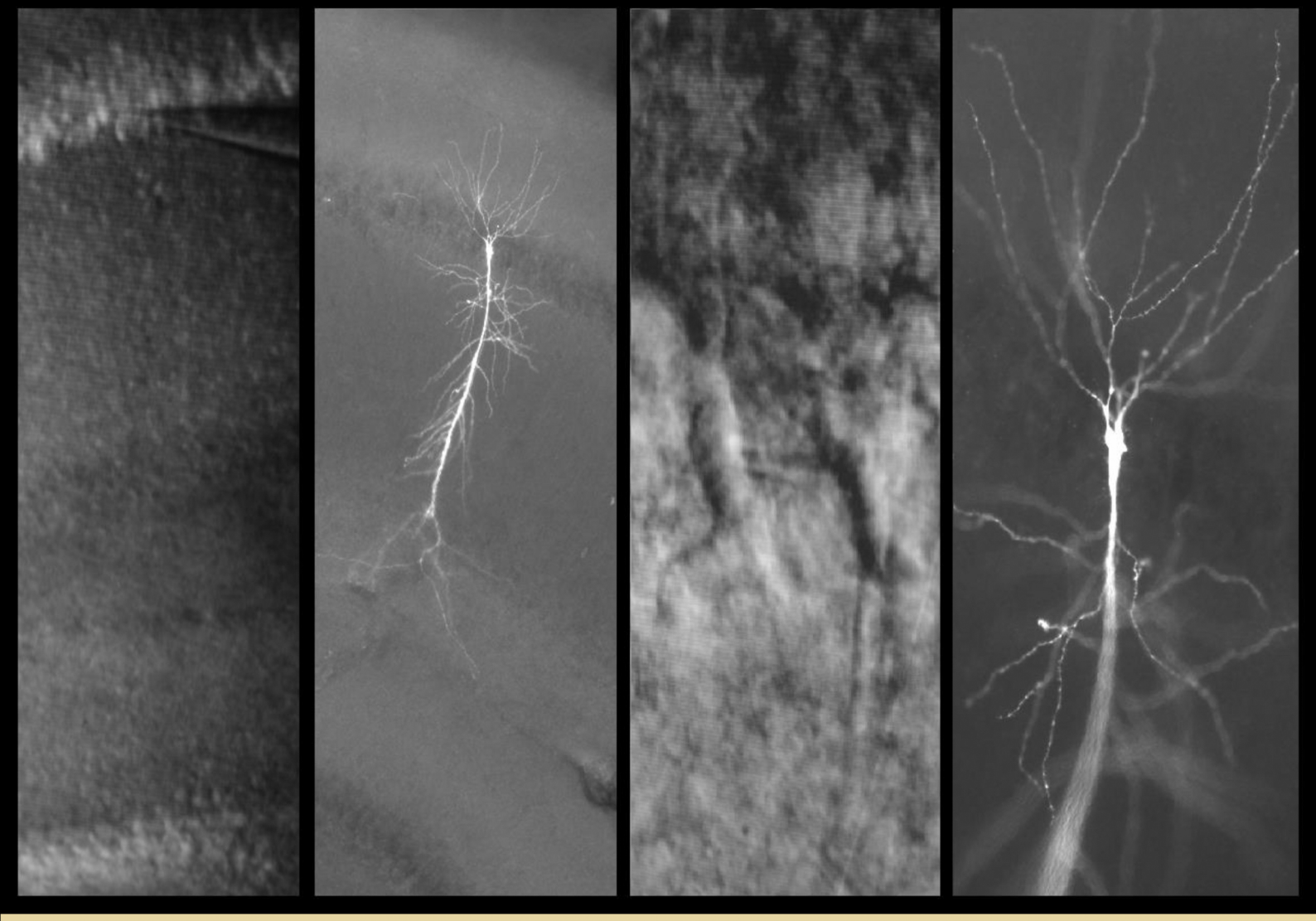

Ana Margarida Cardoso Henriques

MODULATION OF NMDA RECEPTOR CURRENTS BY ADENOSINE A_{2A} RECEPTORS IN THE SCHAFFER COLLATERALS-CA1 SYNAPSES

Dissertação de Mestrado em Biologia Celular e Molecular, Área de Especialização em Neurobiologia, orientada pela Doutora Samira Ferreira e pelo Professor Doutor Ângelo Tomé e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Universidade de Coimbra

Modulation of NMDA receptor currents by adenosine A_{2A} receptors in the Schaffer collaterals-CA1 synapses

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Master in Cellular and Molecular Biology

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Resumo

A adenosina é um neuromodulador capaz de controlar o balanço entre excitação e inibição ao longo de todo o sistema nervoso central. Este controlo é feito através da activação da subfamília de receptores acoplados a proteínas G (GPCRs), os receptores de adenosina $P1$: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R.

O receptor de adenosina do tipo 2A (A_{2A}) é abundantemente expresso no estriado e é menos expresso noutras regiões do cérebro. Uma destas regiões é o hippocampo, uma região cerebral central envolvida nos processos de aprendizagem, memória e reconhecimento espacial. Foi descoberto que os A_{2A} podem modular a memória de referência. Além disso, os A_{2A} estão maioritariamente descritos como facilitadores da transmissão sináptica, sendo que as suas principais funções são o refinamento de outros sistemas neuromodulatórios, o controlo da libertação de neurotransmissores e ainda a modulação da actividade de outros receptores, quer metabotrópicos quer ionotrópicos.

O receptor de NMDA (NMDAR) é um receptor ionotrópico com o qual o A_{2A} pode interagir. Deste modo, dada a particular importância do NMDAR para o fenómeno de neurotransmissão no hippocampo, este trabalho explora de que forma é que os A_{2A} modulam as correntes dependentes de NMDAR nas sinapses entre os neurónios piramidais do CA1 e as colaterais de Schaffer (SC) no hipocampo.

Mostrou-se que a activação exógena do A_{2A} com o seu agonista selectivo CGS21680 diminui as correntes excitatórias pós-sinápticas evocadas (eEPSC) dependentes do receptor de NMDA, um efeito que deixa de ser observado na presença do antagonista selectivo dos A_{2A}, SCH58261. Além disso, SCH58261 diminui a amplitude dos eEPSCs em condições basais o que sugere que os A_{2A} poderão exercer um controlo tónico destas correntes nos neurónios piramidais do CA1. Finalmente, com este trabalho observou-se ainda, que as preparações *in vitro* utilizadas têm uma grande quantidade de adenosina endógena. A sua remoção, porém, não previne o efeito induzido pelo antagonista selectivo dos A_{2A}, continuando a observar-se uma diminuição na amplitude de eEPSC dependentes de NMDAR nas sinapses CA1-SC.

A principal hipótese para explicar esta aparente contradição de resultados tem a ver com a possível existência de duas sub-populações de A_{2A} que podem afectar as correntes dependentes de NMDAR de maneira oposta. Contudo, o estudo de qual a população de A_{2A} que é responsável por cada um dos efeitos, assim como quais os mecanismos por detrás desta evidente modulação dos A_{2A} sobre os NMDAR permanece por clarificsr, o que poderá servir de basede trabalho futuro.

Keywords: Adenosine; Receptor de adenosine A_{2A}; Hipocampo; Receptor de NMDA.

Abstract

Adenosine is a neuromodulator able to control the balance between neuronal excitation and inhibition, throughout the central nervous system (CNS). Adenosine acts onto a G protein–coupled receptors (GPCRs) subfamily called $P1$ adenosine receptors: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R.

Adenosine type 2A receptor ($A_{2A}R$) is highly expressed in striatum, whereas in other brain regions $A_{2A}R$ is expressed at lower levels. One of these regions is the hippocampus, a central brain region enrolled in learning and memory processes as well as spatial recognition. Moreover, it was discovered that $A_{2A}R$ modulate reference memory. Additionally, $A_{2A}R$ can facilitate synaptic transmission since it can fine-tune other neuromodulatory systems, by controlling neurotransmitter release and modulating metabotropic or ionotropic receptors.

It was also described that $A_{2A}R$ can interact with NMDA receptor (NMDAR), that is an ionotropic receptor. Therefore, given the particular importance of NMDAR in the hippocampal neurotransmission phenomena, in this work, we explore how $A_{2A}R$ can modulate NMDAR-dependent currents in hippocampal Schaffer collaterals - CA1 synapses.

We showed that exogenous activation of $A_{2A}R$, with its selective agonist CGS21680, decreases NMDAR-dependent evoked excitatory postsynaptic currents (eEPSCs) and that this effect is no longer observed in the presence of the selective $A_{2A}R$ antagonist, SCH58261. However, the superfusion of SCH58261 alone also decreases NMDAR-eEPSCs, suggesting that $A_{2A}R$ may exert a tonic control of these currents in CA1 pyramidal cells. Finally, this study revealed that our slice preparations contained high levels of endogenous adenosine which, once removed, does not prevent the $A_{2A}R$ antagonist-induced decrease in NMDAR-eEPCSs in SC-CA1 synapses.

Our main hypothesis to explain these results is based in the possibility of existing two sub-populations of $A_{2A}R$ that may have antagonistic effects upon NMDA-dependent currents. Nevertheless, this hypothesis and mechanisms remain to be clarified, which can be the foundation for future work.

Keywords: Adenosine receptors; Adenosine 2A receptors; Hippocampus; NMDA receptors.

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Chapter 1

State of the Art

1.1 Adenosine

1.1.1 Metabolic dynamics of adenosine in the brain

The purine nucleoside adenosine is a molecule released by nearly all cells, since it constitutes the backbone of the ATP molecule (reviewed in Borea et al., 2016), the key energetic source. Adenosine does not accumulate in vesicles and it is not released from nerve terminals upon calcium influx after neuron activation; thus, adenosine is not a classical neurotransmitter. Instead, it is considered a neuromodulator as this molecule controls the flow of information between neurons and glia instead of directly transferring information between neurons, as classical neurotransmitters (reviewed in Cunha, 2005). In fact, adenosine plays such a modulatory role upon a wide variety of systems and situations because it affects the excitability of neurons that release neurotransmitters, such as glutamate, GABA, acetylcholine and dopamine (Arrigoni et al., 2006; Borycz et al., 2007; Ciruela et al., 2006a; Floran et al., 2002; Jeong et al., 2003; Marchi et al., 2002; Quarta et al., 2004; Tanase et al., 2003; Van Dort et al., 2009).

The formation of adenosine is strictly dependent on the metabolic state of cells and adenosine is accumulated in the neurons as a consequence of high metabolic demand. Intracellularly, adenosine formation is dependent on AMP hydrolysis by an intracellular 5-nucleotidase or by S-adenosyl-homocysteine hydrolysis (SAH) (reviewed in Borea et al., 2016). In the extracellular space, adenosine is originated from ATP breakdown (which is released together with some neurotransmitters, but also from other non-vesicular fonts, reviewed in Fields, 2011) by ectoenzymes, including CD39 (ecto-nucleoside triphosphate diphosphohydrolase), which performs the conversion of ATP into ADP/AMP, and CD73 (ecto-5'-nucleotidase), which converts AMP into adenosine (reviewed by Cunha, 2001; Fredholm et al., 2007 and Zimmermann, 2000). The mechanism of extracellular adenosine formation were proved to be essential for adenosine-mediated effects (reviewed in Cunha, 2016).

Adenosine crosses the plasma membrane through bi-directional nucleoside transporters (Ferré et al., 2005) such as the equilibrative nucleoside transporters (ENTs). ENTs transport adenosine according to its concentration gradient (reviewed by Thorn and Jarvis, 1996).

A detailed explanation of the adenosine metabolism is depicted in Figure 1.1.

Normally, the extracellular concentration of adenosine is around the low nanomolar range (10 and 30 nM, reviewed in Sperlágh and Sylvester Vizi, 2011), although a single stimulation pulse at Schaffer collaterals fibers, in the hippocampus, is capable of inducing an increase of adenosine synaptic levels, which are rapidly normalized by adenosine uptake and/or deamination (Mitchell et al., 1993). However, in conditions involving increased metabolic demand and/or lack of oxygen, the levels of extracellular adenosine reach much higher concentrations (during hypoxia or ischemia, adenosine concentrations can reach 20 and

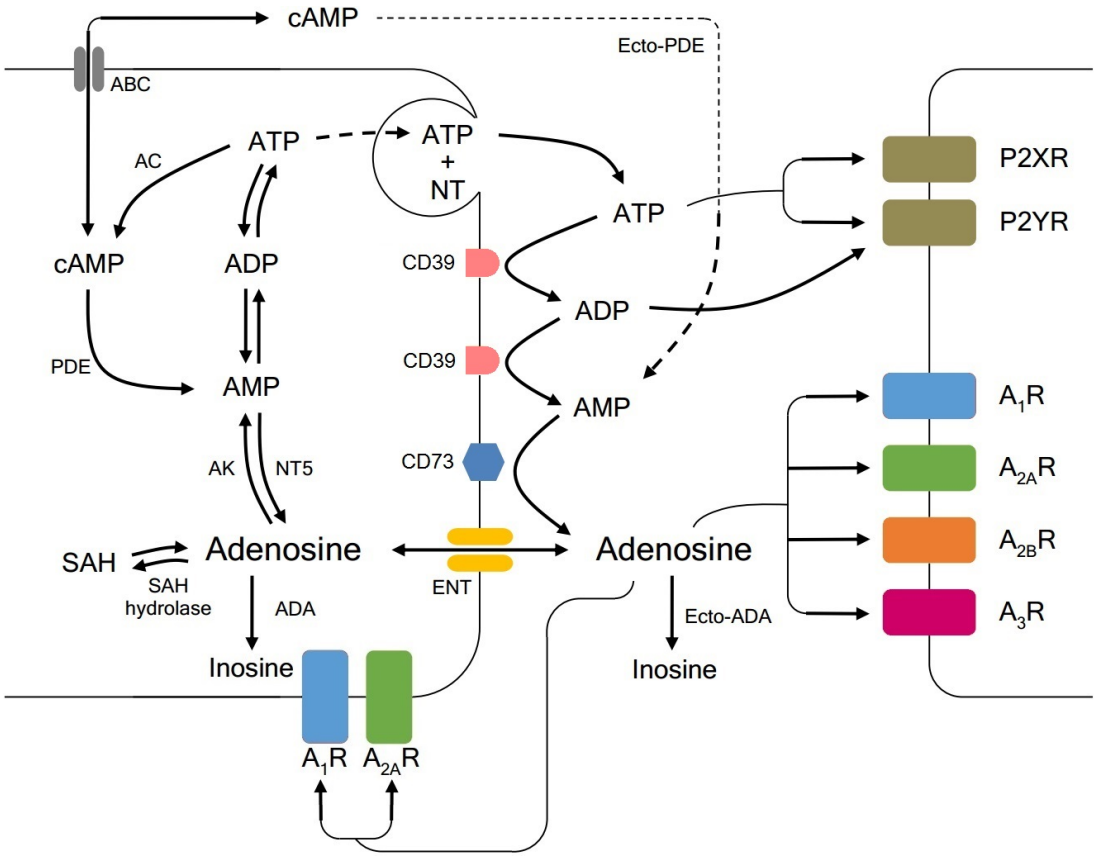

Figure 1.1 **Schematic representation of intra and extracellular adenosine metabolism.** Adenosine can be synthesized intracellularly by the dephosphorylation of AMP. In the extracellular medium, adenosine can also be generated from extracellular cAMP (through the action of phosphodiesterases, PDE) or by the ATP released together with neurotransmitters, or from other fonts, through the action of the enzymes CD39 and CD73. The extracellular adenosine concentrations are mainly regulated by bi-directional transport through equilibrative nucleoside transporters (ENTs), which under physiological conditions usually do the uptake of adenosine. Once in the extracellular space, adenosine acts through four types of metabotropic receptors (named P1Rs: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) while ATP and ADP exert their actions through both ionotropic and metabotropic receptors (named P2Rs: P2X and P2Y). Elimination of adenosine occurs intracellularly, mainly through phosphorylation to AMP by adenosine kinase (AK) and also by the hydrolytic deamination into adenosine catalyzed by adenosine deamninase (ADA), adapted from Rombo, 2015.

30 μM, respectively, in the extracellular space of the hippocampus - reviewed in Sperlágh and Sylvester Vizi, 2011). Therefore, adenosine may be considered an additional marker for unfavorable metabolic conditions such as epilepsy, ischemia, pain and inflammation (reviewed in Borea et al., 2015 and Cunha, 2001).

Due to the influence of adenosine on neuronal transmission and its rapid metabolism, adenosine life-time is highly regulated (reviewed in Borea et al., 2015). Extracellularly, adenosine levels are partially maintained by ENT that, usually mediate the reuptake of adenosine, however, this uptake is thought to be mainly performed by astrocytes since both adenosine kinase and deaminase (ADK and ADA, respectively), the enzymes responsible for the elimination of adenosine, are mainly expressed in these cells (reviewed in Krügel, 2015). Thus, once inside the cell, adenosine is phosphorylated to AMP by ADK or irreversibly deaminated to inosine and ammonia by ADA, which is also found at the extracellular space (reviewed in Sperlágh and Sylvester Vizi, 2011).

Although the synthesis, release and extracellular levels of ATP and adenosine are tightly coupled, these two molecules integrate different signaling systems, which is thought to be intimately linked to the dynamic control of the extracellular levels of these purines (reviewed in Cunha, 2016). The actions of ATP are mediated by P2 nucleotide receptors, which can be subdivided into ionotropic P2X and metabotropic G protein–coupled P2Y receptor subfamilies, whereas adenosine acts through P1 adenosine receptors which integrate the G protein–coupled receptors (GPCRs) family.

1.1.2 Adenosine Receptors and their main characteristics

Adenosine is able to bind to four different subtypes of GPCRs: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R (reviewed in Sperlagh et al., 2012), represented in Figure 1.2.

Adenosine receptors have been implicated in several key physiological processes, ranging from neuromodulation to immune regulation, and from vascular function to metabolic control. The main differences among adenosine receptors are:

***i)* Structure:** $A_{2A}R$ is the biggest adenosine receptor with 412 amino acids and a molecular mass of 44.7 kDa; this size is due to its long carboxyl terminal, which allow a variety of interactions with different proteins and different patterns of phosphorylation. The A_1R has 326 amino acids and a molecular mass of 36.5 kDa. The $A_{2B}R$ has a lower molecular

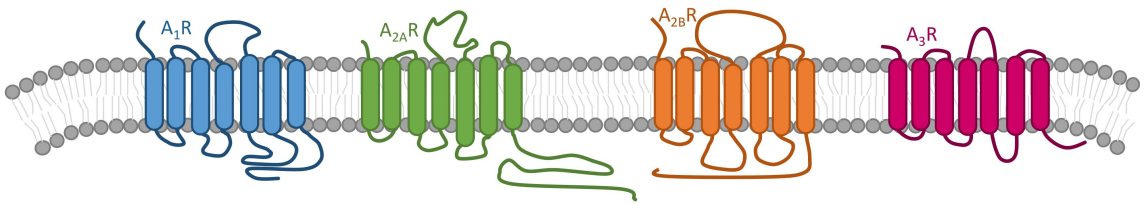

Figure 1.2 **The family of adenosine receptors.** The receptors structures hereby represented were criteriously design attending to the crystallography structures of adenosine receptors: A_1R (van Galen et al., 1992), $A_{2A}R$ (Carpenter et al., 2016; van Galen et al., 1992), $A_{2B}R$ (Feoktistov and Biaggioni, 1997) and A_3R (Almerico et al., 2013).

mass than A_1R, 36.3 kDa, but it has a larger amino acid chain with 332 residues. A_3R is the smaller adenosine receptor with 318 amino acids and a molecular mass of 36.2 kDa (reviewed in Abracchio and Williams, 2013).

***ii)* Affinity for adenosine:** in humans, A_1R, $A_{2A}R$ and A_3R display high to moderate affinity (K_i = 100, 310, and 290 nM, respectively) whereas $A_{2B}R$ has a low affinity ($K_i = 15\ \mu M$). In rodents the affinity is a bit higher: A_1R and $A_{2A}R$ have higher affinity for adenosine ($K_i = 73, 150\ nM$, respectively) than $A_{2B}R$ and A_3R ($K_i = 5100, 6500\ nM$, respectively) (reviewed in Müller and Jacobson, 2011);

***iii)* G-protein type recruited:** A_1R and A_3R preferentially couple to $G_{i/o}$ proteins and inhibit the activity of adenylyl cyclase (reviewed by Hill et al., 2014) which, consequently, causes a decrease in excitatory neurotransmission, both pre- and postsynaptically (Fredholm and Altiok, 1994). However, depending on agonist binding, some studies have claimed a possible interaction of A_1R with $G_{q/11}$ and also with G_s (Cordeaux et al., 2000, 2004). In addition, A_3R can also stimulate PLC via $G_{q/11}$ proteins (reviewed by Fredholm et al., 2001). On the other hand, $A_{2A}R$ and $A_{2B}R$ couple preferentially to $G_{s/olf}$ and G_s proteins, respectively, and stimulate cyclic AMP formation (reviewed by Hill et al., 2014), leading to increased synaptic transmission (Krügel, 2015), although some cases reported that $A_{2A}R$ may inhibit synaptic transmission via the PLC pathway (Wirkner et al., 2000, 2004) and that $A_{2B}R$ may also couple to $G_{q/11}$ (reviewed by Fredholm et al., 2001);

***iii)* Receptor distribution:** The expression of adenosine receptors in the central nervous system (CNS) is region-specific (Ribeiro et al., 2002) and it changes along brain development (Castillo et al., 2009; Shaw et al., 1986). A_1R are the most abundant and widespread adenosine receptors, being highly expressed in the cortex, cerebellum, hippocampus, and dorsal horn of spinal cord (Ribeiro et al., 2002). The $A_{2A}R$ are highly expressed in the striatopallidal GABAergic neurons and olfactory bulb, but also in the hippocampus and other brain regions, however, in less abundance than in the striatum (reviewed in Sebastiao and Ribeiro, 2009). $A_{2B}R$ are expressed in low levels in the brain (Dixon et al., 1996), and the level of expression for the A_3R is apparently moderate in the cerebellum and hippocampus but low in most other areas of the brain (Fredholm et al., 2001).

In conclusion, adenosine receptors are distributed along several areas of the central nervous system (CNS), playing different roles in neurotransmission. The hippocampal formation is a central brain region for learning and memory and therefore the mechanisms of synaptic plasticity thought to underlie these phenomena have been mostly studied in this structure. In addition adenosine receptors have been implicated in such hippocampal-related functions (Kaster et al., 2015; Li et al., 2015; Machado et al., 2017), so, it is of interest to continue to explore the role of adenosine receptors in hippocampal formation.

1.2 The Hippocampal Formation and its circuitries

The Hippocampal Formation is constituted by two major divisions: the hippocampus proper, which includes the dentate gyrus (DG) and the Ammon's horn, or *Cornus Ammonis*

from the Latin, composed by CA1, CA2 and CA3 fields, and the retrohippocampal region constituted by the subiculum, presubiculum/postsubiculum, parasubiculum and finally the entorhinal cortex (EC) (reviewed in Cenquizca and Swanson, 2007 and Li et al., 2009). In general, neocortical areas send projections onto the retrohippocampal areas that surround the hippocampus. Then, neurons from the retrohippocampal regions project their axons to the different subdivisions of the hippocampus allowing the flow of information (Dickerson and Eichenbaum, 2010).

The exact functioning of this network is still the focus of much research but its relevance for to short-term episodic or declarative memory or to the elaboration of a cognitive map of the surrounding environment is well established (reviewed in Paxinos, 2014 and Tonegawa and McHugh, 2008).

A major characteristic of this brain region is the unidirectionality of its excitatory circuits which links each region of the hippocampal formation (Andersen et al., 1966a,b), as represented in Figure 1.3. However some authors defend the possibility of bidirectionality in the hippocampus circuit (Andersen, 2007), although there is scarce evidence supporting this hypothesis.

The CA1 neuronal population is one of the most studied by neuroscientists since there is a low level of heterogeneity and the CA1 pyramidal neurons are well organized and easy

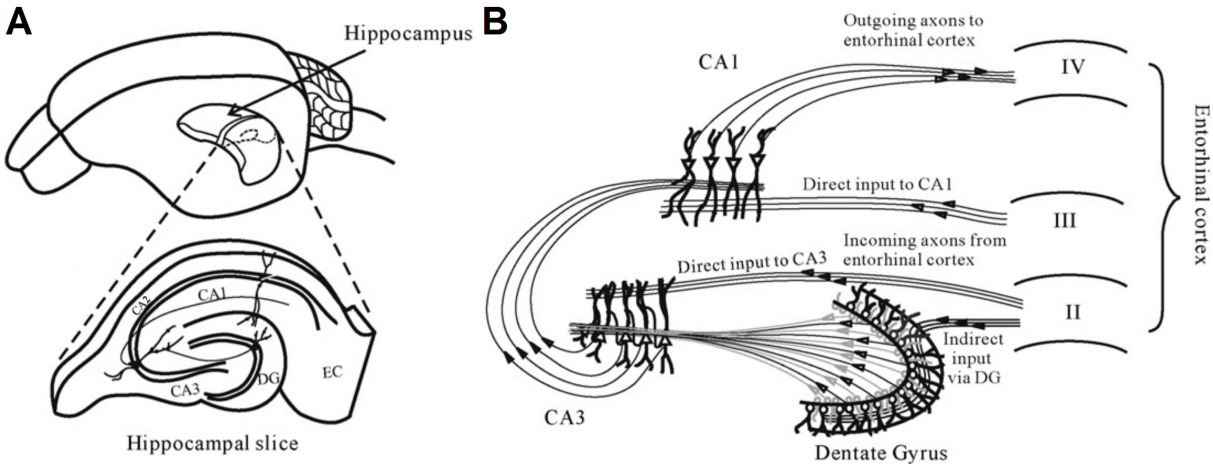

Figure 1.3 **Localization of the hippocampus in the brain and the general layout of the trisynaptic loop. A** – Orientation of the hippocampus in the rat brain showing an hippocampal slice as it would appear after slicing in transverse plain. Each slice includes a portion of the entorhinal cortex (EC), the dentate gyrus (DG), the field CA3 (CA3), CA2 and CA1. Thin lines represent axonal projections. **B** – The trisynaptic loop includes projections from the EC layer II to the DG and to CA3. These projections can be further subdivided into medial and lateral perforant pathways. EC layer III projects to the distal dendrites of pyramidal neurons in CA3. The axons of the DG granule neurons (both mature and immature) stream to the proximal dendrites of CA3. Immature neurons (grey) are typically lined along the inner border of the granule cell layer. The CA3 neurons send axons to CA1 (Schaffer collaterals) and the CA1 neurons send axons to layer IV of the EC. The direction of electrical impulses is shown by the small arrowheads on the axons (adapted from Wojtowicz, 2012).

to identify (reviewed by Szilagyi et al., 2011). Besides, the CA1 hippocampal field is the main output region of the hippocampus and it is part of three different circuits within the hippocampal formation: The trisynaptic circuit $ECII \rightarrow DG \rightarrow CA3 \rightarrow CA1$ and the monosynaptic circuits $ECIII \rightarrow CA1$ and $CA2 \rightarrow CA1$, which then project back to the entorhinal cortex (Dvorak-Carbone and Schuman, 1999).

The most intensely studied is the trisynaptic circuit where the dentate gyrus receives inputs from ECII neurons, via the perforant path (PP), and then these neurons project their axons to the CA3 pyramidal cells, via the mossy fibers (Mf) pathway. Then, through the Schaffer collaterals (SC) fibers, constituted by the axons of the CA3 neurons, the CA1 pyramidal neurons receive their major excitatory input; finally the flow information is then sent back to the entorhinal cortex (Wojtowicz, 2012). In 1966, Andersen et al., 1966c concluded that the stimulation of the perforant path, that comes from ECIII neurons to CA1 pyramidal cells, did not induce firing of the CA1 but only evoked small monosynaptic responses (Andersen et al., 1966c). Consequently this publication led to the hypothesis that the trisynaptic pathway was the major intrinsic pathway of neurotransmission through the hippocampus and, in fact, in 1991 this circuitry was considered the anatomical structure for memory formation (Marr et al., 1991). However, later on, some studies have shown that the monosynaptic excitation of CA1 pyramidal neurons by the entorhinal cortex may contribute to the firing of place cells (Colbert and Levy, 1992; Segal, 1972). The relevance of this pathway is also supported by other evidences suggesting that the trisynaptic circuit is not necessary for some kinds of spatial memory (Jarrard, 1995; Jarrard et al., 1984) and that the inputs from the PP are crucial for temporal association memory (Suh et al., 2011).

The pyramidal cells from CA1 have the typical aspect depicted in Figure 1.4. In these neurons, the proximal portion of the apical dendrites receive preferential input from GABAergic interneurons, while the distal portions receive increasing densities of excitatory inputs (Papp et al., 2001).

The trisynaptic circuit has diffusely widespread connections (Ishizuka et al., 1990) although the basal dendrites and the proximal part of the apical dendrites (*stratum radiatum*) are well defined targets for the trisynaptic path. On the other hand, the monosynaptic circuit constituted, almost exclusively, by the projections from ECIII neurons to the CA1, is organized in a topographical way, forming almost one-to-one connection and targeting the distal part of apical dendrites (*stratum lacunosum moleculare*)(Ishizuka et al., 1990; Li et al., 1994). Moreover, the majority of the entorhinal terminals make asymmetrical synaptic contacts with both dendritic spines (93%) and dendritic shafts (7%) which presumes that the perforant path projection to CA1 provides mainly excitatory input as well as a minor feed-forward inhibitory input onto these neurons (reviewed in Paxinos, 2014). However, ECIII neurons also synapse onto the inhibitory basket and chandelier cells of CA1 (Kiss et al., 1996) and are likely to innervate the interneurons of *stratum lacunosum moleculare* (Lacaille and Schwartzkroin, 1988; Vida et al., 1998). Therefore, these synapses exhibit both long-lasting depression (Dvorak-Carbone and Schuman, 1999) and potentiation, which indicates that it is possible to occur bidirectional synaptic modifications (Remondes and Schuman, 2002).

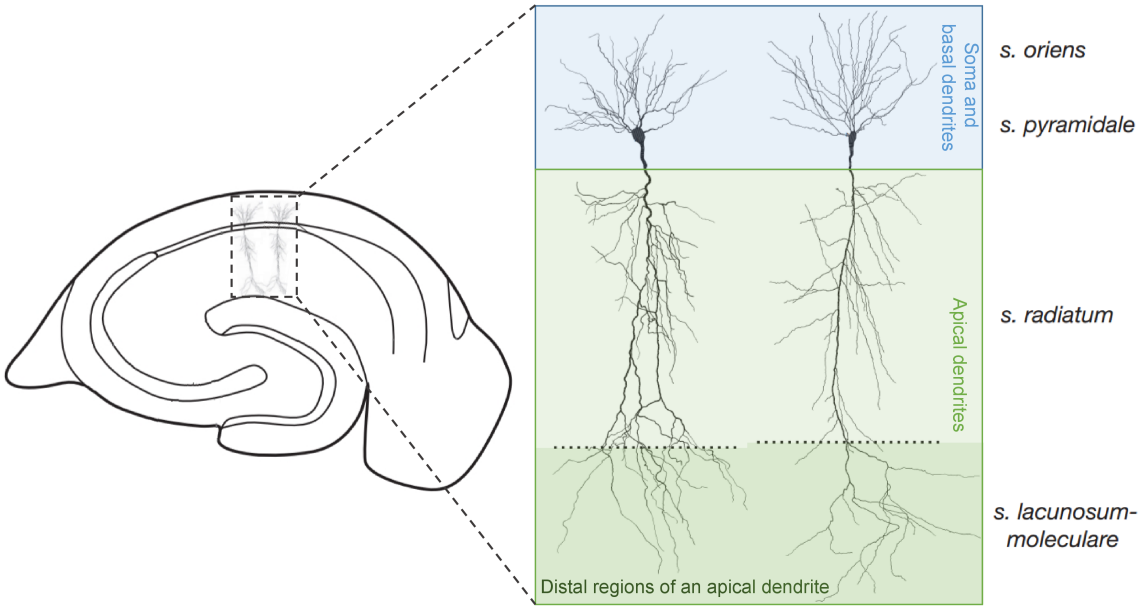

Figure 1.4 **Morphology of CA1 pyramidal neurons of 5-7 week-old Sprague–Dawley rats with representative reconstructed neurons.** Dotted lines represent the borders between *stratum radiatum* and *stratum lacunosum-moleculare* for each cell, adapted from Routh et al., 2009 and from Romand et al., 2011. The dendritic trees of CA1 pyramidal cells have 47% of the dendritic length located in the s*tratum radiatum*, 34% in the *stratum oriens* and 18% in the *stratum lacunosummoleculare* (reviewed in Paxinos, 2014).

Although both the trisynaptic and the monosynaptic pathways converge onto the same neuron, they synapse with different parts of these neurons, which differently influence the local circuitry implicating different electrophysiological responses by CA1 neurons (Colbert and Levy, 1992; Empson and Heinemann, 1995; Otmakhova et al., 2002; Remondes and Schuman, 2002).

Figure 1.5 summarizes the inputs to CA1 pyramidal cells from other CA1 hippocampal cells, from neurons that belong to different hippocampal fields and also from cells in other brain regions.

Concerning the monosynaptic interaction in CA1 neurons (achieved through the excision of CA3 and of the DG), Colbert and Levy, 1992 have shown that stimulation of the perforant path and of the Schaffer collaterals activated distinct populations of synapses. The stimulation of the PP evoked population excitatory postsynaptic potentials (pEPSPs) only in the *stratum lacunosum moleculare*, whereas stimulation of the SC evoked pEPSPs in the *stratum radiatum*.

Also Otmakhova et al., 2002, have shown that the outward NMDAR-mediated current resulting from the stimulation of the perforant path with the postsynaptic neuron depolarized to $+60\ mV$ was approximately six times smaller than the current resulting from the stimulation of the SC, which was not entirely related to the distance between both afferents to the target cells. In fact, the two CA1 pathways have different functional roles (reviewed in Lisman and Otmakhova, 2001) and are differentially controlled by neuromodulators including adenosine (Hasselmo and Schnell, 1994; Lee et al., 1983; Otmakhova and Lisman, 1999), which implicates

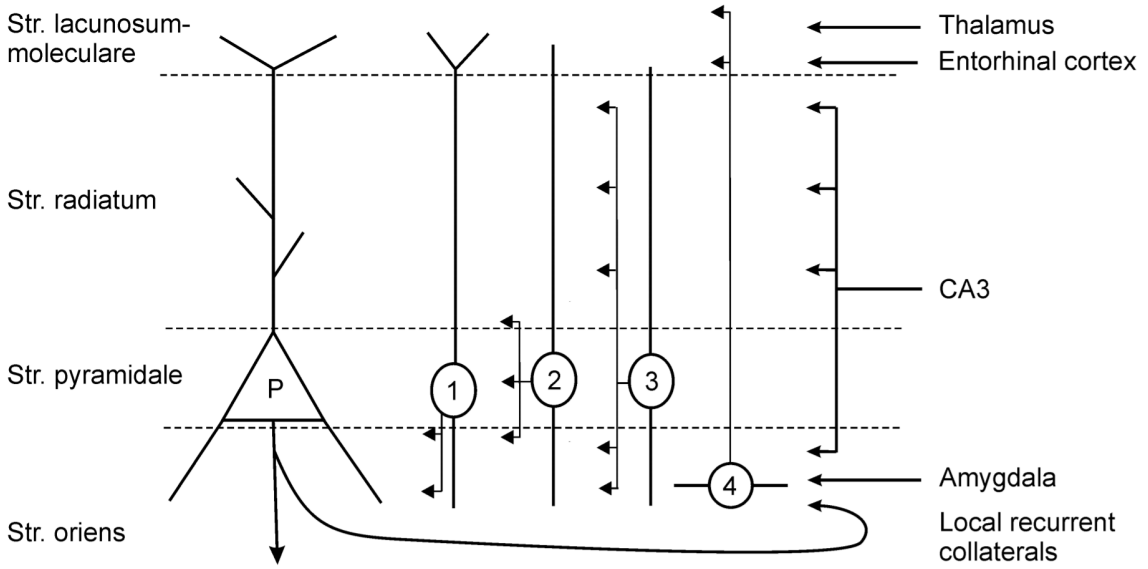

Figure 1.5 **Innervation of pyramidal cells in the hippocampal CA1 area.** The most important glutamatergic inputs to the CA1 pyramidal neurons and their laminar alignment is indicated in the right. In addition, the main interneurons present at this region (1-4) are also represented. The interneuron dendrites are drawn with thick lines and the axons with thin lines. The main synaptic contacts are indicated by arrows. P: Pyramidal cell, 1: Axo-axonic cell, 2: Basket cell, 3: Bistratified cell, 4: O-LM cell (adpted from Szilagyi et al., 2011).

slight physiological differences. In particular, Lee et al., 1983 have shown that the apical dendritic region of CA1 has a differential distribution of A_1R between the *stratum radiatum* (high density of A_1R) and *stratum lacunosum/moleculare* (low density of A_1R), based on the effect of adenosine deaminase substrate. In contrast, Cunha et al., 1994a shows that the density of binding of [3H]CGS21680, an agonist of $A_{2A}R$ was greatest for the *stratum radiatum* of CA1 pyramidal neurons followed by the same region of CA3 field.

Thus the entorhinal cortex connects the hippocampal formation to a variety of multimodal associative areas of the cortex such as the parietal, the temporal, and the prefrontal. In addition, the EC neuros also contain $A_{2A}R$ (Cunha et al., 1994a). Moreover, functionally, the EC complements the hippocampal formation, although the specific functional contributions of the EC to memory remain to be established (reviewed in Canto et al., 2008).

Recently, a new monosynaptic circuit from CA2 to CA1 neurons has been proposed to be enrolled in this interaction since CA2 also receives inputs from the ECII and ECIII (Kohara et al., 2014).

The main outputs of CA1 go to the layer V of the EC, either directly or indirectly through the subiculum (Yau et al., 2015), although intrinsic CA1 innervation is also possible.

Given the important role that the indirect projections from the EC to the CA1, through the CA3 neurons, play in the rapid tuning of the CA1 (and of the CA3) place fields when an animal faces a novel environment (Cheng and Frank, 2008), it would not be wise to ignore the CA3 contribute in this analysis. The CA3 neurons are involved in the following circuits: $ECII \rightarrow Dentate\ gyrus \rightarrow CA3$, through both perforant path and mossy fibers, respectively

and $ECII \rightarrow CA3$, through the perforant path only. Interestingly, CA3 pyramidal neurons may also receive recurrent collateral inputs from other CA3 cells. Previous studies have pointed these two CA3 inputs as important for spatial learning as well (Hagena and Manahan-Vaughan, 2011; Kesner and Warthen, 2010; Neunuebel and Knierim, 2014). Moreover, also the CA3 pyramidal cells are endowed with $A_{2A}R$, which have an important modulatory role onto these neurons. (Cunha et al., 1994a; Rebola et al., 2005, 2008)

As main outputs, the CA3 pyramidal neurons project to the CA1 neurons through Schaffer collaterals fibers and recurrent collaterals from other CA3 cells. In addition, CA3 project back to the granule cells of the DG, through the so-called associational–commissural (AC)–CA3 synapses (Reviewed by Kesner and P, 2007, and Andersen, 2007; Hagena and Manahan-Vaughan, 2015; Yau et al., 2015).

In suma, the hippocampus is responsible for the formation of long-term memories and spatial navigation, having an extraordinary organization of neuronal pathways. In this work, the particular CA1 field of hippocampus will be region of interest and besides several studies about adenosine receptors have been primarily made in the striatum, the hippocampus has also emerge as a region of interest to study the role of adenosine receptors in neurotransmission and neuroplasticity.

1.3 Adenosine A_{2A} Receptor

From all adenosine receptors the A_1R and $A_{2A}R$ are the ones that benefit of a larger number of pharmacological tools, allowing a controlled and selective manipulation. Due to their wide distribution along the CNS, comparing to other adenosine receptors (reviewed in Gomes et al., 2011), A_1R and $A_{2A}R$ are the most explored and studied adenosine receptors.

Consequently, it is almost impossible to mention one without mentioning the other. Nevertheless, the main focus of this dissertation are the $A_{2A}R$, since its role on synaptic transmission is poorly explored.

1.3.1 Distribution and localization of $A_{2A}R$ in the brain

$A_{2A}R$ are in neurons and in non-neuronal cells. In non-neuronal cells they are found in astrocytes (Nishizaki et al., 2002), in microglia (reviewed in Santiago et al., 2014) and in blood vessels, probably in endothelial cells (Coney and Marshall, 1998). In neuronal cells, $A_{2A}R$ may be found in synaptic (pre- or postsynaptic), perisynaptic or extrasynaptic sites (Schubert et al., 1995).

In the striatum of wistar rats, $A_{2A}R$ are abundantly located outside of the active zone, *i. e.* extrasynaptically, being the rest of $A_{2A}R$ mainly located in the postsynaptic density (Rebola et al., 2005).

In the hippocampus, Rebola et al., 2005 have shown that these receptors were mainly located in the presynaptic active zones, for both pups and 6-8 week-old rats. In the same way, the A_1R are mainly located in the presynaptic active zones of the hippocampus. Later on, Rebola et al., 2008 revealed that in the CA3 field of the hippocampus, $A_{2A}R$ are highly

located in postsynaptic sites. In addition, $A_{2A}R$ are preferentially located in glutamatergic neurons (Rebola et al., 2003; Tetzlaff et al., 1987). In support of the differential $A_{2A}R$ expression in the hippocampus is the case of acetylcholine modulation, in which there is a preponderance of A_1R-mediated modulation by endogenous adenosine in both CA1 and CA3 areas, but in the CA3 there is a relatively higher influence of $A_{2A}R$ than in the CA1 region (Cunha et al., 1994b).

So, both A_1R and $A_{2A}R$ are largely located at synapses (Rebola et al., 2005, 2003; Tetzlaff et al., 1987), with particular abundance in the glutamatergic ones (Ciruela et al., 2006a; Rebola et al., 2005; Tetzlaff et al., 1987). Nevertheless, they are also expressed in GABAergic (Cunha and Ribeiro, 2000; Rombo et al., 2015; Shindou et al., 2002), dopaminergic (Borycz et al., 2007; Garção et al., 2013; Pandolfo et al., 2013), cholinergic (Cunha et al., 1995; Rodrigues et al., 2008), noradrenergic (Barraco et al., 1996; Jackisch et al., 1985) and serotoninergic (Barraco et al., 1996; Okada et al., 1999) synapses. Moreover, A_1R and $A_{2A}R$ were shown to interact and even heteromerize in a way dependent on neuronal activity and on the molecular niches relevant for the production or inactivation of the ligand (reviewed in Sebastiao and Ribeiro, 2009).

1.3.2 Importance of Adenosine A_1 and A_{2A} receptors in neuromodulation

Only A_1R and $A_{2A}R$ have a high affinity for endogenous adenosine, being able to exert functions in a wide range of brain structures (reviewed in Jacobson et al., 1996). Several authors considered that A_1R and $A_{2A}R$ control basal synaptic transmission and control synaptic plasticity, respectively. In fact, $A_{2A}R$ was described as a "hub switching presynaptic modulation from inhibitory to excitatory" (reviewed in Cunha, 2016). Thus, the adenosine neuromodulatory system is balanced between the activation of inhibitory A_1 and of facilitatory A_{2A} receptors (reviewed in Cunha, 2001). The inhibitory or excitatory effects of adenosine may be dependent on adenosine concentration, as shown in CA1 pyramidal cells of the rat hippocampus (Li and Henry, 1998b).

These assumptions rely on several studies that have been published throughout the years, which revealed both independent functions of the two adenosinergic receptors as well as their cooperation in the modulation of synaptic activity in different brain circuits. A particularity that may allow the selective activation of each type of receptor is related with source of extracellular adenosine, a topic that is still far from being well understood (reviewed in Cunha, 2016).

In general, adenosine is able to exert different functions through the activation of $A_{2A}R$, namely fine-tune other neuromodulatory systems, control the release of neurotransmitter release, modulate the function of other receptors which heteromerize with $A_{2A}R$ (Ciruela et al., 2006a,b; Ferré et al., 2002), control receptor desensitization (Chiodi et al., 2016; Dixon et al., 1997; Ferreira et al., 2015; Martire et al., 2011) and modulate the function of ionotropic receptors (reviewed in Cunha, 2016 and Sebastiao and Ribeiro, 2009).

In the basal ganglia, the most proeminent function of $A_{2A}R$ is the postsynaptic control of dopaminergic signaling in striatopallidal neurons (reviewed in Ferré et al., 2003 and Fredholm et al., 2003). In addition, in striatal neurons, the majority of $A_{2A}R$ are located in postsynaptic

density and it has been described its ability to inhibiting NMDAR conductance (Nörenberg et al., 1998) as well as NMDA currents (Wirkner et al., 2000). Nevertheless, some studies have described its ability to modulate the release of neurotransmitters such as GABA, acetylcholine (Kirk and Richardson, 1994) and glutamate (Corsi et al., 2003; Rodrigues et al., 2005).

In contrast, in the hippocampus, the best described function of $A_{2A}R$ is the presynaptic control of neurotransmitters release such as glutamate (Lopes et al., 2002), GABA (Cunha et al., 2000), acetylcholine (Jin and Fredholm, 1996; Rebola et al., 2002) or serotonin (Okada et al., 2001) and in fact presynaptic $A_{2A}R$ has been described to facilitate synaptic transmission. However this does not mean that hippocampal $A_{2A}R$ have no role in postsynaptic neuron, as in fact is demonstrated by, for instance Rebola et al., 2008 and Li and Henry, 1998b, who confirm $A_{2A}R$ ability to control postsynaptic responsiveness.

$A_{2A}R$ are more discrete under basal conditions (or at low frequencies of stimulation) but display a gain of function at high frequencies of stimulation, such as those used to induce synaptic plasticity (Costenla et al., 2011; Cunha et al., 1997; Lupica et al., 1990; Rebola et al., 2008). This likely occurs since neuronal firing induced by stimulation at high frequences favors ATP release from these neurons and the consequent formation of adenosine, which is thought to preferentially activate $A_{2A}R$ (Cunha et al., 1996), possibly due to a favorable distribution of this receptor with synaptic ecto-5-nucleotidase (CD73). On the other hand, A_1R-mediated inhibition seems to become less efficient as the neuronal circuits are recruited (reviewed in Cunha, 2016).

Activation of presynaptic A_1R decreases calcium influx, which consequently influences glutamate release (Ambrósio et al., 1997; Banie and Nicholls, 1993; Wu and Saggau, 1994). In addition, activation of postsynaptic A_1R induces a decrease in the activation of ionotropic receptors and of voltage sensitive Ca^{2+} channels (Chen et al., 2014b; de Mendonça et al., 1995; Klishin et al., 1995), and on the other hand, activates K^+ channels, leading to the hyperpolarization of the postsynaptic neuron (Chung et al., 2009; Greene and Haas, 1991; Kim and Johnston, 2015; Wetherington and Lambert, 2002).

Besides this wide range of functions in neuronal cells, we might consider the role of $A_{2A}R$ in non-neuronal cells, since in astrocytes and microglia $A_{2A}R$ is capable of controlling Na^+/K^+-ATPases, the uptake of glutamate and the release of pro-inflammatory cytokines (Cristóvão-Ferreira et al., 2013; Matos et al., 2013, 2012a,b; Nishizaki et al., 2002; Orr et al., 2015; Rebola et al., 2011).

Given the relevance of the adenosinergic system for cell-to-cell communication, the impairment or the imbalance of this system will cause dysfunction and damage.

1.3.3 Ontogenic expression, distribution and function of the $A_{2A}R$

Adenosine receptors are known to be ontogenically regulated, which means that the expression of these receptors is dependent on development factors. A_1R and $A_{2A}R$ are present since conception until elderly, although with different patterns of distribution. Despite of A_1R and $A_{2A}R$ are found early in developmental time points, the expression and density of A_1R and $A_{2A}R$ increases at birth and continues to increase until 9-15 postnatal days (reviewed by Cunha, 2005). Moreover, the rise in the density of receptors is accompanied for a decrease

in the affinity of the receptor for adenosine, reaching constant values around 25 days of age (Doriat et al., 1996).

Not only the pattern of distribution changes within development as well as the role of these two receptors. In particular to $A_{2A}R$, in an immature brain, the absence of $A_{2A}R$ ($A_{2A}R$ KO mice) prejudices brain damage as contrast with the opposite effect in adulthood where the blockade of $A_{2A}R$ is beneficial. However, this opposite roles may not be due to a change in the function of the receptor itself but rather to an opposite effect of intracellular calcium levels in immature comparing to mature neurons (reviewed by Cunha, 2005).

In addition, the metabolism of adenosine seems to be different in newborn animals (Psarropoulou et al., 1990), or in adult animals, which may also contribute to the observed changes in the function of the $A_{2A}R$ through time (reviewed by Cunha, 2005).

Following adulthood, the ageing process leads to a change in the A_{2A} receptor distribution pattern, being the most outstanding characteristic the increase in $A_{2A}R$ expression (Canas et al., 2009b; Cunha et al., 1995; Rebola et al., 2003), Figure 1.6.

A real fascinating fact is that this increase in $A_{2A}R$ intensity in the limbic and neocortex of aged rats occurs in parallel with a decrease in the density of A_1R (Cunha et al., 1995), as well as a decrease in the ability of A_1R agonist to inhibit synaptic transmission in the hippocampus of aged rats (Sebastiao et al., 2000). In addition the low density and function

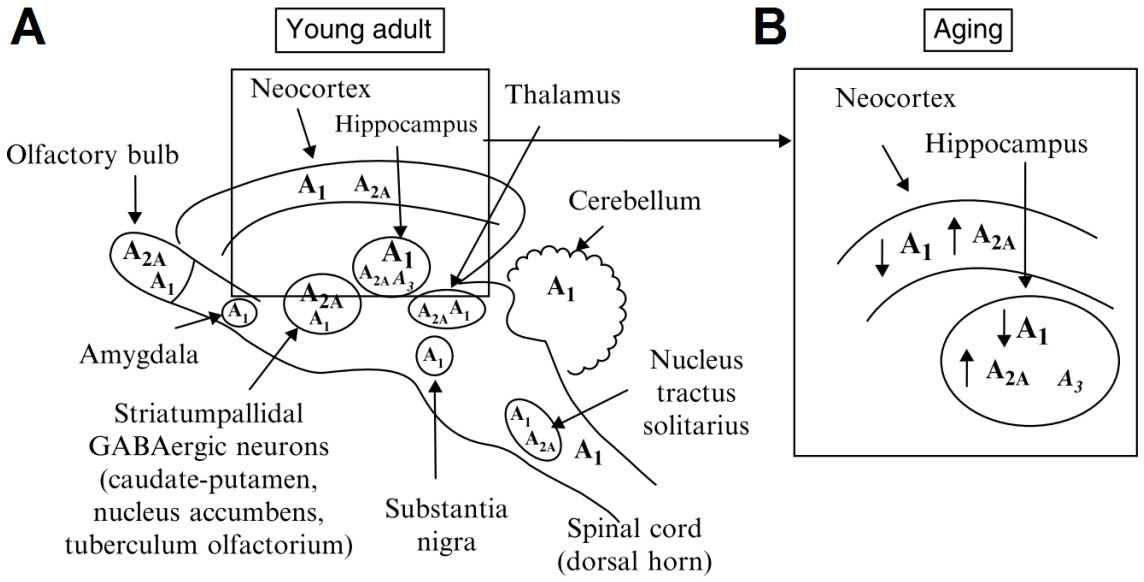

Figure 1.6 **Schematic representation of distribution of Adenosine receptors distribution in the different brain areas together with age-related changes in receptor expression. A** – Illustration of adenosine receptors distribution throughout different brain areas. A bigger size of letter represents greater abundance of that receptor. **B** – illustration of the reported changes in the density of adenosine receptors in the forebrain (including the hippocampus and cortex). In aged rats, the density and functioning of $A_{2A}R$ is increased (upward arrow) in the hippocampus and cortex, whereas the density and functioning of A_1R is decreased (downward arrow). No information, so far, is available for age-related changes in A_{3A} density upon ageing (adapted from Sebastiao and Ribeiro, 2009).

of A_1R may be compensated with higher levels of extracellular adenosine, which keep tonic inhibition high in aged animals (Bauman et al., 1992).

It seems that the role of $A_{2A}R$ in aged rats is rather different from the one in young adults. So, in young adults, this receptor have predominant role in fine-tuning (Sebastiao et al., 2000), being one of the more relevant actions the control of A_1R, whereas in aged rats the activation of $A_{2A}R$ causes a direct facilitation of glutamatergic transmission (Rebola et al., 2003), which constitute a relevant fact for functional A_{2A}-mediated changes. In agreement with this data, there are results showing that this increase ability of A_{2A} to facilitate synaptic transmission is no longer due to PKC-mediated attenuation of A_1R tonic inhibition (Cunha and Ribeiro, 2000; Lopes et al., 2002), but rather to a PKA-mediated direct facilitation of synaptic transmission (Rebola et al., 2003).

1.4 Signaling pathways triggered by A_{2A} Receptor

$A_{2A}R$ are constituted by seven transmembrane subunits. Their long carboxyl terminal allows the binding of several proteins that are able to transmit extracellular signals into the intracellular compartment. One of the most relevant set of these proteins are the heterotrimeric G proteins. The heterotrimeric G proteins is constituted by a guanine nucleotide binding G_α subunit and the dimeric $\beta\gamma$ subunits (reviewed by Pierce et al., 2002). Once activated, the receptor catalyzes the exchange of GTP for GDP in the inactive G_α subunit, which causes a conformational change and dissotiation of the complex. Thus, G_α and $\beta\gamma$ subunits are able to regulate cellular effectors including phospholipases, adenylyl cyclase (AC), and ion channels (Hurowitz et al., 2000). The reverse process, *i.e.*, GTP hydrolysis, which inactivates the G_α subunit, drives re-association of the heterotrimer (Hurowitz et al., 2000).

1.4.1 G-protein-dependent pathways

G-proteins' family are divided into four classes accordingly to sequence homology, gene structure, and regulation of specific effectors: $G_{\alpha s}$ (αs, αolf), $G_{\alpha i}$ ($\alpha i1 - \alpha i3$, αt, $\alpha o1 - \alpha o2$, $\alpha\zeta$), $G_{\alpha q/11}$ (αq, $\alpha 11$, $\alpha 14 - \alpha 16$) and $G_{\alpha 12}$ ($\alpha 12$, $\alpha 13$) (Hurowitz et al., 2000 and reviewed by Moreira, 2014). The $G_{\alpha s}$ and $G_{\alpha i}$ classes modulate positively and negatively, respectively, the activity of adenylyl cyclase, while $G_{\alpha q/11}$ activates phospholipase Cβ and $G_{\alpha 12/13}$ activates small GTPase's families (reviewed by Kamato et al., 2015).

Adenosine A_{2A} receptors were initially described as being coupled to the G_S/adenylate cyclase/cAMP/protein kinase A pathway (reviewed in Fredholm et al., 2007). However, several studies have shown that $A_{2A}R$ may couple to different transducing systems in different preparations, making it clear that $A_{2A}R$ are pleiotropic receptors (reviewed in Cunha, 2001). The signaling downstream to activation of A_{2A} receptors is complex, and it is difficult to understand which signaling pathways are activated in each brain region in order to explain the variety of A_{2A}-associated functions. So, Figure 1.7 comprises all $A_{2A}R$ associated signaling pathways that are dependent on G-proteins described, until now, in the literature.

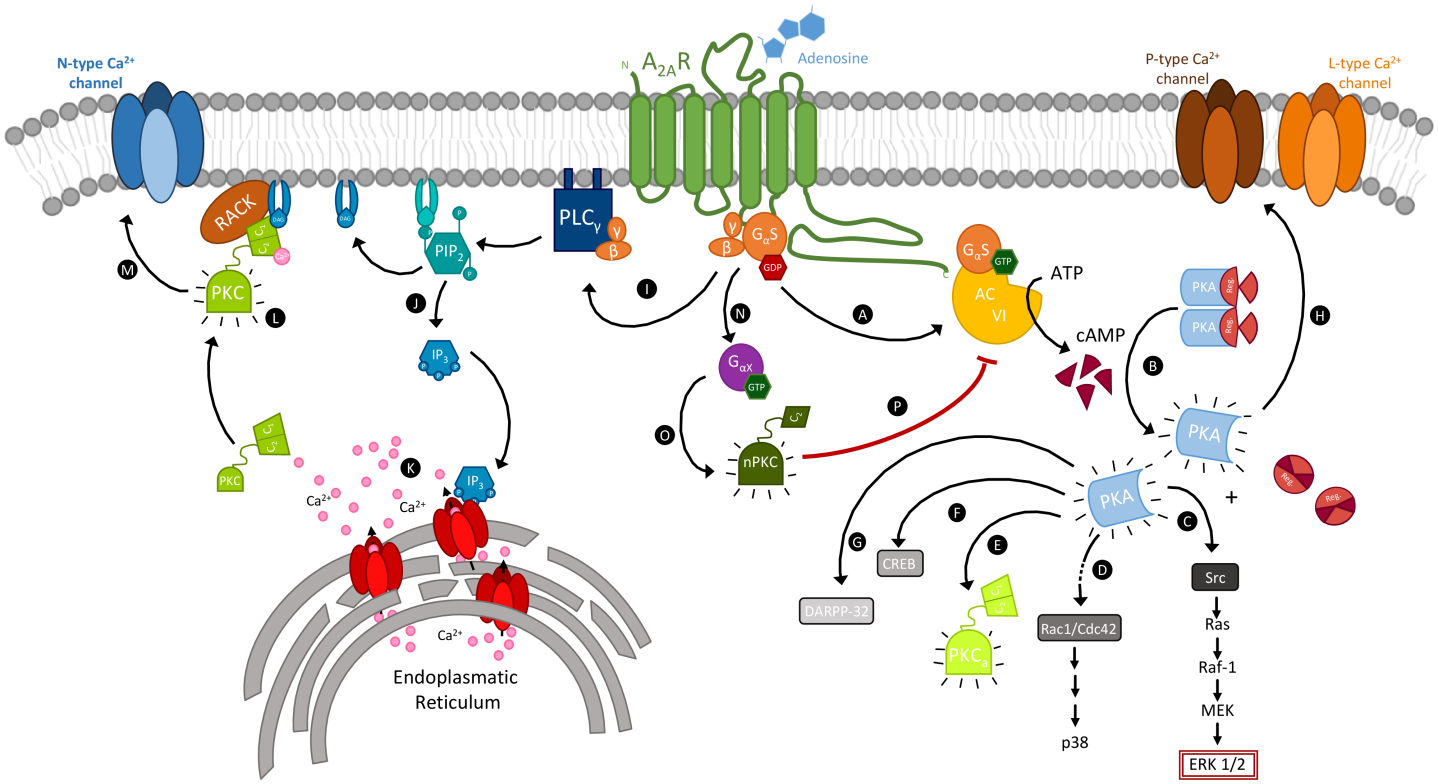

Figure 1.7 **Signaling pathways dependent on G-protein downstream to the activation of A_{2A} receptors.** The detail explanation of the pathways here represented are described in the text.

When $A_{2A}R$ couple to G_s, it induces AC VI activation, which consequently accumulates cAMP (Figure 1.7A). The binding of cAMP to the regulatory subunit of the PKA liberates the catalytic subunit, allowing the PKA phosphorylation of its substrates (Figure 1.7B).

Stimulation of $A_{2A}R$ activates the Ras/Raf-1/MEK/ERK signaling pathway via Src-mediated mechanisms in a PKA-dependent manner (Figure 1.7C). In turn, ERK can phosphorylate a series of different transcription factors in addition to several cytoplasmic proteins which are intimately involved in cell cycle regulation (reviewed by Schulte and Fredholm, 2003).

In addition, $A_{2A}R$ induces the activation of Rac1/Cdc42, (Figure 1.7D), two proteins belonging to the Rho family of GTPases, which have an impact on actin filaments. However Rac1/Cdc42 activation seems not to be directly phosphorylated by PKA, but rather by a PKA-dependent component (Feoktistov et al., 2000).

The atypical protein kinase C (aPKC) (Figure 1.7E) may also be activated by $A_{2A}R$ (reviewed by Schulte and Fredholm, 2003). A study by Huang et al., 2001 suggests that aPKC mediates the protective effect of $A_{2A}R$ against apoptosis in PC12 cells since generic inhibitors of PKC impaired this process but a selective inhibitor of aPKC (found in a high density in the nucleus of these cells) was more effective, which supports an important role for PKC in the signaling mediated by the action of $A_{2A}R$, at least in PC12 cells.

Another key target of PKA is the cAMP responsive element binding protein (CREB) (Figure 1.7F) which is critical for many forms of neuronal plasticity (reviewed by Josselyn and Nguyen, 2005) and long-term memory (reviewed by Benito and Barco, 2010). In fact, new tools, such as the $opto-A_{2A}R$ reveal activation of CREB downstream $A_{2A}R$ activation *in vivo* (Li et al., 2015).

Furthermore, activation of $A_{2A}R$ in striatal medium-sized spiny neurons induce DARPP-32 phosphorylation through PKA-dependent signaling (Svenningsson et al., 2000, 1998); Caffeine, an antagonist of adenosine receptors, controls not only the phosphorylation of DARPP-32 but also its dephosphorylation. So DARPP-32 is extremely important for the *in vivo* stimulant effects of caffeine (Lindskog et al., 2002).

As a consequence of PKA downstream signaling, due to the activation of $A_{2A}R$, there is the enhancement of P-type Ca^{2+} channels in the CA3 field of the hippocampus, in presynaptic nerve terminals of the rat brainstem and others (Gubitz et al., 1996; Mogul et al., 1993; Satoh et al., 1997; Umemiya and Berger, 1994). In addition, $A_{2A}R$ also facilitate the activity of the L-type calcium channel (Figure 1.7H) in the presynaptic neuron of motor synapses (Tarasova et al., 2015).

The PLC/IP_3/DAG pathway is activated by both $G_{q/11}$ and $\beta\gamma$ subunits, Figure 1.7I. After increased levels of IP_3 and DAG, Figure 1.7J, the common known pathway will proceed ending with PKC activation, Figure 1.7L, as result of increased intracellular levels of Ca^{2+} released from the endoplasmic reticulum through IP_3-sensible Ca^{2+} channels, Figure 1.7K, and DAG. One may say that this is a procedure most likely to occur in $A_{2B}R$ than for $A_{2A}R$ mediated signaling because this receptor may couple to $G_{q/11}$, however on medium spiny neurons of striatum, $A_{2A}R$ activation inhibits (rather than facilitates) the conductance of NMDA receptor channels, by a mechanism involving the PLC, IP_3, calmodulin and CaMKII pathway, which results in NMDAR internalization (Wirkner et al., 2000).

In addition, the activation of $A_{2A}R$ induces N-type calcium channels activation through a PKC-dependent manner, Figure 1.7M in striatal cholinergic nerve terminals, although, it is still not clear how $A_{2A}R$ induces PKC activation (Gubitz et al., 1996).

In PC12 cells $A_{2A}R$ stimulates PKC, not via PLC pathways, but rather through the activation of calcium-independent novel PKCs (nPKCs), Figure 1.7N, which in turn phosphorylates AC type VI. However, the G-protein responsible for this process is unknown ($G_{\alpha X}$), Figure 1.7O. Once phosphorylated, AC does not produces cAMP, which causes a negative feedback upon cAMP signal, Figure 1.7P, (Chern et al., 1995, 1993; Gubitz et al., 1996; Lai et al., 1997). Such negative regulation of the $G_{\alpha X}$/AC VI pathway by $G_{\alpha X}$/nPKC signaling during $A_{2A}R$ stimulation seems to have a fundamental role in the fine-tuning $A_{2A}R$ mediated signaling (reviewed by Chen et al., 2014a) and even suggests the bidirectionality of this signaling.

Activation of PKC by $A_{2A}R$ has been shown to be essential for several $A_{2A}R$-mediated functions in the brain. For instance, the selective $A_{2A}R$ agonist CGS21680 (30 nM) enhances ENTs activity in rat hippocampal nerve terminals in a way dependent on the activation of PKC. Therefore, $A_{2A}R$ modulates extracellular levels of adenosine that are available to activate A_1R mediated, consequently controlling A_1R signaling (Pinto-Duarte et al., 2005).

In particular, in the CA1 region of hippocampal slices from male Wistar rats, Cunha and Ribeiro, 2000 showed that despite triggering cAMP production, activation of presynaptic $A_{2A}R$ lead to potentiation of fEPSPs in a way dependent on PKC. Therefore these results indicate that $A_{2A}R$-mediated facilitation of hippocampal synaptic transmission involves PKC rather than PKA activation.

In the striatal medium-sized neurons $A_{2A}R$ were shown to interact with G_{olf}, essential for the coupling of D1 and A_{2A} receptors to AC (Corvol et al., 2001; Kull et al., 2000). On the other hand, in the striatal cholinergic neurons, $A_{2A}R$ trigger two distinct signaling systems to activate different types of calcium channels: G_s/AC/PKA leads to the activation of P-type calcium channels, whereas the pathway involving a cholera toxin-insensitive G ($G_{\alpha X}$) and PKC leads to the activation of N-type calcium channels (Gubitz et al., 1996). Evidences that these mechanisms may, in fact, co-exist were found in hippocampal nerve terminals, where N- and P-type co-localize and are both controlled by $A_{2A}R$ in a way that suggests a bidirectional modulation of synaptic transmission by these receptors (Castillo et al., 1994; Luebke et al., 1993).

Until now, one of the major challenges of the above-mentioned studies was to assess the importance of all of these pathways triggered by the activation of $A_{2A}R$ in an *in vivo* context. The lack of proper tools was the bigger barrier to unveil *in vivo*-signaling mechanisms. However a new optogenetic tool, the *opto*$A_{2A}R$, recently developed by Li et al., 2015, gives the opportunity to study the effects of the activation of $A_{2A}R$ *in vivo* in a precise spatiotemporal manner.

This receptor, specifically designed from channel rhodopsin, retains the extracellular and transmembrane domains of rhodopsin fused with the intracellular loop of $A_{2A}R$, hence maintaining the specificity of the $A_{2A}R$ mediated signaling. Thus, this tool allows the selective activation of $A_{2A}R$ *in vivo* and the study of the signaling pathways triggered in each brain region that underlie certain behavioral outcomes as well as specific learning and memory processes.

For instance, both the cAMP and the MAPK pathway are activated by the selective agonist of $A_{2A}R$, CGS21680. In Li et al., 2015, the activation of *opto*$A_{2A}R$ showed that in the nucleus accumbens, $A_{2A}R$ preferentially trigger the MAPK pathway whereas in the hippocampus it preferentially activates CREB. Furthermore, the unveiling of the transducing pathways relevant for a certain phenotype dependent on the activation of $A_{2A}R$ gives the opportunity to selectively control these phenotypes by targeting the intracellular interacting partners of these receptors in specific brain regions and cell types.

$A_{2A}R$ is a GPCR which interacts not only with G-proteins but also with others non-G-proteins.

1.4.2 G-protein-independent pathways

The C terminus of the $A_{2A}R$ is relatively long ($\sim$ 120 amino acids) and is highly conserved among species. Several studies have demonstrated the existence of interacting proteins at the C terminus of $A_{2A}R$, called GPCR-interacting proteins (GIPs), which mediate the

G protein-independent actions of this receptor (reviewed by Fredholm et al., 2007 and by Keuerleber et al., 2011).

The following proteins were reported to link to the C terminus of $A_{2A}R$: i) α-actinin, an F-actin-cross-linking protein. This protein functions as a bridge between the $A_{2A}R$ and the actin cytoskeleton, hence it is enrolled on $A_{2A}R$ trafficking, which might contribute to receptor desensitization (Burgueño et al., 2003); ii) The deubiquitination enzyme (Usp4) is also associated to this adenosine receptor and simultaneously relaxes ER quality control and enhances cell surface expression of functionally active $A_{2A}R$ (Milojević et al., 2006); iii) translin-associated protein (XTRAX) which mediates $A_{2A}R$ ability to suppress the proliferation of PC12 cells while promoting NGF-induced differentiation (Sun et al., 2006); iv) β-arrestin 2 and 3 which bind to the N-terminal phosphate sensor of $A_{2A}R$, causing a conformational change that exposes the binding site to clathrin molecules, favoring the desensitization and endocytosis of the receptor (reviewed by Zezula and Freissmuth, 2008); v) ARNO, a small GTP-binding protein which is also found attached to C-terminus of $A_{2A}R$. This interaction has been proposed to mediate the sustained activation of MAPK by the $A_{2A}R$, via a pathway that is independent of heterotrimeric G proteins (Gsandtner et al., 2005).

1.5 $A_{2A}R$ interacts with other GPCRs

Besides the direct pre- and postsynaptic effects on neurons triggered by the activation of adenosine receptors, these may also form homo and heterodimers between themselves or interact with receptors for other neurotransmitters and/or neuromodulators (reviewed by Ferré et al., 2007 and Sebastiao and Ribeiro, 2009).

There are a variety of documented interactions between $A_{2A}R$ and others GPCRs, resumed in the Figure 1.8. These include dopamine receptors (namely D_1 and D_2), neuropeptides, metabotropic glutamate receptors ($mGluR$), cannabinoid receptors (namely CB_1), P2 purine receptors (namely $P2Y_1$ and $P2Y_2$) and adenosine receptors themselves (namely A_1, $A_{2A}R$ and A_3R).

1.5.1 $A_{2A}R$ heterodimerize with adenosine receptors

The most studied interaction between $A_{2A}R$ and other adenosine receptors is the $A_{2A}R$-A_1R crosstalk, which has in fact been shown in co-transfected HEK cells. Moreover, in rat striatum the $A_{2A}R$-A_1R heterodimers have been detected (Ciruela et al., 2006b). The prevalent signaling triggered by the activation of these heterodimers is dependent on the levels of adenosine since low concentrations of adenosine preferentially activate A_1R, leading to a decrease in glutamatergic transmission, and the opposite is true for higher levels of adenosine (reviewed in Ferré et al., 2007).

However the nature of this $A_{2A}R$-A_1R interaction may not be physical but rather involve downstream elements in the intracellular signaling cascades triggered by the receptors. One of those elements is PKC, since inhibitors of this kinase affect the $A_{2A}R$-A_1R crosstalk in nerve terminals from the striatum (Dixon et al., 1997), the cortex and the hippocampus of

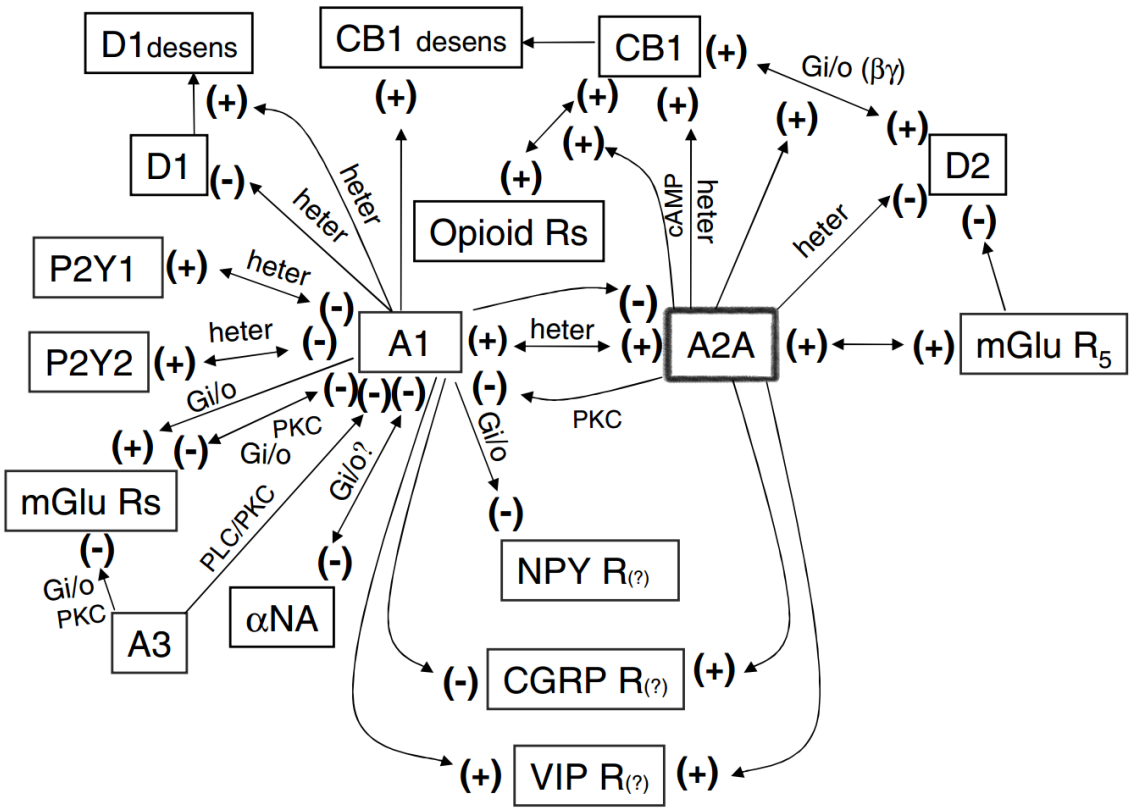

Figure 1.8 **Interactions between adenosine receptors and other G-protein-coupled receptors,** adapted from Sebastiao and Ribeiro, 2009.

young adult rats (Lopes et al., 1999). The mechanism may involve the activation of PLC by $A_{2A}R$ and the consequent formation of PIP_2; the hydrolysis of PIP_2 into DAG and IP_3 may then lead to the activation of PKC which can inactivate the coupling of A_1R to G-proteins, as previously shown to occur in the cholinergic modulation of inhibitory GPCRs in the rat hippocampus (Worley et al., 1987).

Moreover, it was also observed an interaction between A_1R and $A_{2A}R$ at presynaptic motor nerve terminals from the diaphragm of rats, where the inhibition mediated by the activation of A_1R was enhanced in the presence of an $A_{2A}R$ selective antagonist, and in turn, the facilitatory effect of $A_{2A}R$ is increased in the presence of an A_1R selective antagonist (Correia-de Sá et al., 1996).

1.5.2 $A_{2A}R$ and metabotropic glutamate receptors

One of the earliest works revealing an interaction between $mGluRs$ and adenosine receptors was the work by Alexander et al., 1992, where it was shown that agonists of mGluR facilitated the increase in cAMP induced by an agonist of adenosine receptors (NECA), in cerebral cortical slices from guinea-pig. Later, in hippocampus, it was shown that $mGluR$ from the group I, which are coupled to G_q/G_{11} and activate PLC, attenuatd A_1R-mediated inhibition of synaptic transmission in a PKC-dependent manner (De Mendonça and Ribeiro, 1997).

As a matter of fact, in the hippocampus, $mGluR$ are capable of control NMDAR populations (Anwyl, 1999; Benquet et al., 2002) and a co-localization between $mGluR$ and NMDAR in this brain region was found (Aniksztejn et al., 1991; Benquet et al., 2002). This relation is, therefore, modulated by the $A_{2A}R$. Accordingly, CA1 fEPSPs recordings showed that endogenous $A_{2A}R$-mediated tone was required to enable $mGluR_5$ to potentiate NMDA effects (Sarantis et al., 2015; Tebano et al., 2005). Then the authors postulated that hippocampal $A_{2A}R$ and $mGluR_5$ may be co-localize and act synergistically (Köles et al., 2016).

In addition, in the striatum, it was also shown that $mGluR_5$ modulate NMDAR-mediated responses (Pisani et al., 1997). In turn, adenosine was found to modulate $mGluR$ responses in striatum (Kearney and Albin, 1995) which was later supported by the discovery of an heteromerization between $A_{2A}R$ and $mGluR_5$ in the perisynaptic space, adjacent to the postsynaptic density of the glutamatergic terminals (Ferré et al., 2002), where they facilitate glutamate release in a synergistic manner (Pintor et al., 2000; Rodrigues et al., 2005). Moreover, this interaction is required to overcome strong tonic inhibitory effect of dopamine on striatal adenosine $A_{2A}R$ function, since the synergistic action of these receptors modulates the activity of D_2R (Ferré et al., 1999), increasing the phosphorylation of DARP-32 (Nishi et al., 2003).

So in conclusion, the activation of $A_{2A}R$ facilitate the $mGlu_R5$-mediated effects in the striatum (Domenici et al., 2004) and a similar functional interaction between these two receptors has been reported in the hippocampus, where $A_{2A}R$ and $mGlu_R5$ are co-localized and synergistically interact to modulate NMDAR-mediated effects (Tebano et al., 2005).

1.6 $A_{2A}R$ interacts with ionotropic receptors

Adenosine receptors are also capable of interacting with glutamate AMPA and NMDA receptors, with nicotinic acetylcholine receptors (nAChRs) or even with receptor kinases like TrkB (reviewed by Sebastião and Ribeiro, 2009), Figure 1.9. Nevertheless, in this work, we will focus on the interaction between the $A_{2A}R$ and the NMDAR, namely the modulation of NMDAR currents by the $A_{2A}R$. Thus, in the following sections it will be discussed the characteristics of NMDAR and the biological relevance of these currents as well as their modulation by adenosine receptors.

1.6.1 NMDAR Characterization

The NMDA receptor is one of the best studied neuronal receptors. The "launch pad" for this massive exploration was the discovery that many processes of synaptic plasticity throughout the nervous system rely on these receptors. Since then, numerous studies about its expression, splicing, structure, activity and influence upon other molecules have emerged, giving us a broad knowledge about several characteristics of this receptor.

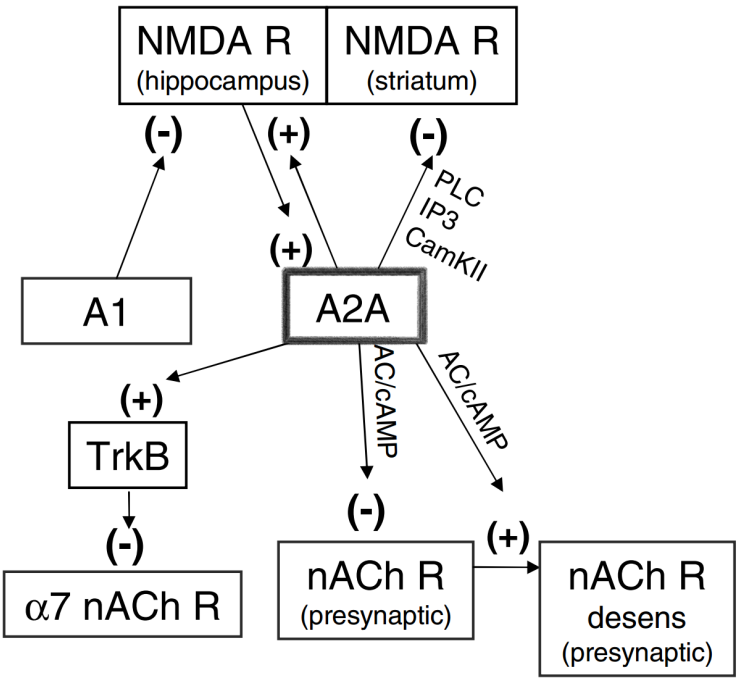

Figure 1.9 **Interactions between adenosine receptors with ionotropic receptors, with special emphasis on $A_{2A}R$**, adapted from Sebastiao and Ribeiro, 2009.

1.6.1.1 NMDAR structure

N-methyl-D-aspartate receptors (NMDAR) are among the most important ionotropic glutamate receptors in the CNS and present unique properties depending on its subunit composition. NMDAR are tetrameric complexes containing two obligatory NR1 subunits (GluN1, with 8 different splice variants) and two regulatory subunits that can be NR2 (GluN2A-D) or NR3 (GluN3A-B) (Cull-Candy et al., 2001; Cull-Candy and Leszkiewicz, 2004); Figure 1.10A shows in more detail the transmembrane structure of this receptor. Each subunit confer unique properties to NMDAR, from sensitivity to glutamate, to pore open probability and kinetics (Zarain-Herzberg et al., 2005).

In the region CA1 of hippocampus, the NMDAR are usually composed by two glycine-binding NR1 subunits, which are essential for a functional NMDAR, and two modulatory glutamate-biding NR2 subunits. The different combinations of these subunits determines the biophysical and pharmacological properties of these receptors (Doherty and Sladek, 2011; Takahashi, 2005), Figure 1.10B.

The long N-terminus of NMDAR is located at the extracellular side whereas the C-terminus is found in the intracellular side. Thus, depending on the subunit composition of NMDAR subunit type, these may interact with several different downstream signaling molecules (reviewed by Cull-Candy and Leszkiewicz, 2004, by Mayer, 2005 and by Paoletti and Neyton, 2007), which not only contribute to signaling events as to NMDAR internalization and desensitization processes (Hardingham and Bading, 2002), Figure 1.10A.

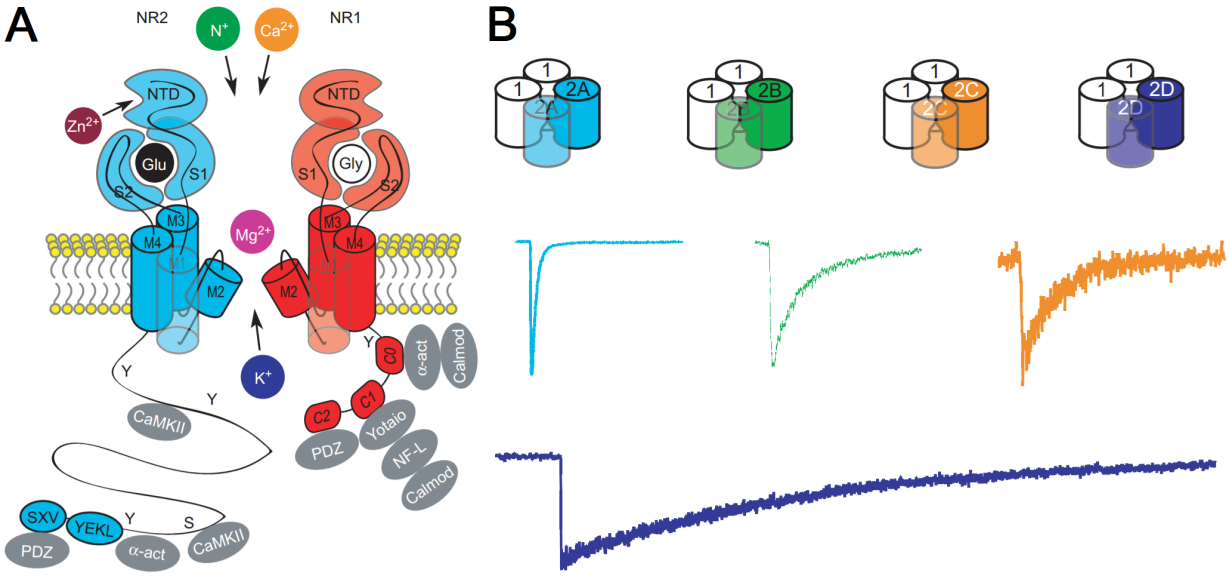

Figure 1.10 **Subunit composition of the NMDA receptor. A -** Display of the transmembrane architecture of glutamate NMDA receptors, permeable to Na^+, K^+ and Ca^{2+}. NR1 and NR2 subunits are composed by several conserved regions. The extracellular N-terminal domain (NTD) of the NR2 subunit contains modulatory sites that bind to zinc (Zn^{2+}). Moreover, the S1 and S2 domains constitute the binding site for Glutamate (Glu) in NR2 subunit (subunit shown in turquoise) whereas in NR1 subunit (subunit in red) this binding site binds to Glycine (Gly). The M2 domain is a channel-lining region that crosses the membrane from the inside. The Mg^{2+} binds deep within the pore causing a voltage-sensitive block. There are other residues that are linked to the C-terminal tail of NMDAR subunits such as synaptic kinases and structural proteins (colored in gray). At the C-terminal of NR1 subunit the proteins assemble at specific domains: C0 link α-actinin and calmodulin; C1 link the neurofilament-Long (NF-L), calmodulin and yotaio; finally, C2 has link the PDZ-containing proteins. Concerning the NR2 subunit, it contains intracellular binding sites for PDZ-containing proteins (SXV motif in blue), α-actinin, Ca^{2+}/calmodulin-dependent protein kinase II (CamKII), AP-2 (YEKL motif in blue), as well as several phosphorylation sites. **B -** In this panel are depicted the whole-cell responses from recombinant NMDAR (GluN2A, turquoise; GluN2B, green; GluN2C: orange; GluN2D: blue) that illustrate the different receptor deactivation kinetics in response to a 1 ms pulse of 1 mM glutamate: GluN2A 100 ms; GluN2B 250 ms; GluN2C 250 ms; GluN2D 4 s. Adapted from Cull-Candy and Leszkiewicz, 2004.

Since changes in NMDAR number, composition and/or function were associated with important physiological and pathological consequences, the interest for studying molecules that are capable of modulating NMDAR (and in particular those that modulate synaptic NMDAR) has risen in the last two decades.

1.6.1.2 Modulation of NMDA receptor function

NMDAR are known as coincident receptors, meaning that to be functional they require both presynaptic activation and postsynaptic depolarization, which can be achieved through the binding of glutamate to its receptors (mainly AMPAR-mediated depolarization). When

the postsynaptic cell is depolarized Mg^{2+} dissociates from its extracellular binding site at the NMDA receptor channel, allowing the flow of Ca^{2+} as well as Na^{+} ions into the dendritic spine (reviewed in Malenka and Bear, 2004 and Kauer and Malenka, 2007). As consequence NMDAR has a low activation-deactivation kinetic.

The extracellular zinc inhibits NMDAR function by binding to the N-terminal domain and by increasing proton inhibition (reviewed in Paoletti et al., 2009). On the other hand, polyamines and redox modulators have also been reported to influence NMDAR functions (Cull-Candy and Leszkiewicz, 2004). In particular, the C-terminal domain of NMDAR contains many serine and threonine residues that may be phosphorylated by kinases such as PKA, PKC, PKB, CaMKII, cyclin-dependent kinase-5 (Cdk5) casein kinase II (CKII) and by members of Src-family kinases (reviewed by Chen and Roche, 2007 and Salter and Kalia, 2004). For instance, the phosphorylation of protein tyrosine kinases (SFKs) by the Src family induces an upregulation of NMDAR function (Salter and Kalia, 2004). Moreover, the activation of PKC and PKA increase NMDAR-mediated currents and Ca^{2+} permeability (Chen and Huang, 1992; Lan et al., 2001; Lu et al., 1999; Raman et al., 1996; Skeberdis et al., 2006). In addition there are several GPCR whose ability to modulate, positively or negatively, the activity of NMDAR are already described, such as muscarinic receptors, $mGluR$, adenosine receptors, etc (Salter and Kalia, 2004).

Nevertheless, all these studies have been done using exogenous activation of NMDAR modulatory pathways, so the physiological conditions underlying such modulations and their consequences on activity-dependent synaptic plasticity have mostly remained elusive and require more studies.

For example, NMDAR-mediated effects are intimately related to the localization of the receptors. In fact, they may have opposite effects (such as promoting cell survival or death) depending on where they are, which is known as the *NMDA receptor paradox*. One of the assumptions for this paradox is that NMDAR at different localizations have distinct subunit composition which leads to the activation of different signaling cascades.

1.6.1.3 Synaptic versus Extrasynaptic NMDA Receptors

From an electrophysiological point of view, synaptic NMDAR are the ones recruited during afferent stimulation at low frequency (less than 0.05 Hz) or in response to spontaneous glutamate release, producing miniature excitatory postsynaptic responses. On the other hand, extrasynaptic NMDAR are not activated in such conditions (reviewed by Papouin and Oliet, 2014).

Despite of this, NMDAR are continuously diffusing between peri- or extrasynaptic locations and the active synaptic zone (reviewed by Köles et al., 2016). The trafficking of this receptors from intracellular components into synaptic or non-synaptic membranes is being intensely studied and it depends upon specific intrinsic trafficking signals for each subunit (reviewed by Lau and Zukin, 2007 and by Groc et al., 2009). The Extrasynaptic NMDAR are organized in clusters at the plasma membrane between the dendrite and a cell process (glial, axonal-like, or dendritic). In fact, in CA1 pyramidal neurons, extrasynaptic NMDAR constitute about 36% of total dendritic NMDAR pool (reviewed by Harris and Pettit, 2007).

The synaptic and extrasynaptic NMDAR found in CA1 pyramidal cells are activated by different co-agonists. Synaptic NMDAR are gated by D-Serine (reviewed by Papouin et al., 2012) and by reducing the endogenous production of this aminoacid it is observed an impairment in NMDAR-mediated processes (Basu et al., 2009; Henneberger et al., 2010; Yang et al., 2003) . On the other hand, extrasynaptic NMDAR are gated by glycine (reviewed by Papouin et al., 2012) and, interestingly, by reducing glycine levels the activity of synaptic NMDAR is not affected. One possible explanation for this rely on the subunit composition of the NMDAR. In particular, the GluN2A subunit is found to be part of the majority of the NMDAR population at CA3-CA1 (Papouin et al., 2012) and it has a higher affinity for D-serine than for glycine (Matsui et al., 1995). In contrast, GluN2B subunit is more sensitive to glycine, but not so proeminently detected in the same population of neurons (Papouin et al., 2012). Accordingly there are evidences that GluN2B-containing NMDARs are enriched at extrasynaptic sites (Groc et al., 2006; Martel et al., 2009; Tovar and Westbrook, 1999) and, that, in fact, this subunit interacts with specific downstream signaling molecules important for the attributed role of extrasynaptic NMDAR (reviewed by Paoletti, 2011; Paoletti et al., 2013; Papouin et al., 2012). In opposition to what was mentioned above, Harris and Pettit, 2007 have shown that the GluN2B is not enriched in extrasynaptic compartments, but it is likely the same for both synaptic and extrasynaptic NMDAR. Nevertheless, the subunit composition of NMDAR change with age, *i.e.* juvenile animals (P14–P22 rats) have less GluN2B-containing NMDAR.

The quantity of calcium allowed through NMDAR apparently underlies the NMDAR paradox. Over activation of these receptors may lead to excessive entrance of calcium to the neuron leading to cellular signaling disruption which contributes to neuronal death.

In this regard, synaptic NMDAR have been linked to cell survival, while extrasynaptic NMDAR have been associated with the disruption of calcium homeostasis and with neuronal death (Stark and Bazan, 2011).

1.6.2 NMDAR-mediated synaptic plasticity and modulation by $A_{2A}R$

Glutamatergic-mediated synaptic transmission and, in particular, plasticity at CA3-CA1 synapses are key players in hippocampus-dependent learning and memory processes (Brown et al., 1990b; Jarrard, 1993). While AMPA receptors mostly define the basal synaptic transmission, NMDAR seem to be critically involved in the activity-dependent changes. So, in order to understand how this information is encoded in CA1 neurons, it is necessary to study NMDA-dependent mechanisms of synaptic plasticity.

Synaptic plasticity can be defined as the activity-dependent modifications of strength or efficacy in excitatory or inhibitory synaptic transmission at preexisting synaptic connections (Benito and Barco, 2010; Citri and Malenka, 2008; Lynch, 2004). The main forms of synaptic plasticity are represented in Figure 1.11.

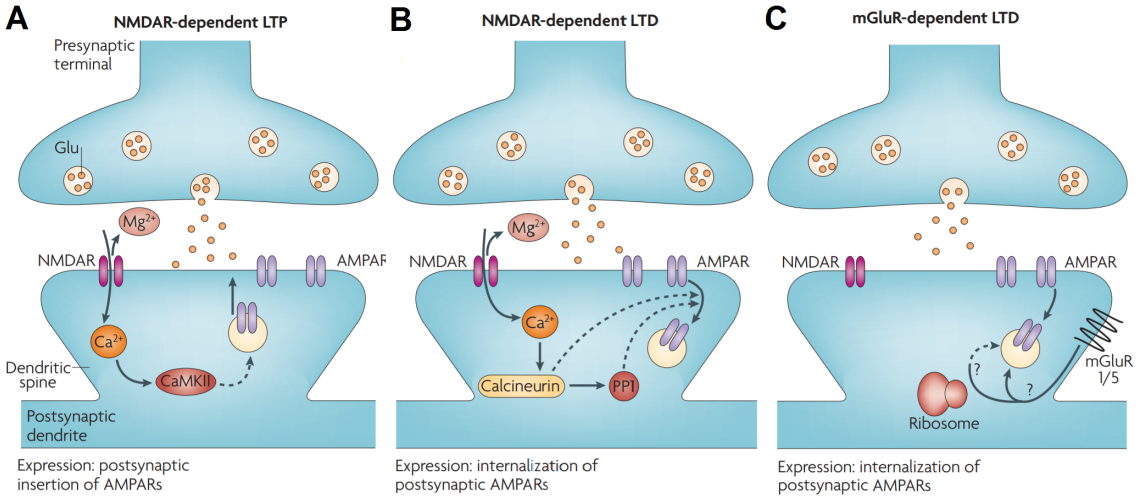

Figure 1.11 **Mechanisms of NMDAR-dependent LTP and LTD. A** – NMDAR-dependent LTP requires activation of NMDAR and consequently of the CaMKII that promote the insertion of AMPAR into the postsynaptic membrane; **B** – NMDAR-dependent LTD is triggered by Ca^{2+} entry through postsynaptic NMDAR channels, leading to increases in the activity of phosphatases that will promote the internalization of AMPAR from the postsynaptic membrane; **C** – $mGluR$-dependent LTD has been best characterized at hippocampal synapses. Activation of postsynaptic $mGluR_{1/5}$ triggers the internalization of postsynaptic AMPARs adapted from Kauer and Malenka, 2007.

1.6.2.1 Influence of $A_{2A}R$ on NMDAR-dependent LTP

Long Term Potentiation (LTP) is one of the most well studied mechanism of synaptic plasticity (Bliss et al., 1993; Larkman and Jack, 1995; Nicoll and Malenka, 1995) and the LTP of CA3-CA1 synapses appears to be identical (or very similar) to the LTP occurring at other glutamatergic excitatory synapses throughout the mammalian brain (Bear and Kirkwood, 1993).

The NMDAR-dependent LTP, described in Figure 1.11A, requires the activation of NMDA receptors, a process dependent of pre- and postsynaptic neuron depolarization. This depolarization is usually accomplished experimentally by repetitive tetanic stimulation of synapses or by directly depolarizing the cell with continuing low-frequency synaptic activation, a "pairing protocol", (Gustafsson and Wigström, 1988; Larkman and Jack, 1995; Malenka and Nicoll, 1999; Nicoll et al., 1988; Nicoll and Malenka, 1995)

The increase in postsynaptic Ca^{2+} concentration through the activation of NMDAR allows the activation of CaMKII (Malenka and Nicoll, 1999) and of other kinases, including PKC, PKA, tyrosine kinase Src and MAPK, which have also been shown to be involved in NMDAR-dependent LTP (Gustafsson and Wigström, 1988; Larkman and Jack, 1995; Malenka and Nicoll, 1999; Nicoll et al., 1988; Nicoll and Malenka, 1995). Different subunit composition of NMDA can also up- or downregulate NMDAR-mediated synaptic currents due to the difference permeability to calcium, thereby altering the induction of synaptic plasticity (reviewed at Lau and Zukin, 2007).

One of the first mechanisms that occurs in order to increase synaptic strength during LTP is the recruitment of more AMPA receptors to the postsynaptic membrane. In NMDAR-dependent LTP, this happens through CaMKII activation, which will promote the insertion of AMPAR into the postsynaptic membrane (reviewed at Lynch, 2004). Also, PKC has been proposed to play an analogous role to CaMKII because PKC inhibitors have been reported to block NMDAR-dependent LTP and conversely, increased postsynaptic PKC activity enhances synaptic transmission (Hrabetova and Sacktor, 1996; Hu et al., 1987; Linden and Routtenberg, 1989; Schwartz, 1993).

Following, the cytoskeleton is rearranged leading to several spine modifications that are observed during the LTP phenomenon, namely the enlargement of dendritic spines and of associated postsynaptic densities (reviewed at Malenka and Nicoll, 1999). Thus, within few hours, the maintenance of LTP requires protein synthesis (reviewed at Lynch, 2004).

Adenosine A_{2A} receptors are known to amplify Ca^{2+} signals and to directly control the release of glutamate (Lopes et al., 2002) as well as the activation of glutamate receptors, including AMPA receptors (Dias et al., 2012) and NMDA receptors (Costenla et al., 2011; Kessey et al., 1997; Rebola et al., 2005, 2008). Thus, $A_{2A}R$ can positively modulate NMDAR-dependent LTP, although the signaling pathways enrolled are still poorly understood.

Some suggested that $A_{2A}R$ promote the insertion of NMDARs in the membrane in a PKC- and Src-dependent mechanism (Sarantis et al., 2015) with possible long-lasting changes (reviewed on Rebola et al., 2010). In agreement with this, a study by Chen and Huang, 1992 showed that intracellular application of PKC potentiated NMDAR response by reducing the voltage-dependent Mg^{2+} block of NMDA-receptor channels and increasing the probability of channel opening in trigeminal neurons.

Many scientists that studied NMDAR-dependent LTP at the hippocampus also observed a potentiation of synaptic AMPAR currents. However, Kwon and Castillo, 2008 and Rebola et al., 2008 described the existence of an NMDAR-dependent LTP that occurred in the absence of a potentiation of the AMPAR EPSCs at mossy fibers (Mf)-CA3 synapses. Moreover, these authors found that the maintenance of this LTP required the influx of Ca^{2+} through the postsynaptic membrane and the activation of not only NMDAR but also type I $mGluR$ (Kwon and Castillo, 2008; Peng et al., 2010; Rebola et al., 2008).

One study showed that $A_{2A}R$ were relevant only during the induction of LTP at CA3-CA1 synapses, having no significant effect on its maintenance (Kessey et al., 1997).

Accordingly, Rebola et al., 2008 also found that $A_{2A}R$ are essential to induce LTP of NMDAR-EPSC in Mf-CA3 synapses, and that these $A_{2A}R$ were localized at the postsynaptic membranes and were not involved in presynaptic forms of synaptic plasticity because *i)* the $A_{2A}R$ antagonists did not affect AMPA-EPSCs; *ii)* $A_{2A}R$ agonist did not induce alterations in basal synaptic transmission nor affected the frequency of mEPSC facilitation; *iii)* $A_{2A}R$ did not affect the release of glutamate.

On the other hand Costenla et al., 2011 studied the impact of $A_{2A}R$ on NMDAR-dependent LTP at SC-CA1 synapses of the hippocampus from young adult to old male wistar rats (fEPSP) and found that the blockade of $A_{2A}R$ decreased both the release of glutamate and the magnitude of the LTP at all ages but had a larger impact in the hippocampus from old

rats. These results were in agreement with the increased expression and density of $A_{2A}R$ in glutamatergic nerve terminals of the hippocampus from aged animals when compared to younger rats. This result was not surprising in light of previous works showing that the activation of $A_{2A}R$ facilitates NMDAR responses and that the expression of these adenosine receptors increases with ageing (Canas et al., 2009a; Cunha et al., 1995; Rebola et al., 2003)

1.6.2.2 Influence of $A_{2A}R$ on NMDAR- and $mGluR$-dependent LTD

Another mechanism of long-term synaptic plasticity with opposite outcome of LTP has been emerging as important for learning and memory. This mechanisms is known as long term depression (LTD) (Bear and Abraham, 1996; Bear and Malenka, 1994; Linden, 1994), and it is depicted in Figure 1.11B.

It has been reported that in rats with 11-35 day-old two distinct forms of LTD may coexist in the CA1 hippocampal pyramidal neurons. One is dependent on the activation of NMDAR and the other is dependent on $mGluR$ activation. Therefore, one may assume that this two forms of LTD use different induction mechanisms (Gladding et al., 2009). However, LTD induction by either $mGluR$ or NMDAR activation isn't mutually exclusive, meaning that one form of LTD does not occlude the other (Fitzjohn et al., 1999; Huber et al., 2001; Oliet et al., 1997; Palmer et al., 1997).

Contrary to what was seen in NMDAR-dependent LTP process, these receptors are only weakly activated (which can be achieved experimentally with stimulation of neurons at a low frequency) which leads to a smaller entrance of calcium through these receptors to the postsynaptic neuron and consequently to a poor membrane depolarization (reviewed in Malenka and Bear, 2004). An interesting fact is that when NMDAR are properly functional, *i.e.* in optimal LTD-conditions, the intracellular calcium is not necessary for LTD expression, although, the intracellular reservoirs of calcium have a crucial role in the spreading of LTD to neighboring synapses (Nakano et al., 2004; Nishiyama et al., 2000).

The small amounts of calcium entering through NMDAR during LTD induction, recruit a different subset of Ca^{2+}-dependent proteins than the ones recruited during the induction of LTP, namely serine/threonine phosphatases which dephosphorylate critical synaptic substrates, including AMPAR, leading to its internalization (reviewed in Malenka and Bear, 2004). Thus after LTD induction, further NMDAR-dependent synaptic plasticity will be limited, at least temporarily (Kauer and Malenka, 2007).

Concerning to $mGluR$-dependent LTD, it has been firstly described at parallel fiber synapses on cerebellar Purkinje cells as reviewed by Ito, 1989 and depicted in Figure 1.11C. This LTD mechanisms may require both postsynaptic Ca^{2+} influx and postsynaptic group I $mGluR$ activation, or only activation of postsynaptic $mGluRs$ alone, being mediated by clathrin-dependent endocytosis of synaptic AMPARs that requires rapid protein synthesis.

On the other hand, this maily associated Group I of $mGluR$-mechanism (Harney et al., 2006; Peng et al., 2010; Yi et al., 1995) have been associated with endocytic events that involve a depression of synaptic responses dependent on NMDAR, since the application of the agonist for the group I of $mGluR$ agonist ((RS)-3,5-dihydroxyphenylglycine, DHPG) induces

long lasting depression of NMDA-dependent currents at the CA1 synapses, in addition to internalization of NMDARs in cultured hippocampal neurons (Snyder et al., 2001).

In the CA1 region of hippocampal slices, Rodrigues et al., 2014 showed that BDNF decreases the amplitude of the LTD through an $A_{2A}R$-dependent mechanism. In this work, the selective agonist of $A_{2A}R$ (CGS21680) promoted, whereas the selective antagonist (SCH58261) blocked, this effect of BDNF. The authors also showed that BDNF-mediated LTD inhibition required tonic activation of $A_{2A}R$, since the clearance of extracellular adenosine with adenosine deaminase also prevented the effect of BDNF. Furthermore, the mechanism underlying this interplay between $A_{2A}R$ and BDNF involves the transphosphorylation of the BDNF receptor TrKB by PKA (Rodrigues et al., 2014).

It has recently been shown that adenosine $A_{2A}R$ activation inhibits the conductance of NMDA receptor channels in a subset of rat neostriatal neurons (Nörenberg et al., 1997). This is not a mechanism of LTD, but rather a decrease in the levels of basal transmission.

1.7 Aims of the dissertation

Considering the relevance of glutamatergic transmission mediated by NMDAR for the control of hippocampal function and the lack of detailed knowledge on the neuromodulatory actions of adenosine onto NMDAR activity, the overall aim of this thesis was to evaluate the influence of adenosine A_{2A} receptors on NMDA-dependent currents.

We do not focus on the influence of the $A_{2A}R$ in processes of synaptic plasticity such us LTP and LTD, but rather in their putative role in conditions of basal neurotransmission in the CA1 pyramidal neurons of the hippocampus, performing patch clamp recordings in the whole-cell configuration mode.

The majority of the studies have dismissed $A_{2A}R$ from a role in basal neurotransmission and lay emphasis on its excitatory role upon processes of synaptic plasticity. In this work, we aimed to reproduce the data so far obtained in the CA1 region of hippocampus concerning to the excitatory role of $A_{2A}R$ upon NMDAR and then unveil the mechanisms underlying this interaction. However, we were not able to reproduce this excitatory effect upon the NMDAR-dependent currents and so, we were rather explore this result in order to understand the $A_{2A}R$-mediated effects observed in CA3-CA1 synapses.

Chapter 2

Techniques

The current chapter will focus on the principles behind slice managing as well as in basic electrophysiological concepts. The knowledge provided will be useful to a better comprehension of the results presented in this dissertation.

2.1 Seeking the perfect slice

The process of making good brain slices is very delicate and there are several aspects that must be considered, taking into account the type of experiment to be done.

Brain slices are an *ex vivo* preparation, obtained from fresh brain sectioning and were first performed by Yamamoto and McIlwain, 1966. The main advantages of using brain slices for neuronal studies are the possibility to study cellular mechanisms and neurotransmission in small networks. This type of biological preparation allows excellent optical access to even small cellular elements such as dendritic spines as well as mechanical stability and the ability to control the extracellular environment for ionic or pharmacological manipulations. Moreover, brain slices have a huge advantage over primary neuronal cultures since they can retain much of the *in vivo* network structure and the connection specificity of cells. Besides, after an electrophysiological experiment it is possible to preserve the slice with a fixant agent (for instance paraformaldehyde, PFA) to posterior immunostainning (reviewed in Covey and Carter, 2015, Chapter 1). Nevertheless, there are some associated disadvantages as well. Brain slices can only be used for a few hours after preparation, and they lack many of the normal activity patterns that can be observed *in vivo*, however for the past years, the methods for slice preparation have been optimized in order to better conserve the intrinsic circuits as well as to prevent degradation of the tissue, allowing long-lasting recordings.

The main factors to pay attention in order to preserve slice viability are the following (reviewed in Covey and Carter, 2015, Chapter 1): (1) prevention of ischemic damage by dissecting and slicing quickly, often in well-oxygenated, ice-cold cutting solution; (2) prevention of excitotoxic damage, in the region of interest, through use of specific solutions; (3) prevention of mechanical damage by avoiding compression or stretching of brain tissue and by using well-tuned vibratomes with appropriate blades.

One of most outstanding improvement for brain slice's quality is the use of modified solutions during brain dissection, sectioning processes and slice recovering period. The solutions are composed by a mixing of salts, pH buffers, energy sources, and divalent ions according to the type of protocol to be performed and to the brain region of interest (reviewed in Covey and Carter, 2015, Chapter 1).

In these solutions, the concentrations of sodium are usually moderate, however, it has been described that the replacement of sodium for methylated organic cations, such as N-methyl-d-glucamine (NMDG), choline and Tris, or for sucrose during slice preparation can improve the survival of the cells in the slice (reviewed by Ting et al., 2014). This prevents the cells from spiking since it decreases the permeability of sodium channels thus reducing not only excitotoxic damage as well as the metabolic demand due to decreased activity of Na^+/K^+ pumps (Hille, 1971 and reviewed in Covey and Carter, 2015, Chapter 1). In terms of calcium concentrations, elevated calcium levels seem to help forming better seals between cell membranes and the pipette glass (explained on the section 2.2). Besides it also increases the probability of release at synapses, which makes synaptic responses larger, which may differ from the real physiological mechanisms (Lorteije et al., 2009). In addition, it is common to add sodium pyruvate, since it provides an alternate entry point into the cellular metabolism, and also ascorbic acid, which acts as a free radical scavenger. These two molecules are described to promote cell survival. Furthermore, the osmolarity and pH are critical to prevent cell lysis or swelling during the cutting process.

After slicing the brain, slices are commonly allowed to incubate for 30–60 minutes at 32–34°C to allow them to recover and re-equilibrate with the aCSF environment. After this, the slices are usually incubated at room temperature. The work of Ting et al., 2014 shows that the period of recover is as important as the sectioning period, and once again, appropriate recovering solutions must suit the experiment.

Once transferred to the recording chamber, slices must be maintained under continuously superfusion of oxygenated aCSF, adding the pharmacological tools to this same solution.

In patch clamp, besides the extracellular solutions (ECS) there is the need of an intracellular solution (ICS) used to fill the pipettes for the whole-cell patch recordings. Considering the pH, while ECS have a pH of about 7.4, the ICS is a little more acidic with a pH *circa* 7.2–7.3. Moreover, the difference in the osmolarity between these two types of solutions is critical for obtaining good seals (explained on section 2.2). However, if the difference is large, the cell will die, but, if small, differences in the osmolarity give better chances of achieving a good seal. For example, if the intracellular osmolarity is a little higher than the extracellular osmolarity, cells with wrinkly membranes will swell slightly so they can be better patched (reviewed in Molleman, 2003, Chapter 4).

2.2 The *Patch Clamp* Technique

2.2.1 Origins of the technique

Our brains have about a hundred billion neurons that fire signals to communicate with each other all the time. These are electrochemical signals that travel from the cell body of a

neuron through its axon, to the next neuron. This electrochemical signals, used to process and to transmit information, may exist in an extended variety of forms and may be recorded using different techniques.

The most simple recordings are measurements of extracellular responses, which are generated by the flow of current between different parts of individual neurons. These recordings are very useful to understand the coding of information in spike trains (spikes are produced in response to stimuli or spontaneously, and each spike typically lasts for 1 millisecond; a spike train is simply a combination of sequences of spikes and silences) and the spatial and temporal organization of synaptic inputs, however they do not reveal the underlying mechanisms of synaptic integration or of spike generation, so the need for a techique that allows the measurement of transmembrane voltage and current with high resolution and low noise was urgent (reviewed in Covey and Carter, 2015, Chapter 1).

By the late seventies, beginning of the eighties, one technique have emerged by the hands of Bert Sakmann and Erwin Neher: the *Patch Clamp* technique. These two scientists were able to monitor the opening and closing of a single ion channel by measuring its conductance. At the beginning, the technique was limited, meaning that when the tip of the pipette touched the surface of the cell membrane only a smooth "seal" would be obtained with a resistance of just *circa* 50 $M\Omega$ of resistance. However this limitation has long been overcome and now the so called "giga-seal" can be reached. This giga-seal considerably reduced the background noise allowing also greater time resolution by ensuring that almost all of the current from the patched membrane flows into the pipette.

Patch clamp is a modern electrophysiological technique which allows the recording of even small sub threshold events in a single neuron, while blocking external currents associated with the electrical activity of surrounding cells. There are several possible configurations in the patch clamp method: *i)* whole-cell recording; *ii)* cell-attached patch recording; *iii)* outside-out patch recording and *iv)* inside-out patch recording.

Besides the different possible configurations there are also two recording methods widely used in the study of electrical signaling in neuronal cells: the *voltage clamp* and the *current clamp*. While voltage clamp is based on a fixed voltage allowing the measurement of neuronal currents, associated with changes in the conductance of the membranes, the current clamp mode allows the recording of the voltage across the membrane of individual neurons. Both techniques can provide us important information about the fire patterns of action potentials, of how synaptic inputs change with time, etc.

2.2.2 Whole-cell recording

The most commonly used configuration in patch clamp is the whole-cell recording, at voltage clamp mode, which was also the configuration used in this thesis. This method allows the total access of the recording pipette to the interior of the cell and therefore the electrode will be in direct electrical contact with the entire neuron (reviewed in Covey and Carter, 2015, Chapter 1), the detailed method is depicted in Figure 2.1. In addition, in this configuration mode it is possible to measure an entire ion-channel population of the cell membrane, through

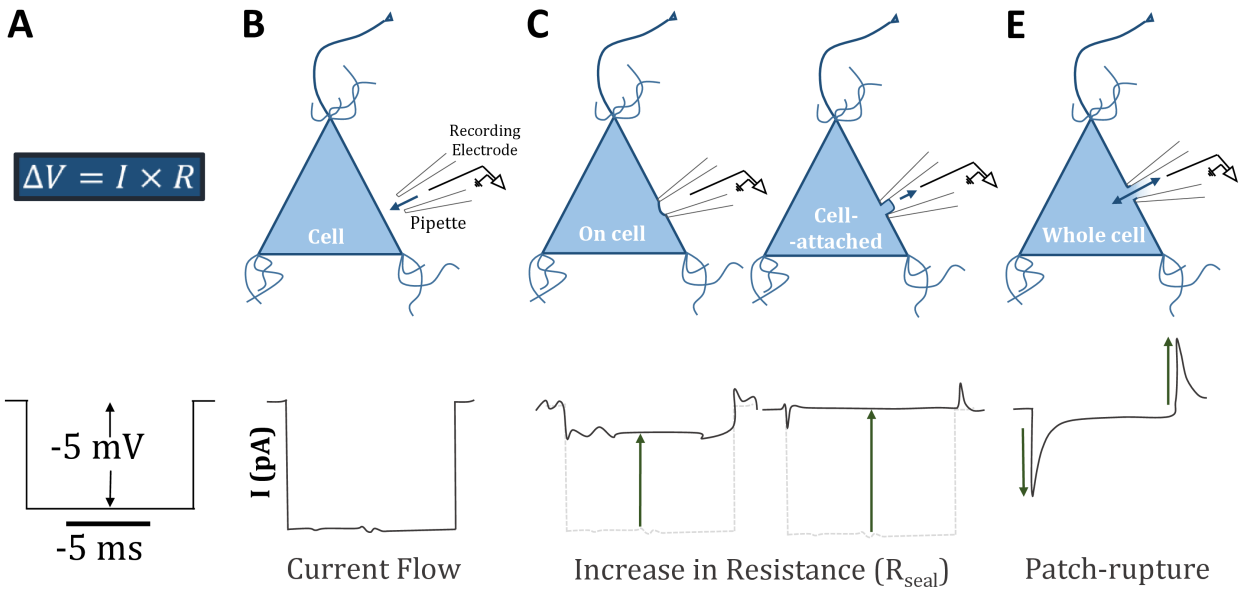

Figure 2.1 **Oscilloscope traces obtained in response to constant test pulses for establishment of whole-cell recording. A -** The law of Ohm states that the electric potential difference between two points on a circuit (ΔV, the square voltage step delivered through the recording electrode, in units of volts) is equivalent to the product of the current between those two points (I, the measured current injected into the cell to induce ΔV, in units of amperes) and the total resistance of all electrical devices present between those two points (R, the total resistance across the patch electrode tip, in units of ohms). During electrode placement, electrode resistance is monitored continuously by applying a small voltage pulse ($5\ mV$) to the electrode. **B -** The resistance is very low (electrode resistance $4-6\ \Omega M$) while the electrode is in the bath and not in direct contact with the cell, in addition the test pulse current is large ($Vh = 0\ mV$). **C -** The size of the current change, produced by the test pulse, goes down as the resistance across the patch electrode tip goes up. Thus, a reduction in test-pulse current indicates closer contact between the electrode tip and the cell (increase in seal resistance, R_{seal}). Once contact is made with the cell, electrode resistance spontaneously increases and application of gentle suction to the electrode, by mouth or a small syringe, quickly results in the formation of a gigaseal (cell attached mode). At this point, seal quality can be improved by changing the amplifier to voltage-clamp mode and applying a negative holding potential to the pipette until reaching the holding current that will be used during the recording ($Vh = -65\ mV$). **D -** Then Whole-cell configuration is achieved with brief pulses of suction that will rupture the membrane patch under the electrode, leaving the seal and the cell intact. This will result in a low-resistance access to the cell and in the appearance of large capacity transient arising from the added membrane capacitance. Based on Rombo, 2015 and Sontheimer and Olsen, 2007, Chapter 2.

the readings of the current flow of all cellular activity mediated by the specific ion-channels (reviewed in Liem et al., 1995).

The potential of the membrane of a neuron must be obtained right after rupturing the membrane (which is performed in the current-clamp mode with $I = 0\ pA$), since after that point, within minutes of establishing the whole-cell configuration, the pipette contents will equilibrate with the cytoplasm of the cell and impose an artificial ionic potential across the

membrane (reviewed in Sontheimer and Olsen, 2007, Chapter 2). In particular, the use of cesium-based intracellular solution will rapidly move the membrane potential towards more positive values due to the blocking of inwardly-rectifying potassium channels (Guide to PHARMACOLOGY, 2017).

Thus the real advantage of the patch clamp technique is to measure the voltage, V_m, and resistance, R_m, of the patched neuron, both intrinsic properties of this type of cells. The V_m is the voltage of the interior of the neuron relative to the bath and it should be more negative than $-50\ mV$. The R_m is the resistance of the cell membrane and it is also called the input resistance. Nevertheless, it is necessary to understand all the electrical contaminations that are simultaneously recorded due to the electrical/physical characteristics of the pipette, (reviewed in Covey and Carter, 2015, Chapter 1 and Molleman, 2003, Chapter 4) and resumed in the Figure 2.2:

It is important to have in mind that to perform patch clamp in the voltage clamp mode, the accuracy of the recordings are highly dependent on the ability of the amplifier to inject the precise amount of current that will compensate any membrane potential alteration

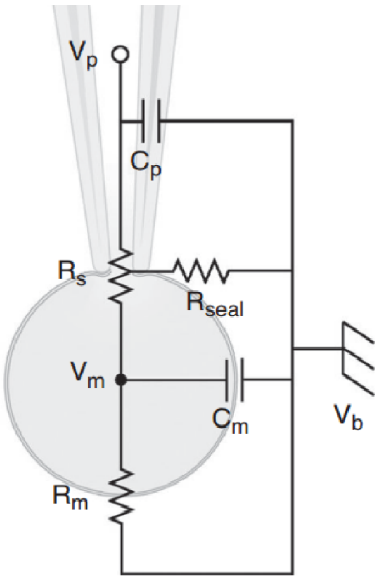

Figure 2.2 **Patched neuron equivalent circuit diagram.** V_p is the voltage inside the pipette; this is the voltage controlled or measured by the amplifer; C_p is the pipette capacitance; typically a few pF; Rs is the series (or access) resistance; this is the resistance separating the pipette from the cell body and is mainly due to the narrow pipette tip and organelles that may be blocking it. Rs must be lower than 20 $M\Omega$ and stay that way throughout the recording; R_m is the resistance of the cell membrane and it is also called the input resistance. R_{seal} is the seal resistance, *i.e.* this is the resistance of the contact region between the pipette and the membrane, a measurement right after the giga seal formation. To make quality recordings, this must be $> 1\ G\Omega$; C_m is the cell membrane capacitance and it give us a idea of the size of the recording neuron, as the bigger the capacitance the bigger the size of the neuron; V_b is the bath voltage, as measured by the ground electrode; V_m is the voltage of the interior of the neuron relative to the bath. Adapted from Sontheimer and Olsen, 2007, Chapter 2.

due to changes in membrane conductivity. This is a known value equal and of opposite direction to that flowing through the ion channels. This negative feedback prevents a change in the membrane voltage and holds the membrane potential in the same value as the command potential.

So, these type of recordings are highly influenced by fluctuations in the series resistance that will affect the passage of current into the cell and consequently the amplitude of postsynaptic currents. Thus, a constant monitoring of the series resistance in voltage-clamp mode is mandatory in order to confirm that the variations seen in the currents recordings are due to biophysical phenomena and not caused by technical issues associated with Rs. The calculation of the Rs is done according to the formula $R_s = V_{step}/I_{peak}$ and explained in Figure 2.3, (adapted from Rombo, 2015).

In addition to this calculation, it is possible to make an approximation of the membrane resistance value. For R_m calculation it is used the steady-state current (I_{ss}) which corresponds to the difference between the holding current before the voltage step and the later part of the voltage step ($R_m = V_{step}/I_{ss}$).

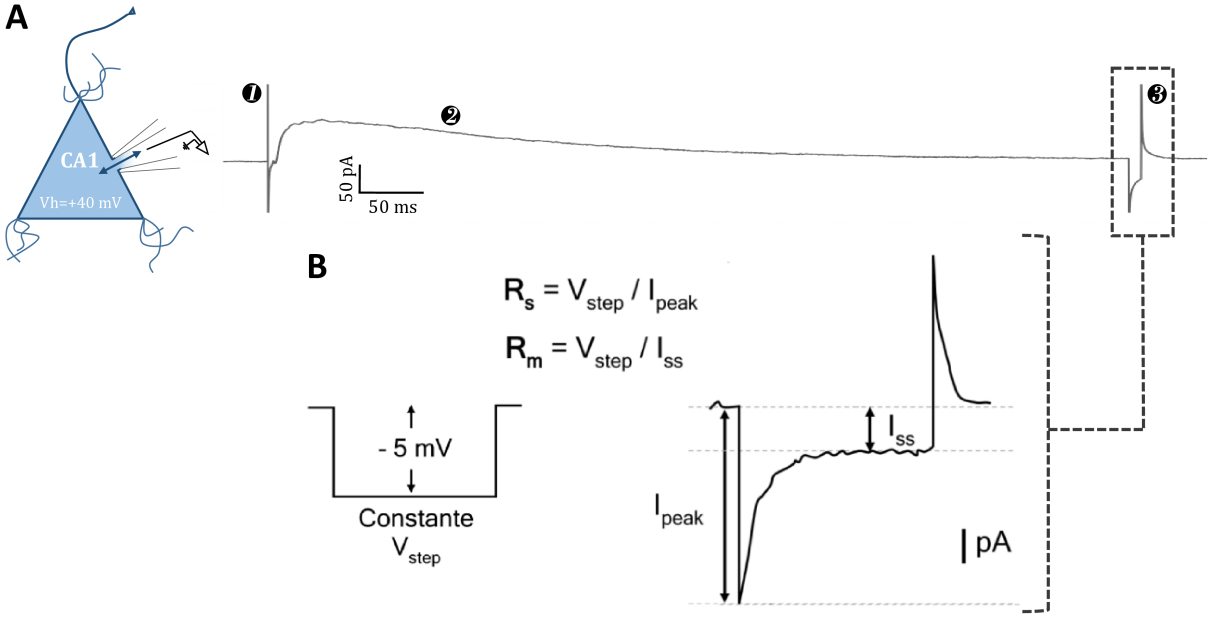

Figure 2.3 **How to calculate series resistance from a neuron recording. A -** CA1 pyramidal neuron being recorded at $Vh = +40\ mV$ and an example of an obtained recording: *1.* Stimulus artifact, *2.* Neuron stimulus response and *3.* Neuron stimulus response to the voltage step of -5 mV, which is depicted in detail at panel B; **B -** V_{step} corresponds to the amplitude of the voltage step, which is constant and has an amplitude of $-5\ mV$. R_s corresponds to the resistance that is in series with the pipette, also observed in the previous Figure (2.2). This is the resistance that is opposing the passage of the current into the cell that maintains the voltage command stable. For the calculation of this parameter, besides V_{step}, it is required the value of the peak amplitude of the transient current immediately after the application of a step, I_{peak}, a value that is constantly monitored throughout the time of the experiment. In order to calculate R_m another parameter is required, the steady-state current I_{ss}.

2.2.3 Isolation of an homogeneous population of channels

The voltage-clamp mode requires some manipulations in order isolate the currents mediated by the ion channels of interest for a given study. This may be done by:

(i) Selectively choosing the ionic composition of the bath solution. Changing ion concentrations in the intra- and extracellular medium can affect ion conductance differentially, depending on their ion specificity;

(ii) Controlling the holding potential of the cell membrane may facilitate or make it harder to activate a specific ion channel. For instance, at positive voltages the NMDAR no longer has the blockage of the magnesium ion, which facilitates its activation;

(iii) The use of pharmacological tools that specifically block or induce the activity of certain ion-channels;

(iv) The shape of the current response allows to infer if more than one population of ion channels is being recruited since two or more phases in response to the stimuli indicate two or more populations of ions with different voltage dependencies and kinetics.

In addition, some drugs can be added directly into the cell, through the recording pipette, in order to study intracellular signaling cascades (reviewed in Sontheimer and Olsen, 2007, Chapter 2 and Molleman, 2003, Chapter 5).

In this thesis, we recorded NMDA-dependent currents, therefore some of the above-described manipulations were implemented, such as the use of specific drugs in order to isolate these particular currents, the control of the holding potential in order to favor NMDAR activation and also the monitoring of the shape of the response in order to see if there were contaminations from other ion channels. In addition, to confirm if the registered current was, in fact, mediated by NMDAR we added a selective antagonist in the last 8 minutes of the experiment, who eliminates almost all of the current, otherwise the experiment was discarded.

Chapter 3

Methods and Materials

3.1 Animals

For the results hereby presented it was used male Wistar rats with two different ages: from 8 to 13 weeks-old (Charles-River Laboratories, Barcelona, Spain) shown in Figure 3.1A (left), and from P24 to P26 (Animal House of CNC facilities) shown in Figure 3.1A (right). All animals were housed in groups of 2 or 3 animals, in a temperature and relative humidity-controlled environment with a regular 12h light/dark cycle with access to food and water *ad libitum*.

For ethical reasons, the experiments were carefully planned and conducted in order to reduce the number of animals used. All procedures were carried out in accordance with the Portuguese national authority for animal experimentation, Direcção Geral de Veterinária (ID: DGV9457) and in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council.

3.2 Slice Preparation

In order to optimize our experimental conditions rats were anaesthetized by two different methods. In one set of animals it was used the xylazine (10 mg/Kg)/ketamine (75 mg/Kg) mixture. To assure that the animals were properly anesthetized their behavioral responses were observed, namely the lack of movement and the slow respiratory rate. The tail and the interdigital webbing were then pinched and the absence of movement reflected deep anesthesia. Immediately after, an incision through the skin and muscle with surgical scissors along the thoracic midline was made to expose the abdominal cage. The diaphragm was carefully separated from the chest wall with scissor cuts. Right after cuts were made through the thoracic musculature and ribcage in each side. The ribcage was pin with a needle to expose the heart (and other thoracic organs). A small incision was made in the right atrium and then a butterfly needle was placed in the left ventricle. The animal was then transcardially perfused with 60 mL of an ice-cold ($0 - 4\ ^oC$) oxygenated (95% O_2 − 5% CO_2) solution composed of 200 mM of sucrose, 20 mM glucose, 0.4mM $CaCl_2$, 8 mM $MgCl_2$, 2 mM KCl, 1.3 mM NaH_2PO_4, 26 mM $NaHCO_3$, 1.3 mM L-ascorbic acid, 0.4 mM sodium pyruvate

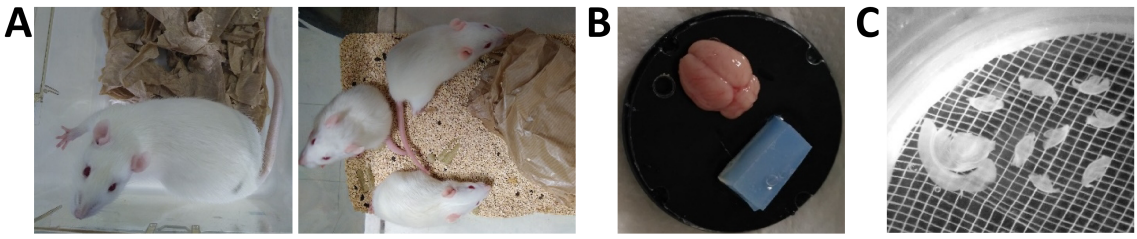

Figure 3.1 **Representative image of the animal's models used for brain dissection and sectioning. A** − representative images of the animals models used in these experiments (adult, left, and young, right, Wistar rats); **B** − representative image of a dissected young Wistar rat brain ready to be sliced; **C** − coronal and transversal slices obtained after brain sectioning.

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(pH 7.3), the volume needed to that the fluid exiting the right atrium was entirely clear. After this procedure, the animal was decapitated and the brain was quickly removed and placed in ice-cold cutting solution with the same composition. Another set of animals, of the same age, were anesthetized with halothane and decapitated with a guillotine. Once the animals were deep anesthetized according to the same standards explained above, the brain was quickly removed and dissected in the same ice-cold oxygenated solution as previously described.

Young wistar rats were anesthetized with halothane and quickly decapitated after lack of behavioral response. The procedure followed as described for adult Wistar rats.

From the brain of young Wistar rats, the hemispheres were separated and one of them was used to make coronal slices (260 μM thick), transverse to the long axis of the brain, while the other hemisphere was used to isolate and dissect the hippocampus to make transversal slices (300 μM thick), Figure 3.1C. Both type of slices were obtained using a Vibratome Leica VT 12005 and placed in a holding chamber with oxygenated (CO_2 5% + O_2 95%) artificial cerebrospinal fluid (aCSF, composed of $NaCl$ 124 mM, KCl 3 mM, $NaH_2PO_4.H_2O$ 1.25 mM, $NaHCO_3$ 26 mM, $MgSO_4$ 1 mM, $CaCl_2$ 2 mM and Glucose 10 mM, at pH 7.4 and 300 mOsm, supplemented with 0.4 mM of L-ascorbic acid and 3 mM of sodium pyruvate). The slices were allowed to recover in the chamber for one hour at $30 - 32^oC$ and used afterwards until a maximum of 7 hours after the cutting while the remaining slices had remain in the resting chamber at the room temperature.

For adult Wistar rats only coronal slices were used, following the same protocol as previous described.

In the Figure 3.1B/C is a picture of the intact brain and of the final slices collected and used for patch clamp recordings.

3.3 Patch Clamp Recordings

Individual slices were then transferred to the recording chamber and fixed with a grid, to minimize agitation.

A bipolar concentric stimulation electrode (SNE-100, Kopf, Germany) was placed on the Schaffer collateral afferents, delivering rectangular pulses of constant current with 0.5 ms

duration, applied through a Digitimer DS3 stimulator (Digitimer LTD, U.K) every 20 s, Figure 3.2A and 3.2B (left). The recording pipette was placed in the *stratum pyramidale* of the CA1 region, Figure 3.2B (right).

The submerged slices were continuously superfused using a gravitational superfusion system at $1-2\ mL/min$ of oxygenated aCSF with bicuculline ($1\ \mu M$, a selective antagonist of $GABA_AR$), at room temperature, in order to discard GABAergic signals, Figure 3.2C and 3.2D. Cells were identified through differential interference contrast microscopy using ZEISS Axioskop 2 FS plus (IR camera VX44) fixed stage upright microscope equipped with an immersion objective of 40x magnification, Figure 3.2B (right).

Whole-cell recordings of NMDA-evoked excitatory postsynaptic currents (eEPSCs) were performed from CA1 pyramidal neurons, clamped at $+40\ mV$ (voltage-clamp mode) to force the exit of magnesium ion blockade from NMDA receptor, Figure 3.2C and 3.2D, using borosilicate glass capillaries (1.5mm outer diameter, 0.86 inner diameter, GC150F-10, Harvard Apparatus, Holliston, MA, USA) with a resistance of 3–7 $M\Omega$. The stimulus intensity was adjust to obtain an amplitude of NMDA-dependent currents in the range of 90 to 150 pA. Pipettes were filled with a mimetic intracellular solution containing 140 mM

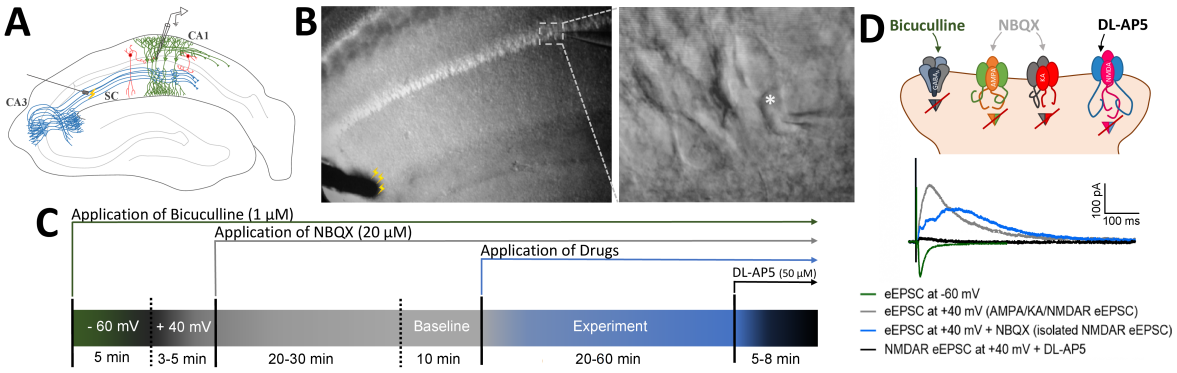

Figure 3.2 **Schematic and real representation of electrodes position in hippocampal formation, together with the general experimental design for drug application in order to isolate NMDA-eEPSCs. A** – schematic representation of electrodes position in hippocampal formation; **B** – real images showing the position of stimulating (at Schaffer collaterals afferents) and recording (at *stratum pyramidale*) electrode (left panel) and a CA1 pyramidal neuron being recorded (right panel); **C** – generic experimental design: after breaking into a neuron a period of 5 to 10 minutes was taken to allow intracellular solution diffusion. The recordings started at voltage holding (Vh) of $-60\ mV$ (a similar voltage to resting potential) and after 5 minutes 10 mv steps were taken, each 20 second, until reach a $Vh = +40\ mV$. Once clamped at $+40\ mV$ the slice was perfused with NBQX. The NMDA-eEPCS stabilize for a while and when a ten minutes baseline was made the drug of interest was diluted in a 15-25 mL aCFS solution containing also bicuculline and NBQX and then put in recirculation until the end of that experiment segment. At the end, it was added DL-AP5 to the previous solution for 5 to 8 minutes, the usual time needed to nearly abolish NMDA-dependent eEPSCs; **D** – Elucidative scheme about the targets of bicuculline, NBQX and DL-AP5 used in cell recordings hereby presented. Representative traces obtained in the different phases of the experiment depicted in panel C.

$CsCl$, 10 mM $HEPES$, 0.2 mM $EGTA$, 2 mM Na_2ATP, 2 mM $MgCl_2$, 4 mM $NaCl$, 5 mM phosphocreatine and 0.33 mM $NaGTP$ at pH 7.3 and 290 $mOsm$. The Cesium ion is a potent channel blocker of inwardly rectifying potassium channels, such as $K_{ir}1.1$, $K_{ir}2.4$, $K_{ir}3.4$, $K_{ir}4.1$ and $K_{ir}5.1$ (Guide to PHARMACOLOGY, 2017), which are typically responsible for repolarizing the cell following an action potential, by allowing positive charges to move into the neuron. Due to this, it was not possible to record the action potential profiles of the neurons before the experiments, however other characteristics were accessed in order to confirm that we clamped a pyramidal neuron, like membrane potential of $(-50 < V_m < -80\ mV)$ and membrane resistance $(65 < R_m < 130\ M\Omega)$.

To determine the action potential profile of the neurons, the potential was adjusted to $-60\ mV$ or $-70\ mV$, and the recordings were performed using an intracellular solution containing 125 mM Gluconate-K^+, 11 mM KCl, 10 mM $HEPES$, 1 mM $EGTA$, 2 mM Na_2ATP, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM phosphocreatine and 0.33 mM $NaGTP$ at pH 7.3 and 290 $mOsm$.

Recordings were made using an axoclamp 200B amplifier filtered at $0.5 - 1\ kHz$. Digital/analog converter Digidata 1322A recording software *Clampex* version 10.4 were the chosen software of interface to use. The recordings do not started before, at least, wait 5 to 10 min after break-in, to enable diffusion of intracellular solution into the soma and proximal dendrites.

Offset potentials were nulled directly before the formation of the giga-seal. Series or access resistance (R_s) was not compensated during voltage-clamp recordings but it was regularly monitored throughout each experiment with a $-5\ mV$, 50 ms pulse, and cells with more than 20% change in series resistance were excluded from the data, moreover R_s was $< 20\ M\Omega$ before normalization. R_s was calculated from the amplitude of the current recorded in response to the test pulses, according to Ohm's law. In addition, the membrane resistance (R_m) was calculated, as described in the section 2.2.2, and monitored throughout the experiment and discarded if it changed up to 20%.

3.4 List of Drugs, Chemicals and Reagents

The pharmacological tools used in this work are detailed in Table 3.1.

Other chemicals and reagents used were acquired from 1) Sigma-Aldrich: $MgCl_2$, NaH_2PO_4, $NaHCO_3$, L-ascorbic Acid, Sodium Pyruvate, CsCl, HEPES, EGTA, Halothane, Na_2ATP, Phosphocreatine; 2) Fluka: Sucrose; Glucose; $CaCl_2$; $NaCl$; $MgSO_4$; 3) Riedel-de Häen: KCl 4) Others: $NaGTP$

3.5 Histochemistry

Histochemistry was performed in order to better visualize the morphology of the recorded neuron. At the intracellular solution was added $0.3 - 0.4\%$ of biocytin, which was allowed to diffuse into the neuron at least 30 minutes. Afterwards, the cell was carefully re-sealed and the slice was washed 5 to 10 minutes through superfusion with aCSF, in the recording

Table 3.1 Pharmacological tools

Drug	Chemichal Name	Biological Activity	Supplier	[Stock]	[working]
Adenosine Deaminase (ADA)	Adenosine Deaminase from calf intestinal mucosa	irreversible conversion of adenosine into inosine	Sigma- Aldrich, St Louis, MO, USA	1 U/L in $(NH_4)_2SO_4$ suspension	2 U/mL
Bicuculline (+) -	$-5-(6,8-Dihydro-8-oxofuro[3,4-e]-1,3-benzodioxol-6-yl)-5,6,7,8-tetrahydro-6,6-dimethyl-1,3-dioxolo[4,5-g]isoquinoliniumiodide$	$GABA_AR$ antagonist	Tocris Bioscience, Bristol, UK	10 mM in DMSO	1 μM
CGS21680	$4-[2-[[6-Amino-9-(N-ethyl-\beta-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoicacid hydrochloride$	Selective $A_{2A}R$ agonist ($Ki = 27\ nM$)	Tocris Bioscience, Bristol, UK	1 mM in DMSO	30 nM
DL-AP5	$DL-2-Amino-5-phosphonopentanoicacid$	Competitive NMDAR glutamate site antagonist	Tocris Bioscience, Bristol, UK	5 mM in ddH_2O	50 nM
NBQX	$2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodiumsalt$	AMPA and Kainate receptor antagonist	Tocris Bioscience, Bristol, UK	20 mM in ddH_2O	20 μM
SCH58261	$2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine$	Potent and selective $A_{2A}R$ competitive antagonist ($Ki = 1.3\ nM$)	Tocris Bioscience, Bristol, UK	5 mM in DMSO	50 nM

chamber, to remove any trace of extracellular biocytin contamination. Then, the slice was fixed overnight in a multi-well plate containing 4% of paraformaldehyde. On the next day, the slice was rinsed in PBS 3x10 minutes and transferred to a criopreservative solution of 30% sucrose (prepared in PBS), overnight. After that, the slice was once more rinsed in PBS 3x10 minutes and then stored in PBS at 4^oC until further processing. Finally, the slice was once more rinsed with PBS 3x10 minutes and incubated at 4^oC in PBS with streptoavidin coupled to a green fluorophore excitable at 488 nm (1:100 or 1:200 dilution), prepared in PBS with 0.3% triton for 90 hours.

Then, the slices were mounted in glass slides with DAKO mounting medium and stored at room temperature. On the next day, nail polish was applied to avoid the formation of air bubbles and images were taken in an Imager.Z2 Zeiss microscope using axiovision as the software of interface.

3.6 Data analysis and Statistics

Data was analyzed offline using the Clampfit software, version 10.6. Data is expressed as mean ± SEM of n cells f**rom different slices**. Statistical significance was accessed using

one-sample t-test comparing to an hypothetical value of 100. A p-value of less than 0.05 was considered to account for significant differences. Analyses were conducted with the GraphPad Software, version 6.01.

Chapter 4

Results

A role for $A_{2A}R$-mediated modulation of the activity of NMDA receptors in the hippocampus has been known for a while (Rebola et al., 2008; Sarantis et al., 2015; Tebano et al., 2005). Particularly, Rebola et al., 2008 have shown that the activation of $A_{2A}R$, by its selective agonist CGS21680 (30 nM), potentiates synaptic NMDA-dependent EPSCs at Mossy Fiber (Mf) synapses, an effect abolished in the presence of the $A_{2A}R$ selective antagonist, SCH58261 (50 nM). Moreover, Rebola et al., 2008 showed that LTP of NMDAR-eEPSCs in this pathway, was blocked in the presence of SCH58261. Accordingly, Mouro, 2012 has also shown in his Master thesis that the selective $A_{2A}R$ agonist CGS21680 (30 nM) increased NMDAR-dependent postsynaptic currents in the Schaffer collaterals-CA1 synapses of the hippocampus. Furthermore, he showed that this effect was no longer observed in the presence of SCH58261 (100 nM).

Thus, in this dissertation, we first intended to reproduce these results and then unveil the mechanisms underlying the modulation of NMDAR currents by adenosine $A_{2A}R$ in the hippocampus. The results obtained are depicted in the following figures.

4.1 Exogenous activation of adenosine $A_{2A}R$ decreases the amplitude of NMDAR-eEPSCs in CA1 pyramidal cells

The results obtained with the superfusion of the selective $A_{2A}R$ agonist CGS21680 30 nM are shown in Figure 4.1.

As can be observed, the exogenous activation of $A_{2A}R$ decreased the amplitude of NMDA-eEPSCs in slices from both adult and young Wistar rats, Figures 4.1D-I.

However, this effect is not observed during the first 10 minutes of superfusion with CGS21680, in the slices from adult rats, as depicted in the first column of the scatter dot plot, in Figure 4.1F (98.55 ± 17.22% of baseline, n=6 cells) but at 30 to 40 minutes of perfusion with the $A_{2A}R$ selective agonist the decrease in NMDAR-mediated eEPSCs is significant (74.35 ± 24.28% of baseline, $p<0.05$, n=6 cells). The effect of CGS21680 is maintained from 15-20 minutes after CGS21680 addition until the end of the experiment, 25 minutes later. Moreover, this is a consistent effect since in five of the six cells tested this decrease in the amplitude of NMDAR-eEPSCs was observed and only one cell maintained the amplitude of

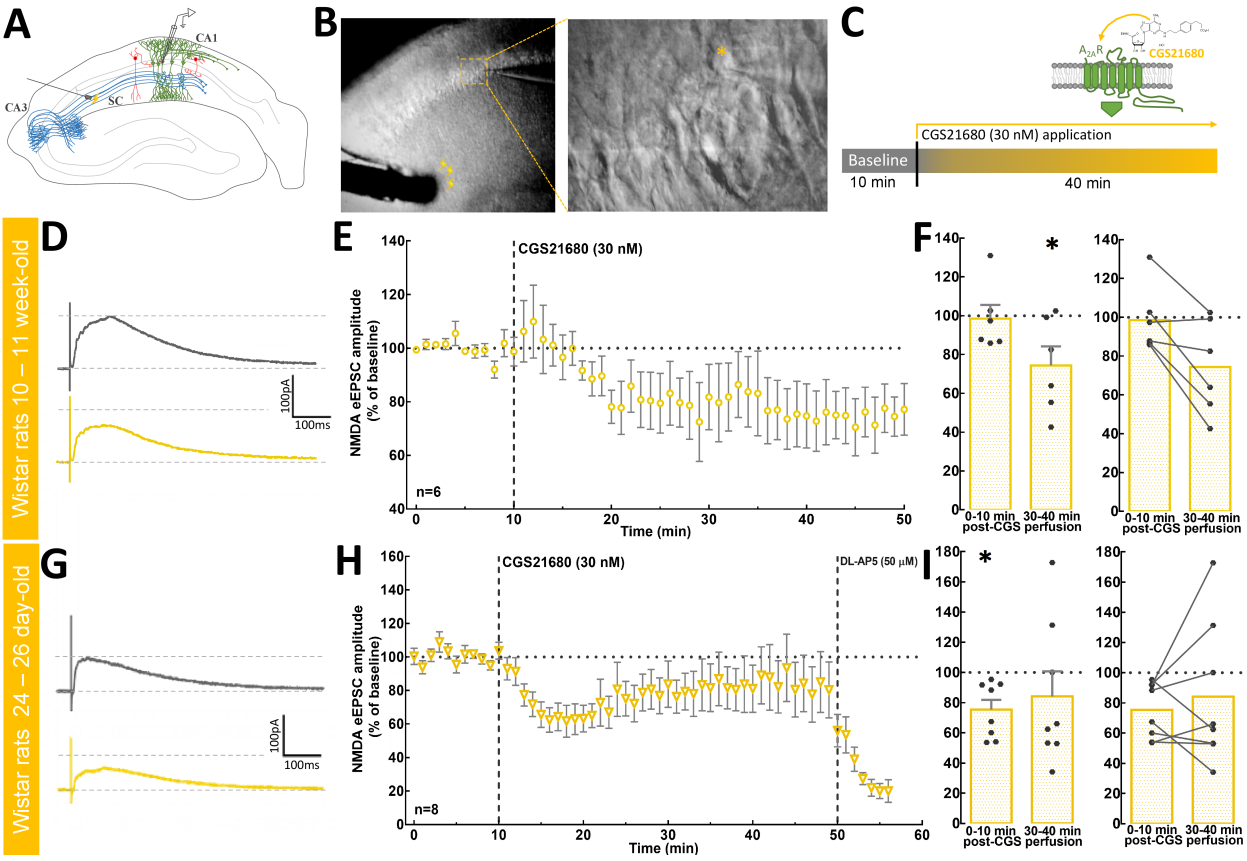

Figure 4.1 **Exogenous activation of $A_{2A}R$ with the selective agonist CGS21680 decreases the amplitude of NMDAR-eEPSCs in CA1 pyramidal cells.** **A** – schematic representation of the positioning of the electrodes at dosomedialhippocampal slice; **B** – photograph of a transversal hippocampal slice with the stimulus electrode placed at the Schaffer collateral afferents and the recording electrode positioned at the pyramidal cell layer of CA1 (left) and an amplified image of a pyramidal neuron being recorded (right); **C** – Experimental design: CGS21680 (30 nM) was applied for 40 minutes after obtaining a stable NMDA-eEPSC for 10 minutes (baseline); **D** – representative traces of the peak effect of CGS21680 on NMDAR-eEPCS (yellow trace), compared to the baseline (gray trace), in 10-11 weeks-old (adult) Wistar rats; **E** – graph of the time course of the experiment showing a decrease in the amplitude of NMDAR-eEPSCs (in percentage of baseline) in slices from adult rats following the superfusion of CGS21680; **F**– scatter dot plot of the effect of CGS21680 from 0-10 min and from 30-40 min the superfusion with the $A_{2A}R$ agonist in adult animals (left panel) and scatter dot plot showing the evolution of the NMDAR-eEPSCs for each neuron throughout the experiment (right panel), where each dot represents one cell; **G** – representative traces of the peak effect of CGS21680 on NMDAR-eEPSCs (yellow trace) comparing to the baseline (gray trace), in 24-26 days-old animals; **H** – graph of the time course graph of the experiment showing a decrease in the amplitude of NMDAR-eEPSC (in percentage of baseline) in slices from young rats following the superfusion of CGS21680; Note that in the presence of DL-AP5, an NMDAR antagonist, the current recorded is nearly abolished confirming that we are indeed recording NMDAR-eEPSC; **I** – scatter dot plot of the effect of CGS21680 from 0-10 min and from 30-40 minutes after superfusion with the $A_{2A}R$ agonist in young animals (left panel) and scatter dot plot showing the evolution of the NMDAR-eEPSCs for each neuron throughout the experiment (right panel), where each dot represents one cell. Data are mean values of the amplitude of NMDAR-eEPSCs calculated as percentage of baseline values (obtained in the absence of CGS21680) ± SEM of n=6 cells for adult rats, and of n=8 cells for young rats; $*p < 0.05$, one-sample t-test comparing to 100.

NMDAR currents (Figure 4.1F, second panel). According to Pearson correlation statistics, there is no correlation between the values obtained in the first 10 minutes and the outcome during the later period of perfusion with CGS21680 (r=0.659 and p>0.05), which means that the initial values of amplitude of evoked NMDAR currents do not predict the behavior of the cell in the last 10 minutes of the experiment.

Interestingly, contrary to what was observed in slices from adult rats, in slices from young animals the effect of CGS21680 is only significant during the first 10 minutes of superfusion with the $A_{2A}R$ agonist (75.39 ± 18.29% of baseline, p<0.05, n=7 cells), Figure 4.1H-I. Afterwards, the effect is gradually lost and it is no longer significant after 30-40 minutes of superfusion with CGS21680 (84.14% ± 47.16% of baseline, n=7 cells). However, in this animals, there is a clear tendency to have two distinct groups of cells, *i.e.*, cells with higher amplitude of eEPSCs in the first ten minutes of superfusion with CGS2168O show a tendency to increase the NMDAR-eEPSCs later on, while cells with lower amplitude of eEPSCs tend to maintain or decrease even more the amplitude of these currents throughout the experiment (r=0.654 and p=0.078, according to Pearson's correlation). Still, more cells will be needed to confirm this correlation.

Notice that for the construction of Figures 4.1H and 4.1I it was used coronal (n=3) and transversal (n=5) slices, however they do not show significant differences between them and so, both slices types were included in the same graph.

Nevertheless, it is important to notice that in both groups of animals the data is consistent, *i.e.*, the exogenous activation of adenosine $A_{2A}R$ decreases the amplitude of NMDAR-dependent eEPSC at CA3-CA1 synapses, which means that $A_{2A}R$ may negatively modulate the activity of NMDA receptors in CA1 pyramidal neurons. Therefore, next it was evaluated whether the selective antagonist of $A_{2A}R$, SCH58261, would produce the opposite effect.

4.2 $A_{2A}R$ exert tonic facilitatory effect on NMDAR-eEPSCs in CA1 pyramidal cells

Superfusion of hippocampal slices for 20 minutes with the selective antagonist of adenosine $A_{2A}R$, SCH58261 (50 nM), decreased the amplitude of NMDAR-eEPSCs in both adult and young animals, Figures 4.2C-F, though the effect is small and transient in adult rats (Figure 4.2D) and more significative and stable in young animals (Figure 4.2G)

In hippocampal slices from adult rats, SCH58261 has a tendency to decrease the amplitude of NMDAR-eEPSCs during the first 10 minutes of superfusion of the antagonist (89.30 ± 14.19% of baseline, n=8 cells, Figure 4.2D and first column of Figure 4.2E). Afterwards, from 20-30 minutes of superfusion with SCH58261, NMDAR currents increase back to basal values (90.97 ± 21.7% of baseline, n=8 cells, Figure 4.2D and 4.2E first panel). In addition, according to Pearson's correlation, neurons can be divided into two groups, *i.e.*, cells displaying higher EPSCs during the first 10 minutes of superfusion with SCH58261 have more probability to maintain or to increase these values whereas cells with lower eEPSCs during the first 10 minutes of superfusion with SCH58261 have a tendency to maintain or further decrease these currents along the experiment (r=0.760 and p<0.05), second panel of Figure 4.2E.

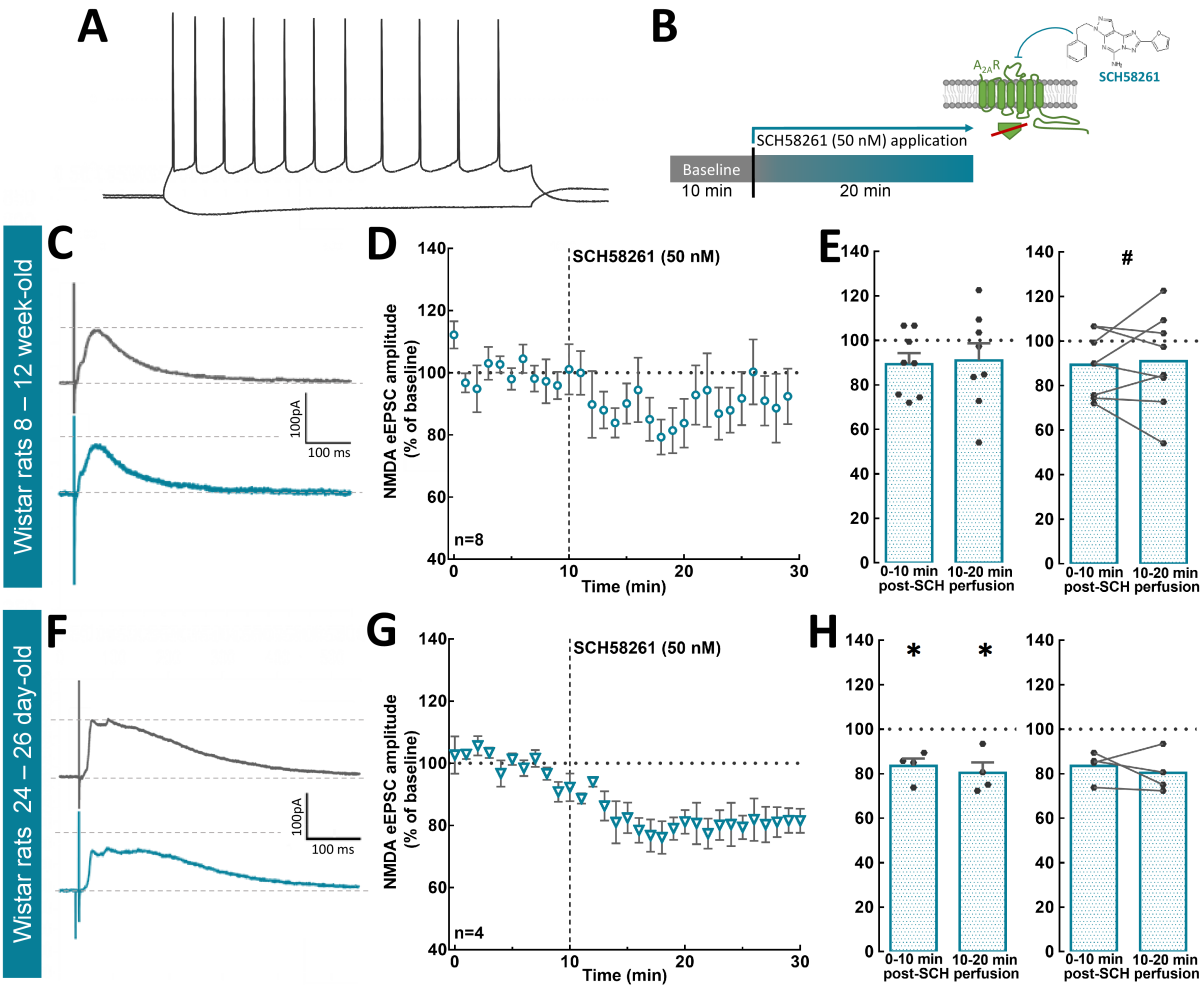

Figure 4.2 $A_{2A}R$ **exerts tonic control of NMDAR-eEPSCs in CA1 pyramidal cells.** **A** – action potential firing pattern of a pyramidal CA1 neuron recorded using a gluconate-based intracellular solution; **B** – Experimental design: $A_{2A}R$ selective antagonist SCH58261 (50 nM) was applied for 20 minutes after obtaining a stable baseline of NMDAR-eEPSC; **C** – representative traces of the peak effect of SCH58261 (blue trace) on NMDAR-eEPSCs, comparing to the baseline (gray trace), in adult Wistar rats; **D** – graph of the time course of the effect of SCH58261 on the amplitude of NMDAR-eEPSCs in hippocampal slices from adult Wistar rats; **E** – scatter dot plot showing the mean values of the amplitude of NMDAR-eEPSCs from 0-10 minutes and 10-20 minutes of superfusion with SCH58261 in slices from adult Wistar rats (left panel) and the scatter plot showing the evolution of these values for each neuron throughout the experiment (rightpanel), each dot represents one cell; **F** – representative traces of the peak effect of SCH58261 (blue trace) on NMDAR-eEPCSs, comparing to the baseline (gray trace), in young Wistar rats; **G** – graph of the time course of the effect of SCH58261 on the amplitude of NMDAR-eEPSCs in hippocampal slices from young Wistar rats; **I** – scatter dot plot showing the mean values of the amplitude of NMDAR-eEPSCs from 0-10 minutes and from 10-20 minutes of superfusion with SCH58261 in slices from young Wistar rats (left panel) and the scatter dot plot showing the evolution of these values for each neuron throughout the experiment (righ panel), each dot represents one cell. Data are mean values of the amplitude of NMDAR-eEPSCs calculated as percentage of baseline values (obtained in the absence of SCH58261) ± SEM of n=8 cells for adult rats, and of n=4 cells for young rats; $*p < 0.05$, one-sample t-test comparing to 100; $\#p < 0.05$ Pearson's correlation statistics.

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No correlation between the age of the animal and the amplitude of NMDAR-eEPSCs was observed.

The effect of SCH58261 in hippocampal slices from younger rats is much more sharp (Figure 4.2G-H). During the first 10 minutes of superfusion with the $A_{2A}R$ antagonist, the amplitude of NMDAR-eEPSCs decreases almost 20% relative to basal values (83.48% ± 6.734% of baseline, $p<0.05$, n=4 cells), Figure 4.2H. Moreover, this effect of SCH58261 is maintained for the remaining time of the experiment (the amplitude values of NMDAR-eEPSCs were 80.37% ± 9.356% relative to the baseline in the last ten minutes interval after superfusion with SCH58261, $p<0.05$, n=4 cells), Figure 4.2H. Finally, there was no differences between the results obtained with transversal hippocampal slices (n=2) and with coronal slices (n=2) which suggests tonic, local effect of $A_{2A}R$ over NMDAR currents.

4.3 $A_{2A}R$ agonist-mediated effect on NMDAR-eEPSCs is prevented in the presence of the $A_{2A}R$ antagonist

If the effect of CGS21680 on NMDAR-eEPSCs is selectively mediated by $A_{2A}R$, then it should be prevented in the presence of the selective antagonist of $A_{2A}R$. Thus, in the next experiment, hippocampal slices from both adult and young rats were superfused with CGS21680 (30 nM) in the presence of the SCH58261 (50 nM), Figure 4.3. First, a stable baseline of NMDAR-eEPSCs was reached during 10 minutes; then the antagonist of $A_{2A}R$ was superfused for, at least, 20 minutes and finally, the agonist of $A_{2A}R$ was added in the presence of SCH58261 for another 40 minutes (Figure 4.3B).

In slices from both adult and young animals, the depression in NMDAR-mediated currents caused by CGS21680 is largely prevented in the presence of SCH58261 (Figure 4.3C-H). In fact, in slices from young rats, the effect is actually reversed (Figure 4.3G and left panel of Figure 4.3H).

In particular, in coronal slices from adult rats, the amplitude of NMDAR-eEPSCs tends to slightly decreases in the first 10 minutes of superfusion with SCH+CGS (93.72 ± 7.978% of the baseline in the presence of SCH58261, n=6 cells), Figure 4.3D and first column of the scatter dot plot depicted in Figure 4.3E. However, the average amplitude of the NMDAR-dependent eEPSCs increases to the values of NMDAR-mediated currents in the presence of SCH58261 alone, throughout the time of exposure of the slice to SCH+CGS (107.2 ± 28.69% of the baseline in the presence of SCH58261, n=6 cells, after 30-40 minutes of superfusion with SCH+CGS).

In hippocampal slices from 24-26 days-old rats, there is no change in the amplitude of NMDAR-EPSCs during the first 10 minutes of co-applying CGS21680 with the antagonist of $A_{2A}R$ (104.8 ±14.99% of the baseline in the presence of SCH58261 alone, n=4 cells); however, afterwards, there is a sustained increase in NMDAR-mediated currents in the presence of SCH+CGS comparing with SCH58261 alone (117.0 ± 8.630% of the baseline in the presence of SCH58261 alone, $p<0.05$, n=4 cells, from 30 to 40 minutes of superfusion with SCH+CGS) Figure 4.3G and left panel of Figure 4.3H.

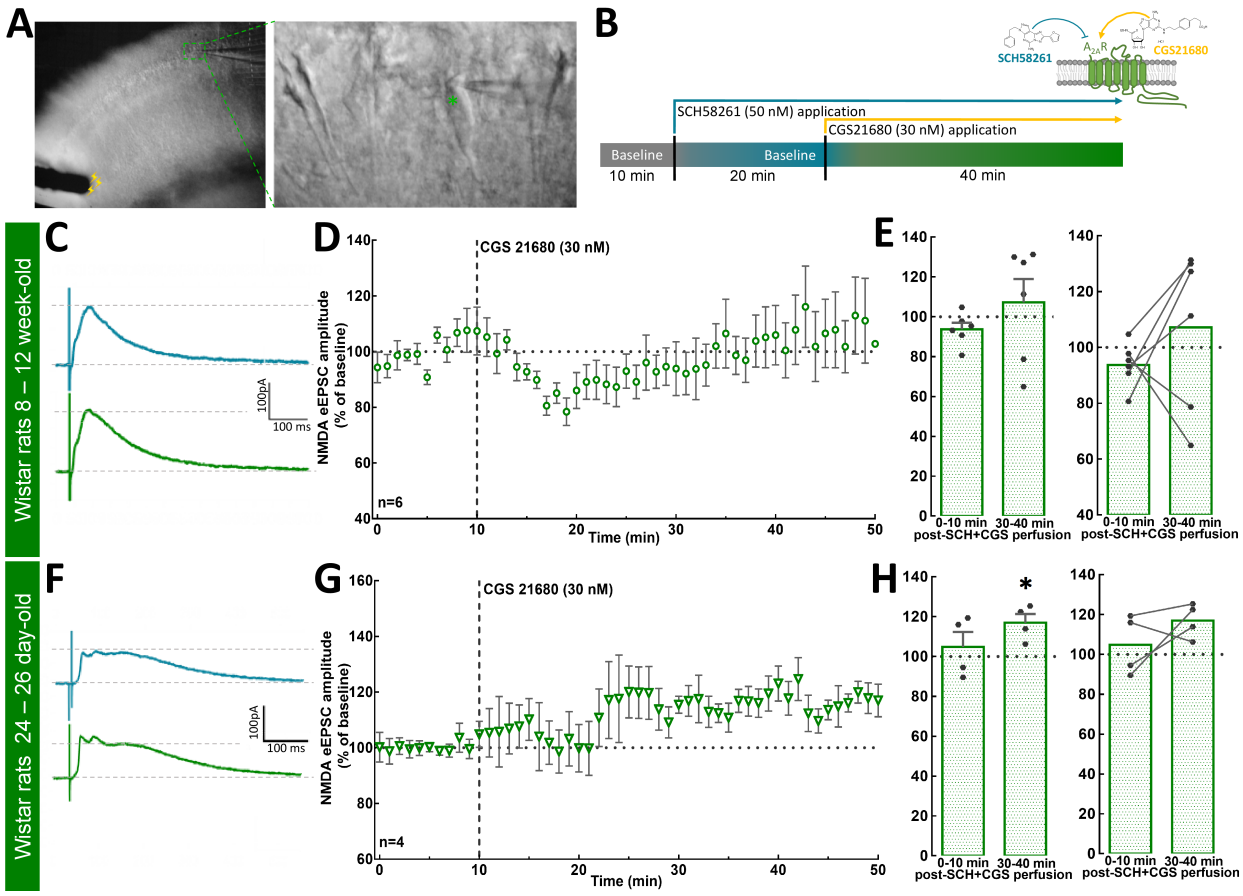

Figure 4.3 **The selective $A_{2A}R$ antagonist prevents CGS21680-induced decrease in NMDAR-eEPSCs. A** – photograph of a transversal hippocampal slice with the stimulus electrode placed at the Schaffer collateral afferents and the recording electrode positioned at the pyramidal cell layer of CA1 (left) and an amplified image of a pyramidal neuron being recorded (right);; **B** – Experimental design: the antagonist of $A_{2A}R$ was superfused for, at least, 20 minutes and after reaching a stable baseline of NMDAR-eEPSCs during 10 minutes, the selective agonist of $A_{2A}R$, CGS21680 30 nM, was superfused for 40 minutes, always in the presence of SCH58261; **C** – representative traces of the peak effect of SCH+CGS (green trace) on NMDAR-eEPSCs, comparing to the baseline with SCH58261 (blue trace), in adult Wistar rats; **D** – graph of the time course of the effect of SCH+CGS on the amplitude of NMDAR-eEPSCs in hippocampal slices from adult animals; **E** – scatter dot plot showing the mean values of the amplitude of NMDAR-eEPSCs from 0-10 minutes and from 30-40 minutes of superfusion with SCH+CGS in slices from adult Wistar rats (left panel) and the scatter dot plot showing the evolution of these values for each neuron throughout the experiment (right panel) where each dot represents one cell; **F** – representative traces of the peak effect of SCH+CGS (green trace) on NMDAR-eEPSCs, comparing to the baseline with SCH58261 (blue trace), in young Wistar rats; **G** – graph of the time course of the effect of SCH+CGS on the amplitude of NMDAR-eEPSCs in hippocampal slices from young Wistar rats; **I** – scatter dot plot showing the mean values of the amplitude of NMDAR-eEPSCs from 0-10 minutes and from 30-40 minutes of superfusion with SCH+CGS in slices from young Wistar rats (left panel) and the scatter dot plot showing the evolution of these values for each neuron throughout the experiment (right panel), each dot represents one cell. Data are mean values of the amplitude of NMDAR-eEPSCs calculated as percentage of baseline values (obtained in the absence of SCH58261) ± SEM of n=8 cells for adult rats, and of n=4 cells for young rats; $*p < 0.05$, one-sample t-test comparing to 100.

In order to understand the effect of endogenous adenosine on NMDAR-eEPSCs, the amplitude of these currents were examined in the presence of adenosine deaminase, an enzyme that converts adenosine into inosine.

4.4 Adenosine exerts a tonic inhibitory effect on NMDAR-eEPSCs in CA1 pyramidal cells

Adenosine deaminase (ADA) is an enzyme that does not cross cell membranes and it is usually an inactive ligand for adenosine receptors (Sebastião and Ribeiro, 2009). Thus, this enzyme is widely used when the removal of endogenous adenosine from preparations is required. Therefore, once a stable baseline of NMDAR-eEPSCs was achieved during 10 minutes, ADA (2 U/mL) was superfused for 30 minutes, as schematized in the Figure 4.4B.

The superfusion of hippocampal slices, from both young and adult rats, with ADA significantly increased the amplitude of NMDAR-eEPSCs (Figure 4.4C-H). This effect had slightly different kinetics in slices from adult *versus* young animals (Figure 4.4D and Figure 4.4G, respectively), reaching a plateau in slices from adult rats (Figure 4.4D), whereas in slices from young Wistar rats the effect of ADA kept increasing throughout the time of experiment (Figure 4.4G) and reached a higher magnitude (Figure 4.4G and left panel of Figure 4.4H).

In particular, in slices from adult animals the amplitude of NMDAR-eEPSCs slightly increased in the first 10 minutes of superfusion with ADA (112.9 ± 11,14% of baseline , n=3 cells), peaked approximately 5 minutes later and remained at the same values until the end of the experiment (148.0 ± 40.28% of baseline, n=3 cells).

On the other hand, in hippocampal slices from younger Wistar rats, ADA also caused a slight increase in the amplitude of NMDAR-eEPSCs in the first 10 minutes (115.3 ± 14.39% of baseline, n=3 cells), Figure 4.4G-H, but these values continuously increased until the end of the experiment (167.8 ± 30.83% of baseline, n=3 cells), Figure 4.4G-H.

4.5 Removal of endogenous extracellular adenosine does not prevent the $A_{2A}R$ antagonist-induced decrease in NMDAR-eEPSCs

In order to know if the removal of adenosine from the extracellular medium would prevent the effect of blocking $A_{2A}R$ on NMDAR-eEPSCs, hippocampal slices were perfused with SCH58261 (50 nM) in the presence of ADA (2 U/mL), after obtaining a stable baseline of 10 minutes with the superfusion of ADA alone, as shown in Figure 4.5B.

Surprisingly, the effect of SCH58261 on NMDAR-eEPSCs after removal of endogenous adenosine is still observed in neurons from the hippocampus of both adult and young rats (Figure 4.5D and 4.5G). In particular, in adult animals, the $A_{2A}R$ antagonist caused an initial decrease of *circa* 20% in NMDAR currents, during the first 10 minutes (83.74 ± 12.64% of baseline in the presence of ADA alone, n=4 cells), Figure 4.5D and first column of 4.5E. Then,

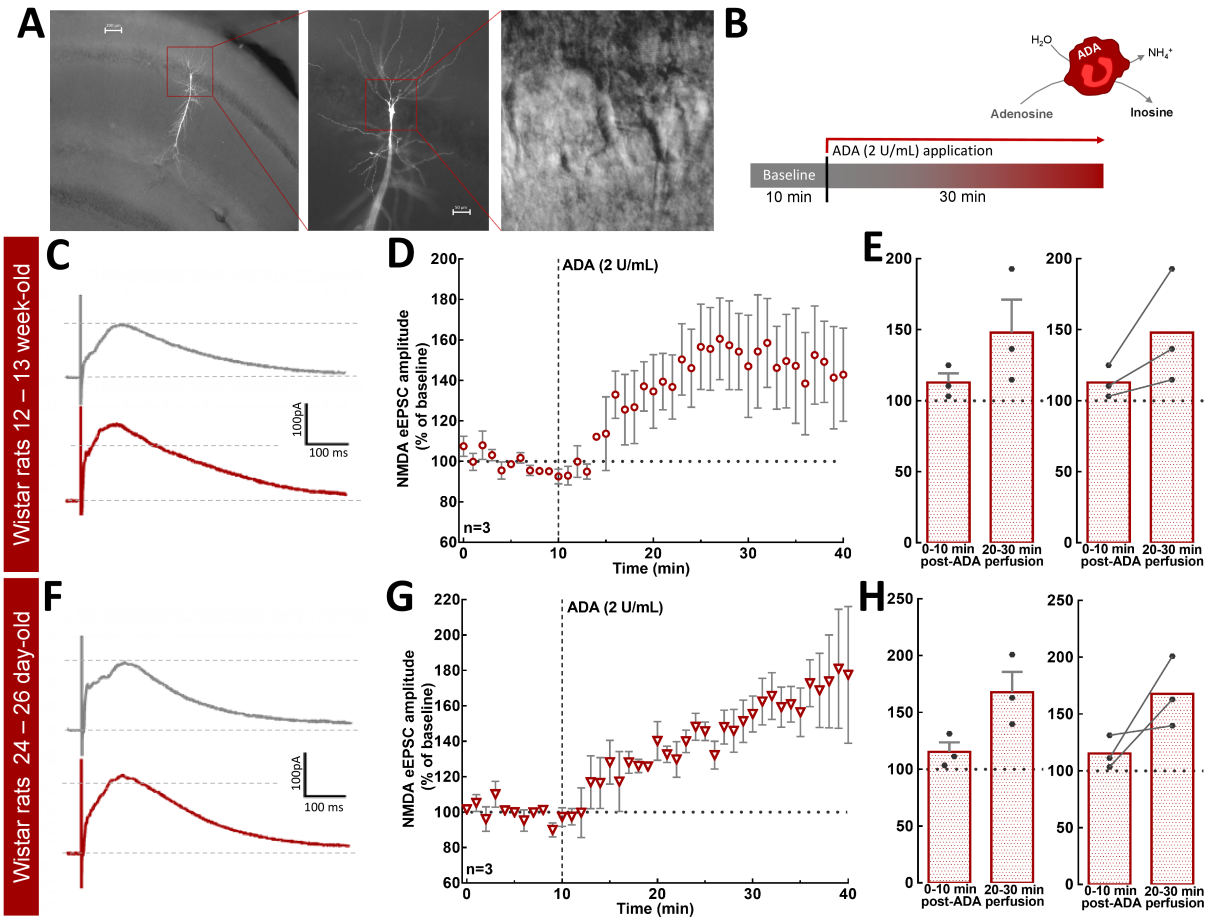

Figure 4.4 **Endogenous adenosine exerts tonic inhibitory effect on NMDA-eEPSCs in CA1 pyramidal cells.** **A** – Biocytin-labeled CA1 pyramidal neuron from an hippocampal slice from an adult rat: the first image shows the entire pyramidal cell then, at the center, the cell body together with the basal and proximal apical dendrites is shown in detailed and finally, at the left, there is an image of the same neuron being recorded; **B** – Experimental design to study the effect of endogenous adenosine on NMDAR-eEPSCs: adenosine deaminase (ADA, 2 U/mL) was applied for 30 minutes after obtaining a stable baseline of NMDAR-eEPSC; **C** – representative traces of the peak effect of ADA (red trace) on NMDAR-eEPSCs, comparing to the baseline (gray trace), in adult Wistar rats; **D** – graph of the time course of the effect of ADA on the amplitude of NMDAR-eEPSCs in hippocampal slices from adult Wistar rats; **E** – scatter dot plot showing the mean values of the amplitude of NMDAR-eEPSCs from 0-10 minutes and 20-30 minutes of superfusion with ADA in slices from adult Wistar rats (left panel) and the scatter plot showing the evolution of these values for each neuron throughout the experiment (right panel), each dot represents one cell; **F** – representative traces of the peak effect of ADA (red trace) on NMDAR-eEPSCs, comparing to the baseline (gray trace), in young Wistar rats; **G** – graph of the time course of the effect of ADA on the amplitude of NMDAR-eEPSCs in hippocampal slices from young Wistar rats; **I** – scatter dot plot showing the mean values of the amplitude of NMDAR-eEPSCs from 0-10 minutes and from 20-30 minutes of superfusion with ADA in slices from young Wistar rats (left panel) and the scatter dot plot showing the evolution of these values for each neuron throughout the experiment (right panel), each dot represents one cell. Data are mean values of the amplitude of NMDAR-eEPSCs calculated as percentage of baseline values (obtained in the absence of ADA) ± SEM of n=3 cells for adult rats, and of n=3 cells for young rats; $*p < 0.05$, one-sample t-test comparing to 100.

this effect is temporarily lost and partially recovered in the last 10 minutes of the experiment (85.77 ± 11,65% of baseline in the presence of ADA alone, n=4 cells), Figure 4.5D-E.

In hippocampal neurons from young rats, the effect of SCH58261 on NMDAR-eEPSCs after removal of endogenous adenosine is even more pronounced than in adult animals (Figure 4.5G-H) and of slightly increased magnitude comparing to the effect of SCH58261 in the absence of ADA (Figure 4.5G-H). During the first 10 minutes of superfusion with ADA+SCH the amplitude of NMDAR-dependent eEPSCs significantly decreased (83.28 ± 5.135% of baseline in the presence of ADA alone, $p<0.05$, n=3 cells) and, within 10-20 minutes, the amplitude of NMDAR-eEPSCs gets even lower (74.37 ± 14.50% of baseline in the presence of ADA alone, n=3 cells), Figure 4.5G-H.

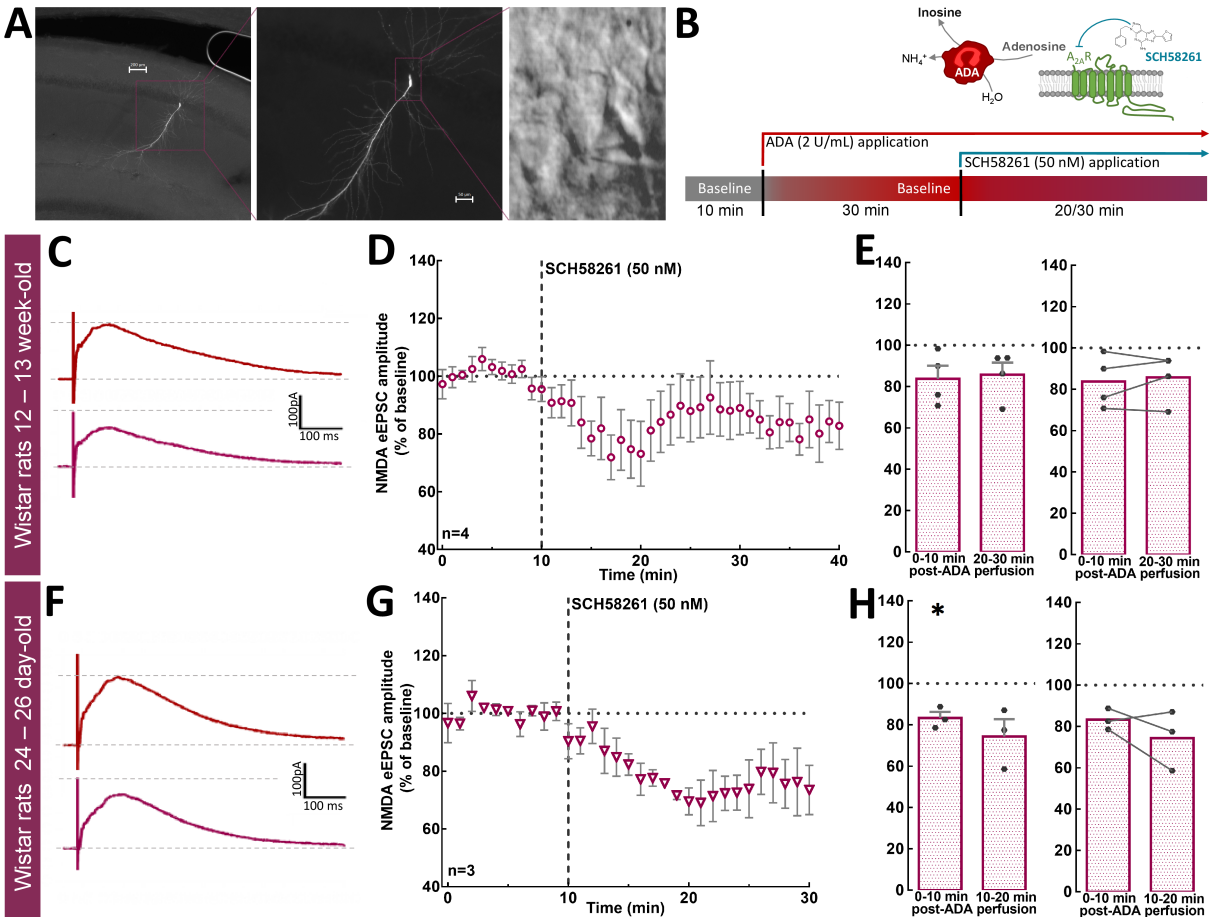

Figure 4.5 **Removal of adenosine does not prevent the $A_{2A}R$ antagonist-induced decrease in NMDAR-eEPSCs in CA1 pyramidal cells. A** – Biocytin-labeled CA1 pyramidal neuron from an hippocampal slice from a young rat: the first image shows the entire pyramidal cell then, at the center, the cell body together with the basal and proximal apical dendrites is shown in detailed and finally, at the left, there is an image of the same neuron being recorded; **B** – Experimental design: after the superfusion of ADA ($2\ U/mL$) for at least 30 minutes until have a baseline of NMDAR-dependent eEPSCs stable for 10 minutes, then the antagonist of $A_{2A}R$, SCH58261 50 nM, was superfused for more 20 or 30 minutes, always in the presence of ADA; **C** – representative traces of the peak effect of ADA+SCH (purple trace) on NMDAR-eEPSCs, comparing to the baseline with ADA alone (red trace), in adult Wistar rats; **D** – graph of the time course of the effect of ADA+SCH on the amplitude of NMDAR-eEPSCs in hippocampal slices from adult Wistar rats; **E** – scatter dot plot showing the mean values of the amplitude of NMDAR-eEPSCs from 0-10 minutes and 30-40 minutes of superfusion with ADA+SCH in slices from adult Wistar rats (left panel) and the scatter plot showing the evolution of these values for each neuron throughout the experiment (right panel), each dot represents one cell; **F** – representative traces of the peak effect of ADA+SCH (purple trace) on NMDAR-eEPSCs, comparing to the baseline with ADA alone (red trace), in young Wistar rats; **G** – graph of the time course of the effect of ADA+SCH on the amplitude of NMDAR-eEPSCs in hippocampal slices from young Wistar rats; **I** – scatter dot plot showing the mean values of the amplitude of NMDAR-eEPSCs from 0-10 minutes and from 20-30 minutes of superfusion with ADA+SCH in slices from young Wistar rats (left panel) and the scatter dot plot showing the evolution of these values for each neuron throughout the experiment (right panel), each dot represents one cell. Data are mean values of the amplitude of NMDAR-eEPSCs calculated as percentage of baseline values (obtained in the absence of ADA) ± SEM of n=4 cells for adult rats, and of n=3 cells for young rats; $*p < 0.05$, one-sample t-test comparing to 100.

Chapter 5

Discussion

In this work we have studied the modulation of NMDA receptor currents by the adenosine A_{2A} receptor in the Schaffer collaterals-CA1 synapses.

The main findings of the study hereby presented are that a) exogenous activation of $A_{2A}R$ by the selective agonist CGS21680 decreases NMDAR-dependent eEPSCs, b) the effect of CGS21680 is no longer observed in the presence of a selective $A_{2A}R$ antagonist, SCH58261, which c) also decreases basal NMDAR-eEPSCs, suggesting that the $A_{2A}R$ may exert a tonic control of these currents in CA1 pyramidal cells. Finally, this study revealed that d) our slice preparations contain high levels of endogenous adenosine which e) once removed does not prevent the $A_{2A}R$ antagonist-induced decrease in NMDAR-eEPSCs in SC-CA1 synapses.

5.1 Contribution of different populations of $A_{2A}R$ may explain the distinct modulatory effects on the activity of NMDAR

The $A_{2A}R$ have been mainly showed to facilitate synaptic transmission, including at Schaffer Collaterals-CA1 synapses (Cunha et al., 1994a; De Mendonça and Ribeiro, 1994; Lopes et al., 2002; Mouro, 2012; Sebastiao and Ribeiro, 1992), but also in other hippocampal synapses (Rebola et al., 2008) or even at other brain regions (Brown et al., 1990a). However, our results show that a selective agonist of $A_{2A}R$ (CGS21680) causes a decrease, instead of an increase, in NMDAR-dependent eEPSCs. Thus, this is at odd with previous studies showing a positive modulation of these currents by $A_{2A}R$ (Cunha et al., 1994a; Lopes et al., 2002; Mouro, 2012; Rebola et al., 2008).

These discrepancies may be explained, in part, by the fact that most of these studies in the CA1 region, were based on extrasynaptic measurements whereas in this work, we used patch clamp to record single-neuron responses. Thus, in our data the recorded NMDAR-mediated currents and the modulatory effects of $A_{2A}R$ may reflect different populations of $A_{2A}R$, which may be asymmetrically distributed and contribute to the variability in the results. In fact, Li and Henry, 1998a found an inhibitory effect of adenosine in all CA1 pyramidal neurons

with exception of two or three cells which may suggest that these neurons were not equally endowed with adenosine receptors.

On the other hand, applying the agonist of $A_{2A}R$ in the presence of a selective antagonist, of these receptors revealed a different effect of the agonist on NMDAR-eEPSCs *i.e.* now CGS21680 potentiated the activity of NMDAR. This not only suggests that not all $A_{2A}R$ were active in basal conditions to be inactivated by SCH58261 but also that these receptors that became active in the presence of CGS21680 potentiate NMDAR-mediated currents. These findings reinforce our previous hypothesis of distinct pools of $A_{2A}R$.

This, however, raises another question: which population of $A_{2A}R$ is being responsible for each of the observed effects? The described presynaptic effects CGS21680 (10 nM) in CA1 pyramidal cells are also of a facilitation of excitatory synaptic transmission, *i.e.* the inhibition of paired pulse facilitation (PPF) and the facilitation of glutamate release in the presence of tonic A_1R-activation (Lopes et al., 2002). Moreover, it is though that the ATP released upon neuronal stimulation will be converted into adenosine that will preferentially activate presynaptic $A_{2A}R$ (reviewed by Lacaille and Schwartzkroin, 1988 and Zimmermann, 1994).

On the other hand, application of adenosine at the CA1 region causes an inhibition of evoked EPSPs but in half of the neurons this is followed by an increase in the excitatory postsynaptic currents and therefore in neuronal depolarization (Dunwiddie et al., 1981; Haas and Greene, 1984).

Pharmacological analysis concluded that the activation of postsynaptic $A_{2A}R$, as assessed in the presence of TTX (tetrodotoxin), a pharmacological tool that blocks the generation of action potentials and spontaneous neuronal activity-related manifestations, is responsible for this late excitatory effect of adenosine Li and Henry, 1998a. Thus, it is possible that pre- and postsynaptic $A_{2A}R$ are differently engaged upon stimulation of the Schaffer collaterals and exert an overall opposite effect on neuron excitability. Also, according to these results not all CA1 pyramidal neurons are endowed with excitatory $A_{2A}R$. Moreover, Mouro, 2012 had seen that activation of $A_{2A}R$ potentiates NMDAR through an increase of NMDA-dependent postsynaptic currents, an effect abolished in slices superfused with the selective $A_{2A}R$ antagonist SCH58261 (100 nM). However, the method of stimulation involved the application of *puffs* of NMDA to directly stimulate the receptor, a method that can activate synaptic NMDAR but that mainly activate extrasynaptic NMDAR.

In our experiments, we are not blocking presynaptic activity as we stimulate the afferent Schaffer collateral fibers, thus NMDAR stimulation is made through the release of its endogenous agonist. Since NMDAR are coincident receptors, being activated only upon both presynaptic stimulation and postsynaptic depolarization, we clamped the neuron at +40 mV in order to release the blockade of NMDAR channels by magnesium and facilitate the activation of these glutamate receptor, however, we are still in basal conditions since no protocols to induce a change in plasticity response were applied. Therefore, to distinguish between pre- and postsynaptic effects of $A_{2A}R$ over NMDAR-mediated currents, we will have to conduct an experiment in the presence of TTX. This is even more relevant considering that in the hippocampus $A_{2A}R$ are more abundant at the presynaptic site than at postsynaptic

(Rebola et al., 2005). The use of TTX in the Mouro, 2012 may explain why the superfusion of SCH58261, did not alter basal neurotransmission, contrary to our results. This, further support our hypothesis that the effect obtained with CGS21680 in our experiments is mediated by presynaptic $A_{2A}R$.

Confirming the theory that different populations of $A_{2A}R$ cause opposite modulation of NMDAR-eEPSCs, there is a tendency to have two different groups of neurons that respond in an opposite way to both SCH58261 (right graph of the Figure 4.2E) and CGS21680 (right graph of the Figure 4.1I), according to the Pearson's correlation analysis. Still, a higher number of cells should be recorded in order to confirm this result. Also, in the medium spiny neurons on the striatum, Wirkner et al., 2000 found that NMDAR-mediated currents are not altered by CGS21680 and confirmed that those neurons were not endowed with $A_{2A}R$.

Another curious result in our data is the fact of the selective blockade of $A_{2A}R$ with SCH58261 decreased NMDAR-mediated currents, suggesting that $A_{2A}R$ contribute to basal neurotransmission, which is at odds with previous findings showing a preferential activation of A_1R in this case (Costenla et al., 2011; Cunha et al., 1997; Lupica et al., 1990; Rebola et al., 2008). At resting potentials ($\leqslant -60\ mV$), the currents dependent of NMDAR are substantially attenuated by the magnesium block, however, by clamping the neuron at $+40\ mV$ this blockade is removed which induces a state of pre-activation of the NMDAR, which became more prone to be activated. We are not applying protocols to induced synaptic plasticity processes although we are forcing somehow the activation of NMDAR. However we are still at basal conditions. Nevertheless, in mossy fibers-CA3 synapses, Rebola et al., 2008 also used electric stimulation with a $Vh = +30\ mV$, however, in this hippocampal region, $A_{2A}R$ are mainly postsynaptic and the presynaptic receptors had no role in the observed effects. Besides, the hippocampal circuit involving CA3 pyramidal cells and mossy fibers pathways is very unique due to the particular constitution of dentate gyrus neuronal population and its particular influence upon internal hippocampal circuit. Contrary to what was observed in the CA1 field, in CA3 region CGS21680 caused a potentiation of NMDAR-dependent EPSCs, although the $A_{2A}R$ had no role in basal transmission and the system was fully saturated since the superfusion of CGS21680 after the antagonist pretreatment had no effect on NMDA-eEPSCs.

5.2 The method of stimulation influences NMDAR-mediated currents

In electrophysiological experiments, the type and pattern of stimulation used to trigger a response will condition that same response.

One of the problems with the electric stimulation of afferent fibers within a certain circuit is that if not perfectly adjusted to the physiological situation, it could result in a spillover of the neurotransmitters, in this case, glutamate, which may also activate extrasynaptic receptors. Extrasynaptic NMDAR are known to have a different composition and function in neurotransmission. Indeed, we get some recordings with smooth changes in the receptor deactivation kinetics, yet this currents, in the presence of DL-AP5 were nearly abolish, thus

they are dependent of NMDAR. Therefore, accordingly to what was reviewed in the Section 1.6.1, the different kinetics conferred to the NMDAR by the GluN2 subunits determine the total charge that is allowed to cross the channel. As a consequence, subunit composition regulates the amount of sodium e calcium that will enter into the neuron and contribute to neuronal depolarization together with ignition of specific signaling cascades. This could potentially explain some of the variability of our results in adult and young Wistar rats.

In addition, the intrinsic characteristics of each of the recorded neurons may differ and require adjustments in order to adjust the intensity of the stimulus needed to achieve the same range of amplitude of NMDAR-eEPSCs (90<NMDAR-eEPSC amplitude<150, in pA), which is important to avoid ceiling effect.

On the other hand, in some of the works here mentioned, the authors applied *puffs* of NMDA instead of electrical stimulation. This method activates mainly extrasynaptic NMDA. Since $A_{2A}R$ are also present at perisynaptic and at extrasynaptic zones, these receptors can also have a different impact on extrasynaptic populations of NMDAR.

It would be interesting to accurate whether $A_{2A}R$ is modulating one or another population of NMDAR despite the type of stimulation. So, continuing with electrical stimulation we could pharmacologically isolate synaptic NMDAR-dependent currents through the use of different strategies, since the precise isolation of one or another population of NMDAR is not unanimous. For instance, memantine, which preferentially blocks extrasynaptic NMDAR (Xia et al., 2010) and, another possibility, the use of the selective antagonist of GluN2B subunit, Ro 04-5595 (Mutel et al., 1998), since this subunit is known to be mainly extrasynaptic (reviewed in Wyllie et al., 2013).

5.3 Adenosine deaminase may not efficiently remove adenosine from the synapses

Endogenous adenosine is known to exert an inhibitory effect on synaptic transmission and thus, to decrease neuronal excitability in hippocampal slices (Dunwiddie et al., 1981; Haas and Greene, 1984). Accordingly, the removal of endogenous adenosine with adenosine deaminase (ADA) caused a significant increase in NMDAR-dependent eEPSCs (De Mendonça and Ribeiro, 1994), however it did not prevent the inhibitory effect of the selective antagonist of $A_{2A}R$.

Adenosine deaminase does not cross the membrane neither associates with adenosine receptors (reviewed in Sebastiao and Ribeiro, 2009). Nevertheless, in order to be sure that ADA was not interfering with adenosine receptors an inhibitor of its enzymatic function could be superfused in the slice before the application of ADA. Thus, if ADA was interfering with the receptors would be seen an alteration of the NMDAR-mediated current, contrary to what is expected in case of ADA do not interfere with the adenosine receptors.

However, a possible explanation for SCH58261 still having an effect in the presence of ADA could be that this enzyme may not efficiently reach and remove all adenosine present in synapses and consequently $A_{2A}R$ may still be activated. Nevertheless, this factor would be difficult to isolate due to the constant generation of adenosine at synapses either through

equilibrative transports or through the extracellular catabolism of ATP, which involves a variety of enzymes (Zimmermann, 2006). Moreover, the evidences of a physical interaction between CD73 and $A_{2A}R$ (Augusto et al., 2013 in addition to preliminary data of our lab) suggest an increased posibility of the activation of the presynaptic $A_{2A}R$, thus the removal of endogenous adenosine by ADA *in loco* directly from the source seems unlikely. There is seen however an increase in NMDAR-dependent eEPSCs with superfusion of ADA alone because A_1R are largely affected by the lower levels of adenosine.

A tool already available is an adenosine sensor that may be placed in the region of interest to continuously measure the extracellular levels of adenosine. Moreover, this tool allows, in parallel, extracellular electrophysiological recordings (Dale et al., 2000), although it is not synaptic specific and so, it is not possible to confirm our hypothesis through the use of this sensor.

Another problem associated with the use of ADA is related to the generation of inosine as a result of the deamination of adenosine. Inosine is known to interact with some of adenosine receptors, namely the A_3R (Jin et al., 1997). In fact, A_3R are detected in the hippocampus CA1 field (Fredholm et al., 2001) and even though the activation/inhibition of A_3R has no effect *per se* on basal synaptic transmission, these are able to reduce the sensitivity of presynaptic A_1R in the CA1 field of hippocampus (Dunwiddie et al., 1997) as well as to modulate phenomena of LTP and LTD in the hippocampus (Costenla et al., 2001). This could explain the attenuation in the effect of SCH58261 after 10 minutes of superfusion of the antagonist in the presence of ADA, in hippocampal slices from adult rats. However, this was not observed in slices from young Wistar rats.

5.4 Distinct hippocampal inputs may differentially impact NMDAR-mediated responses at CA1 pyramidal cells

When using hippocampal slices, especially with the coronal orientation, it is important to mention that the CA1 area receives abundant feed forward excitation from several extrinsic sources as well as relatively sparse recurrent glutamatergic inputs from local pyramidal cells. Then, is not unlikely that one of the reasons for the observed variability in our results may be due to external hippocampal inputs through the entorhinal cortex. Although, we stimulate the Schaffer collaterals pathway (the last step of the trisynaptic circuit) in the coronal slices, CA1 pyramidal neurons still receive inputs directly from the perforant path via ECIII neurons. Moreover, adenosine A_1 and A_{2A} receptors are also present in the EC, where they have a role in epileptogenesis (Hosseinmardi et al., 2007; Rivkees et al., 1995), in addition to other brain regions like amygdala, the thalamus and others that may influence CA1 neurons, depending on the type of coronal slices (Szilagyi et al., 2011).

As previously mentioned, the perforant path and the Schaffer collateral fibers enervate different parts of the CA1 pyramidal cells, and an heterogeneous distribution of adenosine receptors was found along the different parts of these neurons, namely a higher density of A_1R, in the *s. radiatum* relative to the *s. lacunosum/moleculare*, which implicates smooth physiological differences between these regions (Lee et al., 1983).

It is also known that the Schaffer collateral synapse preferentially with basal dendrites (*s. oriens*) and with the proximal part of the apical dendrites (*s. radiatum*) whereas the perforant fibers from ECIII neurons have almost a one-to-one connection with the distal part of apical dendrites (*s. lacunosum moleculare*) (Ishizuka et al., 1990; Li et al., 1994). This means that in coronal slices, CA1 cells receive more inputs onto *s. lacunosum moleculare* compared to CA1 neurons in transversal slices, which may contribute to some of the variability observed in coronal slices.

On the other hand Cunha et al., 1994a showed that the binding of [3H]CGS21680 was higher in the *stratum radiatum* of the CA1 area, which does not receive inputs from the PP but from the SC collaterals.

Moreover, the CA3 region is also under the influence of $A_{2A}R$ (Rebola et al., 2008) which are present at the *stratum radiatum*, even though in a lower density than in the CA1 region Cunha et al., 1994a. Thus, direct modulation of the neurotransmission at the CA3 region by the $A_{2A}R$ is likely to influence the output responses to the CA1 pyramidal neurons. A way to overcome this problem is to make a cut between the CA3 and the CA1 regions, a procedure used previously by other scientists (e.g. Costenla et al., 2001; Lopes et al., 2002; Rombo et al., 2015).

5.5 Desensitization of $A_{2A}R$ and/or unspecific binding of the agonist unlikely accounts for the observed NMDAR-eEPSCs in CA1 pyramidal neurons

It seems unlikely that $A_{2A}R$ desensitization accounts for the CGS21680-mediated decrease in the amplitude of NMDAR-eEPSCs amplitude since this effect happens shortly after the initial superfusion with the agonist. Moreover, the concentration used of 30 nM has been shown to cause an increase of NMDAR-eEPSCs (Jarvis and Williams, 1989; Li and Henry, 1998a; Mouro, 2012; Rebola et al., 2008; Rombo et al., 2015; Sarantis et al., 2015). Nevertheless, lower concentrations of CGS21680 (10 nM) have also been used in hippocampal slices (Cunha et al., 1994a; Lopes et al., 2002; Sebastiao and Ribeiro, 1992).

Also, it is unlikely that the effects observed with CGS21680 are due to the targeting of A_1R since there are several studies reporting that CGS21680 binds poorly to A_1R. In addition, binding studies using labeled CGS21680 indicated that the K_i for A_1R is of 2600 nM whereas the K_i for $A_{2A}R$ is of 15 nM (Jarvis and Williams, 1989). Therefore, only concentrations of CGS21680 above 10 μM are expected to bind to A_1R and to have inhibitory effects (Lopes et al., 2002; Lupica et al., 1990). However Li and Henry, 1998a reported that 20 μM of CGS21680 was a weaker agonist of A_1R in hippocampus CA1 pyramidal neurons.

Nevertheless, other authors reported an inhibition of NMDAR-mediated currents with the superfusion of $0,1$ μM of CGS21680 (Nörenberg et al., 1998; Wirkner et al., 2000). In this work, it was used a concentration of 30 nM of CGS21680 which is far more smaller than the concentration required to activate A_1R and was reported to only target $A_{2A}R$ in the hippocampus (Lopes et al., 2002).

Interestingly, it has been reported that this $A_{2A}R$ agonist may bind at two different high-affinity binding sites on the receptor with different affinities. The "typical" binding site is mainly found in the striatum and at a low density in the hippocampus and cerebral cortex. Moreover, the "atypical" binding site is mainly found at the hippocampus (Cunha et al., 1997). The exact behavior of this two different bindings sites is not well understood, however, this show that $A_{2A}R$ agonist/antagonist response may be region-dependent. In fact, the recent studies with the $opto A_{2A}R$ have shown that the signaling pathway triggered by the $A_{2A}R$ in striatal and hippocampal neurons is distinct and related with different behavioral responses (Li et al., 2015).

5.6 An inhibitory action of CGS21680 was previously reported in the striatum

There are some studies reporting that CGS21680 inhibits the conductance of NMDA receptor channels on striatal medium spiny neurons (Nörenberg et al., 1998, 1997) and that it causes a decrease in NMDAR-dependent eEPSCs (Wirkner et al., 2000).

Wirkner et al., 2000 has indeed identified two groups of neuronal cells, one that was insensitive to CGS21680, and it was, in fact, devoided of $A_{2A}R$, and another group that was CGS21680-senstive in which the presence of $A_{2A}R$ has been confirmed by immunohistochemistry. This approach is hard to take in hippocampal neurons given the much lower density of $A_{2A}R$ comparing to the striatum.

Moreover, in this work, the authors showed that the pathway triggered by $A_{2A}R$ activation with CGS21680 and responsible for the decrease in NMDAR-mediated currents was not the canonical cAMP-PKA but rather the PLC-CaMKII. The activation of CaMKII caused the displacement of α-actinin-2 from NMDAR GluN1 subunits and consequently to the rupture of the NMDAR-cytoskeleton linkage. This resulted in the of NMDAR clusters from the postsynaptic neuron.

Thus, it is possible that our results reflect a similar activation of PLC-IP_3-CaMKII or of other non-canonical pathways by $A_{2A}R$. In fact, Cunha and Ribeiro, 2000 have shown that inhibiting PKA in the CA1 field of hippocampus did not prevented presynaptic $A_{2A}R$-mediated effects and that the activation of PKC may be involved.

5.7 The heteromerization between $A_{2A}R$ and $mGluR_5$ may be relevant to the observed modulatory effects of $A_{2A}R$

The association of adenosine A_{2A} receptors with other brain receptors, especially with the glutamate $mGluR_5$ receptor has been intensely explored and this particular interaction may explain the observed $A_{2A}R$-mediated effects in CA1 pyramidal neurons. Accordingly, these receptors are known to interact and to co-localize in the hippocampus (Aniksztejn et al., 1991; Anwyl, 1999; Benquet et al., 2002; Köles et al., 2016; Sarantis et al., 2015; Tebano et al., 2005). Moreover, it is described a positive modulation of NMDAR-eEPSCs by $mGluR_5$

which is abolished with the activation of $A_{2A}R$ (Domenici et al., 2004), while others have shown a cooperation between $A_{2A}R$ and $mGluR_5$ in the positive modulation of NMDAR currents (Rebola et al., 2008; Sarantis et al., 2015)

In particular, Sarantis et al., 2015 have shown that $mGluR_5$ mediates the enhancement of NMDAR currents in the rat hippocampus through the phosphorylation of its NR2B subunit in a Src-dependent manner and that this requires the activation of $A_{2A}R$. Thus, the authors have shown that ADA abolished the mGlu5R agonist 2-chloro-5-hydroxyphenylglycine (CHPG)-induced phosphorylation of the NR2B whereas the presence of both CHPG and CGS21680 induced a robust increase in NR2B phosphorylation.

However, interestingly, in this study the activation of the NMDAR is made through application of 50 μM of NMDA. As previously mentioned this leads to the activation of extrasynaptic receptors, which are here once more linked to the excitatory effects of $A_{2A}R$ in the CA1 pyramidal neurons. So, it could be that the excitatory effects of $A_{2A}R$ on NMDAR-dependent currents in CA1 pyramidal cells are on extrasynaptic NMDAR whereas the inhibitory effects of $A_{2A}R$ are done on synaptic NMDAR and the different outcome of $A_{2A}R$-mediated modulation may depend on its local interaction with $mGluR_5$.

Even though we did not explore this hypothesis, previous data from our laboratory has showed that the superfusion of hippocampal slices from adult Wistar rats with the $mGluR_5$ agonist CHPG (50 μM) caused a decrease in the NMDAR-dependent eEPSCs, thus if the relationship between $mGluR_5$ and $A_{2A}R$ is synergistic, maybe the activation of $A_{2A}R$ in the same conditions would corroborate our results.

Chapter 6

Conclusions

In the data hereby presented it is clear that the activation of $A_{2A}R$ induces a decrease in the amplitude of NMDAR-eEPSCs at SC-CA1 synapses, in both young and adult rats. However, the inhibition of these receptors has the same effect, which suggests that $A_{2A}R$ have a role in basal transmission. Moreover, in the presence of the selective antagonist of $A_{2A}R$, the agonist increases NMDAR-eEPSCs. Our main hypothesis to explain these results is that exogenous activation of $A_{2A}R$ mainly targets presynaptic $A_{2A}R$ and that these may differentially modulate NMDAR currents, *i.e.* presynaptic $A_{2A}R$ inhibit while postsynaptic $A_{2A}R$ facilitate those currents, which is in agreement with previous works (Mouro, 2012; Sarantis et al., 2015).

Moreover, there is also the possibility of $A_{2A}R$ interact with two different populations of NMDAR, which may be related with the type of stimulation applied, however more studies about this topic are needed.

Nevertheless, the study of which population of $A_{2A}R$ is responsible for each of these effects as well as the mechanisms through which the $A_{2A}R$ exert their influences on NMDAR remains to be done and it will be the basis of future work.

Also, it will be interesting to further explore the ontogenetic modulatory role of adenosine receptors in the hippocampus since there is a clear change in the pattern of distribution of these receptors along development and also with ageing (Canas et al., 2009b; Cunha, 2016; Cunha et al., 1995; Rebola et al., 2003).

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