Chronic Sazetidine-A at Behaviorally Active Doses Does Not Increase Nicotinic Cholinergic Receptors in Rodent Brain

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ABSTRACT

Chronic nicotine administration increases $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptor (nAChR) density in brain. This up-regulation probably contributes to the development and/or maintenance of nicotine dependence. nAChR up-regulation is believed to be triggered at the ligand binding site, so it is not surprising that other nicotinic ligands also up-regulate nAChRs in the brain. These other ligands include varenicline, which is currently used for smoking cessation therapy. Sazetidine-A (saz-A) is a newer nicotinic ligand that binds with high affinity and selectivity at $\alpha 4\beta 2^*$ nAChRs. In behavioral studies, saz-A decreases nicotine self-administration and increases performance on tasks of attention. We report here that, unlike nicotine and varenicline, chronic administration of saz-A at behaviorally active and even higher doses does not up-regulate nAChRs in rodent brains. We used a newly developed method

involving radioligand binding to measure the concentrations and nAChR occupancy of saz-A, nicotine, and varenicline in brains from chronically treated rats. Our results indicate that saz-A reached concentrations in the brain that were ~ 150 times its affinity for $\alpha 4\beta 2^*$ nAChRs and occupied at least 75% of nAChRs. Thus, chronic administration of saz-A did not up-regulate nAChRs despite it reaching brain concentrations that are known to bind and desensitize virtually all $\alpha 4\beta 2^*$ nAChRs in brain. These findings reinforce a model of nicotine addiction based on desensitization of up-regulated nAChRs and introduce a potential new strategy for smoking cessation therapy in which drugs such as saz-A can promote smoking cessation without maintaining nAChR up-regulation, thereby potentially increasing the rate of long-term abstinence from nicotine.

Introduction

Nicotine addiction, which sustains smoking, is a leading cause of morbidity and mortality worldwide [Centers for Disease Control and Prevention (CDC), 2008]. Although the cellular substrates of addiction are not completely understood, neuronal nicotinic acetylcholine receptors (nAChRs) are likely crucial to the mechanisms of addiction. Specifi-

cally, chronic administration of nicotine increases nAChRs in rat and mouse brain (Marks et al., 1983; Schwartz and Kellar, 1983), and a similar increase is found in autopsied brains from smokers (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999). Increased nAChRs are also seen with in vivo imaging techniques in baboon brain after chronic administration of nicotine (Kassiou et al., 2001) and the brains of current smokers (Staley et al., 2006; Wüllner et al., 2008). In addition to nicotine, chronic administration of other nicotinic receptor ligands up-regulate nAChRs in rodent brain, including cytisine (Schwartz and Kellar, 1985), anatoxin (Rowell and Wonnacott, 1990), and varenicline (Turner et al., 2011).

Immunoprecipitation with subunit-selective antibodies and studies in genetic knockout models demonstrate that chronic administration of nicotine increases predominantly the $\alpha 4\beta 2$ subtype of nAChRs in rodent brain (Flores et al.,

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ABBREVIATIONS: nAChR, neuronal nicotinic acetylcholine receptor; [3 H]EB, [3 H]epibatidine; [125 I]EB, [125 I]epibatidine; Nic, nicotine; saz-A, sazetidine-A; Var, varenicline; MK801, (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine.

1992; Mao et al., 2008; Moretti et al., 2010; Marks et al., 2011), and based on the limited spectrum of the radioligand used to image the receptors *in vivo* that is also probably the case in primate brains (Kassiou et al., 2001; Staley et al., 2006; Willner et al., 2008). Consistent with the likely important involvement of $\alpha 4\beta 2$ nAChRs in nicotine addiction, $\beta 2$ subunit knockout mice do not self-administer nicotine (Piciotto et al., 1998). Thus, $\alpha 4\beta 2$ nAChRs have been a major focus of studies related to the cellular mechanisms of nicotine addiction and its treatment.

Sazetidine-A [saz-A; 6-(5-(((S)-azetidin-2-yl)methoxy)pyridin-3-yl)hex-5-yn-1-ol] is a partial agonist at $\alpha 4\beta 2$ nAChRs that may show differential efficacy at this receptor subtype depending on the stoichiometry (Zwart et al., 2008). Saz-A also potently and selectively desensitizes these receptors (Xiao et al., 2006). Saz-A produces several potentially important behavioral effects in rodents: it decreases nicotine self-administration (Levin et al., 2010b; Rezvani et al., 2010; Johnson et al., 2012), reduces alcohol consumption (Rezvani et al., 2010), and increases performance on tasks of attention, even after attention is disrupted by pretreatment with scopolamine or dizocilpine [(5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine] (MK801) (Rezvani et al., 2011). In addition, in preclinical tests saz-A produces behavior indicative of potential antidepressant and/or antianxiety effects (Kozikowski et al., 2009; Turner et al., 2010, 2011; Caldarone et al., 2011).

The roles of up-regulation and desensitization of $\alpha 4\beta 2$ nAChRs in the effects of nicotine, including addiction, are potentially crucial (see *Discussion*); thus, a selective agonist or partial agonist that affects these processes could be an important tool to better understand addiction and might even be a potential therapeutic agent to treat addiction as well as attention and/or affective disorders. In this study we compared the ability of chronic administration of saz-A, nicotine, and varenicline to up-regulate brain nAChRs in rats and mice. We were surprised to find that whereas chronic administration of nicotine and varenicline up-regulated these receptors, saz-A did not, even at doses four times higher than those that decreased nicotine self-administration.

Materials and Methods

Materials. [^3H]epibatidine ([^3H]EB, ~55 Ci/mmol) and [^{125}I]epibatidine ([^{125}I]EB) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). The specific activity of [^{125}I]EB was adjusted to ~500 Ci/mmol. Saz-A dihydrochloride (Xiao et al., 2006) was synthesized by RTI International (Research Triangle, NC) and supplied by the National Institute on Drug Abuse (Rockville, MD). Nicotine hydrogen tartrate was purchased from Sigma-Aldrich (St. Louis, MO). Varenicline tartrate was a gift from Dr. Hans Rollema (Pfizer, Groton, CT). Osmotic minipumps were purchased from Alzet (Cupertino, CA). For primary neuron cultures, Hanks' balanced salt solution buffer was purchased from Invitrogen (Carlsbad, CA). Primary neuron plating media were made from neurobasal media (Invitrogen), B27 supplement (Invitrogen), 500 μM L-glutamine (Sigma-Aldrich), 25 μM L-glutamate (Sigma-Aldrich), 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen), and 100 units/ml penicillin (Invitrogen). Plating media without L-glutamate are referred to as feeding media. Cell culturing flasks and mouse laminin were purchased from Thermo Fisher Scientific (Waltham, MA). Frozen brains from adult Sprague-Dawley rats were purchased from Zivic Miller Laboratories (Portersville, PA).

Primary Cortical Cell Cultures. Timed pregnant Sprague-Dawley rats were purchased from Charles River (Raleigh, NC) and housed at the Georgetown University Research Resource Facility (Washington, DC) with free access to food and water. The pregnant rats were killed by CO_2 followed by decapitation, and the cortices from embryonic day 18 and 19 rats were dissected, washed in Hanks' balanced salt solution buffer, and then treated with 0.15% trypsin for 15 min at 37°C . The trypsin reaction was quenched with 10% fetal bovine serum, and the tissue was washed with plating medium. Tissue was gently triturated and strained to collect single cells. Approximately 2 million cells were plated on poly-D-lysine- and laminin-coated six-well culturing plates. Cells were maintained at 37°C in a humidified incubator under 5% CO_2 for 24 h, and then 50% of the plating medium was replaced with feeding medium and treatment drug. A 50% medium exchange was carried out with fresh feeding medium and drugs every 3 to 4 days.

Animals. Male and female adult Sprague-Dawley rats were purchased from Harlan Laboratories (Frederick, MD) or Hilltop Laboratory Animals, Inc. (Scottsdale, PA). Male 129SvJ;C57BL/6J F_1 hybrid mice were bred and housed at the University of Pennsylvania (Philadelphia, PA). Rats and mice were housed in groups in Association for Assessment and Accreditation of Laboratory Animal Care-approved facilities at Georgetown University (Washington, DC), George Washington University (Washington, DC), Duke University (Durham, NC), or the University of Pennsylvania. All rodents were maintained on a 12-h light/dark cycle with free access to food and water. Treatment, care, and housing were carried out in accordance with the National Institutes of Health guidelines on animal care (Institute of Laboratory Animal Resources, 1996), and all experimental procedures were approved by each university's animal care and use committee.

Drug Administration. All drugs were dissolved in sterile saline, pH 6–8, and doses are reported as the free base. Injections were administered subcutaneously between the shoulders. To insert osmotic minipumps, rodents were anesthetized with isoflurane, and a small incision was made between the shoulders. The pump was then inserted under the skin, and the incision was closed with wound clips. Buprenorphine (0.01 mg/kg) or ketoprofen (0.5 mg/kg) was then injected subcutaneously one time for analgesia. At the end of the treatment period, the animals were anesthetized with isoflurane and then decapitated. Brains were quickly removed, dissected, frozen on dry ice, and stored at -80°C until they were used in assays.

Nicotine self-administration by operant licking was carried out as described previously (Levin et al., 2010a). In brief, rats were placed in test chambers (MED Associates, St. Albans, VT). Two water spouts were available to be licked but only one spout triggered an intravenous infusion of nicotine (0.03 mg/kg/infusion). Both inactive and active licks were recorded. The schedule of reinforcement was Fixed Ratio 1, and each infusion was immediately followed by a 1-min period in which responses were recorded but not reinforced. Each session lasted 45 min. Acute doses of saz-A were administered by subcutaneous injection 10 min before the start of each 45-min session. The doses were given in a repeated-measures counterbalanced design, with at least 2 days between consecutive doses. The full dose-effect function and responses to control injections were measured twice.

Preparing Brain Membranes for Measurement of nAChR Binding Sites. Brain tissues were washed extensively to remove treatment drugs. The tissues were first homogenized in 25 ml of Tris buffer (50 mM Tris-HCl, pH 7.0) with a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland) for ~20 s and then centrifuged at 32,000g for 10 min at 4°C . The membrane pellets were resuspended in 25 ml of fresh Tris buffer, homogenized again for 20 s, incubated at 37°C for 30 min, and then centrifuged again at 32,000g for 10 min at 4°C . This procedure was repeated so that the tissue underwent a total of four washes in fresh Tris buffer and three 30-min incubations at 37°C . The final membrane pellet was resuspended in Tris buffer, pH 7.4. Washed membrane homogenates from

cultured primary neurons were prepared by using this same protocol, with the exception that the cells were washed in 15 ml of Tris buffer and homogenized by sonication.

To measure the concentration of drugs in the brain after treatment, cerebral cortical homogenates were prepared by homogenizing dissected brain tissue in 15 to 20 volumes of Tris-HCl buffer, pH 7.4, with a glass-Teflon homogenizer (six or seven strokes), followed by a brief sonication. This homogenate was not washed or centrifuged before use and therefore is referred to as an unwashed homogenate. The binding kinetics (k_{on} and k_{off}) of [3 H]EB were similar in washed and unwashed tissue (data not shown).

Radioligand Binding. Washed membrane homogenates from 10 mg of brain tissue were incubated with 1.5 to 2 nM [3 H]EB in a total volume of 0.5 ml of Tris buffer, pH 7.4, in the absence or presence of 300 μ M nicotine to measure total and nonspecific binding, respectively. After incubation with gentle shaking at 24°C for 2 to 4 h, the samples were collected by vacuum filtration over GF/C filters presoaked with 0.5% polyethyleneimine. Radioactivity in the filters was measured by scintillation counting. Specific [3 H]EB binding was then calculated as the difference between total and nonspecific binding. This same protocol was used to measure binding of nAChRs in cultured primary neurons, except that [125 I]EB was used instead of [3 H]EB.

Saturation binding assays were carried out with concentrations of [3 H]EB from 5 to 3500 pM. Competition binding assays were carried out by competing increasing concentrations of nicotine, varenicline, or saz-A against 1.0 nM [3 H]EB. These saturation and competition binding assays were incubated at 24°C for 18 h before collecting and measuring bound [3 H]EB as described above. The longer incubation time was used to provide the most accurate measurements of the dissociation constant (K_d) for [3 H]EB and the derived dissociation constants (K_i values) for the competing drugs.

Measuring the Concentration of Drugs in Rat Brain Ex Vivo. Unwashed cortical homogenates from chronically treated rats were incubated for ~18 h with [3 H]EB (5–3500 pM) in a saturation binding assay. The affinity of [3 H]EB in homogenates from saline-treated rats (K_d) and homogenates from drug-treated rats (K_{dA}) were calculated from the fitted saturation binding curves. These K_d and K_{dA} values of [3 H]EB, and the predetermined K_i value of the treatment drug, were used to calculate the concentration of treatment drug in the binding assay ([I]) in a modified version of the Cheng-Prusoff equation:

$$[I] = \frac{K_i(K_{dA} - K_d)}{K_d}$$

To determine the original concentration of drug in the brain, the value of [I] was multiplied by the appropriate factor, resulting from the necessary dilutions in going from brain to binding assay. The derivation of this modified Cheng-Prusoff equation and the validity of this method are described in more detail elsewhere (Hussmann et al., 2011a; Hussmann and Kellar, 2012).

Measuring the Fraction of nAChRs Occupied by Drug in Rat Brain Ex Vivo. Unwashed cerebral cortical homogenates from chronically treated rats were prepared in ice-cold buffer and then aliquoted in a binding assay with 4.0 nM [3 H]EB. Binding was incubated at 24°C and harvested at varying time points (1–240 min). The association rate of [3 H]EB binding was used to indirectly calculate the dissociation rates of treatment drugs from the receptor binding site. These dissociation rates were plotted and extrapolated to time point 0 min to determine the initial fraction of nAChRs occupied by the treatment drugs. This assay method, which is similar to one published for histamine (Malany et al., 2009), has been described in more detail elsewhere (Tuan et al., 2010; Hussmann et al., 2011a).

Measuring the Concentration of Drug in Rat Serum Ex Vivo. The concentrations of nicotinic drugs in serum were measured by a radioligand binding competition assay. This type of assay, which

is similar to a competitive radioimmunoassay, has been described previously to measure plasma concentrations of dopamine antagonists (Creese and Snyder, 1977). In brief, serum was added to binding assay tubes containing 1.0 nM [3 H]EB and unwashed cerebral cortical homogenates as a source of receptors. After incubation for ~18 h, bound [3 H]EB was collected and measured as described above. The percentage of specific [3 H]EB binding in drug-treated serum was normalized to saline-treated serum. Competition binding curves with known concentrations of treatment drugs were used as a standard curve to calculate the unknown concentration of treatment drug in the serum.

Data Analyses. Radioligand binding data from control and drug-treated groups were analyzed with one-way analysis of variance followed by the Bonferroni's multiple comparison test. Behavioral data were assessed by analysis of variance across the dose levels of drug treatment. Pairwise comparisons were made to control with each dose. Saturation binding data were analyzed by using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). A p value <0.05 was considered the threshold for significance in all cases.

Results

Up-Regulation of nAChRs in Rodent Brain. Chronic administration of nicotine is well known to up-regulate neuronal nAChRs in rodent brain (Marks et al., 1983; Schwartz and Kellar, 1983); specifically, nicotine up-regulates the $\alpha 4\beta 2$ nAChR subtype (Flores et al., 1992; Perry et al., 2007; Mao et al., 2008; Moretti et al., 2010; Marks et al., 2011). Recently, varenicline, a drug used for smoking cessation, was also found to increase these nAChRs in mouse brain (Turner et al., 2010). Saz-A, a partial agonist that potentially desensitizes $\alpha 4\beta 2$ nAChRs, decreases nicotine self-administration in rats (Levin et al., 2010b; Johnson et al., 2012); thus, it is important to know if it, too, up-regulates these receptors during chronic administration. We therefore compared the effects of saz-A, nicotine, and varenicline administered for 2 weeks via osmotic minipumps on the density of nAChRs labeled by a saturating concentration (1.5–2 nM) of [3 H]EB, which provides a good estimate of the total number of heteromeric nAChRs.

As shown in Fig. 1, A and B, chronic administration of nicotine at a dose of 6.0 mg/kg/day significantly increased the density of nAChRs in the cerebral cortex and striatum of male rats; in contrast, neither a low dose (1.6 mg/kg/day) nor a high dose (4.7 mg/kg/day) of saz-A, both of which are behaviorally active (Johnson et al., 2012), increased these receptors. In female rat cortex (Fig. 1C), chronic administration of both nicotine (6.0 mg/kg/day) and varenicline (1.2 mg/kg/day) increased nAChRs, but saz-A at a dose of 4.7 mg/kg/day did not. Full saturation curves with [3 H]EB in rat cortex (Fig. 1D) confirmed that treatment with saz-A did not increase the density of the receptors and also demonstrated that the affinity of [3 H]EB was not altered, indicating that the absence of up-regulation of nAChR binding sites after chronic saz-A was not caused by residual drug in the assay.

In mice chronic administration of both nicotine (18 mg/kg/day) and varenicline (1.8 mg/kg/day) via osmotic minipumps significantly increased nAChRs in the cerebral cortex (the higher dose of nicotine was used to account for the very rapid metabolism of nicotine in the mouse; Marks et al., 1983); but again, saz-A at doses of 1.8 and 3.6 mg/kg/day did not increase the receptors (Fig. 1E).

Administration of drugs via osmotic minipumps over a 2-week period allowed the effects of the nicotinic drugs at

Fig. 1. nAChR up-regulation in rodent brains. Adult male rats (A and B), adult female rats (C), and adult male mice (E) received saline, Nic, Var, or a low or high dose of saz-A for 14 days via osmotic minipumps. The density of cortical or striatal nAChRs was measured in washed membrane homogenates with 1 to 2 nM ^3H JEB. The results are reported as mean $\pm$ S.E.M. from four to nine individual animals in each treatment group. Statistical significance of up-regulation was compared with saline-treated animals. **, $p < 0.01$; ***, $p < 0.001$. D, saturation binding of ^3H JEB in cortex dissected from adult female rats that were chronically treated with saline or saz-A for 14 days via osmotic minipumps. Binding was carried out for 18 h. Data were plotted from three individual animals in each treatment group (mean $\pm$ S.E.M.) and were fit by using a nonlinear saturation curve. The K_d values for ^3H JEB were calculated from the fitted curves and are reported as mean $\pm$ S.E.M. Data were also graphed as a Scatchard plot (Inset) to allow easier comparison of the K_d values.

steady-state concentrations to be assessed. Under those conditions, saz-A did not up-regulate nAChRs. To test this apparent resistance to up-regulation further, rats were treated twice daily for 2 weeks with saz-A at doses of 2.3 or 9.4 mg/kg administered as bolus subcutaneous injections, which produce higher peak concentrations of the drug in brain. Comparisons were made to rats treated twice daily with nicotine (0.7 mg/kg) for 2 weeks or varenicline (1.2 mg/kg) for 10 days. The results of these studies are shown in Fig. 2. Both nicotine and varenicline administered twice daily significantly increased cerebral cortical nAChRs; whereas, again in contrast, saz-A even at doses four to six times behaviorally active doses did not up-regulate these receptors. At the subcutaneous dose of 9.4 mg/kg, sedation and ptosis were obvious, so higher doses were not tested.

Saz-A Decreases Nicotine Self-Administration. The doses of saz-A used here reduce self-administration of nicotine (Levin et al., 2010b; Rezvani et al., 2011; Johnson et al., 2012) and alcohol (Rezvani et al., 2011) and improve performance in tasks of attention (Rezvani et al., 2011) in rats with only modest effects on food intake (Levin et al., 2010b; Rezvani et al., 2010). Moreover, similar doses of saz-A exert positive effects in preclinical tests of anxiolytic (Turner et al., 2010, 2011) and antidepressant activity in mice (Kozikowski et al., 2009; Caldarone et al., 2011). Turner et al. (2010) found in addition that these doses of saz-A did not significantly affect the latency of mice to feed or the average amount of

Fig. 2. nAChR up-regulation after twice-daily subcutaneous injections. A, adult male rats received saline, Nic, or saz-A for 14 days via twice-daily subcutaneous injections. B, in a separate study, adult male rats received saline or Var for 10 days via twice-daily subcutaneous injections. The density of nAChRs was measured with 1 to 2 nM ^3H JEB binding in washed cortical homogenates. The results are expressed as the mean $\pm$ S.E.M. from seven to eight individual animals in each treatment group. Statistical significance of nAChR up-regulation was compared with saline-treated rats. *, $p < 0.05$; ***, $p < 0.001$.

food consumed in home environments. To explore these behavioral effects further, saz-A was examined in a model of nicotine self-administration in which rats were placed in a chamber with two water spouts and received an intravenous infusion of nicotine when they licked one spout but not the

other. Consistent with previous studies that showed saz-A reduces nicotine self-administration triggered by pressing a lever, saz-A reduced nicotine-self-administration triggered by licking the correct spout (Fig. 3).

The Concentration of nAChR Ligands in Rat Brain and Blood Serum after Chronic Administration. A possible reason that saz-A does not up-regulate nAChRs at behaviorally active doses (or even higher doses) could be that the drug might not easily cross the blood-brain barrier and thus it might not reach the concentrations necessary to up-regulate the receptors. To examine this possibility, we measured the concentrations of nicotine, varenicline, and saz-A in brain by using a new method based on the shift in affinity of [3 H]EB measured in saturation binding assays in brain; we then compared these brain concentrations with the blood concentrations measured by competition binding assays. A potential confound of this method would be a difference in the binding affinity of [3 H]EB at control and up-regulated nAChRs. This seems unlikely, however, because we have previously shown that [3 H]EB displays the same binding affinity for control and up-regulated $\alpha 4\beta 2$ nAChRs heterologously expressed in human embryonic kidney 293 cells (Husmann et al., 2011b). Furthermore, the affinities of [3 H]acetylcholine and [3 H]nicotine are not altered at up-regulated nAChRs in brains from rats or mice treated chronically with nicotine (Marks et al., 1983; Schwartz and Kellar, 1983).

Examples of the [3 H]EB binding saturation assays are shown in Fig. 4A. The binding curves measured in unwashed homogenates from rats treated chronically with each of the three drugs were shifted to the right, indicating a lower apparent affinity of [3 H]EB compared with its affinity in homogenates from saline-treated rats. This is seen more clearly in the Scatchard plots (Fig. 4A, Inset). Figure 4A also shows that the maximum [3 H]EB binding values in homogenates from nicotine- and varenicline-treated rats, but not from saz-A-treated rats, were increased compared with the

maximum binding from saline-treated rats, reflecting the up-regulation of nAChRs.

Examples of the measurements of drugs in serum are shown in Fig. 4B. Serum from drug-treated rats added to the [3 H]EB binding assays resulted in decreased binding, reflecting competition by the treatment drug for the receptors. The percentage of decrease in binding was then compared with full competition curves generated with known concentrations of each drug (Fig. 4C).

The calculated steady-state concentrations of nicotine, varenicline, and saz-A in brain and serum after 2 weeks of chronic administration via osmotic minipumps are shown in Table 1, and the concentrations of these drugs measured 10 min after the last of twice-daily subcutaneous injections for 10 to 14 days are shown in Table 2.

After 14 days of chronic administration via osmotic pumps, which presumably allows drugs to reach steady-state concentrations, the nicotine concentration in serum was 0.89 μ M, whereas the concentrations of varenicline and saz-A were 0.45 and 0.40 μ M, respectively. The steady-state concentrations of nicotine, varenicline, and saz-A in brain were calculated to be 2.2, 0.66, and 0.032 μ M, respectively. Thus, nicotine and varenicline were concentrated in brain over serum by approximately 2.5- and 1.5-fold, respectively. In contrast, saz-A reached a concentration in brain that was only 8% its concentration in serum (Table 1).

Similar measurements made after twice-daily subcutaneous injections of these drugs revealed that 10 min after the last injection of nicotine (0.7 mg/kg, 14 days), varenicline (1.2 mg/kg, 10 days), saz-A (2.3 mg/kg, 14 days), or saz-A (9.4 mg/kg, 14 days), the concentrations of the drugs in serum were 0.94, 1.5, 1.8, or 4.3 μ M, respectively (Table 2). The brain concentrations of nicotine and varenicline were 8.9 and 4.2 μ M, whereas the brain concentrations of saz-A after the two doses were 0.19 and 0.42 μ M, respectively. Thus, 10 min after injection nicotine and varenicline were concentrated in brain over serum by 9.5- and 2.8-fold, respectively, whereas, again, saz-A only reached a concentration in brain approximately 10% its concentration in serum (Table 2).

Fraction of nAChRs Occupied by Drug. Although these assays demonstrate that saz-A enters the brain, they do not provide direct information about the degree to which the drug reaches and occupies the nAChRs. Therefore, a radioligand binding assay was adapted to measure the fraction of occupied receptors after chronic treatments. This assay was based on one previously developed to measure the dissociation rates of ligand from its receptor (Tuan et al., 2010). Unwashed cortical homogenates from rats chronically treated via osmotic minipumps were quickly prepared in ice-cold buffer, and aliquots of the homogenate were immediately added to a nAChR binding assay containing a high concentration of [3 H]EB (3–4 nM) and incubated at 24°C. The binding was quenched at various time points by vacuum filtration, and the specific [3 H]EB binding was plotted against the incubation time. Figure 5A shows the association rates of [3 H]EB binding to nAChRs in cortical membranes from rats treated via osmotic minipumps for 14 days with saline, nicotine, varenicline, or saz-A. The results demonstrated that in membranes from saline-treated rats 80 to 90% of the nAChRs were bound by [3 H]EB within 2.5 to 5 min. The kinetics of [3 H]EB association were slower in the tissues from rats treated with nicotinic drugs, because [3 H]EB could

Fig. 3. Sazetidine-A decreases nicotine self-administration in rats. Female rats received nicotine (0.03 mg/kg i.v.) upon licking a spout. Nicotine self-administration sessions lasted 45 min each. Saline or increasing doses of sazetidine-A were administered by subcutaneous injection 10 min before a 45-min nicotine self-administration session. The number of infusions per session was recorded and is plotted as the mean $\pm$ S.E.M. from 10 individual animals in each treatment group. Statistical significance was compared with saline-treated rats (0 mg/kg sazetidine-A). *, $p < 0.025$.

Fig. 4. Measured concentrations of nicotinic drugs. A, cortex was dissected from female rats that received saline, Nic, Var, or saz-A for 14 days via osmotic minipumps. Unwashed cortical homogenates were then aliquoted and incubated for 18 h with 2 to 4000 pM [³H]EB. Specific [³H]EB binding was plotted as the mean $\pm$ S.E.M. from three to five individual animals in each treatment group. The K_d and K_{dA} values of [³H]EB, calculated from the fitted curves, were as follows: saline, 72 ± 5 pM; nicotine, 237 ± 17 pM; varenicline 851 ± 93 pM; and sazetidine-A, 302 ± 28 pM. Inset, saturation data graphed as Scatchard plots. B, serum was also collected from these same rats. Volumes of serum (as reported above bars) and 1.0 nM [³H]EB were added to untreated and unwashed cortical homogenates as a source of receptors. The percentage of specific [³H]EB binding was calculated and then normalized to the percentage of specific [³H]EB binding in the presence of serum from saline-treated rats. The data are plotted as the mean $\pm$ S.E.M. from three to five individual animals in each treatment group. C, [³H]EB competition binding curves

TABLE 1
Concentration of nicotinic drugs in rat brain and serum after administration via osmotic minipumps
Data are expressed as the mean $\pm$ S.E.M. from three to five individual animals in each treatment group. Drugs delivered by osmotic minipumps were administered for a total of 14 days. Rats were sacrificed while still on the pumps.

Drug	Dose	Concentration		
		Drug in Brain	Drug in Serum	Ratio, Brain/Serum
	mg/kg/day		μ M	
Nicotine	6.0	2.2 ± 0.9	0.89 ± 0.21	2.47
Varenicline	1.2	0.66 ± 0.14	0.45 ± 0.13	1.46
Sazetidine-A	4.7	0.032 ± 0.008	0.40 ± 0.05	0.08

TABLE 2
Concentration of nicotinic drugs in rat brain and serum after administration via subcutaneous injections
Data are expressed as the mean $\pm$ SEM from three to five individual animals in each treatment group. Drugs delivered by twice-daily subcutaneous injections were administered for 14 days, with the exception of varenicline, which was administered for a total of 10 days. Rats were sacrificed 10 min after the last injection of the chronic study.

Drug	Dose	Concentration		
		Drug in Brain	Drug in Serum	Ratio, Brain/Serum
	mg/kg		μ M	
Nicotine	0.7	8.9 ± 0.5	0.94 ± 0.4	9.5
Varenicline	1.2	4.22 ± 1.13	1.5 ± 0.3	2.8
Sazetidine-A	2.3	0.19 ± 0.01	1.8 ± 0.2	0.1
Sazetidine-A	9.4	0.42 ± 0.03	4.3 ± 0.4	0.09

not bind receptors until the treatment drugs first dissociated from the receptors. The fitted binding maxima from these association curves indicate that [³H]EB eventually bound all nAChR binding sites and that the values reflected the total number of receptors in the homogenate. Thus, the binding maxima in tissues from rats treated with nicotine or varenicline were $\sim$ 2-fold higher than those from rats treated with saline, whereas the values from rats treated with saz-A were nearly the same as those from the rats treated with saline.

Starting at $\sim$ 2.5 min after adding [³H]EB, when all nAChRs not occupied by treatment drug are bound by the radioligand, the percentage of the receptors occupied by the treatment drug (i.e., not bound by [³H]EB) was calculated and plotted (Fig. 5B). These decreasing functions were best fit to a single first-order dissociation curve. When these curves were extrapolated to time 0 (Fig. 5B, Inset), the original fraction of receptors occupied by nicotine, varenicline, and saz-A were estimated to be approximately 51, 69, and 75%, respectively. We consider these estimates to be conservative because some dissociation of the drugs from the receptors almost surely takes place during the $\sim$ 2.5 min required to prepare the tissue.

A potential complication with using this method to measure receptor fractional occupancy would be if [³H]EB bound up-regulated nAChRs at a different rate compared with untreated nAChRs. However, this is not likely in our case because the K_d value of a drug is a function of its on rate (k_{on}) and off rate (k_{off}), and [³H]EB displays the same K_d value for

with 1.0 nM [³H]EB and increasing concentrations of nicotine, varenicline, or sazetidine-A in untreated and unwashed cortical homogenates. Data were plotted from three individual experiments (mean $\pm$ S.E.M.), and curves were best fit to a one-site model. The K_i values, calculated from the fitted curves, were as follows: nicotine, 15.6 ± 1.0 nM; varenicline, 1.13 ± 0.20 nM; and sazetidine-A, 178 ± 10 pM. These values were used in calculating the concentrations of the drugs in brain and serum.

Fig. 5. Fraction of nAChRs occupied by nicotine, varenicline, or sazetidine-A. Female rats received saline, Nic (6.0 mg/kg/day), Var (1.2 mg/kg/day), or saz-A (4.7 mg/kg/day) for 14 days via osmotic minipumps. Cortex was dissected, homogenized in ice-cold buffer, and then immediately aliquoted in a binding assay with 4.0 nM [³H]EB. Binding was incubated at 24°C and then quenched after increasing incubation times. A, the specific [³H]EB binding was plotted as the mean $\pm$ S.E.M. from five animals in each treatment group. Data were fit with a nonlinear regression association curve. B, the percentage of nAChRs not bound by [³H]EB (i.e., occupied by treatment drug) was plotted against binding incubation time. A nonlinear dissociation binding curve was then fit to the data. Inset, the initial percentage of nAChRs occupied by treatment drug was estimated at time 0 min from the extrapolated curve.

both up-regulated and untreated nAChRs (Husmann et al., 2011b). Moreover, the key finding in this experiment was that nAChRs from saz-A-treated rats, which were not up-regulated, were indeed occupied by the drug.

Up-Regulation of nAChRs in Primary Cortical Neurons. Next, up-regulation of nAChRs by nicotine, varenicline, and saz-A was compared in rat primary cortical cultures. Measurements in primary cortical cultures allowed us to directly expose a monolayer of neurons to saz-A to determine whether there are concentrations that, in fact, up-regulate nAChRs. The primary neurons were treated with 10 μ M nicotine, 1 μ M varenicline, or increasing concentrations of saz-A for 10 days to reflect chronic treatment in vivo. After these chronic treatments, membrane homogenates were prepared, and the density of nAChRs was measured with [¹²⁵I]EB. As shown in Fig. 6, pharmacologically relevant concentrations of nicotine and varenicline up-regulated nAChRs by 2.5- to 3-fold, similar to the up-regulation observed in brain after chronic administration of these drugs. We were surprised to find that saz-A also up-regulated nAChRs in primary cortical neurons. The up-regulation by saz-A was concentration-dependent and resulted in an in-

Fig. 6. Nicotine-, varenicline-, and sazetidine-A-induced nAChR up-regulation in primary neurons in culture. Cortical neurons from embryonic day 18 and 19 rats were grown in culture. Cells were treated for 10 days with Nic (10 μ M), Var (1 μ M), or increasing concentrations of saz-A. The density of nAChRs was measured in washed membrane homogenates with 0.5 to 1.0 nM [¹²⁵I]EB. These densities are plotted as the mean $\pm$ S.E.M. from three to six individual experiments. *, $p < 0.05$; **, $p < 0.01$; ns, not significant.

crease in nAChRs that was similar to the increases produced by nicotine and varenicline. Moreover, saz-A up-regulated receptors even at or below the steady-state concentrations measured in the rat brain (30 nM).

Discussion

Chronic administration of nicotine and other nicotinic ligands increases the density of brain nAChRs across several species including rats, mice, and nonhuman primates (Marks et al., 1983; Schwartz and Kellar, 1983; Rowell and Wonnacott, 1990; Kassiou et al., 2001; Turner et al., 2011). A similar increase was found in autopsied brains from humans who smoked (Benwell et al., 1988; Breese et al., 1997; Perry et al., 1999), as well as in vivo by imaging methods (Staley et al., 2006; Wüllner et al., 2008). The predominant nAChR increased by nicotine in rodents is the $\alpha 4 \beta 2$ subtype (Flores et al., 1992; Mao et al., 2008; Moretti et al., 2010; Marks et al., 2011), and based on the ligand used to measure the receptors, that is probably also the case in humans. Moreover, in cultured cells that express nAChRs either endogenously or heterologously these receptors are increased after incubation with those agonists, as well as with a wide variety of other nicotinic ligands (Gopalakrishnan et al., 1996, 1997; Whiteaker et al., 1998; Dávila-García et al., 1999; Xiao and Kellar, 2004; Gahring et al., 2010; Husmann et al., 2011b).

Saz-A has an affinity for $\alpha 4 \beta 2$ nAChRs approximately 25 times higher than nicotine's (Xiao et al., 2006) and would thus be expected to up-regulate these receptors at least as well as nicotine in vivo. And yet neither continuous infusion of saz-A nor bolus injections twice daily for 2 weeks increased the density of nAChRs in rat or mouse brain. In contrast, both nicotine and varenicline reliably increased nAChRs in each of these paradigms. The doses of saz-A used here are behaviorally active, in that they decrease nicotine and alcohol self-administration (Levin et al., 2010b; Rezvani et al., 2010; Johnson et al., 2012; Fig. 3) and increase performance on tasks of attention (Rezvani et al., 2011). Furthermore, in preclinical tests these doses of saz-A produce behaviors that are consistent with potential antidepressant and/or anxiolytic effects (Kozikowski et al., 2009; Turner et al., 2010, 2011; Caldarone et al., 2011). Thus it seems that saz-A acts in the

brain; yet even at doses two to six times these behaviorally active doses saz-A did not increase nAChR density.

To assess how well saz-A enters the brain, we developed an assay that allowed us to measure the brain concentrations of saz-A, nicotine, and varenicline. We then compared the brain concentrations of these drugs with their concentrations in blood during chronic infusion or immediately after chronic bolus injections of the drugs. These studies indicated that during chronic infusion of saz-A for 2 weeks its steady-state concentration in brain was less than 10% of that in blood. Likewise, after 2 weeks of twice-daily injections of saz-A, its brain concentration 10 min after the last injection was also approximately only 10% that of blood, which agrees with a recent study that measured brain and blood concentrations of saz-A in mice after a single intraperitoneal injection (Caldarone et al., 2011). This is in marked contrast to both nicotine and varenicline, which were highly concentrated in brain during both methods of chronic administration; moreover, both the concentrations and the brain-to-blood ratios of nicotine and varenicline were similar to previously reported values (Ghosheh et al., 1999; Doura et al., 2008; Rollema et al., 2010).

Despite its relatively low penetration into brain, saz-A reached a steady-state concentration in brain of ~ 30 nM during chronic infusion and 190 to 400 nM after bolus injections. The affinity of saz-A for $\alpha 4\beta 2$ nAChRs is ~ 200 pM, thus even the lower concentration of saz-A reached during chronic infusion should be sufficient to occupy virtually all of these nAChRs in brain. Moreover, measurements of receptor occupancy indicated that saz-A does, in fact, occupy $\alpha 4\beta 2$ nAChRs at least as well as nicotine and varenicline. Thus, because saz-A enters the brain and occupies $\alpha 4\beta 2$ nAChRs, we considered the possibility that its occupancy of the receptors was in some way different from that of nicotine and varenicline and this difference precluded up-regulation of the receptor. This does not seem to be the case, however, because saz-A up-regulated nAChRs in primary neurons even at concentrations lower than those we found in the brains from rats treated in vivo.

To explain the dichotomy between the behavioral effects of saz-A and its inability to up-regulate $\alpha 4\beta 2$ nAChRs, we propose that saz-A can enter the brain well enough to act at and desensitize cell surface nAChRs and thus affect behaviors, but it does not cross membranes well enough to traverse the plasma membrane to enter neurons and then negotiate the additional membranes to enter sites within the endoplasmic reticulum, where current evidence indicates that nicotine and presumably varenicline act to increase nAChR density by stimulating assembly of subunits into mature receptors (Sallette et al., 2004, 2005; Kuryatov et al., 2005; Lomazzo et al., 2011; Srinivasan et al., 2011). In contrast to neurons surrounded by glial cells in adult brain in vivo, saz-A may up-regulate nAChRs in cultured embryonic neurons because a monolayer of neuronal cells may contain fewer barriers, such as neuropil, and other structural proteins that could prevent saz-A from entering the cell and then the endoplasmic reticulum. Alternatively, because our assay does not differentiate between free drug and that bound to proteins or lipids, it is possible that the free saz-A concentration is high enough to desensitize brain nAChRs, but not high enough to trigger up-regulation in the endoplasmic reticulum.

Nicotine potently desensitizes nAChRs (Sharp and Beyer, 1986; Hulihan-Giblin et al., 1990; Grady et al., 1994; Marks et al., 1994; Lester and Dani, 1995; Pidoplichko et al., 1997; for reviews, see Meyer et al., 2001; Picciotto et al., 2008; Buccafusco et al., 2009); in fact, it is approximately eight times more potent to desensitize these receptors than to activate them in vivo (Hulihan-Giblin et al., 1990) and up to 45 times more potent in vitro (Grady et al., 1994; Marks et al., 1994). Thus, after chronic administration of nicotine receptor-mediated responses are sometimes found to be initially diminished. Subsequently, however, increased responses reflecting the increased receptors are usually found (Rowell and Wonnacott, 1990; Benwell et al., 1995; Gopalakrishnan et al., 1996, 1997; Buisson and Bertrand, 2001; Avila et al., 2003), which probably reflects the resensitization of the receptors once the nicotine concentrations decrease sufficiently. Thus far, however, it has not been possible to measure nAChR up-regulation by electrophysiology methods in brain slices from chronically treated rats, probably because nAChRs are not expressed on every neuron in high enough density to allow reliable electrophysiology measurements in brain slices.

Up-regulation and desensitization are powerful influences on brain nAChR responses and the efficacy of drugs at these receptors. Thus, the dynamics between these two effects of nicotine may play a crucial role in or even underlie nicotine addiction and/or the withdrawal responses that occur during abstinence. For example, in Fig. 7 we propose a model, which is similar to one previously proposed by Dani and Heinemann (1996), that can explain some of the salient features of nicotine withdrawal that sustain the drive to repeatedly self-administer nicotine. In this model, the increased nAChRs in a smoker's brain are relatively silent during periods of low neural activity (e.g., sleep), but when the relevant brain pathways are stimulated by acetylcholine signaling (e.g., in the reticular activating system upon waking), the increased receptors convey excess levels of neural activity, which triggers anxiety pathways that lead to craving. The addicted smoker learns over time that this excess neural activity in the form of craving can quickly be decreased to a more comfortable level by smoking a cigarette. The rapid bolus of nicotine desensitizes the nAChRs, resulting in decreased levels of cholinergic signaling and decreased anxiety, i.e., the craving is satisfied. However, as the nicotine concentration in the brain falls, the nAChRs become resensitized, and again the excess level of activities mediated by the increased number of nAChRs provides the signal for the next cigarette. This establishes a continuous cycle at the cellular level that is reflected in the behavior we call addiction.

According to this model, replacement therapies either with nicotine or the more potent varenicline can diminish to some extent the excess activity mediated by the increased nAChRs, which could account for their modest success in helping people to stop smoking. But if nicotine and varenicline replacement therapies sustain the increased nAChRs in human brain during smoking cessation treatment, as they do in rat and mouse brain, when the replacement therapies are stopped, up-regulated nAChRs, a critical component of the addiction cycle, are still in place. This could partially explain why the success rates for long-term smoking cessation with these replacement therapies are not very high (Jorenby et al., 2006; Gonzales et al., 2010). Finding new strategies for smok-

Fig. 7. A model to explain nicotine withdrawal that contributes to addiction. 1) The “quiet” $\alpha 4 \beta 2^*$ nAChRs (white circles) represent inactive receptors (e.g., during sleep). 2) The acetylcholine-activated nAChRs (red circles) represent the active receptors. Upon awakening, the increased numbers of activated nAChRs in the brains of smokers trigger circuits that lead to arousal and anxiety, which are perceived by the smoker as unpleasant. 3) Receptor desensitization by nicotine. The smoker learns over time that smoking a cigarette reduces those unpleasant feelings. 4) Resensitization of nAChRs: as the nicotine levels fall, nicotine-induced desensitization from that first cigarette wanes and the receptors become active again. When a certain threshold of receptor activity is reached, the increased receptor signaling is the cue for the smoker to smoke another cigarette. Thus the cycle continues. We call this behavior a manifestation of nicotine addiction.

ing cessation that increase long-term nicotine abstinence remains a high priority, because there are still more than 1 billion smokers in the world; and in the United States, for example, a lifelong smoker loses approximately a decade of life [Centers for Disease Control and Prevention (CDC), 2008]. The studies here indicate that drugs such as saz-A that can desensitize nAChRs without increasing their density might provide a means to increase smoking cessation rates during both the short term and, more importantly, over the long term.

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Authorship Contributions

Participated in research design: Hussmann, Turner, Lomazzo, Venkatesh, Cousins, Xiao, Yasuda, Wolfe, Perry, Rezvani, Levin, Blendy, and Kellar.

Conducted experiments: Hussmann, Turner, Lomazzo, Venkatesh, and Cousins.

Performed data analysis: Hussmann, Turner, Lomazzo, Venkatesh, Cousins, Wolfe, Perry, Rezvani, Levin, Blendy, and Kellar.

Wrote or contributed to the writing of the manuscript: Hussmann, Turner, Perry, Rezvani, Levin, Blendy, and Kellar.

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