EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, Vol. 29, pp. 539–550, 2009 doi:10.1111/j.1460-9568.2009.06613.x

NEUROSYSTEMS

α7 and non-α7 nicotinic acetylcholine receptors modulate dopamine release *in vitro* and *in vivo* in the rat prefrontal cortex

Phil D. Livingstone,[1,*] Jayaraman Srinivasan,[1,*,†] James N.C. Kew,[2] Lee A. Dawson,[2] Cecilia Gotti,[3] Milena Moretti,[3] Mohammed Shoaib[4] and Susan Wonnacott[1]

[1]Department of Biology & Biochemistry, University of Bath, Bath BA2 7AY, UK
[2]Neurosciences Centre of Excellence for Drug Discovery, GlaxoSmithKline, New Frontiers Science Park, Harlow, Essex CM19 5AW, UK
[3]CNR, Institute of Neuroscience and Department of Medical Pharmacology, University of Milan, Milan, Italy
[4]Psychobiology Research Laboratories, School of Neurology, Neurobiology and Psychiatry, University of Newcastle, Newcastle Upon Tyne NE2 4HH, UK

Keywords: 5-Iodo-A-85380, compound A, glutamate-dopamine crosstalk, immunoprecipitation, *in vivo* microdialysis, nicotine, PNU-120596

Abstract

Nicotine enhances attentional and working memory aspects of executive function in the prefrontal cortex (PFC) where dopamine plays a major role. Here, we have determined the nicotinic acetylcholine receptor (nAChR) subtypes that can modulate dopamine release in rat PFC using subtype-selective drugs. Nicotine and 5-Iodo-A-85380 (β2* selective) elicited [^{3}H]dopamine release from both PFC and striatal prisms *in vitro* and dopamine overflow from medial PFC *in vivo*. Blockade by dihydro-β-erythroidine supports the participation of β2* nAChRs. However, insensitivity of nicotine-evoked [^{3}H]dopamine release to α-conotoxin-MII in PFC prisms suggests no involvement of α6β2* nAChRs, in contrast to the striatum, and this distinction is supported by immunoprecipitation of nAChR subunits from these tissues. The α7 nAChR-selective agonists choline and Compound A also promoted dopamine release from PFC *in vitro* and *in vivo*, and their effects were enhanced by the α7 nAChR-selective allosteric potentiator PNU-120596 and blocked by specific antagonists. DNQX and MK801 inhibited [^{3}H]dopamine release evoked by choline and PNU-120596, suggesting crosstalk between α7 nAChRs, glutamate and dopamine in the PFC. *In vivo,* systemic (but not local) administration of PNU-120596, in the absence of agonist, facilitated dopamine overflow in the medial PFC, consistent with the activation of extracortical α7 nAChRs by endogenous acetylcholine or choline. These data establish that both β2* and α7 nAChRs can modulate dopamine release in the PFC *in vitro* and *in vivo*. Through their distinct actions on dopamine release, these nAChR subtypes could contribute to executive function, making them specific therapeutic targets for conditions such as schizophrenia and attention deficit hyperactivity disorder.

Introduction

The prefrontal cortex (PFC) is the site of integration of widespread glutamatergic inputs essential for executive function and goal-directed behaviour. Dopamine is a prominent modulator of PFC executive functions, including working memory and attention (Williams & Goldman-Rakic, 1995; Schultz, 2002; Floresco & Magyar, 2006). For example, local infusion of a dopamine D1 agonist into the medial PFC (mPFC) of rats increased attentional performance in animals with low baseline levels of accuracy (Granon *et al.*, 2000). Working memory and attentional performance are also improved by nicotine, in both laboratory animals and humans (Newhouse *et al.*, 2004; Levin *et al.*, 2006; Mansvelder *et al.*, 2006). The ability of nicotine to increase dopamine release from ascending dopamine neurons (Nisell *et al.*, 1994) offers one mechanism to account for the positive influence of nicotine on executive function.

Nicotine facilitates dopamine release by acting at somatodendritic or presynaptic nicotinic acetylcholine receptors (nAChRs) on meso-limbic and nigrostriatal neurons. nAChRs constitute a family of pentameric ligand-gated cation channels that differ in subunit composition and functional properties (Gotti *et al.*, 2006). A multiplicity of β2-containing heteromeric nAChRs reside on cell soma and nerve terminals of midbrain dopamine neurons (Klink *et al.*, 2001; Grady *et al.*, 2007). These can be distinguished into two classes based on the presence or absence of the α6 subunit (α6β2* or α4β2*

Correspondence: Professor S. Wonnacott, as above.
E-mail: bsssw@bath.ac.uk

*P.D.L. and J.S. contributed equally to this work.

†*Present address*: Department of Pharmacology, Schering-Plough Corporation, Newhouse, Motherwell ML1 5SH, UK.

Received 3 September 2008, revised 20 November 2008, accepted 5 December 2008

nAChRs), using the α3/α6β2-selective antagonist α-conotoxin MII (αCnTxMII). Presynaptic nAChRs facilitate dopamine release *in vitro* in the absence of other depolarizing stimuli (Wonnacott, 1997; Grady *et al.*, 2007), and they may be important for the local integration of endogenous cholinergic signals (Exley & Cragg, 2008), with α6β2* nAChRs having a dominant role in the nucleus accumbens (Exley *et al.*, 2007). Homomeric α7 nAChRs are more commonly associated with glutamatergic nerve terminals. Presynaptic α7 nAChRs on glutamate afferents to the ventral tegmental area (VTA; Jones & Wonnacott, 2004) participate in mechanisms of synaptic plasticity (Mansvelder *et al.*, 2002) and influence the probability of burst firing in mesolimbic dopamine neurons, with consequent increases in accumbens dopamine release (Schilstrom *et al.*, 1998). In the striatum *in vitro*, dopamine release is influenced locally by α7 nAChRs that are proposed to reside on glutamatergic afferents (Kaiser & Wonnacott, 2000; Barik & Wonnacott, 2006).

In the PFC, both α4β2* and α7 nAChRs have been implicated in attentional performance and cognition. Nicotine injections into the PFC enhance accuracy in a working memory task (Hahn *et al.*, 2003). Transgenic mice lacking the α7 nAChR subunit have deficits in attentional and working memory tasks (Young *et al.*, 2004; Fernandes *et al.*, 2006; Hoyle *et al.*, 2006; Young *et al.*, 2007), whereas α4β2* and α7 nAChR subtype-selective agonists improve working and reference memory in rats (Chan *et al.*, 2007). However, there is little information on the functional organization of nAChR subtypes within the PFC, especially in relation to mesocortical dopaminergic projections (Mansvelder *et al.*, 2006; Couey *et al.*, 2007). In the present study we have addressed the modulation of dopamine release in the PFC by nAChRs *in vitro* and *in vivo,* using subtype-selective agonists in conjunction with selective antagonists. We show that both α7- and β2-containing nAChRs can influence dopamine release.

Materials and methods

Animals

Male Sprague–Dawley rats (200–300 g, *in vitro* assays; 250–300 g, microdialysis; Charles River Laboratories and University of Bath Animal House breeding colony) were used. Prior to surgery, rats were housed in groups of four per cage in a temperature- and humidity-controlled environment with free access to food and water. Rats were kept on a 12-h light: dark cycle, with lights on at 07.00 h. All experiments were carried out within the guidelines of the United Kingdom Animals (Scientific Procedures) Act 1986.

Drugs

[7,8-^{3}H]Dopamine (43 Ci/mmol) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). 5-Iodo-3-(2(S)-azetidinylmethoxy)pyridine dihydrochloride (5-Iodo-A-85380, 5-I-A-85380), methyllycaconitine (MLA), nisoxetine hydrochloride, GBR12909 dihydrochloride, 6,7-dinitroquinoxaline-2,3-dione (DNQX) and α-CnTxMII were obtained from Tocris Cookson (Bristol, UK). (−)-Nicotine tartrate, dihydro-β-erythroidine (DHβE), chlorisondamine, mecamylamine, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate (MK801), pargyline hydrochloride, choline tartrate, ascorbic acid and nomifensine maleate were purchased from Sigma-Aldrich (Poole, Dorset, UK). (R)-N-(1-azabicyclo[2.2.2] oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide) (Compound A) and 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596) were synthesized by GSK (Harlow, UK). α-Bungarotoxin (α-Bgt) was acquired from Molecular Probes (Pourt Gebaow, the Netherlands). Optiphase Supermix was obtained from Perkin Elmer NV/SA (Zaventem, Belgium) and Optiphase 'Safe' was acquired from Fisher Chemicals (Loughborough, Leicestershire, UK). All other chemicals were of analytical grade and obtained from Sigma-Aldrich (Poole, Dorset, UK). For *in vivo* studies, PNU-120596 was dissolved in dimethylsulphoxide (DMSO) to 100 mM and diluted in artificial cerebrospinal fluid (aCSF) to give the desired final concentration for local administration via the microdialysis probe. All other drugs for administration via this route were dissolved directly in aCSF (pH adjusted to 7.4). For systemic injections, PNU-120596 and chlorisondamine were dissolved in 5% DMSO in saline, and MLA was dissolved directly in saline.

[^{3}H]Dopamine release in vitro

A 96-well filter plate method was used (Anderson *et al.*, 2000; Barik & Wonnacott, 2006). Briefly, two rats were killed by cervical dislocation, and the striatum (160 mg) and PFC (110 mg) were dissected. PFC was defined as 3 mm from anterior, 4 mm from bregma and 1 mm from corpus callosum. Tissue was chopped using a McIlwain tissue chopper to give 150 μm prisms. The prisms were washed in Kreb's buffer [KB; (in mM): NaCl, 118; KCl, 2.4; $CaCl_2$, 2.4; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; $NaHCO_3$, 25; D-glucose, 10; ascorbic acid, 1; pH 7.4 containing 10 μM pargyline) and incubated with 50 nM [^{3}H]dopamine for 30 min at 37°C. After four washes with KB containing 0.5 μM nomifensine, the prisms were distributed between the wells of a 96-well filter plate and incubated with or without antagonist or PNU-120596 in KB (70 μL) at 37°C for up to 20 min. Buffer was removed by filtration and replaced with 70 μL KB containing agonist and/or antagonist or PNU-120596. After a further 5 min at 37°C, buffer containing released [^{3}H]dopamine was collected by filtration. Treatments with DNQX and MK801 were conducted in Mg^{2+}-free KB. Tritium released into the filtrate and that remaining on the filters was determined by scintillation counting in a Wallac 1450 Microbeta Trilux (Perkin Elmer Wallac, Turku, Finland).

Data analysis

Fractional release of [^{3}H]dopamine was calculated as the percent of total radioactivity present before stimulation. Typical basal release of [^{3}H]dopamine in the absence of agonist was 14.0 ± 0.5% (n = 5) for striatal prisms and 13.0 ± 0.7% (n = 4) for PFC prisms; basal values were subtracted from agonist-evoked responses before constructing concentration–response curves. Statistical significance was assessed using unpaired Student's t-test, or one- or two-way ANOVAs with Tukey's *post hoc* test for multiple comparisons, as specified in the figure legends.

[^{3}H]Dopamine uptake

Striatal and PFC prisms were pre-incubated for 10 min in KB alone, 0.5 μM nomifensine, 50 nM GBR12909 or 1 μM nisoxetine, at 37°C or 4°C. Then [^{3}H]dopamine (100 μL; final concentration 50 nM) was added and samples were incubated with intermittent shaking for 5 or 30 min. Tissue was collected by filtration through GF/C glass fibre filters using a Millipore manifold system and washed three times with 3 mL ice-cold KB. Samples were counted in a Packard Tri-carb 1600

liquid scintillation counter with 46% counting efficiency (Perkin Elmer Life and Analytical Sciences).

Immunoprecipitation

nAChR subunits were quantitated by immunoprecipitation using subunit-specific polyclonal antibodies raised in rabbits and generated against peptides corresponding to the C-terminus or major intracellular loop of rat nAChR subunits (Champtiaux *et al.*, 2003; Gotti *et al.*, 2005). Rat striatal and PFC tissue was extracted using 2% Triton X-100 in (in mM): Tris-HCl, 50, pH 7.0; NaCl, 120; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2.5; phenylmethylsulphonyl fluoride, 2; containing the protease inhibitors leupeptin, bestatin, pepstatin A and aprotinin, each at 20 μg/mL (2 h, 4°C). Following preincubation for 2 h with 2 μM αBgt and labelling with 2 nM [^{3}H]epibatidine for 2 h at room temperature, nAChRs were immunoprecipitated by incubating overnight with saturating concentrations of affinity-purified subunit-selective antibodies (20–30 μg) and recovered by incubating the samples with anti-IgG-coated beads (Technogenetics, Milan, Italy). For the α2-, α3-, α4-, β2- and β4-subunits antibodies directed against the intracytoplasmic loop peptide were used, whereas for the α5-, α6- and β3-subunits antibodies directed against both the C-terminal and intracytoplasmic loop peptides were used. Each subunit-specific immunoprecipitate was quantified as a percentage of the total population of [^{3}H]epibatidine-labelled nAChRs precipitated and expressed as femtomoles per mg protein.

Surgery and brain microdialysis

Rats were anaesthetized with ketamine (75 mg/kg i.p.; Ketaset, Fortdodge Animal Health, Southampton, UK) and medetomidine (0.5 mg/kg i.p.; Domitor, Orion Pharma, Newbury, UK), and stereotaxically implanted with homemade unilateral microdialysis probes with a 4-mm active dialysis membrane (polyacrylonitrile/sodium methalyl sulphonate copolymer, internal diameter 0.22 mm, external diameter 0.31 mm; AN 69, Hospal, Bologna, Italy). The co-ordinates of the implantation were A/P + 3.3, L/M + 0.8 and V/D −5.0 from bregma and dura with the incisor bar kept at −3.3 mm (Paxinos & Watson, 1986). Buprenorphine (0.05 mg/kg s.c.; Vetergesic, Alstoe Animal Health, Sheriff Hutton, York, UK) was administered as an analgesic and anaesthesia was reversed with atipamezole (1 mg/kg/s.c.; Antisedan, Orion Pharma, UK). Microdialysis was performed the next day. Probes were perfused with aCSF at 2 μL/min (in mM: NaCl, 150; KCl, 3; $CaCl_2$, 1.4; $MgCl_2$, 0.8, pH 7.4), and 15-min fractions were collected. Following 4 h equilibration, baseline samples were collected for 90 min. Agonist (or vehicle control) in aCSF was then perfused for 30 min. Antagonists or modulators given locally were perfused continuously for 30 min before perfusion of agonist and

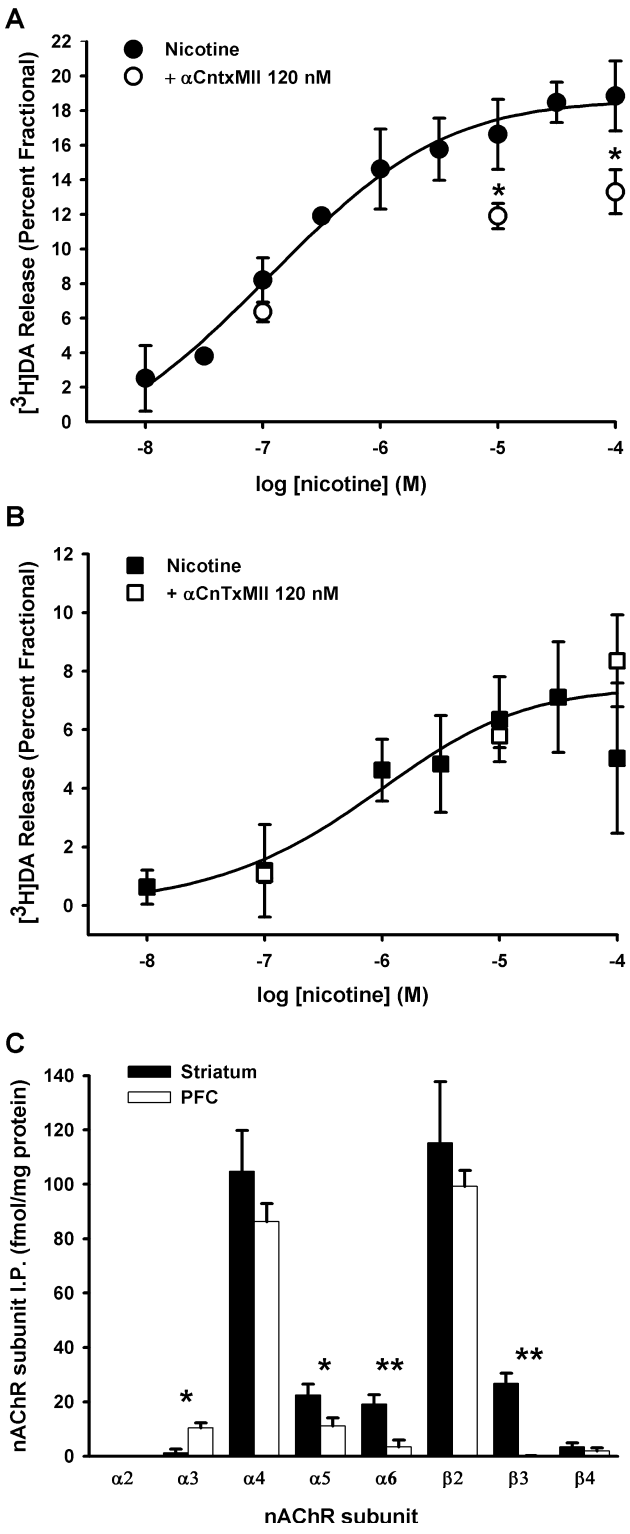


FIG. 1. Contribution of α6 nicotinic acetylcholine receptor (nAChR) subunits to nicotine-evoked [^{3}H]dopamine release from (A) rat striatal and (B) prefrontal cortex (PFC) prisms, and (C) immunoprecipitation of nAChR subunits. Release of [^{3}H]dopamine from rat striatal and PFC prisms (A and B) was stimulated by increasing concentrations of nicotine (0.01–100 μM) and measured by filtration. Fractional release was determined as a proportion of [^{3}H]dopamine present in the tissue at the time of stimulation, as described in the Materials and methods. Responses to nicotine were determined in the presence (open symbols) or absence (closed symbols) of 120 nM α-conotoxin MII (αCnTxMII) in striatal (A) and PFC (B) prisms. Basal release in the absence of agonist has been subtracted. Note the different *y*-axis scales for the two tissues. Data points were fitted to a single site Hill equation. Data points represent mean ± SEM, $n = 4$, *significantly different from response in the absence of αCnTxMII, $P < 0.05$, two-way ANOVA, Tukey's *post hoc* test. (C) nAChR subunits were immunoprecipitated using specific antibodies from Triton X-100-permeabilized rat striatal (filled bars) or PFC tissue (open bars) labelled with [^{3}H]epibatidine as described in the Materials and methods. The results are expressed as fmol/mg protein by reference to the specific radioactivity of the ligand. Each bar represents mean ± SEM, $n = 3$ independent experiments, *significantly different from striatum, $P < 0.05$, unpaired two-tailed Student's *t*-test. **Significantly different from striatum, $P < 0.01$, unpaired two-tailed Student's *t*-test.

remained in the aCSF until the end of the experiment. The vehicle for PNU-120596 was 0.01% DMSO: at higher concentrations (0.1%) DMSO itself increased dopamine overflow. The same syringe changes were carried out on all animals, irrespective of the need to change drug perfusion, so that this manipulation was common to all experiments. For systemic administration of antagonists, chlorisondamine (10 mg/kg i.p.) was given 7 days before surgery, whereas MLA (3 mg/kg i.p.) was injected 45 min before perfusion of agonist. The modulator PNU-120596 (1 mg/kg i.p.) was injected at the time when local perfusion of modulator would otherwise commence. At the end of the experiment, the rat was given an overdose of pentobarbitone (Euthatol, Merial, UK), and the brain was removed and fixed with 4% paraformaldehyde. Coronal sections (40 μm thick) were made, and microdialysis probe placement verified.

Dopamine analysis

Dopamine in the microdialysate samples was quantified by reverse-phase, ion-pair high-pressure liquid chromatography coupled with electrochemical detection (HPLC-ECD). A Jasco (PU-980) HPLC pump was used in conjunction with an ECD (DECADE, Antec, Leiden, the Netherlands) with an amperometric VT-03 cell set at 650 mV. Compounds were separated on a reverse-phase column (HAISIL 100 C-18, 3 μm, 100 × 2.1 mm; Higgins Analytical, Mountain View, California, USA). The mobile phase consisted of a mixture of 100 mmol/L sodium acetate, adjusted to pH 4.0 with perchloric acid, 0.7 mmol/L octanesulphonic acid, 1 mmol/L Na_2EDTA and 120 mL methanol/L HPLC grade water at a flow rate of 0.2 mL/min. The detection limit was about 1 fmol per 30 μL. The mean basal extracellular level of dialysate dopamine from the mPFC samples was 5.02 ± 0.35 fmoles (n = 161). These data have not been corrected for recovery across the microdialysis membrane.

In vitro *recovery of dopamine*

The *in vitro* recovery of dopamine across the microdialysis membrane was determined in randomly selected microdialysis probes. The microdialysis probes were placed in a standard solution of dopamine (10^{-8}–10^{-6} M) and flushed with aCSF at 2 μL/min for 5 min. Levels of dopamine entering the microdialysate were determined. Recoveries were calculated from the concentration of dopamine in the microdialysate as a percentage of the concentration in the standard solution. The recovery of dopamine across a 4-mm active microdialysis membrane was 18.3 ± 1.31% (n = 20).

Data analysis

Values for *in vivo* dopamine overflow are shown as mean ± SEM. The mean dopamine concentration of the initial three baseline samples was taken as 100% (<15% variation). Values for subsequent samples were calculated as a percentage of basal release. Data were analysed using two-way ANOVA for repeated measurements (time × treatment) followed by Bonferroni *post hoc* test for multiple comparisons. Where necessary, data within the same curve were analysed by one-way ANOVA for repeated measures followed by Newman–Keuls *post hoc* test. Peak areas (area under the curve) for multiple groups were compared using one-way ANOVA with Newman–Keuls *post hoc* test. Unpaired two-tailed *t*-test was used for comparing peak areas between two groups.

Results

[^{3}H]Dopamine release from PFC prisms in vitro *is modulated by β2-containing nAChRs that do not include α6-subunits*

Nicotine has been shown previously to elicit [^{3}H]dopamine release from cortical synaptosomes and prisms by interacting with local nAChRs (Whiteaker *et al.*, 1995; Cao *et al.*, 2005). Here, we have used an *in vitro* 96-well plate filter assay (Puttfarcken *et al.*, 2000; Barik & Wonnacott, 2006) to assess the subtypes of nAChR that modulate release from PFC prisms loaded with [^{3}H]dopamine; striatal prisms were assayed in parallel for comparison. Loading experiments in the presence of different selective uptake inhibitors confirmed that [^{3}H]dopamine is predominantly accumulated by dopamine terminals in both the striatum and PFC (data not shown).

Nicotine (0.01–100 μM) induced the concentration-dependent release of [^{3}H]dopamine, with similar potencies in striatal and PFC prisms (striatum pEC_{50} −6.8 ± 0.3; PFC pEC_{50} −6.2 ± 0.3; Fig. 1A and B). However, nicotine-induced fractional release in striatal prisms was 2.5 ± 0.3 times more efficacious than in PFC prisms. In both tissues, responses to 10 μM nicotine were attenuated by mecamylamine (30 μM), and abolished by DHβE (30 μM) and chlorisondamine (100 μM; Table 1).

The β2-selective agonist 5-I-A-85380 (0.001–1 μM; Mukhin *et al.*, 2000; Mogg *et al.*, 2004) also increased [^{3}H]dopamine release (Fig. 2) with comparable potency in both regions (striatum pEC_{50} −7.9 ± 0.2; PFC pEC_{50} −8.1 ± 0.3; Fig. 2). As seen with nicotine, the maximum response to 5-I-A-85380 was greater in the striatum than the PFC. 5-I-A-85380-evoked [^{3}H]dopamine release was inhibited by DHβE that shifted the dose–response curve for 5-I-A-85380 to the right ($F_{3,12}$ = 3.64; P < 0.05 for striatum; $F_{3,12}$ = 3.86; P < 0.05 for PFC; Fig. 2).

A maximally effective concentration of the $\alpha3/\alpha6\beta2$* nAChR antagonist αCnTxMII (120 nM) partially inhibited nicotine-evoked [^{3}H]dopamine release in the striatum by up to 29.5 ± 2.2% ($F_{3,8}$ = 4.32; P < 0.05; Fig. 1A), in agreement with previous studies (Kulak *et al.*, 1997; Kaiser *et al.*, 1998; Grady *et al.*, 2007). In

TABLE 1. Effect of various antagonists on nicotine- and choline-evoked [^{3}H]dopamine release in striatum and PFC minces

	[^{3}H]dopamine release			
	Fractional release (%)		Inhibition (%)	
Agonist and antagonist	Striatum	PFC	Striatum	PFC
(−)-Nicotine (10 μM)				
Control	16.6 ± 2.0	6.32 ± 1.7	–	–
+Mecamylamine (30 μM)	3.4 ± 1.6	1.5 ± 1.1	79.4 ± 2.3	75.5 ± 2.0
+Chlorisondamine (100 μM)	1.5 ± 1.3	0.4 ± 0.6	91.3 ± 3.5	93.0 ± 4.2
+DHβE (30 μM)	2.4 ± 2.0	0.5 ± 0.3	85.5 ± 4.4	92.1 ± 1.76
Choline (3 mM)				
Control	5.6 ± 0.9	5.2 ± 0.3	–	–
+αBgt (40 nM)	0.3 ± 1.0	0.2 ± 0.3	94.0 ± 2.6	97.0 ± 0.9
Choline (3 mM) + PNU-120596 (10 μM)				
Control	9.7 ± 0.6	8.8 ± 0.7	–	–
+αBgt (40 nM)	0.7 ± 1.2	0.6 ± 0.9	93.0 ± 3.2	93.0 ± 4.0

αBgt, α-bungarotoxin; DHβE, dihydro-β-erythroidine; PFC, prefrontal cortex. Data are presented as mean ± SEM, n = 3–5 independent experiments for each condition.

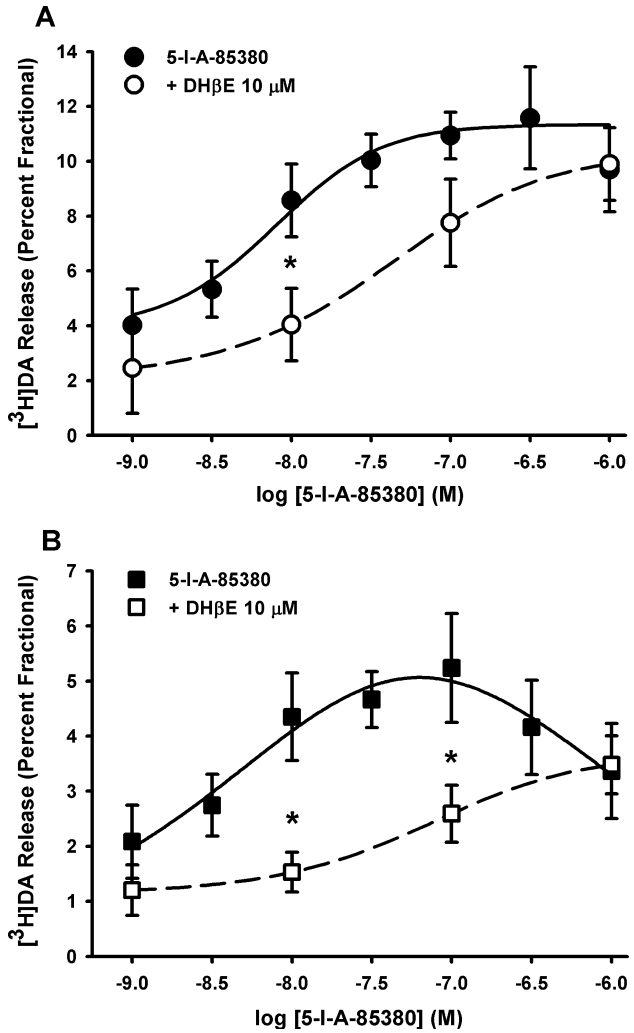


FIG. 2. Contribution of $\beta 2$ subunit-containing nAChRs to [^{3}H]dopamine release from prisms of (A) striatum and (B) PFC. Release of [^{3}H]dopamine from rat striatal (A) and PFC prisms (B) was measured by filtration, and fractional release was determined as a proportion of [^{3}H]dopamine present in the tissue at the time of stimulation, as described in the Materials and methods. The concentration-dependence of [^{3}H]dopamine release evoked by 5-I-A-85380 (0.001–1 μM) was determined in the presence (open symbols) and absence (closed symbols) of 10 μM dihydro-β-erythroidine (DHβE) in striatal (A) and PFC prisms (B). Basal release in the absence of agonist has been subtracted. Note the different *y*-axis scales for the two tissues. Data points represent mean ± SEM, $n = 4$; $^*P < 0.05$, statistically different from response in the absence of DHβE, two-way ANOVA, Tukey's *post hoc* test.

contrast, in PFC prisms this antagonist had no detectable effect on nicotine-evoked [^{3}H]dopamine release ($F_{3,8} = 0.982$; Fig. 1B). To determine if the lack of αCnTxMII-sensitive [^{3}H]dopamine release in the PFC was a reflection of altered subunit expression, a quantitative analysis of nAChR subunits (α2-6 and β2-4) in rat PFC and striatal tissue was performed by immunoprecipitation (Gotti *et al.*, 2005). In the PFC, in contrast to levels in the striatum, β3-containing nAChRs were not detectable and the percentage of α6-containing nAChRs (2.6% of total [^{3}H]epibatidine-labelled nAChRs present in the tissue extract) is lower than the threshold of the immunoprecipitation technique (5%; Fig. 1C). The expression of other nAChR subunits was similar between the two regions, except for a significantly lower level of the α5 nAChR subunit in PFC (Fig. 1C). The level of the α3-subunit in PFC was low, but its expression is specific and significantly higher ($P < 0.05$) than in the striatum, as determined in parallel experiments using the same antibodies.

α7 nAChRs indirectly modulate [^{3}H]dopamine release via ionotropic glutamate receptors

In striatal slices (but not synaptosomes) a portion of anatoxin-a-evoked [^{3}H]dopamine release could be blocked by αBgt, consistent with an indirect effect of α7 nAChRs (Kaiser & Wonnacott, 2000). In the present experiments, αBgt (40 nM) did not decrease [^{3}H]dopamine release evoked by nicotine at any concentration tested (10 nM–100 μM) in either striatum or PFC prisms (Fig. 3). However, co-application of the specific α7 nAChR-positive allosteric modulator PNU-120596 (10 μM; Hurst *et al.*, 2005) with nicotine further increased [^{3}H]dopamine release in response to the higher concentrations of nicotine in both striatal and PFC prisms. This corresponds to an increase in evoked release of ∼40% (after subtraction of basal levels) in both regions. In the presence of αBgt, the enhanced release in the presence of PNU-120596 was reduced to the level of the response to nicotine alone. The ability of α7 nAChRs in both striatum and PFC to influence [^{3}H]dopamine release was confirmed using an α7 nAChR-selective agonist. Choline (3 mM) significantly increased the fractional release of [^{3}H]dopamine from both regions to a similar extent, and this was inhibited by α-Bgt (Table 1). PNU-120596 (10 μM) further augmented responses to 3 mM choline (Fig. 4A), and the responses were completely blocked by α-Bgt (Table 1).

[^{3}H]Dopamine release elicited by choline plus PNU-120596 was partially blocked in the presence of ionotropic glutamate receptor antagonists (tested in Mg^{2+}-free conditions; see Materials and methods). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonist DNQX (200 μM) significantly reduced [^{3}H]dopamine release by 59.8 ± 3.2% (striatum) and 69 ± 4.2% (PFC); the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK801 (5 μM) inhibited [^{3}H]dopamine release by 40.3 ± 3.6% (striatum) and 58.7 ± 4.1% (PFC; Fig. 4B). The effects of DNQX and MK801 were additive when co-applied with choline and PNU-120596: under this condition the amount of [^{3}H]dopamine release did not differ significantly from basal release (Fig. 4B).

To determine if glutamate receptors also mediate [^{3}H]dopamine release evoked by activation of $\beta 2^*$ nAChRs, we used 1 μM nicotine, a concentration not blocked by αBgt (Fig. 3), to selectively activate $\beta 2^*$ nAChRs. Nicotine-evoked [^{3}H]dopamine release in both striatum and PFC was unaffected by the presence of DNQX and/or MK801 (Fig. 4C). Thus, α7 nAChRs can influence dopamine release in the striatum and PFC, via the release of glutamate, whereas β2-containing nAChRs promote [^{3}H]dopamine release without the intervention of ionotropic glutamate receptors.

Nicotine-evoked dopamine overflow in mPFC

To determine if α7 and $\beta 2^*$ nAChRs modulate endogenous dopamine release in mPFC we used *in vivo* microdialysis in conscious freely moving rats. Local perfusion of nicotine (30 μM–3 mM) into the mPFC, delivered via the microdialysis probe by retrograde dialysis for 30 min, produced a concentration-dependent increase in extracellular dopamine levels that rapidly returned to basal levels after removal of nicotine from the perfusate (Fig. 5A). The highest concentration of nicotine (3 mM) produced a large increase in dopamine levels ($F_{9,20} = 12.1$; $P < 0.05$) compared with the response to 1 mM nicotine ($F_{5,30} = 3.88$; $P < 0.05$). The increase in extracellular dopamine in

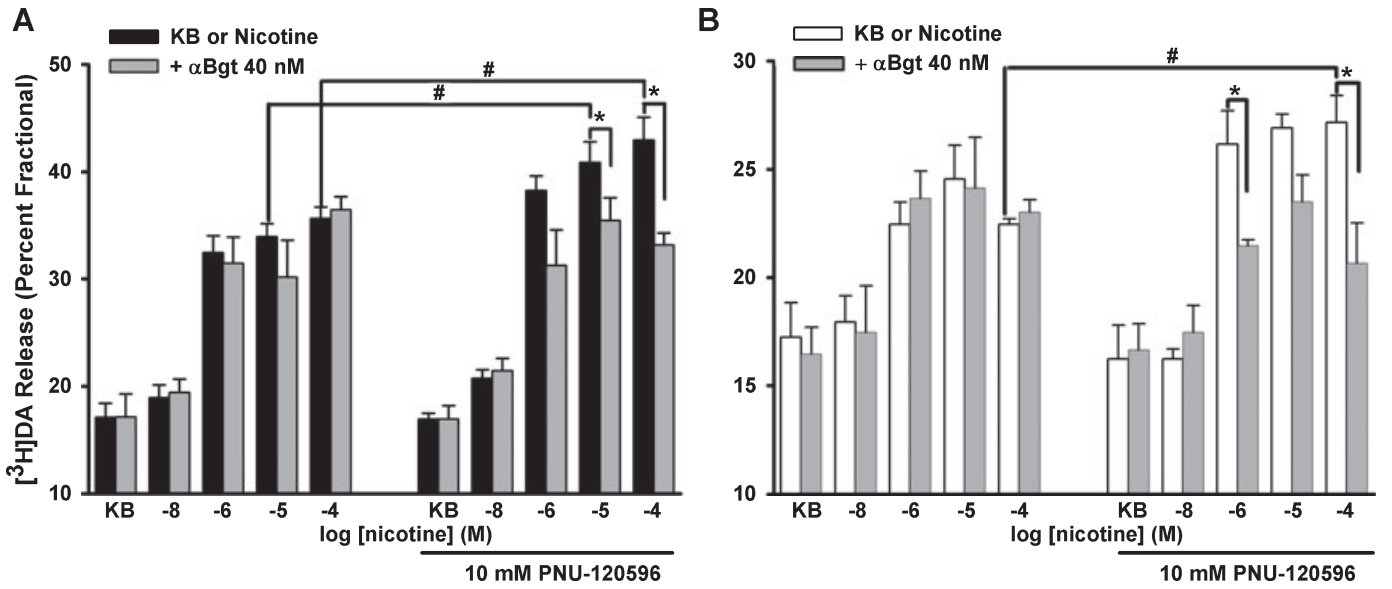


FIG. 3. Effect of PNU-120596 on nicotine-evoked [^{3}H]dopamine release. Release of [^{3}H]dopamine from rat striatal (A) and PFC prisms (B) was measured by filtration, and fractional release was determined as a proportion of [^{3}H]dopamine present in the tissue at the time of stimulation, as described in the Materials and methods. Tissue was preincubated with 40 nM α-bungarotoxin (αBgt; grey bars) for 20 min and/or 10 μM PNU-120596 for 10 min prior to stimulation with nicotine (0.01–100 μM) for 5 min. Bars represent mean ± SEM (n = 4). *$P < 0.05$, statistically significant effect of αBgt; $^{\#}P < 0.05$, statistically significant effect of PNU-120596, by one-way ANOVA with *post hoc* Tukey's test. KB, Kreb's buffer.

response to 1 mM nicotine was blocked by prior systemic administration of the long-acting non-competitive nAChR antagonist chlorisondamine (10 mg/kg i.p.; $F_{9,30} = 7.35$; $P < 0.05$), administered 7 days before microdialysis. Chlorisondamine alone did not affect basal values, in comparison to vehicle-treated controls (4.64 ± 0.36 fmoles and 5.00 ± 0.46 fmoles, respectively; Fig. 5B). The response to 1 mM nicotine was also completely blocked by local administration of chlorisondamine (100 μM; $F_{8,27} = 6.34$; $P < 0.05$) via the microdialysis probe (Fig. 5C), confirming that the effect of nicotine was mediated by nAChRs in the mPFC, consistent with the data from *in vitro* prisms.

To address the subtypes of nAChR responsible for nicotine-evoked dopamine overflow, the subtype-selective antagonists DHβE and MLA were locally applied, before and during the delivery of 1 mM nicotine. DHβE (10 μM) significantly decreased the response to nicotine ($F_{8,27} = 11.1$; $P < 0.05$), whereas the α7 nAChR-selective antagonist MLA (100 μM) failed to affect the nicotine-induced increase in extracellular dopamine (Fig. 5C).

The effect of nAChR subtype-selective agonists on dopamine overflow in the mPFC

Local perfusion of 5-I-A-85380 (100 μM and 1 mM) increased extracellular dopamine overflow above basal (Fig. 6; $F_{13,56} = 17.7$; $P < 0.05$; $F_{13,56} = 23.4$; $P < 0.05$, respectively). The two concentrations tested elicited similar responses, comparable in magnitude to that elicited by 3 mM nicotine (Fig. 5A). The increase in dopamine overflow in response to 100 μM 5-I-A-85380 was abolished by the continuous co-perfusion of DHβE, consistent with 5-I-A-85380 acting at β2* nAChRs (Fig. 6; $F_{13,56} = 7.06$; $P < 0.05$).

To examine if activation of α7 nAChRs can elicit an increase in dopamine overflow, we took advantage of the novel α7 nAChR-selective agonist Compound A (Cilia *et al.*, 2005; Dickinson *et al.*, 2008). Local perfusion of Compound A (100 μM and 1 mM) into the mPFC produced a concentration-dependent increase in extracellular dopamine levels above basal (Fig. 7A and B; $F_{13,42} = 5.93$; $P < 0.05$; $F_{13,112} = 4.85$; $P < 0.05$, respectively); the response to the higher concentration tested was markedly lower than the maximum responses to nicotine and 5-I-A-85380. Increases in dopamine overflow in response to 100 μM or 1 mM Compound A were attenuated by administration of MLA, either systemically (3 mg/kg i.p.; Fig. 7A; $F_{13,42} = 5.53$; $P < 0.05$) or locally (100 μM; Fig. 7B; $F_{13,112} = 11.6$; $P < 0.05$).

To substantiate a role for α7 nAChRs in the modulation of dopamine release in the mPFC, we examined the effect of PNU-120596. Co-perfusion of PNU-120596 (10 μM) with a subthreshold concentration of Compound A (10 μM) resulted in a significant increase in dopamine overflow, comparable to the response to 1 mM nicotine (Fig. 8A; $F_{11,48} = 6.77$; $P < 0.05$). Local perfusion of PNU-120596 (10 μM) or vehicle (0.01% DMSO) in the absence of exogenous agonist did not increase dopamine overflow above basal levels. However, systemic administration of PNU-120596 (1 mg/kg s.c.) in the absence of agonist did elicit a rapid increase in dopamine overflow in the mPFC that declined gradually over the next hour (Fig. 8B; $F_{14,90} = 12.3$; $P < 0.05$). This effect was attenuated by prior systemic injection of MLA (3 mg/kg i.p.; $F_{14,45} = 8.49$; $P < 0.05$).

Discussion

We have used an *in vitro* release assay and *in vivo* microdialysis to characterize nAChR-evoked dopamine release in the rat PFC. Nicotine and 5-I-A-85380 promoted DHβE-sensitive dopamine release *in vitro* and *in vivo,* indicative of the participation of local β2-containing nAChRs. Nicotine-evoked [^{3}H]dopamine release from PFC prisms was insensitive to αCnTxMII, in contrast to release from striatal tissue; the absence of α6-containing nAChRs in the PFC is supported by immunoprecipitation experiments. The ability of α7 nAChRs in the PFC to facilitate dopamine release was demonstrated using choline *in vitro* and Compound A, delivered via the microdialysis probe,

in vivo. These responses were blocked by α7 nAChR-selective antagonists and potentiated by PNU-120596. Ionotropic glutamate receptor antagonists DNQX and MK801 blocked the release of [^{3}H]dopamine from PFC prisms stimulated by choline plus PNU-120596, consistent with α7 nAChRs acting indirectly by promoting glutamate release, as observed in the striatum. Finally, the differential effects of systemic and locally applied PNU-120596 *in vivo* suggest that in the presence of the potentiator extra-cortical cholinergic tone can activate α7 nAChRs to promote dopamine overflow in the mPFC.

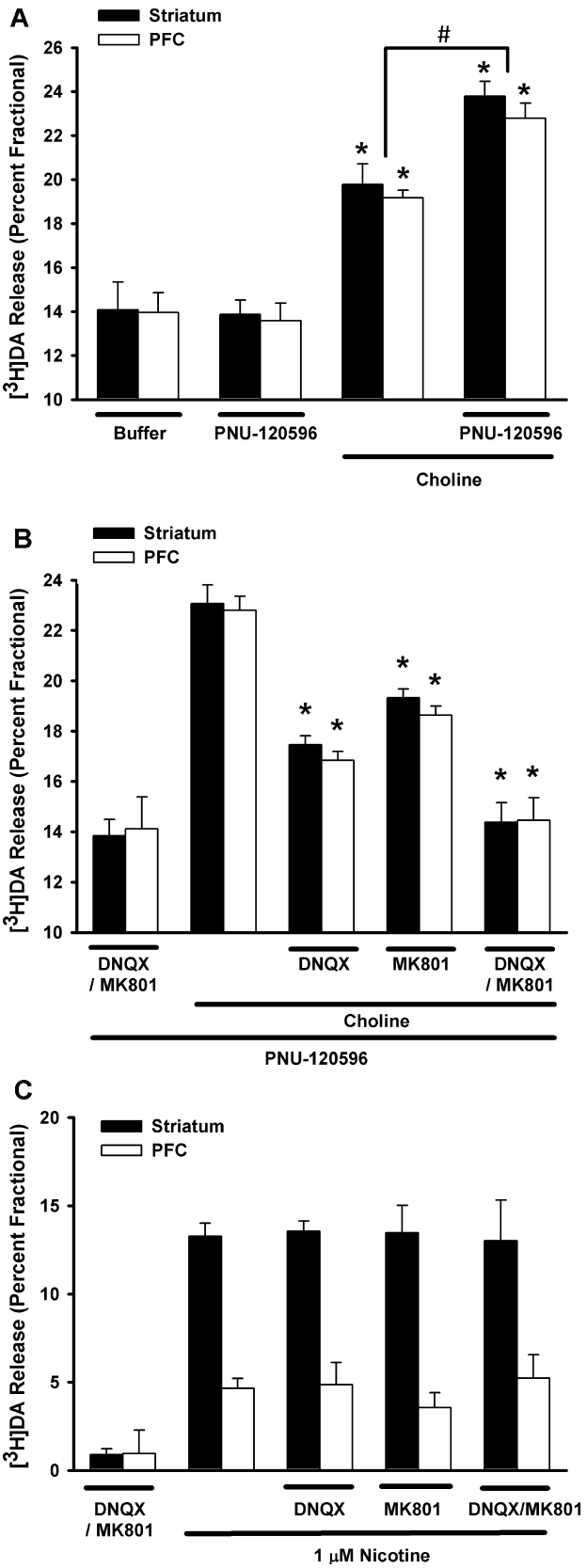


FIG. 4. α7 nAChRs increase [^{3}H]dopamine release in striatum and prefrontal cortex (PFC) via ionotropic glutamate receptors. Release of [^{3}H]dopamine from rat striatal (black bars) and PFC prisms (open bars) was measured by filtration, and fractional release was determined as a proportion of [^{3}H]dopamine present in the tissue at the time of stimulation, as described in the Materials and methods. (A) Tissue was preincubated with or without 10 μM PNU-120596 for 10 min prior to stimulation with 3 mM choline in the presence or absence of PNU-120596 for 5 min. (B) Tissue was preincubated with 10 μM PNU-120596 with or without antagonist for 10 min prior to stimulation with 3 mM choline plus PNU-120596 in the presence or absence of 6,7-dinitroquinoxaline-2, 3-dione (DNQX; 200 μM) and/or (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801; 5 μM) for 5 min. (C) Tissue was preincubated with or without antagonist for 10 min prior to stimulation with 1 μM nicotine in the presence or absence of DNQX (200 μM) and/or MK801 (5 μM) for 5 min. Bars show mean ± SEM. $n = 5$, *significantly different from buffer control (A) or agonist-evoked response (B), $P < 0.05$; $^{\#}P < 0.05$, significant effect of PNU-120596, for both striatum and PFC, one-way ANOVA with *post hoc* Tukey's test.

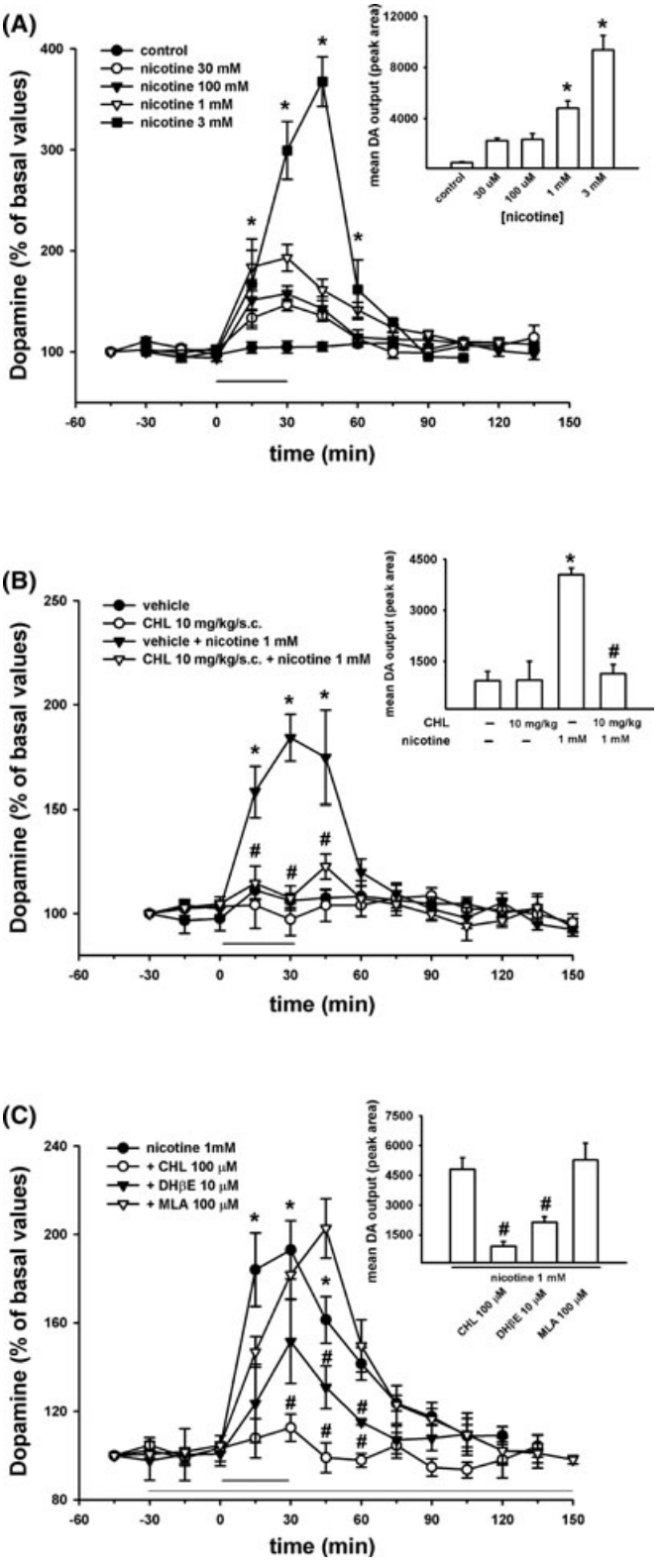
Dopamine in the PFC plays a significant neuromodulatory role in aspects of executive function and goal-directed behaviours, and dopamine levels are critical for optimal performance (Schultz, 2002; Floresco & Magyar, 2006). Prefrontal dopaminergic dysfunction contributes to neuropysychiatric conditions, including schizophrenia (Toda & Abi-Dargham, 2007) and attention deficit hyperactivity disorder (Levy, 2008). nAChR ligands have been advocated as therapeutic candidates for such conditions (Jensen *et al.*, 2005; Mazurov *et al.*, 2006; Arneric *et al.*, 2007). In view of the well-documented ability of various nAChR subtypes to influence striatal and accumbens dopamine release (Klink *et al.*, 2001; Grady *et al.*, 2007), detailed examination of the nicotinic modulation of dopamine release in the PFC was warranted. By exploiting both *in vitro* and *in vivo* assays we have generated a comprehensive picture of the diversity and interplay of local nAChR subtypes capable of influencing dopamine release in the rat PFC.

Heteromeric β2-containing nAChR subtypes modulate dopamine release in the PFC; comparison with striatum

Nicotine-evoked [^{3}H]dopamine release from PFC prisms and dopamine overflow from the mPFC *in vivo* was concentration-dependent and nAChR-dependent, with complete blockade produced by chlorisondamine. Sensitivity to DHβE supports the involvement of β2-containing nAChRs, and this is reinforced by the ability of 5-I-A-85380 to elicit DHβE-sensitive dopamine release *in vitro* and *in vivo* (Mukhin *et al.*, 2000; Mogg *et al.*, 2004).

Dopaminergic projections to the rodent striatum contain a variety of β2-containing nAChRs that include different combinations of α4-, α5-, α6- and β3-subunits (Salminen *et al.*, 2007). Those with an α6β2 interface can be discriminated by blockade with αCnTxMII (Champtiaux *et al.*, 2003; Salminen *et al.*, 2007) and account for about 30% of the total response to nicotine in rat striatal prisms (Fig. 1; Kaiser *et al.*, 1998; Cao *et al.*, 2005). The notable difference between striatal and PFC prisms is the absence of an αCnTxMII-sensitive component of nicotine-evoked [^{3}H]dopamine release in PFC (Fig. 1): this distinction is in agreement with a previous study (Cao *et al.*, 2005). The absence of α6-containing nAChRs from the PFC was

corroborated by immunoprecipitation experiments. Although a small amount of α3 nAChR subunit was detected in the PFC, and α3β2-containing nAChRs are sensitive to αCnTxMII (Cartier *et al.*, 1996), the failure of this toxin to block nicotine-evoked [^{3}H]dopamine release from PFC prisms suggests that α3-subunits are associated with nAChRs that do not influence dopamine release.



Interestingly, in PFC prisms the efficacy of [^{3}H]dopamine release in response to nicotine and 5-I-A-85380 was about 40% of the responses in striatal prisms; a lower response in PFC was also noted by Cao *et al.*, (2005). As release is calculated as a fraction of the total [^{3}H]dopamine in the tissue, this is not a reflection of the lower density of dopaminergic terminals in the PFC; nor is the lower efficacy of release due to intrinsic differences in presynaptic dopaminergic mechanisms because α7 nAChR stimulation provokes fractional release of comparable magnitude in the two tissues. One interpretation is that a smaller proportion of prefrontal dopamine terminals bear nAChRs, compared with the striatum. Alternatively, differences in the density of nAChRs or their coupling to exocytosis could influence the magnitude of [^{3}H]dopamine release. The absence of high-affinity α6β2* nAChRs from PFC dopamine terminals could be a significant factor.

Two recent studies have provided evidence for α6β2* nAChRs making a greater functional contribution in the mesolimbic system (with respect to α6*-mediated currents in VTA neurons and dopamine release in the ventral striatum/nucleus accumbens core) than in the nigrostriatal system (Drenan *et al.*, 2008; Exley & Cragg, 2008). This requires that mesolimbic and mesocortical neurons projecting to the nucleus accumbens and PFC, respectively, must differ in expression of the α6-subunit, or assembly of α6-containing nAChRs, or trafficking of those nAChRs to functionally effective presynaptic destinations in the terminal field. Single-cell polymerase chain reaction analysis indicates that about 15% of dopaminergic neurons in the rat VTA and substantia nigra do not express α6 mRNA (Klink *et al.*, 2001); it is possible that these neurons include a population that projects to the PFC.

Although αCnTxMII was not applied *in vivo* in the present study, due to its large size and limited availability, the correspondence between the pharmacology of the nicotinic modulation of dopamine release *in vitro* and *in vivo* with respect to other drugs, coupled with the data from immunoprecipitation assays, encourage the assumption that α6β2* nAChRs do not contribute to dopamine release in the mPFC *in vivo*.

FIG. 5. Nicotine-evoked dopamine overflow in the mPFC *in vivo*. Rats were implanted unilaterally with a microdialysis probe in the mPFC, as described in the Materials and methods; 15-min (30-μL) serial fractions were collected and dopamine was quantified using HPLC-ECD. (A) Concentration-dependence of nicotine-evoked dopamine overflow was determined, after collection of three–four stable baseline samples, by local infusion of nicotine (30 μM–3 mM) or aCSF (vehicle) by reverse dialysis for 30 min (horizontal bar). $^*P < 0.05$, significant effect of nicotine vs vehicle by two-way repeated-measures ANOVA with Bonferroni *post hoc* test. (B) Antagonism of nicotine-evoked dopamine overflow by systemic chlorisondamine (CHL). Rats received CHL (10 mg/kg) or vehicle s.c. 7 days prior to microdialysis. After collection of three or four stable baseline samples, nicotine (1 mM) or aCSF was given locally by reverse dialysis for 30 min (horizontal bar). $^*P < 0.05$, significant effect of nicotine vs. vehicle, $^{\#}P < 0.05$, significant effect of CHL on nicotine-evoked dopamine overflow, two-way repeated-measures ANOVA with Bonferroni *post hoc* test. (C) Antagonism of nicotine-evoked dopamine overflow by locally applied antagonists. Nicotine (1 mM)-evoked dopamine overflow was determined in the presence and absence of CHL (100 μM), dihydro-β-erythroidine (DHβE; 10 μM) or methyllycaconitine (MLA; 100 μM). Antagonist was delivered by reverse dialysis 30 min before administration of nicotine (1 mM) for 30 min (short horizontal bar) and was maintained in the aCSF throughout the remainder of the experiment (long horizontal bar). $^*P < 0.05$, significant effect of nicotine vs. vehicle, $^{\#}P < 0.05$, significant effect of antagonists on nicotine-evoked dopamine overflow, two-way repeated-measures ANOVA with Bonferroni *post hoc* test. Values are the mean ± SEM, $n = 4$–6. Insets: Dopamine overflow measured as area under the curve. $^*P < 0.01$ vs. vehicle, $^{\#}P < 0.05$ vs. nicotine, one-way ANOVA with Newman–Keuls test.

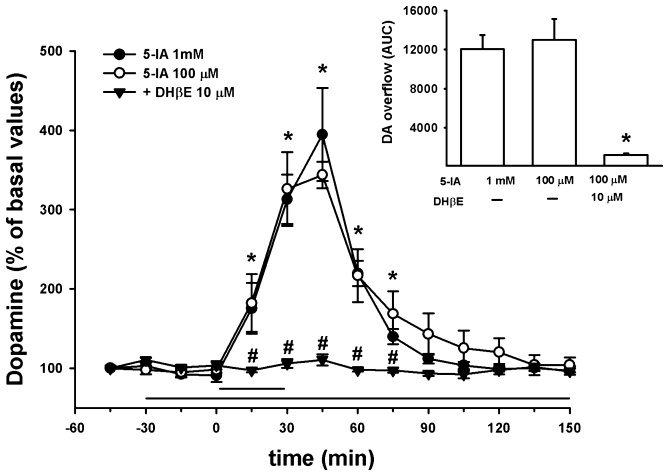


FIG. 6. 5-I-A-85380-evoked dopamine overflow in the mPFC *in vivo*. Rats were implanted unilaterally with a microdialysis probe in the mPFC as described in the Materials and methods; 15-min (30-μL) serial fractions were collected and dopamine was quantified using HPLC-ECD. After collection of four stable baseline samples, 5-I-A-85380 (5-IA, 100 μM; 1 mM) or aCSF was given locally by reverse dialysis for 30 min (short horizontal bar). When present, dihydro-β-erythroidine (DHβE; 10 μM) was introduced into the aCSF 30 min prior to agonist and was maintained throughout the remainder of the experiment (long horizontal bar). $^{*}P < 0.05$, significant effect of 5-I-A-85380, one-way repeated-measures ANOVA with Newman–Keuls *post hoc* test, $^{\#}P < 0.05$, significant effect of DHβE on 5-I-A-85380-evoked dopamine overflow, two-way repeated-measures ANOVA with *post hoc* Bonferroni's test. Values are the mean ± SEM ($n = 5$ per group) Insets: Dopamine overflow measured as area under the curve. $^{*}P < 0.01$ vs. DHβE 100 μM, unpaired Student's *t*-test.

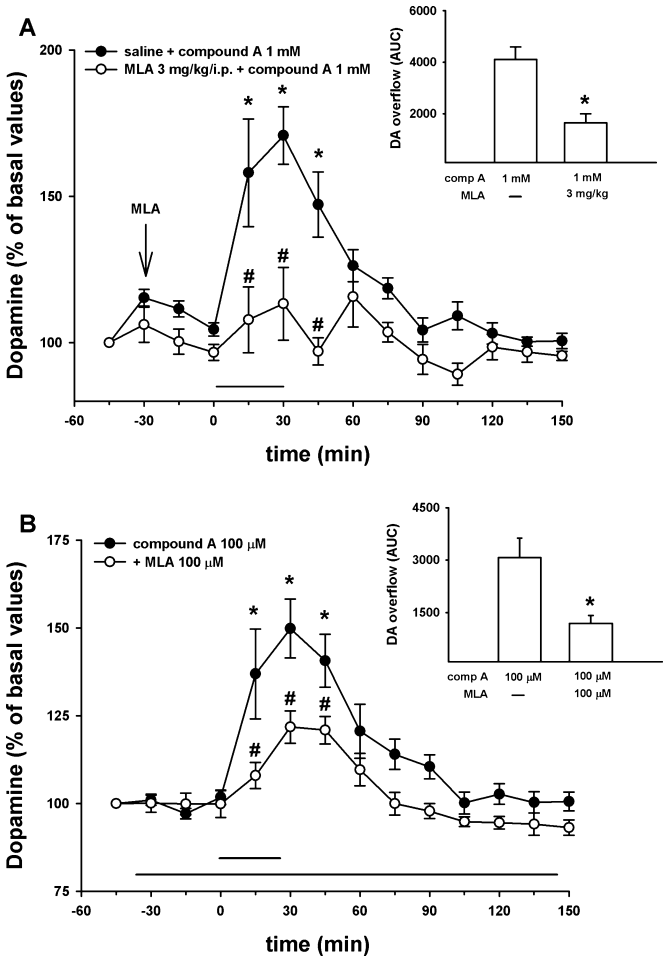


FIG. 7. Dopamine overflow in the mPFC *in vivo* evoked by the α7 nAChR agonist Compound A. Rats were implanted unilaterally with a microdialysis probe in the mPFC as described in the Materials and methods; 15-min (30-μL) serial fractions were collected and dopamine was quantified using HPLC-ECD. After collection of four stable baseline samples, Compound A (A: 1 mM; B: 100 μM) was given locally by reverse dialysis for 30 min (short horizontal bar). Methyllycaconitine (MLA) or vehicle was given (A) systemically (3 mg/kg), 45 min prior to the start of agonist infusion; or (B) locally (100 μM), by reverse dialysis 30 min prior to Compound A and was maintained throughout the remainder of the experiment (long horizontal bar). $^{*}P < 0.05$, significant effect of Compound A, one-way repeated-measures ANOVA with Newman–Keuls *post hoc* test, $^{\#}P < 0.05$, significant effect of MLA on Compound A-evoked dopamine overflow, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. Values are the mean ± SEM, $n = 4$–9 per group. Insets: Dopamine overflow measured as area under the curve. $^{*}P < 0.01$ vs. Compound A, unpaired Student's *t*-test.

α7 nAChRs influence dopamine release in the PFC

Although some 40% of rat dopaminergic neurons in the VTA express the α7 nAChR subunit and respond to choline (Klink *et al.*, 2001), there is little evidence that α7 nAChRs are trafficked to the terminals: [^{3}H]dopamine release from striatal synaptosomes is insensitive to α7 nAChR antagonists (Mogg *et al.*, 2002) and unaffected by deletion of the α7 gene (Salminen *et al.*, 2004). However, in striatal slices, a portion of [^{3}H]dopamine release evoked by higher concentrations of the broad-spectrum nAChR agonist anatoxin-a could be blocked by αBgt, implicating a contribution from α7 nAChRs in more integrated tissue preparations (Kaiser & Wonnacott, 2000). Here we used α7 nAChR-specific agonists, with and without the α7 nAChR-selective positive allosteric modulator PNU-120596, to activate this component. *In vitro*, choline evoked a similar proportion of [^{3}H]dopamine release from both striatal and PFC prisms, and this was enhanced by PNU-120596 and inhibited by αBgt, confirming the involvement of α7 nAChRs. This is also supported by the absence of choline-evoked [^{3}H]dopamine release from striatal prisms from α7 nAChR knockout mice (Quarta *et al.*, 2008) and the sensitivity of choline-induced [^{3}H]dopamine release to the novel α7-selective antagonist α-conotoxin ARIB[V11L, V16D] in the striatum (Innocent *et al.*, 2008). Although nicotine-evoked [^{3}H]dopamine release was insensitive to αBgt in agreement with Cao *et al.*, (2005), the addition of PNU-120596 did elicit an αBgt-sensitive increment in release. This suggests that the allosteric potentiator can unmask a subthreshold interaction of nicotine with α7 nAChRs, possibly by reversing or attenuating nicotine-mediated receptor desensitization (Hurst *et al.*, 2005).

In agreement with the *in vitro* results, local delivery of the α7 nAChR-selective agonist Compound A (Cilia *et al.*, 2005; Dickinson *et al.*, 2008) into the mPFC *in vivo* evoked dopamine overflow that was blocked by local or systemic administration of MLA, whereas nicotine-evoked dopamine overflow was insensitive to MLA. PNU-120596, in conjunction with a subthreshold concentration of Compound A, substantially increased dopamine overflow. In addition to amplifying or unmasking α7 nAChR responses to exogenous agonist, positive allosteric modulators have the potential to augment the effects of endogenous agonist. This is particularly the case for PNU-120596 because it reduces α7 nAChR desensitization (Hurst *et al.*, 2005). Therefore in the continuous presence of agonist it can convert a desensitized nAChR to a conducting state (Gronlien *et al.*, 2007; Young *et al.*, 2008). Hence it is noteworthy that systemic administration of PNU-120596 alone provoked a significant increase in dopamine overflow in the mPFC, and this response was attenuated by

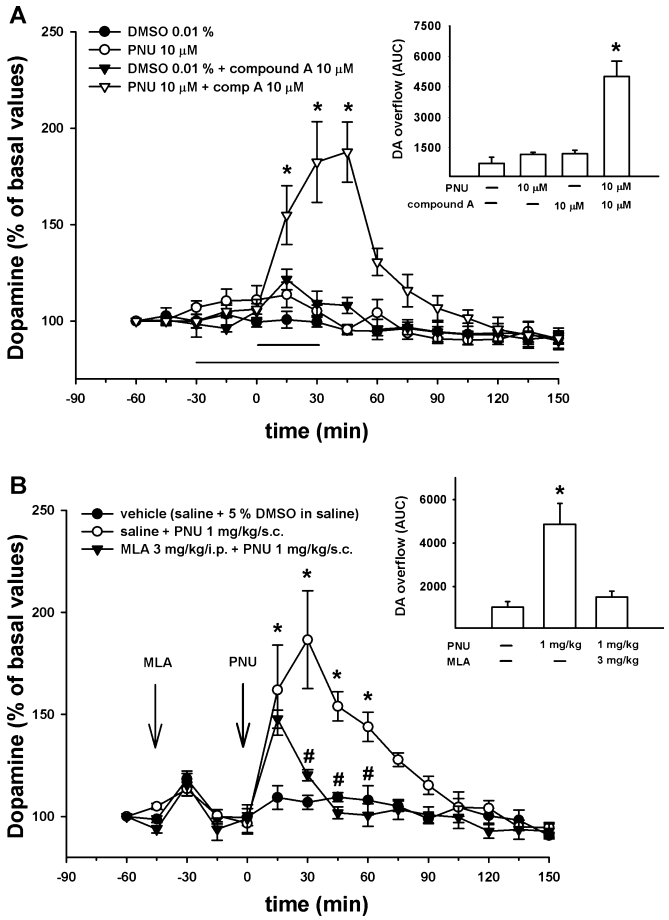


FIG. 8. The effect of PNU-120596 on dopamine overflow in the mPFC *in vivo*. Rats were implanted unilaterally with a microdialysis probe in the mPFC as described in the Materials and methods; 15-min (30-μL) serial fractions were collected and dopamine was quantified using HPLC-ECD. (A) After collection of three stable baseline samples, PNU-120596 or vehicle [0.01% dimethylsulphoxide (DMSO)] was delivered into the mPFC by reverse dialysis 30 min before perfusion of a subthreshold concentration of Compound A (10 μM) for 30 min (short horizontal bar). PNU-120596 or vehicle was maintained in the aCSF throughout the remainder of the experiment (long horizontal bar). $^*P < 0.05$, significant effect of PNU-120596 on Compound A-evoked dopamine overflow, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. (B) After collection of four stable baseline samples, PNU-120596 (1 mg/kg) or vehicle (5% DMSO in saline) was administered systemically. Methyllycaconitine (MLA; 3 mg/kg) or vehicle (saline) was given systemically 45 min prior to PNU-120596. $^*P < 0.05$, significant effect of PNU vs. vehicle, $^{\#}P < 0.05$, significant effect of MLA on PNU-120596-evoked dopamine overflow, two-way repeated-measures ANOVA with Bonferroni's *post hoc* test. Values are the mean ± SEM, $n = 5$ per group Insets: Dopamine overflow measured as area under the curve. $^*P < 0.05$ vs. vehicle, $^{\#}P < 0.01$ vs. PNU-120596, one-way ANOVA with Newman–Keuls test.

MLA. In contrast, delivery of PNU-120596 directly into the mPFC had no effect in the absence of agonist, suggesting that cholinergic tone within the mPFC itself is insufficient to activate α7 nAChRs in the presence of PNU-120596. However, increases in cholinergic activity in the PFC that accompany attention-related tasks (Parikh & Sarter, 2008) should be boosted by PNU-120596. Extra-cortically, it appears that adequate acetylcholine or choline is present in the quiescent animal for the positive modulator to elicit a response, but the site of action of PNU-120596 is a matter of speculation. One candidate region is the VTA where activation of presynaptic α7 nAChRs on glutamate afferents and dopaminergic cell bodies (Mansvelder & McGehee, 2000; Klink *et al.*, 2001; Jones & Wonnacott, 2004), in response to cholinergic tone from the pontine nuclei (Maskos, 2007; Mena-Segovia *et al.*, 2008), could drive midbrain dopamine neurons to increase dopamine release in the terminal fields.

Behaviourally, systemic administration of PNU-120596 alone has been reported to reverse an amphetamine-induced auditory gating deficit (Hurst *et al.*, 2005), consistent with an enhancement of intrinsic cholinergic signalling. Other α7 nAChR-selective positive allosteric modulators have produced cognitive enhancement and improvement in working memory (Ng *et al.*, 2007; Timmermann *et al.*, 2007), effects that could encompass a PFC component consistent with the α7 nAChR-dependent increase in dopamine overflow reported here.

Neurotransmitter crosstalk: glutamate–dopamine interactions

Interactions between dopamine and glutamate systems in the PFC are critical for its integrated functioning, requiring coincident activation of both systems (Jay, 2003). In agreement with our previous report for striatal slices (Kaiser & Wonnacott, 2000), [^{3}H]dopamine release evoked by activation of α7 nAChRs is blocked by ionotropic glutamate receptor antagonists in PFC prisms, suggesting that α7 nAChRs reside on glutamate terminals. This is consistent with the presynaptic localization of α7 nAChRs on glutamatergic afferents in various brain regions including the VTA (Mansvelder & McGehee, 2000). We were unable to explore this relationship *in vivo* because administration of glutamate receptor antagonists via the microdialysis probe increased dopamine release in the absence of any stimulus, consistent with other reports that ionotropic glutamate receptors exert an inhibitory influence on dopamine release in the PFC (Del Arco & Mora, 2005). However, nicotine increases glutamate release from thalamo-cortical afferents to the prelimbic area of the PFC *in vivo* (Gioanni *et al.*, 1999) and layer V of the PFC *in vitro* (Lambe *et al.*, 2003*)*. *β*2* nAChRs appear to underlie these responses, whereas both *β*2** and α7 nAChR-selective agonists increase excitatory amino acid release from PFC synaptosomes (Dickinson *et al.*, 2008), and both subtypes have been proposed to reside on glutamatergic afferents that modulate cholinergic function in the PFC (Parikh *et al.*, 2008). In the present study we found no evidence for any contribution from *β*2** nAChRs to the indirect modulation of [^{3}H]dopamine release: responses to a low concentration of nicotine were insensitive to both αBgt and glutamate receptor antagonists (but were blocked by DH*β*E). This suggests that α7 and *β*2** nAChRs are segregated to distinct populations of glutamatergic terminals and only those bearing α7 nAChRs are strategically localized to influence dopamine release.

Whereas the microarchitecture of the striatum is relatively uniform and well defined (Smith & Bolam, 1990), such that spillover of released glutamate could act on adjacent dopaminergic boutons, the anatomical relationship between these transmitter systems in the PFC is more complex. Dopamine inputs synapse onto pyramidal neurons, as well as γ-aminobutyric acid (GABA)ergic interneurons, and modulate pyramidal cell excitability (Jay, 2003; Tseng & O'Donnell, 2004; Del Arco & Mora, 2005); collaterals from these neurons or glutamatergic afferents may bear α7 nAChRs. The close apposition of dopamine and glutamate terminals in the PFC, seen in some morphological studies, would facilitate reciprocal interactions between these systems (see Del Arco & Mora, 2005). We suggest that this interdependence can be further modulated by α7 nAChRs associated with glutamate terminals.

The ability of *β*2-containing and α7 nAChRs to modulate dopamine release and dopamine–glutamate interactions in the PFC are likely to

contribute to the fine tuning of this circuitry in relation to executive function. Appreciation of the roles of these receptors in regulating transmitter release will facilitate the development of nicotinic strategies to combat cognitive and attentional deficits in neuropsychiatric conditions.

Acknowledgements

This study was supported by grants from the Biological and Biotechnological Sciences Research Council (BBSRC; BBS/B/15600) to S.W.; Fondazione Cariplo (2006/0779/109251) and FP7-Health-2007A (202088-NeuroCypres) from the EC to C.G., and a postgraduate studentship from the BBSRC to P.D.L. We are grateful to Tom Chapman for assistance with data handling.

Abbreviations

5-I-A-85380 (5-Iodo-A-85380), 5-iodo-3-(2(S)-azetidinylmethoxy)pyridine dihydrochloride; aCSF, artificial cerebrospinal fluid; Compound A, (R)-N-(1-azabicyclo[2.2.2]oct-3-yl)(5-(2-pyridyl)thiophene-2-carboxamide); DHβE, dihydro-β-erythroidine; DMSO, dimethylsulphoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; HPLC-ECD, high-pressure liquid chromatography-electrochemical detection; KB, Kreb's buffer; MK801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; MLA, methyllycaconitine; mPFC, medial prefrontal cortex; nAChR, nicotinic acetylcholine receptor; PFC, prefrontal cortex; PNU-120596, 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3yl)-urea; VTA, ventral tegmental area; αBgt, α-bungarotoxin; αCnTxMII, α-conotoxin MII.

References

Anderson, D.J., Puttfarcken, P.S., Jacobs, I. & Faltynek, C. (2000) Assessment of nicotinic acetylcholine receptor-mediated release of [^{3}H]-norepinephrine from rat brain slices using a new 96-well format assay. *Neuropharmacology*, **39**, 2663–2672.

Arneric, S.P., Holladay, M. & Williams, M. (2007) Neuronal nicotinic receptors: a perspective on two decades of drug discovery research. *Biochem. Pharmacol.*, **74**, 1092–1101.

Barik, J. & Wonnacott, S. (2006) Indirect modulation by alpha7 nicotinic acetylcholine receptors of noradrenaline release in rat hippocampal slices: interaction with glutamate and GABA systems and effect of nicotine withdrawal. *Mol. Pharmacol.*, **69**, 618–628.

Cao, Y.J., Surowy, C.S. & Puttfarcken, P.S. (2005) Different nicotinic acetylcholine receptor subtypes mediating striatal and prefrontal cortical [3H]dopamine release. *Neuropharmacology*, **48**, 72–79.

Cartier, G.E., Yoshikami, D., Gray, W.R., Luo, S., Olivera, B.M. & McIntosh, J.M. (1996) A new alpha-conotoxin which targets alpha3beta2 nicotinic acetylcholine receptors. *J. Biol. Chem.*, **271**, 7522–7528.

Champtiaux, N., Gotti, C., Cordero-Erausquin, M., David, D.J., Przybylski, C., Lena, C., Clementi, F., Moretti, M., Rossi, F.M., Le Novere, N., McIntosh, J.M., Gardier, A.M. & Changeux, J.P. (2003) Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J. Neurosci.*, **23**, 7820–7829.

Chan, W.K., Wong, P.T. & Sheu, F.S. (2007) Frontal cortical alpha7 and alpha4beta2 nicotinic acetylcholine receptors in working and reference memory. *Neuropharmacology*, **52**, 1641–1649.

Cilia, J., Cluderay, J.E., Robbins, M.J., Reavill, C., Southam, E., Kew, J.N. & Jones, D.N. (2005) Reversal of isolation-rearing-induced PPI deficits by an alpha7 nicotinic receptor agonist. *Psychopharmacology (Berl)*, **182**, 214–219.

Couey, J.J., Meredith, R.M., Spijker, S., Poorthuis, R.B., Smit, A.B., Brussaard, A.B. & Mansvelder, H.D. (2007) Distributed network actions by nicotine increase the threshold for spike-timing-dependent plasticity in prefrontal cortex. *Neuron*, **54**, 73–87.

Del Arco, A. & Mora, F. (2005) Glutamate-dopamine in vivo interaction in the prefrontal cortex modulates the release of dopamine and acetylcholine in the nucleus accumbens of the awake rat. *J. Neural. Transm.*, **112**, 97–109.

Dickinson, J.A., Kew, J.N. & Wonnacott, S. (2008) Presynaptic alpha7 and beta2-containing nicotinic acetylcholine receptors modulate excitatory amino acid release from rat prefrontal cortex nerve terminals via distinct cellular mechanisms. *Mol. Pharmacol.*, **74**, 348–359.

Drenan, R.M., Grady, S.R., Whiteaker, P., McClure-Begley, T., McKinney, S., Miwa, J.M., Bupp, S., Heintz, N., McIntosh, J.M., Bencherif, M., Marks, M.J. & Lester, H.A. (2008) In vivo activation of midbrain dopamine neurons via sensitized, high-affinity alpha6* nicotinic acetylcholine receptors. *Neuron*, **60**, 123–136.

Exley, R. & Cragg, S.J. (2008) Presynaptic nicotinic receptors: a dynamic and diverse cholinergic filter of striatal dopamine neurotransmission. *Br. J. Pharmacol.*, **153**(Suppl. 1), S283–S297.

Exley, R., Clements, M.A., Hartung, H., McIntosh, J.M. & Cragg, S.J. (2007) Alpha6-containing nicotinic acetylcholine receptors dominate the nicotine control of dopamine neurotransmission in nucleus accumbens. *Neuropsychopharmacology*, **33**, 2158–2166.

Fernandes, C., Hoyle, E., Dempster, E., Schalkwyk, L.C. & Collier, D.A. (2006) Performance deficit of alpha7 nicotinic receptor knockout mice in a delayed matching-to-place task suggests a mild impairment of working/episodic-like memory. *Genes. Brain. Behav.*, **5**, 433–440.

Floresco, S.B. & Magyar, O. (2006) Mesocortical dopamine modulation of executive functions: beyond working memory. *Psychopharmacology (Berl)*, **188**, 567–585.

Gioanni, Y., Rougeot, C., Clarke, P.B., Lepouse, C., Thierry, A.M. & Vidal, C. (1999) Nicotinic receptors in the rat prefrontal cortex: increase in glutamate release and facilitation of mediodorsal thalamo-cortical transmission. *Eur. J. Neurosci.*, **11**, 18–30.

Gotti, C., Moretti, M., Clementi, F., Riganti, L., McIntosh, J.M., Collins, A.C., Marks, M.J. & Whiteaker, P. (2005) Expression of nigrostriatal alpha 6-containing nicotinic acetylcholine receptors is selectively reduced, but not eliminated, by beta 3 subunit gene deletion. *Mol. Pharmacol.*, **67**, 2007–2015.

Gotti, C., Zoli, M. & Clementi, F. (2006) Brain nicotinic acetylcholine receptors: native subtypes and their relevance. *Trends Pharmacol. Sci.*, **27**, 482–491.

Grady, S.R., Salminen, O., Laverty, D.C., Whiteaker, P., McIntosh, J.M., Collins, A.C. & Marks, M.J. (2007) The subtypes of nicotinic acetylcholine receptors on dopaminergic terminals of mouse striatum. *Biochem. Pharmacol.*, **74**, 1235–1246.

Granon, S., Passetti, F., Thomas, K.L., Dalley, J.W., Everitt, B.J. & Robbins, T.W. (2000) Enhanced and impaired attentional performance after infusion of D1 dopaminergic receptor agents into rat prefrontal cortex. *J. Neurosci.*, **20**, 1208–1215.

Gronlien, J.H., Hakerud, M., Ween, H., Thorin-Hagene, K., Briggs, C.A., Gopalakrishnan, M. & Malysz, J. (2007) Distinct profiles of alpha7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. *Mol. Pharmacol.*, **72**, 715–724.

Hahn, B., Shoaib, M. & Stolerman, I.P. (2003) Involvement of the prefrontal cortex but not the dorsal hippocampus in the attention-enhancing effects of nicotine in rats. *Psychopharmacology (Berl)*, **168**, 271–279.

Hoyle, E., Genn, R.F., Fernandes, C. & Stolerman, I.P. (2006) Impaired performance of alpha7 nicotinic receptor knockout mice in the five-choice serial reaction time task. *Psychopharmacology (Berl)*, **189**, 211–223.

Hurst, R.S., Hajos, M., Raggenbass, M., Wall, T.M., Higdon, N.R., Lawson, J.A., Rutherford-Root, K.L., Berkenpas, M.B., Hoffmann, W.E., Piotrowski, D.W., Groppi, V.E., Allaman, G., Ogier, R., Bertrand, S., Bertrand, D. & Arneric, S.P. (2005) A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. *J. Neurosci.*, **25**, 4396–4405.

Innocent, N., Livingstone, P.D., Hone, A., Kimura, A., Young, T., Whiteaker, P., McIntosh, J.M. & Wonnacott, S. (2008) alphaConotoxin ArIB[V11L,V16D] is a potent and selective antagonist at rat and human native alpha7 nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Ther.*, **327**, 529–537.

Jay, T.M. (2003) Dopamine: a potential substrate for synaptic plasticity and memory mechanisms. *Prog. Neurobiol.*, **69**, 375–390.

Jensen, A.A., Frolund, B., Liljefors, T. & Krogsgaard-Larsen, P. (2005) Neuronal nicotinic acetylcholine receptors: structural revelations, target identifications, and therapeutic inspirations. *J. Med. Chem.*, **48**, 4705–4745.

Jones, I.W. & Wonnacott, S. (2004) Precise localization of alpha7 nicotinic acetylcholine receptors on glutamatergic axon terminals in the rat ventral tegmental area. *J. Neurosci.*, **24**, 11244–11252.

Kaiser, S. & Wonnacott, S. (2000) alpha-bungarotoxin-sensitive nicotinic receptors indirectly modulate [^{3}H]dopamine release in rat striatal slices via glutamate release. *Mol. Pharmacol.*, **58**, 312–318.

Kaiser, S.A., Soliakov, L., Harvey, S.C., Luetje, C.W. & Wonnacott, S. (1998) Differential inhibition by alpha-conotoxin-MII of the nicotinic stimulation of [^{3}H]dopamine release from rat striatal synaptosomes and slices. *J. Neurochem.*, **70**, 1069–1076.

Klink, R., de Kerchove d'Exaerde, A., Zoli, M. & Changeux, J.P. (2001) Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. *J. Neurosci.*, **21**, 1452–1463.

Kulak, J.M., Nguyen, T.A., Olivera, B.M. & McIntosh, J.M. (1997) Alpha-conotoxin MII blocks nicotine-stimulated dopamine release in rat striatal synaptosomes. *J. Neurosci.*, **17**, 5263–5270.

Lambe, E.K., Picciotto, M.R. & Aghajanian, G.K. (2003) Nicotine induces glutamate release from thalamocortical terminals in prefrontal cortex. *Neuropsychopharmacology*, **28**, 216–225.

Levin, E.D., McClernon, F.J. & Rezvani, A.H. (2006) Nicotinic effects on cognitive function: behavioral characterization, pharmacological specification, and anatomic localization. *Psychopharmacology (Berl)*, **184**, 523–539.

Levy, F. (2008) Pharmacological and therapeutic directions in ADHD: specificity in the PFC. *Behav. Brain. Funct.*, **4**, 12.

Mansvelder, H.D. & McGehee, D.S. (2000) Long-term potentiation of excitatory inputs to brain reward areas by nicotine. *Neuron*, **27**, 349–357.

Mansvelder, H.D., Keath, J.R. & McGehee, D.S. (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron*, **33**, 905–919.

Mansvelder, H.D., van Aerde, K.I., Couey, J.J. & Brussaard, A.B. (2006) Nicotinic modulation of neuronal networks: from receptors to cognition. *Psychopharmacology (Berl)*, **184**, 292–305.

Maskos, U. (2007) Emerging concepts: novel integration of in vivo approaches to localize the function of nicotinic receptors. *J. Neurochem.*, **100**, 596–602.

Mazurov, A., Hauser, T. & Miller, C.H. (2006) Selective alpha7 nicotinic acetylcholine receptor ligands. *Curr. Med. Chem.*, **13**, 1567–1584.

Mena-Segovia, J., Winn, P. & Bolam, J.P. (2008) Cholinergic modulation of midbrain dopaminergic systems. *Brain Res. Rev.*, **58**, 265–271.

Mogg, A.J., Whiteaker, P., McIntosh, J.M., Marks, M.J., Collins, A.C. & Wonnacott, S. (2002) Methyllycaconitine is a potent antagonist of α-conotoxin-MII-sensitive presynaptic nicotinic acetylcholine receptors in rat striatum. *J. Pharmacol. Exp. Ther.*, **302**, 1–8.

Mogg, A.J., Jones, F.A., Pullar, I.A., Sharples, C.G. & Wonnacott, S. (2004) Functional responses and subunit composition of presynaptic nicotinic receptor subtypes explored using the novel agonist 5-iodo-A-85380. *Neuropharmacology*, **47**, 848–859.

Mukhin, A.G., Gundisch, D., Horti, A.G., Koren, A.O., Tamagnan, G., Kimes, A.S., Chambers, J., Vaupel, D.B., King, S.L., Picciotto, M.R., Innis, R.B. & London, E.D. (2000) 5-Iodo-A-85380, an alpha4beta2 subtype-selective ligand for nicotinic acetylcholine receptors. *Mol. Pharmacol.*, **57**, 642–649.

Newhouse, P.A., Potter, A. & Singh, A. (2004) Effects of nicotinic stimulation on cognitive performance. *Curr. Opin. Pharmacol.*, **4**, 36–46.

Ng, H.J., Whittemore, E.R., Tran, M.B., Hogenkamp, D.J., Broide, R.S., Johnstone, T.B., Zheng, L., Stevens, K.E. & Gee, K.W. (2007) Nootropic alpha7 nicotinic receptor allosteric modulator derived from GABAA receptor modulators. *Proc. Natl Acad. Sci. USA*, **104**, 8059–8064.

Nisell, M., Nomikos, G.G. & Svensson, T.H. (1994) Infusion of nicotine in the ventral tegmental area or the nucleus accumbens of the rat differentially affects accumbal dopamine release. *Pharmacol. Toxicol.*, **75**, 348–352.

Parikh, V. & Sarter, M. (2008) Cholinergic mediation of attention: contributions of phasic and tonic increases in prefrontal cholinergic activity. *Ann. N Y Acad. Sci.*, **1129**, 225–235.

Parikh, V., Man, K., Decker, M.W. & Sarter, M. (2008) Glutamatergic contributions to nicotinic acetylcholine receptor agonist-evoked cholinergic transients in the prefrontal cortex. *J. Neurosci.*, **28**, 3769–3780.

Paxinos, G. & Watson, C. (1986) *The Rat Brain in Stereotaxic Coordinates*, 2nd Edn. Academic Press, London.

Puttfarcken, P.S., Jacobs, I. & Faltynek, C.R. (2000) Characterization of nicotinic acetylcholine receptor-mediated [^{3}H]-dopamine release from rat cortex and striatum. *Neuropharmacology*, **39**, 2673–2680.

Quarta, D., Naylor, C.G., Barik, J., Fernandes, C., Wonnacott, S. & Stolerman, I.P. (2008) Drug discrimination and neurochemical studies in alpha7 null mutant mice: tests for the role of nicotinic alpha7 receptors in dopamine release. *Psychopharmacology (Berl)*, in press. Epub ahead of print.

Salminen, O., Murphy, K.L., McIntosh, J.M., Drago, J., Marks, M.J., Collins, A.C. & Grady, S.R. (2004) Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol. Pharmacol.*, **65**, 1526–1535.

Salminen, O., Drapeau, J.A., McIntosh, J.M., Collins, A.C., Marks, M.J. & Grady, S.R. (2007) Pharmacology of alpha-conotoxin MII-sensitive subtypes of nicotinic acetylcholine receptors isolated by breeding of null mutant mice. *Mol. Pharmacol.*, **71**, 1563–1571.

Schilstrom, B., Nomikos, G.G., Nisell, M., Hertel, P. & Svensson, T.H. (1998) N-methyl-D-aspartate receptor antagonism in the ventral tegmental area diminishes the systemic nicotine-induced dopamine release in the nucleus accumbens. *Neuroscience*, **82**, 781–789.

Schultz, W. (2002) Getting formal with dopamine and reward. *Neuron*, **36**, 241–263.

Smith, A.D. & Bolam, J.P. (1990) The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. *Trends Neurosci.*, **13**, 259–265.

Timmermann, D.B., Gronlien, J.H., Kohlhaas, K.L., Nielsen, E.O., Dam, E., Jorgensen, T.D., Ahring, P.K., Peters, D., Holst, D., Chrsitensen, J.K., Malysz, J., Briggs, C.A., Gopalakrishnan, M. & Olsen, G.M. (2007) An allosteric modulator of the alpha7 nicotinic acetylcholine receptor possessing cognition-enhancing properties in vivo. *J. Pharmacol. Exp. Ther.*, **323**, 294–307.

Toda, M. & Abi-Dargham, A. (2007) Dopamine hypothesis of schizophrenia: making sense of it all. *Curr Psychiatry. Rep.*, **9**, 329–336.

Tseng, K.Y. & O'Donnell, P. (2004) Dopamine-glutamate interactions controlling prefrontal cortical pyramidal cell excitability involve multiple signaling mechanisms. *J. Neurosci.*, **24**, 5131–5139.

Whiteaker, P., Garcha, H.S., Wonnacott, S. & Stolerman, I.P. (1995) Locomotor activation and dopamine release produced by nicotine and isoarecolone in rats. *Br. J. Pharmacol.*, **116**, 2097–2105.

Williams, G.V. & Goldman-Rakic, P.S. (1995) Modulation of memory fields by dopamine D1 receptors in prefrontal cortex. *Nature*, **376**, 572–575.

Wonnacott, S. (1997) Presynaptic nicotinic ACh receptors. *Trends Neurosci.*, **20**, 92–98.

Young, J.W., Finlayson, K., Spratt, C., Marston, H.M., Crawford, N., Kelly, J.S. & Sharkey, J. (2004) Nicotine improves sustained attention in mice: evidence for involvement of the alpha7 nicotinic acetylcholine receptor. *Neuropsychopharmacology*, **29**, 891–900.

Young, J.W., Crawford, N., Kelly, J.S., Kerr, L.E., Marston, H.M., Spratt, C., Finlayson, K. & Sharkey, J. (2007) Impaired attention is central to the cognitive deficits observed in alpha 7 deficient mice. *Eur. Neuropsychopharmacol.*, **17**, 145–155.

Young, G.T., Zwart, R., Walker, A.S., Sher, E. & Millar, N.S. (2008) Potentiation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. *Proc. Natl Acad. Sci. USA*, **105**, 14686–14691.