Effects of TI-299423 on Neuronal Nicotinic Acetylcholine Receptors

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) are pentameric, ligand-gated, cation channels found throughout the central and peripheral nervous system, whose endogenous ligand is acetylcholine, but which can also be acted upon by nicotine. The subunit compositions of nAChR determine their physiological and pharmacological properties, with different subunits expressed in different combinations or areas throughout the brain. The behavioral and physiological effects of nicotine are elicited by its agonistic and desensitizing actions selectively on neuronal nAChRs. The midbrain is of particular interest due to its population of nAChRs expressed on dopaminergic neurons, which are important for reward and reinforcement, and possibly contribute to nicotine dependence. The α6-subunit is found on dopaminergic neurons but very few other regions of the brain, making it an interesting drug target. We assayed a novel nicotinic agonist, called TI-299423 or TC299, for its possible selectivity for α6-containing nAChRs. Our goal was to isolate the role of α6-containing nAChRs in nicotine reward and reinforcement, and provide insight into the search for more effective smoking cessation compounds. This was done using a variety of *in vitro* and behavioral assays, aimed dually at understanding TI-299423's exact mechanism of action and its downstream effects. Additionally, we looked at the effects of another compound, menthol, on nicotine reward. Understanding how reward is generated in the cholinergic system and how that is modulated by other compounds contributes to a better understand of our complex neural circuitry and provides insight for the future development of therapeutics.

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INTRODUCTION

Nicotinic Acetylcholine Receptors (nAChRs)

Nicotinic Acetylcholine receptors are pentameric cation channels found throughout the central and peripheral nervous system. Endogenously, acetylcholine (ACh) activates these receptors, though they can also be activated by nicotine and other pharmacological compounds. nAChRs are made up of alpha (1-10), beta (1-4) and, in muscular junctions, δ, and ε or γ subunits [1, 61]. The neuronal nicotinic subunits are α2-7 and β2-4. The diversity of subunit types means there are many potential combinations for composing α-only or α and β-containing receptors; however, some are far more common than others. The most common heteromeric receptor is α4β2, while the most common homomeric receptor is α7. The exact combination of subunits determines the sensitivity of a receptor to ACh or other ligands [24].

nAChRs in the brain are primarily expressed at presynaptic terminals [24] where they modulate the release of neurotransmitters such as dopamine, glutamate, serotonin, and GABA [13]. The role of any nAChR is determined by the type of neuron on which it is expressed. The expression of the α6 subunit, for example, is limited to dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), as well as to the visual pathway, including the superior colliculus and a population of ganglion

cells in the retina [9, 36, 62]. The α4 unit, for comparison, is found on many different neuron types throughout most of the brain [24].

The α6 nAChR Subunit

α6-containing nAChRs are an interesting focus of study and an interesting drug target because their expression is limited to dopaminergic neurons, which are involved in learning, memory, Parkinson's disease, and reward. While α6 is only present in about half of the nicotinic receptors found on dopaminergic neurons, they have a large functional contribution to mesolimbic dopamine release [44, 48], with almost all of the dopamine release in the nucleus accumbens (NAc) being attributable to α6*-nAChR activation (where * indicates the presence of additional subunits) [48]. Dopamine's role in learning, reward, and reinforcement implicates α6* receptors as a contributor to nicotine dependence. In fact, by using mutant mice with hypersensitive α6 subunits (α6L9'S), α6*-nAChRs have been shown to be sufficient for Pavlovian reward conditioning for nicotine using conditioned place preference (CPP) [16]. Additionally, α6 null mutant mice (α6 KO) fail to self-administer nicotine [46]. This behavior is rescued when α6 is virally reintroduced to the VTA [46]. These data indicate α6*-nAChRs are a potentially useful target for smoking cessation compounds.

However, studying the α6 subunit is challenging. *In vitro* expression of α6*-nAChRs has been hampered by technical difficulties in which receptors either do not form or fail to be trafficked to the cellular membrane [30, 32, 37]. Use of chimeric, concatameric, or mutant

α6 subunits has increased our ability to research these receptors in *Xenopus* oocytes and mammalian cell lines, but the external validity of data obtained using these techniques is still unknown. Additionally, because only dopaminergic neurons are known to express the α6 subunit, and only about half of nAChRs on those neurons contain α6, isolating these receptors in brain slices, or their effects in the whole brain, can be equally difficult.

There are two primary methods used to isolate the functional contribution of α6 *in vivo*: genetic manipulation and selective compounds. Genetic manipulation has been used to create mice with either gene disruption (α6 KO) or the addition of a mutant gain-of-function α6 gene (α6L9'S). α6 KO mice do not express any α6*-nAChRs, whereas transgenic α6L9'S mice express a mutated α6 subunit, in which the Leu 9' residue in the M2 domain has been mutated to a Ser, rendering the subunit hypersensitive. Because of their genotype, α6 KO mice do not self-administer nicotine [46], but they can still show reward for high doses of nicotine using CPP [52]. Mice with the α6L9'S gene, however, show CPP for nicotine at lower doses than their WT litter mates [16], and have an increased locomotor response to acute nicotine administration [52]. In addition to genetic manipulation, compounds that are selective for α6-containing nAChRs can be used. α-Conotoxin MII, for example, is a nicotinic antagonist selective for α6 and α3 containing receptors [31]. Thus, when applied directly to the midbrain or to dopaminergic synaptosomes, which contain very few α3*-nAChRs, α-Conotoxin MII can be used to isolate the response being mediated by α6*. The use of α-Conotoxin MII is thus similar to using α6 KO mice. It would therefore be useful to have a compound that activated only α6-

containing receptors: a pharmacological equivalent to α6L9'S mice. To date, however, such a compound has not been developed.

The α4 nAChR Subunit

α4β2-nAChRs are the most common neuronal nicotinic receptor subtype and are found in two distinct stoichiometries, $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$. The $(\alpha4)_2(\beta2)_3$ receptors have a much higher sensitivity to ACh and other agonists than the $(\alpha4)_3(\beta2)_2$ receptors. Unlike α6*-nAChRs, α4β2-containing receptors are not localized to any single area of the brain; rather, they are spread throughout [24]. As a result, they have been implicated in a number of different effects and behaviors, including nicotine induced conditioned place preference [7], nicotine self-administration [45], nicotine induced dopamine release [15], nicotinic antinociception [60], and nicotine induced hypothermia [57, 58]. Because they are involved in a wide range of behaviors and found in so many brain regions, pharmacologically targeting α4β2*-nAChRs risks many off-target effects, making them a less appealing target than α6*-nAChRs.

Perturbations in α4β2-nAChR expression are linked to disease states, most notably autosomal dominant nocturnal frontal lobe epilepsy, or ADNFLE. ADNFLE is caused by point mutations that shift the expression of α4β2-nAChRs towards the low sensitivity configuration [54]. The motor seizures experienced by affected individuals are attenuated when patients consume nicotine through cigarette smoke. This effect is believed to be mediated by nicotine's actions as a pharmacological chaperone, upregulating the number of

high sensitivity α4β2-nAChRs expressed on the cellular membrane. This implies that the downstream and long-term effects of nicotinic compounds are just as, if not more, important as the immediate effects seen via acute administration.

Thankfully, unlike the α6-containing receptors, α4β2*-nAChRs express readily in oocytes and mammalian cell lines. This makes the effects of nicotine, ACh, and other nicotinic compounds on these receptors much easier to identify. Additionally, certain brain areas, such as the cortex and thalamus, contain almost exclusively α4β2-nAChRs [24]. Thalamic synaptosomes or membranes from cortical neurons can thus be used to identify drug properties for α4β2-nAChRs without needing to block activity at other receptor types. Finally, both null mutant α4 KO mice (that do not express the α4 subunit) and gain-of-function α4L9'A mice (that express hypersensitive α4 subunits) have been developed, aiding the study of α4 in living animals. In the case of the α4 KO mice, we can identify behaviors for which the α4 subunit is necessary, as they are knocked out along with the gene. Because the L9'A mutation renders the α4 subunits of these mice hypersensitive to nicotine, α4L9'A*-nAChRs can be activated at doses too low to activate any other receptors. α4*-nAChRs are thus sufficient for any behavior that can still be elucidated by such a low dose in these mice.

Other nAChR Subunits

Other nAChR subtypes with implications for nicotine dependence, cognition, mood, and disease states include α3β4*-nAChRs, α7-nAChRs, and α5*-nAChRs. The α3-subunit

almost always coexpresses with β4 [32]. α3β4* receptors are primarily found in the medial habenula (MHb) and the interpeduncular nucleus (IPN) [24] and are less sensitive to nicotine than α4β2 receptors. Activation of α3β4*-nAChRs has been linked to nicotine aversion and withdrawal. Null mutant β4 KO mice show fewer withdrawal symptoms [28], and mice with enhanced levels of α3β4*-nAChRs show conditioned place aversion to nicotine and consume less nicotine-containing water [20]. Additionally, α3β4*-nAChRs do not become desensitized to nicotine at the levels found in smokers [50]. This makes them an interesting target for smoking cessation research.

Homomeric α7-nAChRs have recently been investigated as a target for the treatment of negative and cognitive symptoms of schizophrenia, as well as a potential target for cognitive enhancement. Antagonists for α7 induce sensory gating deficits similar to those seen in schizophrenics and schizophrenia model mice [38, 41]. Additionally, post-mortem studies in individuals with schizophrenia have revealed a decrease in α7 receptors [22]. A vast majority (>90%) of schizophrenics smoke [65], a behavior thought to be self-medication. Both agonists and positive allosteric modulators of α7 are currently being investigated for schizophrenia treatments and/or cognitive enhancement.

The α5-nAChR subunit is an auxiliary subunit that is selectively coexpressed with α4β2* and α3β4* receptors. In α4α5β2 receptors, the α5 subunit does not participate in a functional binding site. α4α5β2-nAChRs are more sensitive to nicotine and ACh than low sensitivity α4β2(non-α5) receptors, and are more permeable to Ca2+ [34, 56]. The presence of an α5-subunit also makes α4β2*-nAChRs less susceptible to nicotine-induced

upregulation [33]. About 15% of α3β4*-nAChRs in the MHb and 35% in the IPN contain an α5 subunit [25]. α5 KO mice continue to show conditioned place preference for nicotine at doses high enough to induce aversion in their wild type littermates [27], a response thought to be mediated by α3α5β4-nAChRs. Understanding the roles of a range of nAChRs, and how they are interrelated in this way, allows for better determination of drug targets and improved predictability of drug effects.

Nicotine Dependence

One of the primary motivations for studying nAChRs is to better understand nicotine dependence. Nicotine is the primary addictive component in tobacco products, making nicotine addiction a serious health issue. The US Surgeon General first linked tobacco use to disease over 50 years ago [59]. Regardless, today more than 10% of deaths of individuals over 30 are directly attributable to tobacco use [64], amounting to millions of fatalities each year. In fact, tobacco use is a leading cause of preventable death worldwide [63]. This is partially due to tobacco's addictive properties. While about 40% of smokers report trying to quit in the last year, only 10% successfully abstain for a full year [23]. Success rates are slightly improved with medication and counseling, but they remain quite low. Thus, better understanding the molecular basis for nicotine dependence is imperative for improving smoking cessation therapies.

Nicotine has several of actions in the brain that need to be considered when studying nicotine dependence. First, it directly affects nAChRs expressed on neuronal membranes,

both as an agonist and via desensitization. Second, nicotine is a molecular chaperone, affecting the number and type of receptors expressed on the membrane. Third, nicotine can also affect gene expression. This multitude of effects makes it challenging to achieve a complete understanding of nicotine dependence.

A large proportion of research has focused on the midbrain, which is known to be highly involved in learning, attention, and reward [26, 35, 42, 55]. The dopaminergic system, specifically, is thought to encode reward and/or salience. This suggests nicotine's effect on the dopaminergic system could be responsible for its habit-forming properties. Thus, α6β2* and α4β2*-nAChRs, both of which have been shown to mediate dopamine release [51], are often targeted in nicotine dependence research. Additionally, variations in the CHRNA5 gene, which encodes for the human α5 subunit, have been linked to an increase risk of nicotine dependence and an increase in daily number of cigarettes smoked [3, 4]. This implicates α5-containing receptors, in the development of dependence and addition.

Studying how nicotine changes receptor prevalence and gene expression is more difficult because it requires long term, chronic nicotine exposure. Experiments are longer and harder to accomplish using animal models or *in vitro* methods. The external validity of these studies is also difficult to prove. Chronic nicotine exposure in a smoker may imply decades of nicotine use, which is often infeasible to model or study in a laboratory environment. Shorter term chronic nicotine experiments must be used to determine changes in receptor regulation and gene expression.

Nicotine and Neuroprotection

Evidence suggests that nicotine is neuroprotective against Parkinson's disease (PD) [21, 39, 47]. Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the SNc and their projections. This results in difficulties with voluntary movement, including tremors, rigidity, and changes in gait or speech [29]. Later symptoms can include neuropsychiatric disorders, such as depression [29], impulse control issues [8, 29], dementia [5, 29], and executive dysfunction [5]. PD is the second most common neurodegenerative disorder behind Alzheimer's disease, affecting approximately 7 million people worldwide [39]. Currently, there is no cure for PD. The primary treatment for PD is Levodopa, or L-DOPA, which is metabolized into dopamine, reducing the severity of motor symptoms. In a majority of individuals, PD is idiopathic, though a small number of genetics factors may increase risk. Without knowing what causes PD, and without a cure, it is imperative to find ways to prevent the onset of disease.

Several compounds have been suggested to be neuroprotective against PD, including caffeine [12, 39], non-steroidal anti-inflammatory drugs (NSAIDs) [39], oestrogens [39], and nicotine [10, 21, 39, 47]. Smoking is negatively correlated with PD but the causal directionality is debated. At this time, it is unclear if smoking reduces the risk for PD or if being at risk for PD decreases the likelihood of smoking, but the two are not necessarily mutually exclusive. One study showed that ease of quitting smoking was positively correlated with PD, suggesting that premanifest PD may include smoking cessation [49].

Other studies have shown that any history of smoking at all decreases the probability of developing PD in the future [10, 49].

The mechanism by which nicotine is proposed to be neuroprotective against PD seems to be associated directly with nicotine's activation of nAChRs on dopaminergic neurons, and the long-term potentiation of those connections that can follow. In fact, previous studies have suggested that α6β2*-nAChRs, which are primarily responsible for nicotine induced dopamine release [47], may be necessary for neuroprotection, as their presence is only observed when nicotine neuroprotection has occurred [47]. This suggests that ligands targeting α6β2*-nAChRs and, to a lesser extent, α4β2*-nAChRs in the SNc could be used as potential treatments for the prevention of PD. Nicotine also reduces L-DOPA-induced dyskinesias [47]. Though the exact nAChR subtypes responsible for that are unknown, the prevalence of α6β2* and α4β2*-nAChRs in the brain implicates them as possible targets for this as well.

Nicotinic Acetylcholine Receptors as Pharmacological Targets

There are many drugs known to act on nAChRs as either agonists or antagonists, including nicotine, varenicline, mecamylamine, cytisine, bupropion, and epibatidine. Several of these compounds have analgesic properties, affect mood, or are used for smoking cessation. Alternative nicotine delivery methods (besides tobacco use) have been FDA approved for smoking cessation. As previously mentioned, it can have beneficial effects on individuals

with schizophrenia or ADNFLE. Nicotine also has antinociceptive properties [60], though it is not commonly used for this and, as mentioned, may be neuroprotective against PD.

Nicotine has been known to have antinociceptive properties for centuries [11], an effect mediated α4β2-nAChRs [60]. Epibatidine, a nicotinic compound found on the skin of poison dart frogs [19], is a potent α4β2 agonist and, thus, a potent analgesic. However, epibatidine also affects muscarinic ACh receptors, causing paralysis [17] and its therapeutic dose is very close to its toxic concentration [18], making it effectively unusable. Similar α4β2 agonists, such as ABT-594, have also been studied for their antinociceptive effects [14].

Varenicline is a partial agonist for α4β2-nAChRs, with an efficacy about 13% that of ACh [43]. While it has been FDA approved for smoking cessation, the success rate for people using varenicline to quit smoking was only about 10% [6], which is modest at best. Additionally, varenicline has several negative side effects, including abnormal dreams, depression, and suicidal thoughts and tendencies. In addition to its actions on the cholinergic system, varenicline is a potent agonist of 5-hydroxytryptamine(3) (5-HT(3)) receptors [40]. This off-target effect is thought to be the cause of many of the psychological side effects.

Buproprion is a nicotinic antagonist and weak norepinephrine-dopamine reuptake inhibitor developed for use as an antidepressant. Because it works on a different system than selective serotonin reuptake inhibitors (SSRIs), bupropion is often used in addition to a

more traditional antidepressant regimen, or as the primary treatment for individuals with adverse reactions to SSRIs. Smokers taking bupropion for depression reported a reduction in nicotine craving as well as reduced nicotine withdrawal symptoms, which led to the drug being prescribed for smoking cessation [66]. As a smoking cessation aid, bupropion has the same level of efficacy as nicotine replacement therapy, and slightly lower efficacy than varenicline [66].

Like bupropion, mecamylamine is a nonselective nicotinic antagonist that has proven useful in the treatment of several ailments. Originally, mecamylamine was intended as a treatment for hypertension [2]. More recently, low doses have proven effective as smoking cessation treatments [53]. Mecamylamine is also currently proposed as an antidepressant. Phase II trials are in progress for mecamylamine as a monotherapy antidepressant, and phase III trials are being conducted for approval as an add-on depression treatment. The relationship between mood, smoking cessation, and nAChR activation is not fully understood, but continued study on nicotinic compounds helps form a more complete picture of their interaction.

Present Research

The exact mechanisms behind nicotine addiction are currently not well understood. Studying other compounds, that modulate nicotine reward and dependence, or that are themselves rewarding through similar mechanisms, allows us to break down the complex question of nicotine addiction into smaller, more easily understood pieces. To that end, this

thesis focuses on two such compounds: novel nicotinic agonist TI-299423 and menthol, a popular flavor additive for tobacco.

In the next chapter, TI-299423 is introduced and its effects on a range of nAChRs, including α4β2*, α6β2*, and α3β4* are explored. Its apparent selectivity for α6*-nAChRs at low doses allows us to examine its rewarding properties as well as its effects on nicotine reinforcement. Additional data on TI-299423, not included in Chapter 2, are included in Chapter 3.

Chapter 4 focuses on menthol, a different compound which, through unknown mechanisms, modulates the rewarding effects of nicotine. Menthol is added to some tobacco products, possibly making them more addictive. Chapter 4 uses conditioned place preference to investigate the role of chronic and acute menthol on nicotine reward, as well as the rewarding properties of menthol itself. Finally, Chapter 5 discusses possible directions for future research.

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Chapter 2

NOVEL NICOTINIC AGONIST TI-299423 IS SELECTIVE FOR α6β2*-nAChRs AT LOW DOSES, POTENTIATING NICOTINE SELF-ADMINISTRATION

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Abstract

TI-299423 is a novel nicotinic agonist developed for its potential selectivity for nicotinic acetylcholine receptors (nAChRs) containing the α6 subunit (α6*-nAChRs, where * indicates the presence of additional subunits). Using [^{125}I]-epibatidine binding, $^{86}Rb^{+}$ efflux, [^{3}H]-dopamine release, patch-clamp recordings, and [^{3}H]-ACh release, we looked at the affinity, efficacy, and potency of TI-299423 on α6β2*, α4β2*, and α3β4* receptors. TI-299423 is a partial agonist for α6*-nAChRs with an EC_{50} between 30 and 60 nM, making

it partially selective for this subtype at low doses. Pharmacokinetic studies were done to determine TI-299423's bioavailability in mouse models, and an off-target effects screen was done to rule out effects on other neuronal receptor types. Locomotor behavior was measured in gain of function α6L9'S mice, showing TI-299423 to elicit α6β2*-mediated responses in living mammals. Because of the demonstrated role of α6β2*-nAChRs in nicotine reward and reinforcement, we looked at the similar effects of TI-299423 via conditioned place preference (CPP) and intravenous self-administration of nicotine. TI-299423 was shown to potently elicit conditioned place preference in wild type (WT) mice at a dose 12 ng/Kg. α4 null mutant mice (α4KO) showed CPP at the same dose as WT mice. However, mice lacking the β2 subunit (β2 KO) and mice lacking the α6 subunit (α6 KO) did not show CPP, indicating that TI-299423 is rewarding primarily through its activation of α6(non-α4)-containing receptors. To investigate TI-299423's effect on nicotine reward, rats trained to self-administer nicotine on a fixed ratio 5 schedule were pre-injected with TI-299423 20 min prior to being allowed access to nicotine self-administration. TI-299423 significantly increased IVSA of nicotine compared to pre-injection with saline ($F_{(1,7)}$=7.041, $p < 0.05$). Individually, TI-299423 pre-treatment doses of 0.01 mg/kg ($p < 0.05$) and 0.05 mg/kg ($p < 0.05$) significantly increased IVSA of nicotine compared to saline pre-treatment. Similar trends are seen when looking at active lever presses. We hypothesize TI-299423 is activating α6(non-α4)-nAChRs normally not activated by the administration of nicotine alone, increasing reward, a phenomenon that warrants further investigation. Additionally, TI-299423's anxiolytic and analgesic effects were compared to the effects of nicotine and varenicline using marble burying and hot

plate, respectively. Mice given TI-299423 buried a similar number of marbles to mice given nicotine, significantly less than saline treated mice ($p < 0.001$) or varenicline treated mice ($p < 0.001$). Mice given TI-299423 also showed a similar antinociception response to mice given nicotine, spending significantly more time on the hot plate than when they were administered saline ($p < 0.05$) or varenicline ($p < 0.001$).

Introduction

Nicotine is the primary rewarding and addictive compound in tobacco [2, 30, 39, 43]. In addition, nicotine improves cognitive function and attention [27] and reduces the risk of Parkinson's disease (PD) [35]. These actions of nicotine are via activation and desensitization of nicotinic acetylcholine receptors (nAChRs) in the brain, as well as effects on the number of receptors expressed [32]. Neuronal nAChRs are pentameric, ligand-gated cation channels made up of combinations of α2-7 and β2-4 subunits. These subunits can combine to make heteromeric, most commonly α4β2, or homomeric, α7, receptors. nAChRs are expressed primarily on presynaptic terminals where they modulate the release of other neurotransmitters, such as dopamine [5, 11]. Which subunits combine to form a given receptor determines that receptor's pharmacological and electrophysiological properties, and may be indicative of its location both on the cell and in the brain.

Both the α4 subunit and the α6 subunit co-express with β2 in the brain. β2-containing receptors (β2*) have been shown to play a crucial role in nicotine reward and reinforcement [25, 31, 46]. Rodents with β2 subunit deletions (β2KO) fail to self-

administer nicotine [25, 31] but can be taught to self-administer cocaine [31]. Additionally, β2KO mice do not show conditioned place preference for nicotine [46]. α6-containing receptors are only found on dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc), and in the visual pathway, including the superior colliculus and a population of retinal ganglion cells [7, 47]. Their localization alone suggests a large role in nicotine reward and reinforcement, as well as a possible mechanism of nicotine's neuroprotection against PD. Unsurprisingly, α6* receptors have been shown to be sufficient for Pavlovian reward conditioning for nicotine using conditioned place preference [9], and mice with α6 subunit deletion (α6KO) fail to self-administer nicotine, a behavior that was 'rescued' when α6 was virally introduced to the VTA [33]. These characteristics make α6β2* receptors a prime target for potential new smoking cessation compounds, and possibly PD treatments. In contrast, while α4β2* receptors are also known to be important for nicotine reward and reinforcement [4, 26, 31, 33, 38, 40], they are the most common type of receptor in the brain [40] and are found in many different regions [15]. This implies that targeting α4β2* nAChRs holds a higher risk for unintended side-effects, making those receptors less appealing as a target for smoking cessation aids and PD treatments. Thus, developing a compound that is selective for α6*-nAChRs, or even α6(non-α4)-nAChRs, could provide valuable insight into the cholinergic system, and have possibly beneficial therapeutic effects.

Here, we introduce a novel nicotinic agonist, TI-299423. Using *in vitro* binding and release studies, as well as N2A-cells, we show TI-299423 to be extremely potent and somewhat selective for α6*-nAChRs. Additionally, data was obtained on the pharmacokinetics of TI-

299423 to better understand its bioavailability, and a locomotor assay with mice expressing a hypersensitive α6 subunit (α6L9'S) was used to show that TI-299423 potently activates α6*-nAChRs *in vivo*. We hypothesized that TI-299423 would itself be rewarding, and would modulate the rewarding and reinforcement properties of nicotine. To first test the rewarding properties of TI-299423, we did CPP with several different mouse genotypes to determine which receptors were involved, and created CPP dose response curves for WT and α6L9'S mice. To test TI-299423's effect on the rewarding properties of nicotine, we used it as a pre-treatment for rats trained to self-administer nicotine. Due to TI-299423's effects on β2* receptors, which have previously been implicated in the anxiolytic effects of nicotine [1, 42], we suspected TI-299423 would have anxiolytic properties as well, which we tested using marble burying, and compared the results with those of nicotine and varenicline. Finally, because TI-299423 is an agonist for α4β2* and α3β4*-nAChRs at higher doses, we measured its antinociception properties using a hot plate assay and compared that to nicotine and varenicline as well.

Materials and Methods

Mice **–** Animal breeding, maintenance, and procedures at the California Institute of Technology were conducted in accordance with the guidelines of the National Institutes of Health and the approval of the California Institute of Technology Animal Care and Use Committee. Mice of the C57BL/6 strain, ages 40-180 days, were used in this study. After weaning at 25 days of age, same sex littermates were housed no more than 3 to a cage, with free access to food and water, on a 13/11-h light/dark cycle at 22^0C. Mice of

the α4 subunit null mutant line [24], the α6 subunit null mutant line [6], and the hypersensitive α6L9'S transgenic mice [8] were bred and maintained as above and genotyped as previously described [6, 8, 24].

C57BL/6J strain mice, as well as various subunit null mutant mice on this background, were bred and maintained at the Institute for Behavioral Genetics, University of Colorado, Boulder, CO. Animal care and procedures with these mice were all in accordance with the National Institutes of Health and approved by the Animal Care and Utilization Committee of the University of Colorado, Boulder, CO.

TI-299423, Nicotine, Mecamylamine, Varenicline – TI-299423, (E)-5-(pyrimidin-5-yl)-1,2,3,4,7,8-hexahydroazocine (Figure 1), was synthesized by Targacept, Inc. (Winston-Salem, NC). (-)-Nicotine hydrogen tartrate salt and mecamylamine were purchased from Sigma-Aldrich (St. Louis, MO). Varenicline tartrate was synthesized by Targacept, Inc. (Winston-Salem, NC). All compounds were dissolved in physiological saline (0.9% sodium chloride). Dose concentrations refer to the free base.

Conditioned Place Preference – The conditioned place preference apparatus is a rectangular cage with interior dimensions 46.5(L) x 12.7(W) x 12.7(H) cm, divided into three sub compartments: White and Black (each 16.8cm L) with a steel mesh and steel rod floor respectively, and Grey (7.2 cm L) with a solid plastic floor. Each compartment has a polycarbonate hinged lid for loading the animals. Guillotine doors, which can be fixed in the open or closed position, separate the chambers.

Mice were singly housed and habituated to the experimental room for 3-7 days before the initial testing day, and remained in the experimental room for the duration of the experiment. On day 1 (pre-training) mice were placed into the center chamber and allowed to explore the apparatus freely for 20 min. Time spent in each chamber was recorded, and drug pairing was determined by the least preferred chamber. On days 2, 4, 6, and 8, mice were injected with the drug or dose of interest, and were confined to the drug-paired chamber for a total of 20 min. On days 3, 5, 7, and 9, mice were injected with saline and confined to the opposite chamber. On day 10 (post-training), mice were again given free access to the apparatus for 20 min, and time spent in each chamber was recorded. Conditioned place preference was determined by looking at the change in time spent in the drug paired chamber compared to the saline paired chamber from pre-training to post-training. Mice with a severe initial bias for one chamber, defined as a bias of greater than 65% time spent in one conditioning chamber over the other, were excluded.

Self-administration:

Animals and Housing – All rat procedures were conducted in adherence with the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Mt. Sinai. Male Wistar rats (n=8; Charles River Laboratories, Raleigh, NC) weighing 400-500g were housed in groups of 1-2 per cage in an environmentally controlled vivarium on a 12h reverse light-dark cycle. Prior to the commencement of behavioral testing, all rats were given ad-libitum access to food and water.

Materials – Eight operant chambers purchased from Med-Associates Inc. (East Fairfield, VT, USA) were used for the self-administration experiments. Operant chambers are equipped with an active and an inactive response lever, a cue light, a food pellet dispenser, and an automatic injection pump for administration of compounds via IV catheter.

Surgery – Rats were first anesthetized using inhalation of 1–3% isoflurane in oxygen and then surgically prepared with silastic catheters in the jugular vein (Caine et al, 1993). The catheter was passed subcutaneously to a polyethylene assembly mounted on the animal's back.

Self-administration Experiment – Prior to training, rats were food-restricted such that their body weight was approximately 85% that of free-feeding rats. They were then trained to press an active lever for 45mg food pellets on a fixed ratio 5 time-out 20s (FR5TO20) schedule of reinforcement. An inactive lever was also present in the operant box. Pressing of this lever was recorded but was not associated with scheduled consequence. Rats were trained using food pellets until a stable intake was reached, defined as more than 90 pellets earned per 1h session. Rats then responded for nicotine for 1h daily sessions. In accordance with the FR5TO20 schedule, five active lever presses resulted in delivery of a 1s nicotine infusion (0.03 mg per kg per infusion freebase), followed by a 20s time-out period paired with a light cue above the active lever, during which time the active lever was without consequence.

During experimental sessions, rats were given IP injections of TI-299423 at an injection volume of 1ml per 300g weight. 20 min after this injection, the rats were placed in the operant boxes for a 1h session during which time all active and inactive lever presses were recorded. After each session, catheters were flushed with heparin and checked for leaks or blockages. Each rat was tested with each of the doses (Veh, 0.025 mg/kg, 0.05 mg/kg, and 0.08 mg/kg) using a Latin-square crossover design, with two 1-h daily nicotine self-administration sessions without pre-injection between experimental days to maintain and reinforce self-administration behavior. Several weeks later, rats were tested again with a pre-injection dose of TI-299423 of 0.01 mg/kg.

Marble Burying – In an activity cage with bedding 2 inches deep, 15 marbles were arranged approximately 2 inches apart in a 5 by 3 marble grid. Mice were habituated to the experiment room for at least 2 h prior to testing. Mice were injected IP with the dose of interest and then placed in cage with the marbles. After 10 min, the mice were returned to their home cages. Marbles were counted as buried if they were at least 75% covered in bedding. Each mouse was tested under each experimental condition used a Latin-square crossover design with 3-4 days without testing in between each test day.

Hot Plate – Mice were habituated to the experiment room for at least 2 h prior to testing, and injected intraperitoneally 5 min prior to being placed onto the hot plate apparatus (Harvard Apparatus, Holliston, MA). This is a heated metal plate surrounded by a plexiglass cylinder, within which the mouse is free to move (approximately 10 cm in diameter). The plate is set at 51^0C-54^0C. The animal is removed from the plate once it

exhibits evidence of discomfort (such as paw shaking, paw licking, jumping, or vocalization) or until a cutoff time is reached (60 sec), whichever comes first. If the mouse urinates during the assay, it is immediately removed from the hot plate. The time that the mouse remains on the hot plate prior to showing signs of discomfort is recorded. Doses were administered using a Latin-square crossover design with 3-4 days without testing in between each testing day.

Binding Studies Tissue Preparation – The methods used for preparing brain membranes in hypotonic buffer were similar to the methods of [20, 21]. Brain membrane preparations were either stored as pellets under buffer at -70^{0}C or used immediately.

[^{125}I]-epibatidine binding – Methods for [^{125}I]-epibatidine binding are described in [16]. K_i values for inhibition of binding of 100 pM [^{125}I]-epibatidine by TI-299423 were determined for various nAChR subtypes using WT cortical membranes (α4β2*), IPN from β2KO mice (α3β4*), or striatal (ST) membranes from α4KO mice (α6β2*) by incubation for 2 h at room temperature followed by filtration. 1 mM (-)-nicotine tartrate was used to determine non-specific binding. Bound [^{125}I]-epibatidine was determined by counting at 60% efficiency in a 1450 MicroBeta Trilux scintillation counter after addition of Optiphase SuperMix scintillation cocktail (150 ml/sample) (Perkin Elmer Life Sciences-Wallac Oy, Turku, Finland).

Membrane binding data analysis – After subtraction of non-specific binding, K_i values were determined by a one-site fit to the inhibition equation $B = B_0/(1+([I]/IC_{50}))$ where B

is ligand bound in the presence of inhibitor at concentration [I] and B_0 is ligand bound in the absence of inhibitor [48]. K_i values were calculated from IC_{50} values using the equation $K_i = IC_{50}/(1+(L/K_D))$.

Synaptosome preparation – Regions of interest were dissected from fresh mouse brains and were homogenized in ice-cold isotonic sucrose (0.32 M) buffered with HEPES (5 mM, pH 7.5). The suspension was centrifuged at 12,000 x g for 20 min and then the pellet was resuspended in the appropriate uptake buffer [17, 23, 37] and used immediately.

[^{3}H]-Dopamine uptake and release – Release methods of Salminen et al. (2004, 2007) [36, 37] were followed using crude synaptosomal pellets from striatal tissue. Superfusion was at room temperature with buffer (NaCl, 128 mM; KCl, 2.4 mM; $CaCl_2$, 3.2 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; HEPES, 25 mM; pH 7.5; glucose, 10 mM; ascorbic acid, 1 mM; pargyline, 0.01 mM) containing 0.1% BSA, nomifensine (1 µM), to prevent re-uptake of dopamine, and atropine (1 µM), in order to prevent any possible activation of muscarinic ACh receptors, at 0.7 ml/min for 10 min before stimulation with agonist for 20 s. Selected aliquots were perfused with α-CtxMII (50nM) for 3 min immediately before stimulation. This concentration of α-CtxMII is sufficient to inhibit all α6β2*-nAChR forms present in mouse striatum [31]. Fractions (~0.1 ml) were collected every 10s into 96-well plates using a Gilson F204 fraction collector (Middleton WI). After addition of 0.15 ml of Optiphase SuperMix scintillation cocktail, radioactivity was

determined in a 1450 MicroBeta Trilux counter (Perkin Elmer Life Sciences – Wallac Oy, Turku, Finland).

[^{3}H]-ACh uptake and release **–** Release methods of Grady et al. (2001) [17] were followed using the crude synaptosomes from IPN tissue. Uptake of [^{3}H]-choline was by incubation for 30 min at 37^{0}C in buffer (NaCl, 128 mM; KCl, 2.4 mM; $CaCl_2$, 3.2 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; HEPES, 25 mM; pH 7.5; glucose, 10 mM; 0.1% BSA) at 0.1 ml/mouse. Superfusion was for 10 min at 0.7 ml/min with buffer containing atropine (1 µM) before stimulation by agonist for 20 s. Collection of fractions and determination of radioactivity were as for dopamine release.

$^{86}Rb^+$ efflux **–** Agonist-stimulated $^{86}Rb^+$ efflux from synaptosomes was investigated using the methods of Marks *et al.* (1999, 2007) [22, 23]. Briefly, crude synaptosomes prepared from thalamus were resuspended in uptake buffer (NaCl, 140 mM; KCl, 1.5 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; HEPES, 25 mM; pH 7.5; glucose, 20 mM) (350 µl/mouse thalamus). Aliquots (25 µl) of the suspension were added to 10 µl of uptake buffer containing 4 µCi $^{86}Rb^+$ and incubated at room temperature for 30 min. Each sample was then collected onto filter paper (Type AE, Gelman, Ann Arbor, MI), rinsed once with 0.5 ml of uptake buffer, and superfused with buffer (NaCl, 135 mM; CsCl, 5 mM; KCl, 1.5 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; HEPES, 25 mM; pH 7.5; glucose, 20 mM; tetrodotoxin, 50 nM; atropine 1 µM; BSA 0.1%) at 2.5 ml/min for 5 min before data collection began. Stimulation by agonist was for 5s. Effluent was pumped through a 200

µl Cherenkov cell in a β-Ram HPLC detector (IN/US Systems, Tampa, FL) to continuously monitor radioactivity.

Synaptosomal function data analysis – All synaptosomal function assays were calculated as counts exceeding basal release determined from samples immediately preceding and following stimulation [17, 22, 36]. Stimulated release was normalized to baseline to give units of release as a fraction of baseline. Fractions significantly over baseline for each perfusion were summed. EC_{50} values were calculated by fitting data to the Hill equation, or two Michaelis-Menten equations when data were biphasic. IC_{50} values were calculated from the inhibition equation (release = $R_0/(1+[A_n]/IC_{50})$, where R_0= uninhibited release and $[A_n]$ is the concentration of antagonist) using the nonlinear least squares algorithm in SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA). The errors for the EC_{50}, IC_{50} and efficacy (as maximum activity expressed as % nicotine) are errors generated by the least-squares computational algorithm in SigmaPlot termed "SEM". This "SEM" reflects error of the curve fit for the entire data set rather than an SEM calculated from independent determinations of these parameters.

Neuro-2a Cell Culture – Mouse neuroblastoma 2a (Neuro-2a) cells were cultured using standard techniques [18, 50]. Cells were transfected with 500 ng of each nAChR subunit. Plasmids were mixed with 250 µl of Opti-MEM. Lipofectamine-2000 was separately added to 250 µl of Opti-MEM. After 5 min at 24 ^{o}C, DNA and Lipofectamine solutions were mixed together and incubated for 25 min at 24 ^{o}C. The solutions were then added to

pre-plated Neuro-2a cells and incubated for 24 h. After 24 h, the Opti-MEM was removed and replaced with growth medium.

Patch clamp recordings – For patch clamp electrophysiology, 50,000 Neuro-2a cells were plated onto sterilized 12 mm ø glass coverslips (Deckgläser, Prague, Czech Republic), placed in 35-mm culture dishes, and cultured in a humidified incubator (37 ^{o}C, 95% air, 5% CO_2). Cells were transfected as described above. Recorded cells were visualized with an inverted fluorescence microscope (IX71, Olympus) in either bright field or fluorescence (eGFP) mode using a high-pressure Hg lamp (HB-10103AF, Nikon). Electrophysiological signals were recorded with an Axopatch-1D amplifier (Axon Instruments), Digidata 1440A analog-to-digital converter (Axon Instruments), and pClamp 10.0 software (Axon Instruments). Patch pipettes were filled with solution containing (in mM): 135 K gluconate, 5 KCl, 5 EGTA, 0.5 $CaCl_2$, 10 HEPES, 2 Mg-ATP, and 0.1 GTP (pH was adjusted to 7.2 with Tris-base, and osmolarity was adjusted to 280-300 mOsm with sucrose). The resistance of patch pipettes was 2-4 MΩ for whole-cell recordings. Junction potential was nulled just before forming a gigaseal. All recordings were done at 24 ^{o}C. Data were sampled at 10 kHz and filtered at 2 kHz for whole-cell recordings. ACh was dissolved in extracellular solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose (320 mOsm, pH set to 7.3 with Tris-base), and were puffed (0.3 s, 20 psi) onto voltage clamped Neuro-2a cells (holding potential (V_H), -50 mV). To avoid receptor desensitization by repetitive Ach or TI-299423 application, we applied drugs at ≥3 min intervals, and continually perfused the recording chamber with extracellular solution. For concentration response studies, we

used a rapid superfusion system with 500 ms puffs of agonist (Octaflow II, ALA Scientific Instruments).

Locomotion – Horizontal locomotor activity was recorded using an infrared photobeam activity cage system (San Diego Instruments; San Diego, CA). Ambulations were recorded when two contiguous photobeams were broken in succession, preventing activity from being recorded by sedentary beam breaks. Ambulation events were measured at four 15 s intervals per min, for 45 min. Mice were habituated to the experimental room for 2 h prior to the experiment. For single injection experiments, mice were first moved into activity cages where their baseline activity level was measured. After 7.5 min, mice were removed, injected with the drug of interest (100 μL/30 g body mass), and returned to the activity cage for the duration of the 45 min. For experiments involving two injections, mice were injected with the first drug of interest before being placed in the activity cage where their activity level was monitored. After 8 min, they were removed, injected with the second drug of interest, and returned to the activity cage for the remainder of the 45 min.

Off-Target Effects & PK Data – Data was collected on the potential effects of TI-299423 at 1 μM at 70 different sites in rat brain homogenate by PerkinElmer Waltham, MA (Figure S1). Data on the pharmacokinetics of TI-299423 was generated by Absorption Systems LP Exton, PA.

Results

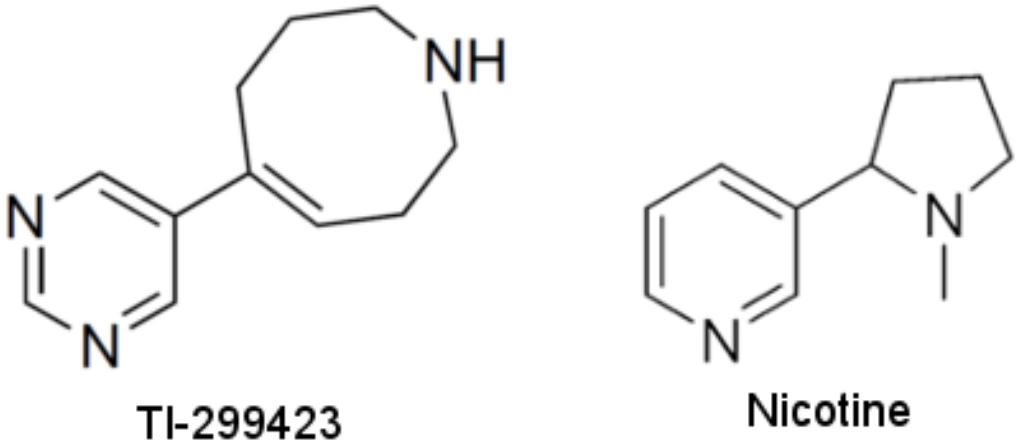

Figure 1: Structure of compound TI-299423 and Nicotine.

Affinity, Efficacy, and Potency of TI-299423 – In order to determine the effects of TI-299423 on α4β2*, α6β2*, and α3β4*-nAChRs, *in vitro* testing using [^{125}I]-epibatidine binding, $^{86}Rb^{+}$ efflux, [^{3}H]-dopamine release and [^{3}H]-ACh release was conducted, the results of which can be seen in Table 1. TI-299423 has a K_i of 1.4 ±0.6 nM for α6β2*-nAChRs. While it is only a partial agonist of these receptors, it is extremely potent, with an EC_{50} between 30 nM and 60 nM depending on the assay, at least an order of magnitude lower than at α4β2*-nAChRs.

TI-299423 has a K_i value of 0.24 ± 0.04 nM at α4β2*-nAChRs, meaning it has a higher affinity for these receptors than any other receptors tested. TI-299423 is a full agonist of high sensitivity (HS) and a partial agonist of low sensitivity (LS) α4β2*-nAChRs. Interestingly, it appears to be more efficacious at receptors that do not contain the α5 subunit. TI-299423 has an EC_{50} in the hundreds of nanomolar range for the HS and tens of

micromolar for the LS α4β2*-nAChRs. TI-299423 has a K_i 18 ± 0.7 nM for α3β4*-nAChRs. It is a full agonist of α3β4*-nAChRs with an EC_{50} of 8 ± 0.4 μM. Based on this data alone, it seemed very low doses of TI-299423 might be selective for α6*-nAChRs.

Table 1: Affinity, Efficacy, and Potency of TI-299423 at α4β2*, α6β2*, and α3β4*-nAChRs

Assay Name	HS α4β2	LS α4β2	α4(non-α5)β2	α6β2*	α6(non-α4)β2	α6β2(α5KO)	α3β4*
[^{125}I]-Epi binding K_i	0.24 ± 0.04 nM				1.4 ± 0.6 nM		18 ± 0.7 nM
$^{86}Rb^+$ efflux Thalamus % Efficacy of Nicotine EC_{50}	139% 600 ± 100 nM	32% 14 ± 12 μM	170% 1,600 ± 500 nM				
$^{86}Rb^+$ efflux Cortex % Efficacy of Nicotine EC_{50}	94% 2,000 ± 2,000 nM	39% 26 ± 21 μM	170% 2,400 ± 700 nM				
[^{3}H]-DA release % Efficacy of Nicotine EC_{50}	55% 50 ± 20 nM	38% 2 ± 2 μM	112% 500 ± 100 nM	30% 60 ± 40 nM		54% 50 ± 20nM	
Neuro-2A Cells EC_{50}	101 ± 15 nM			39 ± 9 nM			
[^{3}H]-ACh release % Efficacy of Nicotine, EC_{50}							100% 8 ± 0.4 μM

Table 1: Table for the comparison of TI-299423's actions at α4β2*, α6β2*, and α3β4*-nAChRs. TI-299423 is a full agonist for high sensitivity α4β2*-nAChRs and α3β4*-nAChRs, and a partial agonist for low sensitivity α4β2*-nAChRs and α6β2-nAChRs. TI-299423 is extremely potent at α6β2*-nAChRs with an EC50 in the tens of nanomolar. Additionally, TI-299423 appears to be less efficacious at α5*-nAChRs than at non-α5-nAChRs.

N2A cells and patch-clamp recordings – In order to examine the functional effects of TI-299423 on α4β2-nAChRs and α6β2β3-nAChRs without the presence of other subunits, a potentially confounding factor in the assays used above, data was collected via whole-cell patch clamp electrophysiology with Neuro-2a cells transiently expressing α4-GFPβ2$_{DM}$ or α6-GFPβ2$_{DM}$β3 nAChRs. (Figure 2A) Fitted curves for this data give an EC_{50} of 101 ± 15 nM for α4-GFPβ2$_{DM}$ nAChRs and an EC_{50} of 39 ± 9 nM α6-GFPβ2$_{DM}$β3 nAChRs. The

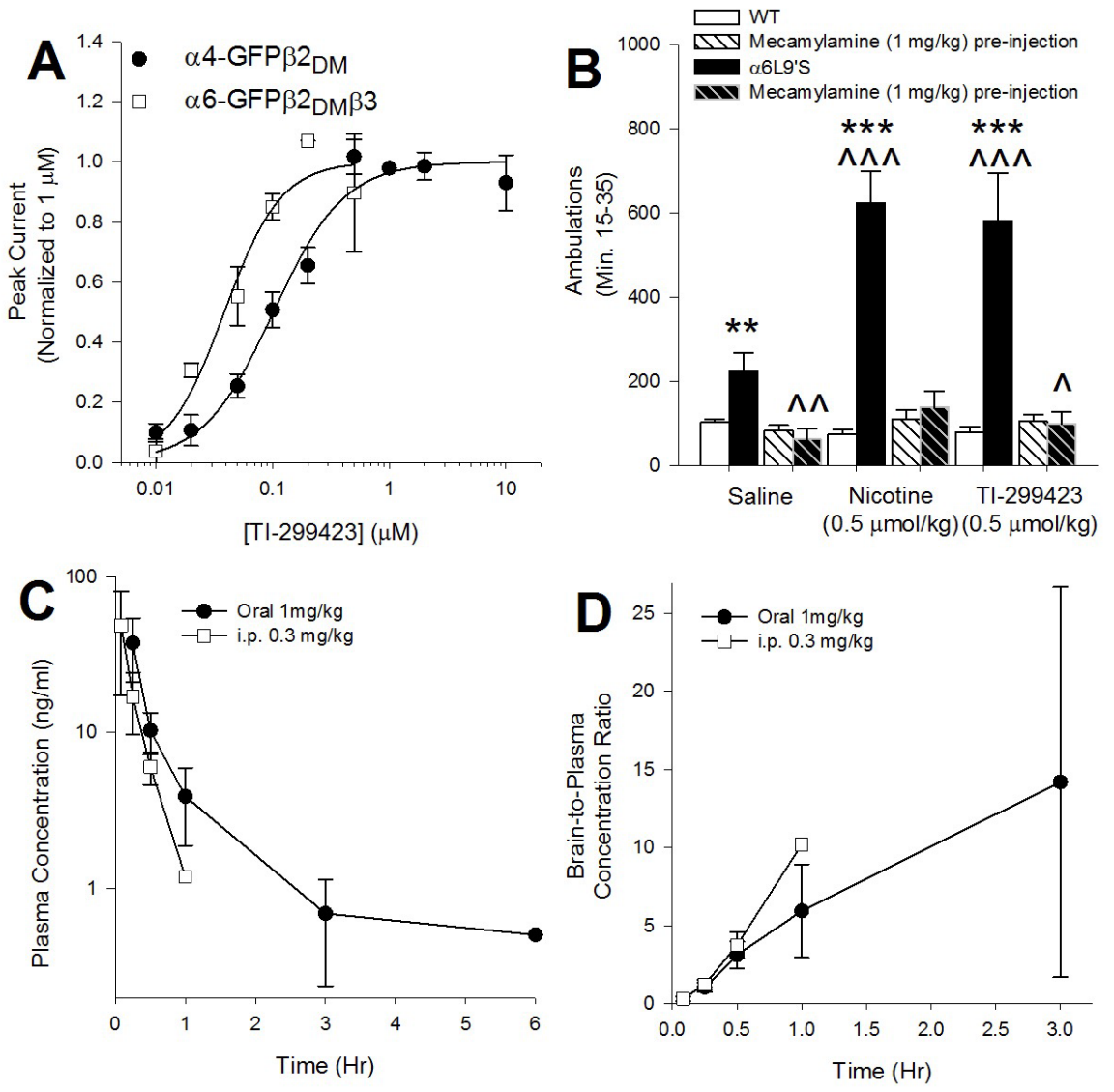

Figure 2: A) Concentration response of TI-299423 on α4-GFPβ2$_{DM}$ nAChRs (n=5-13) and α6-GFPβ2$_{DM}$β3 nAChRs (n=4). Fitted curves give an EC_{50} and n_h of 101 ± 15 nM and 1.43 ± 0.27, respectively for α4-GFPβ2$_{DM}$ and an EC_{50} and n_h of 39 ± 9 nM and 1.78 ± 0.60, respectively for α6-GFPβ2$_{DM}$β3. All data are mean ± SEM. **B)** Locomotor activation assay in WT and α6L9'S mice used to assess the bioavailability and *in vivo* activity at α6*-nAChRs. Average summed locomotor activity (n=8) between min 15-35 of assay in WT and L9'S mice is shown in response to IP injection of saline, 0.5 μmol/kg nicotine, or 0.5 μmol/kg TI-299423, with and without pre-injection of 1 mg/kg mecamylamine. * indicates difference from WT and ^ indicates difference from saline, using two-tailed t-test. **C)** When administered orally, TI-299423 has a maximum plasma concentration of 37.5 ± 9.6 ng/ml at time 0.25 h and a calculated half-life in the plasma is 1.12 h, or about 67 min. When administered intraperitoneally, TI-299423 has a maximum plasma concentration of 48.5 ± 18.0 ng/ml at time 0.08 h. and a calculated half-life in the plasma of 0.17 h, or about 10 min. **D)** The brain-to-plasma concentration ratio increases over time, suggesting TI-299423 enters the brain from the plasma and remains in the brain as the plasma concentration decreases.

calculated EC_{50} for the α6-GFPβ2$_{DM}$β3 nAChRs is significantly lower using a two-tailed t- test than the calculated EC_{50} for the α4-GFPβ2$_{DM}$ nAChRs, further suggesting TI-299423 may be selective for α6*-nAChRs over α4(non-α6)*-nAChRs.

Locomotion – To measure the *in* vivo activity of TI-299423 on α6*-nAChRs, a locomotor assay was used (Figure 2B). Between 15-35 min after being placed in a novel cage, α6L9'S mice showed an average of 224.3 ambulations, which is significantly higher than the average 102.5 ambulations shown by WT mice in that same time period (two-tailed unpaired t-test, $p < 0.01$). When α6L9'S and WT mice were pre-injected with 1 mg/kg of mecamylamine, a nicotinic antagonist, prior to being put in the novel cage, α6L9'S produced 62.3 ambulations between min 15 and 35, while WT mice produced 83.5 ambulations. These activity data were not significantly different from one another, indicating mecamylamine reduces the activity of the hypersensitive receptors.

α6L9'S mice have previously been shown to be hypersensitive to nicotine, showing locomotor activation as a response. Here, after a nicotine IP injection of 0.5 μmol/kg (0.08 mg/kg), α6L9'S mice produced an average of 623.2 ambulations between min 15 and 35. WT mice produced 73.69 ambulations under the same conditions, which is significantly fewer than the L9'S mice (two-tailed unpaired t-test, $p < 0.001$). When pre-injected with mecamylamine 1 mg/kg, and then given nicotine (0.5 μmol/kg), α6L9'S mice produced an average of 139 ambulations and WT mice produced an average of 111 ambulations during min 15-35. These activity levels are not significantly different.

To measure the effects of TI-299423 on α6*-nAChRs, α6L9'S and WT mice were given IP injections of 0.5 μmol/kg. α6L9'S mice produced an average of 581.9 ambulations between min 15 and 35, which is significantly higher (two-tailed unpaired t-test, $p<0.001$) than the WT mice, which produced an average of 104.54 ambulations under the same conditions. When pre-injected with mecamylamine 1mg/kg, and then given TI-299423 0.5 μmol/kg, α6L9'S mice produced an average of 98 ambulations, while WT mice produced an average of 104.5 ambulations between min 15 and 35, showing no significant difference.

Pharmacokinetics **–** To measure the bioavailability of TI-299423 in the brains of mammals, a study was conducted by Absorption Systems LP (Exton, PA) on the exposure and brain-to-plasma ratio of TI-299423 following intraperitoneal and oral administration in mice. Figure 2C shows the plasma concentration over time, and Figure 2D shows the brain-to-plasma ratio over time. These data indicate that TI-299423 enters the plasma and passes into the brain before being metabolized. When administered IP, TI-299423 has a maximum plasma concentration of 48.5 ± 18.0 ng/ml at time 0.083 h and a calculated half-life in the plasma of 0.170 h, or 10.2 min. When administered orally, TI-299423 has a maximum plasma concentration of 37.5 ± 9.59 ng/ml at time 0.25 h and a calculated half-life in the plasma is 1.12 h, or 67.2 min.

CPP by Genotype **–** Because TI-299423 showed a level of selectivity for α6*-nAChRs, we decided to test the rewarding properties of this drug. Mice of different genotypes were tested on CPP for TI-299423 at a dose of 0.012 mg/kg (figure 3A). At this dose, WT mice

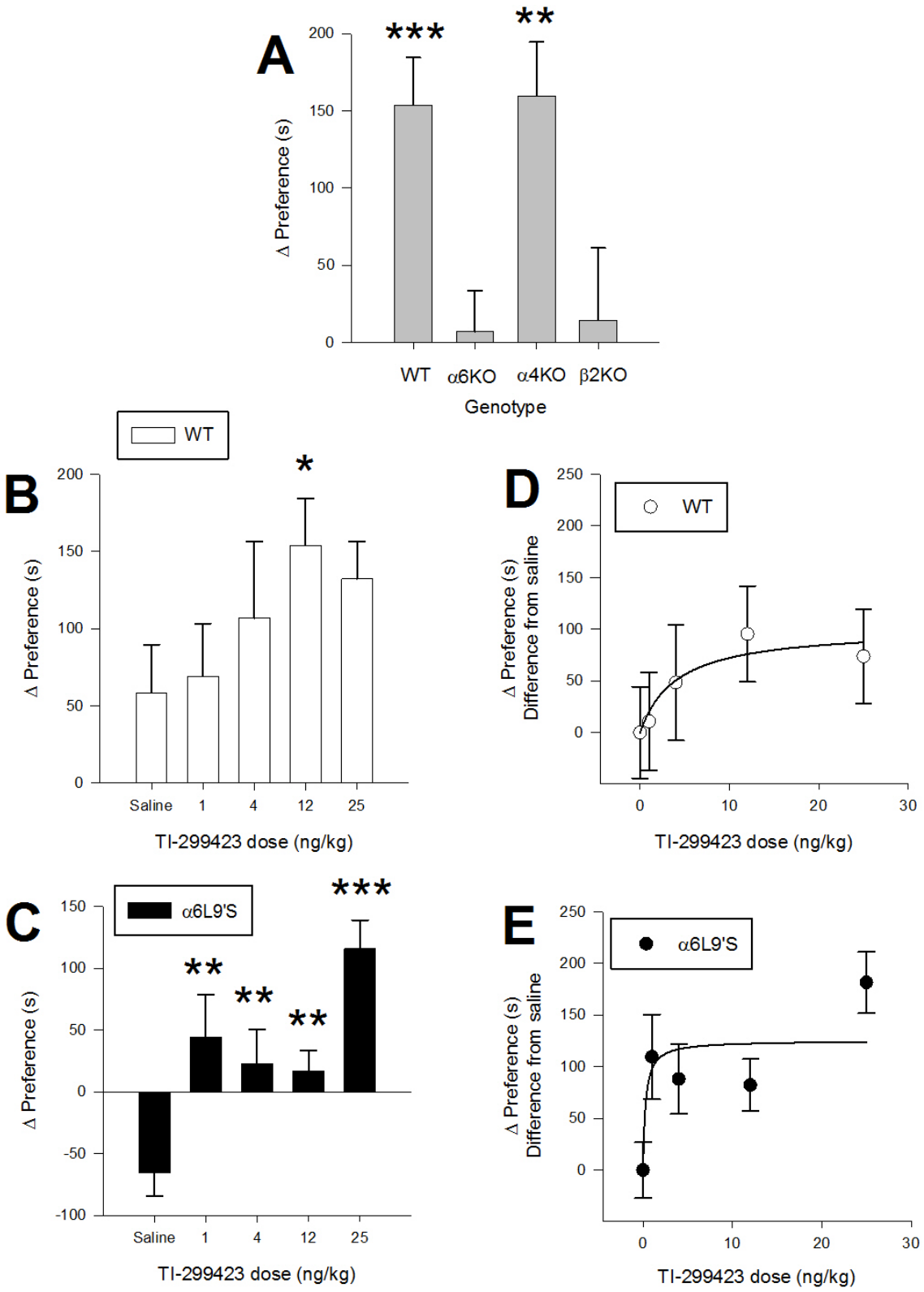
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Figure 3: Conditioned place preference responses by genotype and dose. **A)** Mice were trained on the CPP assay with TI-299423 (12 ng/kg) (n=9-23). WT and α4KO mice showed a significant change in preference towards the drug paired chamber after training (paired t-test between initial and final test, $p < 0.001$ and $p < 0.01$, respectively). Genotype had a significant effect on the conditioned place preference shown ($F_{(3,47)}=4.809$, $p < 0.01$). **B)** WT mice were trained on the CPP assay with TI-299423 at a range of doses (0 ng/kg - 25 ng/kg) (n=8-23). WT mice showed significant CPP when compared to saline controls at a dose of 12 ng/kg (one-tailed t-test, $p < 0.05$). **C)** α6L9'S mice were trained on the CPP assay with TI-299423 at a range of doses (0 ng/kg - 25 ng/kg) (n=8-10). α6L9'S mice showed significant CPP when compared to saline controls at doses of 1 ng/kg (one-tailed t-test, $p < 0.01$), 4 ng/kg (one-tailed t-test, $p < 0.01$), 12 ng/kg (one-tailed t-test, $p < 0.01$), and 25 ng/kg (one-tailed t-test, $p < 0.001$). **D)** The average saline response of WT mice was subtracted from the data, and SEMs was adjusted accordingly, giving a dose response curve with a baseline response of 0. A Michaelis-Menten curve was fit to the data. The calculated maximum response from this fit is 101 ± 23 s ($p < 0.05$) and the calculated EC_{50} is 4 ± 3 ng/kg (n.s.). **E)** The average saline response of α6L9'S mice was subtracted from the data, and SEMs was adjusted accordingly, giving a dose response curve with a baseline response of 0. A Michaelis-Menten curve was fit to the data. The calculated maximum response from this fit is 125 ± 32 s ($p < 0.05$) and the calculated EC_{50} is 0.3 ± 0.7 ng/kg (n.s.). All data shown are mean ± SEM.

(n=23) showed significant average CPP of 153.7 ± 31 s (two-tailed paired t-test between the initial and final test, $p < 0.001$). This reward was still present in α4KO (n=10) mice, who also showed significant CPP of 159.7 ± 35 s (two-tailed paired t-test between initial and final test, $p < 0.01$). α6KO (n=9) mice and β2KO (n=10) mice, however, did not show significant CPP, indicating that the rewarding properties of TI-299423 are α6β2*-mediated. Genotype had a significant effect on the amount of CPP shown ($F_{(3,47)}=4.809$, $p < 0.01$).

CPP Dose Effects – WT mice were tested on CPP at a range of TI-299423 doses. When given saline in both chambers, WT mice habituated to the initially least preferred chamber over the course of training, and showed a CPP of 58.4 ± 31 s (n=14) (Figure 3B). We controlled for this habituation by subtracting the control average from the CPP shown at other doses, and recalculating SEMs accordingly (Figure 3D). A Michaelis-Menten curve was fit to the data to determine the EC_{50} and maximal response. WT mice showed a CPP EC_{50} of 4 ± 3 ng/kg and a maximal response of 101 ± 23 s ($p < 0.05$). α6L9'S mice, however, showed sensitization when trained with saline alone, resulting in CPP of -65.6 ±

19 s (n=9) (Figure 3C). We controlled for this sensitization by subtracting the control average from the CPP shown at other doses, and recalculating SEMs accordingly (Figure 3E). A Michaelis-Menten curve was fit to the data to determine the EC_{50} and maximal response. α6L9'S mice showed a CPP EC_{50} of 0.3 ± 0.7 ng/kg (n.s.) and a maximal response of 125 ± 32 s ($p < 0.05$). Due to the limited number of doses tested, comparing the genotypes with reasonable power is infeasible.

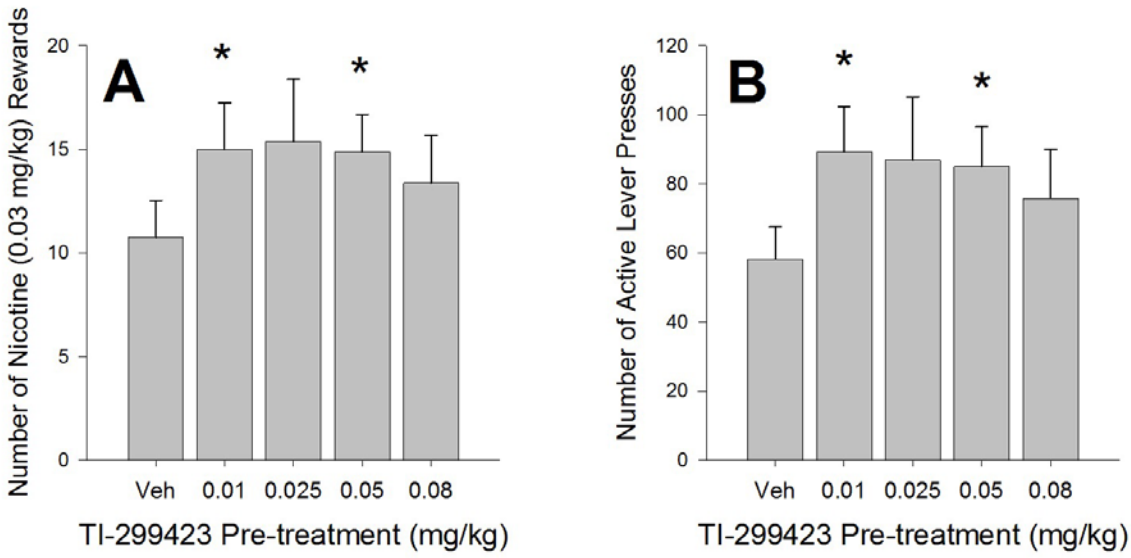

Figure 4: A) Rats (n=8) self-administered more nicotine when pre-injected with TI-299423 than when pre-injected with saline ($F_{(1,7)}$=7.041, $p < 0.05$). This increase was individually significant with pre-injections of 0.01 mg/kg and 0.05 mg/kg (paired t-test, $p < 0.05$). **B)** Rats (n=8) pressed the active lever more times when pre-injected with TI-299423 versus saline ($F_{(1,7)}$=9.737, $p < 0.05$). The increase in lever presses was individually significant at pre-injection doses 0.01 mg/kg and 0.05 mg/kg (paired t-test, $p < 0.05$). Data presented are mean ± SEM.

TI-299423 and Nicotine IVSA in rats– The CPP data suggested that TI-299423 was itself rewarding but provided little information about TI-299423's effects on the rewarding properties of nicotine. To measure that directly, we tested the self-administration of rats pre-injected with TI-299423. When compared with saline pre-injection, rats pre-injected

with TI-299423 significantly increased their intake of nicotine ($F_{(1,7)}$=7.041, $p < 0.05$) (Figure 4A) and the number of times they pressed the active lever ($F_{(1,7)}$=9.737, $p < 0.05$) (Figure 4B). Because each rat was tested at each dose, we can see that on an individual dose level rats significantly increased their nicotine intake and active lever presses after pre-injection with a TI-299423 dose of 0.01 mg/kg (two-tailed paired t-test of rewards: $p < 0.05$; two-tailed paired t-test of active lever presses: $p < 0.05$) and 0.05 mg/kg (two-tailed paired t-test of rewards: $p < 0.05$; two-tailed paired t-test of active lever presses: $p < 0.05$).

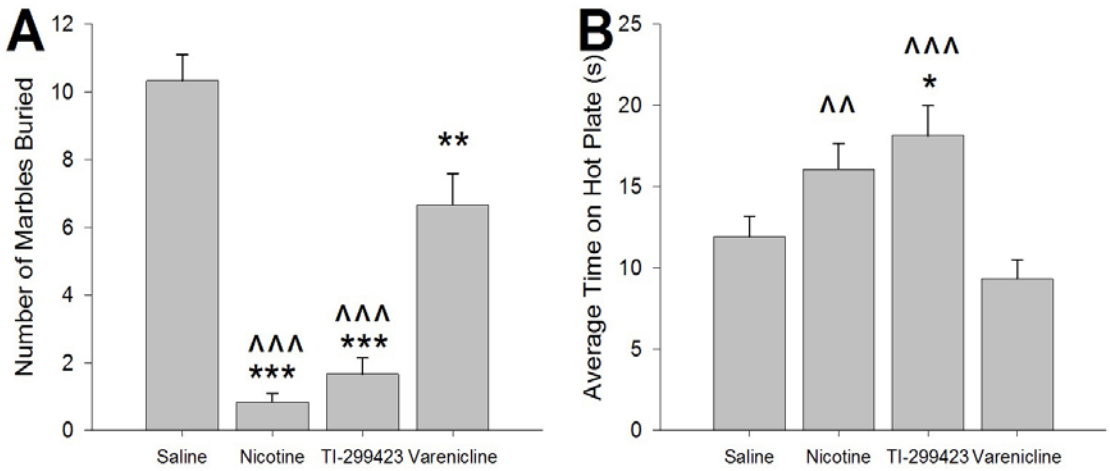

Figure 5: Anxiolytic and analgesic effects of nicotine (0.3 mg/kg), TI-299423 (0.3 mg/kg), and varenicline (0.3 mg/kg) as measured by marble burying and hot plate, respectively. **A)** The compound received had a significant effect on the number of marbles buried ($F_{(3, 33)}$= 45.65, $p < 0.001$). Additionally, comparing pre-injection with saline individually to each drug, there is a significant decrease in the number of marbles buried when nicotine is pre-injected (paired t-test, $p < 0.001$), when TI-299423 is pre-injected (paired t-test, $p < 0.001$), and when varenicline is pre-injection (paired t-test, $p < 0.01$), as denoted by *. Comparing varenicline pre-injection, we see a significant difference from nicotine (paired t-test, $p < 0.001$) and TI-299423 (paired t-test, $p < 0.001$), as denoted by ^. Data presented are mean ± SEM. **B)** The compound received had a significant effect on the number of seconds spent on the hot plate ($F_{(3,27)}$=7.442, $p < 0.001$). Additionally, comparing pre-injection with saline individually to each drug, there is a significant increase in the amount of time spent on the hot plate when TI-299423 is pre-injected (paired t-test, $p < 0.05$), as denoted by *. Comparing varenicline pre-injection, we see a significant increase in the time spent on the hot plate from nicotine (paired t-test, $p < 0.01$) and TI-299423 (paired t-test, $p < 0.001$), as denoted by ^.

Marble Burying – One property of nicotine closely related to reward is its anxiolytic property. To test if TI-299423 had similar effects, mice were given pre-injections of

nicotine, varenicline, or TI-299423 (0.3 mg/kg) and allowed to bury marbles (Figure 5A). The pre-injected compound had a significant effect on the number of marbles buried ($F_{(3, 33)}$= 45.65, p<0.001). When injected with saline, mice buried significantly more marbles when they were injected with nicotine, TI-299423, or varenicline (two-tailed paired t-test: p<0.001, p<0.001, p<0.01, respectively). Additionally, when injected with nicotine or TI-299423, mice buried fewer marbles than when they were given varenicline (two-tailed paired t-test, p<0.001, p<0.001, respectively).

Hot Plate – Nicotine has also been previously shown to have antinociception properties through activation of α4β2*, α3β4*, or α7-nAChRs. Because TI-299423 is an agonist for α4β2* and α3β4*-nAChRs at higher doses, we tested its analgesic properties at a dose of 0.3 mg/kg using a hot plate assay (Figure 5B). TI-299423 produces a similar level of analgesia to nicotine. Mice injected with TI-299423 spent significantly longer on the hot plate than mice injected with saline (paired, two-tailed t-test, $p < 0.05$) or varenicline (paired, two-tailed t-test, $p < 0.001$).

Discussion

Nicotine is considered the primary rewarding component in tobacco products. The US Surgeon General first linked tobacco use to disease over 50 years ago [43], and yet tobacco use is still a leading cause of preventable death worldwide [49], partially due to its addictive properties. While about 40% of smokers report trying to quit at least once in the last 12-months, only about 10% successfully abstain for a full year [14], a rate that is only mildly improved by any current treatments. Nicotine has rewarding and reinforcing

behavioral effects through activation and desensitization of nAChRs in the central nervous system. β2*-nAChRs, which often combine with α4 and α6, are necessary for self-administration and conditioned place preference for nicotine [19, 23, 38]. Additionally, studies have shown that α4*-nAChRs are sufficient for nicotine conditioned place preference [34], but not necessary [4], and are important, if not necessary, for nicotine self-administration [4, 25]. α6*-nAChRs are also sufficient for nicotine CPP [12], but not necessary [31], and are necessary for nicotine IVSA [25]. Being able to isolate α6*-nAChRs over α4*-nAChRs pharmacologically could thus be beneficial in furthering our understanding of nicotine reward and dependence, as well as developing better treatments for smoking cessation.

TI-299423 is a small, two ring compound developed by Targacept as a potentially selective agonist for α6*-nAChRs over α4*-nAChRs. Comparing TI-299423 to nicotine in Figure 1, we see the pyridine ring of nicotine has been replaced by a pyrimidine ring, a change that has been previously shown to increase α6* selectivity [3]. Additionally, nicotine's N-methylpyrolidine ring was modified to a 1,2,3,4,7,8-hexahydroazocine for TI-299423. TI-299423 is slightly heavier with a molecular mass of 189.26 g/mol to nicotine's 162.23 g/mol. Additionally, TI-299423 has a LogP of approximately 1.2 compared to nicotine's LogP of about 1.1. This suggests that TI-299423 will be able to enter the brain and act as an agonist on nAChRs, more specifically α6β2*. In order to screen for possible off-target effects, TI-299423 was put through a novascreen that measured its ability to bind to 70 different receptors sites at 1 μM. The novascreen determined that, as expected, TI-299423 binds to neuronal nicotinic receptors. It also

binds to ATP-sensitive potassium channels, but with a much lower affinity than at nicotinic sites. These were the only two receptor sites to show significant binding, indicating that at the doses we use off-target effects are unlikely to confound our results.

TI-299423's properties with respect to α6β2*, α4β2*, and α3β4*-nAChRs were measured using $^{86}Rb^{+}$ efflux, [^{125}I]-epibatidine binding, [^{3}H]-dopamine release, patch-clamp recordings, and [^{3}H]-ACh release. The results of these tests are presented in Table 1. TI-299423 is a partial agonist for α6β2*-nAChRs and is extremely potent, with an EC_{50} in the low tens of nanomolar range. For α4β2*-nAChRs, TI-299423 is a full agonist of the high sensitivity and a partial agonist of the low sensitivity receptors. It's EC_{50} at α4β2*-nAChRs is in the hundreds of nanomolar, and tens of micromolar, for HS and LS, respectively. Using Neuro-2A cells, we were able to isolate and separate the effects of TI-299423 on α6(non-α4)* and α4(non-α6)*-nAChRs. Previous studies have indicated that nicotine is more potent at α4(non-α6)β2*-nAChRs than it is at α6(non-α4)β2*, though it is even more potent at α4α6β2*-nAChRs [16, 36, 45]. TI-299423, on the other hand, appears to be more potent at α6(non-α4)β2* than at α4(non-α6)β2*-nAChRs. Because its EC_{50} is an order of magnitude lower at α6-GFPβ2$_{DM}$β3 than at α4-GFPβ2$_{DM}$, low doses of TI-299423 may be selective as an agonist for α6*-nAChRs.

To measure this result in behaving animals, we first had to ensure TI-299423 could enter the brain and activate α6*-nAChRs in live mammals. A pharmacokinetics screen was used to measure how TI-299423 enters the brain (Figure 2C and 2D). The pharmacokinetics data indicated that TI-299423 does enter the brain, a result consistent

with its LogP. Next, because α6*-nAChRs are known to modulate nicotine induced changes in locomotion [8], a locomotor assay with α6L9'S mice was used to measure TI-299423's ability to activate these receptors. TI-299423 was found to be as effective as nicotine at eliciting a locomotor response in α6L9'S mice (Figure 2B). These data, along with the *in vitro* and Neuro-2A cell data, indicate that TI-299423 enters the brain and activates α6β2*-nAChRs at low doses, without activating α4(non-α6)β2*.

Because α6*-nAChRs are localized to dopaminergic neurons, we chose to investigate the rewarding properties of TI-299423. α6*-nAChRs have previously been shown to be sufficient for conditioned place preference using α6L9'S mice and a dose of 10 ng/kg [9]. Our understanding of the potency and selectivity of TI-299423 for α6*-nAChRs prompted us to use an equivalent molar dose of TI-299423 in WT mice, without the hypersensitive α6-subunit. Using CPP, TI-299423 is rewarding at a dose of only 12 ng/kg in WT mice. The β2-subunit is known to be critical for conditioned place preference and self-administration mediated by the cholinergic system [46]. When we trained β2KO mice using TI-299423, we did not observe CPP, indicating that the CPP we saw in the WT mice is in fact a cholinergic, and not an off-target, effect. We then tried CPP with TI-299423 with both α6KO and α4KO mice. α6KO mice did not show CPP for TI-299423, but the α4 KO mice did, indicating that this behavior is mediated by α6(non-α4)β2*-nAChRs or, at the very least, is not dependent on α6α4β2*-nAChRs.

Additionally, we created CPP dose response curves for TI-299423 using WT and α6L9'S mice, as seen in Figure 3B-E. To control for potential changes in preference not mediated

by TI-299423, we trained mice with saline administered in both chambers as part of CPP training. WT mice, as expected, habituated to the least preferred chamber during training (Figure 3B). Surprisingly, α6L9'S mice showed sensitization towards the least preferred chamber, preferring it even less after training (Figure 3C). Previous studies have shown that α6L9'S mice do not habituate to novel environments [8]. We believe this behavior is an example of that phenotype. To better compare the WT and α6L9'S dose response curves, we transformed the data so that the saline trained mice had a CPP of 0 (Figure 3D-E), and fitted a Michaelis-Menten curve for each genotype. The limited number of doses tested, unfortunately, makes comparing the coefficients of the two regressions problematic, as the number of degrees of freedom is quite low. However, it is apparent that the α6L9'S mice show CPP at much lower doses than the WT mice, further implicating α6*-nAChRs as the mediating factor.

To better understand TI-299423's effects on reward and reinforcement, we measured its effect on intravenous self-administration (IVSA) of nicotine. Pre-treatment with several different nicotinic compounds, both agonists and antagonists, including nicotine [16, 41], mecamylamine [16, 41], varenicline [17], and sazetidine-A [28], has been shown to reduce nicotine intravenous self-administration (IVSA) in rats. Rats trained to self-administer nicotine were pre-treated via an IP injection 20 min prior to entering the IVSA box. When the rats received TI-299423, they self-administered significantly more nicotine than when they received saline ($F_{(1,7)}=7.041$, $p < 0.05$). This was surprising, given the literature on nicotinic compounds and IVSA; however, such a result is not completely new. A low dose of varenicline has been shown to increase IVSA in certain

rats [13]. While the exact mechanism behind this is unknown, we hypothesize that TI-299423 is activating α6(non-α4)β2*-nAChRs, which are not usually agonized by self-administration due to nicotine's higher EC_{50} at these sites, without altering nicotine's effect on α4β2*-nAChRs, thus compounding nicotine's rewarding effects.

Finally, both nicotine's anxiolytic and analgesic properties are also considered contributing factors to nicotine addiction. We decided to measure how TI-299423 affected anxiety and nociception using a marble burying assay and a hot plate assay, respectively. Nicotine's anxiolytic properties are primarily α4β2*-mediated [1, 42] and its analgesic properties are mediated by α4β2*, α3β4*, and α7-nAChRs [44]. Thus, we used a much higher dose of TI-299423 in these assays (0.3 mg/kg) than in the reward assays, in order to ensure activation of those receptors. TI-299423 is equivalent to nicotine in both its anxiolytic and analgesic properties, and more powerful in each than varenicline. Thus, TI-299423 is not selective for α6*-nAChRs at all doses, a result that allows it to demonstrate potentially useful properties at higher doses.

Developing an α6*-nAChR selective agonist is important for furthering our understanding of the cholinergic system and its interactions with the dopaminergic system in the brain, and for increasing our knowledge of nicotine dependence and how to treat it. TI-299423 is selective for α6*-nAChRs at low doses, resulting in surprising effects on reward and reinforcement, including potentiating nicotine self-administration, a phenomenon that warrants further research. At higher doses, TI-299423 acts as an agonist for other nAChR subtypes, including α4β2* and α3β4*-nAChRs, allowing it to function as both an

anxiolytic and an analgesic in mice. Further study into this and similar compounds may help us develop better smoking cessation compounds, or treatments for other disorders, in the future.

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Table 1.

Competition binding on 70 target proteins tested at a concentration of 1μM TI-299423

Inhibition	Assay Name	Radioligand/Substrate	Hit
12.86%	Adenosine Transporter (h)	[3H]-NBTI	FALSE
7.85%	Adenosine, A1	[3H]CPX	FALSE
1.51%	Adenosine, A2A (h)	[3H]CGS 21680	FALSE
-0.05%	Adrenergic, Alpha 1A	[3H]-7-MeOxy-Prazosin	FALSE
-8.31%	Adrenergic, Alpha 1B	[3H]-7-MeOxy-Prazosin	FALSE
6.75%	Adrenergic, Alpha 2A (h)	[3H]MK-912	FALSE
-14.09%	Adrenergic, Alpha 2B	[3H]MK-912	FALSE
6.28%	Adrenergic, Alpha 2C (h)	[3H]MK-912	FALSE
11.21%	Adrenergic, Beta 1 (h)	[125I] (-) Iodocyanopindolol	FALSE
-8.25%	Adrenergic, Beta 2 (h)	[125]I-Iodo-cyanopindolol	FALSE
-5.87%	Dopamine Transporter	[3H]WIN 35,428	FALSE
-2.94%	Dopamine, D1 (h)	[3H]-SCH23390	FALSE
-21.17%	Dopamine, D2s (h)	[3H]-Raclopride	FALSE
-16.93%	Dopamine, D3	[3H]7-OH-DPAT	FALSE
-2.44%	Dopamine, D4.4 (h)	[3H]-YM-09151-2	FALSE
-2.66%	GABA A, Agonist Site	[3H]GABA	FALSE
11.84%	GABA A, BDZ, alpha 1 site	[3H]Flunitrazepam	FALSE
-2.43%	GABA-B	[3H]CGP 54626A	FALSE
-1.26%	Glutamate, AMPA Site (Ionotropic)	[3H]AMPA	FALSE
-6.93%	Glutamate, Kainate Site (Ionotropic)	[3H]Kainic acid	FALSE
5.46%	Glutamate, MK-801 Site (Ionotropic)	[3H]MK-801	FALSE
5.51%	Glutamate, NMDA Agonist Site (Ionotropic)	[3H]CGP 39653	FALSE
7.71%	Glutamate, NMDA, Phencyclidine Site (Ionotropic)	[3H]TCP	FALSE
4.34%	Glutamate,NMDA,Glycine (Stry-insens Site) (Ionot	[3H]-MDL-105,519	FALSE
-7.63%	Glycine, Strychnine-sensitive	[3H]Strychnine	FALSE
15.56%	Histamine, H1	[3H]Pyrilamine	FALSE
6.50%	Histamine, H2	[125I]-Aminopotentidine	FALSE
5.85%	Histamine, H3	[3H]N-a-MeHistamine	FALSE
-11.73%	Muscarinic, M1 (hr)	[3H]Scopolamine, N-Methyl	FALSE
-3.18%	Muscarinic, M2 (h)	[3H]Scopolamine, N-Methyl	FALSE
-4.77%	Muscarinic, M3 (h)	[3H]Scopolamine, N-Methyl	FALSE
-6.69%	Muscarinic, M4 (h)	[3H]Scopolamine, N-Methyl	FALSE
2.24%	Muscarinic, M5 (h)	[3H]Scopolamine, N-Methyl	FALSE
93.33%	**Nicotinic, Neuronal (a-BnTx insensitive)**	**[3H]Epibatidine**	**TRUE**
23.72%	Norepinephrine Transporter	[3H]Nisoxetine	FALSE
-2.12%	Opioid, Delta 2 (h)	[3H]-Naltrindole	FALSE
-6.43%	Opioid, Mu (h)	[3H]-Diprenorphine	FALSE
7.03%	Serotonin Transporter	[3H]Citalopram, N-Methyl	FALSE
17.60%	Serotonin, 5HT1A (h)	[3H]-8-OH-DPAT	FALSE
9.99%	Serotonin, 5HT1D	[3H]5-CT	FALSE
12.43%	Serotonin, 5HT2A	[3H]Ketanserin	FALSE
-2.21%	Serotonin, 5HT2C	[3H]Mesulergine	FALSE
7.50%	Serotonin, 5HT3	[3H]GR 65630	FALSE
-16.64%	Serotonin, 5HT4	[3H]GR 113808	FALSE
1.24%	Serotonin, 5HT5A (h)	[3H]-LSD	FALSE
8.87%	Serotonin, 5HT6 (h)	[3H]-LSD	FALSE
-2.10%	Serotonin, 5HT7 (h)	[3H]LSD	FALSE
-0.52%	Sigma 1	[3H]-(+)-Pentazocine	FALSE
-0.65%	Sigma 2	[3H]-DTG	FALSE
-3.91%	Calcium Channel, Type L (Dihydropyridine Site)	[3H]Nitrendipine	FALSE
-0.60%	Calcium Channel, Type N	[125I]-Conotoxin GVIA	FALSE
6.83%	GABA, Chloride, TBOB Site	[3H]TBOB	FALSE
52.12%	**Potassium Channel, ATP-Sensitive**	**[3H]Glibenclamide**	**TRUE**
-2.57%	Potassium Channel, Ca2+ Act., VI	[125I]Apamin	FALSE
14.46%	Potassium Channel, I[Kr] (hERG) (h)	[3H]Astemizole	FALSE
6.91%	Sodium, Site 2	[3H]Batrachotoxin A 20-a-Benzo	FALSE
-10.86%	Nitric Oxide, NOS (Neuronal-Binding)	[3H]NOARG	FALSE
29.86%	Leukotriene, LTB4 (BLT)	[3H]LTB4	FALSE
-8.26%	Leukotriene, LTD4 (CysLT1)	[3H]LTD4	FALSE
3.14%	Thromboxane A2 (h) [SQ 29,548]	3H SQ 29,548	FALSE
24.85%	Angiotensin II, AT1 (h)	[125I]-(Sar1-Ile8) Angiotensin	FALSE
32.29%	Bradykinin, BK2	[3H]Bradykinin	FALSE
8.93%	Endothelin, ET-A (h)	[125I] Endothelin-1	FALSE
2.10%	Neurokinin, NK1	[3H]Substance P	FALSE
2.16%	Neuropeptide, NPY2 (h)	[125I]-PYY	FALSE
21.55%	Esterase, Acetylcholine	Acetylthiocholine	FALSE
12.00%	Phosphodiesterase, PDE4A1A (h)	Fluorescent cyclic AMP	FALSE
-3.00%	Phosphodiesterase, PDE5A1 (h)	Fluorescent cyclic GMP	FALSE
-5.00%	Kinase, Protein, PKA (h)	Fluorescein-labeled peptide	FALSE
-1.00%	Kinase, Protein, PKCa (h)	Fluorescein-labeled peptide	FALSE

SI Figure 1: TI-299423 successfully inhibited >50% of the reference radioligand for two sites: Neuronal nAChRs and ATP-sensitive potassium channels. 1μM TI-299423 inhibited 93.33% of the [3H]-epibatidine binding at nAChRs and 52.12% of the [3H]-Glibenclamide binding at the ATP-sensitive potassium channels.

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Chapter 3

A COLLECTION OF ADDITIONAL DATA ON THE PROPERTIES OF TI-299423

This chapter presents data related to TI-299423 (also referred to in this chapter as TC299) and its activities in the brain, which is supplemental to the data presented in the previous chapter. Necessarily, the previous chapter and submitted paper do not contain all of the data collected for the project. Several data sets or types were excluded either due to their confusing and ineffable results, their negative results, or a failure in the experimental procedure.

TI-299423 has previously been shown to be selective for α6*-nAChRs (where * indicates the presence of other subunits) at certain doses. This selectivity results in interesting reinforcement effects and effects on nicotine reward. However, the *in vitro* data on TI-299423 presented in Chapter 2 is not altogether clear. TI-299423 appears to be a full agonist for HS α4β2*-nAChRs with a potency very similar to that of α6β2*-nAChRs. Further investigation into the effects of the α4*-nAChRs and TI-299423 yielded interesting results that we are, so far, unable to explain.

Additional Effects of TI-299423 on α4*-nAChRs

Figure 6A shows the results of a temperature assay used to measure TI-299423's activation of α4β2*-nAChRs *in vivo*. Vital View PDT-4000 activity and temperature telemetric probes (Respironics) were used. For insertion, mice were anesthetized with either

isoflourine or ketamine. A 1 cm incision was made across the back of the neck and the probe was inserted subcutaneously into the animal. The incision was then sealed with surgical glue. Mice were given 4-7 days to recover, before being habituated to the experimental room for an additional day. Temperature data was acquired at 30 s intervals using the Vital View software. Data was collected for an initial 30 min to establish the animals' baseline temperature. Mice were then individually removed, injected with the drug of interest, and returned to their receiver. Post-injection, data was recorded for an additional 90 min. Mice were given 1 week without drug injection between each condition to prevent sensitization effects.

Nicotine induced hypothermia is known to be mediated by α4* nAChRs [9]. The temperature figure shows the resulting maximal change in body temperature within 45 min after IP injection with saline, nicotine, or TI-299423 in WT and mutant α4L9'A mice, which have hypersensitive α4* receptors. WT mice showed no significant change in body temperature after injection with any compound. α4L9'A mice had an average decrease in body temperature of 1.97^0C (two tailed paired t-test $p < 0.001$, compared to saline) after injection with Nicotine at a dose of 0.1 μmol/kg (0.015 mg/kg). When injected with TI-299423 at a dose of 0.1 μmol/kg or a dose of 0.2 μmol/kg, α4L9'A mice did not show a significant change in body temperature. However, when TI-299423 was given at a dose of 0.5 μmol/kg, α4L9'A mice showed an average temperature decrease of 2.0^0C (two-tailed paired t-test $p < 0.001$, compared to Saline). This data backs up the conclusions drawn in Chapter 2, that TI-299423 is selective for α6*-nAChRs and not α4(non-α6)*-

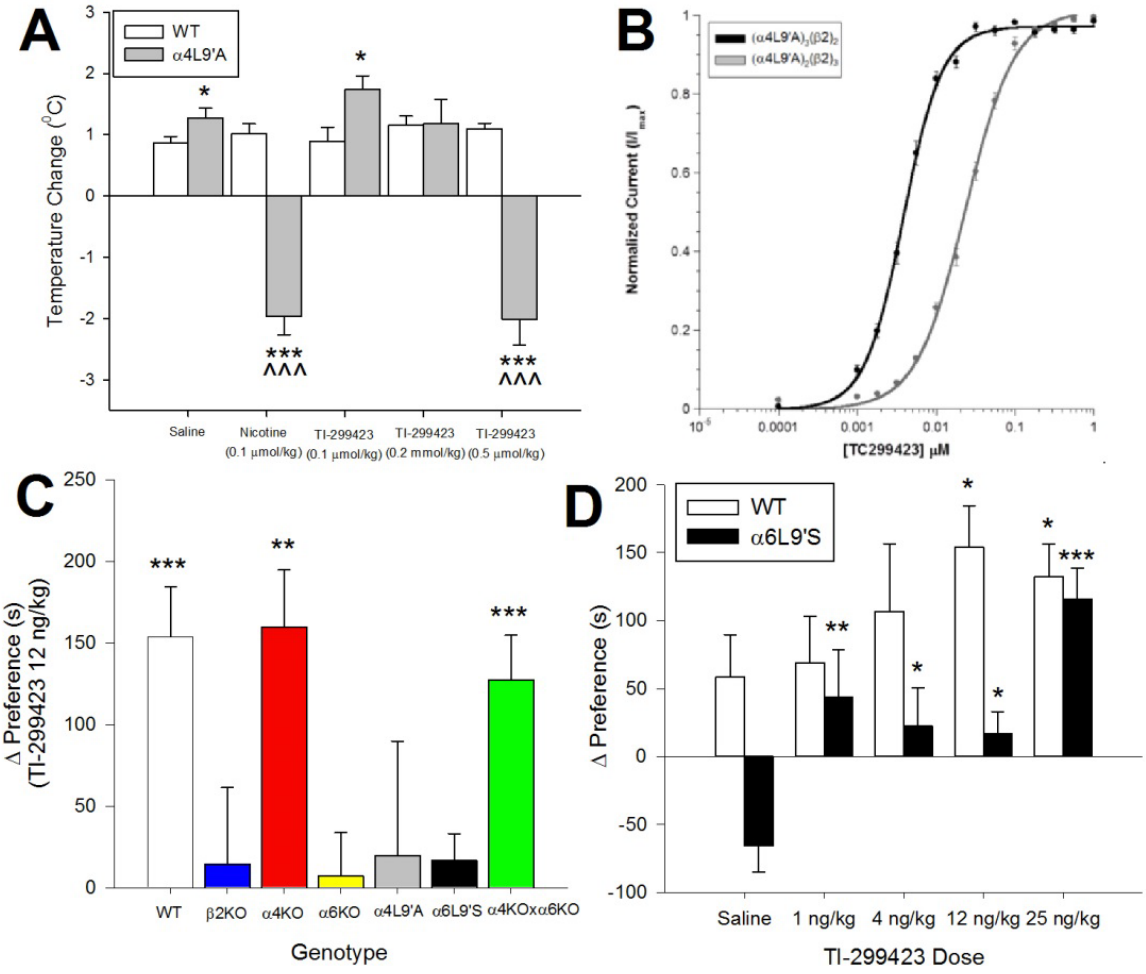

Figure 6: Conditioned Place Preference Data and Temperature data. **A)** Hypothermia assay in WT and α4L9'A mice used to assess the bioavailability and *in vivo* activity at α4*-nAChRs. Average maximum change in temperature (n=9) in WT and L9'A mice following IP injection of saline, nicotine, or TI-299423. **B)** The efficacy and potency of TI-299423 was tested on $(\alpha4L9'A)_2(\beta2)_3$ and $(\alpha4L9'A)_3(\beta2)_2$ receptors expressed in oocytes. TI-299423 had a lower EC_{50} for the three alpha receptors (3.8 ± 0.1 nM) than the two alpha receptors (17.4 ± 0.2 nM). Additionally, TI-299423 was 76% as efficacious as ACh at the three alpha receptors and 35% as efficacious at the two alpha receptors. The hill coefficient for the three alpha receptors is 1.78 ± 0.08 and for the two alpha receptors the hill coefficient is 1.53 ± 0.03. **C)** Mice were trained on the CPP assay with TI-299423 (0.012 mg/kg) (n=9-23). WT, α4KO, and α4KOx α6KO mice showed a significant change in preference towards the drug paired chamber after training (paired t-test between initial and final test, $p < 0.001$, $p < 0.01$, and $p < 0.001$, respectively). Genotype had a significant effect on the conditioned place preference shown ($F_{(6,74)} = 3.697$, $p < 0.01$). **C)** WT and α6L9'S mice were trained on the CPP assay with TI-299423 at a range of doses (0 mg/kg – 0.025 mg/kg) (n=8-23). WT mice showed significant CPP when compared to saline controls at doses of 0.012 mg/kg ($p < 0.05$) and 0.025 mg/kg ($p < 0.05$). α6L9'S mice showed significant CPP when compared to saline controls at 0.001 mg/kg ($p < 0.01$), 0.004 mg/kg ($p < 0.05$), 0.012 mg/kg ($p < 0.05$), and 0.025 mg/kg ($p < 0.001$). Both genotype and treatment had a significant effect on CPP, but the interaction did not (*Genotype*: $F_{(1,99)} = 14.573$, $p < 0.001$; *Treatment*: $F_{(4,99)} = 3.926$, $p < 0.01$; *Interaction*: $F_{(4,99)} = 1.238$, n.s.). All data shown are mean ± SEM.

nAChRs at low doses. However, previous studies have shown that varenicline, a partial agonist for α4β2*-nAChRs with an EC_{50} of 50 nM for the HS and 300 nM for the LS receptors [5], can produce hypothermia in α4L9'A mice at a dose of only 0.001 mg/kg [5]. TI-299423 is a full agonist at high sensitivity and a partial agonist at low sensitivity α4β2*-nAChRs, with an EC_{50} of 101 nM, suggesting that TI-299423 should produce hypothermia at a 0.1 μmol/kg (0.015 mg/kg) if not an even lower dose.

Exactly why TI-299423 does not induce hypothermia at 0.1 μmol/kg and instead requires a higher dose of 0.5 μmol/kg is unknown. One possible hypothesis is that TI- 299423 does not bind as well to the mutant L9'A receptor as nicotine does. This hypothesis was tested using oocytes, as seen in Figure 6B. We can see that TI-299423 activates the three alpha α4L9'Aβ2 receptors quite potently (EC_{50}: 3.8 ± 0.1 nM) with a 76% efficacy compared to ACh, and the two alpha α4L9'Aβ2 slightly less potently (EC_{50}: 17.4 ± 0.2 nM) and with a 35% efficacy compared to ACh. Nicotine, on the other hand, has an EC_{50} of 230 ± 30 nM [4]. Based on this information, we cannot explain why TI-299423 fails to elicit hypothermia at lower doses.

Conditioned Place Preference with Additional Genotypes

The conditioned place preference (CPP) assay also showed interesting results with regards to α4*-nAChRs. In Chapter 2, we showed that CPP for TI-299423 (0.012 mg/kg) was unaffected by knocking out α4, but was eliminated by knocking out α6, leading us to believe that CPP at this dose was mediated by α6(non-α4)*-nAChRs. Figure 6C shows a

more in-depth look at the role of genotype on TI-299423 CPP. Double knock-out (α4KOxα6KO) mice (n=12) showed CPP of 127.3 ± 27.3 s (two-tailed paired t-test between initial and final test, $p < 0.001$). The CPP seen in the α4KOxα6KO, double knock-out mice is likely the most confusing piece of data gathered. Unfortunately, not much is known about these mice and the effects of knocking out both α4 and α6. It is possible that these mice have an abnormally high expression of α3*-nAChRs in the midbrain, a subunit not generally thought to play a role there, but which has been shown to be present in small numbers [1, 2]. Another possibility is that α4KOxα6KO mice habituate to novel environments more quickly than WT mice, the opposite of what is seen in α6L9'S mice, and that the CPP observed is actually habituation. More information on these mice is needed in order to form a well-educated hypothesis.

Mice with hypersensitive α4 (α4L9'A) (n=9) nAChRs averaged CPP of 19.7 ± 70 s (n.s.). The loss of CPP in the α4L9'A mice is confusing because the knock-out data suggests α4 does not playing a role at this dose. One cause of this effect could be the change in the number of α4*-nAChRs found in α4L9'A mice. Mice with the α4L9'A mutation have previously been shown to have significantly fewer α4-nAChRs in the brain [7], meaning dopamine release is even more α6*-dependent, possibly shifting the CPP dose response curve.

α6L9'S mice (n=9) show CPP of only 16.6 ± 16.3 s (n.s.) at a TI-299423 dose of 0.012 mg/kg. If the CPP shown in WT mice is mediated by α6*-nAChRs, we would expect to see a higher level of CPP in the α6L9'S mice, not lower. This contradiction was resolved by

generating a CPP dose response curve for both WT and α6L9'S mice (Figure 6D). When given saline in both chambers, α6L9'S mice did not habituate to their initially least preferred chamber. Instead, their initial preference increased, indicated by negative CPP, or conditioned place aversion (CPA). When compared to saline, α6L9'S mice given TI-299423 (0.012 mg/kg) showed a similar amount of CPP as their WT littermates. When given a TI-299423 dose of 0.025 mg/kg, however, α6L9'S mice showed even more CPP. In fact, looking at the dose response curve, 0.025 mg/kg looks like a secondary peak, possibly mediated by different receptors.

The α4L9'A data, and the α6L9'S dose response curve, suggest a new hypothesis for how TI-299423 is causing CPP. As explained in Chapter 2, we believe that TI-299423 has a similar EC_{50} for α6(non-α4)*-nAChRs as it does for α6α4*-nAChRs in wild type mice. At a dose of 0.012 mg/kg,TI-299423 is able to activate both of these receptor types in WT mice, resulting in the drug's rewarding effects. In the α6L9'S mice, only the α6(non-α4)*-nAChRs are being activated at doses between 0.001 mg/kg and 0.012 mg/kg. At 0.025 mg/kg, the α6α4*-nAChRs are also activating, resulting in even more CPP. What, exactly, is happening in the α4L9'A mice is less clear. As mentioned above, we believe the L9'A mutation is shifting the CPP dose response curve, likely to the left, due to the higher number of α6*-nAChRs in α4L9'A mice [7]. Similarly to the double knock-out mice, additional study is needed both on TI-299423 and on the mutant mice in order to form a better hypothesis.

Chronic TI-299423

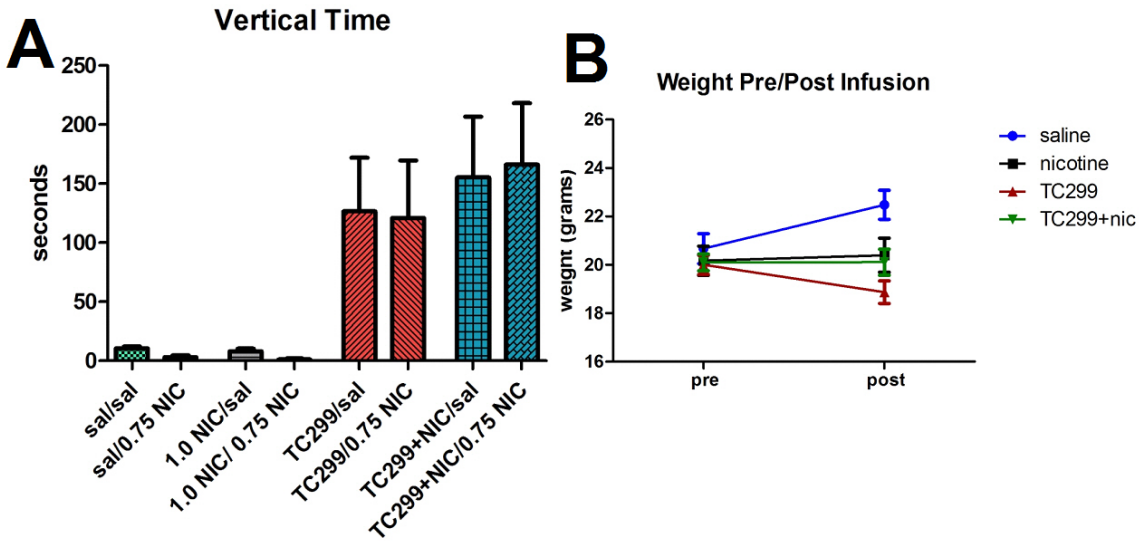

Figure 7: Behavioral effects of chronic Nicotine(1 mg/kg/h), TI-299423 (0.02 mg/kg/h), or TI-299423+Nicotine **A)** Vertical time measured after withdrawal from chronic drug administration in the presence and absence of nicotine. Mice who received TI-299423 as all, or part, of their chronic treatment spent significantly more time vertical (either grooming or rearing) than mice that did not receive any TI-299423 **B)** Average mouse weight before and after chronic treatment. Mice that received chronic infusions of only saline gained weight during the experimental period. Mice that received nicotine, or nicotine and TI-299423 together, had no significant change in weight. Mice that received only TI-299423 chronically lost weight during the course of the treatment. All data shown are mean ± SEM.

In addition to the effects of acute TI-299423 administration, we were interested in the chronic effects of this compound. Several mice were implanted with catheters that allowed chronic administration of saline, nicotine (1 mg/kg/h), TI-299423 (0.02 mg/kg/h), or nicotine+TI-299423 (1 mg/kg/h and 0.02 mg/kg/h, respectively) over the course of 10 days. The mice were then withdrawn from the treatment for several h, and tested on Y-maze and open field assays. Chronic TI-299423 did not have a significant effect on most measures. The two most interesting measures are presented in Figure 7: vertical time and weight. Mice withdrawn from chronic TI-299423, or from a chronic cocktail of TI-299423 and nicotine, spent significantly more time vertical, either grooming or rearing, in the open field

test than mice given saline or nicotine alone (Figure 7A). Additionally, mice given chronic TI-299423 lost weight during the experiment (Figure 7B). Mice given nicotine or TI-299423 and nicotine had no change in weight. Mice given only saline gained weight.

The vertical time measure is not currently understood. Mice given chronic TI-299423 showed no increase in vertical count (the number of times they stood up) or in stereotypy, yet they spent significantly more time standing, even with an acute dose of nicotine. Whether this should be interpreted as either a decrease in anxiety (standing and looking around) or an increase in anxiety (over-grooming) is unclear, as is the molecular basis for such behaviors. Additional study on both the chronic effects of TI-299423, and the representative data from this assay, needs to be conducted.

Chronic TI-299423's effect on weight is not entirely surprising. Nicotine has previously been shown to facilitate weight loss in mice. Nicotine treated mice did not lose weight, but did not gain weight at the same rate as the saline treated mice. TI-299423 may be acting as an appetite suppressant or increasing the activity levels of the mice. Nicotine increases activity in α6L9'S mice via activation of α6*-nAChRs and decrease activity in WT mice through activation of α4*-nAChRs [3]. Because TI-299423 acts on α6*-nAChRs more potently that α4*-nAChRs in mice, the chronically treated mice might have higher activity levels. Observation during chronic treatment is necessary for testing this hypothesis.

Finally, an interesting behavior was noted in the mice withdrawn from chronic TI-299423. These mice were hyperactive in their home cage, jumping and exhibiting behaviors similar

to the phenotype of α6L9'S mice. This behavior was highly unexpected, and therefore currently unquantified. Measuring home cage activity, or locomotor responses to novel environments, could provide better insight into these behaviors. Additional study into the molecular changes caused by chronic TI-299423 is also necessary.

TI-299423 Effects on Receptor Expression

To that end, we tested the effects of TI-299423 on α6β2β3 and α4β2-nAChR expression on the plasma membrane using total internal reflection fluorescence microscopy (TIRFM). TIRFM methods used were similar to those previously completed on cultured cortical neurons [6], here applied to cultured Neuro-2A cells. TIRFM allows for the visualization of fluorescently labeled molecules inside the cell within ~250 nm of the cell-coverslip interface. To obtain TIRF images, we used an inverted fluorescence microscope (IX81; Olympus) equipped with an Olympus Plan Apo 100X 1.45 NA oil objective and a stepper motor (Thorlabs) to control the position of the fiber optic and TIRF evanescent field illumination. TI-299423 was added to the dishes 24 h prior to imaging. Growth medium was exchanged for extracellular solution (ECS) (150 mM NaCl, 4 mM KCl, 10 mM HEPES, 2 mM MgCl2, 2 mM CaCl2, and 10 mM glucose) and adjusted to the appropriate pH (5.4 or 7.4) just prior to imaging. An air-cooled argon laser (IMA101040ALS; Melles Griot) was used to excite SEP at 488-nm. A back-illuminated EMCCD camera (iXON DU-897) was used to capture the images. The imaging dish was acidified to a pH of 5.4 by perfusing the bath, normally at pH 7.4, with an otherwise identical solution adjusted to the lower pH. Images taken at the lower pH demarcated ER-localized nAChRs as opposed to

nAChRs located on the plasma membrane. Thus, PMID was determined by taking the difference of the initial TIRF image of each cell at pH 7.4 and the image at pH 5.4.

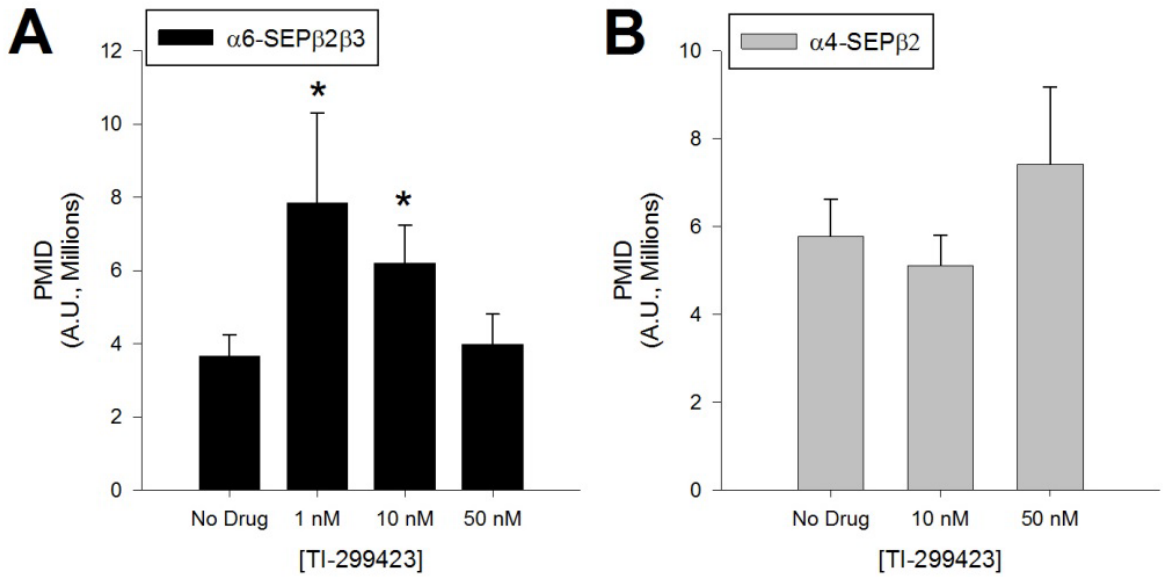

Figure 8: TI-299423 upregulates α6β2β3-nAChRs on the plasma membrane. Neuro-2A cells were transfected with α6-SEPβ2β3 and α4-SEPβ2 nACHRs. TI-299423 was added 24 h before imaging. **A)** PMID was quantified for α6-SEPβ2β3 nACHRs. α6β2β3 nAChRs were significantly upregulated at TI-299423 concentrations of 1 nM (one-tailed t-test, $p < 0.05$) and 10 nM (one-tailed t-test, $p < 0.05$). **B)** PMID was quantified for α4-SEPβ2 nACHRs. α4β2 nAChRs were not significantly up or down regulated at any tested concentration of TI-299423.

Using this method, previous studies have shown that nicotine increases the plasma membrane integrated density (PMID) of α6-SEPβ2β3 at a concentration of 50 nM and ofα4-SEPβ2 nAChR at a concentration of 100 nM. Because TI-299423 is so much more potent than nicotine at both of these receptor types, we measured upregulation of α6-SEPβ2β3 nAChRs using three concentrations (1 nM, 10 nM and 50 nM) and upregulation of α4-SEPβ2 nAChR using two concentration (10 nM and 50 nM). α6-SEPβ2β3 nAChRs treated with 1 nM exhibited a significant, 2-fold upregulation in PMID (one-tailed t-test, $p < 0.05$), and α6-SEPβ2β3 nAChRs treated with 10 nM exhibited a significant, slightly lower, upregulation (one-tailed t-test, $p < 0.05$) (Figure 8A). α4-SEPβ2 nAChRs, however,

did not show significant upregulation at any dose (Figure 8B). The selective upregulation of α6*-nAChRs could be what is causing the behaviors observed in the withdrawn mice. An upregulation of α6*-nAChRs, without an accompanying upregulation of α4*-nAChRs could result in higher levels of dopamine release. Additional study on this is needed.

Additional Analgesia Data

Chapter 2 also presented data on TI-299423's analgesic effects as compared to nicotine and varenicline. Figure 9 elaborates on that data. First, Figure 9 includes tail flick data that was excluded from Chapter 2 simply because it provides no information. The tail flick apparatus used has several issues. It requires the experimenter to hold the mouse during the test, stressing the mouse and making anxiety related, rather than stress related, tail flicks more likely. Additionally, the apparatus is designed to automatically detect when the mouse flicks its tail. This sensor can be tricked by tail movements that do not remove the tail from the beam, resulting in the light going off and the timer stopping, or by cleaning fluid not remaining on the platform, resulting in the light staying on and the timer continuing, even when the mouse has flicked its tail away. Because of the shortcomings of the apparatus and the delicate nature of the experiment, reliable tail flick data was unattainable.

Figure 9A also includes several additional doses of TI-299423 that were tested. 0.01 mg/kg, 0.1 mg/kg, and 0.3 mg/kg were all tested to determine the most effective dose, after which the experiment was repeated with similar doses of nicotine and varenicline (Figure

9B). These doses give us a nice dose response curve with 0.3 mg/kg producing the highest level of analgesia. The exact mechanism of nicotine induced analgesia is much debated. Previously, α4β2*, α3β4*, and α7-nAChRs [8] have been implicated, though recently α6*-nAChRs have been shown to be involved as well [10].

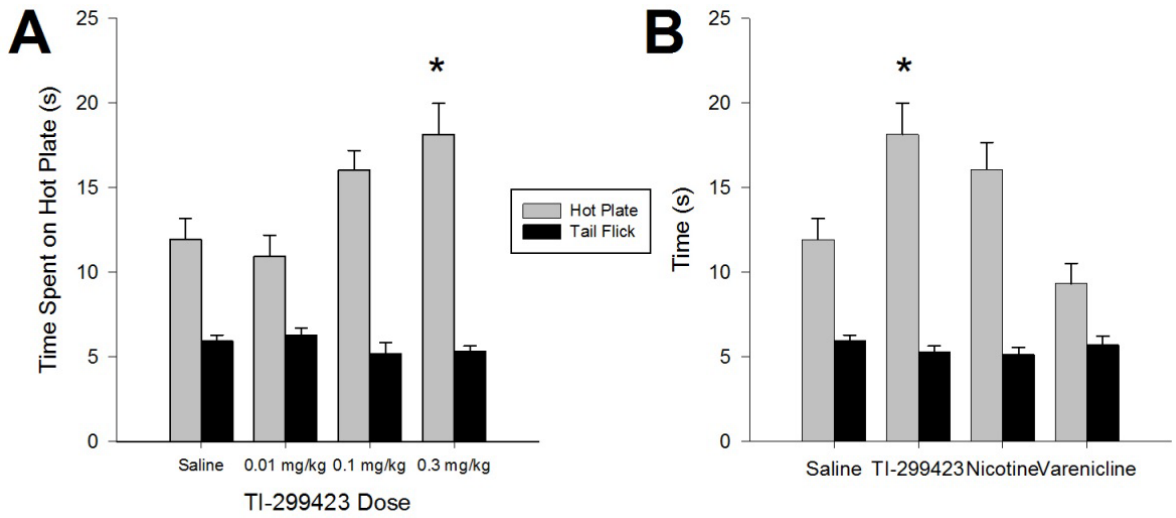

Figure 9: Analgesia Data **A)** Dose response curve for TI-299423 on hot plate and tail flick. A TI-299423 dose of 0.3 mg/kg significantly increases the time spent on the hot plate ($p < 0.05$). No effect was seen at any dose on tail flick. **B)** Hot plate and tail flick with TI-299423 (0.3 mg/kg), nicotine (0.3 mg/kg), and varenicline (0.3 mg/kg). Again, no effect was seen on tail flick for any drug. All data shown are mean ± SEM.

This additional data was excluded from Chapter 2, which was submitted as a manuscript to the journal Neuropharmacology, because it does not provide much insight into how TI-299423 acts in the brain. Instead, much of this data leads us to new, unanswered questions. We still do not understand why TI-299423 fails to elicit hypothermia at low doses, nor do we understand the exact mechanism behind the effects of chronic TI-299423. Continuing to study TI-299423, both *in vitro* and *in vivo*, will hopefully lead to additional information, and a more complete picture of this compounds mechanisms of action in the brain.

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C h a p t e r 4

ACUTE AND CHRONIC MENTHOL AFFECTS REINFORCEMENT OF NICOTINE

This chapter contains behavioral data from the following manuscript:

Menthol alters nicotine reinforcement, nicotinic receptor number, and nicotinic receptor assembly. Henderson BJ, Wall TR, Henley BM, Kim CH, Nichols WA, Xiao C, Lester HA. (Submitted to Neuron, 2015)

Abstract

Smokers of menthol cigarettes have lower cessation rates than smokers of non-menthol cigarettes. This could be due to menthol potentiating the reinforcing effects of nicotine. To test this, I used conditioned place preference to measure reinforcement for nicotine and for a cocktail containing both menthol and nicotine. For both a nicotine dose of 0.5 mg/kg and 0.25 mg/kg, the addition of menthol significantly increased the change in preference towards the drug-paired chamber. Chronic menthol prior to nicotine CPP was also used. Chronic exposure to menthol reduced nicotine CPP.

Introduction

The US Surgeon General first linked tobacco use to disease over 50 years ago [14]. Today, tobacco related disease is still one of the leading causes of preventable death in the world. The primary addictive compound in tobacco is nicotine, which acts on nicotinic acetylcholine receptors (nAChRs) in the brain. Chronic nicotine has been shown to upregulate nAChRs containing the β2 subunit (β2*-nAChRs, where the * denotes the

presence of other subunits) [3, 4, 7, 9, 10, 13], which combines with α4 and α6 subunits on dopaminergic neurons [8, 11, 15]. The increase in these receptors enhances the dopamine pathway, one possible molecular basis for nicotine dependence.

Menthol is the sole remaining flavor additive that is legal in cigarettes in the US. Menthol cigarette smokers have much lower cessation rates than smokers of non-menthol cigarettes [1, 5], though it is unclear exactly what causes this, whether it potentiates dependence or withdrawal, or alters the metabolism of nicotine. Smokers of menthol containing cigarettes have higher levels of β2*-nAChR upregulation than smokers of non-menthol cigarettes [4]. Research is currently being done to determine if menthol itself causes upregulation, or if it is simply potentiating nicotine's effects. One suggestion has been that menthol decreases airway irritation caused by cigarette smoke, allowing smokers of menthol cigarettes to inhale more nicotine. This hypothesis is refuted by Ashley, *et. al.*[2] who found that menthol smokers have no increase in nicotine exposure despite reduced sensory irritation.

In addition to causing upregulation, menthol's effects on β2*-nAChRs could imply that it is itself rewarding without the presence of nicotine. More importantly, since menthol dependence is not a major public health concern, understanding how the addition of menthol affects reward and reinforcement for nicotine could provide insight into why smokers of menthol cigarettes have lower cessation rates. We hypothesized that menthol potentiates the rewarding properties of nicotine. This hypothesis was tested using a Pavlovian reward conditioning assay called conditioned place preference (CPP).

Materials and Methods

Mice – Animal breeding, maintenance, and procedures at the California Institute of Technology were conducted in accordance with the guidelines of the National Institutes of Health and the approval of the California Institute of Technology Animal Care and Use Committee. Mice of the C57BL/6 strain, ages 40-180 days, were used in this study. After weaning at 25 days of age, same sex littermates were housed no more than 3 to a cage, with free access to food and water, on a 13/11-h light/dark cycle at 22^0C.

Nicotine, Menthol – (-)-Nicotine hydrogen tartrate salt and (±)-Menthol were purchased from Sigma-Aldrich (St. Louis, MO). All dose concentrations refer to the free base.

Chronic Administration – Model 1002 or 1004 osmotic mini-pumps were obtained from Alzet (Cupertino, CA). 48 h prior to implantation, pumps were loaded with freshly made vehicle or (±)-Menthol to deliver at 1 mg/kg/hr. The pre-incubation period ensures the pumps reach the desired pump rate before surgery is performed. Surgical procedures for implantation have been previously described [6, 10].

Conditioned Place Preference – The conditioned place preference apparatus is a rectangular cage with interior dimensions 46.5(L) x 12.7(W) x 12.7(H) cm, divided into three sub compartments: White and Black (each 16.8cm L) with a steel mesh and steel rod floor, respectively, and Grey (7.2 cm L) with a solid plastic floor. Each compartment has a polycarbonate hinged lid for loading the animals. Guillotine doors, which can be fixed in the open or closed position, separate the chambers.

Mice were singly housed and habituated to the experimental room for 3-7 days before the initial testing day, and remained in the experimental room for the duration of the experiment. On day 1 (pre-training) mice were placed into the center chamber and allowed to explore the apparatus freely for 20 min. Time spent in each chamber was recorded, and drug pairing was determined by random selection. On days 2, 4, 6, and 8, mice were injected with the drug or dose of interest, and were confined to the drug-paired chamber for a total of 20 min. On days 3, 5, 7, and 9, mice were injected with saline and confined to the opposite chamber. On day 10 (post-training), mice were again given free access to the apparatus for 20 min, and time spent in each chamber was recorded. Conditioned place preference was determined by looking at the change in time spent in the drug paired chamber compared to the saline paired chamber from pre-training to post-training. Mice with a severe initial bias for one chamber, defined as a bias of greater than 65% time spent in one conditioning chamber over the other, were excluded.

Results

CPP was measured for nicotine 0.25 mg/kg and 0.5 mg/kg without and without the addition of 1 mg/kg menthol (Figure 10A). Mice trained with only nicotine showed aversion of 107.6 s to the drug paired chamber at a dose of 0.25 mg/kg and preference of 109.2 s for the drug paired chamber at a dose of 0.5 mg/kg. When Menthol (1 mg/kg) was included in the training cocktail, mice showed a preference of 114.0 s and 244.7 s for the drug-paired chamber at nicotine doses of 0.25 mg/kg and 0.5 mg/kg, respectively.

Mice trained with a nicotine dose of 0.25 mg/kg and menthol (1 mg/kg) showed significantly more conditioned place preference than mice trained with nicotine (0.25 mg/kg) alone (two-tailed t-test, $p < 0.01$). Additionally, mice trained with nicotine at a dose of 0.5 mg/kg and menthol (1 mg/kg) showed significantly more conditioned place preference than mice trained with nicotine (0.5 mg/kg) alone (two-tail t-test, $p < 0.05$).

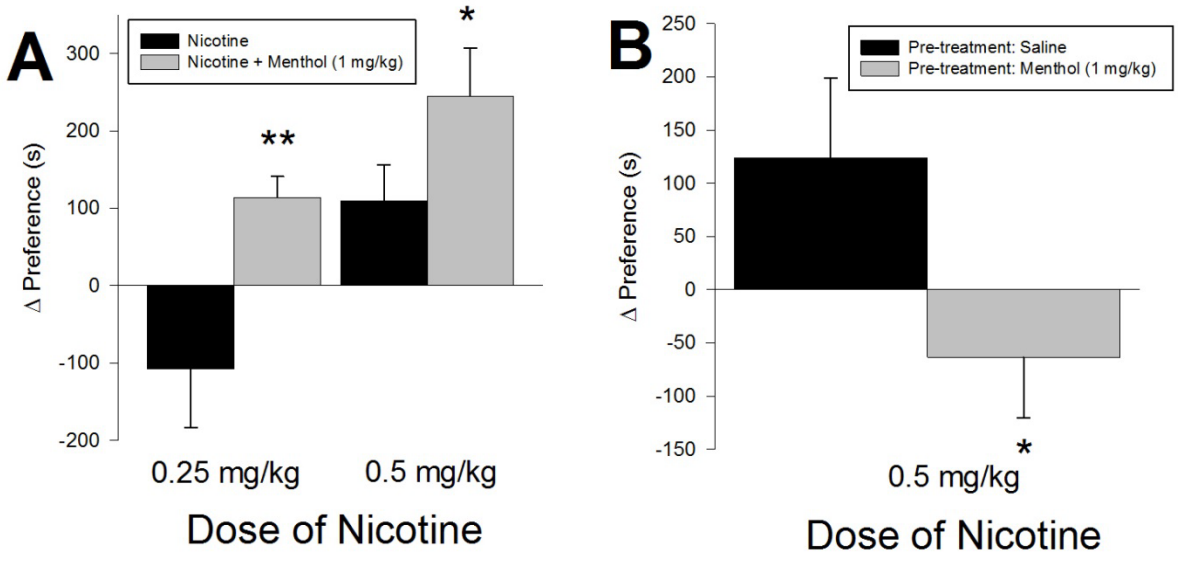

Figure 10: Conditioned Place Preference (CPP) with Menthol and Nicotine. All data shown are mean ± SEM. **A)** CPP was measured for nicotine 0.25 mg/kg and 0.5 mg/kg without and without the addition of 1 mg/kg menthol. **B)** Mice were implanted with osmotic pumps administering either saline or menthol (1 mg/kg/hr) and trained for CPP with nicotine (0.5 mg/kg).

The effect of chronic menthol on nicotine reinforcement (Figure 10B) was measured by implanting mice with osmotic pumps administering a menthol dose of 1mg/kg/hr or saline, and then training mice on the CPP assay using a nicotine dose of 0.5 mg/kg. Mice implanted with saline-containing pumps showed CPP of 123.3 s, similar to what was seen in mice without osmotic pumps trained with nicotine (0.5 mg/kg). Mice with menthol containing pumps showed an aversion of 63.4 s for the nicotine-paired chamber. Thus,

chronic menthol significantly (two-tailed t-test, $p < 0.05$) reduced the amount of CPP seen from training mice with a nicotine dose of 0.5 mg/kg.

Discussion

Menthol cigarette smokers have lower cessation rates than smokers of non-menthol containing cigarettes, suggesting menthol may have a role in nicotine reinforcement. I used the conditioned place preference (CPP) paradigm to measure differences in nicotine reinforcement in the presence and absence of menthol. When trained using nicotine (0.5 mg/kg) and menthol (1 mg/kg) mice showed a two-fold higher preference for the drug paired chamber than mice trained with nicotine (0.5 mg/kg) alone. Previous studies have suggested that a nicotine dose of 0.5 mg/kg elicits near maximal CPP [12]. If 0.5 mg/kg is a 'peak' dose, then the effects of menthol might be obscured. Thus a lower dose of nicotine (0.25 mg/kg) was also tested with and without menthol. Again, the mice that received both nicotine (0.25 mg/kg) and menthol (1 mg/kg) showed significantly higher CPP than mice that received nicotine (0.25 mg/kg) alone. This data shows that menthol can potentiate the reinforcement properties of nicotine when the two are co-administered, one possible explanation for why menthol cigarette smokers have a harder time quitting.

To look at how chronic menthol treatment prior to nicotine exposure affected nicotine reinforcement, we treated mice with menthol (1 mg/kg/hr) or saline for 10-days prior to, and then throughout, the CPP training for nicotine (0.5 mg/kg). Mice that were treated with saline showed similar CPP to those without saline training. However, mice treated with

menthol showed no change in preference from baseline. Compared to the saline treated mice, this is a significantly smaller change in preference. The mechanism by which chronic menthol treatment prior to nicotine exposure blocks the rewarding properties of nicotine is unknown. However, understand this phenomenon more in depth in the future may lead to novel new smoking cessation treatments.

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Chapter 5

CONCLUSIONS

Nicotinic acetylcholine receptors (nAChRs) are spread throughout the central and peripheral nervous system. In the brain, nAChRs are primarily expressed on presynaptic terminals [4] where they modulate the release of other neurotransmitters, including GABA, serotonin, glutamate, and dopamine [2]. Receptors containing the α6-subunit in particular are primarily found on dopaminergic neurons [1, 7, 14], where they mediate dopamine release [9-10]. Because of dopamine's role in learning, reward, and voluntary movement [5, 6, 8, 12], the selective expression of α6*-nAChRs makes them an enticing target for potential new smoking cessation compounds or treatments for Parkinson's disease.

TI-299423 was developed with a number of other compounds by Targacept, Inc, in an attempt to create an agonist selective for α6*-nAChRs over α4*-nAChRs. Because an α6*-nAChR selective agonist has not previously been synthesized, we theorized that such a compound would be useful for smoking cessation, with fewer side effects than varenicline. Several compounds were assayed for their effects on locomotor activity in mice with hypersensitive α6 receptors (α6L9'S). These mice show an increase in locomotion in response to nicotine [3], allowing us to parse out the effects of compounds on α6*-nAChRs. If a compound fails to elicit a locomotor response, it is failing to activate the α6*-nAChRs, and thus is not selective for those receptors. TI-299423 was the most potent compound tested on this assay, leading us to continue researching its effects in the brain.

TI-299423 was then assayed using a battery of *in vitro* tests. TI-299423 was shown to be more potent than nicotine at both α6* and α4*-nAChRs. TI-299423 appears to be a partial agonist at low sensitivity α4β2*-nAChRs and a full agonist of high sensitivity α4β2*-nAChRs. While TI-299423 is only a partial agonist of α6β2*-nAChRs, it is apparently more potent at these receptors. This suggested that, at low doses, we may be able to activate α6*-nAChRs without activating α4(non-α6)*-nAChRs. We tested TI-299423 using Neuro-2A cells expressing α6-GFPβ2$_{DM}$β3 or α4-GFPβ2$_{DM}$ nAChRs. Using this method, past experiments have shown that nicotine has a lower EC_{50} for α4(non-α6)*-nAChRs than it does for α6(non-α4)*-nAChRs. TI-299423, however, has a lower EC_{50} at the α6(non-α4)*-nAChRs.

We then tested TI-299423 on assays intended to measure the effects of compounds selectively on α6* or α4*-nAChRs in behaving animals. We repeated the locomotor assay, using mecamylamine pre-injections to show that the effects were blocked by a nicotinic antagonist. Additionally, we used a temperature assay to measure TI-299423's effects on α4*-nAChRs. Nicotine induced hypothermia is mediated by both α4*-nAChRs [13] and β4*-nAChRs [11]. Mice genetically modified to have a hypersensitive α4-subunit (α4L9'A) thus show hypothermia at much lower doses of nicotine than wild type mice [13], allowing us to selectively measure α4*-nAChR activation by recording the temperature of these mice after compound injection. TI-299423 failed to induce hypothermia at the doses we suspected. Instead, a 5-times higher dose of TI-299423 was needed. This phenomenon is still unexplained, but supports the hypothesis that TI-299423 is selective for α6*-nAChRs over α4*-nAChRs.

Now that we had an apparently α6*-selective compound, we decided to test its rewarding effects and its ability to modulate nicotine reward. TI-299423 was shown to be extremely potent at eliciting conditioned place preference (CPP), a measure of Pavlovian reward conditioning, in WT and α6L9'S mice. This was blocked in mice lacking the α6-subunit (α6KO) and mice lacking the β2-subunit (β2KO), but was unchanged in α4 null mutant mice (α4KO), suggesting these rewarding effects are also α6*-nAChR mediated. Attempts to measure TI-299423's effects on CPP were confounded by the limits of the assay. Pre-injections or osmotic pumps distress the animals, making their learning and behavior less reliable. Additionally, small changes in by CPP may not be measurable, simply due to the experiment design. Instead, we tested TI-299423's effect on intravenous self-administration of nicotine in rats. Pre-treatment with low doses of TI-299423 increased the amount of nicotine that rats self-administered. We hypothesize this is due to TI-299423's activation of α6(non-α4)*-nAChRs not usually activated by the self-administered doses of nicotine, increasing reward. While this indicates that TI-299423 is not a good candidate for smoking cessation, it may be helpful for early Parkinson's treatment. Additional study needs to be done before a stronger claim can be made.

The cholinergic system is extremely complex, as are its interactions with other systems throughout the brain. Discovering new compounds that act selectively on very specific receptors or subunits can give us tremendous insight into how these systems fit together. In the case of nicotine and nicotine addiction, these compounds allow us to reduce complex observed behaviors into smaller, simpler pieces. Looking at the α6 subunit, in particular, helps us to understand how nicotine affects the dopaminergic system, and how that

translates to changes in reward, reinforcement, and drug-seeking behavior downstream. While TI-299423 is not entirely selective, only showing selective properties at low doses, it has been able to offer valuable insight into the activity of the cholinergic system. Studies like this one, which first seek a scientific understanding of how compounds interact with the brain, rather than primarily focusing on discovering their usefulness as therapeutics, will allow us to better understand the complex circuitry of the brain and help us develop treatments more efficiently in the future.

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