**Selective Re-expression of $\beta 2$ Nicotinic Acetylcholine Receptor Subunits in the
Ventral Tegmental Area of the Mouse Restores Intravenous Nicotine Self-
Administration**

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Abstract

Beta-2 ($\beta 2$) nicotinic acetylcholine receptor subunits have been particularly related with nicotine reinforcement. However, the importance of these subunits in the chronic aspects of nicotine addiction has not been established. In this study we evaluated the role of ventral tegmental area (VTA) $\beta 2$ receptor subunits in the acquisition and maintenance of nicotine self-administration. We used an operant mouse model of intravenous self-administration of different doses of nicotine (15, 30, and 60 $\mu\text{g/kg/infusion}$) during 10 days in constitutive knockout mice lacking $\beta 2$ receptor subunits ($\beta 2\text{KO}$), wild-type (WT) controls, mice with $\beta 2$ receptor subunits re-expressed in the VTA using a lentiviral vector ($\beta 2\text{-VEC}$), and control knockout mice with a sham injection (KO-GFP). The results showed that $\beta 2\text{KO}$ mice did not reliably acquire nicotine self-administration at any of the doses tested, while WT controls showed dose-dependent acquisition of this behaviour. $\beta 2\text{-VEC}$ mice readily acquired and maintained nicotine self-administration at the effective dose of 15 $\mu\text{g/kg/infusion}$, while sham KO-GFP mice did not. The recovery of the WT phenotype by the re-expression of $\beta 2$ receptor subunits within the VTA supports the role of this specific population in nicotine reinforcement, and reveals that they are sufficient for the acquisition and maintenance of systemic nicotine self-administration.

Keywords: $\beta 2$ subunits, VTA, chronic nicotine self-administration, lentivirus

1. Introduction

Nicotine addiction is a major social and health problem throughout the world (Hatsukami et al., 2008). Hence, it is of great importance to understand the factors that convey vulnerability to develop nicotine addiction, as well as the neurobiological substrates mediating different aspects of the addictive process, such as initiation and maintenance of nicotine consumption.

The mesocorticolimbic dopamine (DA) system, comprised of dopaminergic cell bodies in the ventral tegmental area (VTA) and their projections to the nucleus accumbens (NAC) and pre-frontal cortex (PFC), has been identified as a crucial substrate for the reinforcing properties of prototypical drugs of abuse, including nicotine (Di Chiara, 2000). In this sense, systemic administration of nicotine increases DA levels in the NAC of rats (Pontieri et al., 1996) and mice (Berrendero et al., 2005). The specific involvement of the VTA in the acute effects of nicotine has been established in studies showing that nicotine increases the burst firing of VTA DA neurons (Grenhoff et al., 1986; Mameli-Engvall et al., 2006), and intra-VTA infusions of nicotine enhances DA release in the NAC (Tizabi et al., 2002; Rahman et al., 2004). In addition, the VTA also appears to be important for the reinforcing effects of nicotine since it has been shown that rats and mice will self-administer nicotine into this structure (Ikemoto et al., 2006; David et al., 2006).

Anatomical studies have determined that the VTA is a complex brain structure containing $\beta 2$ and $\alpha 7$ nicotinic acetylcholine receptor (nAChR) subunits (see Maskos, 2008 for review). However, only $\beta 2$ receptor subunits seem to be involved in the reinforcing effects of nicotine (Picciotto et al., 1998; Epping-Jordan et al. 1999; Pons et

al., 2008). Moreover, recent studies using an intra-cerebral drug self-administration procedure have confirmed the contribution of $\beta 2$ receptor subunits to nicotine reinforcement by showing that $\beta 2$ knockout (KO) mice do not self-administer nicotine into the VTA, while the selective re-expression of $\beta 2$ receptor subunits in the VTA restores this local self-administration behaviour (Maskos et al., 2005). However, this model only evaluates the local effects of nicotine within the VTA, and it is not appropriate when trying to extrapolate the data to human subjects who are exposed to nicotine systemically through cigarette smoke. Using an acute mouse model of nicotine intravenous self-administration, where a rapid assessment of the reinforcing properties of nicotine can be carried out in one single behavioural session, it has been shown that VTA- $\beta 2$ re-expressed mice self-administered more nicotine intravenously in a 30 min session than yoked mice (Pons et al., 2008). Although these results corroborate that $\beta 2$ receptor subunits in the VTA play an important role in acute nicotine reinforcement, they do not take into account the chronic aspects of nicotine addiction, such as the acquisition and maintenance of nicotine self-administration.

The use of transgenic mouse models in chronic operant nicotine self-administration studies has become an important tool for understanding the neurobiological substrates involved in nicotine addiction (Fowler et al., 2011). Therefore, the aim of this study was to evaluate the acquisition of chronic operant intravenous self-administration of nicotine at different doses (15, 30, and 60 $\mu\text{g/kg/infusion}$) during 10 days in constitutive knockout mice lacking $\beta 2$ receptor subunits ($\beta 2\text{KO}$), wild-type (WT) controls, $\beta 2\text{KO}$ mice with $\beta 2$ receptor subunits re-expressed in the VTA using a lentiviral vector ($\beta 2\text{-VEC}$), and control knockout mice injected in the VTA with a GFP-only expressing vector ($\beta 2\text{GFP}$).

2. Methods

2.1. Animals

All the mice used were male (age 10-16 weeks) from a C57Bl/6J background (Charles River, France). The animals were constitutive knockout mice lacking $\beta 2$ receptor subunits ($\beta 2$ KO), wild-type (WT) controls, and intra-VTA injected $\beta 2$ KO mice ($\beta 2$ -VEC and KO-GFP). $\beta 2$ KO mice were backcrossed in a pure C57Bl/6J background for at least 20 generations. Mice were individually housed in a temperature (21 ± 1 °C) and humidity ($55 \pm 10\%$) controlled room with a reversed 12-h light/dark cycle (lights off 08:00 h). Food and water were available *ad libitum*, except during the self-administration studies. All behavioural studies were performed during the dark phase of the reversed cycle. Animal procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEa-IMAS-UPF). Our laboratory has the Statement of Compliance with Standards for Use of Laboratory Animals by Foreign Institutions (#A5388-01), approved by the Office of Laboratory Animal welfare (OLAW) on 06/08/2009.

2.2. Drugs

(-)-Nicotine hydrogen tartrate salt [(-)-1-methyl-2(3-pyridyl)pyrrolidine] (Sigma, Madrid, Spain) was dissolved in physiological saline (0.9%), and the pH adjusted to 7.4 with sodium hydroxide. All nicotine doses (**7.5**, 15, 30, 60 $\mu\text{g/kg/infusion}$), reported as free base concentrations were contingently administered by intravenous route.

2.3. Intravenous self-administration procedure

Mice were implanted with indwelling intravenous silastic catheters in the right jugular vein under anaesthesia (ketamine/xylazine 5:1; 0.10 ml/10 g, **i.p.**), as previously reported (Soria et al., 2005). The intravenous catheter patency was maintained with a daily flush of heparin solution (30 UI/ml), and the patency was evaluated at the end of the experiment by infusing 0.1 ml of thiopental (5 mg/ml) through the catheter. If prominent signs of anaesthesia were not apparent within 3 s of the infusion, the mouse was removed from the experiment. The self-administration experiments were conducted in mouse operant chambers (Model ENV-307A-CT; Med Associates Inc., Georgia, VT, USA) equipped with two holes, one randomly selected as the active hole and the other as the inactive hole. Stimuli lights (cues), one located inside the active hole and the other above it (both with an intensity of 20 mA) were paired contingently with the delivery of the reinforcer. Nicotine was infused via a syringe that was mounted on a microinfusion pump (PHM-100A; Med Associates) and connected, via Tygon tubing (0.96 mm o.d., Portex Fine Bore Polythene Tubing, Portex Ltd, Kent, UK) to a single-channel liquid swivel (Instech Laboratories, Plymouth Meeting, PA, USA) and to the mouse intravenous catheter. Animals were trained during 10 days under a fixed ratio 1 (FR1) schedule of reinforcement, followed by a 10-sec time-out period, as previously reported (Martín-García et al., 2009). At the beginning of each training session, mice received one free nicotine infusion. Nose poking on the active hole resulted in a nicotine infusion (23.5 µl over 2 sec), while nose poking on the inactive hole had no consequences. Stable acquisition of nicotine self-administration behaviour was achieved when mice showed, during three consecutive sessions, less than 20% deviation from the mean of the total number of reinforcers (80% of stability), at least 65% responding on the active hole, and a minimum of five reinforcers per session.

In order to assess the specificity of responding for nicotine infusions in WT mice, we performed a within-subjects dose-response curve including saline and nicotine at the doses of 7.5, 15, 30 and 60 $\mu\text{g/kg/infusion}$. Doses of nicotine were presented in ascending order, and saline was given last. All doses were available during 3 consecutive days.

An additional experiment was carried out in order to evaluate whether WT and $\beta 2\text{KO}$ mice previously trained with the dose of 15 $\mu\text{g/kg/infusion}$ were sensitive to nicotine omission in a saline substitution paradigm with the continued presentation of the cue-light stimulus.

2.4. Stereotaxic delivery of lentiviral vector

$\beta 2\text{KO}$ mice were anesthetized using ketamine and xylazine (5:1; 0.10 ml/10 g) and stereotaxically injected bilaterally in the VTA (AP: -3.4 mm; ML: ± 0.5 mm; DV: - 4.3 mm) (Franklin and Paxinos, 1997), with 2 μl of a bicistronic (b2-IRES2-eGFP) vector (150 ng of P24 per side) reintroducing $\beta 2$ receptor subunits and GFP (45 ng of P24 per side) in the VTA, as previously described (Maskos et al., 2005). Control mice received a GFP-only expressing vector. Following a rest period of 4 weeks after stereotaxic surgery, $\beta 2\text{-VEC}$ and KO-GFP mice were implanted with chronic indwelling intravenous catheters in order to perform nicotine self-administration studies, as described above.

2.5. Verification of $\beta 2$ receptor subunits re-expression in the VTA of $\beta 2\text{KO}$ mice

In order to confirm the re-expression of $\beta 2$ -containing high-affinity receptors in the VTA of KO mice, [^{125}I]-epibatidine binding studies were performed. Brains from re-injected mice were dissected, frozen in dry ice, and stored at -80°C until use. 20 μm

thick coronal sections were cut at -20°C and thaw mounted on Menzel Glaser SuperFrost Plus microscope slides. Slides were incubated at room temperature with 200pM [¹²⁵I]-epibatidine (NEN Perkin Elmer, Boston; specific activity 2200 Ci/mmol) in Tris 50mM pH 7.4 for 30 min. After incubation, sections were rinsed 2 x 5 min in the same buffer and briefly in distilled water. Non-specific binding was not distinguishable from background. Sections were then exposed for 16hrs to Kodak Biomax films. Total [¹²⁵I]epibatidine binding consists of binding to a combination of up to three separate classes of receptor subtypes (Nguyen et al., 2003). It has been previously reported that high affinity binding sites for [³H]nicotine and [¹²⁵I]epibatidine are absent from the brain of $\beta 2$ knockout mice with the exception of structures of the habenulo-peduncular system. We previously demonstrated that after lentivirus injection into the VTA, [¹²⁵I]-epibatidine binding sites were detected at the injection sites and in terminal regions including the NAC (Maskos et al, 2005; see also the Supplement).

2.6. Statistical Analysis

The acquisition data was analysed using two-way repeated measures ANOVA (Statistical Package for the Social Sciences, SPSS) with day and hole as intra-subject factors for the different genotypes followed by estimation of parameters post-hoc test. The between-subjects dose-response data were analysed with one or two-way ANOVA as appropriate followed by the LSD post-hoc test. For the within-subjects dose-response data, repeated measures ANOVA was performed with hole and dose as within-subject factors followed by the estimation of parameters post-hoc test. For the saline substitution data, repeated measures ANOVA was carried out with hole and day as within-subject factors for the nicotine phase and for the saline phase separately, followed by estimation of parameters post-hoc test. One-way ANOVA for paired

samples was used to compare the mean number of infusions earned during the last day of nicotine self-administration and the first and last days of saline self-administration.

3. Results

β 2KO mice and WT controls were trained to self-administer nicotine (15, 30 or 60 μ g/kg/infusion) during 10 days (Figure 1). WT mice significantly acquired nicotine self-administration at the dose of 15 μ g/kg/infusion (87.5 % reached acquisition criteria). Two-way repeated measures ANOVA revealed a significant main effect of hole [$F(1,7) = 53.143$, $p < 0.001$], a non significant effect of day and a significant interaction between both factors [$F(9,63) = 3.912$, $p < 0.05$]. Subsequent post-hoc analysis showed that WT mice discriminated between active and inactive holes from the 3rd to the 10th day of training (day 3 $p < 0.05$; day 5 $p < 0.05$; day 6 $p < 0.001$; day 7 $p < 0.01$; day 8 $p < 0.01$; day 9 $p < 0.001$; day 10 $p < 0.001$) (Figure 1A). In contrast, only 37.5% of β 2KO mice reached acquisition criteria at this dose. Two-way ANOVA revealed a significant main effect of hole [$F(1,7) = 5.653$, $p < 0.05$], but no significant effects of day nor interaction between factors were observed (Figure 1B).

For the dose of 30 μ g/kg/infusion, 50 % of WT mice reached acquisition criteria. Two-way ANOVA revealed a significant main effect of hole [$F(1,5) = 15.014$, $p < 0.05$], but no significant effects of day nor interaction between factors (Figure 1C). In contrast, none of the β 2KO mice reached acquisition criteria at this dose. Two-way ANOVA revealed a significant main effect of hole [$F(1,7) = 16.904$, $p < 0.01$], but no significant effects of day nor interaction between factors were observed (Figure 1D).

For the dose of 60 μ g/kg/infusion, 12.5 % of WT mice reached acquisition criteria. Two-way ANOVA revealed a significant main effect of hole [$F(1,7) = 35.706$, $p < 0.01$], but no significant effects of day nor interaction between factors (Figure 1E). In contrast, none of the β 2KO mice reached acquisition criteria. Two-way ANOVA showed a significant main effect of hole [$F(1,7) = 20.640$, $p < 0.01$], but no significant effects of day nor interaction between factors were observed (Figure 1F).

The mean number of infusions earned during 10 days of training with the three doses of nicotine by WT and β 2KO mice in the between-subjects dose response paradigm is shown in Figure 2A. Both WT and β 2KO mice showed dose-dependent effects ($15 \mu\text{g/kg/infusion} > 30 \mu\text{g/kg/infusion} > 60 \mu\text{g/kg/infusion}$). One-way ANOVA revealed a significant effect of dose in WT mice [$F(2,19) = 7.928$, $p < 0.01$], and β 2KO mice [$F(2,21) = 4.260$, $p < 0.05$]. The post-hoc tests indicated differences between 15 and $60 \mu\text{g/kg/infusion}$ dose in WT ($p < 0.01$) and β 2KO mice ($p < 0.05$).

The within-subjects dose-response curve for WT mice is shown on Figure 2B. Mice were first trained to self-administer nicotine at the dose of $15 \mu\text{g/kg/infusion}$ for 5 days (data not shown). Subsequently, 4 doses of nicotine were tested in ascending order (7.5 , 15 , 30 and $60 \mu\text{g/kg/infusion}$), and saline was presented last. Each dose was tested during three consecutive days. Two-way repeated measures ANOVA revealed non significant main effects of hole or doses, but a significant interaction between dose and hole was observed [$F(4,16) = 12.847$, $p < 0.003$]. Subsequent post-hoc analysis showed that mice discriminated between active and inactive holes at all doses of nicotine ($7.5 \mu\text{g/kg/infusion}$: $p < 0.05$; $15 \mu\text{g/kg/infusion}$: $p < 0.05$; $30 \mu\text{g/kg/infusion}$: $p < 0.01$; $60 \mu\text{g/kg/infusion}$: $p < 0.01$), but not during saline presentation ($p > 0.05$). In addition, responding on the active nose-poke was significantly higher for the dose of $7.5 \mu\text{g/kg/infusion}$ compared to saline ($p < 0.05$).

To determine whether WT and β 2KO mice were sensitive to the omission of nicotine, we performed a saline substitution paradigm (Figure 3). WT mice (85.71 %) acquired nicotine self-administration behaviour at the dose of $15 \mu\text{g/kg/infusion}$ during 10 days of training. Two-way repeated measures ANOVA revealed a significant main effect of hole [$F(1,6) = 21.503$, $p < 0.001$], a non significant effect of day and a significant interaction between both factors [$F(9,54) = 2.252$, $p < 0.05$]. Subsequent

post-hoc analysis showed that WT mice discriminated between active and inactive holes from the 6th to the 10th day of training (day 6 $p < 0.05$; day 7 $p < 0.001$; day 8 $p < 0.001$; day 9 $p < 0.001$; day 10 $p < 0.01$ (Figure 3A). On day 11, saline was substituted for nicotine and mice were tested for an additional 7 days. Under these conditions, WT mice showed a transient (during 2 days), but significant increase in responding for saline compared to responding for nicotine (15 $\mu\text{g/kg/infusion}$). Subsequently, mice gradually decreased responding on the active nose-poke during the 7 days of training with saline. Two-way repeated measures ANOVA revealed a significant main effect of hole [$F(1,6) = 20.841$, $p < 0.01$], day [$F(6,36) = 6.258$, $p < 0.001$] and a significant interaction between both factors [$F(6,36) = 4.230$, $p < 0.05$]. Subsequent post-hoc analysis showed significant differences between active and inactive holes (day 11 $p < 0.001$; day 12 $p < 0.05$; day 13 $p < 0.05$; day 14 $p < 0.05$; day 15 $p < 0.01$; day 15 $p < 0.05$; day 15 $p < 0.01$. Figure 3B shows the mean number of infusions of nicotine received by WT mice during the last training session, and the mean number of infusions of saline received during the first and last days of the saline substitution experiment. One-way repeated measures ANOVA revealed significant differences between days [$F(2,12) = 13.349$, $p < 0.01$]. Post-hoc analysis confirmed a significant increase in the number of saline infusions on the first day of substitution ($p < 0.05$), but a significant decrease was observed on the last day of saline compared to the last day of nicotine self-administration ($p < 0.001$).

In contrast, none of the $\beta 2\text{KO}$ mice acquired nicotine self-administration behaviour at the dose of 15 $\mu\text{g/kg/infusion}$, and did not show discrimination between active and inactive nose-pokes when nicotine was available (Figure 3C). Two-way repeated measures ANOVA revealed non significant main effects of hole, day or

interaction between factors. Additionally, this pattern of responding did not significantly change when saline was substituted for nicotine. Two-way repeated measures ANOVA did not reveal any significant main effects or interaction between factors. Figure 3D shows the mean number of infusions of nicotine earned by $\beta 2$ KO mice during the last training session, and the mean number of infusions of saline received during the first and last days of the saline substitution experiment. One-way repeated measures ANOVA revealed no significant differences between days.

Figure 4 shows nicotine self-administration at the effective dose of 15 $\mu\text{g/kg/infusion}$ in $\beta 2$ -VEC and KO-GFP control mice. The percentage of mice that reached the acquisition criteria was 38.46 % for KO-GFP (Figure 4A) as compared to 89 % for $\beta 2$ -VEC mice (Figure 4B), revealing that re-expression of $\beta 2$ receptors in the VTA “rescues” nicotine self-administration behaviour in $\beta 2$ KO mice. Two-way repeated measures ANOVA revealed a significant effect of hole in KO-GFP mice [$F(1,12) = 8.515$, $p < 0.05$] and in $\beta 2$ -VEC mice [$F(1,8) = 32.183$, $p < 0.001$], but no significant effects of day or interaction between factors were observed for either genotype.

The average number of nose-pokes during 10 days in KO-GFP and $\beta 2$ -VEC mice is shown in Figure 4C. Two-way ANOVA showed significant main effects of genotype [$F(1,40) = 6.219$, $p < 0.05$], hole [$F(1,40) = 27.748$, $p < 0.001$] and significant interaction between both factors [$F(1,40) = 6.491$, $p < 0.05$]. Both KO-GFP and $\beta 2$ -VEC mice responded significantly more on the active than the inactive hole (KO-GFP: $p < 0.05$; $\beta 2$ -VEC: $p < 0.001$). However, $\beta 2$ -VEC mice showed significantly more active nose-pokes during nicotine self-administration training than KO-GFP controls ($p < 0.001$).

Autoradiography of [125 I]epibatidine binding confirmed the re-expression of $\beta 2$ receptor subunits in the VTA in $\beta 2$ -VEC mice (Figure 5). As in previous studies (Maskos et al., 2005), we observed approximately 50% re-expression of these receptor subunits in the VTA of $\beta 2$ -VEC as compared to WT mice.

4. Discussion

In this study, we demonstrate that $\beta 2$ KO mice do not reliably acquire chronic intravenous nicotine self-administration, and that the selective re-expression of this receptor subunit in the VTA restores this behaviour to similar levels than WT control mice.

The dose-response curve for nicotine using a between-subjects design showed that WT mice responded at higher rates for the lowest dose tested (15 μ g/kg/infusion), while higher doses (30 and 60 μ g/kg/infusion) induced lower rates of responding. In addition, the percentage of mice reaching the acquisition criteria was also dose-dependent (87.5 % for the dose of 15 μ g/kg/infusion, 50 % the dose of 30 μ g/kg/infusion, and 12.5 % for the dose of 60 μ g/kg/infusion). In contrast, $\beta 2$ KO mice responded at low rates for all doses of nicotine tested. Although a significant dose-response effect was observed for the mean number of infusions earned in 10 days, only a low percentage of mice (37.5 %) reached acquisition criteria for the dose of 15 μ g/kg/infusion, while none of the mice acquired nicotine self-administration at the doses of 30 μ g/kg/infusion or 60 μ g/kg/infusion. We also performed a within-subjects dose response curve for nicotine in WT mice. This experiment revealed significantly higher rates of responding for the dose of 7.5 μ g/kg/infusion of nicotine with respect to saline. Interestingly, a significant discrimination between active and inactive nose-poke was observed for all doses of nicotine, but not for saline.

These results are in line with previous data obtained under similar experimental conditions showing that the acquisition of nicotine self-administration follows an inverted U-shaped dose-effect curve in C57BL/6J mice (Galeote et al., 2009). In accordance, studies using a within-subjects dose-response paradigm also report an

inverted U-shaped dose-response curve for nicotine doses between 30-400 $\mu\text{g/kg/infusion}$ in this mouse species (Fowler and Kenny, 2011). However, other data show a relatively flat dose-response curve for nicotine doses between 10-60 $\mu\text{g/kg/infusion}$, with the high dose of 100 $\mu\text{g/kg/infusion}$ decreasing response rates (Contet et al., 2010).

In order to evaluate whether WT and $\beta 2\text{KO}$ mice previously trained to self-administer nicotine were sensitive to its omission, we performed a saline substitution paradigm with the continued presentation of the cue-light stimulus. The results indicate that WT mice undergo typical extinction behaviour when saline is substituted for nicotine, i.e. transient increases followed by decreased responding, supporting a clear sensitivity for the reinforcing effects of nicotine. In contrast, this effect is not observed in $\beta 2\text{KO}$ mice corroborating the lack of reinforcing effects of nicotine in these mice.

These results substantiate the role of $\beta 2$ receptor subunits in the reinforcing properties of nicotine identified in previous animal studies (Picciotto et al., 1998; Epping-Jordan et al., 1999; Pons et al., 2008). Moreover, they agree with human genetic studies relating a singular nucleotide polymorphism of the $\beta 2$ receptor subunit gene with early responses to nicotine and alcohol (Ehringer et al., 2007). However, $\beta 2$ receptor subunits are present in several brain structures of the cortico-limbic system where they may mediate the cognitive, rewarding and reinforcing effects of nicotine (Mansvelder et al., 2009). Currently, the specific contribution of each of these structures to nicotine addiction is still under investigation.

The chronic intravenous nicotine self-administration animal model provides a relevant method to evaluate the addictive potential of nicotine (Corrigall, 1999). Using this procedure, we show that $\beta 2\text{-VEC}$ mice reliably acquired and maintained self-administration of an effective dose of nicotine in a manner similar to WT mice, while

KO-GFP mice did not reach acquisition criteria. These results are supported by previous data indicating that $\beta 2$ receptor subunits in the VTA are critical for acute nicotine reinforcement in studies using an acute model of self-administration (Pons et al., 2008), and in studies where mice self-infuse nicotine directly into the VTA (Maskos et al. 2005). Our present data clearly demonstrate that $\beta 2$ receptor subunits in the VTA are sufficient for the establishment of a chronic pattern of nicotine self-administration, similar to that observed in human smokers.

The involvement of VTA $\beta 2$ receptor subunits in the reinforcing effects of nicotine could be due to the modulation of mesocorticolimbic DA activity. In this respect, it has been shown that systemic administration of nicotine decreased the basal firing rate of DA neurons in the VTA and abolished burst firing in $\beta 2$ KO mice (Mameli-Engvall, et al., 2006). Moreover, the selective re-expression of $\beta 2$ receptor subunits in the VTA restores nicotine-induced increases in the firing rate of DA neurons and the enhancement of extracellular DA concentrations in the NAC (Maskos et al. 2005; Reperant et al., 2010). However, the question still remains as to which cell type in the VTA containing $\beta 2$ receptor subunits, including DAergic, GABAergic or glutamatergic neurons specifically contribute to nicotine-induced modulation of DA activity. In this study, $\beta 2$ receptor subunits were re-expressed in most of the VTA using the coordinates detailed in the Methods section. However, there is evidence suggesting the functional heterogeneity of the VTA in terms of nicotine reinforcement since rats will self-infuse nicotine into the posterior, but not the anterior VTA (Ikemoto et al., 2006). Thus, future experiments using the chronic model of nicotine self-administration should be crucial for determining what region- and neurotransmitter-specific neuronal population in the VTA containing $\beta 2$ receptor subunits is involved in the reinforcing properties of nicotine.

In summary, the present study used advanced genetic techniques and behavioural models with high construct and predictive validity to demonstrate the relevance of $\beta 2$ receptor subunits within the VTA in the acquisition and maintenance of systemic nicotine self-administration behaviour. The reliability of these animal models facilitates the extrapolation of the results to the human conditions of nicotine consumption.

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Figure Legends

Figure 1. Time-course of nicotine self-administration during 10 days in WT and β 2KO mice at different doses (15, 30 and 60 μ g/kg/infusion; $n = 6-8$ per group). The data are expressed as mean + SEM number of nose-pokes on the active and inactive holes in 1 h session per day. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; active vs. inactive holes.

Figure 2. Dose-response effects for nicotine self-administration. (A) Between-subjects dose-response curve showing the mean number of infusions + SEM received by WT and β 2KO mice during 10 days of nicotine self-administration at different doses (15, 30 and 60 μ g/kg/infusion; $n = 6-8$ per group). * $p < 0.05$, ** $p < 0.01$; vs. the dose of 15 μ g/kg/infusion). (B) Within-subjects dose-response curve for WT mice ($n = 5$) showing active and inactive responding + SEM in 1 h sessions for nicotine at different doses (7.5, 15, 30, 60 μ g/kg/infusion) and saline. * $p < 0.05$, ** $p < 0.01$; active vs. inactive hole; + $p < 0.05$; vs. saline.

Figure 3. Saline substitution paradigm in WT and β 2KO mice. Active and inactive responding + SEM for nicotine at the dose of 15 μ g/kg/infusion during 10 days of training followed by saline substitution for 7 days in WT ($n = 7$) (A), and β 2KO mice ($n = 5$) (C) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; active vs. inactive hole. Differences between the number of infusions + SEM received by WT (B) and β 2KO mice (D) during the last day of nicotine self-administration, and the first and last days of saline substitution. * $p < 0.05$, *** $p < 0.001$; vs. last day of nicotine self-administration.

Figure 4. Nicotine self-administration at the dose of 15 µg/kg/infusion during 10 days of training in (A) KO-GFP (n = 13) mice and (B) β2-VEC (n = 9) mice. Panel C shows average responding + SEM in the active and inactive holes during the 10 days of training in KO-GFP and β2-VEC mice (* p < 0.05, *** p < 0.001).

Figure 5. Representative coronal sections at AP: -3.4 mm from bregma showing [125I]-epibatidine autoradiography in WT, β2-VEC and KO-GFP control mice. The arrows indicate binding of β2 receptor subunits in the VTA in WT mice, its absence in KO-GFP mice, and re-expression in β2-VEC mice.

References

Van der Geer, J., Hanraads, J.A.J., Lupton, R.A., 2000. The art of writing a scientific article. *J. Sci. Commun.* 163, 51–59.

Berrendero, F., Mendizábal, V., Robledo, P., Galeote, L., Bilkei-Gorzo, A., Zimmer, A., Maldonado, R., 2005. Nicotine-induced antinociception, rewarding effects, and physical dependence are decreased in mice lacking the preproenkephalin gene. *J Neurosci.* 25, 1103-12.

Contet, C., Whisler, K.N., Jarrell, H., Kenny, P.J., Markou, A., 2010. Patterns of responding differentiate intravenous nicotine self-administration from responding for a visual stimulus in C57BL/6J mice. *Psychopharmacology (Berl)* 212, 283-99.

Corrigall, W.A., 1999. Nicotine self-administration in animals as dependence model. *Nicotine Tob. Res.* 1, 11–20.

David, V., Besson, M., Changeux, J.P., Granon, S., Cazala, P., 2006. Reinforcing effects of nicotine microinjections into the ventral tegmental area of mice: Dependence on cholinergic nicotinic and dopaminergic D1 receptors. *Neuropharmacology* 50, 1030-1040.

Di Chiara, G., 2000. Role of dopamine in the behavioural actions of nicotine related to addiction. *Eur. J. Pharmacol.* 393, 295-314.

Ehringer, M.A., Clegg, H.V., Collins, A.C., Corley, R.P., Crowley, T., Hewitt, J.K., Hopfer, C.J., Krauter, K., Lessem, J., Rhee, S.H., Schlaepfer, I., Smolen, A., Stallings,

M.C., Young, S.E., Zeiger, J.S., 2007. Association of the neuronal nicotinic receptor beta2 subunit gene (CHRNA2) with subjective responses to alcohol and nicotine. *Am J Med. Genet. B. Neuropsychiatr. Genet.* 144B, 596-604.

Epping-Jordan, M.P., Picciotto, M.R., Changeux, J.P., Pich, E.M., 1999. Assessment of nicotinic acetylcholine receptor subunit contributions to nicotine self-administration in mutant mice. *Psychopharmacology* 147, 25-26.

Fowler, CD, Lu, Q., Johnson, P.M., Marks, M.J., Kenny, P.J., 2011. Habenular $\alpha 5$ nicotinic receptor subunit signalling controls nicotine intake. *Nature* 471, 597-601.

Fowler, C.D., Kenny, P.J., 2011. Intravenous nicotine self-administration and cue-induced reinstatement in mice: Effects of nicotine dose, rate of drug infusion and prior instrumental training. *Neuropharmacology* May 25. [Epub ahead of print]

Franklin, K.B.J., Paxinos, G., 1997. *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press.

Galeote, L., Berrendero, F., Bura, S.A., Zimmer, A., Maldonado, R., 2009. Prodynorphin gene disruption increases the sensitivity to nicotine self-administration in mice. *Int. J. Neuropsychopharmacol.* 12, 615-25.

Grenhoff, J., Aston-Jones, G., Svensson, T.H., 1986. Nicotinic effects on the firing pattern of midbrain dopamine neurons. *Acta Physiol. Scand.* 128, 351–358.

Hatsukami, D.K., Stead, L.F., Gupta, P.C., 2008. Tobacco addiction. *Lancet* 371, 2027-38.

Ikemoto, S., Qin, M., Liu, Z.H., 2006. Primary reinforcing effects of nicotine are triggered from multiple regions both inside and outside the ventral tegmental area. *J. Neurosci.* 26, 723–730.

Mameli-Engvall, M., Evrard, A., Pons, S., Maskos, U., Svensson, T.H., Changeux, J.P., Faure, P., 2006. Hierarchical control of dopamine neuron-firing patterns by nicotinic receptors. *Neuron* 50, 911-921.

Mansvelder, H.D., Mertz, M., Role, L.W., 2009. Nicotinic modulation of synaptic transmission and plasticity in cortico-limbic circuits. *Semin. Cell. Dev. Biol.* 20, 432-40.

Martín-García, E., Barbano, M.F., Galeote, L., Maldonado, R., 2009. New operant model of nicotine-seeking behaviour in mice. *Int. J. Neuropsychopharmacol.* 12, 343-56.

Maskos, U., Molles, B.E., Pons, S., Besson, M., Guiard, B.P., Guilloux, J.P., Evrard, A., Cazala, P., Cormier, A., Mameli-Engvall, M., Dufour, N., Clöez-Tayarani, I., Bemelmans, A.P., Mallet, J., Gardier, A.M., David, V., Faure, P., Granon, S., Changeux, J.P., 2005. Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors. *Nature* 436, 103-107.

Maskos, U., 2008. The cholinergic mesopontine tegmentum is a relatively neglected nicotinic master modulator of the dopaminergic system: relevance to drugs of abuse and pathology. *Br. J. Pharmacol.* 153, S438-45.

Nguyen, H.N., Rasmussen, B.A., Perry, D.C. 2003. Subtype-selective up-regulation by chronic nicotine of high-affinity nicotinic receptors in rat brain demonstrated by receptor autoradiography. *J. Pharmacol. Exp. Ther.* 307, 1090-1097.

Picciotto, M.R., Zoli, M., Rimondini, R., Lena, C., Marubio, L.M., Pich, E.M., Fluxe, K., Changeux, J.P. 1998. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. *Nature* 391, 173-177.

Plaza-Zabala, A., Martín-García, E., de Lecea, L., Maldonado, R., Berrendero, F., 2010. Hypocretins regulate the anxiogenic-like effects of nicotine and induce reinstatement of nicotine-seeking behavior. *J. Neurosci.* 30, 2300-10.

Pons, S., Fattore, L., Cossu, G., Tolu, S., Pocu, E., McIntosh, J.M., Changeux, J.P., Mascos, U., Fratta, W., 2008. Crucial role of $\alpha 4$ and $\alpha 6$ nicotinic acetylcholine receptor subunits from Ventral Tegmental Area in Systemic nicotine self-administration. *J. Neurosci.* 28, 12318-12327.

Pontieri, F.E., Tanda, G., Orzi, F., Di Chiara, G., 1996. Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature* 382, 255-7.

Rahman, S., Zhang, J., Corrigan, W.A., 2004. Local perfusion of nicotine differentially modulates somatodendritic dopamine release in the rat ventral tegmental area after nicotine preexposure. *Neurochem. Res.* 29, 1687–1693.

Reperant, C., Pons, S., Dufour, E., Rollema, H., Gardier, A.M., Maskos, U., 2010. Effect of the $\alpha 4\beta 2^*$ nicotinic acetylcholine receptor partial agonist varenicline on dopamine release in $\beta 2$ knock-out mice with selective re-expression of the $\beta 2$ subunit in the ventral tegmental area. *Neuropharmacology* 58, 346-350.

Soria, G., Mendizabal, V., Touriño, C., Robledo, P., Ledent, C., Parmentier, M., Maldonado, R., Valverde, O., 2005. Lack of CB1 cannabinoid receptor impairs cocaine self-administration. *Neuropsychopharmacology* 30, 1670-1680.

Tizabi, Y., Copeland, R.L. Jr., Louis, V.A., Taylor, R.E., 2002. Effects of combined systemic alcohol and central nicotine administration into ventral tegmental area on dopamine release in the nucleus accumbens. *Alcohol Clin. Exp. Res.* 26, 394 -399.