UNIVERSIDADE DA BEIRA INTERIOR
**Ciências da Saúde**

# Relationship Between the Adenosine $A_1$ Receptor and cGMP Levels in the Hippocampus: Implications on Neurotransmission and Synaptic Plasticity

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Orientador: Prof. Doutor José Francisco Cascalheira

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# Dedicatory

To my family

“Live as if you were to die tomorrow. Learn as if you were to live forever.”

Mahatma Gandhi

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# List of Publications

## Publications resulting from this doctoral work

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II. **Serpa A**, Correia S, Ribeiro JA, Sebastião AM, Cascalheira JF, 2015. The combined inhibitory effect of the adenosine $A_1$ and cannabinoid CB1 receptors on cAMP accumulation in the hippocampus is additive and independent of $A_1$ receptor desensitization. *Biomed Research International* 2015: 872684.

III. **Serpa A**, Pinto I, Bernardino L, Cascalheira JF, 2015. Combined neuroprotective action of adenosine $A_1$ and cannabinoid CB1 receptors against NMDA-induced excitotoxicity in the hippocampus. *Neurochem International* (in press).

IV. Pinto I, **Serpa A**, Cascalheira JF, Sebastião AM, 2015. The role of cGMP on adenosine $A_1$ receptor-mediated inhibition of synaptic transmission at the hippocampus. *Submitted for publication*.

## Other publications

I. **Serpa A**, Ribeiro JA, Sebastião AM, 2009. Cannabinoid CB1 and adenosine $A_1$ receptors independently inhibit synaptic hippocampal transmission. *European Journal of Pharmacology* 623: 41-46.

II. Cascalheira JF, Gonçalves M, Barroso M, Castro R, Palmeira M, **Serpa A**, Dias-Cabral AC, Domingues FC , Almeida S, 2015. Association of the transcobalamin II gene 776C→G polymorphism with Alzheimer's type dementia: dependence on the 5,10-methylenetetrahydrofolate reductase 1298A→C polymorphism genotype. *Annals of Clinical Biochemistry* 52: 448-455.

III. Soares AO, **Serpa A**, 2007. Interference competition between ladybird beetle adults (Coleoptera: Coccinellidae): effects on growth and reproductive capacity. *Population Ecology* 49: 37-43.

## Publications in proceedings of scientific meetings

I. **Serpa A**, Correia S, Ribeiro JA, Sebastião AM, Cascalheira JF, 2012. Additive inhibitory modulation of cAMP accumulation in the hippocampus by the adenosine $A_1$ and cannabinoid CB1 receptors is independent of $A_1$ receptor desensitization. 22$^{nd}$ IUBMB & 37$^{th}$ FEBS Congress, Sevilla, Spain, 4-9 September. FEBS Journal **279:** S385.

# List of scientific communications

## Poster presentations of this doctoral work

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II. **Serpa A**, Ribeiro JA, Sebastião AM, Cascalheira JF. Inhibition of hippocampal cAMP formation by adenosine $A_1$ and cannabinoid CB1 receptors is additive and independent of $A_1$ receptor desensitization – VIII FENS Forum of Neuroscience, Barcelona, Spain, 4-18 July 2012.

III. **Serpa A**, Sebastião AM, Ribeiro JA, Cascalheira JF. Adenosine $A_1$ and cannabinoid $CB_1$ receptors additively inhibit cAMP accumulation in the hippocampus – XVII Congresso Nacional de Bioquímica, Porto, Portugal, 15-17 December 2010.

## Other poster presentations

I. **Serpa A**, Ribeiro JA, Sebastião AM. $A_1$ adenosine and CB1 cannabinoid receptors independently inhibit synaptic transmission in the rat hippocampus – XXXVII Reunião Anual da Sociedade Portuguesa de Farmacologia e XXIV Reunião de Farmacologia Clínica, Porto, Portugal, 5-7 December 2006.

# Resumo Alargado

Os nucleótidos cíclicos cAMP e cGMP são segundos mensageiros intracelulares cujos níveis são modulados por recetores acoplados a proteínas G, e regulam diversas funções cerebrais, incluindo neurotransmissão, memória e plasticidade sináptica. O cAMP ativa diretamente a cinase A de proteína (PKA), o seu principal efetor, regulando a libertação de neurotransmissores através da ativação de canais de $Ca^{2+}$ ou inativação de canais de $K^{+}$. O cAMP também está implicado na regulação da memória e plasticidade sináptica no hipocampo por meio de ativação direta de EPACs (proteínas permutadoras ativadas pelo cAMP) e através da ativação do CREB (proteína de ligação ao elemento de resposta ao cAMP) mediada pela PKA. O sinal de cAMP é transitório sendo regulado através da ação concertada da ciclase do adenililo e de fosfodiesterases dos nucleótidos cíclicos (PDEs). O cGMP é produzido por duas vias distintas. Uma envolve a ciclase do guanililo solúvel citoplasmática (sGC) cujo agonista, o óxido nítrico (NO), é produzido por ação da sintase do NO (NOS), a qual é ativada pelo cálcio, enquanto a outra envolve a ciclase do guanililo particulada ligada à membrana, a qual é activada por péptidos natriuréticos. A cinase G de proteína (PKG) é o principal efetor direto do cGMP, mediando a maior parte dos efeitos deste no sistema nervoso.

O recetor $A_1$ da adenosina é expresso em abundância no hipocampo onde medeia muitas das ações da adenosina extracelular, como a inibição da libertação de neurotransmissores, proteção contra insultos excitotóxicos e regulação da plasticidade sináptica. O recetor $A_1$ da adenosina está acoplado a proteínas $G_{i/o}$ que regulam negativamente a ciclase do adenililo (AC) e consequentemente a formação de cAMP. Além de inibir a AC, o recetor $A_1$ também inibe canais de cálcio do tipo P/Q, ativa canais de potássio e regula a formação de fosfatos de inositol. A modulação dos níveis de cGMP através de recetores acoplados a proteínas $G_{i/o}$ também foi recentemente referida. Neste trabalho foi investigada a capacidade dos recetores $A_1$ da adenosina para regular os níveis de nucleótidos cíclicos no hipocampo. O papel dos nucleótidos cíclicos como mediadores de algumas das ações dos recetores $A_1$ da adenosina também foi estudado.

Foi anteriormente demostrado que a ativação dos recetores $A_1$ da adenosina diminui a formação de cAMP no córtex cerebral, mas o seu efeito nos níveis de cAMP no hipocampo não está clarificado, nem a sua interação com outros neuromoduladores enquanto reguladores dos níveis de cAMP. Pretendeu-se então determinar o tipo de interação entre os recetores $A_1$ da adenosina e CB1 dos canabinóides como moduladores negativos da acumulação de cAMP. Para além disso, o potencial neuroprotetor combinado dos dois recetores foi também investigado. A quantificação dos níveis de cAMP em fatias de hipocampo foi efetuada através de um ensaio imunoenzimático, e a neuroprotecção contra a toxicidade induzida por N-metil-D-aspartato (NMDA) foi quantificada através da medição colorimétrica da atividade da desidrogenase do

lactato (LDH) libertada e da quantificação, por microscopia de fluorescência, da captação de iodeto de propídio (IP) em culturas organotípicas de fatias de hipocampo. A $N^6$-Ciclopentiladenosina (CPA), agonista seletivo dos recetores $A_1$ da adenosina, diminuiu a acumulação de cAMP estimulada pela forscolina em fatias de hipocampo, com um $EC_{50}$ de 35 ± 19 nM e um $E_{max}$ de 29% ± 5%, enquanto que para o agonista CB1, WIN55212-2, foram obtidos um $EC_{50}$ de 6,6 ± 2,7 μM e um $E_{max}$ de 31% ± 2%. Quer o efeito da CPA quer o efeito do WIN55212-2 foram revertidos na presença de DPCPX (um antagonista seletivo dos recetores $A_1$) e de AM251 (antagonista seletivo dos recetores CB1), respetivamente. O NMDA (50μM) aumentou a atividade da LDH libertada em 92% ± 4% (n=4) quando comparada com o controlo. A aplicação de WIN55212-2 (30 μM) diminuiu a atividade da LDH induzida por NMDA em 53% ± 11% (n=4), enquanto a CPA (100 nM) diminuiu-a em 37% ± 11% (n=4). O efeito inibitório combinado do WIN55212-2 (30 μM) e da CPA (100 nM) na acumulação de cAMP (41% ± 6%, n=4) e na atividade da LDH induzida pelo NMDA (88% ± 14%, n=4) não foi diferente da soma dos efeitos inibitórios individuais de cada agonista (43% ± 8%, n=4, para a acumulação do cAMP e 90 % ± 22%, n=4, para a libertação de LDH), mas foi diferente do efeito da CPA e do WIN55212-2 quando aplicados sozinhos. Similarmente, um efeito inibitório aditivo da co-aplicação de WIN 55212-2 (30 uM) e CPA (100 nM) na captação de IP induzida pelo NMDA (50 uM), foi observado na região CA3 mas não na região CA1 da fatia de hipocampo. Assim, o efeito combinado de agonistas CB1 e $A_1$ na acumulação de cAMP e na neurotoxicidade induzida pelo NMDA mostrou ser aditivo, sugerindo que ambos os agonistas desencadeiam vias de sinalização independentes associadas ao cAMP, e produzem neuroprotecção cumulativa independente face a insultos excitotóxicos no hipocampo.

Estudos prévios indicam que o cGMP produz efeitos similares aos dos recetores $A_1$ da adenosina e recentemente foi referido que o cGMP pode mediar algumas das ações dos recetores $A_1$ da adenosina no sistema nervoso periférico. No entanto, o papel do cGMP na atividade mediada pelos recetores $A_1$ da adenosina no sistema nervoso central permanece obscuro. No presente projeto, pretendeu-se investigar se o cGMP é modulado pelos recetores $A_1$ da adenosina no hipocampo, se essa modulação depende da ativação da ciclase do guanililo solúvel, e se esse mecanismo de modulação é idêntico entre ratos machos e fêmeas. Para além disso, também se investigou o papel do cGMP no efeito inibitório do recetor $A_1$ da adenosina na neurotransmissão no hipocampo. Para atingirmos os nossos objetivos, utilizámos duas abordagens experimentais: realização de ensaios imunoenzimáticos para medir a acumulação de cGMP e recurso a eletrofisiologia extracelular para medir a transmissão sináptica na fatia de hipocampo de rato. Os ensaios imunoenzimáticos revelaram que a aplicação de CPA aumentou a acumulação de cGMP com um $EC_{50}$ de 4,2 ± 1,4 nM e um $E_{max}$ de 17% ± 0,9%. Por outro lado, em ratos machos a presença de um dador de NO, o nitroprosseto de sódio (SNP), aboliu o efeito da CPA na acumulação de cGMP. Em contraste, em ratos fêmeas, o SNP não modificou o aumento na acumulação de cGMP induzido pela CPA, no entanto este aumento foi revertido pelo DPCPX, indicando que os recetores $A_1$ modulam a

acumulação de cGMP mesmo quando a atividade da ciclase do guanililo solúvel é aumentada pelo SNP. Portanto, os recetores $A_1$ aumentam os níveis intracelulares de cGMP no hipocampo, através de mecanismos que dependem do sexo. Em relação aos estudos de eletrofisiologia extracelular, investigámos se o bloqueio de componentes da via do cGMP, através do uso de inibidores da NOS, da PKG e da sGC, interferiria com o efeito inibitório da CPA na transmissão sináptica. A CPA (15 nM) diminuiu de uma forma reversível a transmissão sináptica em 48% ± 2,1% (n=5) nos machos e em 54 % ± 5 % nas fêmeas (n=5). Na presença de L-NAME (300 μM, um inibidor da NOS), da ODQ (10 μM, um antagonista da sGC) e do KT5823 (1 nM, um inibidor da PKG), a inibição da transmissão sináptica pela CPA foi atenuada em 57 % ± 9 % (n=5), 23 % ± 7 % (n=4) e 49 % ± 9 % (n=4), respetivamente. Esta atenuação do efeito da CPA foi similar em machos e fêmeas. Estes resultados sugerem que a atividade neuromodulatória do recetor $A_1$ na transmissão sináptica depende parcialmente da via do cGMP.

# Palavras Chave

Recetor $A_1$ da adenosina, cGMP, cAMP, hipocampo, recetor CB1 dos canabinóides, neurotransmissão, neuroprotecção

# Abstract

The cyclic nucleotides cAMP and cGMP are second messengers whose levels are modulated by G proteins-coupled receptors, and regulate multiple brain functions, including neurotransmission, memory and synaptic plasticity. The adenosine $A_1$ receptor is highly expressed in the hippocampus where it inhibits neurotransmitter release, protects against excitotoxic insults and regulates synaptic plasticity. Adenosine $A_1$ receptor is coupled to $G_{i/o}$ proteins which negatively regulate adenylyl cyclase and thus cAMP formation. Modulation of cGMP levels by $G_{i/o}$ proteins-coupled receptors has also been recently reported. In the present work the ability of adenosine $A_1$ receptors to regulate cyclic nucleotides levels in the hippocampus was investigated. The role of cyclic nucleotides as mediators of some actions of adenosine $A_1$ receptor in the hippocampus was also studied.

Activation of adenosine $A_1$ receptor has been shown to decrease cAMP formation in the cerebral cortex, but its effect on cAMP levels at the hippocampus is not clarified, nor its interaction with others neuromodulators while regulating cAMP levels. We set forth to determine the type of interaction found between adenosine $A_1$ and cannabinoids CB1 receptors as negative modulators of cAMP accumulation. Furthermore, we also intend to explore their combined neuroprotective potential. Quantification of cAMP in hippocampal slices was performed through an enzymatic immunoassay, while neuroprotection against NMDA-induced toxicity was assessed by determination of released LDH activity and by quantification, by fluorescence microscopy, of the uptake of propidium iodide (PI) in cultured organotypical hippocampal slices. The $A_1$ agonist $N^6$-Cyclopentyladenosine (CPA) decreased forskolin-stimulated cAMP accumulation in the hippocampal slice with an $EC_{50}$ of 35 ± 19 nM and an $E_{max}$ of 29% ± 5%, whereas for the CB1 agonist, WIN55212-2, an $EC_{50}$ of 6.6 ± 2.7 μM and an $E_{max}$ of 31% ± 2% were obtained. NMDA (50 μM) increased the release of LDH activity by 92% ± 4% (n=4) when compared with control. Application of WIN55212-2 (30 μM) decreased NMDA-induced LDH activity by 53% ± 11% (n=4), while CPA (100 nM) decreased it by 37% ± 11% (n=4). The combined inhibitory effect of WIN55212-2 (30 μM) and CPA (100 nM) on cAMP accumulation (41% ± 6%, n=4) and NMDA-induced LDH release (88% ± 14%, n=4) did not differ from the sum of the individual inhibitory effects of each agonist (43% ± 8%, n=4, for cAMP accumulation and 90 % ± 22%, n=4, for LDH release), but was different from the effects of CPA or WIN55212-2 alone. Similarly, an additive inhibitory effect of co-application of WIN55212-2 (30μM) and CPA (100nM) on NMDA (50μM)-induced PI uptake was also observed in CA3 but not in CA1 area of the hippocampal slice. Thus, the combined effect of CB1 and $A_1$ agonists on cAMP accumulation and NMDA-induced neurotoxicity is additive suggesting that both agonists trigger independent cAMP signalling pathways and produce independent cumulative neuroprotection against excitotoxic insults in the hippocampus.

Previous studies indicate that cGMP produces similar effects to those triggered by adenosine $A_1$ receptors and, recently, it was reported that cGMP might mediate some actions of adenosine $A_1$ receptor in the peripheral nervous system. However, the role of cGMP on adenosine $A_1$ receptor mediated activity at the central nervous system remains obscure. Our aim was to clarify if cGMP is modulated by $A_1$ receptors at the hippocampus, if this modulation depends on activation of the soluble form of guanylyl cyclase, and if such mechanism is identical between male and female rats. Furthermore, the role of cGMP in mediating the inhibitory effect of adenosine $A_1$ receptor on neurotransmission in the hippocampus was also investigated. To achieve our objectives, we used two approaches, enzymatic immunoassays to measure cGMP accumulation and extracellular electrophysiology to measure synaptic transmission at the rat hippocampal slice. The enzymatic immunoassays tests reveal that application of CPA increased cGMP accumulation with an $EC_{50}$ of 4.2 ± 1.4 nM and an $E_{max}$ of 17% ± 0.9%. Furthermore, in male rats, the presence of sodium nitroprosside (SNP, a nitric oxide donor) abolished the effect of CPA on cGMP accumulation. In contrast, in female rats, SNP failed to modify the increase in cGMP accumulation induced by CPA, but this increase was reversed by DPCPX, stressing that $A_1$ receptors modulate cGMP accumulation despite the increase in soluble guanylyl cyclase activity by SNP. Thus, $A_1$ receptors increase intracellular cGMP levels at the hippocampus, through mechanisms which differ according to gender. Regarding extracellular electrophysiology studies, we investigated in what extent blocking the cGMP pathway using nitric oxide synthase (NOS), protein kinase G (PKG) and soluble guanylyl cyclase (sGC) inhibitors, would interfere with the inhibitory effect of CPA on synaptic transmission. CPA (15 nM) alone reversibly decreased synaptic transmission by 48% ± 2.1% (n=5) in males and by 54 % ± 5 % in females (n=5). In the presence of the NOS inhibitor L-NAME (300 μM), the sGC antagonist ODQ (10 μM) and the PKG inhibitor KT5823 (1 nM), CPA-induced inhibition of synaptic transmission was dampened by 57 % ± 9 % (n=5), 23 % ± 7 % (n=4) and 49 % ± 9 % (n=4), respectively. This attenuation of the effect of CPA was similar in males and females. These findings suggest that $A_1$ receptor neuromodulatory activity on synaptic transmission partially depends on the cGMP pathway.

# Keywords

Adenosine $A_1$ receptor, cGMP, cAMP, hippocampus, cannabinoids CB1 receptor, neurotransmission, neuroprotection

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# List of abbreviations

AKAPs: A-kinase anchor proteins

AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AM251: N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide

ATP: adenosine triphosphate

BAY 60-7550: 2-[(3,4-Dimethoxyphenyl)methyl]-7-[(2*R*,3*R*)-2-hydroxy-6-phenylhexan-3-yl]-5-methyl-1*H*-imidazo[5,1-f][1,2,4]triazin-4-one

BDNF: brain derived neurotrophic factor

CA1/CA3; cornu ammonis 1/ cornu ammonis 2

CaMKs: Ca2+/calmodulin-dependent protein kinases

cAMP: cyclic adenosine monophosphate

cGMP: cyclic guanosine monophosphate

CHA: N6-cyclohexyladenosine

CNS: central nervous system

CO: carbon monoxide

CPA: $N^6$-cyclopentyladenosine.

CPT: 8-cyclopentyltheophylline

CREB: cAMP response element-binding protein

DREAM: downstream regulatory element antagonistic modulator

DPCPX: 1,3-dipropyl-8-cyclopentylxanthine

EPSPs: excitatory postsynaptic potentials

GABA: gamma-aminobutyric acid

GKAPs: guanylate kinase-associated protein

GPCRs: G-protein coupled receptors

GTP: Guanosine-5'-triphosphate

IBMX: 3-isobutyl-1-methylxanthine

KT5823: (9*S*,10*R*,12*R*)-2,3,9,10,11,12-Hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid, methyl ester

L-NAME: L-$N^G$-Nitroarginine methyl ester

LTP: long-term potentiation

LTD: long-term depression

MK-801: Dizocilpine

mRNA: Messenger ribonucleic acid

NMDA: N-methyl-D-aspartate

NO: nitric oxide

NOS: nitric oxide synthase

ODQ: 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one

PDE: phosphodiesterase

PET: Positron emission tomography

PIA: N6-R-phenylisopropyladenosine

PKA/ PKC/ PKG: protein kinase A/ protein kinase C/ protein kinase G

PTX: pertussis toxin

SAH: S-adenosylhomocysteine

sGC: soluble guanylyl cyclase

SNP: sodium nitroprusside

THC: tetrahydrocannabinol

WIN55212-2: (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate

# Chapter 1: General Introduction

## 1.1. The hippocampus

### 1.1.1. Memory

The hippocampus is a region of the mammalian brain formed by a heterogeneous population of neurons that are singularly important in the ability to use spatial, olfactory, auditory, and other contextual cues to learn new tasks and to translate those experiences into memory. It stands out as a crucial structure for the initial encoding and storage of memories. There is a division of labour in memory acquisition between the hippocampus, a fast learner, and the neocortex, slow constructer of connections but with a vast storage capacity (Marr 1970). The hippocampus is required for a form of declarative memory, the episodic memory, but not necessary, according to some authors, for the acquisition of factual knowledge of semantic memory (Vargha-Khadem *et al.* 1997; Verfaellie *et al.* 2000). Episodic memory is the memory of autobiographical events, the collection of past personal experiences that occurred at a particular time and place (see Eldridge 2000). Such distinction between hippocampus-dependent and independent memories was mainly obtained from neuropsychological studies of amnesia in patients with damaged hippocampus (Eichenbaum 1999; Rempel-Clower *et al.* 1996; Zola-Morgan *et al.* 1986). The hippocampus also processes spatial memory. The cognitive mapping theory defends that the activity of hippocampal neurons in both rats and primates reflects information about the spatial organisation of an animal's environment and was based in the discovery that the firing rate of a particular group of hippocampal neurons, the so-called place cells, were correlated with the location of the animal in a test environment (O'Keefe and Dostrovsky 1971) and involved learning the spatial relationships between cues. Indeed, neuroimaging studies in humans have provided evidence that the hippocampus becomes active during spatial navigation and that hippocampal morphology depends on spatial navigation skills (Maguire *et al.* 2000). Accordingly, hippocampal morphology also varies between males and females in rats (Diamond 1987) and across species (West and Schwerdtfeger 1985; Stephan *et al.* 1981) depending on the intensity of spatial processing.

### 1.1.2. The Hippocampus Circuitry

The hippocampus shows an impressive capacity for structural reorganization, remaining plastic throughout life. Plasticity results from the synapses and dendrites of mature neurons which undergo continuous rearrangement, reinforcing or debilitating patterns of neural

activity. Moreover, entirely new neurons are formed by neurogenesis throughout life, particularly in stimulus enriched environments (Kempermann *et al*. 1997). The hippocampus is composed by a trisynaptic circuit, lying within transverse hippocampal lamellae, were the excitatory activity in the entorhinal cortex propagates to the granule cells of the dentate gyrus which project the mossy fibers to the pyramidal neurons of CA3, from which the axons or Schaffer collaterals project to the pyramidal cells of CA1 (Fig 1). With a lamellar organization, a relatively simple structure may mediate complex functions, generating a massive amount of unique outputs. The lamellar hypothesis derives support from the confined modular transverse distribution of the mossy fibers, which innervate dentate hilar neurons, CA3 pyramidal cells and interneurons. However, anatomical studies revealed that hilar mossy cells, CA3 pyramidal cells, and entorhinal cells, along with its lamellar organized fibers, also form axonal projections that cross along the longitudinal axis. Thus, the lamellar organization hypothesis of the hippocampus should be viewed as a convenient simplified model (reviewed in Sloviter and Lomø, 2012).

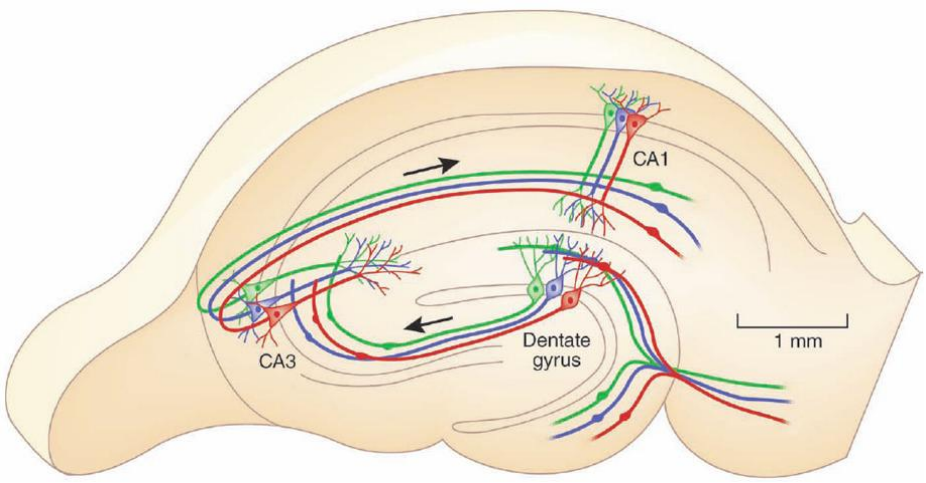


Figure 1. The Hippocampus Circuitry. Each hippocampal lamellae is composed by a trisynaptic circuit, were an excitatory output from the entorhinal cortex depolarizes the granule cells of the dentate gyrus which convey the signal through the mossy fibers to the pyramidal neurons of CA3, from where the signal is transmitted through the Schaffer collaterals to the pyramidal cells of CA1 (adapted from Moser 2011).

### 1.1.3. Synaptic Plasticity

Neurons in the hippocampus have the ability to undergo persistent increases and decreases in synaptic transmission in response to electrical stimuli. Long-term potentiation (LTP) is an enhanced synaptic responsiveness that is elicited by high frequency stimulation of the afferent neurons. A second form of synaptic plasticity, long-term depression (LTD), in which low-frequency stimulation produces a decrease in synaptic responsiveness, is also present in the hippocampus. Both forms of synaptic plasticity are dependent upon N-methyl-D-aspartate

(NMDA*) receptor mediated $Ca^{2+}$ entry (Dudek and Bear 1992; Harris *et al.* 1984). It is thought that these long-term changes in synaptic efficacy may be a mechanism by which enduring neural networks are established during memory acquisition and consolidation. Accordingly, NMDA receptor antagonists have been shown to impair hippocampus dependent learning in rats (Davis *et al.* 1992; Kim *et al.* 1991) and humans (Morris *et al.* 1996).

### 1.1.4. Neurogenesis

In the adult mammalian brain, new neurons are continuously generated in two regions: the subgranular zone of the hippocampal dentate gyrus and the subventricular zone of the lateral ventricles (Ming and Song 2011). A neurogenic niche regulates the sequential steps of adult neurogenesis ensuring continuous neuronal production while maintaining the neural stem cell pool (Kempermann *et al.* 2004). Adult neurogenesis in the hippocampus contributes to learning and memory (Shors *et al.* 2001), and increasing evidence revealed that interference in the process is associated with neurodegenerative and neuropsychiatric diseases, including Huntington's disease (Curtis *et al.* 2003), Parkinson's disease (Winner *et al.* 2011), Alzheimer's disease (Mu and Gage, 2011), schizophrenia and depression (Ouchi *et al.* 2013; Sahay and Hen, 2007). Enriched environment, exercise, or hippocampal-dependent models of learning increase neurogenesis (Kempermann *et al.* 1997; Gould *et al.* 1999; van Praag *et al.* 2000), whereas aging, environmental stress, as well as administration of adrenal glucocorticoids*, decrease neurogenesis in the hippocampus (Kuhn *et al.* 1996; Gould *et al.* 1997; Cameron and McKay, 1999). Neurogenesis may be pharmacologically increased by phosphodiesterase inhibitors which block degradation of cyclic nucleotides. The phosphodiesterase IV (specific for cAMP) inhibitor rolipram and the phosphodiesterase V (specific for cGMP) inhibitors sildenafil and taladafil positively modulate neurogenesis (Palmeri *et al.* 2013; Nakagawa *et al.* 2002; Zhang *et al.* 2006). cGMP, resulting from activation of intracellular pathways by nitric oxide (Puzzo *et al.* 2006), produces its effect on hippocampal neurogenesis by regulating the activity of cyclic nucleotide-gated ion channels (Podda *et al.* 2013), whereas the cAMP effect implicates phosphorylation of the transcription factor CREB* (Nakagawa *et al.* 2002). Downregulation of adult neurogenesis in response to stress and high levels of adrenal glucocorticoids contributes to a decrease in the volume of hippocampus observed in patients with mood disorders (Sheline *et al.* 1996; Steffens *et al.* 2000). Hippocampal neurogenesis is upregulated by antidepressant treatment (Malberg *et al.* 2000), which reverses or blocks the damaging effects of stress. Cyclic nucleotides and their downstream targets contribute to adult neurogenesis and may therefore help to design well-grounded therapies for stimulating neurogenesis for neural repair and to treat or delay neurodegenerative diseases when this process is impaired.

* *see glossary for definition*

### 1.1.5. Synaptic transmission

#### 1.1.5.1. Glutamatergic Signalling

Glutamate is the predominant excitatory neurotransmitter in the brain (Orrego and Villanueva, 1993), and it is synthesized from glucose through intermediates of the tricarboxylic acid cycle. The hippocampus and the surrounding medial temporal lobe cortex are particularly dependent on glutamate signalling. There are two prominent glutamate-mediated systems, which project through hippocampus: one is the unidirectional trisynaptic pathway and the other is the system of direct entorhinal cortex projections to individual subfields (Fig 2). The trisynaptic pathway, a microcircuit with different subfields with specialized memory functions (Rolls 2013), is distinguished by its one-way flow of information, where CA3 neurons are predominantly connected with themselves and with CA1 cells (Amaral *et al*. 1990), while CA1 cells allow for a larger degree of arbitrary associations (Leutgeb and Leutgeb 2007). Glutamate is concentrated in presynaptic vesicles at excitatory synapses and released by calcium-dependent vesicle fusion at the presynaptic terminal following membrane depolarization. Once in the synapse and after binding to glutamate receptors, the transmitter is rapidly recycled by the excitatory amino-acid transporters in astrocytes and neurons (Katsel *et al*. 2011). Stimulation of glutamate receptors causes activation of postsynaptic signalling cascades led by protein kinases (Greer and Greenberg, 2008) which, among other effects, activate transcription of proteins that in turn regulate components of signalling cascades. The excitatory effects of glutamate are exerted via the activation of three major types of ionotropic receptors and several classes of metabotropic receptors linked to G-proteins. The major ionotropic receptors activated by glutamate are the *N*-methyl-*D*-aspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) and kainic acid (KA) receptors. These ionotropic receptors are ligand-gated ion channels permeable to various cations (Danysz and Parsons, 2003), such as calcium, which stands out by its multitude of effects. In fact, more than 300 genes are transcribed in response to increased intracellular calcium, and each gene has a unique time course and magnitude of induction. The transcribed genes play important roles by controlling neuronal survival, dendritic growth and refined regulation of synaptic activity (Greer *et al*. 2009). This regulation is in part achieved by calcium-mediated interference with transcriptional repressors, such as DREAM* (downstream regulatory element-antagonist modulator; Carrion *et al*. 1999). DREAM binds to the promoters of its target genes and represses their transcription unless calcium ions enter the nucleus and bind to DREAM, in which case it is released from the promoters of its target genes, thus relieving transcriptional repression. Furthermore, calcium, after binding to calmodulin, activates calcium-calmodulin activated protein kinases (CaMKs), important in the formation of hippocampus-dependent long-term memory (Mizuno

* *see glossary for definition*

and Giese 2005) and protein phosphatases, such as calcineurin*, which are key players in synaptic plasticity and synaptic transmission (Baumgärtel and Mansuy 2012).

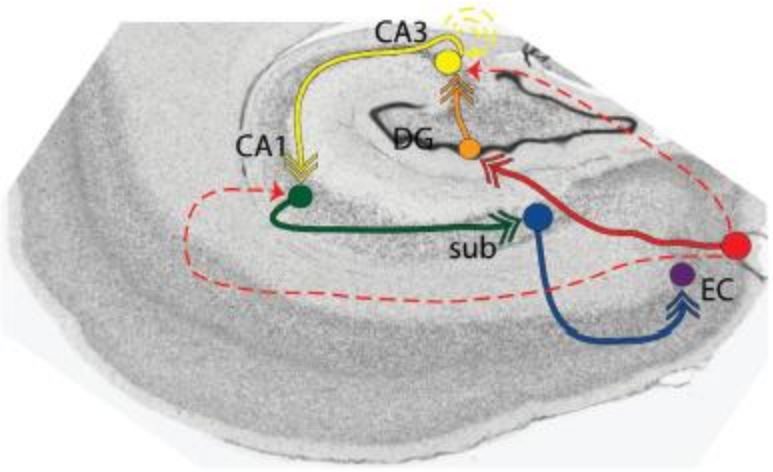


Figure 2. Hippocampus glutamatergic trisynaptic pathway (solid lines). EC: entorhinal cortex; DG: dentate gyrus; CA3: cornu amonius 3; CA1: cornu amonius 1; sub: subiculum. The interrupted line represents the direct projections from EC to CA3 and to CA1.

### 1.1.5.1.1. Excitotoxicity

Alterations in glutamatergic synapse function have been implicated in the pathogenesis of many different neurological disorders including ischemia, epilepsy, Parkinson's disease, Alzheimer's disease and Huntington's disease (Bittigau and Ikonomidou, 1997; Lau and Tymianski, 2010). Excitotoxicity is defined as cell death resulting from the toxic actions of prolonged excitatory amino acid exposure. Because glutamate is the major excitatory neurotransmitter in the mammalian brain, excitotoxicity usually refers to the injury and death of neurons arising from prolonged exposure to glutamate and the associated excessive influx of ions into the cell. Particularly, calcium overload has severe consequences, leading to the activation of enzymes that degrade proteins, membranes and nucleic acids (Berliocchi *et al.* 2005). An overview of the various calcium influx pathways which contribute to excitotoxicity is depicted in figure 3.

* *see glossary for definition*

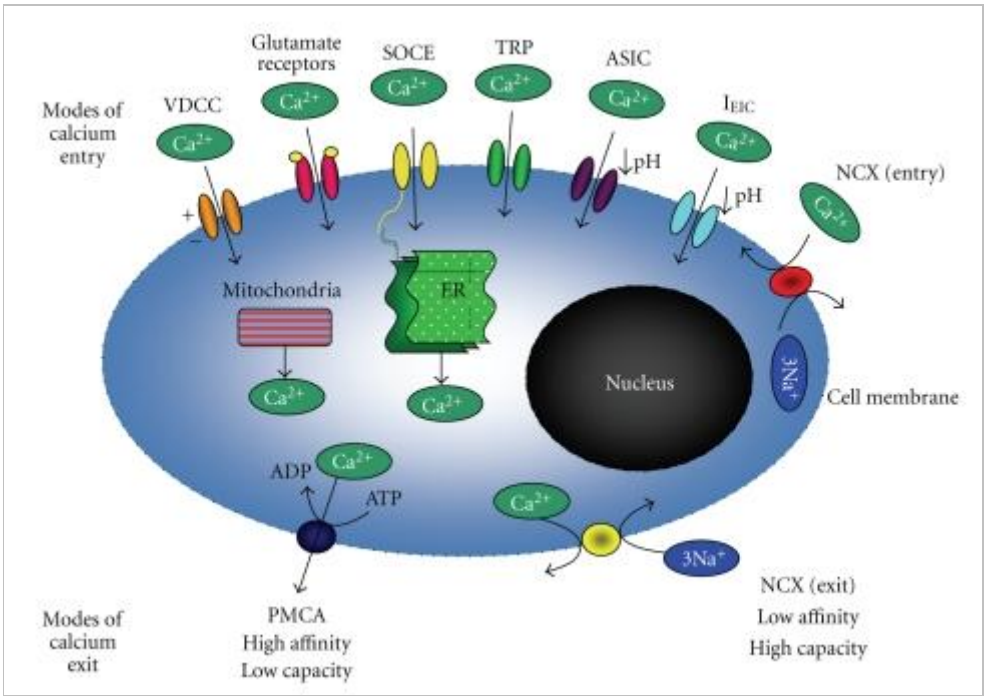


Figure 3. Modes of calcium entry and exit into neurons. Modes of calcium entry are VDCCs (voltage-dependent calcium channel), glutamate receptors (NMDA, AMPA, KA, and mGluR), SOCE (store-operated intracellular calcium entry), TRP (transient receptor potential channels), ASIC (acid-sensing ion channels), $I_{EIC}$ (inward excitotoxic injury current calcium-permeable channels), and NCX (sodium-calcium exchanger operating in entry mode). Calcium can be sequestered intracellularly by the mitochondria and endoplasmic reticulum (adapted from Cross *et al*. 2010). ER- Endoplasmic reticulum; PMCA- Plasma membrane Calcium ATPase.

NMDA receptors are $Ca^{2+}$-favouring glutamate-gated ion channels that are the main responsibles for neuronal injury derived from excessive excitatory activity, due to their high $Ca^{2+}$ permeability and conductance properties (Rothstein 1996). Continuous activation of NMDA receptors leads to increases in intracellular calcium concentration and catabolic enzyme activities, which can trigger a cascade of events eventually leading to apoptosis or necrosis (Ndountse and Chan 2009). Such events include mitochondrial membrane depolarization, caspase activation and production of oxygen and nitrogen free radicals (Fan and Raymond 2007; Jung *et al*. 2009). AMPA-type glutamate receptors have also been implicated in excitotoxicity because assemblies of the receptor are highly permeable to $Ca^{2+}$ and possibly contribute to the delayed neuronal cell death processes induced by $Ca^{2+}$ overload (Friedman 2006).

### 1.1.5.2. GABAergic Signalling

#### 1.1.5.2.1. GABAergic Neurons

Hippocampus interneurons are local circuit neurons represented by an heterogeneous population, with distinct morphologic and physiologic characteristics (Han 1996). In the CA1 region alone, there are at least 16 morphologically distinct interneurons (Parra *et al*. 1998), such as CA1 basket cells with their large soma located near the border of *stratum*

*pyramidale*. A single basket cell contacts more than 1500 pyramidal cells and 60 other basket cells (Sík *et al*. 1995). Interneurons share a neurotransmitter, GABA, which binds $GABA_A$ and $GABA_B$ receptors. These receptors hyperpolarize the post-synaptic membrane, decreasing the probability of an action potential being fired. Comprising only about 10% of the neuronal population of the hippocampus, interneurons are found in all regions of the hippocampal formation, including all strata of areas CA1-CA3 and the dentate gyrus, where they coordinate the activity of large numbers of principal cells thereby regulating the flow of information. Their extensive axons arborize throughout the hippocampus making synaptic contacts with principal cells as well as with other interneurons. In these synaptic contacts different inhibitory interneurons express different combinations of receptors, providing each interneuron with a different set of responses according to biochemical context.

#### 1.1.5.2.2. Inhibition of Inhibitory Feedback Circuitry

Principal neurons represent the major postsynaptic target of most interneurons, and fluctuations in interneuron activity may be partially explained by changes in the excitatory drive received by GABAergic interneurons (Klausberger *et al*. 2003; Maurer *et al*. 2006). However, specific inhibitory mechanisms coordinate the activity of GABAergic interneurons tightly (Chamberland and Topolnik, 2012) as suggested by early anatomical and electrophysiological studies which indicate that GABAergic cells in the hippocampal formation innervate each other (Lacaille *et al*. 1987; Kunkel *et al*. 1988). Thus, interneurons, which inhibit principal neurons and are excited by principal neurons, are themselves further controlled via specific inhibitory mechanisms. These mechanisms may arise from four main sources. First, it was demonstrated that a subgroup of interneurons, the interneuron-specific interneurons, specializes in innervating exclusively other GABAergic cells (Acsády *et al*. 1996; Gulyas *et al*. 1996). Second, interneurons in the hippocampus form synapses with each other besides innervating principal neurons (Vida *et al*. 1998; Cobb *et al*. 1997). Third, long-range GABAergic projections originating from the septum or the entorhinal cortex inhibit interneurons (Freund and Antal 1988; Melzer *et al*. 2012) and fourth, some types of interneurons, such as basket cells or bistratified cells, are also self-connected via functional autapses (Cobb *et al*. 1997; Pawelzik *et al*. 2003). Compared with excitatory inputs onto interneurons, the interneurons inhibitory inputs has received far less attention, but among the four mechanisms described, the interneuron-specific cells are conspicuous, since they are specialized in controlling the firing rate and timing of the hippocampal feedback inhibitory circuitry (Tyan *et al*. 2014), thus providing a higher level of coordination of hippocampal network activity. These neurons are calretinin-expressing or vasoactive intestinal polypeptide (VIP)-expressing GABAergic cells in the CA1 area of the hippocampus, which contact interneurons selectively. The interneuron-specific cells are further subdivided into three subtypes with distinct anatomical and neurochemical features (reviewed in Chamberland and Topolnik, 2012).

### 1.1.5.3. Retrograde Signalling

#### 1.1.5.3.1. Nitric Oxide

Nitric oxide (NO) is a simple diatomic gas that yet plays an intricate role in signal transduction. The main target of NO, mediating most of its downstream effects, is soluble guanylyl cyclase (sGC). NO plays broad roles in vertebrates, some of which have been conserved through millions of years of evolution, dating back to animals with the most primitive nervous systems, such as jellyfish (Moroz *et al*. 2004). The first direct evidence that NO is involved in neurotransmitter release came from electrophysiological recordings showing that NO elicited an enduring increase in the frequency of spontaneous miniature EPSPs*, in cultured hippocampal neurons (O'Dell *et al*. 1991). Subsequent work extended this observation implicating NO as a retrograde messenger, which acts through the cGMP/PKG pathway (Arancio *et al*. 1995) promoting clustering of presynaptic proteins at synapses (Wang *et al*. 2005) and synaptic vesicle recycling (Micheva *et al*. 2003). Retrograde signalling by NO at excitatory synapses begins with an action potential-driven release of glutamate from presynaptic axon terminals, followed by activation of postsynaptic AMPA* and NMDA glutamate receptors, which allow calcium influx triggering the synthesis of NO in the postsynaptic component. NO then retrogradely diffuses to the presynaptic component, where it stimulates cGMP production and consequently PKG activation, leading to increase or decrease of transmitter release (Stanton *et al*. 2003, 2005). NO is thus a retrograde trans-synaptic messenger, which transmits information about NMDA receptor activity to the presynaptic terminal, to coordinate transmitter release (Garthwaite *et al*. 1988; Fig 4). Retrograde signalling as also been implicated in the induction of both hippocampal LTP (Arancio *et al*. 2001) and LTD (Gage *et al*. 1997). In some systems, NO-induced transmitter release occurs independently of an increase in presynaptic $Ca^{2+}$ (Stewart *et al*. 1996).

Some of the NO effects are mediated independently of sGC. For example, NO-dependent LTP at the cerebellum is not affected by blockage of cGMP signalling (Jacoby *et al*. 2001), whereas NO stimulates calcium-independent vesicular release (Meffert *et al*. 1994) possibly via a direct interaction with proteins involved in vesicle docking/fusion (Meffert *et al*. 1996). On the other hand, the NO/sGC/PKG pathway is involved in LTD at the hippocampus (Reyes-Harde *et al*. 1999) whereas at the cerebellum conflicting results were obtain, favouring (Lev-Ram *et al*. 1997) or arguing against such involvement (Linden *et al*. 1995).

* *see glossary for definition*

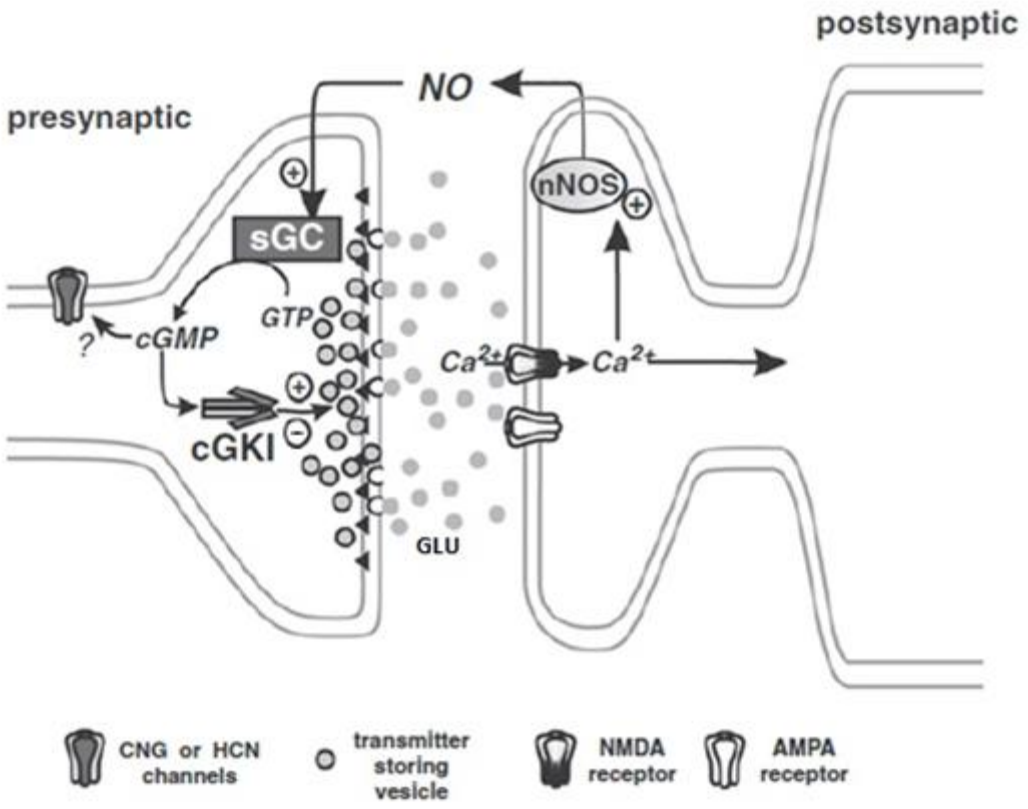


Figure 4. Retrograde NO signalling in a glutamatergic synapse. Glutamate (GLU) released by the presynaptic compartment binds to NMDA and AMPA receptors which allow calcium influx into the postsynaptic compartment. Calcium induces NO production by neuronal NO synthase (nNOS). NO diffuses retrogradly into the presynaptic terminal, where it activates soluble guanylyl cyclase (sGC) and consequently boosts cGMP levels. cGMP directly modulates transmitter release by activating ion channels (CNG and HCN) and by stimulating PKG (cGKI) activity (adapted from Feil and Kleppisch, 2008).

The calcium-dependent nitric oxide synthase (NOS), responsible for NO production, has a widespread distribution in the CNS. In the hippocampus two isoforms have been described: the neuronal NOS (nNOS) and the endothelial NOS (eNOS). Interestingly, localizations of the two enzymes are strikingly different, with eNOS more concentrated in hippocampal pyramidal cells, whereas nNOS is restricted to certain populations of interneurons (Dinerman *et al.* 1994). In addition to NO, CO also activates sGC, and it was demonstrated that the neuronal localization of messenger RNA for CO synthesizing enzyme heme oxygenase is essentially the same as that for sGC messenger RNA (Verma *et al.* 1993). However, carbon monoxide, which is known to play an antinociceptive role, (Carvalho *et al.* 2011) activates sGC with a much lower affinity than NO (Stone and Marletta 1994).

#### 1.1.5.3.2. Cannabinoids CB1 receptors

Like NO, retrograde signalling by endogenous cannabinoids in excitatory synapses initiates with presynaptic depolarization and neurotransmitter release, followed by postsynaptic depolarization and consequent $Ca^{2+}$ influx into the neuron. Endogenous cannabinoids are then rapidly synthesized from postsynaptic membrane phospholipids, and released on-demand in a non-vesicular manner (reviewed in Vaughan and Christie 2005). The activation by the endogenous cannabinoids of pre-synaptic CB1 receptors, coupled to inhibitory heterotrimeric G-proteins ($G_{i/o}$), releases $G_{\beta\gamma}$ subunits which decreases calcium influx into the presynaptic compartment, thus decreasing neurotransmitter release. In fact, CB1 receptor activation inhibits the N-type voltage-gated calcium channel (Pan *et al.* 1996), the P/Q-type calcium channel (Hampson *et al.* 1998), and stimulates outward potassium flux (Childers and Deadwyler, 1996; Mackie *et al.* 1995) allowing CB1 receptors to directly regulate membrane polarization and neuronal function. After interacting with the receptor, the endogenous cannabinoids are captured by a specific transport protein present on both neurons and glia (Beltramo *et al.* 1997; Hillard *et al.* 1997) and inactivated by enzymes such as the membrane-bound fatty acid amid hydrolase (FAAH) located in the endoplasmic reticulum (Gian and Cravatt 1997).

### 1.1.5.4. Excitatory/Inhibitory Balance

#### 1.1.5.4.1. The activity-dependent gene network

The proper balance between excitatory and inhibitory synaptic input is crucial for normal brain function and when this balance is disturbed neurological disorders may occur, such as autism (Snijders *et al.* 2013) or epilepsy (El-Hassar *et al.* 2007). The activity-dependent gene network (genes whose expression is regulated by neuronal activity) has the ability to regulate this balance by controlling excitatory and inhibitory synapses on-demand according with the neuronal network activity. As expected, there is a significant degree of overlap between genes that regulate activity-dependent transcription and genes that are mutated in human cognitive disorders associated with excitatory/inhibitory balance. These genes express transcriptions factors such as NPAS4, MEF2 and MeCP2 that control the balance between synaptic excitation and inhibition within the CNS (Lin *et al.* 2008). NPAS4-mediated transcription is rapidly and transiently induced following calcium influx into neurons and controls the number of inhibitory synapses that form on excitatory neurons, MEF2 has the ability to regulate excitatory synapse number, whereas MeCP2 has been shown to specifically control the strength of excitatory synaptic connections (Dani *et al.* 2005). The regulatory effects of NPAS4, MEF2, and MeCP2 on the balance between neuronal excitation and inhibition are in turn explained by the ability of these factors to regulate transcription of BDNF gene by interacting with its promoter, since activity-dependent transcription of BDNF controls excitatory/inhibitory balance (Hong *et al.* 2008).

#### 1.1.5.4.2. BDNF gene transcription

Before neurotransmitter release at synapses, the BDNF gene promoter is effectively repressed. In this state, despite the presence of multiple transcription factors, the chromatin surrounding the transcription initiation site of BDNF gene is bound by histones that are methylated, and the chromatin is condensed, a state that is correlated with gene repression (Martinowich *et al*. 2003). This likely reflects the ability of transcription factors such as MEF2 to recruit histone deacetylases which remove the acetyl groups from activated histones, allowing its replacement by deactivating methyl groups, thus favouring repression of transcription. Upon neurotransmitter release at a synapse and the activation of calcium signalling to the nucleus, the transcriptional complex that was previously repressing BDNF gene transcription is converted to an activating complex (Chen *et al*. 2003), and histones are demethylated and acetylated. The activation of BDNF gene occurs within several minutes of neurotransmitter release, and BDNF mRNA levels peak 6–8 hr following neuronal stimulation (Hong *et al*. 2008). BDNF may then participate in activity-dependent synaptic plasticity, linking synaptic activity with long-term functional and structural modification of synaptic connections (Poo 2001), essential in establishing the excitatory/inhibitory balance. BDNF expression is also under control of cAMP response element binding protein (CREB*) transcription factor (Murray and Holmes 2011), which is activated by PKA (Nguyen and Woo 2003) and PKG (Lu *et al*. 1999). Consequently, cyclic nucleotides participate in the regulation of BDNF expression.

## 1.2. Cyclic nucleotides

### 1.2.1. Historical Background

Cyclic nucleotides are second messengers, a term originally coined by Sutherland´s group, when cAMP was discovered in studies of glycogen metabolism in skeletal muscle (Rall *et al*. 1957). Sutherland´s group proposed that if the hormone that binds the membrane receptor is the first messenger, then cAMP was the second messenger because the signal was transposed into the cytosol, inside the cell. The principal features of this concept were envisioned to be applicable to all hormonal systems. These features include activation by cyclic nucleotides of a phosphorylation cascade of events catalysed by kinases, which enabled a set of responses ultimately allowing the cell to elicit a specific biological response. More than a decade later Rodbell´s group showed that the transformation of the extracellular hormonal signal into intracellular production of cAMP required GTP and G-proteins (Rodbell *et al*. 1971), and the term "signal transduction" was introduced. Signal transduction involves the binding of an agonist to its receptor, which – in the case of G protein-coupled receptors – promotes G protein activation by facilitating exchange of bound GDP for GTP. The activated G-proteins

* *see glossary for definition*

then act upon adenylyl cyclase or other effector enzymes, modulating second messenger production. Afterwards, different types of G-proteins were identified which could inhibit ($G_i$-proteins) or stimulate ($G_s$-proteins) adenylyl cyclase, according with the type of receptor that was activated. Subsequent studies found other types of G-proteins and that G-protein modulation of second messenger levels was not exclusive for cAMP, but rather triggered other second messenger pathways, namely diacylglycerol and inositol-triphosphate, which originate by cleavage of phosphatidylinositol-4,5-bisphosphate (reviewed in Wettschureck and Offermanns 2005).

Shortly after the discovery of cAMP, Ashman *et al*. (1963) found cGMP in rat urine. Six years later cGMP had been found in all tested tissues and a parallelism was established with cAMP. A striking concept emerged, in which cAMP and cGMP regulated the biological activity of each cell by opposite influence. It was named the "Yin-Yang" hypothesis, and it stated that a hormone or stimulus increased cGMP levels, which then caused a reciprocal decline in cAMP levels and vice-versa (Goldberg *et al*. 1975). This concept rested in the suppositions that guanylyl cyclase was a membrane-bound enzyme and that it was reciprocally interlocked with adenylyl cyclase and thus constituted a bi-directional transduction system. However, the enzyme guanylyl cyclase, responsible for the production of cGMP, had fundamental differences concerning its counterpart adenylyl cyclase. Adenylyl cyclase is a GTP-stimulated membrane-bound enzyme sensitive to $Mg^{2+}$ (Ross and Gilman*et al*. 1980), while guanylyl cyclase is inhibited by ATP, occurs mainly in its soluble form and it´s sensitive to $Mn^{2+}$. Furthermore, several papers determined that transduction pathways operated by cAMP and cGMP were, in many cases, unidirectional in effect, instead of opposite (Conn *et al*. 1971; Glinsmann and Hearn 1969; Glinsmann *et al*. 1969), and the Yin-Yang hypothesis was abandoned.

Between 1974 and 1984 the field of guanylyl cyclase struggled to solve an unclear puzzle generated by contradictive results. However, after determining that soluble guanylyl cyclase responded non-specifically to a variety of stimulus (Arnold *et al*. 1977a; Murad *et al*. 1978), it was proposed that the underlying mechanism of action was the same, via nitric oxide (NO) gas. Additional reports revealed that NO activated guanylyl cyclase in almost every mammalian tissue tested. The response of the cyclase to NO varied, ranging from a five-fold increase in cGMP levels in the rat spleen to 33-fold in the rat cerebellum (Arnold *et al*. 1977b). At the time, PKG was the only known effector of the cGMP transduction pathway. Reports indicated that PKG phosphorylated target proteins with a lower efficacy than PKA, the main effector for the cAMP transduction pathway. This was interpreted by placing the signalling pathway of cGMP as subservient to the cAMP transduction system (reviewed in Gill and McCune 1979). Further research indicated that it was not so. Instead, an autonomous mechanism was revealed, where NO played a key role. It was found that NO is generated from L-arginine by nitric oxide synthase (NOS), and binds soluble guanylyl cyclase in its heme

moiety*. NOS is regulated by $Ca^{2+}$/calmodulin, which in turn depends on intracellular $Ca^{2+}$ levels. Many mechanisms are involved in the regulation of intracellular $Ca^{2+}$ (Cross *et al*. 2010; Fig 3), including mechanism involving G protein-coupled receptors (GPCRs), such as the $A_1$ adenosine and the CB1 cannabinoid receptors, which inhibit $Ca^{2+}$ channels, and the NMDA receptor, sensitive to glutamate, which promotes $Ca^{2+}$ flow into the cell. The NO-dependent pathway was found to specifically stimulate the soluble form of guanylyl cyclase (sGC).

### 1.2.2. Molecular Structure and Signalling

Cyclic nucleotides are composed by three functional groups: a sugar (ribose), a nitrogenous base, and a single phosphate group (Fig 5). The cyclic portion consists of two bonds between the phosphate group and the 3' and 5' hydroxyl groups of the sugar. These hydrophilic second messengers relay and amplify incoming signals from receptors on the cell surface through the cytosol into the nucleus. Signals can vary in timeframe, from milliseconds to hours, and often show a very strict compartmentalization in a specific part of the cell, enabling a wide variety of functions. Such meticulous regulation allows a multitude of synchronous, multifunctional activity which branches out from its roots, the receptors in the plasma membrane. The most important regulation factor is phosphodiesterase activity, responsible for the breakdown of cAMP and cGMP to their respective inactive forms, 5´-AMP and 5´-GMP, thus regulating cyclic nucleotide levels. The relative average amount of cyclic nucleotides levels present in tissues is far from identical since cGMP levels are known to be typically 10-100 fold lower than cAMP levels.

Cyclic nucleotides directly regulate protein kinase activity, ionic channel function (Burns *et al*. 1996) and phosphodiesterase activity (reviewed in Omori and Kotera, 2007; Fig 6). Cyclic nucleotides are produced by the nucleotidyl cyclases adenylyl cyclase and guanylyl cyclase, enzymes which catalyze the cyclization of the adenylyl moiety of ATP and the guanylyl moiety of GTP, respectively. In mammals there are 9 membranous isoforms of adenylyl cyclase and 1 soluble isoform expressed differentially throughout the body (Seifert *et al*. 2012). The cAMP dependent protein kinase (PKA) is a multi-task signalling molecule activated by cAMP and many of the effects of cAMP in synaptic plasticity are thought to be mediated by this kinase.

* *see glossary for definition*

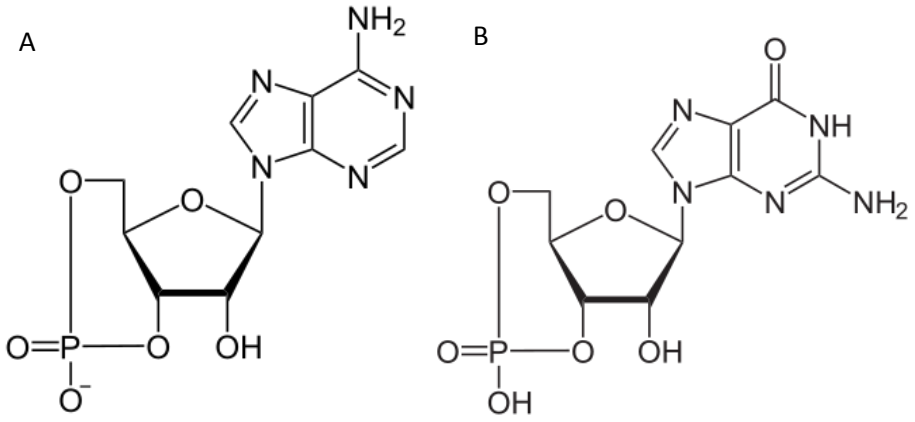


Figure 5. Molecular structure of Cyclic Nucleotides. Both cAMP (A) and cGMP (B) are composed by three distinct functional groups: a sugar, a nitrogenous base, and a phosphate group. These molecules are hydrophilic because of the difference of electronegativity between the atoms that form them and therefore easily diffuse through the cytosol.

Synaptic transmission is enhanced by cAMP signalling, has shown in hippocampal slices, since such enhancement can be induced by application of the AC activator forskolin (Chavez-Noriega and Stevens, 1992) and blocked by PKA antagonists (Woo *et al*. 2002). Accordingly, glutamate release is inhibited by blockers of PKA (Rodríguez-Moreno *et al*. 2004). In the case of guanylyl cyclases there are two types, soluble guanylyl cyclases (sGCs) activated by nitric oxide (NO) and particulate guanylyl cyclases (pGCs) activated by natriuretic peptides. Soluble guanylyl cyclase (sGC) is a cytosolic heterodimer consisting of α and ß subunits, with two isoforms for each subunit (α1, α2, ß1, and ß2). The α2, ß1 sGC heterodimer is expressed in the hippocampus (Mergia *et al*. 2003) were it converts GTP into cGMP when stimulated by NO. The main cGMP effector protein is cGMP dependent protein kinases (PKG), of which there are two subtypes, cGKI and cGKII (reviewed in Kleppisch and Feil, 2009). Contrarily to cAMP, cGMP inhibits synaptic transmission in the hippocampus (Boulton *et al*. 1994) and visual cortex (Wei *et al*. 2002) and suppresses glutamate release in the cerebral cortex (Sistiaga *et al*. 1997) and cerebellum (Kamisaki *et al*. 1995) and acetylcholine* release in the hippocampus (Nordström and Bartfai 1981). Thus, cGMP appears to be negatively coupled to neurotransmitter release, while cAMP has the opposite effect, such as suggested by microiontophoretic studies performed over 35 years ago (Stone and Taylor 1977).

* *see glossary for definition*

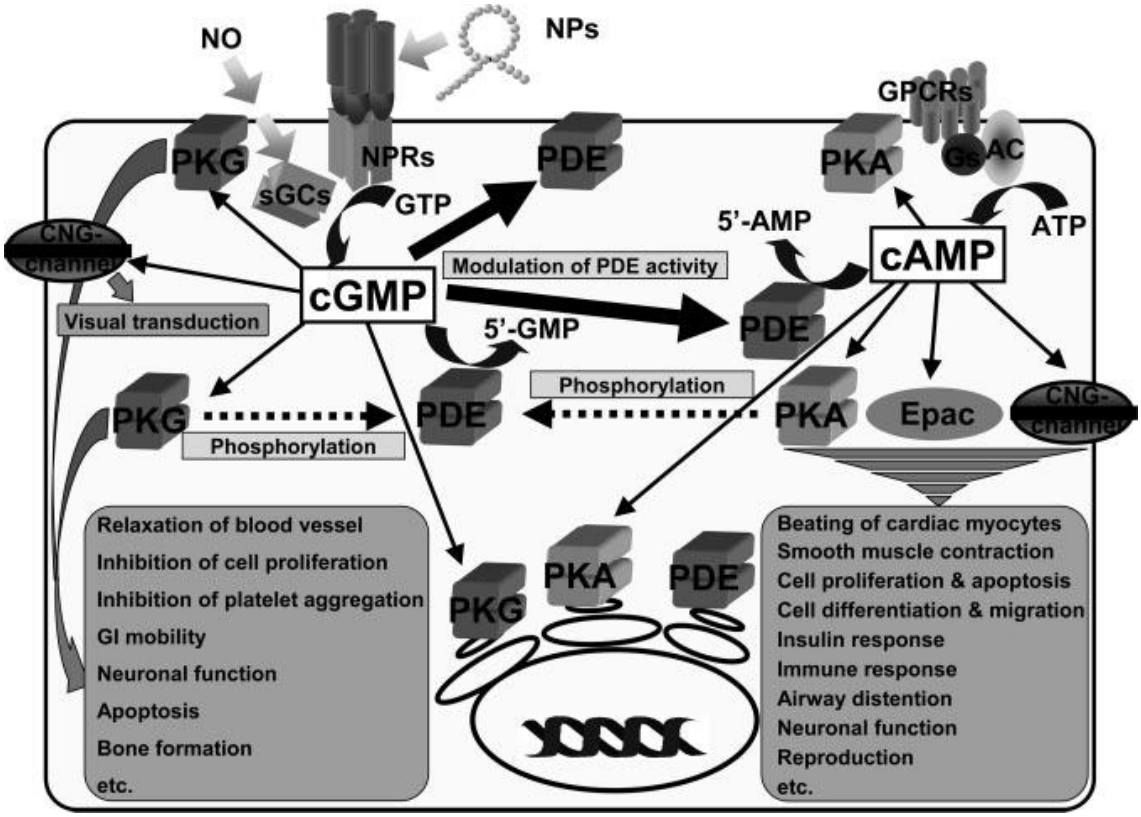


Figure 6. Cyclic nucleotide signalling and regulation. Localization of fundamental molecules involved in cAMP and cGMP signalling is illustrated. Effector molecules of cAMP and cGMP are indicated by arrows from each cyclic nucleotide. Phosphorylation of PDEs by PKA and PKG is demonstrated by dotted arrows. Modulation of PDE activity by cGMP is shown by a thick arrow. Cellular and physiological outputs of cyclic nucleotide signalling are shown in gray-coloured boxes. NPs, natriuretic peptides; NO, nitric oxide; NPRs, natriuretic peptide receptors; sGCs, soluble guanylyl cyclase; AC, adenylyl cyclase; GPCRs, G protein–coupled receptors; Epac, exchange protein directly activated by cAMP; CNG-channel, cyclic nucleotide gated channel (adapted from Omori and Kotera, 2007).

### 1.2.3. The Main Effectors: PKA and PKG

Cyclic nucleotides main effectors are the protein kinases PKA and PKG families, homologous proteins that have evolved from a common ancestor (Takio *et al.* 1984). Many of the known physiological substrates for PKGs are also substrates for PKAs. PKG and PKA have a 50- to 200-fold selectivity for cGMP and cAMP, respectively, and most commonly the cellular effect of cGMP or cAMP is thought to be mediated by the respective kinase for which they are selective. However, in several systems, a physiological level of cGMP or cAMP can cross-activate the other kinase (Jiang *et al.* 1992). PKA and PKG transfer the γ-phosphate of ATP to serine and threonine residues[*] of many cellular proteins, regulating their activity. PKAs are present in most cells and are effectors of many cAMP-elevating first messengers such as hormones and neurotransmitters. PKG is highly expressed in the cerebellum, particularly in Purkinje cells, but is also present in other brain cells (Lohmann *et al.* 1981). Nonetheless, in most tissues, PKGs are much less abundantly expressed than PKAs (reviewed in Francis *et al.* 2005).

[*] *see glossary for definition*

In the absence of its activating ligand cAMP, PKA exists as an inactive holoenzyme* of two regulatory and two catalytic subunits (Cadd and McKnight 1989). Following an increase in intracellular cAMP, the regulatory subunits bind cAMP resulting in the dissociation of the holoenzyme and the release of two free active catalytic subunits, which phosphorylate peptide substrates. Differences between PKA subtypes functions are mainly due to differences in levels of expression in specific cells and the relative ability of each kinase subtype to localize near substrates to specific compartments by binding to scaffolding proteins (AKAPs). A number of PKG anchoring proteins (GKAPs) has also been identified (Corradini *et al.* 2013). In contrast to PKA, the regulatory and catalytic regions of the PKG enzyme are present in one polypeptide, but still the same substrate proteins can be phosphorylated by both kinases in vitro (Subramanian *et al.* 2013). In addition to phosphorylating other proteins (heterophosphorylation), cyclic nucleotide protein kinases have the ability to phosphorylate themselves (Seidler *et al.* 2009; Kotera *et al.* 2003), an intermolecular mechanism which activates the kinases (Dodson *et al.* 2013).

### 1.2.4. Phosphodiesterases

PDEs are enzymes that control the intracellular concentrations of cyclic nucleotides by catalyzing their hydrolysis*. PDEs have been categorized into 11 families and are encoded by at least 21 genes in mammals with a large number of PDE splicing variants, which serve to fine-tune cyclic nucleotide signals (Sanderson and Sher 2013). Their activities are regulated by diverse mechanisms including phosphorylation/dephosphorylation, allosteric binding of cGMP or cAMP, binding of $Ca^{2+}$/calmodulin and various protein–protein interactions (Conti and Beavo 2007). Allosteric binding of cyclic nucleotides occurs in PDE families with a GAF domain (PDE 2, 5, 6, 10 and 11), which constitute approximately half of PDE gene families (Omori and Kotera 2007). The major families involved in cGMP hydrolysis are $Ca^{2+}$-calmodulin-activated PDE1, cGMP-stimulated PDE2 and cGMP-specific PDE5 and PDE9. On the other hand, cyclic AMP is mainly hydrolyzed by PDE2, by cGMP-inhibited PDE3, and by cAMP-specific PDE4, PDE7 and PDE8 (Conti and Jin 1999; Soderling and Beavo 2000). Some phosphodiesterases have dual specificity, such as PDE2 and PDE10, which process both cyclic nucleotides, although PDE10 favours cAMP energetically (Lau and Cheng 2012). The pharmacological effects of rolipram are linked to its ability to increase cAMP concentrations and activate the corresponding signal transduction cascade. Some studies suggest that rolipram may alter NMDA receptor-mediated signalling. In rat retrospenial cortex, MK-801, a noncompetitive NMDA receptor antagonist, induces heat shock protein HSP-70, a marker for reversible neuronal damage. Rolipram, which selectively inhibits PDE4 and leads to an increase in cAMP, reverses the effect of MK-801 on HSP-70 expression (Hashimoto *et al.* 1997). On the other hand, in Mongolian gerbils, neuronal death due to cerebral ischemia, a process that seems to involve NMDA receptors, is prevented by pre-treatment with rolipram (Kato *et al.* 1995). This

* *see glossary for definition*

suggests a role for PDE4 in neurotoxic effects of NMDA receptor antagonists and cerebral ischemia. It also has been shown that chronic treatment with rolipram increases [$^3$H]MK-801 binding in the dentate gyrus of both young and aged rats (Kato *et al*. 1997). Recent behavioural studies have shown that the memory impairments induced by MK-801 are antagonized by rolipram (Zhang *et al*. 2000). Finally, PDE4 inhibitors have been found to potentiate the ability of NMDA to increase cAMP in hippocampal slices and neuronal cultures (Chetkovich and Sweatt, 1993; Zhang *et al*. 2000). Overall, these data suggest that PDE4 is involved in NMDA receptor-mediated signalling; however, potential roles for other PDE families in this signalling pathway have not been investigated. In cultured cerebellar granule cells, NO donors and NMDA induce cGMP formation; this effect is potentiated by IBMX, a nonselective PDE inhibitor (Baltrons *et al*. 1997; Oh *et al*. 1997). Immunocytochemical studies done in cerebellar slice preparations also have shown potentiation by IBMX of NO-dependent cGMP formation in granule neurons (de Vente *et al*. 1990; Southam *et al*. 1992). These studies suggest that PDEs are regulators of cGMP in neurons; however, it is not clear which PDE families are involved in the hydrolysis of cGMP formed by NMDA receptor stimulation. A recent study was conducted to identify the PDE families involved in NMDA receptor signalling. Cultures of rat cerebral cortical and hippocampal neurons were used to evaluate the effects of PDE inhibitors on NMDA receptor-mediated cAMP and cGMP formation. It was found that the PDE4 and PDE2 families regulate the NMDA receptor-mediated enhancement of increases in cAMP and cGMP concentrations, respectively (Suvarna and O´Donnell 2002).

### 1.2.5. Cyclic nucleotides and synaptic plasticity

Following the discovery that long-term potentiation (LTP), the activity-induced strengthening of synapses, is dependent on NMDA receptor activity (Collingridge *et al*. 1983) the list of signalling molecules involved in synaptic plasticity has rapidly increased. One class of signalling that is important for both long term potentiation (LTP) and long-term depression (LTD), is cyclic nucleotide signalling. NMDA receptors boost cyclic nucleotide levels by the following mechanism: the presynaptic terminal releases glutamate which binds postsynaptic NMDA receptors generating a $Ca^{2+}$ influx, which in turn increases cAMP through $Ca^{2+}$-calmodulin-dependent adenylyl cyclase (Chetkovich *et al*. 1991; Chetkovich and Sweatt, 1993) and cGMP through $Ca^{2+}$-calmodulin-dependent nitric oxide synthase activation, which stimulates soluble guanylyl cyclase by producing NO (Garthwaite 1991). Conversely, chemically induced LTP by modulation of cAMP levels is NMDA receptor dependent (Otmakhov *et al*. 2004). cGMP is also involved in LTP produced by strong tetanic stimulation (Son *et al*. 1998), while at low frequency stimulation cGMP is expected not to produce potentiation, but rather depression (Zhuo *et al*. 1994a), which may also be caused by other cGMP-independent mechanisms (Gage *et al*. 1997). In addition, both PKA and PKG have been found to regulate the phosphorylation of cAMP response element binding protein (CREB) in the context of LTP,

and so to regulate gene expression necessary for this form of plasticity (Bourtchuladze *et al*. 1994; Impey *et al*. 1996; Lu *et al*. 1999).

### 1.2.6. Cross-activation of phosphodiesterases and protein kinases by cyclic nucleotides

The cyclic nucleotides pathways are also modulated by the cyclic nucleotides themselves, which, by binding different forms of PDEs, can either facilitate or inhibit their own pathway or the other cyclic nucleotide pathway by attenuating or enhancing phosphodiesterase (PDE) and protein kinase activity (Fig 7). By their substrate specificity, all PDE families fit into three categories: cAMP specific, cGMP specific or PDEs with dual enzymatic activity. One phosphodiesterase expressed by hippocampal neurons, PDE3 (Reinhardt and Bondy 1996), is inhibited by cGMP (Mehats *et al*. 2002). However, hippocampal neurons also express PDE2, a phosphodiesterase that is strongly stimulated by cGMP (Repaske *et al*. 1993; Van Staveren *et al*. 2003). Both PDE3 and PDE2 degrade cAMP, suggesting that increases in cGMP facilitate LTP induction by inhibiting PDE3-catalysed cAMP degradation or suppressing LTP induction by facilitating PDE2-catalysed cAMP degradation (Santschi *et al*. 1999; Monfort *et al*. 2002). Conversely, cAMP may slow down the degradation of cGMP by promoting competitive inhibition of cGMP turnover mediated by PDE10 (Jäger *et al*. 2012). In the case of protein kinases, it is known that many of the physiological substrates for PKGs are also substrates for PKAs and that the binding domains of both protein kinases contain specific cyclic nucleotide binding sites with significant homology. Thus, the PKG binding sites may be occupied by cAMP (Doskeland *et al*. 1986) and the PKA binding sites may be occupied by cGMP, although with a 50-fold lower selectivity than cAMP (Lucas *et al*. 2000). Electrophysiological studies showed that cGMP suppressed PKA-dependent forms of LTP, probably as consequence of modulation of the activity of phosphodiesterases (Makhinson *et al*. 2006). cGMP also plays a more direct role in hippocampal synaptic plasticity through activation of PKG (Zhuo *et al*. 1994a; Wu *et al*. 1998).

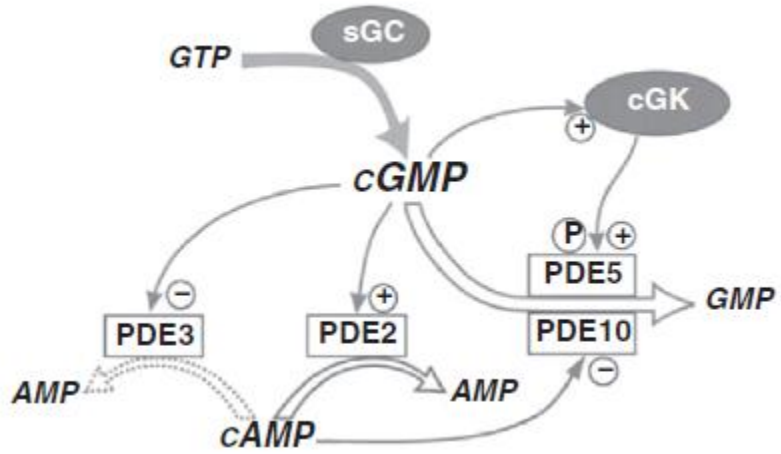


Figure 7. Role of PDEs in cyclic nucleotide signalling. Phosphodiesterases hydrolyze the 3´-cyclic phosphate bond of the cyclic nucleotide yielding inactive 5´-AMP or 5´-GMP. Breakdown of cAMP and cGMP can be catalyzed by PDEs highly specific either for cGMP (PDE5, PDE6, PDE9) or cAMP (PDE4, PDE7, PDE8) or both (PDE1, PDE2, PDE3, PDE10, PDE11). cGMP accelerates its PDE5-dependent degradation by stimulating PKG (cGK) which phosphorylates PDE5. cGMP also stimulates and inhibits the cAMP-hydrolyzing PDE2 and PDE3, respectively, whereas cAMP binds PDE10 inhibiting hydrolysis of cGMP. sGC: soluble guanylyl cyclase (adapted from Kleppisch 2009).

### 1.2.7. Compartmentation of cyclic nucleotide activity

Evidence has been accumulating that cyclic nucleotide signalling is not uniformly distributed in the cytosol, but is rather organized in subcellular signalling microdomains. Each of these microdomains contains a specific subset of GPCRs, protein kinases, specific PDEs and G-kinase and A-kinase anchoring proteins (GKAPs and AKAPs; Kennedy 2000; Sprenger and Nikolaev 2013), which keep cyclic nucleotides in close proximity to the molecular machinery which regulates them or whose activity is modulated by them. The presence of compartments restricts cyclic nucleotide-mediated cross-activation of protein kinases, since PKA and PKG are anchored to specific scaffold proteins integrated in the postsynaptic density, in a way which allows them to properly perform their physiological functions. With regard to PKG, a leucine/isoleucine zipper domain mediates PKG interaction with scaffold protein GKAP, targeting different PKG isoforms to different subcellular compartments (Schlossmann and Desch 2009) where the isoforms phosphorylate specific substrate proteins. In the case of PKA, this anchoring is performed by AKAPs, scaffold proteins which form an assembly with other signalling proteins to coordinate signal transduction, integrating cAMP signalling with other pathways (Sanderson and Dell'Acqua 2011). Both GKAPs and AKAPs are localized at the postsynaptic membrane of excitatory synapses, where neurotransmitter receptors are attached to large protein conglomerates, signalling machines that fine-tune the strength of excitatory synaptic transmission, an assembly designated as the postsynaptic density. At the postsynaptic density the GKAPs and AKAPs scaffold proteins integrate cyclic nucleotide

signalling with glutamatergic transmission (Sanderson and Dell'Acqua 2011) to regulate activity-dependent signalling processes at hippocampal neurons (Lin *et al*. 2011).

### 1.2.8. Therapeutic Potential of Cyclic Nucleotides

The manipulation of cyclic nucleotides pathways constitutes a promising therapeutic target since second messengers simultaneously command many functions which allow general control over neuronal function. Such manipulation may be achieved by drugs which regulate receptor activity. Each brain pathology occurs in a particular neuron assembly with its own receptors associated, and increasing or decreasing their activity will modulate neuronal function to a specific end, to reverse the pathology. A good example is the melatonin receptors. Melatonin is an important component of the body's internal time-keeping system. Melatonin MT1 and MT2 receptors are G protein coupled receptors which are expressed in various parts of the CNS, including the hippocampus. Alterations in melatonin receptor expression as well as changes in endogenous melatonin production comprise changes in cAMP and cGMP and have been shown to be involved in circadian rhythm sleep disorders, Alzheimer's and Parkinson's disease (Pandi-Perumal *et al*. 2008). Phosphodiesterase inhibitors, such as rolipram (Houslay *et al*. 1998) and BAY 60-7550 (Ding *et al*. 2014), are useful therapeutic tools which allow intracellular increases in cyclic nucleotides levels, and thus may be helpful in many disorders, including sleep disorders and neurodegenerative disorders.

#### 1.2.8.1. Traumatic Brain Injury

Traumatic brain injury occurs when the brain is damaged by a physical impact to the head. It’s a critical public health and socio-economic problem throughout the world. It is a major cause of death, and lifelong disability is common in those who survive (Roozenbeek *et al*. 2013). Both cAMP and cGMP neuronal signalling pathways are involved in neuroprotection at the hippocampus, protecting memory-associated mechanisms in traumatic brain injury. Recently Titus *et al*. (2013) examined the effect of rolipram on traumatized animals with a poor performance in the Morris water maze. He found that rolipram significantly improved performance in the Morris water maze, which tests hippocampal spatial memory. Also, he observed that learning increased phosphorylated CREB levels of healthy animals, but not in traumatized animals. This deficit in CREB activation during learning was also rescued by rolipram. Furthermore, hippocampal long-term potentiation was also reduced in traumatized animals. Such condition was reversed by rolipram treatment. Thus, the adequate activity by cAMP pathways rescue learning impairments which result from traumatic brain injury. Turning to cGMP pathways, it was found that nitric oxide, a known agonist for soluble guanylyl cyclase, improves the integrity of neurons of rats subjected to traumatic brain injury. The nitric oxide donor DETA/NONOate promoted the proliferation, survival, migration and

differentiation of neural progenitor cells in the hippocampus, striatum, corpus callosum and cortex of traumatized rats (Lu *et al.* 2003). Both DETA/NONOate and sildenafil, a PDE5 inhibitor, enhanced progenitor cell proliferation and survival in the hippocampal formation in rats (Prado *et al.* 2013), by increasing cGMP levels, what may contribute to improvements in the response of neurons to traumatic brain injury. Thus, new therapies may arise from drugs which modulate cyclic nucleotide levels by repairing the molecular mechanisms of neuronal activity disturbed by traumatic brain injury.

#### 1.2.8.2. Phosphodiesterases in Neurodegenerative Disorders

Since cyclic nucleotide phosphodiesterases are responsible for the breakdown of both cAMP and cGMP, they are crucial regulators of cyclic nucleotide-mediated signalling, which has been associated with neuroplasticity and protection. As such, influencing their levels through inhibition of PDEs has become a much studied target for treatment in a variety of disorders, including neurodegenerative disorders. Alzheimer's disease is associated with changes in PDE4, PDE7 and PDE8 expression in the brain (Perez-Torres *et al.* 2003), while major depressive disorder is associated with altered functioning of PDE4 as well as PDE11 (O'Donnell and Xu, 2012). In multiple sclerosis the myelinated axons in the brain are protected by inhibition of PDE4 (Genain *et al.* 1995) and PDE5 (Nunes *et al.* 2012). In Huntington's disease and Parkinson's disease, most research has focused on PDE1B and PDE10, because of their abundance in striatal neurons (Lakics *et al.* 2010). Thus, regulation of cyclic nucleotide signalling via modulation of PDE activity remains appealing for the treatment of neurodegenerative disorders.

##### 1.2.8.2.1. Alzheimer´s Disease

Alzheimer's disease is a progressive neurodegenerative disorder, characterized by cognitive impairment which results from the overproduction and extracellular deposition of ß-amyloid peptide, intracellular deposition of neurofibrillary tangles (hyperphosphorylated tau protein) and cholinergic* deficit, which cause atrophy of specific brain areas, particularly the hippocampus (Parihar and Hemnani, 2004). These depositions serve as initiating factors for neurotoxicity pathways, which include excitotoxicity. The earliest amnesic symptoms are likely to originate from beta-amyloid assemblies at the synaptic level, which alter mechanisms underlying excitatory neurotransmission at individual synapses (Selkoe 2002). The beta-amyloid has been found to markedly inhibit LTP (Bliss and Collingridge, 1993). The inhibition of hippocampal LTP by beta-amyloid was found to be reversed by both an NO donor and by a cGMP cell permeable analogue (Puzzo *et al.* 2006). These effects were abolished by a sGC antagonist and by a PKG inhibitor, respectively. Furthermore, rolipram and forskolin,

* *see glossary for definition*

agents that enhance the cAMP-signalling pathway, can reverse the beta-amyloid peptide mediated inhibition of hippocampal LTP, an effect blocked by H89, an inhibitor of PKA (Vitolo *et al.* 2002). Thus, the upregulation of cyclic nucleotides pathways in the hippocampus is an interesting approach to counteract the earliest amnesic symptoms of Alzheimer´s disease.

#### 1.2.8.2.2. Multiple Sclerosis

Multiple sclerosis is a chronic autoimmune, inflammatory neurological disease of the central nervous system (Calabresi 2004). It attacks the myelinated axons in the CNS, destroying the myelin and the axons to varying degrees (Weinshenker 1996). The course of multiple sclerosis is highly varied and unpredictable. In most patients, the disease is characterized initially by episodes of reversible neurological deficits, often followed by progressive neurological deterioration over time. cAMP levels were assayed in cerebrospinal fluid obtained from patients with multiple sclerosis and were found to be low in more than half of the patients, while plasma cAMP levels were normal (Maida and Kristoferitsch 1981). The decrease is correlated significantly with the disability of the patient and with the progression of the disease. Thus, low cAMP levels in the cerebrospinal fluid can be considered as prognostically unfavourable, particularly in the early stage of the disease (Maida and Kristoferitsch, 1981). Experimental allergic encephalomyelitis is an autoimmune disease that serves as a model for multiple sclerosis. The effect of rolipram, a PDE4 inhibitor, was tested for its efficacy in preventing experimental allergic encephalomyelitis in monkeys. All of the monkeys that received rolipram were protected against experimental allergic encephalomyelitis autoimmune demyelinating activity (Genain *et al.* 1995). Contrarily to cAMP, an increase of cGMP levels was found in the cerebrospinal fluid of patients in the initial stage of the disease (Maciejek *et al.* 1985), whereas a reduced expression of NO-sensitive guanylyl cyclase was observed in reactive astrocytes of multiple sclerosis brains (Baltrons *et al.* 2004). cGMP-pathways protect oligodendrocytes (Benjamins and Nedelkoska 2007), which produce the myelin sheath. Astroglia and microglia, which play a role in perpetuating multiple sclerosis, are also modulated by cGMP (Baltrons *et al.* 2008). Sildenafil induces cGMP accumulation through PDE5 inhibition. Cuprizone-induced demyelination and neuroinflammation effects in the cerebellum were reversed by sildenafil, which was neuroprotective (Nunes *et al.* 2012). Therefore, phosphodiesterase inhibitors constitute a potential tool for multiple sclerosis treatment.

#### 1.2.8.2.3. Major Depressive Disorder

Major depressive disorder may be considered a neurodegenerative disease, as it is thought to be associated with a decrease in neurogenesis in hippocampal dentate gyrus (Boldrini *et al.* 2009) and neuronal atrophy in the hippocampal CA3 area (Bremner *et al.* 2000; Sheline *et al.* 1996). Rolipram and other PDE4 inhibitors which decrease cAMP hydrolysis produce

antidepressant effects (Jindal *et al*. 2013), such as reversing the effects of chronic, mild stress (Jindal *et al*. 2013) and increasing proliferation and survival of newborn neurons in the hippocampus (Xiao *et al*. 2011). Thus, the ability of antidepressant drugs to induce neurogenesis is important in mediating late-developing antidepressive-effects on behaviour (Santarelli *et al*. 2003). A very recent study quantified in vivo the binding of $^{11}$C-(R)-rolipram to PDE4, using PET scans in unmedicated patients with major depressive disorder. The patients with major depressive disorder showed a significant reduction in PDE4 levels, most likely a compensatory mechanism in response to decreased cAMP signalling (Fujita *et al*. 2012). The neurogenic effect of cAMP appears to be mediated through CREB (Nakagawa *et al*. 2002), a transcription factor that is activated by PKA-mediated phosphorylation (Duman *et al*. 2000). On the other hand, in rats chronically treated with the antidepressants fluoxetine and amitriptyline, cGMP levels were increased at the hippocampus (Reierson *et al*. 2009) suggesting that increased levels of hippocampal cGMP underlie the efficacy of chronic antidepressant treatment. Previous studies have shown that drugs which increase NO demonstrate neuroprotective effects in cultured hippocampal cells *in vitro* and enhance long-term potentiation in rat hippocampal slices (Bon and Garthwaite, 2001; Culmsee *et al*. 2005). The cGMP specific PDE5 inhibitor sildenafil displays antidepressant effects in a behavioural test of depression in rodents only when combined with atropine, a muscarinic acetylcholine receptor antagonist (Brink *et al*. 2008). Although anticholinergic agents are generally disregarded as clinically effective antidepressants (Fritze 1993; Gillin *et al*. 1995), their efficacy in treating resistant depression (Furey and Drevets 2006) emphasizes that disturbances in cholinergic activity play a distinct yet unresolved role in the neurobiology of depression. Sildenafil also decreases deficits in memory in a T-maze passive avoidance task and has been shown to improve object recognition memory in rats (Devan *et al*. 2004; Prickaerts *et al*. 2002). Clinically used for the treatment of erectile dysfunction, sildenafil is safe and effective in male patients in remission from major depressive disorder and self-reports suggest a lowering of depressive symptoms (Muller *et al*. 2001; Tignol *et al*. 2004). These studies point to the possibility that the NO/cGMP cascade might be deregulated in major depressive disorder, and that antidepressant efficacy could be related to the normalization of dysfunctional NO/cGMP signalling in the brain.

## 1.3. Adenosine as an intercellular mediator in the nervous system

Adenosine affects nerve cells directly and influences the action of neurotransmitters and other neuromodulators indirectly, behaving as a modulator of modulators (Sebastião and Ribeiro, 2000). It is produced, released, taken up and metabolized in almost every cell (Ribeiro and Sebastião, 1986). Neuromodulation of synaptic transmission by adenosine occurs through the high affinity receptors $A_1$ and $A_{2A,}$ while the low affinity receptor $A_{2B}$ might

regulate axonal growth and be relevant in pathological conditions (Ribeiro *et al.* 2003b). The $A_3$ receptor is a high affinity receptor present in humans in most tissues but at low densities (Ribeiro *et al.* 2003b). $A_3$ receptors may mediate both cell protection and cell injury, depending upon the degree of receptor activation and/or the pathophysiological condition (Ribeiro *et al.* 2003b).

### 1.3.1. Adenosine Metabolism

Basal concentrations of adenosine reflect equilibrium between the several mechanisms that increase extracellular adenosine and its uptake and metabolism, and are sufficient to tonically activate a substantial fraction of the high-affinity $A_1$ and $A_{2A}$ adenosine receptors (Dunwiddie and Masino, 2001). The basal concentration of adenosine is estimated to be in the range of 25-250 nM in the brain (Ballarin *et al.* 1991; Dunwiddie and Diao, 1994). In stressful situations the intracellular concentration of adenosine increases to micromolar concentrations (Ballarin *et al.* 1991) and adenosine is released to the extracellular medium by carrier-mediated processes to refrain cell metabolism of neighboring cells (Dunwiddie and Masino, 2001). ATP is colocalized with neurotransmitters in vesicles and is coreleased after electrical stimulation (Fredholm *et al.* 1982), and subsequently hydrolyzed to adenosine. Within the cell, adenosine is produced from the hydrolysis of S-adenosylhomocysteine (SAH) and from the hydrolysis of 5´-AMP, catalysed by SAH hydrolase and 5'-nucleotidase, respectively. Inactivation of extracellular adenosine activity occurs by uptake followed by the action of the enzymes adenosine deaminase and adenosine kinase (Ribeiro and Sebastião, 1986). Uptake may be mediated by facilitated diffusion through nucleoside transporters or by active transport (Dunwiddie and Masino, 2001). Although $A_1$ and $A_{2A}$ adenosine receptors coexist, it appears that adenosine formed from adenine nucleotides acts preferentially on $A_{2A}$, while directly released adenosine preferentially binds to $A_1$ receptors (Cunha *et al.* 1996). This could result from different localization of $A_1$ and $A_{2A}$ receptors relatively to the release sites of adenosine and from the location of ecto-nucleotidases, the enzymes that hydrolyses extracellularly adenine nucleotides into adenosine (Sebastião and Ribeiro, 2000).

### 1.3.2. Adenosine high affinity $A_1$ and $A_{2A}$ receptors

Adenosine high affinity receptors have crucial functions in modulating synaptic transmission: they regulate receptors for the calcitonin gene-related peptide, the vasoactive intestinal peptide and NMDA receptors (Sebastião and Ribeiro, 2000). The $A_1$ and $A_{2A}$ receptors are coupled to G-proteins, through which they modulate the activity of adenylyl cyclase and calcium channels. Functional evidence that $A_1$ and $A_{2A}$ receptors can coexist in the same nerve terminal derives from the observation that an $A_1$ receptor agonist presynaptically inhibits and an $A_{2A}$ receptor agonist presynaptically enhances the amplitude of evoked endplate potentials recorded intracellularly from a single endplate (Correia-de-Sá *et al.*

1991). In addition, co-localization and co-expression of mRNA encoding $A_1$ and $A_{2A}$ receptors was detected in the hippocampus (Cunha *et al.* 1994). Both $A_1$ and $A_{2A}$ receptors regulate neuronal excitability in the CA1 region of hippocampus (Cunha *et al.* 1994), along with influencing the activity of several other neuromediators. Activation of $A_{2A}$ receptors decreases $A_1$ receptor binding in hippocampal (Lopes *et al.* 1999) and striatal (Dixon *et al.* 1997) synaptosomes, whereas electrophysiological studies show that upon activation of $A_{2A}$ receptors the ability of adenosine $A_1$ receptor to inhibit neuronal activity is attenuated (Cunha *et al.* 1994; O´Kane and Stone 1998) a cross-talk which is prevented by PKC inhibitors, but not PKA inhibitors (Dixon *et al.* 1997; Lopes *et al.* 1999), whereas PKC activators, such as phorbol esters, mimic the ability of $A_{2A}$ agonists to decrease $A_1$ receptor binding effects (Lopes *et al.* 1999).

#### 1.3.2.1. The adenosine $A_1$ receptor

The $A_1$ receptor is the most abundant adenosine receptor in the central nervous system (Cunha *et al.* 2001), exerting a role in cell growth, survival, death and differentiation (Schulte and Fredholm, 2003). The $A_1$ receptor is also involved in the regulation of the second messenger pathways of cAMP, diacylglycerol and inositol triphosphate. Adenylyl cyclase is responsible for the production of cAMP, activating PKA (Daniel *et al.* 1998), while phospholipase C catalyzes the degradation of phosphatidylinositol bisphosphate, yielding diacylglycerol and inositol trisphosphate (Fig 8). Diacylglycerol is an activator of PKC, whereas inositol triphosphate mobilizes $Ca^{2+}$ from endoplasmic reticulum, also promoting the activity of PKC (Tanaka and Saito 1992). Regulation of the activity of protein kinases allows for the modulation of protein expression, of enzymatic activity and of ionic channel function. The $A_1$ receptor inhibitory effect on synaptic transmission results from a combined postsynaptic hyperpolarization due to activation of a potassium conductance and a presynaptic inhibition of neurotransmitter release (Dunwiddie and Haas 1985; Segal 1982) through blockade of calcium influx (Schubert *et al.* 1986; Wu and Saggau 1994). At hippocampal glutamatergic nerve terminals calcium influx is partly inhibited by adenosine $A_1$ receptor activation which blocks N-, P/Q- and other types of $Ca^{2+}$ channels (Ambrósio *et al.* 1997).

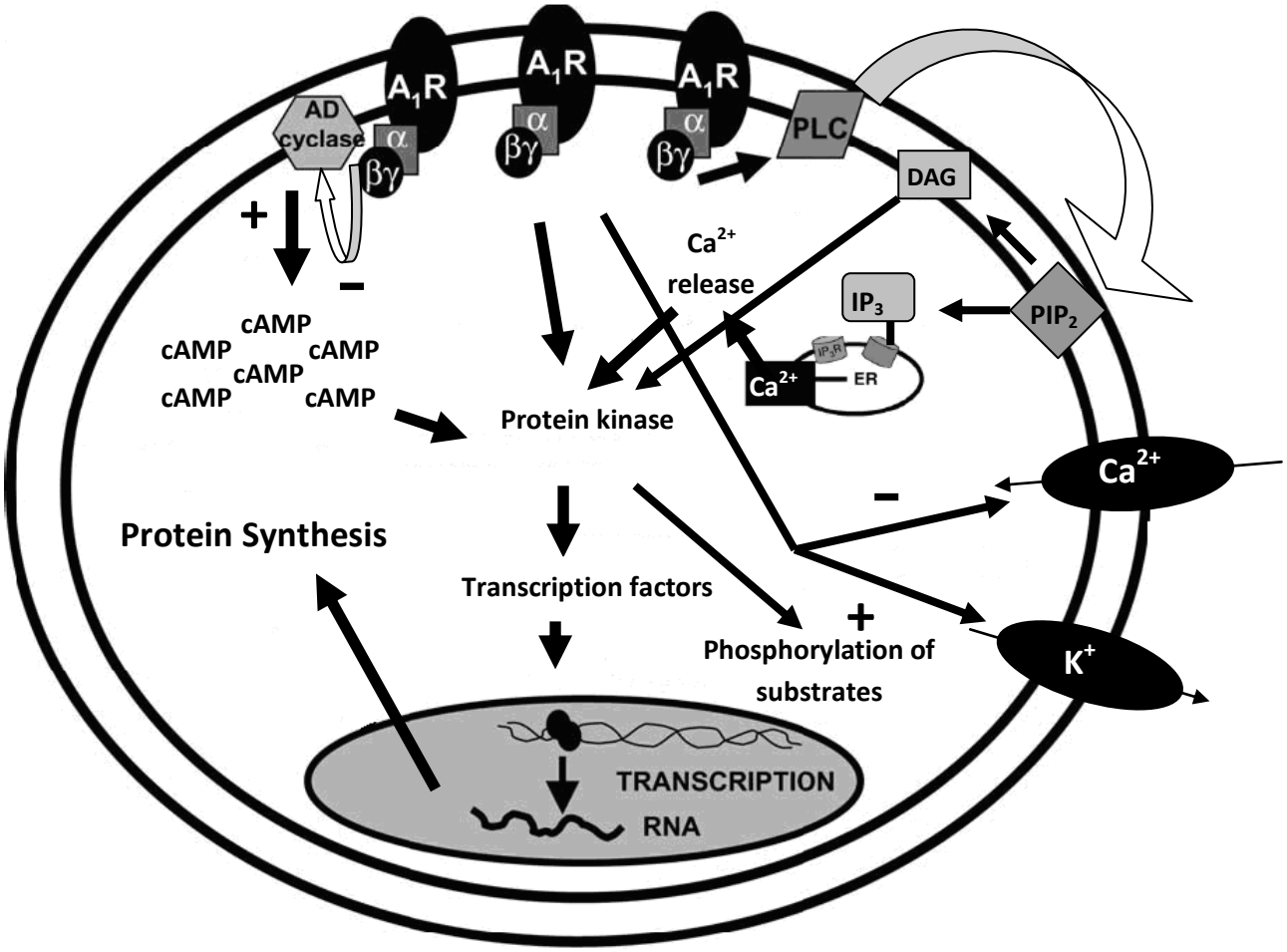


Figure 8. Intracellular signal transduction pathways triggered by activation of the adenosine $A_1$ receptor. Adenosine by acting on $A_1$ receptor activates $G_{i/o}$ proteins, which inhibit adenylyl cyclase, therefore damping the production of cAMP and the consequent activity of protein kinase A. Moreover, activated $G_{i/o}$-proteins promote the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$). Production of $PIP_2$ yields inositol 1,4,5-trisphosphate (IP3), which releases calcium ($Ca^{2+}$) from endoplasmic reticulum (ER), and diacylglycerol (DAG), activating protein kinase C. The subsequent phosphorylation of transcription factors facilitates transcription of genes in the nucleus and protein expression, while phosphorylation of other substrates implicates the modulation of enzymatic activity and ionic channel function. The βγ dimmers may also trigger the MAPK pathway, stimulate inwardly-rectifying $K^+$ channels and inhibit $Ca^{2+}$ channels (adapted from Basheer *et al*. 2004).

The hippocampus is one of the brain regions with the highest density of $A_1$ receptors (Fastbom *et al*. 1987), mainly located on excitatory presynaptic elements (Thompson *et al*. 1992). The most prominent inhibitory actions occur on excitatory glutamatergic systems (Dunwiddie and Hoffer, 1980; Proctor and Dunwiddie, 1987), where synaptic transmission can be fully blocked by $A_1$ agonists (Lorenzen *et al*. 1997; Sebastião *et al*. 1990). Inhibitory modulation of GABAergic systems is less frequent, so that the net effect of adenosine receptor activation in nearly all regions of the brain is to reduce excitability (Dunwiddie and Masino, 2001).

#### 1.3.2.1.1. Therapeutical potential of the adenosine $A_1$ receptor

Endogenous adenosine released by hypoxia (Fowler 1993a, b), ischemia (Lloid *et al*. 1993; Latini *et al*. 1999), electrical activity (Lloyd *et al*. 1993) and hypoglycemia or aglycemia (Calabresi *et al*. 1997; Fowler, 1993b) reduces the subsequent damage to neuronal tissue. Contributing to the increased adenosine concentration is the release of nitric oxide, as demonstrated in hippocampal slices (Fallahi *et al*. 1996) and forebrain neuronal cultures (Rosenberg *et al*. 2000). The neuroprotective ability of adenosine does not confine to damage derived from interference in energy metabolism, since it also acts against mechanical cell-injury (Mitchell *et al*. 1995) and methamphetamine-induced neurotoxicity (Delle Donne and Sonsalla, 1994). There is a consensual notion that the neuroprotective effects of adenosine are mediated primarily via $A_1$ receptor activation (Cunha *et al*. 2001; Dunwiddie and Masino 2001; Ribeiro *et al*. 2003a, b; Sebastião *et al*. 2001b). $A_1$ receptors exert two key actions in what respects neuroprotection: presynaptically inhibition of glutamate release and postsynaptic inhibition of NMDA receptors in hippocampal pyramidal neurons (de Mendonça *et al*. 1995). Excitotoxicity is decreased by limiting $Ca^{2+}$ entry and by reducing metabolic demand, which would help to preserve ATP stores essential for pumping $Ca^{2+}$ out of the cell (Dunwiddie and Masino 2001). In accordance with its role as an inhibitory neuromodulator, adenosine presents anticonvulsant effects in experimental models of epilepsy (reviewed in Dunwiddie 1999), which appear to be mainly mediated by $A_1$ receptors (Murray *et al*. 1992; Zhang *et al*. 1990). A chronic reduction of $A_1$ receptors has been found in epileptic tissue in both humans (Glass *et al*. 1996) and rats (Ochiishi *et al*. 1999), which may lead to a decrease in the tonic inhibitory action of adenosine and contribute to the neuronal hyperexcitability and recurrent epileptic seizures. Drugs that facilitate or block adenosine $A_1$ receptor-mediated actions may also be effective for the treatment of anxiety, pain and neurodegenerative diseases. $A_1$ agonists have anxiolytic activity in rodent models of anxiety, whereas antagonists present anxiogenic properties (Florio *et al*. 1998; Listos *et al*. 2005). In addition, knock-out mice for this receptor showed increased anxiety-related behaviour (Johansson *et al*. 2001). In the spinal cord, $A_1$ receptor activation produces anti-nociceptive responses in acute nociceptive, inflammatory and neuropathic pain tests (Lao *et al*. 2001, 2004; reviewed in Sawynok 1998). Modulation of pain by $A_1$ receptors is probably related to its ability to presynaptically inhibit excitatory transmission to substantia gelatinosa neurones in the spinal cord (Lao *et al*. 2001). Application of an $A_1$ receptor agonist attenuates striatal lesions, as well as dystonia, induced in a rat model of Huntington´s disease (Blum *et al*. 2002). $A_1$ receptors have also been implicated in drug addiction. In isolated guinea-pig ileum preparations, the selective $A_1$ antagonist 8-cyclopenthyl-1,3-dimethylxantine increased the amplitude of the naloxane-precipitated withdrawal response in tissue preparations acutely exposed to μ-opioid receptor agonist (Romanelli *et al*. 2005). Accordingly, the selective $A_1$ receptor agonist N6-cyclopentyladenosine (CPA) inhibits the withdrawal response to naloxane (Capasso *et al*. 2000; Romanelli *et al*. 2005). Other roles for $A_1$ receptor regulating

compounds include sleep regulation (Basheer *et al.* 2004; Elliott *et al.* 2001), neuronal maturation (Turner *et al.* 2002) and modulation of the effects of ethanol (Baptista *et al.* 2005; Gatch 2006).

#### 1.3.2.1.2. Interaction of the adenosine $A_1$ receptor with other receptors

The $A_1$ receptors have been shown, through immunoblotting and immunoprecipitation studies, to pair with each other to form homodimers (Ciruela *et al.* 1995). The formation of $A_1$ receptor heterodimers with mGluR1α (Ciruela *et al.* 2001), dopamine D1 (Franco *et al.* 2000) and $P2Y_1$ or $P2Y_2$ nucleotide receptors (Yoshioka *et al.* 2001) is also documented. Previous studies suggest that the $A_1$ receptors also interact with other G-protein-coupled receptors. Several reports provide evidence favoring an interaction with CB1 receptors in hippocampus (Kouznetsova *et al.* 2002) and cerebellum (DeSanty and Dar 2001; Savinainen *et al.* 2003; Selley *et al.* 2004). Like the $A_1$ receptors, the GABA receptors are also coupled to inhibitory G-proteins. Inhibitory neurotransmission in the hippocampus is mainly mediated by GABA, providing a balance for the excitatory glutamatergic transmission. Adenosine is able to functionally disconnect GABAergic interneurons by inhibiting their glutamatergic input, what could implicate an indirect control of GABAergic functions through the $A_1$ receptors (Lucchi *et al.* 1996). A tonic, low level of occupation of $A_1$ receptors might also be responsible for the regulation of the strength of $GABA_B$ synapses, as suggested by the synergistic interactions detected between low concentrations of $A_1$ and $GABA_B$ agonists (Sodickson and Bean 1998). On the other hand $A_1$ receptors may also interact with the metabotropic glutamate receptors and with the dopamine $D_1$ receptors, coupled to excitatory G-proteins. According to Shahraki and Stone (2003) the group I metabotropic glutamate receptors agonist DHPG depressed the inhibitory effect of adenosine in hippocampal slices, an interaction blocked by the protein kinase C inhibitor chelerythrine, while group II glutamate receptors (mglu2 and mglu3) mediate presynaptic inhibition of retinotectal synaptic transmission possibly by sharing a pertussis toxin (PTX)-sensitive G-protein with $A_1$ receptors (Zhang and Schmidt, 1999), since the sequential application of agonists for both receptors was not additive in effect but occluded each other. At cerebrocortical synaptosomes KCl-evoked glutamate exocytosis can be inhibited by $A_1$ receptor. This inhibition is associated with a decreased KCl-evoked $Ca^{2+}$ level elevation, and it is suppressed by a group I/group II mGluR agonist via protein kinase C (Budd and Nicholls 1995). Strikingly, an antagonic interaction between $A_1$ and $D_1$ receptors was detected in cotransfected fibroblast cells, since $A_1$ receptor agonists shifted the high affinity binding of dopamine to the low-affinity states of the $D_1$ receptor (Ferré *et al.* 1998). In addition, the $A_1$ receptor may contribute to modulate extracellular dopamine levels in the basal ganglia (Okada *et al.* 1996). Activation of adenosine $A_1$ receptors reduces the NMDA currents by a postsynaptic action (de Mendonça and Ribeiro 1993; de Mendonça *et al.* 1995). Other studies found that activation of NMDA receptors can increase the release of adenosine

(Delaney and Geiger, 1998; Manzoni *et al.*, 1994), which in turn may decrease the release of glutamate, and thereby the activation of NMDA receptors (Poli *et al.* 1991).

#### 1.3.2.1.3. Interactions between adenosine $A_1$ and cannabinoids CB1 receptors

Both the adenosine $A_1$ and the cannabinoid CB1 receptors are $G_{i/o}$-protein coupled receptors (Straiker *et al.* 2002) which regulate multiple transduction pathways and therefore may act upon several neuronal mechanisms simultaneously. They inhibit adenylyl cyclase activity and thereby reduce cyclic AMP concentrations in the brain (Fredholm *et al.* 1986; Felder *et al.* 1998). Furthermore, both $A_1$ and CB1 receptors are involved in the regulation of the second messenger pathways of diacylglycerol, the protein kinase C agonist, and inositol triphosphate, which mobilizes intracellular calcium (Tanaka and Saito, 1992; Ho *et al.* 1999; Basheer *et al.* 2004). Both receptors are expressed at high levels in the hippocampus, where they inhibit glutamatergic synaptic transmission (Dunwiddie and Hoffer 1980; Serpa *et al.* 2007; Shen *et al.* 1996), are involved in impairment of learning and memory (Carlini *et al.* 1970; Lu *et al.* 2010) and protect against neurotoxic insults (Monory *et al.* 2006; Sebastião *et al.* 2001). Given the similarity between transducing pathways operated by adenosine $A_1$ and cannabinoid CB1 receptors, as well as the similarity of effects produced by both receptors, clarification of combined activity of these receptors is particularly relevant. Moreover, $A_1$ and CB1 receptors are both targets for widely consumed drugs. Caffeine, an adenosine receptor antagonist, and the psychotropic CB1 receptor agonist $\Delta^9$-tetrahydrocannabinol (THC), the main active component of marijuana plants, are often ingested together. Although consumers are not in any immediate threat after ingestion, it remains wise to understand such mechanism and to determine the long-term side-effects which may arise from prolonged, continuous exposure to these drugs.

*Marijuana and Caffeine*

Prolonged consumption of marijuana or caffeine is known to modify both CB1 and $A_1$-mediated signalling, respectively, interfering with the sensitivity and availability of these receptors at the plasma membrane, because of receptor desensitization* and down-regulation. Accordingly, at the hippocampus of the rat long-term intake of caffeine leads to increased expression of $A_1$ receptors (Johanssen *et al.* 1993), a common compensation mechanism in response to antagonists, whereas prolonged exposure to CB1 agonists THC and WIN55212-2 decreases [$^{35}$S]GTPγS binding (Sim-Selley *et al.* 2006), a measure of CB1-mediated G-protein activation. Moreover, $A_1$ receptors were found to interfere with CB1 receptors activity in the hippocampus, where adenosine $A_1$ receptors located in GABAergic and glutamatergic nerve terminals of the hippocampus exerted a negative modulatory effect

* *see glossary for definition*

on the cannabinoid CB1 receptor-mediated inhibition of GABA and glutamate release; CB1-mediated G-protein activation was also impaired by $A_1$ receptor activation (Sousa *et al.* 2011). Furthermore, CB1 receptor-mediated signalling was unaffected by $GABA_B$ receptor activation, which indicates that not all $G_{i/o}$-coupled receptors interfere with CB1 receptor activity. On the other hand, *in vivo* experiments show that chronic administration of caffeine leads to an $A_1$ receptor-mediated enhancement of the CB1-dependent effects of THC on short-term spatial memory (Sousa *et al.* 2011). This evidence supports the existence of interaction between the effect of chronic caffeine consumption and the effect of THC.

*The Cerebellum and Motor Coordination*

The most solidly established function of the cerebellum is coordination of motor activity by integrating input from sensory systems of the spinal cord. CB1 receptors are localized presynaptically on cerebellar granule cell axonal terminals in the molecular layer of the cerebellum (Mailleux and Vanderhaeghen 1992). There is also evidence for localization of CB1 receptors on the inhibitory basket cells and excitatory climbing fiber terminals which synapse onto cerebellar Purkinje cells (Tsou *et al.* 1998). These receptors inhibit glutamate release from cerebellar neurones maintained in primary culture (Dolphin and Prestwich 1985). In addition, the adenosine $A_1$ receptor appears to be co-localized with CB1 receptors at the cerebellum (Wojcik *et al.* 1985). Previously it was demonstrated with the rotorod treadmill (Fig 9), a device which tests motor coordination, that THC impairs motor coordination through a CB1 receptor-mediated mechanism (Dar 2000). Posteriorly, DeSanty and Dar (2001) performed identical experiments where $A_1$ agonists were also tested. As expected, the CB1 receptor agonist CP55,940 decreased motor coordination, while the $A_1$ agonist cyclohexyladenosine (CHA) had no effect on motor performance. However, when both agonists were simultaneously injected in the cerebellum, a synergy was uncovered, since motor coordination was lower from the one observed with CP55,940 alone. Furthermore, cross-tolerance was also demonstrated, since three days of CHA administration reduced the motor incoordination effect of CP55,940, supporting a modulatory role for the cerebellar adenosine system on CB1-induced motor incoordination dependent of the cerebellum.

Figure 9. The rotorod treadmill measures motor coordination in rodents. In each lane a rodent is placed. The rodents are exposed to drugs or vehicle. Notice the platform beneath the rotating cylinder. When rodents fall off the cylinder into the platform the timer stops. The quicker the rodents fall the lower their motor coordination.

*G protein activation in Brain Membranes*

Receptor-specific activation of G-proteins can be measured using agonist-stimulated binding to membranes of the nonhydrolysable, radioactively labelled GTP analogue [$^{35}$S]GTPγS (Breivogel *et al.* 1997). The CB1 receptor, when activated by THC or WIN 55212-2, and the $A_1$ receptor, when activated by L-phenylisopropyladenosine (L-PIA), both generate [$^{35}$S]GTPγS binding in a concentration-dependent manner in whole-brain membranes (Sim *et al.* 1996) and in hippocampal membranes (Childers *et al.* 2005), respectively. Additional experiments performed by Breivogel *et al.* (2001) show that stimulation of [$^{35}$S]GTPγS binding by maximally effective concentrations of cannabinoid CB1 and adenosine $A_1$ receptor agonists are additive in whole-brain membranes of the rat. Accordingly, [$^{35}$S]GTPγS binding generated by co-application of $A_1$ and CB1 receptor agonists is also additive in membranes of the hippocampus (Childers *et al.* 2005), thus arguing against competition between both receptors at the level of their signal transduction mechanisms. Such result agrees with our own findings with extracellular electrophysiology that show additivity between $A_1$ receptor and CB1 receptor-mediated inhibition of synaptic transmission at the hippocampus (Serpa 2007). On the other hand, in a previous work, where synaptic activity was assessed measuring calcium spikes* using microfluorometry, prolonged exposure of cultured hippocampal neurons to adenosine $A_1$ or cannabinoid CB1 receptors agonists produced cross-desensitization between these receptors (Kouznetsova *et al.* 2002), suggesting that interference between the two

* *see glossary for definition*

receptors might occur for longer treatment with agonist where other downstream effectors might be influenced.

*Learning and Memory*

There is a relationship between glutamatergic activity and learning and memory at the hippocampus. Both $A_1$ and CB1 receptors inhibit glutamatergic transmission at the hippocampus (Dunwiddie and Hoffer 1980; Serpa 2007; Shen *et al*. 1996). Furthermore, CB1 (Nowicky *et al*. 1987) and $A_1$ (Costenla *et al*. 2010) receptor blockade enhanced LTP in hippocampal slices. In a behavioural model of learning and memory, selective $A_1$ receptor agonists decrease latency to drink in a passive-avoidance task (Normile and Barraco 1991), where water-deprived animals were trained to avoid drinking by pairing foot-shock with licks from a water spout. In spatial learning $A_1$ receptor antagonists exhibited an antiamnesic effect (Suzuki *et al*. 1993). In contrast to acute treatment, chronic treatment with an $A_1$ receptor agonist improved spatial memory acquisition in the Morris water maze (Von Lubitz *et al*. 1993). With respect to the CB1 receptor, mice subjected to cannabinoid antagonists show increased memory performance in the radial maze (Lichtman 2000; Fig 10), and also in the Morris water maze (Ferrari *et al*. 1999). When $A_1$ and CB1 receptor antagonists were coadministrated at a dosage which by itself failed to interfere with spatial learning, the acquisition of spatial learning was enhanced (Assini *et al*. 2012). In addition, the effect was blocked by NMDA receptor antagonists, implicating glutamatergic transmission of the hippocampus. Thus, a synergy between $A_1$ and CB1 receptors activity appears to be present at the hippocampus, at least at a high level of organization, such as it occurs in the cerebellum (DeSanty and Dar 2001).

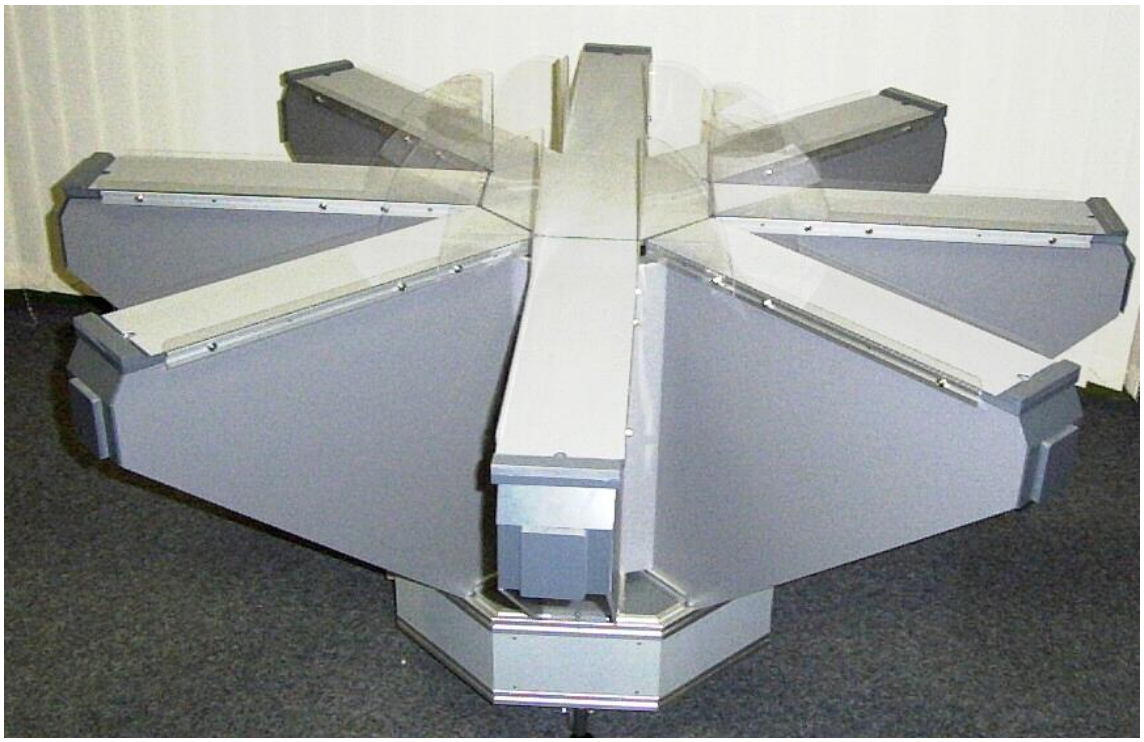

Figure 10. The radial arm maze. Used to assess working and reference memory in rats and mice. When investigating working memory, all arms are usually provided with a reward and the animal should visit each arm only once. When used for assessing reference memory, only some of the arms contain a reward and the animal should only visit those baited arms. Visits to an arm more than once or visits to non-baited arms count as working memory errors or reference memory errors, respectively.

## 1.4. Objectives

Adenosine $A_1$ receptor is coupled to $G_{i/o}$ proteins which negatively regulate adenylyl cyclase and thus cAMP formation (Straiker *et al.* 2002). Modulation of cGMP levels by a $G_{i/o}$ proteins-coupled receptor have also been recently reported (Jones *et al.* 2008). Since cyclic nucleotides play a central role in regulating essential functions in the brain, clarification of adenosine $A_1$ receptor action on cyclic nucleotides will help to better understand how adenosine produce its neuromodulatory and neuroprotective effects. Therefore, the main goal of the present work was to investigate the ability of adenosine $A_1$ receptors to regulate cyclic nucleotides levels, as well as the role of cyclic nucleotides as mediators of some actions of adenosine $A_1$ receptor, in the hippocampus. The hippocampus was chosen because it has the highest density of adenosine $A_1$ receptor in the brain (Fastbom *et al.* 1987) and it plays a central role in learning and memory (Vargha-Khadem *et al.* 1997).

Activation of adenosine $A_1$ receptor has been shown to decrease cAMP formation in the cerebral cortex (Alexander *et al.* 1994), but its effect on cAMP levels at the hippocampus is not clarified, nor its interaction with others neuromodulators while regulating cAMP levels. We first started by determining how adenosine $A_1$ receptors modulate cAMP levels at the hippocampus, by constructing a dose-response curve and calculating the $EC_{50}$ and $E_{max}$ for a selective adenosine $A_1$ agonist. Secondly, the effect of the cannabinoid CB1 receptor, which has similar properties to the adenosine $A_1$ receptor, was compared with the effect of $A_1$ receptor, and it was investigated if combining the effect of both receptors would result in additivity or interaction. Specifically, the combined effect of adenosine $A_1$ and cannabinoids CB1 receptors as negative modulators of cAMP accumulation, as well as their combined neuroprotective potential against an excitotoxic insult was investigated.

Previous studies indicate that cGMP produces similar effects to those triggered by adenosine $A_1$ receptor (Feil and Kleppisch 2008; Dunwiddie and Hoffer 1980; Dias *et al.* 2013) and, recently, it was reported that cGMP might mediate some actions of adenosine $A_1$ receptor in the peripheral nervous system (Lima *et al.* 2010). However, the role of cGMP on adenosine $A_1$ receptor mediated activity at the central nervous system remains obscure. Therefore, another aim of the present work was to clarify if and how cGMP is modulated by $A_1$ receptors at the hippocampus. A dose-response curve was constructed and the $EC_{50}$ and the $E_{max}$ were calculated for a selective adenosine $A_1$ agonist. The occurrence of differences between gender, in the pathway which $A_1$ receptors use to modulate cGMP levels was also assessed. Finally, it was investigated if the inhibitory effect of $A_1$ receptors on synaptic transmission is partially mediated by the NOS/cGC/PKG pathway, and if so to test if such mechanism depends on gender.

# Chapter 2: Methods

## 2.1. The hippocampal slice

As an experimental model, hippocampal slice provide distinct experimental advantages over other in vitro and in vivo preparations of the hippocampus. The most significant advantages are that, except for the absence of afferent input, hippocampal slice preparations retain the cytoarchitecture and synaptic circuits of the intact hippocampus (Lo *et al.* 1994), yet are readily accessible for optical imaging or electrophysiological studies. Hippocampal slices have proven to be a powerful experimental model for investigating the structural and functional features of synaptic connectivity at the molecular, cellular, and circuit levels. We used two different approaches: the acute hippocampal slices and the organotypic hippocampal slices cultures. The acute slices are intended to be used the same day that they are prepared and they remain functional for up to eight hours when immersed in oxygenated artificial cerebrospinal fluid (aCSF). We used two types of acute slices, the cross-chopped and the transverse-cut slice, both dissected from the young adult Wistar rat hippocampus. The cross-chopped slices are adequate to quantify cyclic nucleotides accumulation, since they remain suspended in aCSF with a high contact surface area, which allows efficient metabolic exchanges with the medium and facilitates drug penetration. The transverse-cut slice allows the study of electrophysiological properties and to measure synaptic transmission, since they maintain the hippocampus trisynaptic circuitry intact.

Cultured hippocampal slices were dissected from neonatal Wistar rats and were maintained in vitro for two weeks (Galimberti *et al.* 2006; Nagerl *et al.* 2004). We used slice cultures to investigate the combined neuroprotective potential of adenosine $A_1$ and cannabinoids CB1 receptors agonists against a excitotoxic insult by measuring released LDH activity and propidium iodide staining. The disadvantages of slice cultures relative to acute slices are that they involve more work to prepare and maintain, and the pattern of synaptic connections within the slice is considerably altered relative to the in vivo patterns at the time of harvest. Thus, at hippocampal slice cultures Coltman *et al.* (1995) detected axonal and dendritic remodeling and reorganization of synapses among the remaining neurons. Furthermore, in organotypic cultures glial cell activation leads to the formation of an astrocytic scar that surrounds the healthier tissue in the center of the slice. However, there are several advantages of the organotypic cultures relative to the acute slice preparation. First, the ability to track processes that occur over extended periods of time, second, the dead cells and tissue debris caused by the slicing procedure disappear after 1–2 weeks in vitro (Gahwiler *et al.* 1981), and third, the slice has time to recover from the altered metabolic state caused

by the enzymes and ions released during the cutting of the tissue (De Simoni *and Yu* 2006).

## 2.2. Immunoenzymatic Quantification of Cyclic Nucleotides Accumulation in Hippocampal Slices

### 2.2.1. Incubation Protocol

The experiments were performed using acute hippocampal slices taken from young adult male Wistar rats (6–8 weeks old). The brain was rapidly removed and transferred to ice-cold Krebs-Henseleit buffer with the following composition (mM): NaCl 118, KCl 4.7, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 25, glucose 11.6, gassed with 95% $O_2$ and 5% $CO_2$ (pH 7.4). The brain was cut longitudinally, the two hippocampi were dissected and cross-choped (350x350 µm) with a McIlwain tissue chopper. Sliced hippocampi were then placed in an Erlenmeyer, dispersed and washed twice with buffer. The cross-chopped hippocampal slices were transferred into a conical-bottom falcon tube and 50 µL-aliquots of gravity-packed slices (1-2 mg protein) were pipetted into flat-bottom propylene tubes (1.65x9.5 cm, 20 ml capacity) containing Krebs buffer and pre-incubated for 30 min at 37ºC in a shaking (1 cycle.$s^{-1}$) water bath. When testing the effect of a drug, a parallel control assay was done in which a same volume of vehicle replaced the volume of drug solution added to the tube. Tubes were gassed for 20 s and capped, after slices or drug addition.

### 2.2.2. cAMP

Incubation with drugs started with addition of rolipram (50 µM final concentration). Forty five min after rolipram addition, incubation proceeded in the absence or in the presence of forskolin (10-20 µM) for a further 15-35 min period. When used, WIN55212-2 (0.3-30 µM), adenosine deaminase (2 U/ml) or DPCPX (50 nM) were present simultaneously with the start of incubation with rolipram. CPA (10-150 nM final concentration), when present, was usually added 30 min after rolipram addition. The final volume after all drug additions was 300 µl. In some experiments CPA (100 nM final concentration) was added 50 min before rolipram addition. In another experiment WIN55212-2 (30 µM final concentration) was added 5 h and 15 min prior to rolipram addition. Note that, usually, longer incubation times were used when testing the effect of WIN55212-2 than when testing CPA effect; this was necessary because WIN55212-2 is very lipophilic and therefore needed longer incubations times to equilibrate with hippocampal slices and produce its inhibitory effect (see Serpa 2007). In experiments performed in the presence of AM251 (10 µM final concentration), it was added 30 min prior to addition of rolipram.

### 2.2.3. cGMP

In experiments where cGMP was quantified, incubation with drugs started with addition of Zaprinast and Bay 60-7550 (100 µM and 10 µM final concentration, respectively) to generate cGMP accumulation. Zaprinast is a selective inhibitor of cGMP-specific PDEs V and VI, while Bay 60-7550 is mainly a PDE II inhibitor. When used, adenosine deaminase (2 U/ml) and DPCPX (100 nM) were present since the beginning of incubation, together with Zaprinast and Bay 60-7550. When present, CPA (0.03-300 nM final concentration) was added 30 min after induction of cGMP accumulation by Zaprinast and Bay 60-7550, whereas sodium nitroprusside (SNP, 100 µM final concentration), when present, was added 15 min after CPA addition. The final volume after all drug additions was 400 µl.

### 2.2.4. Stop of Incubation

Incubations were stopped by addition of 133 µL (in cGMP experiments) or 100 µl (in cAMP experiments) of perchloric acid ($HClO_4$, 10% w/v) solution containing EDTA (20 mM). Samples were sonicated for 2 min, placed on ice for 30 min, neutralized by addition (133 µL for cGMP or 100 µl for cAMP experiments) of potassium carbonate ($K_2CO_3$, 0.5 M) and vortexed for 2 minutes, allowing the $CO_2$ to escape. The tubes were then placed on ice for an additional 15 minute period to precipitate potassium perchlorate. Afterwards, 400 uL of each sample was transferred to 1.5 mL eppendorfs and centrifuged (5000 g, 10 min at 4 ºC) and 300 µL aliquots of the supernatants were collected and stored at -20 ºC for further cAMP or cGMP analysis. The pellets were digested with NaOH (1 M) for 90 min at 37ºC, neutralized and individually assayed in duplicate for protein content by the method of Peterson (1977). The samples were analyzed for cGMP or cAMP concentration using cGMP- or cAMP-specific enzyme immunoassay (EIA) kits (Enzo Life Sciences). cGMP or cAMP concentration in each sample was expressed as pmol per mg of protein.

### 2.2.5. Cyclic Nucleotides Quantification

The EIA kit (Cayman Chemical) assay for quantification of cyclic nucleotides is based on the competition between the cyclic nucleotide content from the samples and cyclic nucleotide bound to acetylcholinesterase (CN-Ach), for a limited amount of cyclic nucleotide-specific rabbit antibody binding sites. Because the concentration of CN-Ach is held constant while the concentration of cyclic nucleotide from the samples varies, the amount of CN-Ach that is able to bind to the rabbit antibody will be inversely proportional to the concentration of free cyclic nucleotide in the well. Finally, acetylcholinesterase metabolizes a substrate added to the wells which renders a by-product that absorbs at a particular wavelength that may be spectrophotometrically measured. The higher the absorbance readings the lower the cyclic nucleotide content in the sample.

Briefly, 100 µl aliquots of cyclic nucleotides containing samples (appropriately diluted) or standards were pipetted into the appropriate plate wells coated with goat antibody specific to rabbit IgG. Three control assays, corresponding to maximal CN-Ach binding ($B_0$), non-specific binding (NSB) and blank, were also performed by pipetting 100 µl of assay buffer into the appropriate wells. Fifty µl of CN-Ach solution or assay buffer (blank) were added to each well, followed by addition of 50 µl of cAMP- or cGMP-specific rabbit antibody or assay buffer (NSB and blank). Plate was incubated for 2h at room temperature on a plate shaker. After this period the contents of the wells were emptied and wells were rinsed 3 times with wash solution. Acetylcholinesterase substrate solution (200 µl) was then added and after incubation for 1h at room temperature, absorbance of each well was read at 405 nm against blank. After subtracting the absorbance of NSB from the total absorbance of each well, the net absorbance corresponding to samples or standards (B) was expressed as percentage of the net absorbance corresponding to maximum binding ($B_0$). Finally, cyclic nucleotide concentration was calculated from a calibration curve of $\%B/B_0$ versus cyclic nucleotide concentration.

## 2.3. Organotypic Hippocampal Slice Cultures

Hippocampal slice cultures were prepared from 7-day-old Wistar rats according to the interface culture method (Stoppini *et al*. 1991). As previously described in detail (Bernardino *et al*. 2005), mice were killed by decapitation, their brains removed and the two hippocampi cut in 350 µm coronal sections using a McIlwain tissue chopper. Individual slices were placed in ice-cold Gey's balanced salt solution (Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 25 mM D-glucose, and then positioned on porous insert membranes (Millipore Corp., Bedford, MA, USA). Six slices were put onto each membrane and the inserts were transferred to a 6-well culture tray (Corning Costar, Corning, NY, USA). Each well contained 1 mL culture medium, composed of 50% Opti-minimal essential medium, 25% heat-inactivated horse serum, and 25% Hank's balanced salt solution (all from Invitrogen, Paisley, UK) supplemented with 25 mM D-glucose, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen). The culture trays were placed in an incubator with 5% $CO_2$ and 95% atmospheric air at 37ºC and the medium was changed twice a week for 2 weeks. 24 h before the start of the experiment, the culture medium was replaced with 1 mL of serum free Neurobasal medium containing 1 mM L-glutamine and 20 µL B27 supplement (Invitrogen). Twenty-four hours after the culture medium was replaced by Neurobasal medium we proceeded by incubating hippocampal slices with drugs. Experiments started with addition of WIN55212-2 (30 µM final concentration) or equal volume of its vehicle (DMSO). After 30 min CPA (100 nM final concentration) or its vehicle (water) were applied and 15 min later NMDA (50 µM final concentration) or its vehicle (water) were added and incubation proceeded for 1 h. After this period, medium was replaced with 1 ml of fresh, serum and drug free, Neurobasal medium

and 24 h later 100 µl aliquots of medium were collected. Samples collected from each well were analyzed for lactate dehydrogenase (LDH) activity.

### 2.3.1. Determination of cell death by propidium iodide

Spontaneous and induced cell death in slice cultures was assessed by monitoring the cellular uptake of the fluorescent dye propidium iodide (PI; 3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenylphenanthridinium diiodide; Sigma). As a polar substance, PI enters dead or dying cells only, and interacting with DNA emits red fluorescence (630 nm; absorbance 493 nm). PI is not toxic to cells and has been used widely as an indicator of neuronal membrane integrity and cell damage (Noraberg *et al*. 2005). Three hours before exposure to drugs, 2 µM PI was added to the incubation medium for determination of basal cellular uptake, and the same concentration of PI was added during all subsequent medium changes. Cellular uptake of PI was recorded by fluorescence microscopy (microscope BX61, Hamburg, Germany) using a rhodamine filter and digital camera (F-View 2; Olympus, Hamburg, Germany) with 100 ms exposure time. Digital fluorescent micrographs were taken before drug exposure (basal PI uptake) and 24 h after drug exposure. For quantitative assessment of neuronal damage, the region of interest was delineated in ImageJ program (1.47g, NIH, USA) and used for densitometric measurements of the PI uptake. The effect of treatments was assessed by subtracting the basal PI-uptake value measured in control untreated slices from the corresponding value after treatment.

### 2.3.2. Determination of cell death by quantification of lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from toxic insults such as NMDA application. LDH catalyzes the reduction of $NAD^+$ to NADH and $H^+$ by oxidation of lactate to pyruvate, or the reverse reaction. LDH activity was measured at room temperature by the method of Stolzenback (1966). Briefly, 50µl samples of culture medium were added to 0.6 mM sodium pyruvate and 0.18 mM β-NADH in 50 mM phosphate buffer (pH 7.5, total volume 150 µl). The decrease of absorbance at 340 nm with time was measured and the slope of the linear relationship was calculated. LDH activity was calculated from the slope and expressed as µmol/min/ml. In some cases we used a commercial kit (LDH cytotoxicity assay kit, Cayman Chemical), also a colorimetric quantification method, similar to the method of Stolzenback. Both methods provided reliable quantification of cellular death.

## 2.4. Extracellular Electrophysiology

Extracellular recordings of field excitatory post-synaptic potentials (fEPSPs) measured at area CA1 were conducted in 400 µm hippocampus slices from male Wistar rats (6-8 weeks). The fEPSP can be subdivided into three components: the stimulus artefact, the presynaptic volley and the field synaptic potential. The stimulus artefact occurs when the stimulator ($S_0$) discharges an electric impulse into the slice (e.g. Schaffer-collaterals). The impulse propagates along CA3 pyramidal cells axons causing $Na^+$ influx until it reaches the synaptic terminals of CA1 dendritic region, originating the presynaptic volley. As a consequence, glutamate is released into the synaptic cleft causing postsynaptic depolarization of CA1 pyramidal neurons, generating a field synaptic potential. The field synaptic potential is a wave that results from the sum of inhibitory and excitatory postsynaptic potentials (Alger and Nicoll, 1982), and its V-like shape represents the induced depolarization (descendent phase) followed by a homeostatic repolarization (ascendant phase). The slope of the descendent phase represents an indicator of the strength and efficiency of synaptic transmission conveyed to the CA1 region.

Briefly, the rats were anaesthetized with Halothane before decapitation, the brain was removed into a Petri dish placed on ice and the right hippocampus was dissected free within ice-cold artificial cerebrospinal fluid (aCSF) and gassed with carbogen ($O_2$, 95%; $CO_2$, 5%) mixture (pH 7.4). After removal, the hippocampus was placed in a McIlwain tissue chopper, and cut perpendicularly to its long axis. Slices were then carefully placed in different holding chambers with aCSF at room temperature (22-25ºC) and left to stabilize for at least one hour or until they were required. From the holding chamber they were transferred to a recording chamber (1ml capacity) and continuously perfused at 3 ml/min with aCSF maintained at 32ºC by a TC-202A temperature controller from Harvard Apparatus. The drugs were added to the perfused aCSF. The aCSF contained (mM): NaCl 124; KCl 3; NaH2PO4 1.24; NaHCO3 26; MgSO4 1; CaCl2 2; and glucose 10.

Evoked field excitatory postsynaptic potentials (fEPSP) were recorded through an extracellular pulled glass capillary microelectrode (4 M NaCl, 2–8 MΩ resistance) placed in the stratum radiatum of the CA1 area. Stimulation (rectangular pulses of 0.1 ms) was delivered (once every 15 s) through a semi-micro concentric bipolar stainless steel electrode (0.1 mm contact diameter, Harvard apparatus) placed on the Schaffer collateral/commissural fibres, in the stratum radiatum near the CA3/CA1 border. Distance between the tips of the stimulating and recording electrodes was about 1 mm. The intensity of the stimulus (80-200 µA intensity) was adjusted to obtain a large fEPSP with a minimal population spike contamination. To avoid supramaximal stimulation, the stimulus intensity was also adjusted to obtain a fEPSP slope within 50–80% of its maximum value under supramaximally stimulating conditions. Extracellular recordings were obtained with an Axoclamp 2B amplifier, digitized and continuously stored on a personal computer with the LTP 230d program (Anderson and Collingridge, 2001). Averages of eight consecutive responses were continuously monitored. To

allow comparisons between different experiments, slope and amplitude values were normalized, taking as 100% the averaged five values obtained immediately before applying the test drug. Responses were quantified as the slope of the initial phase of the averaged fEPSPs, since slope measures are considered a more accurate measure of fEPSP magnitude than the amplitude, due to eventual contamination by the population spike.

## 2.5. Drugs

The following drugs were used: N6-cyclopentyladenosine (CPA; Tocris), Bay 60-7550 (Cayman Chemical;), KT5823 (Santa Cruz Biotecnology), 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP; Sigma), 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ; Sigma), NG-nitro-L-arginine methylester (L-NAME; Sigma), Adenosine deaminase (ADA; Roche), (R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55212-2; Tocris), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; Tocris), N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; Tocris), 4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (rolipram; Tocris), 7ß-acetoxy-8,13-epoxy-1a,6B,9a-trihydroxy-labd-14-en-11-one (forskolin; Tocris), N-methyl-D-aspartate (NMDA; Tocris), 5-(2-Propoxyphenyl)-1*H*-[1,2,3]triazolo[4,5-d]pyrimidin-7(4*H*)-one (zaprinast; Tocris). All other reagents used were from analytical grade.

## 2.6. Data Analysis

The values are expressed as mean ± S.E.M. from n independent experiments. The effect of a drug on cyclic nucleotide accumulation was calculated, for each experiment, as: 100x(D-C)/C; where D is the cGMP or cAMP accumulation obtained in the presence of the drug and C is the cGMP or cAMP accumulation obtained in the corresponding control assay performed in the same conditions but in the absence of the drug. The cGMP accumulation that would be expected in the presence of both CPA and SNP if the effect of each drug were additive was calculated as: A+B-C; where A, B and C are, respectively, the cGMP accumulation obtained in the presence of CPA, in the presence of SNP, and in the absence of CPA and SNP (control). The inhibitory effect of a drug(s) on the NMDA-induced LDH activity was calculated, for each experiment, as: 100x(N-D)/(N-C); where N is the LDH activity obtained in the presence NMDA, D the LDH activity obtained in the presence of the drug(s) plus NMDA and C is the LDH activity obtained in the control assay performed in the same conditions but in the absence of the drug(s) and NMDA. The PI uptake obtained in the presence of drug(s) was expressed as percentage of the PI uptake corresponding to control slices incubated in the same conditions but in absence of drug(s). The PI uptake that would be expected in the presence of CPA, WIN55212-2 and NMDA if the effect of CPA and WIN55212-2 were additive, was calculated as: A+B-C; where A, B and C are, respectively, the PI uptake obtained in the presence of

CPA+NMDA, in the presence of WIN55212-2+NMDA, and in the absence of CPA and WIN55212-2 but in the presence of NMDA.

The significance of the differences between the means obtained in two different conditions, or when comparing means with zero, was evaluated by Student's t-test, where the paired Student's t-test was used whenever evaluating the significance of differences between two conditions tested in a paired way in the same experiment. When more than two different conditions were simultaneously considered, the one-way or two-way (when two factor were analyzed) ANOVA were used, followed by the LSD post-hoc test. When analyzing, by ANOVA, differences between the means of cGMP accumulation obtained in different conditions, adjustment for inter-experiment variability was performed. Statistical significance was considered for $P<0.05$. The maximal effect (Emax) and the concentration of agonist producing half-Emax (EC50) were calculated by fitting the agonist concentration-response curve data to a sigmoidal curve equation, through non-linear regression analysis using the PASW for Windows program version 18.0.

# Chapter 3: Modulation of cAMP accumulation by adenosine $A_1$ and cannabinoid CB1 receptors

## 3.1 Introduction

Activation of adenosine $A_1$ receptor has been shown to decrease cAMP formation in the cerebral cortex (Alexander *et al*. 1994), but its effect on cAMP levels at the hippocampus is not fully clarified, nor its interaction with others neuromodulators which regulate cAMP levels. Endogenous cannabinoids have neuromodulatory actions in the brain, mediated by cannabinoids CB1 receptors, which are very similar to adenosine $A_1$ receptor-mediated actions. In fact, both cannabinoid CB1 and adenosine $A_1$ receptors are coupled to Gi/o-proteins and are expressed at high levels in the hippocampus (Herkenham *et al*. 1991; Fastbom *et al*. 1987). Furthermore, both receptors inhibit glutamatergic synaptic transmission (Dunwiddie and Hoffer, 1980; Shen *et al*. 1996), are involved in impairment of learning and memory (Carlini *et al*. 1970; Lu *et al*. 2010) and protect against neurotoxic insults (Monory *et al*. 2006; Sebastião *et al*. 2001b). Given the similarity between the transducing pathways operated by adenosine $A_1$ and cannabinoid CB1 receptors, clarification of the combined activity of these receptors is a particularly interesting issue since both receptors are targets for widely consumed drugs, such as caffeine, an adenosine receptor antagonist, and the psychotropic $\Delta^9$-tetrahydrocannabinol (THC), a cannabinoid CB1 receptor agonist. Interaction between $A_1$ and CB1 receptors have been reported in *in vivo* studies, where an adenosine $A_1$ receptor-mediated enhancement of cannabinoid CB1 receptor-induced impairment of short-term spatial memory and motor incoordination was observed (Sousa *et al*. 2011; DeSanty and Dar 2001). However, the interactions observed *in vivo* might be polysynaptic and dependent on circuitry, not necessarily reflecting receptor interaction at the cellular and molecular levels. Previous studies indicate that acute co-activation of adenosine $A_1$ and cannabinoid CB1 receptors independently inhibit excitatory synaptic transmission in the rat hippocampus (Serpa 2007) and additively stimulate G-protein activation in whole brain and hippocampal membranes (Breivogel *et al*. 2001; Childers *et al*. 2005; but see Selley *et al*. 2004).

In the present work, we started by investigating how adenosine $A_1$ receptors modulate cAMP levels at the hippocampus. The effect of $A_1$ receptors was also compared with the effect of cannabinoid CB1 receptor on cAMP formation. Furthermore, we also investigated if combining the effect of both receptors would result in an additive inhibitory action on cAMP formation in the hippocampus, similar to what has been observed for G-protein activation in brain

membranes and inhibition of synaptic transmission, or if receptor interaction occurs. On the other hand, since interaction between adenosine $A_1$ and cannabinoid CB1 receptors can be consequence of heterologous receptor desensitization (Kouznetsova *et al.* 2002), the effect of adenosine $A_1$ receptor desensitization on the combined action of adenosine $A_1$ and cannabinoid CB1 receptors, was also investigated.

## 3.2 Results

### 3.2.1 Maximal effect, potency and specificity of adenosine A1 and cannabinoid CB1 agonists on forskolin-stimulated cAMP accumulation

Since basal intracellular levels of cAMP in hippocampal slices are low and hard to quantify, most experiments were performed in the presence of forskolin, which directly stimulates adenylyl cyclase, and rolipram, which inhibits phosphodiesterase 4 (Kelly and Brandon, 2009), to increase basal cAMP concentration. In the presence of rolipram (50 μM), the cAMP accumulation was 40 ± 11 pmol/mg protein, whereas the further addition of 20 μM forskolin increased basal cAMP accumulation by about five fold to 202 ± 46 pmol/mg protein ($P<0.05$, paired Student's t-test).

Concerning the adenosine $A_1$ receptor selective agonist CPA (10-150 nM) and the cannabinoid CB1 receptor selective agonist WIN55212.2 (0.3-30 μM), we observed a dose-dependent inhibition of forskolin-stimulated cAMP accumulation in the hippocampus (Fig 11). Based on this data, we calculated an $EC_{50}$ for CPA of 35 ± 19 nM and a maximal decrease of cAMP accumulation (Emax) of 29% ± 5%, whereas in the case of CB1 receptors an $EC_{50}$ for WIN55212-2 of 6.6 ± 2.7 μM and an Emax of 31% ± 2% were obtained. Application of CPA (100 nM) caused a 21% ± 3% (n=8) inhibition of cAMP accumulation, whereas when WIN55212-2 (30 μM) was applied the cAMP accumulation decreased by 25% ± 4%. We found these concentrations adequate to test the combined effect of CPA and WIN55212-2 on cAMP accumulation, since we obtained a near-maximal effect without losing specificity of applied agonists.

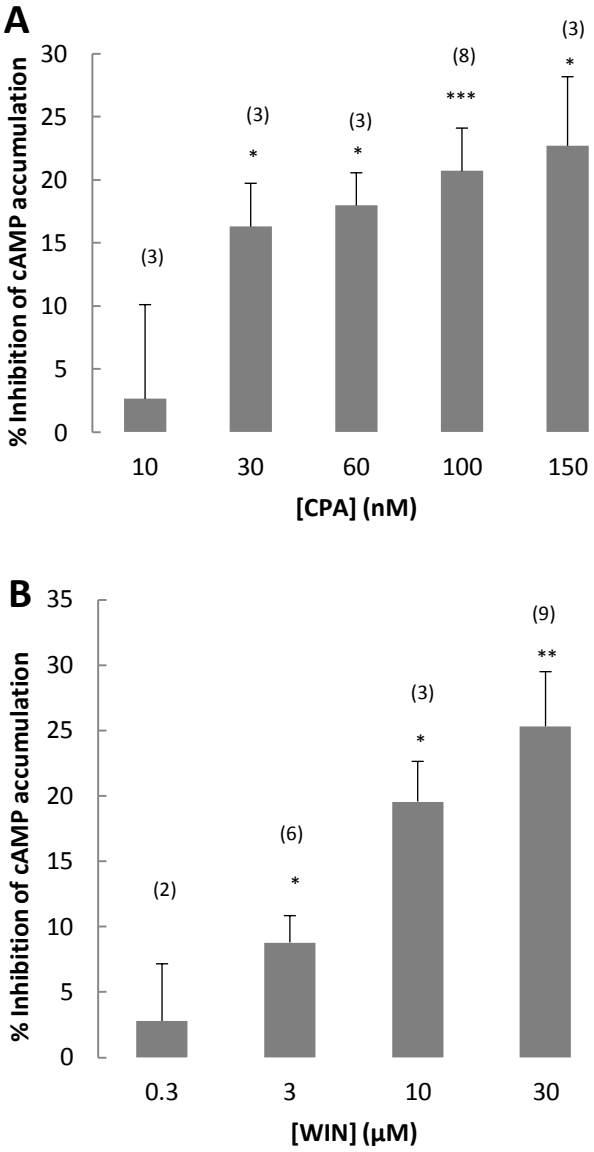


**Figure 11.** Inhibition of forskolin-stimulated cAMP accumulation by CPA (A) and WIN55212-2 (B) in rat hippocampal slices. **(A):** Slices were incubated for 30 min in the presence of rolipram (50 μM) and adenosine deaminase (2 U/ml). After this period, incubation continued for a 15 min period in the absence (control) or in the presence of CPA (10-150 nM). Finally incubation proceeded in the presence of forskolin (20 μM) for a further 15 min period. **(B):** Slices were incubated for 45 min in the presence of rolipram (50 μM) and in the absence (control) or in the presence of WIN55212-2 (0.3-30 μM). After this period, incubation continued for a further 35 min period in the presence of forskolin (10 μM). Data are mean ± SEM of the % inhibition of control cAMP accumulation, corresponding to 3-9 independent experiments run at least in triplicate. * $P<0.05$ and *** $P<0.001$, when compared with zero, Student´s t-test. The number of experiments corresponding to each concentration is indicated in brackets above the bars.

The inhibitory effect of 100 nM CPA on cAMP accumulation was blocked by the adenosine $A_1$ receptor selective antagonist DPCPX (50 nM; Fig 12A), whereas the effect of 10 μM WIN55212-2 was blocked by the cannabinoid CB1 receptor selective antagonist AM251 (10 μM; Fig 12B), indicating that the inhibitory effect of CPA and WIN55212-2 are mediated by adenosine $A_1$ and cannabinoid CB1 receptors, respectively. Accumulation of cAMP was not affected by either DPCPX or AM251 alone (Fig 12).

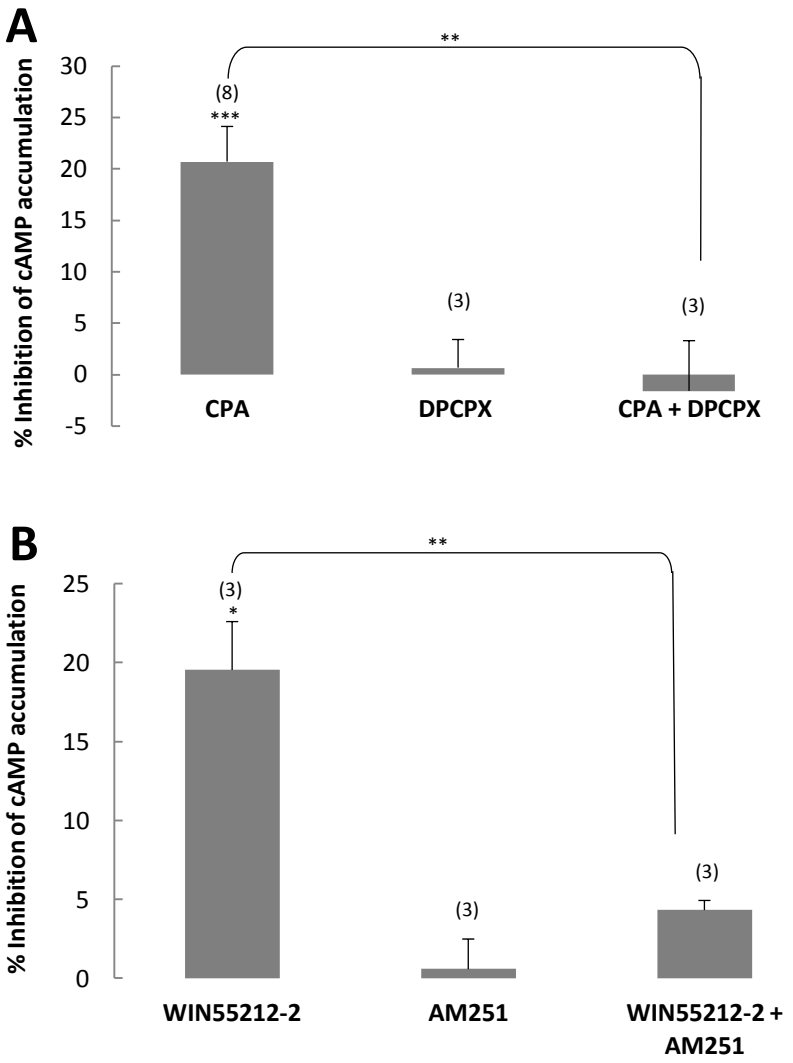


**Figure 12.** Reversal of the inhibitory effects of CPA (A) and WIN55212-2 (B) on forskolin-stimulated cAMP accumulation, by selective $A_1$ receptor and CB1 receptor antagonists, respectively. **(A):** Slices were incubated for 30 min in the presence of rolipram (50 μM), adenosine deaminase (2 U/ml) and in the absence (control) or in the presence of DPCPX (50 nM). After this period, incubation continued for 15 min in the absence (control) or in the presence of CPA (100 nM). Finally incubation proceeded in the presence of forskolin (20 μM) for a further 15 min period. The solid bars represent the % inhibition of control cAMP accumulation produced by (from left to right) CPA, DPCPX, and CPA plus DPCPX. **(B):** Slices were incubated for 30 min in the absence (control) or in the presence of AM251 (10 μM). After this period, the incubation continued for 45 min in the presence of rolipram (50 μM) and in the absence (control) or in the presence of WIN55212-2 (10 μM). Finally incubation proceed in the presence of forskolin (10 μM) for a further 35 min period. The solid bars represent the % inhibition of control cAMP accumulation produced by (from left to right) WIN55212-2, AM251, and WIN55212-2 plus AM251. Data are mean ± SEM from 3-8 independent experiments run at least in triplicate. * $P<0.05$ and *** $P<0.001$ when compared with zero; ** $P<0.01$ when compared with the effect obtained in the absence of antagonist, Student's t-test. The number of experiments performed at each concentration is indicated in brackets above the bars.

### 3.2.2 Combined effect of adenosine $A_1$ and cannabinoid CB1 agonists on cAMP accumulation

The combined effect of CPA (100 nM) and WIN55212-2 (30 μM) on forskolin-stimulated cAMP accumulation was investigated. When CPA (100 nM) and WIN55212-2 (30 μM) were applied to slices, respectively, 15 min and 45 min before forskolin, the combined application of WIN55212-2 and CPA produced a higher inhibition of cAMP accumulation than that produced either by WIN55212-2 (30 μM) or CPA (100 nM) alone (Fig 13). In this case, the combined action of 100 nM CPA and 30 μM WIN55212-2 produced a 41% ± 5.3% inhibition of the forskolin-stimulated cAMP accumulation, which did not differ from the sum of the individual effects of each agonist (43% ± 6%; P>0.7, paired Student´s t-test, Fig. 13).

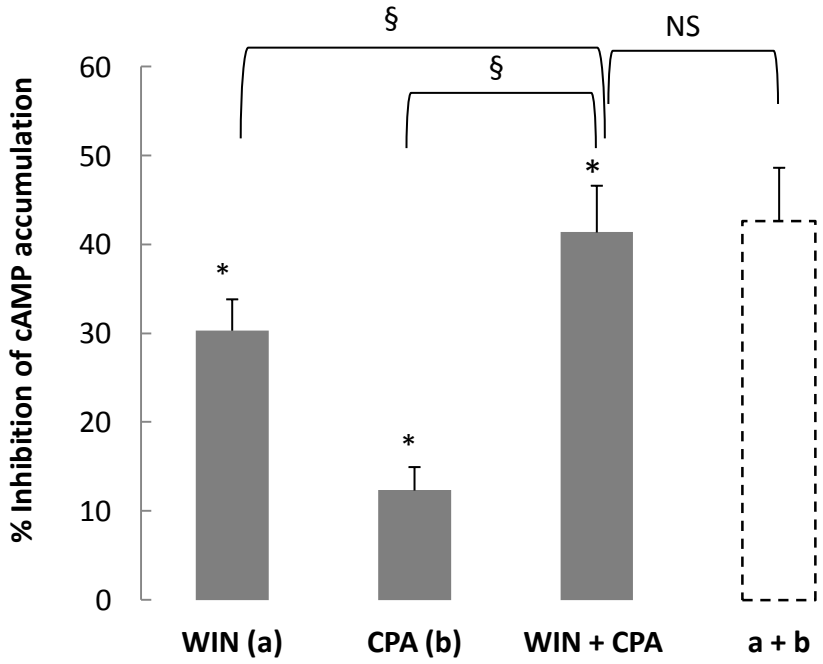


**Figure 13.** Combined effect of WIN55212-2 and CPA on forskolin-stimulated cAMP accumulation in rat hippocampal slices. Slices were incubated for 45 min in the presence of rolipram (50 μM) and in the absence (control) or in the presence of WIN55212-2 (30 μM). After this period, incubation proceeded in the presence of forskolin (10 μM) for a further 35 min period. CPA (100 nM final concentration) or vehicle (control) was added 15 min before forskolin. In each experiment four parallel assays were performed, corresponding respectively to: incubation with WIN55212-2, CPA, WIN55212-2 + CPA and incubation in the absence of WIN55212-2 and CPA (control). Solid bars represent the % inhibition of control cAMP accumulation produced by (from left to right) WIN55212-2, CPA, and WIN55212-2 plus CPA; the dashed bar represents the arithmetical sum of the values represented in the 1st and 2nd columns. Data are mean ± SEM from 4 independent experiments run at least in triplicate. * Statistically different from zero (P<0.05). § Statistically different from the effect of WIN55212-2 (a) or CPA (b) alone (P<0.05; One-way ANOVA). NS: Difference not statistically significant.

### 3.2.3 CB1 modulation of cAMP accumulation remains unaffected by desensitization of adenosine $A_1$ receptors

As we may observe in Figure 14A, increasing the pre-incubation period with CPA, from 15 to 95 min before forskolin addition, caused a significant ($P<0.05$) attenuation of the CPA effect on forskolin-stimulated cAMP accumulation in the hippocampal slice, suggesting adenosine $A_1$ receptor desensitization. In fact, when 100 nM CPA was applied 95 min before forskolin, no significant effect of CPA was observed ($P>0.39$, Fig 14A and 14B). The possibility that adenosine $A_1$ receptor desensitization could cross-desensitize cannabinoid CB1 receptors and consequently modify the cannabinoid CB1-mediated action on cAMP accumulation, was investigated. When adenosine $A_1$ receptors were desensitized by pre-incubation with CPA (100 nM) for 95 min before forskolin addition (50 min before WIN55212-2), the inhibitory effect of WIN55212-2 (30 μM) on forskolin-stimulated cAMP accumulation (37% ± 11% inhibition in the absence and 40 % ± 13% inhibition in the presence of CPA; $P>0.2$, paired Student´s t-test; Fig 14B) was not modified, suggesting absence of heterologous cannabinoid CB1 receptor desensitization by adenosine $A_1$ receptor.

Contrasting with CPA, the WIN55212-2 (30 μM) inhibitory effect on forskolin-stimulated cAMP remained virtually unchanged even when slices were pre-incubated with WIN55212-2 (30 μM) for up to six hours (31% ± 6% inhibition for 45 min pre-incubation and 30 % ± 5 % inhibition for 6 h pre-incubation with WIN55212-2; $P>0.05$, paired Student´s t-test). Longer incubation periods were not used to avoid losing slice integrity.

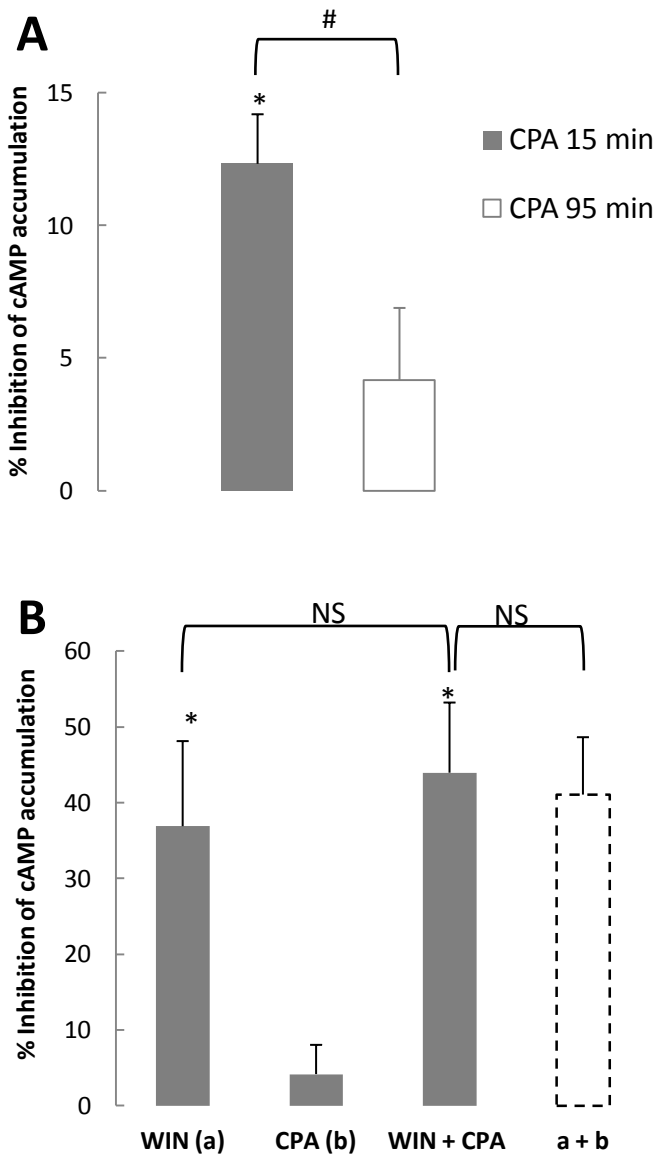


**Figure 14** Combined effect of WIN55212-2 and CPA on forskolin-stimulated cAMP accumulation in rat hippocampal slices; influence of the pre-incubation period with CPA. **(A):** Time-dependent attenuation of the CPA effect. Slices were incubated for 45 min in the presence of rolipram (50 μM). After this period, incubation continued in the presence of forskolin (10 μM) for a further 35 min period. CPA (100 nM final concentration) or vehicle (control) was added 15 min (solid bar) or 95 min (open bar) before forskolin. Bars represent the % inhibition produced by CPA of control cAMP accumulation. # Statistically different from CPA added 15 min before forskolin ($P<0.05$, Student's t-test). **(B):** Effect of WIN55212-2 under $A_1$ receptor desensitization. Slices were incubated for 45 min in the presence of rolipram (50 μM) and in the absence (control) or in the presence of WIN55212-2 (30 μM). After this period, incubation proceeded in the presence of forskolin (10 μM) for a further 35 min period. CPA (100 nM final concentration) or vehicle (control) were added 95 min before forskolin. In each experiment four parallel assays were performed, corresponding respectively to: incubation with WIN55212-2, CPA, WIN55212-2 + CPA and incubation in the absence of WIN55212-2 and CPA (control). Solid bars represent the % inhibition of control cAMP accumulation produced by (from left to right) WIN55212-2, CPA, and WIN55212-2 plus CPA; the dashed bar represents the arithmetical sum of the values represented in the 1st and 2nd columns. Data are mean ± SEM from 4 independent experiments run at least in triplicate. * Statistically different from zero ($P<0.05$). NS: Difference not statistically significant.

## 3.3 Discussion

***Chara**cterization of the effect of adenosine $A_1$ and cannabinoid CB1 agonists on cAMP accumulation in the hippocampus*

The present work showed that both adenosine $A_1$ and cannabinoids CB1 receptors are negative regulators of cAMP formation in the rat hippocampus. The $E_{max}$ obtained in the present work (29%) for the inhibitory effect of CPA on cAMP accumulation, when applied 15 min before forskolin, was lower than the value previously reported in guinea-pig cerebral cortex (91%), probably reflecting differences in species and/or brain regions (Alexander *et al*. 1994), whereas the $EC_{50}$ (36 nM) was similar to that obtained in guinea-pig cerebral cortex (22 nM; Alexander *et al*. 1994). The maximal inhibitory effect of WIN55212-2 on forskolin-stimulated cAMP accumulation obtained in the present work (31% inhibition) was very similar to the value previously obtained in rat *globus pallidus* slices (35% inhibition; Maneuf and Brotchie 1997) and in mouse cerebellar membranes (37% inhibition; Selley *et al*. 2004). The CB1 agonist WIN55212-2 potency for inhibition of cAMP accumulation, obtained in the present work (EC50 of 6.6 μM), was also similar to that reported for rat *globus pallidus* slices (EC50 between 3-10 μM; Maneuf & Brotchie 1997) and slightly higher than that found in mouse cerebellar membranes (EC50 of 1.4 μM; Selley *et al*. 2004). This indicates that the inhibitory potential of CB1 receptors is conserved in different brain regions of the rat, as supported by CB1 receptor quantification studies which indicate that the amount of CB1 receptors at the hippocampus, cerebellum and striatum is roughly the same (Horti *et al*. 2006). The inhibitory effect of the $A_1$ and CB1 receptor agonists CPA and WIN55212 on forskolin-stimulated cAMP accumulation was prevented by the respective antagonists, DPCPX and AM251, indicating that the effect of the agonists were specific for the corresponding receptors.

*Combined actions of A1 and CB1 receptors*

We aimed to measure the dampening of adenylyl cyclase activity by co-stimulation of $A_1$ and CB1 receptors and found that, when both receptors are simultaneously operated they exert cumulative modulation of adenylyl cyclase activity, implying that part of the transduction pathways operated by both receptors does not compete or interfere with each other, if so the combined effect of $A_1$ and CB1 agonists would be less-than-additive. These findings agree with previous observations obtained on hippocampal excitatory synaptic transmission of the rat (Serpa 2007; but see Hoffman *et al*. 2010) where inhibition of the fEPSP slope by simultaneous activation of both receptors was identical to the sum of the effect of each receptor alone. Also in hippocampal membranes and whole brain membranes of the mouse, co-application of $A_1$ and CB1 receptors agonists additively stimulated [$^{35}$S]GTPγS binding (Breivogel *et al*. 2001; Childers *et al*. 2005; but see Sousa *et al*. 2011). These reports, together with the results obtained in the present study, support a cumulative and additive effect, in the hippocampus, at three different levels when $A_1$ and CB1 receptors are co-stimulated: G-proteins, adenylyl cyclase and excitatory synaptic transmission. In mouse

cerebellar membranes, both [$^{35}$S]GTPγS binding and inhibition of forskolin-stimulated cAMP accumulation by combined application of $A_1$ and CB1 receptors agonists were only partially additive, but still the combined effect was higher than the maximal individual effects (Selley *et al.* 2004). In one study $A_1$ receptors attenuate CB1 receptor-mediated GABA and glutamate release from rat hippocampal synaptosomes (Sousa *et al.* 2011), which contrasts with the mutually independent inhibitory action of $A_1$ and CB1 receptors on hippocampal excitatory synaptic transmission found in brain slices (Serpa 2007), probably because in synaptossomes availability of signaling molecules is lower than in slices (Lipton 1986). Furthermore, different timings of agonist application could result in impairment of the CB1 receptor response by $A_1$ receptors, since in Sousa *et al.* (2011) CPA was present in the incubation medium before WIN55212-2 for over 30 minutes, enough to trigger desensitization of $A_1$ receptors (Nie *et al.* 1997), which in turn could induce heterologous desensitization of CB1 receptors (Chu *et al.* 2010). In C57BL/6J mice (which have high levels of endogenous adenosine) sustained tonic activation of $A_1$ receptors prevented CB1-mediated inhibition of excitatory synaptic transmission, but not in the rat, suggesting differences between species (Hoffman *et al.* 2010). However, the occurrence of heterologous CB1 receptor desensitization due to sustained activation of $A_1$ receptors by high endogenous adenosine levels cannot also be discarded.

Since adenosine $A_1$ and cannabinoid $CB_1$ receptors mostly couple to identical $G_{\alpha i}$ subunits (Straiker *et al.* 2002) and are both expressed at pyramidal glutamatergic neurons in the hippocampus (Kawamura *et al.* 2006; Ochiishi *et al.* 1999) it is not surprising that receptor interference could occur. In fact, $A_1$ receptors have a mutually occlusive response when interacting with other Gi/o-coupled receptors, such as group II metabotropic glutamate receptors (Di Iorio *et al.* 1996), α2-adrenergic receptors (Limberger *et al.* 1988) and neuropeptide Y receptors (Qian *et al.* 1997) in the hippocampus, whereas in superior cervical ganglions the expression of human CB1 cannabinoid receptors can sequester Gi/o-proteins from a common pool and make them unavailable to other Gi/o-coupled receptors (Vásquez and Lewis, 1999). On the other hand, additive actions between adenosine $A_1$ and μ-opioid or $GABA_B$ receptors agonists has been described for receptor-mediated $G_{i/o}$ protein activation in hippocampal membranes (Childers *et al.* 2005). Therefore, the cumulative additive inhibitory effects of $A_1$ and CB1 receptors on adenylyl cyclase activity, observed in the present work, suggest that availability not only of $G_{i/o}$ proteins (Childer *et al.* 2005), but also adenylyl cyclase shared by both receptors, might not be limiting in the rat hippocampus. Another possibility is that spatial distribution of $A_1$ and CB1 receptors in different cell types or in the same cell type but in different locations due to receptor compartmentalization, might occur. The scaffold proteins A-kinase anchoring proteins (AKAPs; Lin *et al.* 2011; Sík *et al.* 2000) and the lipid raft caveolae (Bu *et al.* 2003) have been identified in the hippocampus, were they improve the spatial precision of cAMP-related activity (Scott and Pawson, 2009).

*Desensitization of $A_1$ receptors*

When applied 95 min before forskolin, CPA failed to modify forskolin-stimulated cAMP accumulation. Therefore, 95 min is a sufficient time period to induce sub-acute $A_1$ receptor homologous desensitization*. In fact, rapid (<90 min) homologous desensitization of the $A_1$ receptor-mediated inhibition of excitatory neurotransmission, induced by hypoxia which releases copious amounts of adenosine, has been reported in the rat hippocampus (Coelho *et al*. 2006). In smooth muscle $DDT_1$ MF-2 cells, uncoupling of $A_1$ receptors from G proteins was obtained after 30 min exposure to agonist, an effect involving receptor phosphorylation and arrestin binding (Nie *et al*. 1997). The rapid desensitization, by CPA exposure, of the $A_1$-mediated inhibition of cAMP accumulation observed in the present work, must be consequence of receptor uncoupling (Ramkumar *et al*. 1993) since receptor internalization requires several hours to occur (Jajoo *et al*. 2010).

Sousa *et al*. (2011) reported unidirectional attenuation by $A_1$ receptors of CB1 receptor-mediated inhibition of glutamate release from hippocampal synaptosomes, while CB1 receptors did not affect the $A_1$-mediated effect. To evaluate if the apparent unidirectional action of $A_1$ receptor on the CB1 receptor-mediated effect could be a consequence of heterologous desensitization by the $A_1$ receptor, we studied the influence of the CPA incubation period on the WIN55212-2 inhibitory effect. Addition of CPA either 15 min before forskolin (acute stimulation) or 95 min before forskolin (enough to induce sub-acute homologous desensitization of $A_1$ receptors) did not modify the inhibitory effect of WIN55212-2 on forskolin-stimulated cAMP accumulation, therefore excluding heterologous desensitization of CB1 receptors by acute or sub-acute adenosine $A_1$ receptor activation, at least at the level of cAMP production. However it does not precludes the hypothesis of heterologous desensitization of the receptor response by longer treatment with receptor agonists (see Kouznetsova *et al*. 2002; Selley *et al*. 2004), where other downstream effectors may be influenced.

Contrasting with adenosine A1 receptors, pre-incubation with WIN55212-2 for up to 6h did not induce desensitization of the cannabinoid CB1 receptor-mediated inhibition on cAMP production, in agreement with a previous report where a 18- to 24-h exposure of cultured hippocampal neurons to WIN55212-2 was necessary to produce a marked desensitization of the CB1 receptor-mediated inhibition of neurotransmission (Kouznetsova *et al*. 2002).

* *see glossary for definition*

# Chapter 4: Modulation of cGMP accumulation by adenosine $A_1$ receptors

## 4.1. Introduction

Cyclic GMP, a cyclic nucleotide with second messenger action, is produced by two distinct pathways. One involves cytoplasmic soluble guanylyl cyclase (sGC), whose agonist, nitric oxide (NO), is produced by calcium-activated NO synthase (NOS), while the other involves the membrane-bound particulate guanylyl cyclase, which is stimulated by natriuretic peptides (reviewed in Lucas *et al.* 2000). cGMP immunostaining revealed cGMP accumulation, induced by the association of an allosteric enhancer of soluble guanylyl cyclase and an inhibitor of cGMP-degrading phosphodiesterases, in pyramidal cells and astrocytes of the hippocampus (Bartus *et al.* 2013).

Adenosine $A_1$ receptor activation and cGMP mediate similar actions at the central nervous system, both inhibit glutamatergic synaptic transmission (Dunwiddie and Hoffer, 1980; Feil and Kleppisch, 2008; Serpa 2007), protect against excitotoxic insults (Montoliu *et al.* 2001; Orio *et al.* 2007; Ribeiro, 2005; Sebastião *et al.* 2001b) and regulate synaptic plasticity (see Dias *et al.* 2013; Feil and Kleppisch, 2008) at the hippocampus. On the other hand, modulation of cGMP levels by a $G_{i/o}$ protein-coupled receptor have also been recently reported (Jones *et al.* 2008). However, the relationship between adenosine $A_1$ receptors and cGMP remains to be clarified. In the present work we investigated the adenosine $A_1$ receptor ability to modulate cGMP accumulation at the hippocampus. The gender dependence of the adenosine $A_1$ receptor-dependent effect on cGMP levels was also investigated since estrogen receptors are present at the hippocampus (Petersen *et al.* 1998) where estradiol increases the cGMP content (Palmon *et al.* 1998).

## 4.2. Results

### 4.2.1. Basal cGMP accumulation

Since basal intracellular levels of cGMP in hippocampal slices are low, and therefore hard to quantify, experiments were performed in the presence of Zaprinast, a selective inhibitor of cGMP-specific phosphodiesterase (PDE) V and VI but also inhibiting other cGMP-hydrolyzing PDEs (Marte *et al.* 2008), and Bay 60-7550, mainly a PDE II inhibitor (Bender and Beavo, 2006), in order to induce cGMP accumulation. In the presence of both Zaprinast (100 μM) and Bay 60-7550 (10 μM), the cGMP accumulation was 15 ± 7 pmol/mg protein (n=5); when ADA (2 U/mL) was added to remove endogenous adenosine the cGMP accumulation (9 ± 0.9 pmol/mg

protein; n=18) tended to be lower, although it was not significantly different from the accumulation in the absence of ADA (P>0.14 Vs accumulation in the absence of ADA, Student's t-test). In experiments where soluble guanylyl cyclase was directly stimulated by SNP (100 µM), in the presence of ADA (2 U/ml), cGMP accumulation was increased by about four-fold to 34 ± 5 pmol/mg protein (n=10, P<0.001 Vs absence of SNP, Student´s t -test), this increase being identical in male and female Wistar rats.

### 4.2.2. Adenosine deaminase is required to unmask the effect of the adenosine $A_1$ receptor agonist CPA on cGMP accumulation.

Application of the adenosine $A_1$ receptor selective agonist CPA (100 nM) alone to acute hippocampal slices failed to modify (n=5; P>0.6, paired Student´s t-test, while comparing absence and presence of CPA in the same experiments) the cGMP accumulation obtained in the presence of zaprinast (100 µM) and Bay 60-7550 (10 µM) (Fig 15A). We proceeded by removing endogenous adenosine with adenosine deaminase, which could be interfering with the effect of CPA. As shown in Figure 15A, a stimulatory effect of 100 nM-CPA (17% ± 5%; n=7; P<0.016 Vs zero, Student´s t-test) on cGMP accumulation was unmasked by the presence of ADA (2 U/mL). All subsequent experiments involving cGMP quantification were conducted in the presence of ADA (2 U/mL).

To assess if the influence of ADA on the CPA effect was specific for cGMP, we also tested if ADA modified the CPA-induced inhibition of forskolin-stimulated cAMP accumulation. As can be observed in Figure 15B, inhibition of cAMP accumulation by CPA was not affected by the presence of ADA, although ADA (2U/ml) did inhibit cAMP accumulation in the absence of CPA by 64% ± 6% (n=3; P<0,05 Vs zero, Student's t-test).

Contrasting with the findings obtained for cAMP formation, the cGMP accumulation in hippocampal slices was not modified by incubation with the cannabinoid CB1 receptor selective agonist, WIN55212-2 (30 µM) for 80 min (% effect -3.0 ± 3.1, n=3, P>0.4 Vs zero, Student's t-test).

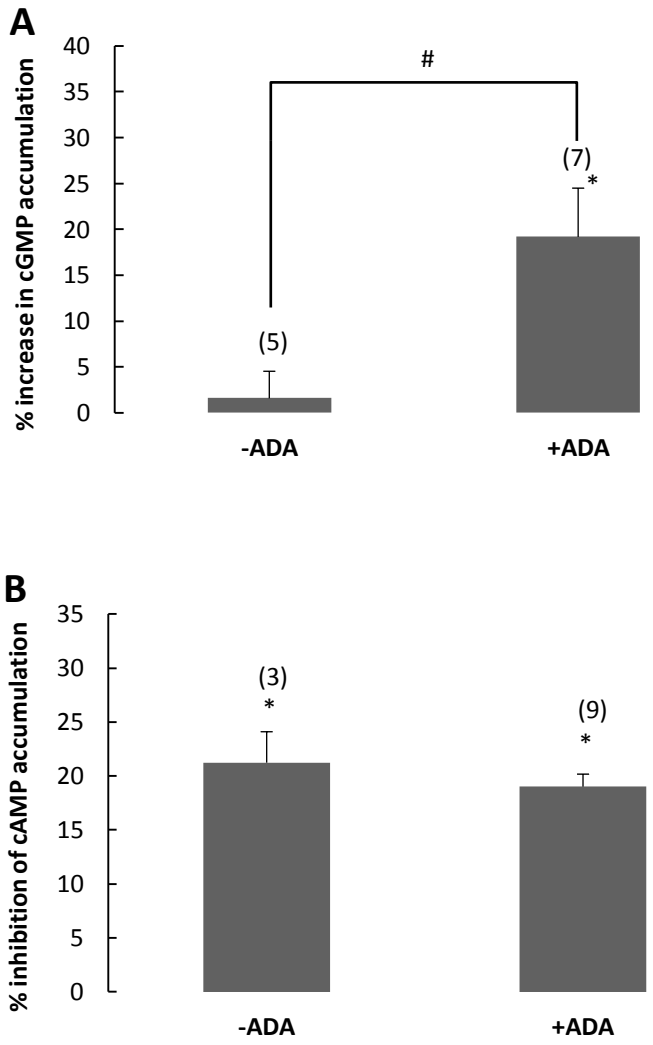


**Figure 15: Adenosine deaminase is required for the expression of the effect of CPA on cGMP accumulation, but not on cAMP accumulation. A:** Hippocampal slices (from male Wistar rats) were incubated for 30 min in the presence of zaprinast (100 µM), Bay 60-7550 (10 µM) and in the absence or in the presence of ADA (2 U/ml). After this period CPA (100 nM final concentration) or vehicle (control) was added and incubation continued for a further 50 min period. Columns represent mean ± SEM of the percentage of CPA-induced increase in control cGMP accumulation obtained in the absence (left column) or in the presence (right column) of ADA. **B:** Slices were incubated for 30 min in the presence of rolipram (50 µM) and in the absence or in the presence of ADA (2 U/ml). After this period CPA (100 nM final concentration) or vehicle (control) was added and 15 min later forskolin (20 µM final concentration) was added and incubation proceeded for a further 35 min period. Columns represent mean ± SEM of the percentage of CPA-induced inhibition of control cAMP accumulation obtained in the absence (left column) or in the presence (right column) of ADA. The number of experiments performed, run at least in quadruplicate, is shown in brackets above the bars. (*): $P<0.05$ Vs zero, Student´s t-test. (#): $P<0.05$ Vs absence of ADA, Student´s t-test.

### 4.2.3. Maximal effect and potency of the adenosine $A_1$ receptor agonist CPA on cGMP accumulation.

As shown in Figure 16 the adenosine $A_1$ receptor selective agonist CPA (0.03-300 nM) dose-dependently increased cGMP accumulation at the hippocampus. The highest concentration of CPA tested (300 nM) produced a nearly maximal increase in cGMP accumulation (16% ± 3%, n=4; Student´s t-test, P<0,02 Vs zero, Student's t-test). Nonlinear curve fitting to the data shown in Figure 16 gave an $EC_{50}$ for CPA of 4.2 ± 1.4 nM and an Emax of 17% ± 0.9%.

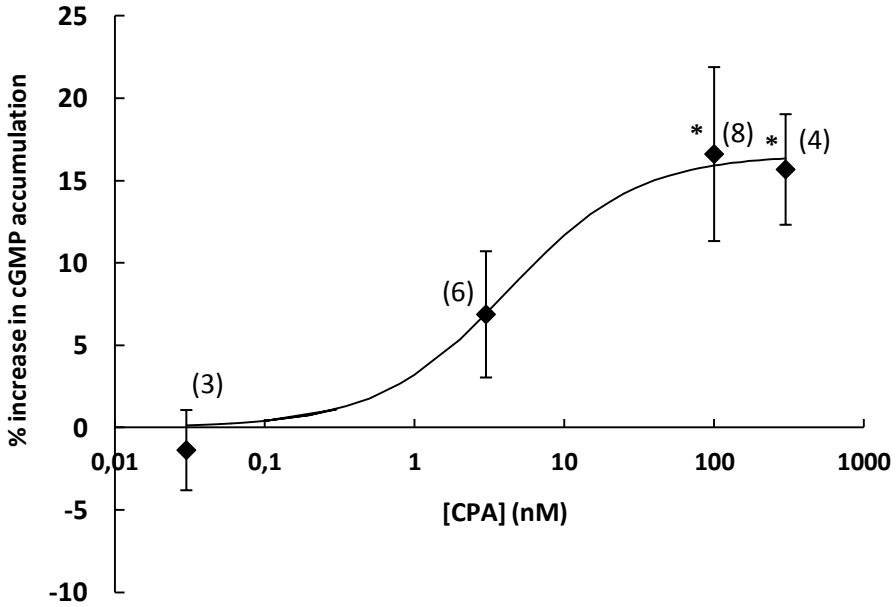


**Figure 16: CPA dose-dependently increases cGMP accumulation**. Hippocampal slices (from male Wistar rats) were incubated for 30 min in the presence of zaprinast (100 µM), Bay 60-7550 (10 µM) and ADA (2 U/ml). After this period CPA (0.03-300 nM final concentration) or vehicle (control) was added and incubation continued for a further 50 min period. Data points are expressed as mean ± SEM of the percentage increase of control cGMP accumulation. Number of experiments, each run at least in quadruplicate, is shown in brackets above the data points. The solid line corresponds to the nonlinear regression curve obtained by fitting a Michaelis-Menten type equation to the experimental points. (*****): P<0.05 Vs zero.

### 4.2.4. Effect of the adenosine $A_1$ receptor agonist CPA on cGMP accumulation in the presence of a nitric-oxide donor: gender dependence.

In order to evaluate if the CPA effect on basal cGMP accumulation is still observed under increased concentrations of cGMP, its production was stimulated using sodium nitroprusside (SNP), a NO donor and activator of soluble guanylyl cyclase (see Lucas *et al*. 2000). The effect of CPA on cGMP accumulation, in the absence or in the presence of SNP, was evaluated in both males and females Wistar rats. While gender did not influence neither basal cGMP levels nor the effect of CPA upon basal cGMP accumulation (absence of SNP, Fig 17A), in the

presence of SNP (100 μM) there was a marked gender influence both upon cGMP accumulation in the absence of CPA, and upon the effect of CPA (Fig 17B, 17C). In male Wistar rats, in the presence of SNP (100 μM), CPA (100 nM) failed to modify cGMP accumulation (% effect of 3.1%±2.4%, n=5; P>0.25 Vs zero, Student's t-test; Fig 17C). In fact, in males, the cGMP accumulation obtained in the presence of both CPA (100 nM) and SNP (100 μM) (31.2 ± 3.4 pmol/mg protein, n=5) was not significantly different from the cGMP accumulation obtained in the presence of SNP alone (30.3 ± 2.9, n=5, see Fig 17B), but was also not different (P>0.9, Student´s t-test) from the cGMP accumulation expected in the presence of both CPA and SNP if the effect of each drug were additive (31.7 ± 2.9 pmol/ mg protein). This is probably consequence of the effect of SNP on cGMP accumulation (236% ± 34%, n=5) being much higher than the CPA effect (16.8% ± 5.1%, n=8, in the absence of SNP), therefore the SNP effect would mask/obscure the CPA effect when measured in the presence of SNP (see Fig 17).

In contrast, in female rats, the presence of SNP did not prevent CPA (100 nM) from increasing cGMP accumulation by 37%±11%, (n=4, P<0.05 Vs zero, Student's t-test, Fig 17C). Although in the absence of SNP the effect of CPA on cGMP accumulation did not differ between males and females (P>0.22, one-way ANOVA followed by LSD test; Fig 17C), in the presence of SNP the effect of CPA observed in females rats (37%±11%, n=4) was significantly different (P<0.001, one-way ANOVA followed by LSD test) from the effect observed in males (3.1%±2.4%, n=5), suggesting that the pathway through which $A_1$ receptors modulate cGMP levels differs according to gender (Fig 17C), at least under NO-increased cGMP levels. The stimulatory effect of CPA on cGMP accumulation in the presence of SNP, observed in females was completely reversed by DPCPX, an adenosine $A_1$ receptor selective antagonist (see Fredholm *et al*. 2001) (Fig 18). DPCPX alone had no significant effect on cGMP accumulation (Fig 18), which could be expected since endogenous adenosine had been removed by addition of ADA.

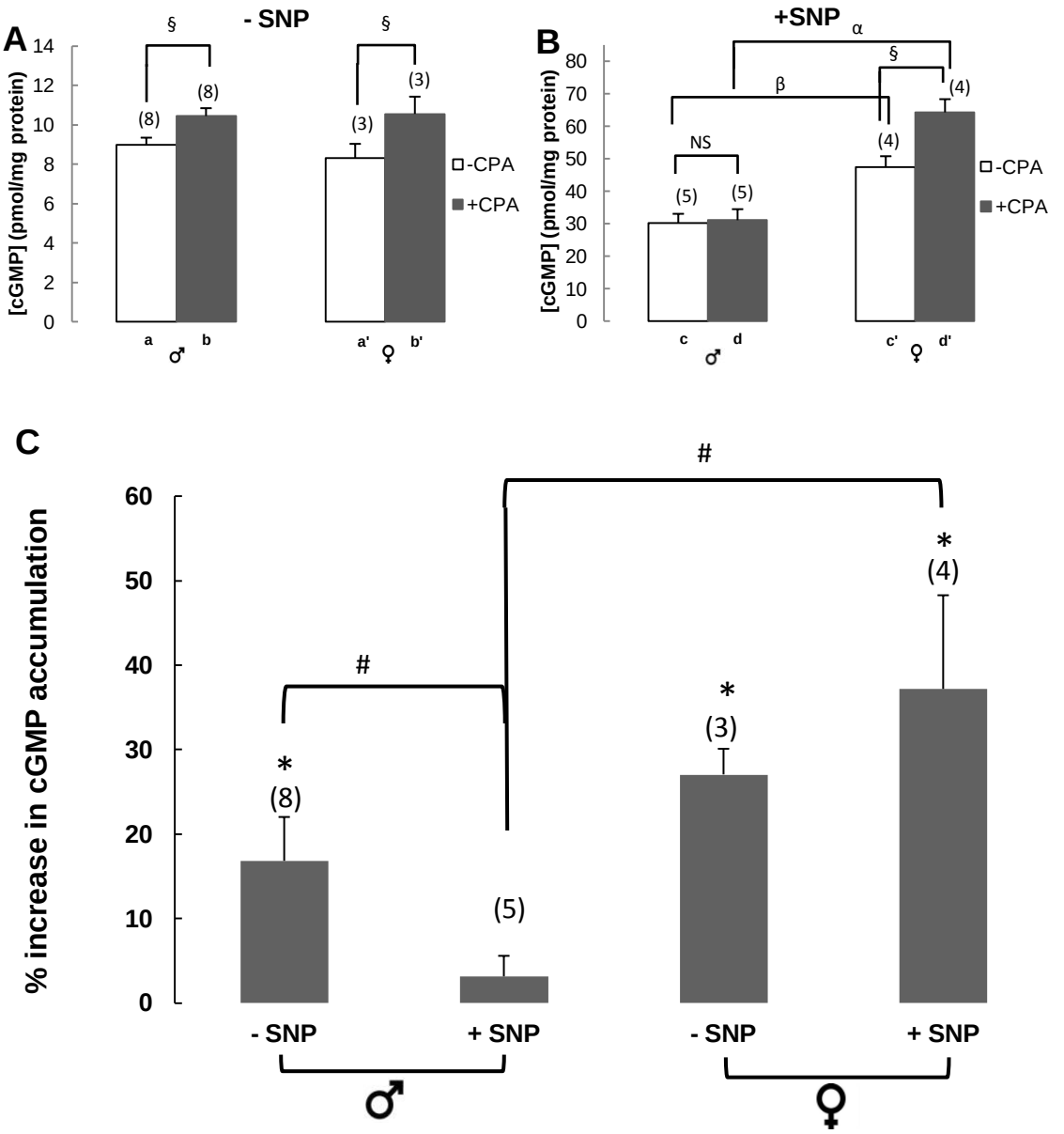


Figure 17: **Influence of SNP on the increase in cGMP accumulation induced by CPA: gender dependence.** Hippocampal slices were incubated for 30 min in the presence of zaprinast (100 µM), Bay 60-7550 (10 µM) and ADA (2 U/ml). After this period incubation continued for 25 min in the absence (control) or in the presence of CPA (100 nM). Finally SNP (100 µM final concentration) or its vehicle was added and incubation proceeded for a further 25 min period. **A, B:** cGMP accumulation obtained in the absence (A) or in the presence (B) of SNP (100 µM). Columns represent mean ± SEM of cGMP accumulation obtained in the absence (controls, open columns: a, a', c and c') or in the presence (solid columns: b, b', d and d') of CPA (100 nM) in males and females rats. **C:** CPA effect on cGMP accumulation. Columns represent mean ± SEM of the percentage of CPA-induced increase in cGMP accumulation when compared with the appropriate controls, obtained (from left to right) in males in the absence (b vs a, in A) or in the presence of SNP (d vs c, in B), and in females in the absence (b' vs a', in A) or in the presence of SNP (d' vs c', in B). The number of experiments performed, run at least in quadruplicate, is shown in brackets above the bars. (§): $P<0.05$ Vs control; (α): $P<0.05$ Vs d; (β): $P<0.05$ Vs c; NS: Non significantly different; One-way ANOVA followed by LSD test. (*): $P<0.05$ Vs zero; Student's t-test. (#): $P<0.05$ Vs males in the presence of SNP; One-way ANOVA, followed by LSD test.

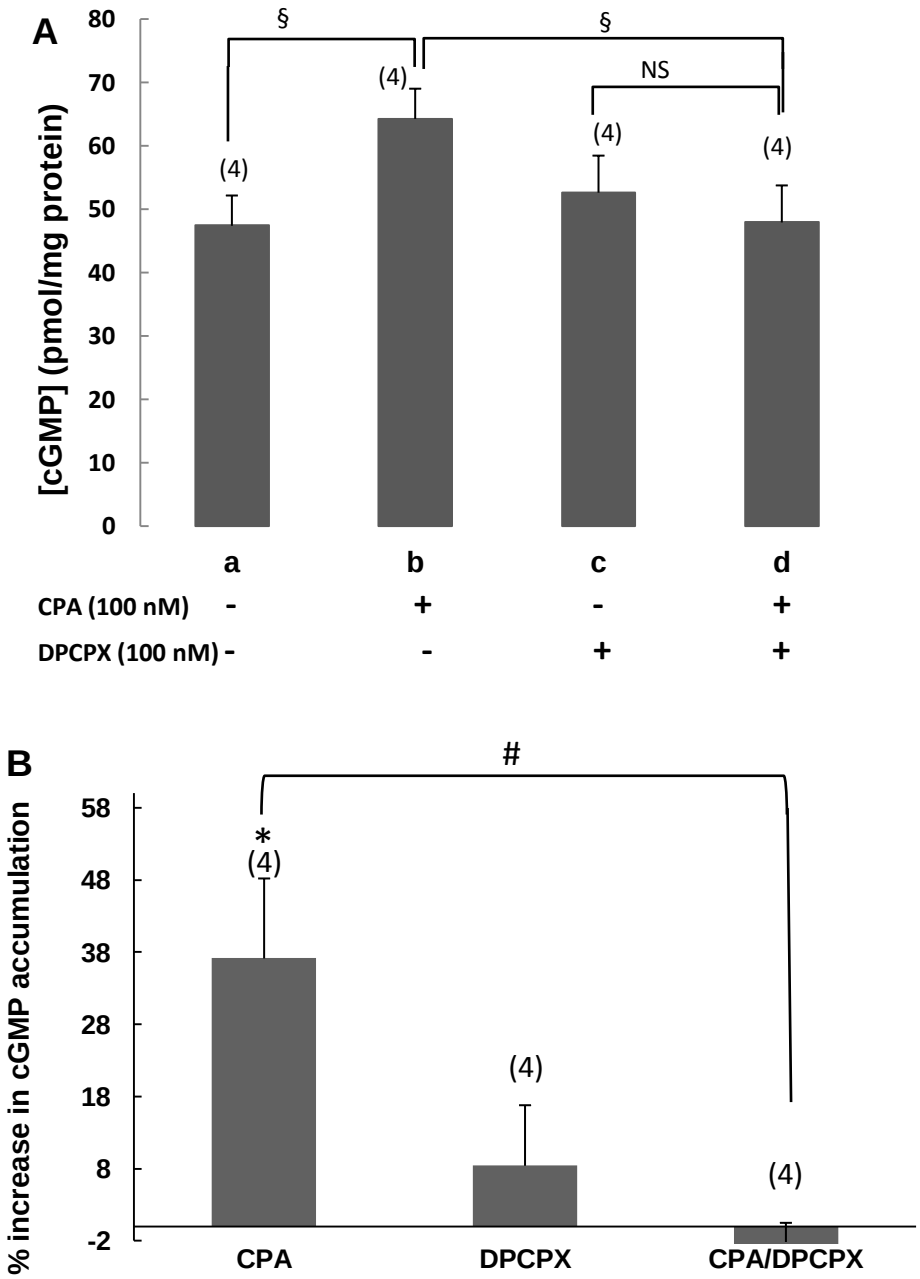


**Figure 18:Specificity of the effect of CPA, in the presence of SNP.** Hippocampal slices from female Wistar rats were incubated for 30 min in the presence of zaprinast (100 µM), Bay 60-7550 (10 µM), ADA (2 U/ml) and in the absence or in the presence of DPCPX (100 nM). After this period CPA (100 nM final concentration) or vehicle (control) was added and 25 min later SNP (100 µM final concentration) was added and incubation proceeded for a further 25 min period. **A:** Columns represent mean ± SEM o cGMP accumulation obtained (from left to right) in the absence of CPA and DPCPX (a), in the presence of CPA (b), in the presence of DPCPX (c) and in the presence of CPA and DPCPX (d). **B:** CPA effect on cGMP accumulation. Columns represent mean ± SEM of the percentage increase in cGMP accumulation, when compared with the corresponding control, produced by (from left to right) CPA (b vs a, in A), DPCPX (c vs a, in A), and CPA in the presence of DPCPX (d vs c, in A). The number of experiments performed, run at least in quadruplicate, is shown in brackets above the bars. (§): $P<0.05$ Vs CPA in the absence of DPCPX (b); NS: non significantly different; One-way ANOVA, followed by LSD test. (*): $P<0.05$ Vs zero; Student's t-test. (#): $P<0.05$ Vs CPA in the presence of DPCPX; One-way ANOVA, followed by LSD test.

## 4.3. Discussion

*Effect of the adenosine $A_1$ receptor on cGMP levels*

In the present study, we found evidence that the adenosine $A_1$ receptor increases cGMP accumulation at the rat hippocampus, through a mechanism which depends on gender. Previous studies had already addressed the influence of adenosine $A_1$ receptors on cGMP levels but in peripheral tissues, an effect which we now confirm to be also present at the hippocampus. An increase in cGMP levels by adenosine $A_1$ receptor activation was observed in cardiac atrium, where cGMP mediates the adenosine $A_1$ receptor-induced decrease in contractibility (Sterin-Borda *et al*. 2002), in vascular smooth muscle cells of rat aorta (Kurtz, 1987) and in rat kidney cells (Kurtz *et al*. 1988). At the central nervous system, previous studies reported an adenosine-induced increase in cGMP levels in guinea pig slices of cerebellum and cerebral cortex (Ohga and Daly, 1977; Saito, 1977), but no selective agonists or antagonists were used to firmly identify the receptor involved. Subsequent work showed that this effect of adenosine was mediated by the $A_{2B}$ receptor, at least in the cerebellum (Hernández *et al*. 1993). Reports of interaction between effects mediated by adenosine $A_1$ receptor and cGMP, has been described at the hippocampus. The activation of adenosine $A_1$ receptor together with the simultaneous increase in cGMP concentration elicited by zaprinast, was enough to induce chemical LTD (Santschi *et al*. 2006). On the other hand, the potentiation of $A_1$ receptor-mediated inhibition of synaptic transmission by a NO donor was blocked by the sGC antagonist ODQ, suggesting a facilitatory effect of cGMP on the adenosine $A_1$ receptor effect at the hippocampus (Fragata *et al*. 2006). In addition, it was shown that a nitric oxide donor depressed the fEPSP slope and that this depression was blocked by DPCPX and was cGMP-independent (Broome *et al*. 1994; Arrigoni and Rosenberg 2006). However, none of these studies directly address how $A_1$ receptors modulate cGMP levels at the hipocampus.

An indirect evidence of adenosine $A_1$ receptor action on cGMP levels at the nervous system comes from a recent study, where the inhibitory effect of peripheral adenosine $A_1$ receptors on inflammatory hypernociception was blocked by sGC and PKG inhibitors, suggesting a cGMP-mediated effect of the adenosine $A_1$ receptor (Lima *et al*. 2010) but a direct effect of adenosine $A_1$ receptor on cGMP levels was not evaluated. Direct observation of adenosine $A_1$ receptor-mediated effect on cGMP levels is difficult to observe due to low intracellular levels and high compartmentalization of cGMP (see Arora *et al*. 2013). In the present work, we used a selective inhibitor of PDE II (Bay 60-7550), the main enzyme responsible for cGMP degradation at the hippocampus (Bartus *et al*. 2013), together with a more general inhibitor of cGMP-degrading PDEs (zaprinast) to induce cGMP accumulation. A 100 μM concentration of zaprinast was chosen because it was necessary to inhibit most cGMP-degrading PDEs present at the hippocampus, namely PDEs I, III, VI and IX (see Marte *et al*. 2008; van Staveren *et al*. 2001), though at this concentration zaprinast does not inhibit efficiently PDE II, thus we used,

in addition, a more potent PDE II inhibitor, Bay 60-7550. A previous study also showed that at least a 100 μM concentration of zaprinast was necessary to produce significant increases of cGMP accumulation at the hippocampal slice (van Staveren *et al*. 2001). Using these conditions, we were able to observe a stimulatory effect of the adenosine $A_1$ receptor on cGMP formation at the rat hippocampal slice: a maximal increase of 17% and an EC50 of 4.2 nM were obtained for the selective adenosine $A_1$ receptor agonist CPA on basal cGMP accumulation. The EC50 obtained in the present study was very similar to those reported for the CPA-induced increase of cGMP accumulation at the atrium (between 1 and 10 nM, Sterin-Borda *et al*. 2002), for CPA inhibition of neurotransmission (EC50 ≈ 12 nM; de Mendonça and Ribeiro, 1997) and inositol phosphates accumulation (EC50 = 10 nM; Cascalheira *et al*. 1998) at the hippocampus.

*ADA unmasks the effect of CPA on cGMP accumulation*

After initial attempts to measure the effect of CPA on cGMP accumulation failed, we decided to test the effect of CPA in the presence of ADA, the enzyme which irreversibly deaminates adenosine into inosine. Since basal levels of endogenous adenosine might activate adenosine $A_1$ and $A_{2A}$ receptors, which could interfere with the adenosine $A_1$ receptor-mediated effect of CPA on cGMP formation, the effect of CPA was assessed both in the absence and in the presence of ADA to remove endogenous adenosine. In fact, ADA was required to unmask the effect of CPA as modulator of intracellular cGMP levels, most likely because it removed interference by endogenous adenosine acting at $A_1$ receptors. However, one should not discard other possibilities, less straightforward, regarding the influence of ADA on the activity of $A_1$ receptors. These possibilities include: i) adenosine $A_1$ receptor desensitization by endogenous adenosine; ii) inhibition of adenosine $A_1$ receptor by tonic activation of the adenosine $A_{2A}$ receptor by endogenous adenosine, which has the ability to directly attenuate the activity of adenosine $A_1$ receptors (Lopes *et al*. 1999); iii) direct influence of ADA on adenosine $A_1$ receptor, independent of adenosine removal. The first and second hypothesis seem unlikely since ADA did not affect the adenosine $A_1$ receptor-mediated inhibition of cAMP accumulation – although ADA alone did decrease cAMP accumulation, reflecting a tonic activation of adenylyl cyclase by $A_{2A}$ receptors. However, if either of these hypotheses were correct we should observe increased adenosine $A_1$ receptor-mediated inhibition of cAMP accumulation upon removal of endogenous adenosine by ADA. The third hypothesis seems more likely since previous studies showed that extracellular ADA binds adenosine $A_1$ receptor, increasing its affinity towards agonists, and acts as a co-stimulatory molecule facilitating specific signalling events, independently from its enzymatic activity (reviewed in Franco *et al*. 1997, 2005).

*Nitric oxide and gender dependence of the $A_1$ receptor-mediated increase in cGMP accumulation*

To determine if the CPA effect on cGMP accumulation depended on the NOS/sGC pathway we increased cGMP levels using SNP, a NO donor which activates sGC. In our perspective, if we saturate sGC activity with a moderately high concentration of SNP, then the effect of $A_1$ receptors acting through the NOS/sGC pathway to increase cGMP levels would be occluded by SNP. On the other hand if CPA has any measurable effect in the presence of SNP, then its suggestive that $A_1$ receptors act by another pathway, such as the pGC pathway, or more likely by more than one pathway. In male Wistar rats, the presence of SNP suppressed the increase in cGMP accumulation induced by CPA, thus sugesting a sGC-dependent effect, in agreement with a report were adenosine $A_1$ receptors were found to increase NO production in cortical astrocytes (Janigro *et al.* 1996). On the other hand, when female Wistar rats were tested, SNP did not modify the CPA-induced increase in cGMP accumulation. Furthermore, DPCPX reversed the effect of CPA, stressing that adenosine $A_1$ receptors kept modulating cGMP accumulation despite the increase in soluble guanylyl cyclase activity by SNP. Thus, the persistence in females of an adenosine $A_1$ receptor effect on SNP-stimulated cGMP levels, suggests that this effect could depend on pGC or even on non nitric oxide-mediated mechanisms of sGC activation, as it was described in cardiomyocites, were translocation of sGC to the membrane induced by a divalent cation ionophore* potentiated cGMP production through calcium-dependent changes on the phosphorylation status of sGC (Agulló *et al.* 2005). The results therefore suggest that the mechanism of adenosine $A_1$ receptor-mediated modulation of cGMP levels at the hippocampus depend on gender.

* *see glossary for definition*

# Chapter 5: Combined neuroprotective effect of adenosine $A_1$ and cannabinoid CB1 agonists against NMDA-induced excitotoxicity in organotypic hippocampal slices

## 5.1. Introduction

Sustained activation of glutamate NMDA receptors, following traumatic or ischemic brain injury, or caused by the pathological mechanisms of neurodegenerative diseases, generates neuronal death due to excitotoxicity, a secondary brain damage mechanism which involves excessive release of the neurotransmitter glutamate, the continuous influx of calcium and depletion of intracellular ATP stores (Cross *et al.* 2010). The $G_{i/o}$-protein coupled cannabinoid CB1 and adenosine $A_1$ receptors are both expressed at high levels in the hippocampus, were they inhibit glutamatergic synaptic transmission (Serpa 2007; Han *et al.* 2012) and protect against neurotoxic insults (Koch *et al.* 2011; Sebastião *et al.* 2001b). Given the similarity between transducing pathways operated by adenosine $A_1$ and cannabinoid CB1 receptors, as well as the identical effects produced by both receptors on neuronal activity, clarification of the combined activity of these receptors is particularly relevant, since the harmful effects of neurotoxic insults might be cumulatively dampened. *In vivo* studies indicate a synergy between adenosine $A_1$ and cannabinoids CB1 receptors effects in a motor coordination paradigm (DeSanty and Dar, 2001) and an enhancement of cannabinoid CB1 receptor-induced impairment of short-term spatial memory by adenosine $A_1$ receptors (Sousa *et al.* 2011). However, the interactions observed *in vivo* involve global brain circuitry mechanisms, not necessarily reflecting receptor interaction at the cellular and molecular levels, where previous studies show that acute co-activation of adenosine $A_1$ and cannabinoid CB1 receptors generates additive effects when inhibiting excitatory synaptic transmission (Serpa 2007) and cAMP formation (chapter 3 of the present work, see also Serpa *et al.* 2015) in the rat hippocampus (but not in mouse; Hoffman *et al.* 2010), and when stimulating G-protein activation in rat hippocampal membranes (Childers *et al.* 2005). We now further tested how the combined action of $A_1$ and CB1 receptor agonists modulates NMDA-mediated excitotoxic insult at the rat organotypic hippocampal slice.

## 5.2. Results

### 5.2.1. Combined effect of adenosine $A_1$ and cannabinoids CB1 receptors agonists on synaptic transmission

Previously it was shown that co-application of adenosine $A_1$ and cannabinoids CB1receptors agonists independently decreased excitatory neurotransmission, assessed by recording electrically-evoked fEPSPs at the CA1 area of acute hippocampal slices (Serpa 2007). In this previous study the combined effect of CPA and WIN55212-2 was determined by applying CPA twice to the same slice. First CPA was applied alone and its effect quantified, then after CPA washout, WIN55212-2 was applied and its effect quantified after it produced it full inhibitory action, finally CPA was applied again and the combined effect of WIN55212-2 and CPA determined (Fig 19A). To exclude the possibility that some degree of $A_1$ receptor desensitization might occur during the second CPA application, that could be influenced by WIN55212-2 presence, and also to assess CPA and WIN55212-2 combined effect in similar conditions to those used to study their combined neuroprotective potential in organotypical slice cultures, the effect of CPA in the absence and presence of WIN55212-2 was also tested in different slices from the same hippocampus. As can be seen in Figure 19B, the CPA effect was not affected by the presence of WIN55212-2, whether this effect is studied in the same slice or in different slices from the same animal. Note that, in either case, the time course of the inhibition by CPA was also not affected, but CPA washout in the presence of WIN55212-2 was hampered. Indeed, after removing CPA from the bath while keeping WIN55212-2 in the perfusion solution, the fEPSP slope did not return to the value recorded immediately before CPA addition in the presence of WIN555212-2.

After confirming the additive action of adenosine $A_1$ and cannabinoids CB1 receptors agonists on excitatory synaptic transmission in the hippocampus, we set forth to investigate if this additive action would also apply to counteracting the neurotoxic effects of excessive glutamatergic activity.

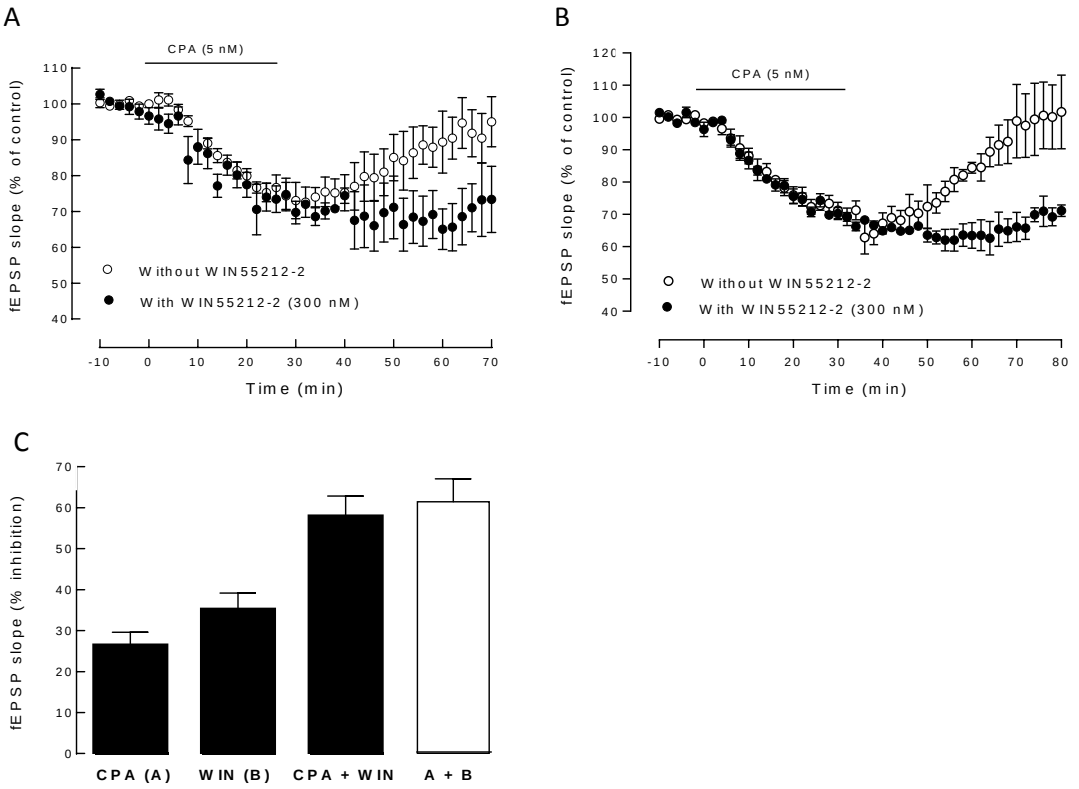


Figure 19. The effect of CPA in the absence and presence of WIN55212-2 was tested in the same hippocampal slice (A) or in different slices (B). **A), B)**: Activation of CB1 receptors with WIN55212-2 did not influence the inhibition of fEPSPs caused by the selective $A_1$ receptor agonist, CPA. Averaged time course changes of fEPSP slope induced by CPA (5 nM) in the absence (○) and in the presence (●) of WIN55212-2 (300 nM). **A)** CPA (5 nM) was applied twice to each hippocampal slice; WIN55212-2 (300 nM) was applied after the first wash out of CPA and remained in the bath up to the end of the experiments; time was allowed (at least 80 min) for the full effect of WIN55212-2 to inhibit the fEPSPs, before starting the second application of CPA. Each point in the ordinates corresponds to the averaged fEPSP slopes normalized for its value before addition of CPA. **B)** In each experiment the effect of CPA (5 nM) in the absence and presence of WIN55212-2 (300 nM) was tested in different slices from the same hippocampus. When WIN55212-2 was present, it was added to the slices at least 80 min before addition of CPA, which started to be perfused only after a full effect of WIN55212-2 was established. Each point in the ordinates corresponds to the averaged fEPSP slopes normalized for its value before addition of CPA. **C)** The filled bars represent the averaged full effects of (from left to right) CPA (1$^{st}$ application), WIN55212-2, and CPA plus WIN55212-2 (taking as control the fEPSP slope before WIN55212-2); the open bar represents the arithmetical sum of the data represented in 1$^{st}$ and 2$^{nd}$ columns. Data in (A), (B) and (C) are the average ± SEM from 4 experiments.

## 5.2.2. Neuroprotective action of adenosine $A_1$ and cannabinoids $CB_1$ receptors co-activation on NMDA-induced citotoxicity in the whole hippocampal slice assessed by LDH release

To investigate the neuroprotective potential of $A_1$ and CB1 receptors co-activation we first tested the effect of $A_1$ and CB1 agonists on the release of LDH activity from organotypic hippocampal slice cultures exposed to an excitotoxic insult. Exposure to NMDA alone (50 μM) for 1 h, increased the released LDH activity from 0.0070 ± 0.0013 μmol/min/ml (control) to

0.0124 ± 0.0023 µmol/min/ml corresponding to a 92% ± 16% increase (n=4, See fig. 20A). Further application of the cannabinoid CB1 receptor selective agonist WIN55212-2 (30 µM), from 45 min before the NMDA insult, decreased the NMDA-induced release of LDH activity by 53% ± 10% (n=4, fig. 20B). In the same way, the adenosine $A_1$ receptor selective agonist CPA (100 nM), when applied 15 min prior to NMDA, decreased the NMDA-induced release of LDH activity by 37% ± 11% (n=4, fig. 20B). Note that a longer pre-incubation time was used when testing the effect of WIN55212-2 than when testing the CPA effect; this was necessary because WIN55212-2 is very lipophilic and therefore needed longer incubation time to equilibrate with hippocampal slices and produce its inhibitory effect (see Serpa 2007). Interestingly, when WIN55212-2 (30 µM) and CPA (100 nM) were applied together, prior to NMDA (50 µM), the released LDH activity was reduced nearly to the levels obtained in the absence of NMDA (see Fig. 20A). The combined application of WIN55212-2 (30 µM) and CPA (100 nM) produced an 88% ± 12% (n=4) inhibition of the NMDA-induced LDH release from cultured hippocampal slices, which was higher than the inhibition produced by either WIN55212-2 (30 µM) or CPA (100 nM) alone but not different from the sum of the individual inhibitory effect of each agonist (90% ± 15%; n=4, fig. 20B), indicating additivity of effects of both agonists. Neither WIN55212-2 (30 µM) nor CPA (100 nM) modified basal (control) release of LDH activity. The presence of the adenosine $A_1$ receptor selective antagonist DPCPX (50 nM) reduced the inhibitory effect of 100 nM CPA to 1% ± 5% (n=3, $P<0.05$, Student's t-test, compared with the effect in the absence of DPCPX), whereas the cannabinoid CB1 receptor selective antagonist AM251 (10 µM) decreased the effect of 30 µM WIN55212-2 to 11% ± 2% (n=3, $P<0.05$, Student's t-test, compared with the effect in the absence of AM251), indicating that the inhibitory effect of CPA and WIN55212-2 are mediated by adenosine $A_1$ and cannabinoid CB1 receptors, respectively.

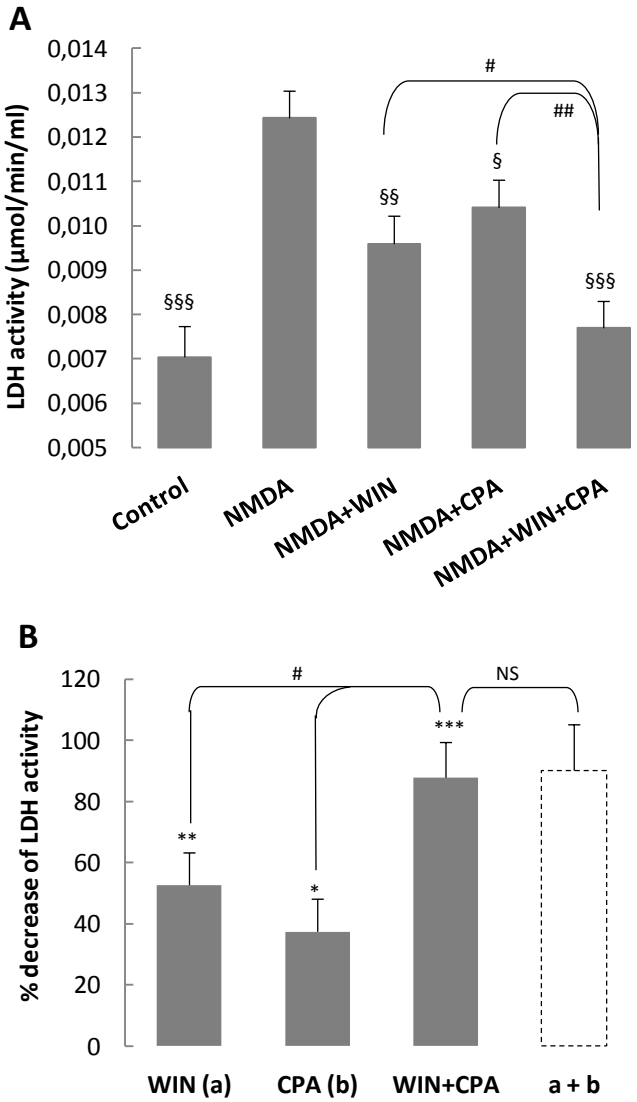


**Figure 20:** Combined neuroprotective effect of WIN55212-2 and CPA on cell death induced by NMDA insult at the rat hippocampus evaluated by released lactate dehydrogenase (LDH). Two weeks old cultured organotypic hippocampal slices were exposed to WIN55212-2 (30 µM) or its vehicle (control). After 30 min CPA (100 nM final concentration) or its vehicle (control) were applied and 15 min later NMDA (50 µM final concentration) or its vehicle (control) were added and incubation continued for 1 h. Medium was replaced to remove drugs and released LDH activity was quantified 24 h later. In each experiment five parallel assays were performed, corresponding respectively to: incubation with NMDA, NMDA + WIN55212-2, NMDA + CPA, NMDA + WIN55212-2 + CPA and incubation in the absence of drugs (control). A) Bars represent released LDH activity obtained (from left to right) in the absence of drugs (control), in the presence of NMDA, NMDA + WIN55212-2, NMDA + CPA and NMDA + WIN55212-2 + CPA B) Bars represent % decrease in NMDA-induced LDH activity produced (from left to right) by WIN55212-2, CPA and WIN55212-2 plus CPA; the dashed bar represents the arithmetical sum of the values represented in the 1st and 2nd columns. Data are mean ± SEM from 4 independent experiments run at least in triplicate. §, §§, §§§: $P<0.05$, $P<0.02$ and $P<0.00005$, respectively, Vs NMDA alone ( One-way ANOVA followed by LSD test). #, ##: $P<0.05$ and $P<0.02$, respectively, Vs WIN55212-2 (a) or CPA (b) alone ( One-way ANOVA followed by LSD test). NS, Non statistically different ($P>0.90$, Student's t-test). *, **, ***; $P<0.05$, $P<0.02$ and $P<0.005$, respectively, Vs zero (Student´s t-test).

### 5.2.3. Combined neuroprotective action of adenosine $A_1$ and cannabinoids $CB_1$ receptors on NMDA-induced cell injury in different areas of the hippocampal slice evaluated by fluorescence microscopy

We further investigated which hippocampal areas were more susceptible to the NMDA challenge by assessing cell injury measuring the PI uptake through fluorescence microscopy. Figures 21A and 21B depict the effect of NMDA (50 μM) application for 1h on the PI uptake in different areas of the rat hippocampal slice; the areas analyzed were those defined by Amaral and Witter (1989) and Andersen *et al*. (1971). As can be observed (Fig. 21B), NMDA-induced cell injury was highest in the CA3 area, where the PI uptake obtained was increased to 291% ± 19% (n=4) of the control PI uptake obtained in the absence of NMDA, while for the CA1 area the increase in PI uptake was less pronounced. No significant effect of NMDA ($P>0.05$) on PI uptake was observed in CA4 and *dentate gyrus* areas (Fig. 21A and B).

As can be observed in Fig 21C, for the CA3 area, WIN55212-2 (30 μM) and CPA (100 nM) when applied simultaneously produced a decrease of the NMDA (50 μM)-induced PI uptake, which was higher than the decrease produced by either WIN55212-2 (30 μM) or CPA (100 nM) alone but which was not different from the sum of the individual effects of each agonist. Note that this effect of WIN55212-2 and CPA was specific for the CA3 area. In CA1 area, the PI uptake obtained in the presence of NMDA (50 μM) alone (187% ± 22% of control PI uptake obtained in the absence of NMDA, n=4), was not different from the PI uptake obtained in slices incubated with NMDA but in the presence of WIN55212-2 (30 μM), CPA (100 nM) or WIN55212-2 (30 μM) plus CPA (100 nM), respectively 182% ± 22%, 214% ± 20% and 180% ± 19% of the control PI uptake ($P>0.05$, when compared with NMDA alone, One-Way ANOVA followed by LSD test).

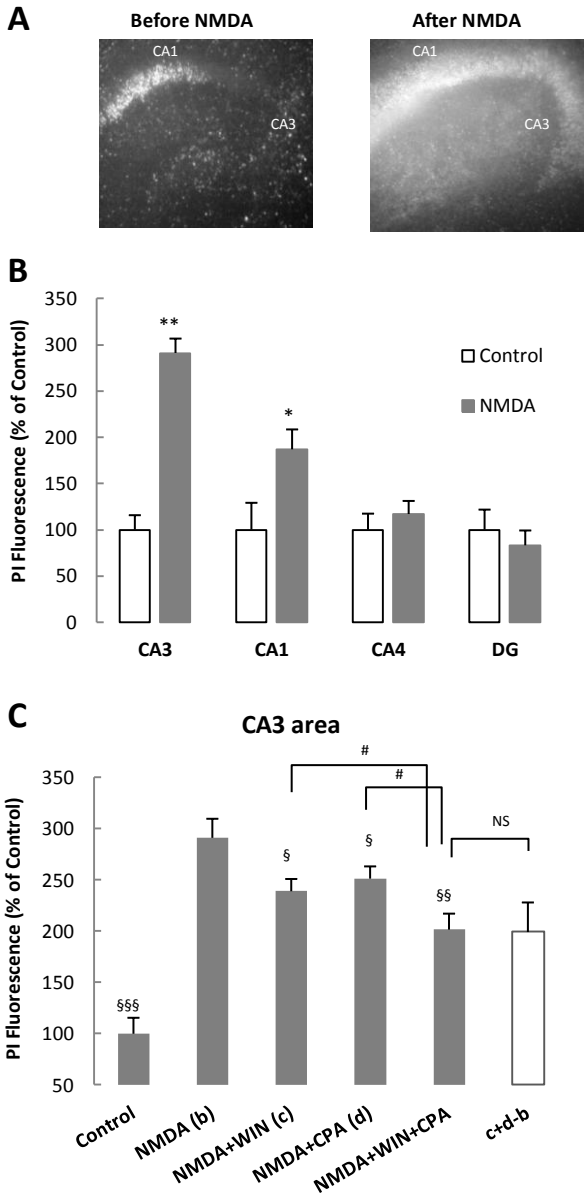


**Figure 21:** Combined neuroprotective effect of WIN55212-2 and CPA on cell injury produced by NMDA insult at the rat hippocampus assessed by propidium iodide (PI) uptake. Two weeks old cultured organotypic hippocampal slices were exposed to WIN55212-2 (30 µM) or its vehicle (control). After 30 min CPA (100 nM final concentration) or its vehicle (control) were applied and 15 min later NMDA (50 µM final concentration) or its vehicle (control) were added and incubation continued for 1 h. Medium was replaced to remove drugs and incubation continued for a 24 h period. PI (2 µM final concentration) was added 3 hours before exposure to drugs and after medium replacement. In each experiment five parallel assays were performed, corresponding respectively to: incubation with NMDA, NMDA + WIN55212-2, NMDA + CPA, NMDA + WIN55212-2 + CPA and incubation in the absence of drugs (control). Cellular uptake of PI was assessed by measuring emitted fluorescence before drugs addition (basal PI uptake) and 24 h after exposure to NMDA (total PI uptake). After subtracting basal PI uptake to total PI uptake, the net PI uptake was expressed as % of the net PI uptake obtained for control slices. **A)** Representative digital fluorescent micrographs, taken before (left panel) and 24h after (right panel) exposure of one slice to NMDA. B) Bars represent PI uptake obtained in the absence (open bars) or in the presence of NMDA (solid bars) corresponding to (from left to right) the CA3, CA1, CA4 and dentate gyrus (DG) areas of the rat hippocampal slice. C) Bars represents PI uptake obtained (from left to right) in the absence of drugs (control), in the presence of NMDA, NMDA + WIN55212-2, NMDA + CPA and NMDA + WIN55212-2 + CPA; the open bar represents the PI uptake that would be obtained if the effects of CPA and WIN were additive. Data are mean ± SEM from 4 independent experiments run at least in triplicate. §, §§, §§§: P<0.05, P<0.01 and P<0.0001, respectively, Vs NMDA (b) alone (One-way ANOVA followed by LSD test). #: P<0.05 Vs NMDA+WIN55212-2 (c) or NMDA+CPA (d) ( One-way ANOVA followed by LSD test). NS, Non statistically different (P>0.9, Student's t-test). *, **; P<0.05 and P<0.0001, respectively, Vs control (Student´s t-test).

## 5.3. Discussion

The present study provides evidence, for the first time, of an independent, cumulative inhibitory effect of adenosine $A_1$ and cannabinoid CB1 receptors against NMDA-mediated excitotoxic insult at the rat hippocampus. Previous studies showed that, when applied alone, both $A_1$ and CB1 receptor agonists decrease NMDA-dependent neuronal activity and injury in hippocampal slices (Koch *et al*. 2011; Sebastião *et al*. 2001b), neurons (Zhuang *et al*. 2005; Oku *et al*. 2004) and in live rodents (Von Lubitz *et al*. 1993; Zani *et al*. 2007). In the present work we observed that both $A_1$ and CB1 agonists not only had individual neuroprotective action but cumulatively dampened NMDA-mediated excitotoxicity, with an additive combined effect higher than the one obtained activating each receptor alone. This results are in agreement with previous studies where acute co-activation of adenosine $A_1$ and cannabinoid CB1 receptors have been shown to generate additive effects in inhibiting glutamatergic synaptic transmission (Serpa 2007) and cAMP formation (chapter 3 of the present work, see also Serpa *et al*. 2015) in the rat hippocampus, and in stimulating G-protein activation in rat hippocampal membranes (Childers *et al*. 2005).

The combined additive neuroprotective action of adenosine $A_1$ and cannabinoids $CB_1$ receptors on NMDA-induced excitotoxicity was evident when cell injury was assessed either by measuring LDH release – which reflects cell necrosis and late apoptosis – or quantifying PI uptake – which measures mainly cell necrosis and late apoptosis but also early apoptosis in a smaller degree (Parhamifar *et al*. 2013; Vitale *et al*. 1993; Cebers *et al*. 1997).

Using PI fluorescence microscopy we were able to analyze NMDA-induced cell injury in different hippocampal regions. The NMDA-induced excitotoxic effect varied across different hippocampal areas, the NMDA effect being stronger in CA3 area than in the CA1 area, while no excitotoxic effect was observed at either CA4 or dentate gyrus, indicating different susceptibilities of the neuronal populations across different hippocampal areas. The extent of NMDA-induced cell injury in CA3 and CA1 areas observed in the present work was similar to that reported in Sprague Dawley rats in a previous study using the same NMDA concentration and exposure period (Mayer *et al*. 2002), however they refer a significant neurotoxic effect of NMDA in dentate gyrus, which was not observed in the present work. The different results for the NMDA effect on dentate gyrus obtained in the present work and the study of Mayer *et al*. (2002), might be due to differences in rat strains and/or composition of incubation media.

Interestingly, a neuroprotective action against NMDA-induced excitotoxicity, assessed by PI uptake, produced by both adenosine $A_1$ and cannabinoids $CB_1$ receptors activation was only observed in the CA3 area, which was the most susceptible to NMDA citotoxicity, but not the CA1 area. Since both adenosine $A_1$ and cannabinoids $CB_1$ receptors are expressed at CA3 and CA1 areas, the absence of protective effect of $A_1$ and $CB_1$ receptors on NMDA-induced cell injury at the CA1 area might reflect different receptor expression/distribution between the two areas. In fact immunoreactivity for $A_1$ receptor was highest at post-synaptic sites of

stratum pyramidale of CA2/CA3 areas than in other regions of the hippocampus (Ochiishi *et al*. 1999). However for the CB1 receptor, the immunoreactivity is more homogenous across hippocampal areas (Egertová and Elphick, 2000), suggesting that lower receptor expression is not the cause for the lack of neuroprotective action of CB1 receptor in the CA1 area.

Since $A_1$ and CB1 receptors mainly couple to identical $G\alpha_i$ proteins, the additive effect of both receptors on NMDA-mediated excitotoxicity, observed in the present work, suggests high availability of shared signaling molecules, or the presence of compartmentalization which would physically separate the transduction pathways operated by these receptors. Furthermore, the robust additive inhibitory effect of combined $A_1$ and CB1 receptor agonists on the NMDA-mediated excitotoxicity, suggests that a relevant neuroprotective role is played by $A_1$ and CB1 receptors against excitotoxic damage at the hippocampus. CB1 and $A_1$ receptors might use complementary mechanisms to afford neuroprotection against NMDA-mediated neurotoxic insults since CB1 receptor-mediated protection against NMDA-induced cell death depends on adenylyl cyclase and ryanodine receptor inhibition (Zhuang *et al*. 2005), whereas $A_1$ receptor-mediated protection did not depend on cAMP levels (Oku *et al*. 2004) but might involve direct inhibition of NMDA receptors (Sebastião *et al*. 2001b).

# Chapter 6: $A_1$ receptor and Synaptic Transmission at the Hippocampus: role of cGMP

## 6.1. Introduction

Adenosine $A_1$ receptor activation and cGMP have similar regulatory actions on synaptic transmission. Both inhibit the pre-synaptic release of glutamate (Prast and Philipu 2001; Shen *et al*. 1996) and facilitate induction of LTD (Dias *et al*. 2013; Feil and Kleppisch, 2008; Santschi *et al*. 2006). However, the possibility that cGMP mediate some of the adenosine $A_1$ receptor actions in the hippocampus remains to be clarified.

Interaction between the adenosine $A_1$ receptor and the NO/sGC/cGMP pathway, in regulating synaptic transmission, have been reported. Previous studies have shown that NO donors inhibit synaptic transmission in hippocampal slices, and that this inhibition was blocked by adenosine $A_1$ receptor antagonists (Broome *et al*. 1994), however the inhibitory effect of NO donors was not affected by inhibition of sGC (Arrigoni and Rosenberg 2006). On the other hand, NO was shown to increase the inhibitory effect of adenosine $A_1$ receptor activation in synaptic transmission and this increase was blocked by inhibitors of sGC (Fragata *et al*. 2006). In another study, the activation of adenosine $A_1$ receptor together with the simultaneous increase in cGMP concentration elicited by zaprinast, was enough to induce chemical LTD (Santschi *et al*. 2006).

In the present study, the activation of the adenosine $A_1$ receptor has shown to increased cGMP levels in the hippocampus (chapter 4 of the present work, see also Serpa *et al*. 2014). However, it is not clarified if cGMP plays any role in mediating the inhibitory effect of adenosine $A_1$ receptor on neurotransmission. To answer this question we tested if interfering with components of the NOS/sGC/PKG pathway would modify the inhibitory effect of adenosine $A_1$ receptors in synaptic transmission at the hippocampus.

## 6.2. Results

We proceeded by measuring synaptic transmission conveyed to area CA1, after inducing depolarization of the Schaffer collaterals, with the purpose of clarifying if $A_1$ receptor-mediated inhibition of synaptic transmission in the hippocampus is partially mediated by the NOS/sGC/PKG pathway. To this end we elaborated experiments were each slice was exposed

twice to CPA (15 nM). The second exposure to CPA was conducted in the presence of drugs which modulate NOS/sGC/PKG pathway components, to determine if it would interfere with CPA-mediated inhibition of synaptic transmission. We then compared the first CPA-induced inhibition with the second one. We first had to perform the appropriate control experiments, were the second application of CPA was performed in the absence of any additional drugs. We observed that the first application of CPA (15 nM) inhibited the fEPSP slope by 40 % ± 2 %, whereas the second application was identical to the first, with 41 % ± 2 % inhibition (n=3; P>0.05, paired Student t-test; Fig 22). The time interval between applications of CPA was the same as when NOS/sGC/PKG pathway modulator drugs were applied (about 90 min).

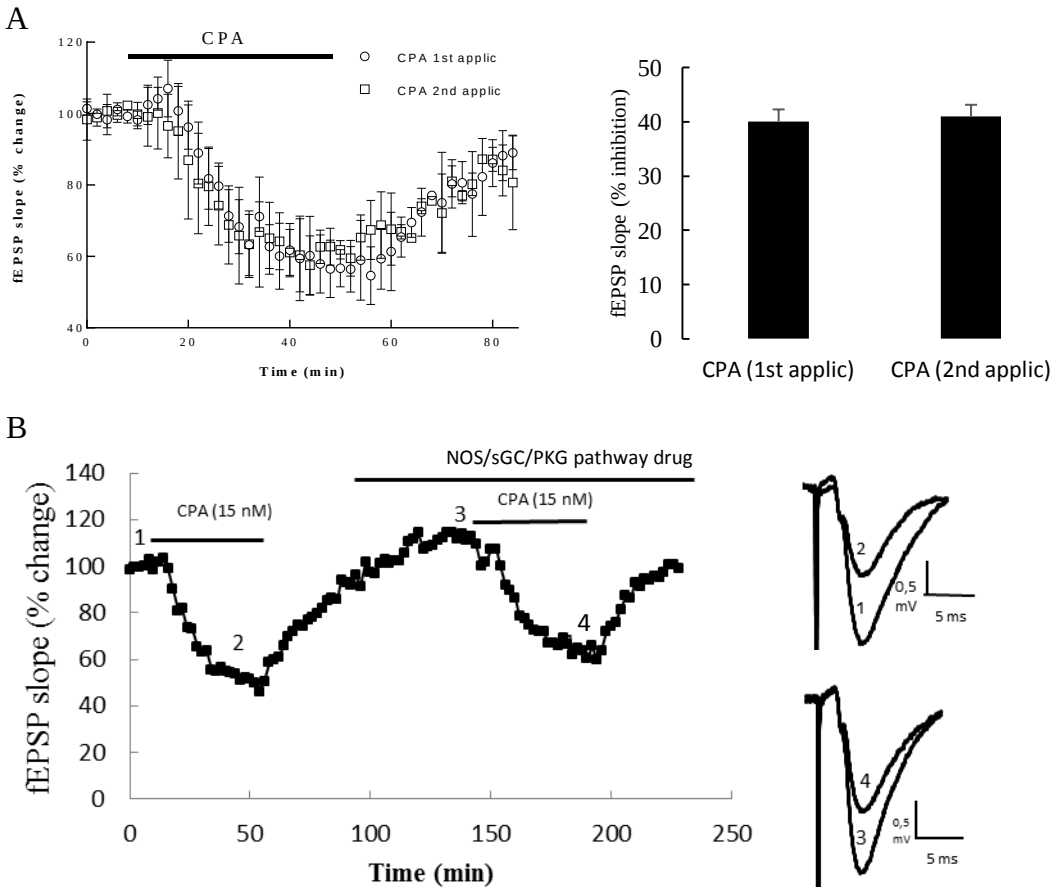


Figure 22. Control experiment and generic model for all experiments performed. **A)** The first application of CPA (15 nM), and the time of perfusion before second application, did not modify the effect of second application of CPA on fEPSP slope. Left panel: Averaged time course changes of fEPSP slope induced by CPA. Each point in the ordinates corresponds to the average ± SEM of fEPSP slopes, normalized for its value before addition of CPA, of three experiments. The horizontal bar indicates the time of perfusion of CPA. Right panel: Bars represent average ± SEM inhibition by CPA of fEPSP slope, of three independent experiments. The effect of CPA was obtained after 40 minutes of exposure to the drug, when the CPA effect stabilized. The time interval between CPA applications was identical to the one used when NOS/sGC/PKG pathway modulator drugs were applied (90 min). **B)** Left panel: Model of a generic time course representative of all experiments performed, where time-distance between points corresponds to two minutes. Model was used for testing if CPA-mediated inhibition of fEPSP slope is dependent on the NOS/sGC/PKG pathway. Right panels: Represent the fEPSPs measured in the hippocampus slice whose slope from the descending phase was used to quantify synaptic transmission.

### 6.2.1. Effect of a phosphodiesterase inhibitor and a cGMP analogue

We began testing the hypothesis that if the $A_1$ receptors effect on synaptic transmission was mediated, at least partially, by the activation of the NOS/sGC/PKG pathway, then preventing basal cGMP degradation would increase the effect of CPA. For that purpose we used BAY 60-7550 to decrease cGMP degradation in hippocampal slices of male rats; BAY 60-7550 is mostly a selective inhibitor of PDE2, the main enzyme responsible for cGMP hydrolysis at the hippocampus (Bartus *et al.* 2013). We also tested the membrane permeable cGMP analogue 8-pCPT-cGMP which directly mimics cGMP activity; in this case the CPA effect was expected to be attenuated by the analogue, since the cGMP generated by CPA would compete with the cGMP analogue to produce its regulatory effect. As one may observe in Figure 23, both drugs failed to modify the activity of $A_1$ receptors. In the case of BAY 60-7550 (100 nM), the effect of CPA alone was 48 % ± 2 % inhibition of the fEPSP slope, whereas in the presence of the drug it was 50 % ± 3 % (n=5; $P>0.05$, paired Student t-test). BAY 60-7550 (100 nM) alone increased the fEPSP slope by 25 % ± 4 % (n=5; $P>0.05$ when compared with zero, Student´s t-test). As for 8-pCPT-cGMP (10 μM), CPA alone decreased the fEPSP slope by 59 % ± 5 % while after exposure to the drug the CPA effect was slightly lower, 50 % ± 7 % (n=4), although not significantly different from the effect of CPA alone ($P>0.05$, paired Student t-test). 8-pCPT-cGMP alone failed to consistently modify the fEPSP slope (% decrease of 3 ± 15, n=4; $P>0.05$ when compared with zero, Student t-test).

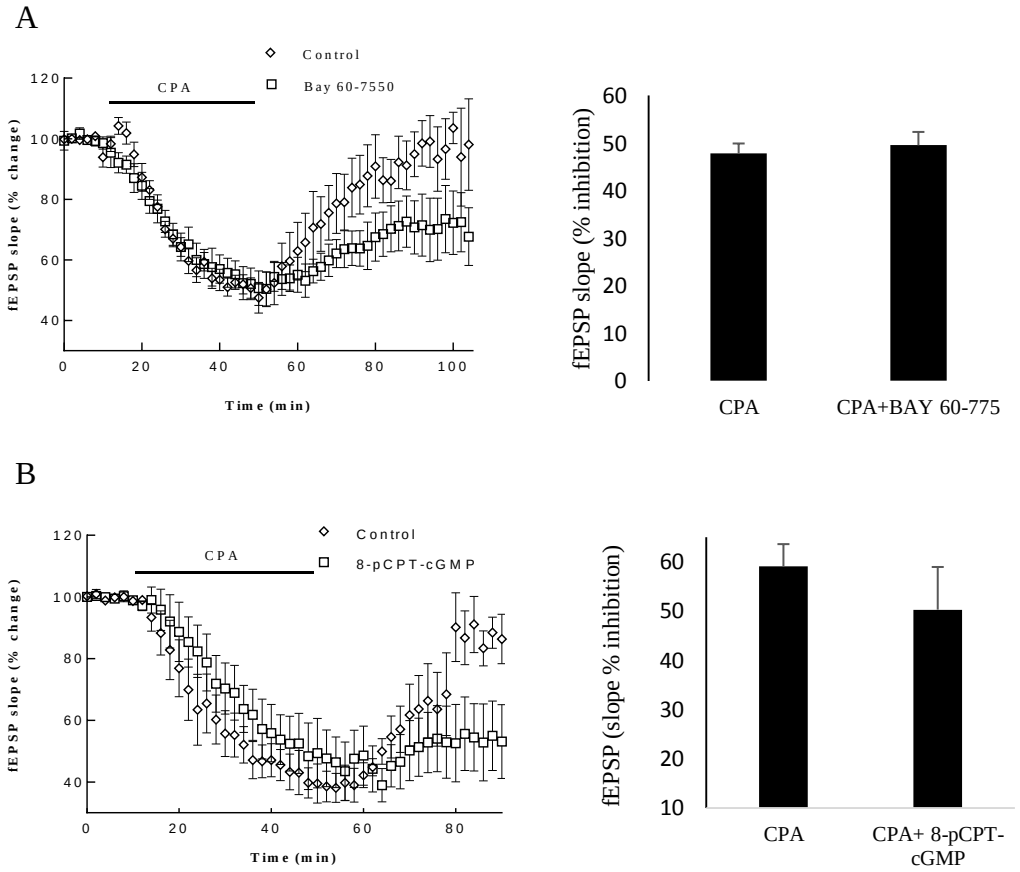


Figure 23: Increasing cGMP related activity by inhibition of cGMP-degrading phosphodiesterase (A) or by direct application of a cGMP analog (B) failed to modify CPA-mediated inhibition of synaptic transmission in male rats. CPA (15 nM) was first applied for 40 minutes and washed-out for a minimum of 40 minutes more (control time course). BAY 60-7550 (100 nM) or 8-pCPT-cGMP (10 μM) were then applied for 30 minutes and throughout the second application of CPA (test time course). Control and test time courses were performed in the same hippocampal slice. (A) and (B) Left Panels: Superimposition of the time courses. Each point in the ordinates corresponds to the average ± SEM of 5 experiments; in each experiment a point corresponds to the average of eight consecutive fEPSP slopes; the time-distance between points corresponds to two minutes. As one may observe in the superimposition of time courses, the effect of CPA was not modified by the presence of either drug, but both drugs tended to decreased the washout of CPA. (A) and (B) Right panels: Comparison between average of percentage inhibition produced by CPA in the absence (left) or in the presence (right) of drugs. The bars represented the average ± SEM of 5 independent experiments.

## 6.2.2. Effect of the NOS/sGC/PKG pathway inhibitors

We proceeded by examining how $A_1$ receptor activity reacted to antagonists of the NOS/sGC/PKG pathway. We tested if blocking NOS, the enzyme which produces NO, with L-NAME would decrease CPA-mediated inhibition of synaptic transmission. In fact, when female Wistar rats were tested, the inhibitory effect of CPA on fEPSP slope was dampened from 54 % ± 5 % to 24 % ± 6 % by the presence of L-NAME (300 μM; n=5; $P<0.05$; paired Student´s t-test), which corresponds to a 57 % ± 9 % decrease of the CPA effect (Fig 24). L-NAME (300 μM) alone showed a tendency to increase the fEPSP slope (50% ± 25%) but this tendency was not

statistically significant (n=5; P>0.05 when compared with zero, Student´s t-test). Male Wistar rats were also tested, and like in females, L-NAME showed a tendency to dampen the effect of CPA, but the number of experiments were low (n=2), and more must be performed to clarify this issue.

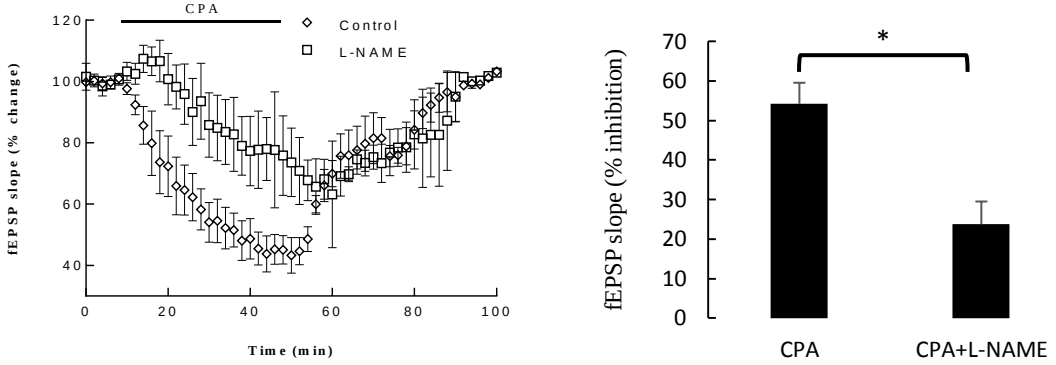


Figure 24 Blockage of NOS by L-NAME dampened CPA-mediated inhibition of synaptic transmission in female rats. CPA (15 nM) was first applied for 40 minutes (control time course) and washed-out for a minimum of 40 minutes more. L-NAME (300 µM) was then applied for 30 minutes and throughout the second application of CPA (test time course). Control and test time courses were performed in the same hippocampal slice. Left panel: Superimposition of the time courses. Each point in the ordinates corresponds to the average ± SEM of 5 experiments performed; in each experiment a point corresponds to the average of eight consecutive fEPSP slopes; the time-distance between points corresponds to two minutes. As one may observe in the superimposition of time courses, the effect of CPA was dampened by the presence of L-NAME, without interfering with the washout of CPA. Right panel: Comparison between percentage inhibition produced by CPA in the absence (left) or in the presence (right) of L-NAME. Bars represent average ±SEM percentage inhibition of fEPSP slope after stabilization of the inhibitory effect of CPA. * P<0.008 (Paired Student´s t-test).

To elucidate if the effect of $A_1$ receptors in synaptic transmission depended on PKG activity, the CPA effect was evaluated in the absence and in the presence of a selective PKG inhibitor (KT5823) in male and female rats. We found that, in male Wistar rats (Fig 25A), the presence of KT5823 (1 nM) decreased the inhibitory effect of CPA on synaptic transmission from 44% ± 11% to 22% ± 3%, corresponding to a 49% ± 9% attenuation in effect (n=4; P<0.05 when compared with zero, paired Student´s t-test) whereas application of KT5823 (1 nM) alone tended to increase the fEPSP slope (23 % ± 14.2 % increase, n=4) although this increase was not statistically significant (P>0.05 when compared with zero, Student´s t-test). In a similar way, in female Wistar rats (Fig 25B), the inhibitory effect on fEPSP slope produced by CPA, 50 % ± 5 %, was dampened to 33 % ± 3% by the presence of KT5823 (1 nM), corresponding to a 32 % ± 9 % decrease in effect (n=5; P<0.05 when compared with zero, paired Student´s t-test), whereas the effect of KT5823 (1 nM) alone was more reproducible than in males, increasing the fEPSP slope by 25 % ± 5 % (P<0.05 when compared with zero, Student´s t-test).

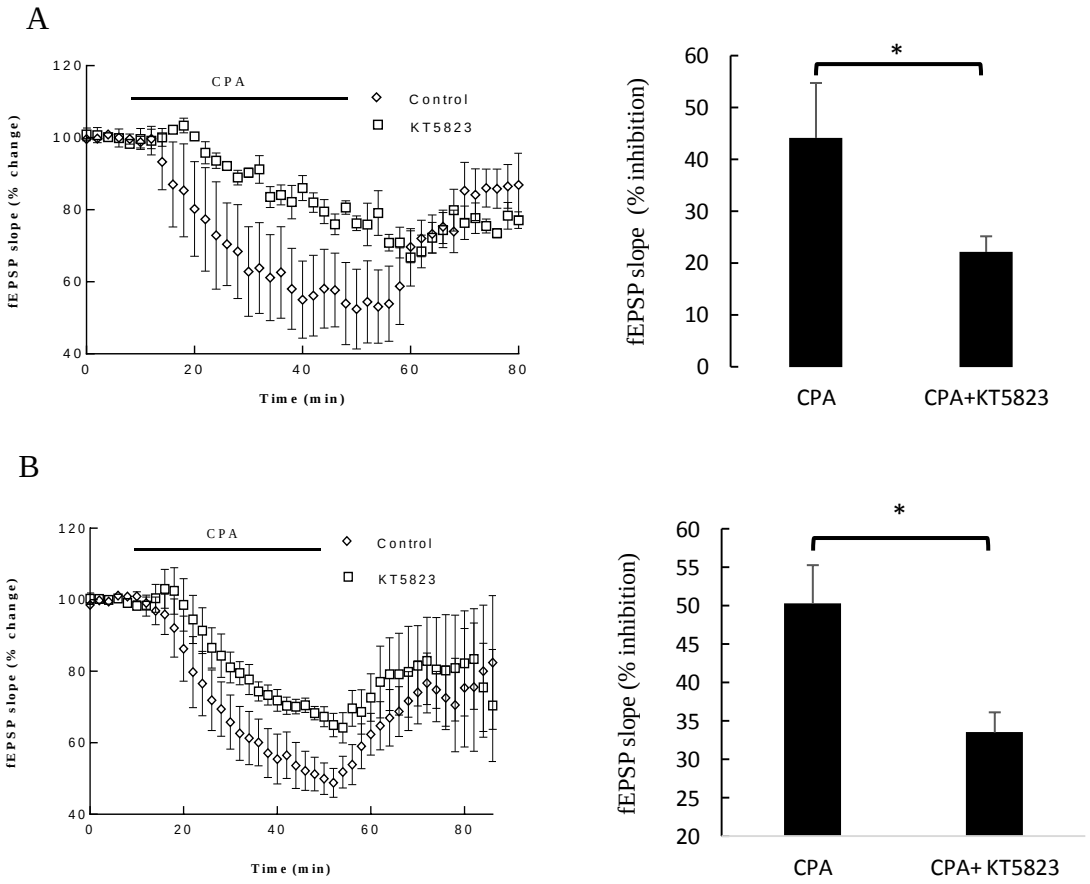


Figure 25 Inhibition of PKG by KT5823 decreased CPA-mediated inhibition of synaptic transmission in male (A) and female (B) rats. CPA (15 nM) was applied for 40 minutes and washed-out for a minimum of 40 minutes more (control time course). KT5823 (1 nM) was then applied for 30 minutes and throughout the second application of CPA (test time course). Control and test time courses were performed in the same hippocampal slice. (A) and (B) Left Panels: Superimposition of the time courses. Each point in the ordinates corresponds to the average ± SEM of 5 experiments performed; a point corresponds to the average of eight consecutive fEPSP slopes; the time-distance between points corresponds to two minutes. As one may observe in the superimposition of time courses, the effect of CPA decreased in the presence of KT5823, without interfering with the washout of CPA. (A) and (B) Right Panels: Comparison between percentage inhibition produced by CPA in the absence (left) or in the presence (right) of KT5823. Bars represent average ± SEM percentage inhibition of fEPSP slope after stabilization of the inhibitory effect of CPA. * P<0.05 (Paired Student´s t-test).

Since it was observed in the present work, that the effect of $A_1$ receptors in synaptic transmission was strongly attenuated by inhibiting NOS-mediated NO production, it was then investigated if the effect of $A_1$ receptors could be mediated through NO-activated sGC in adult male and female rats. We proceeded by using ODQ to block the activity of sGC, the enzyme which generates cGMP, to assess its role on CPA-mediated inhibition of synaptic transmission. In male rats (Fig 26A), the effect of CPA (15 nM) in the presence of ODQ (35 % ± 6 %) did not differ from the effect of CPA alone (32 % ± 3 %; n=3; P=0.7; paired Student t-test, compared with CPA in the presence of ODQ). When we tested female Wistar rats (Fig 26), CPA alone inhibited the fEPSP slope by 39 % ± 7 % and ODQ (10 μM) again failed to interfere with

the effect of CPA (35 % ± 5 %; n=4; P>0.05; paired Student t-test, compared with CPA alone). ODQ (10 µM) alone increased synaptic transmission by 17 % ± 3 % in males (n=3; P<0.05 when compared with zero, Student´s t-test), but females showed only a non-significant tendency for increasing the fEPSP slope (19 % ± 10 %; n=4; P>0.05 when compared with zero, Student´s t-test). We then questioned if endogenous adenosine was interfering on $A_1$ receptor activity in a way that it was masking the effect of ODQ on CPA-mediated inhibition of the fEPSP slope. We used adenosine deaminase (ADA), the enzyme which irreversibly deaminates adenosine converting it to the related nucleoside inosine. In the presence of ADA (1 U/ml), ODQ (10 µM) successfully decreased the inhibitory effect of CPA on the fEPSP slope from 50 % ± 4 % to 39 % ± 6 % in males (n=5; P<0.05; paired Student´s t-test) and from 51 % ± 5 % to 39 % ± 6 % in females (n=4; P<0.05; paired Student´s t-test), corresponding to a decrease of effect of 23 % ± 6 % in males and 23 % ± 7 % in females (Fig 26C, 26D). The effect of CPA alone, such as the dampening of the effect of CPA by ODQ, were identical between genders, both in the presence and absence of ADA (P>0.05, One-Way ANOVA, post hoc LSD test). In addition, ODQ (10 µM) alone but in the presence of ADA increased the fEPSP slope by 23 % ± 5 % in males (n=4; P<0.05 when compared with zero; Student´s t-test), whereas in females again ODQ (10 µM) only showed a non-significant tendency for increasing the fEPSP slope (29 % ± 13 %; n=4; P>0.05 when compared with zero, Student´s t-test).

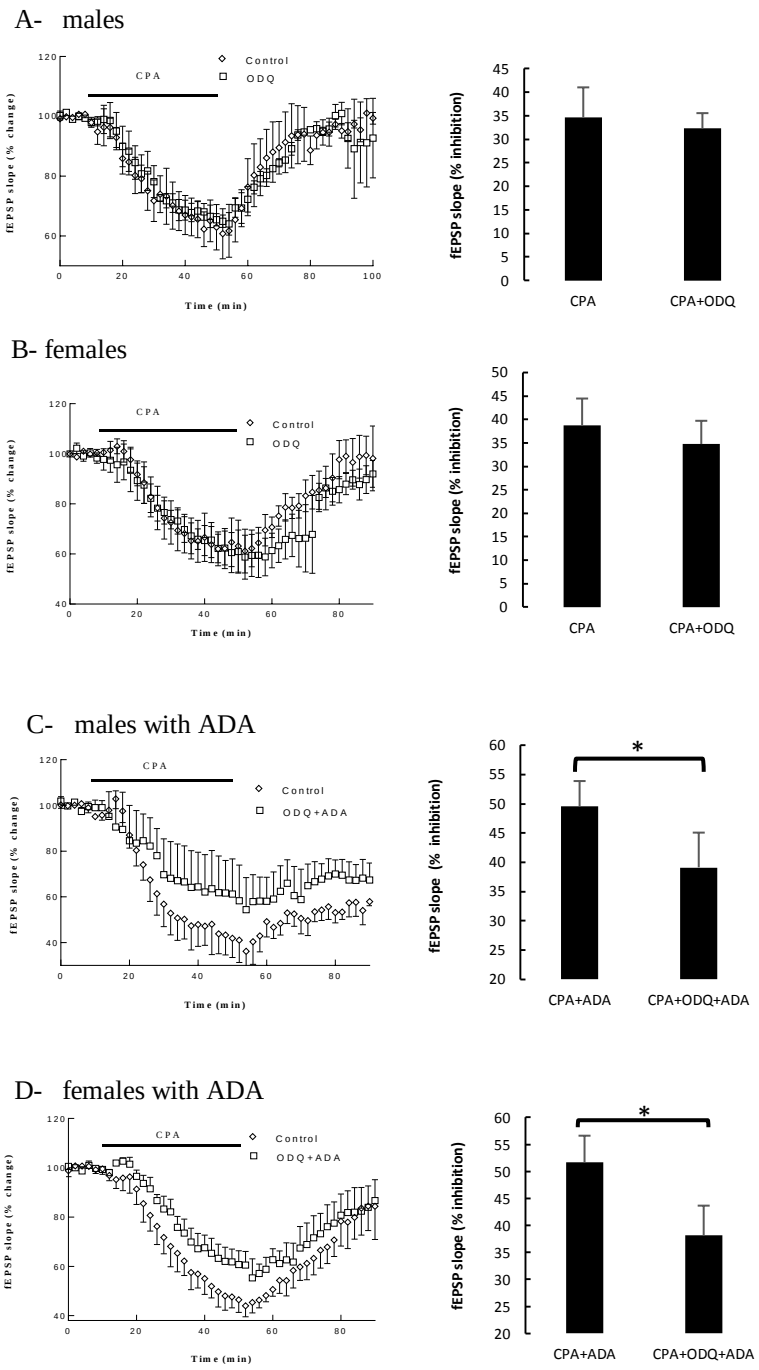


Figure 26 The decrease of CPA-mediated inhibition of synaptic transmission by the sGC antagonist ODQ in males (A, C) and females (B, D) rats is unmasked by the presence of ADA (C, D). CPA (15 nM) was applied for 40 minutes and washed-out for a minimum of 40 minutes more (control time course). ODQ (10 μM) was then applied for 30 minutes and throughout the second application of CPA (test time course). Control and test time courses were performed in the same hippocampal slice. (A), (B), (C) and (D) Left Panels: Superimposition of the time courses. Each point in the ordinates corresponds to the average ± SEM of 5 experiments performed; a point corresponds to the average of eight consecutive fEPSP slopes; the time-distance between points corresponds to two minutes. As one may observe in the superimposition of time courses in A and B, the effect of CPA and washout was not modified by the presence of ODQ. The presence of ADA unmasked the dampening effect of ODQ on CPA-mediated inhibition of synaptic transmission (C, D), and interfered with washout in male rats hippocampal slices (C). (A), (B), (C) and (D) Right Panels: Comparison between percentage inhibition produced by CPA in the absence (left) or in the presence (right) of ODQ. Bars represent average ± SEM percentage inhibition of fEPSP slope after stabilization of the inhibitory effect of CPA. * $P<0.05$ (Paired Student´s t-test).

## 6.3. Discussion

*The effect of CPA on synaptic transmission and the NOS/sGC/PKG pathway*

It is long known that $A_1$ receptors inhibit synaptic transmission at the hippocampus by activating $G_{i/o}$ proteins. This effect of adenosine $A_1$ receptor is primarily consequence of presynaptic inhibition of neurotransmitter release by reduction of $Ca^{2+}$ entry trough N-type calcium channels (Wu e Saggau 1994). Decrease of cAMP levels does not seems to mediate the $A_1$ receptor effect on neurotransmitter release (see Fredholm and Dunwiddie 1988). However, blockade of N-type calcium channels only partially attenuates the inhibitory effect of $A_1$ receptor on synaptic transmission, suggesting that other mechanism might be involved (Fredholm and Dunwiddie 1988).

It is not so clear the role played by cGMP in $A_1$ receptor-mediated inhibition of synaptic transmission. The function of the NOS/sGC/PKG pathway in the CNS has been studied most extensively in synaptic plasticity. Nitric oxide plays a main role in LTP, as indicated by experiments showing that LTP is eliminated or blocked significantly by inhibitors of nitric oxide synthase (Bohme *et al*. 1991; O'Dell *et al*. 1991; Schuman and Madison, 1991), including L-NAME (Lessmann *et al*. 2011). Furthermore, inhibitors of sGC or PKG also block the induction of LTP (Zhuo *et al*. 1994b; Blitzer *et al*. 1995; Boulton *et al*. 1995), while direct application of 8-Br-cGMP to the bath or presynaptic injection of cGMP produces LTP in cultured hippocampal neurons (Arancio *et al*. 1995). Regarding LTD, it was found that the NO donor SNAP enhances it through activation of sGC and that the PKG inhibitor KT5823 blocked the induction of LTD by low frequency stimulation in hippocampal slices (Reyes-Harde *et al*. 1999). On the other hand the $A_1$ receptor antagonist DPCPX facilitates both LTP (de Mendonça and Ribeiro 2000) and LTD (de Mendonça *et al*. 1997) suggesting that endogenous adenosine, acting through adenosine $A_1$ receptors, is able to attenuate synaptic plasticity in the hippocampus. Thus, the NOS/sGC/PKG pathway promotes, while $A_1$ receptors usually attenuate synaptic plasticity. However, in one study, the activation of adenosine $A_1$ receptor together with the simultaneous increase in cGMP concentration elicited by zaprinast, was enough to induce chemical LTD (Santschi *et al*. 2006). These apparently contradictory results probably reflects the influence of $A_1$ receptors on multiple pathways, thus it does not exclude the NOS/sGC/PKG pathway participation in the inhibitory effect of $A_1$ receptors on synaptic transmission. In fact, inhibitors of NOS, sGC and PKG decreased $A_1$ receptor-mediated antinociceptive effects in the rat (Lima *et al*. 2010). In addition, the inhibitory effect of $A_1$ receptor on basal synaptic transmission – studied at frequencies of stimulation not inducing synaptic plasticity – is similar to the effect of activating the NOS/sGC/PKG pathway, since stimulating nitric oxide synthase, activating soluble guanylyl cyclase or elevating concentrations of intracellular cGMP depressed synaptic transmission in CA1 hippocampal neurons (Lei *et al*. 2000).

*Raising cGMP activity did not modify CPA-mediated inhibition of synaptic transmission*

We tested the hypothesis that, if $A_1$ receptors partially inhibit synaptic transmission at the hippocampus through the NOS/sGC/PKG pathway, then increasing cGMP-mediated activity should interfere with the inhibitory effect of $A_1$ receptors. We used two approaches, inhibition of phosphodiesterase 2 (PDE2) and a membrane-permeable cGMP analogue, to increase cGMP related activity. Inhibition of PDE2 has been reported to enhance hippocampal long-term potentiation (LTP) and to restore spatial working memory disrupted by a NMDA antagonist (Boess *et al*. 2004). In the of case of 8-pCPT-cGMP, its presence was required for LTP to occur after a weak tetanus (Son *et al*. 1998), whereas presynaptic injection of cGMP produces activity-dependent LTP in cultured hippocampal neurons (Arancio *et al*. 1995). However, both approaches failed to modify the inhibitory effect of CPA. Regarding the effect of Bay 60-7550, although it primarily elevates cGMP levels, it also increases cAMP in hippocampus slices (Boess *et al*. 2004), which has the opposite effect of cGMP on synaptic transmission. Despite the fact Bay 60-7550 requires a 10-fold higher concentration to induce increases in cAMP levels compared with cGMP, as determined by radioimunoassays in hippocampal slices (Boess *et al*. 2004), it is noticeable that Bay 60-7550 by itself increased synaptic transmission in the present study, suggesting that the effect of cAMP (stimulating neurotransmission) surpassed the effect of cGMP. The effect of 8-pCPT-cGMP alone did not modify synaptic transmission in the present study, as confirmed by Son *et al*. (1998), while others reported either inhibition or facilitation of synaptic transmission by cGMP (see Arrigoni and Rosenberg 2006; Russwurm *et al*. 2013). These discrepancy between different studies probably reflects the fact that cGMP has many potential direct targets, including phosphodiesterases, kinases, and cyclic nucleotide gated channels (MacFarland 1995), therefore the intricate nature of cGMP signaling could have masked the effect of 8-pCPT-cGMP. Note also that 8-pCPT-cGMP could activate its multiple targets located at different cellular locations, which might have opposing effects on synaptic transmission, while the cGMP-mediated adenosine $A_1$ effect on neurotransmission could probably be a more localized phenomenon.

*$A_1$ receptors inhibitory activity is partially mediated by cGMP*

We were prompt to determine if the $A_1$ receptor inhibitory effect on basic synaptic transmission is partially dependent on the NOS/sGC/PKG pathway. Previously it was observed that the NOS/sGC/PKG pathway plays a role in basic synaptic transmission in hippocampus slices, since NO donors depressed fEPSPs via the NOS/sGC/PKG pathway (Boulton *et al*. 1994). Other studies have shown that NO stimulates adenosine release in the ventral striatum, where inhibition of NOS activity decreases extracellular adenosine levels (Fischer *et al*. 1995). Moreover, inhibition of hippocampal fEPSPs by NO may, at least in part, be due to the release of adenosine that subsequently acts to depress neurotransmission through $A_1$ receptors (Fallahi *et al*. 1996). Accordingly, nitric oxide-mediated inhibition of synaptic transmission in hippocampus slices was blocked by an adenosine $A_1$ receptor antagonist (Boulton *et al*. 1994;

Broome *et al*. 1994), but the NO effect did not depend on sGC activity (Arrigoni and Rosenberg 2006). Conversely, nitric oxide was shown to increase the inhibitory effect of the $A_1$ agonist 2-chloroadenosine in synaptic transmission and this increase was blocked by inhibitors of sGC (Fragata *et al*. 2006). However the possibility that the NOS/sGC/PKG pathway might mediate the adenosine $A_1$ inhibition of synaptic transmission has not been investigated. The results obtained in the present study demonstrated that the sGC antagonist ODQ and the PKG inhibitor KT5823, when applied alone, significantly increased the fEPSP slope, indicating that, such as it occurs with $A_1$ receptor antagonists, inhibition of components of the NOS/sGC/PKG pathway increased synaptic transmission. Moreover, when components of the NOS/sGC/PKG pathway were blocked by L-NAME, an inhibitor of NOS, by ODQ, a sGC antagonist, and by the PKG inhibitor KT5823, the effect of $A_1$ receptor in synaptic transmission was strongly attenuated in hippocampal slices. Such results indicate that, part of the inhibitory effect of $A_1$ receptors on synaptic transmission is also mediated by cGMP signalling in the hippocampus.

# Chapter 7: General Discussion

The results obtained in the present work clearly showed that adenosine $A_1$ receptors have a modulatory action on cyclic nucleotides levels in the hippocampus, decreasing cAMP formation and increasing cGMP generation. Since cyclic nucleotides play a central role in regulating essential functions in the brain, understanding how adenosine $A_1$ receptor regulates cyclic nucleotides will help to better understand the neuromodulatory and neuroprotective effects of adenosine.

Regarding cAMP, the effect of adenosine $A_1$ receptor on cAMP accumulation was compared with the effect of another $G_{i/o}$ protein-coupled receptor, the cannabinoid CB1 receptor. Previous findings based on [$^3$H]-cAMP quantification indicate that the adenosine $A_1$ receptor acts as negative modulator of adenylyl cyclase activity in the hippocampus (Fredholm *et al.* 1989), similarly to what has been found in the cerebral cortex (Alexander *et al.* 1994). Experiments performed with CB1 agonists in hippocampal slices demonstrated inhibition of adenylyl cyclase in the hippocampus, frontal cortex and striatum (Bidaut-Russell et al., 1990) but the effect was assessed by quantifying cAMP using a protein kinase binding assay susceptible to several interferents (Brostrom and Kon, 1974). We used up-to-date immunoenzymatic methods to measure accurate cAMP levels and determine the individual and the combined effect of adenosine $A_1$ and cannabinoid CB1 receptors on cAMP formation at the hippocampus. The results showed that the adenosine $A_1$ receptor and the cannabinoid CB1 receptor acted as negative modulators of adenylyl cyclase and that co-activation of the receptors produced an additive, independent inhibition of cAMP formation in the hippocampus. Thus, the results suggest that receptor cross-talk between adenosine $A_1$ and cannabinoid CB1 receptors, due to heterologous receptor desensitization, limited availability of shared signalling molecules involved in the transduction pathways operated by these receptors, or another type of interference originated by receptor activity does not play a role on acute inhibitory actions of $A_1$ and CB1 receptors on cAMP production at the rat hippocampus. This indicates that modulation of adenylyl cyclase activity by $A_1$ and by CB1 receptors is cumulative rather than occlusive or synergistic. In fact, adenosine $A_1$ and cannabinoid CB1 receptors, can produce cumulative additive actions in the hippocampus at different levels of cell organization: activation of $G_{i/o}$ proteins in membranes (Childers *et al.* 2005; but see Sousa *et al.* 2011), inhibition of adenylyl cyclase activity (chapter 3 of the present work, see also Serpa *et al.* 2015), inhibition of synaptic transmission (Serpa 2007, 2009) and protection against excitotoxic insults (chapter 5 of the present work). Therefore, despite having similar actions, adenosine $A_1$ and cannabinoid CB1 receptors might use complementary rather than competitive mechanisms to produce their neuromodulatory and

neuroprotective actions in the hippocampus. In the case of neuroprotection, there is in fact some evidence for the use of complementary mechanism by adenosine $A_1$ and cannabinoid CB1 receptors, since CB1 receptor-mediated protection against NMDA-induced cell death depends on adenylyl cyclase (Zhuang *et al*. 2005), whereas $A_1$ receptor-mediated protection did not depend on cAMP levels (Oku *et al*. 2004) but might involve direct inhibition of NMDA receptors (Sebastião *et al*. 2001b).

The results obtained in the present study do not preclude however that adenosine $A_1$ and cannabinoid CB1 receptors might interact in specific situations. In fact synergic actions of the two receptors have been reported in *in vivo* studies (Sousa *et al*. 2011; DeSanty and Dar 2001), but these interactions probably depend on polysynaptic contacts and neuronal circuitry. Less than additive interactions between adenosine $A_1$ and cannabinoid CB1 receptors might also occur under prolonged exposure to receptor agonists (Selley *et al*. 2004; Kouznetsova *et al*. 2002) or to high levels of endogenous adenosine (Hoffman *et al*. 2010), where other downstream effectors may be influenced which might induce heterologous receptor desensitization (Kouznetsova *et al*. 2002), or in situations of reduced availability of signaling molecules shared by both receptors.

The results obtained in the present work demonstrate that, along with $G_{i/o}$ protein-dependent regulation of the second messenger pathways of cAMP (Dunwiddie and Fredholm 1989), diacylglycerol and inositol trisphosphate (Cascalheira and Sebastião 1998), part of the neuromodulatory activity of $A_1$ receptors is also mediated by cGMP signalling in the hippocampus. Although the involvement of $G_{i/o}$ proteins in mediating the effect of adenosine $A_1$ receptor on cGMP levels was not investigated in the present work, it was previously found that pertussis toxin treatment, which inactivates specifically $G_{i/o}$ proteins, abolished the adenosine $A_1$ receptor-mediated inhibition of cAMP formation, but failed to modify inhibition of fEPSP mediated by $A_1$ receptors in hippocampal slices (Fredholm *et al*. 1989), suggesting that other signalling mechanisms might be involved in the inhibitory action of adenosine $A_1$ receptor in synaptic transmission.

The similar actions of adenosine $A_1$ receptor activation and cGMP at the nervous system, and the present findings that the adenosine $A_1$ receptor increases cGMP formation in the hippocampus (chapter 4 of the present work, see also Serpa *et al*. 2014) and that cGMP might contribute to mediate adenosine effects on neurotransmission (chapter 6 of the present work), opens the possibility that modification of cGMP levels – either pharmacologically or by activation of other receptors – could allow modulation of adenosine $A_1$ receptor-mediated effects in the nervous system, thus increasing the therapeutic potential of the $A_1$ receptor. On the other hand, the finding of a cumulative neuroprotective action of adenosine $A_1$ and cannabinoid CB1 receptors against neurotoxic insults might be promising in ameliorating pathological conditions involving excessive excitatory activity. These findings have many potential applications in pathologies which involve $A_1$ and CB1 receptors, cyclic nucleotides and the hippocampus. These include regulating the activity of $A_1$ and CB1 receptors to

ameliorate the symptoms, or even contribute in healing Alzheimer´s disease, multiple sclerosis, Huntington´s disease and traumatic brain injury. It is hoped the data obtained here will provide insight into these life debilitating illnesses and improve life quality and expectancy of the afflicted. In addition, new subjects of research were opened by this work. Since $A_1$ receptors modulate cGMP levels and contribute to the mechanisms of neuroprotection, the role of cGMP in mediating the neuroprotective action of adenosine $A_1$ receptor is a relevant issue which deserves future investigation. On the other hand, the relative contribution of both sGC and pGC in mediating the actions of adenosine $A_1$ receptors on cGMP formation, modulation of neurotransmission and possibly in neuroprotection needs to be evaluated. Likewise, the combination of the effects of different receptors to obtain higher neuroprotective and neuromodulatory responses is also an interesting approach which can be further explored.

# Conclusions

The results obtained in the present work indicate a cumulative, additive, inhibition of cAMP accumulation by adenosine $A_1$ and cannabinoid CB1 receptors at the rat hippocampus. Furthermore, the effect of CB1 was not affected by sub-acute adenosine $A_1$ receptor desensitization. Therefore, the results suggest that receptor cross-talk between adenosine $A_1$ and cannabinoid CB1 receptors, due to heterologous receptor desensitization, limited availability of shared signaling molecules involved in the transduction pathways operated by these receptors, or another type of interference originated by receptor activity does not play a role on acute inhibitory actions of $A_1$ and CB1 receptors on cAMP production at the rat hippocampus.

The results obtained in the present work also indicate an additive and therefore independent combined neuroprotective action of adenosine $A_1$ and cannabinoid CB1 receptors against NMDA-mediated excitotoxicity. This cumulative dampening of excitotoxicity is highly relevant since a potential therapeutical role of combined $A_1$ and CB1 agonists would be very valuable in promoting neuronal survival and mitigating secondary brain damage in pathologies with associated excitotoxicity, such as ischemia, trauma or neurodegenerative disorders.

In the present study it was also found that adenosine $A_1$ receptor is upstream modulator of cGMP levels at the hippocampus and that both NO-mediated and non-mediated mechanisms might be involved in the adenosine $A_1$ receptor action, depending on gender. Moreover, antagonism of components of cGMP pathway dampened $A_1$ receptor inhibitory activity on synaptic transmission, revealing that part of the effect of $A_1$ receptor depends on the NOS/sGC/PKG pathway. These results provide a more complete picture of $A_1$ receptor-mediated activity and associated mechanisms in synaptic transmission at the hippocampus.

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# Glossary

Acetylcholine: Acetylcholine acts as part of a neurotransmitter system and plays a role in attention and arousal. In the peripheral nervous system, this neurotransmitter plays a major role in the autonomic nervous system and works to activate muscles.

AMPA receptors: Major mediators of fast glutamatergic excitatory synaptic transmission in the central nervous system

Calcium Spike: Short-lived change in the membrane potential caused by calcium entering the neuron.

Calcineurin: calcium-dependent serine-threonine phosphatase, also known as protein phosphatase 3.

CREB: It is a cellular transcription factor. It binds to DNA sequences increasing or decreasing the transcription of target genes, such as BDNF gene.

Dendritic spine: Small membranous protrusion from a neuron's dendrite that typically receives input from a single synapse of an axon, increasing the number of possible contacts between neurons

Desensitization: Homologous desensitization involves adaptive changes at the level of the GPCR itself, whereas heterologous desensitization may also involve changes in signalling components downstream of the GPCR.

DREAM: In the nucleus, DREAM binds to specific downstream regulatory element (DRE) to repress transcription of target genes. Outside the nucleus, DREAM interacts with potassium channels, and modulate calcium release from the endoplasmic reticulum.

ERK: Extracellular signal-regulated kinase. Protein kinase intracellular signalling molecules involved in induction of long-lasting forms of hippocampal synaptic plasticity and in hippocampus-dependent associative and spatial learning.

GEFs: Guanine nucleotide exchange factors activate monomeric GTPases by stimulating the release of guanosine diphosphate (GDP) to allow binding of guanosine triphosphate (GTP)

Glycogenolysis: Is the conversion of glycogen polymers to glucose monomers, which takes place in the muscle and liver tissues, regulated by the hormones glucagon and epinephrine.

Glucocorticoids: Steroid hormones that bind to the glucocorticoid receptor which is present in almost every vertebrate animal cell. They regulate the metabolism of glucose, and are part of the feedback mechanism in the immune system that turns immune activity (inflammation) down.

GTPases: Molecular switches in intracellular signalling pathways. The most well known GTPases comprise the Ras superfamily and are involved in essential cell processes such as cell differentiation and proliferation, cytoskeletal organization, vesicle trafficking, and nuclear transport. GTPases are active when bound to GTP and inactive when bound to GDP, allowing their activity to be regulated by GEFs and the opposing GTPase activating proteins (GAPs).

Holoenzyme: An active, complex enzyme consisting of an apoenzyme and a cofactor. An apoenzyme is the protein component of an enzyme, to which the cofactor, a nonproteinaceous organic component, attaches to forming the holoenzyme.

Hydrolysis: When a molecule of a target substance is split into two parts by the addition of a molecule of water. One fragment of the target molecule gains a hydrogen ion ($H^+$) while the other fragment collects the hydroxyl group ($OH^-$).

Ionophore: An ionophore is a lipid-soluble molecule which transports ions across the lipid bilayer of the cell membrane.

late LTP: LTP induced by stronger multi tetanic stimulation protocols, which persist for many hours in hippocampal slices and is dependent on new protein synthesis in addition to PKA.

MAGUKs: Membrane-associated guanylate kinases (MAGUKs) form a family of scaffolding proteins, engaged in the organisation of multiprotein complexes. Guanylate kinases are phosphotransferase enzymes that catalyzes the interconversion of adenine nucleotides.

Methylation: Methylation denotes the addition of a methyl group to a substrate by enzymes. It´s involved in regulation of gene expression, protein function, and RNA processing.

Moiety: A part of a molecule that may include either whole functional groups or parts of functional groups as substructures.

Miniature EPSPs: The release of neurotransmitter vesicles from the presynaptic cell is probabilistic. Even without stimulation of the presynaptic cell, a single vesicle will occasionally be released into the synapse, generating miniature EPSPs.

Muscarinic cholinergic system: Composed by acetylcholine receptors activated by acetylcholine which form G protein-receptor complexes in the plasma membrane of cells, including neurons.

NMDA: N-Methyl-D-aspartate, binds to NMDA receptors, which belong to the glutamate receptor family and are ligand-gated ion channels permeable to $Ca^{2+}$ and $Na^{+}$ ions.

Principal cells of the hippocampus: Pyramidal neurons from CA3 and CA1 and granule cells from dentate gyrus.

Serine-threonine residues: Kinases and posphatases phosphorylate and dephosphorylate proteins at serine and threonine residues.