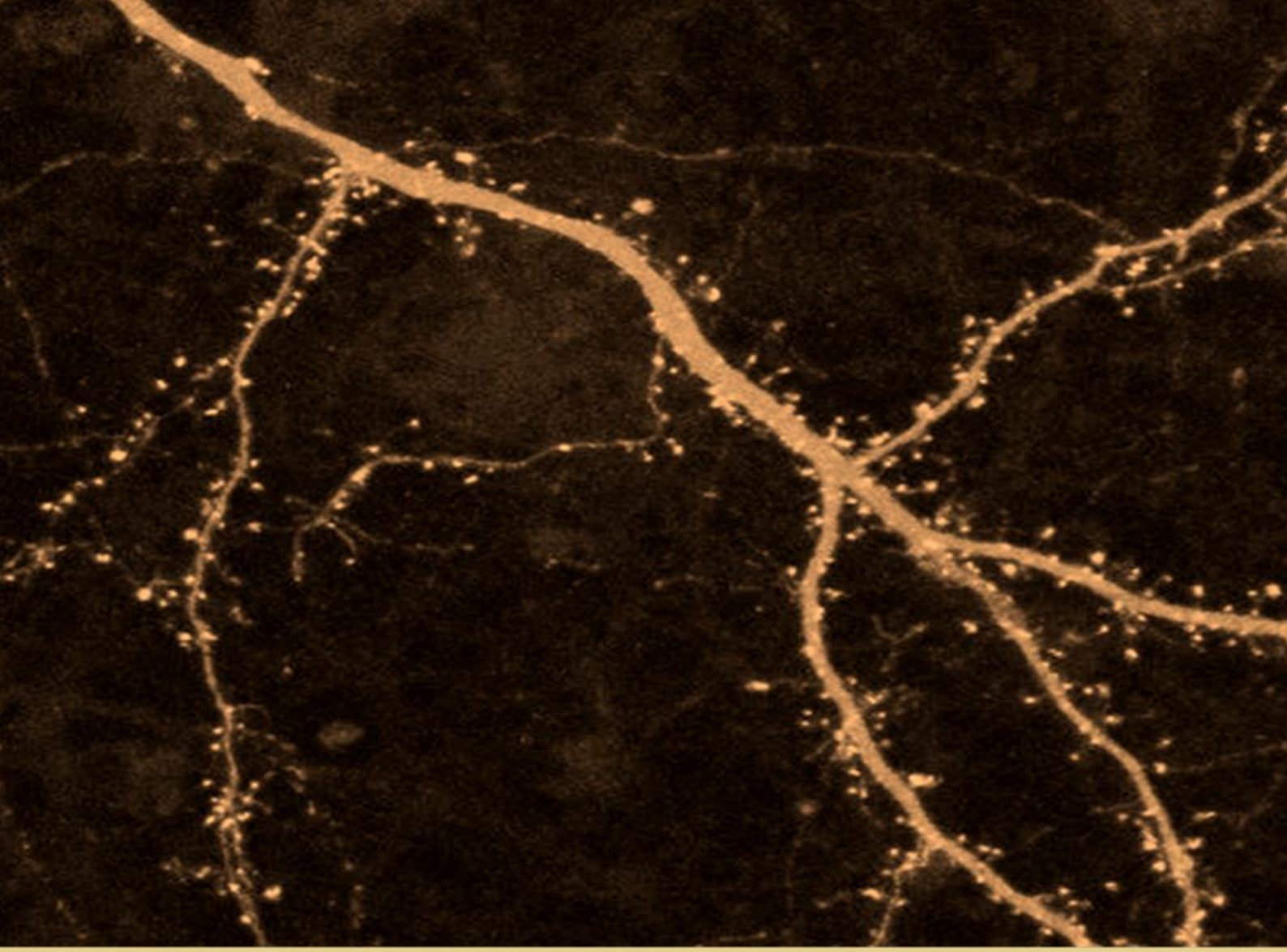

Pedro Manuel Venâncio Garção

Functional interaction between presynaptic nicotinic and adenosine receptors in the control of dopamine release in the striatum

Tese de doutoramento em Ciências da Saúde, ramo de Ciências Biomédicas, orientada pela Doutora Paula Agostinho e co-orientada pela Professora Doutora Catarina R. Oliveira, apresentada à Faculdade de Medicina da Universidade de Coimbra

2013

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UNIVERSIDADE DE COIMBRA

University of Coimbra

Faculty of Medicine

Functional interaction between presynaptic nicotinic and adenosine receptors in the control of dopamine release in the striatum

Interação funcional entre receptores nicotínicos e de adenosina pré-sinápticos no controlo da libertação de dopamina no estriado

Pedro Manuel Venâncio Garção

Tese apresentada à Faculdade de Medicina da Universidade de Coimbra para prestação de provas de doutoramento na área das Ciências da Saúde, ramo de Ciências Biomédicas, sob orientação da Doutora Paula Agostinho e co-orientação da Professora Doutora Catarina R. Oliveira.

Este trabalho foi realizado no Centro de Neurociências e Biologia Celular de Coimbra (CNC) e na Faculdade de Medicina da Universidade de Coimbra.

Este trabalho foi financiado por uma bolsa de doutoramento concedida pela Fundação para a Ciência e a Tecnologia (FCT) com a referência SFRH/BD/28722/2006.

Coimbra 2013

Agradecimentos/ Acknowledgements

Agradeço especialmente à minha orientadora, Doutora Paula Agostinho, por tudo o que tem feito desde o início da minha participação no mundo da investigação científica. Pela inestimável contribuição para a elaboração deste trabalho, pelo seu exemplo, conselhos, exigência, paciência e amizade. Estou grato por todas as oportunidades que proporcionou de trabalharmos juntos e que contribuiram de forma marcante para a minha formação científica e pessoal.

À minha co-orientadora, Professora Doutora Catarina Oliveira por me ter possibilitado realizar este trabalho no Centro de Neurociências e Biologia Celular de Coimbra (CNC), pelo seu contributo para o meu percurso científico e pela gentileza e disponibilidade que sempre demonstrou.

Ao Professor Doutor Rodrigo Cunha pela oportunidade e privilégio de trabalhar no seu grupo, por toda a contribuição para o meu trabalho e desenvolvimento científico, pelas perspectivas que oferece sobre a ciência e por proporcionar todas as condições para que este trabalho fosse realizado.

Ao Doutor Attila Köfalvi pela gentileza de me permitir trabalhar no seu laboratório. Pela sua criatividade, conhecimento e paciência, que foram essenciais para este trabalho e para a minha formação científica e técnica. Pela amizade e conversas futebolísticas. Köszönöm! Aos elementos do seu grupo, Samira, Bárbara, Carla, Rafael, com os quais tive o prazer de trabalhar e conviver.

Ao Professor Doutor Rui Prediger pela sua participação no desenvolvimento deste trabalho, pela acessibilidade e simpatia, bem como aos elementos do seu grupo.

A todos os colegas do grupo “Purines at CNC” (presentes e passados) com os quais tive (breve ou longamente) o privilégio de compartilhar o laboratório: Adalberto, Alex, Ana, Ana Paula, Ângelo, Anna, Betty, Bibi, Carolina, Catarina, Cristina, Daniel, Diana, Eszter, Francisco, Geanne, Gonçalo, Henrique, Jimmy, Jessié, Joana, Marco, Marta, Nuno, Nélio, Pablo, Paula, Patrícia, Ricardo, Rui S, Rui B, Stefania, Tiago A, Tiago C, Tiago S. Obrigado pelo conhecimento partilhado, pela ajuda e pelo convívio.

À Giana Cognato e Manuella Kaster pela maneira amável como partilharam os seus conhecimentos técnicos. À Dani Pochmann pela ajuda, amizade e pelos risos que proporcionou.

À Caroline Veloso pelo modo gentil como coloca toda a gente na ordem e mantém as coisas a funcionar no laboratório.

À dona Isabel pela simpatia e boa disposição e a todo o pessoal técnico pela ajuda e disponibilidade.

A todos os membros do CNC, com os quais partilhei momentos de trabalho e convívio ao longo deste percurso.

Ao Professor Doutor Cláudio da Cunha e aos membros do seu grupo na Universidade Federal do Paraná em Curitiba pela forma cordial como me receberam e por tudo o que me ensinaram. Um grande abraço a todos os amigos que tive a felicidade de fazer no Brasil.

À Fundação para a Ciência e a Tecnologia (FCT) pelo financiamento indispensável para a realização deste trabalho.

Aos colegas e amigos de longa data, Ana Luísa Cardoso, Bruno Silva, João Lopes e Rui Costa, pelo apoio e pelos inesquecíveis momentos passados dentro e fora do laboratório.

Aos companheiros da velha guarda, João, Ricardo e Rita, pela amizade, pelos muitos e bons momentos e experiências de vida partilhados.

Aos amigos de Portalegre e Coimbra, aquele abraço.

Deixo um agradecimento especial e sentido à familia Caldeira pelo apoio e amizade inabalável ao longo dos anos.

Dedico esta tese à minha familia, aos meus pais, irmãos, à memória dos meus avós, aos sobrinhos e todo o restante povo. Tudo o que pudesse dizer aqui seria pouco, fica apenas o meu sentido e sincero obrigado por tudo.

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Abbreviations list

A_1R – Adenosine A_1 receptor

A_{2A}R – Adenosine A_{2A} receptor

A_{2B}R – Adenosine A_{2B} receptor

A_3R – Adenosine A_3 receptor

ACh – Acetylcholine

ADP – Adenosine-5´-diphosphate

AMP – Adenosine-5´-monophosphate

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

AP – Alkaline phosphatase

ARRIVE – Animals in Research: Reporting In Vivo Experiments

ATP – Adenosine-5´-triphosphate

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

α-BTX – α-Bungarotoxin

Ca^{2+} – Calcium ion

CAMP – Ciclic adenosine-5´-monophosphate

CAPS – 3-(Cyclohexylamino)-1-propanesulfonic acid

CB_1R – Cannabinoid receptor subtype 1

CGS21680 – 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofu-ranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzenepropanoic acid

CNS – Central nervous system

α-CTX MII – α-Conotoxin MII

α-CTX PIA – α-Conotoxin PIA

D_{1-5}R – Dopamine receptor subtypes 1-5

DHβE – Dihydro-β-erythroidine

DMSO – Dimethyl sulfoxide

DPM – Disintegrations per minute

DTT – Dithiothreitol

EC50 – Concetration of a ligand eliciting 50% of the maximal response

EDTA – Ethylenediaminetetraacetic acid

EGTA – Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid

EHNA – Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride

ENTs – Equilibrative nucleoside transporters

EXTRA – Extrasynaptic (outside active zone) fraction

FELASA – Federation for Laboratory Animal Science Associations

GABA – γ-Aminobutyric acid

GDNF – Glial cell line-derived neurotrophic factor

GPe – External segment of the globus pallidus

GPi – Internal segment of the globus pallidus

[^{3}H]DA – Tritiated dopamine

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Halothane – 2-Bromo-2-chloro-1,1,1-trifluoroethane

HPLC – High-performance liquid chromatography

IC_{50} – Concentration of a ligand that causes 50% of the maximal inhibition

K^+ – Potassium ion

kDa – Kilodaltons

KW-6002 – (E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dhydro-1H purine-2,6-dione

mGluR5 – Metabotropic glutamate receptor 5

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRS1754 – N-(4-Cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide

MSNs – Medium spiny neurons

Na^+ – Sodium ion

Na^+/K^+-ATPase – Sodium-potassium adenosine triphosphatase pump

nAChR – Nicotinic acetylcholine receptor

NAc – Nucleus accumbens

NMDA – *N*-methyl-$_D$-aspartate

PD – Parkinson's disease

PKA – Protein kinase A

PKC – Protein kinase C

PMSF – Phenylmethanesulfonylfluoride

POST – Postsynaptic fraction

PPN – Pedunculopontine nucleus

PRE – Presynaptic fraction

PSD-95 – postsynaptic density protein 95

3Rs – Replacement, Refinement and Reduction of Animals in Research

RT – Room temperature

6-OHDA – 6-hydroxydopamine

SCH58261 – 2-(2-Furanyl)-7-(2-phenylethyl)-7*H*--pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-amine

SDS – Sodium dodecyl sulfate

SEM – Standard error of the mean

SNAP-25 – Synaptosomal-associated protein 25

SNc – Substantia nigra *pars compacta*

SNr – Substantia nigra *pars reticulata*

STN – Subthalamic nucleus

TBS – Tris buffered saline medium

TBS-T – Tris buffered saline medium with 0.1% Tween-20

Triton X-100 – 4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol solution

VTA – Ventral tegmental area

VOCCs – Voltage-operated calcium channels

ZM241385 – 4-(2-[7-amino-2-(2-furyl)]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol

Publications

The experimental work presented in this thesis was carried out by the author. In section 4.3, the adenosine release experiments were done in collaboration with Eszter Szabó, Attila Köfalvi and Ângelo Tomé (CNC; Center for Neuroscience and Cell Biology, University of Coimbra). In section 4.4, analysis of the locomotor sensitization experiments was performed in collaboration with Rui Prediger, Sandro Wopereis and Adalberto Castro (Department of Pharmacology, Federal University of Santa Catarina, Florianópolis, Brazil). The dopamine release studies were done under the supervision of Attila Köfalvi. The work presented in this thesis was performed in the group lead by Rodrigo A. Cunha, under the supervision of Paula Agostinho and Catarina R. Oliveira.

The following papers (I-IV) were published in peer-reviewed international scientific journals during the development of this thesis. The studies presented in this thesis were published (IV) or are being prepared for submission (V):

I. Melo JB, Sousa C, **Garção P**, Oliveira CR, Agostinho P (2009) Galantamine protects against oxidative stress induced by amyloid-beta peptide in cortical neurons. European Journal of Neuroscience 29: 455-464.

II. Ferreira SG, Teixeira FM, **Garção P**, Agostinho P, Ledent C, Cortes L, Mackie K, Köfalvi A (2012) Presynaptic CB_1 cannabinoid receptors control frontocortical serotonin and glutamate release – Species differences. Neurochemistry International 61: 219-226.

III. Silva D, Chioua M, Samadi A, Agostinho P, **Garção P**, Lajarín-Cuesta R, de Los Ríos C, Iriepa I, Moraleda I, Gonzalez-Lafuente L, Mendes E, Pérez C, Rodríguez-Franco MI, Marco-Contelles J, Carmo Carreiras M (2013) Synthesis, Pharmacological Assessment, and Molecular Modeling of Acetylcholinesterase/Butyrylcholinesterase Inhibitors: Effect against Amyloid-β-Induced Neurotoxicity. ACS Chemical Neuroscience 4: 547-565.

IV. Garção P, Szabó EC, Wopereis S, Castro AA, Tomé AR, Prediger RD, Cunha RA, Agostinho P, Köfalvi A (2013) Functional interaction between pre-synaptic α6β2-containing nicotinic and adenosine A_{2A} receptors in the control of dopamine release in the rat striatum. British Journal of Pharmacology 169: 1600-1611.

V. **Garção P**, Oliveira CR, Cunha RA, Agostinho P. Subsynaptic localization of nicotinic acetylcholine receptor subunits in mouse and rat striatum (*in preparation*).

Abstract

Presynaptic nicotinic acetylcholine receptors (nAChRs) and adenosine A_{2A} receptors (A_{2A}Rs) are key modulators of striatal neurotransmitter release. The A_{2A}Rs were shown to control the rate of desensitization of different nAChRs in the peripheral nervous system and have also been reported to interact, in the central nervous system, with other striatal receptors, such as adenosine A_1, metabotropic glutamate type 5 and CB_1 cannabinoid receptors, suggesting the possibility for a functional interaction between nAChRs and A_{2A}Rs in the striatum, with possible relevant implications in dopaminergic disorders like Parkinson´s disease (PD). The striatum is the main relay nucleus of the basal ganglia, a brain network essential in sensorimotor, cognitive and motivational functions. The glutamatergic corticostriatal projections are the leading excitatory inputs to the striatum and are controlled by a coordinated interaction among several neuromodulators, where the dopaminergic inputs from the substantia nigra play a prominent role. The main modulators of striatal functions include adenosine, acting at the metabotropic A_{2A}Rs, and acetylcholine, which acts at ionotropic nAChRs. These two neuromodulation systems have particular interest since they are targeted by some of the most widely consumed psychoactive drugs worldwide, namely nicotine (a nAChR agonist) and caffeine (a nonselective adenosine receptor antagonist); and albeit these two drugs are often consumed simultaneously little is known about their combined effects. Moreover, epidemiological studies showed that nicotine and caffeine use is related with a lower incidence of PD. Notwithstanding, the relation between nAChRs and A_{2A}Rs is poorly understood, and thus it is important to investigate the possible interaction between these types of receptors in striatal neurons.

The main goal of this study was to investigate the possible functional interaction between nAChRs and A_{2A}Rs in terms of neurotransmission in the striatum. To achieve this goal it was first detailed the synaptic and subsynaptic distribution of different nAChR subunits, such as α7, α6, α4 and β2 in stratial nerve terminals (synaptosomes), and it was observed that all these subunits, which make part of the main nAChRs subtypes, were present in nerve terminals. The subsynaptic levels and distribution of α7, α6, α4 and β2 were

investigated in the presynaptic active zone, postsynaptic density and in extra-synaptic fractions (outside the active zone), since the nAChRs might have different roles in neurotransmission depending on their localization in the different subsynaptic components. The possible presynaptic functional interaction between different nAChR subtypes and A_{2A}Rs in rat dopaminergic nerve terminals was investigated by measuring the *in vitro* dopamine release from striatal nerve terminals and *in vivo* by assessing the rat locomotor sensitization in response to nicotine, in the presence or absence of an A_{2A}R antagonist.

In the rodent striatum we found that the α7 subunit, which predominantly makes part of the homomeric α7 nAChR subtype sensitive to α-bungarotoxin, was mainly present in the presynaptic active zone. The α4 and β2 subunits displayed a similar distribution, being primarily present in presynaptic and in extrasynaptic zones, which was expected since these two subunits together form heteropentameric nAChRs. By contrast to the former, the α6 subunit was mainly present in the postsynaptic fraction, albeit being also present in pre- and extra-synaptic fractions. These results underline the possible relevance of striatal nAChRs in controlling neurotransmission.

The *in vitro* dopamine release studies showed that nicotine elicited the release of [^{3}H]dopamine in a concentration-dependent manner from rat striatal nerve terminals. Notably, it was observed that the selective A_{2A}R agonist CGS21680, significantly inhibited, while ZM241385, an A_{2A}R antagonist, significantly potentiated the nicotine-stimulated [^{3}H]dopamine release. The A_{2B}R antagonist MRS1754 did not potentiate the release. Adenosine receptor ligands CGS21680 and caffeine also stimulated the release of [^{3}H]dopamine *per se*. Antagonists of α6-containing (α6*) and of β2-containing nAChRs, but not of the α7 nAChR subtype, significantly inhibited release of [^{3}H]dopamine. Under the blockade of the α6* nAChRs the remaining nicotine-stimulated [^{3}H]dopamine release was no longer subject to modulation by A_{2A}R ligands, indicating that the α6β2* nAChRs function is coupled to A_{2A}Rs.

The *in vivo* relevance of the interplay between α6β2* nAChRs and A_{2A}Rs was also assessed. In locomotor sensitization experiments it was observed that nicotine significantly augmented the locomotor activity of rats from the day 7 of nicotine injection, but it no longer persisted after 8 days of abstinence.

ZM241385-injected rats developed locomotor sensitization to nicotine already on day 2 of injection, which was not blunted by the 8 day drug-free period, reinforcing the contention for a functional interaction between the α6β2* nAChRs and the A_{2A}Rs. Altogether, this work details the striatal subsynaptic density of some of the main nAChR subunits and provides the first evidence for a functional interaction between nicotinic and adenosine receptors in striatal dopaminergic terminals, with likely therapeutic consequences for Parkinson's disease, nicotine addiction and other dopaminergic disorders.

Resumo

Os receptores colinérgicos nicotínicos (nAChRs) e os receptores A_{2A} de adenosina (A_{2A}Rs) pré-sinápticos são moduladores importantes da libertação de neurotransmissores no estriado. Foi demonstrado que os A_{2A}Rs controlam a taxa de desensitização de diferentes nAChRs no sistema nervoso periférico e foi também relatado que, no sistema nervoso central, interagem com outros receptores estriatais, tais como receptores A_1 de adenosina, metabotrópicos de glutamato do tipo 5 e canabinóides CB_1, sugerindo a possibilidade de uma interação funcional entre nAChRs e A_{2A}Rs no estriado, com possiveis implicações relevantes em distúrbios dopaminérgicos como a doença de Parkinson (PD). O estriado é o principal núcleo retransmissor dos gânglios da base, uma rede cerebral essencial em funções sensorimotoras, cognitivas e motivacionais. As projecções glutamatérgicas cortico-estriatais são a principal fonte de entrada excitatória no estriado e são controladas por uma interação coordenada entre diversos neuromoduladores, onde as entradas dopaminérgicas provenientes da substância nigra desempenham um papel fundamental. Os principais moduladores de funções estriatais incluem a adenosina, atuando nos A_{2A}Rs metabotrópicos, e a acetilcolina, que atua nos nAChRs ionotrópicos. Estes dois sistemas neuromoduladores têm particular importância já que são alvo de algumas das drogas psicoactivas mais consumidas mundialmente, nomeadamente a nicotina (um agonista dos nAChRs) e a cafeína (um antagonista não-selectivo dos receptores de adenosina); e embora estas duas drogas sejam frequentemente consumidas em simultâneo, pouco se sabe acerca dos seus efeitos combinados. Para além disso, estudos epidemiológicos mostraram que o uso de nicotina e cafeína está relacionado com uma menor incidência da PD. Contudo, a relação entre nAChRs e A_{2A}Rs é deficientemente conhecida, e portanto é importante investigar a possivel interação entre estes tipos de receptores em neurónios estriatais.

O principal objectivo deste estudo foi investigar a possivel interação funcional entre nAChRs e A_{2A}Rs em termos de neurotransmissão no estriado. Para atingir este objectivo foi primeiro detalhada a distribuição sináptica e sub-

sináptica de diferentes subunidades de nAChRs, tais como α7, α6, α4 e β2 em terminais nervosos estriatais (sinaptossomas), e foi observado que todas estas subunidades, que formam parte dos principais subtipos de nAChRs, estavam presentes em terminais nervosos. Foram investigados os niveis sub-sinápticos e a distribuição das α7, α6, α4 e β2, na zona activa pré-sináptica, densidade pós-sináptica e em fracções extra-sinápticas (fora da zona activa), dado que os nAChRs podem ter diferentes papéis na neurotransmissão dependendo da sua localização nos diferentes componentes sub-sinápticos. A possível interação funcional pré-sináptica entre diferentes subtipos de nAChRs e A_{2A}Rs em terminais dopaminérgicos de rato foi investigada *in vitro* através da medição da libertação de dopamina de terminais nervosos estriatais e *in vivo* pela avaliação da sensitização locomotora de ratos em resposta a nicotina, na presença ou ausência de um antagonista dos A_{2A}Rs.

No estriado de roedores observou-se que a subunidade α7, que faz predominantemente parte do subtipo α7 de nAChRs homomérico sensível à α-bungarotoxina, estava principalmente presente na zona activa pré-sináptica. As subunidades α4 e β2 exibiram uma distribuição semelhante entre si, estando primariamente presentes em zonas pré-sinápticas e extra-sinápticas, o que é esperado dado que estas duas subunidades juntas formam nAChRs heteropentaméricos. Em contraste com as anteriores, a subunidade α6 estava principalmente presente na fracção pós-sináptica, embora estando também presente em fracções pré- e extra-sinápticas. Estes resultados sublinham a possível relevância de nAChRs estriatais em controlar a neurotransmissão.

Os estudos *in vitro* de libertação de dopamina mostraram que a nicotina provocou a libertação de [^{3}H]dopamina de um modo dependente da concentração, de terminais nervosos estriatais de rato. De modo relevante, foi observado que o agonista selectivo dos A_{2A}Rs CGS21680, inibiu significativamente, enquanto que ZM241385, um antagonista dos A_{2A}Rs, potenciou significativamente a libertação de [^{3}H]dopamina estimulada por nicotina. O antagonista dos A_{2B}Rs, MRS1754, não potenciou a libertação. Os ligandos dos receptores de adenosina CGS21680 e cafeína também estimularam a libertação de [^{3}H]dopamina *per se.* Antagonistas de subtipos de nAChRs que contém a subunidade α6 (α6*) e de subtipos que contém a subunidade β2, mas não do subtipo α7 de nAChRs, inibiram significativamente

a libertação de [^{3}H]dopamina. Sob bloqueio dos α6* nAChRs a restante libertação de [^{3}H]dopamina estimulada por nicotina já não foi sujeita a modulação por ligandos dos A_{2A}Rs, indicando que a função dos α6β2* nAChRs está acoplada aos A_{2A}Rs.

A relevância *in vivo* da cooperação entre os α6β2* nAChRs e os A_{2A}Rs foi também avaliada. Em experiências de sensitização locomotora foi observado que a nicotina aumentou significativamente a actividade locomotora de ratos a partir do dia 7 de injecção de nicotina, mas que o aumento já não persistiu depois de 8 dias de abstinência. Ratos injectados com ZM241385 desenvolveram sensitização locomotora à nicotina logo no dia 2 de injecção, que não foi diminuída pelo período de 8 dias livre de fármacos, reforçando os indícios quanto a uma interação funcional entre os α6β2* nAChRs e os A_{2A}Rs.

No seu conjunto, este trabalho detalha a densidade estriatal sub-sináptica de algumas das principais subunidades de nAChRs e providencia a primeira evidência para uma interação funcional entre receptores nicotínicos e de adenosina em terminais dopaminérgicos estriatais, com prováveis consequências terapêuticas para a doença de Parkinson, dependência nicotínica e outros distúrbios dopaminérgicos.

1. Introduction

1.1 THE STRIATUM

The striatum is a subcortical structure of the brain that constitutes one of the main components of the basal ganglia, a neural network essential in sensorimotor, cognitive and motivational brain functions (Bolam *et al.*, 2000; Schiffmann *et al*., 2007). The striatum acts as a major relay nucleus between the neocortex and the deeper nuclei of the basal ganglia. Based on connectivity and function the striatum is divided into a dorsal part, the neostriatum, which consists of the caudate nucleus and the putamen (dorsal striatum), and a ventral portion that is mainly composed by the nucleus accumbens (NAc) and also by the olfactory tubercle (ventral striatum). Other main constituents of the basal ganglia are the globus pallidus (GP, also called paleostriatum), which in primates is divided into external (GPe) and internal (GPi) segments, the entopeduncular nucleus (EP, in rodents, equivalent to primates GPi), subthalamic nucleus (STN) and the substantia nigra (SN) (see Fig. 1.1). The SN is divided into two main parts, the dorsal *pars compacta* (SNc) where the dopaminergic nigrostriatal neurons are located and the more ventral *pars reticulata* (SNr), containing GABAergic neurons (Bolam *et al*., 2000; Havekes *et al*., 2011).

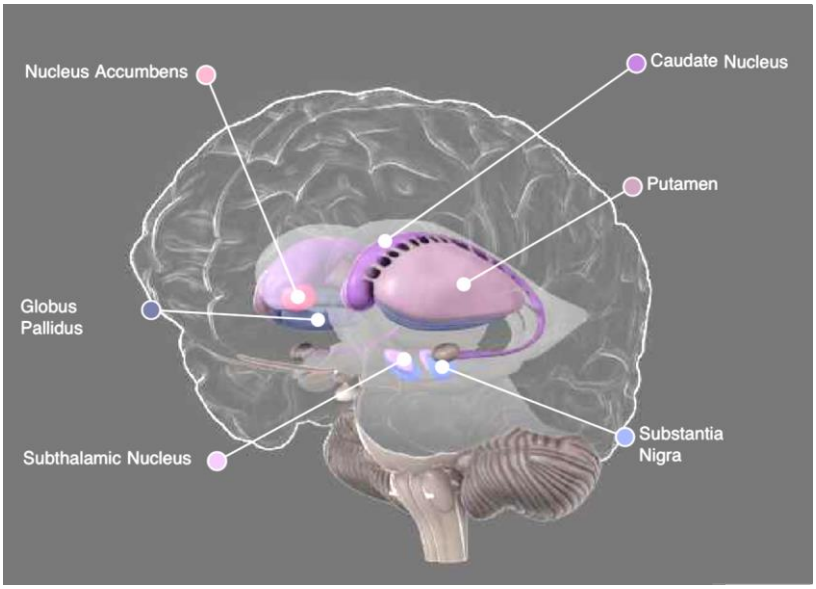


Figure 1.1 - Location of the neostriatum (caudate nucleus-putamen) and other main structures of the basal ganglia in the human brain (source: http://www.g2conline.org/#Brain?aid=2092).

The striatum receives extensive thalamo-cortical inputs which, after being integrated at the striatal level, are processed by the basal ganglia output nuclei and subsequently sent back to thalamic and cortical areas (see Fig. 1.3) (Graybiel 1991). This processing occurs through an orchestrated interaction among several neuromodulators at the pre- and post-synaptic levels (Girault 2012), where the dopaminergic inputs from the SN play a prominent role (Gerfen and Surmeier, 2011). Accordingly, the dopaminergic system is crucial in different functions processed through striatal circuits, such as locomotor activity, habit formation or associative and mnemonic functions (Wickens *et al*., 2007; Dagher and Robbins, 2009; Lovinger 2010; Cools 2011). Also, the manipulation of different neuromodulators, including acetylcholine (ACh) and adenosine, can affect the striatal dopaminergic system and hence is a potential strategy to manage striatal-related brain diseases associated with dopaminergic dysfunction such as Parkinson's disease (PD) and addiction (Schiffmann *et al*., 2007; Quik *et al.*, 2011).

The majority of striatal neurons are efferent inhibitory GABAergic *medium sized spiny neurons* (MSNs) (Fig.1.2), which constitute about 95% of the neuronal population of the striatum (Rymar *et al*., 2004). MSNs have dendrites densely covered with dendritic spines, giving them their name, and are divided into two major subpopulations, the enkephalinergic and the dynorphinergic neurons, based on their neurochemical content and different projection regions. These distinct neuronal types originate two different pathways (see Fig. 1.3) that link the striatum with the output nuclei of the basal ganglia: i) the direct pathway, formed by dynorphinergic neurons that express the neuropeptide substance P and ii) the indirect pathway constituted by enkephalinergic neurons that express enkephalin (Ferré *et al*., 1997; Gerfen 2004). The MSNs of the direct pathway

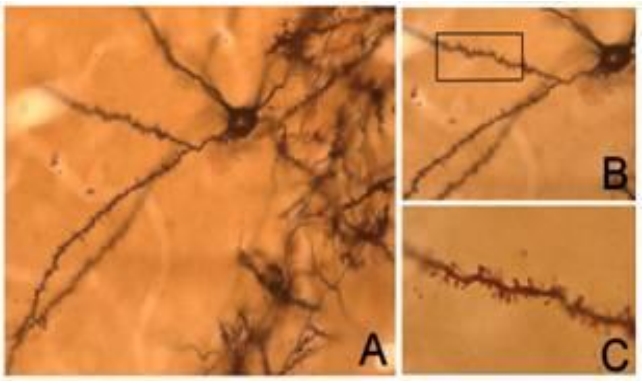


Figure 1.2 – (A) Photomicrograph of Golgi-impregnated medium spiny neurons from monkey putamen. (B) high-magnification of dendritic branches (C) detail of a dendritic branch with dendritic spines (source: http://www.emory.edu/NEUROSCIENCE/Smith/heyneindex.htm).

are directly connected to output nuclei of the basal ganglia, such as the internal segment of the globus pallidus (GPi, or entopeduncular nucleus in rodents), and their stimulation results in motor activation, while the MSNs of the indirect pathway project almost exclusively to the external section of the globus pallidus (GPe or globus pallidus in rodents) and when stimulated originate motor inhibition (Fig. 1.3) (see Smith *et al*., 1998 for review).

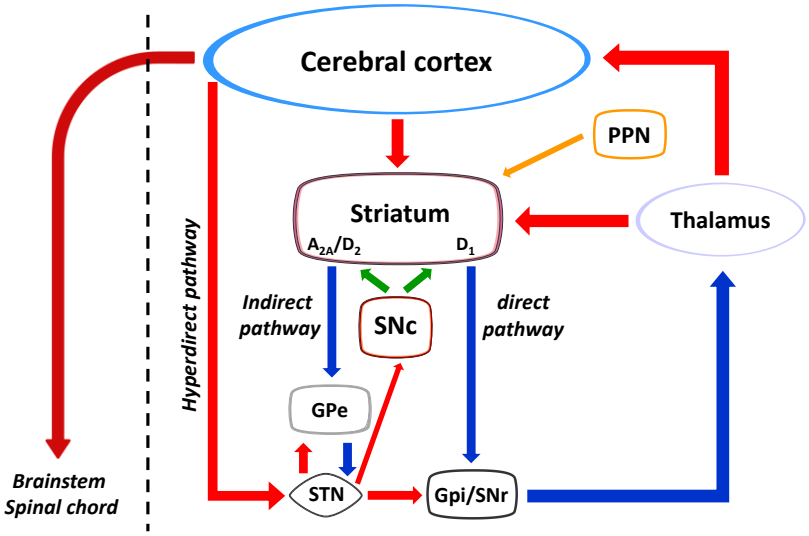


Figure 1.3 - Schematic model of the major connections of basal ganglia circuits. Blue arrows indicate GABAergic connections (inhibitory), red arrows indicate glutamatergic connections (excitatory), green arrows indicate dopaminergic connections and the orange arrow represents cholinergic connections. Cortical activation of the direct pathway (striatum–GPi/SNr) promotes movement while stimulation of the indirect pathway (striatum–GPe–STN–GPi/SNr–thalamus) has the opposite effects. SNc, substantia nigra *pars compacta*; SNr, substantia nigra *pars reticulata*; GPi, internal segment of globus pallidus or entopeduncular nucleus in rodents; GPe, external segment of globus pallidus; PPN, pedunculopontine nucleus; STN, subthalamic nucleus.

The striatum (caudate-putamen) is the main processing unit of the basal ganglia, and receives direct input from glutamatergic efferents from virtually all regions of the neocortex and also from limbic and thalamic areas (see Fig. 1.3). The striatum functioning implies an interface role between the neocortex and the deeper nuclei of the basal ganglia. That is, cortical inputs are processed via

changes in synaptic plasticity at cortico-MSNs glutamatergic synapses. The neuromodulators adenosine, dopamine and acetylcholine are essential elements in the control of striatal synaptic plasticity (for review see Lerner and Kreitzer, 2011) and, therefore, anything that alters the delicate balance among these neuromodulators will have profound effects on motor functions, behaviour and personality.

The striatum receives substantial dopaminergic efferents from the mesencephalon, the majority originating in the substantia nigra *pars compacta*, and in the ventral tegmental area (VTA) to a lesser extent (Gerfen 2004). Both the glutamatergic and the dopaminergic sets of inputs target the dendritic spines of MSNs, but converge at different locations. Cortical projections form mainly asymmetrical synapses with the head of dendritic spines, whereas dopaminergic terminals primarily form symmetric synaptic contacts with the necks of dendritic spines (see Ferré *et al*., 2007) (Fig. 1.4).

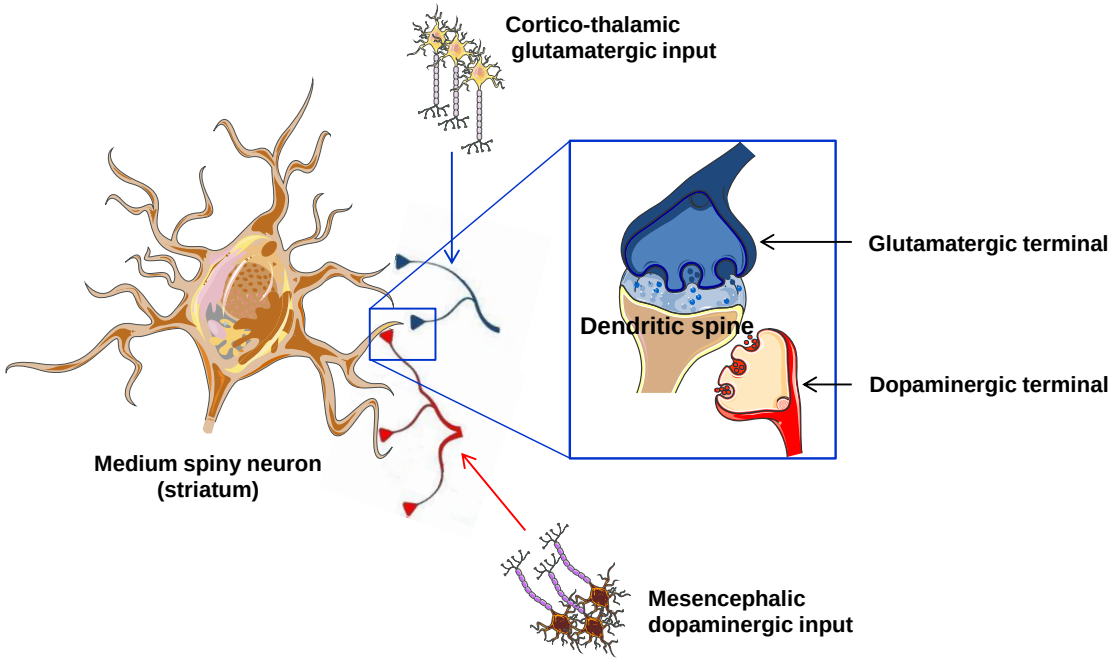


Figure 1.4 - Representation of a striatal medium spiny neuron (MSN) and dendritic spine detail. This type of neuron receives glutamatergic afferent inputs from cortical and thalamic areas, and dopaminergic afferents from the mesencephalon. The enlarged scheme shows a dendritic spine of the MSN and glutamatergic and dopaminergic terminals, which make synaptic contact with the head and neck of the dendritic spine, respectively (adapted from Ferré *et al.,* 2007).

This morphological organization results in different functions: the glutamatergic input acts as a trigger of striatal circuits, while the dopaminergic input has an essential modulatory role, given that it is incapable of triggering electrical responses in MSNs without glutamatergic inputs (Bolam *et al*., 2000; Gerfen 2004). Besides the MSNs, the neuronal population of the striatum is composed by cholinergic interneurons (~2%) and at least three types of GABAergic interneurons (~5%) (Kawaguchi *et al.*, 1995; Ferré *et al*., 2007). These GABAergic interneurons receive input from the cortex and are strategically positioned to integrate and modulate the arriving cortical information. The large cholinergic interneurons are responsible almost exclusively for the neostriatal cholinergic innervation (Havekes *et al*., 2011).

1.2 NEUROMODULATION IN THE STRIATUM

1.2.1 Dopaminergic neuromodulation

As described in the previous section, the structural organization of the striatal neurons allows the modulation of glutamatergic excitatory inputs from the cortex, limbic system and thalamus by mesencephalic dopaminergic inputs (mainly from the SN). Dopaminergic neurons display two general and well-defined firing modes *in vivo*; single-spike firing in regular or irregular patterns (~1–10 Hz) and burst firing of 3–5 spikes at a frequency of ~15–100 Hz (Grace and Bunney, 1984a,b; Hyland *et al*., 2002) and the switch in firing mode of these neurons, from low to higher firing frequencies, is thought to be responsible for the encoding of information (Threlfell and Cragg, 2011). Although dopaminergic neurons constitute a relatively small population, a single dopaminergic neuron projecting to the striatum forms a very dense arbor (Matsuda *et al*., 2009; Moss and Bolam, 2010). An individual dopaminergic neuron is believed to make several hundred thousand synapses, at least an order of magnitude higher than most other neurons of the central nervous system (CNS) (Matsuda *et al*., 2009). Thus, dopaminergic neurons are well-positioned to serve as modulators of striatal functions.

Dopamine (DA) acts via the activation of 5 subtypes of metabotropic G protein-coupled receptors, classified as D_1, D_2, D_3, D_4, and D_5. The receptors D_1 (D_1Rs) and D_5 (D_5Rs) are classified as D_1R-like, and activate the enzyme adenylate cyclase; the D_2Rs, D_3Rs and D_4Rs are termed D_2R - like subtypes, and those receptors inhibit adenylate cyclase (Missale *et al*., 1998). The two populations of MSNs, enkephalinergic and dynorphinergic, have marked differences in the types of dopamine (and also adenosine) receptors that they express, which underlies their function in the modulation of striatal information flow. The dynorphinergic neurons of the direct pathway express principally D_1Rs and A_1 subtype adenosine receptors (A_1Rs), while the enkephalinergic neurons that form the indirect pathway display mainly dopamine D_2Rs (D_{2L}R isoform) and adenosine A_{2A} receptors (A_{2A}Rs) (Ferré *et al*., 1997). Therefore, dopamine will induce motor activation in two different ways: by activating D_1Rs of the direct pathway and on the other hand by depressing the indirect pathway through the action at inhibitory D_2Rs in enkephalinergic neurons (Gerfen 2004). Besides acting at synaptic contacts in dendritic spines of MSNs, dopamine can spill over from the synapses (process called volume transmission) and activate extrasynaptic dopamine receptors (Ferré *et al*., 2007). Since approximately 60-70 % of dopaminergic axon terminals do not form synaptic contacts (Descarries *et al.,* 1996; Vizi *et al*., 2010) dopamine can also diffuse away from non-synaptic dopaminergic terminals and activate the extrasynaptic dopamine receptors (mainly localized in the vicinity of glutamatergic synapses on MSNs) (Yung *et al*., 1995; Venton *et al*., 2003). Presynaptic $D_{2/4}$Rs can be localized in glutamatergic terminals (Tarazi *et al*., 1998; Bamford *et al*., 2004) and in dopaminergic terminals of synaptic contacts made with enkephalinergic MSNs, behaving as autoreceptors (Sesack *et al*., 1994; Usiello *et al*., 2000).

1.2.2 Cholinergic neurotransmission

It has been recognized for some time that the striatum is enriched in cholinergic innervation (Satoh 1983) suggesting that acetylcholine (ACh) has an important function in this brain region (Ferré *et al*., 2007; Havekes *et al*., 2011). The striatal concentrations of ACh and of cholinergic enzymes, acetylcholinesterase and choline acetyltransferase, are among the highest in the brain (Hoover *et al*., 1978); and the striatum also contains high densities of muscarinic (mAChRs) and nicotinic acetylcholine receptors (nAChRs) (Aubert *et al*., 1996; Grady *et al*., 2007). Apart from a minor projection arising from the pedunculopontine nucleus (PPN) (Woolf and Butcher, 1986; Nakano *et al*., 1990) the neostriatal cholinergic innervation is performed by the intrinsic large aspiny cholinergic interneurons. These cells possess smooth dendrites and are distributed in a characteristic pattern throughout the neostriatum and probably correspond to the tonically active neurons recorded *in vivo* (Apicella 2007). Only a relatively small number (~10%) of striatal cholinergic synaptic specializations have been identified, targeting MSNs, which indicates that ACh in the striatum acts mainly via nonsynaptic (paracrine or nonjunctional) and diffuse (volume) transmission (Descarries *et al*., 1997). The basal levels of ACh in the striatum (DeBoer *et al*., 1993) appear to be high enough to continuously activate the cholinergic receptors (Kurosaki *et al*., 1987; Quirion *et al*., 1989), establishing a baseline and tonic level of cholinergic neurotransmission.

The ACh-mediated modulation of dopamine transmission in the striatum notably involves presynaptic mechanisms, mediated by nAChRs localized mainly in dopaminergic but also in glutamatergic nerve terminals. The cholinergic and dopaminergic neurons have dense overlapping axonal arborizations and a high density of dopaminergic and cholinergic terminals exists in the striatum, often separated only by about 1 µm (Descarries *et al*., 1997). Therefore, striatal ACh (or an exogenous agonist such as nicotine) acting at nAChRs is able to effectively modulate dopamine release by direct and indirect (glutamate mediated) mechanisms (Ferré *et al*., 2007).

1.3 CLASSIFICATION AND STRUCTURE OF NICOTINIC ACETYLCHOLINE RECEPTORS

Nicotinic acetylcholine receptors (nAChRs) are widely distributed in the central and peripheral nervous systems. The neuronal nAChRs belong to a large superfamily of ligand-gated ion channels. This superfamily of homologous ionotropic receptors also includes: muscle-type acetylcholine receptors, gama-aminobutyric acid receptors ($GABA_AR$ and $GABA_CR$) glycine receptors and the serotonin (5-HT) receptors, $5\text{-}HT_3R$ (Le Novere *et al*., 2002; Gotti *et al*., 2007).

The neurotransmitter ACh, firstly identified in 1914, functions as the endogenous agonist of nAChRs, while the alkaloid nicotine (from where the name of the receptors derive), is one of the best know exogenous agonists. Functionally, the nAChRs in the brain mediate classical excitatory cholinergic neurotransmission at selected *loci* but also, and perhaps more globally, play important roles in the modulation of neurotransmission by other chemical messengers (Wu and Lukas, 2011). Therefore, nAChRs are involved in a number of higher brain functional processes, including cognition, learning and memory, arousal, reward, neuronal development and in several pathological conditions, such as Parkinson´s disease, Alzheimer's disease, depression, epilepsy, schizophrenia and addiction (Levin and Simon, 1998; Paterson and Nordberg, 2000; Hogg *et al*., 2003; Graef *et al*., 2011).

The nAChRs consist of five subunits assembled in the plasma membrane to form a barrel like channel (Fig. 1.5) that is permeable for the monovalent Na^+ and K^+ ions, and also for Ca^{2+}. The many possible combinations in which the different nAChR subunits can potentially co-assemble lead to a diversity of distinct nAChR subtypes. The cation permeability of the different subtypes is influenced by their subunit composition (Fucile 2004). Twelve neuronal nAChR

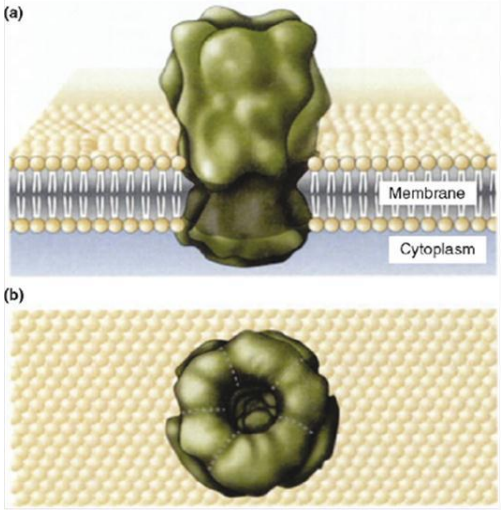


Figure 1.5 – Representation of a nicotinic acetylcholine receptor showing the side (a) and synaptic (b) views (taken from Halliwell 2007).

subunit genes have so far been cloned and classified into two subfamilies of nine alpha (α2– α10) and three beta subunits (β2– β4). To date, two main classes of nAChR subtypes have been identified: the homomeric or heteromeric α-bungarotoxin (α-BTX) sensitive receptors, which are made up of the α7, α8, α7-α8, α9 and/or α10 subunits, and the heteromeric α-BTX-insensitive receptors consisting of the α (α2–α6) and β (β2–β4) subunits (Gotti *et al*., 2007; Albuquerque *et al*., 2009). It should be noted that some studies report that the α7 subunit can also form functional heteromeric receptors with β subunits (Palma *et al*., 1999; Khiroug *et al*., 2002; Liu *et al*., 2009, 2012). Heteromeric subtypes generally assemble according to a 2α3β stoichiometry, with the possibility of existing more than one α subunit type within the pentamer. Homomeric α7, α8 and α9 are able to form functional homopentamers composed by a single subunit subtype (the α8 subtype only being found in aviary tissue). Presently, six α (α2– α7) and three β (β2– β4) subunits have been identified in mammalian brain (Paterson and Nordberg, 2000; Gotti *et al*., 2006b) (see Fig. 1.6). These receptors share a common basic structure, but have specific pharmacological and functional properties that are consequence of the very different subunit combinations that make distinctive subtypes.

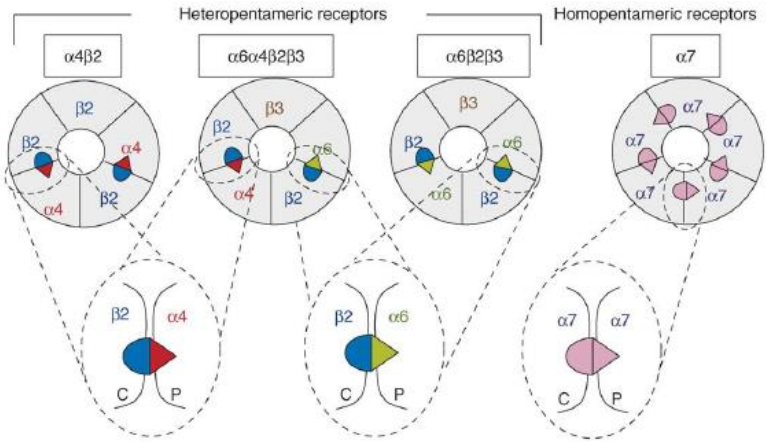


Figure 1.6 – Organization and structure of homopentameric and heteropentameric subtypes of nAChRs. Localization of the subunit interfaces of the ACh-binding site are shown below. The homomeric α7 subtype has five identical acetylcholine (ACh) binding sites per receptor molecule (one on each subunit interface) while the heteropentameric receptors have two identical binding sites per receptor molecule (taken from Gotti *et al.,* 2006b).

The assembly of nAChRs is a strongly regulated and well-organized process, which requires appropriate subunit–subunit interactions (see Millar and Harkness, 2008). In that way, although there is potential for the formation of nAChRs with different subunit combinations, the native nAChRs identified are assembled into subtypes containing a relatively restricted number of subunit combinations, which nonetheless originate a substantial diversity of nAChRs. The basic structure of nAChR subunits (Fig. 1.7) consists of a relatively hydrophilic extracellular amino terminal domain of ~200 amino acids that contains the ACh binding site and faces the synaptic cleft, followed by three segments of 19-27 amino acids that form compact hydrophobic transmembrane domains (M1–M3), a large intracellular loop, a fourth hydrophobic transmembrane domain (M4) and a final small carboxyl-terminal sequence. The M1–M4 transmembrane domains are arranged in concentric layers around the central aqueous pore: the M2 domain lines the pore membrane, M1 and M3 shield M2 from the surrounding lipid bilayer and M4 is the most exposed to lipids (Unwin 2005; Albuquerque *et al*., 2009, Gotti *et al*., 2009).

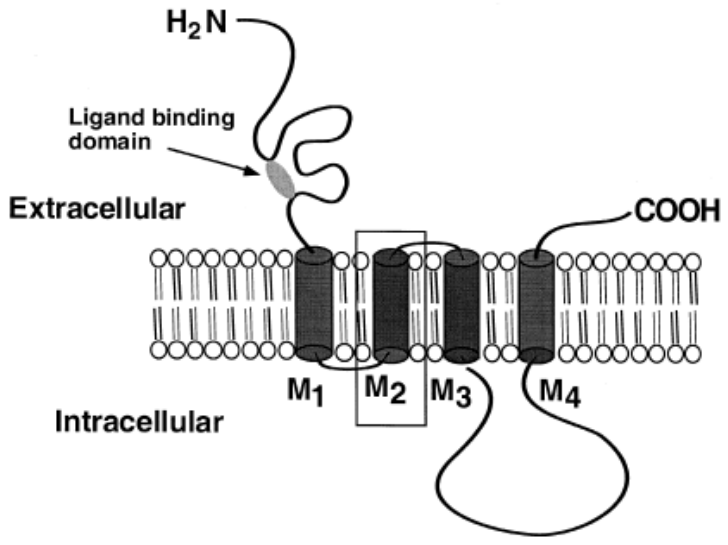


Figure 1.7 – Nicotinic receptor structure showing the hydrophilic extracellular domain containing the ACh binding site, the four transmembrane segments M1-M4, the intracellular loop and the small C terminal domain. Highlighted in the box is the transmembrane segment M2 which is thought to form the lining of the ion channel (taken from Paterson and Nordberg, 2000).

Both α and β subunits contribute to the pharmacological properties of the receptor binding site(s). The binding site has principal and complementary components, and is located at the interface between two identical subunits in homomeric α7 receptors, or between an α and a β subunit in the case of heteromeric α-BTX-insensitive receptors (see Fig. 1.6). The α5 and β3 subunits do not have the primary or the complementary components of the ACh binding site, and it is generally believed that they do not directly participate in its formation. It is thought that homomeric receptors have five identical ACh-binding sites for each receptor molecule (one on each subunit interface), whereas the heteromeric receptors have two binding sites *per* receptor (Corringer *et al*., 2000).

Neuronal nicotinic receptors can be localized at the nerve terminal boutons, at the presynaptic active zone; at the terminal boutons but outside the active zone, or in preterminal axon segments, where they directly modulate the release of almost all neurotransmitters or influence the probability of exocytosis of neurotransmitters. However, these receptors can also be present at somatodendritic postsynaptic locations (see Fig. 1.8) (Wessler 1992; Wonnacott 1997; Dani and Bertrand, 2007; Albuquerque *et al*., 2009). There are also increasing evidences that several neuronal-type nAChR subunits are expressed in non-excitable cells such as microglia, astrocytes and endothelial cells (Wessler and Kirkpatrick, 2008; Albuquerque *et al*., 2009). Many different nAChR subtypes have been identified in various regions of the CNS (see Fig 1.9) and while those subtypes can be localized in different parts of the neuronal cells currently the prevalent idea is that nAChRs present at presynaptic or preterminal sites are physiologically more relevant (Sher *et al*., 2004; Jensen *et al*., 2005).

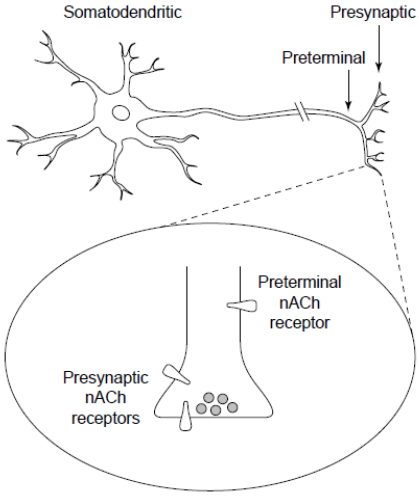


Figure 1.8 – Putative locations of neuronal nicotinic acetylcholine receptors (taken from Wonnacott 1997).

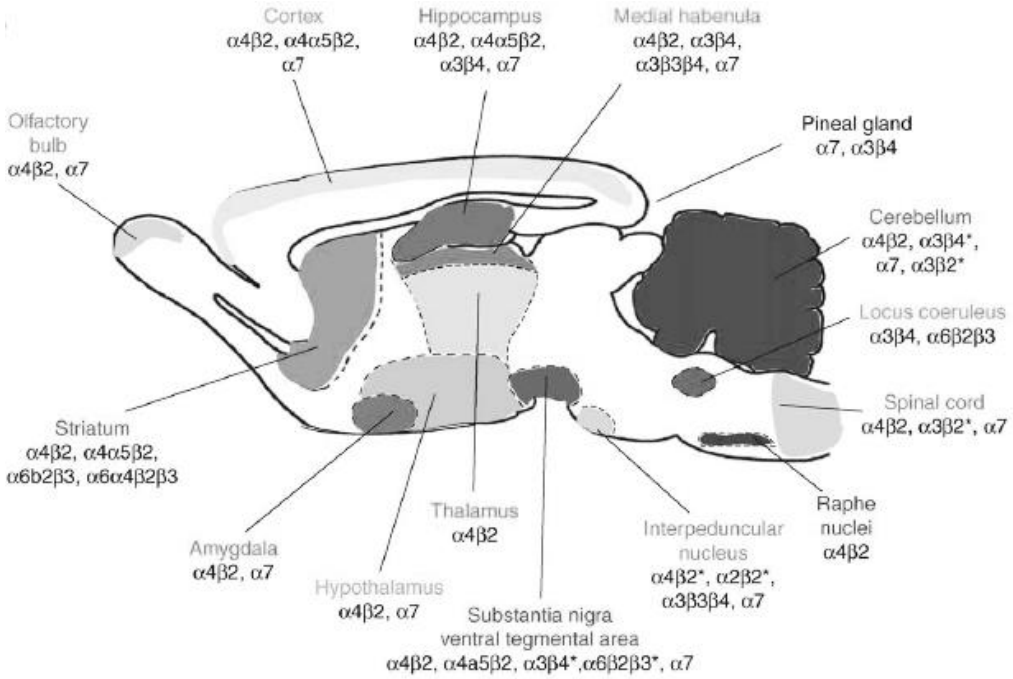


Figure 1.9 – Regional distribution of some of the main nicotinic receptor subtypes in the rodent central nervous system (taken from Gotti *et al.,* 2006b).

1.4 DISTRIBUTION AND LOCALIZATION OF NICOTINIC RECEPTOR SUBUNITS

1.4.1 Alpha 7 (α7) subunit

The α7 subunits form mainly homomeric receptors, designated as the α7 nAChR subtype, that are found in vertebrate central and autonomic nervous systems and have the ability to bind the curaremimetic neurotoxin α-bungarotoxin (Morley *et al*., 1979; Clarke *et al*., 1992; Lukas *et al*., 1992). This nAChR subtype has a high Ca^{2+} permeability (Dajas-Bailador and Wonnacott, 2004), undergoes rapid activation and desensitisation (Wu and Lukas, 2011) and has a low affinity for agonists (Köfalvi *et al*., 2000; Albuquerque *et al*., 2009; Toyohara and Hashimoto 2010). In the CNS, α7 nAChRs are located primarily in the hippocampus, thalamus, prefrontal cortex, subcortical basal ganglia, ventral midbrain and dorsal raphe nucleus (Gotti *et al*., 2007; Bencherif and Lippiello, 2010). This nAChR subtype has been implicated in processes such as

control of neurotransmitter release (Albuquerque *et al*., 2000), development and maintenance of neurites and synapses (Freeman 1977; Pugh and Berg, 1994), long-term potentiation (Hunter *et al*., 1994), neuronal viability/death (Renshaw 1994; Hory-Lee and Frank 1995) and modulation of immunological responses (Conejero-Goldberg *et al*., 2008).

The α7 nAChRs are predominant in the hippocampus, where they can be found postsynaptically on the soma and dendrites of pyramidal neurons and GABAergic interneurons (Albuquerque *et al*., 2009). In the basal ganglia there is also a relevant expression of α7 nAChRs, with higher levels detected in the SN, and lower in the caudate nucleus and putamen, as demonstrated by radioligand binding of [^{125}I]α-bungarotoxin in human brain (Court *et al*., 2000). At the cellular level α7 nAChRs were identified in the cell bodies of dopaminergic and GABAergic neurons of the VTA (Klink *et al*., 2001; Jones *et al*., 2004), of GABAergic neurons of the prefrontal cortex (Lubin *et al*., 1999) and in postsynaptic sites of cortical excitatory synapses (Levy and Aoki, 2002). A presynaptic localization of α7 nAChRs can also occur and this receptor subtype has been identified in glutamatergic and GABAergic terminals in several brain regions such as striatum (Marchi *et al*., 2002), hippocampus (Fabian-Fine *et al*., 2001; Maggi *et al*., 2003), olfactory bulb (Alkondon *et al*., 1996), VTA (Jones and Wonnacott, 2004), medial habenula (Girod and Role, 2001) and frontal cortex (Rousseau *et al*., 2005).

There is expression of α7 nAChR subunit mRNA in some midbrain dopaminergic neurons (Azam *et al*., 2002), and it was also suggested the presence of α7 nAChRs in dopaminergic terminals (Soliakov *et al*., 1995); however, the inability of these receptors to influence [^{3}H]dopamine release from isolated nerve terminals (Grady *et al*., 1992; Mogg *et al*., 2002; Salminen *et al*., 2004) seems to indicate that this subtype may be restricted to somatodendritic regions of dopamine neurons.

Accumulating evidences also support the existence of heteromeric nAChRs formed by α7 and β2 or β3 subunits (Palma *et al*., 1999; Khiroug *et al*., 2002; Liu *et al*., 2009, 2012).

1.4.2 Beta 2 (β2) subunit

The β2 subunit of nAChRs is the most widespread in the brain and can form different nAChR subtypes when it assembles with other subunits, namely with the α4 subunit (forming the α4β2* receptors, where the asterisk denotes the possible presence of other subunits in the receptor complex) or with the α6 subunit (forming α6β2* receptors). These subtypes are present in numerous brain areas, such as the striatum, hippocampus, SN, VTA or NAc (Gotti *et al*., 2007, Albuquerque *et al.,* 2009).

The β2 subunit can also assemble with α3 and α2 subunits, forming nAChRs containing, α2, α3 and β2 subunits, designated as α2β2* and α3β2*, respectively. The α3β2* subtype was identified in rodent retina, cerebellum and habenulo-interpeduncular pathway (Marritt *et al*., 2005; Turner and Kellar, 2005; Grady *et al*., 2009). This receptor subtype was also identified in monkey striatum (Quik *et al*., 2005), but does not appear to be significantly present in rodent striatum as previously thought (Zoli *et al*., 2002). *In situ* hybridization studies have shown that the α2β2* subtype is highly expressed in several areas of primate brain (Han *et al*., 2003) but its expression in rat is limited to retina and interpeduncular nucleus (Moretti *et al*., 2004; Grady *et al*., 2009), suggesting that there are differences between the subtypes of nAChRs expressed in primate and rodent brains.

1.4.3 Alpha 4 (α4) subunit

Nicotinic receptors that contain the α4 subunit (α4* nAChRs) are highly abundant in the brain, displaying a wide distribution throughout many regions, that include the neocortex, striatum, hippocampus, ventral midbrain, most of the thalamic nuclei and the dorsomedial hypothalamic nucleus (Gotti *et al*., 2007; Wu and Lukas, 2011). Immunoassays have shown that the predominant naturally expressed form of α4* nAChRs in the vertebrate brain contains α4 and β2 subunits, forming the α4β2 nAChR subtype (Whiting *et al*., 1987; Flores *et al*., 1992). The α4 colocalizes with the β2 subunit in most brain areas and knocking down either one abolishes high affinity nicotinic agonist binding (Picciotto *et al*., 2001). The α4β2 receptors are expressed in two different

stoichiometries: i) the (α4)2(β2)3 stoichiometry that binds to ACh and nicotine with high affinity, and ii) the (α4)3(β2)2 stoichiometry that binds to ACh and nicotine with low affinity (Zwart *et al*., 1998; Khiroug *et al*., 2004). The α4β2 nAChRs have been implicated in nicotine self-administration, reward, dependence and in brain diseases, such as Alzheimer's disease and epilepsy (Wu and Lukas, 2011).

In the striatum the α4β2 subtype is present in dopaminergic and GABAergic neurons, while a subtype that also contains the α5 subunit (α4α5β2 nAChR subtype) has distinct properties and is mostly expressed by dopaminergic terminals (Zoli *et al*., 2002), but may also exist in GABAergic interneurons (McClure-Begley *et al*., 2009). The α4 subunit is also included in subtypes of more complex nAChRs composition, which contain the α6* subunit (α4α6β2β3 nAChR subtype), localized on striatal dopaminergic nerve terminals (see Figs. 1.6 and 1.9). Pharmacological profiles compatible with that of α4β2 nAChRs were also identified on cholinergic terminals in the human neocortex and in the rat midbrain (Diaz-Hernandez *et al*., 2004). In addition to being expressed on presynaptic terminals, α4β2 nAChRs have been reported in axonal pre-terminal regions of GABAergic neurons in the VTA and hippocampus and in thalamocortical neurons (Alkondon *et al*., 1999; Mansvelder *et al*., 2002; Kawai *et al*., 2007).The α4β2* nAChRs are also present in somatodendritic sites in several brain areas, such as the SN and VTA, where they are located in dopaminergic and GABAergic cell bodies and in hippocampal neurons (Klink *et al*., 2001; Albuquerque *et al*., 2009; Livingstone and Wonnacott, 2009).

The α4 subunit can also assemble with β4 subunits to form α4β4 nAChRs, which have comparably high nicotine affinity (Moaddel *et al*., 2005; Hamouda *et al*., 2009). The β4 subunit mRNA colocalizes with α4 subunit mRNA in several brain regions (Winzer-Serhan and Leslie, 1997; Quik *et al*., 2000) and the presence of α4β4 nAChRs has been suggested in the interpeduncular nucleus (Klink *et al*., 2001), cerebellum and retina (Marritt *et al*., 2005; Turner and Kellar, 2005).

1.4.4 Alpha 6 (α6) subunit

Nicotinic receptors that contain the α6 subunit (α6-containing nAChRs, α6*) were one of the last to be characterized, with the cloning of the α6 nAChR subunit only achieved in the 1990s (Deneris *et al*., 1991; Sargent, 1993; Quik *et al*., 2011). These receptors appear to be excluded from the peripheral nervous system and exhibit a relatively restricted distribution in the mammalian CNS (Quik *et al*, 2006; Calabresi *et al*., 2008; Yang *et al*., 2009; Millar *et al*., 2009). Studies with *in situ* hybridization, in rodent and primate brain, done to identify the presence of α6 nAChR subunit mRNA, showed that the strongest α6 mRNA signal is in the retina and catecholaminergic nuclei, including the locus coeruleus, VTA and SN, with a weaker signal in the superior colliculus, medial habenula, interpeduncular nucleus, visual cortex and a few other small nuclei (Le Novere *et al*., 1996; Quik *et al*., 2000; Han *et al*., 2000; Champtiaux *et al*., 2002). This anatomical distribution suggests that α6* nAChRs may play a role in behaviours linked to reward, motor activity and vision. Studies with α6 nAChR-subunit directed antibodies and the snail toxin α-conotoxin MII (α-CTX MII), which binds to α6β2* nAChRs, revealed that the α6* nAChRs are present in the mesolimbic and nigrostriatal dopaminergic pathways, and are consistent with the existence of α6 mRNA in catecholaminergic nuclei (Whiteaker *et al*., 2000; Quik *et al*., 2001; Zoli *et al*., 2002; Gotti *et al*., 2005). Immunoprecipitation experiments found that α6* nAChRs account for 30% of [^{3}H]epibatidine binding sites in striatum (Champtiaux *et al*., 2003), and quantitative immunoprecipitation showed that most of α6* nAChRs (87%) disappeared in 6-hydroxydopamine (6-OHDA) lesioned striatum (Zoli *et al*., 2002), suggesting a regulatory function of presynaptic α6* nAChRs in dopamine release (Salminen *et al*., 2004; Azam and McIntosh, 2005). These results indicate that α6* nAChRs appear to be preferentially addressed to dopaminergic nerve terminal compartments since the majority of dopaminergic neurons in the SN project to the striatum (Zoli *et al*., 2002; Champtiaux *et al*., 2003; Yang *et al*., 2009). Recent findings indicate that there are also functional α6* nAChRs located on GABAergic presynaptic boutons (Yang *et al*., 2011) and that α6* nAChRs may also be located on somatodendrites of VTA dopaminergic neurons (Drenan *et al*., 2008), but the presence of postsynaptic α6* nAChRs in the striatum has not been clearly

reported yet. The α6* nAChRs (α-CTX MII-binding nAChRs) localized at nerve terminals appear to be expressed almost exclusively in dopaminergic neurons (Quik *et al*., 2011).

Immunological approaches using a battery of subunit specific antibodies to measure the nAChR subtypes identified three α6* nAChR subtypes: α4α6β2β3, α6β2β3 and α6β2 in rodent striatum (Zoli, 2002; Champtiaux *et al*., 2003; Gotti *et al*., 2005), that have different pharmacological properties. These α6* nAChR subtypes are probably the ones that bind [^{125}I]-α-CTX MII with high affinity (Grady *et al*., 2007). Immunological methods have also identified these subtypes of receptors in striatal tissue obtained from squirrel monkeys (Quik *et al*., 2005) and humans (Gotti *et al*., 2006a).

Studies with parkinsonian animal models, including mice, rats and monkeys, suggest that nicotine or nAChR agonist treatment may afford therapeutic benefit in PD by interacting with nAChRs, especially the α4α6β2β3 subtype (O'Neill *et al*., 2002; Quik *et al*., 2007; Picciotto *et al*., 2008; Huang *et al*., 2009). Binding studies in striatal tissue revealed that the α4α6β2β3 is a subtype that is preferentially vulnerable, since it is lost with mild to moderate nigrostriatal damage in animal models and human patients of PD, whereas the α6β2β3 was only decreased with more severe damage (Bordia *et al*., 2007; Gotti *et al*, 2009). Several studies with rodents and monkeys have also showed that nicotine administration reduces abnormal involuntary movements caused by the administration of levodopa, also named L-dopa (L-3,4-dihydroxyphenylalanine) (Quik *et al*., 2007; Bordia *et al*., 2008; Huang *et al*., 2011) and recent results suggest that the neuroprotective nicotine effects are mediated through the α6β2* nAChRs (Quik *et al*., 2012a).

Extensive work has been done to investigate the role of a6* subtypes in dopaminergic activity in the striatum, the terminal region of the nigrostriatal pathway. The nAChRs were shown to be able to modulate dopamine release from striatal synaptosomes (Rapier *et al*., 1988; Grady *et al*., 1992), a function which was thought to be regulated only by α4β2* (non-α6) nAChRs. However, it was later revealed that activation of the subtype α6β2β3* also stimulated [^{3}H]dopamine release from striatal nerve terminals and that these two nAChR subtypes appear to promote dopamine release in similar proportions in rodent striatum (Kulak *et al*., 1997; Kaiser *et al*., 1998). Interestingly, the α6β2β3*

nAChR subtype seems to be even more predominant in nonhuman primate striatum, with 80% of [^{3}H]dopamine release being mediated by α6β2β3 nAChR subtypes and only 20% by the α4β2* nAChR population (McCallum *et al*., 2005, 2006).

Overall, α6* nAChRs appear to play a prominent role in modulating striatal dopamine release. Recently it has become apparent that presynaptic nAChRs may act through a "filtering" action, modulating the activity of dopaminergic neurons during bursting and tonic firing patterns, influencing dopamine release probability instead of simply enhancing neurotransmitter release from dopaminergic neurons (Zhou *et al*., 2001; Rice *et al*., 2004; Zhang and Sulzer, 2004, Exley and Cragg, 2008). These receptors seem to play a crucial role in controlling motor function, and may therefore represent novel therapeutic targets for the treatment of movement disorders, such as PD (Quik *et al*., 2011).

1.5 ADENOSINE METABOLISM

Adenosine is a purine nucleoside that is present ubiquitously in the body, and is permanently produced both intra- and extracellularly in normal conditions (Cunha 2005) (Fig. 1.10). Intracellularly, it is formed from adenosine monophosphate nucleotides (AMP) by the activity of an endo-5'-nucleotidase enzyme or from S-adenosyl-homocysteine by S-adenosyl-homocysteine hydrolase. Adenosine can be integrated back in S-adenosyl-homocysteine, metabolized into inosine by adenosine deaminase or back into AMP by adenosine kinase. Adenosine of intracellular source can be released to the extracellular space by bi-directional equilibrative nucleoside transporters (ENTs) (Latini and Pedata, 2001). Adenosine can also be formed in the extracellular space through the catabolism

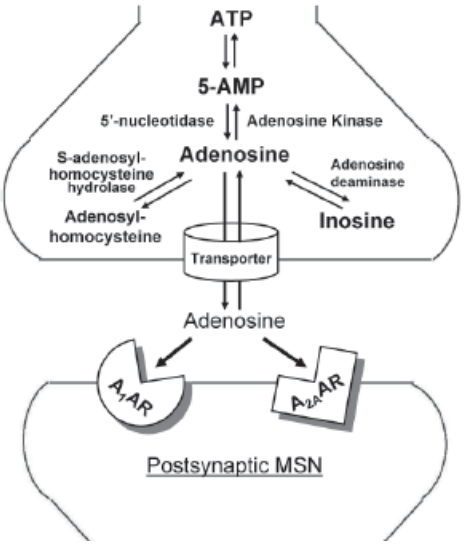


Figure 1.10 – Adenosine intracellular synthesis and metabolism (taken from Xie *et al.,* 2007).

of exocytotic-released ATP (Cunha *et al*., 1996; Dunwiddie *et al*., 1997). In this pathway, ATP is converted into AMP by an ATPase and/or ATP diphosphohydrolase enzyme and then the AMP is subsequently converted to adenosine by the activity of an ecto-5'-nucleotidase enzyme (see Zimmermann 1996; Cunha 2001). Extracellular adenosine is removed, by neurons and astrocytes, through the uptake mediated by specific nucleoside transporters, *i.e.* the ENTs and the concentrative nucleoside transporters (CNTs) (Latini and Pedata, 2001; Nam *et al*., 2012). The intracellular concentration of adenosine in energetically non-deprived cells is estimated to be between 10-50 nM and the basal extracellular concentration is predicted to range between 50-200 nM (Latini and Pedata, 2001). The functions played by adenosine in the CNS are diverse and include cell-to-cell signaling, modulation of cellular metabolic status, metabolism of nucleotides, nucleosides and amino acids (Cunha 2005).

1.6 ADENOSINE RECEPTORS: STRUCTURE, DISTRIBUTION AND LOCALIZATION

Adenosine acts through adenosine receptors which are classified into four different subtypes: A_1, $A_{2A,}$ A_{2B} and A_3 (designated as A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R). All of them are membrane spanning proteins with seven transmembrane domains (see Fig. 1.11), belonging to the superfamily of metabotropic G protein-coupled receptors. In the CNS, adenosine receptors are widely distributed. The A_1Rs are the most abundant and widespread, being highly expressed in the cortex, hippocampus, cerebellum, spinal cord and thalamus, on neurons and also on glial cells (Fastbom *et al*., 1987; Schindler *et al*., 2001; Cunha 2005). A_1Rs are also expressed in the basal ganglia at lower densities (Fastbom *et al*., 1987). The $A_{2A}Rs$ (Fig. 1.11) are the second most abundant adenosine receptor subtype in the CNS and are predominantly concentrated in the basal ganglia, although also being present throughout the brain at considerably lower levels, in areas such as the cortex, hippocampus and cerebellum (Svenningsson *et al*., 1999; Fredholm *et al*., 2003). In the basal ganglia, $A_{2A}Rs$ are mainly expressed in the caudate-putamen, NAc, olfactory tubercle and globus pallidus (Svenningsson *et al*., 1999; Rosin *et al*., 2003;

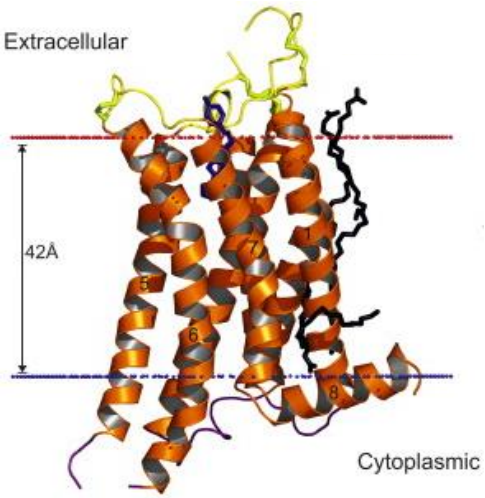


Figure 1.11 – Representation of the crystallographic structure of the human adenosine A_{2A} receptor, bound to the antagonist ZM241385 (dark blue) (taken from Jaakola and Ijzerman, 2010).

Fredholm *et al*., 2005). At the cellular level A_{2A}Rs are not only expressed in neurons but also in astrocytes (Nishizaki *et al*., 2002) and microglia (Küst *et al*., 1999). The A_{2B}Rs and A_3Rs are the less abundant in the brain. The A_{2B}Rs are reported to have a widespread distribution in the brain, although at low levels (Dixon *et al*., 1996), and have been identified in diverse locations such as the median eminence of the hypothalamus, eye, blood vessels and pituitary gland. A_{2B}Rs appear to be mainly expressed in astrocytes but are also present in neurons (Allaman *et al*., 2003; Fredholm *et al*., 2005). Due to their low expression and to the lack of selective pharmacological tools, the distribution and function of this subtype is still largely unknown. The A_3Rs are expressed at low density in several brain regions, including the striatum, cortex, hippocampus and cerebellum, in neuronal and glial cells (Dixon *et al*., 1996, Cunha 2005).

In striatum A_1Rs and A_{2A}Rs are found both pre- and post-synaptically, predominantly localized at the contacts between cortico-thalamic glutamatergic projections and GABAergic medium spiny neurons, but also in the neck of dendritic spines of these MSNs (Hettinger *et al*., 2001; Ferré *et al.,* 2007). The A_{2A}Rs are more densely expressed postsynaptically in the MSNs, but are also present presynaptically in the active zone of the glutamatergic nerve terminals (Rebola *et al*., 2005). Aside from MSNs, the A_{2A}Rs are also expressed in cholinergic interneurons (Preston *et al*., 2000; Tozzi *et al*., 2011). Notably, postsynaptic A_{2A}Rs are almost exclusively expressed by the enkephalinergic subtype of MSNs that form the indirect pathway in the striatum (Svenningsson *et al*., 1999; Gerfen *et al*., 2004). Adenosine receptors are also localized at dopaminergic synapses, where A_1Rs are found presynaptically (Borycz *et al*., 2007). Given that A_{2A}Rs do not control directly the release of dopamine in the striatum the presence of functional A_{2A}Rs in the dopaminergic striatal afferents has been contentious until recently, but compelling data, including work from

our laboratory, provided experimental evidence for the existence of A_{2A}Rs in dopaminergic terminals of the striatum (see Chowdhury and Fillenz, 1991; Gomes *et al*., 2006, 2009 and Garção *et al*., 2013).

1.7 NEUROMODULATION BY ADENOSINE RECEPTORS IN THE STRIATUM

Adenosine receptors are thought to play an essential role in the regulation of neurotransmission in the striatum, which is emphasized by their cellular and subcellular distribution in this brain region (Rebola *et al*., 2005; Morelli *et al*., 2009). The modulation of cortico-thalamic excitatory glutamatergic transmission is a main focus for adenosine receptors in controlling striatal functions (Schiffmann *et al.*, 2007). Activation of A_1Rs leads to a decrease in probability of neurotransmitter release (Lovinger and Choi, 1995; Flagmeyer *et al*., 1997), and on the contrary, activation of A_{2A} receptors increases the probability of neurotransmitter release through cAMP-PKA or PKC dependent mechanisms (Fredholm *et al*., 2005; Leenders and Sheng, 2005), such as occurs in the A_{2A}Rs localized in striatal glutamatergic terminals, leading to the increase of evoked glutamate release (Ciruela *et al*., 2006). Activation of A_{2A} receptors has also been reported to lead to the stimulation of striatal dopamine and acetylcholine release (Kurokawa *et al*., 1994; Okada *et al*., 1996), although these actions are still not fully understood (Schiffmann *et al.*, 2007).

Presynaptic A_{2A}Rs were described to colocalize with metabotropic glutamate type 5 (mGluR5) receptors in striatal glutamatergic terminals and it was observed that there is a synergistic effect between these receptors on the facilitation of glutamate release (Rodrigues *et al*., 2005). Moreover, presynaptic A_{2A}Rs participate in an inhibitory interaction with cannabinoid CB_1 receptors (CB_1Rs) in the striatum, where they generate opposite effects on glutamate release and on signal transduction pathways (Martíre *et al*., 2011). The co-localization and functional interaction between A_{2A}Rs and CB_1Rs in corticostriatal glutamatergic terminals was also confirmed by work developed in our research group (Ferreira *et al*., unpublished results). It was also reported that presynaptic A_{2A}Rs and A_1Rs in glutamatergic terminals can form receptor heteromers that modulate glutamate release through antagonistic reciprocal

interactions (Ciruela *et al*., 2006; Quiroz *et al*., 2009). Postsynaptically, the enkephalinergic MSNs that express predominantly facilitatory A_{2A}Rs also express inhibitory D_2 dopamine receptors (D_2Rs), while the dynorphinergic MSNs express predominantly inhibitory A_1Rs and facilitatory D_1 dopamine receptors (D_1Rs) (Gerfen 2004). There are evidences supporting a close functional interaction between the adenosine and dopamine receptors in the MSNs of both the direct and indirect pathways. It was shown that the function of the dynorphinergic neurons is modulated by antagonistic interactions between A_1Rs and D_1Rs, whereas the enkephalinergic neurons are modulated by antagonistic interactions between A_{2A}Rs and D_2Rs (Ferré *et al*., 1997, 2005), which were reported to be responsible for most of the locomotor depression and activation caused by A_{2A}R agonists and antagonists, respectively (Ferré *et al*., 2008). The A_{2A}Rs localized in the enkephalinergic MSNs can also form heteromeric complexes with the inhibitory receptors, D_2Rs and $mGlu_5$R, which contribute to modulate postsynaptic plastic changes at the glutamatergic synapses (Ferré *et al*., 2007). In this way, both presynaptic and postsynaptic adenosine receptors, in close interaction with other receptors types work to insure an efficient regulation of the motor function.

1.8 ROLE OF STRIATAL NICOTINIC AND ADENOSINE RECEPTORS IN PATHOPHYSIOLOGY AND NEUROPTOTECTION

The presence of nicotinic and adenosine receptors in neuronal afferents to the striatum, as well as the well-known epidemiological data obtained from consumers of nicotine and caffeine (a general adenosine receptor antagonist) (Ascherio *et al.,* 2001; Schwarzschild *et al.,* 2003; Quik et al., 2012b) position the nicotinic and adenosine receptors as potential therapeutic targets in brain pathologies, such as PD.

PD is a neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra *pars compacta* and consequent loss of function of the nigrostriatal dopaminergic system (Schapira and Jenner, 2011). The major clinical symptoms of this devastating disease include reduced spontaneous movement, rigidity, bradykinesia (slowness of movements), akinesia (lack of movement) and resting tremor (Armentero *et al*., 2011;

Halliday *et al*., 2011). The reduction in striatal DA transmission that occurs in the parkinsonian brain is believed to increase the inhibitory output from the basal ganglia to the thalamus, leading to impaired motor function (Lester *et al*., 2010). To date, only palliative interventions are available to the majority of parkinsonian patients.

Data obtained from epidemiological studies were essential in identifying potentially protective agents against PD. Two of the most noteworthy, and relevant to our study, are nicotine and caffeine. Nicotine constitutes approximately 0.6-3% of the dry weight of a tobacco plant, and is a potent natural insecticide (Hurst *et al*., 2013). About 1 billion people worldwide are tobacco smokers and consumption of tobacco products is increasing globally (World Health Organization, 2013). The recognition that tobacco use is related with a lower incidence of PD led to the consideration of nicotine as a possible therapeutic agent (see Quik *et al*., 2012b) and studies with animal models reinforced the idea that nicotine or other nAChR agonists may be an efficient treatment for the motor symptoms of PD. Activation of nAChRs was shown to modulate locomotion in non-lesioned animals and to improve motor dysfunction symptoms in animals with nigrostriatal damage (Schneider *et al*., 1998; Meshul *et al*., 2002). Moreover, nicotine treatment was shown to protect the nigrostriatal dopaminergic neurons from degeneration after administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or 6-OHDA (Costa *et al*., 2001; Parain *et al*., 2003), two toxins widely used to induce parkinsonian-like symptoms. The most relevant nAChRs to PD pathophysiology are probably the ones in the nigrostriatal pathway, although receptors in other brain areas could also play important roles (Quik *et al*., 2009). The loss of dopaminergic presynaptic terminals in PD is accompanied by a decrease in nAChRs (Quik *et al*., 2004; Bohr *et al*., 2005); and although the identity of the affected nAChR subtypes is not well-defined, a number of studies have indicated a prominent involvement of α4β2* and α6β2* receptors (Gotti and Clementi, 2004; Quik and McIntosh, 2006). Notably, these nAChR subtypes seem to be differentially affected by nigrostriatal damage since studies with parkinsonian rodents and monkeys indicate that the α6α4β2* nAChR subtype is more prone to damage than the α6(non-α4)β2* or α4β2* nAChR population, and similar observations were obtained in the brain of PD patients (Bordia *et al*., 2007).

Caffeine is the most widely consumed psychoactive drug worldwide (Fredholm *et al*., 1999). The consumption of caffeine, a non-specific adenosine A_1/A_{2A} receptor antagonist, was also associated with a reduced risk of developing PD (Ascherio *et al*., 2001; Schwarzschild *et al*., 2003). Moreover, it was reported that caffeine ameliorates the loss of striatal dopamine induced by acute MPTP administration in mice (Chen *et al*., 2001) and conferred neuroprotection in a model of unilaterally intrastriatal 6-OHDA-lesioned rats (Joghataie *et al*., 2004). Other studies with more selective adenosine receptor ligands highlighted the major neuroprotectant role for the blockade of A_{2A}Rs (see Cunha 2005; Schwarzschild *et al*., 2006). Genetic deletion of A_{2A}Rs prevented striatal dopamine depletion in mice intraperitoneally injected with MPTP (Chen *et al*., 2001) and A_{2A}R antagonists were shown to be neuroprotective in a PD rat model where 6-OHDA was infused inside the striatum (Ikeda *et al*., 2002). The A_{2A}R antagonist ZM241385 was shown to protect PC12 cells against the dopaminergic neurotoxins 1-methyl-4-phenylpyridinium (MPP+) and methamphetamine (Scatena *et al*., 2011) and to inhibit oxidative stress in the striatum of rats with nigrostriatal neurons damaged by 6-OHDA (Gołembiowska and Dziubina, 2012). Also, the use of the selective A_{2A}R antagonist SCH58261 potentiated rotational behavior produced by levodopa in 6-OHDA-lesioned rats (Fenu *et al*., 1997) and prevented the death of nigral dopaminergic neurons caused by sub-chronic MPTP administration in mice (Carta *et al*., 2009). In non-human primates intraperitoneally injected with MPTP it was shown that the administration of another selective A_{2A}R antagonist, istradefylline (KW-6002), also increased locomotor activity (Kanda *et al*., 1998), and the co-administration of KW-6002 with a threshold dose of levodopa enhanced the motor response of animals without increasing dyskinesias (Kanda *et al*., 2000), which are a side-effect caused by levodopa treatment. Therefore, the blockade of the A_{2A}Rs appears to provide a functional protection of striatal dopamine transmission, and also prevent the loss of dopaminergic pathway neurons caused by neurotoxins (*e.g.* MPTP and 6-OHDA).

2. Aim

2. AIM

The main objective of this work was to detail the possible functional interaction between nAChRs and A_{2A}Rs in the striatum.

To accomplish this goal the following aims were addressed:

- Investigate the synaptic and subsynaptic distribution of the α7, α6, α4 and β2 subunits of nAChRs in the striatum of rodents.

- Characterize *in vitro* the functional interaction between nAChRs and A_{2A}Rs in the control of dopamine release in striatal nerve terminals.

- Assess the *in vivo* impact of the interaction between nAChRs and A_{2A}Rs by analyzing the locomotor activity of rats.

3. Material and Methods

3.1 BIOLOGICAL MATERIAL AND PREPARATIONS

All studies were conducted in accordance with the principles and procedures outlined as “Replacement, Refinement and Reduction of Animals in Research” (3Rs) in the guidelines of EU (86/609/EEC), FELASA, the National Centre for the 3Rs (the ARRIVE; Kilkenny *et al.,* 2010), and the Animal Care Committee of the Center for Neuroscience and Cell Biology of Coimbra. Moreover, we also applied the principles of the ARRIVE guideline for the design and the execution of *in vitro* pharmacological experiments (see below) as well as for data management and interpretation, according to McGrath *et al*., 2010. It should be referred that different tissues from the animals employed in our study were used in other on-going projects at our research centers.

3.1.1 Animals

The animals used in the experiments presented in this thesis were male Wistar rats (10-14 weeks old) (Fig. 3.1) and male C57BL/6 mice (10-12 weeks old) (Fig. 3.2) obtained from Charles River (Barcelona, Spain). The animals were housed under controlled temperature (23±2 ºC), subject to a fixed 12 h light/dark cycle, with free access to food and water. All efforts were made to reduce the number of animals used and to minimize their stress and discomfort.

Figure 3.1 – *Wistar rat*

Figure 3.2 – *C57BL/6 mouse*

The animals used to perform the *in vitro* studies were deeply anesthetized with halothane (no reaction to handling or tail pinch, while still breathing) before decapitation with a guillotine.

3.1.2 Synaptosomal preparations

Isolated nerve terminals, termed synaptosomes (Whittaker *et al*., 1964; Whittaker 1993) (Fig. 3.3), represent an excellent standard tool to study presynaptic processes free of polysynaptic and glial influences (for review see Raiteri and Raiteri, 2000). Synaptosomes are formed when brain tissue is homogenized in an isotonic solution of appropriate viscosity and using suitable shear force. The nerve terminals, or synaptic boutons, are this way detached from their axons and from the postsynaptic terminals and glial cells to which they were connected (Dunkley *et al*., 2008). Their membranes then reseal, forming a closed vesicular structure, ranging in size between 0.5-2 µm of diameter (Dunkley *et al.,* 1988) that maintains the nerve terminal contents, including functional ion channels, carriers and receptors, synaptic vesicles, cytoplasm, mitochondria and cytoskeleton together with their functionality (Bai and Witzmann, 2007).

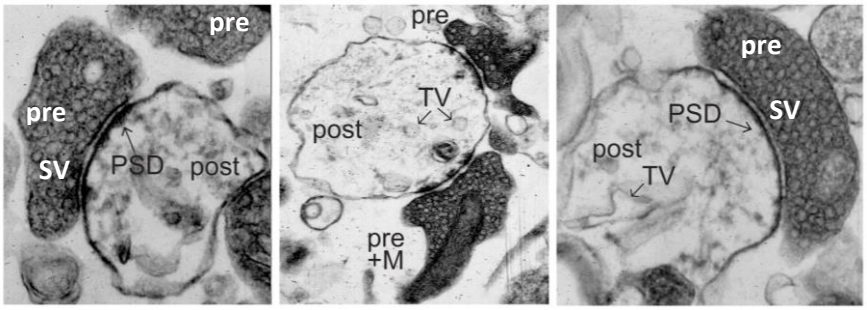


Figure 3.3 – High resolution views of intact, sealed synaptosomes (pre), containing synaptic vesicles (SV) and mitochondria (pre+M). Adjacent postsynaptic elements (post) containing electron dense elements (PSD) are also visible, along with tubular and vesicular structures (TV) (taken from Corera *et al.,* 2009).

Synaptosomal preparations thus retain Ca^{2+}-buffering capacity and physiological plasma membrane potential (-60 to -70 mV), which is regulated by a Na^{+}/K^{+}-ATPase. The application of depolarizing stimuli (*e.g.*, potassium or 4-aminopyridine) leads to the entry of Ca^{2+} into the synaptosomes through voltage-operated Ca^{2+} channels (VOCCs) and triggers exocytosis of docked synaptic vesicles, releasing neurotransmitters into the extracellular medium

(Breukel *et al*., 1997). Similarly, nAChRs agonists such as nicotine are also able to induce neurotransmitter release from synaptosomes by increasing cytosolic Ca^{2+}, both directly (*e.g*. through the α7 nAChR channel) and indirectly by recruiting VOCCs activated by the depolarization caused by Na^{+} influx through predominantly non-α7 nAChR subtypes (Dickinson *et al.*, 2008). Synaptosomal preparations are viable and retain their function for several hours if maintained at low temperatures (0-4 ºC), supplemented with glucose.

Frequently, a part of the postsynaptic membrane and its attached postsynaptic density will remain attached to the presynaptic nerve terminal (see Figure 3.3). However, the use of sucrose or Percoll density-gradient centrifugation techniques allows the obtention of preparations enriched in presynaptic nerve terminals. Synaptosomes are therefore an excellent preparation to study expression of neuronal synaptic proteins and investigate synaptic functions such as neurotransmitter release (Whittaker 1993).

3.1.2.1 Partially purified synaptosomes (P2 fraction)

The animals were decapitated and their brains quickly removed into ice-cold sucrose solution (0.32 M, containing 5 mM HEPES, pH 7.4). The caudate-putamen region of the striata, without the nucleus accumbens (from now simply designated as striatum) were quickly dissected out into 2 ml ice-cold sucrose solution, homogenized with a Teflon homogenizer, and centrifuged at 5,000 *g* for 5 min. The supernatant was centrifuged at 13,000 *g* for 10 min to obtain the P2 synaptosomal fraction (Ferreira *et al*., 2009).

3.1.2.2 Synaptosomes purified by a 45% percoll gradient

Rodent striatal nerve terminals were prepared using a combined sucrose/Percoll centrifugation protocol as previously described (Rebola *et al.*, 2005). Briefly, the two striata from one animal were homogenized with a Teflon homogenizer in an ice-cold sucrose-HEPES medium containing 0.32 M sucrose, 1 mM EDTA, 0.1% BSA and 10 mM HEPES (pH 7.4). The homogenate was spun at 3,000 *g* for 10 min at 4ºC and the supernatant spun

again at 14,000 *g* for 12 min. The pellet (P2 fraction) was resuspended in 1 ml of Percoll 45% (v/v) made up in Krebs-HEPES-Ringer (KHR) medium (in mM: NaCl 140, EDTA 1, KCl 5, glucose 5 and HEPES 10, pH 7.4) and spun again at 14,000 *g* for 2 min. Synaptosomes were then removed from the top layer and washed once with KHR medium at 14,000 *g* for 2 min. The synaptosomal pellet obtained was solubilized in 5% SDS supplemented with 100 µM PMSF, 2 mM DTT and a protease inhibitor cocktail (containing 1 µg/ml of leupeptin, pepstatin A, chymostatin, and antipain). The protein concentration was then determined using the bicinchoninic acid (BCA) protein assay reagent.

3.1.2.3 Subsynaptic fractionation of nerve terminals

The presynaptic active zone, postsynaptic and extrasynaptic fractions from rodent striatal synaptosomes were separated according to the method previously described by Phillips *et al*., 2001, with some modifications introduced by our group (Rebola *et al.*, 2005) (Fig. 3.4). It was observed that this subsynaptic fractionation method allows an over 90% effective separation of the presynaptic active zone (enriched in SNAP-25 and syntaxin), postsynaptic density (enriched in PSD95) and the non-active fraction or extrasynaptic zone (synaptophysin). By determining the levels of these synaptic proteins (SNAP 25, syntaxin, PSD95 and synaptophysin) it is possible to estimate the relative purity of the different isolated subsynaptic fractions (Rebola *et al*., 2005). These subsynaptic fractions can be useful to assess the subsynaptic

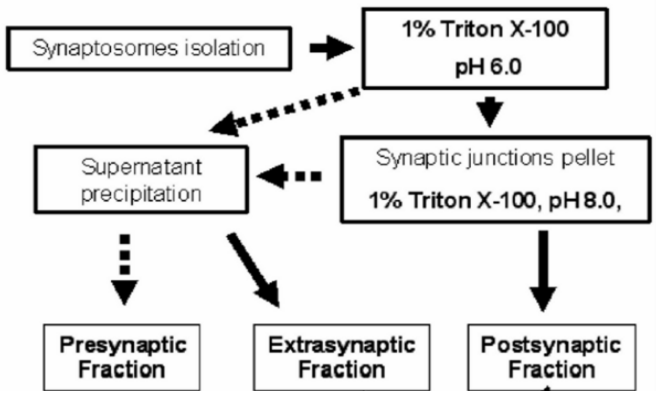


Figure 3.4 – Schematic representation of the procedure for subcellular fractionation of nerve terminals. Isolated synaptosomes are first solubilized with Triton at pH 6.0, separating the soluble fraction (Extrasynaptic Fraction) from the synaptic junctions (insoluble). This pellet is then solubilized with Triton at pH 8.0 which dissociates the presynaptic active zone (Presynaptic Fraction) from the postsynaptic density (Postsynaptic Fraction) (taken from Rodrigues *et al.,* 2005).

distribution of receptors by Western blot analysis (see Rebola *et al*., 2005; Rodrigues *et al.*, 2005).

Briefly, striata from 5-8 rats or 10-12 mice were homogenized at 4°C in 2.5 ml of isolation solution [0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. The concentration of sucrose was raised to 1.25 M by addition of 12 ml of 2 M sucrose and 5 ml of 0.1 mM $CaCl_2$ and the suspension divided into 2 ultracentrifuge tubes. The homogenate was overlaid with 2.5 ml of 1.0 M sucrose and 0.1 mM $CaCl_2$, and the volume of the tubes adjusted with isolation solution and centrifuged at 100,000 *g* for 3 h at 4°C. Synaptosomes were collected at the 1.25/1.0 M sucrose interface, diluted 1:10 in cold 0.32 M sucrose with 0.1 mM $CaCl_2$ and pelleted (15,000 *g* for 30 min at 4°C). Pellets were resuspended in 1 ml of isolation solution and a small sample taken for gel electrophoresis, and this synaptosomal suspension was then diluted 1:10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2× concentrated solubilization buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The synaptosomal membranes were then incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junctions) pelleted (40,000 *g* for 30 min at 4°C). The supernatant (extrasynaptic fraction) was decanted and proteins precipitated with 6 volumes of acetone at -20°C and recovered by centrifugation (18,000 *g* for 30 min at -15°C). The synaptic junctions pellet was washed twice in pH 6.0 solubilization buffer, resuspended in 5 ml of 1% Triton X-100 and 20 mM Tris (pH 8.0), incubated for 30 min on ice with mild agitation, centrifuged (40,000 *g* for 30 min at 4°C) and the supernatant (presynaptic active zone fraction) processed as above. The increase in pH from 6.0 to 8.0 leads to the dissociation of the extracellular web filaments that strongly connect the presynaptic active zone to the postsynaptic density. In this way the active zone is solubilized while the postsynaptic density is mostly maintained, because the amount of detergent is not sufficient to achieve its solubilization (see Phillips *et al*., 2001). PMSF (1 mM) was added to the suspension in all extraction steps to minimize proteolysis. The pellets from the supernatants and the final insoluble pellet (postsynaptic density fraction) were solubilized in 5% SDS, the protein concentration determined by the BCA method.

3.2 NEUROCHEMICAL ANALYSIS

3.2.1 Protein quantification by the bicinchoninic acid (BCA) method

The quantification of protein in the biological samples was performed by using the bicinchoninic acid (BCA) method, a colorimetric detergent-compatible procedure. In brief, a standard curve was first prepared by diluting BSA with milliQ water to several concentrations: 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 µg/µl. The samples were diluted 1/10 in milliQ water and the buffer previously used to lyse the samples was also diluted 1/10. The standard curve was then prepared in a 96-multiwell plate by pipetting 25 µl of each concentration of the diluted BSA, in triplicate, followed by 25 µl of the diluted lysis buffer in each of the wells. The diluted samples in triplicate were prepared in the same way, but 25 µl of milliQ water were added to each well instead of the diluted lysis buffer. Next, the reagent A (containing bicinchoninic acid and tartrate in an alkaline carbonate buffer) was mixed with the reagent B (containing 4% copper sulfate pentahydrate solution) of the kit in a 50:1 proportion, for 1 min, protected from light, thus forming the BCA reagent. After the mixing, 200 µl of the BCA reagent were added to each well, the plate was protected from light and placed for 30 minutes in an incubator at 37ºC. Lastly, the plate was cooled to RT and the protein read at 570 nm in a plate reader spectrophotometer.

3.2.2 Western blot

The samples for Western blot analysis were prepared with SDS-PAGE sample buffer composed of a 4x Tris.Cl/SDS solution (0.5 M Tris and 0.4% SDS, pH 6.8 corrected with HCl and filtered with 0.45 µM pore filters), 30% (v/v) glycerol, 0.6 M dithiothreitol (DTT), 10% (w/v) SDS and 0.012% of 3',3",5',5"-tetrabromophenolsulfonphthalein (bromophenol blue), and the volume adjusted with milliQ water to normalize for a maximum of 2 µg/µl. The SDS-PAGE sample buffer increased the denaturation of the protein in the samples to a primary conformation, assuring a near uniform negative charge and also increased the sample density, facilitating gel loading and preventing convective migration out of the sample wells. The samples were denaturated by boiling at

95ºC for 5 min and separated by SDS-PAGE electrophoresis, using 10% polyacrylamide resolving gels and 4% polyacrylamide concentrating (stacking) gels (see Table 1), under reducing conditions at 80-120 mV. Prestained precision protein standards were run simultaneously with the samples to help identify the proteins of interest. The proteins in the gel were then electrophoretically transferred (1A current, for 1.5 hours at 4ºC with constant agitation) to previously activated polyvinylidene difluoride (PVDF) membranes (0.45 µm). After blocking for 1 h at RT with 5% bovine serum albumin (BSA) in Tris-buffered saline (Tris 20 mM, NaCl 140 mM, pH 7.6) containing 0.1% Tween 20 (TBS-T), to prevent nonspecific binding, the membranes were incubated overnight at 4°C with the primary antibody diluted in TBS-T with 1% BSA (the antibodies and dilutions used are described in Table 2). After three washing periods of 15 min with TBS-T, the membranes were incubated with the appropriate alkaline phosphatase-tagged secondary antibody (dilution 1:20,000 or 1:3000 - see table 2) diluted in TBS-T containing 1% BSA, for 2 h at RT. After three 15-min washes in TBS-T, the membranes were incubated with Enhanced Chemi-Fluorescence (ECF) substrate and visualized in a VersaDoc 3000 imaging system (Biorad, Amadora, Portugal) with the assistance of Quantity one software. When applicable the membranes were then re-probed and tested for β-actin immunoreactivity to confirm that similar amounts of protein were applied to the gels.

TABLE 1: Gel formulations in Western blot

GEL Formulation (1 GEL)	4 %	10 %
Tris-buffer, 1.5 M, pH 8.8 (resolving gel)	- - - - - - - -	2.5 ml
Tris-buffer, 0.5 M, pH 6.8 (stacking gel)	2.5 ml	- - - - - - - -
Acrylamide 30 %	1.3 ml	3.3 ml
Ultrapure water	6.1 ml	4.1 ml
SDS 10 %	100 µl	100 µl
TEMED	10 µl	5 µl
APS 10 % (freshly prepared in water)	50 µl	50 µl

3.2.3 [^{3}H]Dopamine release assays from rat striatal nerve terminals

The experiments were carried out as previously reported (Ferreira *et al.*, 2009; Martíre *et al.*, 2011). The P2 synaptosomal fraction was diluted to 0.5 mL with Krebs-HEPES solution (in mM: NaCl 113, KCl 3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 10, HEPES 15, pH 7.4, 37ºC), containing the monoamine oxidase B (MAO-B) inhibitor, pargyline (10 µM). Synaptosomes were then incubated for 10 min in the presence of [^{3}H]dopamine (~60 Ci/mmol, final concentration, 150 nM). A 16-microvolume chamber superfusion setup was filled with the preloaded synaptosomes (see Fig. 3.5), which were trapped by layers of Whatman GF/C filters and superfused continuously at a rate of 0.8 mL/min at 37ºC until the end of the experiment. This system allows testing seven pharmacological treatments in duplicate (each averaged as n=1) to reduce the number of animals utilized, in accordance with the ARRIVE guidelines. After a 10-min washout period, nine 2-min samples were collected for liquid scintillation assay.

The radioactivity content of each sample and of the filters with the trapped synaptosomes was counted by a Tricarb β-counter (PerkinElmer, Waltham, Massachusetts, USA). Disintegrations per minute (DPM) values were expressed as fractional release (FR%), *i.e.* the percent of actual content in the effluent as a function of the total synaptosomal content of radioactivity.

After collecting four 2-min samples as a baseline, nicotine and adenosine receptor ligands, alone or in combination, were applied through the superfusion solution. nAChR antagonists were present since the beginning of the 10-min washout period, until the end of the experiment.

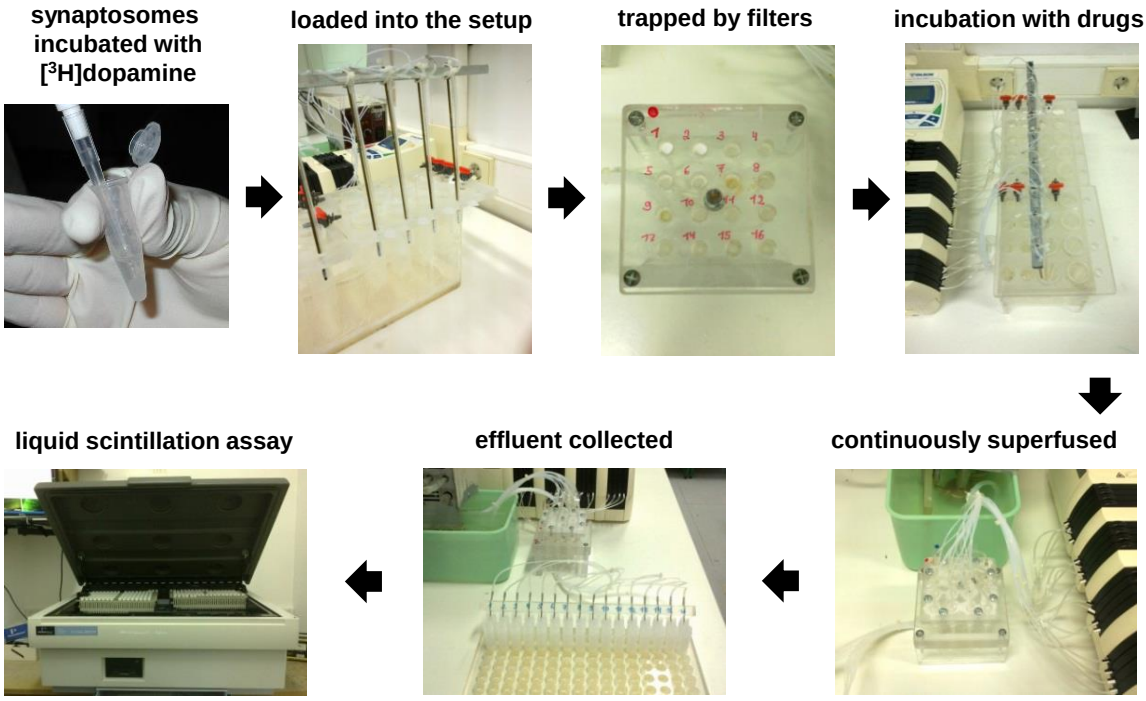


Figure 3.5 - **Images of the superfusion setup illustrating steps in the [^{3}H]dopamine release assays from rat striatal nerve terminals (synaptosomes).** Synaptosomes were incubated for 10 min at 37ºC in the presence of [^{3}H]dopamine, after which they were trapped by layers of Whatman GF/C filters, in microvolume chambers, and superfused continuously at a rate of 0.8 mL/min at 37ºC until the end of the experiment. 2-min samples were collected for liquid scintillation assay.

3.2.3.1 Calculations and statistics for [^{3}H]dopamine release assays

In each individual experiment various treatments were performed, including paired vehicle controls and various pairs of treatments with ligands alone or in combination. To obtain the net effect of each tested drug on the amount of [^{3}H]dopamine released, the averaged duplicates of the respective controls (not shown) were subtracted from the averaged treatment. Application of this principle originates the graphs (A-D) pictured in Figure 3.6. In this simulated case, where nicotine and an adenosine receptor ligand such as CGS21680 are represented to be tested separated and together, the release elicited by nicotine alone (corresponding to the area under the curve) (graph A) amounts to “X” fractional release % (FR%) and in the same way for CGS21680 the value “Y” FR% is obtained (graph B). The combination of

nicotine+CGS21680 yields a total value of area under the curve of "V" FR% (graph C). Relevantly, the "V" value is understood as the sum of "Y"+"Z" (graph D), where "Z" is the modified effect of nicotine, after discounting the effect of CGS21680 *per se* (area "Y" in graph D). In the [^{3}H]dopamine release essays presented in this thesis, the data corresponding in Figure 3.6 to "X", "Y" and "V" were used for the following comparisons: "X", "Y" and "V" were compared to zero FR% (*i.e* no effect on dopamine release) and if statistical difference was detected the graphs were marked with *, ** or ***, depending on the value of statistical significance. Furthermore, "X" was also compared to "Z" to assess whether CGS21680 (or any other adenosinergic ligand alone or in combination) altered the effect of nicotine. In these cases, if statistical difference was detected the graphs were marked with $, $$ or $$$.

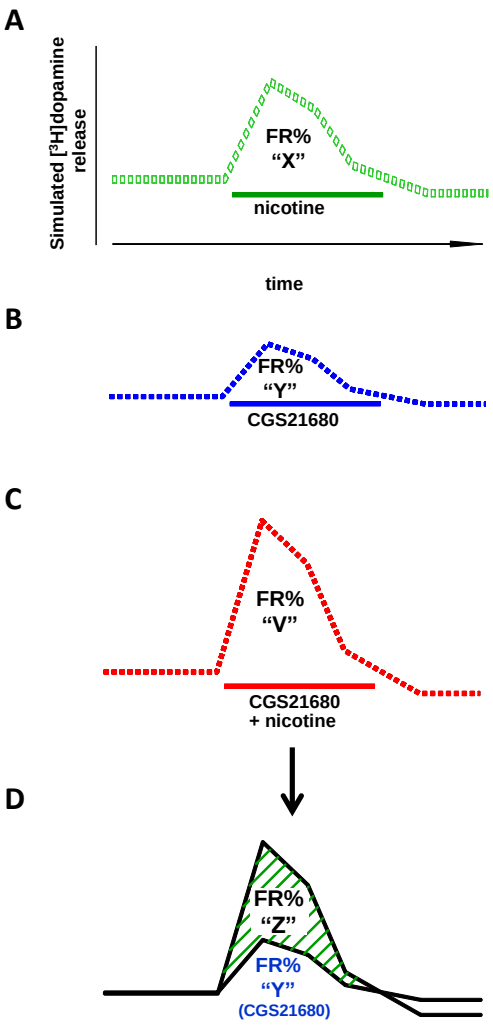


Figure 3.6 - **An explanatory figure for the calculations of [^{3}H]dopamine release assays**. In this scenario, modelled release curves are displayed for a hypothetical drug effect alone, *e.g*. nicotine (A) or CGS21680 (B) and in combination (*e.g*. CGS21680+nicotine) (C). For instance, if nicotine (A) and an adenosine receptor ligand such as CGS21680 (B) are tested separated and together (C), the release from untreated synaptosomes (vehicle control) is subtracted from the release in the presence of CGS21680 alone and of nicotine alone, and the release in the presence of CGS21680 alone is also subtracted from the release in the combined presence of CGS21680 plus nicotine (C and D). "X" represents the release induced by nicotine; "Y" represents the release induced by CGS21680 *per se*; "V" represents the release induced by nicotine+CGS21680 ("Y"+"Z") where "Z" is the release corresponding to the modified effect of nicotine, after discounting the effect of CGS21680 *per se* ("Y").

3.2.4 Adenosine release from rat striatal synaptosomes

Adenosine release was assayed both in batch-like conditions as well as upon superfusion of synaptosomes. In batch-like conditions, striatal synaptosomes (~1.2 mg protein × mL^{-1}) were incubated at 37ºC for 15 min in the presence of the adenosine deaminase inhibitor, EHNA (20 µM). Half of the synaptosomal samples were challenged with nicotine (1 µM) for 8 min at 37ºC and the other half served as control. The mixtures were then centrifuged, at 14000 *g* for 10 min at 4ºC, and the supernatant was used for high-performance liquid chromatograph (HPLC) analysis (see below), in duplicate. In superfusion conditions, the synaptosomes were superfused for 15 min in a manner similar to the [^{3}H]dopamine release assay. The synaptosomes were then exposed during 5 min to EHNA (20 µM) or EHNA combined with nicotine (30 nM or 1 µM), and the effluents were collected for HPLC analysis.

The separation and quantification of adenosine and its metabolites was carried out by HPLC, as previously described (Cunha and Sebastião, 1993), employing a LiChroCart-RT 125-4 C-18 reverse-phase column (particle size, 5 µm), combined with an UV detector set to 254 nm. The mobile phase consisted of KH_2PO_4 (100 mM) and methanol (85/15 v/v%) at pH 6.50, with the flow rate of 1 mL × min^{-1}, and a loop volume of 50 µL. The identification and quantification of adenosine and its metabolites was achieved by calculating the peak areas then converted to concentration values (expressed as µmol × mg $protein^{-1}$) by calibration with known adenosine standards ranging from 0.1 to 10 µM.

3.3 RAT LOCOMOTOR BEHAVIOURAL ANALYSIS: NICOTINE SENSITIZATION

During behaviour test periods adult male Wistar rats (10-14 weeks old) were maintained in a room under controlled temperature (23±2 ºC) subject to a 12 h light/dark cycle (lights on 7.00 a.m.) with free access to food and water. All manipulations were carried out between 9:00 a.m. and 6:00 p.m. to avoid influences from circadian rhythms. The behavioural test was performed in a sound-attenuated room with low-intensity light maintained constant during the

testing period. During the tests, the experimenter stayed in a room adjacent to the one where the test was performed. To remove the odor traces left by the previous animal the floor and walls of the equipment were cleaned with 10 % ethanol before testing the next animal.

3.3.1 Open field test

The open field is a well-established test to measure locomotor activity in rodents, among other behavioural responses (Walsh and Cummins, 1976; Prut and Belzung, 2003).

Locomotor behaviour was monitored in a square open field arena, with 100X100 cm and 60 cm height, made of dark grey PVC plastic (Fig. 3.7). The locomotor activity of the rats was evaluated by measuring the total distance traveled over a period of 30 min. Analysis of the data was performed using the Any-maze video tracking software.

Figure 3.7 – Image of the open-field arena during the behavioural test. A Wistar rat is present in the apparatus.

3.3.2 Drug administration

The nicotine solution was prepared fresh each day by dissolving nicotine bitartrate in an isotonic saline solution (0.9% NaCl) neutralized to pH 7.2. ZM241385 was dissolved in a saline solution with 5% DMSO. Nicotine (0.5 mg/kg, as nicotine-tartrate salt) or saline were injected subcutaneously (s.c.) in a volume of 1 mL/kg of body weight immediately before the test period, whereas ZM241385 (1 mg/kg) or vehicle solution were administered intraperitoneally (i.p.) 30 min before the test period. The dose of each drug was chosen based on previous studies in rats showing the induction of a robust and long-lasting locomotor sensitization by nicotine (Schoffelmeer *et al.*, 2002; Le Foll *et al.*,

2003; Kayir *et al.*, 2009) and the efficient antagonism of A_{2A}Rs by ZM241385 (Poucher *et al.*, 1996; Prediger and Takahashi, 2005; Montandon *et al.*, 2008), at the current doses used. Non-water soluble materials were dissolved in DMSO, and further diluted in H_2O, aliquoted and kept at -20ºC until use.

3.3.3 Experimental procedures

The nicotine-induced locomotor sensitization was assessed based on previous reports (Werling *et al*., 2009; Wellman *et al*., 2011). For this assay, the rats were randomly divided into four experimental groups. On the first two days, all the rats were adapted to the open field arena for 30 min each day (habituation). For the next eight consecutive days, all rats received two daily injections before the test period. ZM241385 or its vehicle (0.9% saline plus 5% DMSO), was injected 30 min before the test, while nicotine or its vehicle (0.9% saline) 30 min later, immediately before the behavioural test. The rats were grouped based on the following injection scheme: vehicle-vehicle (n=3), vehicle-nicotine (n=4), ZM241385-vehicle (n=4) and ZM241385-nicotine (n=5).

Following the 8 day-test period, the rats from all groups remained drug-free for seven consecutive days. On drug-free day 8, all rats were injected with nicotine (0.5 mg/kg) and immediately placed in the open field arena (challenge day) (see Fig. 3.8).

It should be referred that in Figure 4.14 (Results section) the same set of symbols (* and $) is used on the graph corresponding to the locomotor activity assay, instead of dopamine release.

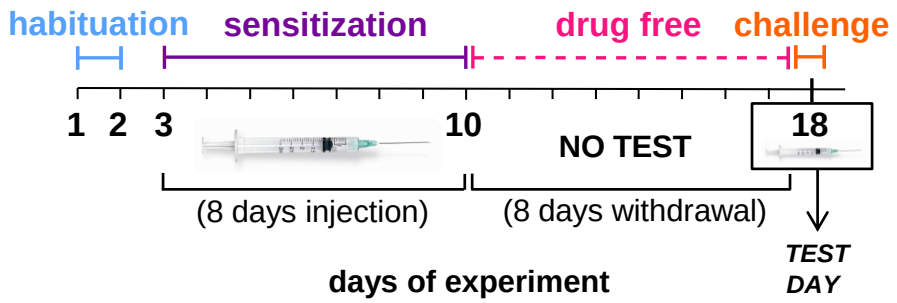


Figure 3.8 - **Timeline of the experimental procedure: nicotine sensitization.** The rats were first adapted to the open field arena in the first two days (habituation) and then daily injected and tested for the next eight consecutive days (sensitization). After the eight day test period the rats received no treatment and no test was performed, for the next eight days (drug free). On the eighth day of withdrawal (day 18 of experiment) all the rats were injected with nicotine and tested in the open field (challenge).

3.4 MATERIALS

3.4.1. Chemicals

Phenylmethanesulfonyl fluoride **(PMSF)**, DL-dithiothreitol **(DTT)**, protease inhibitor cocktail (leupeptin, pepstatin A, chymostatin and antipain), 2-bromo-2-chloro-1,1,1-trifluoroethane **(halothane)**, pargyline, bovine serum albumin **(BSA)**, (-)-nicotine hydrogen tartrate salt and Whatman GF/C filters were obtained from Sigma (Sintra, Portugal). Sodium dodecyl sulphate **(SDS)** and the Quantity one software were from Bio-Rad (Amadora, Portugal). Polyvinylidene difluoride **(PVDF)** membranes, prestained precision protein standards and enhanced chemifluorescence substrate **(ECF)** were purchased from Amersham Biosciences (Amadora, Portugal). Bicinchoninic acid **(BCA)** protein assay reagents were from Thermo Scientific (Pierce Biotechnology, Rockford, IL, USA). 3,4-[ring-2,5,6-^{3}H] dihydroxyphenylethylamine **([^{3}H]DA)** was from American Radiolabeled Chemicals, Inc, (St. Louis, MO, USA). α-bungarotoxin, α-conotoxin-PIA, dihydro-β-erythroidine, 2-[*p*-(2-carboxyethyl)phenethylamino]-5´-*N*-ethylcarboxamido adenosine **(CGS21680)**, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride **(EHNA)** and N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide **(MRS1754)**

were from Tocris Bioscience (Bristol, UK) and 4-(2-[7-amino-2-(2-furyl)]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol **(ZM241385)** was from Abcam Biochemicals (Cambridge, UK). Any-maze was from Stoelting (IL, USA). Other general reagents, such as salts, were obtained from Sigma (Sintra, Portugal).

3.4.2 Antibodies

All the primary and secondary antibodies used during the studies presented are described in table 2.

Table 2: Primary and secondary antibodies for Western blot

Antibodies	Supplier	Host	Type	Dilution
α7	Abcam	rat	IgG1	1:3000
α4	Abcam	rabbit	IgG	1:3000
α6	Merck Millipore	rabbit	N/A	1:3000
	Abcam	rabbit	IgG	1:600
β2	Merck Millipore	rabbit	N/A	1:15000
SNAP 25	Sigma	mouse	IgG1	1:60000
Synaptophysin	Sigma	rabbit	IgG1	1:60000
Syntaxin	Sigma	mouse	IgG1	1:60000
PSD 95	Sigma	mouse	IgG2a	1:100000
β-Actin	Sigma	mouse	IgG2a	1:100000
Rabbit alkaline phosphatase conjugate (AP)	GE Healthcare	goat	IgG (H+L)	1:20000
Mouse AP	GE Healthcare	goat	IgG+IgM (H+L)	1:20000
Rat AP	Santa Cruz	chicken	IgG	1:3000

3.5 DATA PRESENTATION

All data are expressed as means ± SEM of the indicated number of independent observations (n). Raw effect data from release and sensitization experiments were normalized to the respective vehicle control except when noted. Normalized data were tested for normality by the Kolmogorov-Smirnov normality tests and statistical significance was calculated by one-sample *t*-test against a hypothetical value of 100 or 0 (when normalized). If more than two groups were compared, one-way ANOVA with Dunett's *post-hoc* test was performed. Data from paired experiments were compared with the pairwise version of the above mentioned tests, and a value of $p < 0.05$ was accepted as a significant difference.

4. Results

4.1 SYNAPTIC AND SUBSYNAPTIC LOCALIZATION OF NICOTINIC RECEPTOR SUBUNITS IN RODENT STRIATUM

It has become increasingly clear that one of the main functions that nicotinic acetylcholine receptors (nAChRs) play in the striatum is the modulation of neurotransmitter release. The activation of presynaptic striatal nAChRs was clearly shown to be able to induce the release of dopamine, among other neurotransmitters, in rats and mice (Wonnacott *et al*., 2000; Salminen *et al*., 2004; Exley and Cragg, 2008). However, it is still not clearly known if particular nAChR subtypes are predominant in the nerve terminals, and if so, what is their relative distribution at different subsynaptic sites, such as the pre- post- and extra-synaptic zones (see Fig. 4.1). The present study aimed to determine if some relevant subunits of nAChRs, such as α7, α4, α6 and β2, were present in the terminals of striatal neurons and describe the localization and density of these subunits at the subsynaptic level. To address these objectives we used striatal synaptic and sub-synaptic fractions obtained from rodents and gauged the density of nAChR subunits by immunoblot using commercially available antibodies against these proteins. It was also our goal to asses if the distribution of the nAChR subunits is similar in Wistar rats and C57BL/6 mice, two animal models extensively used in neuroscience research (see Takahashi *et al.,* 2008; Blandini and Armentero, 2012). This detailed mapping of the nAChR subunits provided insights about the putative involvement of the different nAChR subtypes in the release of neurotransmitters, in particular of dopamine, and also in postsynaptic signal reception in the striatum (Windels and Kiyatkin, 2003; Hamada *et al*., 2004). Apart from its intrinsic general relevance this data helped set the stage for the subsequent work exploring the interaction between nAChRs and adenosine A_{2A}Rs.

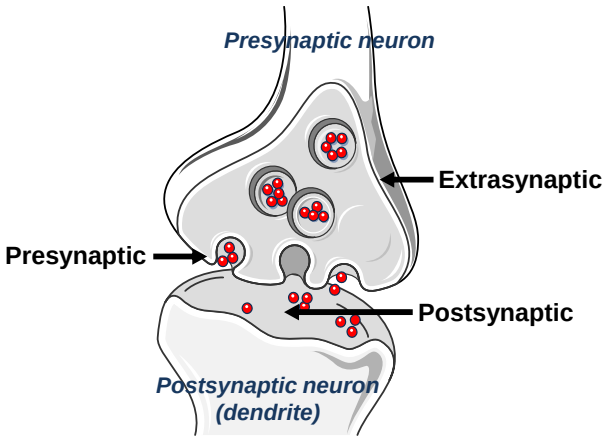


Figure 4.1 – Schematic representation of a synapse detailing the localization of nAChRs in the neuronal membrane, at presynaptic, postsynaptic and extrasynaptic sites.

4.1.1 Synaptic and subsynaptic levels of nAChRs in the striatum of Wistar rats and C57BL/6 mice

The synaptic and subsynaptic levels of α7, α6, α4 and β2 subunits of nAChRs were evaluated in the striatum of male adult Wistar rats and C57BL/6 mice. The synaptic densities of these subunits were determined in synaptosomes (membranes from resealed nerve terminals) by Western blot, in which were applied three different concentrations of protein of synaptosomes to ensure that the signal was not saturated, obtained from the same preparation. We confirmed that the synaptosomal membrane preparations indeed corresponded to isolated nerve terminals by analyzing the immunoreactivity of synaptophysin, a synaptic protein (not shown). To investigate the subsynaptic distribution of the nAChR subunits we employed a fractionation method that achieves an efficient separation of the pre-, post- and extra-synaptic regions. The immunoreactivity of different subunits was evaluated by Western blot in the fractions enriched in the presynaptic active zone (PRE), postsynaptic density (POST) and areas outside the active zone (EXTRA) and in the initial synaptosomal fraction (SYN) (Fig. 4.2). This fractionation technique facilitates the access of the antibodies to epitopes located in the synapse, that otherwise could be prevented by the proteic matrix that keeps the synapse together (see Phillips *et al*., 2001). It also has the possible advantage of concentrating in the samples receptors which have low abundance. The validation of the subsynaptic fractionation procedures was performed by quantifying the immunoreactivity of specific protein synaptic markers: i) SNAP-25 and syntaxin in the presynaptic active zone fraction, ii) PSD-95 in the postsynaptic density fraction and iii) synaptophysin (a synaptic vesicles protein) in the extrasynaptic fraction. As can be seen in Figure 4.2 A and B, the presynaptic fraction displayed higher levels of SNAP-25 and syntaxin as compared with the post- or extra-synaptic fractions, whereas the postsynaptic fraction exhibited higher levels of PSD-95, being this protein almost undetectable in the other subsynaptic fractions. The density of synaptophysin was higher in extrasynaptic than in presynaptic fractions, and it was almost inexistent in the postsynaptic density. The labeling of synaptophysin observed in the presynaptic fraction is possibly due, at least partially, to the presence of docked synaptic vesicles

(Pinheiro *et al*., 2003). These data allow assessing the efficiency of separation of the subsynaptic fractions used in this study.

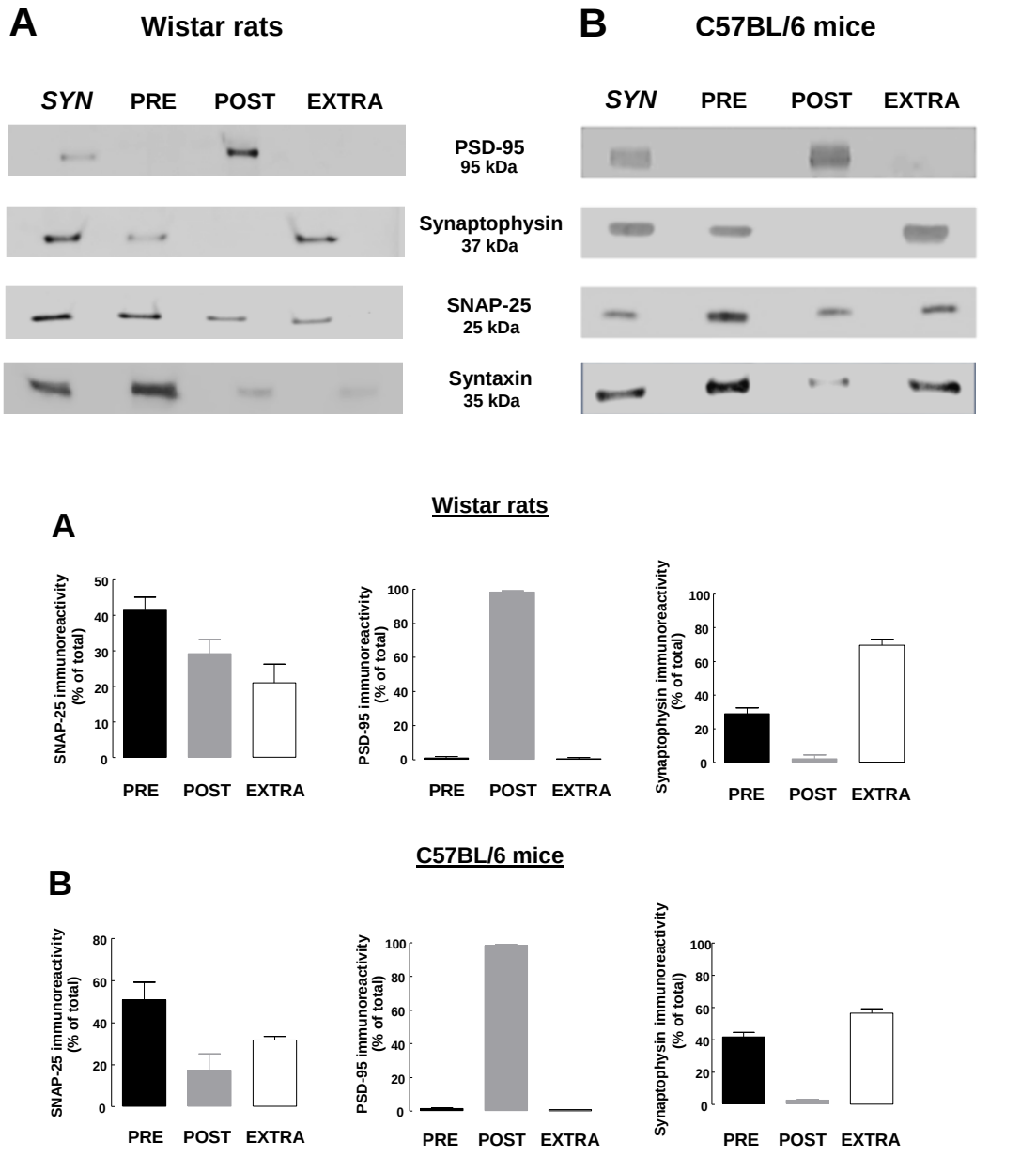


Figure 4.2 – Assessment of the efficiency of separation of the PRE-, POST- and EXTRA-synaptic fractions of rat and mouse striatum nerve terminals. The purity of the fractions enriched in the presynaptic active zone (PRE), postsynaptic density (POST) and areas outside the active zone (EXTRA) and in the initial synaptosomal fraction (SYN) was gauged in Wistar rats **(A)** and C57BL/6 mice **(B)** by the segregation of the active zone markers (SNAP-25 and syntaxin), postsynaptic density marker (PSD-95) and of the marker exterior (vesicular) to the active zone (synaptophysin). Representative Western blot images displaying the subsynaptic densities of the markers are shown. The analysis of each marker was performed in different blot gels with samples from each of the fractions from the same fractionation using 10 µg of each fraction per gel. The percentage of immunoreactivity was obtained taking as reference the total value of reactivity in each separate experiment. Graphs exhibit the mean ± SEM of 3-4 experiments derived from separate fractionations.

4.1.1.1 nAChRs α7 subunit synaptic and subsynaptic levels

By Western blot analysis it was found that the α7 subunit of nAChRs was localized in Wistar rats and in C57BL/6 mice striatal synaptic membranes, as displayed by the specific bands of three different concentrations of protein. The antibody recognized bands of identical molecular weight (63 kDa), in rats (n = 3) and mice (n = 4). Immunoreactivity increased with higher amounts of loaded protein, assuring the signal was not saturated (Fig. 4.3 A and B).

As showed in Figure 4.3 C, in rat striatum the α7 subunit was localized mainly at the presynaptic active zone (51.5 ± 4.4% of total immunoreactivity, n = 3) and was also present in extrasynaptic locations, outside the active zone (36.7 ± 3.3% of total immunoreactivity, n = 3), but it had a much smaller presence in the postsynaptic density (11.8 ± 2.7 % of total immunoreactivity, n = 3). In subsynaptic fractions isolated from the striatum of mice, it was observed that the α7 subunit (Fig. 4.3 D) was localized mainly at the presynaptic active zone (63.6 ± 6.1 % of total immunoreactivity, n = 4), similarly to what was observed in rat striatum. It was also identified in the postsynaptic density (24.5 ± 5.6 % of total immunoreactivity, n = 4) and had a smaller presence outside the active zone (11.9 ± 1.6 % of total immunoreactivity, n = 4).

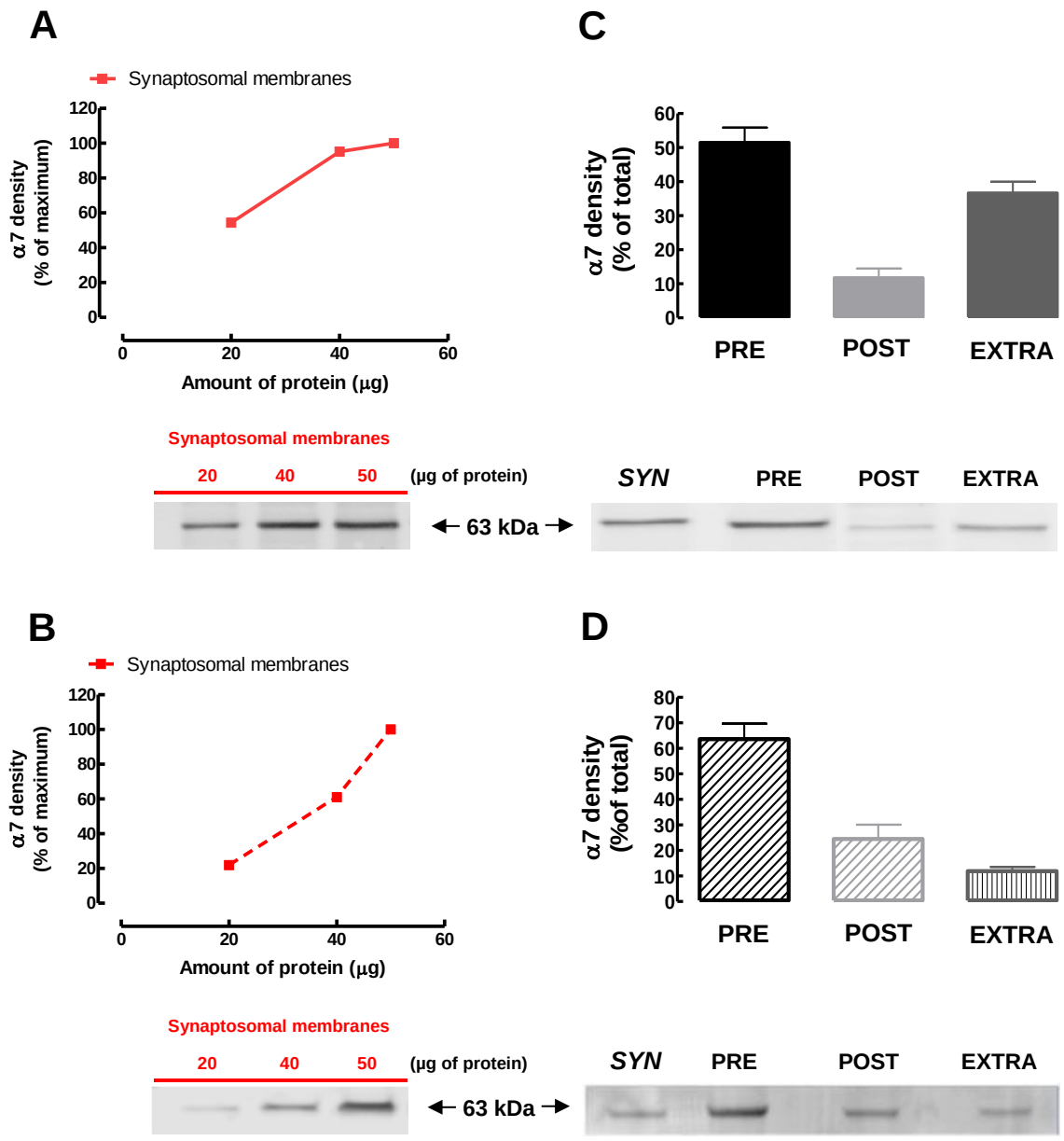


Figure 4.3 - Synaptic and subsynaptic distribution of the α7 subunit of nAChRs in the striatum of Wistar rats and C57BL/6 mice. (A and B) Levels of α7 subunits of nAChRs in membranes of isolated nerve terminals (synaptosomes), from the striatum of adult male rats (A) and mice (B). The graphs represent the percentage of α7 immunoreactivity for different amounts of loaded protein, taking as reference the maximum value of immunoreactivity in each blot. The data are the means ± SEM of 3-4 independent experiments. Representative Western blots, where 3 different protein amounts (20, 40 and 50 µg) of synaptosomes were applied, are shown. (C and D) Graphs and representative Western blots show the subsynaptic levels of α7 subunits of nAChRs in striatum of rats (C) and of mice (D). The α7 immunoreactivity was compared after fractionation of striatal total nerve terminals (synaptosomes, SYN) into fractions enriched in the presynaptic active zone (PRE), postsynaptic density (POST) and areas outside the active zone (EXTRA). The amount of loaded protein for each fraction was 20 µg and the fraction corresponding to total synaptosomes was used as an internal control. The percentage of immunoreactivity of the subunits was obtained taking as reference the total value of reactivity in each separate experiment. Graphs exhibit the means ± SEM of 3-4 independent experiments derived from three or four fractionations from different groups of 5-8 rats or 10-12 mice.

4.1.1.2 nAChRs β2 subunit synaptic and subsynaptic levels

The signal for the β2 subunit in Wistar rats and C57BL/6 mice was identified in synaptic membranes by Western blot, showing the localization of the subunit in striatal nerve terminals (Fig. 4.4 A and B). The antibody recognized bands of identical molecular weight (55 kDa), in rats (n = 3) and mice (n = 3). Immunoreactivity increased with higher amounts of loaded protein.

The results obtained showed that the striatal subsynaptic localization of the β2 subunit in Wistar rats was predominantly in extrasynaptic sites, outside the active zone (Fig 4.4 C) (86.1 ± 2.5 % of total immunoreactivity, n = 3), and that it had a significantly lower density in the presynaptic active zone (9.6 ± 1.4 % of total immunoreactivity, n = 3). The immunoreactivity of this subunit was less abundant in the postsynaptic enriched fraction (4.4 ± 2.6 % of total immunoreactivity, n = 3). In C57BL/6 mice (Fig. 4.4 D) the β2 subunit was localized mainly at the presynaptic active zone (51.8 ± 12.3 % of total immunoreactivity, n = 3) and also had a strong component in extrasynaptic sites (47.4 ± 12.5 % of total immunoreactivity, n = 3). It showed a negligible presence in the postsynaptic density (2.5 ± 0.03 % of total immunoreactivity, n = 3).

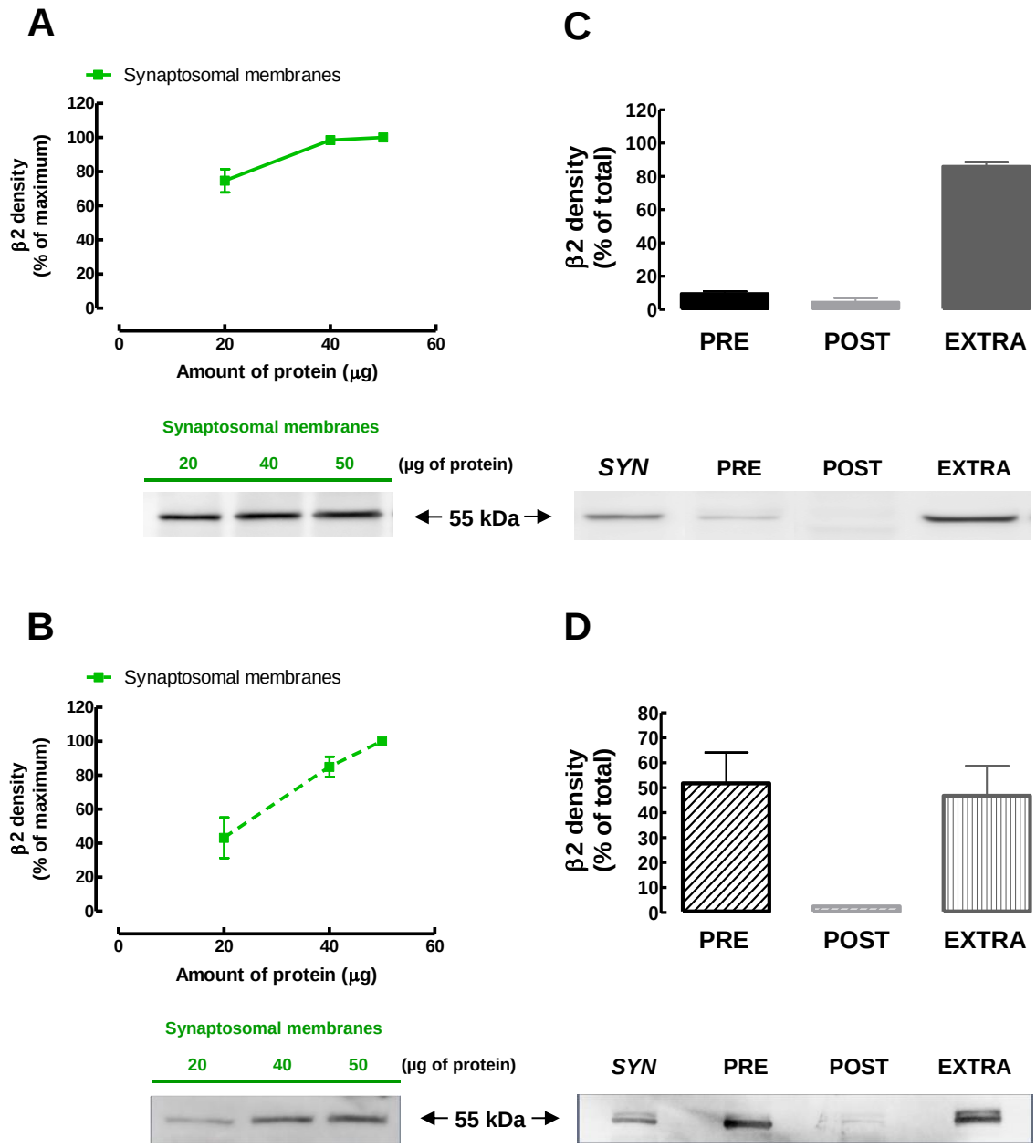


Figure 4.4 - Synaptic and subsynaptic distribution of the β2 subunit of nAChRs in the striatum of Wistar rats and C57BL/6 mice. (A and B) Levels of the β2 subunit of nAChRs in membranes of isolated nerve terminals (synaptosomes), from the striatum of adult male rats (A) and mice (B). The graphs represent the percentage of β2 immunoreactivity for different amounts of loaded protein, taking as reference the maximum value of immunoreactivity in each blot. The data are the means ± SEM of 3 independent experiments. Representative Western blots, where 3 different protein amounts (20, 40 and 50 µg) of synaptosomes were applied, are shown. (C and D) Graphs and representative Western blots show the subsynaptic levels of the β2 subunit of nAChRs in striatum of rats (C) and of mice (D). The β2 immunoreactivity was compared after fractionation of striatal total nerve terminals (synaptosomes, SYN) into fractions enriched in the presynaptic active zone (PRE), postsynaptic density (POST) and areas outside the active zone (EXTRA). The amount of loaded protein for each fraction was 20 µg. The percentage of immunoreactivity of the subunits was obtained taking as reference the total value of reactivity in each separate experiment. Graphs exhibit the mean ± SEM of 3 experiments derived from three fractionations from different groups of 5-8 male Wistar rats or 10-12 C57BL/6 mice.

4.1.1.3 nAChRs α4 subunit synaptic and subsynaptic levels

Results for the α4 subunit showed its localization in striatal synaptic membranes of rats (n = 3) and mice (n = 4), evaluated by Western blot (Fig. 4.5 A and B). The density of the α4 subunit increased when progressively higher amounts of protein were loaded. The antibody recognized bands of identical molecular weight (60 kDa) in rats and mice.

In rat striatum the α4 subunit was localized predominantly in extrasynaptic sites, outside the active zone (88.8 ± 9.5 % of total immunoreactivity, n = 3), and had a significantly lower density in the presynaptic active zone (10.8 ± 9.3 % of total immunoreactivity, n = 3). The immunoreactivity of this subunit was lowest in the postsynaptic enriched fraction (0.5 ± 0.2 % of total immunoreactivity, n = 3) (Fig. 4.5 C). In C57BL/6 mice the α4 subunit (Fig. 4.5 D) was mostly located at the presynaptic active zone (69.1 ± 5.7 % of total immunoreactivity, n = 4) and had a significant presence in extrasynaptic sites (30.6 ± 5.6 % of total immunoreactivity, n = 4). In the postsynaptic density the level of α4 subunit was almost null (0.4 ± 0.4 % of total immunoreactivity, n = 4).

As can be seen in Figures 4.4 (C and D) and 4.5 (C and D) the α4 and β2 subunits displayed a similar subsynaptic distribution, which is in agreement with the notion that these subunits together form abundant heteromeric nAChRs (see Whiting *et al*., 1987; Flores *et al*., 1992). Interestingly, there is a significant difference between animal species, since in samples obtained from Wistar rats the α4 and β2 subunits are predominantly localized in extrasynaptic sites (Figs. 4.4 and 4.5 C) outside the active zone, whereas in C57BL/6 mice the subunits are mainly in the presynaptic fraction, at the synaptic active zone (Figs. 4.4 and 4.5 D).

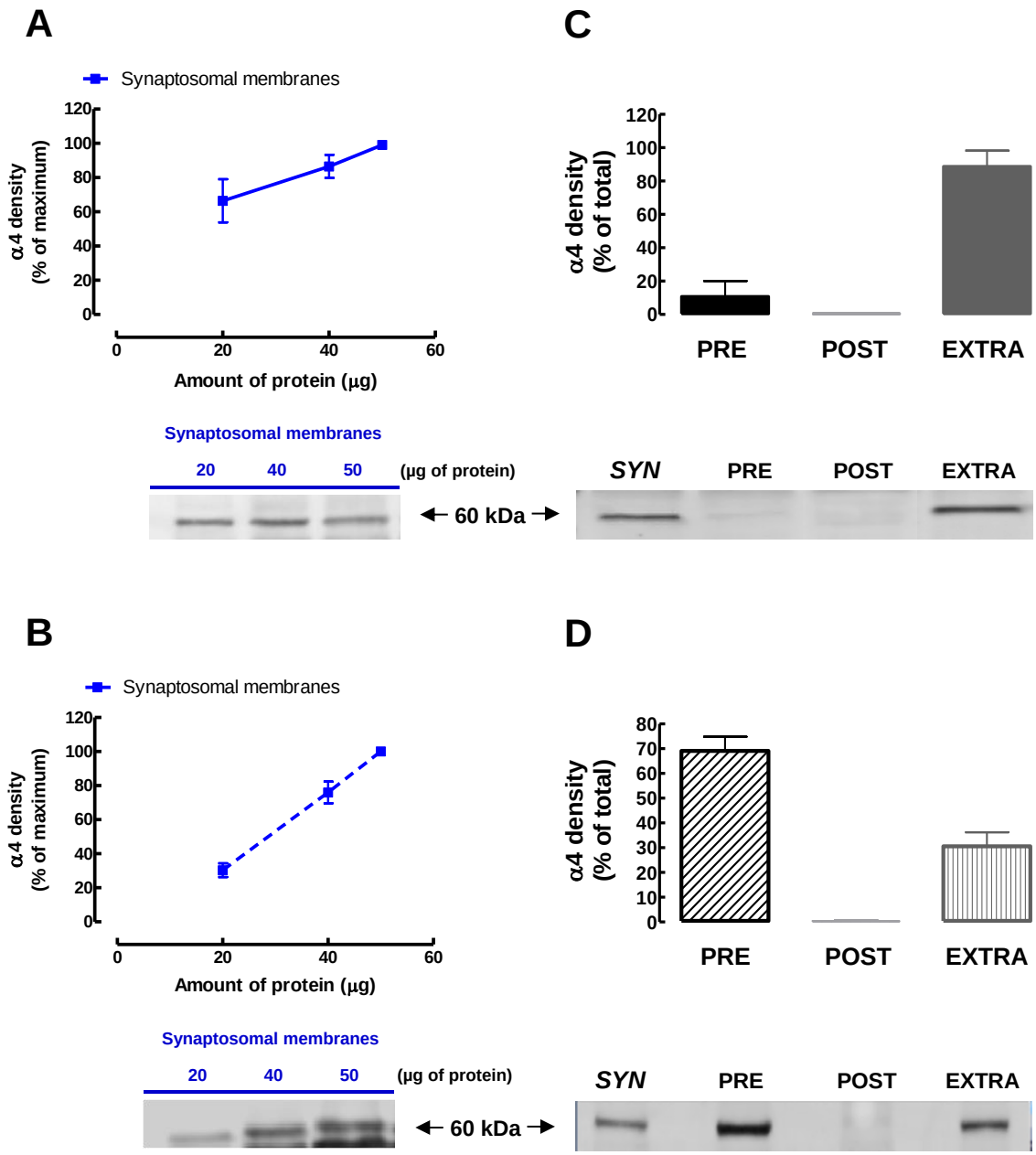


Figure 4.5 - Synaptic and subsynaptic distribution of the α4 subunit of nAChRs in the striatum of Wistar rats and C57BL/6 mice. (A and B) Immunoreactivity of the α4 subunit of nAChRs in membranes isolated from the striatum of rats (A) and mice (B). The graphs represent the percentage of immunoreactivity, for different amounts of loaded protein (20, 40 and 50 µg), taking as reference the maximum value of immunoreactivity in each blot. The data are the means ± SEM of 3-4 independent experiments. Representative Western blots are displayed below. (C and D) Graphs and representative Western blots show the subsynaptic levels of the α4 subunit of nAChRs in the striatum of rats (C) and mice (D). The α4 immunoreactivity was compared after fractionation of striatal total nerve terminals (synaptosomes, SYN) into fractions enriched in the presynaptic active zone (PRE), postsynaptic density (POST) and areas outside the active zone (EXTRA). Loaded protein for each fraction was 20 µg. The percentage of immunoreactivity was obtained taking as reference the total value of reactivity in each separate experiment. Representative Western blots are displayed below. Graphs exhibit the mean ± SEM of 3-4 experiments derived from fractionations from different groups of 5-8 rats or 10-12 mice.

4.1.1.4 nAChRs α6 subunit synaptic and subsynaptic levels

The α6 subunit was localized in the synaptic membranes from striatum of Wistar rats (n = 4) and C57BL/6 mice (n = 3) (Fig. 4.6 A and B). The Western blot analysis revealed specific bands of increasing immunoreactivity signal, with higher amounts of loaded protein. In rats and mice, the antibody recognized bands of identical molecular weight (56 kDa).

The results obtained for the α6 subunit in the striatum of rats (Fig. 4.6 C) revealed that it was clearly predominant in the postsynaptic density (56.4 ± 3.9 % of total immunoreactivity, n = 3) albeit also being extensively located in extrasynaptic sites and in the presynaptic active zone (27.6 ± 1.1 % and 16.0 ± 5.1 % of total immunoreactivity, respectively, n = 3). Like in the rat, the α6 subunit was prevalent in the postsynaptic density in mice (4.6 D) (76.8 ± 5.5 % of total immunoreactivity, n = 3) albeit also being present in extrasynaptic sites and in the presynaptic active zone (16.9 ± 8.5 % and 9.4 ± 1.5 % of total immunoreactivity, respectively, n = 3).

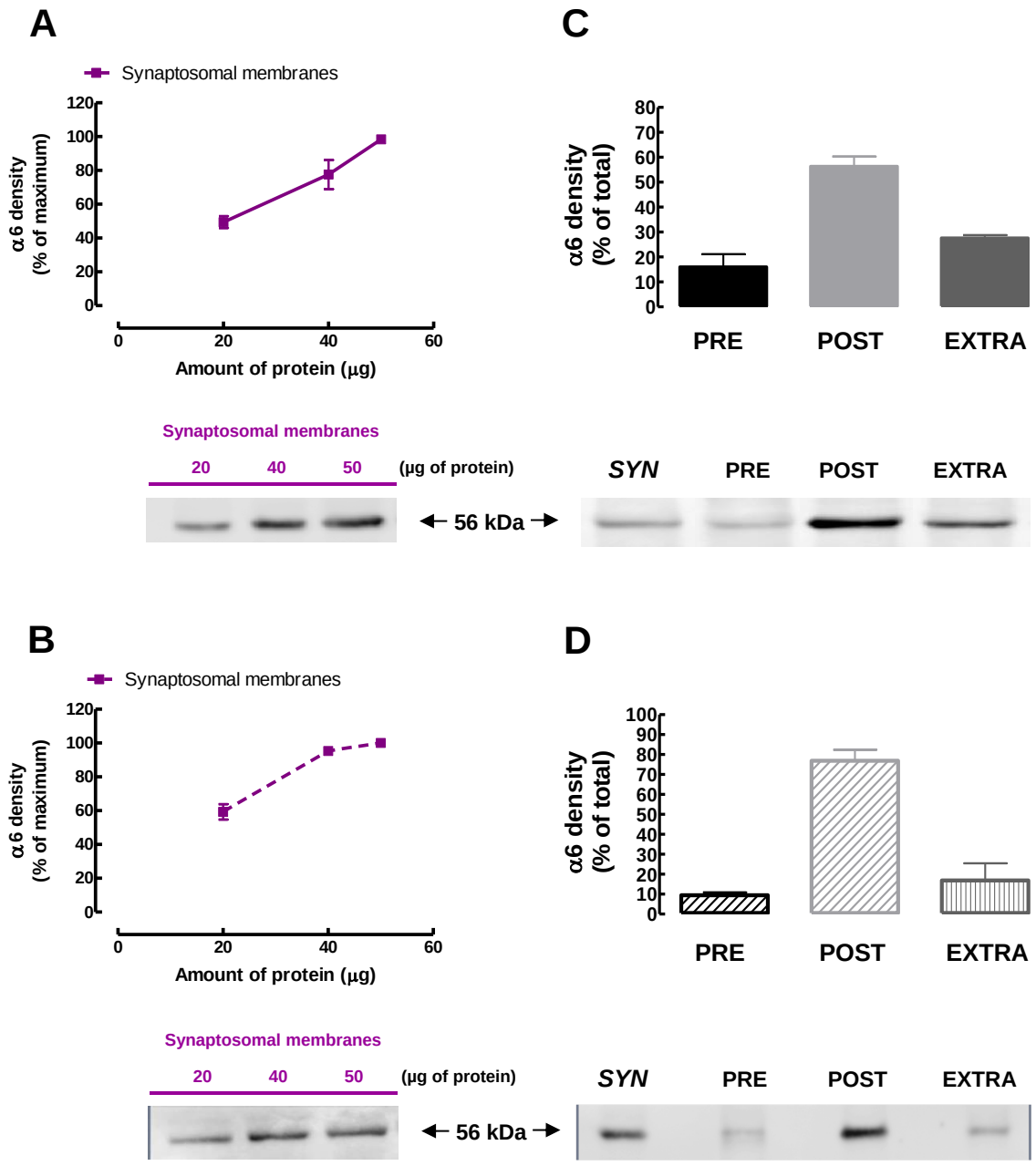


Figure 4.6 - Synaptic and subsynaptic distribution of the α6 subunit of nAChRs in the striatum of Wistar rats and C57BL/6 mice. (A and B) Density of the α6 subunit of nAChRs in membranes of purified synaptosomes isolated from the striatum of adult male rats (A) and (B) mice. Graphs represent the percentage of immunoreactivity for each quantity of protein taking as reference the maximum value of immunoreactivity in each blot and are the means ± SEM of 3-4 independent experiments. Representative Western blots, where 3 different amounts of protein of synaptosomes were applied, are shown below. (C and D) Subsynaptic levels of the α6 subunit of nAChRs in striatum of rats (C) and mice (D). The α6 immunoreactivity was compared after fractionation of striatal total nerve terminals (synaptosomes, SYN) into fractions enriched in the presynaptic active zone (PRE), postsynaptic density (POST) and areas outside the active zone (EXTRA). Loaded protein for each fraction was 20 µg. The percentage of immunoreactivity was obtained taking as reference the total value of reactivity in each separate experiment. Representative Western blots are shown below. Graphs exhibit the means ± SEM of 3 independent experiments derived from three fractionations from different groups of 5-8 rats or 10-12 mice.

4.2 CHARACTERIZATION OF NICOTINE-INDUCED [^{3}H]DOPAMINE RELEASE IN RAT STRIATAL SYNAPTOSOMES - MODULATION BY ADENOSINE RECEPTORS

To investigate the purported functional interaction between presynaptic nicotinic acetylcholine and adenosine A_{2A} receptors, we used a golden standard tool, the synaptosomal preparation, which consists of resealed nerve terminals, free from polysynaptic and glial interferences (Raiteri and Raiteri, 2000) (see section 3.1.2). Striatal synaptosomes were loaded with tritium-labeled dopamine ([^{3}H]dopamine), in the presence of the MAO-B inhibitor, pargyline (10 µM), to avoid degradation of the dopamine; trapped in filters, superfused and stimulated either by nicotine at various concentrations, by adenosine receptors ligands, or by a combination of them.

We performed assays of nicotine-induced [^{3}H]dopamine release using synaptosomes from rat and from mouse (not shown), and it was observed that in both preparations there was [^{3}H]dopamine release induced by nicotine. Considering that the results we obtained from the synaptic and subsynaptic localization of the nAChR subunits showed that their striatal distribution was to some extent similar in rats and mice; that the studies indicating the presence of adenosine A_{2A}Rs in striatal dopaminergic terminals were performed in rat (see Borycz *et al*., 2007; Gomes *et al*., 2006, 2009) and taking into account the previous neurotransmitter release studies of our research group (*e.g.* Köfalvi *et al*., 2007; Ferreira *et al.,* 2009), the following studies were performed using only Wistar rats.

4.2.1 Nicotine stimulates the release of [^{3}H]dopamine from rat striatal synaptosomes

Nicotine (1, 30, 300 nM and 3 µM) stimulated the release of [^{3}H]dopamine ([^{3}H]DA) in a concentration-dependent manner (Fig. 4.7 A and B). With this dose-response curve it was determined: i) the EC_{50} (half maximal effective concentration) that represents the concentration of the agonist (nicotine) which produces 50% of the maximum possible response, indicating the potency of the agonist; and ii) the E_{max}, which represents the maximum possible effect for the agonist, describing its efficacy. Thus, for nicotine-induced dopamine release it was determined a value of EC_{50}= 68.0 ± 17.2 nM and a E_{max}= 6.80 ± 0.67 fractional release (FR%), n = 10-21 rats, in duplicate.

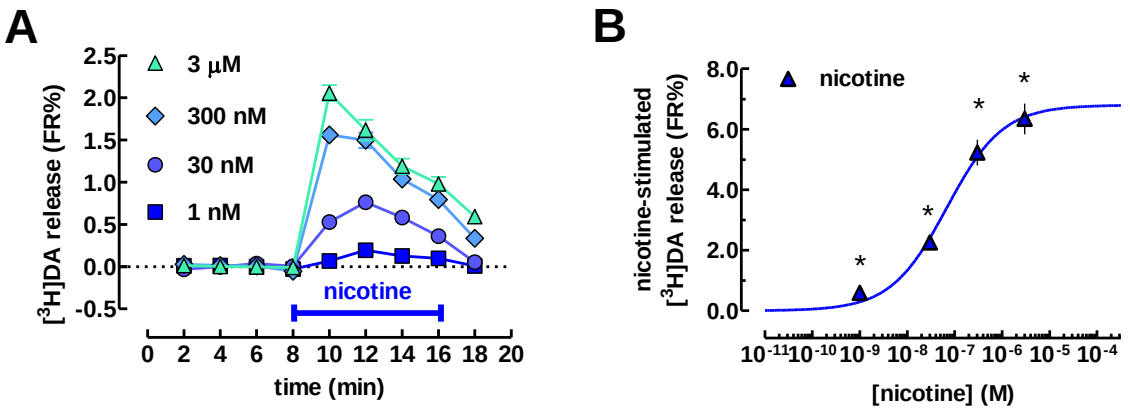


Figure 4.7 - Nicotine stimulates [^{3}H]dopamine ([^{3}H]DA) release from rat striatal synaptosomes. (A) Time course of the averaged release of [^{3}H]DA. Synaptosomes were treated with various concentrations of nicotine (1, 30, 300 nM or 3 µM) for 8 min, as indicated by the horizontal bar. (B) Concentration-response curve for nicotine to trigger the release of [^{3}H]DA. The values of EC_{50}= 68.0 ± 17.2 nM and [E_{max}]= 6.80 ± 0.67 FR%; were determined for nicotine. * p<0.001 *vs*. non-stimulated control; n = 10-21 independent observations in duplicate.

4.2.2 Putative involvement of adenosine receptor ligands on the release of [^{3}H]dopamine

Given that the major goal of this part of the study was to investigate whether nicotine receptors and adenosine receptors act in concert to regulate dopamine release in the striatum, we evaluated the effect of A_{2A}R ligands *per se* on the release of [^{3}H]dopamine: the A_{2A}R agonist CGS21680 (30 nM) stimulated the release of [^{3}H]DA (0.63 ± 0.25 FR%, n = 10, $p < 0.05$, t = 2.503, df = 9) (Fig. 4.8), while the A_{2A}R antagonist ZM241385 (100 nM) was without effect (0.16 ± 0.24 FR%, n = 18, $p > 0.5$, t = 0.6823, df = 17, Fig. 4.8). Since ZM241385 can also antagonize A_{2B}Rs (Lasley *et al.*, 2007; Li *et al.*, 2007), although with lower potency than A_{2A}Rs (Poucher *et al*., 1995; Ji and Jacobson, 1999), we also tested the A_{2B}R antagonist MRS1754 (200 nM), which was found without effect *per se* (0.53 ± 0.22 FR%, n = 7, $p > 0.05$, t = 0.2383, df = 6; Fig. 4.8). Interestingly, the combined application of ZM241385 together with MRS1754 stimulated the release of [^{3}H]DA (1.61 ± 0.51 FR%, n = 9, $p < 0.05$, t = 3.165, df = 8) (Fig. 4.8), suggesting a cooperative interaction between A_{2A}Rs and A_{2B}Rs similar to that observed in splenocytes (Moriyama and Sitkovsky, 2010). Additionally, the nonselective adenosine receptor antagonist, caffeine (50 µM) also stimulated the release of [^{3}H]DA (0.53 ± 0.13 FR%, n = 38, $p < 0.0001$, t = 3.903, df =37) (Fig. 4.8).

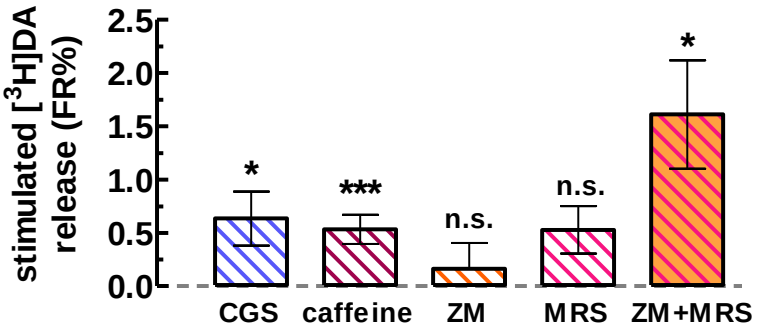


Figure 4.8 - The adenosine A_{2A}R agonist, CGS21680 (CGS, 30 nM) but neither the A_{2A}R antagonist, ZM241385 (ZM, 100 nM) nor the A_{2B}R antagonist, MRS1754 (MRS, 200 nM) stimulates [^{3}H]DA release from rat striatal synaptosomes. The non-selective adenosine receptor antagonist, caffeine (50 µM) also stimulates [^{3}H]DA release, which may be explained by the observation that the simultaneous blockade of A_{2A}Rs and A_{2B}Rs by ZM241385 and MRS1754 also facilitated [^{3}H]DA release. Data are mean ± SEM of 7-38 experiments performed in duplicate. * $p < 0.05$, *** $p < 0.01$ vs. 0 FR% (*i.e.* no change in baseline); n.s., not significant.

4.2.3 Nicotine stimulates the release of [^{3}H]dopamine from rat striatal synaptosomes in an adenosine A_{2A} receptor-dependent manner

In this part of the study we aimed to define the functional interaction between nicotinic and adenosine receptors by evaluating the impact of A_{2A}Rs on modulating the nicotine-stimulated dopamine release in striatal nerve terminals.

In experiments combining A_{2A}R ligands with nicotine, we observed that the selective A_{2A}R agonist CGS21680 (30nM) inhibited the effect of nicotine on [^{3}H]DA release at the two lowest concentrations of nicotine (1 and 30 nM) (Figs. 4.9 A, B and E), while it had no significant impact on the effect of higher nicotine concentrations (300 nM and 3 µM) (Fig. 4.9 E). In the presence of CGS21680, the effect of nicotine (1 nM) was abolished (-0.54 ± 0.28 FR%, n = 8, $p < 0.05$ *vs*. CGS21680 alone), while the effect of nicotine (30 nM) was reduced, by 53.1 ± 11.6% ($p < 0.01$, t = 4.457, df = 6), to a value of 1.47 ± 0.43 FR%, n = 7. The effect of CGS21680 was concentration-dependent (Figs. 4.9 B-F), displaying an IC_{50} of 11.0 ± 6.3 nM (IC_{50} = half maximal inhibitory concentration; provides a measure of the effectiveness of the inhibition) and an I_{max} of 53.8 ± 7.4% for the inhibition of 30 nM nicotine-induced [^{3}H]DA release (I_{max} = maximum value of inhibition) (n = 9-11).

In agreement with the involvement of A_{2A}Rs to inhibit the action of nicotine, the A_{2A}R antagonist ZM241385 (100 nM), facilitated the action of different concentrations of nicotine: at 30 nM by 63.4 ± 10.0%, increased the release to 3.66 ± 0.22 FR%, n = 7, $p < 0.05$, t = 3.169, df = 6), at 300 nM by 26.2 ± 7.6% (to 6.33 ± 0.44 FR%, n = 8, $p < 0.01$, t = 3.623, df = 7), and at 3 µM by 26.9 ± 5.3% (to 8.55 ± 0.58 FR%, n = 6, $p < 0.01$, t = 4.835, df = 5) (Fig. 4.10 B-E); whereas at 1 nM no significant facilitation was observed (Fig. 4.10 A and E).

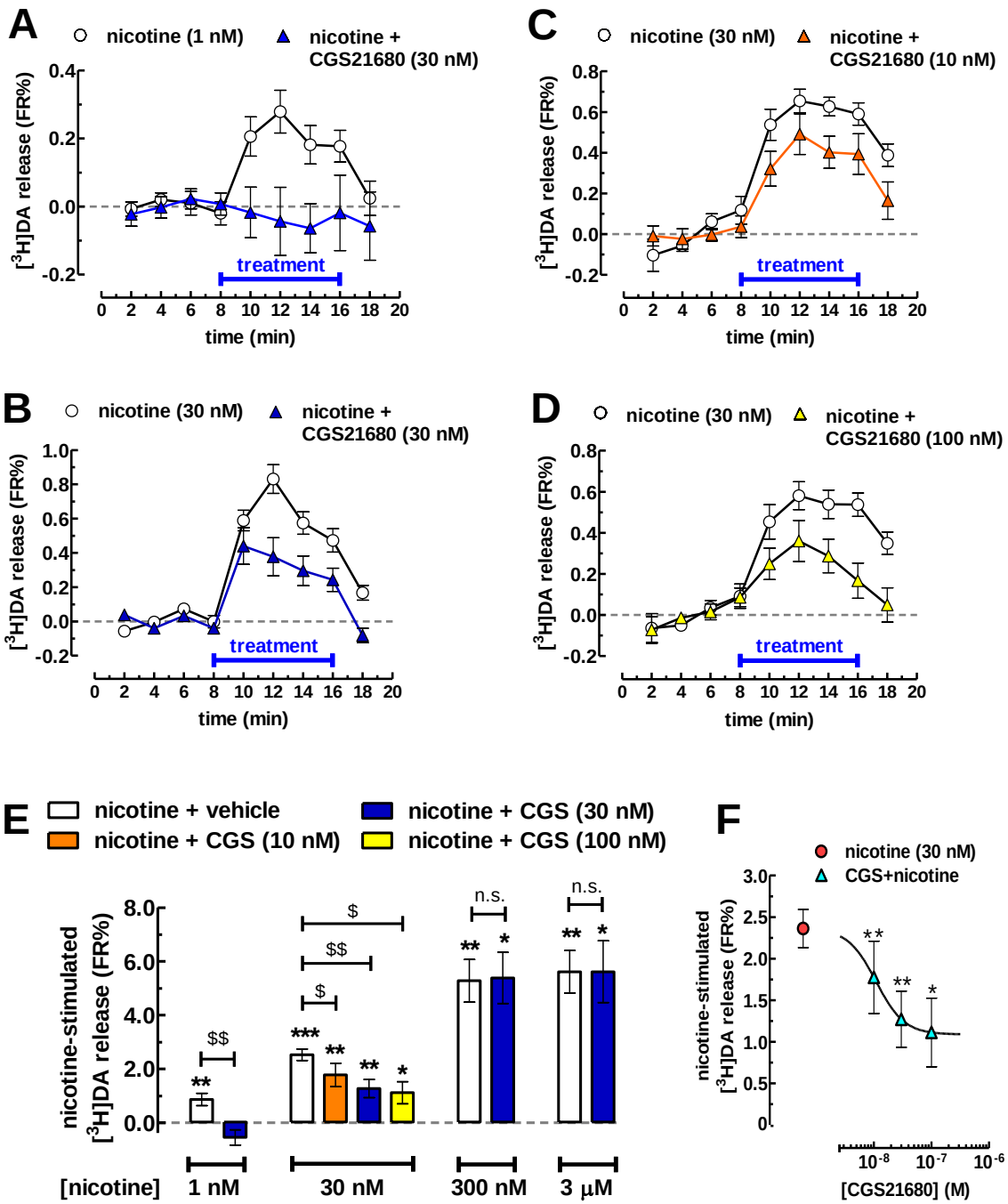


Figure 4.9 - Adenosine $A_{2A}R$ activation inhibits the nicotine-induced [^{3}H]DA release from rat striatal synaptosomes. (A-D) Time course and (E) bar graph displaying the averaged release of [^{3}H]DA induced by various concentrations of nicotine alone or in presence of the $A_{2A}R$ agonist, CGS21680 (CGS, 10-100 nM). The co-administration of CGS21680 and nicotine occurred as indicated by the horizontal bars (treatment). (F) Concentration-response curve for CGS21680 (CGS, 10-100 nM) to inhibit the nicotine (30 nM) induced release of [^{3}H]DA. Data are mean ± SEM of 6-18 experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs*. 0 FR% (*i.e*. no change in baseline); $ $p < 0.05$ and $$ $p < 0.01$ between nicotine alone and with CGS21680; n.s., not significant.

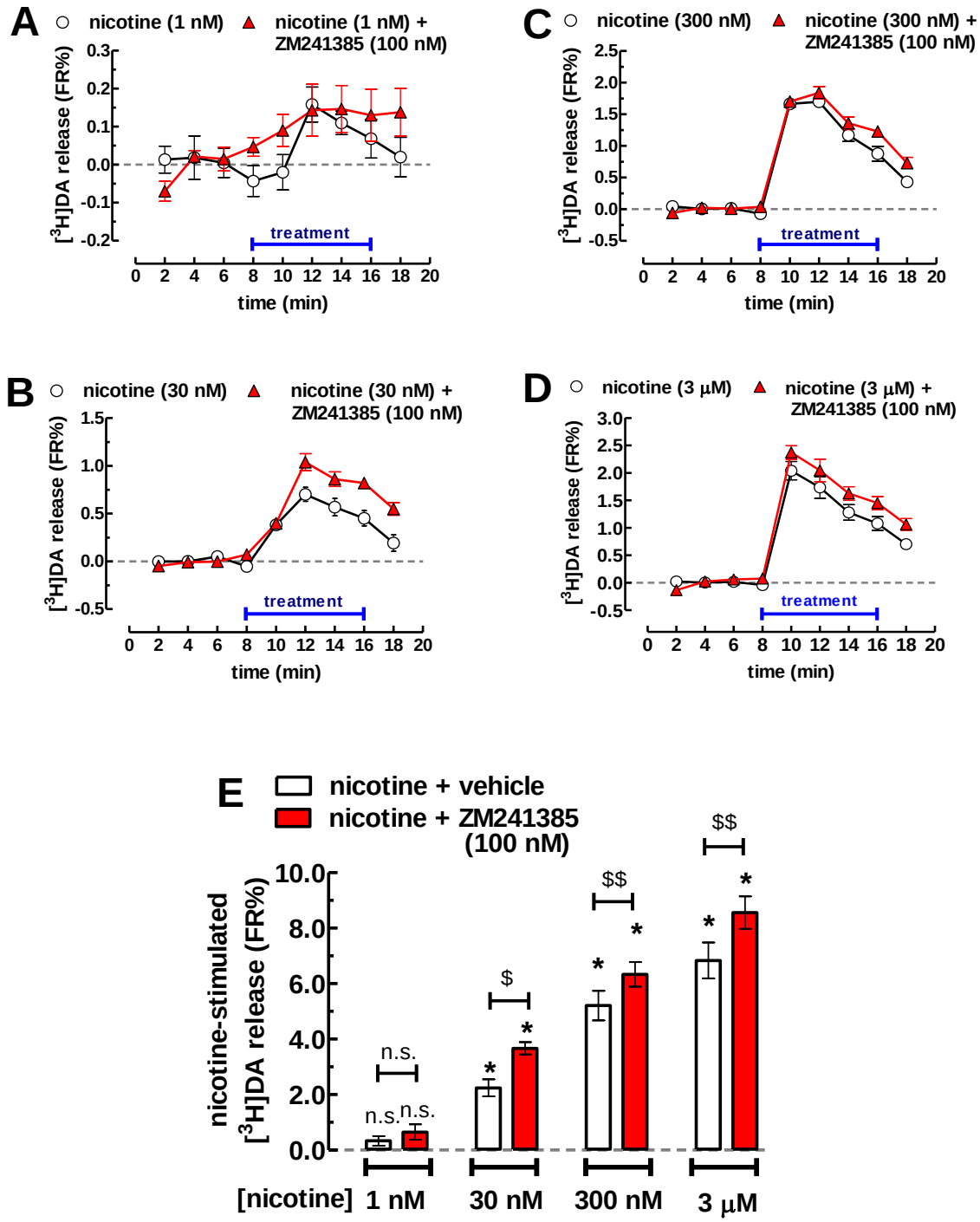


Figure 4.10 - Adenosine A_{2A}R blockade increases the nicotine-induced [^{3}H]DA release from rat striatal synaptosomes. (A-D) Time courses and (E) bar graph displaying the averaged release of [^{3}H]DA induced by nicotine alone (1, 30, 300 nM and 3 µM) or in the presence of the A_{2A}R antagonist, ZM241385 (100 nM). The co-administration of ZM241385 and nicotine occurred as indicated by the horizontal bar (treatment). Data are mean ± SEM of 6 experiments performed in duplicate. * $p < 0.05$ *vs*. 0 FR% (*i.e*. no change in baseline); \$ $p < 0.05$ and \$\$ $p < 0.01$ between the indicated bars, when compared to control (nicotine in the absence of any adenosine receptor antagonist, displayed in the left bars).

Considering that caffeine, a major component of beverages such as coffee and tea, is the most widely consumed psychoactive drug worldwide (Fredholm *et al*., 1999) and has been correlated with the incidence of brain pathologies, such as PD, we next tested the impact of caffeine on nicotine-induced [^{3}H]DA release. The acute administration of 10 µM (data not shown) and 50 µM of caffeine failed to facilitate the action of nicotine at lower concentrations (30 and 300 nM) and actually diminished the effect caused by a higher concentration of 3 µM nicotine (to 4.06 ± 0.73 FR%, n = 8, $p < 0.01$, t = 3.795, df = 7) (Fig. 4.11 A). Since caffeine (Fredholm *et al.*, 1999) as well as ZM241385 have also been reported to antagonize the A_{2B}Rs of rat and human (Lasley *et al.*, 2007; Li *et al.*, 2007) albeit with a lower potency than A_{2A}Rs (Poucher *et al*., 1995; Ji and Jacobson, 1999), we next tested the effects of a selective A_{2B}R antagonist, MRS1754, in our assay. MRS1754 (200 nM) antagonized the 30 nM nicotine-stimulated release of [^{3}H]DA by 58.7 ± 11.3% (0.89 ± 0.24 FR%, n = 6, $p < 0.05$, t = 3.716, df = 5) (Fig. 4.11 B). When combined with ZM241385, the two antagonists (MRS1754 and ZM241385) did not alter the 30 nM nicotine-induced release of dopamine (1.33 ± 0.89 FR%, n = 7, $p > 0.05$ vs. nicotine alone; Fig. 4.11 B). These results indicated that the facilitation of nicotine's action by ZM241385 was not mediated by A_{2B}Rs antagonism, although it appears that A_{2B}Rs may also be involved in the control (qualitatively opposite to the role of A_{2A}Rs) of nicotinic receptor function in dopaminergic terminals of the striatum.

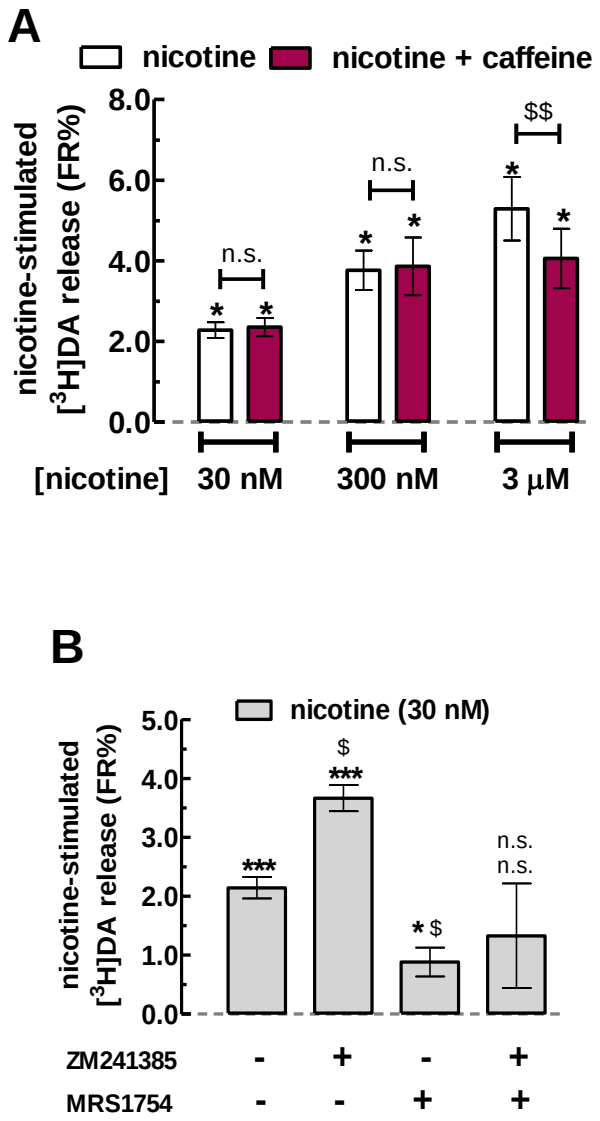


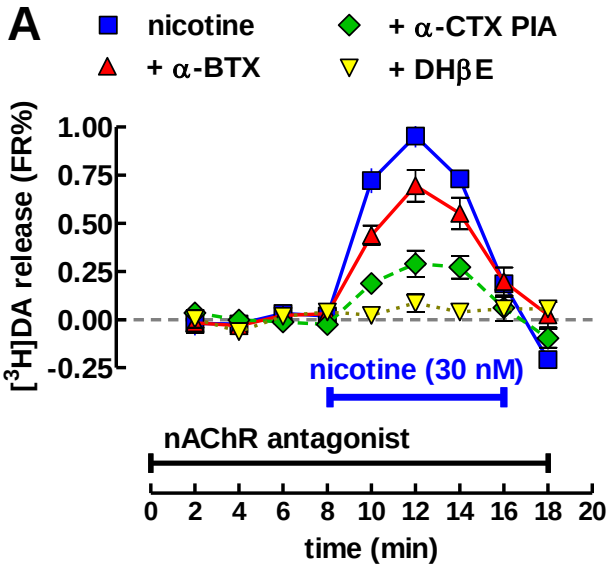
Figure 4.11 - Adenosine A_{2A}R blockade by caffeine does not facilitate the nicotine-induced [^{3}H]DA release from rat striatal synaptosomes. (A) In a similar experimental paradigm to the previous assay, the non-selective adenosine receptor antagonist caffeine (50 μM) failed to mimic the action of ZM241385, *i.e.* to facilitate the effect of nicotine. (B) This lack of caffeine effect may be due to the involvement of A_{2B}Rs, which are also antagonized by caffeine, since the selective A_{2B}R antagonist, MRS1754 (200 nM) inhibited the effect of nicotine and prevented the facilitatory action of ZM241385 when the two antagonists were combined. Data are mean ± SEM of 6-13 experiments performed in duplicate. * $p < 0.05$ and *** $p < 0.001$ *vs*. 0 FR% (*i.e*. no change in baseline); $ $p < 0.05$ and $$ $p < 0.01$ between the indicated bars in (A) and when compared to control (nicotine in the absence of any adenosine receptor antagonist, displayed in the leftmost bar) in (B); n.s., not significant.

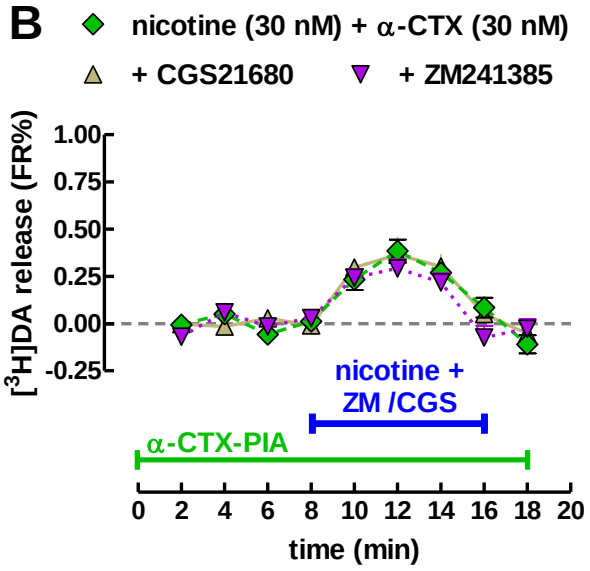
4.2.4 Blockade of the α6-containing nAChRs abolishes the action of A_{2A}R ligands on nicotine-stimulated [^{3}H]DA release

Our previous studies about the distribution of nAChR subunits α7, α6, α4 and β2 revealed that they are localized at striatal nerve terminals (section 4.1), which is in accordance with previous studies (Kaiser and Wonnacott, 2000; Grady *et al.*, 2002, 2007; Zoli *et al*., 2002; Meyer *et al.*, 2008; Livingstone and Wonnacott, 2009). Moreover, all these subunits were identified in the presynaptic active zone of the striatal nerve terminals, thus providing insights about which nAChR subtypes might underlie the nicotine-stimulated [^{3}H]DA release from striatal synaptosomes. The functional relevance of these subunits was next probed using nAChRs antagonists tested against the concentration of nicotine (30 nM) that was found to be sensitive to both CGS21680 and ZM241385 (see Figs 4.9 and 4.10).

As illustrated in Fig. 4.12 A and C, the antagonist of α7 nAChRs α-bungarotoxin (α-BTX, 100 nM) (Marchi *et al*., 2002), failed to significantly affect the action of nicotine (30 nM) on [^{3}H]DA release (mean difference, 16.9 ± 15.1%, $p > 0.05$ by repeated measures ANOVA with Dunett's post-hoc test). The majority of nicotine binding sites in the brain contains the β2 subunit (Grady *et al.,* 2002; Toyohara and Hashimoto, 2010); accordingly, the β2 subunit-preferring competitive antagonist dihydro-β-erythroidine (DHβE, 10 μM) (Grady *et al*., 2007) prevented the nicotine stimulated [^{3}H]DA release by 88.1 ± 5.0% ($n = 5$, $p < 0.001$, by repeated measures ANOVA with Dunett's post-hoc test) (Fig. 4.12 A and C). The α6-containing nAChR antagonist α-conotoxin PIA, that discriminates between the closely related α6 and α3 subunits (α-CTX PIA, 30 nM) (Azam and McIntosh, 2005), largely inhibited the action of nicotine by 69.8 ± 7.5% ($n = 6$, $p < 0.001$, by repeated measures ANOVA with Dunett's post-hoc test) (Fig. 4.12 A and C). This suggests that ~70% of the nicotine (30 nM) stimulated [^{3}H]DA release involves the activation of α6β2-containing receptors. Upon blockade of α6-containing nAChRs (in the presence of α-CTX PIA, 30 nM), the nicotine (30 nM)-induced [^{3}H]DA release was no longer affected by either CGS21680 (30 nM, $n = 9$, $p > 0.05$ vs. α-CTX PIA + nicotine) or ZM241385 (100 nM, $n = 9$, $p > 0.05$ vs. α-CTX PIA + nicotine) (4.12 B and C). Altogether, these data advocate that A_{2A}Rs are selectively coupled to the

inhibition of α6β2-containing nAChRs in dopaminergic terminals of the rat striatum.





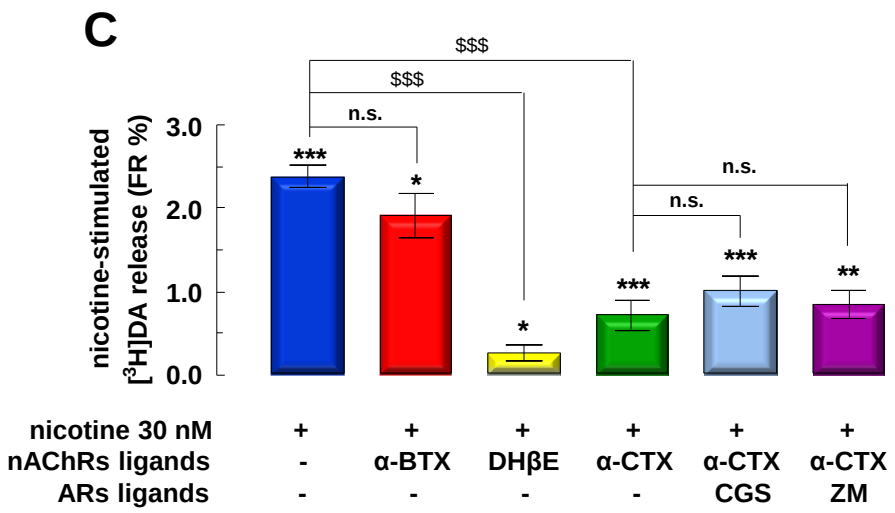


Figure 4.12 - The adenosine A_{2A}R-mediated inhibition of the nicotine-induced [^{3}H]DA release mainly depended on α6β2-containing nAChRs. (A) Time course displaying the averaged [^{3}H]DA release, induced by nicotine (30 nM) in the absence or in the presence of nAChRs ligands: the α6 subunit antagonist α-conotoxin PIA (α-CTX PIA, 30 nM), the α7 subunit antagonist α-bungarotoxin (α-BTX, 100 nM) or the β2 subunit antagonist dihydro-β-erythroidine (DHβE, 100 nM). (B) Time course displaying the averaged [^{3}H]DA release, induced by nicotine (30 nM) in the presence of α-conotoxin-PIA (α-CTX-PIA or α-CTX, 30 nM), when combined with adenosine receptors (ARs) ligands: either the A_{2A}R agonist CGS21680 (CGS, 30 nM) or the A_{2A}R antagonist ZM241385 (ZM, 100 nM). (C) Bar graph summarizing the sensitivity of 30 nM nicotine-stimulated [^{3}H]DA release under the different conditions tested in (A) and (B). Data are mean ± SEM of 9 experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs*. 0 FR% (*i.e*. no change in baseline); $$$ $p < 0.001$ between nicotine alone (blue bar) and nicotine with antagonists of nicotinic acetylcholine receptors; n.s., not significant. Note that neither CGS21680 nor ZM241385 affected the non-α6 subunit-containing nAChR-induced release of [^{3}H]DA, suggesting that it is these α6 subunit-containing nAChRs that are modulated by A_{2A}Rs to control striatal dopamine release.

4.3 EVALUATION OF ADENOSINE RELEASE BY STRIATAL SYNAPTOSOMES IN THE ABSENCE OR PRESENCE OF NICOTINE

The fact that $A_{2A}R$ and $A_{2B}R$ antagonists modify dopamine release implies the existence of endogenous adenosine presumably released from the synaptosomes both tonically and upon nAChR activation. To evaluate this hypothesis, we directly quantified the levels of adenosine in incubated (batch conditions) or superfused synaptosomes. Fig. 4.13 A shows that adenosine and its metabolites (inosine and hypoxanthine) were present in concentrations of 6 nmol/mg protein in incubated synaptosomes (n = 4) whereas their levels were below the limit of detection in the superfusate (n = 4; figure not shown). Additionally, we found that nicotine 1 µM (and 30 nM, not shown) failed to modify the extracellular levels of adenosine or its metabolites either in incubated or in superfused synaptosomes (n = 4, p> 0.05) (Fig. 4.13 A and B).

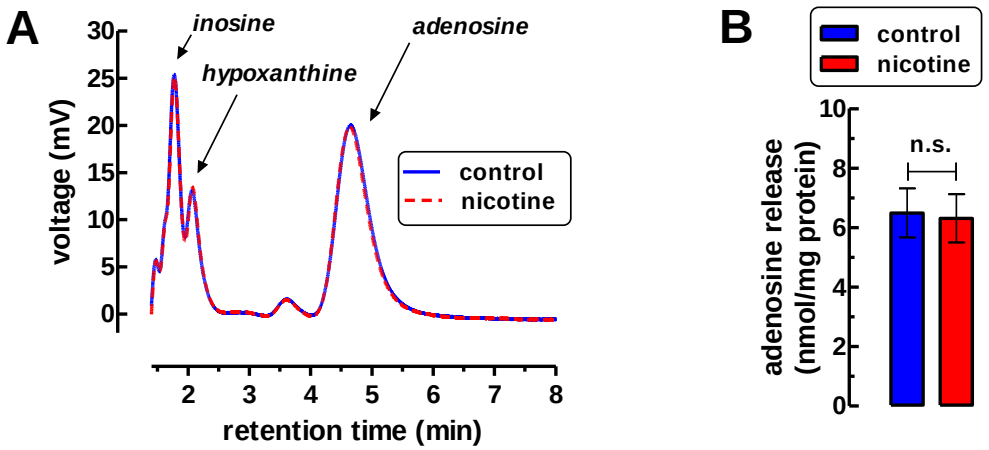


Figure 4.13 - HPLC analysis reveals that striatal synaptosomes release adenosine. (A) Representative chromatograms and (B) mean ± SEM values for the quantified extracellular levels of adenosine and its metabolites in the presence of the adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) (20 µM), upon incubation of synaptosomes (~1.2 mg protein/ml) in the absence or in the presence of nicotine (1 µM); similar results were obtained with 30 nM nicotine (not shown). Data are mean ± SEM of 4 experiments; n.s., not significant.

4.4 NICOTINE-INDUCED MOTOR SENSITIZATION IS FACILITATED BY IN VIVO A_{2A}R BLOCKADE

The interplay between nAChRs and A_{2A}Rs controlling dopamine release from striatal nerve terminals prompted us to test the *in vivo* relevance of this A_{2A}Rs-α6β2-containing nAChRs interaction. One simple measure of nicotine action is its ability to induce hyperlocomotion (Grottick *et al.*, 2000), which can be rated using the open field test (Walsh and Cummins, 1976; Prut and Belzung, 2003).

Before the test period the rats were adapted to the room were the assays were performed and to the open field arena, to insure that the novelty of the environment did not influence the test results. To that effect, the rats were placed in the open field arena for 30 min, in the first two consecutive days of experiment (habituation). After the two days of habituation to the apparatus, the rats were daily injected with the drugs (or vehicles) and tested for locomotor activity in the open field for 30 min, for eight consecutive days (sensitization), after which they remained in withdrawal (drug-free) for the following eight days. On day 8 of the drug-free period (day 18 of the experiment) all the rats received a single injection of nicotine and were tested in the open field (challenge), to assess the development of sensitization.

In the test period, one daily injection of nicotine (0.5 mg/kg of animal body weight), significantly ($p < 0.05$) enhanced locomotor activity (*i.e.* sensitization) at the 7th day, as compared to control rats (*i.e.* vehicle-vehicle-treated) (Fig. 4.14). However, the nicotine-induced sensitization was blunted by the subsequent drug-free period (withdrawal) (Fig. 4.14), as indicated by similar locomotor activity between nicotine-vehicle and vehicle-vehicle-treated groups ($n = 4$, $p > 0.05$) in the challenge day. The ZM241385 dose used (1 mg/kg of animal weight) did not significantly alter *per se* the locomotor activity of the animals (Fig. 4.14) since it remained similar to the locomotor activity of control animals throughout the duration of the experiment. Remarkably, ZM241385-pretreated rats developed a sensitization to nicotine already at day 2 of injection, which was no longer blunted by nicotine withdrawal in the drug-free period ($n = 5$, $p < 0.05$), *i.e.* locomotor activity of the ZM241385-pretreated rats remained higher

until the challenge day when compared to the ZM241385-vehicle group (Fig. 4.14).

Therefore, the results obtained showed that treatment with an antagonist of the A_{2A} adenosine receptors (ZM241385) enhanced the locomotor sensitization to nicotine, and this sensitization was maintained even after a period of several days of drug abstinence, reinforcing the contention for a functional interaction between the nAChRs and the A_{2A}Rs.

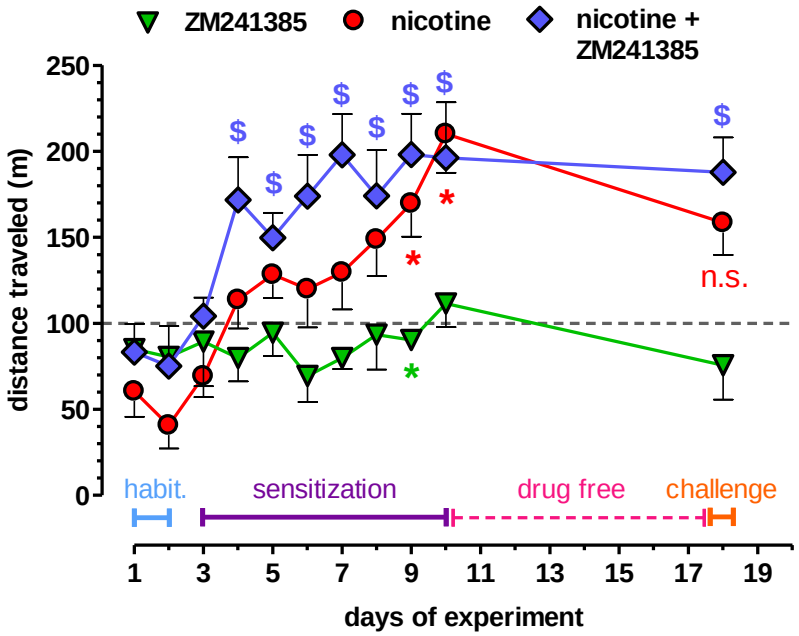


Figure 4.14 - Adenosine A_{2A}R blockade *in vivo* facilitates the sensitization to nicotine stimulated hyperlocomotion. Sixteen rats were used to create factorial × groups for vehicle-vehicle, ZM241385-vehicle, vehicle-nicotine and ZM241385-nicotine injections. The A_{2A}R antagonist ZM241385 (1 mg/kg) or its vehicle was injected 30 min before the test, while nicotine (0.5 mg/kg) or its vehicle was injected immediately before the test. After the first two days of 30 min habituation to the open field arena each day, the rats received daily injections for 8 days. Significant sensitization to nicotine developed on day 7, which was blunted by the subsequent week of abstinence, as determined by a single injection of nicotine (challenge) on the day 8 of abstinence (last day of the experiment), resulting in no statistically significant difference between the vehicle-vehicle group (to which all data points were normalized, represented by the dashed line) and the nicotine-vehicle group, represented by the red circle, at day 18 of the experiment. ZM241385 had no or a minimal hypolocomotor effect throughout the duration of the experiment (green triangles). However, ZM241385-injected rats developed sensitization to nicotine already on day 2 (blue diamonds), which was not blunted by the drug-free period, as indicated by a statistically significant difference

between the ZM241385-vehicle group (green upside-down triangles) and the ZM241385-nicotine group (blue diamonds) at day 18 of the experiment. Data are mean ± SEM of distance traveled during each 30 min of open field observation. A *p < 0.05 representing statistical differences between the nicotine injected (red circles) or ZM241385-injected (green triangles) and the vehicle-injected (dashed line) rats; and $^{\$}$p < 0.05 representing statistical differences between the ZM241385-injected and the ZM241385+nicotine-injected rats (blue diamonds), as determined with ANOVA of repeated measures and Dunett's post-hoc analysis.

5. Discussion and Main Conclusions

DISCUSSION

The present study provides the first pharmacological evidence for adenosine A_{2A}Rs exerting a negative control on the α6β2-containing nAChR-mediated stimulation of dopamine release from striatal dopaminergic terminals. This observation strengthens the notion that A_{2A}Rs mainly act as fine-tuners of different other neurotransmitters systems (Sebastião and Ribeiro, 2009). In fact, striatal presynaptic A_{2A}Rs can negatively control metabotropic receptors such as adenosine A_1Rs (Ciruela *et al*., 2006) and cannabinoid CB_1 receptors (CB_1Rs) (Martíre *et al*., 2011), as well as potentiate glutamate metabotropic group 5 receptors (mGluR5, Rodrigues *et al*., 2005) or catalytic receptors, such as glial cell line-derived neurotrophic factor (GDNF) receptors (Gomes *et al*., 2006, 2009). In other brain areas, A_{2A}Rs also have been shown to control the function of ionotropic receptors, such as *N*-methyl-$_D$-aspartate (NMDA) (Rebola *et al*., 2008), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) (Dias *et al*., 2012) or γ-aminobutyric acid $(GABA)_A$ receptors (Roseti *et al*., 2008). Additionally, A_{2A}Rs also control the rate of desensitization of different nAChRs in peripheral preparations, including the myenteric plexus (Duarte-Araújo *et al*., 2004) and the carotid body (Fitzgerald *et al*., 2009), or in heterologous expression systems (Di Angelantonio *et al*., 2011). The present work extends the understanding of control of the function of ionotropic receptors to the CNS, in particular to α6β2-containing nAChRs in striatal dopaminergic terminals, which are shown to be controlled by A_{2A}Rs.

The Western blot results obtained in this study, detailing the synaptic and subsynaptic distribution of some of the main subunits of nAChRs, demonstrated that in rodent striatum the α7, α6, α4 and β2 nAChR subunits are localized at nerve terminals, were they are able to modulate neurotransmitter release. The localization of these nAChR subunits in nerve terminals of the striatum has already been reported (Jones *et al*., 2001; Marchi *et al*., 2002; Zoli *et al*., 2002; Grady *et al*., 2007), but very little data was previously available on their subsynaptic localization, particularly the presence in the presynaptic active zone. Indeed, the use of a fractionation methodology that accomplishes the separation of pre-, post- and extra-synaptic fractions allows a comprehensive

discernment of the distribution of the various nAChR subunits in the different synaptic zones of the nerve terminals.

The α7 nAChRs, commonly composed by a pentamer of α7 subunits, have been shown to be present in glutamatergic terminals of rat and human striatum (Marchi *et al*., 2002), among other brain areas (Jones and Wonnacott, 2004) where they modulate glutamate release. However, α7 nAChRs seem to be absent from mesolimbic dopaminergic neurons (Zoli *et al*., 2002). Our results support these observations since it was detected α7 subunit immunoreactivity in the rat striatal nerve terminals and a predominant presynaptic localization at the subsynaptic level, both in the active zone and in extrasynaptic sites. However, using an antagonist against the α7 nAChRs, we observed that these receptors are not involved in mediating the nicotine-stimulated dopamine release from isolated nerve terminals. This is probably due to α7 nAChRs being predominantly present in glutamatergic rather than dopaminergic terminals (Kaiser and Wonnacott, 2000; Marchi *et al*., 2002; Zoli *et al*., 2002; Grady *et al*., 2007) or because of their low affinity for nicotine (Köfalvi *et al*., 2000; Toyohara and Hashimoto 2010). The α7 nAChRs have also been localized in glial cells, such as microglia and astrocytes, playing a role in modulating immunological responses (Conejero-Goldberg *et al*., 2008) and in that way these non-neuronal α7 nAChRs could tentatively be included in the synaptosomal membrane samples, adding to the immunoreactivity of the α7 subunit observed in the Western blot assay. However, the isolation procedure using percoll yields purified synaptosomal membranes, which have a low content of glial membranes (Turner and Backelard, 1987), thus assuring the predominant neuronal origin of the synaptosomal samples. Although our observations clearly highlight the possible importance of the α7 nAChRs as presynaptic modulators of neurotransmitters release, our data obtained using isolated subsynaptic fractions also show the presence of the α7 subunit in striatal postsynaptic terminals (albeit at low levels), where they might play a role in fast direct synaptic transmission. Indeed, this process of fast synaptic transmission was shown to be mediated by α7 nAChRs in the hippocampus (see Dani and Bertrand, 2007; Albuquerque *et al*., 2009).

Our data also showed that the β2 subunit of nAChRs was localized in synaptosomal membranes, supporting the notion that this subunit is

predominant in striatum nerve terminals. The β2 subunit is not able to form functional nAChRs by itself, as does the α7 subunit, so it assembles with other nAChR subunits like the α4 and α6 (Gotti *et al.,* 2007). Consequently, the β2 subunit is a component of different main nAChR subtypes identified in the striatum, such as the α4β2* and α6β2* nAChRs (see Grady *et al*., 2007). In fact, immunoprecipitation studies suggest that all nAChRs present in dopamine axon terminals contain the β2 subunit (Champtiaux *et al.,* 2003; Salminen *et al.,* 2004).

Regarding the distribution of α4 subunit it was observed that it is localized in nerve terminals as demonstrated by the immunolabeling of the subunit in purified synaptosomes. Notably, the subsynaptic distribution of the α4 subunit closely resembles that of the β2 subunit since in the striatum of rats both subunits were predominantly detected in the extrasynaptic fraction, outside the active zone, and in the striatum of mice the two subunits were mainly found in the presynaptic fraction, at the synaptic active zone. This is not surprising considering that together they can form α4β2* nAChRs subtypes, which are the most abundant in the rodent striatum (Grady *et al*., 2007), and also in the mammalian brain (Millar and Gotti, 2009). The synaptic and subsynaptic distribution observed for the α4 and β2 subunits is in agreement with several studies that have reported that the α4 and β2 subunits are localized in striatal dopaminergic and GABAergic nerve terminals, and participate in the modulation of dopamine and GABA release (Kaiser and Wonnacott, 2000; Zoli *et al*., 2002; Grady *et al*., 2007; Exley and Cragg, 2008, Exley *et al*., 2012). The predominant localization of these subunits in the synaptic active zone and in synaptic regions outside the active zone, observed in the present study, highlights the reported capacity of β2* nAChR subtypes to modulate neurotransmitter release both directly and indirectly. Indeed, the α4β2* (non-α6) nAChRs likely mediated a fraction of the nicotine-induced [^{3}H]dopamine release observed in this study.

The results obtained for the localization of the α6 subunit reveal that it is present in striatal nerve terminals, as determined by the labeling of striatal synaptic membranes with a subunit selective antibody. Surprisingly, at the subsynaptic level the α6 subunit was mostly observed postsynaptically, albeit being also significantly present in the presynaptic and extrasynaptic fractions. In a different way, previous studies concerning the localization of the α6* nAChRs

strongly suggested a predominant presynaptic localization of these receptor subtypes in dopaminergic nerve terminals (see Zoli *et al*., 2002; Yang *et al*., 2009; Quik *et al*., 2011) which represent only about 1/5 of the total number of nerve terminals in the striatum (Borycz *et al*., 2007; Gomes *et al*., 2009). We confirmed our results by using a second α6 subunit specific antibody (raised against the carboxyl-terminus of human CHRNA6, not shown) and the data obtained confirm the predominant postsynaptic localization of the α6 subunit. Recent studies suggest that functional postsynaptic α6* nAChRs are located on somatodendrites of dopaminergic neurons in the ventral tegmental area (Drenan *et al*., 2008). Our results prompt us to contend for an unreported role of postsynaptic α6* nAChRs in mediating cholinergic transmission; or for the putative existence of an unidentified α6-containing nAChR subtype in striatal postsynaptic regions. Although our data showed a main localization of the α6 subunit in postsynaptic fractions, this subunit was also identified at presynaptic and extrasynaptic locations, consistent with the α6* nAChRs function to modulate neurotransmitter release (see Yang *et al*., 2009; Quik *et al*., 2011). Consequently, we observed that α6* nAChRs are responsible for modulating ~70% of the dopamine release induced by nicotine, in isolated striatal nerve terminals, thus confirming the reports that indicate the α6-containing nAChRs as main players in regulating dopamine release in the striatum (see Yang *et al*., 2009; Quik *et al*., 2011).

The subsynaptic distribution of the α7, α4, α6 and β2 subunits of nAChRs was assessed in nerve terminals from both Wistar rats and C57BL/6 mice, allowing us to conclude that the localization of the subunits is similar between the two species. The results highlight however a substantial difference in the localization of the α4 and β2 subunits within the nerve terminals (presynaptic active zone and extrasynaptic locals), between Wistar rats and C57BL/6 mice.

Taken together the data obtained about the synaptic and subsynaptic distributon of different nAChR subunits, combined with the pharmacological characterization of nAChRs that control dopamine release induced by nicotine, indicate an important role of the α6β2-containing nAChRs to mediate the dopamine releasing action of nicotine. This is in agreement with previous findings showing that the absence of β2 subunits abrogates the ability of nicotine to trigger dopamine release from synaptosomes obtained from three

different mouse brain regions**,** striatum, nucleus accumbens and olfactory tubercle (Grady *et al*., 2002). Moreover, it was also demonstrated that the α6 subunit has an important role in the regulation of mesolimbic dopamine release (Calabresi and Di Filippo, 2008; Drenan *et al*., 2008; Meyer *et al*., 2008; Quik *et al*., 2011).

In the present study, the contention for the involvement of A_{2A}Rs in controlling nAChRs function was based on the antagonism of the effect of CGS21680 (inhibition) by ZM241385 (facilitation) on nicotine-induced dopamine release in striatal synaptosomes. Since ZM241385 can also block A_{2B}Rs, although with a 10-fold lower potency to inhibit A_{2B}Rs compared to A_{2A}Rs (Poucher *et al*., 1995; Ji and Jacobson, 1999), we also probed the possible involvement of A_{2B}Rs in facilitating the nicotine-stimulated dopamine release. The data obtained showed that the selective A_{2B}R antagonist, MRS1754 largely inhibited (contrarily to the potentiation observed with ZM241385) the effect of nicotine at a concentration (200 nM) three-fold lower than its IC_{50} at A_{2A}Rs. Furthermore, our data showed that the concomitant inhibition of both A_{2A}Rs and A_{2B}Rs extinguished each other's effect. This may mean two subsets of dopaminergic terminals bearing either A_{2A}Rs or A_{2B}Rs, leading to a lack of net change, or alternatively that the two receptors may reside and interact in the same nerve terminals. Intriguingly, neither ZM241385 nor MRS1754 affected dopamine release *per se*, but when combined they synergistically stimulated dopamine release. This was also observed when testing the non-selective adenosine receptor antagonist, caffeine, an observation that may be pertinent to the understanding of the addictive profile of caffeine (Svikis *et al*., 2005). Although the underlying mechanism for how A_{2A}R activation or the simultaneous A_{2A}R/A_{2B}R blockade increases basal dopamine outflow is unclear, these data provide the first demonstration of a functional interaction between the two A_2R subtypes in the CNS, in a manner similar to that previously reported to occur in splenocytes (Moriyama and Sitkovsky, 2010). Apart from the ability of A_{2A}Rs to control the α6β2-containing nAChR-induced release of dopamine, the present results also showed that CGS21680 *per se* stimulated the release of dopamine; this is in agreement with functional and morphological data identifying the presence of A_{2A}Rs in dopaminergic nerve endings in the striatum (Chowdhury and Fillenz, 1991; Gomes *et al*., 2006, 2009) and also,

with the ability of striatally microinfused CGS21680 to increase basal dopamine levels in freely moving rats (Gołembiowska and Zylewska, 1997).

In order to assess if extrasynaptosomal adenosine might be responsible for tonic activation of adenosine receptors, the levels of this nucleoside were measured in the synaptosomal preparation. Extracellular adenosine was detected, which shows that adenosine is indeed released by synaptosomes. Furthermore, we found that nicotine did not modify the extracellular levels of adenosine. These results clearly support the notion that, in our system, there is always a tonic activation of adenosine receptors by endogenous extracellular adenosine, which explains the ability of the antagonists of adenosine receptors to modify the dopamine release from superfused synaptosomes.

The ability of presynaptic A_{2A}Rs to control the α6β2-containing nAChR-induced release of dopamine was extended to an *in vivo* setting, by showing that A_{2A}Rs also control the locomotor sensitization induced by nicotine. This locomotor sensitization to nicotine is known to involve the recruitment of β2-containing nAChRs (Picciotto *et al*., 1998) and a differential participation of α4* and α6* nAChRs, but not of α7 nAChRs (Kempsill and Pratt, 2000; Tapper *et al*., 2004; Gotti *et al*., 2010; Smith *et al*., 2010), controlling the release of dopamine in different regions of the basal ganglia. This differential adaptation of different α4-containing and α6-containing nAChRs upon repeated nicotinic exposure (Tapper *et al*., 2004; Perry *et al*., 2007; Perez *et al*., 2008; Smith *et al*., 2010) is a likely explanation for the potentiation of nicotinic locomotor sensitization by the tested A_{2A}R antagonist. This is in general agreement with previous studies showing that the non-selective adenosine receptor antagonist, caffeine, bolsters the nicotine-induced increase of locomotor activity (Cohen *et al*., 1991). However, it is worth noting that the rewarding properties of nicotine, tested in a place-conditioning paradigm, were decreased in global A_{2A}R knockout mice (Castañé *et al*., 2006); heralding the hypothesis that different subtypes of nAChRs might be differently controlled by A_{2A}Rs.

In conclusion, this work details the diverse distribution of the subunits that constitute some of the main nAChR subtypes in the striatum. The localization of the receptors in the various compartments at the synapse relate to the function they perform in the neuronal cells, and our results relevantly show the presence of nAChR subtypes located presynaptically, and especially in the presynaptic

active zone, where they are able to directly modulate neurotransmitter release. Accordingly we present, to our knowledge, the first report of a functional interaction between nicotinic and adenosine receptors in the striatum *i.e,* that A_{2A}Rs curtail the function of α6β2-containing nAChRs in striatal dopaminergic nerve terminals, an effect that seems relevant for the ability of A_{2A}R antagonists to potentiate the psychomotor effects resulting from a repeated exposure to nicotine. These observations provide a mechanistic insight to explain the frequent correlation in nicotine and caffeine abuse (Swanson *et al*., 1994). This A_{2A}R-nAChR interaction also paves the way to foster novel therapeutic opportunities to manage motor diseases related with dysfunctional dopamine signalling such as Parkinson's disease, where both caffeine and nicotine provide a combined prophylactic benefit (Powers *et al*., 2008) and where A_{2A}R antagonists are a leading non-dopaminergic therapeutic strategy (Prediger, 2010; Hickey and Stacy, 2012).

Interestingly, the mechanisms underlying the A_{2A}R-mediated amelioration of Parkinson's disease symptoms are not fully understood and it is also possible that it may involve a rescuing of nAChRs-stimulated phasic dopamine release (see Threlfell *et al*., 2012), inviting further investigation.

MAIN CONCLUSIONS

1. The subunits α7, α4, β2 and α6 of nAChRs were identified by Western blot in the terminals (synaptosomes) of striatal neurons from Wistar rats and C57BL/6 mice.

2. At the synapse of rodent striatal neurons the α7 subunit was enriched in the presynaptic active zone, the α4 and β2 subunits were mostly present in the presynaptic active zone and in extrasynaptic sites whereas the α6 subunit was primarily localized in the postsynaptic region.

3. Nicotine induced the release of dopamine from rat striatal nerve terminals in a concentration-dependent manner. The $A_{2A}R$ agonist CGS21680 inhibited whereas the $A_{2A}R$ antagonist ZM241385 facilitated the nicotine-stimulated release of dopamine, revealing a previously unknown functional interaction between nAChRs and $A_{2A}Rs$ in the striatum.

4. The blockade of the α6-containing nAChRs by a selective antagonist abolished the action of the $A_{2A}R$ ligands CGS21680 and ZM241385 on the nicotine-stimulated dopamine release from nerve terminals of the striatum, implicating the α6-containing nAChR subtypes as the targets for control by $A_{2A}Rs$.

5. The locomotor activity of rats increased when treated with the $A_{2A}R$ antagonist ZM241385 in addition to nicotine. The increase in locomotor activity persisted even after a withdrawal period, thus demonstrating the *in vivo* relevance of the interaction between nAChRs and $A_{2A}Rs$.

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