Genetic deletion of the adenosine A_{2A} receptor prevents nicotine-induced upregulation of $\alpha 7$, but not $\alpha 4\beta 2^*$ nicotinic acetylcholine receptor binding in the brain

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Abstract

Considerable evidence indicates that adenosine A_{2A} receptors ($A_{2A}Rs$) modulate cholinergic neurotransmission, nicotinic acetylcholine receptor (nAChR) function, and nicotine-induced behavioural effects. To explore the interaction between A_{2A} and nAChRs, we examined if the complete genetic deletion of adenosine $A_{2A}Rs$ in mice induces compensatory alterations in the binding of different nAChR subtypes, and whether the long-term effects of nicotine on nAChR regulation are altered in the absence of the $A_{2A}R$ gene. Quantitative autoradiography was used to measure cytosine-sensitive [^{125}I]epibatidine and [^{125}I] α -bungarotoxin binding to $\alpha 4\beta 2^*$ and $\alpha 7$ nAChRs, respectively, in brain sections of drug-naïve ($n=6$) or nicotine treated ($n=5-7$), wild-type and adenosine $A_{2A}R$ knockout mice. Saline or nicotine (7.8 mg/kg/day; free-base weight) were administered to male CD1 mice via subcutaneous osmotic minipumps for a period of 14 days. Blood plasma levels of nicotine and cotinine were measured at the end of treatment. There were no compensatory developmental alterations in nAChR subtype distribution or density in drug-naïve $A_{2A}R$ knockout mice. In nicotine treated wild-type mice, both $\alpha 4\beta 2^*$ and $\alpha 7$ nAChR binding sites were increased compared with saline treated controls. The genetic ablation of adenosine $A_{2A}Rs$ prevented nicotine-induced upregulation of $\alpha 7$ nAChRs, without affecting $\alpha 4\beta 2^*$ receptor upregulation. This selective effect was observed at plasma levels of nicotine that were within the range reported for smokers (10-50 ng ml $^{-1}$). Our data highlight the involvement of adenosine $A_{2A}Rs$ in the mechanisms of nicotine-induced $\alpha 7$ nAChR upregulation, and identify $A_{2A}Rs$ as novel pharmacological targets for modulating the long-term effects of nicotine on $\alpha 7$ receptors.

Keywords: Adenosine A_{2A} ; knockout mice; nicotine; nAChRs; upregulation; quantitative autoradiography

1. Introduction

Neuronal nicotinic **acetylcholine** receptors (nAChRs) are a heterogeneous family of pentameric ligand-gated cation channels, classified according to subunit composition and pharmacology into α -bungarotoxin sensitive, homopentameric $\alpha 7$ receptors, and into α -bungarotoxin insensitive, heteropentameric, non- $\alpha 7$ nAChRs (Albuquerque et al., 2009). The latter can be assembled by combinations of different α and β nicotinic subunits, among which the most prevalent is the $\alpha 4\beta 2$ combination ($\alpha 4\beta 2^*$, with the asterisk indicating the possible participation of additional subunits). The prolonged exposure to nicotine has been consistently shown to increase nAChR binding, both in cigarette smokers (Mukhin et al., 2008; Staley et al., 2006) and in animal models of nicotine intake (Marks et al., 1983; Schwartz and Kellar, 1983). This neurochemical adaptation has been associated with several nicotine-induced behaviours, including self-administration (Metaxas et al., 2010), reward sensitisation (Hilario et al., 2012), locomotor activity tolerance (McCallum et al., 2006), and nicotine withdrawal (Gould et al., 2012).

Adenosine is an ubiquitous purine neuromodulator, which exerts its effects by activating four types of G-protein coupled receptors, namely A_1 , A_{2A} , A_{2B} , and A_3 adenosine receptors. Among them, adenosine A_{2A} receptors ($A_{2A}Rs$) have attracted much interest as potential modulators of the effects of nicotine, both because of their predominant localization in brain circuitries that are involved in reinforcement (Ferre et al., 2007), and due to the altered behavioural phenotype that $A_{2A}R$ knockout (KO) mice exhibit in response to nicotine. For instance, nicotine-induced conditioned place preference and dopamine release in the nucleus accumbens are attenuated in mice with genetic ablation of the adenosine $A_{2A}R$ gene, suggesting that $A_{2A}Rs$ contribute to nicotine reward (Castane et al., 2006). Moreover, the aversive response to nicotine withdrawal is blocked in $A_{2A}R$ KO mice, indicating that $A_{2A}Rs$

modulate the motivational states associated with the cessation of chronic, but not acute, nicotine intake (Grieder et al., 2012).

There is considerable *in vitro* evidence suggesting that A_{2A}Rs regulate cholinergic neurotransmission in the central nervous system. Functional A_{2A}Rs are expressed on cholinergic nerve terminals (Preston et al., 2000; Tozzi et al., 2011), and their activation increases the release of acetylcholine (ACh) in the hippocampus (Rebola et al., 2002; Rodrigues et al., 2008) and the striatum (Gubitz et al., 1996; Kirkpatrick and Richardson, 1993). A_{2A}R-mediated ACh release in the rodent striatum has also been demonstrated *in vivo*, suggesting that striatal cholinergic neurotransmission is tonically influenced by adenosine acting on A_{2A}Rs (Kurokawa et al., 1996). Moreover, electrophysiological recordings from cell lines or neuronal cultures co-expressing A_{2A} and nAChRs, reveal that tonic A_{2A}R activation is required for sustaining the functional response of nAChRs to repeated ACh or nicotine exposure, indicating that A_{2A}Rs may confer a gain of function to nAChRs (Di Angelantonio et al., 2011).

Despite the prominent role of adenosine A_{2A}Rs in regulating cholinergic neurotransmission, nAChR function, and nicotine-induced behaviour, the potential modulatory effects of A_{2A}Rs on the expression of distinct nAChR subtypes remain unknown. Based on the evidence suggesting that A_{2A}R activation tonically regulates cholinergic neurotransmission and nAChR function, we hypothesized that compensatory neuroadaptations might occur in the nicotinic cholinergic system when A_{2A}Rs are compromised. To test this hypothesis, the distribution and binding densities of $\alpha 4\beta 2^*$ and $\alpha 7$ nAChRs were quantified in brain sections of drug-naïve, wild-type (WT) and homozygous (-/-) A_{2A}R KO mice, using quantitative receptor autoradiography. In addition, as A_{2A}R activity regulates the functional and behavioural response to nicotine, we reasoned that adenosine A_{2A}Rs may also modulate the neuroadaptive

changes that occur in nAChR binding, following chronic nicotine treatment. To investigate the effects of A_{2A}Rs on nicotine-induced upregulation, $\alpha 4\beta 2^*$ and $\alpha 7$ nAChRs binding levels were measured in brain sections of WT and A_{2A}R KO mice, following 14 days of nicotine administration.

2 Materials and methods

2.1 Generation of the adenosine A_{2A}R KO mouse and experimental conditions

All animal care and experimental procedures complied with protocols approved by the UK Home Office, Animals (Scientific Procedures) Act, 1986. All studies are reported in accordance with the ARRIVE guidelines (McGrath et al., 2010), and were designed to minimise suffering and to reduce the number of animals used. CD1 adenosine A_{2A}R KO mice, originally generated by Ledent et al. (1997), were obtained from the breeding colony of the University of Surrey, as previously detailed (Bailey et al., 2004a). Briefly, mutant mice were obtained from heterozygote breeding pairs, which originated from the individual crossing of a number of heterozygote males from the existing colony, each with a WT female of the CD1 strain, which was purchased from an external supplier (Charles River, UK). All mice were genotyped at weaning using a polymerase chain reaction (PCR) based method. Tail tip samples were collected at 3 weeks of age, and DNA was extracted using the DNeasy tissue kit, according to the manufacturers' instructions (QIAGEN, Germany).

Adult, 8-12 weeks old, male CD1 mice were used in all experiments (total number: 36). The animals were individually housed in a temperature controlled environment, under a 12 h light/dark cycle (lights on: 7.00 am). Food and water were available *ad libitum*.

2.2 Confirmation of genotype with autoradiographic binding

To confirm the genotype of the animals used in the present studies, brain sections of WT and KO mice were processed for A_{2A}R binding according to Bailey et al. (2004b). Briefly, sections were pre-incubated for 30 min in 170 mM Tris-HCl buffer, containing 1 mM EDTA (pH 7.4, room temperature). Sections were then incubated for 2 h at room temperature, in 170 mM Tris-HCl buffer, containing 10 nM [³H]CGS21680 (PerkinElmer; specific activity 30 Ci/mmol), 10 nM MgCl₂, and 2 U/ml adenosine deaminase (pH 7.4). Incubations were terminated by 3 x 5 min washes into ice cold 50 mM Tris buffer (pH 7.4), and a rapid rinse in ice cold water. Radioligand bound sections were apposed for a period of 3 weeks to Kodak BioMax MR-1 film (Sigma-Aldrich, UK).

2.3 Quantitative autoradiography of $\alpha 7$ and $\alpha 4\beta 2^*$ nAChRs in drug-naïve WT and adenosine A_{2A}R KO CD1 mice

To examine whether compensatory changes occur in different subtypes of nAChR after the genetic deletion of adenosine A_{2A}Rs, quantitative autoradiography of $\alpha 7$ and $\alpha 4\beta 2^*$ nAChRs was performed using brain sections from drug-naïve, WT and adenosine A_{2A}R KO CD1 mice (n=6). Animals were killed by cervical dislocation and brains were immediately removed and frozen in isopentane on dry ice. The brains were stored at -80 °C for a maximum period of 1 month before being processed for quantitative autoradiography of $\alpha 7$ and $\alpha 4\beta 2^*$ nAChRs, as detailed in Metaxas et al. (2012). Tissue sectioning was carried out at -21 °C, using a Microm HM505E cryostat (Carl Zeiss, Germany). Multiple, adjacent 20 µm coronal brain sections were collected at 300 µm intervals, from rostral to caudal levels.

For heteromeric nAChRs, sections were pre-incubated for 10 min at room temperature in Tris-HCl buffer (pH 7.4), containing 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, and 1 mM MgCl₂. Sections were then incubated for 2 h at room temperature in the same buffer,

containing 100 pM of [125 I]epibatidine (specific activity 2,200 Ci mmol⁻¹, PerkinElmer). Determination of subtype-specific heteromeric nAChR 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 cytosine (Sigma-Aldrich, UK). To determine non-specific binding (NSB), further adjacent sections were incubated with [125 I]epibatidine in the presence of 300 μ M of (–) nicotine hydrogen tartrate salt (Sigma-Aldrich, UK). Competition of [125 I]epibatidine binding by ‘cold’ cytosine has been used to reveal two subpopulations of nAChRs with high and low affinity for cytosine (Whiteaker et al., 2002). The epibatidine binding sites with high affinity for cytosine (cytosine-sensitive [125 I]epibatidine binding sites) correspond primarily to $\alpha 4\beta 2^*$ nAChRs, and were calculated following the subtraction of cytosine-resistant from total [125 I]epibatidine binding values. Incubations were terminated by 2 x 10 min 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 BioMax MR-1 film for 24 h.

For homopentameric $\alpha 7$ nAChRs, sections were pre-incubated for 30 min at room temperature in assay buffer (50 mM Tris-HCl, 1% w/v BSA; pH 7.4). Adjacent, non-specific binding sections were pre-incubated in the same buffer, containing 1 mM of (–) nicotine hydrogen tartrate. Sections were then incubated for a period of 3 h at room temperature in assay buffer, containing 3 nM of [125 I] α -bungarotoxin (specific activity 250 Ci mmol⁻¹, GE Healthcare). Adjacent sections were incubated in the same buffer, in the presence of 1 mM (–) nicotine hydrogen tartrate. Specific $\alpha 7$ nAChR binding was calculated after the subtraction of NSB from total binding values. Incubations were terminated by 3 x 10 min washes into ice cold 50 mM Tris buffer (pH 7.4), and a rapid rinse in ice cold water. Sections were apposed to Kodak BioMax MR-1 film for a period of 7 days.

Quantitative analysis of receptor binding was performed by video-based computerised densitometry using an MCID image analyser (InterFocus Imaging, UK), as detailed previously (Kitchen et al., 1997). Briefly, optical density values were quantified from ^{14}C microscscales, which had been cross-calibrated to ^{125}I standards of known radioactive concentration (GE Healthcare, UK). The optical density values were subsequently entered with their corresponding radioactivity values into a calibration table, and the relationship between radioactivity and optical density was determined using the MCID software. All brain structures were identified by reference to the mouse atlas of Franklin and Paxinos (2001).

2.4 Chronic nicotine treatment

2.4.1 Minipump preparation and implantation

Saline or nicotine hydrogen salt (Sigma-Aldrich, UK) were administered to WT and adenosine $\text{A}_{2\text{A}}\text{R}$ KO CD1 mice, using mini osmotic pumps (n=5-7; ALZET[®]2002 model, Charles River, UK). The concentration of the nicotine salt solution was adjusted according to animal weight, to achieve a daily nicotine dose of 7.8 mg kg^{-1} (free-base weight), which was delivered for a period of 14 days, at a rate of $0.5 \mu\text{l h}^{-1}$. This dose was chosen for its ability to induce upregulation of nAChR binding in the mouse brain (Davis et al., 2005; Marks et al., 2004), and to produce blood plasma levels of nicotine comparable with the human condition (see results).

For minipump implantation, animals were anaesthetised with an isoflurane/oxygen vapour mixture (3.5%-4.5%; Isoflo, Abbott Laboratories Ltd, UK), which was delivered at a flow rate of 450 ml min^{-1} (U400 unit, Royem Scientific, UK). Under aseptic conditions, a single incision was made along the midline of the back, to reveal the subcutaneous layer. Blunt ended scissors were used to form a subcutaneous compartment, in which an osmotic minipump was placed parallel to the spine, with the flow moderator pointing away from the

incision, which was subsequently closed using 1-2 Michelle clips. Mice were allowed to recover from the operating procedure in a heated chamber before being transferred to their home cage, where they remained for the duration of this study.

2.4.2 Quantitative autoradiography of $\alpha 7$ and $\alpha 4\beta 2^*$ nAChRs in nicotine treated WT and adenosine $A_{2A}R$ KO CD1 mice

Saline and nicotine treated, WT and $A_{2A}R$ KO mice were killed by cervical dislocation on day 14 after minipump implantation. The brains were immediately removed, rapidly frozen in isopentane at $-20\text{ }^{\circ}\text{C}$, and processed for quantitative autoradiography of $\alpha 7$ and $\alpha 4\beta 2^*$ nAChRs, using [^{125}I] α -bungarotoxin (specific activity 152 Ci mmol^{-1} , PerkinElmer) and [^{125}I]epibatidine (specific activity $2,200\text{ Ci mmol}^{-1}$, PerkinElmer) respectively, as detailed in section 2.3.

2.4.3 Determination of plasma nicotine and cotinine levels

Following cervical dislocation, $\sim 800\text{ }\mu\text{l}$ of trunk blood were collected from each mouse into ice-cold, heparinized tubes, and plasma was immediately obtained by 20 min centrifugation at 2,500 rpm in a refrigerated centrifuge. Samples were subsequently frozen and transported in dry ice to ABS Laboratories Ltd, UK, for analysis of nicotine and cotinine levels using capillary column gas chromatography with nitrogen-phosphorus detection (Feyerabend and Russell, 1990). The detection limit for nicotine and cotinine was 0.1 ng ml^{-1} .

2.5 Statistical analysis

Two-way ANOVA for the factors genotype and region was used for the comparison of quantitative measures of $\alpha 4\beta 2^*$ and $\alpha 7$ nAChRs in brain regions of drug naïve, WT and adenosine $A_{2A}R$ KO mice.

Three-way ANOVA for the factors treatment, genotype and brain region was used to compare receptor binding levels in saline and nicotine treated, WT and adenosine A_{2A}R KO animals. Where ANOVA yielded significant main effects, Fisher's LSD *post hoc* analysis was used to investigate differences in radioligand binding between groups in individual brain regions. Plasma levels of nicotine and cotinine were compared between nicotine treated WT and A_{2A}R KO mice using two-tailed Student's t-tests. Statistical significance was set at alpha level of <0.05. All data were analysed using the Statistica software (Statsoft Inc., France), and are presented as the mean ± SEM of n animals/group.

3. Results

3.1 Confirmation of genotype

Labeling of A_{2A}R binding sites by [³H]CGS21680 was dense in the striatum of WT mice, and completely absent in the brains of homozygous knockout mice, confirming the genotype of the animals used in this study (Supplementary Figure S1).

3.2 Lack of genotype effect on neuronal nAChR binding levels in naïve CD1 mice

To investigate whether deletion of the gene encoding for the adenosine A_{2A}R alters the constitutional expression of nAChRs, quantitative autoradiography of heteromeric and homopentameric nAChRs was carried out in brain sections of drug-naïve WT and A_{2A}R KO mice. The pattern of regional distribution of all nAChR subtypes analysed was identical between WT and adenosine A_{2A}R KO mice. The majority of CD1 mouse brain [¹²⁵I]epibatidine binding sites were of the cytosine-sensitive, α4β2* nAChR subtype. High levels of α4β2* nAChR binding were observed in thalamic regions, followed by moderate levels in cortical and dopaminergic regions, and low levels in the medial habenula and the hippocampus (Figure 1A). Exceptionally high levels of cytosine-resistant [¹²⁵I]epibatidine binding were observed in the medial habenula and the fasciculus retroflexus (Figure 1B).

[¹²⁵I]α-bungarotoxin binding to α7 nAChRs was dense in nuclei of the hypothalamus, hippocampus, and the amygdala, but virtually absent in the thalamus (Figure 1C). Two-way ANOVA for the factors genotype and brain region showed no significant genotype effect and no significant genotype by region interaction effects on cytosine-sensitive [genotype: $F_{(1,196)}=0.11$, $p>0.05$; interaction: $F_{(19,196)}=0.67$, $p>0.05$] or cytosine-resistant [¹²⁵I]epibatidine binding levels [genotype: $F_{(1,108)}=1.83$, $p>0.05$; interaction: $F_{(10,108)}=1.13$, $p>0.05$]. Similarly, there was no effect of genotype on [¹²⁵I]α-bungarotoxin binding levels [genotype: $F_{(1,165)}=0.04$, $p>0.05$; interaction: $F_{(18,165)}=0.30$, $p>0.05$].

3.3 Plasma levels of nicotine and cotinine following chronic nicotine treatment

14 days of nicotine administration at a daily dose of 7.8 mg kg⁻¹ produced similar plasma levels of nicotine and cotinine in WT and adenosine A_{2A}R KO mice. The mean plasma levels of nicotine were 38.1±5.1 ng ml⁻¹ for WT mice and 31.2±3.2 ng ml⁻¹ for A_{2A}R KO animals [$t_{(1,10)}=1.8$, $p>0.05$; Student's t-test]. Plasma levels of cotinine were 77.7±8.5 ng ml⁻¹ for WT and 66.3±7.9 ng ml⁻¹ for KO mice [$t_{(1,10)}=0.9$, $p>0.05$]. In saline treated mice, nicotine and cotinine concentrations were < 1 ng ml⁻¹.

3.4 The genetic deletion of adenosine A_{2A}Rs prevents nicotine-induced upregulation of α7, but not α4β2* neuronal nAChRs

For heteromeric nAChRs, the density of cytosine-sensitive and cytosine-resistant [¹²⁵I]epibatidine binding sites is detailed for all brain areas analysed in Tables 1 and 2, respectively. Administration of nicotine for 14 days resulted in a significant overall regions increase of α4β2* nAChR binding in the brains of nicotine treated mice compared to saline controls [treatment: $F_{(1,515)}=14.47$, $p<0.001$], irrespective of genotype [genotype: $F_{(1,515)}=0.73$, $p>0.05$; genotype by treatment interaction: $F_{(1,515)}=1.2$, $p>0.05$; 3-way ANOVA]. In WT mice, nicotine-induced changes in binding varied from region to region, with areas of the

hippocampus (65.4%), cortex (43.3%) and hypothalamus (22.8%) showing the largest increases, and nuclei of the thalamus being resistant to nicotine-induced alterations in $\alpha 4\beta 2^*$ nAChR binding (Table 1). In both genotypes, cytisine-sensitive [125 I]epibatidine binding sites were significantly elevated in the frontal association cortex, primary motor cortex, secondary motor cortex, visual cortex, and auditory cortex of nicotine treated animals, compared with their respective saline controls (LSD *post hoc* tests).

No main effects of genotype [$F_{(1,320)}=0.17$, $p>0.05$], treatment [$F_{(1,320)}=3.84$, $p>0.05$] or interaction combination [$F_{(1,320)}=0.40$, $p>0.05$] on cytisine-resistant [125 I]epibatidine binding levels were observed following chronic nicotine administration (3-way ANOVA). Representative autoradiograms of heteromeric nAChR binding in brain sections of WT and mutant mice are shown in Figure 2.

For $\alpha 7$ nAChRs, there were significant treatment [$F_{(1,360)}=11.82$, $p<0.001$] and genotype by treatment interaction effects [$F_{(1,360)}=5.57$, $p<0.01$] on [125 I] α -bungarotoxin binding levels, with no significant overall main effects of genotype [$F_{(1,360)}=0.46$, $p>0.05$; 3-way ANOVA]. Thus, chronic nicotine administration increased $\alpha 7$ nAChR binding compared with saline, but this effect was modulated by the genetic deletion of adenosine A_{2A} Rs, and was not observed in the brains of A_{2A} R KO animals (Table 3). In WT mice, nicotine-induced upregulation of $\alpha 7$ nAChRs was less pronounced than that of $\alpha 4\beta 2^*$ nAChRs, and no significant region-specific increases in [125 I] α -bungarotoxin binding sites were observed between nicotine and saline treated animals ($p>0.05$; LSD *post hoc* tests). The mean percentage of $\alpha 7$ nAChR upregulation across all brain areas analyzed was 32.6% for WT and 6.2% for adenosine A_{2A} R KO mice. Between-group comparisons across all brain regions showed higher [125 I] α -bungarotoxin binding levels in nicotine treated WT animals, compared both with saline treated WT ($p<0.001$) and nicotine treated mutant mice ($p<0.05$; LSD *post hoc* tests).

Moreover, no difference in $\alpha 7$ nAChR binding sites was observed between nicotine and saline treated $A_{2A}R$ KO animals ($p>0.05$), or between saline treated control and mutant mice ($p>0.05$; LSD *post hoc* tests). Representative autoradiograms of $\alpha 7$ nAChR binding are shown in Figure 3.

4. Discussion

The current study highlights a novel role for $A_{2A}Rs$ in modulating the long term effects of nicotine treatment on $\alpha 7$ nAChRs. We show that the genetic deletion of adenosine $A_{2A}Rs$ prevents nicotine-induced upregulation of $\alpha 7$, but not $\alpha 4\beta 2^*$ nAChRs, indicating that $A_{2A}Rs$ regulate the nicotinic cholinergic response to chronic nicotine administration via an $\alpha 7$ receptor-specific mechanism.

In control CD1 mice, 14 days of nicotine administration via osmotic minipumps increased cytosine-sensitive [^{125}I]epibatidine and [^{125}I] α -bungarotoxin binding to $\alpha 4\beta 2^*$ and $\alpha 7$ nAChRs, respectively. The pattern and extent of nicotine-induced upregulation differed between the two receptor subtypes, with $\alpha 4\beta 2^*$ nAChR upregulation showing marked regional variability and being more pronounced than that of $\alpha 7$ nAChRs. These observations are in complete agreement with a substantial number of reports, which have established that nAChR upregulation is a time and dose dependent phenomenon, varying quantitatively and qualitatively according to nAChR subunit composition (Marks et al., 1985; Marks et al., 1986; Pauly et al., 1989; Sanderson et al., 1993), and cell-specific neuronal distribution (Nashmi et al., 2007). Thus, $\alpha 4\beta 2^*$ nAChRs, which have high affinity for nicotine, are more readily upregulated compared with low-affinity $\alpha 7$ nAChRs, a differential regulation that occurs in mice (Sparks and Pauly, 1999), rats (Mugnaini et al., 2002) and humans (Teaktong et al., 2004). For $\alpha 7$ nAChRs, in particular, we observed a 30% overall increase in [^{125}I] α -bungarotoxin binding sites in nicotine treated WT mice, which is consistent with the

magnitude of nicotine-induced upregulation previously reported for the $\alpha 7$ receptor subtype by *in vitro* (Barrantes et al., 1995; Peng et al., 1997; Ridley et al., 2001) and *in vivo* studies (Pauly et al., 1991; Rasmussen and Perry, 2006). These results render the dose of chronic nicotine used appropriate for investigating the effects of adenosine $A_{2A}R$ deletion on nicotine-induced upregulation, and confirm that the overall effects of nicotine on the binding of distinct nAChR subtypes are conserved, not only among inbred (Marks et al., 1991), but also in outbred strains of animals. In addition, increased nAChR binding was measured at plasma levels of nicotine that are within the range reported for smokers (10-50 ng ml⁻¹; Benowitz and Jacob, 1984; Russell et al., 1980), and similar to those achieved in mice after long-term treatment with comparable doses of nicotine, administered either via osmotic minipumps (Metzger et al., 2007), constant i.v. infusion (Marks et al., 2004), or in the drinking water (Pekonen et al., 1993). The current experimental design thus resulted in blood plasma levels of nicotine that are physiologically relevant to the human condition.

Unlike $\alpha 4\beta 2^*$ nAChR binding, which was increased both in nicotine treated WT and adenosine $A_{2A}R$ KO mice, the upregulation of $\alpha 7$ nAChRs by chronic nicotine was abolished in $A_{2A}R$ KO animals. This effect is unlikely to be caused by genotype-induced differences in the availability of nicotine to the systemic circulation, as plasma nicotine and cotinine concentrations were similar in WT and KO mice, indicating that the deletion of $A_{2A}Rs$ does not affect nicotine metabolism. Moreover, the specific effects of $A_{2A}R$ deletion on the upregulation of $\alpha 7$ nAChRs cannot be related to developmental compensatory alterations in nAChR binding levels, as the distribution and densities of both $\alpha 7$ and $\alpha 4\beta 2^*$ nAChRs were identical in drug-naïve or untreated control and mutant mice. These observations suggest that the effects of $A_{2A}Rs$ on $\alpha 7$ receptor regulation are not exerted tonically, but require activation of the nicotinic cholinergic system by nicotine. Our results thus point towards the existence

of specific, nicotine-induced interactions of adenosine A_{2A} Rs with $\alpha 7$, but not $\alpha 4\beta 2^*$ nAChRs.

Although the upregulation of nAChR binding constitutes a profound and well-documented neurochemical adaptation to prolonged nicotine exposure, the exact mechanisms underlying the phenomenon are still the subject of intense investigation. It is becoming increasingly accepted, however, that distinct molecular mechanisms underpin the regulation of $\alpha 4\beta 2^*$ vs. $\alpha 7$ nAChRs by chronic nicotine. Thus, while changes in conformation, stoichiometry, or $\beta 2$ nicotinic subunit chaperoning have been implicated in the upregulation of $\alpha 4\beta 2^*$ nAChRs (Govind et al., 2009; Govind et al., 2012; Lester et al., 2009), similar nicotine-induced alterations do not mediate the upregulation of homopentameric $\alpha 7$ nAChRs. Instead, increases in $\alpha 7$, but not $\alpha 4\beta 2^*$ nAChRs, have been shown to require protein synthesis and glycosylation in cultured cortical neurons (Kawai and Berg, 2001), further suggesting that different molecular mechanisms may underlie the upregulation of distinct nAChR subtypes. In this context, the present study clearly demonstrates that A_{2A} Rs are specifically involved in the mechanism of nicotine-induced $\alpha 7$, but not $\alpha 4\beta 2^*$ receptor upregulation.

The reason for this discrepancy is unclear, but it is highly likely to involve alterations downstream of the adenosine A_{2A} R. One such mechanism involves the brain-derived neurotrophic factor (BDNF). The link between A_{2A} Rs and BDNF is well-documented, with evidence showing that A_{2A} R activation is necessary both for regulating the expression and release of BDNF in the brain, and mediating BDNF's effects on synaptic plasticity (reviewed in Sebastiao and Ribeiro, 2009). In neurons cultured from the hippocampus and the ciliary ganglion, BDNF is known to upregulate the intracellular and surface pools of $\alpha 7$, but not $\beta 2^*$ nAChRs (Massey et al., 2006; Zhou et al., 2004). In addition, the interplay of A_{2A} Rs with BDNF and $\alpha 7$ nAChRs is evidenced in the fact that adenosine deaminase or A_{2A} R antagonists abolish the effects of BDNF on $\alpha 7$ nAChR function (Fernandes et al., 2008). Taken together,

these studies suggest that mice lacking adenosine A_{2A}Rs may have deficits in BDNF-dependent mechanisms that are specifically involved in nicotine-induced $\alpha 7$ nAChR upregulation. Interestingly, chronic nicotine treatment has been shown to elevate the expression or the levels of BDNF in the cortex, hippocampus and the amygdala (Aydin et al., 2012; Czubak et al., 2009; Kenny et al., 2000). We observed that in the above brain regions nicotine-induced $\alpha 7$ nAChR upregulation was more pronounced in WT vs. A_{2A}R KO mice, whereas in the ventral tegmental area, where chronic nicotine administration does not affect BDNF levels (Kivinummi et al., 2011), the effects of A_{2A}R deletion were less evident, thus providing further support to our suggestion.

Although the involvement of adenosine A_{2A}Rs in nicotine reinforcement (Castane et al., 2006) and withdrawal (Grieder et al., 2012) has been illustrated using mice with complete and permanent inactivation of the A_{2A}R gene, little is currently known about the contribution of $\alpha 7$ nAChR upregulation to nicotine's long-term behavioural effects. Neuroplastic alterations involving $\alpha 7$ and not $\beta 2^*$ nAChRs, have been shown to mediate the effects of prolonged nicotine exposure on novelty-seeking behaviour (Besson et al., 2007). In addition, $\alpha 7$ nAChRs play a pivotal role in sustaining nicotine's persistent effect on dopamine neuron excitability and, consequently, on nicotine reinforcement (Mansvelder et al., 2002; Mansvelder and McGehee, 2000). Moreover, the manifestation of the somatic symptoms of nicotine withdrawal requires the expression of $\alpha 7$, but not $\beta 2^*$ nAChRs (Besson et al., 2006; Salas et al., 2007). As a result, the discovery that A_{2A}Rs modulate the response of $\alpha 7$ nAChRs to chronic nicotine is likely to have important implications for intervening in nicotine-induced behaviours that involve this receptor subtype, including reinforcement and dependence. In addition to nicotine-induced behaviour, $\alpha 7$ nAChRs have been aetiologically implicated in human neuropathology, most notably in schizophrenia (Olincy and Freedman, 2012). Considerable evidence indicates that the high prevalence of smoking in schizophrenics can be

partly attributed to the cognitive enhancing properties of nicotine, and thus to nicotine's efficacy in alleviating the cognitive symptoms of schizophrenia (Ochoa and Lasalde-Dominicci, 2007). Nicotine's effects on cognition are known to be mediated via $\alpha 7$ nAChRs (Mansvelder et al., 2009). Moreover, smoking has been shown to restore $\alpha 7$ receptor expression in the hippocampus of schizophrenic patients to control levels (Mexal et al., 2010), suggesting a prominent role for $\alpha 7$ nAChR upregulation in the procognitive effect of nicotine. Indeed, the beneficial effects of various $\alpha 7$ agonists on cognition have been closely associated with their ability to induce $\alpha 7$ nAChR upregulation (Christensen et al., 2010). It is thus intriguing to postulate that a pharmacotherapeutic approach which would enhance $\alpha 7$ nAChR upregulation, such as A_{2A} R agonism, might further improve the effectiveness of $\alpha 7$ agonists in controlling the cognitive deficits in schizophrenia. In support of this suggestion, independent clinical and preclinical studies reveal that both dysfunction of the $\alpha 7$ nAChR (AhnAllen, 2012), and a reduced adenosinergic tone at A_{2A} Rs (Boison et al., 2012) contribute to the cognitive symptoms of the disorder.

In conclusion, we have shown that the genetic deletion of adenosine A_{2A} Rs prevents nicotine-induced upregulation of $\alpha 7$, but not $\alpha 4\beta 2^*$ nAChRs. This modulatory effect is specific to $\alpha 7$ nAChRs, it occurs at plasma levels of nicotine that are relevant to the human condition, and it is not due to compensatory developmental alterations in nAChR binding levels. These findings highlight the role of A_{2A} Rs in regulating the long term effects of nicotine treatment on $\alpha 7$ nAChRs *in vivo*, and suggest that A_{2A} Rs may comprise targets for novel pharmacological interventions, aimed at modulating the effects of chronic nicotine on $\alpha 7$ nAChRs.

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Legends to Figures

Figure 1 Quantitative autoradiography of heteromeric and homomeric nAChRs in drug-naïve, wild-type and adenosine A_{2A} receptor knockout mice

Compensatory alterations in nAChR binding levels following the genetic deletion of adenosine A_{2A} receptors (A_{2A}Rs) were examined using male, drug-naïve, wild-type (WT) and A_{2A}R knockout (KO) CD1 mice. No genotype-induced alterations in heteromeric, cytosine-sensitive (A) or cytosine-resistant (B) [¹²⁵I]epibatidine binding levels were observed. Similarly, there was no effect of genotype on homopentameric, α7 nAChR binding levels. Values represent the mean±SEM of 6 animals/group. Abbreviations: FrA: Frontal Association Cortex, M1: Primary Motor Cortex; MS: Medial Septum; Cg: Cingulate Cortex; CPu: Caudate Putamen; AcbC: Nucleus Accumbens, Core; AcbSh: Nucleus Accumbens, Shell; Tu: Olfactory Tubercle; AV: Anteroventral Thalamic Nucleus; LPMR: Lateral Posterior Thalamic Nucleus; Po: Posterior Thalamic Nuclear Group; DLG: Dorsal Lateral Geniculate Nucleus; VLG: Ventral Lateral Geniculate Nucleus; SuG: Superficial Gray Layer of the Superior Colliculus; SNc: Substantia Nigra, pars Compacta; VTA: Ventral Tegmental Area; MG: Medial Geniculate Nucleus; MHb: Medial Habenula; fr: Fasciculus Retroflexus; CA1: Hippocampus, CA1 field; DEn: Dorsal Endopiriform Nucleus; Cl: Claustrum; CA3: Hippocampus, CA3 field; DG: Dentate Gyrus; BLA: Basolateral Amygdaloid Nucleus; PLCo: Posteromedial Cortical Amygdaloid Nucleus; MAN: Medial Amygdaloid Nucleus; DM: Dorsomedial Hypothalamic Nucleus; VM Ventromedial Hypothalamic Nucleus; LH: Lateral Hypothalamus; ZI: Zona Incerta; SN: Substantia Nigra.

Figure 2 Representative autoradiograms of [¹²⁵I]epibatidine binding in saline and nicotine treated, wild-type and adenosine A_{2A} receptor knockout mice

Nicotine was delivered to male CD1 mice at a daily dose of 7.8 mg kg⁻¹, for a period of 14 days. On day 14 animals were killed by cervical dislocation, and their brains were

immediately removed and processed for quantitative receptor autoradiography. The panels show adjacent, 20 μ m-thick coronal brain sections, cut at the level of the caudate putamen (Bregma 0.74 mm). Sections were labeled for 2 h using 100 pM [125 I]epibatidine, alone or in the presence of 20 nM cytisine. Cytisine-sensitive, $\alpha 4\beta 2^*$ nAChR binding was calculated following the subtraction of cytisine-resistant from total [125 I]epibatidine binding values. Non-specific binding (NSB) was indistinguishable from film background. Sections were apposed to Kodak BioMax MR-1 film for 24 h. The degree of nicotine-induced $\alpha 4\beta 2^*$ nAChR upregulation was identical in wild-type (WT) and adenosine A_{2A} R knockout (KO) mice.

Figure 3 Representative autoradiograms of [125 I] α -bungarotoxin binding in saline and nicotine treated, wild-type (WT) and adenosine A_{2A} receptor knockout (KO) mice

To label $\alpha 7$ nAChRs, adjacent brain sections from saline or nicotine-treated mice were incubated for 3 h with 3 nM [125 I] α -bungarotoxin, alone or in the presence of 1mM of nicotine salt to calculate non-specific binding (NSB). Sections were apposed to Kodak BioMax MR-1 film for a period of 7 days. The genetic ablation of adenosine A_{2A} Rs prevented nicotine-induced $\alpha 7$ nAChR upregulation.

Brain Region	Cytisine-sensitive [¹²⁵ I]epibatidine binding (fmol/mg tissue equivalent)				% change in binding	
	WT Saline	WT Nicotine	KO Saline	KO Nicotine	WT Nicotine vs. WT Saline	KO Nicotine vs. KO Saline
Frontal association cortex	9.6 ± 0.8	16.8 ± 1.2**	11.4 ± 0.9	14.2 ± 1.3 [#]	75.2	25.2
Prelimbic cortex	8.8 ± 0.5	13.8 ± 0.9*	10.7 ± 1.0	13.0 ± 2.3	57.0	21.8
Cingulate cortex	11.7 ± 1.1	15.2 ± 1.6	12.8 ± 1.1	15.0 ± 2.2	29.3	17.2
Primary motor cortex	10.6 ± 0.8	14.6 ± 1.6*	10.8 ± 0.9	14.1 ± 1.9 [#]	37.7	30.7
Secondary motor cortex	10.3 ± 0.7	14.5 ± 1.2*	10.9 ± 0.8	14.0 ± 2.1 [#]	40.5	28.5
Visual cortex	11.6 ± 0.5	16.4 ± 0.8*	11.7 ± 1.2	16.5 ± 1.5 ^{##}	41.2	40.9
Primary somatosensory cortex	10.4 ± 0.6	12.8 ± 1.3	9.7 ± 0.8	12.5 ± 1.8	23.3	29.5
Subiculum	15.1 ± 1.3	15.2 ± 1.5	15.3 ± 1.5	16.1 ± 1.4	0.5	5.2
Auditory cortex	9.3 ± 0.8	13.2 ± 1.1*	9.3 ± 0.7	13.4 ± 1.0 [#]	42.0	44.3
Hippocampus	3.4 ± 0.3	5.7 ± 0.5	3.6 ± 0.2	5.7 ± 0.7	65.4	60.3
Hypothalamus	10.6 ± 1.1	13.0 ± 1.2	10.4 ± 0.7	13.3 ± 1.2	22.8	28.2
Nucleus accumbens						
Core	10.6 ± 0.5	11.5 ± 1.8	11.2 ± 0.9	12.3 ± 1.9	8.7	10.2
Shell	10.5 ± 0.6	12.8 ± 1.6	11.3 ± 0.9	13.3 ± 2.2	21.8	17.4
Caudate putamen	13.4 ± 0.9	15.3 ± 1.9	13.6 ± 1.1	15.0 ± 2.2	14.2	11.0
Olfactory tubercle	11.7 ± 0.7	13.7 ± 1.6	12.1 ± 0.4	13.7 ± 2.0	17.3	13.6
Anteroventral thalamic nucleus	29.9 ± 2.6	31.3 ± 3.0	31.1 ± 3.0	27.6 ± 3.0	4.7	-11.4
Medial habenula	7.3 ± 1.1	5.7 ± 1.9	7.4 ± 0.6	7.8 ± 2.3	-21.9	5.4
Fasciculus retroflexus	7.8 ± 1.7	9.3 ± 1.9	8.5 ± 1.4	8.4 ± 1.8	19.2	-1.2
Lateral posterior thalamic nucleus	26.2 ± 2.9	24.2 ± 3.3	28.1 ± 2.6	23.8 ± 2.2	-7.6	-15.1
Posterior thalamic nuclear group	25.2 ± 2.3	25.0 ± 2.5	23.6 ± 2.7	23.1 ± 2.0	-0.8	-2.2
Dorsal lateral geniculate nucleus	25.6 ± 3.4	24.4 ± 3.1	25.4 ± 3.2	25.5 ± 2.6	-4.8	0.2
Ventral lateral geniculate nucleus	22.0 ± 2.3	22.7 ± 3.6	22.5 ± 2.7	22.2 ± 1.9	2.9	-1.5
Medial geniculate nucleus	22.5 ± 2.5	23.1 ± 1.3	25.0 ± 1.2	24.9 ± 2.2	2.4	-0.5
Superficial gray layer of the superior colliculus	17.5 ± 1.6	18.4 ± 2.5	24.5 ± 2.0	21.8 ± 0.9	5.0	-11.1
Ventral tegmental area	22.5 ± 2.5	21.6 ± 1.2	20.8 ± 1.6	21.2 ± 1.8	-3.9	2.0
Substantia nigra, pars compacta	23.1 ± 2.5	22.3 ± 1.2	22.9 ± 1.8	22.6 ± 2.4	-3.6	-1.2

Table 1 Quantitative autoradiography of α4β2* nAChRs in WT and adenosine A_{2A}R KO mice, following chronic nicotine administration.

Values represent the mean±SEM of 5-7 animals/group. **p*<0.05, ***p*<0.01 vs. WT saline; [#]*p*<0.05, ^{##}*p*<0.01 vs. KO saline, LSD *post-hoc* tests.

Brain Region	Cytisine-resistant [¹²⁵ I]epibatidine binding (fmol/mg tissue equivalent)			
	WT Saline	WT Nicotine	KO Saline	KO Nicotine
Subiculum	2.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	2.3 ± 0.1
Nucleus accumbens				
Core	2.9 ± 0.1	2.2 ± 0.4	2.5 ± 0.1	2.5 ± 0.1
Shell	2.7 ± 0.2	2.2 ± 0.3	2.5 ± 0.1	2.4 ± 0.1
Caudate putamen	2.5 ± 0.1	2.3 ± 0.2	2.4 ± 0.1	2.4 ± 0.1
Olfactory tubercle	1.4 ± 0.4	1.9 ± 0.2	2.0 ± 0.2	1.7 ± 0.2
Anteroventral thalamic nucleus	4.7 ± 0.2	4.8 ± 0.5	5.0 ± 0.1	4.8 ± 0.3
Lateral posterior thalamic nucleus	3.9 ± 0.2	4.2 ± 0.2	3.8 ± 0.2	4.6 ± 0.2
Posterior thalamic nuclear group	2.9 ± 0.3	3.3 ± 0.2	2.6 ± 0.3	3.2 ± 0.2
Dorsal lateral geniculate nucleus	12.9 ± 0.5	12.2 ± 1.1	12.9 ± 1.0	11.9 ± 0.9
Ventral lateral geniculate nucleus	16.9 ± 1.2	13.8 ± 1.4	15.1 ± 1.7	12.9 ± 1.4
Medial geniculate nucleus	2.7 ± 0.1	3.3 ± 0.1	2.4 ± 0.2	3.6 ± 0.3
Medial habenula	46.8 ± 3.9	42.2 ± 3.9	48.6 ± 3.1	43.5 ± 2.9
Fasciculus retroflexus	42.4 ± 3.3	38.3 ± 2.3	41.1 ± 2.3	37.0 ± 2.4
Superficial gray layer of the superior colliculus	17.3 ± 2.3	16.6 ± 2.8	15.6 ± 1.6	15.5 ± 2.4
Ventral tegmental area	5.7 ± 0.6	6.1 ± 0.5	6.9 ± 1.7	6.5 ± 0.6
Substantia nigra, pars compacta	4.7 ± 0.6	4.8 ± 0.4	3.3 ± 0.5	5.1 ± 0.5

Table 2 Quantitative autoradiography of cytisine-resistant [¹²⁵I]epibatidine binding in WT and adenosine A_{2A}R KO mice, following chronic nicotine administration. Values represent the mean±SEM of 5-7 animals/group.

Brain Region	¹²⁵ I]α-bungarotoxin binding (fmol/mg tissue equivalent)				% change in binding	
	WT Saline	WT Nicotine	KO Saline	KO Nicotine	WT Nicotine vs. WT Saline	KO Nicotine vs. KO Saline
Frontal association cortex	12.5 ± 2.7	18.7 ± 3.9	17.8 ± 3.3	17.0 ± 3.6	50.3	-4.4
Cingulate cortex	9.5 ± 2.8	15.3 ± 3.1	12.5 ± 0.8	10.8 ± 4.3	61.0	-13.8
Caudate putamen	16.6 ± 3.8	18.9 ± 5.0	14.7 ± 0.8	12.8 ± 2.8	13.3	-13.3
Endopiriform nucleus, dorsal	21.5 ± 3.7	27.7 ± 6.5	21.1 ± 0.7	21.8 ± 3.5	28.6	3.3
Primary motor cortex	15.2 ± 3.8	20.4 ± 5.7	15.4 ± 1.8	16.7 ± 3.8	34.1	8.5
Clastrum	21.9 ± 3.7	29.5 ± 7.3	22.1 ± 1.6	18.7 ± 3.3	34.4	-15.6
Posterior thalamic nuclear group	7.6 ± 2.2	13.5 ± 2.9	8.8 ± 1.9	9.9 ± 2.3	76.7	12.0
Hippocampus, CA1	16.5 ± 4.0	23.3 ± 4.4	18.4 ± 4.6	21.5 ± 4.0	41.1	17.0
Hippocampus, CA3	20.6 ± 3.5	28.6 ± 5.5	23.6 ± 4.5	26.9 ± 4.5	38.6	14.1
Dentate gyrus	12.6 ± 2.8	19.0 ± 3.2	14.3 ± 3.1	17.0 ± 2.9	50.4	18.4
Basolateral amygdaloid nucleus	14.5 ± 2.9	17.3 ± 3.1	16.8 ± 2.1	19.9 ± 3.5	19.3	18.3
Posteromedial cortical amygdaloid nucleus	15.8 ± 2.1	23.3 ± 4.5	19.6 ± 3.5	17.4 ± 2.3	47.5	-11.1
Medial amygdaloid nucleus	46.1 ± 8.0	48.2 ± 6.8	43.2 ± 2.4	47.6 ± 6.6	4.6	10.4
Dorsomedial hypothalamic nucleus	17.3 ± 2.7	20.4 ± 2.1	23.2 ± 3.1	23.5 ± 4.5	18.1	1.0
Ventromedial hypothalamic nucleus	19.4 ± 2.1	24.4 ± 4.7	26.5 ± 6.8	25.6 ± 2.8	25.6	-3.4
Lateral hypothalamus	8.7 ± 1.4	11.1 ± 1.2	11.4 ± 1.4	12.0 ± 1.6	26.9	5.3
Zona incerta	52.0 ± 5.5	61.6 ± 8.7	50.5 ± 3.5	53.4 ± 6.4	18.4	5.8
Ventral tegmental area	26.6 ± 2.1	30.5 ± 5.0	25.8 ± 5.1	29.4 ± 2.7	14.3	13.7
Substantia nigra	7.0 ± 1.0	8.1 ± 1.0	5.7 ± 1.0	8.6 ± 1.6	16.3	51.2
mean increase (%)					32.6	6.2

Table 3 Quantitative autoradiography of α7 nAChRs in WT and adenosine A_{2A}R KO mice, following chronic nicotine administration. Values represent the mean±SEM of 5-7 animals/group.

(A)

(B)

(C)

Supplementary Material

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