Addiction Biology

PRECLINICAL STUDY

doi:10.1111/j.1369-1600.2010.00246.x

Differential region-specific regulation of α4β2* nAChRs by self-administered and non-contingent nicotine in C57BL/6J mice

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ABSTRACT

Neuronal nAChR upregulation is the hallmark of chronic nicotine exposure. Neuroplasticity to abused drugs, however, depends on whether their administration is forced by the experimenter or is under the control of the experimental animal. Neuroadaptation to chronic nicotine self-administration was examined with a yoked-control paradigm, using nose-poking as the operating procedure. Freely moving C57BL/6J mice that responded for 0.03 mg/kg/infusion of intravenous nicotine under a continuous schedule of reinforcement (FR-1), had control over the rate and amount of drug intake that a yoked littermate passively received ($n = 11$). The impact of response dependency on neurobiological changes in nicotinic and dopaminergic systems was subsequently assessed using quantitative autoradiography. Cytisine-sensitive [^{125}I]epibatidine binding, [^{3}H]SCH23390, [^{3}H]raclopride and [^{3}H]mazindol were used to label nAChRs with α4β2* subtype properties, D1 and D2 dopaminergic receptors, and dopamine transporters, respectively. During a period of 12 days, self-administration was reliably initiated and maintained in animals receiving response-contingent nicotine. Region specific changes in the density of α4β2* nAChRs were found to be dependent on the contingency of nicotine treatment. Higher levels of α4β2* receptor binding were observed in the dorsal lateral geniculate nucleus and the ventral tegmental area of self-administering mice, compared to non-contingent animals. Moreover, response-independent increases in D2 binding were observed following chronic nicotine administration. No change in D1 and DAT binding was observed among groups. These findings indicate regional specific alterations in the regulation of the nicotinic cholinergic system following contingent and non-contingent nicotine exposure, and underline the importance of response dependency on the development of nicotine addiction.

Keywords Autoradiography, mouse, nicotine, operant contingency, self-administration.

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INTRODUCTION

Nicotine exerts its reinforcing effects by binding in the brain to its corresponding pharmacological targets, the neuronal nicotinic receptors (nAChRs). These are a heterogeneous family of pentameric structures, formed by the assembly of five α and β subunits. To date, 12 different subunits have been identified (α2–α10, β2–β4), whose combinations give rise to nAChRs with unique pharmacological properties and distinct topographical distribution (reviewed in Gotti & Clementi 2004). This prominent heterogeneity mediates nicotine's abuse-related potential, as the drug has been shown to differentially modulate the function of distinct nAChR subtypes, thereby stimulating and coordinating reward-related signaling in areas of the brain that mediate reinforcement (Pidoplichko *et al.* 1997; Mansvelder & McGehee 2000; Mansvelder, Keath & McGehee 2002). Nevertheless, the transition from the activation of individual nAChR subpopulations to addiction is a complicated and multistage process, which involves the acquisition, maintenance, extinction and reinstatement of drug-taking. Therefore, behavioral paradigms that model distinct stages of addictive behavior provide

important methodological tools for determining the neurobiological substrates that mediate the progression to nicotine addiction.

The observation that the administration of nicotine leads to increased brain nAChR density in cigarette smokers (Perry *et al*. 1999) and in animal models of nicotine consumption (Marks, Burch & Collins 1983; Schwartz & Kellar 1983) constitutes the hallmark of chronic nicotine exposure. This unusual phenomenon of agonist-induced receptor upregulation is considered to form the basis for many of nicotine's abuse-related effects, including tolerance and behavioral sensitization (Tapper *et al*. 2004; Nashmi *et al*. 2007). Of the multiple nicotinic subtypes that exist in the mammalian brain, α4β2* receptors are abundantly expressed throughout the central nervous system, and they readily upregulate following exposure to nicotine, compared with other nAChR subtypes (Nguyen, Rasmussen & Perry 2003; Marks *et al*. 2004). As shown in a conditioned place preference paradigm, a mutation that renders α4 subunits hypersensitive to agonists creates mice that are more susceptible to nicotine reinforcement than wild type animals (Tapper *et al*. 2004). Moreover, deletion of the β2 subunit gene attenuates nicotine self-administration (Picciotto *et al*. 1998) and its re-expression in the VTA of β2 subunit knockout animals re-establishes the behavior (Maskos *et al*. 2005). Altogether, these data demonstrate an important role for α4β2* nAChRs in nicotine addiction.

Among the paradigms used to model different aspects of drug reinforcement, self-administration has been favored as the most direct way of investigating the behavioral and neurobiological processes that lead to addiction. Its face validity is supported by increasing evidence, which suggests that drugs of abuse exert different effects, depending on whether their administration is forced by the experimenter or is under the control of the experimental animal (reviewed in Jacobs *et al*. 2003). The yoked-control paradigm has been particularly designed to examine differences produced by the active or passive drug administration. In this model, self-administering animals have control over the rate and amount of drug intake that a yoked littermate passively receives in an identical environment. Thus, potential differences in the consequences of active versus passive administration can be attributed to having control over the pattern of drug exposure. To determine the extent to which nicotine-induced neuroadaptation is the consequence of the drug's direct pharmacological action or the result of the contingency between a subject's response and the delivery of a reinforcer, a yoked-control paradigm of chronic nicotine administration in mice was used, using nose-poking as the operating response. The latter is considered to more naturally mimic smoking behavior in mice, requiring less motor and motivational output than lever pressing responding (Pons *et al*. 2008). Subsequently, we examined the consequences of active versus passive nicotine exposure on the nicotinic cholinergic and dopaminergic systems of C57BL/6J mice by means of quantitative autoradiography of cytisine-sensitive heteromeric nicotinic receptors, D1 and D2 dopaminergic receptors, and of dopamine transporters, in an effort to clarify the neurochemical determinants that mediate the acquisition of nicotine self-administration.

MATERIALS AND METHODS

Self administration paradigm

Animals

C57BL/6J male mice (Charles River, L'Arbresle, France), weighing 24–26 g at the beginning of the study were used. Animals were housed individually in controlled laboratory conditions under a reversed 13 hours/11 hours dark/light cycle (lights off at 7:30 a.m., lights on at 8:30 p.m.), with the temperature maintained at 21 ± 1°C, and humidity at 55 ± 10%. The circadian cycle used allowed us to maximize the time required to perform the experimental manipulations without substantial modifications of the 12 hours/12 hours cycle commonly used. Mice were tested during the dark phase of the dark/light cycle. Food and water were available *ad libitum*, except during the experimental sessions. 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).

Surgery and self-administration procedure

Mice were anesthetized with a mixture of ketamine (100 mg/kg; Imalgène 1000; Rhône Mérieux, Lyon, France) and xylazine (20 mg/kg; Sigma, Madrid, Spain), and then implanted with indwelling i.v. catheters as previously described (Soria *et al*. 2005). Following surgery, all incisions were sutured and coated with antibiotic ointment (Bactroban, GlaxoSmithKline, Madrid, Spain), and animals were allowed to recover for 3 days. Mice were subsequently randomly assigned to contingent or yoked groups.

Drug self-administration experiments were conducted in mouse operant chambers (Model ENV-307A-CT, Medical Associates, Georgia, VT, USA), housed in sound- and light-attenuated boxes that were equipped with fans to provide ventilation and white noise. The house light was on at the beginning of the session for 3 seconds and off during the remaining time. The chambers were equipped with two holes, of which one was selected as the active and the other as the inactive hole. Responding in both holes was recorded for all groups, throughout the

experiments. The contingent group was trained to self-administer nicotine (0.03 mg/kg/infusion delivered in a volume of 23.5 µl over 2 seconds; Sigma) in single daily 1-hour sessions for a period of 12 days. Even though nicotine self-administration is already acquired after 10 days of training (Martín-García *et al.* 2009, Plaza-Zabala *et al.* 2010), we run the animals for two additional days in order to maximize stability in the observed behavior and in the possible neural modifications under study. Previous experiments in our laboratory indicated that a priming injection at the beginning of the session facilitates the acquisition of nicotine self-administration (Martín-García *et al.* 2009; Plaza-Zabala *et al.* 2010), so each session started with a priming injection of nicotine or saline (depending on the group). Acquisition of nicotine self-administration was conducted under a fixed ratio 1 (FR-1) schedule of reinforcement; one poke in the active nose hole resulted in one nicotine infusion, while nose poking in the inactive hole had no programmed consequences. A 10-second time-out period was established following each nicotine infusion. During this period, responses on the active and inactive holes were recorded but had no programmed consequences. The session was terminated after 50 infusions had been delivered, or after 1 hour, whichever occurred first. Each contingent mouse was connected to two animals, one in the yoked nicotine and one in the yoked saline groups. Yoked mice passively received the same number of drug infusions as compared with their self-administration partners, at identical times during each session. Their nose-poking activity on the active and inactive holes had no consequences. A stimulus light located above the active hole was paired with the delivery of nicotine or saline, according to the response of the contingent mouse. The patency of the intravenous catheters was evaluated periodically, and whenever drug self-administration behavior appeared to deviate dramatically from that observed previously, by the infusion of 0.1 ml of thiobarbital (5 mg/ml) through the catheter. If prominent signs of anesthesia were not apparent within 3 seconds of the infusion, the mouse and its corresponding data were removed from the experiment. The criteria for the acquisition of self-administration behavior have been described previously (Orejarena *et al.* 2009; Trigo, Zimmer & Maldonado 2009), and were achieved when all of the following conditions had been met: a stable response, with less than 20% deviation from the mean of the total number of reinforces earned in three consecutive sessions (80% of stability), at least 65% of response on the active hole and a minimum of four nicotine infusions earned per experimental session. After each session, animals were returned to their home cages. At the end of the study, mice were killed and brains were rapidly removed and frozen for storage at −80°C for subsequent quantitative autoradiography studies.

Quantitative receptor autoradiography

Tissue sectioning was carried out at −21°C using a Zeiss Microm HM505E cryostat (Carl Zeiss, Microm Laborgërate, GmbH, Waldörf, Germany). Frozen coronal sections (20 µm) were cut at 300 µm intervals, from rostral to caudal levels. Quantitative autoradiography was performed in order to measure nicotinic receptors with α4β2* subtype properties, dopaminergic D1 and D2 receptors, and the dopamine transporters (DAT). On the day of each experiment, sections were thawed and processed according to established protocols, with minor modifications (Javitch, Blaustein & Snyder 1984; Marks *et al.* 2002; Lena *et al.* 2004; Bailey *et al.* 2008). All mice were used for quantitative autoradiography of nAChRs. Dopaminergic receptor and transporter binding was performed in three self-administering mice that achieved acquisition criteria, three that did not and their corresponding yoked controls ($n = 6$ per group). Multiple, adjacent sections from all groups were processed together in a paired binding protocol. Radioligand bound sections were apposed to Kodak BioMax MR-1 film (Sigma-Aldrich, Gillingham, UK), along with appropriate microscale standards, to allow quantification. All structures were identified by reference to the mouse brain atlas of Franklin & Paxinos (2001), and analyzed using a MCID image analyzer (Image research, Linton, UK).

For nicotinic receptor binding, sections were pre-incubated for 10 minutes at room temperature in Tris–HCl buffer, containing 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4, followed by incubation with 100 pM [^{125}I]epibatidine (specific activity, 2200 Ci/mmol; PerkinElmer Life Sciences, Boston, MA, USA) for 2 hours at room temperature. Determination of subtype-specific binding was performed using adjacent sections from each brain, in order to measure total [^{125}I]epibatidine binding (no competing ligand), and [^{125}I]epibatidine binding in the presence of 20 nM cytisine (Sigma-Aldrich). Competition of [^{125}I]epibatidine binding by unlabeled cytisine has been used to reveal two subpopulations of nAChRs with high and low affinity for cytisine, termed cytisine-sensitive and cytisine-resistant [^{125}I]epibatidine binding sites, respectively. The cytisine-sensitive [^{125}I]epibatidine binding sites correspond to α4β2* nicotinic receptors (Marks, Smith & Collins 1998; Whiteaker *et al.* 2000), and were calculated after the subtraction of cytisine-resistant from total [^{125}I]epibatidine binding. To determine non-specific binding, further adjacent sections were incubated with [^{125}I]epibatidine in the presence of 300 µM of (–) nicotine hydrogen tartrate (Sigma-Aldrich). Incubations were terminated by two 10-minute washes into ice-cold 50 mM Tris–HCL buffer (pH 7.4) and a rapid rinse in ice-cold water. Radioligand bound sections were apposed to film for 24 hours. Structures of

exceptionally high nAChR density, including the medial habenula, fasciculus retroflexus, and the interpeduncular nucleus were apposed for 6 hours, to avoid film saturation.

For D1 and D2 receptor binding, all sections were first pre-incubated for 20 minutes in 50 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, at room temperature. For D1 binding, sections were then incubated for 90 minutes in the same buffer, at room temperature, in the presence of 4 nM [^{3}H]SCH23390 (specific activity, 70.3 Ci/mmol; PerkinElmer Life Sciences) and 1 μM mianserin, in order to avoid the binding of [^{3}H]SCH23390 to 5-HT_2 and 5-HT_{1c} receptors. To label D2 receptors, incubation was carried out for 60 minutes in the presence of 4 nM [^{3}H]raclopride (specific activity, 60.1 Ci/mmol; PerkinElmer Life Sciences), under identical pH and temperature conditions. Non-specific binding was determined on adjacent sections in the presence of 10 μM of *cis*-flupenthixol for D1 receptors or 10 μM of sulpiride for D2 receptors. The incubations were terminated by rapid rinses (6 × 1 minutes) in ice-cold 50 mM Tris–HCl buffer (pH 7.4) followed by a dip into ice-cold distilled water. For D1 and D2 receptors, radioligand bound sections were apposed to film for 5 and 6 weeks, respectively.

For the DAT, all slides were pre-incubated for 5 minutes at 4°C in 50 mM Tris-HCl buffer, pH 7.9, containing 300 mM NaCl and 5 mM KCl. The slides were subsequently incubated at 4°C for 45 minutes in the same buffer, containing 4 nM [^{3}H]mazindol (specific activity, 20.6 Ci/mmol; PerkinElmer Life Sciences) and 0.3 μM desipramine, to block the binding to norepinephrine uptake sites. Non-specific binding was determined in the presence of 10 μM mazindol. After incubation, sections were rinsed twice for 1 minute in ice-cold Tris buffer, and briefly dipped in ice-cold distilled water. Sections were apposed to film for 5 weeks.

Statistical analysis

Behavioral results were analyzed using a mixed-design multivariate analysis of variance (MANOVA), with group (contingent, non-contingent, and saline-treated) as the between-subjects variable. Daily sessions and hole (active vs. inactive) represented the within-subjects variable. Overall interactions were further analyzed using the Newman-Keuls *post hoc* test, when the initial *P* value was smaller than 0.05. All the results are expressed as mean ± standard error of the mean (SEM).

For quantitative autoradiography, the mean ± SEM of radioligand binding was calculated in each region for all three treatment groups, as previously described (Kitchen *et al.* 1997). Two-way ANOVA for the factors treatment and region was carried out in order to compare quantitative measurements of autoradiographic binding in brain areas of contingent, non-contingent, and saline-treated animals. Structures of the habenulo-interpeduncular pathway were quantified using the 6 hours exposure to film, due to the high densities of heteromeric nAChRs in this cholinergic system. Therefore, comparisons between regions of exceptionally high nicotinic receptor density were performed using a separate two-way ANOVA (independent factors treatment and region). Quantitative differences in nicotinic and dopaminergic binding sites between self-administering mice that achieved acquisition criteria and those that did not were analyzed using a separate two-way ANOVA, for the factors acquisition and region. LSD *post hoc* analysis was applied, when appropriate, to investigate differences in radioligand binding between groups in individual regions.

All data were analyzed using the Statistica software (StatSoft Inc., Maisons-Alfort, France).

RESULTS

Nicotine self-administration

Figure 1 shows the mean number of nose-pokes in the active and inactive holes, carried out by nicotine contingent, non-contingent and saline-treated animals under an FR-1 schedule of reinforcement, for each of the 1-hour daily self-administration sessions. At the nicotine dose of 0.03 mg/kg/infusion, contingent mice started to discriminate between the active and inactive holes from the first training session (Fig. 1a). The percentage of animals that proceeded to complete all of the criteria for stable nicotine self-administration was 54.5% (6 out of 11), and the mean time of acquisition was 8.83 ± 1.19 days. On the contrary, nicotine- and saline-yoked mice did not discriminate between holes in any of the experimental sessions (Fig. 1b and c). On days 1 and 2, the number of nose pokes was enhanced in all groups, compared to days 3–12. The mean nicotine intake decreased from 0.38 ± 0.01 mg/kg/hour on days 1 and 2, to 0.22 ± 0.01 mg/kg/hour for days 3–12. Contingent mice showed a mean cumulative nicotine intake of 0.25 ± 0.04 mg/kg/hour throughout the training sessions. In the case of the animals that achieved acquisition criteria, the mean intake of nicotine during the last 3 days of self-administration was 0.35 ± 0.09 mg/kg/hour. The overall ANOVA revealed a statistically significant effect of the factors group ($F_{2,58} = 12.0, P < 0.001$), time ($F_{11,638} = 15.2, P < 0.001$), and nose-poke ($F_{1,58} = 22.6, P < 0.001$), as well as significant group × nose-poke interaction effects ($F_{2,58} = 7.8, P < 0.001$). Newman-Keuls *post hoc* analysis confirmed that the responses on the active and inactive nose-pokes were significantly higher only in the contingent group and not in the yoked groups.

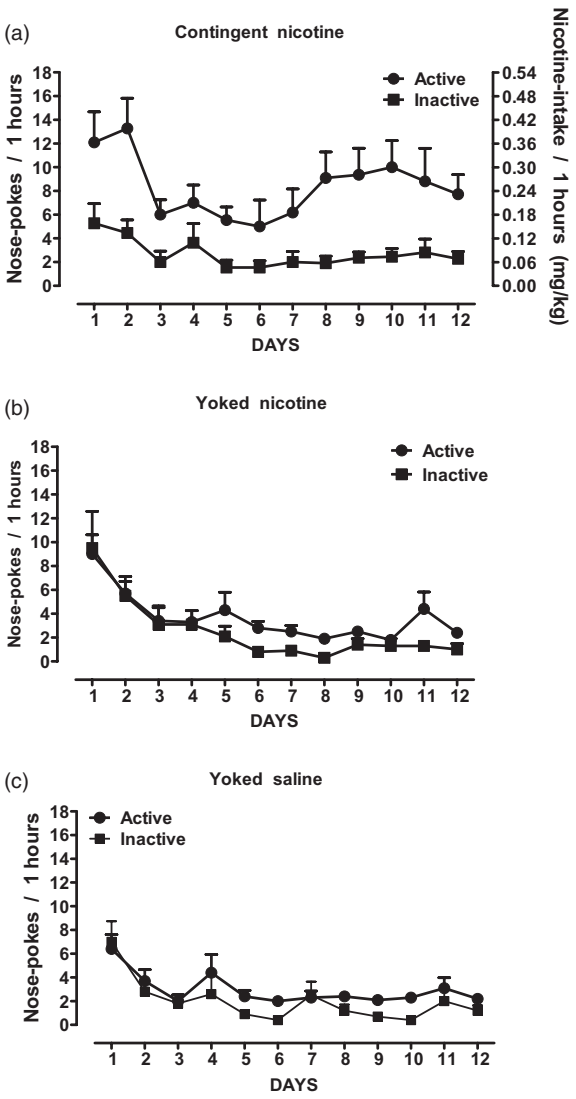

Figure 1 Acquisition and maintenance of nicotine self-administration in nicotine contingent and non-contingent C57BL/6J mice. Data are expressed as mean ± SEM of the number of nose pokes in the active and the inactive holes during the 1-hour sessions performed over 12 days at 0.03 mg/kg/infusion. Following day 7, reliable drug-taking behavior was observed in animals receiving response-dependent nicotine, which was maintained throughout the duration of this experiment. The mean cumulative nicotine intake throughout the training sessions was at 0.25 ± 0.04 mg/kg/hour. $n = 11$ for nicotine-treated animals, $n = 10$ for saline-yoked mice

Quantitative receptor autoradiography

Effects of response contingency on nAChR binding

Quantitative autoradiography was conducted for α4β2* nAChRs, using 100 pM of [^{125}I]epibatidine alone or in the presence of cytisine (20 nM). The majority of mouse brain [^{125}I]epibatidine binding sites were of the cytisine-sensitive, α4β2* subtype. Non-specific [^{125}I]epibatidine binding was indistinguishable from film background (Fig. 2). The density of cytisine-sensitive and cytisine-resistant [^{125}I]epibatidine binding in brain regions of saline, contingent and non-contingent mice is detailed for all areas analyzed in Tables 1 and 2, respectively. Figure 3 shows representative autoradiograms of [^{125}I]epidatidine binding sites in coronal brain sections from saline and nicotine treated mice, cut at the level of the thalamus and of the ventral tegmental area.

Significantly higher levels of α4β2* nAChRs were observed in the dorsal lateral geniculate nucleus (DLG) and the VTA of self-administering mice, compared to non-contingent animals and to saline controls. Contingent nicotine also increased cytisine-sensitive [^{125}I]epibatidine binding in the superficial gray layers of the superior colliculus (SuG) and certain thalamic nuclei, such as the ventral lateral geniculate nucleus (VLG), the posterior thalamic nuclear group (Po) and the lateral posterior thalamic nucleus (LPMR), compared to saline treatment. In non-contingent animals, higher levels of α4β2* receptor binding were observed in the VLG and SuG compared to saline controls. No differences in cytisine-sensitive [^{125}I]epibatidine binding sites were detected between contingent mice that achieved acquisition criteria, and animals that did not stably respond for nicotine ($F_{1,140} = 1.8$, $P > 0.05$). For nAChRs with α4β2* nAChR properties, two-way ANOVA showed significant main effects of treatment ($F_{2,463} = 20.4$, $P < 0.001$) and region ($F_{17,463} = 48.3$, $P < 0.001$), with no treatment-region interaction effects ($F_{34,463} = 1.2$, $P > 0.05$). LSD *post hoc* analysis confirmed significant, region-specific differences in cytisine-sensitive [^{125}I]epibatidine binding between self-administering and nicotine-yoked mice in the DLG and the VTA. Comparisons of cytisine-sensitive [^{125}I]epibatidine binding measurements between structures of high nicotinic receptor density revealed no treatment ($F_{2,43} = 0.3$, $P > 0.05$), and no treatment-region interaction effects between groups ($F_{4,43} = 0.2$, $P > 0.05$).

Cytisine-resistant [^{125}I]epibatidine binding sites represented the major nAChR population in areas of the habenulo-interpeduncular pathway, and the quantitative comparison of nAChR density in these brain regions revealed no treatment ($F_{2,45} = 1.0$, $P > 0.05$) or treatment-region interaction effect ($F_{4,45} = 0.5$, $P > 0.05$). Cytisine-resistant binding in the rest of brain regions analyzed was significantly lower in the SuG, the intermediate gray layer of the superior colliculus (InG), and the substantia nigra pars compacta (SNs) of nicotine yoked animals, compared to saline controls. A lower level of cytisine-resistant binding was also observed in the InG of self-administering animals, compared to controls. Two way ANOVA confirmed significant main effects of treatment ($F_{2,463} = 3.0$, $P < 0.05$) and region ($F_{17,463} = 216.8$, $P < 0.001$), with significant treatment-region interaction effects on cytisine-resistant receptor density ($F_{34,463} = 1.5$, $P < 0.05$). No differences were detected between

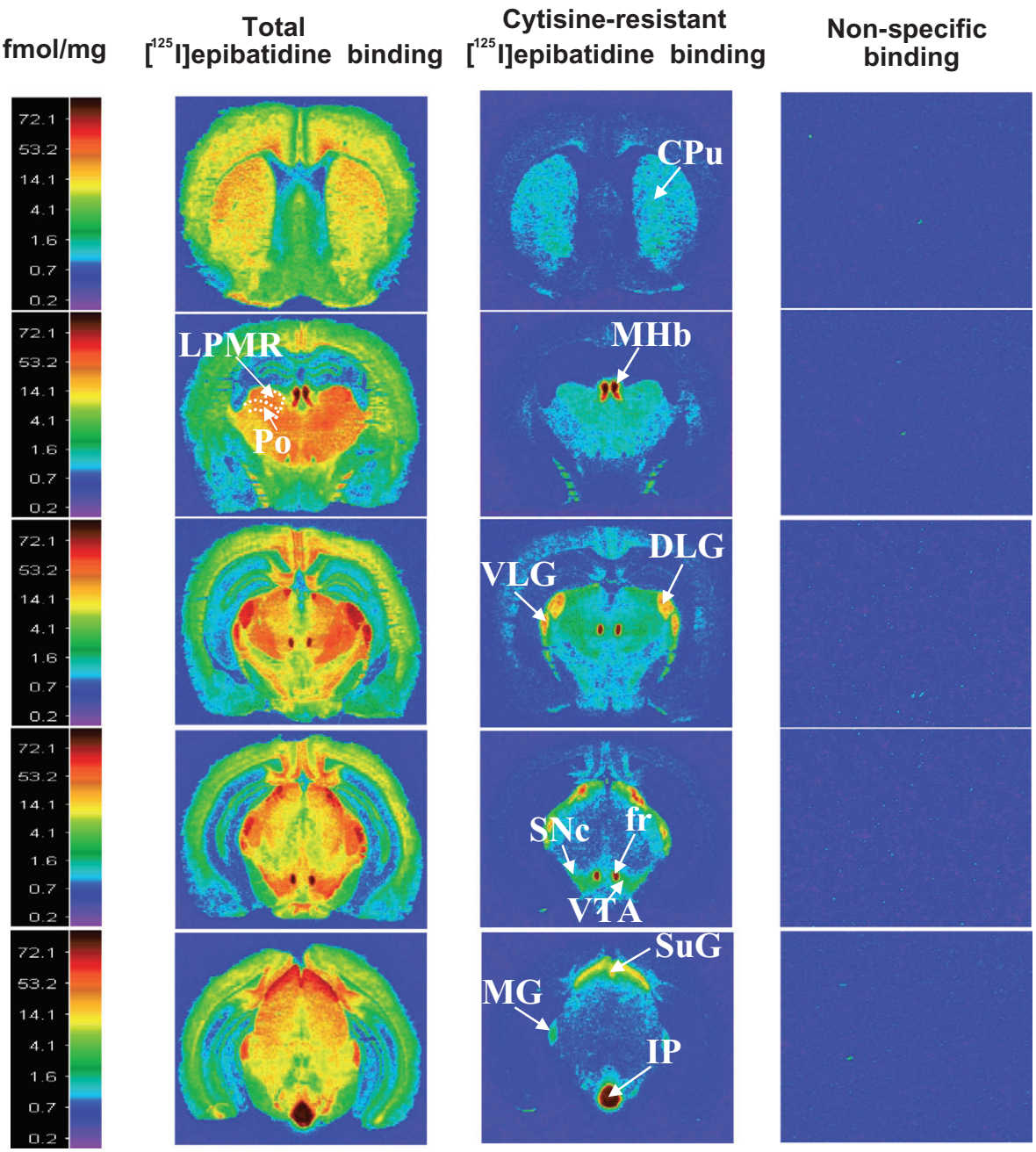

Figure 2 Computer-enhanced color autoradiograms of total, cytisine-resistant, and non-specific [^{125}I]epibatidine binding in coronal sections from saline-treated C57BL/6J mouse brain. Adjacent sections were incubated for 2 hours with 100 pM of [^{125}I]epibatidine alone (first column) or in the presence of 20 nM cytisine (second column), and 300 μM (–) nicotine hydrogen tartrate (third column). Non-specific binding was indistinguishable from film background. The majority of [^{125}I]epibatidine binding sites were of the cytisine-sensitive, α4β2* subtype. High levels of cytisine-resistant [^{125}I]epibatidine binding were observed in the medial habenula, fasciculus retroflexus, and interpeduncular nucleus. Sections were apposed to Kodak BioMax MR-1 film for a period of 6–24 hours. The color bar indicates a pseudo-color interpretation of black and white image density, calibrated in fmol/mg of tissue equivalent. The arrows point to areas of measurement for the Caudate Putamen (CPu), Medial Habenula (MHb), Lateral Posterior Thalamic Nucleus (LPMR), Posterior Thalamic Nuclear Group (Po), Dorsal and Ventral Lateral Geniculate Nuclei (DLG & VLG), Ventral Tegmental Area (VTA), Substantia Nigra, pars compacta (SNc), Fasciculus retroflexus (fr), Interpeduncular Nucleus (IP), Superficial Gray layer of the Superior Colliculus (SuG), and the Medial Geniculate Nucleus (MG)

Table 1 Quantitative autoradiography of cytisine-sensitive [^{125}I]epibatidine binding in nicotine contingent, non-contingent and saline-treated animals.

	Cytisine-sensitive [^{125}I]epibatidine binding (fmol/mg tissue)			*% change in binding*	
Region	*Saline*	*Contingent*	*Non-contingent*	*Saline versus contingent*	*Saline versus non-contingent*
Habenulo-interpenducular tract					
Medial habenular nucleus ($n = 6$)	7.1 ± 1.8	8.6 ± 2.1	5.9 ± 1.5	20.6	–17.0
Fasciculus retroflexus ($n = 6$)	9.2 ± 1.3	11.2 ± 2.1	11.3 ± 3.4	22.6	23.5
Interpeduncular nucleus ($n = 6$)	10.8 ± 6.0	12.3 ± 3.6	9.9 ± 3.4	13.5	–8.1
Dopaminergic regions					
Caudate Putamen ($n = 10$–11)	10.7 ± 0.6	11.4 ± 0.5	10.8 ± 0.4	6.6	0.9
Nucleus accumbens, core ($n = 9$–11)	7.3 ± 0.6	8.0 ± 0.4	7.3 ± 0.3	9.9	1.1
Nucleus accumbens, shell ($n = 9$–11)	7.6 ± 0.6	7.9 ± 0.4	7.5 ± 0.3	3.9	–1.3
Substantia nigra, pars compacta ($n = 9$–11)	17.2 ± 1.3	18.7 ± 1.0	17.9 ± 1.2	8.7	4.1
Ventral tegmental area ($n = 9$–11)	16.4 ± 1.2	20.4 ± 1.0*	17.0 ± 1.0$^{\#}$	24.3	3.4
Optic tract regions					
Visual cortex ($n = 10$–11)	9.2 ± 0.4	9.3 ± 0.4	8.8 ± 0.6	1.5	–3.9
Superficial gray layer of the superior colliculus ($n = 9$–11)	13.4 ± 1.2	18.2 ± 2.1*	17.2 ± 2.3*	36.2	28.7
Dorsal lateral geniculate nucleus ($n = 10$–11)	20.6 ± 0.9	27.8 ± 2.4***	23.4 ± 1.5$^{\#}$	35.0	13.6
Ventral lateral geniculate nucleus ($n = 10$–11)	17.3 ± 0.7	24.6 ± 1.8***	21.5 ± 1.5*	42.2	24.3
Other regions					
Accessory olfactory bulb ($n = 6$–11)	6.5 ± 0.8	9.3 ± 2.0	9.2 ± 1.0	43.1	41.5
Insular cortex ($n = 10$–11)	8.8 ± 0.3	9.4 ± 0.5	9.1 ± 0.4	6.8	3.4
Cingulate cortex ($n = 10$–11)	9.5 ± 0.5	9.7 ± 0.5	9.1 ± 0.4	2.6	–3.4
Olfactory tubercle ($n = 10$–11)	8.9 ± 1.0	9.8 ± 0.7	9.9 ± 0.8	10.5	11.6
Lateral posterior thalamic nucleus ($n = 10$–11)	18.8 ± 0.9	22.6 ± 1.1*	20.6 ± 1.1	19.8	9.6
Posterior thalamic nuclear group ($n = 10$–11)	18.3 ± 0.9	21.4 ± 1.8*	20.7 ± 1.6	17.2	13.2
Medial geniculate nucleus ($n = 8$–11)	19.7 ± 1.1	21.4 ± 1.0	20.3 ± 1.3	8.7	2.8
Intermediate gray layer of the superior colliculus ($n = 9$–11)	12.7 ± 1.0	15.8 ± 0.8	13.8 ± 1.2	24.2	8.9
Anteroventral thalamic nucleus ($n = 9$–11)	30.4 ± 0.8	31.2 ± 1.6	29.1 ± 2.1	2.5	–4.4
Mean				17.2	7.3

Values of cytisine-sensitive [^{125}I]epibatidine binding represent mean ± SEM in brain regions of 6–11 animals per group. Self-administered nicotine region-specifically increased α4β2* nAChRs in the dorsal lateral geniculate nucleus (DLG) and the ventral tegmental area (VTA) of self-administering mice, compared to non-contingent animals and to saline controls. *$P < 0.05$, ***$P < 0.001$ versus saline controls, $^{\#}P < 0.05$ versus contingent values, LSD *post hoc*.

Table 2 Quantitative autoradiography of cytisine-resistant [^{125}I]epibatidine binding in nicotine contingent, non-contingent and saline-treated animals.

	Cytisine-resistant [^{125}I]epibatidine binding (fmol/mg tissue)			*% change in binding*	
Region	*Saline*	*Contingent*	*Non-contingent*	*Saline versus contingent*	*Saline versus non-contingent*
Habenulo-interpenducular tract					
Medial habenular nucleus	84.1 ± 7.8	85.9 ± 7.2	81.1 ± 8.2	2.1	−3.5
Fasciculus retroflexus	65.7 ± 5.6	64.6 ± 6.9	63.8 ± 5.3	−1.7	−2.9
Interpeduncular nucleus	101.2 ± 6.1	99.3 ± 6.5	95.1 ± 6.9	−1.9	−6.0
Dopaminergic regions					
Caudate Putamen	2.1 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	0.5	2.8
Nucleus accumbens, core	2.0 ± 0.3	2.2 ± 0.2	2.0 ± 0.2	11.1	−1.5
Nucleus accumbens, shell	2.2 ± 0.2	2.1 ± 0.2	2.0 ± 0.2	−0.9	−5.6
Substantia nigra, pars compacta	5.5 ± 0.9	4.0 ± 0.7	3.7 ± 0.5*	−26.2	−32.1
Ventral tegmental area	6.8 ± 0.3	6.2 ± 0.5	5.3 ± 0.5	−9.7	−22.6
Optic tract regions					
Visual cortex	0.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	4.8	−29.0
Superficial gray layer of the superior colliculus	19.1 ± 1.0	19.3 ± 1.5	15.1 ± 1.2***	1.2	−20.6
Dorsal lateral geniculate nucleus	12.2 ± 0.5	11.4 ± 0.9	12.7 ± 1.0	−5.8	4.3
Ventral lateral geniculate nucleus	12.4 ± 0.6	11.1 ± 1.1	12.7 ± 1.4	−10.4	2.6
Other regions					
Accessory olfactory bulb	17.2 ± 1.0	15.9 ± 1.2	18.2 ± 3.3	−7.6	6.4
Insular cortex	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	14.6	−17.1
Cingulate cortex	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	−8.7	−13.0
Olfactory tubercle	1.2 ± 0.2	1.8 ± 0.2	1.4 ± 0.2	46.7	13.3
Lateral posterior thalamic nucleus	4.6 ± 0.3	4.7 ± 0.4	4.1 ± 0.3	2.9	−11.0
Posterior thalamic nuclear group	2.8 ± 0.2	2.9 ± 0.2	2.8 ± 0.3	5.5	1.8
Medial geniculate nucleus	3.2 ± 0.3	3.2 ± 0.3	2.8 ± 0.5	0.9	−13.1
Intermediate gray layer of the superior colliculus	5.3 ± 1.1	2.7 ± 0.3**	2.3 ± 0.5***	−49.5	−56.9
Anteroventral thalamic nucleus	4.4 ± 0.6	4.9 ± 0.5	4.8 ± 0.4	11.6	8.6
Mean				−1.1	−10.1

Values of cytisine-resistant [^{125}I]epibatidine binding represent mean ± SEM in brain regions of 6–11 animals per group. A significant treatment × region interaction effect on cytisine-resistant nAChR density was observed following the administration of nicotine. No differences were observed between animals that stably responded for nicotine, and mice that did not achieve acquisition criteria. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. saline controls, LSD post hoc.

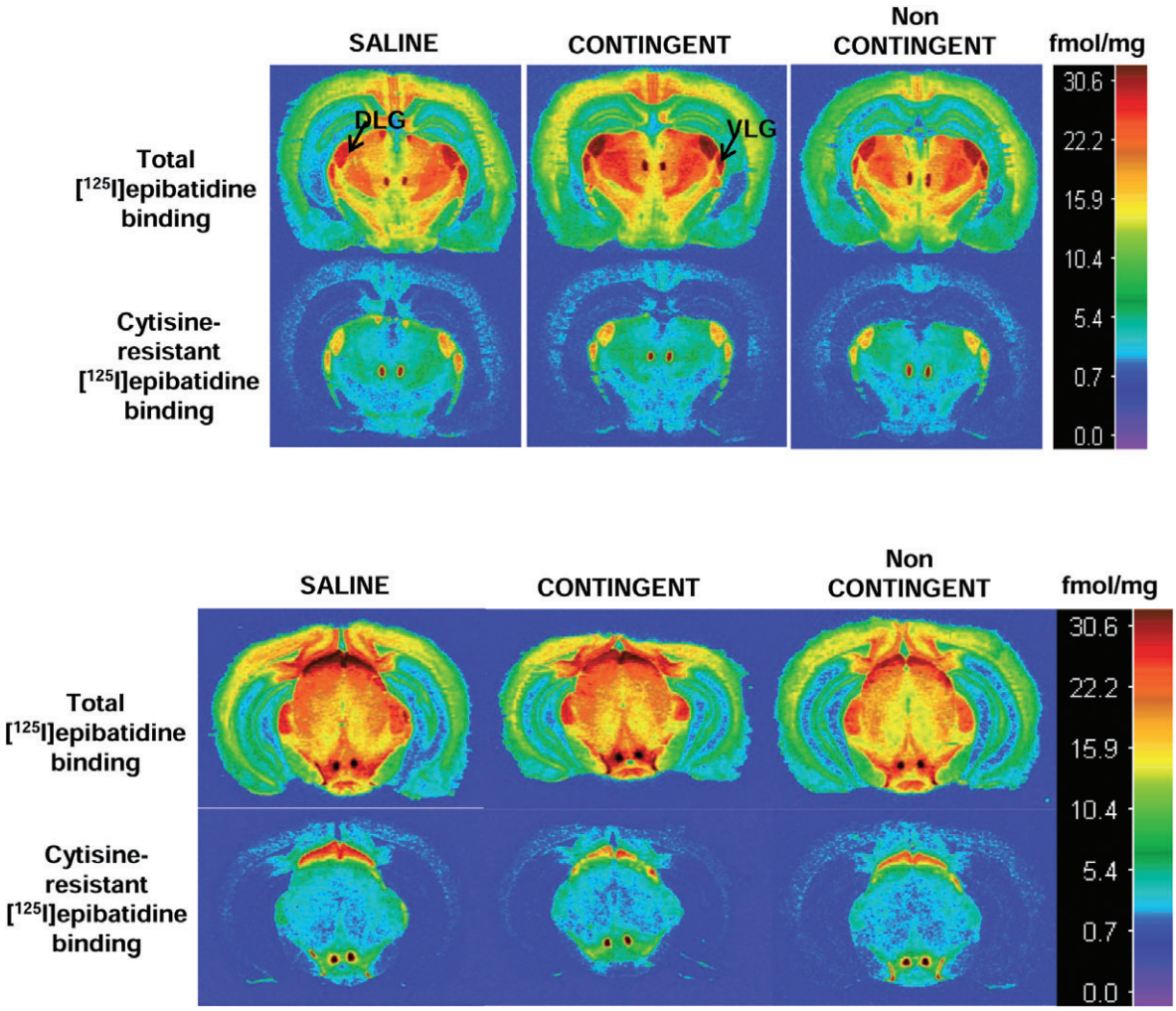

Figure 3 Representative autoradiograms of heteromeric nAChR binding in coronal brain sections from nicotine contingent, non contingent, and saline treated animals. The effects of response-contingency on nicotinic receptor binding populations are shown at the levels of thalamus (bregma –2.06 mm) and the ventral tegmental area (bregma –3.16), following self-administered or passively received nicotine. α4β2* receptor expression was calculated after subtraction of specific cytisine-resistant [^{125}I]epibatidine binding from total [^{125}I]epibatidine binding. Response-dependent nicotine increased α4β2* receptor binding in the dorsal geniculate nucleus and the ventral tegmental area of self-administering mice, compared to yoked animals. The color bar indicates a pseudo-color interpretation of black and white image density, calibrated in fmol/mg of tissue equivalent

nicotine-contingent and yoked mice in any of the brain regions analyzed ($F_{1,140} = 2.0$, $P > 0.05$).

Effects of response contingency on dopaminergic D1 and D2 receptor, and on dopamine transporter binding

Full quantification of dopaminergic D1 and D2 receptor binding, and of the dopamine transporters (DAT) was carried out on brain sections from all treatment groups, using [^{3}H]SCH23390, [^{3}H]raclopride, and [^{3}H]mazindol, respectively. For D2 receptor binding, two-way ANOVA revealed significant main effects of treatment ($F_{2,95} = 6.1$, $P < 0.01$) and region ($F_{6,95} = 74.4$, $P < 0.001$), with no significant treatment × region interaction effect ($F_{12,95} = 0.3$, $P > 0.05$) (Table 3). There was an overall increase in D2 receptor binding across all regions, in both nicotine contingent and non-contingent mice, compared to saline controls. However, *post hoc* comparison revealed no individual region significant increase among groups. The small level of increase in D2 receptor density was observed in self-administering animals, as well as in contingent mice that did not fulfill acquisition criteria ($F_{1,24} = 0.0$, $P > 0.05$).

Neither response-dependent, nor passively received nicotine had any significant effects on dopamine D1 receptor ($F_{2,133} = 2.6$, $P > 0.05$) and dopamine transporter (DAT) binding ($F_{2,105} = 2.0$, $P > 0.05$), compared with saline treatment (Tables 4 and 5). Moreover, no changes in D1 receptor density ($F_{1,36} = 0.2$, $P > 0.05$) and DAT binding ($F_{1,28} = 1.1$, $P > 0.05$) were detected in brain

Table 3 Quantitative autoradiography of D2 receptor binding in nicotine contingent, non-contingent and saline-treated animals.

	[^{3}H]raclopride specific binding (fmol/mg tissue)			*% change in binding*	
Region	*Saline*	*Contingent*	*Non-contingent*	*Saline versus contingent*	*Saline versus non-contingent*
Olfactory tubercle	88.9 ± 9.9	108.9 ± 7.0	104.4 ± 4.6	22.5	17.4
Nucleus accumbens					
Core	76.3 ± 7.6	83.9 ± 12.1	85.7 ± 5.2	10.0	12.4
Shell	74.3 ± 8.5	94.1 ± 6.1	90.6 ± 5.7	26.6	22.0
Caudate Putamen					
Rostral part	122.7 ± 12.7	143.0 ± 10.7	143.7 ± 4.2	16.6	17.1
Caudal part	127.1 ± 6.2	137.3 ± 13.5	148.6 ± 5.1	8.0	16.9
Ventral tegmental area	32.3 ± 2.7	38.5 ± 1.9	33.9 ± 2.8	19.4	4.9
Substantia nigra, pars compacta	33.6 ± 2.6	43.0 ± 4.3	38.9 ± 2.2	28.0	16.0
Mean				18.7	15.2

Values represent the mean specific binding of [^{3}H]raclopride ± SEM in brain regions of 6 animals per group. A small level of D2 receptor upregulation was observed following self-administered and passively received nicotine, but no individual region significant increases were observed between groups.

Table 4 Quantitative autoradiography of D1 receptor binding in nicotine contingent, non-contingent and saline-treated animals.

	[^{3}H]SCH23390 specific binding (fmol/mg tissue)			*% change in binding*	
Region	*Saline*	*Contingent*	*Non-contingent*	*Saline versus contingent*	*Saline versus non-contingent*
Nucleus accumbens					
Core	186.4 ± 24.7	248.4 ± 30.0	246.4 ± 48.2	33.3	32.2
Shell	193.6 ± 23.8	237.0 ± 27.4	236.0 ± 44.9	22.5	21.9
Caudate Putamen	328.1 ± 16.4	359.2 ± 24.5	363.0 ± 37.5	9.5	10.6
Olfactory tubercle	309.1 ± 22.6	335.2 ± 31.0	339.9 ± 48.2	8.5	10.0
Endopiriform nucleus, dorsal	65.3 ± 3.5	80.2 ± 6.0	68.2 ± 6.4	22.8	4.5
Claustrum	51.2 ± 5.5	55.6 ± 2.2	48.3 ± 6.5	8.7	−5.6
Amygadala, total	27.6 ± 2.3	30.9 ± 2.1	28.7 ± 1.8	12.0	3.8
Ventral tegmental area	27.1 ± 3.9	37.6 ± 2.9	36.8 ± 2.8	38.5	35.7
Substantia nigra	168.7 ± 15.3	165.3 ± 14.1	190.3 ± 19.2	−2.0	12.9
Mean				17.1	14.0

Values represent the mean specific binding of [^{3}H]SCH23390 ± SEM in brain regions of 6 animals per group. Nicotine self-administration had no effect on D1 receptor density.

sections from animals that stably responded for nicotine, compared to mice that did not achieve acquisition criteria. Representative autoradiograms of dopaminergic receptor and transporter bindings in brain sections of nicotine contingent, yoked, and saline-treated animals are shown in Fig. 4.

DISCUSSION

Previous studies have shown that nicotine, at a concentration range between 0.01 and 0.05 mg/kg/infusion, supports self-administration in diverse species, including humans (Henningfield, Miyasato & Jasinski 1983), non-human primates (Goldberg, Spealman & Goldberg 1981) and rodents (Martellotta *et al.* 1995; Donny *et al.* 1998). In agreement with this literature, we report here that freely moving C57BL/6J mice will chronically self-administer an optimal dose of response-dependent nicotine, using nose-poking as the operating procedure. The yoked-control model of self-administration allows discriminating the neurobiological response that occurs as a result of the direct pharmacological effects of a drug, from neuroplasticity that is due to the contingency between a behavioral reaction and drug delivery. By successfully applying this operant paradigm in mice, we reveal that differential neuroadaptation takes place in the nicotinic cholinergic system, depending on whether nicotine is administered actively or passively. Indeed, cytisine-sensitive [^{125}I]epibatidine binding differed between self-administering and nicotine-yoked animals in the ventral tegmental area (VTA) and the dorsal lateral geniculate nucleus (DLG), suggesting that these brain regions are

Table 5 Quantitative autoradiography of dopamine transporter binding in nicotine contingent, non-contingent and saline-treated animals.

	[^{3}H]mazindol specific binding (fmol/mg tissue)			*% change in binding*	
Region	*Saline*	*Contingent*	*Non-contingent*	*Saline versus contingent*	*Saline versus non-contingent*
Olfactory tubercle	281.6 ± 41.5	251.0 ± 19.2	298.2 ± 54.6	–10.9	5.9
Nucleus accumbens					
Core	159.1 ± 25.7	173.0 ± 22.1	219.3 ± 34.3	8.7	37.8
Shell	193.2 ± 33.0	198.5 ± 14.0	227.6 ± 42.4	2.8	17.8
Caudate Putamen					
Rostral part	375.2 ± 52.2	447.8 ± 35.4	461.8 ± 38.0	19.4	23.1
Caudal part	367.3 ± 55.3	421.2 ± 29.7	367.9 ± 30.8	14.7	0.2
Ventral tegmental area	121.1 ± 21.2	165.8 ± 10.5	162.7 ± 16.2	36.9	34.4
Substantia nigra, pars compacta	118.2 ± 13.0	128.4 ± 17.7	117.8 ± 11.6	8.6	–0.3
Mean				11.9	19.9

Values represent the mean specific binding of [^{3}H]mazindol ± SEM in brain regions of six animals per group. Nicotine self-administration had no effect on dopamine transporter density.

critically involved in the processes relating to the acquisition and maintenance of nicotine self-administration.

The increased rate of self-administration observed on days 1 and 2 may be partly attributed to the novelty of the experimental procedure. Similar patterns of increased intake during the initial phase of drug exposure have also been observed elsewhere (Mendizabal, Zimmer & Maldonado 2006). As early as on day 1, however, contingent mice were discriminating between the active and inactive hole, suggesting that the enhanced initial consumption may partly constitute a specific, nicotine-maintained effect on behavior. In line with our findings, a pattern of escalated drug intake that levels off in subsequent sessions has also been observed in rats that chronically self-administer nicotine (O'Dell *et al.* 2007). Out of a wide range of doses, acute self-administration of nicotine in mice occurs around a narrow window of approximately 0.03 mg/kg/infusion, indicating that rodents will tightly regulate their amount of nicotine intake (Paterson *et al.* 2003; Pons *et al.* 2008). Indeed, titration seems to be an important feature of nicotine self-administration across species, as both adult (Benowitz & Jacob 1985) and adolescent (Kassel *et al.* 2007) smokers carefully control their nicotine levels in order to experience the reinforcing effects of the drug. The observed enhanced initial rate of nicotine consumption may thus reflect a learning response at a given level of titration, which rapidly occurred within the first two experimental sessions.

Following day 7, reliable drug-taking behavior was observed in animals receiving response-dependent nicotine, which was maintained for the remainder of this experiment. Response-contingent animals adjusted their behavior to obtain a mean drug intake of approximately 0.25 mg/kg/session, which is within the range of nicotine chronically self-administered by rats (Donny *et al.* 1995; Kenny & Markou 2006). As demonstrated by cytisine-sensitive [^{125}I]epibatidine autoradiography, exposure to this nicotinic dose led to region-specific increases of $\alpha4\beta2^*$ nAChRs in the brains of self-administering animals. The upregulation displayed marked regional variability, with different areas of the brain participating and showing different sensitivity to upregulation. In this respect, our findings are consistent with previous observations that experimenter-administered nicotine in rodents (Marks *et al.* 1983; Schwartz & Kellar 1983; Nguyen *et al.* 2003), as well as nicotine self-administration by rats (Parker *et al.* 2004), produces regional-specific increases in the density of nicotinic receptors. The brain areas where contingent nicotine upregulated nAChRs have been shown to be affected by nicotine exposure following a variety of drug administration regimes, including tail vein infusion (Pauly *et al.* 1991), osmotic minipumps (Nguyen *et al.* 2003), oral administration (Sparks & Pauly 1999), and continuous self-administration in the rat (Parker *et al.* 2004). This suggests that the paradigm of nicotine self-administration we have used is a valid model for the investigation of nicotine-induced neuroadaptation. Substantial experimental work has established nAChR upregulation as the hallmark of chronic nicotine treatment. Using intravenous nicotine infusion, it has been consistently demonstrated that nicotine-induced receptor upregulation is a dose and time dependent phenomenon, with the half maximal dose required to induce 50% of region-specific nAChR upregulation in mice being approximately 0.5 mg/kg/hour (Marks *et al.* 1991; Pauly *et al.* 1991; Marks *et al.* 2004). In our paradigm, the mean daily amount of self-administered and passively received nicotine was at 0.25 mg/kg/hour. Therefore, the

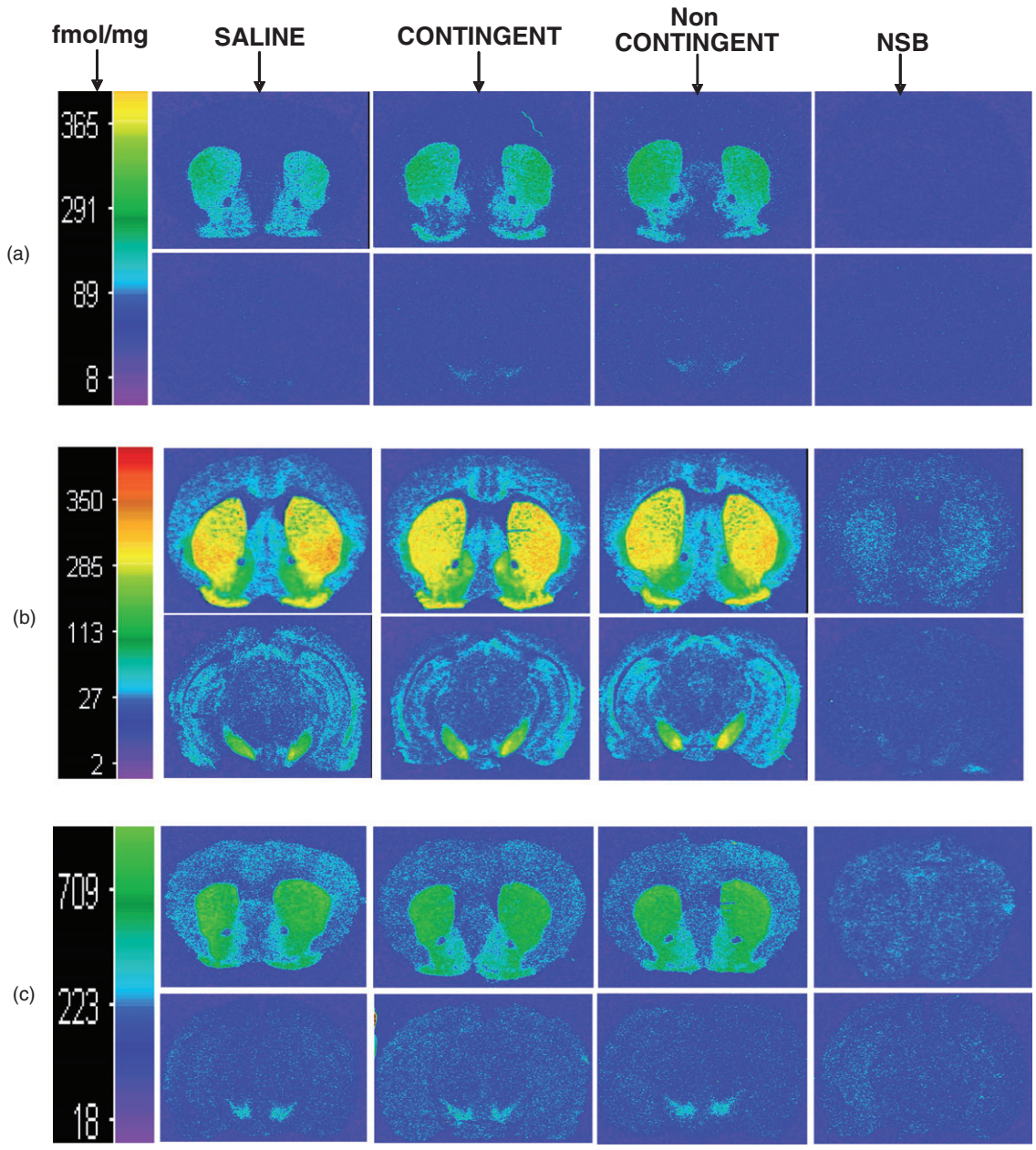

Figure 4 Computer-enhanced color autoradiograms of dopamine D2 (A) and D1 receptors (B), and of DA transporters (C), in coronal brain sections from nicotine contingent, non contingent, and saline treated animals. Sections were labeled with 4 nM [^{3}H]raclopride, [^{3}H]SCH23390 and [^{3}H]mazindol, for D2 and D1 receptors, and for the dopamine transporters, respectively. The horizontal panels show adjacent sections cut at the level of the caudate putamen, and of the ventral tegmental area. Sections were apposed to Kodak BioMax MR-1 film for a period of 5–6 weeks. Specific binding was calculated after subtraction of non-specific binding images from total binding images. No change in D1 and DAT binding was observed among groups. The color bars indicate a pseudo-color interpretation of black and white image density, calibrated in fmol/mg of tissue equivalent

lack of pronounced upregulation in nicotine-yoked mice is in agreement with the dosing profile for drug induced nAChR increases, and underlines the significance of contingency in nicotinic neuroadaptation, since we observed α4β2* nAChR upregulation with smaller, albeit behaviorally relevant doses of nicotine. Although similar increases might occur in non-contingent animals after longer exposure to nicotine, our data suggest that the sensitivity of nAChRs to upregulation is particularly enhanced during the phase of initial exposure to the drug. The finding that cytisine-sensitive [^{125}I]epibatidine binding sites were increased in response-dependent mice that were stably self-administering nicotine, as well as in animals that did not achieve the acquisition criteria, further supports that it is the contingent relationship between a behavioral response and drug delivery, rather than the amount of

nicotine-intake, that crucially mediates nicotine-induced neurochemistry during the establishment of self-administration behavior.

Among the regions that responded differently to active and passive nicotine administration, the VTA is the biological substrate most likely to be involved in the initiation and maintenance of drug-taking behavior. The VTA is source of the dopaminergic projection to the nucleus accumbens (NAc), and it has been implicated in mediating the reinforcing properties of many drugs of abuse, including nicotine (Di Chiara 2000). Rats will self-administer nicotine directly into the VTA through intracranial injections, a behavior that is attenuated by co-infusion of the β2 subunit antagonist dihydro-β-erythroidine, indicating that nicotine's rewarding effects are exerted through nAChRs in this brain region (Corrigall, Coen & Adamson 1994). Moreover, evidence from studies on gain-of-function α4 nAChR subunit knock-in animals (Tapper *et al.* 2004), as well as data from β2 subunit knock-out mice (Picciotto *et al.* 1998), further reveals the critical role of VTA α4β2* receptors in mediating nicotine reinforcement. Therefore, our data are not only in keeping with several other lines of evidence, but further suggest that the upregulation of α4β2* nAChRs in the VTA is not simply an epiphenomenon of nicotine administration, but a key neurobiological feature that is critical for mediating nicotine-intake.

A large body of literature suggests that nicotine reinforcement is not only the result of the drug's primary rewarding effects, but also stems from nicotine's ability to establish concurrent stimuli as conditioned reinforcers (reviewed in Chaudhri *et al.* 2006). In rodents, environmental stimuli promote the rapid acquisition of drug-seeking behavior (Caggiula *et al.* 2002), and the maintenance of nicotine self-administration during saline substitution (Donny *et al.* 1999; Martín-García *et al.* 2009). Although the dissociation of nicotine's primary reinforcing, from its reinforcement enhancing effects was not an aim of our study, our autoradiography data are suggestive of a role for the visually paired stimulus in the acquisition of nicotine self-administration. Increased cytisine-sensitive [^{125}I]epibatidine binding was observed in self-administering mice compared to nicotine yoked animals in the DLG, a brain region that is dedicated to the processing of visual information. In cells of the DLG, β2 subunits have been associated with fast latencies of visual responding, and high visually evoked firing rates (Grubb & Thompson 2004). Moreover, mouse DLG neurons are capable of switching from tonic to burst firing, in order to facilitate signal detection (Grubb & Thompson 2005). The enhanced density of α4β2* nAChRs in this brain area may, therefore, underlie intense visual information processing, which might contribute to the acquisition of self-administration. In support of this, we have previously shown that saline-infused mice that receive a visual cue for active nose-poking do not acquire self-administration, implying that nicotine-induced effects on visual processing are important for the acquisition of this behavior (Trigo *et al.* 2007; Martín-García *et al.* 2009).

Neuronal nAChRs constitute a heterogeneous family of receptors, which, depending on their subunit composition, exhibit distinct physiological and pharmacological properties (reviewed in Dani & Bertrand 2007). From the present results, we cannot exclude that contingent nicotine preferentially increases α4β2* receptor binding, while its passive administration regulates other nicotinic subtypes in a similar manner. Indeed, cytisine-resistant [^{125}I]epibatidine binding sites correspond to structurally diverse nAChRs subtypes, which require expression of α3, α4, α6, α2, β2 and β4 subunits (Gotti *et al.* 2005; Marks, Whiteaker & Collins 2006). Although cytisine-resistant nAChR density did not differ between animals receiving nicotine in a response-dependent or passive manner, subtle differences in the regulation of subunit-specific responses to contingent or passive nicotine remain to be examined. Nevertheless, evidence argues against the role of α7 nAChRs in nicotine reinforcement (Walters *et al.* 2006; Pons *et al.* 2008). Furthermore, although α6 subunits are involved in acute nicotine self-administration (Pons *et al.* 2008), autoradiographic studies on the effects of nicotine administration on α6* nAChRs have not yet produced conclusive results (Nguyen *et al.* 2003; Parker *et al.* 2004; Mugnaini *et al.* 2006; Even *et al.* 2008). In addition, α3β4* nAChRs are relatively resistant to up-regulation, both *in vivo* and *in vitro* (Wang *et al.* 1998; Davila-Garcia, Musachio & Kellar 2003; Nguyen *et al.* 2003). It thus seems probable that the effects of operant contingency are depicted as changes in cytisine-sensitive nAChR density in self-administering mice.

Whatever produces neurobiological differences between response-dependent and passively received nicotine, it does not seem to engage dopaminergic D1 and D2 receptors or the dopamine transporters. In the current study, drug administration did not alter D1 receptor or DAT density, and it equally upregulated D2 receptors in contingent and non-contingent animals. As shown by *in vivo* microdialysis, extracellular dopamine levels do not differ between rats self-administering nicotine and nicotine-yoked animals, further suggesting that dopaminergic responses to chronic nicotine administration are independent of response contingency (Rahman *et al.* 2004). D2 receptors have been implicated in nicotine dependence in both human (McEvoy *et al.* 1995) and animal experiments (Ikemoto, Qin & Liu 2006). Radioligand studies, however, have produced conflicting results as to the effect of chronic nicotine on D2 receptor density.

When delivered through osmotic minipumps, nicotine has been shown to decrease (Janson *et al.* 1992) or produce no differences (Kirch *et al.* 1992) in D2 receptor density. Therefore, D2 receptor regulation seems to depend on the paradigm of nicotine administration employed, as well as on the drug dose and the duration of treatment.

In conclusion, our results demonstrate the influence of response-dependency on the neurobiological output of nicotine. Contingent nicotine in self-administering animals produced the typical pattern of region-specific upregulation of $\alpha4\beta2^*$ nAChRs, whereas the administration of nicotine in a passive manner produced little change in the density of cytisine-sensitive receptors. Importantly, the observation that differential regulation of nAChRs takes place in the VTA and the DLG of self-administering animals, compared with nicotine-yoked mice, suggests that nAChR upregulation in these brain regions is not a by-product of nicotine exposure, but a neurobiological feature that crucially determines the acquisition and maintenance of nicotine self-administration. Our data add to the increasing evidence that experimenter and self-administered drugs of abuse produce different effects (Hemby *et al.* 1997; Kuzmin & Johansson 1999; Donny *et al.* 2000; Lecca *et al.* 2007), and suggest that nicotine-induced neuroadaptation crucially depends on the contingent relationship between a subject's response and the delivery of the reinforcer.

Acknowledgments

This work was supported by the European Commission (LSHM-CT2004-005166).

Authors Contribution

All behavioral experiments were conducted by M. F. Barbano and L. Galeote in R. Maldonado's group, at the University of Pompeu Fabra, Barcelona, Spain. The quantitative autoradiography experiments were conducted in I. Kitchen's group by A. Metaxas and A. Bailey, at the University of Surrey, Guildford, UK.

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