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Selective $\alpha_4\beta_2$ Nicotinic Acetylcholine Receptor Agonists Target Epigenetic Mechanisms in Cortical GABAergic Neurons

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Nicotine improves cognitive performance and attention in both experimental animals and in human subjects, including patients affected by neuropsychiatric disorders. However, the specific molecular mechanisms underlying nicotine-induced behavioral changes remain unclear. We have recently shown in mice that repeated injections of nicotine, which achieve plasma concentrations comparable to those reported in high cigarette smokers, result in an epigenetically induced increase of glutamic acid decarboxylase 67 (GAD_{67}) expression. Here we explored the impact of synthetic $\alpha_4\beta_2$ and α_7 nAChR agonists on GABAergic epigenetic parameters. Varenicline (VAR), a high-affinity partial agonist at $\alpha_4\beta_2$ and a lower affinity full agonist at α_7 neuronal nAChR, injected in doses of 1–5 mg/kg/s.c. twice daily for 5 days, elicited a 30–40% decrease of cortical DNA methyltransferase (DNMT)1 mRNA and an increased expression of GAD_{67} mRNA and protein. This upregulation of GAD_{67} was abolished by the nAChR antagonist mecamylamine. Furthermore, the level of $MeCP_2$ binding to GAD_{67} promoters was significantly reduced following VAR administration. This effect was abolished when VAR was administered with mecamylamine. Similar effects on cortical DNMT1 and GAD_{67} expression were obtained after administration of A–85380, an agonist that binds to $\alpha_4\beta_2$ but has negligible affinity for $\alpha_3\beta_4$ or α_7 subtypes containing nAChR. In contrast, PNU–282987, an agonist of the homomeric α_7 nAChR, failed to decrease cortical DNMT1 mRNA or to induce GAD_{67} expression. The present study suggests that the $\alpha_4\beta_2$ nAChR agonists may be better suited to control the epigenetic alterations of GABAergic neurons in schizophrenia than the α_7 nAChR agonists.

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INTRODUCTION

Protracted and repeated administration of nicotine to laboratory animals, and the inhalation of large amounts of nicotine from tobacco smoking in healthy human subjects and schizophrenia (SZ) patients results in an increased attention span and in an improvement of sensory/cognitive functions (Freedman *et al*, 2008; Evans and Drobes, 2009; Hasselmo and Sarter, 2011). However, the precise molecular correlates of the behavioral actions of nAChR stimulation have not been extensively studied.

It has now been established that when the postmortem brain of SZ patients is compared with that of non-psychiatric subjects, a decrease of high- ($\alpha_4\beta_2$) and low (α_7)-affinity nAChR subtypes is detected in the hippocampus and cortex (Freedman *et al*, 1995; Olincy *et al*, 1997; Breese *et al*, 2000) along with GABAergic neuropathologies in the same brain areas (Akbarian *et al*, 1995; Guidotti *et al*, 2000, 2005; Lewis *et al*, 2005; Benes *et al*, 2007). These neuropathologies are characterized by the decreased expression of glutamic acid decarboxylase 67 (GAD_{67}) and the increased expression of DNA methyltransferases (DNMTs) (Ruzicka *et al*, 2007; Veldic *et al*, 2005; Zhubi *et al*, 2009; Guidotti *et al*, 2010). DNMTs are a family of enzymes that catalyze the methylation of the carbon 5′ of cytosines embedded in cytosine phosphodiester guanine (CpG) islands of many gene promoters (Van Emburgh and Robertson, 2008). Evidence suggests that GABAergic promoter hypermethylation, mediated by the overexpression of DNMTs, may be an underlying pathophysiological mechanism leading to a downregulation of GABAergic transmission in SZ patients (Guidotti *et al*, 2010). Downregulation of GABAergic transmission may in turn lead to

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an impairment of the cortical gamma oscillations related to sensory/cognitive deficits in these patients (Lewis and Gonzalez-Burgos, 2008).

We have recently shown in mice that repeated injections of nicotine, which achieve plasma concentrations comparable to those reported by heavy cigarette smokers, result in: (1) a downregulation of DNMT expression, (2) a decrease of GAD_{67} promoter methylation, and (3) an increased expression of GAD_{67} in cortical or hippocampal but not in striatal GABAergic neurons (Satta *et al*, 2008).

As these effects of nicotine are blocked by mecamylamine but not by hexamethonium, the data support the notion that nAChR ligands active at central $\alpha_4\beta_2$ and α_7 nAChR may represent an effective pharmacological tool to correct the epigenetic GABAergic neuropathology present in SZ patients.

Nicotine, acting on a variety of central and peripheral nAChRs, also elicits unwanted side effects such as cardiovascular toxicity, gastrointestinal toxicity, tremor, and addiction. Furthermore, if nicotine is administered by smoking, serious comorbid health conditions are likely to develop. Hence, the potential of $\alpha_4\beta_2$ and/or α_7 nAChR full or partial agonists for increasing attention and cognitive performance (Potter *et al*, 1999; Wilens *et al*, 2006; Wilens and Decker, 2007; Dunbar *et al*, 2007; Howe *et al*, 2010; Olincy *et al*, 2006; Freedman *et al*, 2008) with minimal adverse side effects may represent an innovative and efficient way to improve cognitive deficits in patients with neuropsychiatric disorders (Freedman *et al*, 2008; Smith *et al*, 2009; Hasselmo and Sarter, 2011).

To test whether the epigenetic GABAergic events induced by the administration of high and repeated doses of nicotine can be replicated with specific $\alpha_4\beta_2$ and/or α_7 nAChR full or partial agonists endowed with a lower toxic liability than nicotine, we compared the action of varenicline (VAR) (a high-affinity partial agonist at $\alpha_4\beta_2$ and a lower affinity full agonist at α_7 neuronal nAChR) with that of A–85380 (a high-affinity full agonist at $\alpha_4\beta_2$ nAChR) and PNU–282987 (a high-affinity full agonist at α_7 nAChR) on GABAergic epigenetic parameters (Rollema *et al*, 2007; Mihalak *et al*, 2006; Smith *et al*, 2007; Bodnar *et al*, 2005).

VAR has been recently approved for smoking cessation treatment (Faessel *et al*, 2010). Unlike nicotine, VAR fails to increase sympathetic tone, such as the increase of respiratory rate, locomotor activity, and tremor, but at the same time it reduces the level of smoking addiction in patients, and blocks nicotine intake in rats with access to nicotine self-administration (Rollema *et al*, 2007; George *et al*, 2010).

Post-marketing reports of neuropsychiatric symptoms, including agitation, depressed mood, and suicidal ideation in subjects attempting to quit smoking using VAR have led to the addition of a 'boxed warning' to the product labeling (Faessel *et al*, 2010; Gunnell *et al*, 2009), nevertheless in a large clinical study (Gunnell *et al*, 2009), there was no evidence that VAR was associated with an increased risk of self-harm or suicidal ideation. Hence, despite early negative reports, VAR may represent an innovative and relatively safe way to control cognitive dysfunction, anxiety, and mood disorders through nAChR stimulation in psychotic patients (Smith *et al*, 2009).

We recently reported that 8 weeks of VAR treatment improves cognitive function while decreasing DNMT1 mRNA expression in the peripheral blood lymphocytes of SZ patients (Smith *et al*, 2010).

This has provided a rationale for testing whether VAR or the more selective $\alpha4\beta2$ (ie, A–85380) or α_7 (ie, PNU–282987) nAChR agonists with reduced nicotine-like side-effect liability, elicit an epigenetic-GABAergic gene upregulation in cortical or in hippocampal GABAergic neurons.

MATERIALS AND METHODS

Animals and Drug Administration

Male Swiss albino mice (Harlan Breeders), 60–80 days old and weighing 20–25 g were used in this study. The following drugs were used: mecamylamine hydrochloride and 3-(2(S)-azetidinyl methoxy)pyridine dihydrochloride (A–85380), from Sigma–Aldrich. *N*-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride (PNU–282987) and VAR were a generous gift from Dr Mihaly Hajos (Department of Neuroscience, Pfizer Global Research and Development, Groton, CT). All drugs were dissolved in a volume of saline solution corresponding to 0.05 ml/10 g body weight and were injected s.c. or i.p. as indicated.

We studied the effect of VAR, A–85830, and PNU–289987 in Swiss albino mice because in a previous study (Satta *et al*, 2008), we observed that nicotine injected into these mice in doses ranging from 0.75 to 3.5 mg/kg elicited a significant dose-related decrease of DNMT1 and an increase of GAD_{67} expression in the frontal cortex (FC) and hippocampus if injected protractedly and repeatedly for at least 4 days. Therefore, in this study, to mimic the central effects of nicotine, selective nAChR agonists and antagonists were administered for 5 days. Unless otherwise indicated, five mice per group were used for the analyses.

Frontal Cortex Dissection

To collect FC samples free of striatal tissue, brains were sectioned coronally 2 mm anterior to the bregma. The tissue anterior to the section was frozen immediately and kept at $-80^{\circ}C$ until assays were carried out.

Fear-Conditioning Apparatus

The fear-conditioning apparatus consisted of a transparent acrylic chamber measuring 25 cm wide, 18 cm high and 21 cm deep (San Diego Instruments, San Diego, CA). The cage floor was composed of stainless steel rods connected to a shock generator (San Diego Instruments). The chamber was surrounded by a frame with 16 infrared photo beams. A computer controlled the delivery of foot shocks (unconditioned stimulus (US)) and auditory stimuli (CS), and recorded beam interruptions and latencies to beam interruptions (freezing time). A small fan was located on the top wall of the enclosure. A speaker placed on a sidewall of the conditioning chamber delivered the CS.

Training Test

Animals were placed into a training chamber (San Diego Instruments) and allowed to explore it for 2 min. After this time, they received an acoustic CS (30 s, 85 dB with noise) followed by a US (electric shock 2 s, 0.5 mA) three times every 2 min. After the last shock, animals were allowed to

explore the context for an additional minute before removal from the training chamber.

Contextual Test

After 24 h, the animals were placed in the contextual cage and freezing behavior was measured for 5 min (Freeze Monitor System, San Diego Instrument) without foot shock presentation.

Freezing behavior was measured for 5 min without foot shock presentation.

Cue Test

After 24 h, mice were tested for freezing responses to the CS. For this purpose, the conditioning chamber was modified (by locating a smaller Plexiglas box, measuring 18 cm wide, 9 cm high, and 11 cm deep in the contextual chamber and adding a few drops of lemon scent). Mice were allowed to explore the new environment for 2 min. After this time, an acoustic CS (30 s, 85 dB with noise) was presented and the expression of freezing behavior was measured during the following 5 min.

Freezing was defined by the absence of any movement except for those related to respiration while the animal was in a stereotypical crouching posture (for further details see Pibiri *et al*, 2008).

Open Field Test

To assess the general behavior and locomotor activity level, each mouse was placed in an open field apparatus lined with photobeams ($40 \times 40 \times 30$ cm; Accuscan Instruments, Columbus, OH) and partitioned into quadrants. We had three fields and six available quadrants allowing us to run six animals simultaneously. Horizontal and vertical activity was recorded over a 10-min period.

RNA Isolation and Quantitative RT-PCR Analysis

Total RNA was extracted by cesium chloride density gradient centrifugation as described (Ruzicka *et al*, 2007). DNMT1 and NSE mRNA content were measured in each sample by competitive RT-PCR. The following amplification primers were used: DNMT1, forward, base pairs 4231–4254; reverse, base pairs 4667–4644; GenBank accession no. X14805.1 and NSE, forward, base pairs 382–405; reverse, base pairs 769–792; GenBank accession no. M22349.1. This technique is based on the simultaneous amplification of the target mRNA and a specific internal standard having the same sequence as the target template except for a deletion of 58 bp for DNMT1 (between 4461 and 4518 bp) and 85 bp for NSE (between 552 and 636 bp) (Ruzicka *et al*, 2007; Auta *et al*, 2007).

Western Blot Analysis

Extraction of $GAD_{65/67}$. Briefly, proteins were extracted directly from the brain tissue in Laemmli buffer (100 µl/10 mg tissue) and were separated by 4–12%. SDS/PAGE (Invitrogen), blotted onto nitrocellulose membranes (Invitrogen) and developed overnight at 4°C with anti-$GAD_{65/67}$ rabbit polyclonal antibody diluted to 1 : 2000 (Chemicon). Membranes were then washed and re-blotted with a β-actin mAb (1 : 5000; Sigma-Aldrich) for 2 h (Tremolizzo *et al*, 2005).

Changes in $GAD_{65/67}$ expression induced by various treatments were expressed as the ratio of the OD of $GAD_{65/67}$ *vs* the OD of β-actin. Approximately, 3 to 5 serial dilutions were run for every sample to identify the linear range for protein quantification.

$MeCP_2$ Chip Assay

To indirectly assess the cytosine methylation level of GAD_{67} promoters, we used the $MeCP_2$ ChIP assay method as previously described (Dong *et al*, 2005, 2008).

Approximately 10 mg of FC tissue were used for this procedure. Tissue slices (0.3×0.3 mm) were incubated at 37°C for 15 min with 400 µl of PBS containing 1% formaldehyde supplemented with protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml pepstatin) (Upstate Cell Signaling Solutions, Lake Placid, NY) to crosslink $MeCP_2$ with the target-methylated genomic DNAs. After being washed six times with cold PBS containing protease inhibitors, slices were homogenized in 200–400 µl of SDS lysis buffer (supplied by ChIP kit; Upstate Cell Signaling Solutions) with minor modifications as previously described (Dong *et al*, 2005).

Genomic DNA was extracted from the mouse FC and sonicated to produce fragment sizes of 200–600 bp. An aliquot of the solution (50 µl) was removed and stored at −20°C to be used as input. The remaining solution was incubated overnight at 4°C with $MeCP_2$ antibody (Upstate Cell Signaling Solutions). The DNA–antibody complex was then added to 50 µl of protein A agarose beads (Invitrogen) and incubated on a rotating platform for 2 h at 4°C. The resulting DNA–antibody–bead complex was isolated after reverse crosslink and the DNA was released by proteinase K digestion. After phenol–chloroform extraction and ethanol precipitation, the DNA pellet was resuspended in 20 µl of diethylpyrocarbonate water.

A CpG-rich GAD_{67} promoter fragment (base pairs –760 to −311) (Dong *et al*, 2008) was measured by quantitative PCR analysis. The following amplification primers were used: forward, base pairs 760–737; reverse, base pairs 311–334 (Dong *et al*, 2008). The internal standard used for the quantification was an oligonucleotide with the same sequence as the target except for a deletion of 101 bases (between 390 and 490 bp) (Dong *et al*, 2008). The level of methylation of the GAD_{67} promoter is expressed as percentage of the input DNA that is immunoprecipitated by the $MeCP_2$ antibody. In Figure 1, a representative example of a competitive PCR for the quantitative measurement of GAD_{67} promoter in a sample immunoprecipitated with an antibody directed against $MeCP_2$ and the corresponding input is depicted.

Statistical Analyses

A one-way repeated ANOVA was used to analyze the treatment effects. *Post-hoc* analyses of variance were determined by the Student–Newman–Keuls test with multiple range comparisons. Data are expressed as mean ± SE.

RESULTS

Varenicline Reproduces the Action of Nicotine on Cortical DNMT and GAD_{67} Expression

In the doses used in this study (1–5 mg/kg, i.p.), VAR fails to produce the tremor, piloerection or loss of motor

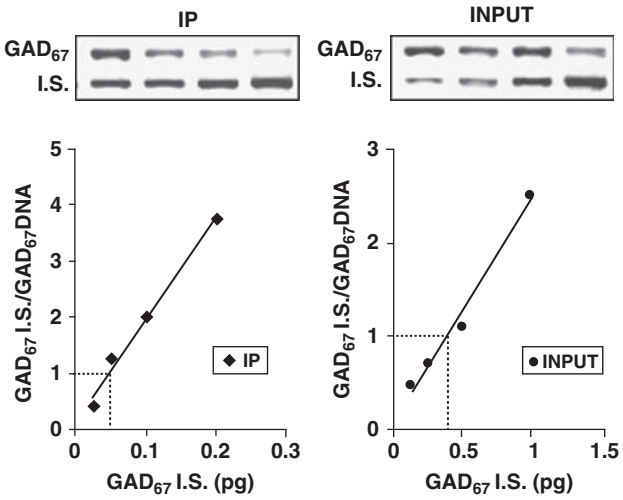

Figure 1 Example of competitive PCR analysis for glutamic acid decarboxylase 67 (GAD_{67}) promoter in a frontal cortex (FC) sample immunoprecipitated with an antibody directed against $MeCP_2$ (IP) and in the corresponding INPUT. For each sample, four solutions were prepared, each containing the same amount of DNA and increasing amounts of GAD_{67} internal standard (IS). The upper panels show ethidium bromide gel electrophoresis for the IP and INPUT samples. The two bands in the upper panel correspond to the amplification products of GAD_{67} IS and GAD_{67} DNA promoter fragments in each solution. The ratio between the optical density of IS and GAD_{67} promoters is plotted (lower panels) *vs* the IS concentrations. At the equivalence point (ratio = 1), the amount of GAD_{67} promoter fragment in the sample is equal to the amount of GAD_{67} IS. The ratio of IP/INPUT represents the percentage of GAD_{67} promoter immunoprecipitated by the $MeCP_2$ antibody. For details of the method, see Dong *et al* (2007).

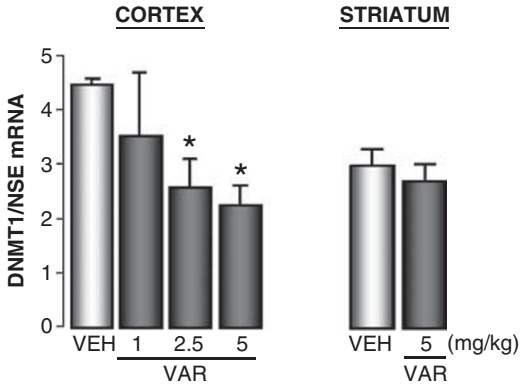

Figure 2 Varenicline (VAR) reduces DNA methyltransferase (DNMT)1 mRNA expression in the FC but not in the striatum. VAR was injected in mice i.p. twice a day for 5 days. FC samples were collected 2 h after the last VAR injection. Data are expressed as fmol DNMT1 mRNA/0.1 pmol NSE mRNA. Overall one-way ANOVA ($F_{3,14} = 5.0$) provided a $p < 0.01$; $^*p < 0.01$ for Student–Newman–Keuls multiple comparison between vehicle (VEH)- and VAR-treated groups.

coordination that are observed (Satta *et al*, 2008) after 0.75–3.5 mg/kg of nicotine administration. Furthermore, VAR (5 mg/kg i.p., 2 h before testing) fails to alter locomotor activity. The distance traveled was 2010 ± 228 counts/10 min in vehicle (VEH)-treated mice and 1879 ± 238 counts/10 min in VAR-treated mice; $n = 10$; nonsignificant. A single injection of VAR (5 mg/kg i.p., 2 h or 24 h before testing) fails to change cortical DNMT1 mRNA levels. However, like nicotine, repeated VAR injections (1–5 mg/kg i.p., twice daily for 5 days) elicit a significant 30–40% decrease of cortical DNMT1 mRNA (Figure 2), but fail to significantly decrease DNMT1 mRNA expression in the striatum (Figure 2).

In addition to DNMT1, DNMT3a has been identified in the mouse brain (Satta *et al*, 2008). Unlike DNMT1, FC levels of DNMT3a mRNA failed to change following VAR administration (fmol DNMT1/0.1 pmol NSE mRNA: 4.6 ± 0.5 in VEH and 4.8 ± 0.35 in VAR (5 mg/kg s.c. twice daily for 5 days) treated mice; $n = 5$). DNMT3b was not expressed in significant amounts in the FC of adult mice.

In the same mice in which VAR induced a decrease of cortical DNMT1 mRNA, the expression of GAD_{67} mRNA (fmol GAD_{67}/0.1 pmol NSE mRNA: 2.4 ± 0.38 in VEH- and 4.1 ± 0.48 in VAR-treated mice) and protein (Figure 3) was significantly increased. The upregulation of GAD_{67} induced by VAR was abolished by the nAChR antagonist mecamylamine (2 mg/kg twice a day just before VAR administration) (Figure 3).

In the same sample, we failed to observe a significant increase of GAD_{65} protein expression; the GAD_{65} β-actin OD ratio expressed as a percentage of the control is 112 ± 15; $n = 10$; nonsignificant.

A typical example of Western blot of $GAD_{65/67}$ and β-actin after 4–12% SDS-PAGE from VEH- and VAR-treated mice is shown in the insert of Figure 3.

Varenicline Reduces the Binding of $MeCP_2$ to a CpG-Rich GAD_{67} Promoter Region

To test the hypothesis that the VAR-induced increase of GAD_{67} expression is a consequence of the reduction of DNMT1 and the reduction of GAD_{67} promoter methylation, we studied the effect of VAR on the methylation status of a specific GAD_{67} promoter region enriched with CpG dinucleotides (see Materials and Methods section). In mouse FC extracts, we quantified the fraction of the GAD_{67} promoter, immunoprecipitated by a specific $MeCP_2$ antibody (Dong *et al*, 2005, 2007, 2008), using competitive PCR with a genomic internal standard, as detailed in Figure 1. We showed that protracted VAR treatment (5 mg/kg i.p., twice daily for 5 days) significantly reduces the level of $MeCP_2$ bound to the GAD_{67} promoter. This effect was abolished when VAR was administered with the nAChR antagonist mecamylamine (Figure 4).

A–85380 Reproduces the Action of Varenicline on Cortical DNMT And GAD_{67} Expression

As mentioned, in small concentrations (2 μM), VAR is a partial agonist at $\alpha_4\beta_2$ and in higher concentrations (18 μM), is a full agonist at α_7 nAChRs (Mihalak *et al*, 2006). Hence, the experiments with high doses of VAR do not allow the identification of the nAChR subtype that is responsible for the downregulation of cortical DNMT1 or the increase of cortical GAD_{67} expression.

To establish whether $\alpha_4\beta_2$ nAChR stimulation is responsible for the epigenetic modifications in mice caused by nicotine and VAR, we administered A–85380, an agonist

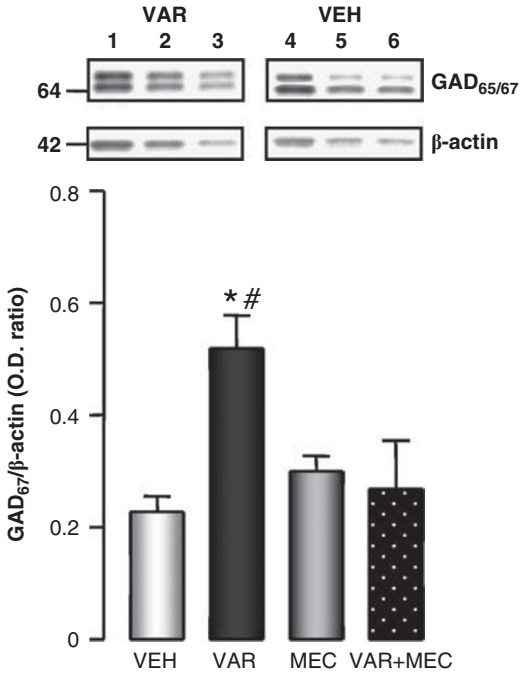

Figure 3 Varenicline (VAR) increases glutamic acid decarboxylase 67 (GAD_{67}) protein expression in mouse frontal cortex (FC). The effect of VAR (5 mg/kg, i.p. twice a day for 5 days) was blocked by co-administration of mecamylamine (MEC) (2 mg/kg, i.p.). A one-way ANOVA for GAD_{67} in vehicle (VEH), VAR MEC, and VAR + MEC ($F_{3,16} = 4.8$) provided a $p < 0.01$. $^{*}p < 0.01$ for Student–Newman–Keuls multiple comparison between VEH- and VAR-treated groups. $^{\#}p < 0.01$ for Student–Newman–Keuls multiple comparison between VAR- and VAR + MEC-treated groups. In the insert is a typical Western immunoblot of $GAD_{65/67}$ and β-actin after 4–12% SDS-PAGE. Comparison between FC extracts of VAR- (lanes 1–3 serial dilution of the same sample) and VEH- (lanes 4–6 of the same sample) treated mice.

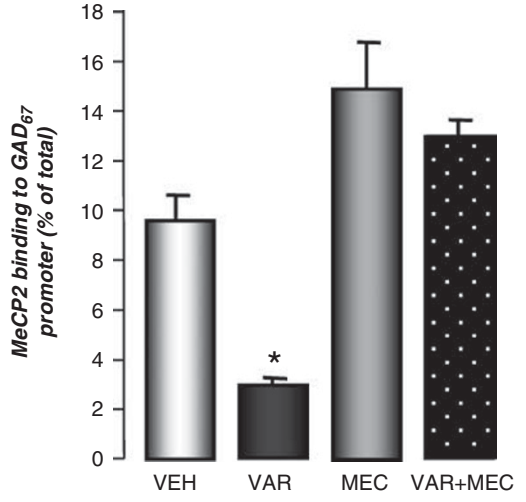

Figure 4 Varenicline (VAR) reduces $MeCP_2$ binding to GAD_{67} promoters in the mouse frontal cortex (FC). The effect of VAR (5 mg/kg, i.p. twice a day for 5 days) was blocked by the co-administration of mecamylamine (MEC) (2 mg/kg, i.p.). A one-way ANOVA for GAD_{67} promoter methylation in vehicle (VEH), VAR, MEC, and VAR + MEC ($F_{3,8} = 5.6$) provided a $p < 0.01$. $^{*}p < 0.01$ for Student–Newman–Keuls multiple comparison between VEH- and VAR- treated mice.

Table 1 A–85380, A Selective $\alpha_4\beta_2$ nAChR Agonist, Reduces DNMT1 mRNA Expression In The Frontal Cortex But Not In The Striatum

Drug	Dose (mg/kg)	DNMT1 mRNA[a]	
		FC	Striatum
VEH	—	3.8 ± 0.3	3.0 ± 0.3
A–85380	0.5	2.4 ± 0.1*	2.5 ± 0.1
	1.0	2.7 ± 0.2*	2.6 ± 0.1
	2.5	2.5 ± 0.1*	2.2 ± 0.2
PNU–282987	1.0	3.4 ± 0.2	2.7 ± 0.5
	2.5	3.8 ± 0.4	3.1 ± 0.5
	5.0	3.9 ± 0.4	3.3 ± 0.5

Mice received s.c. injections of A–85380 or PNU–282987 for 5 days, twice daily and were killed 2 h after the last injection. Overall one-way ANOVA for DNMT1 mRNA in VEH-, A–85380-, and PNU–282987-treated mice ($F_{6,28} = 5.4$) provided a $p < 0.01$ in the FC.
*$p < 0.01$ for Student–Newman–Keuls multiple comparison between VEH- and A–85380-treated mice.
[a]DNMT1 mRNA is expressed as fmol DNMT1mRNA/0.1 pmol NSE mRNA.

that binds with subnanomolar affinity to $\alpha_4\beta_2$ but has negligible affinity for $\alpha_3\beta_4$- or α_7 subtype-containing receptors (Smith *et al*, 2007; Rueter *et al*, 2006). In pharmacologically relevant antidepressant, anti-nociceptive, and anti-allodynic doses (0.1–1 mg/kg), A–85380 shows considerably lower toxicity than nicotine and epibatidine (Curzon *et al*, 1998; Rueter *et al*, 2003, 2006; Buckley *et al*, 2004). This drug easily penetrates the blood–brain barrier, and in mice has a relatively long half-life (more than 1 h) (Curzon *et al*, 1998) compared with nicotine that has a half-life of few minutes (Damaj *et al*, 2007).

In the doses used in this study, ie, 0.5 to 2.5 mg/kg s.c. twice a day, A–85380 fails to produce the hypercholinergic peripheral symptomatology of nicotine. However, similar to nicotine (Satta *et al*, 2008) or VAR (Figure 2), A–85380 elicits a 30–40% decrease of cortical DNMT1 mRNA and fails to significantly decrease DNMT1 mRNA in the striatum (Table 1).

Mice exposed to A–85380 in doses that reduce the expression of DNMT1 mRNA exhibit a significant increase of GAD_{67} but fail to increase GAD_{65} protein expression in the FC (Table 2).

PNU–282987 Fails to Decrease Cortical DNMT mRNA or to Induce GAD_{67} Expression in the Mouse FC

PNU–282987 is a benzamide derivative functioning as a selective agonist of the homomeric α_7 nAChR (Bodnar *et al*, 2005). It evokes a whole-cell current sensitive to the selective α_7 nAChR antagonist methylcaconitine and enhances GABAergic synaptic activity when applied to rat hippocampal slices or dissociated rat hippocampal neurons in culture (Hajós *et al*, 2005). In rats injected with PNU–282987 at doses of 1 mg/kg i.v., the disruption of auditory gating elicited by amphetamine is reversed (Hajós *et al*, 2005).

PNU–282987 has a half-life of several hours in rodents (Hajós *et al*, 2005). On the basis of this information, we administered PNU–282987 every 12 h.

Table 2 A–85380 But Not PNU–282987 Increases GAD_{67} Protein Expression in Mouse Frontal Cortex

A–85380 (mg/kg)	GAD_{67}[a]	GAD_{65}[a]
VEH	0.23 ± 0.05	0.45 ± 0.07
0.5	0.52* ± 0.05	0.47 ± 0.10
1	0.37* ± 0.03	0.41 ± 0.05
2.5	0.35* ± 0.02	0.53 ± 0.08
PNU–282987 (mg/kg)	**GAD_{67}[a]**	**GAD_{65}[a]**
VEH	0.21 ± 0.03	0.47 ± 0.06
1	0.26 ± 0.02	0.41 ± 0.03
2.5	0.17 ± 0.01	0.54 ± 0.05
5	0.25 ± 0.02	0.38 ± 0.04

Mice were treated s.c. with A–85380 or PNU–282987 two times a day for 5 days. GAD_{67} and GAD_{65} were measured 2 h after the last injection of drug. Overall one-way ANOVA for GAD_{67} in VEH-, A–85380-, and PNU–282987-treated mice ($F_{6,\ 6} = 5.4$) yielded a $p < 0.01$ in the FC.
*$p < 0.01$ for Student–Newman–Keuls multiple comparison between VEH- and A–85380- treated mice.
[a]Data is expressed as GAD_{67} or GAD_{65}/β-actin OD ratio.

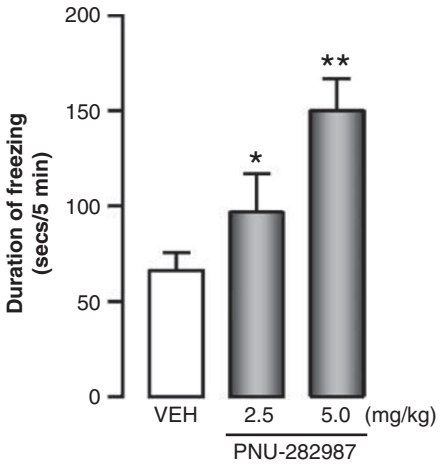

Figure 5 PNU–282987 increases the contextual fear conditioning response in mice. PNU 282987 was injected s.c. 2 h before the training session. Total duration of freezing time was measured 24 h after a training session. The training session consisted of a conditioned stimulus (CS) (acoustic tone, 30 s, 85 dB) paired with an unstimulated stimulus (US) (electric foot shock, 2 s, 0.5 mA) three times every 2 min. Each value is the mean ± SEM of five animals. An overall one-way ANOVA in VEH- and PNU–282987-treated mice ($F_{3,12} = 6$) provided a $p < 0.01$. *$p < 0.05$ for Student–Newman–Keuls multiple comparison between VEH- and PNU–282987- (2.5 mg/kg) treated mice. **$p < 0.01$ for Student–Newman–Keuls multiple comparison between VEH- and PNU–282987- (5 mg/kg) treated mice.

Because most of the experiments with PNU–282987 were conducted in rats, to optimize the pharmacological intervention in mice with PNU–282987, we first studied the effect of PNU–282987 injected in doses of 0.5 to 5 mg/kg s.c. on contextual fear conditioning.

It is important that PNU–282987, despite its lack of effects on gross behavior and locomotor activity, enhances cortico-limbic-dependent forms of learning (LeDoux, 2000), inducing a dose-dependent enhancement of the contextual fear condition (Figure 5). There was no effect of PNU–282987 on freezing from the acoustic CS. In these experiments, PNU282987 was injected s.c. at 2 h before the training test.

In doses ranging from 1 to 5 mg/kg, PNU–282987 fails to induce significant changes in DNMT1 mRNA expression in the FC (Table 1). Furthermore, no significant GAD_{67} or GAD_{65} protein increase was observed in the FC of PNU–282987-treated mice (Table 2).

DISCUSSION

We have recently reported that mice treated protractedly with nicotine show a cortical and hippocampal decrease of DNMT, reduced GAD_{67} promoter methylation and increased GAD_{67} expression (Satta *et al*, 2008).

The present study (Figures 2–4) shows that VAR injected in mice in doses that failed to alter gross behavior or locomotor activity but acted as a partial agonist at the $\alpha_4\beta_2$ nAChR subtype and as a full agonist at the α_7 nAChR subtype (Mihalak *et al*, 2006) decreased DNMT mRNA, reduced the binding of $MeCP_2$ to GAD_{67} promoters, and increased the levels of GAD_{67} in the FC in a manner similar to that of nicotine (Figure 1). These changes are apparently mediated through central nAChR stimulation because they are blocked by mecamylamine.

The downregulation of DNMT expression induced by nAChR stimulation in GABAergic neurons of the FC appears to be brain region-specific because it fails to occur in the GABAergic medium spiny neurons of the striatum. These neurons primarily express muscarinic AChR (Zhou *et al*, 2002).

The decrease of DNMT and the increase of GAD_{67} induced by VAR in FC can be mimicked by injections of A–85380, an $\alpha_4\beta_2$ nAChR-selective agonist that fails to replicate the unwanted peripheral side effects of nicotine. In contrast, PNU–282987, an α_7 nAChR-selective agonist injected in doses that enhance the cortico-limbic-dependent form of learning (Figure 5), is inactive on DNMT1 and GAD_{67} expression. Hence, the data suggest that VAR may regulate DNMT and GAD_{67} expression in cortical and hippocampal GABAergic neurons acting at $\alpha_4\beta_2$ nAChR subtypes.

Several lines of investigation suggest that DNMT, which is highly expressed in telencephalic GABAergic neurons, is responsible for the epigenetic regulation of selective GABAergic gene promoters, including GAD_{67} and reelin (Veldic *et al*, 2007; Ruzicka *et al*, 2007; Zhubi *et al*, 2009; Grayson *et al*, 2009). In fact, the effect of $\alpha_4\beta_2$ nAChR stimulation on GABAergic gene promoter regulation is not generalized to all genes because in the same mice in which VAR and A–85380 induce an increase of FC GAD_{67} expression, GAD_{65} expression is not increased.

At present time, the cascade of molecular events that through nAChR stimulation downregulates DNMT1 and upregulates GAD_{67} expression in cortical and hippocampal GABAergic neurons is unknown. However, there is ample evidence obtained in *in vitro* (Grayson *et al*, 2009) and *in vivo* (Day and Sweatt 2010; Tremolizzo *et al*, 2005; Zhang *et al*, 2010; Meaney, 2010) experiments that changes in DNMT levels are cause-related to changes in target gene expression, including the expression of GAD_{67}.

Given that DNMT promoters contain consensus sequences for inducible transcription factors such as c-jun and c-fos (Bigey *et al*, 2000; Slack *et al*, 2001), it could be hypothesized that nAChR stimulation can regulate DNMT expression by altering the availability of these transcription factors. These factors could include the 'growth arrest and DNA damage-inducible protein 45b,' an inducible immediate early gene funtioning as a molecular factor in the DNA demethylation process in the brain (Ma *et al*, 2009).

Although the data indicate a decrease of DNMT as the cause of decreased GAD_{67} promoter methylation, we cannot exclude that nAChR stimulation reduces the GAD_{67} promoter methylation activating DNA demethylation processes. Studies of the characterization of DNA demethylase are presently in progress in our laboratory (Dong *et al*, 2010).

Current research in SZ suggests that the overexpression of DNMT in telencephalic GABAergic neurons is responsible for the epigenetic hypermethylation of specific GABAergic gene promoters, including GAD_{67} and reelin (Veldic *et al*, 2007; Ruzicka *et al*, 2007). The expression downregulation of these genes in SZ brains likely leads to a GABAergic transmission defect, which presumably has an important role in the pathogenetic mechanisms that underlie the cognitive, behavioral, and auditory gating system impairments expressed in psychotic patients (Guidotti *et al*, 2005; Lewis *et al*, 2005). This evidence suggests that a reversal of the epigenetically induced transcriptional downregulation of GAD_{67} and other genes in cortical GABAergic neurons of SZ patients should be attempted by using drugs that directly or indirectly target DNMT.

The present study and independent investigations (Martin *et al*, 2004; Adams and Stevens, 2007; Ochoa and Lasalde-Dominicci, 2007; Hasselmo and Sarter, 2011) suggest that full and partial $\alpha_4\beta_2$ nAChR agonists are promising pharmacological agents that deserve to be tested for the treatment of cognitive deficits in SZ and in related psychiatric disorders.

Hence, the use of VAR to selectively downregulate DNMT in GABAergic interneurons of the cortex but not in the striatum may represent an innovative attempt to control the hypermethylation of GAD_{67} and other gene promoters operative in selected populations of telencephalic GABAergic neurons of SZ patients while leaving the function of DNMT intact in cells that do not express nAChRs.

Interestingly, VAR repeatedly administered to patients, with SZ or schizoaffective disorders, who are tobacco smokers produced significant anti-smoking effects and improvements in some cognitive test scores, primarily associated with verbal learning and memory (Smith *et al*, 2009). Contrary to early reports that VAR may increase suicidal ideation or depression (Gunnell *et al*, 2009), subsequent studies have shown that VAR is well tolerated in animals and humans, and is an effective smoking cessation agent (Faessel *et al*, 2010; Jorenby *et al*, 2006).

Varenicline, unlike nicotine, fails to produce profound tachyphylaxis and it is only partially reinforcing in animal studies (Rollema *et al*, 2007; George *et al*, 2010). Hence, one may infer that this drug represents a better pharmacological tool than nicotine to selectively increase GAD_{67} expression in cortico-limbic inhibitory interneurons.

On the basis of our results, we suggest that the possible molecular mechanism underlying the cognitive-enhancing action of nAChR stimulation in SZ patients is the control of the epigenetic alteration of cortico-limbic GABAergic neurons. Furthermore, the present study suggests that $\alpha4\beta_2$ nAChR agonists may be better suited than the α_7 nAChR agonists to control epigenetic alterations of GABAergic neurons in SZ. This difference is in keeping with studies showing that selective $\alpha_4\beta_2$ nAChR agonists produce more robust and perhaps more clinically relevant increases in attention and cognitive performance than the α_7 nAChR agonists, which show rapid tachyphylaxis on cognition (Olincy *et al*, 2006; Hasselmo and Sarter, 2011).

DISCLOSURE

The authors declare no conflict of interest.

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