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Effect of the α4β2* nicotinic acetylcholine receptor partial agonist varenicline on dopamine release in β2 knock-out mice with selective re-expression of the β2 subunit in the ventral tegmental area

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ABSTRACT

We studied the effects of 1 mg/kg doses of nicotine and the α4β2* nicotinic acetylcholine receptor (nAChR) partial agonist, varenicline, on extracellular dopamine (DA) levels in the nucleus accumbens (NuAcc) of lentivirally vectorized male mice. Three separate experimental groups were injected with a lentiviral vector transducing the ventral tegmental area (VTA): wild-type C57BL/6J mice with a vector expressing eGFP only, β2 knock-out mice (β2KO) with the eGFP-only vector, and β2KO mice with a bicistronic vector reintroducing β2 and eGFP into the VTA as recently described (Maskos et al., 2005). Our results suggest that the neurochemical effects of varenicline as measured by using microdialysis in awake, freely moving mice are mainly mediated via β2* nAChR subunits localized in the VTA.

1. Introduction

The recently introduced α4β2* neuronal nicotinic acetylcholine receptor (nAChR) partial agonist varenicline has demonstrated increased efficacy for smoking cessation over the other first line treatments bupropion and nicotine replacement therapy (Lancaster et al., 2008). The improved efficacy is likely due to its dual mechanism of action: when quitting smoking, varenicline can reproduce the subjective effects of smoking by partially activating α4β2* nAChRs, and can prevent full activation of these receptors by nicotine when smoking again (Rollema et al., 2007b). Since varenicline binds selectively with sub-nanomolar affinity to α4β2* nAChRs, it is believed that the varenicline-induced increase in dopamine (DA) release from rat nucleus accumbens (Coe et al., 2005; Rollema et al., 2007a) is mediated via interactions with α4β2* nAChRs in the ventral tegmental area (VTA), similar to nicotine-evoked DA release. Convincing evidence for the crucial role of mesolimbic β2* nAChRs in the actions of nicotine came from our previous study in which elimination of the β2 subunit attenuated pharmacological and behavioral effects of nicotine, while targeted expression of the β2 subunit in the VTA of β2 knock-out (β2KO) mice reinstated nicotine self-administration and nicotine-induced DA release (Maskos et al., 2005). The present study examined whether the effects of varenicline on mesolimbic DA release in mice are also dependent on activation of β2 subunit containing nAChRs in the VTA, using the same protocols as in our nicotine study (Maskos et al., 2005).

2. Methods

2.1. β2KO mice and β2KO mice with re-expressed®2

2.1.1. Lentiviral vectors

The lentiviral vectors used in this work were derived from the pHR' expression vectors first described by Naldini and colleagues (Naldini et al., 1996a,b) with several subsequent modifications. Vectors are based on the previously described pTRIPΔU3 (Sirven et al., 2001), in which the U3 region of the 3′ long terminal repeat (LTR) was deleted (ΔU3), rendering the integrated viral DNA replication-incompetent. The central polypurine tract (cPPT) (Charneau et al., 1992) and the central termination sequence (CTS) (Charneau et al., 1994) of the wild-type HIV-1 were added, creating the 99-base pair central DNA "flap" (Zennou et al., 2000) that enhances infection of non-dividing cells by facilitating transport of the preintegration complex through the nuclear membrane pores. The woodchuck hepatitis B virus post-transcriptional regulatory element (WPRE) was added to increase RNA stability and transgene expression (Zufferey et al., 1999).

2.1.2. Re-expression lentivector

The re-expression lentivector [PGK-Beta2-Ires2-eGFP], see Fig. 1A, is a bicistronic β2-IRES2-eGFP construct, previously described (Maskos et al., 2005). Briefly,

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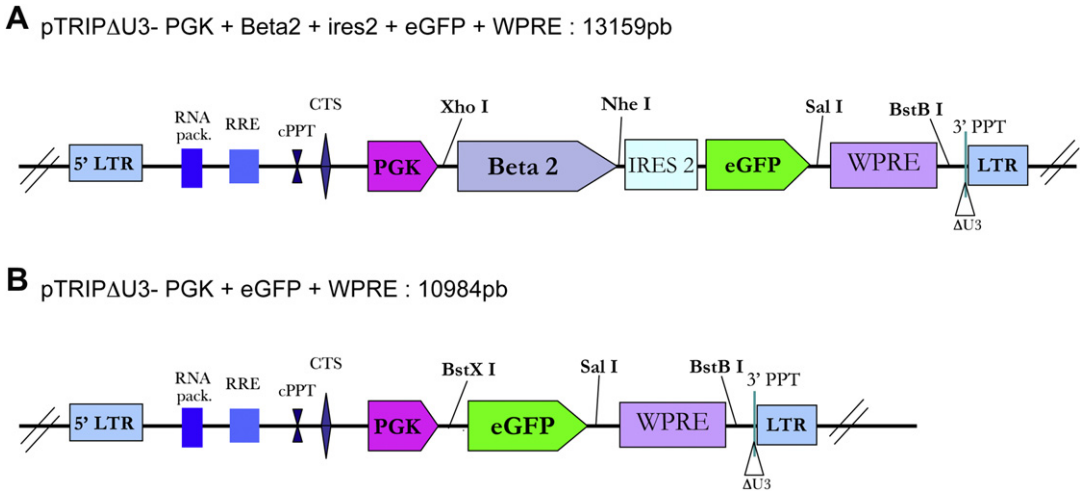

Fig. 1. Maps of lentiviral expression vectors. Diagrams of the two lentiviral vectors used in this study, between and including the LTR regions. *LTR*, long terminal repeat; *RNA pack.*, genomic RNA packaging signal; *RRE*, *rev* response element; *cPPT*, central polypurine tract; *CTS*, central termination sequence; *PGK*, promoter of the mouse phosphoglycerate kinase gene; *IRES2*, internal ribosome entry sequence; *eGFP*, enhanced green fluorescent protein; *WPRE*, woodchuck hepatitis B virus post-transcriptional regulatory element; *3'PPT*, 3′-polypurine tract; Δ*U3*, deletion of the U3 portion of 3′-LTR.

the mouse phosphoglycerate kinase (PGK) promoter was PCR amplified from a PGK-nls lacZ expression vector, M48, and ligated into pTRIPΔU3. To generate the β2-IRES2-eGFP construct a cloning site was created in the pIRES2-EGFP expression plasmid (Clontech) by mutagenesis (QuikChange, Stratagene), and the wild-type mouse β2 subunit, containing a consensus Kozak translation initiation site (Maskos et al., 2002), was then ligated into plasmid pIRES-EGFP. The β2-IRES2-eGFP cassette was then ligated into the pTRIPΔU3-PGK vector using *Xho*I-*Sal*I sites. Finally, the WPRE sequence was added. The PGK-eGFP control lentivector, Fig. 1B, is identical to the bicistronic version, but lacks the β2-IRES2 portion.

2.1.3. Lentivirus production

HEK-293T cells (at 80–85% confluence) were co-transfected with the vector plasmid (either the pTRIPΔU3 [PGK-Beta2-Ires2-EGFP] or the pTRIPΔU3 [U6-shRNA-Ubiq-EGFP]), together with a packaging plasmid (CMVΔ8.9) and an envelope plasmid (CMV-VSVg), using Lipofectamin Plus (Invitrogen) according to the manufacturer's instructions. Two days after transfection, viral particles were harvested from the supernatant, treated with DNaseI (1/5000; 0.2 μl/ml) and $MgCl_2$ 2 M (1 μl/ml), filtered through 0.45 μm pores and concentrated by ultracentrifugation at 24.000 rpm for 2 h ($\omega^2t = 4.55$). Viruses were stored in 10 μl aliquots at −80 °C.

Viral titers were determined by quantification of the p24 capsid protein using an HIV-1 p24 antigen immunoassay (Beckman Coulter, Villepinte, France). As all lentivectors contained the eGFP sequence, transfecting units were estimated by Fluorescence Activated Cell Sorting (FACS) after infection of HEK-293T cells with increasing doses of each viral production. Immediately before stereotaxic injections lentiviruses were diluted in PBS to achieve a dose of injection of $0.5–1x10^6$ TU in 2 μl (equivalent to 150 ng p24 protein for bicistronic viruses, 70–80 ng p24 for monocistronic viruses).

2.1.4. Mice

β2KO mice were backcrossed to the C57BL/6J@Ico strain for 19 generations. β2KO and WT (C57BL/6J@Ico) mice, 7 weeks old, were kept under standard laboratory conditions with ad libitum food and water and in a 12 h light/dark period (on at 8:00 AM).

2.1.5. Stereotaxic procedure

Mice aged 8–10 weeks (weight 25–30 g) were deeply anaesthetized using ketamine/xylazine and introduced into a stereotaxic frame adapted for mice (Cunningham and McKay, 1993). 2 μl of virus were injected bilaterally, for VTA at the following coordinates (Paxinos and Franklin, 2001): −3.4 mm antero-posterior, ±0.5 mm lateral, and −4.3 mm dorso-ventral. All procedures were in accordance with European Commission directives 219/1990 and 220/1990, and approved by Animalerie Centrale and Médecine du Travail, Institut Pasteur. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.2. In vivo microdialysis

The effects of nicotine and varenicline on basal DA release were determined by intra-cerebral microdialysis in the nucleus accumbens of awake, freely moving mice as previously described (Maskos et al., 2005). Briefly, mice were anaesthetized with chloral hydrate (400 mg/kg, i.p.), placed in a stereotaxic frame and concentric dialysis probes (cuprophan fiber, 1.0 mm, outer diameter 0.30 mm) were implanted unilaterally into the ventral striatum. Twenty hours after surgery the probe was continuously perfused with an artificial CSF solution (composition in mmol/L: NaCl 147, KCl 3.5, $CaCl_2$ 1.26, $MgCl_2$ 1.2, NaH_2PO_4 1.0, pH7.4) at a flow rate of 1.5 μL/min using a CMA/100 pump (Carnegie Medicin, Stockholm, Sweden) and dialysates were collected at 15 min intervals. Measurements of dialysate DA concentrations ([DA]ext) were made by HPLC (XL-ODS 4.6 × 75 mm, 3 μm column, Beckman) with amperometric detection (1049A, Hewlett–Packard, Les Ulis, France). Eight samples were collected to determine basal values (means ± SEM) before systemic administration of nicotine or varenicline. The limit of sensitivity for [DA]ext was 0.5 fmol per sample (signal-to-noise ratio = 2). Mice were injected *i.p.* with drugs dissolved in 0.9% NaCl (10 ml/kg) and control animals received an i.p. injection of 0.9% NaCl (10 ml/kg). Responses to drug administration were determined over a 120-min period. Probe placement was verified histologically after completion of the microdialysis experiment according to Bert et al. (2004)

2.3. Drugs and chemicals

Varenicline tartrate (MW: 361; MW base: 211; Pfizer, Groton, CT, USA) and nicotine bitartrate (MW: 462; MW base:162; Sigma–Aldrich, St Quentin Fallavier, France) were dissolved in 0.9% NaCl. Both compounds were dosed at 1 mg/kg active drug (free base). All chemicals were analytical grade and purchased from Sigma–Aldrich, St. Quentin, Fallavier, France.

2.4. Statistical analyses

Data were analyzed by repeated measures ANOVAs using StatView version 5.0 (Abacus Concepts, Berkeley, CA, USA), followed by post-hoc Student-T test. The level of statistical significance was set at $P < 0.05$.

3. Results

Three groups of mice were vectorized with a lentiviral vector as previously described (Maskos et al., 2005; Mameli-Engvall et al., 2006; Pons et al., 2008): WT mice obtained an injection of the eGFP-only expressing vector, Fig. 1B, and β2 knock-out (β2KO) mice

Table 1
Basal extracellular levels of dopamine (fmol/sample; Bo at t0) in the nucleus accumbens of wild-type (WT), β2 knock-out (KO) and β2 vectorized β2 knock-out (KO) mice (6–9 mice per group). No statistically significant differences were observed in the three groups of mice ($P > 0.05$). Stereotaxic coordinates from Bregma (in mm): anterior: +3.4; lateral: 0.7; ventral: −5.4.

	C57BL6 WT	β2KO	β2vect. KO
Bo	9.7	6.5	6.0
±SEM	1.1	1.5	1.0

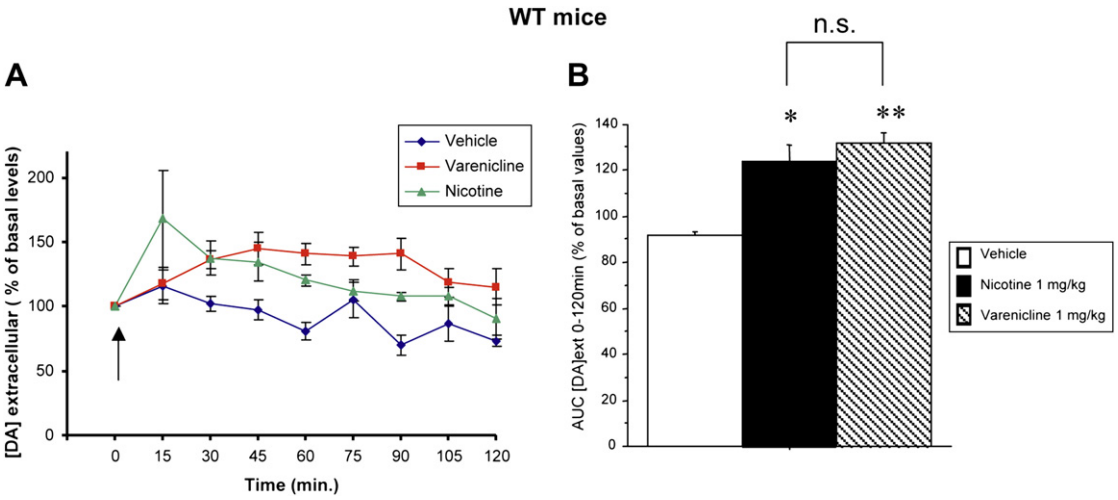

Fig. 2. Effects of nicotine and varenicline *in vivo* on dopamine release in wild-type mice. Extracellular dopamine levels in the nucleus accumbens shell showing (A) time course (means ± SEM) and (B) area under the curve (AUC values, means ± SEM as % of baseline) during a 2-hr post-treatment period. Both nicotine and varenicline increased DA release in wild-type mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle. n.s., not statistically significant.

were injected either with the same eGFP-only vector, or with a bicistronic vector expressing also the mouse WT β2 subunit, Fig. 1A. Several months after vectorization, microdialysis probes were stereotaxically implanted to sample from the NuAcc. After a recovery period of 20 h, basal DA overflow in the NuAcc was measured in the three groups of mice as described before (Maskos et al., 2005).

Table 1 summarizes the mean basal levels of DA in microdialysates from the NuAcc for each group of mice, which were not significantly different from each other ($P > 0.05$). After a stabilization period of about 2 h half of the mice in each group were given an *i.p.* injection of 1 mg/kg nicotine, the other half an *i.p.* injection of 1 mg/kg varenicline, and DA overflow in the NuAcc was measured for an additional 2 h.

Fig. 2A shows the time courses of the effects of varenicline and nicotine in eGFP vectorized WT mice on DA release, expressed as percentage of basal levels. In Fig. 2B these data are summarized as area under the curve (AUC) values over 2 h following drug administration for all groups. In WT mice, 1 mg/kg nicotine and 1 mg/kg varenicline both increased DA release compared to saline, and their respective increases expressed as AUC were not significantly different between the two treatments. In eGFP vectorized KO mice, nicotine or varenicline treatment did not produce any significant increase in DA release, as shown by the time courses (Fig. 3A) and by the AUC values (Fig. 3B).

Subsequently, administration of 1 mg/kg nicotine or varenicline to β2KO mice vectorized with a β2 expressing lentivirus elicited increases in DA release in the NuAcc, as shown in the time courses (Fig. 4A.) Expressed as the AUC, the drug treatments were significantly different from saline treatment (Fig. 4B).

The increases were not statistically different between the varenicline and nicotine-treated groups, as determined by more detailed analysis of the microdialysis data: AUC values for extracellular DA levels (DAext) were calculated for each successive 30 min period in WT and β2 KO-vectorized? mice. Thus, we calculated AUC 0–30, AUC 30–60, AUC 60–90 and AUC 90–120 min in nicotine-treated mice as well as in varenicline-treated mice. We then compared these values in nicotine-treated mice to those calculated in β2-KO mice, between both genotypes. One-way ANOVA with repeated measures showed no significant differences between nicotine and varenicline effects on DAext from 60 to 90 min in WT mice (Fig. 2A: $F(1,30) = 1.97$; $p = 0.19$). In addition, no

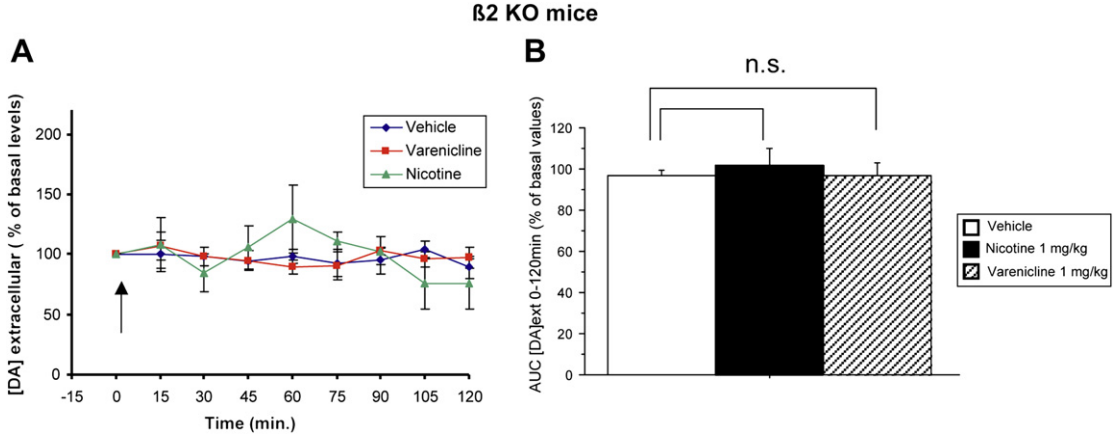

Fig. 3. Effects of nicotine and varenicline *in vivo* on dopamine release in knock-out mice. Extracellular dopamine levels in the nucleus accumbens shell showing (A) time course (means ± SEM) and (B) area under the curve (AUC values, means ± SEM as % of baseline) during a 2 h post-treatment period. Nicotine and varenicline failed to increase DA release in β2 knock-out mice. n.s., not statistically significant.

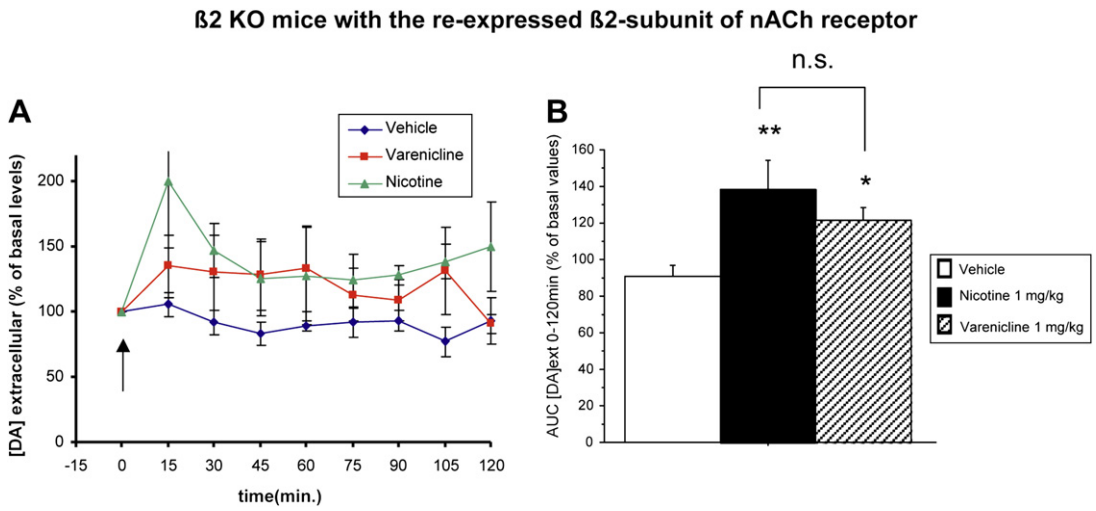

Fig. 4. Effects of nicotine and varenicline *in vivo* on dopamine release in β2 vectorized KO mice. Extracellular dopamine levels in the nucleus accumbens shell showing (A) time course (means ± SEM) and (B) area under the curve (AUC values, means ± SEM as % of baseline) during a 2 h post-treatment period. Both nicotine and varenicline similarly increased DA release in β2 vectorized β2KO mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to vehicle. n.s., not statistically significant.

statistically significant differences were observed between all the sub-groups studied. Similarly, no significant differences were found between nicotine and varenicline effects on DAext from 0 to 30, 30–60, 60–90 and 90–120 min in β2KO mice (Fig. 4A $F(1,33) = 1.63$; $p = 0.23$). Thus, the time course did not show differences in allowing a differentiation of varenicline *versus* nicotine effects on DA release between mice that re-expressed β2KO nAChR in VTA-NuAcc only and those expressing the β2 nAChR subunit all over the brain (i.e., WT mice).

4. Discussion

We have compared the actions of nicotine and varenicline on DA release in the NuAcc of freely moving mice, in three different groups vectorized in the VTA. Both drugs did not elevate DA levels in GFP vectorized KO mice and produced a similar DA increase in GFP vectorized WT mice. Most importantly, both drugs elicited comparable increases in extracellular DA concentrations in β2 VTA vectorized KO mice. These results demonstrate that the DA increases in mouse NuAcc produced by both drugs are mediated by the same nAChR subtypes in the VTA. The fine stoichiometry of nAChRs in the DAergic system is complex, as is their contribution to behaviour and central DA release: most of the nine neuronal nAChR subunits, α2-α7 and β2-β4, are expressed in the VTA on DAergic and GABAergic neurons, and are present in cell bodies, dendrites, and in axons projecting to the NuAcc (Klink et al., 2001; Exley and Cragg, 2008). The β2 and α4 subunits had been implicated in nicotine-elicited DA release before, with an absence in the corresponding KO mice (Picciotto et al., 1998; Marubio et al., 2003). In the case of the β2KO mouse, this DA response to systemic nicotine could be restored by the targeted expression of the β2 subunit (Maskos et al., 2005; Maskos, 2007), demonstrating the necessary and sufficient nature of this subunit in mediating nicotine responses in the VTA. In the case of the α6 nAChR subunit KO mouse, no difference in the effects of nicotine on DA release was observed (Champtiaux et al., 2003). This is surprising, as this subunit is highly enriched in the dopaminergic system, confers very high affinity to nicotine, and is specifically recognized by a cone snail toxin, α-conotoxin MII (Champtiaux et al., 2002).

The α7 KO mouse is still under study (Besson et al., in preparation), but has so far been shown to only slightly modulate DA neuron firing patterns (Mameli-Engvall et al., 2006). The importance of the remaining subunits in modulating DA overflow *in vivo* has not yet been established. As for the β3 subunit (Cui et al., 2003), synaptosomal preparations have been analyzed for DA release, which is increased in β3 KO mice, although β3, like α5, does not contribute to the nicotine binding site (Exley and Cragg, 2008).

Since the β2 subunit is the most completely characterized nAChR subunit in mice, we examined its participation in the action of varenicline in the VTA. At a maximally effective dose of 1 mg/kg in rats (Rollema et al., 2007a), varenicline did not increase DA overflow in the NuAcc of β2KO mice, similar to the lack of effect of nicotine observed before (Picciotto et al., 1998; Maskos et al., 2005). In the wild-type, both drugs showed a comparable DA response.

The slow onset and long lasting effect of varenicline, with DA increases maintained at about 130–140% of basal levels for several hours in mouse NuAcc, is very similar to the effect of varenicline on DA release in rat NuAcc described previously (Coe et al., 2005; Rollema et al., 2007a). The maximal nicotine-induced DA increases in WT mice and KO mice with reintroduced β2 to about 160–200% of basal levels, are consistent with previously reported maximal nicotine-evoked mesolimbic DA release in mouse (Maskos et al., 2005) and rat (Coe et al., 2005; Rollema et al., 2007a). When expressed as the AUC over 2 h after dosing, the effects of nicotine and varenicline were not significantly different, due to the longer lasting effect of varenicline and the higher peak effect of nicotine. We can however not exclude the possibility that affinities and/or functional efficacies of varenicline for mouse and rat nAChRs vary, or that the mechanism of varenicline-induced DA release in mice is different than in rats.

In conclusion, nicotine and varenicline both elevate DA release in the NuAcc of the mouse via an interaction with α4β2* nAChRs located in the VTA. Further studies on other subunits that are co-expressed with β2 and thought to play a role in modulating DA release in mouse NuAcc, such as the α6 subtype (Champtiaux et al., 2003), will provide further insight in the underlying mechanisms of varenicline's effects on mesolimbic DA release.

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