stimulated  $^{86}\text{Rb}^+$  efflux. The goodness of fit ( $R^2$ ) of the data was highest using a two site regression analysis when fitting the data. No significant difference in  $\text{EC}_{50}$  values were detected within brain regions between genotype for the high and low sensitivity components. Therefore, for curve-fitting,  $\text{EC}_{50}$  values were constrained to be equal between genotypes for each brain region. The constrained  $\text{EC}_{50}$  for the high sensitivity component and low sensitivity component were as follows in hippocampus, striatum, midbrain, and thalamus:  $2.45 \pm 1.24$  and  $110.50 \pm 86.42$ ,  $1.24 \pm 1.02$  and  $83.59 \pm 43.92$ ,  $2.21 \pm 1.16$  and  $198.30 \pm 79.77$ , and  $2.31 \pm 1.64$  and  $43.46 \pm 45.12$   $\mu\text{M}$  ACh, respectively.

## Results

### Verification of the *Chrna4* T529A knockin mutation

*Chrna4* T529A knockin mice, hereafter referred to as A529 knockin mice, were generated by converting a threonine codon at position 529 of *Chrna4* to an alanine codon as described in the methods. To confirm that the RNA for *Chrna4* from the knockin mice differed from wild-type *Chrna4* allele for only codon 529, RT-PCR was performed on RNA isolated from the knockin mice and their littermate controls. Sequencing results from the PCR products established that the alanine for threonine codon substitution was the only sequence difference in the RNAs between the knockin animals and controls (data not shown). RT-PCR products from primers designed to span intron-exon boundaries also indicated that splicing of the nAChR  $\alpha 4$  subunit RNA was not affected by the engineered mutation (data not shown).

### Influence of the *Chrna4* T529A polymorphism on measures of nicotine sensitivity

The mouse *Chrna4* T529A polymorphism originally was found to be associated with strain differences in nicotine induced changes in body temperature and locomotor activity [20]. To more directly assess whether the *Chrna4* T529A polymorphism was responsible for these observed associations the *Chrna4* A529 knockin mice and T529 littermate controls were tested for these measures of the acute effects of nicotine. Consistent with previous data, a significant effect of the polymorphism ( $p < 0.05$ ) was observed for nicotine induced hypothermia (figure 1b) with *Chrna4* A529 animals exhibiting greater sensitivity to the body temperature depressant effects of nicotine than *Chrna4* T529 animals. In contrast to previous data, no effect of the polymorphism was observed on Y-maze activity (figure 2b). These results indicate that the *Chrna4* T529A polymorphism measurably affects sensitivity to the hypothermic effects of nicotine but not sensitivity to the locomotor depressant effects of nicotine.



Another measure that consistently has been associated with the *Chrna4* T529A polymorphism is free choice oral nicotine consumption [12,13]. Consequently, the *Chrna4* A529 knockin mice and T529 littermates were evaluated for an effect of the T529A polymorphism on nicotine consumption. The animals were given access to 25, 50 and 100 µg/ml nicotine incrementally for 12 days in a standard two-bottle choice paradigm. Results confirmed that the T529A polymorphism influences oral nicotine consumption (figure 3). Two-way ANOVA indicated that there was an effect of *Chrna4* genotype ( $p < 0.05$ ) and concentration ( $p < 0.005$ ) on nicotine consumption but not a significant interaction between the variables ( $p = 0.47$ ). Over the twelve day test period, *Chrna4* A529 knockin mice drank less nicotine and consumed a lower average dose (mg/kg) of nicotine than their T529 wildtype littermates.

The observation that *Chrna4* T529A genotype affects free choice nicotine consumption suggests that the polymorphism might alter sensitivity to the reinforcing effects of nicotine. In addition, there is evidence that  $\alpha 4^*$  nAChRs are necessary and sufficient for modulating the reinforcing effects of nicotine [27]. Therefore, to assess whether the *Chrna4* T529A polymorphism alters sensitivity to the reinforcing properties of nicotine, *Chrna4* T529A knockin animals and wildtype controls were examined for differences in nicotine induced conditioned place preference. Results indicated that the polymorphism influences sensitivity to the reinforcing properties of nicotine (figure 4). Wildtype mice (T529 littermates) exhibited a significant place preference for nicotine while *Chrna4* A529 knockin mice did not ( $p < 0.01$ ). On average, the *Chrna4* A529 knockin animals spent no more time in the drug-paired chamber on the test day relative to the preconditioning day whereas T529 animals spent significantly more time in the drug paired chamber on the test day relative to the preconditioning day.

### Influence of the *Chrna4* T529A polymorphism on the function of $\alpha 4\beta 2^*$ nAChRs

Studies have suggested that the *Chrna4* T529A polymorphism influences the function of  $\alpha 4\beta 2^*$  nAChRs in both a heterologous expression system and in mouse brain [29–31]. Therefore, the observed effect of the polymorphism on nicotine sensitivity might be explained by an effect of the polymorphism on receptor function. In order to directly test the effect of the *Chrna4* T529A polymorphism on receptor function, acetylcholine-stimulated  $^{86}\text{Rb}^+$  efflux was measured from synaptosomes prepared from four brain regions of the *Chrna4* A529 animals and T529 littermates. The brain regions tested were hippocampus, striatum, midbrain, and thalamus. Results indicate that ACh elicited  $^{86}\text{Rb}^+$  efflux in midbrain was different between genotypes. Concentration response curves from midbrain synaptosomes indicated that nAChRs with the T529 variant of  $\alpha 4$  produce a greater maximal response to ACh than nAChRs with the A529 variant of  $\alpha 4$  ( $p < 0.05$ ) (Figure 5). In addition, multiple reports over the past decade have shown that  $\alpha 4^*$  containing receptors can exist as two populations differing in sensitivity to activation by nicotinic agonists [36–39]. Therefore, the potential influence of the *Chrna4* T529A polymorphism on the proportion of high and low sensitivity  $\alpha 4^*$  nAChRs was assessed (figure 6). Similar to the results of the concentration-response curves, the polymorphism affected this measure of nAChR function in midbrain ( $p < 0.05$ ) but not in other brain region. In midbrain, the percent of the response to ACh that could be attributed to the high sensitivity component was significantly greater for nAChRs containing the A529 variant of  $\alpha 4$  than for nAChRs possessing the T529 variant of  $\alpha 4$ .

The *Chrna4* T529A genotype-dependent difference in maximal response to acetylcholine that was detected in midbrain could be the result of differences in receptor function, receptor expression or a combination of the two. Therefore,  $^{125}\text{I}$ -Epibatidine binding was measured in the hippocampus, midbrain, striatum and thalamus of *Chrna4* A529 knockin and T529 wildtype animals to determine if the polymorphism affects nAChR expression. Results

(figure 7) indicated no genotypic difference in  $^{125}\text{I}$ -epibatidine binding in hippocampus, striatum, and midbrain, but did detect a genotypic effect in thalamus. However, T529 control mice exhibited significantly ( $p < 0.05$ ) higher binding in thalamus than did *Chrna4* A529 knockin animals.

## Discussion

Previous studies reported that the *Chrna4* T529A polymorphism is associated with a wide range of behavioral and physiological responses to nicotine in mice [12,13,19–21]. However, it could not be determined whether these reported associations were specifically due to the T529A polymorphism, a different polymorphism in *Chrna4* or a polymorphism in a gene linked to *Chrna4*. In the data reported here, *Chrna4* A529 knockin mice were utilized to confirm that the T529A polymorphism does influence nicotine-induced hypothermia and free-choice oral nicotine consumption. *Chrna4* A529 knockin mice were found to be more sensitive to the hypothermic effects of nicotine and to consume less nicotine by choice than wildtype controls. The A529 knockin mice also were found to differ from their control littermates for the reinforcing properties of nicotine. Mice possessing the T529 allele exhibited a clear reinforcement to a dose of 0.09 mg/kg nicotine that was absent in animals carrying the A529 allele. The positive reinforcement to nicotine seen in mice possessing the T529 allele of *Chrna4* may explain why these animals consume more nicotine by choice than do T529A knockin animals. However, since only a single dose of nicotine was tested in the place preference paradigm, it remains to be determined if the A529 knockin mice completely lack positive reinforcement to nicotine or have a shifted dose-response to nicotine reinforcement.

A529 knockin mice, however, did not differ from controls in sensitivity to the locomotor depressant effects of nicotine across several doses of nicotine (only the data for saline and the 0.5 mg/kg dose are shown in figure 2). This finding suggests that the previous reported association between the T529A polymorphism and nicotine-induced hypolocomotion in the Y-maze may be due to a different polymorphism in either *Chrna4* or a linked gene. Alternatively, an effect of the T529A polymorphism on nicotine-induced hypolocomotion may be immeasurable in T529A knockin mice because  $\alpha 4\beta 2^*$  nAChRs are only one of at least three different nAChR subtypes that mediate the effects of nicotine on locomotor activity [40–43]. It should be noted that locomotor activity was only assessed for three minutes in the Y-maze to allow for direct comparisons with previously published data. To more thoroughly assess the role of the *Chrna4* T529A polymorphism on locomotor activity a full time course of the effect of nicotine on locomotion is required.

Results of this study demonstrated that the *Chrna4* T529A polymorphism alters the function of  $\alpha 4\beta 2^*$  nAChRs in a brain region-specific manner. In the midbrain, two effects of the polymorphism were observed. First, maximal acetylcholine-stimulated  $^{86}\text{Rb}^+$  efflux from  $\alpha 4\beta 2^*$  nAChRs was greater in mice possessing the T529 variant of *Chrna4* relative to  $\alpha 4\beta 2^*$  nAChRs from mice carrying the A529 allele of *Chrna4*. Second, the fraction of the total acetylcholine-stimulated  $^{86}\text{Rb}^+$  efflux that could be attributed to the high sensitivity population of  $\alpha 4\beta 2^*$  nAChRs was significantly greater in A529 knockin mice. The observation that there is a functional effect of the polymorphism on midbrain  $\alpha 4\beta 2^*$  nAChRs as well as individual differences in oral nicotine consumption, conditioned place preference and nicotine induced hypothermia is consistent with previous studies that have shown that  $\alpha 4\beta 2^*$  nAChRs in the midbrain are essential for nicotine self-administration [25,26,44,45], nicotine place preference [27,28] and the effects of nicotine on body temperature [40,41].

Acetylcholine-stimulated  $^{86}\text{Rb}^+$  efflux data from midbrain also confirmed previous reports that the *Chrna4* T529A polymorphism leads to a shift in the proportion of  $\alpha 4\beta 2^*$  nAChRs with high and low sensitivity to activation by agonists. The phenomena that  $\alpha 4\beta 2^*$  nAChRs can exist in two pharmacologically distinct forms, one activated at low concentrations of agonist and the other activated only at higher agonist concentrations, has been described both in heterologous expression systems [36,37,39,46] and in mouse brain synaptosomes [33,47]. Moreover, it has been speculated that alterations in the ratio of high sensitivity to low sensitivity  $\alpha 4\beta 2^*$  nAChRs might be important for the addiction process [46]. The data reported here provide the first evidence that innate differences in the ratio of high to low sensitivity  $\alpha 4\beta 2^*$  nAChRs might influence susceptibility to nicotine addiction by altering sensitivity to the reinforcing effects of nicotine.

Although an effect of the *Chrna4* T529A polymorphism on  $\alpha 4\beta 2^*$  function was observed in the midbrain, no detectable effect of the polymorphism was observed in the hippocampus, striatum or thalamus. The reason for the region-specific effect of the polymorphism on receptor function is not clear. However, one possible explanation is that these regions, but not the midbrain, express functional  $\alpha 4\beta 2^*$  nAChRs that include  $\alpha 5$  [48–50] or other subunits [51]. Inclusion of these other subunits may mask the effect of the polymorphism on receptor function. Studies that examine the effect of the polymorphism on the function of  $\alpha 4\beta 2$  nAChRs in mice that lack these other subunits are needed to address this possibility. In addition, assessing the function of subpopulations of  $\alpha 4\beta 2^*$  nAChRs, such as those that contribute to dopamine and GABA release, may uncover functional effects of the *Chrna4* T529A polymorphism not detected by the  $^{86}\text{Rb}^+$  efflux assay.

One unexpected finding is that the T529A polymorphism affected the expression of  $\alpha 4\beta 2^*$  nAChRs in the thalamus. Previous studies provided little evidence that the T529A polymorphism affects receptor expression or  $\alpha 4$  protein levels in mouse brain [31,52] or transiently transfected cells [29]. Although the basis for the apparent effect of the T529A polymorphism on the expression of thalamic  $\alpha 4\beta 2^*$  nAChRs is not known, one possible explanation is that the level of  $\alpha 4\beta 2^*$  function in the thalamus is under tight homeostatic control. This hypothesis is based upon the observation that, despite the differences in expression of thalamic  $\alpha 4\beta 2^*$  nAChRs, there is no difference in the functional response of these receptors between T529 littermates and A529 knockin animals. Because the T529 variant is the normal allele expressed in C57BL/6J mice, these data suggest that  $\alpha 4\beta 2^*$  nAChRs made from the A529 allele of *Chrna4* are down regulated in expression in the thalamus in order to maintain a functional response indistinguishable from the normally expressed  $\alpha 4\beta 2$  nAChRs in this mouse strain. If this is the case, then the effect of the T529A polymorphism on receptor expression in the thalamus is not a direct effect of the polymorphism, but rather an indirect effect via homeostatic regulation of receptor function.

The finding that the T529A polymorphism affects the function of  $\alpha 4\beta 2^*$  nAChRs in the A529 knockin mice confirms that the polymorphism is within a novel functional domain of the receptor that influences the ratio of high to low sensitivity  $\alpha 4\beta 2^*$  nAChRs. As described previously [29,31], the polymorphism is within a region of the large cytoplasmic loop of the  $\alpha 4$  subunit that is highly conserved in mammals. Understanding the role of this region in regulating the function of  $\alpha 4\beta 2^*$  nAChRs and how the T529A polymorphism alters this activity provides unique insight into the molecular regulation of  $\alpha 4\beta 2^*$  nAChR function.

Although the *Chrna4* T529A polymorphism has not been found in human populations to date, the results of this study are, nonetheless, relevant for understanding the genetics of nicotine dependence in humans. For example, several groups have used genetic approaches to establish that  $\alpha 4\beta 2^*$  nAChRs are critical for nicotine self-administration, conditioned place preference and nicotine-induced hypothermia [26–28,40,41,44,45]. However, these



studies utilized knockout mice in which expression of  $\alpha 4\beta 2^*$  nAChRs was eliminated or hypersensitive knockin mice that express  $\alpha 4\beta 2^*$  nAChRs that exhibit a nearly twenty-fold increase in sensitivity to nicotine relative to native  $\alpha 4\beta 2^*$  nAChRs. Such dramatic changes in gene expression and function are not likely to be common among human genetic variants that influence nicotine dependence and other complex phenotypes. Results from the T529A knockin mice demonstrate that substantially smaller changes in the function or expression of  $\alpha 4\beta 2^*$  nAChRs, similar to those one would expect to occur with modest frequency in the human population, are sufficient to affect nicotine sensitivity in a meaningful way.

In summary, the data reported here are the first to demonstrate that a naturally-occurring polymorphism in the  $\alpha 4$  nAChR subunit contributes to individual variability in sensitivity to several effects of nicotine, including positive reinforcement, oral consumption and hypothermia. The effect of the polymorphism on these behavioral and physiological phenotypes is accompanied by an effect of the polymorphism on the function of  $\alpha 4\beta 2^*$  nAChRs in the midbrain, a brain region that modulates these behavioral and physiological phenotypes in an  $\alpha 4\beta 2^*$  dependent manner. Moreover, results from these studies provide the first evidence that the ratio of high to low sensitivity  $\alpha 4\beta 2^*$  nAChRs may be behaviorally and physiologically relevant. However, the mechanism by which the *Chrna4* T529A polymorphism affects the ratio of high to low sensitivity  $\alpha 4\beta 2^*$  nAChRs remains to be elucidated. Finally, these results along with the recently published data from Mague *et al.*, [53] demonstrate the utility of using the knockin mouse strategy in understanding the functional consequence of naturally occurring polymorphisms in complex behaviors related to drug addiction.

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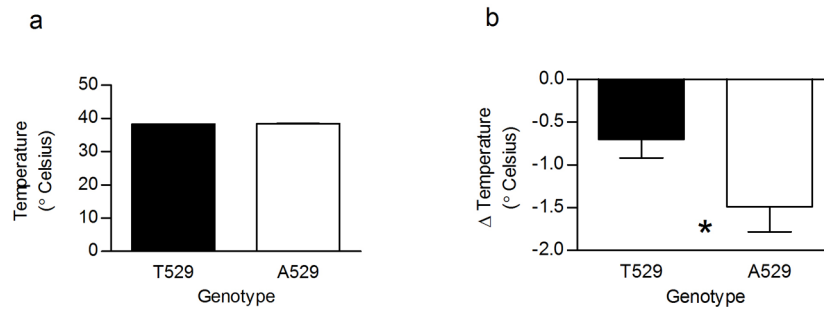
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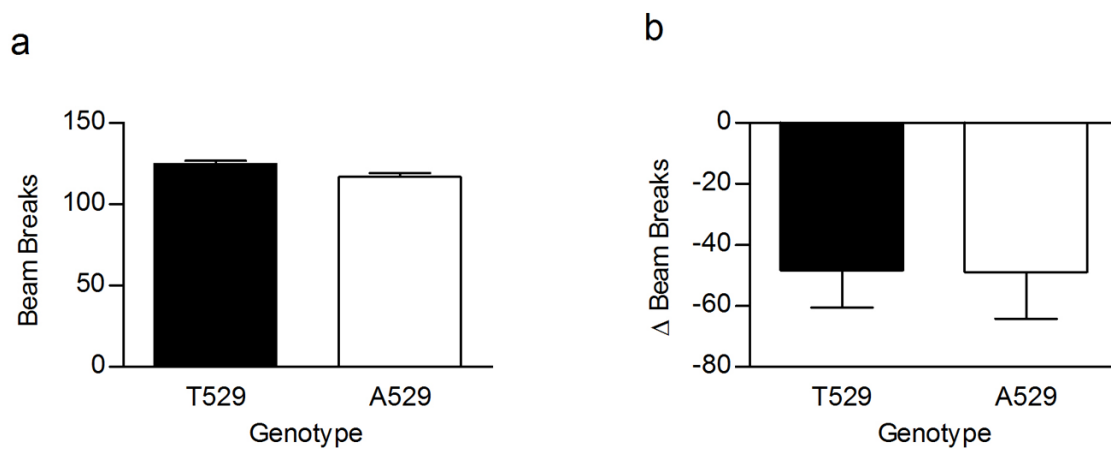
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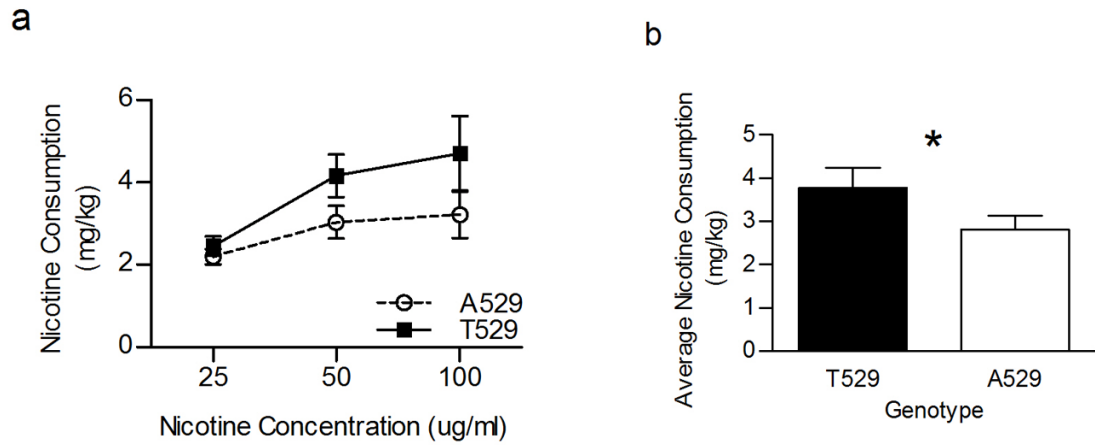


**Figure 1.** Effect of *Chrna4* T529A polymorphism on saline (a) and 0.5 mg/kg nicotine induced hypothermia (b). A529 knockin animals (n = 22) were more susceptible to the hypothermic effects of nicotine compared to T529 littermates (n = 25). The mean decrease in body for A529 knockin animals compared to control animals was  $1.49 \pm 0.29$  and  $0.70 \pm 0.22$  degrees Celsius, respectively. Asterisk (\*) indicates data values are significantly different ( $p < 0.05$ ) between genotype. In some instances, the error bars are too small to be seen.



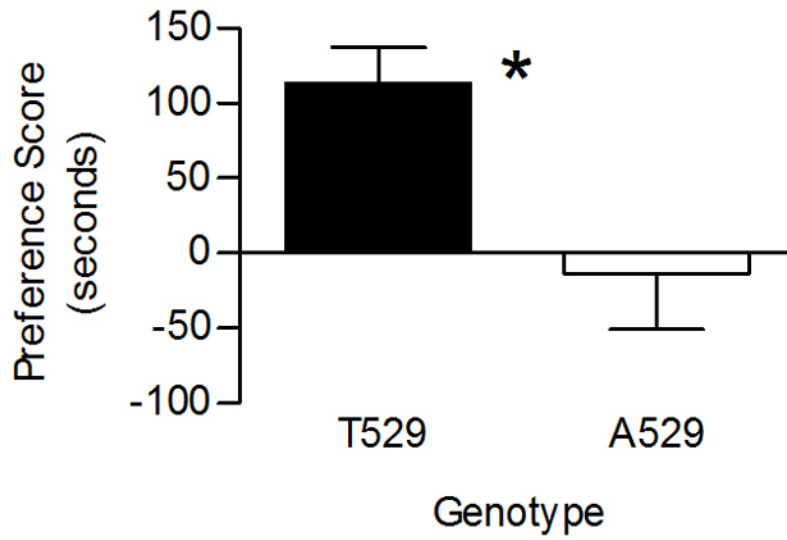


**Figure 2.** Effect of saline (a) and 0.5 mg/kg nicotine (b) on Y-maze activity in *Chrna4* T529A knockin mice. A529 animals (n = 22) and T529 littermates (n = 25) displayed no difference in nicotine-induced hypolocomotion. The average number of beam breaks for A529 knockin and T529 animals were  $48.86 \pm 15.28$  and  $48.32 \pm 12.20$ , respectively.

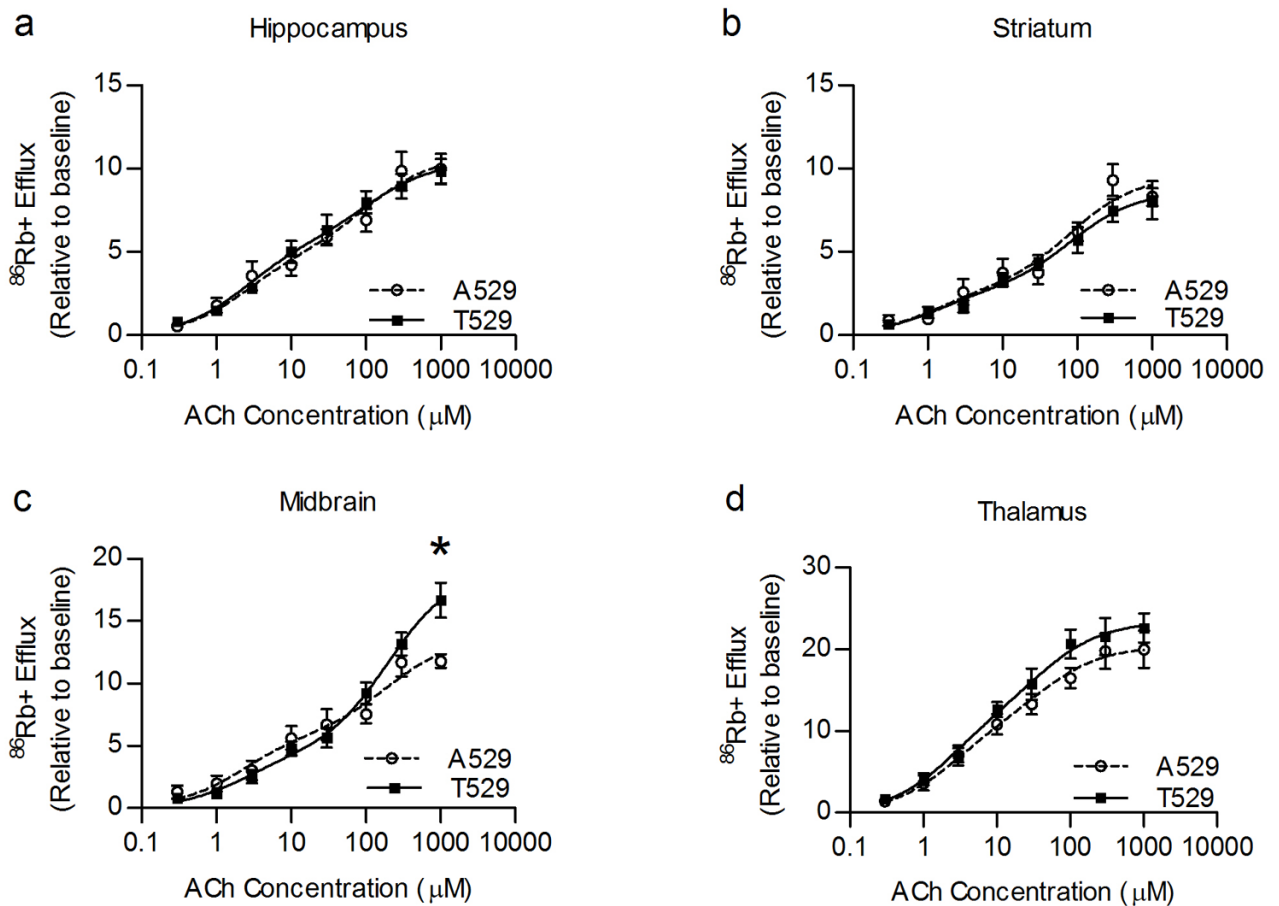


**Figure 3.**

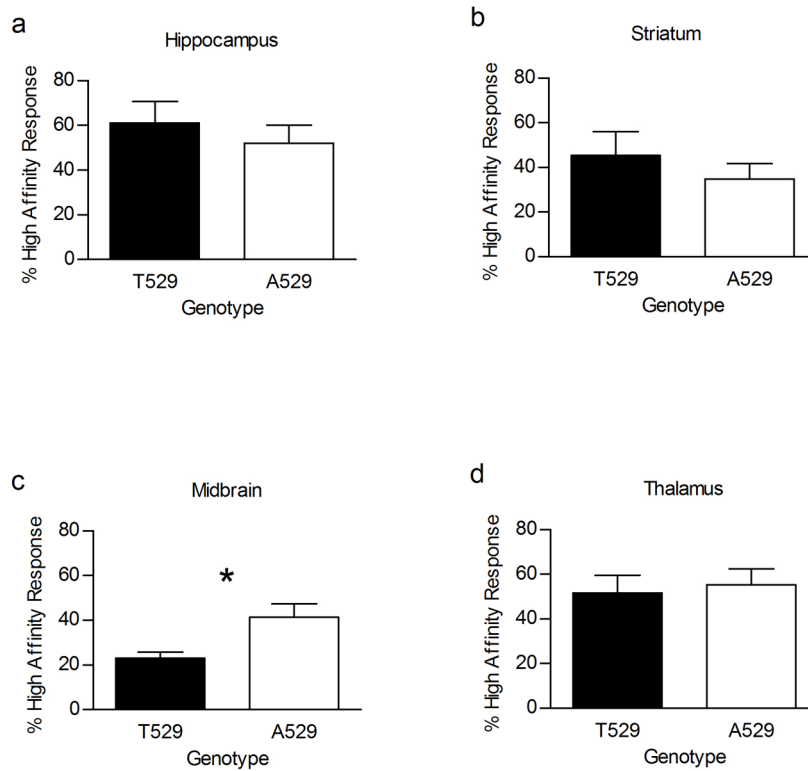
The influence of the T529A polymorphism on oral nicotine consumption. Overall nicotine consumption in *Chrna4* A529 mice (n = 29) was significantly less relative to T529 littermates (n = 26). A529 knockin animals consumed  $2.19 \pm 0.19$ ,  $3.03 \pm 0.39$ , and  $3.21 \pm 0.56$  mg/kg/day nicotine at the 25, 50 and 100  $\mu\text{g/ml}$  concentrations, respectively. In contrast, T529 littermates consumed  $2.45 \pm .023$ ,  $4.16 \pm 0.52$  and  $4.71 \pm 0.90$  mg/kg/day nicotine at the same nicotine concentrations. In addition, the average daily amount of nicotine consumed (figure 3b) was significantly less for *Chrna4* A529 animals ( $2.82 \pm 0.32$  mg/kg/day) relative to T529 controls ( $3.78 \pm .047$  mg/kg/day). Asterisk (\*) indicates  $p < 0.05$ .



**Figure 4.** Effect of *Chrm4* T529A polymorphism on unbiased nicotine induced conditioned place preference. T529 control animals (n = 12) exhibited significantly increased place preference to nicotine (0.09 mg/kg) relative to A529 knockin animals (n = 9). The average preference score for T529 control animals was  $113.17 \pm 24.32$  seconds compared to  $-13.56 \pm 36.96$  seconds for A529 knockin mice. Asterisk (\*) indicates  $p < 0.05$ .

**Figure 5.**

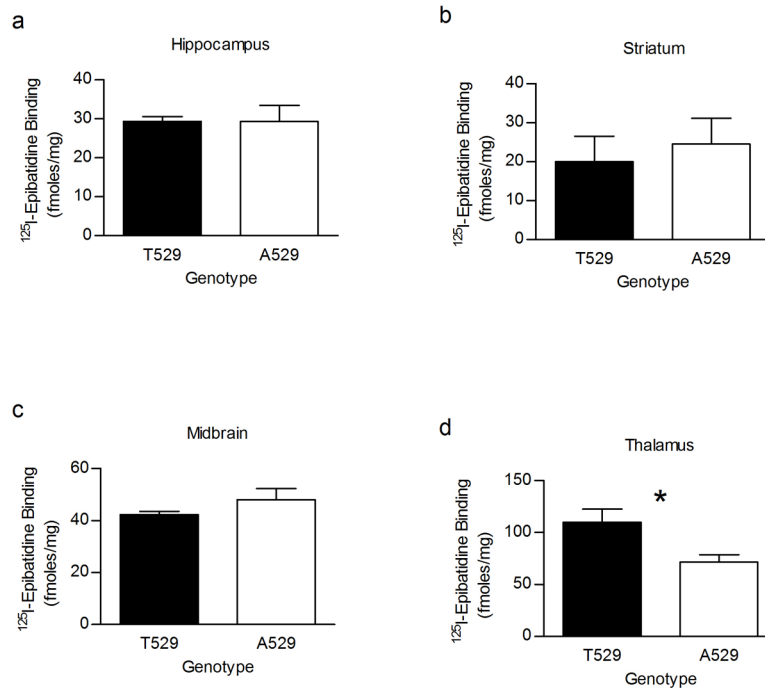
Effect of *Chrna4* T529A polymorphism on ACh elicited  $^{86}\text{Rb}^+$  efflux in hippocampus, striatum, midbrain, and thalamus. Genotypic differences in the high sensitivity component were detected in midbrain (c) but not hippocampus (a), striatum (b), and thalamus (d). Additionally, a significant difference in maximal response was observed in midbrain with T529 control animals exhibiting greater function ( $16.69 \pm 1.41$  units above baseline,  $n = 8-9$ ) than A529 knockin animals ( $11.79 \pm 0.55$  units above baseline,  $n = 6-8$ ). Data were fit to a two site curve with the  $\text{EC}_{50}$  values constrained for each component (high and low sensitivity). Asterisk (\*) indicates data are significantly different ( $p < 0.05$ ) for a genotypic comparison at the maximal response.



**Figure 6.**

Influence of the *Chrna4* T529A polymorphism on the percentage of high sensitivity  $\alpha 4\beta 2^*$  nAChRs. The contribution of the high sensitivity  $\alpha 4\beta 2^*$  component to the acetylcholine concentration response curve was calculated for hippocampus (a), striatum (b), midbrain (c), and thalamus (d) in A529 knockin animals ( $n = 6-8$ ) and T529 control animals ( $n = 8-9$ ). There was no effect of genotype for this measure of receptor function in hippocampus, striatum, and thalamus. However, a significant difference in the percent of high sensitivity was observed in midbrain where  $41.4 \pm 6.1\%$  of the response to acetylcholine was due to the high sensitivity component in A529 knockin mice and  $23.0 \pm 2.8\%$  of the acetylcholine response was due to the high sensitivity component in controls. Asterisk (\*) indicates  $p < 0.05$ .





**Figure 7.**

<sup>125</sup>I-Epibatidine binding in hippocampus, striatum, midbrain and thalamus of *Chrna4* T529A knockin mice. <sup>125</sup>I-Epibatidine (200 pM) binding, which measures high affinity nAChR expression, did not differ between A529 knockin mice and T529 littermates in membrane fractions prepared from hippocampus (a), striatum (b), and midbrain synaptosomes. However, there was a significant difference ( $p < 0.05$ ) in ligand binding in membrane fractions prepared from thalamic synaptosomes (d). T529 thalamus exhibited higher levels of binding ( $109.87 \pm 7.43$  fmol/mg) than thalamus from A529 knockin animals ( $71.50 \pm 7.43$  fmol/mg).