Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Effect of Purines in the Developing Hippocampus - Consequences for the Establishment of Circuits Related to Learning and Memory

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Abbreviations

AC: Adenylyl cyclase

aCSF: Artificial cerebrospinal fluid

ADA: Adenosine deaminase

ADK: Adenosine kinase

ADP: Adenosine 5'-diphosphate

AMP: Adenosine 5'-monophosphate

AMPA: (2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid)

ANOVA: Analysis of variance

AP: Alkaline phosphatase

AP-2: Adaptor Protein-2

APAF1: Apoptotic protease activating factor 1

ARNO: ARF (ADP-ribosylation factor) nucleotide-binding site opener

ATP: Adenosine 5'-triphosphate

Bak: Bcl2-antagonist/killer 1

Bax: Bcl2-associated X protein

Bcl-2: B-cell lymphoma 2

Bcl-xL: B-cell lymphoma-extra large

BDNF: Brain-derived neurotrophic factor

bFGF: basic fibroblast growth factor

BH3: Bcl-3 homology domain 3

Bid: BH3 interacting domain death agonist

BIG2: Brefeldin A-inhibited guanine nucleotide-exchange protein 2

Ca^{2+}: Calcium

CamKII: Ca^{2+}/calmodulin-dependent protein kinase

cAMP: Cyclic adenosine monophosphate

CGE: Caudal ganglionic eminence

CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid

CHP: Cortico-hippocampal preparation

Cm: Membrane capacitance

CMF-HBSS: Ca^{2+}- Mg^{2+}-freeHank's solution

CNS: Central nervous system

cGMP: Cyclic guanosine monophosphate

CP: Cortical plate

CPA: N^6-cyclopentyladenosine

CRE: cAMP response element

CREB: cAMP response element-binding

Cx-43: Connexin 43

DAB: 3,3'-diaminobenzidine

DAG: Diacylglicerol

D-APV: D-2-amino-5-phosphonovaleric acid

DCC: Deleted in colorectal cancer

DG: Dentate gyrus

DIABLO: Direct IAP binding protein with low pI

DISC: Death-inducing signaling complexes

DIV: Days *in vitro*

DMSO: Dimethylsulfoxide

DNA: Deoxyribonucleic acid

EDTA: Ethylenediamide tetraacetic acid

EGTA: Ethylene-bis(oxyethylenenitrilo)tetraacetic acid

EGFR: Epidermal growth factor receptor

E-NPP: Ecto-nucleotide pyrophosphatase/phosphodiesterases

ENT: Equilibrative nucleoside transporter

e-5NT: Ecto-5'-nucleotidase

E-NTPDase: Ecto-nucleotide triphosphate diphosphohydrolase

EPSCs: Excitatory postsynaptic currents

ER: Endoplasmic reticulum

ERK1/2: Extracellular signal regulated kinase 1 and 2

ERNI: Early response to neural induction

FADD: Fas-associated protein with death domain

FGF: Fibroblast growth factor

GABA: Gamma-aminobutyric acid

GABARAP: GABA receptor-associated protein

GAD: Glutamate decarboxylase

GAT-1: GABA transporter-1

GD: Gestational day

GDNF: Glial-derived neurotrophic factor

GFAP: Glial fibrillary acidic protein

GDP(s): Giant depolarizing potential(s)

GRIP1/ABP: Glutamate Receptor Interacting Protein 1/AMPA binding protein

GRK-2: G protein-coupled receptor kinase 2

GSK3β: Glycogen synthase kinase 3

^{3}H-CHA: Tritiated cyclohexyldenosine

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

HtrpA2: High temperature requirement protein A2

IAP: Inhibitor of apoptosis proteins

ILE(s): Interictal-like event(s)

IP_3: Inositol triphosphate

IPSC(s): Inhibitory postsynaptic current(s)

IZ: Intermediate zone

JAK: Janus kinase

K^+: Potassium

KCC2: K^+Cl^- cotransporter 2

KPBS: potassium-phosphate solution

LGE: Lateral ganglionic eminence

LM: Lacunosum moleculare

LRD(s): Late recurrent discharge(s)

LTP: Long-term potentiation

MAP-2: Microtubule-associated protein 2

MAP kinase: Mitogen-activated protein kinase

MAPKAP: MAPK activated protein kinase

MEM: Minimal essential medium

mEPSC(s): Miniature excitatory postsynaptic current(s)

Mg^{2+}: Magnesium

MGE: Medial ganglionic eminence

mIPSC(s): Miniature postsynaptic current(s)

mRNA: messenger ribonucleic acid

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MZ: Marginal zone

Na+: Sodium

NBQX: 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline

NECA: 5'-N-ethylcarboxamido adenosine

NF-κB: Nuclear factor –κB

NGF: Nerve growth factor

NMDA: N-methyl-D-aspartate

NMP: Neuronall plating medium

NGS: Normal goat serum

NHS: Normal horse serum

NSF: N-ethylmaleimide-sensitive factor

NRSF: Neuron-restrictive silencer factor

O-LM: Oriens-lacunosum moleculare

PAF: Paraformaldehyde

PB: Phosphate buffer

PBS: Phosphate buffered saline

PD: Postnatal day

PDGF: Platelet-derived growth factor

PI: Propidium iodide

PICK1: Protein interacting with PRKCA

PI3K: Phosphatidylinositol-3'-kinase

Pir Cx: Piriform cortex

PKA: Protein kinase A

PKB: Protein kinase B

PKD: Protein kinase D

PLC: Phospholipase C

PMSF: Phenylmethanesulfonylfluoride

POA: Preoptic Area

PSD-93: Postsynaptic density 93

PSD-95: Postsynaptic density 95

RA: Retinoic acid

REST: RE1-silencing transcription factor

Rm: Membrane resistance

RRP: Readily releasable pool

SAP97: Synapse-associated protein 97

SAP102: Synapse-associated protein 102

SDS: Sodium dodecyl sulfate

SEM: Standard error of the mean

SEP: Super ecliptic phluorin

SMAC: Second mitochondria-derived activator of caspases

SNAP-25: Synaptosomal-associated protein-25

SPL: Subplate

STAT: Signal transducer and activator of transcription

STS: Staurosporine

SVZ: Subventricular zone

TBS: Tris buffered saline

TE: Tris-EDTA

TEMED: Tetramethylethylenediamine

TGN: Trans golgi network

TNF: Tumor Necrosis Factor

TRAF-2: TNF receptor-associated factor 2

TrkA: Tyrosine receptor kinase A

TrkB: Tyrosine receptor kinase B

UDP: Uridine diphosphate

VGLUT1: Vesicular glutamate transporte 1

VZ: Ventricular zone

Resumo/Abstract

O sistema nervoso desenvolve-se seguindo uma série precisa de etapas que fazem parte de um programa de desenvolvimento. O sistema purinérgico - que compreende os neuromoduladores/neurotransmissores denominados purinas (dos quais se destacam a adenosina e o ATP), respectivos receptores, enzimas e transportadores que promovem a sua produção ou remoção – está presente no sistema nervoso central desde fases muito iniciais do desenvolvimento. A versatilidade deste sistema manifesta-se pela sua capacidade em modificar a expressão e localização dos seus receptores, enzimas e transportadores de acordo com o nível de maturação do animal. O aperfeiçoamento de métodos de isolamento de fracções membranares permitiu observar que o receptor A_{2A} para a adenosina, cuja expressão é muito reduzida no hipocampo adulto onde é conhecido controlar a libertação pré-sináptica de neurotransmissores, tem uma expressão abundante em domínios pós- e extrasinápticos no hipocampo de ratinhos com 5 a 7 dias pós-natais. Em fases mais tardias do desenvolvimento, parece haver uma diminuição progressiva da sua expressão. A activação deste receptor no hipocampo em desenvolvimento parece depender já nesta fase da libertação pré-sináptica de ATP como sugere a presença do enzima ecto-5'-nucleotidase envolvida na produção de adenosina a partir do ATP em regiões igualmente pós-sinápticas.

Durante a primeira semana pós-natal, a adenosina endógena parece ser necessária para a estabilidade de receptores do tipo AMPA/kainato e $GABA_A$ uma vez que o bloqueio de receptores A_1 e A_{2A}, os dois principais subtipos de receptores de adenosina presentes no hipocampo, conduz ao "enfraquecimento" da transmissão sináptica GABAérgica e glutamatérgica. Técnicas de biologia celular e molecular confirmaram a endocitose dependente de clatrina dos receptores ionotrópicos para o GABA e glutamato após bloqueio de receptores A_1 e A_{2A}. Este efeito modulador sobre a actividade sináptica parece ser reversível em sinapses glutamatérgicas mas de difícil recuperação em sinapses GABAérgicas. Embora se desconheçam os mecanismos subjacentes, estas observações sugerem um papel relevante dos receptores de adenosina durante a sinaptogénese.

A disrupção do sistema de modulação mediado pelos receptores de adenosina revelou-se prejudicial ao desenvolvimento de circuitos de aprendizagem e memória no hipocampo, uma vez que o bloqueio crónico de receptores de adenosina pela cafeína (que bloqueia não especificamente receptores A_1 e A_{2A} a baixas concentrações) induziu uma série de modificações morfológicas e fisiológicas no hipocampo de ratinhos com 6 dias pós-natais, essencialmente observadas em

interneurónios. Algumas destas modificações são stresse celular, síntese anormal de GAD 67 e aglomeração de proteínas do sistema GABAérgico bem como uma desregulação do balanço entre a transmissão sináptica GABAérgica e glutamatérgica. Os animais adultos expostos a antagonistas de receptores de adenosina durante o desenvolvimento revelaram défices cognitivos quando sujeitos a testes comportamentais que avaliam a memória espacial e não espacial, o que mostra que houve uma violação do programa de desenvolvimento desencadeada pelos tratamentos. Se no animal imaturo as principais modificações após exposição crónica a cafeína foram observadas no sistema GABAérgico, no adulto o sistema glutamatérgico parece ser morfológica e fisologicamente mais afectado.

O GABA e o glutamato foram descritos como moléculas importantes agindo como factores tróficos durante o desenvolvimento. A adenosina surge então, como um poderoso modulador que parece alterar alterar a sinalização mediada por receptors ionotrópicos para o GABA e glutamato, podendo interferir em processos como diferenciação, migração e morte celular, sinaptogénese e neurotransmissão. Uma violação das leis que governam estes processos poderá estar na base das modificações observadas a longo-termo e ao estabelecimento de circuitos de aprendizagem e memória no hipocampo que funciona de forma aberrante.

The nervous system develops following a series of steps respecting a developmental programme. The purinergic system - comprising the neuromodulators/neurotransmitters called purines (the most famous being ATP and adenosine), their receptors, enzymes and transporters that promote their production or removal – is present in the central nervous system since initial stages of development.

The versatility of this system relies on its ability to modify the expression and localization of their receptors enzymes and transporters according to the stage of the development. Improvement of methods for isolation of membrane fractions allowed the observation that A_{2A} receptors, whose expression is very low in the adult hippocampus where they control the presynaptic release of neurotransmitters, has an abundant expression in post and extrasynaptic fractions of hippocampal synaptic membranes from 5-7 days-old mice. In later stages of development, A_{2A} receptors suffer a progressive down-regulation . The activation of this receptor subtype in the developing hippocampus seems to depend on the presynaptic release of ATP, suggested by the presence of the enzyme ecto-5 '-nucleotidase, involved in the production of adenosine from ATP, in postsynaptic membranes.

During the first postnatal week, endogenous adenosine seems to be necessary for the stability of AMPA/kainate and $GABA_A$ receptors, since the blockade of A_1 and A_{2A} receptors, the two main subtypes of adenosine receptors present in the hippocampus induced the weakening of the GABAergic and glutamatergic synaptic transmission. Techniques of cellular and molecular biology confirmed the clathrin-dependent endocytosis of ionotropic GABA and glutamate receptors after A_1 and A_{2A} receptor blockade. This effect on synaptic activity seems to be reversible in glutamatergic synapses and long-lasting in GABAergic synapses. Although the intracellular mechanisms linking adenosine receptor blockade to the internalization of $GABA_A$ and AMPA/kainate receptors are not understood, these observations suggest a role of adenosine during synaptogenesis.

The disruption of the modulation afforded by adenosine receptors seems to have detrimental effects in the establishment of hippocampal circuits related to learning and memory, since the chronic blockade of adenosine receptors by caffeine (which is a non-selective antagonist of A_1 and A_{2A} receptors at low concentrations) changed several physiological and morphological parameters in hippocampus of 6 days-old mice, particularly evident in interneurons. Some of these modifications are cellular stress, abnormal synthesis of GAD 67 and agglomeration of proteins belonging to the GABAergic system, as well as an imbalance between the GABAergic and glutamatergic neurotransmission. Adult animals exposed to adenosine receptor antagonists during development showed cognitive deficits when subjected to behavioral tests that assess spatial and non spatial memory, suggesting that there was a violation of the normal developmental program. If in immature animals the main changes observed after chronic exposure to caffeine were observed in the GABAergic system, in the adult, the glutamatergic system seems to be the most affected.

GABA and glutamate were identified as capable of exerting trophic actions during brain development. Adenosine, having a powerful control over signaling cascades activated/inactivated by ionotropic receptors for GABA and glutamate can interfere with fundamental stages of neural development such as the differentiation of progenitor cells, migration and cell death, synaptogenesis and neurotransmission, which can be the cause or contribute to the genesis of the long-term modifcations observed in the adult hippocampus.

INTRODUCTION

1. The Purinergic System - Overview

The **purinergic system** corresponds to the assembly of several types of ligands, generally called **purines** and responsive **receptors** as well as proteins (**enzymes** and **transporters**) responsible for production and/or reuptake of purines that mediate intra/intercellular physiological events (see **Fig.1**). **Adenosine triphosphate** (ATP) is one of the best known molecules belonging to this system (Burnstock, 1972). ATP binds to and activates two subfamilies of receptors, **P2X** and **P2Y**. P2X receptors comprise ligand-gated channels, permeable to sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}). Until now, seven receptor subtypes have been characterized - $P2X_1$ through $P2X_7$ (Ralevic and Burnstock, 1998; North, 2002). P2X receptors are composed by transmembranar subunits containing an extracellular site for binding to the ligand and intracellular domains involved in the modulation of the channel. P2Y receptors are G-protein-coupled receptors. Depending on their type of coupling, they can be subdivided into two groups, G_q preferring receptors - $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ - and G_i preferring receptors -$P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$ (Abbracchio *et al.*, 2006). P2 receptors can be activated by other purines such as **adenosine diphosphate** (ADP), **uridine triphosphate** (UTP), **uridine diphosphate** (UDP), **UDP glucose** and other nucleotide sugars (Neary and Zimmerman, 2009). Some P2 receptors can have greater affinity for other purines than for ATP itself (Neary and Zimmerman, 2009).

ATP can reach the extracellular space using ATP release channels – hemichannels, anion channels including P2X7 receptor, ATP-bindig cassette transporters - (Sabirov and Okada, 2005), facilitated diffusion through nucleotide transporters such as ADP/ATP exchange carrier and vesicular exocytosis (Pankratov *et al.*, 2006). Recently, the protein responsible for the accumulation of ATP into synaptic vesicles released by exocytosis, the **vesicular nucleotide transporter** (VNUT), was identified and characterized in human and mouse tissues (Sawada *et al.*, 2008). Once outside the cell, ATP can be metabolized into **adenosine** which is another signaling molecule belonging to the purinergic system. Several enzymes called generically **ecto-nucleotidases** are involved in this process – ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) (Robson *et al.*, 2006), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) (Goding *et al.*, 2003; Stefan *et al.*, 2006), ecto-5'-nucleotidase (Hunsucker *et al.*, 2005; Colgan *et al.*, 2006) and alkaline phosphatases (APs) (Millan, 2006).

Adenosine can bind to and activate four metabotropic subtypes of receptors, **A_1**, **A_{2A}**, **A_{2B}** and **A_3** - A_1 and A_3 receptors inhibiting via $G_i/_0$ and G_i/G_q respectively and A_{2A} receptors activating adenylate cyclase via G_s/G_{olf} –A_{2A} receptors- or G_s/G_q –A_{2B} receptors- (Jacobson and Gao, 2006; Ryzhov *et al.*, 2006). All adenosine receptors share a similar structure, a polypeptide chain forming seven transmembrane helices, with the N-terminus placed outside the cell and the C-terminus inside the cell (Constanzi *et al.*, 2007). Adenosine receptors display different affinities for the ligand: A_{2B} receptor subtype having the lowest affinity for adenosine and A_1 and A_{2A} receptor subtypes having higher affinity for the ligand. A_3 receptor has intermediate affinity for adenosine comparing with the other adenosine receptor subtypes (Jacobson *et al.*, 1996; Dunwiddie and Masino, 2001), although it is a high affinity subtype of adenosine receptor in human tissue (Ribeiro and Sebastião, 2010). They desensitize also after agonist binding and it can occur rapidly in the case of A_3 receptor subtype and more slowly in the case of the A_{2A} receptor subtype, being associated to receptor down-regulation, internalization and degradation (Klaase *et al.*, 2008).

Adenosine actions are terminated when extracellular adenosine is transported into the cell via **nucleoside transporters** (ENTs). Since adenosine is neither stored nor released as a classical neurotransmitter since it does not use synaptic vesicles for storage and release, the direct release of cytosolic adenosine can occur *via* the same transporters. It constitutes an alternative way of adenosine release independently of the exocytosis of ATP. This adenosine that can be directly released by transporters, is formed by the action of an AMP selective 5'-nucleotidase and the rate of production is dependent on the amount of available AMP (Schubert *et al.*, 1979; Zimmermann *et al.*, 1998) or by the hydrolysis of S-adenosyl-homocysteine (Broch and Ueland, 1980) by the action of S-adenosyl-homocysteine hydrolase being L-homocysteine the limiting reagent of the reaction (Reddington and Pusch, 1983). An alternative source of adenosine is the release of cyclic AMP (cAMP) (Rosenberg and Li, 1995).

The direction of the transport of adenosine in or out of the cells is dependent upon the concentration gradient across biological membranes (Gu *et al.*, 1995). Two groups of transporter proteins were identified, **equilibrative** – ENT1-4 - that use facilitated diffusion driven from the concentration gradient and **concentrative nucleoside transporters** – CNT1-3 - which use proton gradients to drive concentrative fluxes of nucleosides (Podgorska *et al.*, 2005). Their widespread distribution includes the central nervous system (Jennings *et al.*, 2001).

Adenosine is more concentrated inside the cells (Fredholm *et al.*, 2005). Extracellular adenosine concentration in the brain was estimated to be 20 nM (Fredholm *et al.*, 2005). Two enzymes constitute the major pathways for adenosine removal – **adenosine kinase** (ADK) and **adenosine deaminase** (ADA). ADK phosphorylates adenosine into AMP intracellularly, decreasing the available pool of free adenosine and adenosine deaminase cleaves adenosine into inosine (Arch and Newsholme, 1978; Lloyd and Fredholm, 1995). Adenosine deaminase seems to be relevant when very high amounts of adenosine have to be cleared (Fredholm *et al.*, 2005).

Once adenosine binds to responsive receptors in the plasma membrane, an intricate network of intracellular signaling pathways is recruited. The modulation of cAMP levels is one of the most important pathways triggered by adenosine receptors. cAMP-dependent protein kinase A (PKA) which will be activated and able to phosphorylate cytoplasmic and nuclear targets, including ion channels and transcription factors. A major target of PKA is cAMP response element binding protein (CREB) that binds to the cAMP response element CRE (Mayr and Montminy, 2001), regulating gene transcription. The phospholipase C (PLC)/inositol triphosphate (IP_3) pathway can also be activated by adenosine receptors. The action of phospholipase C produces molecules that will act as second messengers such as inositol triphosphates (IP_3) and diacylglicerol (DAG). IP_3 binding to ryanodine receptors, located in the endoplasmic reticulum (ER), increasing cytoplasmic calcium concentration. This signal increases the activity of calcium-dependent protein kinases (PKC) or other calcium-binding proteins, including calmodulin and even adenylyl ciclase (AC). PKC can be recruited to the plasma membrane after binding to DAG. In PC12 cells, upon stimulation of A_{2A} receptor, PKC seems to be involved in a negative loop that regulates the cAMP signal triggered by A_{2A} receptors (Lai *et al.*, 1997). Also, PKC activation facilitates activities of adenosine transporters in the hippocampus, reducing the adenosine available for A_1 receptor activation (Pinto-Duarte *et al.*, 2005). Phosphatidylinositol-3'-kinase (PI3K) can also be recruited if βγ subunits of G-protein are activated (Yart *et al.*, 2002). These kinases are involved in the production of phosphatidylinositolphosphates that activate several signaling proteins, such as protein kinase B (PKB)/Akt pathway (Vanhaesebroeck and Alessi, 2000). PKB/Akt activation leads to phosphorylation of glucogen synthase 3β (GSK-3β), nuclear factor-κB (NF-κB), B-cell lymphoma 2 (Bcl-2) protein (involved in apoptotic mechanisms and S6 kinase associated to ribosome. Adenosine can also activate mitogen-activated kinases (MAP kinases) (Schulte and Fredholm, 2003). In the classical model proposed for the activation of MAP kinases, some of the final targets are transcription factors (Turjanski *et al.*, 2007) and other proteins including the Signal Transducer and Activator of Transcription (STAT),

tyrosine hydroxylase, MAPK activated protein kinase (MAPKAP) and S6 kinase, relevant in cell differentiation, proliferation and apoptosis (Jacobson and Gao, 2006; Che *et al.*, 2007). For example, A_3 receptor activates Akt to inhibit apoptosis. A_{2A} receptor can also recruit β-arrestin via GRK-2 (Khoa *et al.*, 2006) and the transduction pathways are independent on G-proteins. The arrestin pathway can be involved in signal transmission and internalization of the receptor (Klaasse *et al.*, 2008). The A_{2A} receptor subtype was also found also to be able to bind proteins such as α-actinin, ARF nucleotide-binding site opener (ARNO) and translin-associated protein-X (Zezula and Freissmuth, 2008).

Even P2XRs which main feature in to increase intracellular Ca^{2+} levels can be coupled to signaling molecules not directly related to Ca^{2+}. They can activate PKC (Heo and Han, 2006), PKB, PKD (Bradford and Soltoff, 2002), extracellular signal regulated kinase 1 and 2 (ERK1/2) (Amstrup and Novak, 2003), p38 MAPK, caspases (Bulanova *et al.*, 2005), PI3-K and phospholipases (Alzola *et al.*, 1998; Pérez-Andrés *et al.*, 2002; Andrei *et al.*, 2004;. Some of these signaling pathways are equally triggered by P2Y subtype of receptors (see Abbracchio *et al.*, 2006).

A characteristic that increases even more the complexity and versatility of the purinergic system is the ability of both adenosine and ATP receptors to establish interactions with other proteins. Adenosine receptors can be assembled in homo/heterodimers such as A_1R/D_1R and $A_{2A}R/D_2R$, with dopamine receptors, $A_1R/P2Y_1R$ and $A_1R/P2Y_2R$ (Franco *et al.*, 2006). P2X receptors can form homo- and hetero-oligomers (Torres *et al.*, 1999), metabotropic ATP receptors can also associate with NMDA, dopamine and β-adrenergic receptors (Volonté *et al.*, 2006). Interactions between metabotropic and ionotropic ATP receptors were also reported however classified as indirect interactions (Gerevich *et al.*, 2007). It also seems that ecto-nucleotidases can establish direct interactions between them and with metabotropic and ionotropic ATP receptors (Schicker *et al.*, 2008).

In the middle of this complex network of molecules and pathways, the correct coupling of the signaling in response to purines may be achieved by compartmentation in lipid rafts at the level of biological membranes (Garcia-Marcos *et al.*, 2008). In fact there is evidence that purine receptors may exist in restricted domains of biological membranes (Lasley *et al.*, 2000; Vacca *et al.*, 2004; Barth and Volonté, 2009). Also, since both adenosine and ATP receptors may be present together and some of the effects of different signaling pathways may oppose, the rapid conversion of ATP into adenosine may determine which effect will prevail.

Fig.1

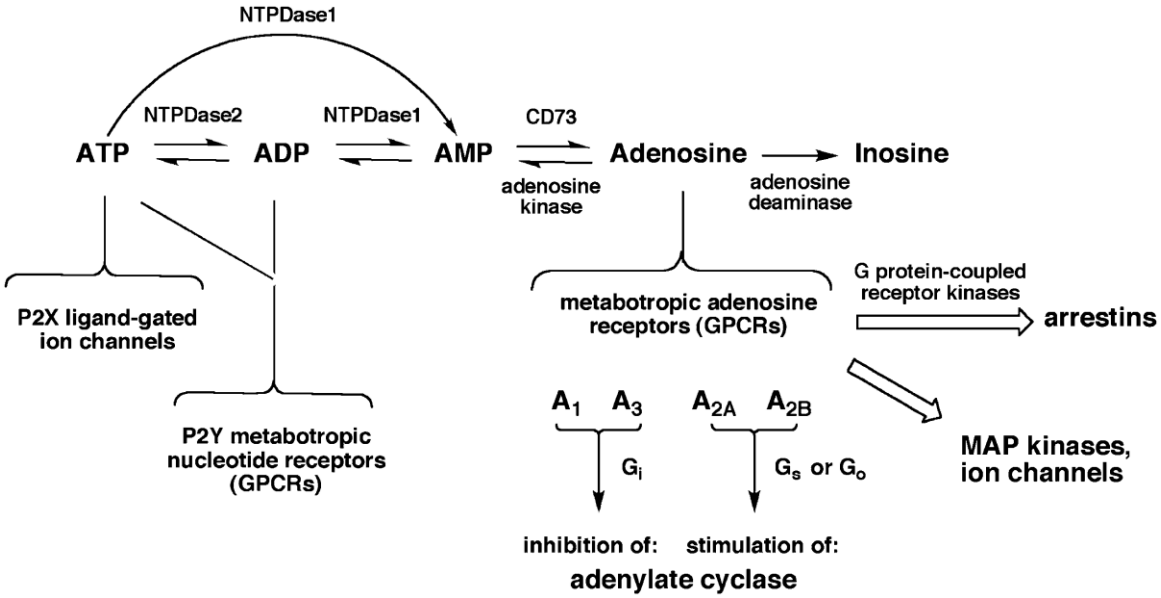


Fig. 1 – Purinergic System – Summary of the principal components of the purinergic system: ATP, adenosine, ATP and adenosine receptors, ecto-nucleotidases (nucleoside transporters are not depicted in the scheme) and relation between them as well as some of important signaling pathways, such as MAP kinases and their targets (*e.g*. ion channels and adenylate cyclase). *From Introduction to Adenosine Receptors as Therapeutic Targets by Jacobson, KA. Handbook of Experimental Pharmacology 4-19 (2009)*

2. Neural Development

In this section, the steps involved in the generation, shaping and reshaping of the maturating nervous system will be briefly described. The most important steps acting in brain development will be presented with the purpose of making familiar some concepts and facilitating the understanding and interpretation of some results that will be presented later on. This presentation will emphasise the rodent hippocampal formation which is the "brain target structure" explored in this thesis.

The succession of stages in which the nervous system develops from embryogenesis throughout life is called **neural development**. It includes **neural induction**, **neurulation, regionalization**, **patterning**, **differentiation**, **cell migration**, **synapse formation** and **elimination**, functional (morphological and physiological) **network formation**, **network refinement**. Each of these steps will be presented in the following paragraphs.

2.1. Embryogenesis, Neural Induction, Neurulation and Regionalization

After fertilization, successive steps of cell division without significant cell growth produce successive clusters of cells, zygote, morula, blastula and gastrula which has two or three layers, ectoderm and endoderm or ectoderm, mesoderm and endoderm, respectively. Ectoderm folding is the basis of the formation of the neural groove that deepens and forms the neural tube (this step is known as **neurulation)**. The neural tube assumes the form of three vesicles that correspond to the future forebrain, midbrain and hindbrain. This stage is known as **regionalization**. The remainder of the neural tube will generate the spinal cord. This step involves neural inducers which are molecules responsible for the turning on and off genes that lead to the specialization of ectoderm in neural tissue, the process known as **neural induction**. Spemann and Mangold (Spemman and Mangold, 1924) proposed that neural induction probably occurs at the gastrula stage. Examples of inducers are fibroblast growth factor 8 (FGF8) that induce the sequential expression of ERNI, Sox 3 and Churchill (pre-neural markers – see review of Stern, 2007). These three factors are needed to the formation of neural plate. As soon as neural tube is formed, it acquires a dorso-ventral organization and the region between the top and bottom of this structure is occupied by neural precursors (Politis *et al.*, 2008).

Patterning corresponds to the formation of axes. The ventral part of the neural plate is controlled by the notochord generated by the endoderm and the dorsal axis is controlled by the ectoderm. The

rostrocaudal axis generation is dependent on molecules such as FGF and retinoic acid (RA) (Diez del Corral and Storey, 2004). Patterning leads to segmentation of the neuroepithelium into progenitor domains for neurons and glia (Rowitch and Kriegstein, 2010). Each domain has a distinct developmental potential and differentiation programs for neurons, which leads to the generation of different neuronal subtypes. The decision of maintaining their proliferative capacity or differentiation in post-mitotic neurons seems to be related with the control of their cell cycle (Ohnuma and Harris, 2003). Neurons and microglia generate from the neuroepithelial cells that line the ventricles and the spinal canal, a process named **neurogenesis**. Radial glial cells are the first progenitors appearing (Malatesta *et al.*, 2000; Myiata *et al.*, 2001; Noctor *et al.*, 2001). They also line the ventricles and their asymmetrical cell division produces neurons, oligodendrocytes and intermediate progenitor cells (Haubensak *et al.*, 2004; Miyata *et al.*, 2004; Noctor *et al.*, 2004; Noctor *et al.*, 2008), which are the main source of neurons and glia in the telencephalon (Haubensak *et al.*, 2004; Noctor *et al.*, 2004).

Several molecules and signaling pathways are implicated in the self-renewal, quiescence or differentiation of stem cells (see **Fig. 2**). For example, Notch signaling pathway and Hes proteins (effectors) are involved in keeping progenitors undifferentiated (Hatakeyama *et al.*, 2004; Mizutani and Saito, 2005). Endothelial cells are proposed to be the cells that secrete soluble factors to trigger Notch signaling pathway (Shen *et al.*, 2004). The FGF pathway also seems to be involved in stem cells renewal (Yoon *et al.*, 2004). In the cortex, Wnt/β-catatenin pathway is both implicated in self-renewal (Chenn and Walsh, 2002) and neuronal differentiation (Hirabayashi *et al.*, 2004), however, the type of response seems to be dependent on the stage of cortical development (Hirabayashi and Gotoh, 2005). Cross-talk between FGF and Wnt/β-catatenin pathways can also determine if cells undergo (in the absence of FGF) or not (in the presence of FGF) differentiation (Israsena *et al.*, 2004). The signaling mediated by of Notch, FGF and Wnt involve transcription activators (Hirabayashi *et al.*, 2004; Israena *et al.*, 2004; Miyata *et al.*, 2004) as well as transcription repressors, such as REST/NRSF that represses neuronal genes in non-neuronal cells (Ballas *et al.*, 2005). When stem cells differentiate in neurons there is a decrease of the binding of REST to neuronal promoters (Ballas *et al.*, 2005).The generation of astrocytes occurs after neuronal generation and it also requires instructive signals and activation of signaling pathways such as bone morphogenic protein BMP/ Sma and Mad related proteins (Smad), JAK/STAT and Notch (Kamakura *et al.*, 2004; Hirabayashi and Gotoh, 2005).

Some genes involved in neurogenesis are also implicated in neuronal progenitor specification to a particular identity. One example is *Neurogenin 1* and *2*, apart from its role in neurogenesis, are also involved in the specification of the glutamatergic transmission phenotype and dendritic morphology of telencephalic neurons (Schuurmans *et al.*, 2004).

Fig. 2

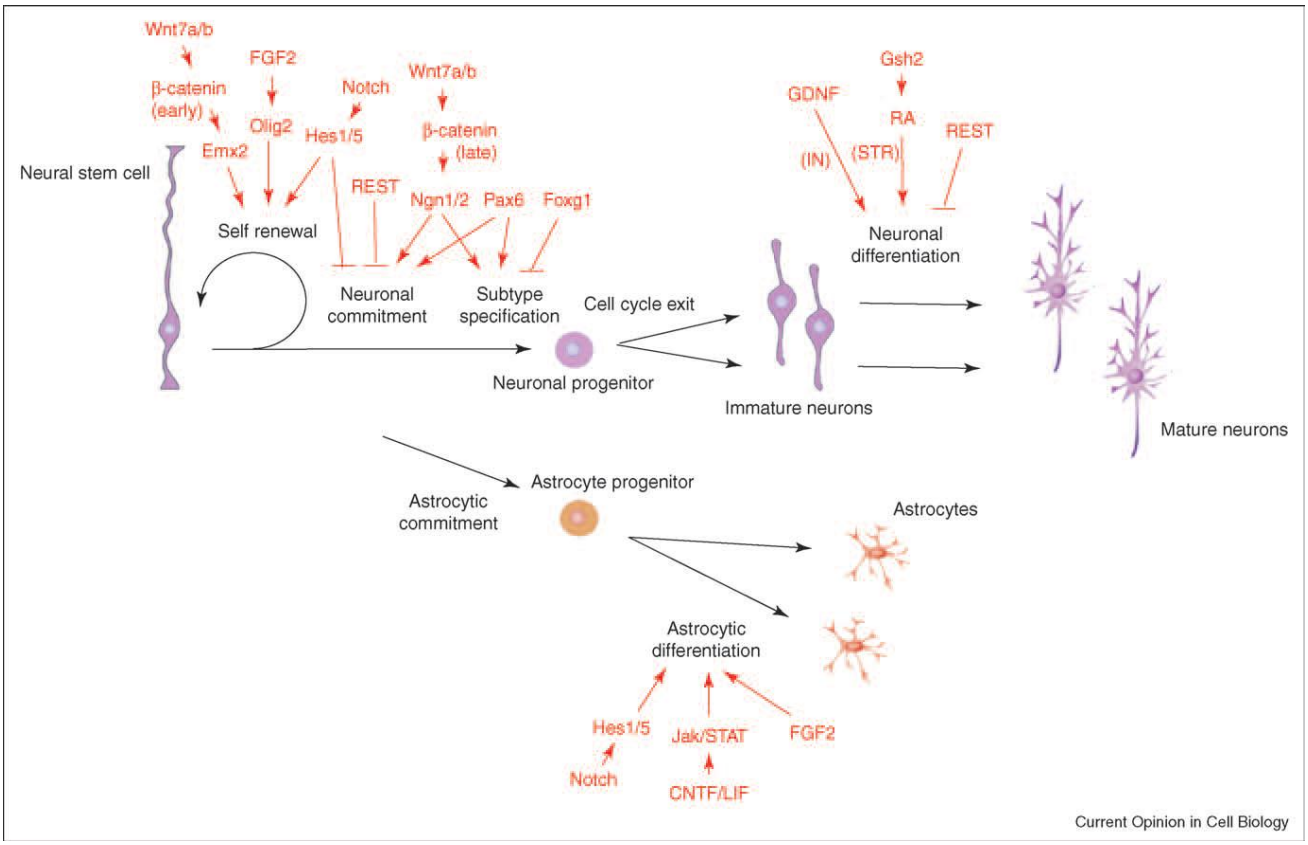


Fig.2 - Signaling pathways shown to promote self-renewal of telencephalic stem cells such as FGF and Notch pathways or FGF and Wnt/β-catatenin. Glial-derived neurotrophic factor (GDNF) seems to be required for cortical interneuron differentiation and Gsh2/RA pathway is necessary for striatal interneuron differentiation. *From Cellular and Molecular Control of Neurogenesis in the Mammalian Telencephalon by Guillemont, F. Current Opinion in Cell Biology 17: 639-647 (2005)*

2.2. Cell Death

Both undifferentiated and differentiating cells, including neurons and glia need to be eliminated during pre- and post-natal development. In fact, cell death during nervous system development seems to be important for the formation or elimination of structures, in the control of the cell numbers and in the elimination of abnormal cells (Glücksmann, 1951; Jacobson, 1997).

In conformity with the nomenclature committee on cell death, a cell should be considered death when a point-of-no-return is achieved; this may correspond to a loss of plasma membrane integrity, formation of apoptotic bodies resultant from deoxyribonucleic acid (DNA) condensation or engulfment by neighboring cells (Kroemer *et al.*, 2009). The "death signals" may arise intrinsically from the cell that will undergo death (autonomous specification) or from other cells (conditional specification) and they can be cell-lineage information, extracellular survival factors, steroid hormones, membrane-bound receptors and DNA-damaging agents. Furthermore, newly generated neurons compete for neurotrophic molecules, avoiding cell death (Hamburger and Levi-Montalcini, 1949).

Cells may die by **apoptosis**, a regulated process, also known as programmed cell death. Several morphologic changes characterize this type of cell death, such as cytoplasmic and nucleus condensation, followed by the fragmentation of the nuclear content. These fragments are encapsulated and give origin to the apoptotic bodies that are eliminated by adjacent cells (Kerr *et al.*, 1972). This type of remodeling is regulated and interacts with the cytoskeletal proteins such as actin, lamins and tubulin – (Cryns and Yuan, 1998). Also, phosphatidylserine migrates to the external layer of the plasma membrane and it may serve as a death signal. Indeed, a phosphatidylserine receptor is present in the membrane of phagocytes and recognizes phosphatidylserine at the membrane of dying cells (Fadok *et al.*, 2000). In mammals, there are at least two major pathways of apoptosis, called **extrinsic** and **intrinsic**. The first one is triggered by death receptors, like Fas, Tumor Necrosis Factor Receptor - 1 (TNFR-1) and TNF-related apoptosis inducing ligand (TRAIL), expressed in the plasma membrane in response to external signals (Lavrik *et al.*, 2005). Protein interaction assemble these receptors with intracellular proteins involved in signaling pathways that lead to death, forming death-inducing signaling complexes (DISC) into which are recruited and activated regulatory enzymes including pro-caspases that will give origin to the death executioners called caspases. Fas-associated protein with death domain (FADD) and Apoptotic protease activating factor 1(Apaf-1) are two examples of regulatory proteins responsible for the aggregation of procaspase-8 and procaspase-10

and conversion into active caspases by auto- or trans-activation (Martin *et al.*, 1998; Muzio *et al.*, 1998). Caspases are a class of cysteine proteases constitutively expressed in virtually all cells in an inactive form. When an inactive caspase is cleaved, two subunits of different molecular weight are produced. The association of two light and two heavy subunits gives rise to the tetrameric structure of active caspases.

When cell death is triggered by factors belonging to the cell that will die such as inadequate cytokine support or intracellular damage, cytochrome c and death-promoting proteins are released from mitochondria as a consequence of mitochondrial membrane permeabilization (Newmeyer and Ferguson-Miller, 2003). Several regulatory proteins play a role in cytochrome c release, as explained latter in this chapter. Cytochrome c induces a conformational change in Apaf-1 which is then able to recruit procaspase-9 (Acehan *et al.*, 2002). Altogether, procaspase-9 and Apaf-1 form a giant structure (~ 1 megaDalton) called apoptosome. It is in the apoptosome that caspase-9 is activated by allosteric change and dimerization (Rodriguez and Lazebnik, 1999; Boatright *et al.*, 2003). In many cells the apoptosome seems not to be required for apoptosis (Hoppe *et al.*, 2002; Kilic *et al.*, 2002). The observation that in the absence of the apoptosome the activity of caspases is very low, led to the proposal that the apoptosome is also an amplifier of the caspase cascade (Fumarola and Guidotti, 2004). Caspases- 8, -9 and -10 are the proposed initiators that activate the effector caspases-3 and -7. They may act upstream or independently of mitochondria (Marsden *et al.*, 2002).

Several substrates may be cleaved by caspases during the process of cell death (Cryns and Yuan, 1998) and there is biochemical and functional consequences, like the activation of dormant pro-apoptotic factors, elimination of endogenous death antagonists and disruption of the cell apparatus as well as structural dismantling (Cryns and Yuan, 1998) – see also **Table II**.

Several reports suggest that caspase activation may not be a crucial step for the ongoing process of apoptosis. For example, the second mitochondria-derived activator of caspases /Direct IAP Binding Protein with low pI (Smac/Diablo) and Omi/High temperature requirement protein A2 (HtrA2) are capable of blocking the activity of proteins involved in the inhibition of active caspases activity but it seems to be ineffective in the arrest of apoptosis (Okada *et al.*, 2002) and as well, the inhibition of endogenous inhibitors of caspase activity does not trigger apoptosis *per se* (Verhagen *et al.*, 2000).

A caspase-independent mechanism was proposed in an attempt to solve the inconsistency of this caspase-dependent model for the process of cell death. According to this mechanism, the genetic

material of cells is affected - chromatin condensation and DNA degradation – (Penninger and Kroemer, 2003) and protein Apoptosis Inducing Factor (AIF) and endonuclease G which promote DNA condensation seem to contribute to this type of cell death.

Table II

Caspase	Proposed Funtion
Caspase 1	Processing the precursors of Interleukin- 1β (IL-1β) and IL-18 (Kuida *et al.*, 1995); processing executioner caspases *in vitro* (Van de Craen *et al.*, 1999); promotes death of neutrophils in culture (Rowe *et al.*, 2002) and of macrophages (Hilbi *et al.*, 1998)
Caspase 2	Contributes to death of thymocytes and neuronal cells triggered by several stimuli (O'Reilly *et al.*, 2002); is required for Bcl2-associated X protein (Bax) translocation to mitochondria and cytochrome c release (Lassus *et al.*, 2002) can cleave BH3 interacting domain death agonist (Bid) and initiate mitochondrial disruption through truncated Bid or Bax translocation (Guo *et al.*, 2002); Locates into the nucleus and Golgi apparatus (O'Reilly *et al.*, 2002) and it has been argued that it can trigger apoptosis from the nucleus cleaving Bid which is small enough to pass through nuclear pores and translocate into the cytoplasm (Paroni *et al.*, 2002)
Caspase 3	Cleavage of most apoptotic substrates
Caspase 6	Lamin cleavage
Caspase 7	Cleavage of most apoptotic substrates
Caspase 8	Required for the apoptosis triggered by death receptors (Strasser *et al.*, 2000; Ashkenazi *et al.*, 2002); it can directly process caspase-3 and in hepatocytes it can also cleaves Bid, which is related to mitochondria permeabilization (Yin *et al.*, 1999) and capase-9 activation; a possible function in stress-induced death was also proposed (Breckenridge *et al.*, 2003; Jimbo *et al.*, 2003)
Caspase 9	Caspase-3/7 activation after mitochondrial stress
Caspase 11	Activation of caspase-3, caspase-7 and caspase-1 (Kang *et al.*, 2002); in oligodendrocytes avoids their refractoriness to cytotoxic cytokines (Hisahara *et al.*, 2001)
Caspase 12	It is placed on the cytoplasmic side of ER and is activated by ER stress (Nakagawa and Yuan, 2000; Nakagawa *et al.*, 2000); it may activate caspase-9 that leads to caspase-3 activation (Rao *et al.*, 2002); TRAF2, a factor associated to death receptors, may interact with caspase-12 promoting it self-processing (Yoneda *et al.*, 2001)

Table II – Summary of proposed functions for different caspases.

Other players in the cell death process are the Bcl-2 family of proteins. The members of this family interact with cellular membranes of healthy cells, including in the outer mitochondrial membrane, ER and nuclear membrane (Janiak *et al.*, 1994). Other members of this family, like Bcl2-antagonist/killer 1 (Bak), Bax, Bcl-w and B-cell lymphoma extra large (Bcl-X_L) move to one or all of these compartments during the process of cell death (Hsu *et al.*, 1997; Kaufmann *et al.*, 2003). Bax and Bak are bound to membranes and can undergo conformational changes and oligomerize, forming large complexes which can permeabilize mithocondria allowing the passage of several cytosolic "poisons", including cytochrome c. Interestingly proteins of the Bcl-2 family seems also to have protective roles. For example, embryonic stem cells that lack Apaf-1 are protected by Bcl-2 from stress-induced death (Haraguchi *et al.*, 2000).

Beyond mitochondria, other organelles play a role in apoptosis. The ER may play a role both in apoptosis as well as in necrosis (described latter). In normal conditions, ER verifies the folding of proteins. The continuous stress, induced by the misfolding and perturbed glycosylation of proteins and deficits in calcium homeostasis, are factors that affect ER function (Ferri and Kroemer, 2001; Kaufman, 2002). ER stress can promote calcium release and this process is mediated by Bax and Bak – (Scorrano *et al.*, 2003; Zong *et al.*, 2003) that is channeled, for example, into mitochondria, inducing "mitochondrial stress". Blockade of Bcl-2 located in ER may interrupt the cross-talk between the two organelles (Annis *et al.*, 2001; Rudner *et al.*, 2001). Bax and Bak have also direct actions on ER, promoting activation of caspase-12 (Marsden *et al.*, 2002). Although there is a link between ER and mitochondria, apoptotic pathways involving ER and not mitochondria were proposed, which involve caspase-12 activation; in this model, caspase-12 activates caspase-9, which can then cleave caspase-3 (Rao *et al.*, 2002)**.**

Necrosis is other type of cell death that can also occur during development and is characterized by morphological and ultra-structural features that contrast to those characterizing apoptosis. Swelling of the cytoplasm, distension of cellular organelles, random DNA degradation, extensive plasma membrane endocytosis and autophagy (Ferri and Kroemer, 2001) are some of them. Intracellular components may be spilled out the cell and initiate inflammatory and immune responses in the extracellular space. The most remarkable characteristic that distinguishes apoptosis from necrosis is the fact that, during apoptosis, there is a regulated removal of cells in the absence of inflammation. This necrotic death does not require new protein synthesis, the energy requirements are minimal and there are no homeostatic processe to regulate it. Necrosis can be triggered by physiological ligands, which implies that specific signal transduction pathways are connected to its

induction. Thus, necrosis may be regulated genetically or epigenetically (several strains of mice are more sensitive to cell death by necrosis). Necrosis seems to be triggered by extreme conditions like toxins and reactive oxygen species, hypoglycemia and nutrient deprivation, hypoxia, ischemia and high temperature (Walker *et al.*, 1988; Nicotera *et al.*, 1999).

Fig.3

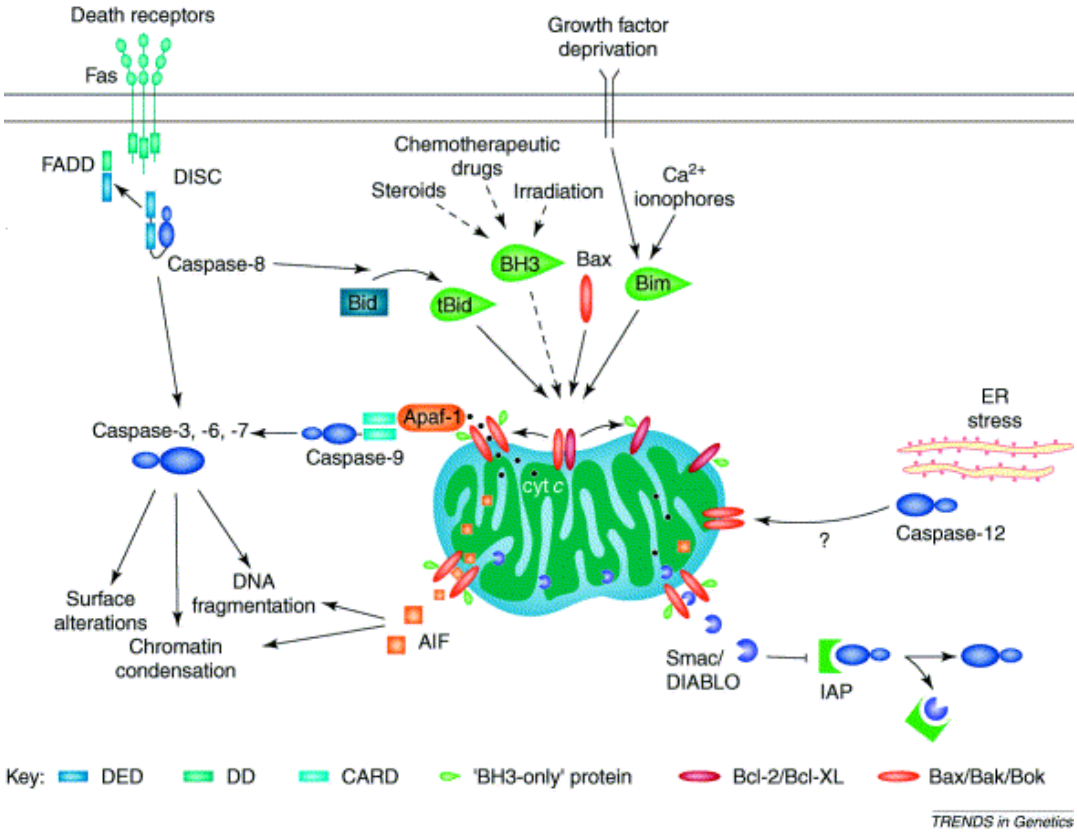


Fig.3 - Overview of apoptotic signaling in mammalian cells. Fas recruits FADD and caspase-8, which activates caspases-3, -6 and -7. Those effector caspases are responsible for surface alterations, chromatin condensation and DNA fragmentation. Bid, Bcl-2 Homology Domain 3 (BH3) and Bim may induce mitochondrial stress leading to the release of cytochrome c, which will form the apoptosome with Apaf-1 and caspase-9. AIF induces caspase-independent apoptosis. Smac/DIABLO inactivates the inhibitor of apoptosis proteins (IAP) derepressing caspases. ER stress leads to caspase-12 activation that may also have a role in mitochondrial damage. *From Genetic Analysis of the Mammalian Cell Death Machinery by Joza, N; Kroemer, G. and Penninger, J.M. Trends in Genetics 18: 142-149 (2002)*

There is evidence that Fas and TNF family of death receptors are required for necrosis induction (Henriquez *et al.*, 2008). Normally, the death receptors bind to FADD and caspase-8 and from this assembly results active caspase-8 which in turn activates downstream caspases like caspase-3 (Cryns and Yuan, 1998), in apoptosis. However, in the absence of caspases, the same death receptors may trigger a type of cell death with necrotic features - swelling of the mitochondria and ER, intracellular vacuolization and dilation of the nuclear membrane, loss of mitochondrial transmembrane potential without loss of cytochrome c (Van Cruchten and Van den Broeck, 2002).

Developing cells may die by processes whose characteristics are mixed between apoptosis and necrosis (Nelson and White, 2004). For example, in some cells the diameter of the cytoplasm increases due to mitochondrial swelling with little nuclear alterations which suggests the occurrence of necrosis; other cells die tipically by apoptosis (with nuclear chromatin condensation) or may die later, exhibiting cytoplasmic features of necrotic cell death when caspases have been inhibited or deleted.

2.3. Hippocampal Formation and Migration of Neurons to this Structure

A representation of the rodent hippocampal formation is shown below in **Fig. 4** and a brief description of this brain structure is required to later understanding of how newly generated neurons migrate from proliferative regions to occupy and form this structure. The term hippocampus refers only to the portion comprising CA1, CA2 and CA3 fields (Lorente de Nó, 1934). The hippocampal formation includes the hippocampus and the adjacent regions, dentate gyrus (DG), subiculum, presubiculum, parasubiculum (the three regions forming the called “subicular complex”) and the entorhinal cortex.

The pyramidal cell layer, also named *Stratum pyramidale*, contains principal or pyramidal glutamatergic cells, the most numerous cell types in the hippocampus. The narrow and cell-free layer located above the pyramidal cell layer is called *Stratum oriens*. The basal dendrites from pyramidal cells are located in this layer. Alveus is a thin fiber-containing layer and the most extreme layer of the hippocampal formation. Below the pyramidal cell layer and in the region of CA3, is present the layer containing mossy fibers originating from the DG. It is called *Stratum lucidum*. The *Stratum radiatum* is located more internally to the *Stratum lucidum* in CA3 and immediately below the

pyramidal cell layer in CA1 and CA2. The most internal layer of the hippocampus is called *Stratum lacunosum-moleculare*.

Fig. 4

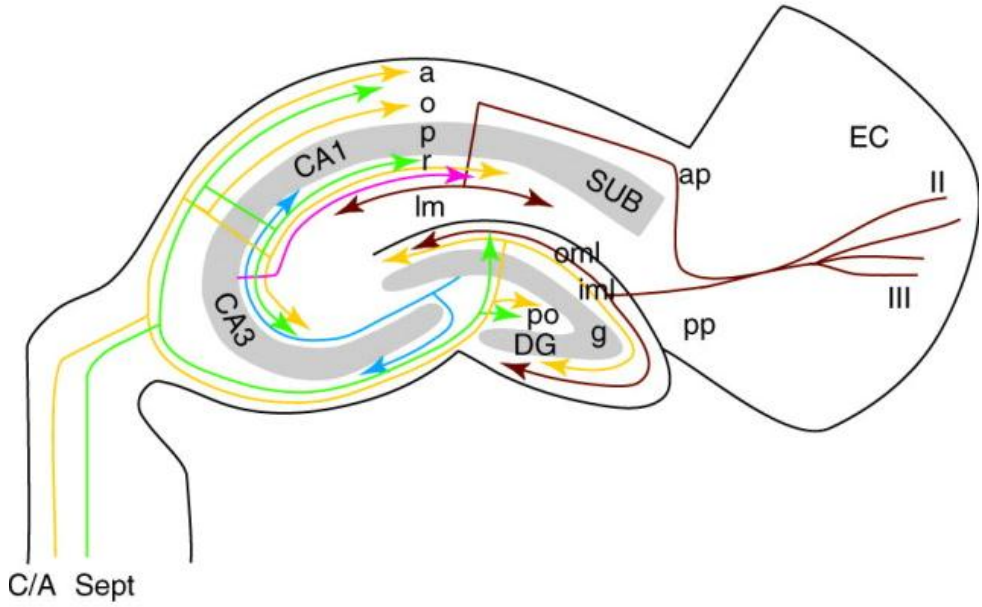


Fig.4 – Main axonal pathways of the hippocampal formation. Perforant (pp) and alvear path (ap) are shown in brown. Ipsilateral afferents originating from layer II and III of the entorhinal cortex (EC) innervate the outer molecular layers (oml) of the DG and the *Stratum lacunosum moleculare* (lm) of Ammon's horn. The commissural/associational projection (C/A) originating from mossy cells of ipsilateral and contralateral polymorphic layer (po) and terminating in the inner molecular layer (iml) of DG and *Stratum radiatum* (r) of Ammon's horn is shown in yellow. The septal projection (Sept) is shown in green, consisting of fibers from the medial septum and diagonal band of Brocca terminating underneath granular cell layer (g) and in both molecular layers of DG, r and *Stratum oriens* (o) of Ammon's horn. The mossy fiber projection (Mf) is shown in blue. These fibers connect the DG with CA3. Schaffer collaterals (Sch) are shown in pink. These fibers connect CA3 with CA1. Abbreviations: a, alveus; p, *Stratum pyramidale*; SUB, subiculum. *From New Molecules for Hippocampal Development by Skutella, T. and Nitsch, R. Trends in Neurociences. 24: 107-113 (2001).*

Interneurons or gamma aminobutyric (GABA)-releasing cells can be found in all layers (Freund and Buzsaki, 1996) and display a high heterogeneity of types. For example, basket cells are located close to the pyramidal cell layer and the dendrites locate in *Stratum oriens*, *radiatum* and *lacunosum-moleculare* (Lorente de Nó, 1934). They receive projections mostly from pyramidal cells but they also receive GABAergic projections (Jones, 2002). They innervate mostly dendrites from pyramidal cells. Axo-axonic or chandelier interneurons are also located close to the pyramidal cell layer and

their dendrites, similarly to basket cells, can be found in all layers, inervating the initial segment of pyramidal cells axons (Woodruff *et al.*, 2010). Oriens lacunosum-moleculare cells (O-LM) cells have a dense axonal arborization restricted to the *Stratum lacunosum-moleculare* and in CA3, the cell body and dendrites of this type of interneuron can be in the *Stratum oriens* or *radiatum* whereas in CA1 the cell body is located specifically in the *Stratum oriens* (Tort *et al.*, 2007). Bistratificated cells have the cell body located close to pyramidal cell layer and the dendrites never reach the *Stratum lacunosum-moleculare*. The axon projects in the *Stratum oriens* or *radiatum*. Some interneurons from the *Stratum radiatum* have the dendrites confined to this layer and the axon ramify there and terminate on the dendrites of pyramidal cells (Freund and Buszaki, 1996). Interneurons located in the *Stratum lacunosum-moleculare* (LM neurons) or in the border between *Stratum radiatum* and *lacunosum-moleculare* have dendrites oriented horizontally and some branches extend into the pyramidal cell layer and the axon projections and ramifications are also restricted to *Stratum radiatum* and *lacunosum-moleculare* (Kunkel *et al.*, 1988). The interneuron-selective cells are located in all layers and their dendrites form bundles with dendrites of other IS neurons and their axons terminate in the axons of other interneurons (Freund and Gulyás, 1997).

In the hippocampus, pyramidal neurons are generated between GD 14 and 16 in the mouse and between GD 16 and 19 in the rat brain (Soriano *et al.*, 1986). The generation of pyramidal cells that will later occupy CA3 region precede the generation of future CA1 pyramidal cells (Altman and Bayer, 1990). Stem cells of pyramidal neurons originate from the ammonic neuroepithelium located in the ventricular zone of the dorsal telencephalon (Altman and Bayer, 1990). Pyramidal cells start migrating one day after their generation and after 48 hours they start migrating towards the future pyramidal cell layer forming the hippocampal plate (Altman and Bayer, 1990). It takes 4 days to reach CA1 region and even more days to reach CA3 (Altman and Bayer, 1990). The pyramidal cell layer is formed at GD 20 for CA1 and GD 22 for CA3. Some pyramidal cells are still migrating at birth (Nakahira and Yuasa, 2005).

Granule cells are mainly generated during the post-natal period (Bayer, 1980a;) from the dentate neuroepithelium. The structure of DG can be recognized at around GD 21-22 (Altman and Bayer, 1990c).

Interneurons are generated prenatally in rodents (Amaral and Kurz, 1985; Lübbers *et al.*, 1985; Soriano *et al.*, 1986, 1989a,b). They are produced from both dentate and ammonic neuroepithelia located in the subpallial telencephalon generated from medial ganglionic eminence (MGE) at GD

13.5 – 14.5 (Pleasure *et al.*, 2000). There is also a heterogeneity in the day of birth of different types of interneurons, the ones belonging to the *S. radiatum* and *S. oriens* being generated before than the ones that will occupy the *S. pyramidale* and in this region, the interneurons that occupy the inferior portion of the stratum are the latest to be produced (Bayer, 1980a; Soriano *et al.*, 1989a). MGE also gives rise to cortical (Lavdas *et al.*, 1999; Wichterle *et al.*, 1999) and striatal (Marin *et al.*, 2000; Wichterle *et al.*, 2001) interneurons. The caudal ganglionic eminence (CGE) will generate interneurons that will be placed in DG, however some interneurons that have as destination the Ammon's horn can also be generated in this region (Nery *et al.*, 2002).

Newly generated neurons experience **migration** and generate different brain structures. Fibers of radial glia serve as scaffolding for migrating cells or differentiate itself into astrocytes or neurons (Rakic, 1972; Myiata *et al.*, 2001). Rakic (Rakic, 1988a) proposed a hypothesis supporting that post-mitotic cells migrate from the neuroepithelium toward the cortical surface along the same radial glial fascicle – **radial migration**. Other studies revealed that some neurons can migrate using the known **tangential migration** (Fishell *et al.*, 1993; O'Rourke *et al.*, 1997), not remaining associated to a radial glial fascicle. In rodents, 20% to 35% of all the cortical neurons are produced in the ganglionic eminence and arrive to the cortex by tangential migration independently of radial glial cells. In primates, virtually all the interneurons use this type of migration to reach the cortex (Anderson *et al.*, 1997; Tamamaki *et al.*,1997). Migration depends on the expression of genes that mediate chemoattraction/repulsion, cell adhesion, motility and cytoskeletal dynamics (Métin *et al.*, 2008). Environmental factor may also have an impact in neuronal migration (Ang *et al.*, 2006).

Pyramidal cells and interneurons from the hippocampus seem to originate from different precursors and they adopt different types of migration. Glutamatergic cells move by radial migration and GABAergic cells adopt tangential migration (Danglot *et al.*, 2006) – see **Fig. 5**.

2.4. S ynaptogenesis

The formation of the presynaptic terminal precedes the postsynaptic maturation and it happens earlier in CA3 region of the hippocampus, comparing with CA1 region (Altman and Bayer, 1990a) – see **Fig. 6**, where is presented the staged of formation of a glutamatergic synapse.including the contact between the pre and postsynaptic sites, the recruitment of pre and postsynaptic specializations, the increase of synaptic size and accumulation of receptors in the postsynaptic sites.

Fig. 5

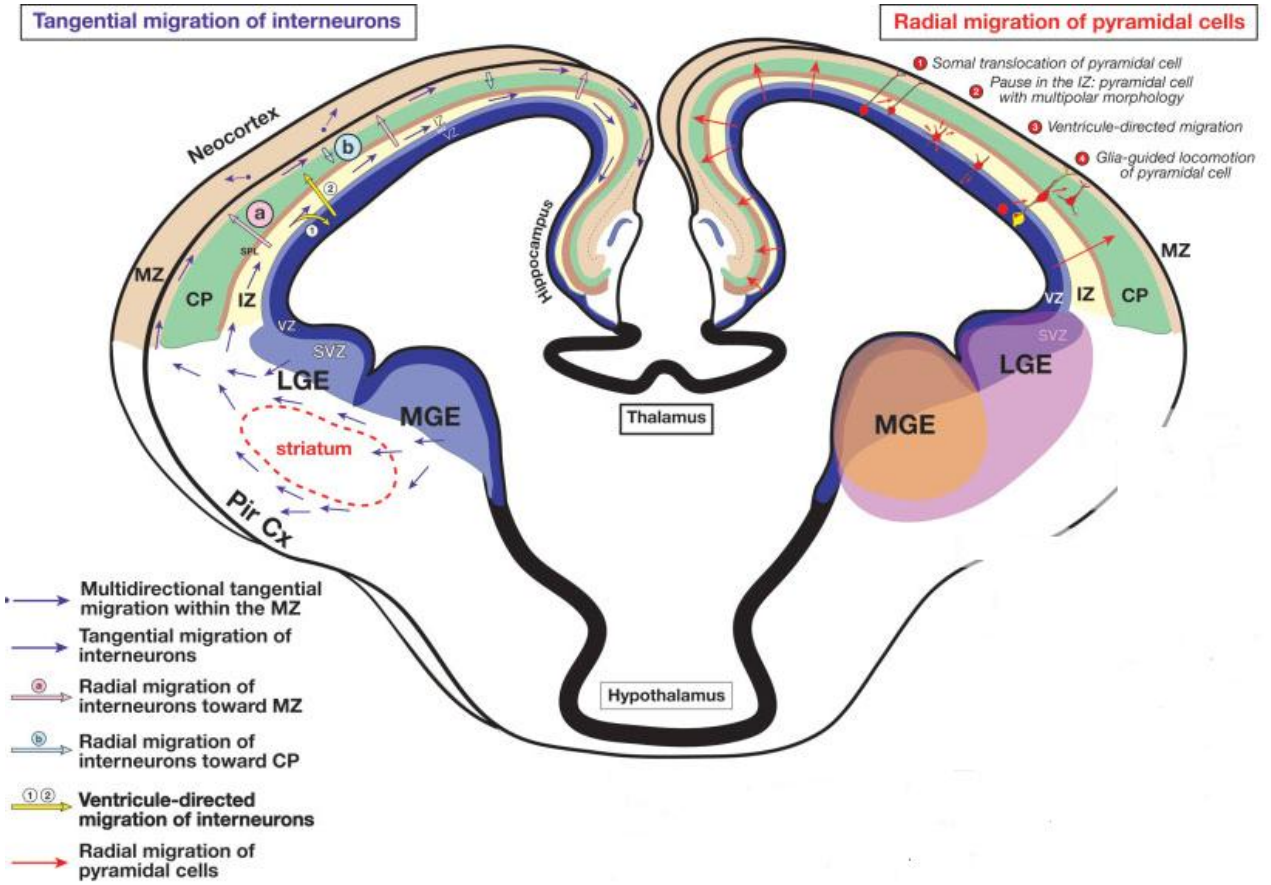


Fig. 5 – Types of migration of interneurons and pyramidal cells from the telencephalon. Pyramidal cells originate in the neuroepithelium and migrate orthogonally toward the pial surface (right part of the figure, red plain arrows). They can adopt four different modes of migration: somal translocation during early corticogenesis, glia-guided locomotion, multipolar migration at the intermediate zone/subventricular zone (IZ/SVZ) border and ventricule-directed migration. Most of all interneurons are believed to come from the ganglionic eminence by tangential migration (left part of the figure — violet plain arrows). Interneurons from the medial ganglionic eminence (MGE) migrate to the piriform cortex, the neocortex, or the striatum. Interneurons migrating tangentially follow two different streams: one in the SVZ/IZ and another one in the marginal zone (MZ). Interneurons in the MZ migrate tangentially to the plane of the cortex. They can adopt various directions and thus spread all over the cortex. Some neurons in the IZ can switch to radial migration and reach the MZ. Conversely MZ neurons can move radially toward the cortical plate (CP) but display a prolonged pause at the MZ/CP interface. Some interneurons can also adopt ventricule-directed migration (yellow arrows): they migrate toward the ventricular zone (VZ) (1), pause in the VZ, and then turn back toward the pial surface to their final destination. Interneurons can reach the hippocampus either by the SVZ/IZ or by the MZ stream.
Abbreviations: CGE, caudal ganglionic eminence; CP, cortical plate; IZ, intermediate zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; MZ, marginal zone; Pir Cx, piriform cortex; POA, preoptic area; SPL, subplate; SVZ, subventricular zone; VZ, ventricular zone. *Adapted from Development of Hippocampal Interneurons in Rodents by Danglot, L., Triller, A, Marty, S. Hippocampus. 16: 1032-1060 (2006)*

GABAergic and glutamatergic contacts constitute the major types of synapses in the hippocampus. Several molecules are important in different steps of synapse formation. For example, ephrins play important roles directing axons to a proper location (Dufour *et al.*, 2003; O'Leary *et al.*, 1999) – see also **Table II**. This process is known as **axon guidance**. Adhesion molecules such as cadherin are though to be important in establishing the initial axodenditic contact (Benson *et al.*, 2001; Togashi *et al.*, 2002). Neuroligins and neurexin which are transsynaptic molecules are fundamental for the bidirectional signaling and recruitment of pre and postsynaptic proteins (Scheifelle, 2003; Levinson and El-Husseini, 2005) and finally, neuronal activity seems to be another key regulator in formation of new synapses (Alzenman and Cline, 2007; Cline and Haas, 2008).

The process of axon guidance seems not to be random. Some axons establish contacts with specific subcellular compartments such as cell body, dendrites, axons and spines (Chédotal and Richards, 2010). For example, in the developing hippocampus, axons from the entorhinal cortex establish synaptic contacts with the distal part of granule cells dendrites and commissural/associational axons establish synapses with the proximal parts of these dendrites (Super and Soriano, 1994; Forster *et al.*, 2006).

The major afferent projections in the hippocampus are **entorhino-hippocampal** pathway in which synapses are formed with the granule cells at the level of dendrites, the **commissural/associational system** and the **septal projection** (Amaral and Witter, 1995). The intrinsic fibers are **mossy fibers** that connect the DG to CA3 pyramidal cells and **Schaffer collaterals** that connect CA3 to CA1 (see **Fig.4**). Further away in the text, it will be presented the molecules that serve as chemoattractant/repulsive molecules intervenient in the process of synaptogenesis.

Ca^{2+} signaling promotes the production of cyclic nucleotides and these molecules can control the activity of Ca^{2+} channels in the plasma membrane or induce Ca^{2+} release from intracellular stores (Gomez and Zheng, 2006; Zheng and Poo, 2007). Disruption of these systems leads to guidance defects. Ca^{2+} levels in growth cones are increased after exposure of these structures to guidance cues (Gomez and Zheng, 2006; Henley and Poo, 2004). Moderate increase in Ca^{2+} levels, normally achieved by its release from intracellular stores, favors attraction whereas low or high levels favors repulsion (Gomez and Zheng, 2006; Henley and Poo, 2004). Attractant cues such as brain derived neurotrophic factor (BDNF) and netrin depolarize the growth cone membrane and repulsive cues, such as slit and semaphorin have an opposite effect (Henley *et al.*, 2004; Li *et al.*, 2005). High levels of cAMP or cGMP promote attraction and low levels favor repulsion (Nishiyama *et al.*, 2003; Song

et al., 1997). These effects are independent on the effects of receptors that they modulate. Some effectors of Ca^{2+} and cyclic nucleotides are kinases (PKA, PKC, Src, CamKII) phosphatases (calcineurin and PP1 protein phosphatases), calpains and Rho family small GTPases (Bashaw and Klein, 2010). Rho GTPases are also regulated by semaphorins, ephrins, netrins and slits (Moore *et al.*, 2008a; Swiercz, *et al.*, 2002; Jurney *et al.*, 2002; Wahl *et al.*, 2000). Some of the molecules involved in axon guidance are also involved in the other stages of synaptogenesis, like the contact between the pre and postsynaptic sites and pre and postsynaptic specialization and maturation.

Synapse maturation which is characterized by an increase in the morphological size and transmission strength of the synapse dependent on both pre and postsynaptic sites (Chiu and Cline, 2010). For example immature glutamatergic synapses can be silent or have low activity because N-methyl-D-aspartate (NMDA) receptor is their major component in terms of glutamate receptors. As the synapse mature, 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid (AMPA) receptors are also incorporated in synapses providing fully functional synaptic activity (Wu *et al.*, 1996). Synapses are maintained according to their activity. Weaker synapses are eliminated (Hashimoto and Kano, 2003). Synapse elimination can be a process of refining the brain circuits during development (Rakic *et al.*, 1986).

2.4.1. The GABAergic synapse

GABAergic synapses are established before glutamatergic synapses in the hippocampus (Tyzio *et al.*, 1999). Interneurons appear at GD18 in the rat hippocampus (Bayer and Altman, 1974; Rozenberg *et al.*, 1989). Glutamate decarboxylase (GAD)-containing terminals are already present *in utero* and they can be found in the *Stratum radiatum* at birth (Rozenberg *et al.*, 1989). Immunolabeling with antibodies against GABAergic neurons, such as GAD (Dupuy and Houser, 1996), GABA (Rozenberg *et al.*, 1989), calbindin (Super *et al.*, 1998), and synaptic terminals, synapsin-1 and synaptophysin (Tyzio *et al.*, 1999), showed that the first synapses formed in the hippocampus are peridendritic preceding the perisomatic ones that are established later on when pyramidal cells occupy the S*tratum pyramidale*. These morphological observations are supported by electrophysiological recordings made in CA1 area of the hippocampus at P0. Three populations of interneurons were identified - 5% of the recorded interneurons were silent, 17% of neurons had only GABAergic currents and 78% had both GABAergic and glutamatergic currents (Ben-Ari *et al.*, 2004; Hennou *et al.* 2002). Those groups of cells corresponded to cells at different developmental

stages. Silent neurons had few or no dendrites, neurons containing only GABAergic currents displayed more extended dendritic arbors and cells with GABAergic and glutamatergic synapses were the most complex having extended and ramified dendrites (see **Fig. 7**)..

$GABA_A$ receptors are formed by combinations of subunits forming a pentameric structure (Stephenson, 2006). In an immunohistochemical study, the $GABA_A$ receptor composition in terms of subunits was analyzed during postnatal development (Fritschy *et al.*, 1994). The α-1 subunit immunoreactivity is low at birth in the neocortex, hippocampus, thalamus and cerebellum and α-2 subunit decreases in expression after the onset of α-1 subunit. β-2,3 subunits show a widespread localization throughout all periods analyzed. Previous studies using Western blot and messenger ribonucleic acid RNA (mRNA) analysis demonstrated a high density of α-2, α-3, α-5, β-3 and γ-2 (*e.g.* Fuchs and Sieghart, 1989; Gambarana, *et al.*, 1990; Vitorica *et al.*, 1990; Zhang *et al.*, 1991b, 1992b; Laurie *et al.*, 1992b; Poulter e*t al.*, 1992, 1993). The subunit combination α2/β2,3/γ2 is the most prevalent in neurons in the neonatal brain (Benke *et al.*, 1991b) and α1/β2,3/γ2 is the most prevalent in the adult brain (Benke *et al.*, 1991b,c).

Fig.6

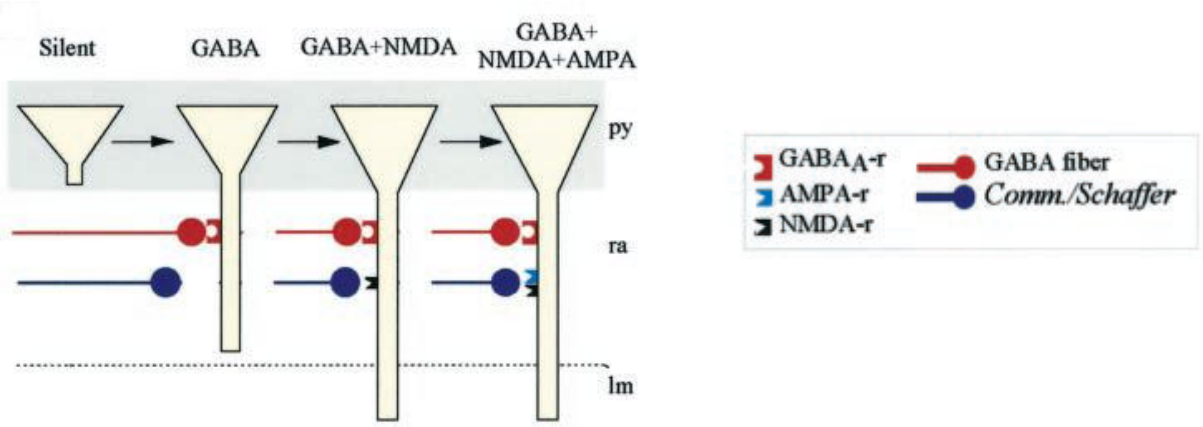


Fig.6– Representation of the stages of maturation of pyramidal cells at PD 0 in CA1 region of the hippocampus. Silent pyramidal cells have an apical dendrite restricted to the *Stratum pyramidale* and do not receive axonal projections. Pyramidal cells possessing dendrites in the *Stratum radiatum* receive GABAergic fibers and glutamatergic fibers (such as Schaffer/commissural projections) only project onto synapses that have dendrites in the *Stratum lacunosum moleculare*. NMDA receptor containing synapses precede AMPA receptor-containing synapses. Abbreviations: py-Stratum pyramidale, ra-Stratum radiatum, lm-Stratum lacunosum moleculare, $GABA_A$-r –$GABA_A$ receptor, AMPA-r – AMPA receptor, NMDA-r –NMDA receptor, Comm/Schaffer-Schaffer/commissural synapses. Adapted from *The Establishment of GABAergic and Glutamatergic Synapses on CA1 Pyramidal Neurons is Sequential and Correlates with the Development of the Apical Dendrite. Journal of Neuroscience.19:10372-10382 (1999).*

$GABA_A$ receptors assemble in the ER. After being inserted into vesicles, the intracellular domain of $GABA_A$ receptor γ subunit interacts with GABA receptor-associated protein (GABARAP) which is concentrated in the Golgi apparatus and in intracellular vesicles (Wang *et al.*, 1999). Recently it was shown that GABARAP is necessary for increasing cell surface of GABA receptors after NMDA receptor activation (Marsden *et al.*, 2007). N-ethylmaleimide-sensitive factor (NSF) and GABARAP can promote trafficking of receptors from the Golgi apparatus (Goto *et al.*, 2005). Brefeldin A-inhibited guanine nucleotide-exchange protein 2 (BIG2) is another protein having a function in the intracellular trafficking of $GABA_A$ receptors namely in their delivery in the plasma membrane (Chen and Olsen, 2007). Lateral diffusion within the plasma membrane allows for continual exchange of receptors between synaptic and extrasynaptic sites (Danglot *et al.*, 2003; Jacob *et al.*, 2005). Most receptors are delivered to extrasynaptic locations (Bogdanov *et al.*, 2006).

The scaffolding protein gephyrin is involved in synaptic targeting of $GABA_A$ receptors containing α2 and γ2 subunits at inhibitory synapses (Essrich *et al.*, 1998). However, gephyrin-independent synaptic targeting was shown for α5 subunit involving radixin protein (Kneussel *et al.*, 2001).

2.4.1.1. The Fundamental Role of GABA and GABA Receptors in the Neural Development

GABA can be released at an early developmental stage even before synapse formation and cell precursors and maturating neurons express functional GABA receptors (Nguyen *et al.*, 2001; Owens and Kriegstein, 2002; Demarque *et al.*, 2002). At this stage, GABA can be released by pionner and migrating neurons (Ben-Ari *et al.*, 2007), growth cones (Gordon-Weeks *et al.*, 1984) and possibly glia (Barakat and Bordey, 2002) and diffuses even at long distance, exerting paracrin functions, acting as a trophic factor and modulating events as neuronal formation, migration, differentiation, synapse formation, neuronal growth and network formation (Ben-Ari *et al.*, 2007). In this case, the release may involve exchangers, gap junction hemichannels or volume-sensitive Cl^- channels (Demarque *et al.*, 2002).

Fig.7

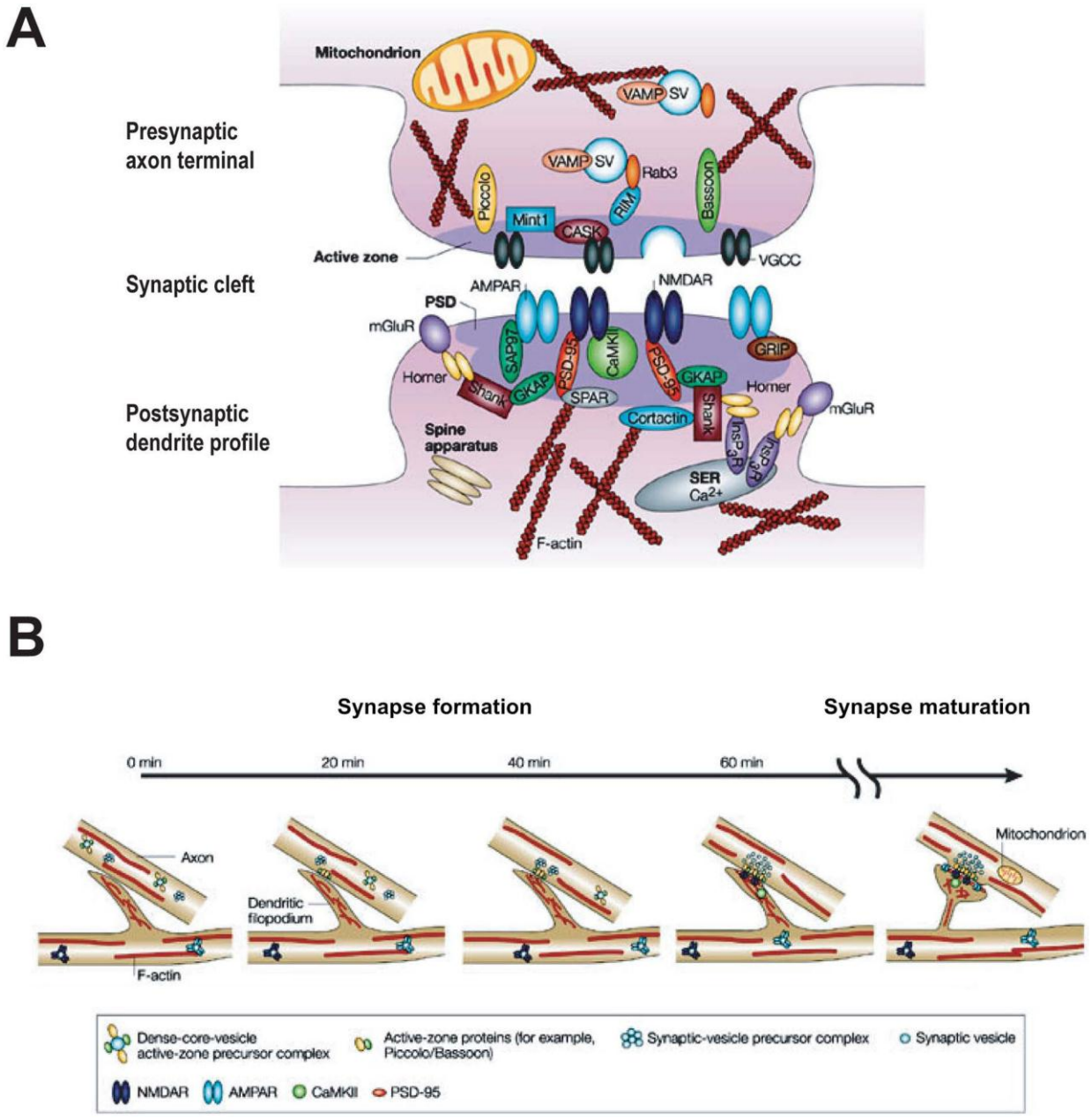


Fig.7 - Schematic diagram of an excitatory synapse and the temporal sequence of synapse formation and maturation. (**A**) Synapses are specialized junctions between neurons composed of complex membrane and proteins. A synapse can be divided structurally into three parts: a presynaptic axon terminal packed with synaptic vesicles (SV) and release machinery, a synaptic cleft, and a postsynaptic dendritic counterpart filled with neurotransmitter receptors, scaffold proteins and signaling machinery. (**B**) Synapse formation is initiated by the contact between dendrites and axons, followed by the recruitment of presynaptic and postsynaptic specializations. Increases in synapse size and synaptic strength by accumulation of AMPA receptors at synapses are characteristics of synapse maturation. AMPAR, a-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; CaMKII, Calcium calmodulin dependent kinase type II; CASK, calcium calmodulin-dependent serine kinase;GKAP, guanylate kinase-associated protein; GRIP, glutamate receptor-interacting protein; InsP3R, inositol triphosphate receptor; mGluR,metabotropic glutamate receptor; NMDAR, N-methyl-D-aspartate receptor; PSD, postsynaptic density; PSD-95, postsynaptic density protein-95;RIM, Rab3-interacting molecule; SAP, synapse-associated protein; SER, smooth endoplasmic reticulum; SPAR, spine-associated Rap GTPase activating protein; VAMP, vesicle-associated membrane protein; VGCC, voltage-gated calcium channel. *From Insulin receptor signaling in the development of neuronal structure and function by Chiu, S-L., Cline, HT. Neural Development.7(5): 1-18*

Table II

LIGAND	***LOCALIZATION***	***FUNCTION***
Semaphorin-3A	DG, border Sub/CA1	Repulsion of EC, DG, CA1–3
Semaphorin-3C	DG, CA	Repulsion of MS
Semaphorin-3F	DG, CA	Repulsion of DG, CA1–3
Netrin-11	DG, CA	Attraction of DG, CA3
Slit-2	EC, Cajal–Retzius cells, DG, CA	Repulsion of DG
Ephrin-A1	LS	Inhibition of outgrowth in medial hippocampus
Ephrin-A3	DG, CA	Repulsion of EC, DG
Ephrin-A5	CA, LS	Inhibition of outgrowth in medial hippocampus
RECEPTOR	***LOCALIZATION***	***FUNCTION***
Neuropilin-1	EC, DG, CA	Repulsion of EC, DG, CA
Neuropilin-2	DG, CA, MS	Repulsion of DG, CA, but not EC
Robo	EC, DG, CA	Repulsion of DG
EphA5	EC, DG, CA	Repulsion of EC, DG

Abbreviations: CA, cornu ammonis; DG, dentate gyrus; EC, entorhinal cortex; LS, lateral septum; MS, medial septum; Sub, subiculum.

Table II – Molecules involved in hippocampal pathfinding. *Adapted from New Molecules for Hippocampal Development by Skutella, T. and Nitsch, R. Trends in Neurociences. 24: 107-113 (2001)*

Fig. 8

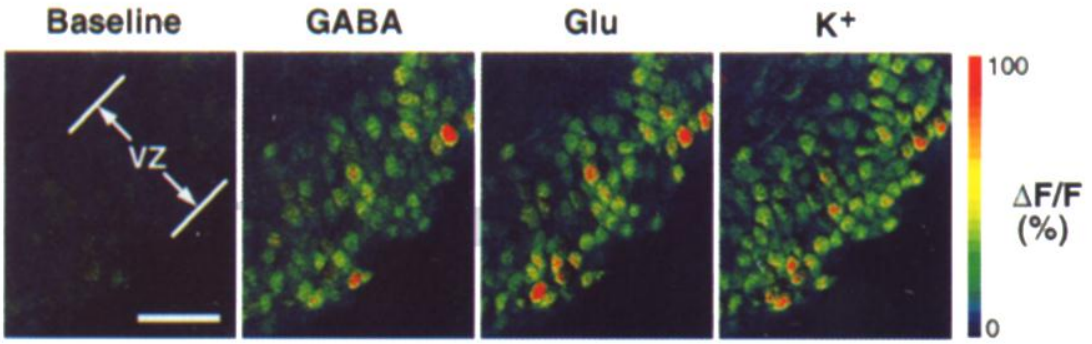


Fig.8 – Embryonic cortical cells in a brain slice at the GD 16 loaded with Fluo 3, a Ca^{2+} indicator, before and after GABA, glutamate and potassium (K^+) application. All of the molecules tested have depolarizing properties increasing the intracellular calcium concentration in these cells. *From GABA and Glutamate depolarize Progenitor Cells and Inhibit DNA Synthesis by LoTurco, J.L., Owens, D.F., Heath, M.J.S., Davis, M.B.E, Kriegstein, A.R. Neuron 15:1287-1298 (1995)*

GABA acting on $GABA_A$ receptors was shown to induce the activation of protein kinase (MAPK) pathway (Borodinsky *et al.*, 2003) which is involved in the regulation of cell cycle progression in neuronal progenitor cells (Li *et al*, 2001) thus controlling neuronal progenitors proliferation. For example, indirect activation of MAPK cascade by GABA promotes immature cerebellar granule cell proliferation (Fiszman *et al.*, 1999).

GABA can also have trophic actions upon neuronal precursors. GABA enhanced the survival of rat embryonic (GD18) striatal neurons *in vitro* and this effect can be indirect, through the ability of GABA to modulate basic fibroblast growth factor (bFGF), a factor involved in the proliferation of cortical progenitors (Antonopoulos *et al.*, 1997). GABA or the $GABA_A$ receptor agonist muscimol co-applied with bFGF blocked that effect.

Apparently, GABA acting both in $GABA_A$ and $GABA_B$ receptors can accelerate or decrease motility of post-mitotic precursors. Chemoattractant or repulsive actions of GABA seem to depend on the concentration. Cortical neurons cultured at GD18 and evaluated one day after plating move randomly – **chemokinesis** - in the presence of micromolar concentration of GABA whereas femtomolar GABA concentration induced directed migration – **chemotaxis** – (Behar *et al.*, 1996). *In vivo* it was observed that GABA is released near the target destinations of migratory neurons (Behar *et al.*, 2000). Apparently, the cells that respond to different GABA concentrations have different

origins. Femtomolar GABA induces ventricular cells to migrate and micromolar GABA stimulates migration of cortical plate cells (Behar *et al.*, 1998).

GABA-induced chemotaxis and chemokinesis depends on the activation of G_i/G_0 proteins because pertussis toxin blocked those movements (Behar *et al.*, 1998). The G-protein recruitment may serve as an amplifying system making low concentrations of GABA physiologically relevant (Barceló *et al.*, 2007). Also, GABA-induced motility is attenuated by cell depolarization. For example, glutamate and elevated K^+ concentration decrease migration triggered by micromolar GABA (Behar *et al.*, 1998). This is in agreement with other reports that show that *in vivo* excitatory signals, for example GABA released by GABAergic cells, high K^+ concentration from the interstitial fluid, encountered near the target destination of migrating neurons may be important in signaling approaching cells to stop moving (LoTurco *et al.*, 1995).

Behar and others (Behar *et al.*, 2000) showed that activation of $GABA_A$ receptors can also arrest cell movement because its blockade with bicuculline, an antagonist of $GABA_A$ receptors, resulted in an increase in the number of bromodeoxyuridine positive ($BrdU^+$) cells, considered progenitor cells. The intracellular mechanisms underlying cell arrest seem to involve modulation of intracellular Ca^{2+} levels (Behar *et al.*, 1998). However, different GABA concentrations elicit distinct patterns of intracellular Ca^{2+} levels which mean that this ion can promote or retard cell movement (Gomez *et al.*, 1995; Horgan and Copenhaven, 1998; Komuro and Rakic, 1998; Gomez and Spitzer, 1999).

Molnar and co-workers (López-Bendito *et al.*, 2003) found that $GABA_B$ receptor antagonist produced a dose-dependent accumulation of tangentially migrating neurons in the ventricular/subventricular zones of the cortex and those effects are most likely to be presynaptic once they do not generate currents in neurons.

It was shown in mammalian neurons that bicuculline reduced the dendritic outgrowth of cultured hippocampal neurons (Barbin *et al.*, 1993), an effect also observed in other brain regions, (*e.g.* Borodinski *et al.*, 2003; Maric *et al.*, 2001; Tapia *et al.*, 2001). Again, this GABA-mediated effect seems to increase Ca^{2+} influx and subsequent activation of Ca^{2+}-dependent kinases (Borodinski *et al.*, 2003) see **Fig. 8**.

2.4.2. The Glutamatergic Synapse

The first functional glutamatergic synapses require the participation of NMDA receptors rather AMPA receptors (Durand *et al.*, 1996; Wu *et al.*, 1996; Hsia *et al.*, 1998; Petralia *et al.*, 1999). The synaptic appearence of AMPA receptors is modulated by activity, involving the activation of NMDA receptors and an increase in the intracellular Ca^{2+} levels (Malenka and Nicoll, 1997). During hippocampal development, depolarizing actions of GABA are able to remove the magnesium (Mg^{2+}) block from NMDA channels (Leinekugel *et al.*, 1997) and NMDA receptor activity may strongly contribute to the maturation of glutamatergic synapses. NMDA receptor seems to be essential for synapse formation and maturation. Thus, the long-term blockade of NMDA receptor during development in culture leads to a substantial increase in synaptic number and to the formation of a more complex dendritic arborization of CA1 pyramidal cells (Luthi *et al.*, 2001). Both excessive and weak NMDA receptor activation can be harmfull for developing neurons (Lipton and Nakanishi, 1999).

The NMDA receptor is a tetramer containing an obligatory NR1 subunit and one or more NR2 subunits (Ishii *et al.*, 1993; Meguro *et al.*, 1992; Monyer *et al.*, 1992). Four different genes encode NR2 subunits (NR2A-D) and their expression is developmental- and regionally-dependent (Monyer *et al.*, 1994; Sheng *et al.*, 1994). NR3 subunits can also be found in the composition of NMDA receptors (Cavara and Hollmann, 2008). Only NR2 subunits have binding sites for glutamate (Lynch and Guttmann, 2001). Assembly of NMDA receptors occurs in ER, being then exported for processing in the Golgi and transported into vesicles along dendrites using microtubules and adaptor proteins that couple the vesicle to kinesin motors. Kinesin KIF17 interacts directly with NMDA receptors (Setou *et al.*, 2000). At the early development the targeting is made to extrasynaptic sites and these receptors contain both NR1 and NR2B subunits (Tovar and Westbrook, 1999). Extrasynaptic receptors which have NR2B subunit have the ability to move in and out the synapses. NR2B-containing receptor is delivered to the synapse in a constitutive form and NR2A incorporation requires ligand binding to pre-existing receptors and this incorporation is done directly from intracellular compartments (Barria and Malinow, 2002). NMDA receptors are stabilized at the synapse by postsynaptic density-95(PSD-95), postsynaptic density-93 (PSD-93), synapse-associated protein 97 (SAP97) and synapse-associated protein 102 (SAP102) (Sheng and Sala, 2001).

Phosphorylation of NMDA receptor by PKC increases cell surface expression of the receptor in COS7 cells (Scott *et al.*, 2001) and increases NMDA-elicited currents and channel open probability

in neurons (Chen and Huang, 1992; Xiong *et al.*, 1998; Zheng *et al.*, 1999). PKA-mediated phosphorylation increases the activity of NMDA receptors (Leonard and Hell, 1997) and it induces the targeting of the receptor to the cell surface (Crump *et al.*, 2001). CaMKII seems to be able to phosphorylate NR2B (Omkumar *et al.*, 1996) and its anchoring to NR2B receptor may also contribute to phosphorylation of synaptic AMPA receptors (Barria *et al.*, 1997), increasing their conductance (Derkach *et al.*, 1999), assembly or delivery in the plasma membrane (Lisman *et al.*, 2002). Dephosphorylation of residues on the C-terminus of NMDA receptor allows the Adaptor Protein-2 (AP-2) binding, leading to clathrin-mediated endocytosis (Vissel *et al.*, 2001). NMDA receptors that are bound to PSD-95 protein are stabilized and its internalization is more difficult (Sturgill *et al.*, 2009).

AMPA receptors are tetramers (Rosenmund *et al.*, 1998) composed of different combinations of GluR1, GluR2, GluR3 and GluR4 subunits (Hollmann and Heinemann, 1994). GluR4-containing subunits are more abundant during post-natal development (Zhu *et al.*, 2000), in spite of mature synapses that contain mainly GluR1-GluR2 or GluR2-GluR3 combinations (Wenthold *et al.*, 1996). The assembly of the subunits occurs in the ER (Leuschner and Hoch, 1999) and they pass to the Golgi compartment to undergo fully glycosylation (Greger *et al.*, 2002). The exit of AMPA receptors from the Golgi seems to require interaction with PDZ domain-containing proteins through the PDZ consensus motif of C-terminus of GluR2 subunit. Protein interacting with PRKCA 1 (PICK1) (Dev *et al.*, 1999) and SAP97 (Leonard *et al.*, 1998) are two of these proteins. Cargo containing AMPA receptor subunits are recognized by motors that transport it by an unknown mechanism along the cytoskeleton. The link between the cargo and microtubular motor proteins is Glutamate Receptor Interacting Protein 1/AMPA binding protein (GRIP1/ABP) (Dong *et al.*, 1997). Several types of kisenin motors can transport GRIP1-AMPAR complex (Esteban, 2003b). GluR2-GluR3 oligomers are continuously delivered into synapses independently of synaptic activity (Passafaro *et al.* 2001; Shi *et al.* 2001) – constitutive pathway whereas GluR1-GluR2 (Hayashi *et al.*, 2000) and GluR4 (Zhu *et al.*, 2000) containing synapses are inserted depending on NMDAR activation – regulated pathway.

Several intracellular pathways are involved in AMPARs trafficking. For example, during long-term potentition (LTP), CaMKII activation activates Ras that leads then to synaptic delivery of AMPARs via p42-44 MAPK or PI3K (Man *et al.*, 2003). Also, phosphorylation of GluR1 by PKA is required for AMPA receptor synaptic delivery (Esteban *et al.*, 2003) and controls the recycling of receptors between the plasma membrane and endosomal compartments (Ehlers, 2000). During early

development, cAMP-dependent protein kinase (PKA)-mediated phosphorylation of GluR4 is necessary for synaptic delivery (Esteban *et al.*, 2003). During long-term depression (LTD), the removal of AMPA receptors seems to involve phosphorylation by PKC of the non-phosphorylated GluR2 that is interacting with the PDZ domain-containing protein with glutamate receptor interacting protein 1/associated binding protein (GRIP1/ABP), which results in its dissociation and interaction with PICK1 that keep AMPA away from the synapse (Daw *et al.*, 2000; Chung *et al.*, 2003). During LTD, NMDA receptor opening and Ca^{2+} entry leads to activation of Rap1 (Kawasaki *et al.*, 1998) that then activates p38 kinase resulting in removal of AMPA receptors from synapses. Removal of receptors from synapses can also be a constitutive or regulated process. During the first process, AMPA receptors may continuously move in and out of synapses to extrasynaptic sites (Lüscher *et al.*, 1999). During processes of LTD, AMPARs can also move out of synapses. It seems that all AMPARs subunits can be affected by LTD (Lee, 2006).

Kainate receptors are ion gated channels activated by the endogenous glutamate formed by several combinations of subunits (GluR5-7, KA1 and KA2) (see the review of Jane e*t al.*, 2009). Bahn and collaborators (Bahn *et al.*, 1994) identified the kainate receptors in the rat brain during development. They found that all genes coding kainate receptor subunits undergo up-regulation in the late embryonic/postnatal period, suggesting a role in brain development. Molecular determinantes and differences between the traffick of kainate and AMPA and NMDA receptors can be found in Coussen 2009.

2.4.3. Network Activity in the Immature *in vitro* Hippocampal Slice and Neural Circuit Refinement

During the first postnatal week, hippocampal activity is characterized by synchronous events network-driven, called giant depolarizing potentials (GDPs) (Ben-Ari *et al.*, 2007). GDPs result from the synchronization of neuronal network discharge from the cooperation between GABAergic and glutamatergic synaptic connections. A more detailed description of this pattern of activity in the immature slice can be found in the **Introduction** of the the **Chapter 3**.

Some synapses are modified or removed according to their activity. **Networks** or **neural circuits** form which can underlie brain function and that be **refined** throughout life.

3. The Purinergic System in the Context of the Nervous System Development – Why to Study It?

The development of the mammalian central nervous system (CNS) depends on **intrinsic** and **extrinsic** factors. Amongst the extrinsic factors are signaling molecules like neurotransmitters/neuromodulators. They are components of the *milieu* in which developing cells grow and they may act as trophic factors controlling cell survival, growth and migration (Miranda-Contreras, *et al.*, 1998, 1999, 2000). Progenitor cells are endowed with receptors for the surrounding molecules even before the formation of established synapses (Sah *et al.*, 1997; Belachew *et al.*, 1998a; Flint *et al.*, 1998; Haydar *et al.*, 2000; Ma *et al.*, 1998, 2000; Maric *et al.*, 2000d, Maric *et al.*, 2001), which shows the importance of the extrinsic factors on nervous system formation. Abundant data support the trophic roles of GABA and glutamate (as discussed previously) but less reports focus on their action of other neurotransmitters/modulators. Most of the available evidence on a putative role of the purinergic system during development was gathered in simple models such as cultured cells.

The study of the role of ATP and adenosine during brain development can be relevant. Rivkees and collaborators (Rivkees *at al.*, 2001) emphasized the powerful role of adenosine during brain maturation. Adenosine can be released by all cells in all structures and tissues independently on their level of maturation, such as GABA and glutamate; this endows adenosine with the ability to modulate or affect important developmental processes such as proliferation of precursors, migration and synapse formation. The same should be true for other molecules belonging to the purinergic system such as ATP, which can be released independently of synaptic activity – see the section **Introduction – The Purinergic System Overview**. Purine-mediated signaling "adapts" to the maturation level of the nervous system due to their ability to detect its state of activity, being able to change the location and/or expression of their components (Rivkees *et al.*, 2001).

A summary of the actual knowledge of the ontogenic location/function of the principal components of the purinergic system will be briefly presented next. The ontogenic profile of expression/function of transporters for purines will not be presente here.

3.1. Adenosine Receptors

3.1.1. A_1 Receptor

Rivkees and collaborators (Rivkees, 1995a) performed an ontogenic study using *in situ* hybridization, autoradiography and binding experiments to characterize the ontogenic profile of expression of A_1 receptors in the rat brain. They found detectable levels of this adenosine receptor subtype in the rat brain at GD 14; however a more prominent and widespread expression was observed at GD 17 and at GD 21 the levels were at about five times higher than at GD 17. The higher level of expression was found in the adult. Sites of moderate to high A_1 receptor mRNA levels at GD 17 included the brain stem, pons, dorsal root ganglia, trigeminal ganglia, midbrain, thalamus, hypothalamus and hippocampus. A lower pattern of mRNA expression coding A_1 receptor was found in the cortex and cerebellum.

Weaver (Weaver, 1996) presented a more detailed study about the expression of this receptor in rat embryos, using also *in situ* hybridization. Similarly to the previous study, A_1 receptor mRNA was not detectable in embryonic tissues before GD 14. An intense labeling was detected in neuroepithelia at GD 14 however, a direct correlation between neurogenesis and A_1 receptor expression could not be made because high levels mRNA were also found after neurogenesis and migration of neuroblasts out of proliferative regions and ventricular zone generating the cerebral cortex, hippocampus, striatum and cerebellum do not express high levels of mRNA for A_1 receptor subtype. At GD 18, the pattern of A_1 receptor gene expression was more similar to that observed in the adult (Reppert *et al.*, 1991).

Daval and co-workers (Daval *et al.*, 1991b) carried out the first quantitative autoradiographic study to detect A_1 receptors in the postnatal brain development in rodents. At birth, A_1 receptor levels were found to be at around 10% of the adult levels and the pattern of labeling was quite homogeneous throughout the brain. The regions less intensely labeled corresponded to subcortical regions which is in agreement with previous studies made using membranes from different brain structures (Marangos *et al.*, 1982). Another study of Rivkees's group, brought more detailed information about the expression of A_1 receptor in the hippocampus (Rivkees *et al.*,1995b). It was observed that A_1 receptors first appeared in the *Stratum radiatum* of CA3 at PD 1. The strongest labeling was found on granule cell bodies and dendrites, mossy fibers, and pyramidal neurons. In the

cerebellum basket cells are the most heavily labeled and in the cerebral cortex pyramidal cells were the most heavily labeled type as well as some interneurons. Neurons in the globus pallidus and in the caudate-putamen also displayed some labeling.

In the adult, A_1 receptors couples to G_i or G_0 protein (Olah and Stiles, 1995) as mentioned previously. G_i protein expression has been detected in rodent fetuses at GD 6.5 (Jones *et al.*, 1991) suggesting that the coupling between A_1 receptor and the effector G-protein is possible during uterine life but probably less effective than in the postnatal period (Musser *et al.*, 1993). Daval and colleagues (Daval *et al.*, 1991) used a non-hydrolysable GTP analog in the incubation medium for binding assays, which binds to G- proteins, shifting the affinity of metabotropic receptors to a low affinity; it lowered the tritiated cyclohexyldenosine (^{3}H-CHA)-specific binding to A_1 receptor in all regions and in all ages tested (PD 0, 1, 5, 10, 15, 25 and adult). This suggests that during the first post-natal week of life, A_1 receptors are already functional. Interestingly, this reduction of the specific binding of CHA was at about 90 % throughout the brain at PD 5, whereas at PD 0 and PD 1, this reduction was only about 70% and 60%, respectively. This observation was interpreted as if some A_1 receptors were not linked to G-proteins or simply, some A_1 receptors could have a lower affinity for the ligand. Another proposed explanation was that, in the immature brain, A_1 receptors can associate with different subunits of G-proteins less sensitive to the GTP analog used.

In another study (Turner *et al.*, 2002), particular attention was devoted to the physiological function(s) of A_1 receptors in the brain. Neonate and young rats - from PD 3 to PD 14 – were treated with N^6-cyclopentyladenosine (CPA), a selective agonist of A_1 receptors subtype. It was observed that the brains from CPA-treated animals had lower weight and the volume of the cortex and hippocampus and *corpus callosum* was also inferior to that of control animals. Observation of brain sections revealed also ventricular enlargement (see **Fig. 10**) in treated animals. The reduction of volume in the cortex and hippocampus of CPA-treated animals was correlated with a decrease in the number of neurons and glia in these structures. In contrast, in the *corpus callosum*, a region comprising a high amount of white matter, no difference in the cell number was registered, comparing treated versus control animals. Instead, a reduction of the myelin basic protein (MBP) in this region was noticed. A more detailed analysis using electron microscopy revealed that CPA-treated animals had a lower cortical axonal volume.

Data obtained by Othman and colleagues (Othman *et al.*, 2003) using primary mixed glial cultures from PD 1 rat pups, showed that oligodendrocytes, responsible for formation of myelin

sheets, express functional A_1 receptors whose activation decreases cAMP accumulation at different stages of development.

Fig. 9

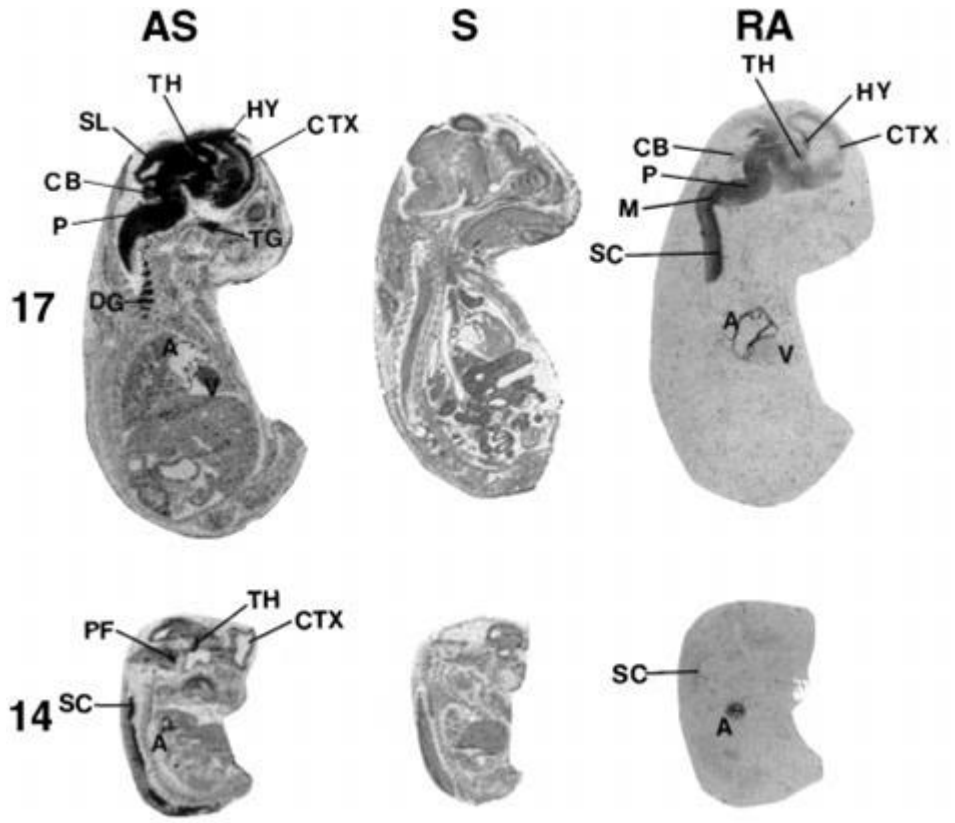


Fig. 9 – A1 receptor expression in the rat embryo – Antisense (AS) and sense (S) labeling for *in situ* hybridization and receptor labeling autoradiography for A_1R mRNA and protein, respectively. Areas of specific labeling of A_1R mRNA appear dark in the image. Sagital images shown at GD 14 and GD 17. **SC**, spinal cord; **A**, atria; **V**, ventricles; **P**, pons; **PF**, pontine flexure; **TH**, thalamus; **CTX**, cortex; **SL**, superior colliculus; **TG**, trigeminal ganglia; **DG**, dorsal root ganglia; **HT**, hypothalamus; **HY**, hippocampus; **BG**, basal ganglia. *Adapted from Influences of Adenosine on the Fetus and Newborn by Rivkees, S.A., Zhao, Z., Porter, G., Turner, C. Molecular Genetics and Metabolism 74: 260-171(2001)*

Another studies focused on the function of A_1 receptors in axons and dendrites. Studies in PC12 cells, that differentiate into sympathetic-like neurons in a medium containing nerve growth factor (NGF) (see for example Gunning *et al.*, 1981) as well as studies in cultured neurons, showed that neurite growth seems to be dependent on MAPK activation (Traverse *et al.*, 1994) and on small GTPase activation (Govek *et al.*, 2005). GTPases interfere with the cytoskeleton via activation of

Rho kinase which seems to induce neurite retraction (see for example Jalink *et al.*, 1994). A_1 receptor activation in cultured PC12 cells and hippocampal and cortical neurons decreased the length of axons and this effect was mediated by Rho-kinase (Thevananther *et al.*, 2001).

Fig.10

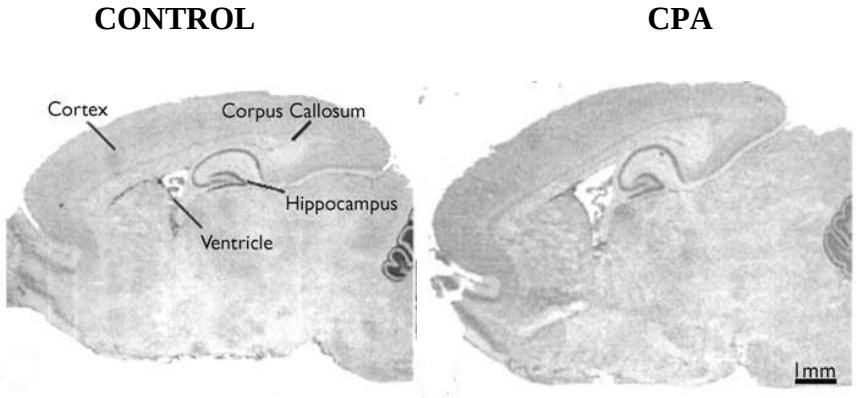


Fig.10– Sections of PD 14 rat brains showing a ventricular enlargement induced by the chronic (from PD 3 to PD 14) treatment of animals with CPA (A_1R agonist). Interestingly, some features of CPA-treated animals correspond to those observed in humans with periventricular leukomalacia. *Adapted from A1 Adenosine Receptor Activation Induces Ventriculomegaly and White Matter Loss by Turner, C.P., Yan, H., Schwartz, M., Othman, T., Rivkees, S.A. Neuroreport 13: 1199-1204 (2002)*

In the adult, A_1 receptors are highly expressed in the brain cortex, cerebellum, hippocampus and spinal cord (Ribeiro *et al.*, 2003) an located in neurons (Rivkees *et al.*,1995b) , astrocytes (Biber *et al.*, 1997), microglia (Gebicke-Haerter *et al.*, 1996) and oligodendrocytes (Othman *et al.*, 2003). In neurons, A_1 receptors seem to be present in nerve terminals of rat hippocampus, both in the active presynaptic site as well in the postsynaptic density (Tetzlaff *et al.*, 1987; Rebola *et al.*, 2003a). A classical effect of A_1 receptors is the decrease of release of presynaptic release of glutamate, acetylcholine and serotonin from hippocampal neurons (Dunwiddie and Masino, 2001).

It was shown that A_1 receptors are located together with NMDA receptor subtype at the postsynaptic density and that the activation of A_1 receptors inhibits NMDA receptor-mediated currents in hippocampal neurons (de Mendonça *et al.*, 1995). It was proposed that A_1 receptor antagonism recruits silent NMDA receptors at synapses (Klishin *et al.*, 1995). This ability to inhibit NMDA-mediated currents together with its role on the inhibition of glutamate release may have

implications for synaptic plasticity (de Mendonça and Ribeiro, 1997b) and seem to contribute to end the epileptiform firing in CA1 pyramidal cells (Li and Henry, 2000).

3.1.2. A_{2A} Receptor

Schiffmann and Vanderhaeghen (Schiffmann and Vanderhaeghen, 1993) studied the ontogeny of A_{2A} receptor mRNA. They detected mRNA encoding A_{2A} receptors in the striatum at GD14. Weaver (Weaver, 1993) found the presence of A_{2A} receptor mRNA in extrastriatal areas in fetal period with a peak expression at GD 20.

Johansson and co-workers (Johansson *et al.*, 1997) made an ontogenic autoradiographic study using membranes from several cerebral structures including caudate-putamen, nucleus accumbens, olfactory tubercle, globus pallidus and cortex. Using tritiated 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofu ranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl] benzene propanoic acid hydrochloride or [^{3}H]CGS 21680, a selective A_{2A} receptor agonist, they found that A2A receptors were present in the striatum at birth. The levels of A_{2A} receptors in the newborn were about 20% of the levels found in the adult. At PD 25, the levels of A_{2A} receptors were already as high as in older animals in all structures studied. These authors used the same experimental approach as Daval and collaborators (Daval *et al.*, 1991) to study the interaction between the receptor and the related G-protein. Using a GTP analog, they observed that the binding of [^{3}H]CGS 21680 to A_{2A} receptors was equally affected in newborns, one-week old and in adult animals which suggests that A_{2A} receptors may be functional already at birth and no increased interaction with G-protein is observed in older animals. In contrast with this work, Doriat *et al.*, 1996 found that this GTP analogue was not as effective in reducing the [^{3}H]CGS 21680 binding from the receptor in PD 0 and PD 1 as in striatal membranes from more mature animals, suggesting a lower interaction of the receptor with the G-protein in more immature animals or the coupling to a different G-protein. They also analyzed the developmental profile of A_{2A} receptor density, coupling an autoradiographic study to receptor binding assay in striatal membranes using [^{3}H]CGS 21680. The ages tested were PD 0, PD 1, PD 5, PD 10, PD 15, PD 25 and adult. The density of A_{2A} receptors in the striatum of the newborn animals was at around 3% of the adult value, however the affinity of the ligand for the receptor was higher in the more immature stage. At PD5, the number of binding sites in the striatum corresponds to 15.5% of the adult density and the affinity for [^{3}H]CGS 21680 increased also, compared to the newborn. The number of A_{2A} receptors in the

striatum continued to increase at P10 and, at P15, the density of the A_{2A} receptors reached a value of about 33% of adult values; however the affinity to the A_{2A} receptors ligand remained stable from P10 until P25. From PD 5 to PD 15, the binding of [^{3}H]CGS 21680 was detected in all structures with similar values and, at P25 and in adults, the structures more intensely labeled were caudate-putamen, nucleus accumbens and olfactory tubercle.

It was shown that in pre-mielynating Schwann cells, A_{2A} receptor phosphorylates ERK1/2 inhibiting its proliferation (Stevens *et al.*, 2004).

Although first studies targeting A_{2A} receptors showed their clear expression and location in basal ganglia, other works (see for example Lupica *et al.*, 1990 and Cunha *et al.*, 1994) also showed the presence of A_{2A} receptors in extrastriatal areas. In adults, A_{2A} receptors are expressed in neurons (Rebola *et al.*, 2003), astrocytes (Nishizaki *et al.*, 2002) and microglial cells (Küst *et al.*, 1999). In neurons A_{2A} receptors are enriched in the active zone of presynaptic terminals in the hippocampus, whereas in the striatum A_{2A} receptors are mainly postsynaptic (Rebola *et al.*, 2005a). The presynaptic location of A_{2A} receptors controls the release of glutamate, GABA, acetylcholine and serotonin (Jin and Fredholm, 1997; Cunha and Ribeiro, 2000; Lopes *et al.*, 2002; Rebola *et al.*, 2002). A_{2A} receptors can also control glutamate release and clearance from astrocytes (Cunha, 2005). This facilitation of glutamate release may play a role in noxious conditions and the blockade of A_{2A} receptors was shown to be protective (O'Regan *et al.*, 1992; Popoli *et al.*, 2002; Melani *et al.*, 2003).

In the hippocampus, high frequency of neuronal firing leads to ATP release which will lead to the preferential activation of A_{2A} receptors (Cunha *et al.*, 1996) probably due to the relative close distribution of ecto-5'-nucleotidase and of A_{2A} receptorss in the plasma membrane. A_{2A} receptors seem to be able to attenuate A_1 receptor function through several mechanisms. During high frequency of neuronal firing A_{2A} receptors activate adenosine inward transport, reducing the extracellular levels of adenosine thus reducing the probability of activation of A_1 receptors (Pinto-Duarte *et al.*, 2005). A_{2A} receptor-mediated attenuation of A_1 receptors may also occur (Cunha *et al.*, 1994a). A gain of function of A_{2A} receptors over A_1 receptors was also found in the hippocampus. Indeed, it was observed a decrease in the density of A_1 receptor subtype (Pagonopoulos and Angelatou, 1992; Meyer *et al.*, 2007), especially evident in the cortex and hippocampus of aged rats. In contrast, A_{2A} receptor density seems to increase in the cortex and hippocampus and increases in the striatum in aged rats (Cunha *et al.*, 1995). The up-regulation of A_{2A} receptors in cortex and hippocampus seems to be responsible for an enhanced facilitation of glutamatergic synaptic

transmission (Rebola *et al.*, 2003b) and acetylcholine release (Lopes *et al.*, 1999) in the hippocampus and may also be related with the maintenance of tyrosine receptor kinase B (TrkB) receptors function since its expression is decreased in aged animals (Diogenes *et al.*, 2007). A_{2A} receptors are able to transactivate TrkB receptors in the absence of the ligand (Lee and Chao, 2001) and it was also shown that A_{2A} receptors seem to be necessary for the proper functioning of TrkB (Diógenes *et al.*, 2004; Tebano *et al.*, 2008). The effect of TrkB activation is fully lost when A_{2A} receptors are blocked (Diógenes *et al.*, 2007) or in A_{2A} receptor knockout mice (Tebano *et al.*, 2008) and A_{2A} receptors required for normal BDNF levels in the hippocampus (Tebano *et al.*, 2008). A_{2A} receptors seem also to promote the survival of hippocampal neurons after BDNF withdrawal (Lee and Chao, 2001) and be necessary for BDNF-induced LTP (Fontinha *et al.*, 2008).

A_{2A} receptors also seem to inhibit NMDA receptors in the striatum in Mg^{2+}-free conditions (Wirkner *et al.*, 2000) and inhibit GABA release in the presence of Mg^{2+} (Wirkner *et al.*, 2004). In the hippocampus, A_{2A} receptors seem to have a fundamental role on NMDA receptor-dependent LTP, in synapses between mossy fibers and CA3 pyramidal cells (Rebola *et al.*, 2008). This subtype of adenosine receptor was also found to mediate a form of NMDA receptor- independent LTP in CA1 region (Kessey and Mogul, 1997).

A_{2A} receptors can interact with dopamine D_2 receptors decreasing their affinity for dopamine in rat striatal membranes (Ferré *et al.*, 1991) and control α-7-nicotini-mediated current at GABAergic interneurons in the hippocampus (Fernandes *et al.*, 2008) which relieves GABAergic inhibition. Co-activation of A_{2A} receptors seems necessary to mediate BDNF-induced inhibition of currents in GABAergic hippocampal neurons (Fernandes *et al.*, 2008). The inhibition of GABA transporter-1 (GAT-1) by BDNF is facilitated by A_{2A} receptor activation (Vaz *et al.*, 2008), contributing to an increase in GABA maintenance at the synapse.

3.1.3. A_{2B} Receptor

A_{2B} receptors seem to have a role in netrin-1 mediated effects in growth cones. The first work showing an interaction of netrin-1 and A_{2B} receptors was published in 2000 by Corset and colleagues (Corset *et al.*, 2000). Indeed, A_{2B} receptors can be a receptor for netrin-1 and the binding must be done in a different site from adenosine binding site. The complex A_{2B} receptor/netrin-1 should interact with Deleted in colorectal cancer (DCC) receptor. Apparently this interaction increases the

affinity to DCC because the co-immunoprecipitation of DCC by the pull-down of netrin was fivefold higher when A_{2B} receptors were co-expressed in 293T (human renal epithelial cell line) cells. A_{2B} receptors bind DCC in the intracellular domain. Interaction of A_{2B} receptors with netrin-1 mediates the increase in cAMP levels needed for netrin-1-mediated growth cone attraction, because an A_{2B} receptor antagonist, enprophylline, abolished the increase in cAMP levels induced by netrin-1. The authors also inhibited A_{2B} receptor function in commissural axons in the hippocampus and an absence of response in axonal outgrowth was detected after exposure to netrin-1.

In other study (Shewan *et al.*, 2002), the involvement of A_{2B} receptors in netrin-1-mediated responses was confirmed since bath application of an A_{2B} receptor antagonist converted attraction to repulsion and an A_{2B} receptor agonist, applied to cultures of older retinal explants, blocked the repulsive response to netrin-1, an effect mimicked by a membrane permeable cAMP analog. The selectivity of 5'-N-ethylcarboxamido adenosine (NECA) towards A_{2B} receptors was assessed using antagonists for the other adenosine receptor subtypes, A_1, A_{2A} and A_3 receptors. DCCR function blockade had the same effect as the agonist and the cAMP analog. This should mean that DCCR and A_{2B} receptors may mediate netrin-1 responsiveness, acting independently or as co-receptors.

However, Stein and co-workers, (Stein *et al.*, 2001) did not observe a dependence of netrin-1 and DCC interaction mediated by A_{2B} receptors in rat commissural axons (the same type of neurons used in Corset *et al.*, 2000) or in *Xenopus* spinal axon. This discrepancy of results may be related with the age of growth cones. Old growth cones seem to have a lower content in cAMP levels as well as levels of DCC protein which make them less responsive to netrin-1 (Shewan *et al.*, 2002). Interestingly, A_{2B} receptor expression is also decreased in old growth cones (see **Fig. 11**).

In the adult A_{2B} receptor seems to be present in neurons and glia (Fredholm *et al.*, 2005b).

3.1.4. A_3 Receptor

No studies were done concerning adenosine A_3 receptors expression/location/function during development. However, A_3 receptors have a low expression level in the rodent brain in the cortex, amygdala, striatum, olfactory bulb, nucleus accumbens, hippocampus, hypothalamus, thalamus and cerebellum (Linden *et al.*, 1993; Salvatore *et al.*, 1993; Dixon *et al.*, 1996). It has also a neuronal,

glial and microgial expression (Lopes *et al.*, 2003; Wittendorp *et al.*, 2004; Hammarberg *et al.*, 2003).

Fig. 11

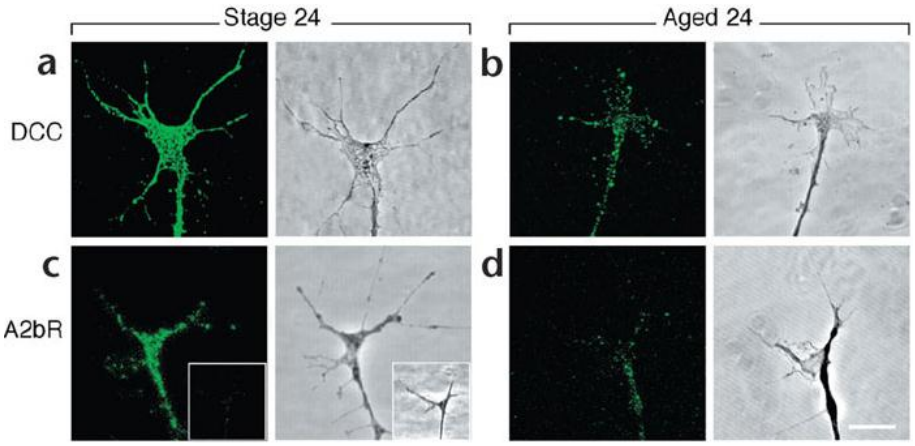


Fig. 11 – Confocal images of retinal growth cones from stage 24 retinal explants cultured overnight (aged 24). "Younger" growth cones have higher levels of DCCR and A_{2B} receptors than aged 24 growth cones. It may be implicated in the loss of responsiveness of "old" growth cones to netrin 1. *From Age-Related Changes Underlie Switch in Netrin-1 Responsiveness as Growth Cones Advance Along Visual Pathway by Shewan, D., Dwivedy, R., Holt, A., Holt, C.E. Nature Neuroscience 5: 955-962 (2002)*

3.2. ATP Receptors

3.2.1. P2Y Receptors

Important trophic effects were observed in different types of cells in the central nervous system. In the study of Cheung and co-workers (Cheung *et al.*, 2003) evidence was found for the presence of mRNA for $P2Y_1$ receptor at GD 18 and the protein was detected at PD 1 in the rat brain. The expression of $P2Y_1$ receptor subtype was restricted to the cerebral peduncle. In the periphery, $P2Y_1$ receptors were detected at GD 12 and the extension of labeling increased until GD18.

Other studies about the function of $P2Y_1$ receptors were made. For example, in Scemes *et al.*, 2003, the authors studied calcium oscillations during the development of neural progenitors from striata at GD 14 and compared it with calcium oscillations in connexin 43 null-mice (Cx43-null mice). They found that in neural progenitors, calcium oscillations depended on the activation of metabotropic ATP receptors and in Cx43 knock out mice, $P2X_7$ receptors seemed to compensate for the lost of activity of $P2Y_1$ receptors. The lack of $P2Y_1$ receptor function seems to impair neuronal

migration of the progenitors. More evidence came from the fact that the treatment with 2'-Deoxy-N^6-methyladenosine 3',5'-bisphosphate tetrasodium salt or MRS2179, a selective $P2Y_1$ receptor antagonist, reduced the migration of wild type neural progenitors out of neurospheres and expression of exogenous $P2Y_1$ receptor subtype restored this function in neurospheres from Cx43-null mice. The authors did not dissect the mechanism that links a decrease in Cx43 and $P2Y_1$ receptors but they propose the involvement of Cx43-interacting proteins such as c-Src (Giepmans *et al.*, 2001) and β-catenin (Ai *et al.*, 2000).

Weissman and colleagues (Weissman *et al.*, 2004) demonstrated that spontaneous calcium waves propagate through radial glial cells (involved in cell migration) in the embryonic VZ and their propagation seems to be mainly dependent on $P2Y_1$ receptors. Indeed, immunohistochemical staining of $P2Y_1$ receptors at GD 16 show a distinct band in the VZ (see **Fig.12**) that in the late embryonic cortical development contains especially radial glia (Noctor *et al.*, 2001; Götz *et al.*, 2002; Malatesta *et al.*, 2003). The IP_3-sensitive stores were the target of $P2Y_1$ receptor action, as confirmed by incubating cells with 2-aminoethoxydiphenyl borate (2-APB), a drug that blocks IP_3-sensitive calcium release. The authors tried to understand which cells could be involved in the initiation of waves and it is known that radial glial neuronal progenitor cells are strongly coupled by this type of gap junctions (Lo Turco and Kriegstein, 1991; Bittman *et al.*, 1997; Nadarajah *et al.*, 1997). The fact that VZ cells fill with small extracellular dyes led them to argue that maybe they were involved in the initiation of waves through opening of hemichannels.

Fig.12

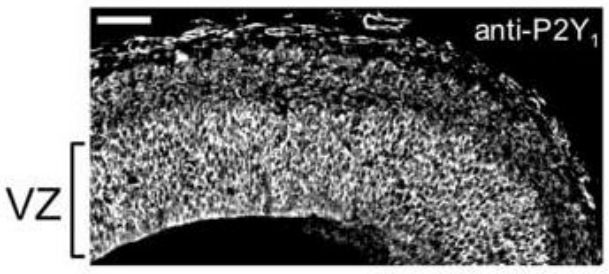


Fig.12 – Immunohistochemistry for $P2Y_1$ receptor subtype in the ventricular zone (VZ) at ED 16 from a coronal slice from the rat. *From Calcium Waves Propagate Through Radial Glial Cells and Modulate Proliferation in the Developing Neocortex by Weissman, T,A., Riquelme, P.A., Ivic, L., Flint, A.C., Kriegstein, A.R. Neuron 43: 647-661(2004)*

Also, cells of the oligodendrocyte lineage respond to a wide range of molecules that initiate signaling cascades leading to Ca^{2+} mobilization (Soliven, 2001). Amongst these molecules is ATP (Kastritsis and McCarthy, 1993; James and Butt, 2002). Agresti and co-workers (Agresti *et al.*, 2005) investigated the expression and function of P2 receptors in oligodendrocyte precursors purified from rat brain cultures. They found that oligodendrocyte precursors express several P2X and P2Y receptor subtypes and that $P2Y_1$ receptor-mediated responses is mainly responsible for their migration and inhibition of response to platelet-derived growth factor (PDGF), a chemoattractant for oligodendrocyte precursors (Armstrong *et al.*, 1990).

The developmental expression profile for $P2Y_2$ receptors was studied in rat embryos (Cheung *et al.*, 2003). Using real time polymerase chain reaction (RT-PCR) analysis, $P2Y_2$ receptors were detected at GD 11 but the expression is very weak and absent from the brain. In the spinal cord $P2Y_2$ receptors were detected in the neural tube at GD 12. At GD 14, $P2Y_2$ receptors are abundantly expressed in the white matter of the intermediate and ventral horns and the dorsal column of the spinal cord. At GD 18, $P2Y_2$ receptors are also found in the dorsal root ganglia.

$P2Y_2$ receptors seem to be able to activate phospholipase C, which can induce a rise in intracellular Ca^{2+} levels; this can trigger the generation of intracellular Ca^{2+} waves that play important roles during brain development as revised by Webb and Miller (Webb and Miller, 2003). ATP seems to be the coupling messenger between cells that are propagating calcium waves and one of the receptors involved in this process is $P2Y_2$ receptors. Also, $P2Y_2$ receptors can promote recruitment and activation of Src protein kinases is important in the stem-cell renewal (Annéren *et al.*, 2004). Apparently, $P2Y_2$ receptor activation increases Src kinase-dependent clustering with the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor-2 (VEGFR-2) receptors (Arthur *et al.*, 2005). The authors proposed a model for the interaction between NGF acting on tyrosine receptor kinase A (TrkA) and $P2Y_2$R-mediated signaling. TrkA activation is responsible for increasing $P2Y_2$ receptor levels, increasing the probability of interaction of neurotrophin receptors with $P2Y_2$ receptors. This interaction leads to increased activation of ERK1/2 by P-TrkA and it results in an increase in neurite formation. $P2Y_2$ receptor activation can increase also the level of ERK1/2 phosphorylation but this not results in an increase in neurite formation if TrkA is not activated by a ligand. The authors used PC12 cells and dorsal root ganglion neurons. The involvement of $P2Y_2$ receptors in the amplification of NGF action was confirmed silencing $P2Y_2$ receptors. $P2Y_2$ receptors are also implicated in cell cycle control in astrocytes. This cellular process

is relevant in the context of development because astrocytes can act as neural stem cells (Doetsch *et al.*, 1999).

Erb and colleagues (Erb *et al.*, 2001) documented an interaction between $P2Y_2$ receptors and integrins. When interaction with integrins occurs, large signaling complexes with integrin-binding proteins can be formed in the membrane. Proteins like Src, focal adhesion kinase, Pyk2, EGFR, PDGFR and actin can be found composing these complexes (Liu *et al.*, 2004).

Amongst trophic actions afforded by $P2Y_2$ receptors is the stimulation of α-secretase activity which cleaves the amyloid precursor protein within a soluble form of the peptide (sAPP) that is in normal conditions present in the brain and in the cerebrospinal fluid (Palmert *et al.*, 1989). Mattson (Mattson, 1997) reviewed the physiological functions of sAPP in the enhancement of synaptogenesis, neurite outgrowth, cell survival and adhesion and proliferation of neural stem cells isolated from the embryonic brain. The MAPK/ERK inhibitor U0126 blocked partially the release of sAPP but activity of metalloproteases is largely implicated in this process. α-Secretase has neurotrophic and mitogenic functions in non-pathological conditions, $P2Y_2$ receptors regulating α-Secretase levels can be also implicated in the modulation of such functions.

Similarly to $P2Y_1$ and $P2Y_2$ receptors, $P2Y_4$ receptors are also present in the neural tube and peripheral nervous system from GD 12 (Cheung *et al.*, 2003). Both mRNA and protein were detected at GD14 in several regions including olfactory nerve and lateral olphactory tract, anterior hypothalamus, dorsal geniculate nucleus and lateral hypothalamic area, cortical amygdaloid nucleus, brain stem, pons, midbrain and medulla. This expression seems to be age-dependent since it disappears from midbrain, isthmus and medulla at PD 1. Also, regions where the $P2Y_4$ receptors were not detected such as septum and the neuroepithelium adjacent to the ventricules become immunopositive postnatally. Crain and collaborators (Crain *et al.*, 2009) studied the role of purine receptors in the regulation of microglia during the ontogenic development of C56BL/6 mice, from PD 3 to 12 months. They found that the expression of all P2Y receptors increased between PD 3 and 21, with exception for $P2Y_{13}$ and $P2Y_{14}$. This profile of development is coincident with a significant synaptic re-remodeling and pruning, functions that require microglial activity (Tremblay *et al.*, 2010). They found a decline in P2 receptors whose expression is necessary for microglial migration and phagocytosis and they speculate that this may contribute to their senescence and with the loss of the ability to perform neuron-protective and –supportive functions (Conde and Streit, 2006; Streit *et al.*, 2008) at around PD 30.

3.2.2. P2X Receptors

The ontogenic expression of $P2X_1$ receptors was studied in rat cochlea. The neural structures involved in sensorial perception (Nikolic *et al.*, 2001). The neural structures were weakly immunopositive at GD 16 for $P2X_1$ receptors. The immunolabeling increases at GD 18 and, at PD 2 is present in synaptic regions associated with the inner and outer hair cells. $P2X_1$ receptors, and also $P2X_2$ and $P2X_3$ receptors (Cheung and Burnstock, 2002), seem to be involved in the establishment of synaptic connections between the primary auditory neurons and hair cells due to the strong labeling observed during the first postnatal week. Hair and supporting cells are, by oppositions, immunonegative for $P2X_1$ receptors. A down-regulation of $P2X_1$ receptors is observed prior to the onset of hearing which happens between GD 8 and GD 18. Cheung and Burnstock (Cheung and Burnstock, 2002) found that $P2X_2$ receptors are expressed at GD 14 in nucleus tractus solitarius. The same authors studied the ontogeny of $P2X_3$ receptors in the rat embryonic nervous system. Using immunohistochemistry they did not find detectable reactivity until GD 10.5. More robust expression of $P2X_3$ receptor in the central nervous system was found at GD 14.5. In Cheung *et al.*, 2005, cultures of neural tube explants were used to study if ATP participated in motor axon outgrowth. They found that ATP reduced the extent of axon outgrowth as well as neurite length and area. This effect is mimicked by α,β-meATP, a stable ATP analog and an antagonist of $P2X_3$ receptors. The authors confirmed the presence of $P2X_3$ receptors by immunofluorescence in neurons and outgrowing neurites. Kidd and co-workers (Kidd *et al.*, 1998) reported $P2X_3$ receptor expression in several nuclei involved in peripheric sensorial perception in late rat embryonic development. Again, down-regulation of the protein was observed in adulthood.

mRNA transcripts encoding $P2X_4$ receptors are detected at GD 14 (Cheung *et al.*, 2005), however immunoreactivity was not detected until PD 1 in supraotic nucleus, striatal subventricular zone and in cuneate nucleus. The expression of $P2X_4$ receptors was found to be restricted to regions like striatal subventricular zone, nuclei involved in sensorial perception and lateral deep cerebellar nucleus.

$P2X_6$ receptor transcripts are found in low abundance at PD 1 but restricted to the lateral hypothalamus (Cheung *et al.*, 2005). Maybe $P2X_6$ receptors follow the same function in the adult hypothalamus, modulating hormone release from axon terminals and membrane recycling of the granular and microvesicles in the hypothlamo-neurohypophysal system (Loesch and Burnstock, 2001).

$P2X_7$ receptors are also present in the inner ear which suggests a role between sensory cells and primary auditory system (Cheung *et al.*, 2003). $P2X_7$ receptor was shown to be involved in cytotoxicity and it was shown that it plays a role in programmed cell death that occurs spontaneously on retinal cholinergic neurons during development (Resta *et al.*, 2005). $P2X_7$ receptors can also be involved in the induction of intracellular Ca^{2+} transients (Agresti *et al.*, 2005).

3.3. Enzymes from the Purinergic System

3.3.1. Adenosine Kinase (AK)

Maturation of ADK was investigated in the mouse immature brain by Studer and colleagues (Studer *et al.*, 2006). Using double immunolabelling with ADK-specific antiserum and neuronal (NeuN) or astrocytes (Glial fibrillary acidic protein - GFAP) marker, they found that at PD 4, immunoreactivity was widespread in neuronal nuclei throughout the brain although the most intense labeling was observed in the striatum, pyramidal and granular cells in the hippocampus, in the lateral ventricle and cerebral cortex. 2 Weeks after birth, a loss of ADK immunoreactivity was observed in principal cells from CA1 and CA2 regions, appearing simultaneously in astrocytes. A similar down-regulation of this enzyme was observed in the DG 3 weeks after birth. They also observed a co-expression between ADK and nestin, a marker for endothelial cells, radial glia, neural precursor cells and immature astrocytes. ADK expression correlates with maturation of hippocampal cells. Interestingly, myelination in the hippocampus follows down-regulation of ADK, suggesting that ATP is needed for the myelination process (Stevens *et al.*, 2002).

In the adult brain, ADK seems to be expressed by astrocytes and its function is to produce a tonic inhibition (Gouder *et al.*, 2004).

3.3.2. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases)

NTPDase density at the plasma membrane controls the expression extracellular nucleotide concentration (Zimmermann, 2000). NTPDase 1 and NTPDase 3 hydrolyze ATP and ADP and NTPDase 2 has no affinity for ADP but only for ATP. NTPDase 1 hydrolyses ATP directly to adenosine monophosphate (AMP) and the accumulation of ADP is possible with NTPDase 2.

NTPDase 3 produces ADP and AMP from ATP hydrolysis (Heine *et al.*, 1999) and suffers down-regulation only in myelinating cells, favoring $P2Y_1$ receptor signaling and depressing $P2Y_2$ receptor signaling (Braun *et al.*, 2004).

It is believed that NTPDases follow the widespread distribution of P2 receptor subtypes in nervous tissues (Zimmermann, 2000) being expressed in neurons, astrocytes, oligodendrocytes and microglia. In neurons, the activity of NTPDases was found in nerve terminals from the hippocampus and biochemical studies suggests a major role for NTPDase 3 since ADP was accumulated after addition of ATP (Cunha, 2001). NTPDase 3 was found being expressed in axon-like structures in various regions of the brain (Belcher *et al.*, 2006).

NTPDase 2 is expressed by neural stem cells in the subventricular zone and ventricles (Braun *et al.*, 2003). At PD 3, astrocytes that appear in the VZ and SVZ express also NTPDase 2, however the identity of the cells expressing this enzyme before was not determined. In the hippocampus, a strong immunoreactivity was detected in the Ammon's horn and fimbria which was reduced and completely disappeared at PD 21. At PD 21 a very low signal was detected in the *Stratum lacunosum moleculare* until adulthood. In the DG the immunolabeling was strong between PD 0 and PD 3, the peak period for production of granule cells (Altman and Bayer, 1990c). The expression was astrocytic. At PD 6 the immunoreactivity was reduced and distributed throughout the DG and from PD 14 until adulthood it assumed a very low expression in the subgranular zone (Braun *et al.*, 2003).

3.3.3. 5'-Nucleotidase

There is considerable amount of evidence showing that ecto-5'-nucleotidase (e-5NT) has distinct role during brain development and plasticity. e-5NT was found located on the surface of migrating nerve cells during postnatal development (Fenoglio *et al.*, 1995; Schoen *et al.*, 1988) and it becomes transiently associated with synapses during synaptogenesis and synapse remodeling (Bailly *et al.*, 1995; Fenoglio *et al.*, 1995; Schoen and Kreutzberg, 1995). The enzymatic activity of e-5NT increases up to five-fold in the cerebral cortex (dePaula Cognato *et al.*, 2005; Mackiewicz *et al.*, 2006), hippocampus (Cunha, 2001; de Paula Cognato *et al.*, 2005), spinal cord (Torres *et al.*, 2003) and in most brain regions of aged compared to young rats (Fuchs, 1991).

Enzyme histochemical staining and biochemical studies revealed broad distribution of e-5NT and its association with myelin, astrocytes, activated microglia and neurons (for review, see

Zimmermann, 1992; Zimmermann,1996; Langer *et al.*, 2008). However, immunohistochemical methods demonstrated more restricted enzyme localization at glial structures (Schoen *et al.*, 1988), oligodendroglia and myelinated fibers (Cammer *et al.*, 1985), whereas neuronal localization was rarely observed (Nacimiento and Kreutzberg, 1990; Bjelobaba *et al.*, 2007). Several biochemical studies demonstrated presence of AMP hydrolyzing activity in the presynaptic elements (Cunha *et al.*,1992; Zimmermann, 1992; James and Richardson, 1993; Cunha, 2001; de Paula Cognato *et al.*, 2005; Schmatz *et al.*, 2009; Sigueira *et al.*, 2010), however, immunocytochemical studies have shown only sporadic association of e-5NT with nerve terminals (Schoen and Kreutzberg, 1997; Zimmermann *et al.*, 1993).

Recently Stanojevi'c and collaborators (Stanojevi'c *et al.*, 2011) found that e-5NT protein is enriched in the presynaptic compartment in early postnatal development (PD 15) and decreases with maturation, whereas its activity follows the reverse pattern of expression.

CHAPTER 1

Evaluation of the Presence of Different Components of the Purinergic System in the Immature Hippocampus

As described in the first part of the introduction, the purinergic system is composed of ligands, its responsive receptors, transporters and enzymes involved in the production/degradation and/or uptake of purines. As described in the introduction, the postnatal period in rodents corresponds to the time window in which important events such as synaptogenesis and appearance of the first forms of hippocampal activity mainly occur. Knowing the presence of some components of this system can give a hint about the possibility and relevance they have to be intervenient in such events.

This chapter describes the presence of some components of the purinergic system during the postnatal ontogeny of the hippocampus in mice hippocampal (neuronal and glial) membranes using both immunohistochemistry and Western blotting analysis. Occasionaly, the subsynaptic location of some proteins was investigated. An attempt of integrating such results with the synaptic development can be found in the **Conclusion** section of this Chapter.

CHAPTER 1 - Material and Methods

Animals: FVB strain mice, genetically modified (FVB-GIN mice – see Oliva *et al.*, 2000) to express somatostatin interneurons labeled with GFP fluorescence, were used to isolate pre, post and extrasynaptic fractions. Pups were sacrificed by decapitation without anesthesia. C57BL6 mice (purchased from Charles River, Barcelona, Spain) were used for the ontogenic characterization of the components of the purinergic system. Pups from this strain were also sacrificed by decapitation without anesthesia and young/adult mice (up to 15 days-old) were anesthetized under halothane atmosphere before decapitation. Pups from both strains were used immediately after separation from the mothers. FVB-GIN pups and adults were also used for immunohistochemistry analysis, pups being anesthetized in ice and adults injected with 7% chloralhydrate before perfusion.

Purification of the Total Membrane Fraction: According to the size of the hippocampi, 4 or 2 hippocampi were placed in a vial with 5 mL of sucrose solution containing 0.32 M sucrose, 1 mM Ethylenediaminetetraacetic acid (EDTA), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mg/mL bovine serum albumin (BSA), pH 7.4 at 4 ºC. Tissue was homogeneized with 10 up and down strokes, using a potter. This step was made in the ice. The volume was completed to 10 mL. The homogenate was centrifuged first at 3 000 *g* for 10 min and the supernatant from this centrifugation was then centrifuged at 25 000 *g* for 30 min. Both centrifugations were made at 4 ºC in a SIGMA 3-18K centrifuge. The supernatant resultant from the second centrifugation was discarded and the pellet containing the membranes was resuspended in an appropriate volume of 5 % sodium dodecyl sulfate (SDS) for Western blot analysis.

Obtention of Pre, Post and Extra Fractions: The procedure for the purification of pre, post and extrasynaptic membranes was based in Phillips *et al.*, 2001, with some modifications. 20 Hippocampi (the amount of tissue required for one experiment) were isolated from FVB-GIN strain mice pups (5 PD to 7 PD) into ice-cold artificial cerebrospinal fluid or aCSF (126.0 mM NaCl, 3.5 mM KCl, 1.2 mM $NaH_2PO_4.H_2O$, 1.3 mM $MgCl_2.6H_2O$, 2.0 $CaCl_2.2H_2O$, 10.0 mM glucose, 25.0 mM $NaHCO_3$, pH 7.2-7.4). The pool of tissue was homogeneized using a potter (10 up and down movements) in 2.5 mL of ice-cold incubation buffer (IB) containing 0.32 M sucrose, 0.1 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 1.0 µg/µL protease inhibitors chimostatin, leupeptin, antipain and pepstatin (CLAP) cocktail as well as 1.0 mM phenylmethanesulfonylfluoride (PMSF), a serine protease inhibitor. Twelve mL of a solution containing 2.0 M sucrose and 5 mL of 0.1mM $CaCl_2$ were added

to this homogenate. After gentle agitation, the homogenate was placed into ultracentrifuge tubes and on top of each tube, more 2.5 mL of sucrose and 0.1 mM $CaCl_2$ were added to the homogenate forming a gradient with the homogenate. The weight of the tubes was calibrated with IB solution. The tubes were centrifuged in an Optima L 100XP centrifuge at 100 000 *g* for 3 hours, at 4 ºC. After centrifugation the fraction between the top layer (myelin) and the bottom pellet (containing nuclei, large fragments of membrane) was collected and it corresponded to the putative **synaptosomal fraction**. The synaptosomes were diluted in a 10x higher volume of IB and centrifuged in a SIGMA 3-18K centrifuge at 15 000 *g*, 4 ºC, for 30 min. The pellet was resuspended in 1.1 mL of IB. 100 µL of this synaptosomal fraction were centrifuged at 12 000 rpm in an eppendorf centrifuge (Eppendorf-5415R) at 4 ºC for 5 min. The pellet was resuspended in 5% SDS and frozen at -80 ºC. The remaining synaptosomal fraction (1 mL) was diluted 10x with 0.1 mM $CaCl_2$. After adding the same volume (10 mL) of 2x solubilization buffer (40 mM Tris, 2% triton X-100, pH 6.0) the mixture was incubated in ice for 30 min under gentle agitation. The solution was centrifuged at 40 000 *g* for 30 min. The pellet was washed with solubilization buffer, pH 6.0 and resuspended in 5 mL of 1x solubilization buffer, pH 8.0. The solution was incubated in ice for 30 min under gentle agitation before centrifugation at 40 000 *g* for 30 min at 4 ºC. The pellet obtained corresponds to the **postsynaptic fraction** and the supernatant to the presynaptic fraction. The postsynaptic fraction was diluted in a minimal amount of 5% SDS for posterior Western blot analysis. The supernatants corresponding to pre and extrasynaptic fractions were submitted to a protein concentration step using acetone at -20 ºC overnight. The supernatant-acetone solution was centrifuged at 18 000 *g* for 30 min at -15 ºC. After centrifugation the acetone was discarded and the tubes dried. The pellets attached to the walls of the tubes were resuspended in a minimal amount of 5% SDS.

Western Blotting: The amount of protein in total membranes, synaptosomal and pre, post and extrasynaptic fractions was determined using the bicinchoninic acid method (Pierce, Dagma, Portugal). Electrophoresis was carried out using a 7.5% SDS-PAGE gel after loading 60 µg of the different fractions. Composition of the resolving gel was 7.5% acrylamide (purchased from Bio-Rad), 0.5 M Tris pH 8.8, 0.2% SDS, 0.2% ammonium persulfate (APS), 6 µL tetramethylethylenediamine (TEMED), water up to 8.7 mL. Composition of stacking gel was 4% acrylamide, 0.125 M Tris pH 6.8, 0.1% SDS, 0.05% APS, 10 µL TEMED, water up to 10mL. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes (from GE Healthcare, Buckingamshire, UK). Membranes were blocked for 1 h at room temperature with 5%

low-fat milk in Tris-buffered saline or 3% bovine serum albumin (depending on the antibodies used), pH 7.6, and containing 0.1% Tween 20 (TBS–T). Membranes were then incubated overnight at 4 °C with primary antibodies. After washing with TBS-T, membranes were incubated IgG secondary antibodies and after removel of non-bound antibody with TBS-T, membranes were revealed by an enhanced chemifluorescence (ECF) kit (GE Healthcare, Buckingamshire, UK) and visualized in a VersaDoc 3000 (Bio-Rad, Portugal). Quantity One Software was used to quantify the density of the bands. The **Table I** and **II**, presented in the next page, lists the antibodies used.

Tissue Preparation for Light Microscopy: Under anesthesia, animals were perfused intracardially with a fixative solution containing 4% paraformaldehyde (PAF) in phosphate buffer (PB) containing 120.0 mM $NaH_2PO_4.2H_2O$, 190.0 mM NaOH, pH 7.4. Mice received 10 mL of fixative solution for each 10 g of body weight. After perfusion, the brains were removed from the skull, post-fixed in the same fixative for 2 hours (PD 13 up to 1 month-old) or overnight (pups) and rinsed 3 times for 30 min in 0.12 M of PB. Blocks of the forebrain were immersed in a cryoprotective solution of 20% sucrose in PB overnight at 4 ºC, quickly frozen on dry ice and sectioned coronally at 100 µm (pups up to PD 9) or 40 µm (PD 13 up to 1 month-old) with a cryostat. The sections were rinsed in phosphate-buffered saline (PBS) containing 13.7 mM NaCl, 0.27 mM KCl, 0.8 mM $Na_2HPO_4.2H_2O$, NaOH to adjust the pH to 7.4; they collected sequentially in tubes containing autoclaved ethylene glycol-based cryoprotective solution containing 500 mM sucrose, 180 mL ethylene glycol, 0.3% (v/v) PBS 10×, 300 µl diethylpyrocarbonate (DEPC). They were then stored at -20 ºC until histological processing. Every 8 (for pups) or 10 (for PD 13 up to 1 month-old) sections were stained using the cresyl violet methodology to determine the general morphology of the tissue.

Cresyl Violet Staining: After dehydration of slices in a solution of 95% (v/v) ethanol for 20 min, chloroform-ether solution for 15 min and solutions of ethanol of decreasing concentration, from 95% (v/v) to 70% (v/v), 2 min each exposure, slices were stained with Cresyl violet for 3 min at 60 ºC after removal of the excessive ethanol with bidistiled water. Cresyl violet solution had the following composition: 0.005% cresyl violet, 0.006% (v/v) acetic acid, 0.0004% sodium acetate. After removal of the excess of cresyl violet with a gentle washout with bidistiled water, slices were exposed again to ethanol solutions of increasing concentration, 2 min in 80% ethanol, 4 min in 95% ethanol and 4

min in 100% ethanol. After incubation for 6 min in histolemon solution (from Carlo Erba Reagents), slices were mounted with Eukitt mounting medium (from Bioblock).

Table I – Primary Antibodies for Western Bloting

Antibodies	Supplier	Host	Type	Dilution
Anti-$P2X_1$	Alamone labs	Rabbit	Polyclonal	1:500
Anti-$P2X_2$	Alamone labs	Rabbit	Polyclonal	1:500
Anti-$P2X_3$	Alamone labs	Rabbit	Polyclonal	1:500
Anti-$P2X_4$	Alamone labs	Rabbit	Polyclonal	1:500
Anti-$P2X_5$	Santa Cruz Biotechnology	Goat	Polyclonal	1:200
Anti-$P2X_6$	Santa Cruz Biotechnology	Goat	Polyclonal	1:200
Anti-$P2X_7$	Alamone labs	Rabbit	Polyclonal	1:500
Anti-$P2Y_1$	Alamone labs	Rabbit	Polyclonal	1:500
Anti-$P2Y_2$	Alamone labs	Rabbit	Polyclonal	1:500
Anti-$P2Y_4$	Alamone labs	Rabbit	Polyclonal	1:1000
Anti-$P2Y_6$	Santa Cruz Biotechnology	Goat	Polyclonal	1:500
Anti-$P2Y_{12}$	Alamone labs	Rabbit	Polyclonal	1:500
Anti-NTPDase1	A kind gift by Jean Sévigny, Univ. Laval, Canada	Mouse	Monoclonal	1:1000
Anti-NTPDase2	A kind gift by Jean Sévigny, Univ. Laval, Canada	Mouse	Monoclonal	1:1000
Anti-NTPDase3	A kind gift by Jean Sévigny, Univ. Laval, Canada	Rat	Polyclonal	1:1000
Anti-5'-Nucleotidase	A kind gift by Jean Sévigny, Univ. Laval, Canada	Rabbit	Polyclonal	1:1000
Anti-Synaptophysin	SIGMA-Aldrich	Mouse	Monoclonal	1:20000
Anti-KCC2	Santa Cruz Biotechnology	Goat	Polyclonal	1:500
Anti-VGLUT1	Milipore	Guinea Pig	Polyclonal	1:10000
Anti-PSD-95	SIGMA-Aldrich	Mouse	Monoclonal	1:20000

Table I – Secondary Antibodies for Western Bloting

Antibodies	Supplier	Host	Type	Dilution
Rabbit-alkaline phosphatase conjugate (AP)	Amersham Biosciences	Goat	IgG (H+L)	1:20000
Mouse-alkaline phosphatase conjugate (AP)	Amersham Biosciences	Goat	IgG+IgM (H+L)	1:20000
Goat-alkaline phosphatase conjugate (AP)	Santa Cruz	Rabbit	IgG	1:2500
Guinea-Pig-alkaline phosphatase conjugate (AP)	SIGMA	Goat	IgG	1:5000

Immunohistochemistry (3,3'-diaminobenzidine or DAB protocol): Slices were rinsed once in 0.12 M PB and twice with a potassium-phosphate solution (KPBS) containing 2.7 mM KCl, 1.5 mM KH_2PO_4, 137.0 mM NaCl, 6.5 mM Na_2HPO_4, pH 7.2, to remove the cryoprotection solution and incubated for 30 min in 1 % H_2O_2 (SIGMA) diluted in 0.12M PB for 30 min. Slices were then incubated for 1hour in 1/30 normal horse serum (NHS, from Vector) or normal goat serum (NGS, from Vector) diluted in 0.02 M KPBS supplemented with 0.3% Triton X100 (SIGMA), after removal of H_2O_2 by washing 3 times 30 with KPBS. All the manipulations were made under agitation. Slices were incubated overnight at room temperature in primary antibodies diluted in 0.02M KPBS supplemented with 0.3% Triton X100 plus 1:100 NHS or NGS and after washout of non-bound antibody with 0.02M KPBS, slices were incubated for 1 hour in secondary antibodies 1:200 (biotinylated horse anti-mouse or goat anti-rabbit) diluted in 0.02M KPBS plus 1/30 NGS or NHS. The non-bound secondary antibody was removed by washing the slices 3 times for 30 min in 0.02 M KPBS. Slices were incubated for 1 hour in avidin-biotin-peroxydase complex ABC Elite (Vector Laboratories, Burlingame, CA, USA), 10% (v/v) solution A+10% (v/v) solution B diluted in 0.02M KPBS and in DAB (SIGMA) for 15 min after a 3x 30 min washing step (using 0.02M KPBS). The slices were washed again 3 times for 30 min in KPBS after exposure to DAB. Slices were mounted on superfrost slides and dried overnight before dehydration and coverslip mounting in Permount medium before observation in a microscope. In the table **III** and **IV** are the shown the primary and

secondary antibodies used in the immunohistochemical procedure. It was not performed a quantification of the immunoreactivity. Images corresponding to slices from different experimental conditions were acquired using the same settings.

Immunohistochemistry (double-labeling with fluorescent antibodies): Slices were rinsed once in 0.12 M PB and 2 times in KPBS to remove the cryoprotection solution under agitation. Pre-treatment for permeabilization of the slices consisted of incubation for 1h in NGS 1:30 in 0.02 M KPBS containing 0.3% Triton X-100 at room temperature. Slices were incubated overnight at room temperature in primary antibodies rabbit anti-GFAP 1:1000 (SIGMA), mouse anti-A_{2A} receptor 1:780 (Upstate Biotechnology), rabbit anti-vesicular transporter 1 (VGLUT1) 1:10000 diluted in KPBS containing 0.3% Triton X100 and 1:100 NGS; the next day, non-bound antibody was removed by washing the slices 3 times for 30 min in KPBS 0.02 M under agitation. The slices were then incubated for 2h in a mixture of secondary antibodies, Alexa 488 goat anti-mouse and CY5 donkey anti-rabbit, both diluted to 1:200 in KPBS 0.02 M containing NGS diluted 1:30. After washout of the excess of secondary antibody by washing 3 times for 30 min in KPBS 0.02 M, slices were mounted in Superfrost slides and let drying overnight. They were hydrated in distilled water for 2-3s and coverslipped in Fluoromount Gel. For the labeling with anti-vesicular gamma-aminobutyric transporter (VGAT) and anti-A_{2A} receptor antibodies, both produced in mice, a different protocol was used. The steps were similar prior antibody incubation. Slices were incubated with one antibody at a time and after washout of the amount of the non-bound antibody, the second incubation with the remaining primary antibody was done. The same procedure was adopted for secondary antibodies. CY5 goat anti-mouse and Alexa 488 goat anti-mouse were used in a dilution of 1:200. In the table **III** and **IV** are the shown the primary and secondary antibodies used in the immunohistochemical procedure. It was not performed a quantification of the immunoreactivity. Images corresponding to slices from different experimental conditions were acquired using the same settings.

Table III – Primary Antibodies for Immunohistochemistry

Antibodies	Supplier	Host	Type	Dilution
Anti-Adenosine receptor 1	Abcam	Rabbit	Polyclonal	1:1000
Anti-Adenosine receptor 2	Upstate Biosystems	Mouse	Monoclonal	1:1500
Anti-VGLUT1	Chemicon	Guinea Pig	Polyclonal	1:10000
Anti-VGAT	Chemicon	Mouse	Monoclonal	1:5000

Table IV – Secondary Antibodies for Immunohistochemistry

Antibodies	Supplier	Host	Type	Dilution
Biotinylated-Rabbit	Vector	Goat	IgG	1:200
Biotinylated-Mouse	Vector	Horse	IgG (H+L)	1:200
Alexa Fluor 488-Mouse	Invitrogen	Goat	IgG (H+L)	1:200
CY5-Rabbit	Beckman Immunotech	Donkey	IgG (H+L)	1:200
CY5-Mouse	Beckman Immunotech	Donkey	IgG (H+L)	1:200

Statistical Analysis: Comparison between densities of bands in Western blot analysis was made performing one-way analysis of variance (ANOVA) followed by Tukey test. The age 5 days-old (or 10 days-old for other conditions for the ecto-nucleotidases) was considered 100%. All the other values were expressed in % of increase/decrease in relation to this reference. When two means were compared, a two-tailed *t*-test was used. In both cases, results are mean +/- standard error of the mean (SEM). Statistical significance is indicated in the figures as follows: *$p<0.05$; **$p<0.001$; ***$p<0.0001$.

Drugs: All the drugs whose companies are not indicated in the text were purchased from SIGMA and Tocris.

CHAPTER 1 – Results

1.1. Adenosine Receptors

The ontogenic profile of density/pattern of distribution of both A_1 and A_{2A} receptors, the two main adenosine receptor subtypes in the brain (Cunha 2005), were investigated using Western blot and immunohistochemical analysis. Western blot analysis provides a quantitative measure of the density of proteins in the tissue whereas immunohistochemical analysis allows assessing the distribution of proteins in different structures of the brain.

Fig.1.1A shows the modification of A_1 receptor density throughout the developmental period. Considering the density of A_1 receptors at PD 5 as reference (100%), it was observed that the density of A_1 receptor was nearly constant throughout the ontogenic period. The density of A_1 receptors in membranes from neurons and glia was 173.6 +/- 36.2% at PD 10, 163.5 +/- 17.2% at PD 15, 229.2 +/- 42.5% at PD 20, 237.1+/- 54.0% at PD 30, 227.8 +/- 32-0% at PD 40, 160.7 +/- 19.6% at PD 50, 132.6 +/- 6.5% at PD 65 and 149.3% +/- 45.0% at PD 60.The means were compared using One-way ANOVA followed by Tukey test, n=3, $p>0.05$.

The profile of localization of A_1 receptors is also displayed in **Fig. 1.1C**; A_1 receptors seem to be widely expressed at PD 3, being located in molecular layers of the hippocampus and in cell bodies up to PD 6. From PD 9 onward the immunoreactivity of A_1 receptor in the pyramidal cell layer become stronger and the labeling in the cell bodies is more defined. From PD 9 to PD 30 all cells seem to become edowed with A_1 receptors, confirming the previous data that A_1 receptors are virtually present in all neurons in the brain (Fredholm *et al.*, 1999). This pattern of distribution was maintained until the adulthood.

The ontogenic profile of A_{2A} receptor density in mice hippocampal total membranes is not presented in this thesis due to the absence of a selective antibody to target it. A selective antibody against A_{2A} receptors, developed later, allowed studying the ontogenic profile of distribution of this protein by immunohistochemistry. **Fig. 1.2** shows that the pattern expression of A_{2A} receptors is highly dynamic; they undergo a progressive down-regulation, which occurs first in the cortex at PD 9 with a propagating decrease throughout all cortical regions and hippocampus from PD 13 onward. Interestingly, at PD 30, an intense labeling of A_{2A} receptors in the striatal region was observed, which corresponds to the typical pattern of expression of the protein in the mature brain.

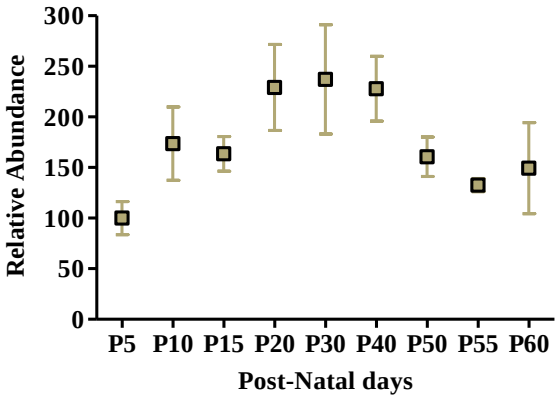
Although there is an almost complete absence of evident immunohistochemical signal for A_{2A} receptors in the adult, several reports show relevant physiological evidence for the presence of the A_{2A} receptors in the hippocampus of mature animals (Cunha *et al.*, 1995; Lopes *et al.*, 1999; Cunha and Ribeiro, 2000; Cunha, 2001; Lopes *et al.*, 2002; Rebola *et al.*, 2002, 2003). This is probably due to the fact that A_{2A} receptors are concentrated in the presynaptic fraction of hippocampal membranes in adult animals (Rebola *et al.*, 2005). This not seems to occur in hippocampal membranes from mice pups. Between PD 5 and PD 7, a time window when a strong immunoreactivity of A_{2A} receptors was detected in the hippocampus (see **Fig. 1.2**), A_{2A} receptors seem to be more concentrated in post and extrasynaptic fractions. Interestingly, A_{2A} receptors seem to be absent or have a low density in the presynaptic fractions (see **Fig. 1.3**).

A comparison with the sub-synaptic location of A_1 receptors was made using the same method of fractioning and it seems that A_1 receptors are located in pre and postsynaptic sites maintaining the distribution observed in the mature hippocampus (Sebastião and Ribeiro, 2010).

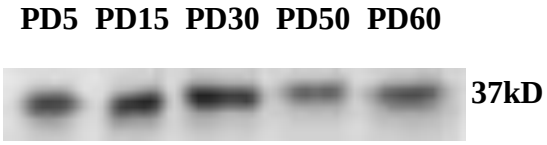
The purity of the fractions was assessed using antibodies against PSD-95 (postsynaptic marker), synaptosomal-associated protein-25 or SNAP-25 (presynaptic marker) and synaptophysin (protein enriched in extrasynaptic fraction) (see section **1.5** from **Chapter 1**).One aspect of this method must be taken into account. PSD-95 is a protein exclusively present in glutamatergic post-synaptic density (Kennedy, 1997). It means that in glutamatergic synapses, both A_1 and A_{2A} receptor subtypes were found located postsynaptically. However, it does not necessarily mean that in GABAergic synapses the same would be observed. In fact, extrasynaptic fraction can contain the post-synaptic fraction from GABAergic synapses since the procedure for postsynaptic isolation was developed and confirmed in glutamatergic synapses. A control experiment investigating the presence of gephyrin, a protein located in the "GABAergic post-synaptic fraction" must be done to clarify this aspect. However, it must also be taken into account that gephyrin can also occupy extrasynaptic sites in immature tissues.

Fig. 1.1

A)



B)



C)

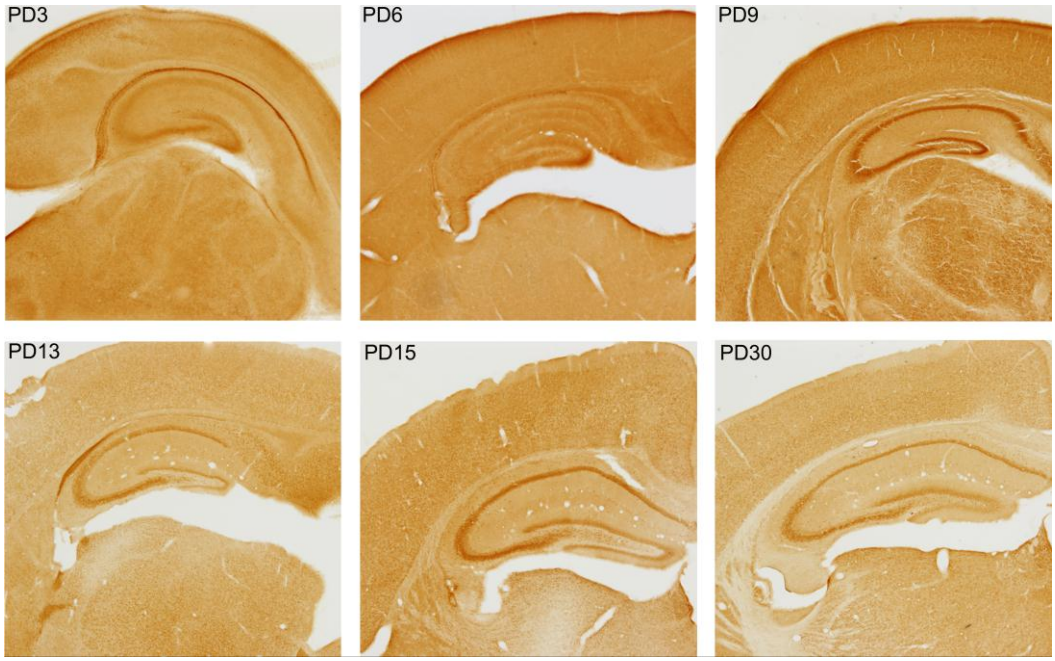


Fig.1.1 – Pattern of expression of A_1 receptors (37 kD) throughout the ontogenic period. **A)** The density of A_1 receptors does not seem to change significantly throughout the ontogenic period. **B)** Western blot showing the density of A_1 receptors at PD 5, PD 15, PD 30, PD 50 and PD 60 when 60 µg of protein corresponding to the total membrane fraction of the hippocampus were loaded in a polyacrylamide gel. **C)** A_1 receptors seem to be abundantly present in more immature animals (PD 3 to PD 9) and a more defined pattern of distribution (in cell bodies) becomes evident from PD 9 onward. Results presented in **A)** are means +/- SEM of 3 independent experiments/age and densities at different ages were analysed by One-way ANOVA followed by Tukey test. No significant differences ($p>0.05$) in the density of A_1 receptors were found in hippocampal membranes belonging to animals of different ages (5 -60 days-old).

Fig.1.2

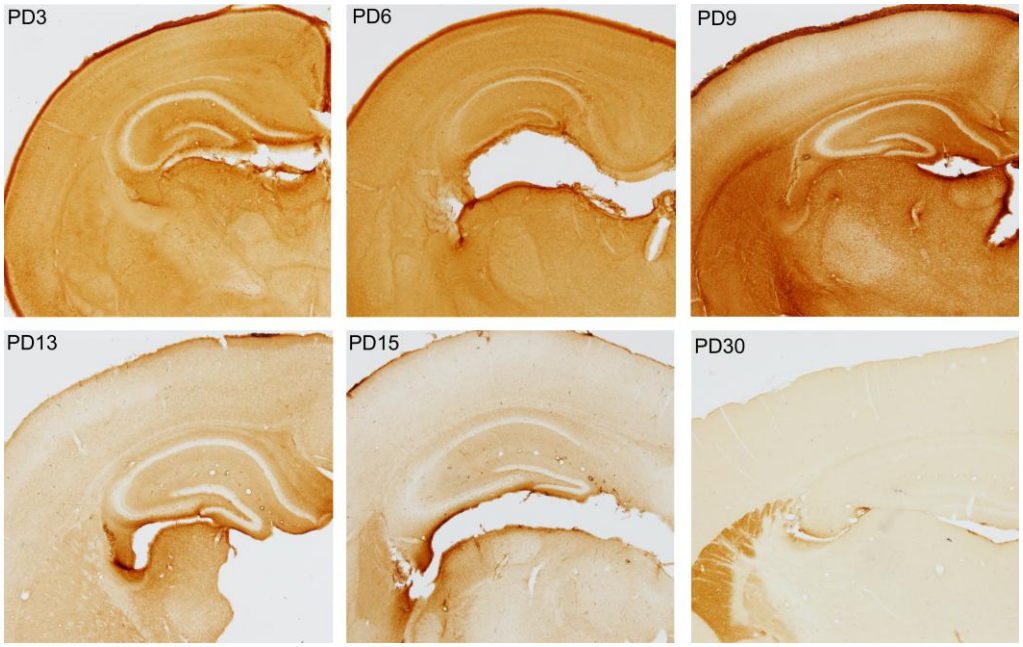


Fig. 1.2 – Immunohistochemistry labeled sections showing the developmental pattern of localization of A_{2A} receptors in the brain from pups with ages ranging from 3 to 30 days-old. A_{2A} receptors present a wide pattern of distribution in more immature tissues, occupying molecular and dendritic layers of the hippocampus. A progressive down-regulation leads to the appearance of the adult pattern of expression of the receptor (PD 30), corresponding to a strong labeling in the striatum and almost absence of labeling in extrastriatal regions. Images are representative or 3-4 independent experiments.

To confirm the absence of A_{2A} receptors in presynaptic sites, a double labeling of A_{2A} receptors with either VGLUT1 or VGAT, presynaptic markers for glutamatergic and GABAergic synapses (see **section 1.5** from **Chapter 1**), was made (see **Fig. 1.4**). No evidence for a presynaptic location of A_{2A} receptors was found in glutamatergic synapses, however A_{2A} receptors seem to be co-localized with VGAT (see the yellow color in the figure indicating co-localization).

Fig.1.3

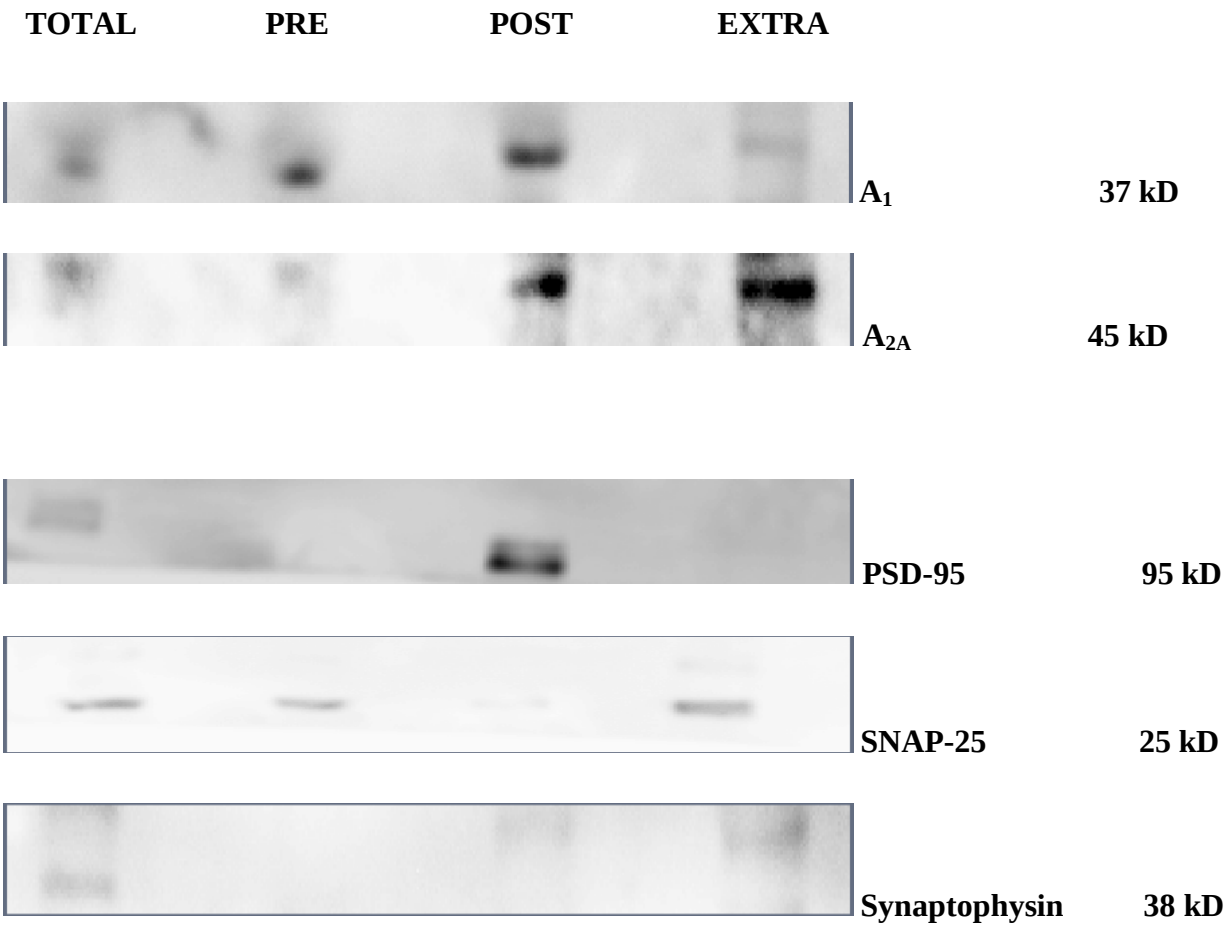


Fig. 1.3 – Western blot images showing the presence of A_1 and A_{2A} receptors in pre, post and extrasynaptic fractions of the hippocampus of 5 to 7 days-old pups. A_1 receptors are located in pre and postsynaptic sites and A2A receptors do not seem to be present in presynaptic sites but are abundantly located in post and especially in putative extrasynaptic sites. The control of purity of each fraction was made using PSD-95, SNAP-25 and synaptophysin. Blots are representative of 2 independent experiments. Molecular weights of the proteins are indicated on the right in the figure.

Fig. 1.4

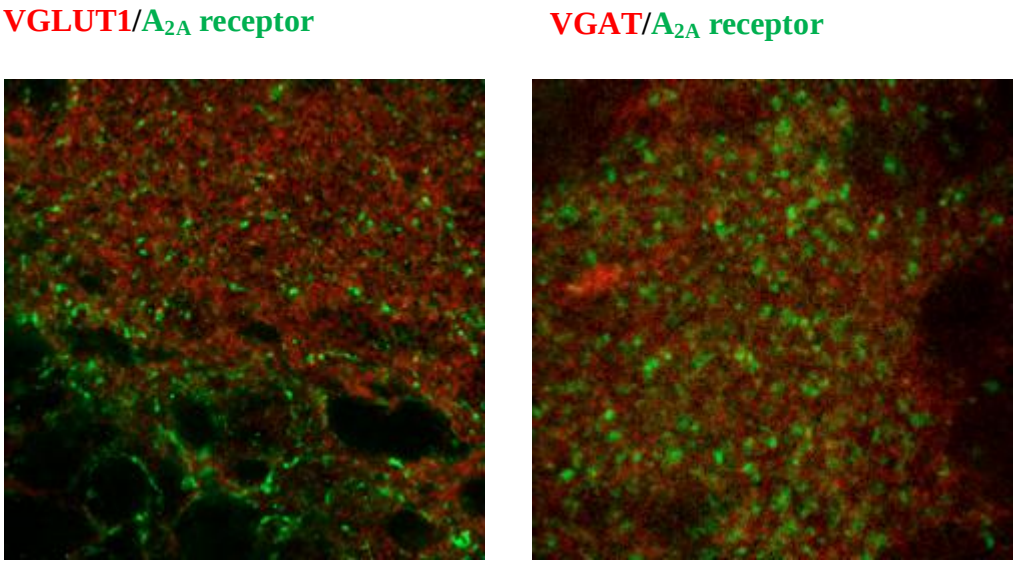


Fig. 1.4 – Confocal images of double immunohistochemically labeled sections with A_{2A} receptor antibody (green) and VGLUT1 or VGAT antibodies (red) in the hippocampus from a PD 6 mice pup in regions where both proteins are present. No co-localization was found between the labeling of A2A receptor and the VGLUT1, however it seems to exist a co-localization between A_{2A} receptors and VGAT, suggesting the location of this subtype of adenosine receptor in presynaptic sites of GABAergic synapses. Magnification: 60×. Images are representative of 3 independent experiments. It was not performed a quantification of the fluorescence.

A_{2A} receptors were also found to label progenitor cells in the cingulate cortex as well as migrating cells (see **Fig. 1.5**). **Fig. 1.6** is a confocal microscope picture showing newly generated neurons intensely labeled with A_{2A} receptors (green) in close proximity to glial cells (red), probably radial glial cells involved in migration of neurons (see the section **2.3** of the **Introduction**). Interestingly, A_{2A} receptors do not seem to be present in glial cells. The presence of A_{2A} receptors in progenitor and migrating cells suggests a possible role in processes of differentiation, migration and synaptogenesis (see the section **2.4** of the **Introduction**). Chapter 4 provides data on the effect of the chronic treatment with adenosine receptor antagonists during the embryonic development that supports a role for A_{2A} receptors in steps of neural development.

Fig. 1.5

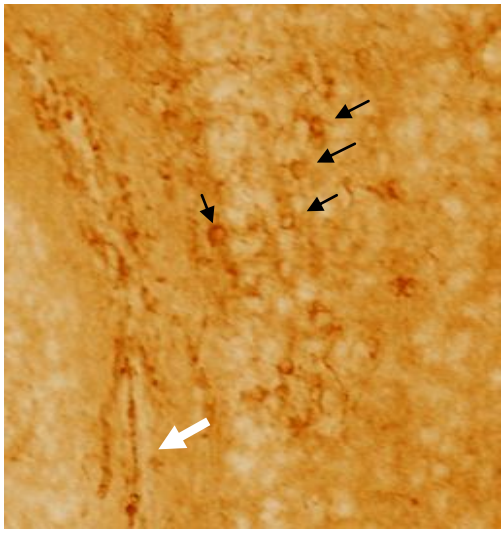


Fig. 1.5 – Immunohistochemistry images showing A_{2A} receptor immunoreactivity in progenitor cells (indicated by black arrows) and migrating cells (white arrow) in the region of cingulate cortex of a 6 days-old mice pup. This image is representative of 4 independent experiments.

Fig. 1.6

A) **B)**

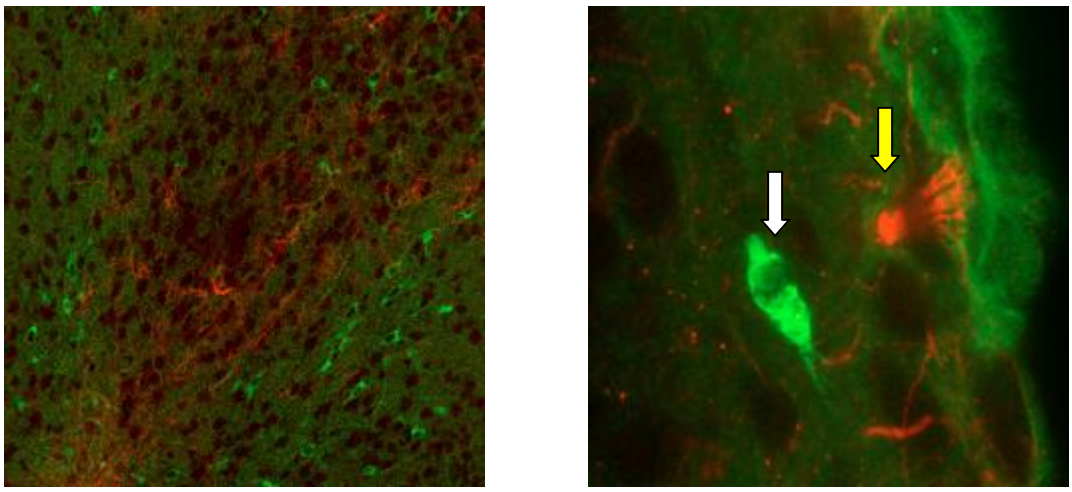


Fig. 1.6 – Confocal images showing proliferative regions from where new generated neurons, labeled in green with the anti-A_{2A} receptor antibody, had been produced and start migrating. Close to these proliferative regions a high concentration of glial cells labeled in red with the anti-GFAP antibody can also be observed (**A** – Magnification 20×). (**B**) High magnification (60 ×) of a newly generated neuron strongly immunopositive for anti-A_{2A} receptor antibody (green) in the cell body (indicated with the white arrow) in close proximity to glial cells labeled with anti-GFAP (red) and indicated with the yellow arrow.

1.2. ATP Receptors

The selectivity of the antibodies against P2X and P2Y ATP receptor subunits was validated in Rodrigues *et al.*, 2005. This study showed that all P2X ($P2X_{1-7}$) are expressed in the adult (6 to 8 weeks old) rat hippocampus. Here we studied the postnatal ontogenic immunoreactivity of the same ATP receptor, using hippocampal membranes of mice hippocampus.

1.2.1. P2X Receptor Subunits

P2X receptor subunits can be separated into different groups which are characterized by a distinct profile of immunoreactivity. In the first group are included $P2X_1$ and $P2X_4$ subunits (see **Fig. 1.7A-D**). The densities of $P2X_1$ receptor subunit were 100 +/- 43.6% at PD 5, 176.9 +/- 62.4% at PD 10, 286.3 +/- 55.7% at PD 15, 266.6 +/- 63.4% at PD 20, 418 +/- 71.3% at PD 25, 365.9 +/- 55.3% at PD 30, 252.3 +/- 22.6% at PD 40, 286.4 +/- 52.8% at PD 50 and 294.0 +/- 8.1% at PD 60. These quantifications were made using 3 independent purifications of total membrane fractions and the means were compared using One-way ANOVA followed by Tukey test. Differences were found between the average density observed at PD 5 and the average densities observed at PD 25 and PD 30. The density of $P2X_4$ receptor was 100 +/- 12.1% at PD 5, 290.9 +/- 22.6% at PD 10, 223.5 +/- 31.4% at PD 20, 163.3 +/- 23.2% at PD 25, 176.1 +/- 9.9% at PD 30, 183.2 +/- 7.4% at PD 35, 177.5 +/- 35.6% at PD 40, 206.5 +/- 25.0% at PD 50 and 171.3 +/- 22.4% at PD 60. At PD 10 it was observed an up-regulation ($p<0.05$) of $P2X_4$ subunit when the comparison was made with the age of 5 days-old. For the other ages tested, it was not observed any significant difference between the relative values of density. The results are means from 3-9 independent purifications of total membranes and the comparison between groups was made using One-way ANOVA followed by Tukey test.

The second group consists of $P2X_5$, $P2X_6$ and $P2X_7$ subunits. These proteins share a similar ontogenic profile – a sharp (in the case of $P2X_6$ subunit – **Fig. 1.8A** and **B**) or a gradual (in the case of $P2X_7$ subunit – **Fig. 1.8C** and **D**) but stable increase in immunoreactivity throughout the postnatal ontogeny.

The density of $P2X_6$ receptor subunit at different ages was 100.0 +/- 7.3% at PD 5, 325.0 +/- 23.1% at PD 10, 319.9% +/- 22.5% at PD 15, 397.8 +/- 65.4% at PD 20, 350.4 +/- 42.0% at PD 25,

366.0 +/- 14.7% at PD 30, 350 +/- 61.0% at PD 60. Significant differences were observed between the relative values of density of $P2X_6$ at the age of 5 days-old and 10 days-old ($p<0.05$) and between the age of 5 days-old and the ages of 15, 20, 25, 30 and 60 days-old ($p<0.001$), using One-way ANOVA followed by a Tukey test (n=3-4).

Fig. 1.7

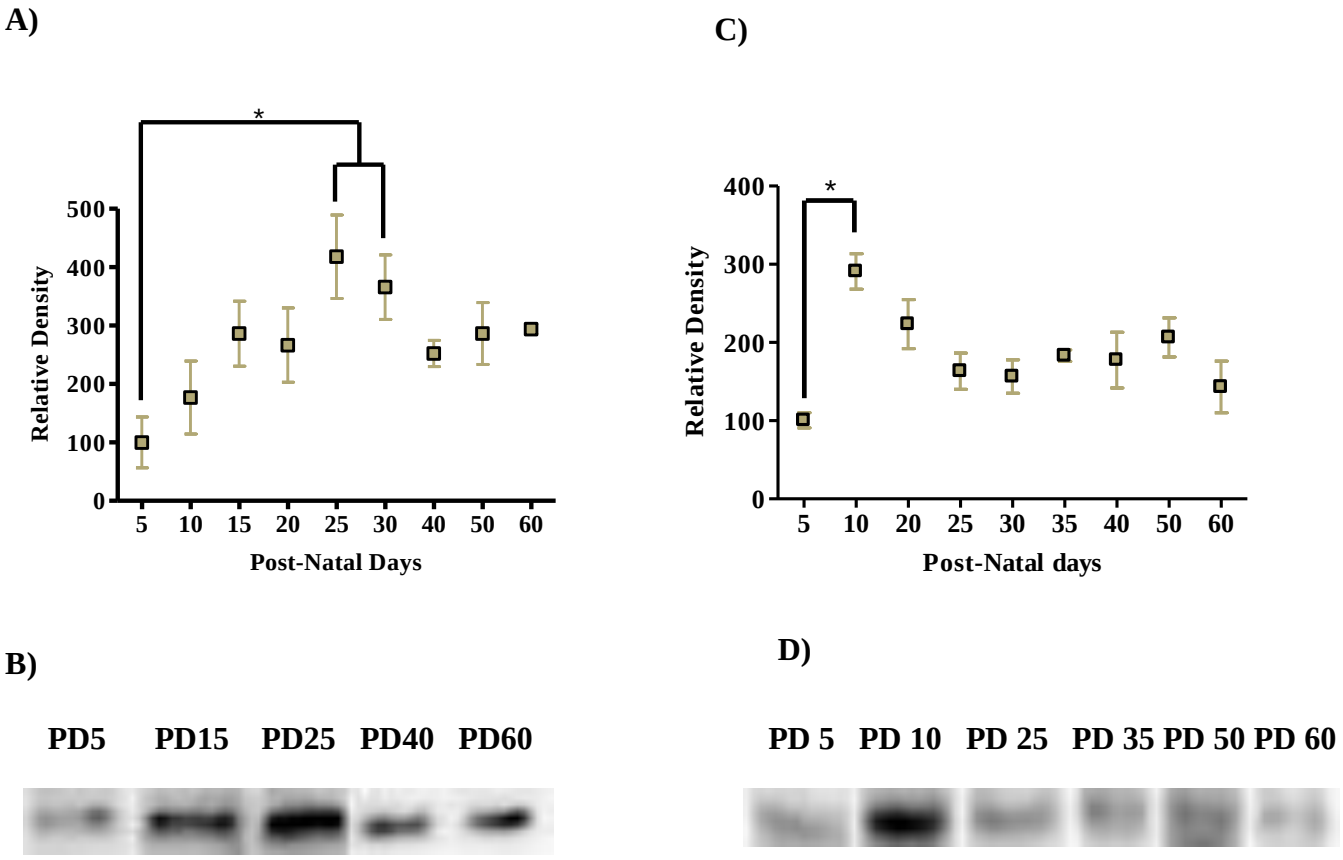


Fig. 1.7 – Postnatal ontogenic profile of the immunoreactivity of $P2X_1$ and $P2X_4$ subunits of ATP receptors in mice hippocampal membranes. **A)** The graphic shows the variations in the density of the $P2X_1$ subunit (62 kD) in hippocampal membranes from 5, 10, 15, 20, 25, 30, 40, 50 and 60 days-old mice. The density of $P2X_1$ receptors increased from PD 5 to PD 25 and PD 30. This period of up-regulation was transient since from PD 40 to PD 60, the differences in the density of $P2X_1$ receptors were not statistically significant, when the comparison wass made with the age 5 days-old (using One-way ANOVA followed by Tukey test, n=3). **B)** Western blot showing the variation of density of $P2X_1$ receptors during the postnatal period, more precisely at PD 5, 15, 25, 40 and 60. **C)** In the case of $P2X_4$ receptors (57 kD), the period of up-regulation seems to occur earlier, at PD 10. The same statistical analysis was performed as for $P2X_1$ subunit and 3-9 experiments were considered. **D)** Western blot corresponding to the density of $P2X_4$ receptors in hippocampal membranes from 5, 10, 25, 35, 50 and 60 days-old mice. Statistical significance is indicates as *$p<0.05$.

Fig. 1.8

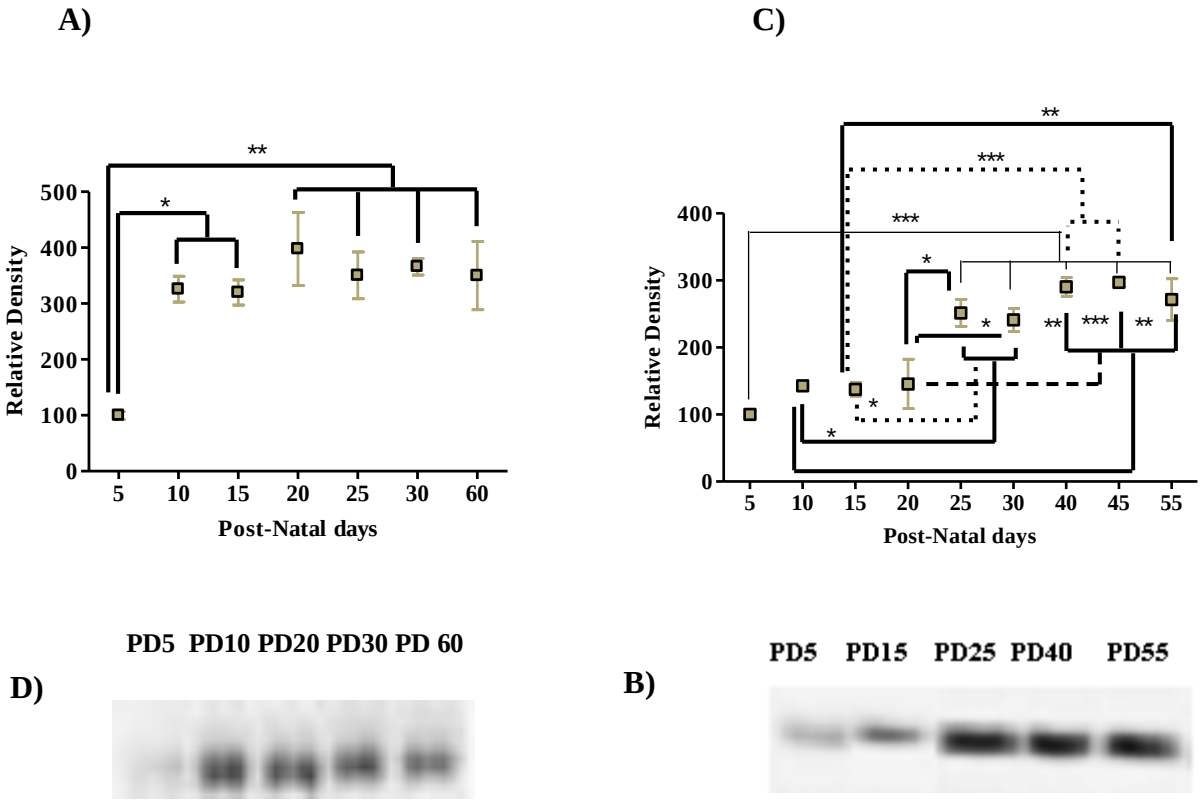


Fig. 1.8 – Postnatal ontogenic profile of the immunoreactivity of $P2X_5$, $P2X_6$ and $P2X_7$ subunits of ATP receptors in mice hippocampal membranes. **A)** The graph shows the average density of the $P2X_6$ subunit immunoreactivity in hippocampal membranes from 5, 10, 15, 20, 25, 30 and 60 days-old mice. The density of the $P2X_6$ receptor increased from PD 5 to PD 10 and remained stable in membranes from older animals. **B)** Western blot showing the variation of density of the $P2X_6$ receptor (51 kD) at PD 5, PD 10, PD 20, PD 30 and PD 60. **C)** In the case of $P2X_7$ receptor subunit, the period of up-regulation seems to occur later at PD 25. **D)** Western blot showing the relative density of $P2X_7$ receptor subunit (75 kD) at PD 5, PD 15, PD 25, PD 40 and PD 55. **E)** Graph showing the ontogenic pattern of immunoreactivity of $P2X_5$ subunit showing that there is an up-regulation of this protein from PD 40 onward. This increase in the density with age observed for $P2X_5$ was however subtle. **F)** Western blot image showing the variation of the density of $P2X_5$ (68 kD) in hippocampal membranes from 5, 15, 25, 40, 55 days-old mice. For all receptor subunits, the comparison between the densities of the ATP subunits at the different ages tested was made using One-way ANOVA followed by Tukey test. Statistical significance is indicated in the graphics by *p<0.05; **p<0.01 and ***p<0.001. 3-4 independent purifications of total membranes were used for the analysis of each receptor subunit.

The density of $P2X_7$ receptor subunit was observed to increase gradually from PD 5 to PD 25, becoming stable from PD 25 onward. The values of the relative density of $P2X_7$ receptors at different ages were: 100.0 +/-5.6% at PD 5, 142.9 +/- 1.7% at PD 10, 137.6 +/-10.2% at PD 15, 178.6 +/- 28.2% at PD 20, 251.6 +/- 20.2% at PD 25, 241.0 +/- 17.24% at PD 30, 335.4 +/- 36.2% at PD 35, 290.6 +/- 14.0% at PD 40, 297.1 +/- 6.2% at PD 45, 271.4 +/- 31.01% at PD 55 (**see Fig. 1.8C** and **D**). Tukey test detected significant differences in the levels of $P2X_7$ receptors density between the ages 5 days-old and the ages 25, 30, 40, 45 and 55 days-old ($p<0.001$), between the density of $P2X_7$ receptors at PD 10 and PD 25 and PD 30 ($p<0.05$) and PD 40 and PD 55 ($p<0.001$); PD 10 and PD 45 ($p<0.0001$); between PD 15 and PD 25 and PD 30 ($p<0.05$); between PD 15 and PD 40 and PD 45 ($p<0.0001$) and PD 15 and PD 55 ($p<0.001$); between PD 20 and PD 25 and PD 30 ($p<0.05$); between PD 20 and PD 40 and PD 55 ($p<0.001$) and PD 20 and PD 45 ($p<0.0001$).

$P2X_5$ subunit seems to suffer an up-regulation at PD 40 that was still observed at PD 60. The values of the density of $P2X_5$ receptor subunit throughout the postnatal period were: 100 +/- 20.1% at PD 5, 91.5 +/- 16.78% at PD 10, 31.8 +/- 1.6% at PD 15, 66.2 +/- 11.0 % at PD 25, 177.3 +/- 13.9% at PD 40, 176.1 +/- 23.4% at PD 50. Statistical significance indicating a difference between the means of relative density of $P2X_5$ receptor subunit were observed between the ages of 5 days-old and 40 days-old ($p<0.05$); 10 days-old and 40 and 50 days-old ($p<0.05$); between the age of 15 days-old and 40 and 50 days-old ($p<0.0001$) and between 25 days-old and 40 and 50 days-old ($p<0.001$). The magnitude of the up-regulation was only of 1.5 fold (at PD 50) relatively to the levels observed at PD 5.

$P2X_2$ subunit was included into a third group (see **Fig. 1.9A** and **B**). The density of $P2X_2$ receptors was found to be stable throughout the postnatal ontogenic period. The values of the average densities of $P2X_2$ receptor subunit were 100 +/- 6.7% at PD 5, 117.4 +/- 8.7% at PD 10, 72.0 +/- 10.2% at PD 15, 76.3 +/- 8.3% at PD 20, 106.2 +/- 13.2% at PD 25, 93.3 +/- 8.5% at PD 30 and 87.3 +/- 23.2% at PD 45. Statistical analysis of 3-7 independent experiments did not show significant difference ($p>0.05$) in the density of $P2X_2$ receptor subunit analysed in hippocampal membranes from animals at different ages.

Fig. 1.9

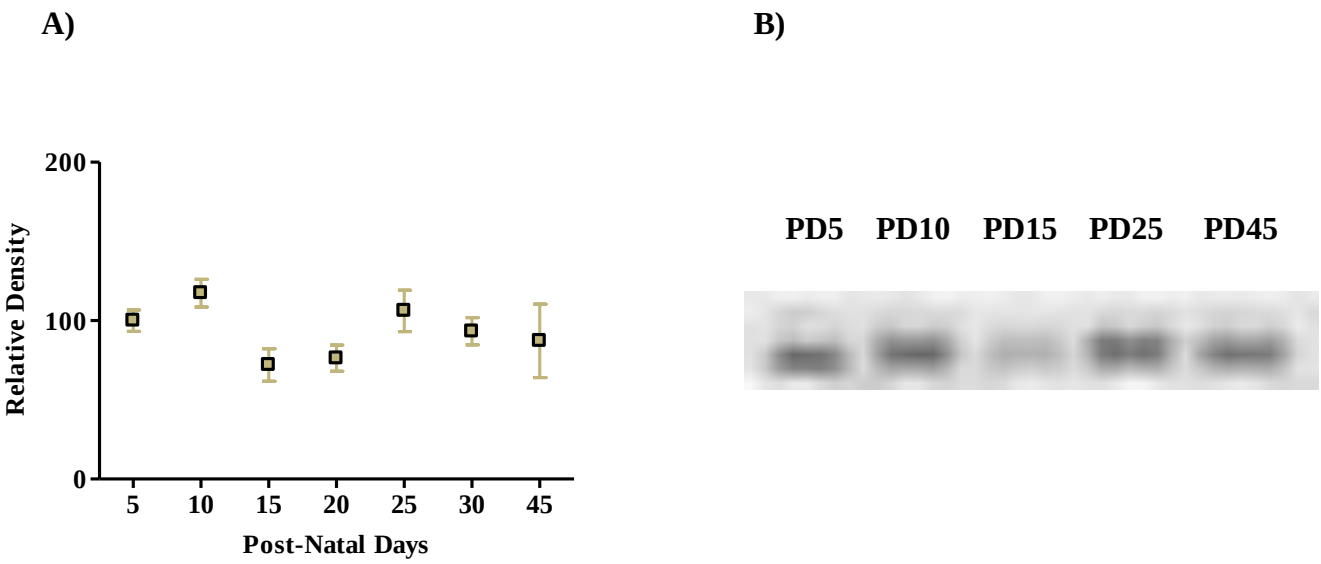


Fig. 1.9 – Postnatal ontogenic profile of the immunoreactivity of $P2X_2$ subunit of ATP receptors in mice hippocampal membranes. **A)** No significant differences were found between the densities of $P2X_2$ (60 kD) at the ages tested, 5, 10, 15, 20, 25, 30 and 45 days-old. Comparisons were made using an One-way ANOVA followed by a Tukey test (n=3-7). **B)** Western blot image showing the relative density of $P2X_2$ at PD 5, 10, 15, 24 and 45 when 60 μg of protein were loaded into a gel.

The last group includes $P2X_3$ receptor subunits. **Fig. 1.10** presents the ontogenic profile of this protein. Densities of $P2X_3$ subunit at PD 5, PD 10 and PD 15 and at PD 25, PD 30 and PD 40 were significantly higher than the density measured in PD 20 mice hippocampal membranes, indicating a down-regulation, $p<0.05$, $p<0.001$, $p<0.0001$, n=3-6, using One-way ANOVA followed by Tukey test. The values od density of $P2X_3$ were the following: (density at PD 20 was 42.9 +/- 6.5% *versus* densities at PD 5, 100.0 +/- 9.9%, 104.5 +/- 10.2% at PD 10, 126.4 +/- 7.4% at PD 15, 42.9 +/- 6.5% at PD 20, 100.4 +/- 25.2% at PD 25, 116.3 +/- 8.3% at PD 30 and 142.8 +/- 8.7% at PD 40.

1.2.2. P2Y Receptors

Several patterns of immunoreactivity could also be established for P2Y receptors. The first group defined includes $P2Y_1$ receptor subtype (**Fig. 1.10A** and **B**) and $P2Y_2$ (**Fig. 1.10C** and **D**). It was not detected any between PD 5 and PD 25. The densities of $P2Y_1$ receptor subtype were 100.0 +/-4.6% at PD 5, 147.7 +/- 7.8% at PD 15, 64.9 +/- 6.3% at PD 20 and 283.8 +/- 116.2% at PD 25. A

marked up-regulation ($p<0.0001$, one-way ANOVA followed by Tukey test, n=3-4) was observed at PD 30, an age at which the relative density of the protein reached a value of 752.6 +/- 38.8%, followed by a recovery back to the initial levels of immunoreactivity in membranes from more mature animals. The average density of the protein corresponding to the $P2Y_1$ receptor subtype was 57.1 +/- 4.2% at PD 40 and 135.1 +/- 10.8% at PD 60, not statistically different from the levels of density in hippocampal membranes from animals between 5 to 20 days-old ($p>0.05$, using One-way ANOVA followed by Tukey test, n=3-4).

Fig. 1.10

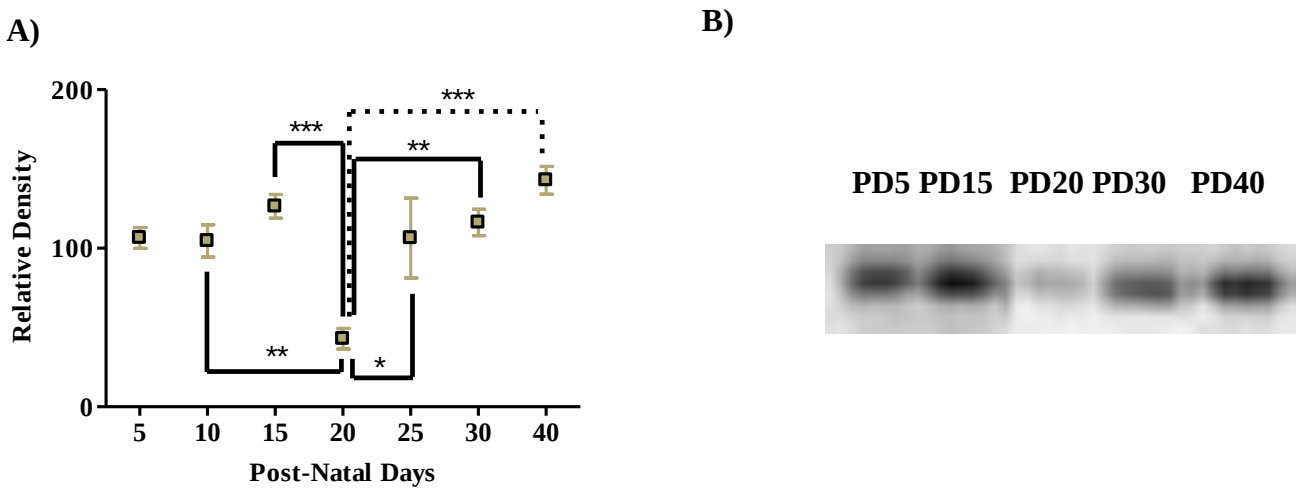


Fig. 1.10 – Postnatal ontogenic profile of the immunoreactivity of $P2X_3$ subunit of ATP receptors in mice hippocampal membranes A) It was observed a down-regulation of the protein at PD 20 followed by a recovery to the initial levels of immunoreactivity between PD 30 and PD 40. Results are mean +/- SEM of 3-6 independent purifications of hippocampal membranes. Means were compared using One-way ANOVA followed by Tukey test. Significant differences are indicated in the graphic as *$p<0.05$; **$p<0.001$ and ***$p<0.0001$. **B)** Western blot showing the dynamic variations of the density of $P2X_3$ subunit in hippocampal membranes at different time points (5, 15, 20, 30 and 40 days-old) of the postnatal development, upon application of 60 μg of protein from hippocampal membranes of each age group, into a polyacrylamide gel.

The density of $P2Y_2$ receptor subtype increased gradually from PD 5 to PD 20, age at which the highest immunoreactivity was observed. A significant difference ($p<0.0001$) was observed between the densities observed at PD 5 (100.0 +/- 8.5%), PD 10 (251.8 +/- 66.8%), PD 15 (244.9 +/- 40.0%) and the density at PD 20 (538.1 +/- 25.6%). This increase was also transient since from PD 30 onward the density of $P2Y_2$ was similar to the density observed at more immature stages, $p>0.05$ (except the density at PD 60, which is different from the density at PD 5, $p<0.05$). The density of

$P2Y_2$ at PD 30 was 244.5 +/- 9.0%, at PD 40 was 311.4 +/- 64.1%, at PD 45 was 238.3 +/- 19.5%, at PD 50 was 273.5 +/- 32.1% and at PD 60 was 304.3 +/- 18.03%. The comparison between the average immunoreactivities was was performed using one-way ANOVA followed by Tukey test over data from 3-4 independent isolations of hippocampal membranes.

A less dynamic change of density was observed for $P2Y_6$ and $P2Y_{12}$ receptors. **Fig. 1.12** ilustrates the ontogenic profile of the relative densities of $P2Y_6$ and $P2Y_{12}$ receptor subtypes in hippocampal membranes. Considering the density at PD 5 as the lowest (100 +/- 11.4%), the densities observed at the other ages investigated were 204.2 +/- 12.4% at PD 10, 179.7 +/- 25.1% at PD 15, 192.8 +/- 19.9% at PD 20, 195.7 +/- 15.1% at PD 25, 195.5 +/- 8.6% at PD 30, 180.6 +/- 8.6% at PD 35, 153.9 +/- 15.4% at PD 40, 225.5 +/- 16.6% at PD 45 and 171.8 +/- 14.6% at PD 55. One-way ANOVA analysis followed by Tukey test indicated a significance difference between the densities at PD 5 and densities at PD 10, 20, 35, 40 ($p<0.05$) and between the densities at PD 5 and PD 25 and PD 45 ($p<0.001$ using One-way ANOVA followed by Tukey test, n=3-9). In the case of $P2Y_{12}$ receptor subtypes, the densities at the ages investigated were 100 +/- 19.4% at PD 5, 95.4 +/- 25.5% at PD 10, 100.7 +/- 38.8% at PD 15, 137.5 +/- 24.7% at PD 20, 112.0 +/- 11.2% at PD 30, 73.8 +/- 5.8% at PD 35, 126.8 +/- 14.0% at PD 40, 138.7 +/- 6.6% at PD 50 and 204.1 +/- 10.4% at PD 55. The same statistical analysis revealed differences between the densities at PD 5 and PD 55 ($p<0.05$, One-way ANOVA followed by Tukey test, n=3-9), between the densities at PD 10 and PD 55 ($p<0.05$, One-way ANOVA followed by Tukey test, n=3-9), between densities at PD 15 and PD 55 ($p<0.05$, One-way ANOVA followed by Tukey test) and between densities at PD 35 and PD 55 ($p<0.001$, One-way ANOVA followed by Tukey test, n=3-9).

The third group includes the $P2Y_4$ receptor subtype. The expression of $P2Y_4$ seems to be constant throughout the postnatal period, $p>0.05$, One-way ANOVA followed by Tukey test, n=3-6. The density observed at PD 5 was 100 +/- 16.9%, at PD 10 was 95.6 +/- 6.0%, at PD 15 was 50.5 +/- 8.4%, at PD 20 was 78.3 +/- 9.8%, at PD 30 was 79.3 +/- 14.9%, at PD 45 was 95.2 +/- 13.7%, at PD 50 was 108.4 +/- 19.7%, at PD 60 was 133.9 +/- 18.0%.

1.3. Ectonucleotidases

The ontogenic profile of immunoreactivity of ecto-nucleotidases through the postnatal development was also investigated. **Fig. 1.14** presents the graphic showing the pattern of density of ecto-5'-nucleotidase (**A**), ecto-nucleoside triphosphate diphosphohydrolase 1 – eNTPDase 1 (**C**), ecto-nucleoside triphosphate diphosphohydrolase 2 – eNTPDase 2 (**D**) and ecto-nucleoside triphosphate diphosphohydrolase 3 – eNTPDase 3 (**E**).

Hippocampal membranes (containing membranes from neurons and glia) were used again. A detectable immunoreactivity for ecto-5'- nucleotidase was observed at PD 10 (applying into a gel 60 µg of protein) and it was used as the reference (100%) for comparison with densities observed at other ages. It was observed a marked up-regulation ($p<0.0001$, One-way ANOVA followed by Tukey test, n=3-4) between PD 10 (100 +/- 8.4%) and PD 20 (483.4 +/- 55.3%) and a fast recovery at PD 25 (186.3 +/- 25.1%) - (see **Fig. 1.14A** and **B**). From PD 30 onward the densities of ecto-5'-nucleotidase were similar to the densities measured at PD 10 (densities at PD 30=126.2+/- 9.4%, at PD 40=125.0 +/- 13.6%, at PD 45=119.6 +/- 7.9%, at PD 50=121.0 +/- 23.9%).

Because ecto-5'-nucleotidase is a key enzyme for the conversion of ATP into adenosine which will bind and activate adenosine receptors, a sub-synaptic purification was made from hippocampal synaptic terminals and the presence of this enzyme in each of these fractions was investigated. Interestingly, ecto-5'-nucleotidase was already present in fractions from hippocampal membranes from pups with 5 to 7 days-old and it was found to be exclusively located in postsynaptic sites (see **Fig. 1.14C**). As previously showed, A_1 and A_{2A} receptors were also present in the postsynaptic fractions. The presence of ecto-5'-nucleotidase in the same fraction may be viewed as an argument that links ATP release from presynaptic terminals to postsynaptic activation of adenosine receptors. It means that during the first postnatal week it is possible that postsynaptically acting adenosine.

eNTPDase 1 density (see **Fig. 1.15 A** and **B**) increased with age (densities at PD 10, considered the reference value was 100 +/- 23.7%, at PD 20 was 80.2 +/- 11.4%, at PD 25 was 116.1 +/- 5.7%, at PD 35 was 130 +/- 5.8%, at PD 40 was 125.3 +/- 12.4%, at PD 45 was 196.5 +/- 7.3%, and at PD 60 was 192.5 +/- 17.9%. The densities of eNTPDase 1 observed in hippocampal membranes from animals younger than 45 days-old were significantly lower ($p<0.05$, $p<0.001$ or $p<0.0001$, using one-way ANOVA followed by Tukey test to compare means obtained from 3 independent purifications of hippocampal membranes) than the densities observed in hippocampal membranes from animals

older than 45 days-old. The same was observed for eNTPDase 3 (**Fig. 1.15E** and **F**). The relative densities of eNTPDases were 100 +/- 8.2% at PD 10, 122.4 +/- 9.6% at PD 15, 177.7 +/- 23.9% at PD 20, 200.8 +/- 15.4% at PD 25, 236.5 +/- 26.5% at PD 30, 190.0 +/- 5.7% at PD 35, 179.2 +/- 9.1% at PD 40, 179.3 +/- 16.2% at PD 50 and 175.2 +/- 12.3% at PD 60. Densities at PD 10 and PD 15 were significantly lower than these measured at PD 30 and at PD 60 ($p<0.05$ and $p<0.001$, one-way ANOVA followed by Tukey test performed to compare means from 3-6 independent purifications of hippocampal membranes).

Fig. 1.11

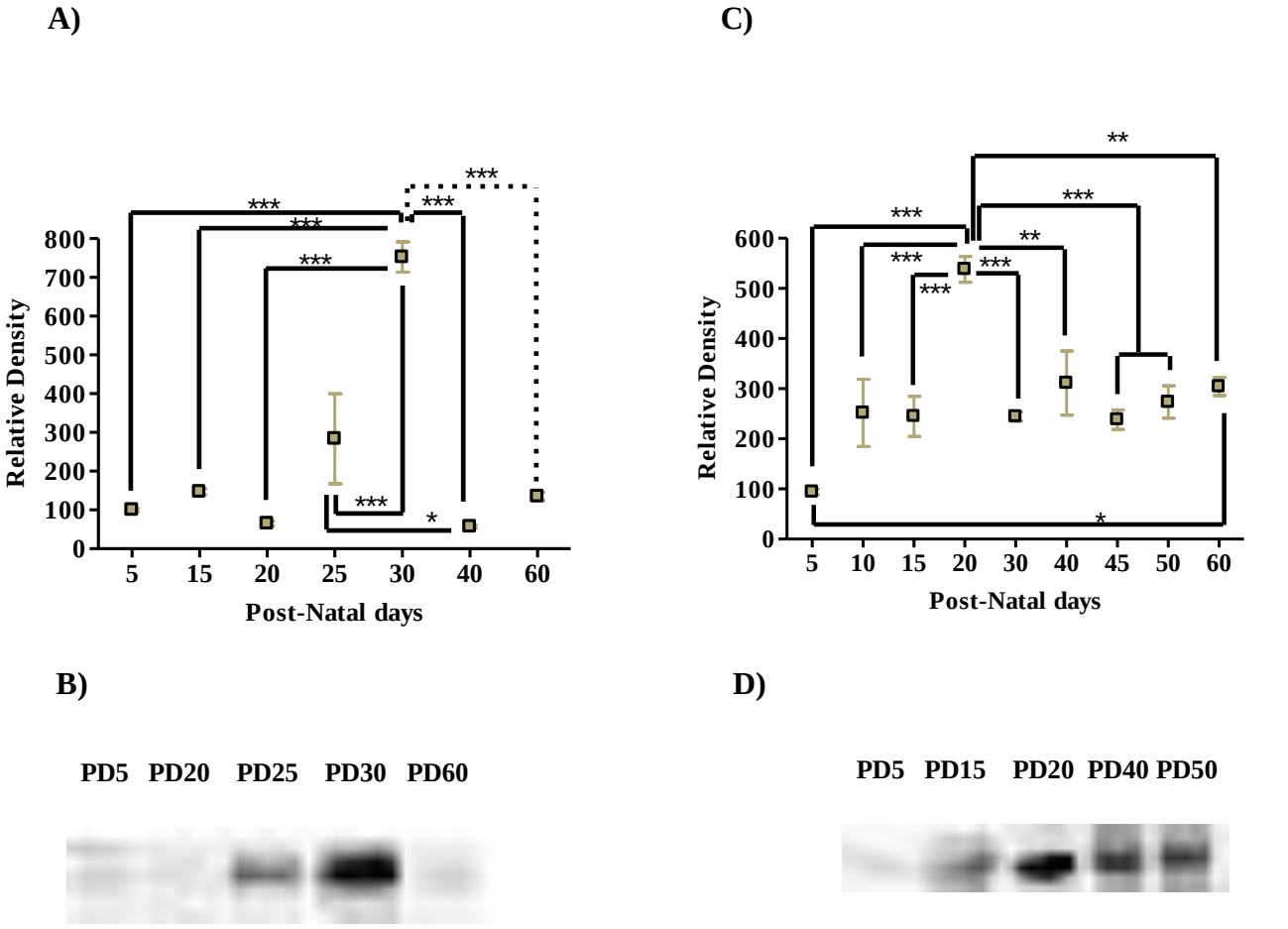


Fig. 1.11 – Postnatal ontogenic profile of the immunoreactivity of $P2Y_1$ and $P2Y_2$ subtype of ATP receptors in mice hippocampal membranes. It was observed a marked and transient up-regulation of $P2Y_1$ protein at PD 30. **B)** Western blot corresponding to the graph represented in **A)** at PD 5, 20, 25, 30 and 60. **C)** Graph showing the ontogenic profile of $P2Y_2$ subunit immunoreactivity, showing an up-regulation at PD 20 and again, a recovery from PD 30, 40, 45, 50 and 60 onward. **D)** Western blot showing the variation of the expression of $P2Y_2$ receptors represented in **C)**. The ages represented are PD 5, PD 15, PD 20, PD 40 and PD 50. Results are mean +/- SEM and the comparison between the densities was made using One-way ANOVA (n=3-4). Statistical significante is indicated in the graphic as *$p<0.05$; **$p<0.001$ and ***$p<0.0001$.

Fig. 1.12

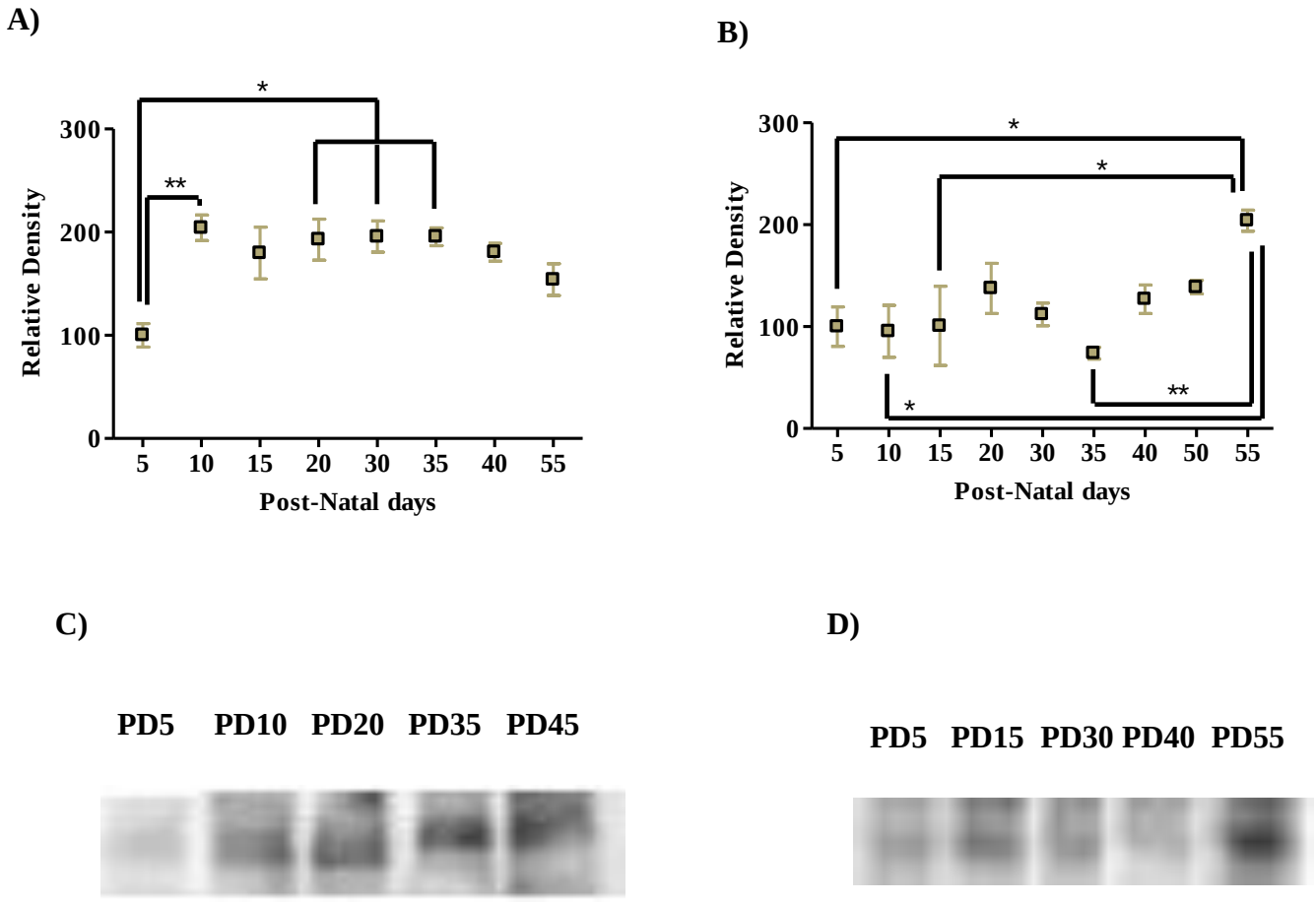


Fig. 1.12 – Postnatal ontogenic profile of the immunoreactivity of $P2Y_6$ and $P2Y_{12}$ ATP receptors in mice hippocampal membranes. Graph shows the ontogenic profile of $P2Y_6$ (**A** and **B**) and $P2Y_{12}$ (**C** and **D**) proteins in mice hippocampal membranes. In the case of $P2Y_6$ receptor there is an up-regulation of this protein as soon as PD 10. This increase in the density of the protein was maintained in hippocampal membranes at certain ages, 20, 25, 35, 40 and 45 days old. The maximal density of $P2Y_{12}$ receptors (**B**) in hippocampal membranes was found in 55 days-old animals. **C** and **D** correspond to the Western blots of $P2Y_6$ and $P2Y_{12}$ receptor analysis, respectively, and served as reference to the analysis of the density of these proteins. Results depicted in the graphs are mean +/- SEM of 3-9 independent purifications of hippocampal membranes. One-way Anova followed by Tukey test was performed to compare the densities in hippocampal membranes at different ages. Significant difference is indicated in the graphic as *p<0.05 and **p<0.001.

Fig. 1.13

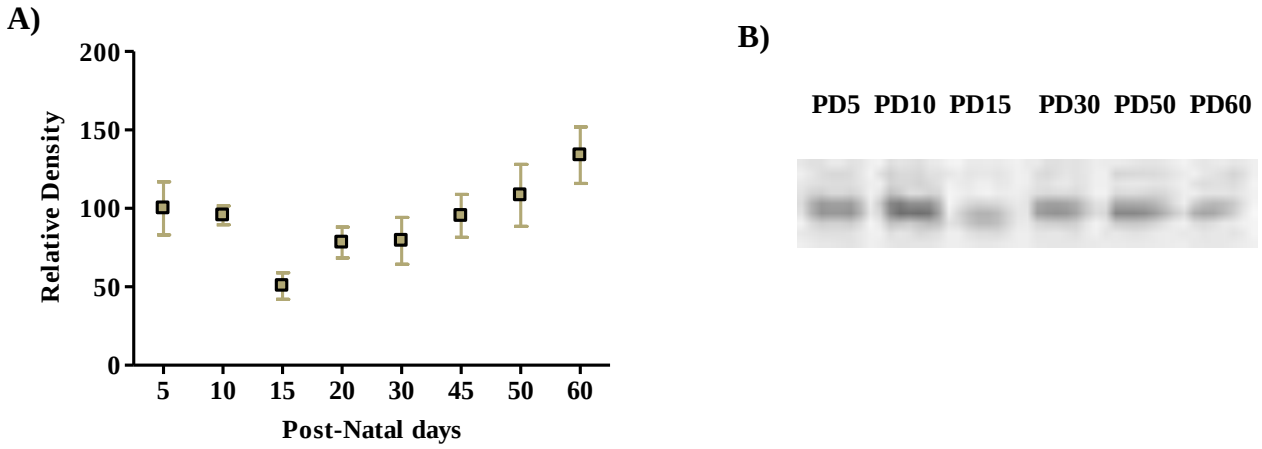


Fig. 1.13 – Postnatal ontogenic profile of the immunoreactivity of $P2Y_4$ ATP receptors in mice hippocampal membranes. A) Profile of $P2Y_4$ receptor density during hippocampal development. The density of this protein is constant throughout the developmental period. **B)** Western blot showing the density of $P2Y_4$ receptors in hippocampal membranes from 5, 10, 15, 30, 50 and 60 days-old. One-way ANOVA followed by Tukey test (n=3-6) revealed no statistical difference between the means +/- SEM values presented in **A**.

Fig. 1.14

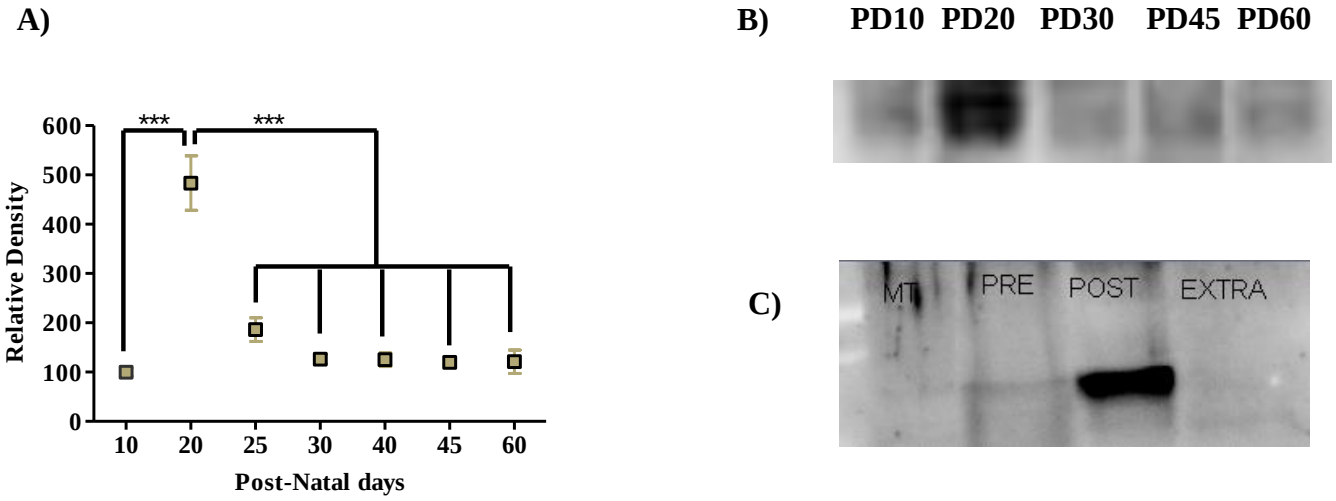


Fig. 1.14 – Postnatal ontogenic profile of the immunoreactivity of ecto-5'-nucleotidase in mice hippocampal membranes. A) There is a marked up-regulation of ecto-5'-nucleotidase (64 kD) at PD 20 which is transient since it is not detectable from PD 25 onward. Statistical analysis: One-way ANOVA followed by Tukey test; *** $p<0.0001$, n=3-4 independent experiments. **B)** Western blot analysis showing the density of ecto-5'-nucleotidase in hippocampal membranes from 10, 20, 30, 45 and 60 days-old animals. The **i**mmunoreactivity was obtained using 60 µg of protein. **C)** Sub-synaptic fractioning of putative synaptosomes obtained from PD 5 to PD 7 mice pups. The protein was found to be concentrated in postsynaptic fractions where A_1 and A_{2A} receptor subtypes are also present.

Fig. 1.15

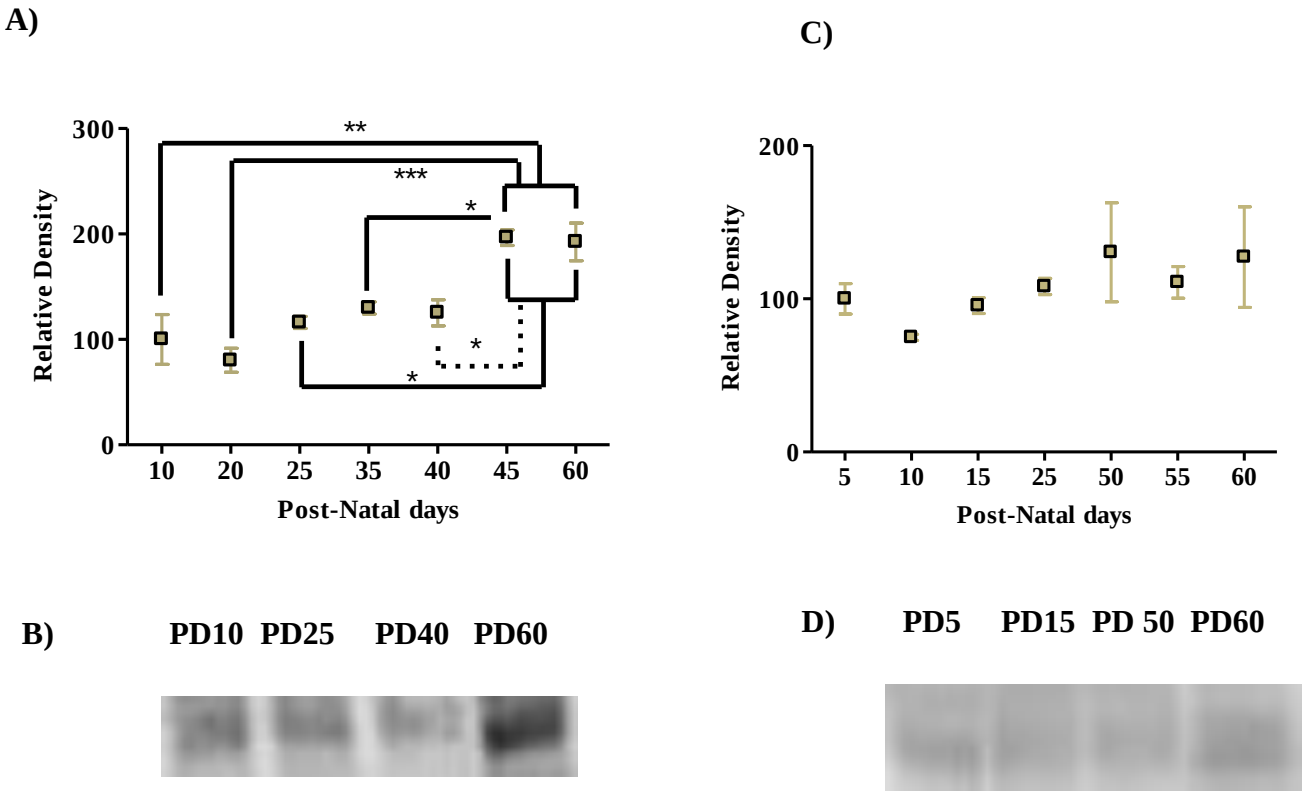


Fig. 1.15 – Postnatal ontogenic profile of the immunoreactivity eNTPDase-1. eNTPDase-2 and eNTPDase-3. Graphs showing the ontogenic profiles of immunoreactivity of eNTPDase-1 (**A**), eNTPDase-2 (**C**) and eNTPDase-3 (**E**) in the postnatal period. Only the density of eNTPDase-1 and eNTPDase-3 seems to change at some ages. eNTPDase-2 immunoreactivity seems to be constant throughout the postnatal period. (**B**, **D**, **F**) show Western blots corresponding to the immunoreactivity of eNTPDase-1, eNTPDase-2 and eNTPDase-3, when 60 µg of protein corresponding to the hippocampal membrane fraction were separated into a polyacrylamide gel. Result presented in the graphs are means +/- SEM and means were compared using One-way ANOVA followed by Tukey test. n=3-4 independent experiments. Statistical significance is indicated in the graphs by *p<0.05, **p<0.001 and ***p<0.0001.

Expression of eNTPDase-2 (**Fig. 1.15C** and **D**) does not seem to change in the postnatal period ($p>0.05$, One-way ANOVA followed by Tukey test, n=3-4). The average density determined at PD 5 was 100+/-2.0%, at PD 10 was 75.0 +/- 2.0%, at PD 15 was 95.6 +/- 5.2%, at PD 25 was 108.1 +/- 5.3%, at PD 50 was 130.4 +/- 32.4% at PD 55 was 110.8 +/- 10.3% and at PD 60 was 127.2 +/- 32.8%.

1.4. Nucleoside Transporters

It was evaluated the presence of the equilibrative nucleoside transporter 1 (ENT1), the most widespread of the four equilibrative transporters and considered the most important to control adenosine levels in the physiological range (Bone *et al.*, 2007). Jennings and collaborators (Jennings *et al.*, 2001) demonstrated a co-localization between ENT1 and A_1 receptor reinforcing the importance attributed to ENT1 this protein in the regulation of adenosine levels. **Fig. 1.16** displays Western blots from synaptosomal preparation (enriched in synaptic terminals) and total membranes (containing both neuronal and glial membranes) fractions. Total membranes from 5 to 7 days-old mice pups are enriched (111.9 +/- 1.5%) in this protein in comparison to the detectable signal in membranes from synaptosomes (100 +/- 1.6% - see **Fig. 1.16A**). Interestingly, in the adult of 30 days-old, ENT1 was enriched in synaptosomal membranes (see **Fig. 1.14B**). The relative density of ENT1 in synaptosomal membranes was 168.8 +/- 25.8% (n=2) and in total membranes, containing also membranes from glial cells, the relative density of ENT1 was 100 +/- 6.2% (n=2). In younger animals (pups), the transporter seems to be expressed mainly in glial membranes and there was a switch during development, making ENT1 more abundantly expressed by neurons.

1.5. Other Synaptic Components

An analysis of other pre and postsynaptic markers was also made in order to put into a context the changes observed for some receptors and enzymes belonging to the purinergic system (see **Conclusions** section From **Chapter 1**). For example, K^+Cl^- cotransporter 2 (KCC2) (Payne *et al.*, 1996) is a cation chloride transporter selectively expressed in neurons and up-regulated during development. This protein plays a key role controlling Cl^- concentration and is responsible for

producing the negative shift of $GABA_A$ responses from depolarizing to hyperpolarizing during neuronal maturation. KCC2 expression correlates with synaptogenesis, synchronizes functional maturation of excitatory and inhibitory synapses independently of the presence of synaptic activity since neither neuronal spiking nor GABAergic or glutamatergic transmission are required (Ludwig *et al.*, 2003). **Fig. 1.17A** represents the pattern of density for KCC2 protein from PD 10 to PD 55. There was a progressive increase in the expression of this protein. Density of KCC2 at PD 55 was 354.0 +/- 20.0% (n=3), which is at about 3.5 fold higher than the density at PD 5 (100.0 +/- 28.2%, n=3). At around PD 25 there was a stabilization of the levels of the protein, which was maintained until PD 55. The density of KCC2 at PD 40 was 331.0 +/- 28.8%, at PD 50 was 432.0 +/- 48.2% and at PD 55 was 354.0 +/- 20.9%.

Fig. 1.16

Fig. 1.16 – **Relative abundance of ENT1 in synaptosomal and total membranes from the hippocampus of pups and adult mice.** Western blots showing the immunoreactivity for ENT1 protein (50 kD-55 kD) in both hippocampal synaptosomal (SYN) and total membranes (MT) from 5-7 days-old pups (**A**) and 30 days-old mice (**B**). In young animals ENT1 is mainly located in glial membranes and in PD 30 animals it is more abundant in membranes from synaptic terminals than older animals. Results are representative of 2 independent experiments.

Postsynaptic density protein 95 (PSD-95) associates with receptors and cytoskeletal components in hippocampal neurons, driving maturation of glutamatergic synapses and is also involved in presynaptic terminal maturation, orchestrating the glutamatergic synaptic development (El-Husseini *et al.*, 2000). Its density also stabilizes at around PD 25: density at PD 25 was 638.6 +/- 91.3% (n=6), density at PD 35 was 950 +/- 71.6% (n=3) and density at PD 40 was 766.9+/- 42.7% (n=3) (see **Fig. 1.17B**). In membranes from younger animals it was observed a fast increase in the levels of the protein from PD 5 up to PD 20. The density of PSD-95 was 100 +/- 1.5% (n=3) at PD 5, 148.3 +/- 1.3% (n=3) at PD 10, 175.9 +/- 5.0% (n=3) at PD 15 and 223.3 +/- 16.1% (n=3) at PD 20. Densities were analysed using One-way ANOVA followed by Tukey test and 3-6 experiments were considered

for this analysis. Significant differences are indicated by asterisks in the graph presented in the **Fig. 1.17B**.

Fig. 1.18A) shows the relative density of synaptophysin which is abundant in presynaptic vesicles in almost all neurons and has a role in activity-dependent synapse formation (Tarsa and Goda, 2002), used here as a presynaptic marker. Similarly to what was observed for postsynaptic proteins KCC2 and PSD-95, the expression of synaptophysin reached a *plateau* at around PD 25, suggesting that the stabilization of presynaptic development may occur at this age. The density of synaptophysin in hippocampal membranes at different ages was 100 +/- 4.0% at PD 5, 109.0 +/- 1.8% at PD 10, 128.0 +/- 0.3% at PD 15, 136.4 +/- 0.5% at PD 20, 234.5 +/- 0.6% at PD 25, 238.1+/- 47.3% at PD 40, and 285.7 +/- 51.9% at PD 55, n=3 for all ages tested. The increase in the immunoreactivity observed in hippocampal membranes from PD 5 to PD 55 was nearly three-fold, $p<0.0001$ using one-way ANOVA followed by Tukey test. Densities observed in hippocampal membranes from animals older than 25 days-old were not significantly different from the density at PD 25, $p>0.05$ using one-way ANOVA followed by Tukey test.

Fig. 1.19C and **Fig. 1.19D** shows the ontogenic profile of immunoreactivity of VGAT and VGLUT1, respectively, in the postnatal development. Stabilization of VGAT levels was achieved at around PD 20 and for VGLUT1 at around PD 30. The density of VGAT in mice hippocampal membranes was 100 +/- 8.4% at PD 5, 68.7 +/- 3.4% at PD 10, 126.6 +/- 2.4% at PD 15, 131.7 +/- 16.7% at PD 20 (n=3-6). From PD 20 onward, the values of relative density of VGAT detected in hippocampal membranes were 212.8% +/- 25.82% at PD 25 (n=6), 354.2 +/- 79.5% at PD 30 (n=7), 211.8% +/- 13.14% at PD 35 (n=6) 180% +/- 16.76% at PD 40 (n=12). The levels of significance for the differences between the means of density after performing one-way ANOVA analysis are indicated in the graphics as $*p<0.05$, $**p<0.001$, and $***p<0.0001$. The density of VGLUT1 in mice hippocampal membranes at PD 5 was 100 +/- 15.9% at PD 5, 396.3 +/- 14.8% at PD 10, 374.7 +/- 70.8% at PD 15, 378.8 +/- 39.2% at PD 20, 711.9 +/- 39.4% at PD 25, 1077.0 +/- 41.4% at PD 30, 1010.0 +/- 155.6% at PD 40 and 651.7 +/- 147.0% at PD 55. The density of VGLUT1 increased significantly from from 5 days-old to 30-40 days-old ($p<0.001$, One-way ANOVA followed by Tukey test, n=3-9).

Fig. 1.17

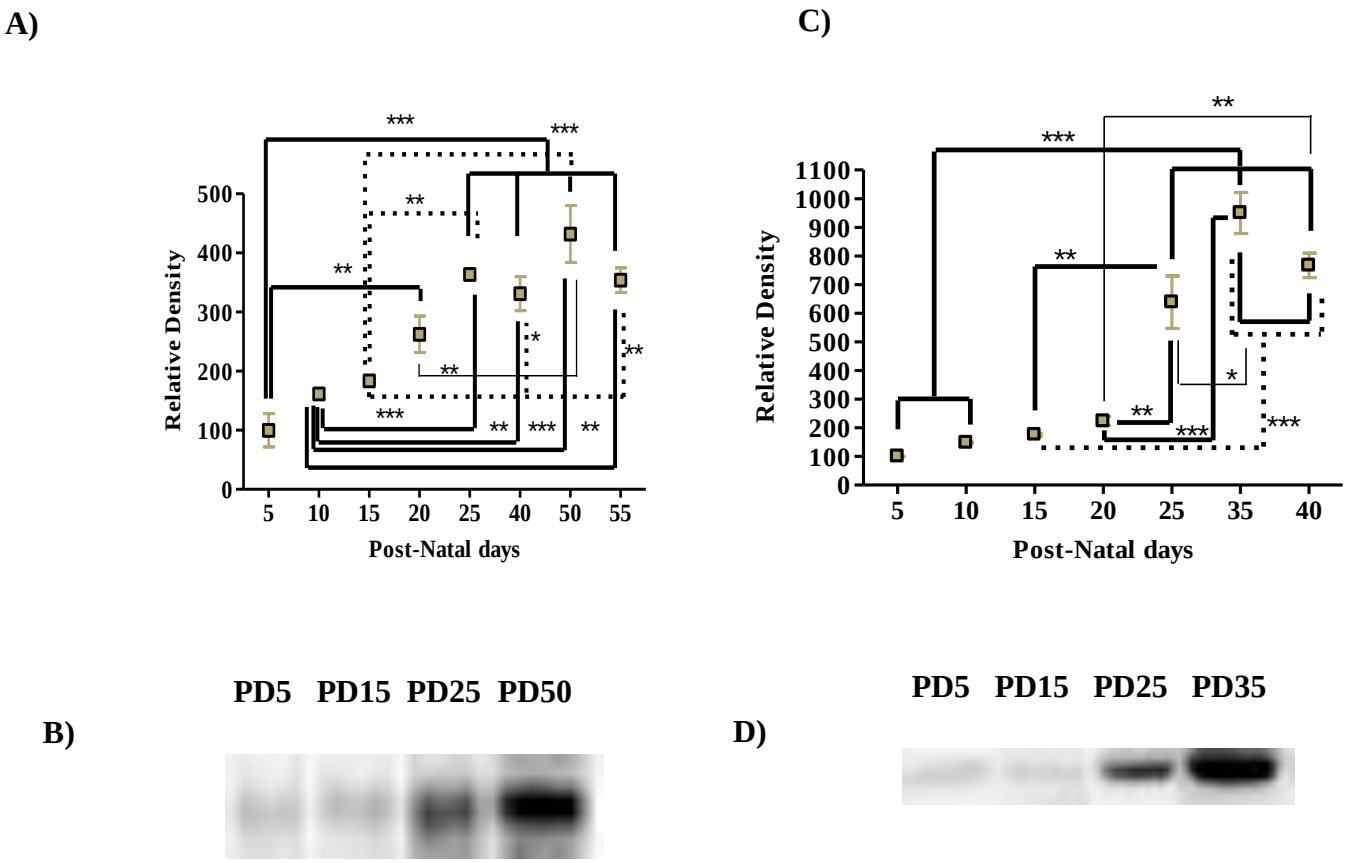
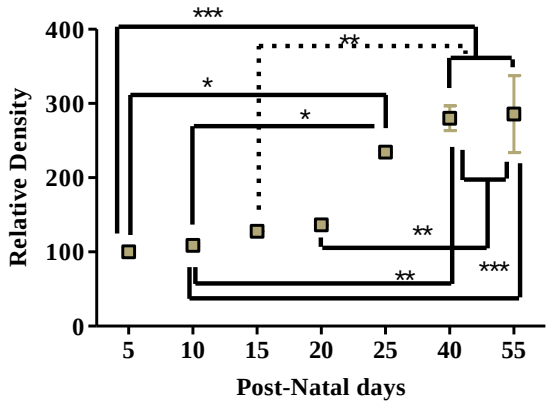


Fig. 1.17 – The postnatal ontogenic profile of the immunoreactivity of KCC2 and PSD-95 in hippocampal membranes. Graphs show the variations in the density of KCC2 (**A**) and PSD-95 (**C**) with age. Both proteins increase gradually until PD 25. From PD 25 onward, their density stabilize suggesting the achievement of a functional maturity of synapses. Results are mean +/- SEM of 3-6 independent purifications of hippocampal membranes. The comparison between the means was made using one-way ANOVA followed by Tukey test and significant differences were indicated in the graphics by *p<0.05; **p<0.001; ***p<0.0001. (**B** and **D**) Western blots representative of the data in **Fig. 1.17A** and B for KCC2 (123 kD) and PSD-95 (95 kD) proteins, respectively. The signal corresponds to the application of 60 µg of protein from the membrane fraction into a polyacrylamide gel.

Fig. 1.18

A)



B)

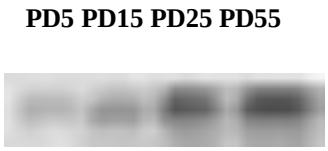


Fig. 1.18 – **Postnatal ontogenic profile of synaptophysin immunoreactivity in hippocampal membranes.** The expression of synaptophysin (37 kD) increased during the postnatal development until reaching a *plateau* at PD 25 (**A**). Results are mean +/- SEM of 3 independent experiments. Statistical significance is indicated in the graphs with *p<0.05; **p<0.001; ***p<0.0001 after performing One-way ANOVA followed by Tukey test. **B)** Western blot showing the density of synaptophysin in hippocampal membranes from 5, 15, 25 and 55 days-old mice. The signal corresponds to the application of 60 µg of protein from the fraction of hippocampal membranes.

Fig. 1.19

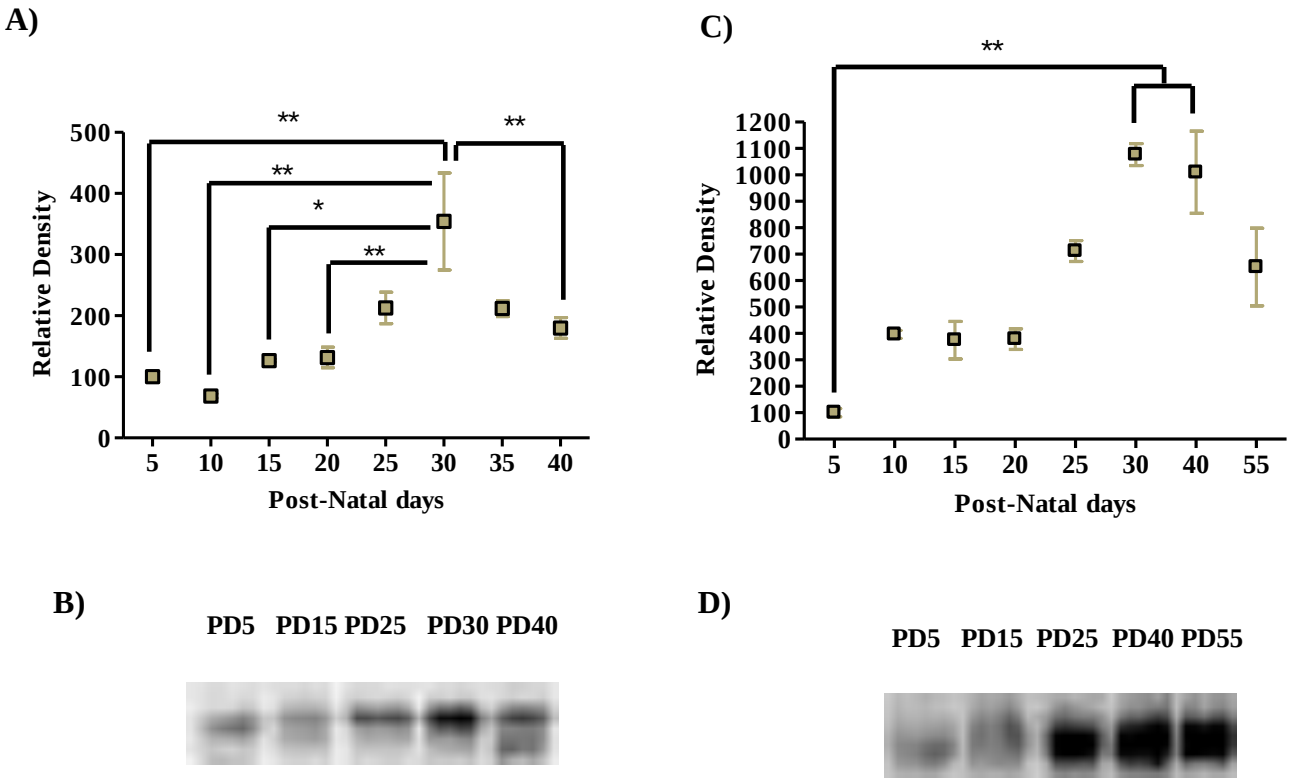


Fig. 1.19 – **Postnatal ontogenic profile of VGAT and VGLUT1 immunoreactivity in mice hippocampal membranes.** The immunoreactivity (obtained with 60 µg of protein) of VGAT (**A** and **B**) and VGLUT1 (62 kD) (**C** and **D**) found in hippocampal membranes from mice was progressively stronger reaching a maximum at PD 30. The density of VGAT (60 kD) decreased in membranes of older animals. Results are mean +/- SEM of 3 to 12 independent samples of hippocampal membranes. Statistical significance is indicated in the graphic as *p<0.05; **p<0.001. One-way ANOVA followed by Tukey test was used to compare means. **B)** and **D)** correspond to the Western blots from VGAT and VGLUT1, respectively, showing the density of both proteins detected at different ages (from 5 to 55 days-old) in hippocampal membranes.

CHAPTER 1 – Conclusions

A first observation that emerges from the results obtained is that the purinergic system is highly dynamic. Adenosine receptors change in density during postnatal development. Both A_1 and A_{2A} receptors are present in the immature hippocampus. With maturation, A_1 receptor immunoreactivity becomes more defined, being present in virtually all cell bodies whereas A_{2A} receptor density decreases with time: the density of A_{2A} receptors becomes weaker in the hippocampus and cortex whereas becomes more abundant in the striatum of adult mice. A_{2A} receptors were also found in progenitor and migrating cells and in immature pyramidal and granular cells at PD 6. Also, new neurons separating from the neuroepithelium and starting migrating were shown to be strongly immunopositive for A_{2A} receptors, which can be considered an argument that correlates the presence of A_{2A} receptors with some of these early steps of neural development.

Subsynaptic fractioning revealed that A_1 receptors kept their classical location in pre and postsynaptic compartments, and A_{2A} receptors were unexpectedly absent from axon terminals (at least from the active zone) and were concentrated at post and extrasynaptic sites. These extrasynaptic sites can be pre or postsynaptically located. Ecto-5'-nucleotidase was present as soon as PD 5 and PD 7 and occupied exclusively post-synaptic sites, reinforcing the possibility that links postsynaptic A_1 and A_{2A} receptor activation to the functioning of the presynaptic terminal that release ATP, which will be converted later into adenosine by the action of this enzyme. Interestingly, the peak of ecto-5'-nucleotidase density corresponded to the peak of synaptogenesis, suggesting that the adenosine-mediated signaling may have an important role at this stage. This type of signaling and the fact that adenosine receptors and the enzyme involved in adenosine production are all in close proximity at post-synaptic sites would guarantee a high specificity of action of adenosine at the synaptic level. Other enzymes involved in the formation of adenosine from ATP are also present as soon as PD 5 and they exhibit different patterns of ontogenic variation, either increasing continuously until PD 55 or decreasing in more mature stages.

Based on the profile of expression of density of pre and postsynaptic markers, one can separate postnatal development into two major periods, before and after PD 20 – PD 30. Before PD 20 – PD 30, an intense remodeling of synapses occurs and it correlates with an increase in the expression of synaptic proteins; after that period of time, there is a stabilization of these events. For example, the density of $P2X_2$, $P2X_3$ and $P2Y_4$ proteins decreases between PD 20 and PD 30. The density of $P2X_4$

decreases later but its density is elevated during the second postnatal week. The density of $P2Y_6$ receptor increases and stabilizes in the second postnatal week and the density of $P2Y_1$ and $P2Y_2$ receptor subtypes suffer a strong up-regulation followed by a massive down-regulation after PD 20-PD 30. Although this study has been done using total membranes and it was not possible to dissect between the density of these subunits by neurons and glia and their sub-synaptic location, their density varies in the periods of intense synaptogenesis and network formation in the hippocampus. The observation that they are present and their density is dynamic during these specific periods may open the possibility they are putative relevant players. The down-regulation of some of these subunits at around PD 20 and PD 30 opposing to the peak of expression of ecto-5'-nucleotidase would also favor adenosine-mediated signaling instead of an ATP-mediated signaling. The up-regulation of $P2Y_1$ and $P2Y_2$ receptors in this period would be involved in Ca^{2+} - wave generation and activation of astrocytes, helping the coordination of astrocyte-related events with the remodeling of synaptic contacts.

As soon as PD 5, ENT1 was already present and abundant in glial membranes in contrast to the pattern observed in the adult, which is mainly neuronal; this suggests that the purinergic system is highly dynamic and that its constituents may change their localization and their density observed during development, thus putatively playing different roles.

CHAPTER 2

Modulation by Adenosine A_{2A} Receptor of Apoptotic Cell Death of "Young" Cultured Hippocampal Neurons

This chapter documents the ability of adenosine A_{2A} receptors to control the cell death process. For this purpose, dissociated hippocampal cultures of embryonic (GD 17-19) rat hippocampi were used. This experimental model has the advantage to allow the direct observation and manipulation of neurons and the low-density of cells make it a less complex system well suited to study morphologic and cellular parameters (Kaech and Banker, 2006). Embryonic tissue contains few glial cells and the development of cultures maintained in a serum-free culture medium (Brewer *et al.*, 1993) guarantee the development of a neuronal-enriched population *in vitro* (Kaech and Banker, 2006). As described in Benson *et al.*, 1994, embryonic cell cultures will give rise to a population comprised in the vast majority by pyramidal cells. Only about 6% of the neurons were identified as interneurons. These pyramidal cells express their major phenotypic characteristic in culture, such as development of well identified axons and dendrites and, in more developed stages, the presence of dendritic spines and synaptically connected networks (Kaech and Banker, 2006).

Cultures with 7 days *in vitro* (7 DIV) containing "young" neurons were used, when the first glutamatergic synaptic contacts are made (Grabucker *et al.*, 2009). Mature synapses on dendritic spines were only visualized from day 10 onward and the number of synapses increased significantly in the third week. At this stage, hippocampal cultures have formed a dense network of dendrites containing spines. Also, the study of Lin and collaborators (Lin *et al.*, 2002) focusing on physiological maturation of cortical neurons in culture, showed that there are development-dependent changes in the excitatory synapses of cultured neurons. For example, at DIV7, the excitatory postsynaptic potentials were almost only non-NMDAR mediated and at DIV 14, these events were mediated by NMDAR and non-NMDAR, indicating that DIV 7 cultures are physiologically more immature than cultures at DIV 14.

DIV 7 cultures, composed of a morphological and physiological "younger" population of neurons, however presenting relatively well developed dendrites are well suited to monitor some of the morphological changes induced by the process of cell death. To focus on a particular form of cell death, it was decided to use staurosporine (STS), which is well known to induce apoptotic-like cell death (Bertrand *et al.*, 1994; Koh *et al.*, 1995; Prehn *et al.*, 1997).

The process of cell death is a normal and important physiological event occurring during nervous system development (see the **Section 2 of Introduction**). Receptors promoting or blocking cell death can have an impact upon brain development and in the adult pattern of brain circuits. Here we tested if A_{2A} receptors which seem to be present in embryonic hippocampal neurons maintained in culture (Tebano *et al.*, 2005) and present in synaptic contacts are a key modulatory system potentially playing a role in the control of apoptotic-like neuronal death.

CHAPTER 2 - Material and Methods

Primary cultures of hippocampal neurons: Hippocampal neurons were cultured from 17- to 19-day-old Wistar rat embryos, handled according to European guidelines (86/609/EEC), as previously described (Rebola et al., 2005a,b), and plated on poly-D-lysine-coated 16-mm-diameter coverslips or 6-well dishes at densities of 5×10^4/coverslip (viability and immunocytochemistry assays) or 1×10^6/well (Western blot analysis). Neurons were grown at 37 °C in a 5% CO_2 humidified atmosphere in neurobasal medium with B27 supplement, glutamate (25 μM), glutamine (0.5 mM) and gentamicin (0.12 mg/ml).

Staurosporine-Induced Cell Damage: Staurosporine (STS)-induced neuronal damage was evaluated after culturing the neurons for 7 days by directly adding STS (30 nM) to the medium followed by incubation for periods between 6 and 24 h. To test the ability of two selective A_{2A} receptor antagonists, SCH58261 or ZM241385, to modify the effects of STS, they were added 15 min before the addition of STS onwards.

Cell viability assays: Viability assays were performed by double labelling (3-min incubation) with the fluorescent probes Syto-13 (4 μM) and propidium iodide (PI, 4 μg/ml) (Molecular Probes, Leiden, The Netherlands) followed by fluorescence microscopy cell counting. As previously described (Rebola *et al.*, 2005b), viable neurons present nuclei homogenously labelled with Syto-13 (green fluorescent nuclei), whereas apoptotic neurons show condensed and fragmented nuclei labelled with Syto-13 (primary apoptosis) or with Syto-13 plus PI (secondary apoptosis) and necrotic neurons present intact nuclei labelled with PI (red fluorescent nuclei). In parallel, the nuclear morphology of hippocampal neurons was analyzed by fluorescence microscopy using Hoechst 33342 (2 μg/ml for 10 min; from Molecular Probes), as previously described (Almeida *et al.*, 2004). Each experiment was repeated using different cell cultures in duplicate, and cell counting was carried out in at least six fields per coverslip, with a total of approximately 300 cells. Results are expressed as mean ± SEM and statistical significance ($P < 0.05$) was evaluated by one-way ANOVA followed by Newman–Keuls multiple comparison test.

Immunocytochemical analysis: Immunocytochemistry assays were performed as previously described (Rebola *et al.*, 2005a and Rebola *et al.*, 2005b) after incubation with drugs, as described for the viability assays. For the double labelling with mitotracker-red and anti-cytochrome *c* antibody (Almeida et al., 2004), the neurons were incubated for 1 h in Krebs buffer with 500 nM mitotracker-red (Molecular Probes), a mitochondrial marker that is insensitive to the mitochondrial potential (Krohn et al., 1999). The following steps had to be performed protected from light. After fixation in 4% paraformaldehyde, the neurons were washed three times with PBS, incubated for 10 min with 20 mM glycine, permeabilized with 0.1% saponin, incubated for 30 min with a mouse anti-cytochrome *c* antibody (1:100; PharMingen, San Diego, USA) and, after washing, with an Alexa Fluor 488-labelled anti-mouse secondary antibody (1:200; Molecular Probes). For the immunocytochemical double labelling of microtubule-associated protein 2 (MAP-2) and synaptophysin, the fixed permeabilized neurons were incubated for 1 h with rabbit anti-MAP-2 antibody (1:400; Santa Cruz Biotechnologies, Freelab, Lisbon, Portugal) and mouse anti-synaptophysin antibody (1:200; Sigma, Sintra, Portugal). The secondary antibodies used were Alexa Fluor 488-labelled anti-rabbit and Alexa Fluor 594-labelled anti-mouse antibodies (1:200; Molecular Probes). The labelled neurons were visualized using either a fluorescence microscope (Zeiss Axiovert 2000, PG-HITEC, Portugal) or a confocal microscope (MRC 600, Bio-Rad, Hercules, USA). Immunoreactivities were evaluated using 3 representative fields per coverslip (magnification × 600). Quantitative assessment of synaptophysin immunoreactivity was calculated by comparison of the total number of labelled dots in control *versus* test conditions (approximately one thousand dots per field, with 3 fields counted per experimental condition). Results are expressed as mean ± SEM and statistical significance ($P < 0.05$) was evaluated by one-way ANOVA followed by Newman–Keuls multiple comparison test.

Western blot analysis: Hippocampal neurons, plated on 6 wells dishes, were gently scraped in lysis buffer (50 mM KCl, 50 mM 1,4-Piperazinediethanesulfonic acid (PIPES), 10 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), 2 mM $MgCl_2$, 0.5% Triton X-100, 1 mM PMSF, 1 mM dithiothreitol (DTT) and 5 μg/ml of a mixture of protease inhibitors containing chymostatin, leupeptin, pepstatin A and antipain) and subject to 3 freezing cycles at − 80 °C. Neuronal extracts were diluted at the final concentration of 1 μg protein/μl in SDS–PAGE buffer and 20 μg were separated by SDS–PAGE (7.5% with a 4% concentrating gel), as previously described (Rebola *et al.*, 2005a). After electro-transfer, the membranes were incubated overnight at 4 °C with mouse anti-

synaptophysin antibody (1:1000) or rabbit anti-MAP-2 antibody (1:400), washed and incubated with an alkaline phosphatase-conjugated anti-mouse secondary antibody (1:2000; Calbiochem, PG-HITEC). The membranes were then analysed with a VersaDoc 3000 (Biorad) after incubation with ECF (Amersham, Buckinghamshire, UK). The membranes were then re-probed and tested for tubulin immunoreactivity using a mouse anti-α-tubulin antibody (1:1000; Zymed, Lisbon, Portugal), as previously described (Rebola *et al.*, 2005a).

Hippocampal synaptosomes: Male Wistar rats (8 weeks old, 150–160 g, obtained from Charles River, Barcelona, Spain) the rats being anesthetized under halothane atmosphere before being sacrificed by decapitation. Membranes from Percoll-purified hippocampal synaptosomes were prepared as previously described (Rebola *et al.*, 2005a). Briefly, the two hippocampi from one rat were homogenized at 4 °C in sucrose solution (0.32 M) containing 10 mM HEPES, 1 mM EGTA and 1 mg/ml BSA (fatty acid-free), pH 7.4, centrifuged at 3000×*g* for 10 min at 4 °C; the supernatants were collected, centrifuged at 14,000×*g* for 12 min at 4 °C and the pellet was resuspended in 1 ml of a 45% (v/v) Percoll solution made up in a Krebs solution (composition: 140 mM NaCl, 5 mM KCl, 10 mM HEPES, 1 mM EDTA, 5 mM glucose, pH 7.4). After centrifugation at 14,000×*g* for 2 min at 4 °C, the top layer was removed (synaptosomal fraction), washed in 1 ml Krebs solution and resuspended in Locke's buffer (with 154 mM NaCl, 5.6 mM KCl, 5 mM HEPES, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, pH 7.2).

The mitochondrial reduction status of the synaptosomes (Mattson *et al.*, 1998) was measured by a colorimetric assay for cell survival, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, from Sigma), as previously described (Almeida *et al.*, 2004). The synaptosomes (1 mg/ml) were incubated for 2 h at 37 °C in Locke's buffer in the absence or presence of STS (100 nM) and/or SCH58261 (50 nM). We used a higher concentration of STS in the experiments using synaptosomes to compensate for the shorter exposure periods imposed by the limited period of viability of this preparation, which does not exceed 4 h. MTT (0.5 mg/ml) was then added and incubated for 1 h at 37 °C in the dark. As MTT is converted to a water-insoluble blue product (formazan) by viable terminals, the precipitated dye can be spectrophometrically (570 nm) quantified after exposing the synaptosomes to isopropanol containing 0.04 M HCl. Values were expressed as the percentage of optical density of control synaptosomes, i.e. in the absence of drugs.

Caspase-3 activity assessment: Caspase-3 activity was assessed in synaptosomes using a colorimetric pseudo-substrate of caspase-3, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA; Calbiochem), as previously described for cultured neurons (Almeida *et al.*, 2004). After incubation for 2 h at 37 °C in Locke's buffer in the absence or presence of STS (100 nM) and/or SCH58261 (50 nM), the synaptosomes were pelleted and lysed by addition of lysis buffer before quantification of protein. The suspension (50 μg protein) was then incubated for 2 h at 37 °C in the dark with 100 μM Ac-DEVD-pNA in 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid or CHAPS buffer containing 25 mM HEPES–Na, 10 mM dithiothreitol, 10% (w/v) sucrose and 0.1% (w/v) CHAPS, pH 7.4 before reading the optical density at 405 nm.

Drugs and solutions: STS was purchased from Sigma and made up as a 5 mM stock in dimethylsulfoxide before diluting into working solutions at the desired concentration. The selective antagonists of adenosine A_{2A} receptors, SCH58261 (provided by S. Weiss, Vernalis, UK) or ZM241385 (Tocris, Northpoint, UK) were prepared as 5 mM stock solutions in dimethylsulfoxide and then dissolved (< 0.001% dimethylsulfoxide) in the working solutions. All culture media were from GIBCO BRL (Life Technologies, Scotland, UK).

CHAPTER 2 – Results

The prototypic inductor of apoptosis STS (a non-selective protein kinase inhibitor) was used at 30 nM, near its EC50 value (see Koh *et al.*, 1995; Prehn *et al.*, 1997). In our model of DIV 7 hippocampal cultures, STS reduced the number of viable neurons from a control value of 84% +/- 3% to 57% +/- 2% of cells ($p<0.05$, One-way ANOVA followed by Newman-Keuls test, n=6) (see **Fig. 2.1A**), increasing the number of primary apoptotic cells (0% to 6% +/- 1%, n=6) and secondary apoptotic cells (15% +/- 3% to 36% +/- 2%, n=6), after 24h of incubation, $p<0.05$, One-way ANOVA followed by Newman-Keuls test (see **Fig. 2.1B**). There was also a decrease of 33% +/- 4% (n=6) of co-localization of cytochrome c with mitotracker red, a mitochondrial dye that allows the detection of loss of mitochondrial membrane potential (see **Fig. 2.1C**), indicating that STS induced the release of cytochrome c from this organelle, which is another feature of apoptotic cell death, as described in the **Section 2 of Introduction** (Krohn *et al.*, 1999; Ahlemeyer *et al.*, 2002).

The evaluation of the modulatory role of A_{2A} receptors in the process of this apoptotic-like cell death was made pharmacologically using two selective A_{2A} receptor antagonists, ZM241385 and SCH58261 in a concentration range shown to be selective for this subtype of receptor (Poucher *et al.*, 1995; Zocchi *et al.*, 1996). The presence of each of the antagonists (50 nM) significantly attenuated STS-induced death, (n=6, $p<0.05$, One-way ANOVA followed by Newman-Keuls test) and the incubation with SCH58261 abrogated the loss of co-localization between cytochrome c and mitotracker red observed in STP-treated cells (see **Fig. 2.3**). Furthermore, SCH 58261 or ZM 243185 were devoid of effect when added to cultured neurons in the absence of STS (see **Fig. 2.3**).

STS decreased the density of synaptophysin - a pre-synaptic marker (Wiedenmann and Franke, 1985) - and of MAP-2 - a dendritic marker (Caceres *et al.*, 1986). 6h after application of STS, a statistically significant decrease ($p<0.05$, One-way ANOVA followed by Newman-Keuls test, n=3) of synaptophysin levels observed in the absence of an increase in the number of death cells. Synaptophysin levels were measured counting the number of dots by immunocitochemistry (there was a decrease of about 17.1 +/- 3.1%, n=4, in STS-treated cells). With more prolonged incubation with STS, the decrease of synaptic markers and viable cells increased progressively. Thus, after addition of STS, a quantitative analysis made by Western blot showed a decrease of 33.8% +/- 9.9% (n=3) of the levels of synaptophysin and a decrease of 36.3 +/- 13.8%, n=3 in MAP-2 reactivity accompanied by a simultaneous decrease of 33.7 +/- 2% (n=3) of the number of viable cells, as mentioned previously in the text (see **Figure 2.3**).

Fig. 2.1

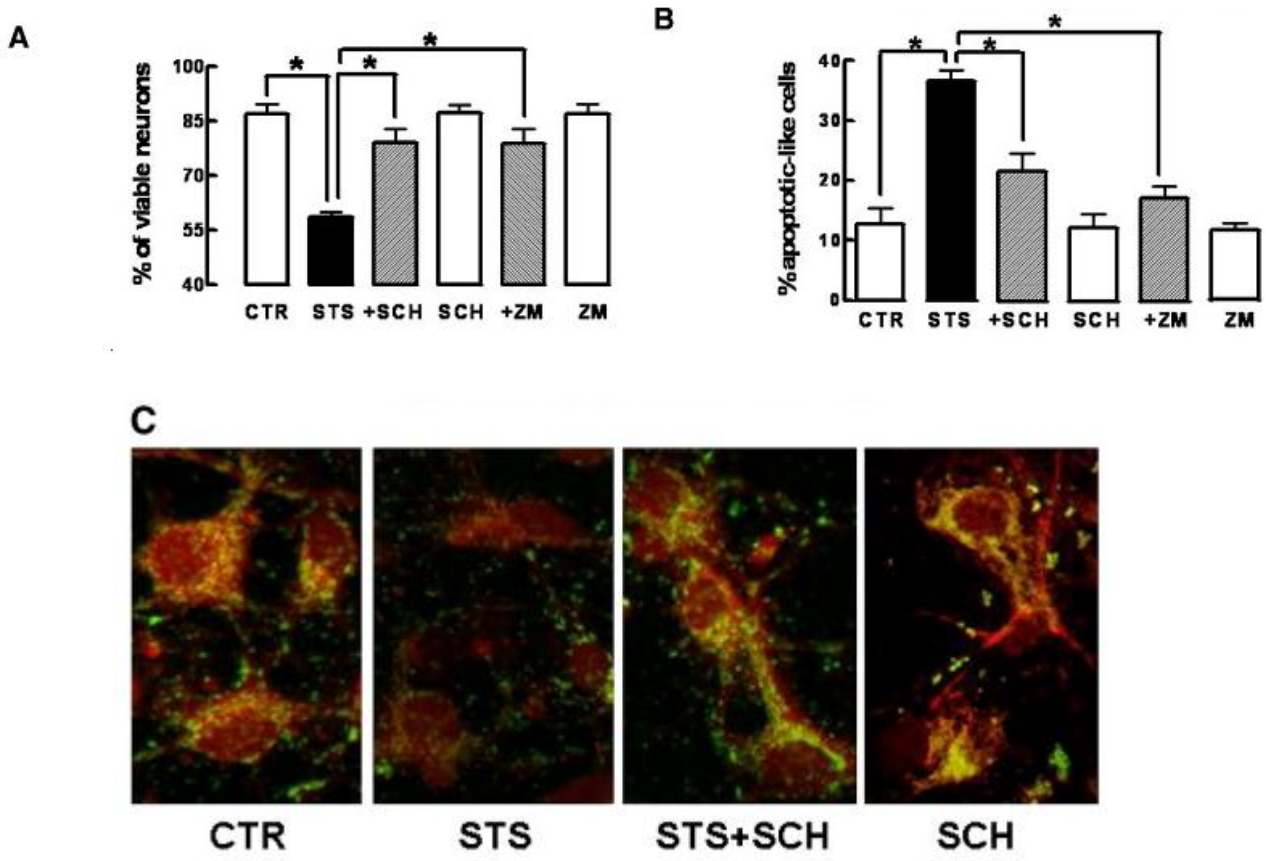


Fig. 2.1 – Effect of A_{2A} receptor blockade upon apoptotic cell death features. Blockade of A2A receptors prevents the apoptosis induced by staurosporine (STS) in cultured hippocampal neurons. 24h of incubation with STS decreased neuronal viability (**A**) and increased the number of apoptotic-like neurons (i.e. displaying a condensed nucleus simultaneously labeled with Syto-13 and PI) (**B**). This was prevented by the A_{2A} receptor antagonists, SCH58261 (SCH) or ZM241385 (ZM) that were devoid of effects in the absence of STS. Results are from 6 independent hippocampal cultures and a total of at least 300 neurons per coverslip were counted. $*P<0.05$ versus control (One-way ANOVA followed by Newman–Keuls test). Blockade of A_{2A} receptors also prevented STS-induced release of cytochrome c from mitochondria in cultured hippocampal neurons (**C**) as shown by the loss of co-localization of mitotracker-red (red) and cytochrome c (green) immunoreactivities in the displayed merged confocal images (magnification ×600). These results correspond to mean +/- SEM and are representative of 4 experiments.

Fig. 2.2

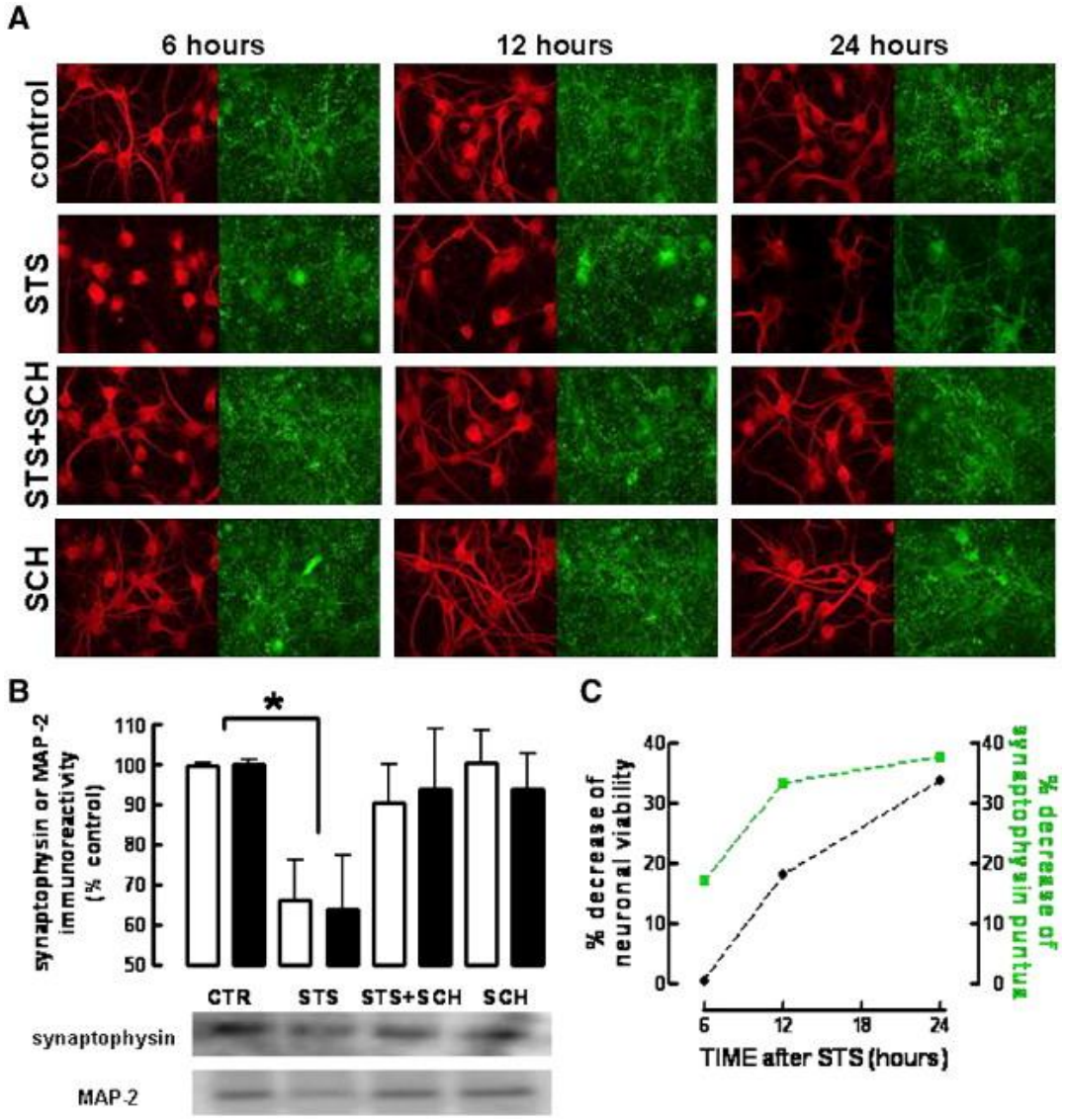


Fig. 2.2 – Effect of A_{2A} receptors blockade upon the early synaptic degeneration. Staurosporine (STS, 30 nM) causes a precocious synaptic degeneration, before overt neuronal loss occurred, which is prevented by A_{2A} receptor blockade. Panel A shows that STS decreased MAP-2 (red) and synaptophysin (green) immunoreactivity in cultured hippocampal neurons after 6 h (first column of paired photographs from the left), 12 h (second column) or 24 h (third column) of incubation with STS. This was abrogated at all time points upon blockade of A_{2A} receptor with SCH58261 (SCH). Similar qualitative results were obtained in 4 experiments. This STS-induced synaptic degeneration was confirmed by Western blot quantification, showing that the density of synaptic proteins (synaptophysin and MAP-2) in cultured hippocampal neurons is decreased by STS and this decrease is prevented by SCH58261 (50 nM) (B). The results, presented as percentage of the densitometric values of control conditions from 3 independent experiments. Results are mean +/- SEM $*P<0.05$ compared to control (One-way ANOVA followed by Newman–Keuls test). Panel C shows that at the early time point evaluated (6 h), STS caused a decrease of synaptic markers without evident modification of neuronal viability (n=5–6).

Fig. 2.3

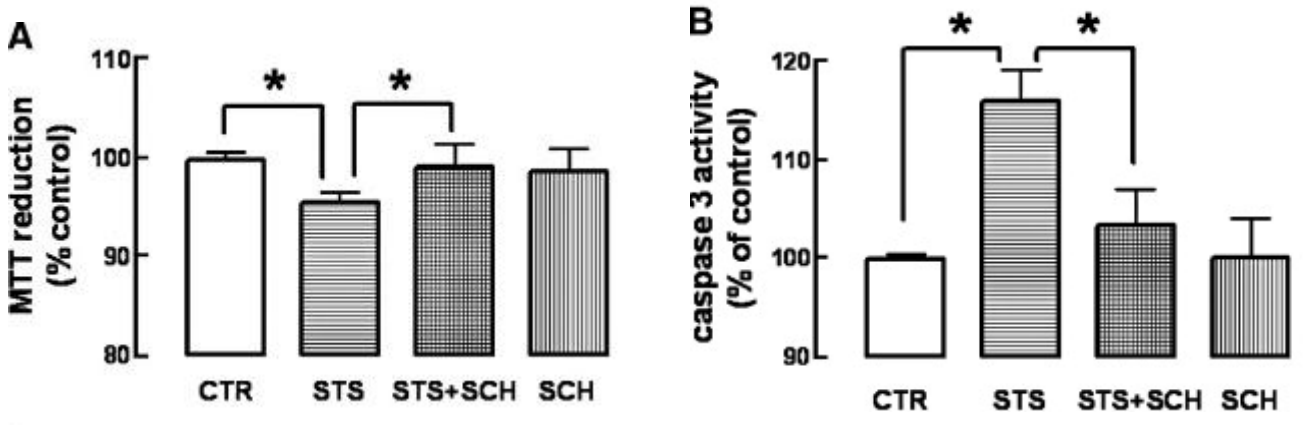


Fig. 2.3 – Effect of A_{2A} receptor blockade upon mitochondria function and caspases 3 activity. STS decreased mitochondria function (evaluated by the reduction of MTT in panel A) and induced caspase-3 activation (evaluated by the increased fluorescence of its pseudo-substrate Ac-DEVD-pNA, panel B) in hippocampal nerve terminals (2 h of incubation). Both effects were prevented upon blockade of A_{2A} receptor with SCH58261 (SCH, 50 nM). Results are mean±SEM of 8–15 experiments. ∗p<0.05 using the paired Student's *t* test.

A_{2A} receptors were reported to be concentrated in synaptic sites in cultures of hippocampal neurons (Tebano *et al.*, 2005). STS also induced a synaptic degeneration before overt neuronal loss. It was tested the hypothesis that A_{2A} receptor-mediated protection was due to the control of viability of synaptic terminals. Using the synaptosomal preparation, which allows studying nerve terminals independently of other brain elements (Nicholls, 2003), it was found that exposure of synaptic terminals to 100 nM of STS for 2h, decreased the MTT reduction to 95.9 +/- 0.8% (n=15, p<0.05), comparing with control levels (100 +/- 0.2%, n=15) and increased caspase-3 activity, a protease implicated in the process of apoptotic cell death (**Section 2 of Introduction**) by 11.5% +/- 2.3% (n=16, p<0.05). This effect of STS was no longer observed when synaptosomes were incubated also with 100 nM SCH58261 (see **Fig. 3**). Synaptosomes were prepared from tissue of adult animals since A_{2A} receptor in the adult hippocampus shown to be in presynaptic terminals (Rebola *et al.*, 2005a).

It was not done the study of the intracellular signaling pathway(s) used by A_{2A} receptors to afford neuroprotection. However, the ability of this subtype of receptor to control protein phosphatases(Revan *et al.*, 1996; Murphi *et al.*, 2003), which play a relevant role in the control of mitochondrial function and neuronal viability after noxious stimuli (Agostinho and Oliveira, 2003;

Almeida *et al.*, 2004) or control of MAPKs (Seidel *et al.*, 1996; Schulte and Fredholm, 2003), are possible pathways.

CHAPTER 2 – Conclusions

In this Chapter it was shown that A_{2A} receptor blockade was able to prevent cell death by apoptosis, controlling the mitochondrial function and caspase 3 activity. This control over cell death may have positive or negative consequences dependending on the factors triggering it. If cell death is triggered by agents that induce neuronal damage, A_{2A} receptor blockade may have beneficial effects however if it avoids the spontaneous cell during critical periods where cell death is necessary, an abnormal number of cells may affect neural function (Simonati *et al.*, 1997).

A critical window of neonatal brain development occurs around 6-9 postnatal days in rat pups. This period is coincident with the peak rates of brain growth (Rakic, 1998b) and a production of synaptic sites (Rakic *et al.*, 1986). At this time, there are decreased thresholds for NMDA- or kainate-induced seizures (Wasterlain and Shirasaka, 1994) and a greater susceptibility of brain cells to NMDA induced toxicity (Ghosh and Greenberg, 1995; McDonald and Johston, 1990; Lipton and Nakanishi, 1999). At this time point, the the density of A_{2A} receptor is high in the hippocampus (see **Chapter 1**, **Results Section 1.1**). This observation suggests that this subtype of adenosine receptor may play relevant modulatory functions upon cell death during the two first postnatal weeks, when the brain is more prone to damage.

Interestingly, apoptotic signaling cascades can exert local functions and structural dynamics of growth cones and synapses (Gilman and Mattson, 2002). One example is the activation of caspases that can cleave some ionotropic glutamate-receptors subunits, thus modifying synaptic plasticity or cleaving cytoskeletal protein substracts that regulate the development of growth cones and neurite outgrowth. In our model, it was observed a decrease in the level of dendritic (MAP-2) and axonal (synaptophysin) markers indicating, according with was proposed by the authors, that synapses may be affected in the process of cell death. They identified neurotrophic factor-activated kinase cascades, calcium-mediated actin depolymerization and activation of the transcription factor NF-kappaB as anti-apoptotic signals that regulate the plasticity of growth cones and dendrites.

CHAPTER 3

Modulation by Adenosine A_1 and A_{2A} Receptors of the Spontaneous Activity in the Immature Hippocampal Slice

The spontaneous activity observed in the immature hippocampus was vastly studied (see for example, Menedez de la Prida *et al.*, 1998; Leinekugel *et al.*, 1998; Sipilä *et al.*, 2005; Wong *et al.*, 2005). In the *in vitro* model of hippocampal slice, giant depolarizing potentials (GDPs) provide most of the synaptic activity (Ben-Ari, 2001). GDPs are recurrent network-driven events lasting several milliseconds in duration. In the rat sagital hippocampal slice, GDPs are both observed in CA1 and CA3 regions (Khazipov *et al.*, 1997; Garaschuk *et al.*, 1998). The CA3 subfield was indicated as the pacemaker of generation of GDPs and they were also observed in the intact hippocampal preparation (Khalilov *et al.*, 1997) which preserves the entire hippocampal network. The septal pole of the hippocampus seems to be the generator of GDPs that will propagate towards the temporal pole. In the origin of GDPs, depolarizing actions of GABA were proposed (see for example Sipilä *et al.*, 2005). This unexpected action of GABA was attributed to the higher concentration of chloride (Cl^-) stored inside immature neurons resulting from the delayed expression of chloride transporters (Rivera *et al.*, 1999) that extrude Cl^- ions out of cells in the adult. Since $GABA_A$ receptors are activated, the drop in membrane potential voltage due to the movement of Cl^- ions out of neurons can exceed the threshold for Na^+ channels which leads to the activation of voltage-gated Ca^{2+} channels and increase in the intracellular Ca^{2+} levels (Leinekugel *et al.*, 1997). GABA also reduces the voltage-dependent Mg^{2+} block, a step that is necessary for NMDA receptor activation Leinekugel *et al.*, 1997). This also contributes to a rise in intracellular Ca^{2+} levels (Leinekugel *et al.*, 1997). Blockade of $GABA_A$ receptor function revealed that GDP can be also AMPA receptor- and NMDA receptor-mediated (Bolea *et al.*, 1999). The formation of IP_3 inositol that diffuses through gap junctions to adjacent cells can be also in the generation of GDPs because it also trigger an increase of intracellular Ca^{2+} levels (Owens and Kriegstein, 1998; Kandler and Katz, 1998). When both GABA and glutamate contribute to the generation of GDPs, $GABA_A$ receptor- mediated currents shunt glutamate receptor-mediated currents, preventing abnormal and pathological excitation (Khalilov *et al.*, 1999; Bolea *et al.*, 1999; Wells *et al.*, 2000).

The spontaneous activity in the immature hippocampal slice comprises also spontaneous synaptic currents. As previously described in the **Section 2.2 of the Introduction**, there is a maturational degree of functioning of synapses in the hippocampus. The first functional synapses are only GABAergic; maturation then leads to the formation of GABAergic and NMDA receptor-containing synapses and in a last stage of maturation, AMPA receptors are also incorporated in it (Tyzio *et al.*, 1999).

This chapter presents data showing that A_1 and A_{2a} receptors control AMPA receptor- and GABA receptor-mediated synaptic currents as well as GDPs in the hippocampal slice from PD 5 to PD 7 mice pups. This was concluded using the cortico-hippocampal preparation (CHP) because it preserves the integrity of the hippocampal network thus being an ideal model to study the physiological effect of the tonic activation of these subtypes of adenosine receptors.

CHAPTER 3 – Material and Methods

Animals: Animals used for cell culture were Sprague-Dawly rats purchased from Charles River, France. For electrophysiology in the hippocampal slice, FVN-GIN mice pups were used (from PD 5 to PD 7). For recordings in the CHP, the same strain of mice was used from PD 5 to PD 15. Pups were killed by decapitation without anesthesia.

Glial Cell Culture: The purpose of preparing a glial cell culture was to make a monolayer of cells to be cultured together with developing neurons. These astrocytes function as a source of trophic support for neurons, increasing their probability of survival, which is especially relevant when low-density cultures are made (Kaech and Banker, 2006). Isolation of hippocampal neurons was made according to the protocol found in Kaech and Banker, 2006. One day-old (3 or 4/culture) were killed by decapitation and brains were removed in a laminar flow hood and maintained in a dish containing calcium-, magnesium- and bicarbonate-free Hanks' solution (CMF-HBSS , from Invitrogen) of the following composition: 137.0 mM NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, buffered at pH 7.4 with 10.0 mM HEPES. Cerebral hemispheres were removed under the microscope, the meninges removed and the remaining tissue chopped in small pieces. Chopped tissue was placed in a 50 mL centrifuge tube in a solution of CMF-HBSS with 2.5% trypsin and 1% DNAse and incubated in a 37 ºC water bath for 5 min. The tissue was triturated using a 10 mL pipette. After filtering the cell suspension, cells were added to Glial Medium, a mixture of minimal essential medium (MEM), composed of aminoacids, vitamins, inorganic salts, ribonucleosides and deoxyribonucleosides (detailed composition in Invitrogen product information sheet) and supplemented with 0.6% glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % (vol/vol) horse serum. Cells were centrifuged for 5-10 min at 120 *g* to remove enzymes and lysed cells. The supernatant was discarded and the pellet resuspended in 15-20 mL of Glial Medium. Cell density was assessed using a hemacytometer and 7.5×10^6 cells per 75 cm^2 were plated in a flask with Glial Medium. Astrocytes were grown at 37 ºC in a 5% CO_2 humidified atmosphere. New medium was added to the cultures each 2-3 days. Confluence was achieved in nearly one week. After that period, cells were dissociated using a liquid solution containing 0.05 % trypsin/0.02 % EDTA from Invitrogen, at 37 ºC. Trypsinization was stopped by adding 5 mL of Glial Medium and, after centrifugation at 120 *g* for 7 min, the pellet was resuspended in Glial Medium and divided in 60-mm dishes.

Neuronal Cell Culture: Neuronal cultures were derived from embryonic day (ED) 17-18 embryos. After euthanasia of a pregnant female, the uterus was dissected out and fetuses removed in a laminar flow hood. Hippocampi were dissected and collected in a HBSS-containing dish and then incubated for 15 min in a water bath at 37 ºC with HBSS containing 0.5% of trypsin. Trypsin was removed from the tissue by removing the trypsin solution and washing the hippocampi with HBSS medium for 2 or 3 times. Tissue was dissociated using a Pasteur pipette. After filtering cells to discard undissociated tissue, cell density was determined using a hemacytometer and cells labeled with 0.4% trypan blue in HBSS were considered non-viable and excluded from the counting. 150,000 cells were plated over polylysine-treated coverslip in Neuronal Plating Medium (NPM), which consistes of MEM with Earle's salts and L-glutamine (detailed information in product information sheet from Invitrogen) supplemented with 0.6 % (wt/vol) glucose, 10 % (vol/vol) horse serum and 5 % (vol/vol) fetal bovine serum. After attachment of cells to polylysine-covered coverslips (3-4 hours after), the coverslips were transfered to the 60 mm dishes, facing the glial feeder layer, in 1 mL Neurobasal Medium (see detailed composition in the product information sheet from Invitrogen), supplemented with B27 medium (to replace serum) and Glutamax-I (containd L-Alanyl-L-glutamine) supplements (both supplements were purchased from Invitrogen). Three days after plating, 5 µM of cytosine arabinoside were added to prevent glial proliferation. Neurons were grown at 37 °C in a 5% CO_2 humidified atmosphere. One-third of the volume of the culture was replaced by fresh medium every 7 days.

Purification of cDNA for SEP-γ-2 and SEP-GluR1: The cDNA for Super Ecliptic Phluorin associated to γ-2 subunit of $GABA_A$ receptors (SEP-γ-2) and to GluR1 subunit of AMPA receptors, SEP-GluR1, were purified from 100 mL of *E. coli* culture (DH5alpha, Invitrogen) using the EndoFree Plasmid Kit from Qiagen, following the manufacturer instructions. *E. coli* was used because it presents several advantages over other cloning systems, namely, rapid growth rate, low cost and less prone to errors, possibility of dealing with large vector and fusion choices. The genic sequencies of SEP, γ-2 and GluR1 were the following:

SEP-5'→3'

AGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGG
TGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTCTGACCTATGGTGTTC
AATGCTTTTCAAGATACCCAGATCATATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTCAAAGAT
GACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACAT
TCTTGGACACAAATTGGAATACAACTATAACGATCACCAGGTGTACATCATGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAATTAGACACA
ACATTGAAGATGGAGGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGGCCCGTCCTTTTACCAGACAACCATTACCTGTTTACA
ACTTCTACTCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATA
CAAA

γ-2- 5'→3'

ATGAGTTCGCCAAATACATGGAGCACTGGAAGCACAGTCTACTCTCCTGTATTTTCACAGAAAATGACGCTGTGGATTCTGCTCCTGCTATCGCTCTACCC
AGGCTTCACTAGCCAAAAGTCAGATGATGACTATGAAGATTATGCTTCTAATAAAACATGGGTGTTGACTCCAAAAGTTCCCGAGGGTGATGTCACTGTCA
TCTTAAACAACCTTCTGGAAGGGTACGACAACAAACTTCGGCCCGACATAGGAGTGAAACCAACATTAATTCATACAGATATGTACGTGAACAGCATTGGT
CCAGTGAATGCTATCAATATGGAATACACAATTGATATTTTTTTTGCCCAAACCTGGTATGACAGACGTTTGAAATTTAACAGTACCATTAAAGTTCTCCG
ATTGAATAGCAATATGGTGGGGAAAATCTGGATTCCAGACACTTTCTTCAGGAACTCCAAAAAAGCGGATGCTCACTGGATCACGACTCCCAACAGGATGC
TGAGAATTTGGAATGACGGTCGAGTTCTCTACACCTTAAGGCTAACAATTGATGCCGAGTGCCAGTTGCAATTACACAACTTCCCAATGGATGAACACTCC
TGCCCCCTGGAGTTCTCCAGTTATGGTTATCCTCGTGAAGAAATTGTTTATCAATGGAAGCGCAGTTCTGTTGAAGTGGGAGACACAAGGTCATGGAGGCT
GTATCAGTTTTCCTTTGTTGGATTGAGGAATACCACTGAAGTAGTGAAGACAACTTCTGGTGACTATGTGGTTATGTCCGTGTACTTTGATCTGAGCAGAA
GAATGGGGTACTTTACCATCCAGACCTACATTCCCTGCACACTCATTGTGGTTCTGTCCTGGGTGTCCTTCTGGATCAATAAGGATGCTGTCCCTGCAAGA
ACATCTTTAGGAATCACGACTGTCCTGACGATGACCACTCTCAGCACCATAGCCCGGAAGTCTCTGCCCAAGGTCTCCTATGTCACAGCAATGGATCTCTT
CGTCTCTGTTTGCTTCATCTTTGTGTTTTCAGCTTTGGTGGAGTATGGTACCCTGCACTATTTTGTGAGCAACCGGAAACCAAGCAAGGATAAAGACAAAA
AGAAGAAAAACCCTGCCCCTACCATTGATATCCGTCCCAGATCAGCAACGATCCAAATGAACAATGCCACCCACCTTCAAGAGAGGGATGAAGAATATGGC
TATGAGTGTTTGGATGGCAAGGACTGTGCCAGTTTCTTTTGCTGTTTTGAAGACTGCCGAACAGGAGCCTGGAGACACGGGAGGATACACATTCGCATTGC
CAAAATGGACTCCTATGCTCGGATCTTCTTCCCTACCGCCTTCTGCTTGTTCAATCTTGTTTACTGGGTCTCCTATCTTTATCTGTGA

GluR1- 5'→3':

GGTGCCAATTTCCCCAACAATATCCAGATAGGGGGGTTATTTCCAAACCAACAATCACAGGAACATGCGGCTTTTAGGTTTGCTTTGTCACAACTCACGGA
GCCCCCCAAGCTGCTTCCCCAGATCGATATTGTGAACATCAGCGACAGCTTTGAGATGACTTACCGTTTCTGTTCCCAGTTCTCCAAAGGAGTCTATGCCA
TCTTTGGATTTTATGAACGAAGGACTGTCAACATGCTGACCTCCTTCTGTGGGGCCCTCCATGTGTGCTTCATTACTCCAAGTTTTCCTGTTGACACATCC
AATCAATTTGTCCTTCAGCTACGCCCGGAACTACAGGAAGCTCTCATTAGCATTATCGACCATTACAAGTGGCAAACCTTTGTCACATTTATGATGCTGAC
CGGGGCCTGTCAGTCCTGCAGAGAGTCTTGGATACAGCCGCAGAGAAGAACTGGCAGGTAACGGCTGTCAACATTCTGACAACCACCGAGGAAGGATACCG
GATGCTCTTTCAGGACCTGGAGAAGAAAAAGGAGAGGCTGGTGGTGGTTGACTGTGAATCAGAACGCCTCAACGCCATCCTGGGCCAGATCGTGAAGCTAG
AAAAGAATGGCATCGGGTACCACTACATCCTCGCCAATCTGGGCTTCATGGACATTGACTTAAATAAGTTCAAGGAGAGCGGAGCCAATGTGACAGGTTTC
CAGCTGGTGAACTACACAGACACGATCCCAGCCAGAATCATGCAGCAATGGAGGACAAGTGACTCCCGAGACCATACCAGGGTGGACTGGAAGAGGCCAAA
GTACACTTCTGCTCTCACCTATGATGGTGTCAAGGTGATGGCTGAGGCCTTCCAAAGCCTGCGGAGGCAGAGGATTGACATATCCCGCCGGGGGAATGCTG
GGGACTGTCTGGCTAACCCAGCTGTGCCCTGGGGTCAAGGGATCGACATCCAGAGAGCCCTGCAGCAGGTGCGCTTCGAAGGTTTGACAGGAAATGTGCAG
TTCAACGAGAAAGGGCGCCGGACCAATTACACCCTCCACGTGATCGAAATGAAACATGATGGAATCCGAAAGATTGGTTACTGGAATGAAGACGATAAATT
TGTCCCCGCAGCCACCGACGCTCAGGCTGGAGGGGACAACTCAAGCGTCCAGAATAGGACCTACATCGTCACTACTATCCTCGAAGATCCTTACGTGATGC
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CTTGAGATTGTCAGCGACGGCAAATATGGAGCCCGGGATCCCGACACAAAGGCTTGGAATGGCATGGTGGGAGAACTGGTCTATGGAAGAGCAGACGTGGC
TGTGGCTCCCTTGACCATAACCTTGGTCCGGGAGGAAGTCATCGACTTCTCCAAGCCATTCATGAGTTTGGGAATCTCCATTATGATTAAGAAGCCACAGA
AGTCCAAGCCAGGTGTCTTCTCCTTTCTTGACCCTTTGGCCTATGAGATCTGGATGTGTATAGTGTTTGCCTACATTGGAGTGAGCGTCGTCCTCTTCCTG
GTCAGCCGTTTCAGCCCCTACGAATGGCACAGCGAAGAGTTTGAAGAGGGACGAGACCAGACAACCAGTGACCAGTCAAATGAGTTTGGCATATTCAACAG
CCTGTGGTTCTCCCTGGGGGCCTTCATGCAGCAAGGATGTGACATTTCCCCCAGGTCCCTGTCCGGACGCATCGTCGGCGGCGTCTGGTGGTTCTTCACTT
TGATCATCATCTCCTCGTACACAGCAACCTGGCTGCCTTCCTGACTGTGGAGAGGATGGTGTCTCCCATTGAGAGTGCAGAGGACCTGGCAAAGCAGACGG
AAATTGCTTATGGGACATTGAGCAGGCTCCACTAAGGAGTTCTTCAGGAGATCTAAAATCGCTGTGTTTGAGAAGATGTGGACATACATGAAGTCTGCAGA
ACCATCCGTGTTTGTTCGGACCACAGAGGAAGGCATGATCAGAGTGAGAAAATCTAAAGGCAAATACGCCTACCTCCTGGAGTCCACCATGAATGAGTATA
TTGAGCAACGAAAGCCCTGTGACACCATGAAAGTGGGAGGTAACTTGGATTCCAAAGGCTATGGCATTGCGACACCCAAGGGGTCCGCCCTGAGAAATCCA
GTAAACCTGGCAGTGTTAAAACTGAACGAGCAGGGGCTTTTGGACAAATTGAAAAACAAATGGTGGTACGACAAGGGCGAGTGCGGCAGCGGGGGAGGTGA
CTCCAAGGACAAGACCAGCGCTTTGAGCCTCAGCAATGTGGCAGGCGTGTTCTACATCCTGATTGGAGGGCTGGGACTGGCCATGCTGGTTGCCTTAATCG
AGTTCTGCTACAAATCCCGTAGCGAGTCGAAGCGGATGAAGGGTTTCTGTTTGATCCCACAGCAATCCATCAATGAAGCCATACGGACATCGACCCTCCCC
CGGAACAGTGGGGCAGGAGCCAGCGGAGGAGGCGGCAGTGGAGAGAATGGCCGGGTGGTCAGCCAGGACTTCCCCAAGTCCATGCAATCCATTCCCTGCAT
GAGTCACAGTTCAGGGATGCCCTTGGGAGCCACAGGATTG

Transfection of Hippocampal Neurons: Transfection was made using 1 µg DNA corresponding to the subunit Gamma 2 (SEP-Gamma2) or GluR1 (SEP-GluR1) per 60 mm dish. Effectene (Qiagen) was used as a chemical transfection reagent at 4 DIV. 1 µg DNA was dissolved in Tris-EDTA (TE) buffer (10 mM Tris-Cl, 1 mM EDTA), pH between 7.0-8.0, to achieve a DNA concentration of 0.1 µg/µl. A final volume of 150 µL was completed with DNA-condensation buffer (Buffer EC) from Qiagen, to which were added 8 µL of Enhnancer from Qiagen. After incubation at room temperature (15–25°C) for 2-5 min and spin down of the mixture, 25 µl Effectene Transfection Reagent were added to the DNA-Enhancer mixture. Samples were incubated with these final mixture for 5-10 min at room temperature to allow transfection-complex formation. In another Petri dish it was added 5 mL of Neurobasal medium supplemented with B27 and Glutamax (Invitrogen) equilibrated with CO_2

for at least 4 hours. Transfection was made at DIV 4. After transfection completion, coverslips containing the transfected neurons were transferred to the new Petri dish, with neurons facing up. 1 mL of growth medium was added to the tube containing the transfection complexes. The transfection complexes were drop-wise onto the cells and cells were left with the complexes for 1 hour. After the incubation period coverslips were transferred to the original dish maintaining neurons facing the glial cells and maintained into an incubator until the experimental day.

Image Acquisition: The evaluation of changes in the fluorescence of the SEP-γ2 and SEP-GluR1 after exposure to A_1 and A_{2A} receptor antagonist was made using a Nikon Eclipse TE 2000U microscope with an EMCCD camera upon illumination by a Mercury lamp. The filters used were 465-500 nm for excitation, 515-555 nm for emission and 506 nm for the dichroic mirror. Images were captured with a Nikon Plan Apo objective, using a magnification of 100 ×. Cells were kept at 34 ºC using as heating systems, the air blowing system (Air-Therm ATX from World Precision Instruments) and objective heating control by a Heater Controller from (Biopetch) and incubated in 1 ml of artificial cerebrospinal fluid (aCSF) with the following composition: 113.0 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10.0 mM D-glucose, 10.0 mM HEPES, pH adjusted to 7.4 with NaOH (1M).

Electrophysiology in Hippocampal Slices: Transverse hippocampal slices (450 μm) were prepared from male Wistar rat pups (PD 5 – PD 7) with a vibroslicer Leica VT 1200S in a cold (lower than 4 ºC) cutting solution containing 140.0 mM potassium gluconate, 10.0 mM HEPES, 15.0 mM sodium gluconate, 0.2 mM EGTA, 4.0 mM NaCl, pH 7.2. After recovery for at least 1 hour in aCSF containing 126.0 mM NaCl, 25.0 mM $NaHCO_3$, 10.0 mM D-glucose, 3.5 mM KCl, 2.0 mM $CaCl_2$, 1.3 mM $MgCl_2.6H_2O$, and 1.2 mM NaH_2PO_4 equilibrated with 5% CO_2 in 95% O_2 at room temperature. Slices were transferred to a chamber containing the same aCSF and kept at a temperature between 33 ºC to 35 ºC, where the recordings were made. Pharmacologically isolated GABAergic postsynaptic currents were measured in the presence of 10 μM 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX) and 40 μM D-2-amino-5-phosphonovaleric acid (D-APV) to block AMPA/kainate and NMDA receptors, respectively. Glutamatergic currents were isolated using 10 μM biccuculine. Miniature currents were recorded in the same conditions after addition of 1 μM tetrodotoxin (TTX) (Latoxan). Neurons were visualized by using differential interference contrast microscopy and were recorded in whole-cell voltage-clamp mode with a solution of 140.0 mM CsCl, 8.0 mM CsCl, 5.0 mM, 10.0 mM Hepes, 10.0 mM EGTA, 2.0 mM MgATP, 0.5 Na.GTP, 2.0 mM Na_2ATP, and 0.5% biocytin (pH 7.3, 275 milliosmolar). Synaptic currents were recorded at a holding

potential of -60 mV, filtered at 2 kHz and collected at 10 kHz. When currents were recorded using a solution of 120.0 mM gluconate, 20.0 mM CsCl, 1.1 mM EGTA, 0.1 mM $CaCl_2.2H_2O$, 10.0 mM HEPES, 2.0 mM ATP.Mg, 0.4 mM GTP.Na, $MgCl_2.6H_2O$, $CsOH.H_2O$ (pH 7.3, 280 miliosmolar), IPSCs/mIPSCs were recorded at a holding potential of + 10mV, the reversal potential forglutamatergic events (Esclapez *et al.*, 1997) and EPSCs/mEPSCs were recorded at -60mV, the reversal potential for GABAergic events (Esclapez *et al.*, 1997). When series resistance varied by more than 20% experiments were terminated. Clampfit 10.2 and Minianalysis software (Synaptosoft, Decatur, GA) were used to analyze synaptic events (GDPs, spontaneous synaptic currents and miniature currents). *Post hoc* identification of recorded cells was made after recording, according with the protocol described below. The comparison of the activity of cells was made between cells displaying similar values of membrane capacitance (Cm) and membrane resistance (Rm) to avoid a false interpretation of the results due to factor directly related to the maturity of the cells analysed.

Identification of Recorded Cells: The recorded slices were placed in 4 % PFA at 4 ºC overnight. Slices were rinsed 3 times for 30 min in 0.12 M PB and then stored at 4 ºC in a 20 % sucrose solution made in 0.12 M PB overnight. Slices were rinsed 3 times in 0.12 M PB and incubated under agitation in 1 % H_2O_2 (purchased from SIGMA as a 35 % stock) and 2 times in 0.02 M KPBS, pH 7.3. Slices were incubated overnight and under agitation in ABC complex (Vecta Stain Elite, Vector) diluted in 0.02 M KPBS (10 µl of reagent A + 10 µl of reagent B for 1 ml of KPBS). Slices were then washed 3 times in KPBS before being incubated for 15 min in filtered 3',3'-Diaminobenzidine (DAB) solution (1 tablet SIGMAFast DAB plus 1 tablet of H_2O_2 in 5 ml of bidistiled water). After removal of the unbound solution, slices were rinsed 3 times in 0.12 M KPBS and mounted in Superfrost slides with Crystal Mount-Biomeda for posterior observation. All steps of incubation and rinsing were made under agitation.

Recording of evoked responses to pressure-applied GABA and AMPA: 50 µM of GABA were applied at intervals of 60s using a picospritzer. GABA was dissolved in aCSF with a similar composition to the aCSF used to maintain and superfuse the hippocampal slices. Delivery pressure was fixed at 8 psi and the duration (5-25 ms) was adjusted to yield initial currents of between 200 and 500 pA. It was previously shown that GABA-evoked currents had stable amplitudes and kinetics for >45 min (Keros and Hablitz, 2005) and pressure application of the puff solution without GABA yielded no detectible current (DeFazio et al. 2000), using this methodology. For pressure-applied AMPA (20 µM) the same parameters were used.

Electrophysiology in the CHP: After sacrifice of the pups, the hemispheres were separated after removal of the skin and skull into a Petri dish containing cold (4 ºC) aCSF bubbled with 5% CO_2 in 95% O_2. After dissection of each CHF, they were placed in oxygenated aCSF (with similar composition to the one used to maintain hippocampal slices for electrophysiology) equilibrated with 5% CO_2 in 95% O_2 at room temperature. After recovery for 1 hour at room temperature, the CHF was fully submerged and superfused with aCSF at 32 +/- 1 ºC at a flow rate of 5.0 +/- 0.2 ml/min. The recordings were performed with glass extracellular electrodes filled with aCSF. Data were acquired by using a Digidata 1200B card (Axon Laboratory). Electrodes were put in CA3 region of the hippocampus at the same deepness (around 50 µm) between the experiments. After recording of a stable baseline for at least 20 min, drugs were superfused for at least 30 min to 1 hour.

Drugs and Solutions: Dynasore hydrate (20 µM) (SIGMA) and Brefeldin A (10 µg/mL) (SIGMA) were added to patch solution and prepared from a stock 1000× concentrated and solubilized in dimethylsulfoxide and ethanol, respectively. D-APV (Tocris Bioscience) and NBQX (Tocris Bioscience) were used from a stock 1000× concentrated made in bidistyled water. The selective antagonist of adenosine A_{2A} receptors, 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine or SCH58261 (provided by S. Weiss, Vernalis, UK) was prepared as 5 mM stock solutions in dimethylsulfoxide and then dissolved (< 0.001% dimethylsulfoxide) and used at a working solution of 100 nM. The selective antagonist of A_1 receptors, 8-cyclopentyl-1,3-dipropylxanthine or DPCPX (Tocris Bioscience) was used from a stock solution of 100 µM in DMSO and used at a concentration of 100 nM. All the drugs used for cell culture whose company is notexplicitly indicated were purchased from SIGMA or Tocris. The selective A_{2A} receptor agonist 3-[4-[2-[[6-amino-9-[(2 R, 3R, 4S, 5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl] propanoic acid or CGS21680 was used at a concentration of 30 nM from a stock 1000× concentrated in DMSO.

Statistical Analysis: Kolmogorov-Smirnov test was used to compare distributions which were considered different if p value > 0.05. For comparison between means, two tailed *t*-test or One-way ANOVA were used, depending on the number of groups to compare. If One-way ANOVA was used, Tukey test was chosen as the *post hoc* test. One-sample *t*-test was used to compare variations relative to a reference value of 100%. Again, means were considered different if p > 0.05. Statistical significance was indicated as *p<0.05, ** p<0.001, *** p<0.0001.

Chapter3 - Results

3.1. Modulation Afforded by A_1 and A_{2A} Receptor Antagonists in Hippocampal Slices

To study the impact of the blockade of adenosine receptors upon the spontaneous activity in hippocampal slices, a pharmacological approach was used. DPCPX, a selective antagonist on A_1 receptors (Lohse *et al.*, 1987) and SCH58261 an A_{2A} receptors selective antagonist (Zocchi *et al.*, 1996) were chosen and used at a concentration of 100 nM, which was shown to be a supra-maximal and selective concentration to block each receptor subtype. Antagonists were used instead of agonists with the purpose of probing the role of each receptor without inducing artificial and non-physiological effects due to over-activation of signaling pathways controlled by these receptors.

In Safiulina *et al.*, 2005, the modulatory role of A_1 receptor subtype in the immature hippocampal slice of the rat was described. They found that adenosine acting on this adenosine receptor subtype, which was localized on glutamatergic terminals projecting mainly interneurons, was able to reduce the frequency of GDPs. However, no information about the role of A_{2A} receptors in the immature hippocampal slice is available in the literature. Data concerning A_1 receptor-mediated effects was mainly used for comparison with A_{2A} receptor-mediated effects.

Fig. 3.1 presents the effects of DPCPX (**A**) and SCH58261 (**B**) upon the frequency of GDPs recorded from interneurons belonging to the *Stratum radiatum* of PD5 to PD7 hippocampal slices. This population of cells was selected because at this stage of development they display a robust spontaneous activity. Both drugs had a similar effect, increasing the frequency of GDPs. The A_{2A} receptor antagonist changed the basal frequency of GDPs from 0.014 +/- 0.004Hz to 0.039 +/- 0.006Hz (n=4-8, $p<0.05$, one-way ANOVA followed by Tukey test), 20 min after exposure to the drug, the time where the maximal effect was observed (**Fig. 3.1B**). During washout, the frequency of GDPs did not return to the basal levels (frequency after 30 min of washout was 0.0345 +/-0.012Hz), however this was not statistically different from the baseline value nor from the value of treatment period ($p>0.05$, One-way ANOVA followed by Tukey test). Exposure for 20 min to the A_1 receptor antagonist changed the basal frequency of GDPs from 0.020 +/- 0.003Hz to 0.064 +/- 0.012Hz (n=4-8, $p<0.05$, one-way ANOVA followed by Tukey test). The washout period reduced the frequency of GDPs to 0.040 +/- 0.007 Hz and again it was not observed a complete recovery to the baseline levels since the frequency of GDPs after 30 min of washout is not statistically different from the frequency of GDPs during treatment with the A_1 receptor antagonist (**Fig. 3.1A**).

The co-application of DPCPX (100 nM) and SCH58261 (100 nM) increased the frequency of GDPs to a similar extent (of about 300%) as when each drug was applied individually, indicating that there is not an additive effect of both drugs. The frequency of GDPs recorded under the baseline was 0.054 +/- 0.018 Hz, 0.150 +/- 0.024 Hz during co-application of both drugs (n=5, $p<0.05$, one-way ANOVA followed by Tukey test) and 0.108 +/- 0.022Hz after 30 min of washout (see **Fig. 3.2**).

The observation that the blockade of A_{2A} receptors potentiated the increase in the frequency of GDPs (**Fig. 3.1A**) suggests that the activation of NMDA receptors might be an important factor contributing to this effect. Also, it was demonstrated by Rebola and co-workers (Rebola *et al.*, 2008) that A_{2A} receptors control the function of NMDA receptors. To test this hypothesis, the same type of recording was performed in the presence of 50 µM D-APV, a selective antagonist of NMDA receptors shown to fully block the effect of exogenously applied NMDA (King *et al.*, 1989). Blockade of NMDA receptor indeed abrogated the SCH58261-induced increase in GDPs frequency in 5 independent experiments (n=5, $p>0.05$, two-tailed *t*-test - see **Fig. 3.3**).

Ben-Ari and collaborators (Ben-Ari *et al.*, 1989), proposed a mechanism to explain the possible role of NMDA receptors in the control of GDPs. NMDA receptors are present in interneurons that receive glutamate from pyramidal cells. It will make them fire and release GABA that will behave as an excitatory neurotransmitter upon pyramidal cells. This will form a loop that will synchronize the activity of clusters of cells linked directly or indirectly by synaptic terminals.

An increase in NMDA receptor function was observed in some experimental conditions such as chronic exposure to ethanol that results in an increase of NMDA-mediated Ca^{2+} increase (Hu and Ticku, 1995). This increase of NMDA receptor function was found to be dependent on the state of phosphorylation of the receptor (Chen and Roche, 2007), which seems to change its function, in particular Src family (including Fyn) of protein tyrosine kinases were identified as enzymes involved in NMDA receptor phosphorylation (Wang and Salter, 1994; Yu *et al.*, 1997). This tyrosine phosphorylation of the NMDA receptor is increased upon synaptic plasticity, such as upon in LTP (Kojima *et al.*, 1997; Salter, 1998). Phosphorylation by Fyn can also target the NMDA receptor to the plasma membrane (Zito and Scheuss, 2009).

Fig. 3.1

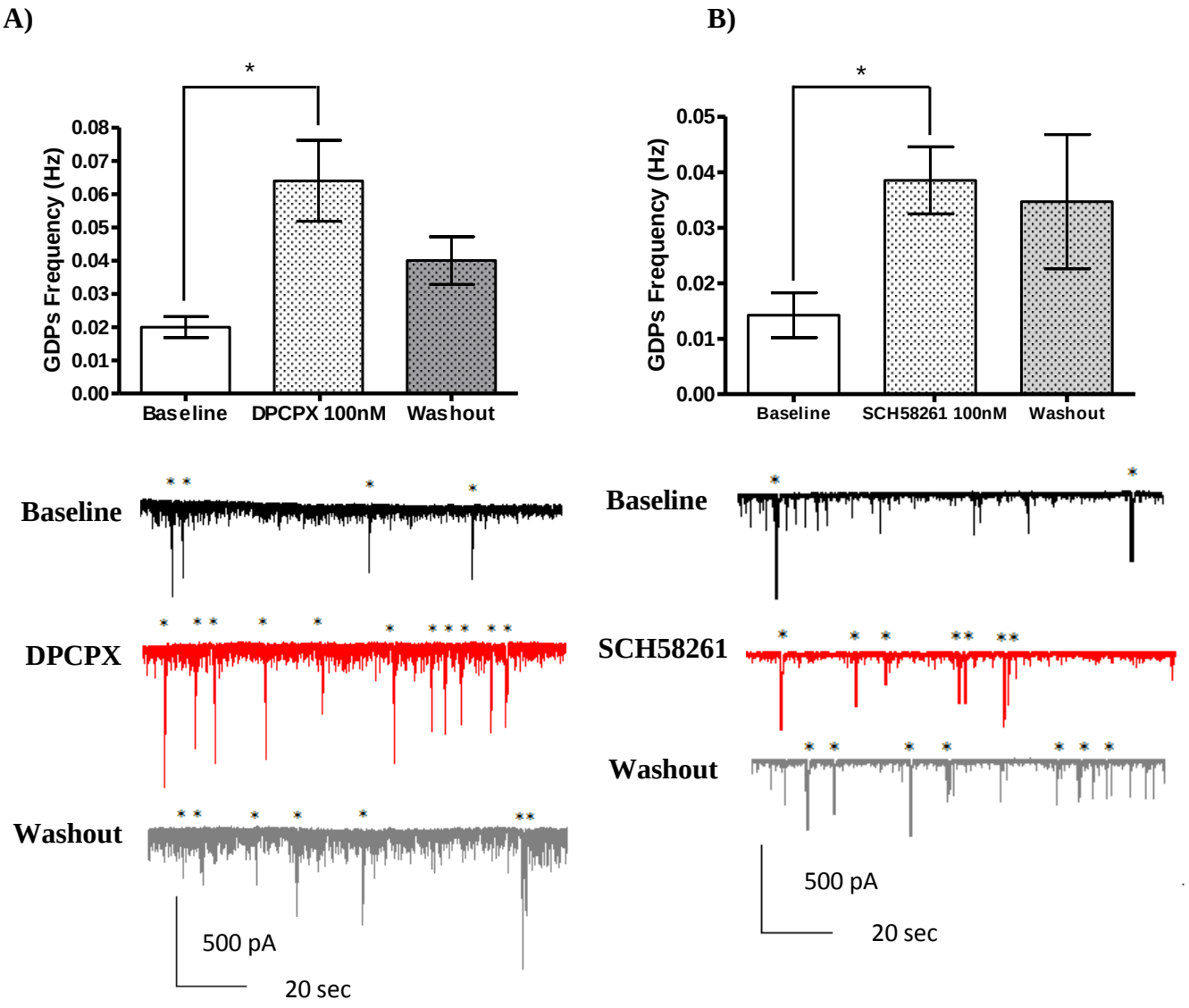


Fig 3.1: Effect of the blockade of the two main adenosine receptor subtypes upon the profile of the spontaneous activity in hippocampal slices. Effect of 100 nM of DPCPX (**A**) or 100 nM of SCH58261 (**B**) in the frequency of GDPs recorded in hippocampal slices from PD 5 to PD 7 mice pups. Both drugs seem to "excite" hippocampal slices, increasing the frequency of GDPs recorded in the *Stratum radiatum* of CA3 region. The washout period (30 min) does not seem to be enough to restore the frequency of GDPs to initial levels. Bellow each graphic there is a trace representative of the recording obtained in each condition, Control, Drug treatment (DPCPX or SCH58261) and Washout. In the recordings, GDPs are indicated by asterisks (*). Results are mean+/-SEM of 4-8 independent experiments. Difference between means was evaluated using One-way ANOVA followed by Tukey test. Significant differences are indicated as *p<0.05.

NMDA receptor may also be phosphorylated by PKC, PKA, CamKII and Src (see Zito and Scheuss, 2009 for a review) with several consequences. For example, phosphorylation by PKC reduces the affinity for extracellular Mg^{2+}, increasing the open probability of the receptor and this potentiation seems to be triggered by Ca^{2+} influx by the receptor itself (Zito and Scheuss, 2009). However PKC may have an opposite effect since it prevents NMDA receptor clustering (Zito and Scheuss, 2009). CaMKII can directly bind to NMDA receptor and this interaction is involved in some forms of plasticity (Zito and Scheuss, 2009). Phosphorylation by PKA seems to increase the influx of Ca^{2+} and phosphorylation by Src enhances NMDA receptor function reducing the block of Zn^{2+} (Zito and Scheuss, 2009).

Fig. 3.2

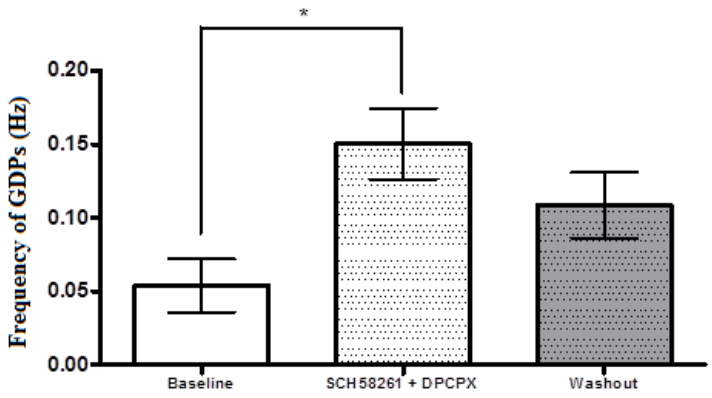


Fig. 3.2 – Effect of the simultaneous blockade of A_1 and A_{2A} receptors on the spontaneous activity of hippocampal slices is not cumulative. A_1 and A_{2A} receptor antagonists do not seem to have a cumulative effect on GDPs frequency. Both drugs, when co-applied increase the frequency of GDPs of about 300%, similarly to what was observed when drugs were applied separately. The frequency of GDPs recorded after 30 min of washout was intermediate between the frequency recorded during baseline and treatment period indicating a partial recovery. The recordings were made in the *Stratum radiatum* of CA3 region. Results are mean+/-SEM of 5 independent experiments. Significant differences between the means is indicated in the graph as *$p<0.05$ using One-way ANOVA followed by Tukey test, n=5.

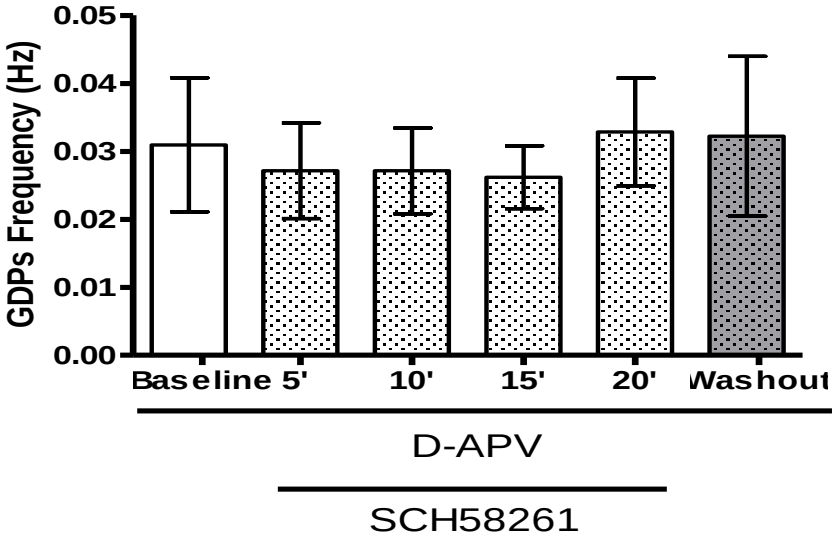
Interestingly, the increase in the frequency of GDPs triggered by A_{2A} receptor antagonism resembled a phenomenon of potentiation whereas its effect on the amount of charge (area) transported through the plasma membrane on the course of each GDP was similar to an effect of depression. A_{2A} receptor antagonist decreased the area of GDPs in about 70% of the 10 cells

recorded. The mean area of GDPs in those cells (where a decrease in the surface was observed) during baseline was 103,954 +/- 26,815 pA×ms and after 20 min of incubation with 100 nM SCH58261 was 53,573 +/- 19,480pA×ms, which corresponds to a decrease of about 50% (**Fig. 3.4A**). This difference was statistically significant (n=7, $p<0.05$, two-tailed *t*-test). Superfusion of hippocampal slices with 100 nM DPCPX did not seem to change the surface of GDPs (**Fig. 3.4B**), during the period of drug application; the average area of GDPs was initially 88,852 +/- 22,796 pA×ms and was 86,126 +/- 22,776 pA×ms 20 min after perfusion with 100 nM DPCPX (n=5, $p>0.05$, two-tailed *t*-test). The blockade of NMDA receptors did no prevent the effect triggered by SCH58261 upon the area of GDPs. The area of GDPS recorded in the presence of D-APV changed from a value of 11,630 +/-920.4 pA×ms to 4,346 +/-358.6 pA×ms 20 min after exposure to 100 nM SCH58261 (n=4, $p<0.001$, two-tailed *t*-test).

Taking all together, both A_1 and A_{2A} receptor antagonists seem to "excite" hippocampal slices, increasing the frequency of GDPs. This seems to be related to the activation of NMDAreceptors at least in the case of the SCH58261-mediated effect. A_{2A} receptor blockade seems also to modulate AMPA receptor- and $GABA_A$ receptor-mediated events independently of NMDA receptor activation and it may lead to the reduction of the surface of GDPs. The A_1 receptor antagonist had similar effects upon the frequency of GDPs but failed to significantly affect the area of GDPs.

Fig. 3.3

A)



B)

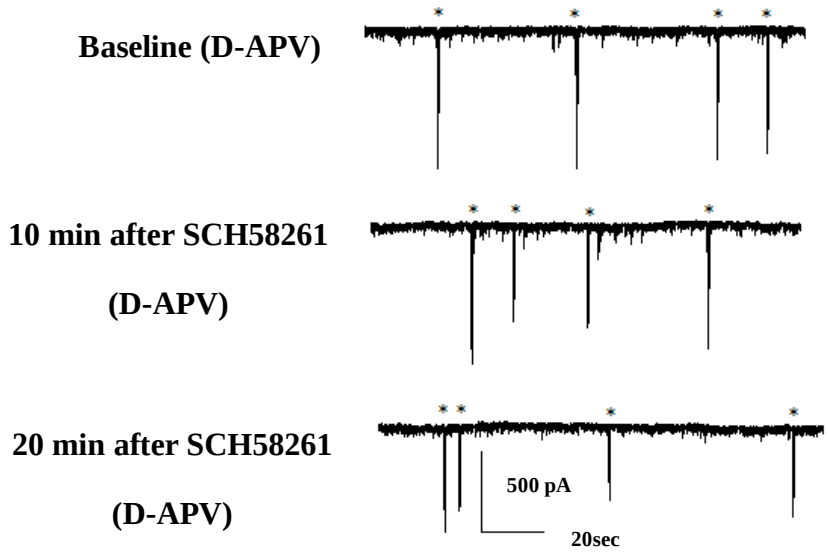


Fig. 3.3 – Role of NMDA receptors in the SCH58261-induced effect upon GDPs frequency. The increase in the frequency of GDPs caused by A_{2A} receptor blockade seems to be dependent on NMDA receptor activation since co-application of D-APV prevents the effect triggered by SCH58261. Results are mean +/- SEM of 3-7 experiments and comparisons between means were done using One-way ANOVA followed by Tukey test. It was not observed any significant difference ($p>0.05$) between means of frequencies of GDPs in the different experimental conditions.

Fig. 3.4

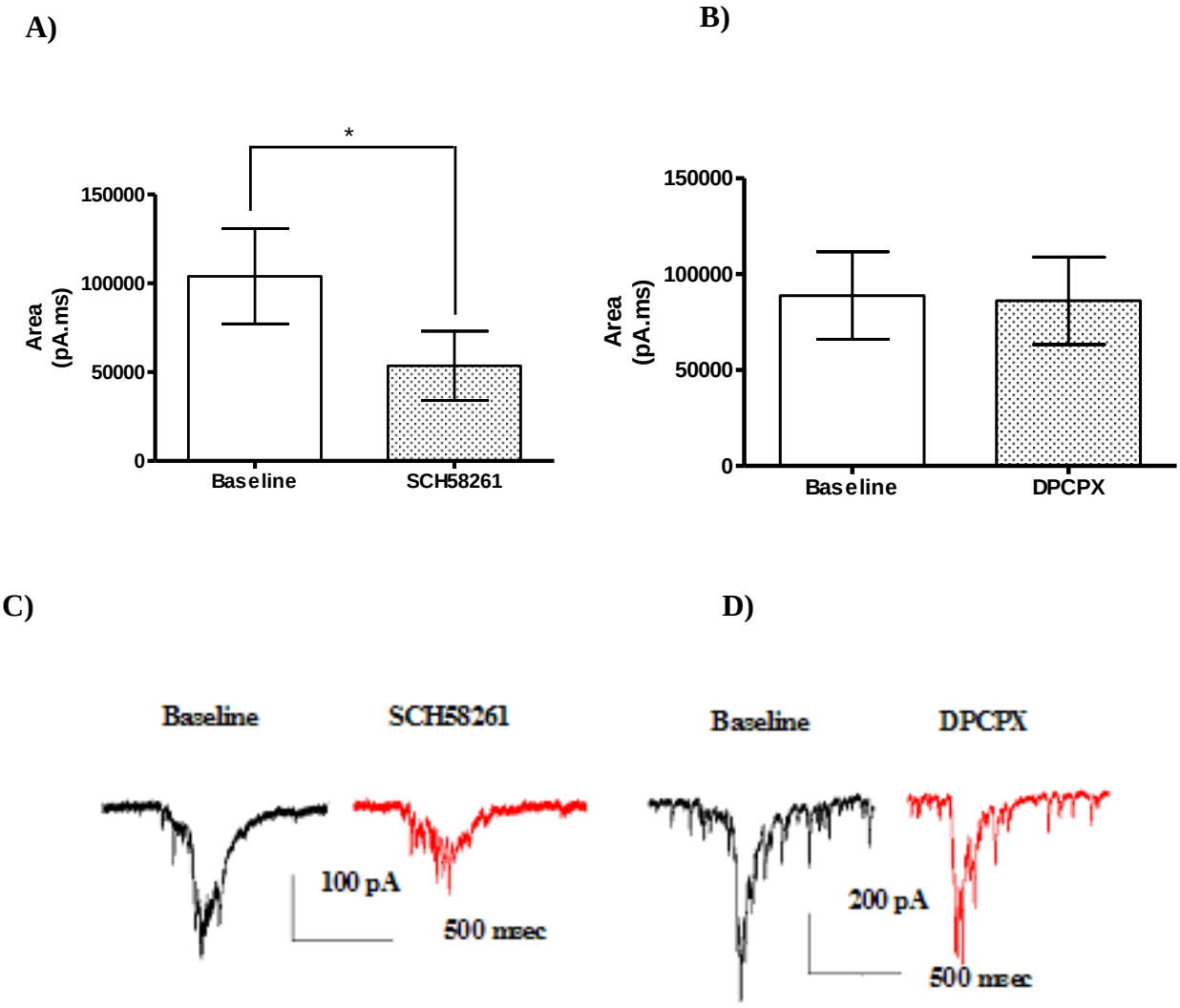


Fig. 3.4 – Effect of A_1 or A_{2A} receptor antagonists upon the area of GDPs. A) Acute application of **the** A_{2A} receptor decreased the surface of GDPs to about half of the initial value. **B)** DPCPX failed to significantly affect the surface of GDPs. Results are mean +/-SEM of 5-7 experiments. *p<0.05 using a two-tailed *t*-test for comparison between means. **C)** and **D)** ilustrative traces of GDPs recorded during baseline and 20 min after incubation with either SCH58261(100 nM) or DPCPX (100 nM), respectively.

3.2. Modulation of AMPA- and $GABA_A$ Receptor-Mediated Synaptic Events by Antagonists of A_1 and A_{2A} Receptor Subtypes

In an attempt to simplify the interpretation of the modulation afforded by A_1 and A_{2A} receptors upon synaptic events, tetrodotoxin (TTX) was used to block spontaneous activity. Synaptic currents would be difficult to analyze due to the high frequency of GDPs generated by the adenosine receptor antagonists. This allows testing wheather DPCPX and SCH58261 could directly modulate ionotropic glutamate and $GABA_A$ receptor function.

Fig. 3.5I and **Fig. 3.5II** illustrate the effect of A_1 receptor blockade by DPCPX on the properties of miniature IPSCs (here called miniature inhibitory currents or mIPSCs). This analysis was made in principal cells and interneurons from the *Stratum radiatum* of CA3 and the effect of 100 nM DPCPX had a similar effect upon both types of cells. DPCPX (100 nM) seems to increase the interevent interval as shown in **A** and **B**, indicating that the drug reduces the frequency of mIPSCs in the recorded cells. The average interevent interval analysed in fractions of the recording corresponding to the baseline was 556.7 +/- 63.4 ms, a value significantly different from the interevent interval observed 20 min of exposure to 100 nM DPCPX, 867.7 +/- 62.5 ms ($n=10$, $p<0.05$, one-way ANOVA followed by Tukey test). The effect of DPCPX was long-lasting since after 30 min of washout, the average interevent interval increased to 1608.0 +/- 196.4 ms ($n=10$, $p<0.0001$ compared to baseline, One-way ANOVA followed by Tukey test). This effect can be better seen observing the cumulative probability plots of interevent intervals (see the shift to right in the graph) to higher values which is even more remarkable in the washout condition (**A**). The comparison between the distributions was made using Kolmogorov-Smirnov test (** $p<0.001$ between the baseline and treatment distributions and *** $p<0.0001$ between the treatment and washout distributions).

Generally, a decrease of the amplitude of currents corresponds to a loss of receptors from the postsynaptic membrane (Nusser *et al.*, 1997). The average amplitude of mIPSCs recorded in these cells was 45.3 +/- 2.3 pA and DPCPX (100 nM) decreased the amplitude of mIPSCs to 32.6 +/- 1.2 pA ($n=10$, $p<0.0001$using one-way ANOVA, followed by Tukey test - see **Fig. 3.5IC** and **D**). The washout period did not allow the recovery to the initial levels of amplitude of mIPSCs. The average amplitude after 30 min of washout was 32.0 +/- 1.9 pA ($n=10$, $p<0.001$ compared to the period of treatment with 100 nM DPCPX, One-way ANOVA followed by Tukey test).

Fig. 3.5I

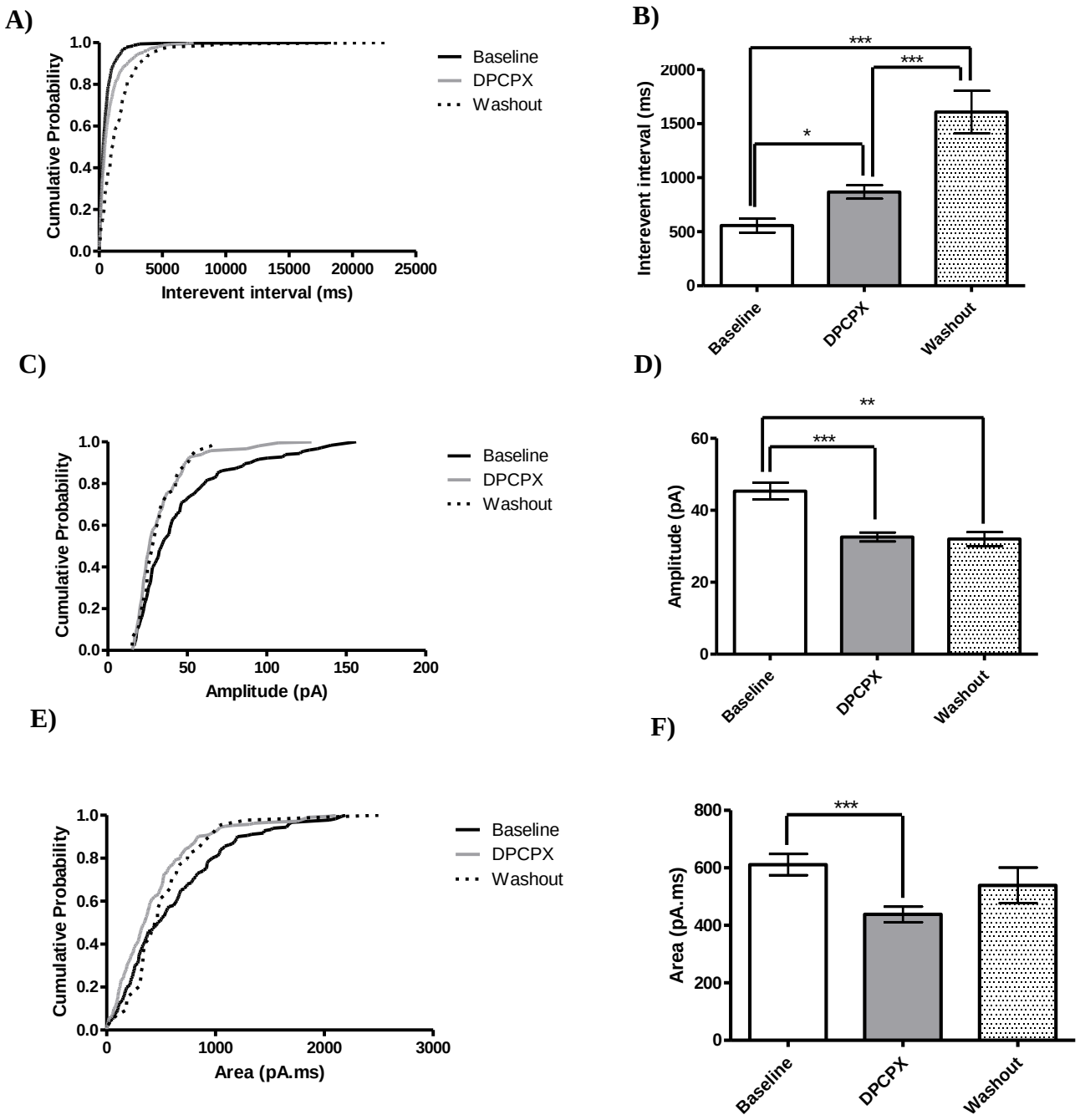


Fig. 3.5I – **A) Effect of A_1 receptor antagonism upon the frequency, amplitude and area of mIPSCs. A** and **B**) Application of the A_1 receptor antagonist DPCPX (100 nM) increased the interevent interval of mIPSCs and this effect was long-lasting since it was visible and more robust 30 min after washout. Blockade of A_1 receptors also resulted in a decrease in the amplitude of these currents and this effect also seems to be long-lasting **C)** and **D)**. The currents recorded 20 min after exposure to 100 nM DPCPX were significantly smaller in area (**E** and **F**). Results are mean +/- SEM of 10 independent experiments and both pyramidal cells and interneurons from the *Stratum radiatum* of CA3 region ploted togheter for the analysis. Comparison between cumulative probability distributions was made using the Kolmogorov-Smirnov test and the comparison between means (mean +/- SEM) was made using One-way ANOVA followed by Tukey test. Results are relative to data observed in 10 cells including pyramidal cells and interneurons from *Stratum radiatum* of CA3. Significant differences are indicated by *p<0.05; **p<0.001 and ***p<0.0001.

Fig. 3.5II

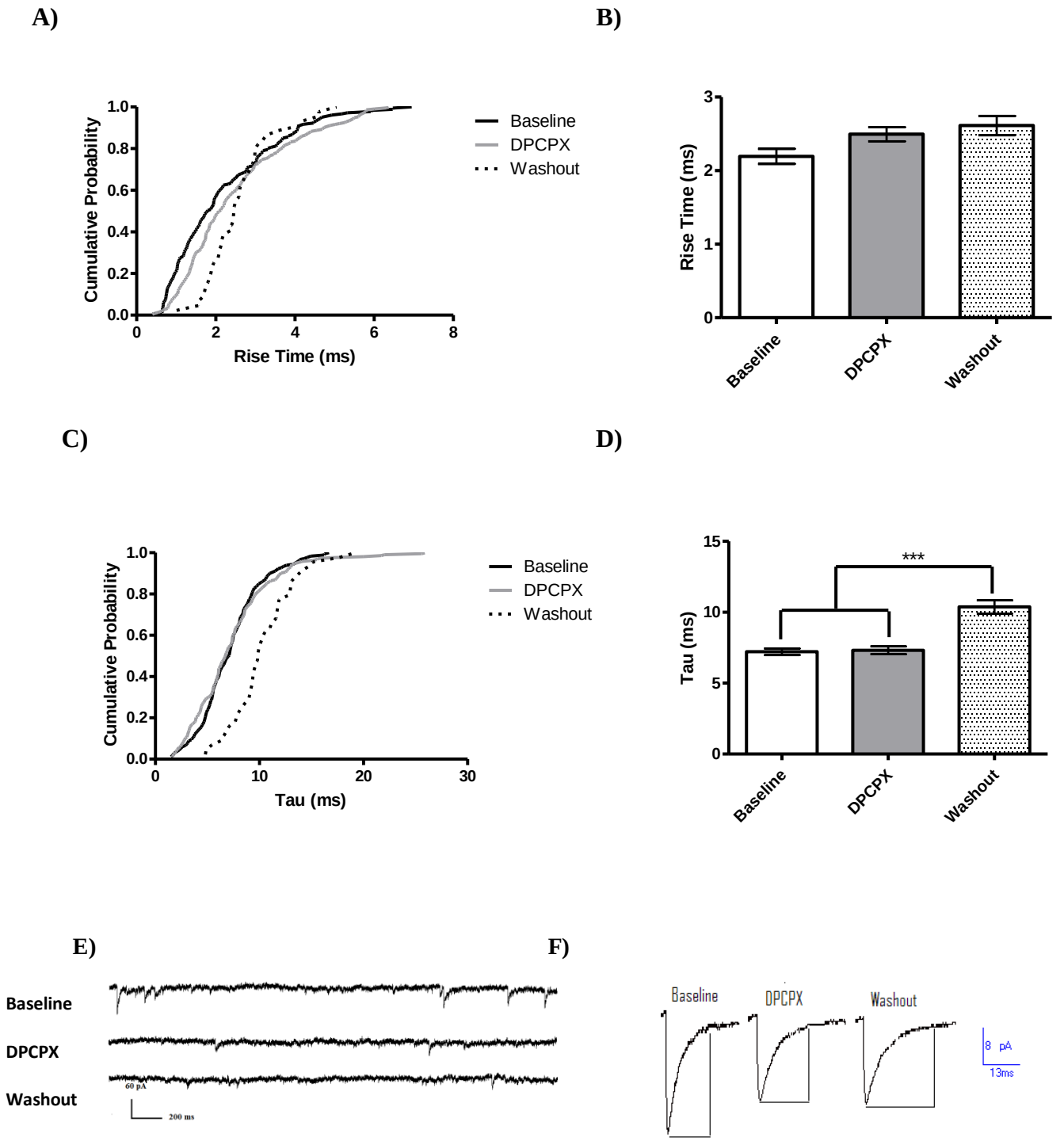


Fig. 3.5II – Effect of A_1 receptor antagonism on the kinetic properties of mIPSCs. Superfusion of the the slices with A_1 receptor antagonist DPCPX (100 nM) seemed to increase the rise time (**A** and **B**) of mIPSCs (see the shift to the right on the cumulative probability distributions) and an increase of the decay time constant *tau* (**C** and **D**) of these currents, which was only visible 30 min after washout. **E)** Representation of mIPSCs recorded during baseline, 20 min upon DPCPX application and 30 min after washout. **F)** Representation of the effect of 100 nM DPCPX on the amplitude and decay time of mIPSCs. Results are mean +/- SEM of 10 independent experiments and both pyramidal cells and interneurons from the *Stratum radiatum* were plotted together for the analysis. Comparison between cumulative probability distributions was made using the Kolmogorov-Smirnov test and the comparison between means (mean +/- SEM) was made using One-way ANOVA followed by Tukey test. Significant differences between mean values are indicated as ***p<0.0001.

The area of mIPSCs was also decreased from 611.1 +/- 36.9 pA×ms to 438.0 +/- 27.0 pA×ms ($p<0.0001$, n=10, one-way ANOVA followed by Tukey test) by DPCPX 100 nM. There was a partial recovery after 30 min of washout to a value of 538.9 +/- 62.2 pA×ms, which was only detected analysing the cumulative probability distributions (n=10, $p<0.05$, using the Kolmogorov-Smirnov test - see **Fig. 3.5I E** and **F**). The average rise time for currents recorded during baseline was 2.2 +/- 0.1 ms, and tended to increase to 2.5 +/- 0.1 ms, 20 min after application of DPCPX and was 2.6 +/- 0.1 ms, 30 min after washout (see **Fig. 3.5II A** and **B**). A more detailed analysis of the cumulative distributions for rise time showed that a higher fraction of the currents displayed higher rise time values in the presence of DPCPX ($p<0.05$, n=10, Kolmogorov-Smirnov test). After 30 min of washout, the fraction of the population of currents displaying rise time values lower than 2.7ms was higher. The opposite was observed for currents with rise times higher than 2.7ms. The decay time constant (**Fig. 3.5II C** and **D)** was not modified by the A_1 receptor antagonist. The values of decay time were 7.2 +/- 0.2 ms before DPCPX application, 7.3 +/- 0.3 ms 20 min after DPCPX application; however currents recorded 30 min after washout of 100 nM DPCPX displayed higher decay time constants, 10.4 +/- 0.5 ms ($p<0.0001$, n=10, one-way ANOVA and Kolmogorov-Smirnov tests).

A_1 receptor blockade seems to change $GABA_A$ receptor properties and stability in the plasma membrane and these effects are long-lasting since the washout period was not enough to restore the parameters of the currents observed before DPCPX application. As previously presented in the **Chapter 1**, A_1 receptors can be present at pre or postsynaptic sites in the immature hippocampus. Normally, a decrease in the frequency is considered a presynaptic mechanism. In this case, as both amplitude and kinetic properties of mini IPSCs were changed, it is not possible to clearly address a specific site of action for the drug. The decrease of the frequency of mIPSCs may result from the absence of detection during the analysis, since parameters as amplitude and surface were decreased by DPCPX application or because $GABA_A$ receptors may change their subcellular distribution upon administration of DPCPX, eventually being removed from synaptic sites.

Treatment with DPCPX also had an effect upon miniature glutamatergic currents (see **Fig. 3.6I** and **Fig. 3.6II**). After 20 min of superfusion, DPCPX (100 nM) significantly decreased the frequency of mEPSCs (n=3, $p<0.001$, two-tailed *t*-test, from an initial value of 6199.4 +/- 1770.8 ms to 3116.3 +/- 340.6 ms – see **Fig. 3.6I B** and **C**). The washout period was not investigated. The mean amplitude recorded during baseline was 30.9 +/- 2.1 pA and mean amplitude recorded during drug application was 26.4 +/- 1.8 pA ($p>0.05$, n=3, two-tailed *t*-test). Kolmogorov-Smirnov test did not

show a significant difference between the distributions of the amplitudes before and after the treatment ($p>0.05$) (**Fig. 3.6I D** and **E**).

Exposure to 100 nM DPCPX did not change the area of these glutamatergic currents. The average area of mEPSCs recorded during baseline was 249.1 +/- 27.9 pA×ms and was 277.0 +/- 35.5 pA×ms 20 min after DPCPX superfusion (see **Fig. 3.6II A** and **B**). Cumulative probability distributions between areas from currents recorded during baseline and during the period of treatment with the DPCPX also failed to reveal significant differences between the areas of the currents. However, a difference in the kinetic parameters was detected after exposure to this A_1 receptor antagonist. The average rise time decreased from 1.769 +/- 0.203 ms to 1.219 +/- 0.142 ms, 20 min after exposure to DPCPX (**Fig. 3.6IIC** and **D**). Decay time (**Fig. 3.6IIE** and **F**) was also decreased after DPCPX exposure ($p<0.05$, n=3, Kolmogorov-Smirnov test), although the average values of decay time were not significantly modified (decay time from currents recorded in the baseline was 4.6 +/- 0.5 ms and 3.7 +/- 0.5 ms 20 min after exposure to DPCPX, $p>0.05$, n=3, two-tailed *t*-test).

The effect of SCH58261 upon the properties of mIPSCs is presented in **Fig. 3.7I**, **II** and **III**. SCH58261 (100 nM) induced both an increase (**A** and **B**) or a decrease (**C** and **D**) in the interevent interval of mIPSCs. When an increase in the interevent interval of mIPSCs was observed, the average value of 801.0 +/- 37.5 ms was modified to 1397.6 +/- 73.4 ms (n=10, $p<0.001$, One-way ANOVA followed by Tukey test). This effect triggered during SCH58261 application became more marked 30 min after washout (n=9, $p<0.0001$, One-way ANOVA followed by Tukey test). The interevent interval in the other group of cells containing both pyramidal cells and interneurons from the *Stratum radiatum* decreased from an initial value of 599.9 +/- 37.2 ms to 404.5 +/- 12.0 ms (n=5, $p<0.0001$, One-way ANOVA followed by Tukey test). This effect upon the frequency triggered by 100 nM SCH58261 seemed to be also long-lasting since the average interevent interval after 30 min of washout was 296.0 +/- 22.7 ms (n=5, $p<0.0001$, One-way ANOVA followed by Tukey test).

Fig. 3.6I

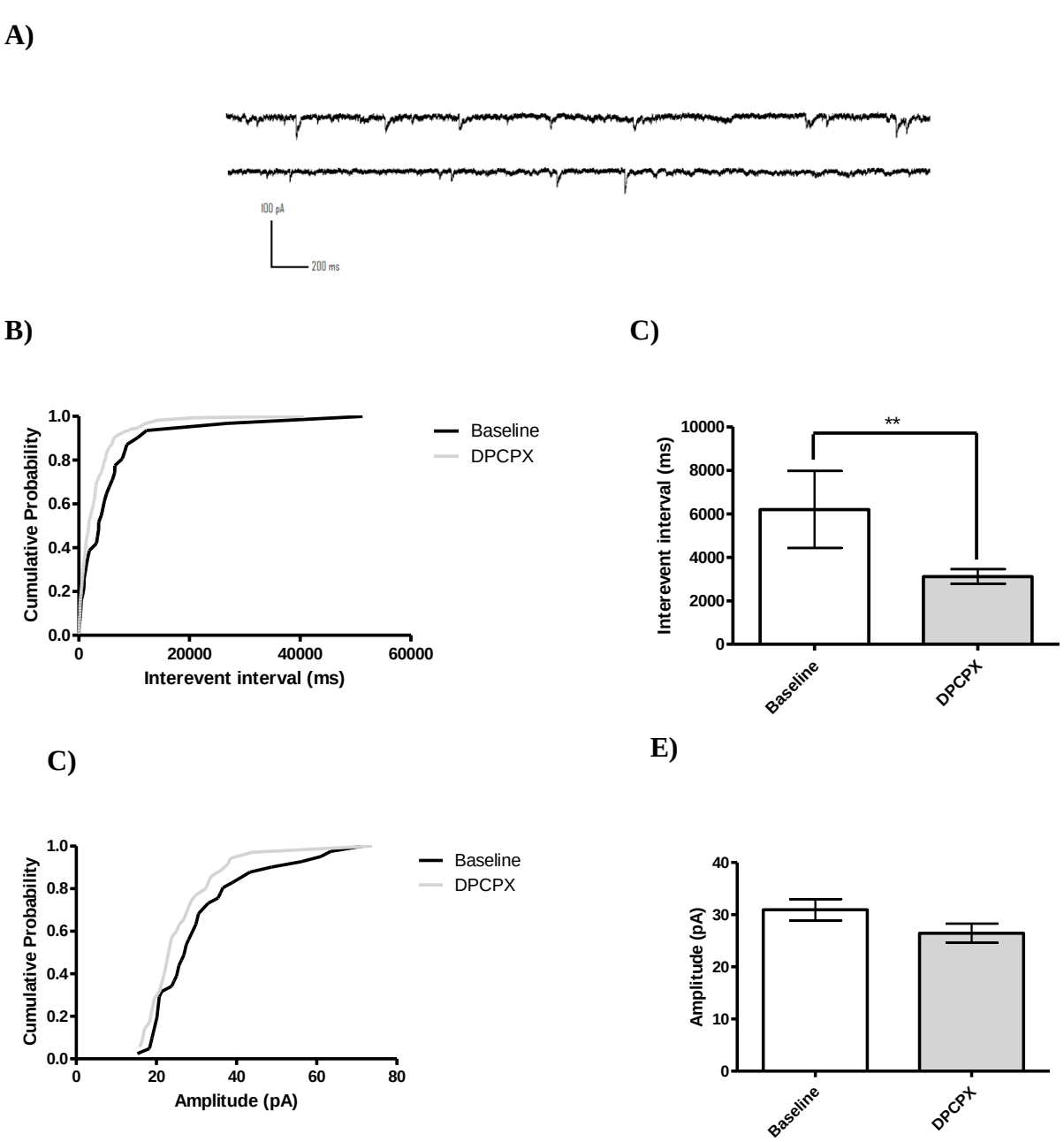


Fig. 3.6I - Effect of A_1 receptor antagonism on the frequency and amplitude of mEPSCs. A) Representative trace correspondind to mEPSCcurrents recorded in the absence (baseline) or 20 min after the exposure to 100 nM DPCPX. DPCPX seemed to decrease the interevent interval of mEPSCs (**B** and **C**), decreasing also their amplitudes (**D** and **E**), an effect that was only detected analysing the cumulative probability distributions (n=3, $p<0.05$, Kolmogorov-Smirnov test). The washout period was not investigated. Results depicted in **C)** and **E)** are mean +/- SEM of 3 independent recordings made in both pyramidal cells and interneurons from the *Stratum radiatum*.Two tailed *t*-test was used to compare the means. Significant differences are indicated in the graphs as **$p<0.001$.

3.6II

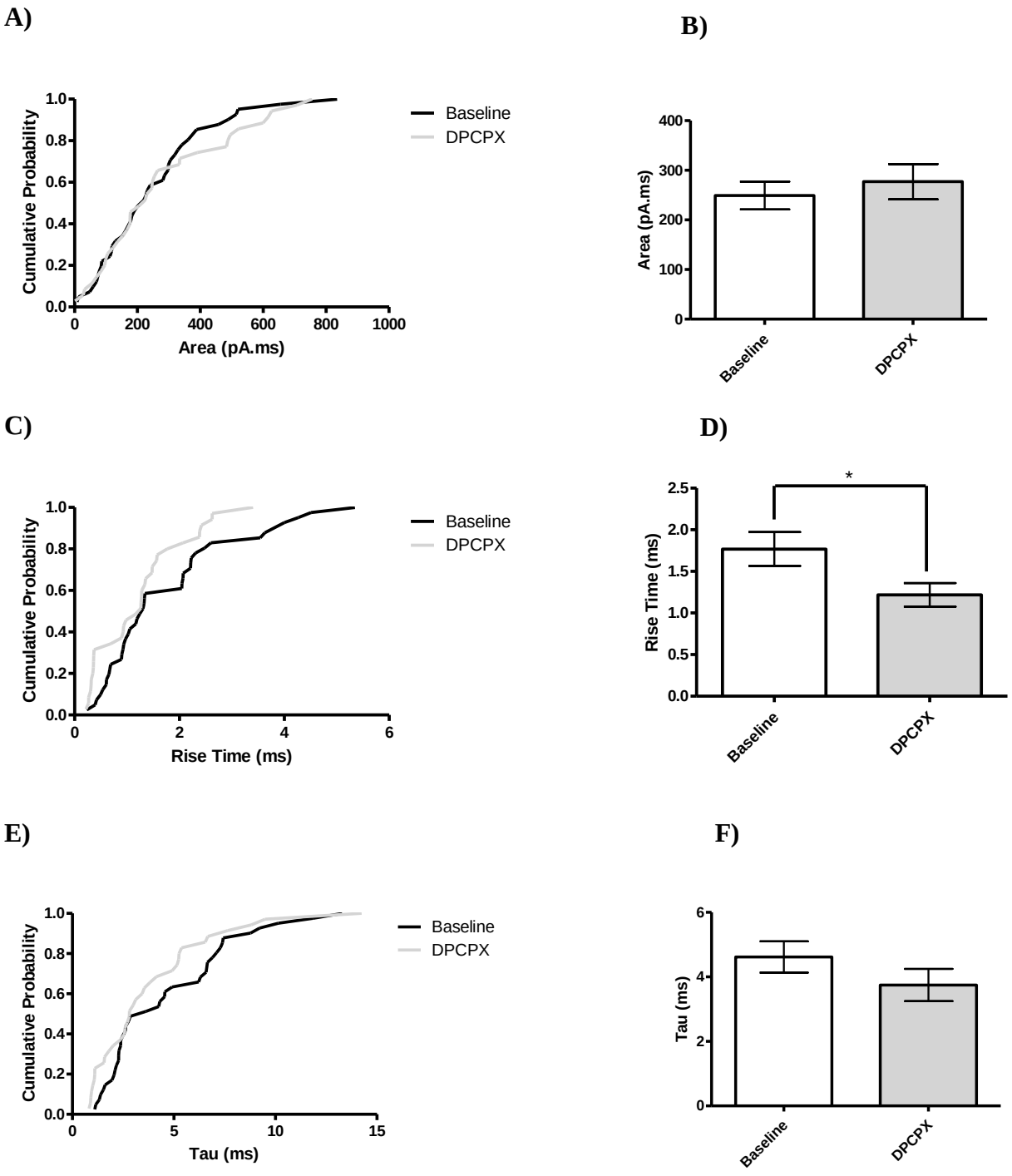


Fig. 3.6II - **Effect of A_1 receptor antagonism on the area and kinetic properties of mEPSCs.** The A_1 receptor antagonist DPCPX (100 nM) did not seem to change the area of mEPSCs (n=3, p>0.05 two-tailed *t*-test and Kolmogorov-Smirnov test - **A** and **B**). The treatment with DPCPX (100 nM) decreased the rise time (n=3, p<0.05, two-tailed *t*-test) and had no effect upon the decay time constant *tau* (n=3, p>0.05, two-tailed *t*-test). The washout period was not investigated. Results are mean +/- SEM of 3 independent recordings made in both pyramidal cells and interneurons from the *Stratum radiatum*.Two tailed *t*-test was used to compare the means. Significant differences are indicated in the graphs as *p<0.05.

Currents belonging from both profiles were plotted together for the analysis of amplitude, area and kinetic properties, since they displayed similar profiles of response to 100 nM of SCH58261. The amplitude of mIPSCs decreased from 32.0 +/- 1.3 pA to 28.6 +/- 0.9 pA, 20 min after SCH58261 (n=14, $p<0.05$, One-way ANOVA followed by Tukey test). After the period of washout the amplitude of mIPSCs decreased even more to 23.2 +/- 1.0 pA (n=14, $p<0.001$, compared to period of treatment with SCH58261, One-way ANOVA followed by Tukey test) – see **Fig. 3.7II A** and **B**).

SCH58261 induced a small effect upon the area of the currents which was only detected comparing the cumulative distributions of the areas of the currents in the three experimental conditions (before, 20 min after exposure to SCH58261 and washout period). The average area of mIPSCS recorded before 100 nM SCH58261 application was 429.1 +/- 27.02 pA×ms and decreased to 390.9 +/- 20.33 pA×ms 20 min after application of this A_{2A} receptor antagonist (n=14, $p<0.05$, Kolmogorov-Smirnov test). The effect was long-lasting since the area of the currents after 30 min of washout was 309.4 +/- 46.27 pA×ms (n=14, $p<0.05$, Kolmogorov-Smirnov test) – see (**Fig. 3.7II C** and **D**).

A subtle effect upon the kinetic parameters (**Fig. 3.7 III A-**D) of mIPSCs was also detected comparing the cumulative probability distributions of these currents and it was noticeable only 30 min after washout period (n=14, $p<0.05$, Kolmogorov-Smirnov test). The effect of SCH58261 was however significant 30 min after of washout comparing to the period of treatment with SCH58261 (n=14, $p<0.05$, Kolmogorov-Smirnov test). The average value of the decay time constant *tau* (**Fig. 3.7III C** and **D**) was 11.0 +/- 0.7 ms during baseline, 10.8 +/- 0.6 ms 20 min after SCH58261 application and 11.1 +/- 2.4 ms 30 min after washout. The treatment with SCH58261 failed to modify the decay time constant (n=14, $p>0.05$, Kolmogorov-Smirnov test). The fraction of mIPSCs displaying lower values of decay time constant was higher 30 min after washout (n=14, $p<0.05$, Kolmogorov-Smirnov test). Again, a putative presynaptic effect (change in the frequency) can be explained by postsynaptic effects.

SCH58261 was also found to modulate the properties of mEPSCs (see **Fig. 3.8I** and **II**). Exposure to 100 nM SCH58261 decreased the frequency displayed by mEPSCs (see the effect upon the intereven interval in **Fig. 3.8I A** and **B**. Both pyramidal cells and interneurons from the *Stratum radiatum* of CA3 were analized. The interevent interval from these currents increased from 1,091 +/- 77.2 ms to 5,379 +/- 536.3 ms 20 min after the exposure to 100 nM of SCH58261 (n=5, $p<0.0001$,

one-way ANOVA followed by Tukey test). Interestingly, this effect was reversible since after 30 min of washout, the average interevent interval of mEPSCs was 976.0 +/- 208.2 ms (n=5, p>0.05, compared to the baseline, one-way ANOVA followed by Tukey test).

The amplitude of mEPSCs was decreased from 26.9 +/- 0.5 pA to 24.2 +/- 0.5 pA 20 min after exposure to SCH58261 (n=5, p<0.0001, one-way ANOVA followed by Tukey test). This effect was eliminated after washout (n=5, p>0.05, one-way ANOVA followed by Tukey test) - see (**Fig. 3.8II A** and **B**). The area of mEPSCs was also affected by the treatment with the A_{2A} receptor antagonist, SCH58261. The average area of mEPSCs before SCH58261 (100 nM) application was 194.4 +/- 9.2 pA×ms and decreased to 156.9 +/- 8.5 pA×ms 20 min after exposure to SCH58261 (n=5, p<0.05, one-way ANOVA followed by Tukey test). The effect of SCH58261 upon the area of these currents was also reversible since an increase to 185.0 +/- 14.9 pA×ms was observed 30 min after washout (n=5, p<0.05, compared to the period of exposure to the SCH58261, one-way ANOVA followed by Tukey test) - see (**Fig. 3.8II C** and **D)**.

Both A_1 and A_{2A} receptor antagonists have an impact in the frequency of mini IPSCs. Normally, an effect in the frequency is attributed to a presynaptic modulation. In fact, A_1 receptors seem to be present presynaptically (see **Chapter 1**, **section 1.1**) and A_{2A} receptors seem also to be present in presynaptic sites co-localizing with VGAT in GABAergic synapses. Both A_1 and A_{2A} receptor antagonists have similar effects upon the frequency of mIPSCs and mEPSCs. In glutamatergic synapses, the increase in the interevent interval triggered by the A_{2A} receptor antagonist if there is a strong postsynaptic effect that would modify the ability of the recorded cell to sense the pre-synaptic release of glutamate, since no evidence was found for the presence of presynaptic A_{2A} receptors in the immature glutamatergic synapse.

Fig. 3.7I

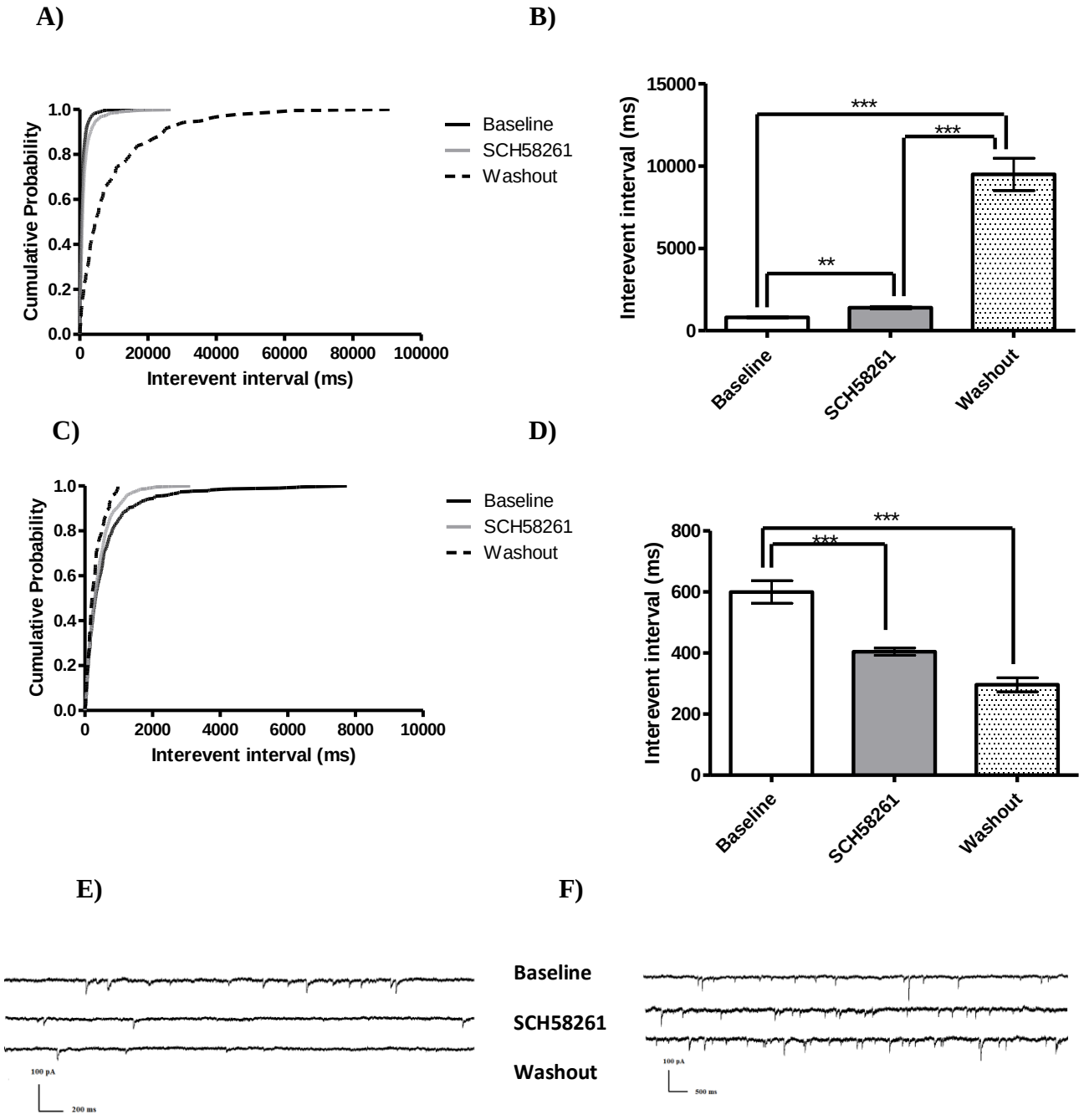


Fig. 3.7I-Effect of A_{2A} receptor antagonism on the frequency of mIPSCs. SCH58261 (100 nM) had a double effect on the frequency of mIPSCs, decreasing (**A** and **B**) or increasing it (**C** and **D**). **E)** and **F)** Representation of mIPSCs recorded during baseline, 20 min of SCH58261 application and 30 min after washout and displaying the profile represented in **A)** and **B)** or **C)** and **D)**, respectively. Results are mean +/- SEM of 10 independent recording made using in both pyramidal cells and interneurons from the *Stratum radiatum* of 5 to 7 days-old mice pups. Comparison between cumulative probability distributions was made using the Kolmogorov-Smirnov test and the comparison between means was made using One-way ANOVA followed by Tukey test. Significant differences are indicated as **p<0.001 and ***p<0.0001.

Fig. 3.7II

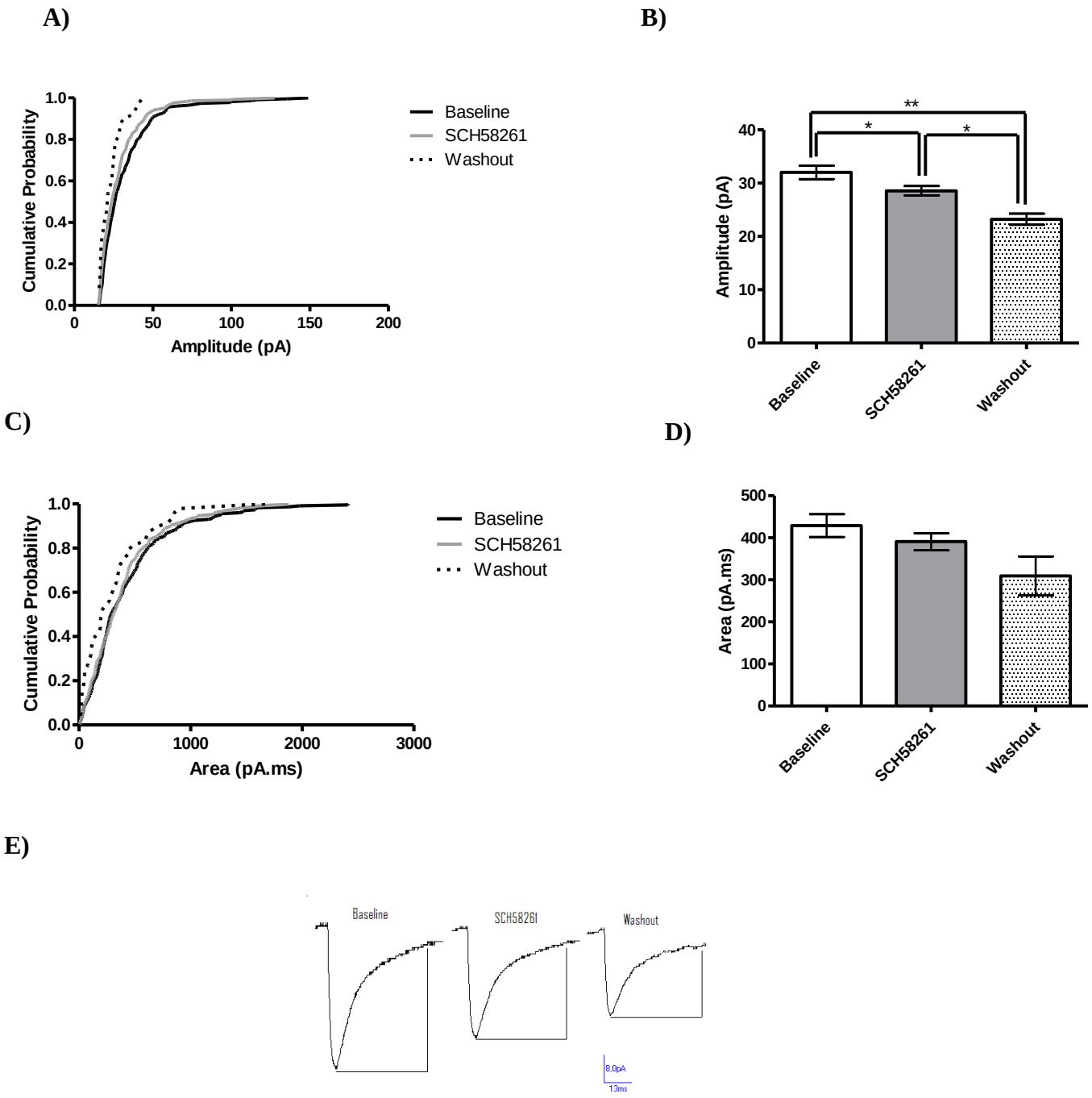


Fig. 3.7II-Effect of A_{2A} receptor antagonism on the amplitude and area of mIPSCs. SCH58261 (100 nM) seemed to decrease the amplitude of mIPSCs (**A** and **B**), an effect that is long-lasting since it was more robust 30 min after washout. The antagonist of A_{2A} receptor seemed to decrease the area of mIPSCs (**C** and **D**). **E)** Representation of mIPSCs showing a decrease in their amplitude after 20 min of exposure to SCH58261 and after 30 min of washout. A decrease in the area of mIPSCs was only detected 30 min and analysing the currents with distribution of areas of the currents with the Kolmogorov-Smirnov test. Results are mean +/- SEM of 14 independent recordings made in both pyramidal cells and interneurons from the *Stratum radiatum* of 5 to 7 days-old mice pups. Comparison between cumulative probability distributions was made using Kolmogorov-Smirnov test and the comparison between means (mean +/- SEM) was made using One-way ANOVA followed by Tukey test. Significant differences are indicated as *p<0.05 and **p<0.001.

Fig. 3.7III

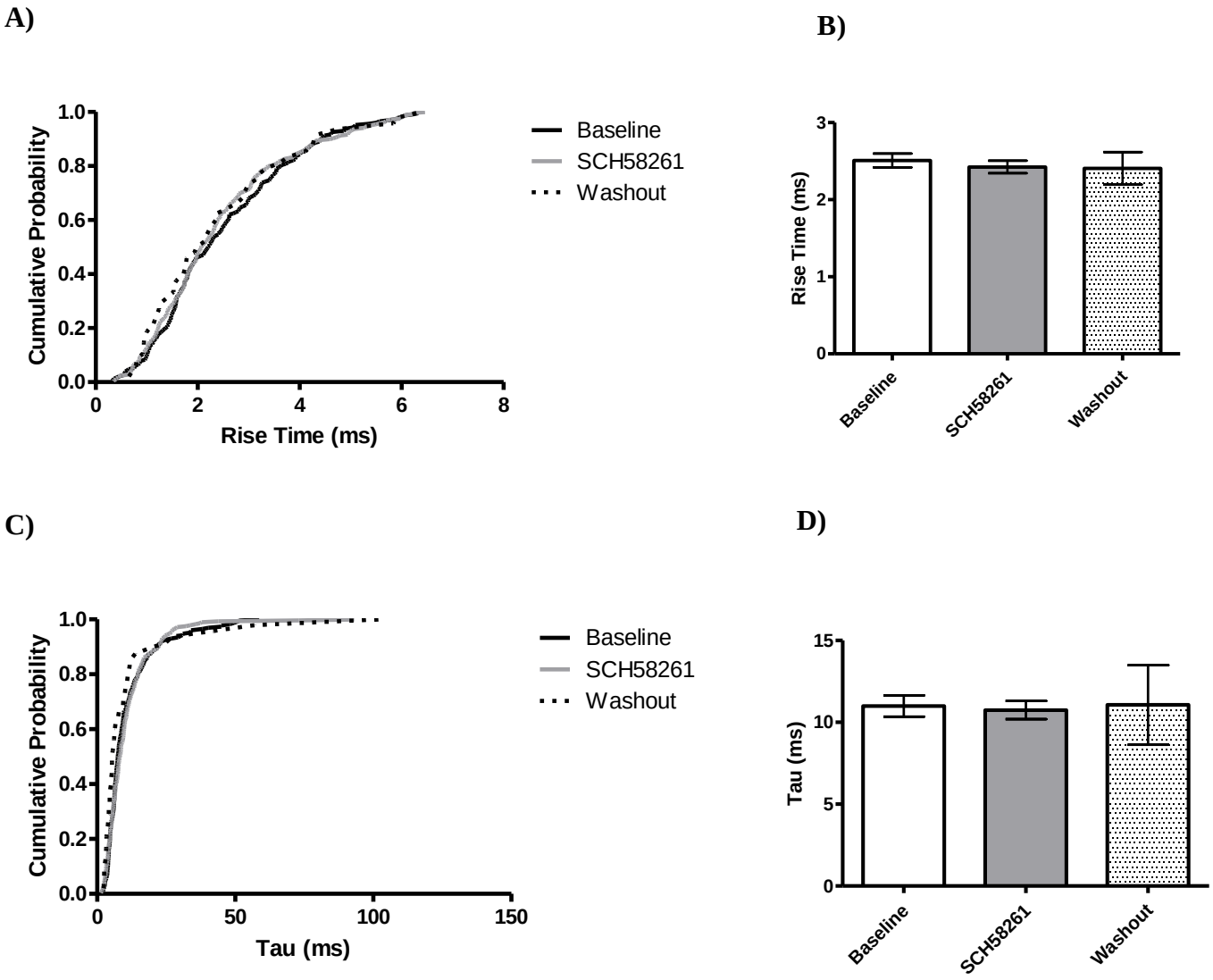


Figure 3.7III – Effect of A_{2A} receptor antagonism on the kinetic parameters of mIPSCs. Graphs showing the effect SCH58261 (100 nM) in the rise time (**A** and **B**) and decay time (**C** and **D**) of mIPSCs. SCH58261 triggered the decrease in the rise time and decay time of mIPSCs wich was only detectable 30 min after the washout of SCH58261 (n=14, $p<0.05$, Kolmogorov-Smirnov test). Results are mean +/- SEM of 14 independent recordings made in both pyramidal cells and interneurons from the *Stratum radiatum* of 5 to 7 days-old mice pups. Comparison between cumulative probability distributions was made using Kolmogorov-Smirnov test and the comparison between means (mean +/- SEM) was made using One-way ANOVA followed by Tukey test.

SCH58261 had also an effect upon the kinetic properties of mEPSCs. The average rise time of mEPSCs was 1.4 +/- 0.05 ms before SCH58261 exposure, 1.5 +/- 0.09 ms 20 min after perfusion with 100 nM SCH58261 and 1.2 +/- 0.08 ms after 30 min of washout. The fraction of mEPSCs displaying higher rise time values increased after exposure to SCH58261 ($n=5$, $p<0.0001$, Kolmogorov-Smirnov test - see **Fig. 3.8III A** and **B**). After 30 min of washout of SCH58261 however, the fraction of mEPSCs displaying lower rise time values increased ($n=5$, $p<0.0001$, Kolmogorov-Smirnov test). Decay time constant decreased significantly 30 min after the washout period ($n=5$, $p<0.05$, One-way ANOVA followed by Tukey test). The average decay time constants were 4.9 +/- 0.2 ms before SCH58261 application, 4.9 +/- 0.3 ms 20 min after SCH58261 application and 4.5 +/- 0.2 ms after 30 min of washout (**C** and **D**).

The reduction of the amplitude of the currents may suggest another postsynaptic mechanism which would contribute to a reduction of the frequency of the currents. The amplitude of the currents is correlated with the number of receptors for the ligand. A_1 and A_{2A} receptor-mediated signaling can make $GABA_A$ and AMPA/kainate receptors unstable at the plasma membrane, which could result in receptors removal. The lower the number of these postsynaptic ionotropic receptors could therefore contribute to a lower the probability of sensing the neurotransmitters present in the synapse.

DPCPX seems to have a more evident effect upon the area and kinetics of the mini GABA and mEPSC when compared with SCH58261. It is possible that both drugs lead to the same phenomenon by different signaling mechanisms, DPCPX by eventually changing the level of phosphorylation of the post-synaptic GABAergic and glutamatergic receptors and the A_{2A} receptor antagonism making these receptors unstable in the plasma membrane through a phosphorylation-independent mechanism.

Fig. 3.8

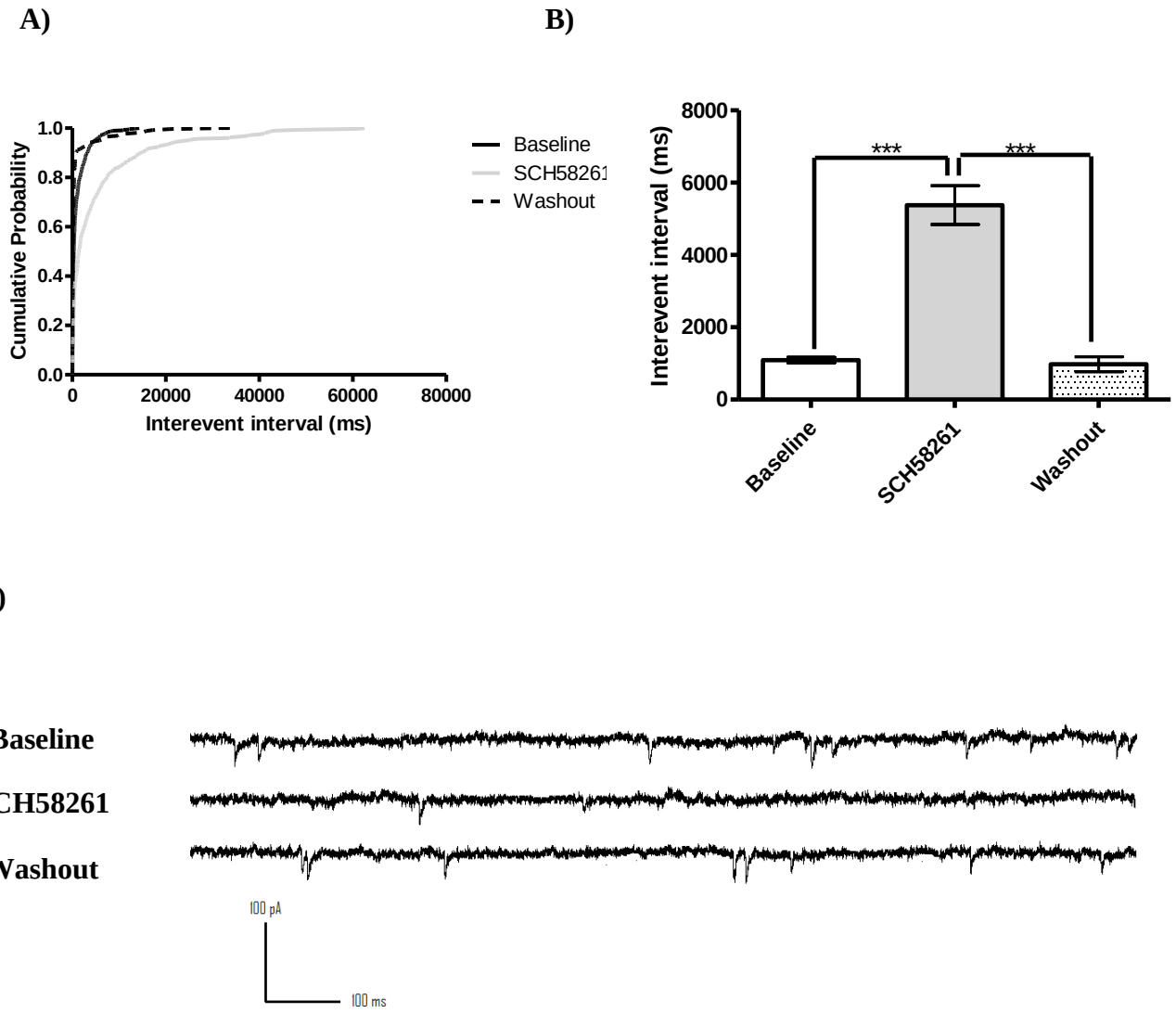


Figure 3.8I – Effect of A_{2A} receptor antagonism on the frequency of mEPSCs. SCH58261 (100 nM) significantly increased the interevent interval (**A** and **B**), however the effect seems to be reversible since a recovery of the basal interevent interval values corresponding to mEPSCs was observed after washout of this A_{2A} receptor antagonist. In **C)** is depicted a fraction of the recording made before, 20 min after the exposure to 100 nM SCH58261 and 30 min after the washout of SCH58261. Results are mean +/- SEM of 5 independent experiments made using both pyramidal cells and interneurons from the *Stratum radiatum* in the CA3 region of the hippocampus of 5 to 7 days-old mice pups. Comparison between cumulative probability distributions was made using Kolmogorov-Smirnov test and the comparison between means was made using One-way ANOVA followed by Tukey test. Statistical significance is indicated in the graphs as ***p<0.0001.

Figure 3.8II

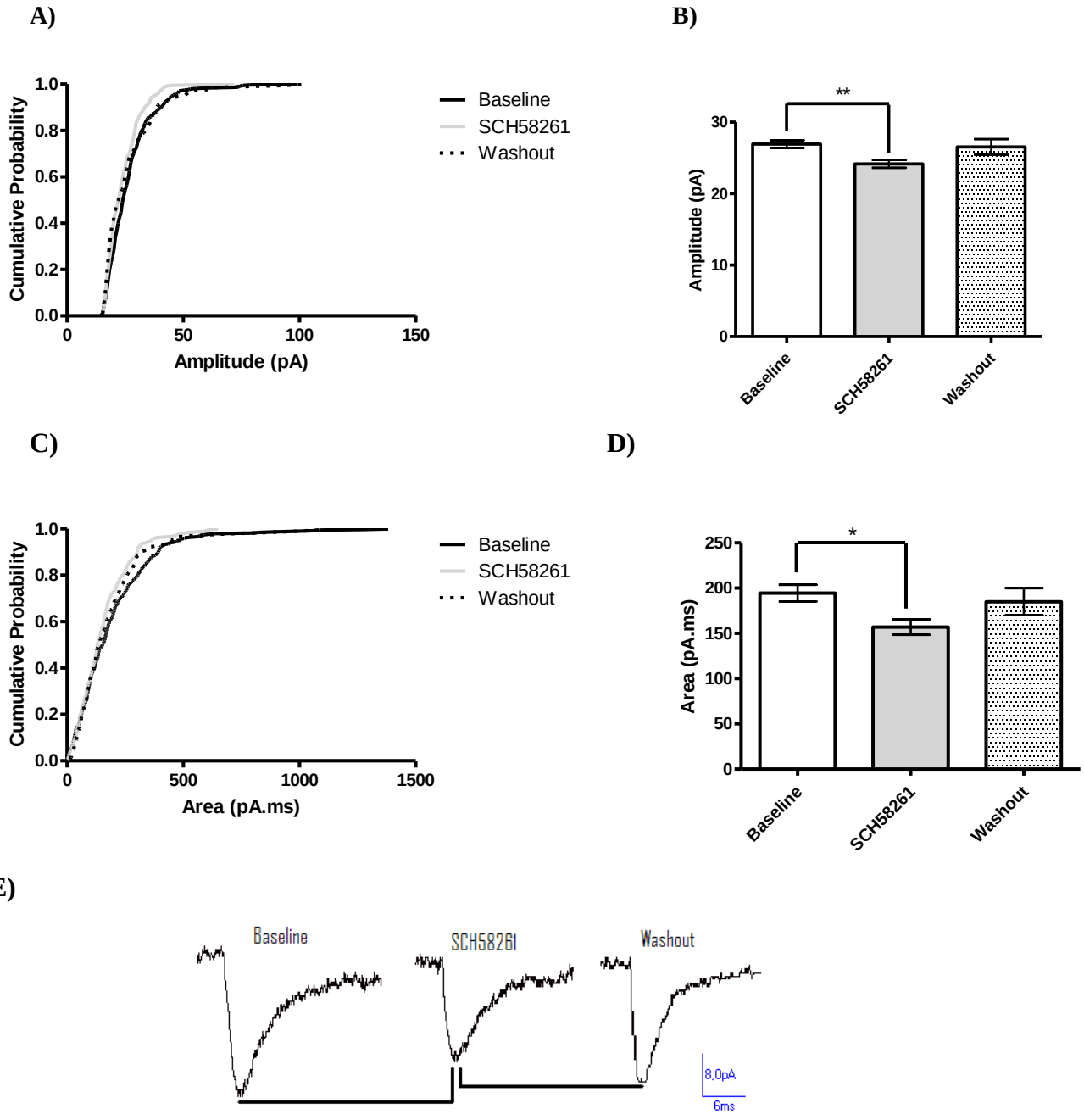


Figure 3.8II – **Effect of the A_{2A} receptor antagonism on the amplitude and area of mEPSCs.** SCH58261 (100 nM) significantly decreased the amplitude (**A** and **B**), and area (**C** and **D**) of mEPSCs. Both effects seem to be transient since 30 min after washout it was observed a recovery of these parameters to initial values (determined in the baseline) **E)** Representative representation of mEPSC currents showing the effect of SCH58261 upon the amplitude of mEPSCs and the recovery after washout. Results are mean +/- SEM of 5 independent recordings made in both pyramidal cells and interneurons from the *Stratum radiatum* in the CA3 region from slices belonging to 5 to 7 days-oldmice pups. Comparison between cumulative probability distributions was made using Kolmogorov-Smirnov test and the comparison between means was made using One-way ANOVA followed by Tukey test. Statistical significance is indicated in the graphs as *p<0.05 and **p<0.001.

Fig. 3.8III

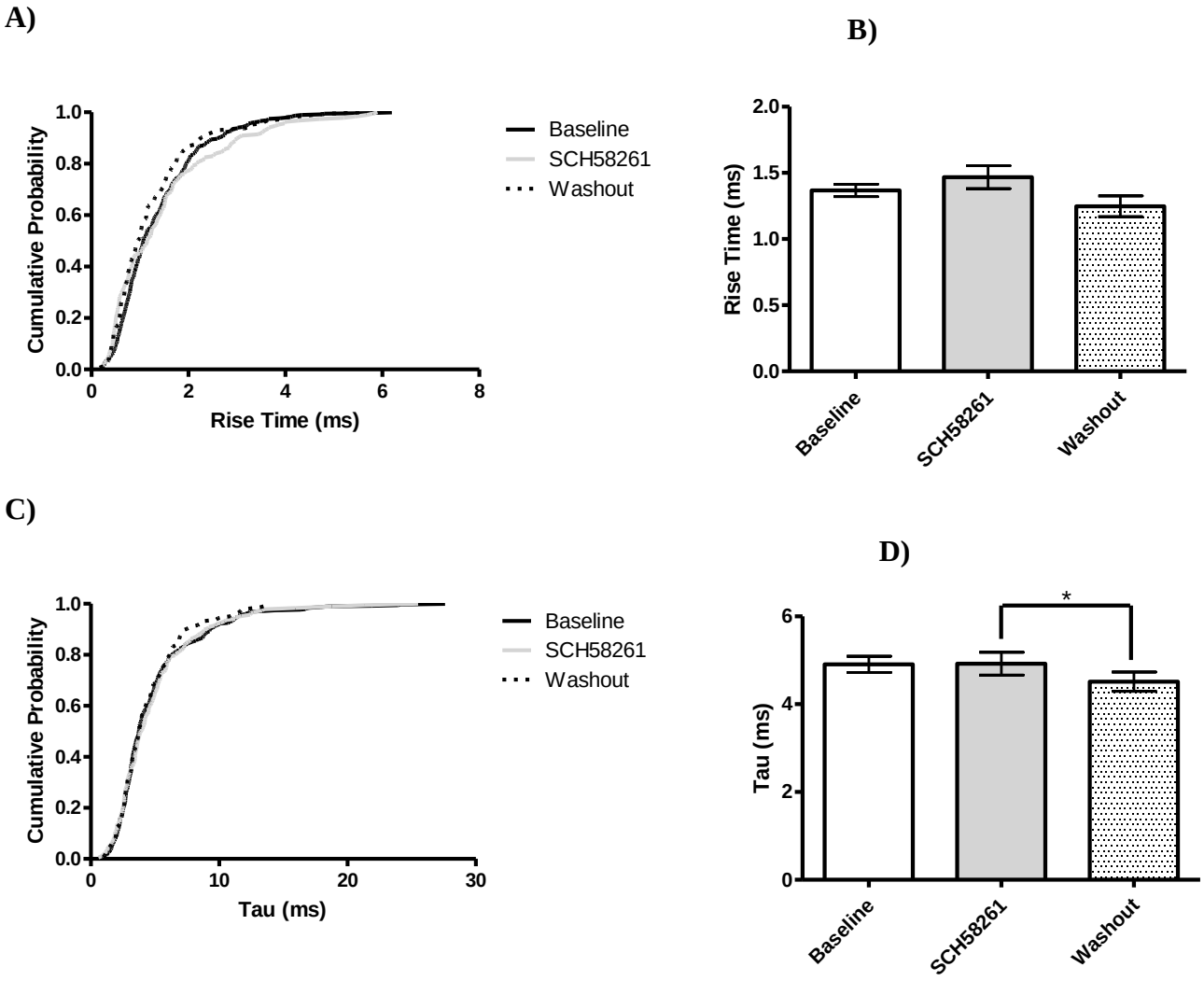


Fig. 3.8III – Effect of the A_{2A} receptor antagonism on the kinetic parameters of mEPSCs. Exposure of recorded cells (pyramidal cells and interneurons from the *Stratum radiatum* of the CA3 region of the hippocampus of 5-7 days-old mice pups) to SCH58261 (100 nM) triggered the increase in the rise time value of mIPSCs and the opposed effect was observed after 30 min of washout of SCH58261. These effects were subtle and detected only comparing the probability distributions for the rise time values with the Kolmogorov-Smirnov test (n=5, $p<0.05$). SCH58261 triggered a decrease in the decay time constant *tau* which was only detected in the washout period (n=5, $p<0.05$, one-way ANOVA followed by Tukey test). Results are mean +/- SEM of 5 independent experiments. The comparison between cumulative probability distributions was made using Kolmogorov-Smirnov test and the comparison between means was made using One-way ANOVA followed by Tukey test. Statistical significance is indicated in the graphs as*$p<0.05$.

The next section describes results providing evidence that AMPA receptors and $GABA_A$ receptors are lost from the plasma membrane. However, phosphorylation of $GABA_A$ receptors which is relevant for its trafficking and function (Lüscher and Keller, 2004), is not always associated to receptor instability. It may change the channel kinetics (Jones and Westbrook, 1997), rate of desensitization (Hinkle and Macdonald, 2003), mean open time or opening probability (Moss *et al.*, 1995). *In vitro* studies showed that the large intracellular loop domain of β1-3 and γ2 subunits are substrates for phosphorylation by serine/threonine and tyrosine kinases, such as PKC (Browning *et al.*, 1993; Moss *et al.*, 1992; Krishek *et al.*, 1994; Brandon *et al.*, 2000), PKA (Moss *et al.*, 1992; McDonald and Moss, 1997; Hinkle and Macdonald, 2003), PKG (McDonald and Moss, 1994, 1997), Ca^{2+}/calmodulin-dependent protein kinase II (McDonald and Moss, 1994, 1997), Akt/PKB (Wang *et al.*, 2003b) and Src (Brandon *et al.*, 2001). For example, phosphorylation of β3 subunit by PKA in residues Ser^{408}/Ser^{409} results in a potentiation of recombinant $GABA_A$ receptor response and phosphorylation of β1 subunit at Ser^{409} results in an inhibition (McDonald *et al.*, 1998). In neuronal cultures, PKA-mediated phosphorylation is only effective in the presence of PKC inhibitors (Brandon *et al.*, 2000) and is facilitated by A-kinase anchoring protein (AKAP), an adaptor protein that interacts with β1 and β3 subunits (Brandon *et al.*, 2003). β3 subunits is also phosphorylated by PKC in cultured neurons at Ser^{408}/Ser^{409} (Brandon *et al.*, 2002). PKC cand interact directly with β subunits or through the receptor for activated C-kinase (RACK-1) (Brandon *et al.*, 2002).

As referred previously, A_{2A} receptors may eventually act through phosphorylation independent-mecanisms. One possibility would be through modulation of cytoskeleton dynamics. A_{2A} receptor blockade could disorganize the scaffolding network that maintains $GABA_A$ receptors stable at the synaptic membrane. In inhibitory synapses, $GABA_A$ receptors co-locate with the tubulin-binding protein gephyrin (*e.g.* Sassoe-Pognetto *et al.*, 2000). Gephyrin is considerer a scaffolding protein required for clustering and/or stabilization of post-synaptic receptors. It seems that γ2 subunit is essential for initiation and maintenance of $GABA_A$ receptor clusters associated with gephyrin (Essrich *et al.*, 1998; Schweizer *et al.*, 2003). However, other reports show evidence for the formation of $GABA_A$ receptor clusters in the absence of gephyrin (Kneussel *et al.*, 2001; Danglot *et al.*, 2003). Receptors that are tagged with one or few ubiquitin moities can also be endocyted, deubiquitinated and recicled back to the plasma membrane. However, polyubiquitinated receptors are tagged irreversibly to degradation (Hicke and Dunn, 2003). Ubiquitination seems to play an important role in $GABA_A$ receptor trafficking (Saliba and Moss, 2003). Ubiquitination can also lead to AMPA receptor internalization (Colledge *et al.*, 2003; Bingol and Schuman, 2004). Palmitoylation

of some subunits of $GABA_A$ receptors also seems to be necessary to their trafficking and accumulation in the synapses, like as occurs with γ2 subunit (Rathenberg *et al.*, 2004). It is not known whether adenosine receptors may control any of these effects.

3.3. $GABA_A$ and AMPA Receptors are Internalized by Exposure to A_1 and A_{2A} Receptor Antagonists

The observation that A_1 and A_{2A} receptor antagonists decreased the AMPA/kainate and $GABA_A$ receptor-mediated responses led to the formulation of the hypothesis that maybe the adenosinergic signaling is necessary for the stabilization of those receptors at the plasma membrane. Its absence would lead to AMPA and $GABA_A$ receptor internalization or removal from synaptic sites.

To answer this question, two different approaches were used. The first one was an electrophysiological approach. Endocytosis of AMPA receptors is a clathrin-dependent process (Man *et al.*, 2000). Clathrin is recruited to the plasma membrane through adaptor proteins that bind transmembrane proteins (Slepnev and de Camilli, 2000). The assembly of clathrin molecules induces an invagination of the plasma membrane that is pinched off as a vesicle by dynamin, a GTPase, and transported by an actin-dependent manner to early endosomes, where proteins will be sorted to recycling endosomes, late endosomes or back to plasma membrane (Maxfield and McGraw, 2004). $GABA_A$ receptors can also be found in clathrin-coated vesicles, suggesting that they are removed from the surface by endocytosis through clathrin-coated pits (Tehrani and Barnes, 1993; Kittler *et al.*, 2000). Endocytosis seems to occurs when β and γ subunits bind to the clathrin adaptor AP-2 (Kittler *et al.*, 2000), which also seem to favor its interaction with GluR2 (Lee *et al.*, 2002). It seems that both GluR1- and GluR2-containing synapses have equivalent endocytosis (Ehlers, 2000). A clathrin-independent mechanism of endocytosis for $GABA_A$ receptors was also reported in HEK293 cells (Cinar and Barnes, 2001); however there is no evidence that it is relevant in neurons (Lüscher and Keller, 2004). Endocytosis was shown to be increased by PKC activation (Chapell *et al.*, 1998; Connolly *et al.*, 1999; Filippova *et al.*, 2000).

Since endocytosis of both AMPA and $GABA_A$ receptors seems to be a clathrin-dependent mechanism, cells were recorded with a patch pipette containing dynasore, a dynamin inhibitor that blocks the formation of endocytic vesicles (Macia *et al.* 2006). SCH58261 was used as the putative inductor of endocytosis. **Fig. 3.9**, shows the effect of dynasore in the amplitude of mEPSC currents

(**B**) and mIPSCs (**D**). The aim was to compare the effect of SCH58261 on the amplitude of miniature currents. Cells used in this experiment were interneurons from the *Stratum radiatum* of the CA3 area. As it can be seen in **Fig. 3.9B**, dynasore abrogated AMPA receptor internalization after exposure to SCH58261 (100 nM). In fact, the currents recorded during SCH58261 application, in the presence of dynasore, displayed higher amplitudes (27.0 +/- 1.0 pA by comparison with 19.9 +/- 0.7 pA before administration of 100 nM SCH58261, n=8, $p<0.0001$, two-tailed *t*-test) which is also visible by the shift to the right of the cumulative distribution of cells treated with SCH58261 in the presence of dynamin inhibitor. The same seemed to happen with mini GABA curents – see **Fig. 3.9D**. The mean amplitude of mIPSCs before SCH58261 application was 28.0 +/- 0.7 pA and after drug application was 30.5 +/- 0.9 pA (n=8, $p<0.05$ and using two-tailed *t*-test for comparison between the two means).

Receptors can move from one location to another through lateral difusion in the plasma membrane, for example, from synaptic to extrasynaptic sites or between sites of endocytosis and exocytosis (Triller and Choquet, 2005). In fact, extrasynaptic receptors constitute a pool of receptors that can be rapidly incorporated in the synapse (Bogdanov *et al.*, 2006; Tretter and Moss, 2008). Endocytosis is necessary for maintaining receptors in a cycling pool and to keep a constant number of receptors at synaptic locations. This can happen constitutively, or be regulated by synaptic activity or external stimuli. In the present example, the blockade of the recycling pathway with blocked using dynasore, causes more receptors to acumulate at synaptic sites and this can explain the increase in amplitude observed in cells, that have the endocytic pathway blocked and were treated with SCH58261.

As presented in **section 3.2**, SCH58261 was able to reduce the frequency of both mIPSCs and mEPSCs. **Fig 3.10** shows the the effect of dynasore on the interevent interval of mEPSCs and mIPSCs. Blocking endocytosis resulted in an increase of the number of receptors present at synaptic sites. It *per se* can decrease the interevent interval or increase the frequency of both excitatory (**B**) and inhibitory (**D**) miniature currents. These results can be viewed as an aditional evidence supporting the hypothesis that A_{2A} receptor blockade changes the frequency of the currents by postsynaptic mechanisms.

Fig. 3.9

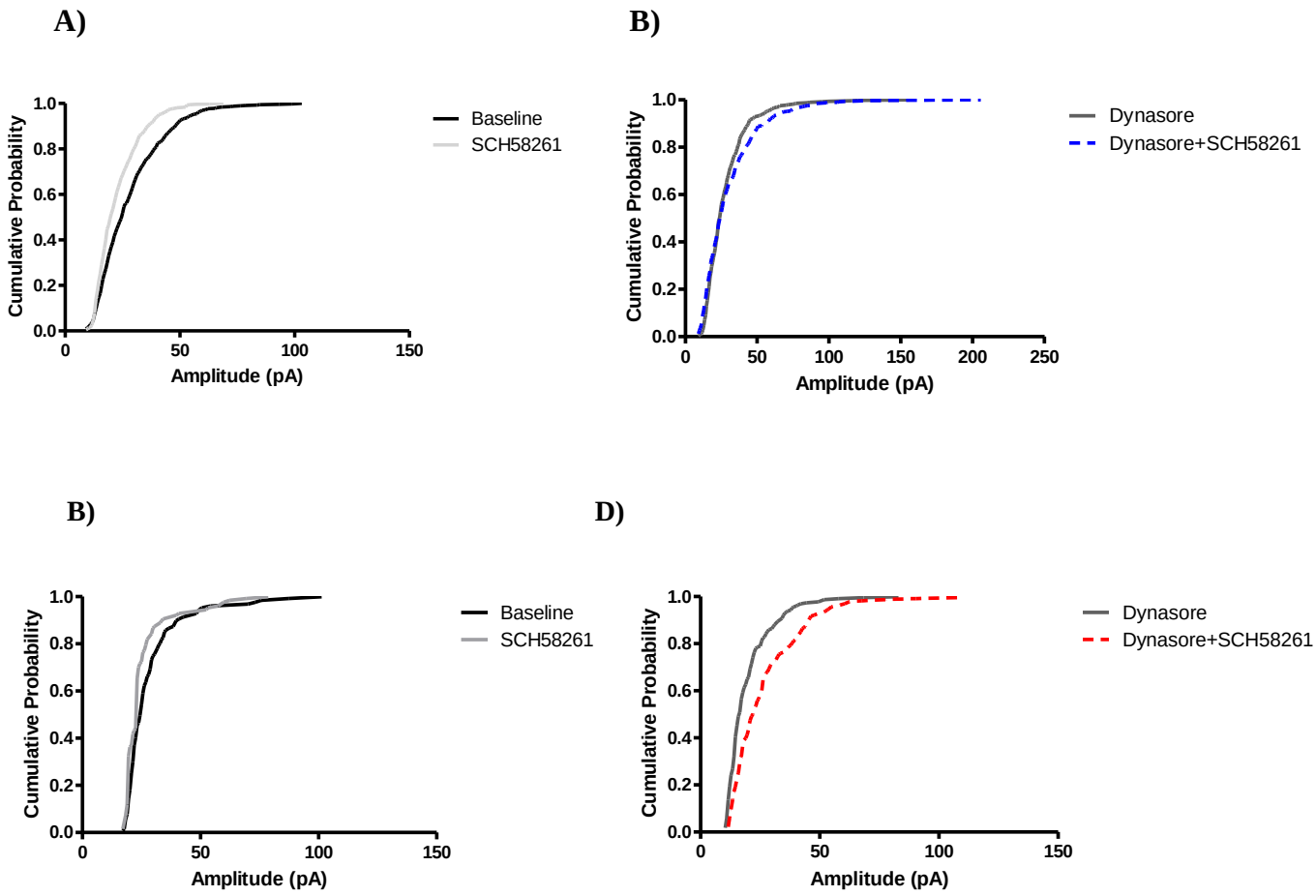


Fig. 3.9 – Effect of inhibiting clathrin-mediated endocytosis on the impact of A_{2A} receptor blockade on the amplitude of miniature GABAergic and glutamatergic-mediated currents. Blockade of clathrin-mediated endocytosis with dynasore (20 µM) applied through the patch pipette, seems to abrogate A_{2A} receptor antagonist-induced decrease in amplitude of mEPSC (**A** and **B**) and miniGABA (**C** and **D**) currents recorded in the *Stratum radiatum* interneurons of the CA3 area. This suggests that the decrease of the number of these ionotropic receptors at the plasma membrane triggered by SCH58261 involves their internalization by a clathrin-dependent mechanisms. Comparing with the distributions depicted in (**A**) and (**C**), it also seems that the blockade of the endocytic pathway increases the amplitudes of mEPSC and mIPSCs (see the shift of the distributions to the right), indicating the presence of a higher number of receptors in the membrane and suggesting that the recycling of the receptors in synaptic membranes is a clathrin-dependent process. Depicted results were analysed with the Kolmogorov-Smirnov test to compare differences in the cumulative probability distributions (n=8).

Fig. 3.10

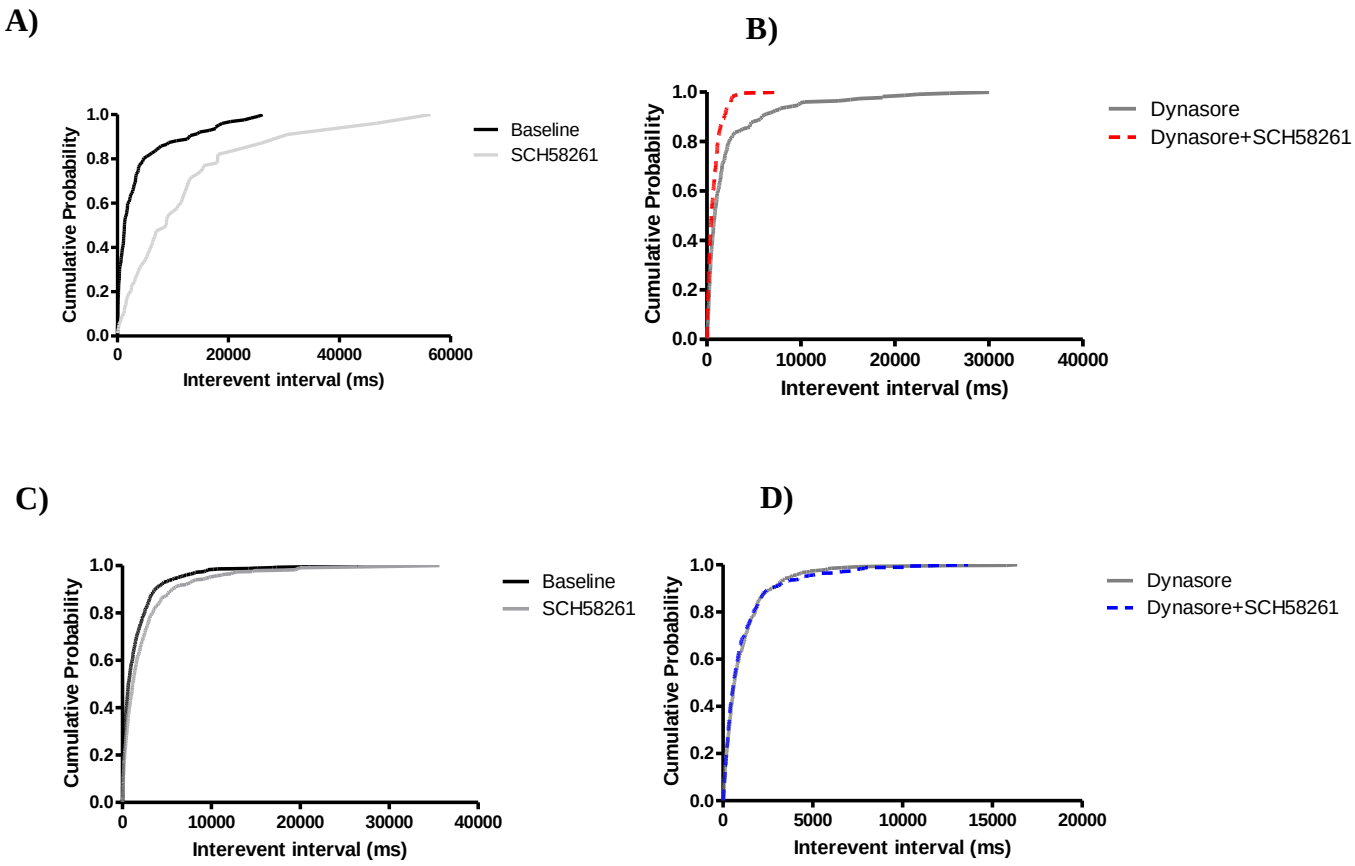


Fig. 3.10 - Effect of inhibiting clathrin-mediated endocytosis on the impact of A_{2A} receptor blockade on the frequency of miniature GABAergic and glutamatergic-mediated currents. Effect of dynasore in the interevent interval of mEPSC (**B**) and mIPSC (**D**). Blockade of clathrin-mediated endocytosis abrogates SCH58261-induced increase in the interevent interval of these currents, supporting the hypothesis that A_{2A} receptor can modulate the frequency at which a cell receive glutamatergic and GABAergic currents, by changing the number of AMPA- and GABA-responsive receptors in the synapse. 8 interneurons from the *Stratum radiatum* of the CA3 area were used in this analysis and cumulative probability distributions were analysed by Kolmogorov-Smirnov test.

The second strategy used to strenghten the hypothesis that the blockade of adenosine receptors is involved in AMPA and $GABA_A$ receptor endocytosis was based on a cell biology approach. Hippocampal neurons were transfected with γ2 (a $GABA_A$ receptor subunit) or with GluR1 (an AMPA receptor subunit). The constructs consisted of the coding sequence for the subunits atached to a sequence coding mutated GFP, called supecliptic fluorin (SEP) (Ashby *et al.*, 2004). This mutation confers the protein, once expressed, the property of being sensitive to pH. SEP was tagged to the N-terminus of both γ2 and GluR1 subunits, facing the extracellular space in which the pH is around 7.0.

Subunits are fluorescent at physiological ranges of pH. During the process of internalization, subunits are inserted into endocytic vesicles, where pH drops to approximately to 5.0-6.0 shortly after formation (Tycko and Maxfield, 1982). In this case, fluorescence is lost. This ability of SEP to sense changes in pH was used to monitor the movement of $GABA_A$ receptor containing γ2 subunit and AMPA receptor containing GluR1, from the membrane to intracellular compartments or vice-versa. Both A_1 and A_{2A} receptor antagonists were tested in cells transfected with γ2-SEP or GluR1-SEP subunits. In almost all cells, γ2-SEP as well as GluR1-SEP expression was restricted to the cell body from neurons maintained in culture for seven days. Both DPCPX (**Fig. 3.11** and **Fig. 3.15**) or SCH58261 (**Fig. 3.12** and **Fig. 3.16**) decreased the fluorescence of γ2-SEP, suggesting that they triggered an internalization of the $GABA_A$ and AMPA receptors. Interestingly, SCH58261 caused an aditional effect: it increased the fluorescence of γ2-SEP in some cells albeit this effect was observed less frequently than the decrease of fluorescence – see **Fig. 3.13**. The phenotype of cells was not evaluated, which percludes the possibility of establishing a correlation between the type of response and the cell type or degree of maturation. As referred in the **Introduction** of the **Chapter 2**, hippocampal neuronal cultures are mainly constitued by pyramidal cells, but a low percentage of interneurons can also be present, which may account for the distinct characteristics observed (Benson *et al.*, 1994). Another possibility is that, in some cells, SCH58261 stimulated exocytotic pathway leading to insertion of *de novo* subunits in the plasma membrane (this possibility will be discussed further away in the text).

The selective agonist of A_{2A} receptors, CGS21680, used at a concentration of 30 nM, did not increase the fluorescence of γ2-SEP. In fact, the drug seems to decrease the intensity of fluorescence, thus causing an effect qualitatively similar to that caused by the A_{2A} receptor antagonist (see **Fig. 3.14**). This may be due to the fact that exposure to A_{2A} receptor agonists can cause a rapid desensitization and internalization of A_{2A} receptors and, from the functional point of view, acting as an antagonist (Palmer *et al.*, 1994; Brand *et al.*, 2008).

Fig. 3.11

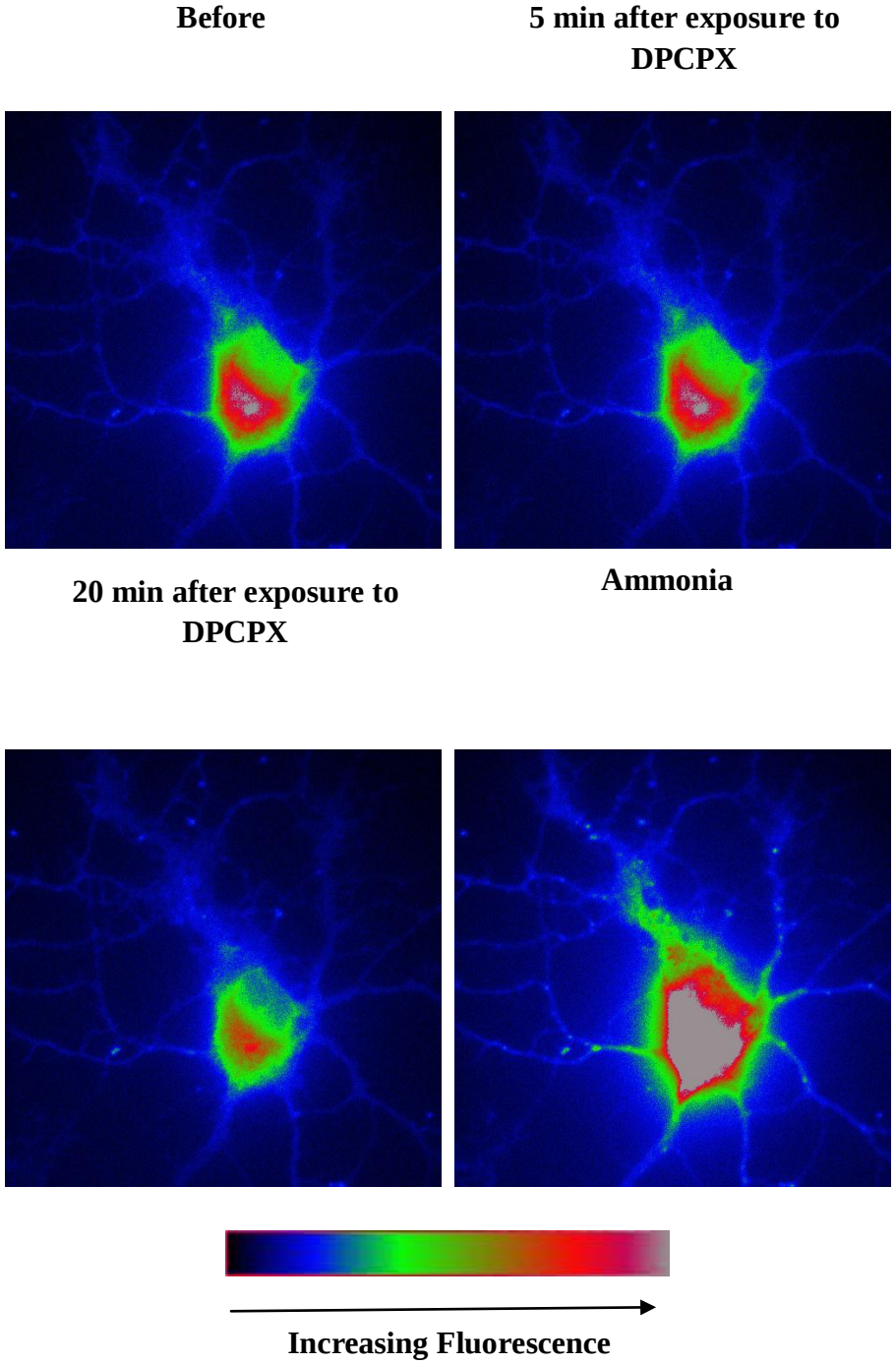


Fig. 3.11 – Effect of the A_1 receptor antagonism in the internalization of γ2 subunit of $GABA_A$ receptor-SEP. The images are representative of the effect exerted by 100 nM DPCPX, an A_1 receptor antagonist, in the fluorescence of γ2-SEP (visible in the figure especially in the cell body of the neuron) in 1 out of 3 cells analyzed. The transfection with the γ2-SEP subunit was done at DIV4 and the effect of the drug evaluated at DIV7. The reduction of the fluorescence was visible 20 min after incubation with DPCPX and suggests an incorporation of the receptor in acidic endocytic vesicles (since the fluorescence of SEP is lost at acidic pH). The maximum of fluorescence corresponds to the white color in the scale bar of intensity and the lowest level of fluorescence corresponds to the black color. It was made a qualitative analysis of the variation of the fluorescence and ammonia was used to increase intra and extracellular pH in order to show the maximum of fluorescence. The neuronal phenotype was not defined in tese experiments.

Fig. 3.12

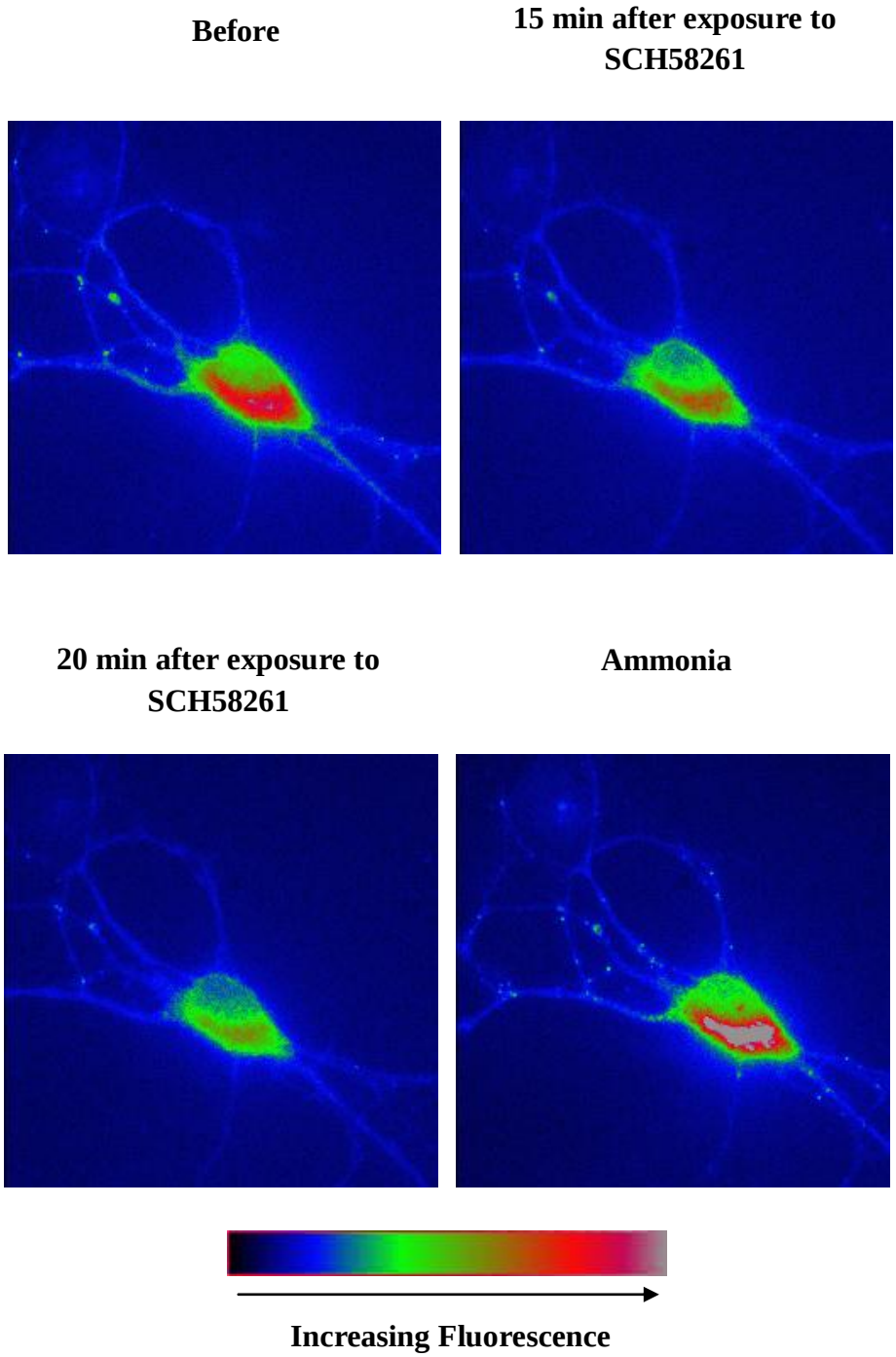


Fig. 3.12- Effect of the A_{2A} receptor antagonism in the internalization of γ2 subunit of $GABA_A$ receptor-SEP. The images are representative of the effect exerted by 100 nM SCH58261, an A_{2A} receptor antagonist, in the fluorescence of γ2-SEP (visible in the figure especially in the cell body of the neuron) in 1 out of 5 cells analyzed. The transfection with the γ2-SEP subunit was done at DIV4 and the effect of the drug evaluated at DIV7. The reduction of the fluorescence was visible 20 min after incubation with SCH58261 and suggests an incorporation of the receptor in acidic endocytic vesicles (since the fluorescence of SEP is lost at acidic pH). The maximum of fluorescence corresponds to the white color in the scale bar of intensity and the lowest level of fluorescence corresponds to the black color. It was made a qualitative evaluation of the variation of fluorescence and ammonia was used to increase intra and extracellular pH in order to show the maximum of fluorescence. The neuronal phenotype was not defined in tese experiments.

Fig. 3.13

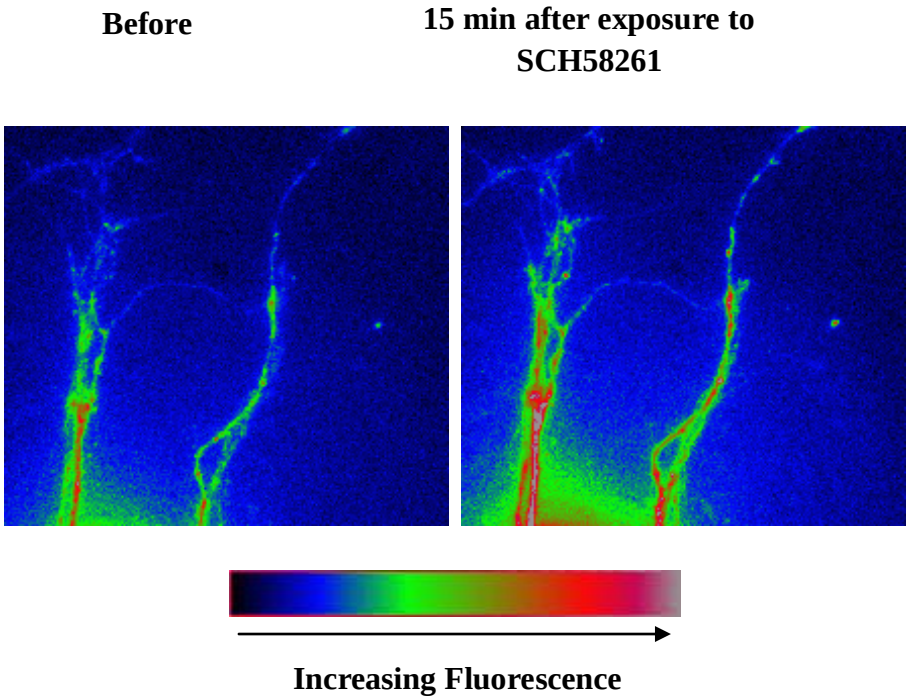


Fig. 3.13 – Effect of the A_{2A} receptor antagonism in the internalization of γ2 subunit of $GABA_A$ receptor-SEP: another profile. The images are representative of the effect exerted by 100 nM SCH58261, an A_{2A} receptor antagonist, in the fluorescence of γ2-SEP (visible in the figure especially in the cell body of the neuron) in 1 out of 3 cells analyzed. The transfection with the γ2-SEP subunit was done at DIV4 and the effect of the drug evaluated at DIV7. The reduction of the fluorescence was visible 20 min after incubation with SCH58261 and suggests an incorporation of the receptor in acidic endocytic vesicles (since the fluorescence of SEP is lost at acidic pH). The maximum of fluorescence corresponds to the white color in the scale bar of intensity and the lowest level of fluorescence corresponds to the black color. It was made a qualitative evaluation of the variation of fluorescence and ammonia was used to increase intra and extracellular pH in order to show the maximum of fluorescence. The neuronal phenotype was not defined in tese experiments.

The results from another set of experiments increased the complexicity of the effects attributed to A_{2A} receptors. When 50 μM GABA were applied to neurons in the presence of TTX (1 μM), SCH58261 (100 nM) caused either an increase or a decrease of the amplitude of GABA-evoked responses (**Fig. 3.19**). In **A**) it can be seen that the mean amplitude of GABA-evoked responses changed from a baseline value of 100% to 71.2 +/- 5.6% (n=3, $p<0.05$, one sample *t*-test) after 20 min of exposure to SCH58261.

Fig 3.14

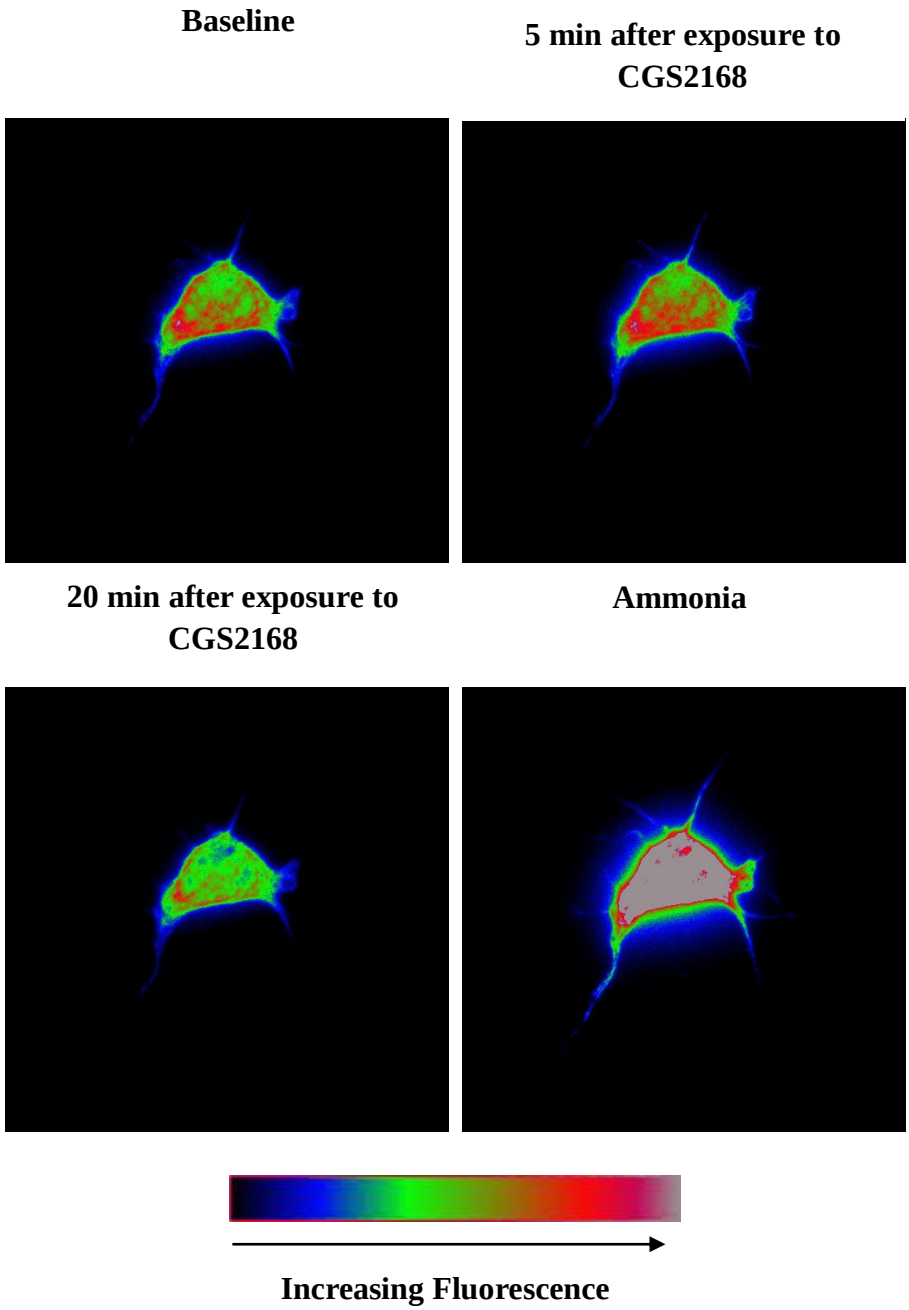


Fig 3.14 – Effect of the A_{2A} receptor agonism in the internalization of γ2 subunit of $GABA_A$ receptor. The images are representative of the effect exerted by 30 nM CGS6180, an A_{2A} receptor agonist, in the fluorescence of γ2-SEP (visible in the figure especially in the cell body of the neuron) in 1 out of 3 cells analyzed. The transfection with the γ2-SEP subunit was done at DIV4 and the effect of the drug evaluated at DIV7. The reduction of the fluorescence was visible 20 min after incubation with SCH58261 and suggests an incorporation of the receptor in acidic endocytic vesicles (since the fluorescence of SEP is lost at acidic pH). The maximum of fluorescence corresponds to the white color in the scale bar of intensity and the lowest level of fluorescence corresponds to the black color. It was made a qualitative evaluation of the variation of fluorescence and ammonia was used to increase intra and extracellular pH in order to show the maximum of fluorescence. The neuronal phenotype was not defined in tese experiments.

Fig 3.15

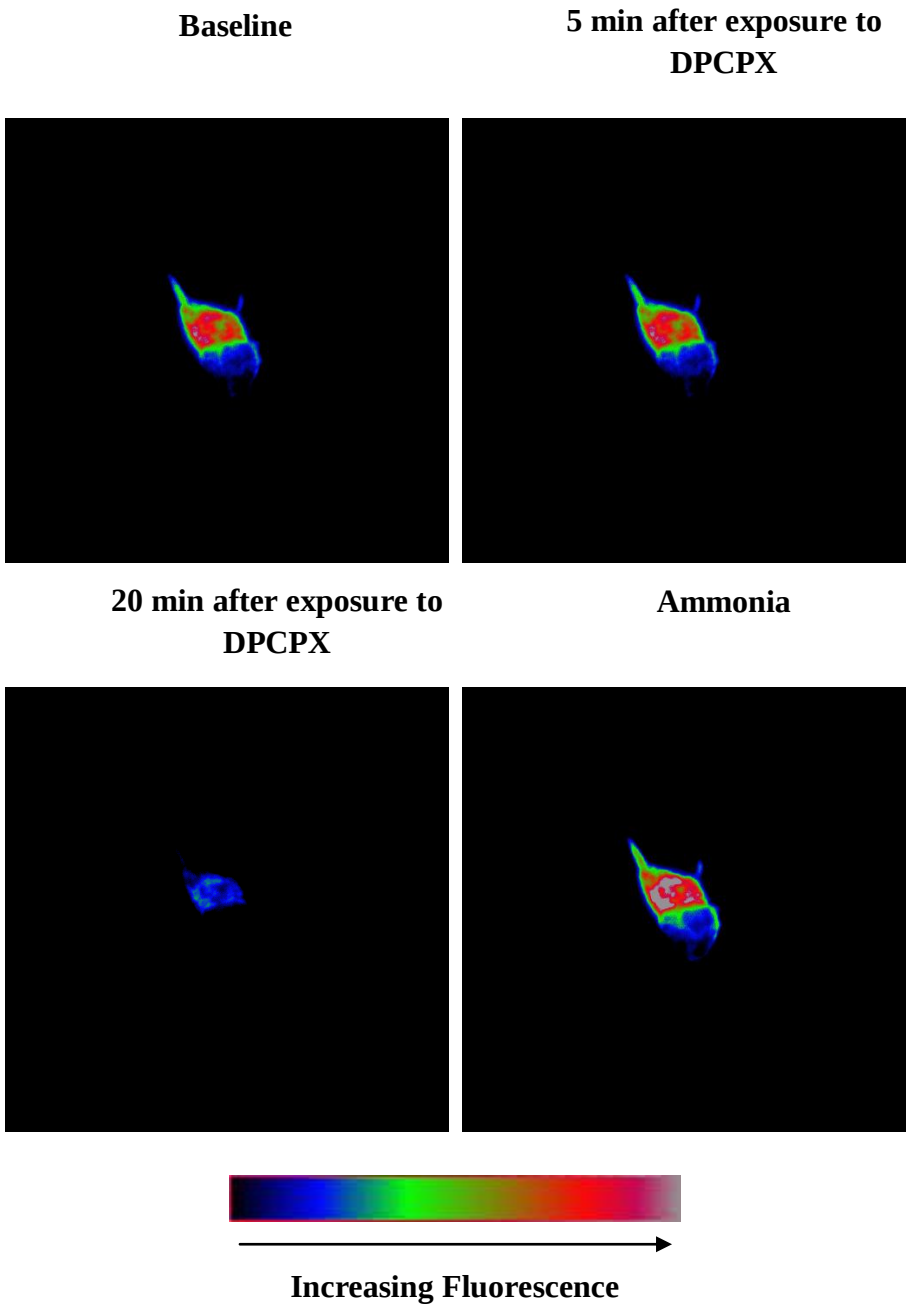


Fig. 3.15 – Effect of the A_1 receptor blockade in the internalization of GluR1 subunit of AMPA receptor-SEP. The images are representative of the effect exerted by 100 nM DPCPX, an A_1 receptor antagonist, in the fluorescence of GluR1-SEP (visible in the figure especially in the cell body of the neuron) in 1 out of 3 cells. The transfection with the GluR1-SEP subunit was done at DIV4 and the effect of the drug evaluated at DIV7. The reduction of the fluorescence was visible 20 min after incubation with SCH58261 and suggests an incorporation of the receptor in acidic endocytic vesicles (since the fluorescence of SEP is lost at acidic pH). The maximum of fluorescence corresponds to the white color in the scale bar of intensity and the lowest level of fluorescence corresponds to the black color. It was made a qualitative evaluation of the variation of fluorescence and ammonia was used to increase intra and extracellular pH in order to show the maximum of fluorescence. The neuronal phenotype was not defined in tese experiments.

Fig. 3.16

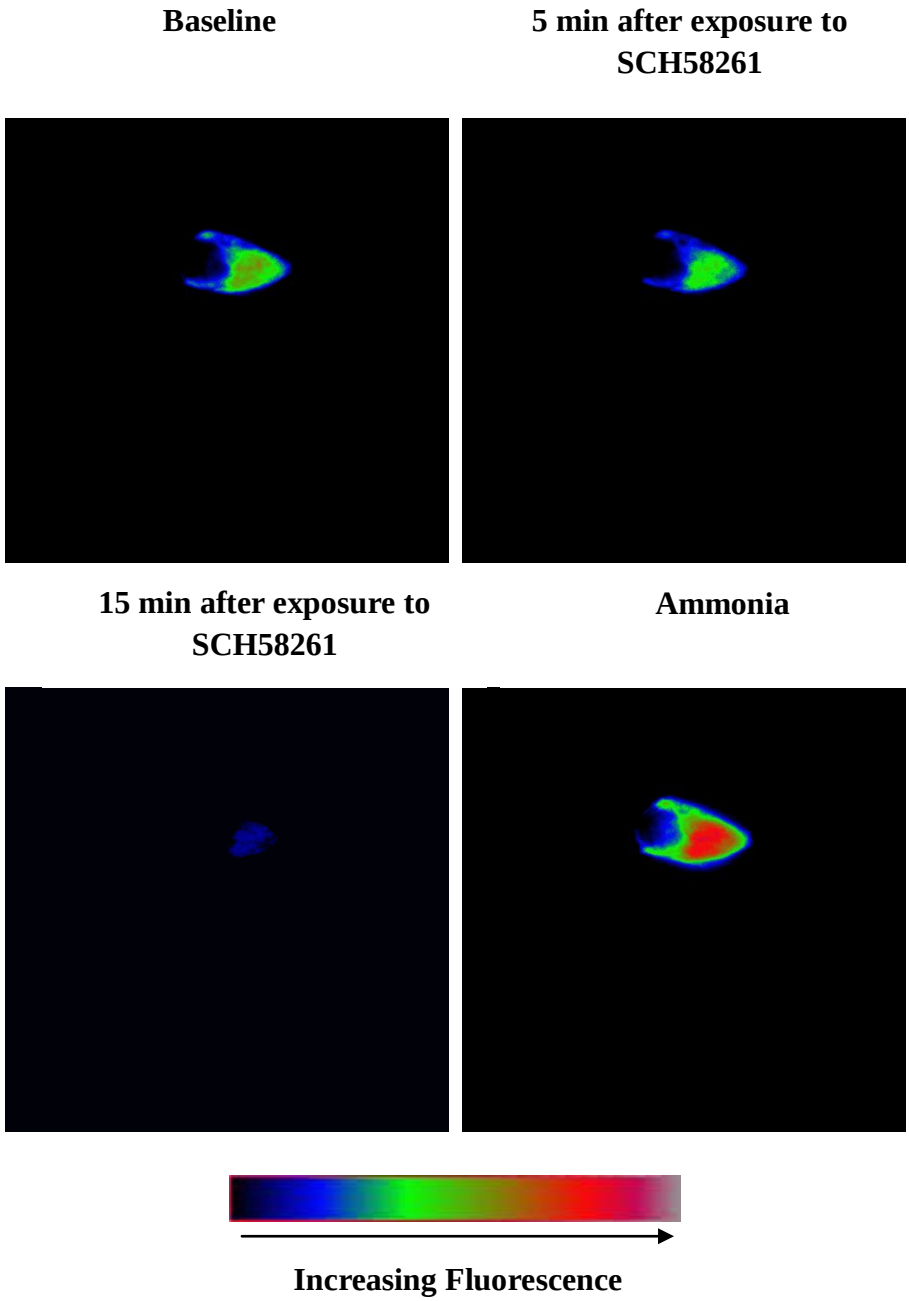


Fig. 3.16 - Effect of the A_{2A} receptor antagonism in the internalization of GluR1 subunit of AMPA receptor-SEP. The images are representative of the effect exerted by 100 nM SCH58261, an A_1 receptor antagonist, in the fluorescence of GluR1-SEP (visible in the figure especially in the cell body of the neuron) in 1 out of 3 cells. The transfection with the GluR1-SEP subunit was done at DIV4 and the effect of the drug evaluated at DIV7. The reduction of the fluorescence was visible 20 min after incubation with SCH58261 and suggests an incorporation of the receptor in acidic endocytic vesicles (since the fluorescence of SEP is lost at acidic pH). The maximum of fluorescence corresponds to the white color in the scale bar of intensity and the lowest level of fluorescence corresponds to the black color. It was made a qualitative evaluation of the variation of fluorescence and ammonia was used to increase intra and extracellular pH in order to show the maximum of fluorescence. The neuronal phenotype was not defined in tese experiments.

Fig. 3.17

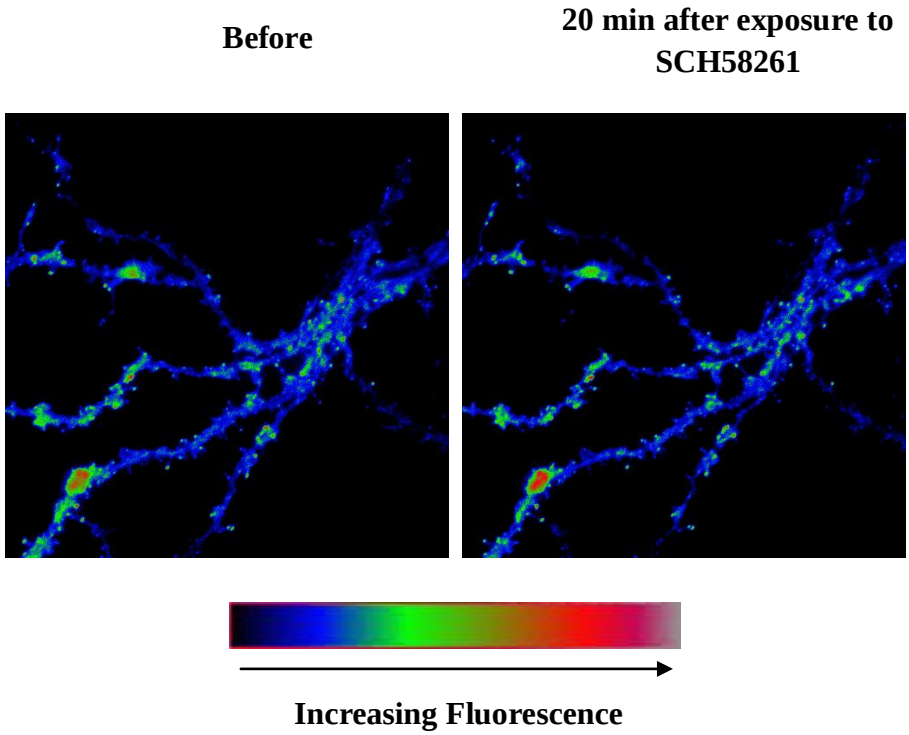


Fig. 3.17 - Effect of the A_{2A} receptor antagonism in the internalization of GluR1 subunit of AMPA receptor-SEP in "mature" neurons. The images are representative of the effect exerted by 100 nM SCH58261, an A_1 receptor antagonist, in the fluorescence of GluR1-SEP (visible in the dendrites) in 1 out of 3 cells. The transfection with the GluR1-SEP subunit was done at DIV4 and the effect of the drug evaluated at DIV21. It was not observed any effect in the fluorescence of GluR1-SEP even 20 min after the incubation with SCH58261 which suggests that A2A receptor only modulates the dynamics of GluR1 receptor in the plasma membrane in young neurons (DIV 7) not in mature neurons. The maximum of fluorescence corresponds to the white color in the scale bar of intensity and the lowest level of fluorescence corresponds to the black color. The neuronal phenotype was not defined in tese experiments.

This effect was similar to the typical effect observed for mIPSCs. In **B**), a faster effect was seen after exposure to SCH58261. After an initial decrease (from 100% in the baseline from 65.0 +/- 1.8%, (n=3, $p<0.05$, one sample *t*-test) of the GABA-evoked response generated between 5 to 10 min of exposure to SCH58261, a sustained increase from 10 to 25 min was observed. The increase in GABA-evoked response from 10 to 15 min after exposure to SCH58261 (100 nM) was 400.8 +/- 115.1% (n=4, $p<0.05$, one sample *t*-test) and 221.5 +/- 19.5%, between 20 to 25 min of SCH58261 (100 nM) administration (n=3, $p<0.05$, one sample *t*-test).

The pressure application of a neurotransmitter is able to activate both synaptic and extrasynaptic receptors (see for example Suzuki *et al.*, 2008). It is thus possible that this new effect is mainly due to extrasynaptic $GABA_A$ receptors since the amplitude of mIPSCs was never seen to increase.

Several explanations can be proposed to justify the present observation. One of them is that, in some cells, $GABA_A$ receptors moving away from the synapse will occupy extrasynaptic sites; in fact receptors can be endocyted and recycled back to the plasma membrane in extrasynaptic sites. Another possibility is that synaptic $GABA_A$ receptors are endocyted and new receptors produced in the Golgi will begin occupying extrasynaptic sites. Receptors are synthesized and procesed in the ER and then trafficked to the Golgi for final processing, sorting and packaging for delivery to the final destination (Horton and Ehlers, 2004). ER, Golgi apparatus, ER-Golgi intermediate compartment (ERGIC) and trans-Golgi network (TGN), form the **exocytic system**. A network of tubulo-vesicular structures composed of early endosomes, recycling endosomes, late endosomes and lysosomes (Maxfield and McGraw, 2004), is involved in the reception of internalized receptors from the plasma membrane; it is here where the "decision" of recycling or degrading a receptor is made – **endocytic system**. Cargo is kept in early endosomes just for a few minutes. When they acidify and maturate they constitute a late endosome. Endosomes can be found in the soma or in dendrites (Ehlers, 2000) of neurons. Proteins that are targeted for degradation receive a tag of ubiquitin (monoubiquitination) and they are recognized by late endosomes (*e.g.*Katzmann *et al.*, 2001, 2002). Vesicles (intralumenal multivesicular body vesicles) where these proteins are accumulated then fuse with lysosomes leading to receptor degradation.

There is a crosstalk between the exocytic and endocytic systems. The endosomal compartment receives both endocytic cargoes from plasma membrane and TGN (Shin *et al.*, 2004). Proteins that are not targeted to late endosomes give rise to the **recycling endosome compartment**. A direct transition from early endosomes to the plasma membrane can take only few minutes (Maxfield and McGraw, 2004), however, some proteins may take a longer time (15 min-30 min) to reach the membrane again and it is the activity of the synapse that may determine if a receptor will be recycled or degraded (Kittler *et al.*, 2005). Recycling process can occur several times. GluR1-, GluR2/3-containing subunits are recycled according to synaptic activity (Ehlers, 2000). This constitutive process is accelerated with neuronal activity (Ehlers, 2000). For example, activation of NMDA receptors promotes AMPA receptors internalization (Beattie *et al.*, 2000; Ehlers, 2000) that will be recycled later (Ehlers, 2000) through a process dependent on Ca^{2+}, PP1, calcineurin and PKA (Ehlers, 2000). If endocytosis is triggered by agonist exposure (in the absence

Fig. 3.18

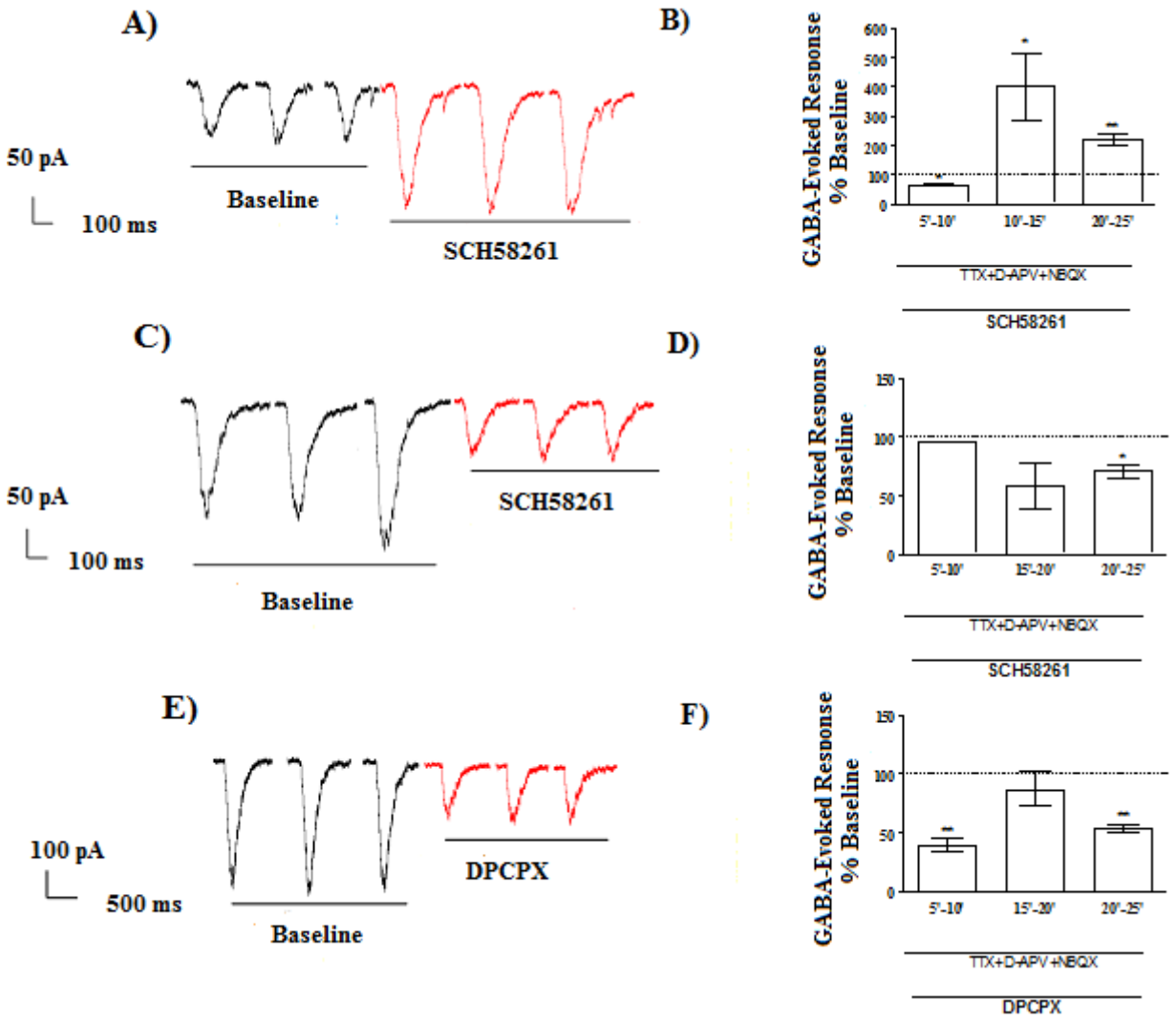


Fig. 3.18 – **Modulation of the evoked-GABA response by A_1 and A_{2A} receptor antagonists.** The antagonist of A_{2A} receptors SCH58261 (100 nM) induced two different types of response when 50 µM GABA were pressure applied close to cell body of neurons (pyramidal cells and interneurons from the *Stratum radiatum* of the CA3 region. In **A**), a significant increase in the amplitude of GABA-evoked response was observed as soon as 10 min after the beginning of application SCH58261 (see the average response in % of baseline depicted in **B)**). In contrast, other cells (**C**), show a significant decrease of the amplitude of the GABA-evoked response that can be observed 15 min after SCH58261 application (see the average response in % of baseline depicted in **D)**). The type of response was not cell-type dependent. In **E**) is depicted the effect of 100 nM of DPCPX, an antagonist of A_1 receptor, in the evoked-GABA response. Blockade of A_1 receptor seems to significantly decrease the amplitude of the response after 5 min and 20 min of exposure to the drug (see the average response in % of baseline in **F)**). Results are mean +/-SEM of 3-5 independent experiments and correspond to the percentage of increase or decrease of the evoked response above or bellow the baseline level (100%). Statistical significance is indicated in the graphs as *p<0.05; **p<0.001 One sample *t*-test was used to compare between the means.

of NMDA receptor activation), AMPA receptors seem to be inserted in late endosomes. A similar process between the signals and trafficking occurs for kainate receptors (Martin and Henley, 2004).

Fig. 3.18F) shows the variation of the mean amplitude of GABA-evoked response in the absence and in the presence of the 100 nM DPCPX. As observed for the mIPSCs, the mean amplitude of evoked-GABA response during the baseline was again considerer 100% and it decreased to 39.6 +/- 5.9% (n=3, $p<0.05$, one sample *t*-test) 5 min after exposure to DPCPX; this effect was not constant since between 15 to 20 min the mean value of GABA-evoked response (87.1% +/- 14.7%, n=3, $p>0.05$, one sample *t*-test) was not different from the mean amplitude value for the GABA-evoked responses recorded at baseline. Between 20 to 25 min of exposure to DPCPX, a new depression of GABA-evoked responses was observed; mean amplitude values, at this period of time were 53.2 +/- 3.5% (n=3, $p<0.0044$, one sample *t*-test) (see also representative responses recorded during baseline and DPCPX application depicted in **E**). Independently on the type of GABA-evoked response, the antagonists of A_1 (n=6, $p<0.05$, Kolmogorov-Smirnov test) and A_{2A} (n=6, $p<0.05$, Kolmogorov-Smirnov test) receptors decreased the amplitude of mIPSCs (see **Fig. 3.19**).

Brefeldin A, a fungal metabolite that disrupts Golgi apparatus, was used in the pipette solution to test if the increase in the amplitude of GABA-evoked response induced by A_{2A} receptor blockade would correspond to an insertion of new $GABA_A$ receptors arriving from vesicles generated in the Golgi apparatus in the plasma membrane. In **Fig. 3.20**, is depicted the effect of brefeldin A on GABA-evoked response (**A**) and in mIPSCs (**B**). Disruption of the exocytotic pathway with brefeldin A avoided the increase or significant decrease in the extrasynaptic response. The comparison was made between mean values at all time intervals with a hipothetical value of 100% corresponding to the mean amplitude values of GABA-evoked responses recorded during baseline. The average amplitude of GABA-evoked response were 80.8 +/- 10.5% (n=3, $p>0.05$, one sample *t*-test), 5 min to 10min after the beginning of the recording, 66.6 +/- 14.6% (n=3, $p>0.05$, one sample *t*-test) 10min to 15 min after the beginning of the recording, 77.8 +/- 11.4% (n=3, $p>0.05$, one sample *t*-test) 15 min to 20 min after the beginning of the recording and 80.2 +/- 27.1% (n=3, $p>0.05$, one sample *t*-test) 20 min to 25 min after the beginning of the recording. As shown in **Fig. 3.20B**), brefeldin A decreased the rate of loss of mIPSCs (compare with **Fig. 3.20A**): after 10 min of superfusion with 100 nM SCH58261 in the presence of brefeldin A it was not observed a significant change of the cumulative probability distribution for the amplitude of miniature GABAergic currents (n=3, $p>0.05$, Kolmogorov-Smirnov test). However, 20 min after the beginning of the experiment, the classical

effect of SCH58261 upon mIPSCs was observed (n=3, $p<0.05$, Kolmogorov-Smirnov test) – compare also with the effect depicted in **Fig. 3.19**..

Fig. 3.19

A) **B)**

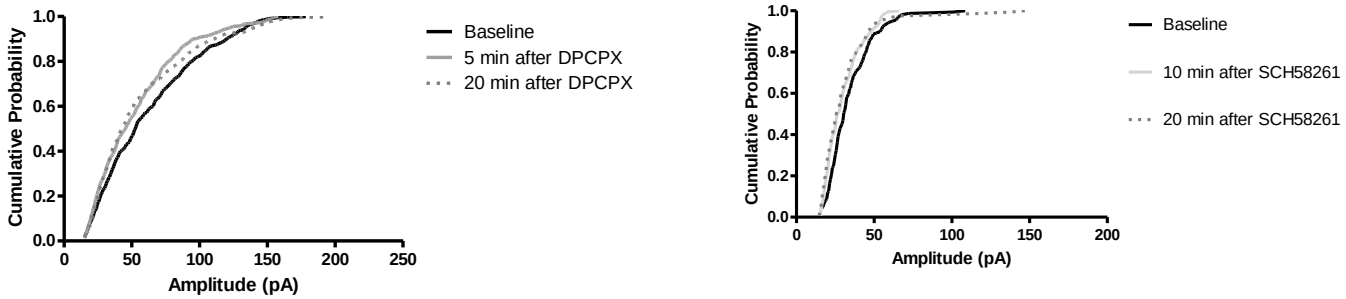


Fig. 3.19 – Effect of the A_1 and A_{2A} receptor antagonism on the amplitude of miniGABA events in cells where an increase in the evoked-GABA response was observed. Cumulative probability distribution from mIPSCs recorded during baseline, 10 min and 20 min after exposure to 100 nM SCH58261 (**A**). Independently on the profile of evoked-GABA response, the A_{2A} receptor antagonist decreased the amplitude of GABAergic synaptic events (n=6) **B)** Cumulative probability distribution from mIPSCs recorded during baseline, 5 min and 20 min after 100 nM DPCPX during application of 50 μM of GABA (n=5). DPCPX decreases the amplitude of mIPSCs (see the shift to the left of the distributions of amplitudes of mIPSCs in the presence of DPCPX).

These results suggest that A_{2A} receptors may also be involved in the regulation of the exocytotic processes, controling the arrival of new GABA subunits to the plasma membrane, probably at extrasynaptic sites. As brefeldin A affects both the exocytotic and the endocytotic processes, the delay of dissapearence of GABA receptors from synaptic sites may mean that the formation of endocytic vesicles are needed to remove receptors from the synapse; this can be viewed as an adittional arguments supporting the hypothesis presented in this chapter that A_{2A} receptor signaling controls the endocytosis of $GABA_A$ receptors. However, 20 min after perfusion with SCH58261, synaptic $GABA_A$ receptors seem to "disapear" from their synaptic location. Maybe at that time point, the cytoskeleton is dismantled with the destruction of the synapse.

Since adenosine A_1 and A_{2A} receptors mediate their physiological effects acting on G-proteins (see **Section 1** and **3** of the **Introduction**), it was next explored if GDPβS, a general blocker of G-protein (Oberdisse and Lapetina, 1987) would allow confirming that the modulation by adenosine receptors of mIPSCs as well as GABA-evoked events is mediated by G-protein. **Fig. 3.21** shows in **A**) a representative profile of GABA-evoked response in the presence of GDPβS. All cells recorded presented the same profile. This profile is similar to that generated by exposure of neurons to 100 nM SCH58261 represented in the **Fig. 3.18B**. The reason for the appearance of two different profiles of GABA-evoked responses triggered only by the presence of the A_{2A} receptor antagonist is unknown. It should be determined if any additional effect upon the application of SCH58261 (100 nM) in the presesnce of GDPβS.

Fig. 3.20

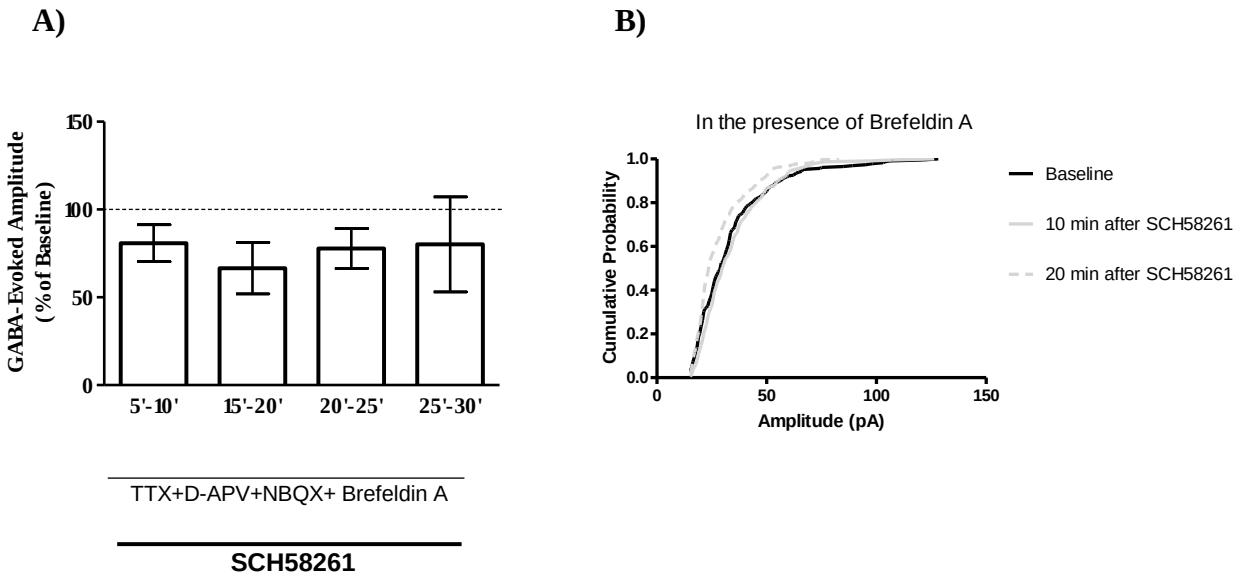


Fig.3.20 – Brefeldin A prevents the impact of A_{2A} receptor blockade on synaptic and extrasynaptic $GABA_A$ receptor-mediated currents. GABA-evoked response does not seem change significantly when interneurons from the *Stratum radiatum* were recorded in the presence of 10 µg/ml of brefeldin A, a drug that blocks the insertion of *de novo* receptors in extrasynaptic locations during superfusion with 100 nM SCH58261. Brefeldin A slows down the decrease in the amplitude of mIPSCs which was only visible 20 min after the beginning of the experiment. Results depicted in **A)** are mean +/- SEM and statistical analysis was one sample *t*-test. The amplitude of mIPSCs was analysed using Kolmogorov-Smirnov test to compare the distributions. A significant difference was found 20 min after the beginning of exposure to brefeldin A ($n = 3$, $p<0.05$).

It was also investigated if AMPA-evoked response would be modulated similarly by A_{2A} receptor antagonist. The results obtained are presented in **Fig. 3.22**. Application of 20 µM of AMPA elicited a response that does not seem to be modulated by 100 nM SCH58261. Taking the value of amplitudes of AMPA-evoked responses as 100% in the absence of SCH58261, 15 min to 20 min of superfusion

of the cells with SCH58261 resulted in a response 117.7 +/- 16.81% of control (n=3, p>0.05, one sample *t*-test). This may mean that A_{2A} receptor-mediated signaling is able to modulate purely glutamatergic synaptic events but it is not involved in the modulation of mainly extrasynaptic effects of AMPA receptor activation.

Fig. 3.21

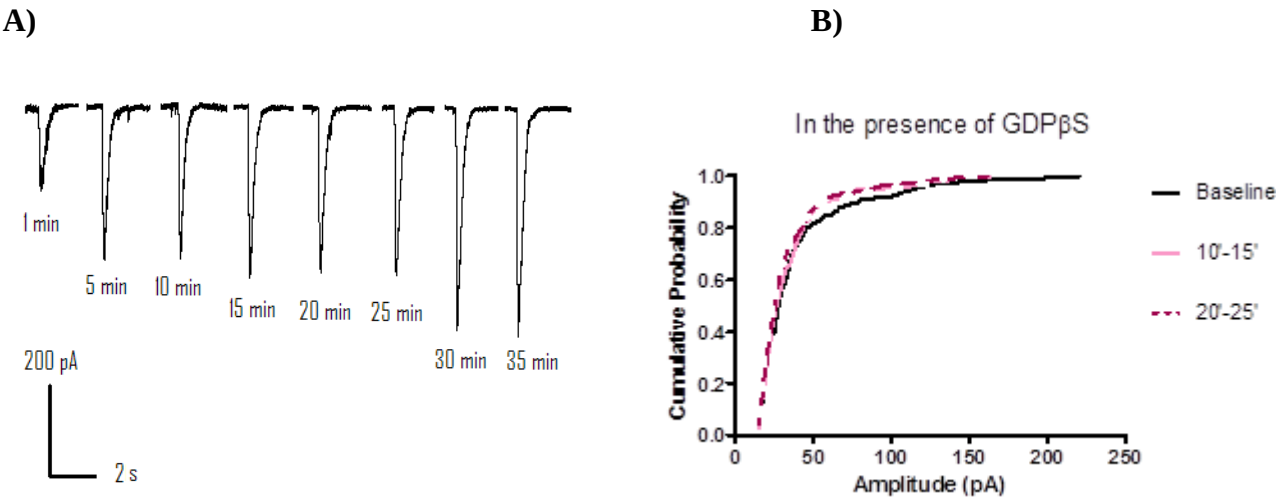


Fig. 3.21 – Effect of GDPβS in the amplitudes of GABA-evoked and mIPSCs. A) Trace showing that 500 µM GDPβS *per se* (in the pipette solution) increased the amplitude of the GABA-evoked response throughout the experimental time (about 35 min; GABA concentration is 50 µM), mimicking the profile of response of 100 nM SCH58261 on mIPSCs depicted in the **Fig. 3.19B**. **B**) GDPβS also mimicked the effect of SCH58261 on mIPSCs, decreasing their amplitude, suggesting that the effect upon synaptic events and GABA-evoked response are mediated by a G-protein-mediated process. 5 independent experiments were carried out in *Stratum radiatum* and pyramidal cell layer of the CA3 region of the hippocampus of 5-7 days-old mice pups. The cumulative probability distributions of the amplitudes displayed by mIPSCs where compared by the Kolmogorov-Smirnov test.

Fig. 3.22

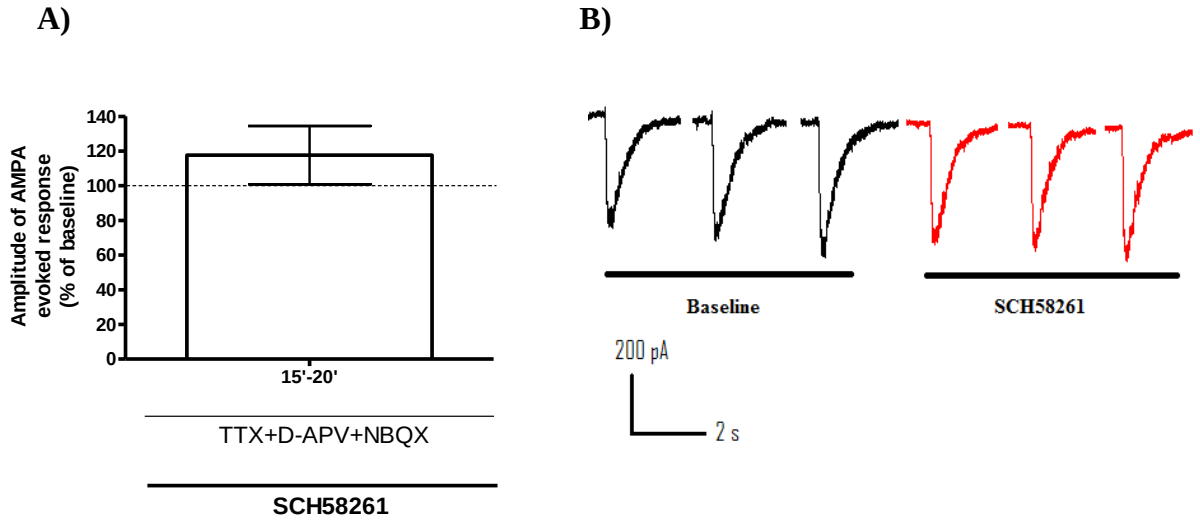


Fig. 3.22 – **Effect of an A_{2A} receptor antagonist on AMPA-evoked response. A)** SCH58261 (100 nM) was devoid of effect upon the amplitude of AMPA-evoked response. Results are mean +/-SEM (% of baseline) of 3 independent experiments performed in the *Stratum radiatum* and pyramidal cell layer of the hippocampus of 5-7 days-old mice pups. One-sample *t*-test was used for comparison between an hypothetical baseline of 100% and the mean of the amplitudes of AMPA-evoked events, 20 min after exposure to the drug (n=3, p>0.05). **B)** Representation of the the evoked AMPA response in the absence and in the presence of SCH58261

3.4. Effect of A_1 and A_{2A} receptor antagonism on spontaneous activity in a more integrated model: the cortico-hippocampal preparation

The selective and non-selective A_1 or A_{2A} receptor antagonists are able "excite" the hippocampal slice increasing the frequency of GDPs. However, whether or not such A_1 and A_{2A} receptor antagonists can trigger the generation of a pathological profile of activity is unknown due to the low connectivity between neuronal elements present in a brain slice. Experimental models that preserve better neuronal networks, like the *in vitro* cortico-hippocampal formation (Quilichini *et al.*, 2002) based on the intact hippocampus preparation (Khalilov *et al.*, 1997), allow an easier evaluation of abnormal and pathological types of activity, such as seizures.

Seizure profile (induced by low Mg^{2+}) in young animals (one post-natal week) was previously studied using this experimental preparation (Quilichini *et al.*, 2002). It was found that removing Mg^{2+} from bath solution induces the generation of interictal like events (ILE) lasting between 80-

120s. These events consist of an initial interictal-like burst, a tonic phase and recurrent clustered bursts separated by silent intervals of increasing duration. This last phase is named clonic phase. ILEs are followed by late recurrent discharges (LRDs) which persist even when physiological levels of Mg^{2+} are added to the bathing solution (Quilichini *et al.*, 2002). ILEs seem to be required for LRDs appearance which are age-dependent events since in this experimental protocol they can only be observed at PD 6 in the rat.

ILEs seem to be dependent on NMDA receptor activation and the maintenance of this activity seems to be dependent on AMPA receptor function. It seems that LRDs can be (in immature tissues) or not (in mature tissues) completely blocked by D-APV whereas AMPA receptor antagonists only impact in the amplitude and duration of LRDs. ILEs are only blocked using high concentrations of $GABA_A$ receptor agonist and low concentrations seem to be effective for the reversible block of LRDs. This dependence on high concentrations of the agonist to prevent the appearance of ILEs may indicate a failure or loss of efficacy of GABAergic synapses (Pfeiffer *et al.*, 1996) or a modification of the properties of $GABA_A$ receptors. These modifications may be induced by phosphorylation by Ca^{2+}-dependent kinases activated by Ca^{2+} that enters the cell through NMDA receptors which were shown to induce desensitization of $GABA_A$ receptors (Stelzer *et al.*, 1987).

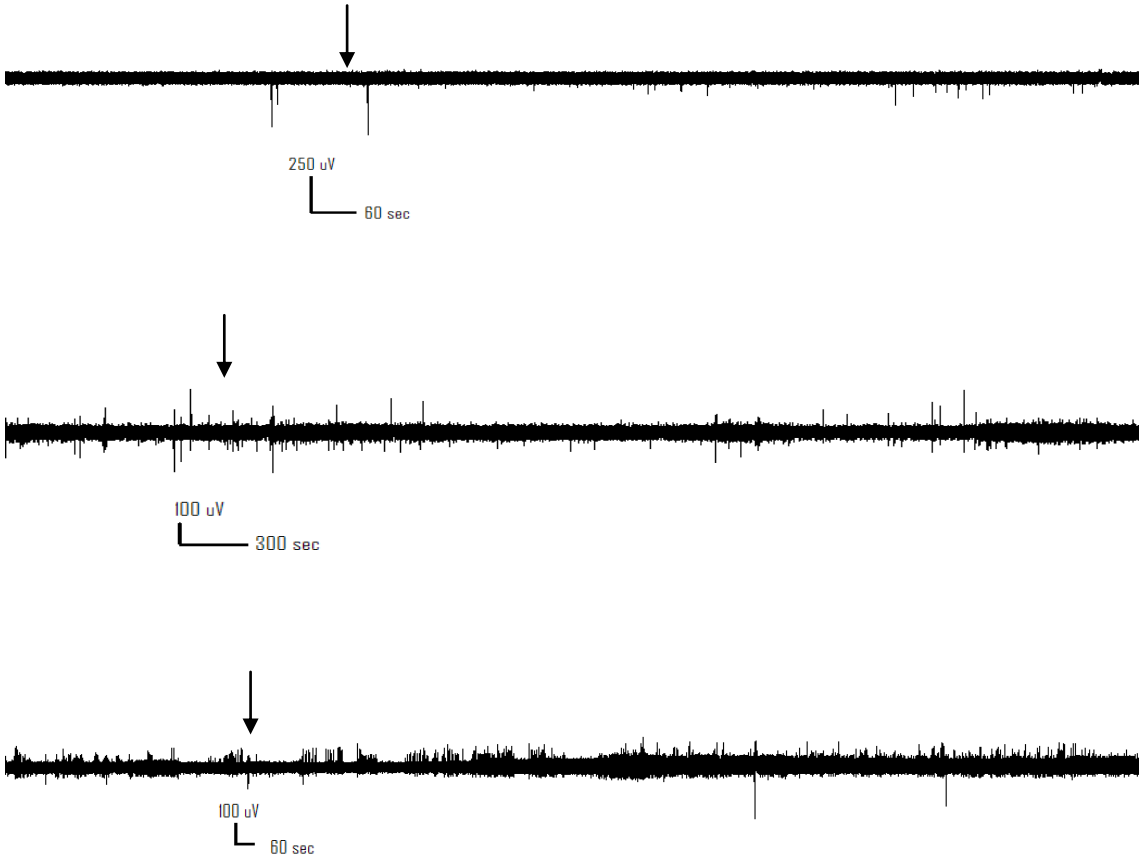
In the adult brain, adenosine receptors A_1 receptor blockade by itself is not able to elicit seizures (Dunwiddie, 1999), however, it can lead to seizure prolongation (Dragunow and Robertson, 1987) or lead to the generation of a *status epilepticus* (Young and Dragunow, 1994). Using this cortico-hippocampal preparation at several ages it was tested if selective and non-selective antagonists of adenosine A_1 or A_{2A} receptors might be able to induce an abnormal pattern of activity and if this is dependent on the degree of maturation of the preparation.

As we can see in **Fig. 3.23**, caffeine, a non-selective antagonist of A_1 and A_{2A} receptor subtypes, used at a concentration of 50 µM, does not change the pattern of spontaneous activity in a cortico-hippocampal preparation from PD 6 mice (**A**). A 10 times higher concentration of caffeine was also unable to trigger the generation of an abnormal pattern of activity at the same stage of development (**B**). At PD 7 (**C**), this higher caffeine concentration only generated a slight increase in the amplitude of the rhythmic spontaneous events recorded by the field electrodes. At PD 8 (**Fig. 3.24 A**), caffeine at 500 µM induced several ILEs with posterior development of LRDs. At PD 14 (**Fig. 3.24 B**), caffeine was not able to induce a seizure-like pattern of activity although it was still able to excite this biological preparation, increasing its spontaneous physiological rhythm. This means that the

blockade of both A_1 and A_{2A} receptors generated a pathological pattern of activity which is age-dependent. It is neither observed in very immature (PD 5 to PD 7) tissue nor at PD 14.

Fig. 3.23

A)



B)

C)

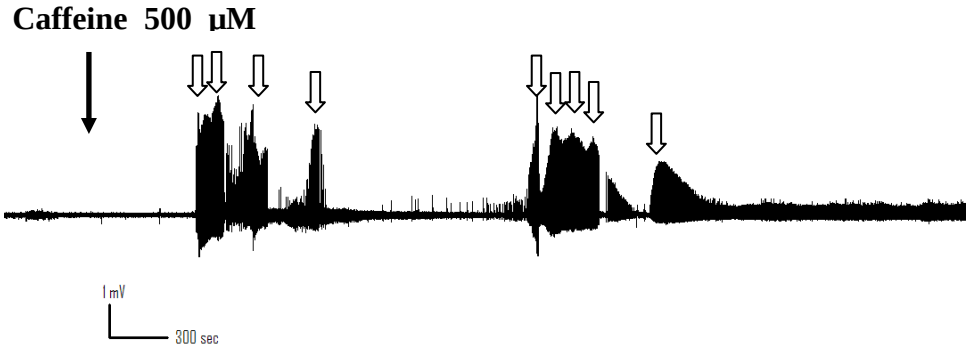
Fig. 3.23 – Effect of the non-selective antagonist of A_1 and A_{2A} receptors, caffeine, on the spontaneous activity recorded in the CHP from PD 6 – PD 7 mice pups. Caffeine application (application time indicated by the black arrow) at a concentration of 50 µM (**A**) or 500 µM (**B** and **C**) was not able to elicit any modification of the pattern of spontaneous activity in the CHP of PD 6 (**A** and **B**) or PD 7 (**C**). The drug had no effect upon the spontaneous activity of CHP at these ages and only increased slightly the amplitude of the rhythm in the the CHP from the PD 7 pup (this effect is visible from the time indicated by the white arrow onward. Results are illustrative of 3-4 independent experiments giving similar results. It was not performed a quatitative analysis of the rhytms recorded in the the CHP.

The non-selective antagonist of A_1 and A_{2A} receptors, caffeine, only had effect at PD8, this age was chosen to test the effect of selective A_1 and A_{2A} receptor antagonists. **Fig. 3.25 A)** and **B**) shows

the effect of an acute application of the A_1 receptor antagonist DPCPX (100 nM) or of the A_{2A} receptor antagonist SCH5826181 (100 nM) in the cortico-hippocampal preparation. Both drugs induced a pathological pattern of activity in this tissue, however, the effect of the A_{2A} receptor antagonist was more evident, generating a classical pattern of tonico-clonic seizures. CGS21680 (30 nM), a selective A_{2A} receptor agonist, CHS61820 (30 nM) also induced a series of ILEs (**Fig. 3.25 C**). It may be related with episodes of receptor internalization after prolonged exposure to the agonist (Palmer *et al.*, 1994; Brand *et al.*, 2008).

Fig. 3.24

A)



B)

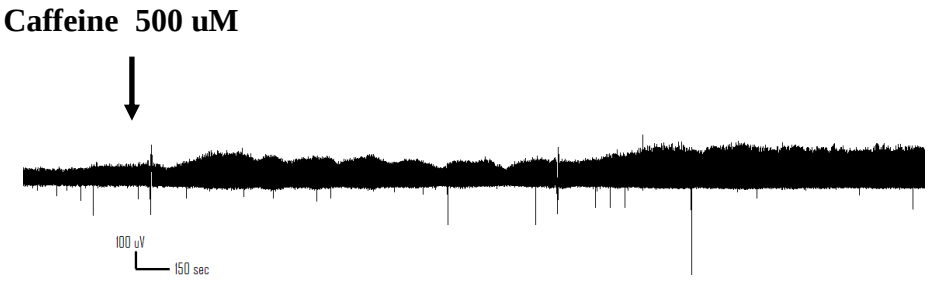


Fig. 3.24 – Effect of the non-selective antagonist of A_1 and A_{2A} receptors, caffeine, in the spontaneous activity recorded in the CHP from PD 8 – PD 14 mice pups. Caffeine application (application time indicated by the black arrow) at a concentration of 500 µM in aCSF used in the superfusion of CHPs from a PD 8 (**A**) and a PD 14 (**B**) mice pup elicited a series of pathological-like events indicated by the white arrows. This effect was however not observed in older pups (**B**), suggesting that there is a time window when caffeine is able to elicit a pathological pattern of activity. At PD 14 (**B**) caffeine only triggers an increase in the amplitude of the spontaneous oscillations recorded in the CHP. Results are illustrative of 5 independent experiments with similar results. It was not performed a quatitative analysis of the rhytms recorded in the the CHP.

Fig. 3.25

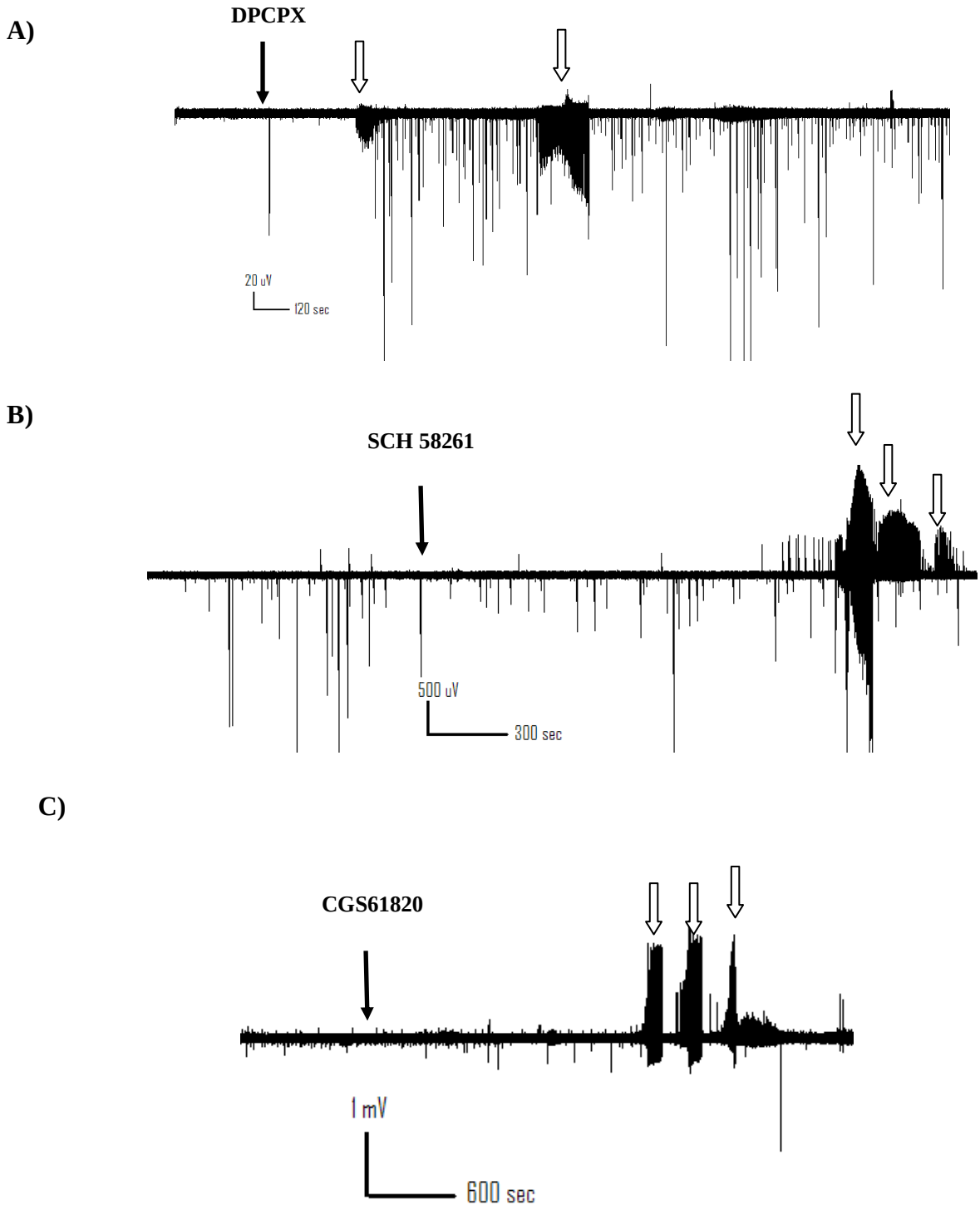


Fig. 3.25 - Effect of the selective antagonists of A_1 or A_{2A} receptors on the spontaneous activity recorded in the CHP from PD 8 mice pups. Different profiles of abnormal activity induced by the selective antagonists of adenosine receptors, 100 nM DPCPX (**A**) or 100 nM SCH58261 (**B**). Both drugs were able to elicit a pathological-like pattern of activity (indicated with white arrows) in CHP from PD 8 mice pups. The overactivation of A_{2A} receptor with the selective agonist CGS61820 (30 nM) seems to have an effect identical to that of the selective antagonist of A_{2A} receptors, triggering pathological-like events (**C**). Results are illustrative of 3-5 independent experiments with similar results. It was not performed a quatitative analysis of the rhytms recorded in the the CHP.

CHAPTER 3 - Conclusions

The results presented in this chapter show that both A_1 and A_{2A} receptors control the spontaneous activity in the immature hippocampal slice. This control can be observed at several levels. Thus, adenosine receptors seem to be relevant in the maintenance of both GABAergic and glutamatergic synapses in principal cells and interneurons. Blockade of these adenosine receptor subtypes make cells less sensitive to the presynaptic activity. Electrophysiological and molecular data seem to indicate that this depression is due to internalization of $GABA_A$ and AMPA receptors. The increase in the frequency of GDPs may be related with an increased function of NMDA receptors by an unknown and unexplored mechanism. Interestingly, the blockade of NMDA receptors does not change the basal frequency of generation of GDPs but abrogates the effect elicited by SCH58261.

Differently from what happens in the adult hippocampus (Cunha, 2005), during development, adenosine receptors seem to have a prominent postsynaptic location and action, supported by the morphological data presented in the **Chapter 1**. A_1 receptor subtype also seems to be located at presynaptic sites in the immature slice. Blockade of A_1 receptors may increase the release of neurotransmitters, whereas in the case of A_{2A} receptors, there is no evidence for its location in glutamatergic axon terminals, the increase in the frequency may be related to over-activation of NMDA receptors and to an increase in intracellular Ca^{2+}. Analysis of properties of miniature GABAergic and glutamatergic currents can also be interpreted as evidence supporting a postsynaptic site of action for these receptors, which can have an impact in the frequency at which cells receive synaptic currents. In fact, blocking clathrin dependent endocytosis abrogates the decrease in the interevent interval of miniGABA and mEPSCs induced by the tested A_{2A} receptor antagonist.

This depression of synaptic activity may have physiological consequences, for example, induction of LTD which is mediated by clathrin-mediated endocytosis of AMPA receptors (Carroll *et al.*, 1999; Beattie *et al.*, 2000). It was shown that long-lasting changes in glutamatergic synapses have to be parallelled by concomitant changes at GABAergic synapses to prevent hyperexcitability or silencing of the network (Tyagarajan *et al.*, 2010). It was proposed that a remodeling of the cytoskeleton is on the basis of such modifications (Steiner *et al.*, 2008; Tyagarajan *et al.*, 2010).

Two hypothesis can be raised to justify the increase in the amplitude of elicited GABA response (which is mainly mediated by the non-synaptic $GABA_A$ receptors): receptors being taken from synaptic sites and incorporated in primary endocytic vesicles will be later fused with membranes in

extrasynaptic sites or receptors newly produced and processed in the Golgi can be inserted in extrasynaptic sites. Maybe the absence of A_{2A} receptor-mediated signaling may "tag" the synaptic site to be destroyed and receptors will ocupy different placements in an attempt for the cell to find a new location to establish a new synaptic contact. It could mean that A_{2A} receptor signaling may be involved in the selection of synaptic contacts and once a synapse is lost by the absence of an "adenosinergic" tonus it will constitute a stimulus to the establishment of new synapses elsewhere. More speculative arguments can be raised to justify this difference of response to GABA applied close to cell bodies of the recorded cells. A_{2A} receptors seem to be located in post and extrasynaptic locations. Another possibility would be that cells expressing A_{2A} receptor in extrasynaptic sites will have an increased response to GABA throughout time. Cells that do not have this type of signaling will have a decreased response to GABA.

Acute application of SCH58261 and DPCPX induced the generation of a pathological-like pattern of activity in the CHP at PD 8 but not at PD 5-PD7. These pathologic-like patterns of activity were only observed from PD 8 onward. Phenomena of AMPA and GABA internalization seem to happen between PD 5 and PD 7. Whether it is observed at PD 8 is not known.

From these observations, it appears that A_1 and A_{2A} receptors are physiologically relevant to stabilize synapses and control excitability in the hippocampus.

CHAPTER 4

Short- and Long-Term Consequences of A_1 and A_{2A} Receptor Blockade During Brain Development

Since A_1 and A_{2A} receptors modulate events like synaptic stability, cell death of immature neurons, excitability in the hippocampal slice and in the cortico-hippocampal formation, it seemed relevant to study the impact of pharmacologically blocking A_1 and A_{2A} receptors during brain development. It was used a classic and non-invasive protocol in which the female mother is the vehicle for the delivery of the drug to embryos and pups. Caffeine was used as a non-selective adenosine receptor antagonist and it seems that caffeine can reach easily the embryos due to the facility of crossing the hematoencephalyc barrier and the placenta (Lachance *et al.*, 1983; Tanaka *et al.*, 1984). In view of the fact that caffeine is also an adenosine receptor antagonist and it is the most widely consumed psychoactive drug worldwide (Fredholm *et al.*, 1999), there is a particular interest in exploring a possible effect of caffeine action(s) during pregnancy. Caffeine absorption from the gastrointestinal tract is complete in the rodent (Arnaud, 1976, 1985) and it is metabolized in the liver being demethylated into mono- and dimethylxanthines, mono- and dimethyl uric acids, dimethyl- and trimethylallantoin and uracil derivatives (Arnaud 1987). Some of these metabolites such as theophylline (1,3-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine) have also physiological relevant effects (Fredholm *et al.*, 1999). Paraxanthine is the major metabolite in the plasma of rats (Fredholm *et al.*, 1999). Fetuses are unable of metabolizing caffeine due to the lack of the major enzyme cytochrome P450 1A2 (CYP1A2) (Kalow and Tang, 1991) and clearance of caffeine is slower throughout pregnancy in the rat (Nakazawa *et al.*, 1985). Fetuses have renal elimination pathways for xanthines but this route of elimination is not as efficient as the liver clearance (Ginsberg *et al.*, 2004). All these factors contribute to a higher half-life of caffeine in younger animals. At moderated doses in rodents, the half-life of caffeine is at about 1.5-2h (Latini *et al.*, 1980).

Several biological actions can be attributed to caffeine. The predominant effect is as an endogenous antagonist of **adenosine receptors** (Daly *et al.*, 1981; Snyder and Sklar, 1984) which is the only known effect of caffeine at concentrations that mimic the daily average intake, being in the range between 1-10 μM and corresponding to a plasma concentration of 0.2-2 mg/L (Fredholm *et al.*,

1999). Inhibition of **phosphodiesterase** and **mobilization of calcium stores** (Snydar and Sklar, 1984) were observed essentially *in vitro*. It seems that even high doses of caffeine fail to increase the levels of cAMP in native tissues (Snyder and Sklar, 1984). Also, release of calcium from intracellular stores requires milimolar concentrations (Snyder and Sklar, 1984). The inhibition of phosphodiesterase activity by caffeine can increase the levels of cyclic AMP (Francis *et al.*, 2011), which may interfere with processes of cell division (Soyka, 1979) and neuronal migration (Valiente and Marín, 2010). Eventually, blockade of the low-affinity A_3 receptor can occur (Áden, 2011). The effects upon $GABA_A$ receptors and intracellular calcium stores are considered toxic. Shi and collaborators (Shi *et al.*, 2003), using binding assays, studied the modulation of $GABA_A$ receptor function and mobilization of intracellular calcium from calcium stores by caffeine. They describe that the ability of caffeine (500 μM) to cause inhibition of [^{3}H]diazepam binding site of $GABA_A$ receptor, depressing $GABA_A$ receptor function and cause stimulatory effects upon radioactive t-butylbicyclophosphorothionate or [^{35}S]TBPS which occupies the picrotoxin binding site of this receptor. All these effects can occur for example, when preterm infants are treated with caffeine for apnea. Those treatments usually increase more than 10 times the serum levels of caffeine (Áden, 2011).

As mentioned before, the model of maternal exposure to caffeine has been widely used (see Guillet, 2003 for a review). However, the duration of caffeine exposure varies in different works - prior to conception, through gestation and lactation (Dunlop and Court, 1981; Tanaka *et al.*, 1987; Butcher *et al.*, 1984), gestation and lactation (Schneider *et al.*, 1990; Concannon *et al.*, 1983; Yazdani *et al.*, 1988) or during gestation only (Gilbert and Pistey, 1973; Driscoll *et al.*, 1990; West *et al.*, 1986; Swenson *et al.*, 1990; Hughes and Beveridge, 1990; Sinton, 1989; Enslen *et al.*, 1980). Other studies limited caffeine exposure to the neonatal period only (Fuller *et al.*, 1982; Fuller and Wiggis, 1981; Quinby and Nakamoto, 1984; Guillet, 1990a,b; Guillet and Kellogg, 1991). One advantage of these models is that the female is not disturbed before and after the pregnancy period and it can mimic the human consumption of caffeine and the way fetuses are exposed to this drug, although parameters like caffeine metabolism and clearance can vary between humans and rodents.

The parameters most often used to evaluate the impact of caffeine in the embryo and newborn were generally the fetal body weights (Butcher *et al.*, 1984; West *et al.*, 1986; Quinby and Nakamoto, 1984; Fuller and Wiggis, 1981; Fuller *et al.*, 1982; Holloway, 1982) and brain weights (Yazdani *et al.*, 1988), delay in physiological development (West *et al.*, 1986), alteration of behavioral parameters (Butcher *et al.*, 1984; Concannon *et al.*, 1983; Kaplan *et al.*, 1989; Sobotka *et*

al., 1979; West *et al.*, 1986) and alterations in the litter size (Gilbert and Pistey, 1973). In Guillet 2003 a range of effects and observations, sometimes conflicting between works, can be found.

Less data exploring neurochemical, molecular and morphologic modifications is available. In Enslen *et al.*, 1980, the first and second generation offspring were analyzed and a decrease in dopamine content was found after exposure to 2.9, 5.7 or 21.5 mg/day of caffeine throughout gestation. Concannon and co-workers (Concannon *et al.*, 1983) exposed rats to 14 mg/kg/day of caffeine throughout gestation and to 48 mg/kg/day of caffeine during lactation (until PD 25). They found regional effects of cAMP in whole brain and cerebellum.

Léon and colleagues (Léon *et al.*, 2002) also treated pregnant female rats with 1 g/L of caffeine or theophylline in the drinking water and found a decrease of about 30% in the density of A_1 adenosine receptor subtype in maternal brain. The effect was more marked in fetal brains. These results were in opposition to previous results (Fredholm, 1982; Ramkumar *et al.*, 1988; Green and Stiles, 1986,) in which brief or longer periods of perinatal exposure to caffeine changed the density of different components of adenosine receptor-adenylate cyclase system. The same group (Léon *et al.*, 2005) found that chronic treatment with caffeine or theophylline during gestation caused a down-regulation of components of the metabotropic glutamate receptor and PLC (mGluR/PLC) transduction pathway in maternal and fetal brain, decreasing the presynaptic glutamate release, however in immature brain the responsiveness of mGluR/PLC pathway was lower.

This chapter describes in more detail, the physiological and morphological alterations present in the hippocampus of animals treated chronically with caffeine *via* the mother during the developmental period. KW6002 or istradefylline was developed as a selective A_{2A} receptor antagonist to be used in *in vivo* studies (Yang *et al.*, 2007). Animal data suggest that doses higher than 5mg/day are able to achieve more than 90% of A_{2A} receptor occupancy in adults (Brooks *et al.*, 2008). A daily concentration of 2 mg/kg/day of caffeine was used dissolved in a vehicle solution given used as drinking solution with the purpose to evaluate whose effects were specifically mediated by A_{2A} receptors.

Both treatments were started 15 days prior conception and were continued throughout pregnancy until PD 15. The study was made at two distinct time points, PD 6 and PD 90 in order to evaluate short- and long-term consequences induced by those treatments.

CHAPTER 4 – Materials and Methods

Animals: Caffeine or KW6002 administration (described below) was made in FVB-GIN adult (older that 60 days-old) female mice never mated or exposed to these drugs before. Females belonging to control and caffeine or vehicle and KW6002 groups were chosen from the same litter to decrease the variability among individuals. Electrophysiology was made using 6 days-old male pups and 90 days-old male.

Caffeine and KW6002 treatment: Caffeine (SIGMA) was dissolved in tap water at a concentration of 0.1% and 0.3%. Females were exposed to caffeine in the drinking water 15 days before mating, during pregnancy up to 15 days after delivery. Water consumption was measured regularly and fresh caffeine-containing water was given every two days to avoid caffeine degradation/modification. KW6002 was prepared daily in a vehicle solution (0.4% methylcellulose from SIGMA and 0.9% NaCl). For total solubilization, the KW6002 solution was subjected to ultra sounds for 20 min. Females were exposed to 2 mg/kg/day of compound added to 5 mL of vehicle. The treatment started 15 before mating and was continued inninterruptly until when pups were 15 days-old. KW6002 was given daily to females and after total consumption of the product, water was given during the rest of the day.

Electrophysiology in the hippocampal slice: Electrophysiology was made according to the protocol described in the **Section Material and Methods** of **Chapter 3**. In slices of 6 days-old pups both interneurons from the *Stratum radiatum* and pyramidal cells were recorded and in slices from 90 days-old mice, pyramidal cells from CA1 were recorded. Comparisons between recordings was made using the same cell type and degree of maturation (evaluated by the parameter Cm and Rm).

Immunohistochemistry (DAB protocol): Immunhistochemistry was performed as described in the **Section Material and Methods** from **Chapter 1**. The antibodies used, supplier, host, type and dilution can be found in the **Table I** presented below. Secondary antibodies biotinylated anti-mouse and biotinylated anti-rabbit used are the same used already in morphological experiments presented in **Chapter 1**.

Table I

Antibodies	Supplier	Host	Type	Dilution
Anti-α1 subunit	Synaptic Systems	Rabbit	Polyclonal	1:5000
Anti-α3 subunit	Milipore	Rabbit	Polyclonal	1:2000
Anti-γ2 subunit	Synaptic Systems	Rabbit	Polyclonal	1:2000
Anti-GAD 65	Chemicon	Mouse	Monoclonal	1:500
Anti-GAD 67	Chemicon	Mouse	Monoclonal	1:2000
Anti-gephyrin	Synaptic Systems	Rabbit	Polyclonal	1:1000
Anti-GFP	Molecular Probes	Rabbit	Polyclonal	1:3000
Anti-GluR1	Milipore	Rabbit	Polyclonal	1:100
Anti-GluR2,3	Milipore	Rabbit	Polyclonal	1:100
Anti-NR1	Chemicon	Rabbit	Polyclonal	1:500
Anti-NR2	Chemicon	Rabbit	Polyclonal	1:500
Anti-VGLUT1	Chemicon	Guinea Pig	Polyclonal	1:20000

Behavioral Tests: Locomotor activity was monitored in two consecutive days in an acrylic open field arena (30 x 30 cm, divided in 9 squares, 30 cm tall) and the exploratory behaviour of the animals was evaluated by counting the total number of line crossings for 5 min during two sessions.

Object Recognition Test was used to evaluate memory performance and consisted in two 5-min sessions (24 hours after habituation to the open field arena): the first with two identical objects (training session) and the second (test session, 2-hour later) with two dissimilar objects (a familiar and a novel one); recognition object index was calculated by the ratio of the time spent exploring the novel object minus time spent exploring the familiar object over the total exploration time of both objects, as previously described (Costa *et al.*, 2008; Bevins and Besheer, 2006).

Y-maze test was used to evaluate spatial memory performance and it was performed using a Plexiglas apparatus consisted of 3 arms in a Y-shape, separated by equal angles. The test consisted in subjecting animals to two 8-min sessions (trial and test) separated by a 2-hour inter-trial interval (Akwa *et al.*, 2001; Dellu *et al.*, 1992, 2000). The involvement of the hippocampus in Y-maze performance is supported by several modifications observed in this brain structure that paralleled a Y-maze deficit (see for example Wetzel *et al.*, 1980; Ainge *et al.*, 2007; Featherby *et al.*, 2008. During the trial session one arm was separated by a guillotine wall that made it inaccessible to the animals so they were allowed to explore only two arms; during the test session the guillotine was

removed so all arms could be explored. Memory performance was evaluated measuring the percentage of time spent exploring the novel arm over the time spent exploring all arms.

The **elevated plus-maze test** was done using an apparatus of four arms of the same size (40 cm × 5 cm) arranged in the form of a cross and raised 50 cm above the floor. Two opposed arms were surrounded by a short (0.5 cm) Plexiglas edge (open arms) while the other two by 30 cm high opaque Plexiglas walls, except for the entrance (closed arms). Each animal was placed on the central square of the maze facing a closed arm and was allowed to explore the maze for 5 min. The number of entries and the time spent in both open and closed arms were recorded. The exploratory behavior upon the open arms was expressed as the mean percentage of entries into the open arms over total entries and the time spent inside the open arms over the total time spent in all arms (the time in the central square was excluded from the analysis). Any animal that fell off the maze was excluded from the experiment (Handley and Mithani, 1984; Walf and Frye, 2007).

All the experiments were carried out during the light phase of the cycle between 10 a.m. and 5 p.m., and the testing room was illuminated with dim red light. The mazes were cleaned with wet (ethanol 10%, v/v) and dry cloths.

Statistical Analysis: Statistical Analysis: Kolmogorov-Smirnov test was used to compare distributions which were considered different if p value < 0.05. For comparison between means, two tailed *t*-test or one-way ANOVA were used, depending on the number of groups to compare. If one-way ANOVA was used, Tukey test was chosen as the *post hoc* test. Again, means were considered different if $p < 0.05$. Statistical significance was indicated as $*p<0.05$, $** p<0.001$, $*** p<0.0001$.

Drugs and Solutions: Drugs used in this chapter were prepared and used at concentrations already described in **Chapter 3**. KW6002 ([(E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6,dione]) was a kind gift of Dr. Christa Müller, Germany.

CHAPTER 4 – Results

Dams were treated with 0.1 g/L (concentration used only in the first experiments to evaluate its impact in the physiology of the hippocampal slice) or 0.3 g/L of caffeine in drinking water or 2 mg/kg/day of KW6002 in a vehicle solution containing methylcellulose. The serum caffeine concentration in the plasma after a chronic treatment with 1 g/L of caffeine in drinking water was quantified to be around 50 µM (Duarte *et al.*, 2009). To avoid unspecific effects of caffeine due to the longer time required for caffeine clearance and increased accumulation the tissue from immature animals, caffeine concentrations were chosen to be below that value of 1 g/L. This concentration of 0.3 g/L was the concentration used in other protocols since it was shown to elicit stronger effects than the concentration of 0.1g/L and it corresponds to a consumption of 75 mg/kg/day throughout the pregnancy, taking into account the volume ingested and the weight of the animals. When caffeine is administered in small amounts during the day, which is the case when caffeine is given through drinking water to animals, a concentration of 330 mg/kg/day is needed to reach teratogenicity in rats (Nehlig and Debry, 1994) and the concentration of 75 mg/kg/day is well below this value. This absence of toxicity and teratogenicity of the treatments was supported by the measured rate of weight gain throughout pregnancy. The comparison was made between the first week of treatment (reference or 100%) and the weight registered in the last week of gestation. In the **Fig. 4.1** it is shown that the females belonging to different groups had a similar variation of the body weight. Control females increased body weight to 144.6 +/- 7.0 % (n=5), and caffeine-treated females increased to 143.9 +/- 2.6 % (n=5) (**A**). Body weight from vehicle-treated females increased from 100.0 % to 163.6 +/- 14.7 % (n=3), and from KW6002-treated dams to 154.6 +/- 7.5% (n=3) (**B**). Comparing means (Control *versus* Treatment) of both groups, no significant difference ($p>0.05$) was detected using a two-tailed *t*-test.

Maternal behavior did not seem to be changed by the treatments. Treated females were not observed to be either aggressive or to kill or to neglect their pups. The litter size of different groups was also analyzed and was not different in groups receiving different treatments. **Fig. 4.2** presents the mean litter size in control and caffeine-treated (**A**) and vehicle and KW6002-treated animals (**B**). Control females had a mean of 10.5 +/- 1.8 animals (n=4) and caffeine-treated females 10.0 +/- 0.5 (n=5, $p>0.05$, two-tailed *t*-test). Litter size from vehicle dams was 8.0 +/- 1.0 (n=3) and from KW6002-treated animals was 10.5 +/- 0.5 (n=3, $p>0.05$ two-tailed *t*-test).

Fig. 4.1

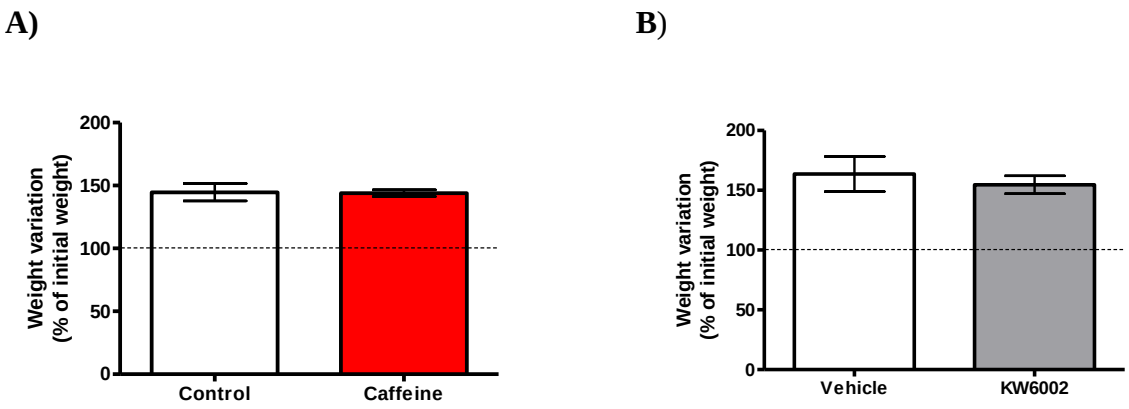


Fig. 4.1 – **Effect of caffeine or KW6002 treatments upon the weight gain in pregnant females.** Evaluation of the increase in body weight in control and caffeine-treated females (0.3 g/l) (**A**) and in control or KW6002-treated (2 mg/kg/day) dams (**B**), throughout gestation and lactation (up to PD 15). Treatments did not change the weight gain in treated animals, suggesting that caffeine, KW6002 and vehicle were not toxic to them. 100% corresponds to the weight in the beginning of the treatments (2 weeks before mating).Values are mean +/- SEM of the weight of 5 (control and caffeine-treated) or 3 (vehicle and KW6002-treated) females, measured in the last week of gestation. Comparison between means was made using a two-tailed *t*-stest. It was not found significant difference between the means ($p>0.05$).

The weight of pups (males) was determined only at PD 8 and PD 12. Weight at earlier stages was not determined to avoid disturbing the link between the mother and pups. Results are represented in **Fig. 4.3**. In younger pups, the treatment did not change ($p>0.05$, two-tailed *t*-test) the weight gain (weight of control animals, n=21 at PD 8 was 4.7 +/- 0.2 g and weight of 8 days-old pups, n=16, born from mothers treated with 0.3 g/L of caffeine was 4.5 +/- 0.07 g; the weight of 8 days-old pups treated with the vehicle solution, n=9, was 4.2 +/- 0.05 g and the weight of 8 days-old pups from mothers treated with KW6002, n=9, was 4.2 +/- 0.1 g). At PD 12, pups from mothers treated with KW6002 were heavier (n=15 for each group, $p<0.05$, two-tailed t-test) than vehicle animals – body weight of vehicle animals was 5.0 +/- 0.06 g and body weight of 12 days-old pups born from KW6002-treated mothers was 5.4 +/- 0.1 g. However, no difference (n=16 and n=35 in control and caffeine groups, respectively, $p>0.05$, two-tailed *t*-test) was observed between the body weight of control and animals belonging to caffeine-treated mothers. The average weight of 12 days-old control pups was 6.2 +/- 0.2 g and of pups belonging to caffeine-treated mothers was 6.4 +/- 0.05 g.

Fig. 4.2

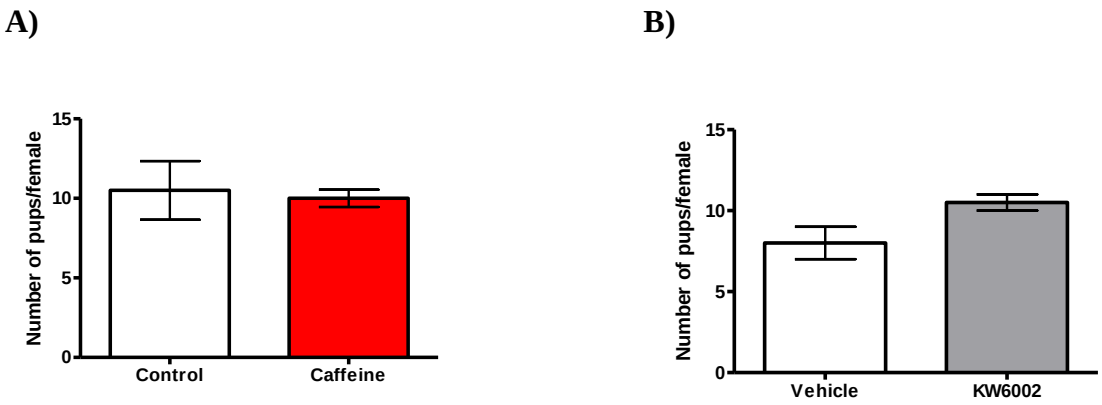


Fig. 4.2 – **Effect of caffeine or KW6002 treatments in the litter size.** Litter size in different experimental conditions, **A**) control and caffeine-treated animals and **B**) vehicle and KW6002-treated animals. Treatments do not seem to change the litter size *per* female. Results are mean +/- SEM and comparisons between the experimental groups presented in **A)**, (n=4 females and n=5 females in control and caffeine groups, respectively) and **B)** (n=3 females in both groups) were made using a two-tailed *t*-test. No statistical difference was found between groups, ($p>0.05$).

Fig. 4.3

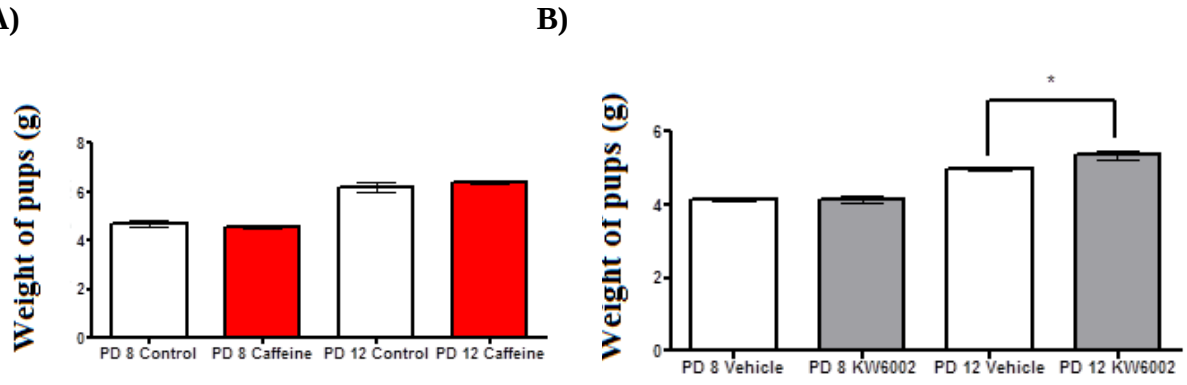


Fig. 4.3 – **Effect of caffeine or KW6002 treatments in the weigh of male pups.** Caffeine does not seem to change the weight of pups at PD 8 or at PD 12 (**A**) (n=21 in control group PD 8; n=16 in caffeine group PD8; n=16 in control group PD12 and n=35 in caffeine group PD 12). However, the weight of 12 days-old KW6002-treated animals seemed to be higher comparatively to the weight of 12 days-old vehicle pups (**B**) (n= 15 in 8 days-old vehicle and KW6002 groups and n=9 in the 12 days-old vehicle and KW6002 groups). Results are mean +/- SEM. Comparison between groups was made using a two-tailed *t*-student. The only significant difference ($p<0.05$) was observed between the weight of 12 days-old pups born from vehicle and KW6002-treated mothers.

A first analysis of the physiology of the hippocampus from control and treated animals with caffeine and KW6002 was made evaluating the frequency of synaptic currents and GDPs recorded in interneurons from the *Stratum radiatum* of the CA3 region. **Fig. 4.4** shows that interneurons of pups from caffeine-treated dams (0.1g/L or 0.3g/L) receive more GABAergic and glutamatergic currents. 79.4 % of the neurons from the control group displayed synaptic currents at frequencies lower than 10 Hz, 20.6% of the neurons displayed synaptic currents in the range of 10 to 20 Hz and there were no neurons displaying frequencies higher than 20 Hz. In the case of neurons from the caffeine-treated group, 12.1% (0.1 g/L) and 29.4% (0.3 g/L) of the neurons displayed synaptic currents at a frequency lower than 10 Hz and there was an increase in the amount of the neurons displaying higher frequencies of synaptic currents. 20.6% (0.1 g/L) and 41.2% (0.3 g/L) of the neurons displayed frequencies ranging from 10 Hz to 20 Hz, 17.6% (0.1 g/L) and 23.5% (0.3 g/L) of the neurons displayed frequencies of synaptic currents varying between 20 Hz to 30 Hz. The distribution of synaptic currents from KW6002-treated animals was similar to the vehicle group (75% of the neurons recorded displaying synaptic currents with frequencies up to 10 Hz, 25% of the neurons between 10 Hz to 20 Hz and no neurons displaying frequencies of synaptic currents above 20Hz.

76.1% of the cells from control animals had a frequency of GDPs inferior to 0.05 Hz. 21.95% of the population displayed a higher frequency of GDPs, from 0.05 Hz to 1 Hz and a smaller fraction of the population displayed even higher frequencies of GDPs ranging from 0.1 Hz to 0.15 Hz. In slices from pups whose dams were chronically treated with caffeine 0.1 g/L or 0.3 g/L, the percentage of neurons displaying lower frequencies of GDPs decreased (38.5% and 56.1% respectively with frequencies of GDPs below 0.05Hz) and more cells were recorded receiving higher frequencies of GDPs (46.2% of cells receiving between 0.05 Hz and 0.1 Hz and 7.1% of cells receiving between 0.1 Hz and 0.15 Hz, for both doses of caffeine). The highest fraction of neurons with higher frequencies of GDPs, 71.4%, (between 0.05 Hz and 0.1 Hz) was found in slices from pups of KW6002-treated animals, where 28.6% of the neurons received GDPs at a frequency higher than 0.05 Hz. No statistical analysis was carried out. The comparison was made only between the distributions of the frequencies in the different experimental groups of animals. These distributions were constructed analysing 30-40 interneurons *per* group.

Fig. 4.4

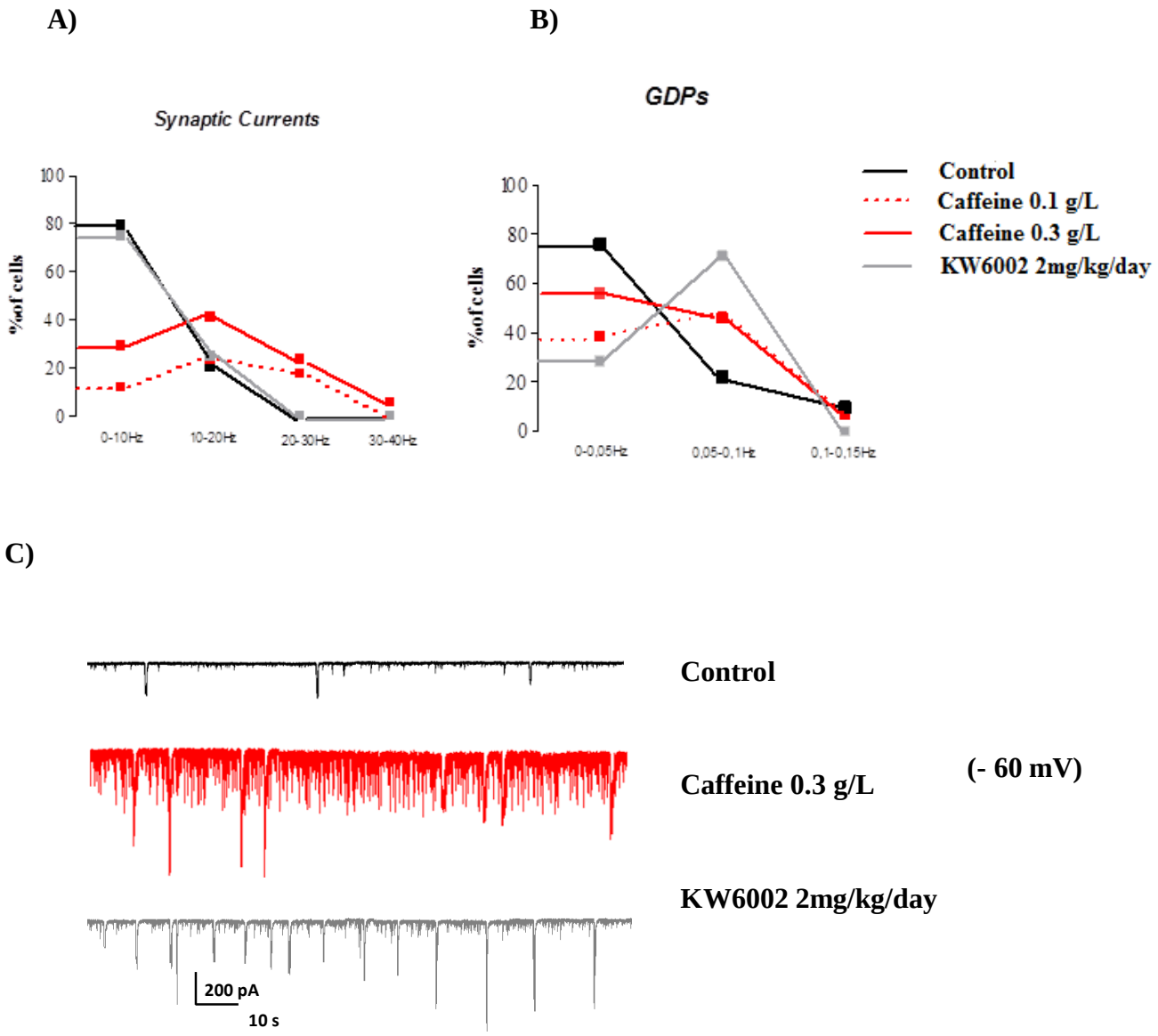


Fig. 4.4 – Effect of caffeine or KW6002 administered *via* the mother in the pattern of spontaneous activity recorded in hippocampal slices from 5 to 7 days-old pups. The graphs show the distribution of frequencies of synaptic currents (**A**) and GDPs (**B**) received by interneurons recorded in the *S. radiatum* from CA3 region of hippocampal slices from mice pups. A higher fraction of cells recorded in slices from animals treated with 0.1 g/L or 0.3 g/L of caffeine displayed higher frequencies of synaptic currents and GDPs. In the case of KW6002-treated animals, an increase in the frequency of GDPs was observed whereas synaptic currents did not seem to be more frequent. **C)** Representative traces of 2 min of recording of spontaneous activity in interneurons from a control (black), a pup born from a 0.3 g/L caffeine-treated mother (red) and a pup born from a KW6002-treated mother (gray) recorded at a holding potential of – 60 mV. Age of the pups: 6 days-old. The analysis was made over 30-40 interneurons *per* group. No statistical analysis was carried out.

It was investigated in further detail if the increased excitability present in hippocampal slices from pups whose mothers were treated with caffeine or KW6002 during gestation and lactation, was associated specifically to modifications of GABAergic or glutamatergic synapses and if it was cell type-dependent. **Fig. 4.5** presents the results concerning the frequency of $GABA_A$ receptor – (**A**) and glutamatergic (AMPA/kainate receptor-mediated) (**B**) synaptic currents recorded in pyramidal cells from pups belonging to control/vehicle (slices from control and vehicle pups where analysed together since no significant differences were found in previous experiments in terms of spontaneous activity), caffeine- and KW6002-treated dams. 75% of the pyramidal cells (n=17 cells) received GABAergic currents at a frequency inferior to 5 Hz. 6.25% of the total number of cells (n=17) had currents varying between 5 Hz and 10 Hz and the same fraction of the population had frequencies ranging from 10 Hz to 15 Hz and between 15 Hz and 20 Hz (n=17). In contrast, a smaller fraction of the population of pyramidal cells from treated animals had a lower frequency of GABAergic currents up to 5 Hz - 50% (n=18 cells) in the case of pups from caffeine-treated dams and 58% (n= 18 cells) in pups from KW6002-treated dams - but higher frequencies in the ranges of 5 Hz to 10Hz (33.3%, n=18, in the case of pups from caffeine-treated dams and 35.3%, n=18, in the case of pups from KW6002-treated dams) and 25 Hz to 30 Hz (16.7% of the 18 cells analyzed only in slices from pups belonging to caffeine-treated dams) was detected. Glutamatergic currents recorded in pyramidal cells had a frequency in the range of 0 Hz to 10 Hz. 92.9% of the population of control pyramidal cells (n=17) displayed glutamatergic currents occurring at a frequency lower or equal to 5 Hz and 7.1% of the rest of the population of 17 cells had frequencies of glutamatergic currents varying from 5 Hz to 10 Hz. The proportion of cells showing glutamatergic currents in a frequency lower than 5 Hz was decreased to 66.7% and 85.7% of the population of 18 cells analyzed in when cells from pups belonging to caffeine- and KW6002-treated dams were analyzed, respectively. By opposition, the fraction of cells showing higher frequencies (5 Hz-10 Hz) of glutamatergic currents increased in these groups of animals to 33.3% (n=18) in the case of caffeine-treated pups *via* the mother) and 14.3% (n=18) in the case of KW6002-treated pups *via* the mother.

The mean frequency of GABAergic currents received by pyramidal cells from control animals was 3.7 +/- 1.2 Hz (n=17), 6.6 +/- 4.1 Hz (n=18) in caffeine-treated animals and 3.9 +/- 0.8 Hz (n=18) in KW6002-treated animals and the frequency of glutamatergic currents was 1.8 +/- 0.7 Hz (n=17) in control pyramidal cells and 2.7 +/- 1.3 Hz (n=18) and 2.1 +/- 0.6 Hz (n=18) in caffeine- and KW6002-treated animals, respectively.

Fig. 4.6A) presents the same type of analysis for the population of interneurons from the *S. radiatum*. 94.1% of the population of interneurons (n=19) had lower frequencies of GABAergic currents (0 Hz-5 Hz) and the maximal frequency observed was 10 Hz which was observed in 5.9% of the 19 interneurons analyzed. In caffeine-treated animals a wide range of frequencies was noticed: 41.2% of the cells (n=19) had frequencies lower than 5 Hz, 29.4% (n=19) displayed frequencies between 5 Hz to 10 Hz, 11.8% (n=19) displayed frequencies between 10 Hz and 15 Hz, 5.9% between (n=19) displayed frequencies between 15 Hz to 20 Hz and the same fraction (n=19) displayed frequencies between 20 Hz to 25 Hz. Interneurons from KW6002-treated animals also diaplayed higher frequencies of IPSCs, however the effect of the treatment was not so evident as observed in caffeine-treated animals.

All neurons (n=19) from control animals received glutamatergic currents at a frequency lower than 5 Hz. In the case of caffeine-treated animals, 61.5% of the cells (n=19) had frequencies lower than 5 Hz, 30.8% (n=19) displayed frequencies varying from 5 Hz to 10 Hz and a smaller fraction, 7.7% (n=19) displayed frequencies ranging from 15 Hz to 20 Hz. The distribution of frequencies from KW6002-treated animals was more similar to the same distribution made with cells from control animals: 84.6% of the neurons (n=19) showing frequencies lower than 5 Hz and 15.4% (n=19) receiving glutamatergic currents at a range between 5 Hz to 10 Hz.

The mean frequency of GABAergic and glutamatergic currents received by interneurons from the *S. radiatum* in caffeine-treated animals was significantly higher compared to frequencies received by interneurons from control/vehicle animals. The frequency of IPSCs was 1.8 +/- 0.6 Hz (n=19) in control animals versus 8.2 +/- 2.0 Hz (n=19) in caffeine-treated animals ($p<0.05$, two-tailed *t*-test) and 2.6 +/- 0.7 Hz (n=19) in KW6002-treated animals ($p>0.05$, two-tailed *t*-test). The frequency of glutamatergic currents from interneurons belonging to control animals was 1.3 +/- 0.3 Hz (n=19), 4.6 +/- 1.4 Hz (n=19) in caffeine-treated animals ($p<0.05$, two-tailed *t*-test) and 2.5 +/- 0.7 Hz (n=19) in KW6002-treated animals.

It seems that caffeine treatment has a strong effect upon the frequency of GABAergic and glutamatergic currents in both interneurons from the *S. radiatum* and pyramidal cells.

Generally an increase in the frequency of synaptic currents is associated with a higher probability of neurotransmitter release and it is related with an increased frequency of action potential generation. However, it may be related to an increase in the size of the readily releasable pool (RRP) of neurotransmitters which would result in an increase in the frequency of miniature currents (Tyler

and Pozzo-Miller, 2001). Increasing RRP would sustain long periods of higher frequency of transmitter release before depletion (Dobrunz and Stevens, 1997; Murthy *et al.*, 1997). This could facilitate LTP induction (Murthy *et al.*, 1997; Gottschalk *et al.*, 1998; Pozzo-Miller *et al.*, 1999b). PKC seems to be the pathway involved in the increase in RRP (Stevens and Sullivan, 1998; Waters and Smith, 2000), which is one of the pathways controlled by adenosine receptors (see **Introduction-Purinergic System Overview**).

The analysis of the quantal release (miniature currents) is presented in **Fig. 4.7** and **Fig. 4.8**. Observing the distributions of frequencies of both mIPSCs and mEPSCs in pyramidal cells, (**Fig. 4.7A** and **Fig. 4.7B**, respectively), it is visible that the quantal release of GABA recorded in pyramidal cells occurs at a higher frequency. For example, 50% of the cells analysed receive mIPSCs at a frequency ranging from 1-1.5 Hz in slices from pups born from caffeine-treated dams, whereas only 10% of the pyramidal cells from pups of control dams receive mIPSCs at this frequency range and the higher amount of control cells (80%) receive mIPSCs at a frequency lower than 0.05 Hz. KW6002 had a more modest effect (**Fig. 4.7A**). Interneurons from the *S. radiatum* of slices from caffeine- and KW6002-treated animals (**Fig. 4.8**) also received a higher amount of mEPSCs (**B**). All the interneurons analyzed in hippocampal slices from control animals received mEPSCs at a frequency lower than 0.05 Hz. However, 66.7% of the interneurons from pups of caffeine- treated and 25% of the interneurons from pups of KW6002-treated dams receive mEPSCs at frequencies between 0.05-1 Hz and between 1-1.5 Hz, respectively (see **Fig. 4.8B**).

Interestingly, the effect of acute application of A_1 and A_{2A} receptors in both pyramidal cells and interneurons from the *S. radiatum*, resulted in a decrease or had no effect upon glutamatergic and GABAergic miniature currents (see **Chapter 3**, **section 3.2**). The chronic treatment of dams with caffeine or KW6002 resulted in an increase in the frequency of these events. Maybe a reorganization of glutamatergic and GABAergic network occurred, sustaining a higher frequency level of synaptic activity. These results should, however, be considered with some care because the slices were incubated and analized superfused with an aCSF free of A_1 and A_{2A} receptor antagonists, as well as other metabolites. The real activity of cells born from caffeine and KW6002-treated mothers may be different *in vivo*. What this experiment tells is that the spontaneous activity recorded in the hippocampal slice from pups of caffeine and KW6002-treated animals is changed due to the treatments maybe revealing compensatory mechanisms, the construction of a different network or a different level of maturation of the brain of these animals.

Fig. 4.5

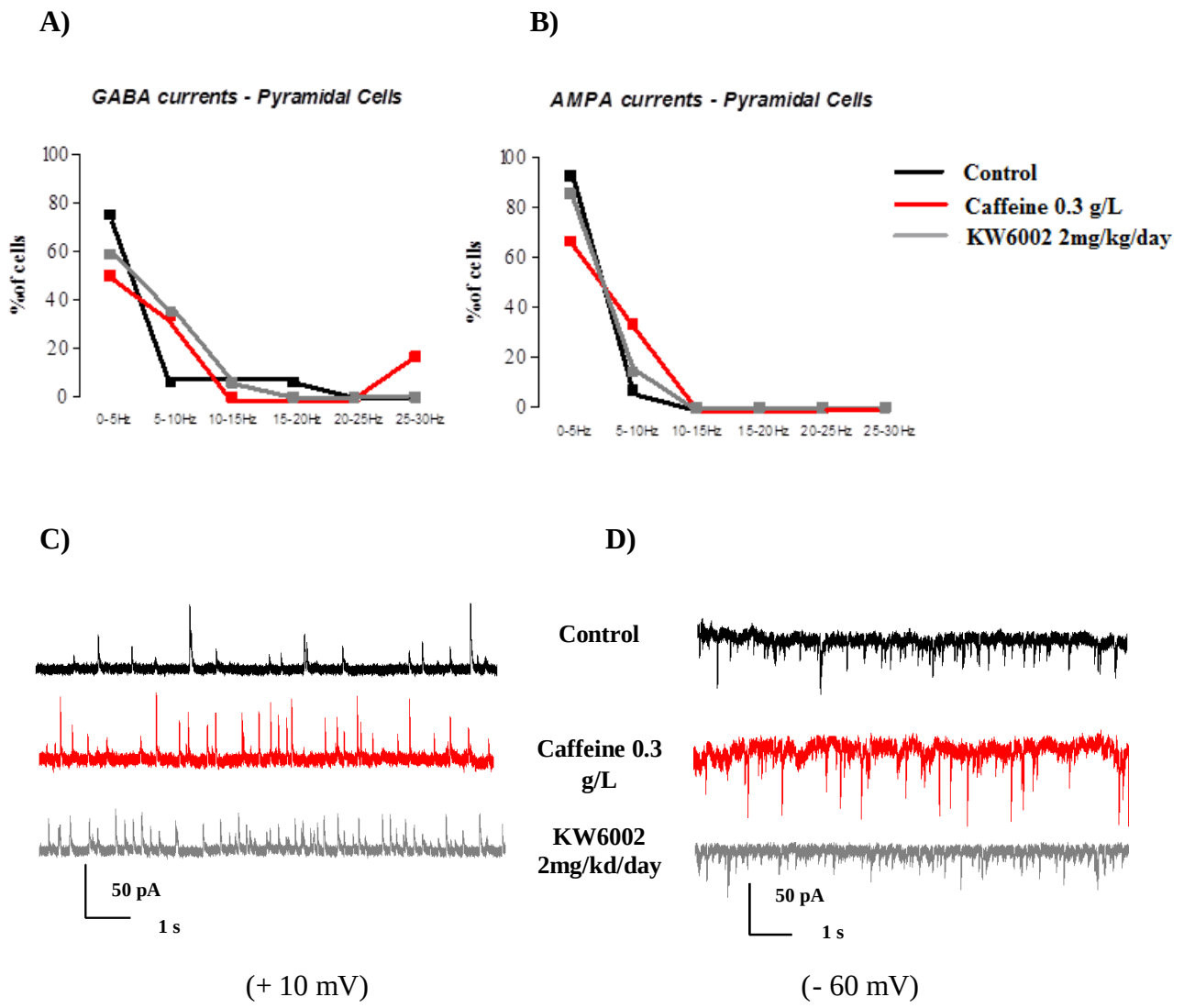


Fig. 4.5 – Effect of caffeine or KW6002 treatments during gestation and beginning of lactation on the frequency of GABAergic and glutamatergic currents received by pyramidal cells of CA3 from 5 -7 days-old pups. The graphs show the distribution of frequency of $GABA_A$ receptor-mediated (A) and AMPA/kainate receptor-mediated (**B**) synaptic currents received by pyramidal cells from CA3 region of PD 5 to PD 7 mice hippocampal slices. **C)** and **D)** Display representative traces showing 10 sec of recording made in pyramidal cells from CA3 region corresponding to GABAergic (holding potential + 10 mV) and glutamatergic (holding potential – 60 mV) activity, respectively. Caffeine (0.3 g/L) and KW6002 (2mg/kg/day) treatments provided to the dams seemed to increase the frequency of GABAergic currents recorded by pyramidal cells from 5 to 7 days-old pups. The effect of the treatments upon glutamatergic currents seemed to be less remarkable in this cell type. 17-18 pyramidal cells from 9-12 pups were used in this analysis.

Fig. 4.6

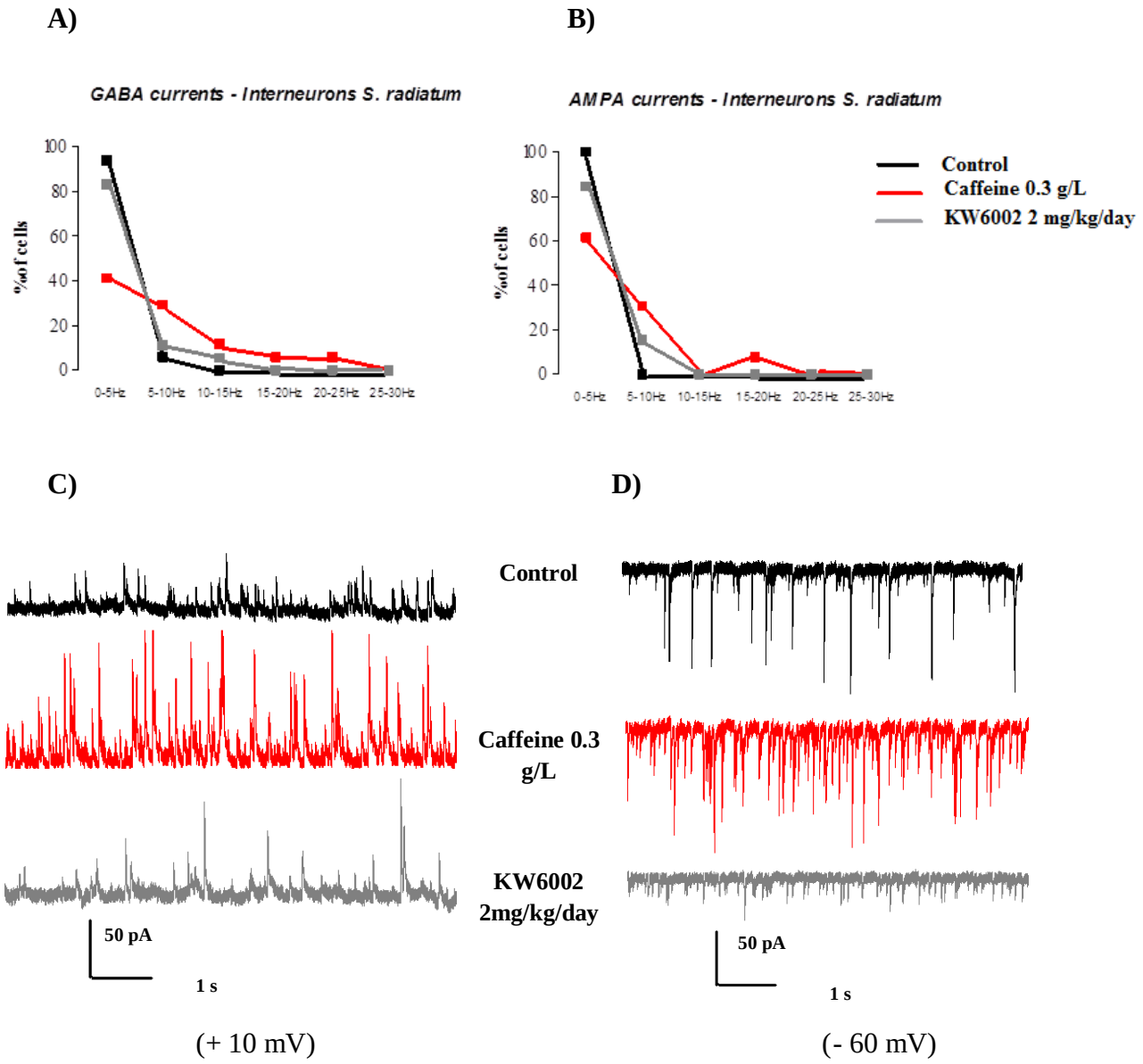


Fig. 4.6 - Effect of caffeine or KW6002 treatments during gestation and beginning of lactation in the frequency of GABAergic and glutamatergic currents received by interneurons from the *Stratum radiatum* of CA3 from 5-7 days-old pups. The graphs show distributions of frequency of $GABA_A$ receptor-mediated (**A**) and AMPA receptor-mediated (**B**) synaptic currents received by interneurons from the *S. radiatum* from CA3 region of PD 5 to PD 7 mice hippocampal slices. The more remarkable effect was observed in slices from pups whose mothers were treated with caffeine 0.3 g/L during gestation and lactation. Caffeine-treatment increased significantly the frequency of GABAergic ($p<0.01$, two-tailed *t*-test) and glutamatergic currents ($p<0.05$, two-tailed *t*-test). 19 interneurons from the *Stratum radiatum* from 9-10 pups were used in this analysis. **C)** and **D)** display representative traces of 10 s of the recording made upon these type of cells in control animals (black), animals born from caffeine-treated dams (red) and animals born from KW6002-treated dams (gray), indicating GABAergic (holding potential was + 10 mV) and glutamatergic currents (holding potential was – 60 mV), respectively.

Fig. 4.7

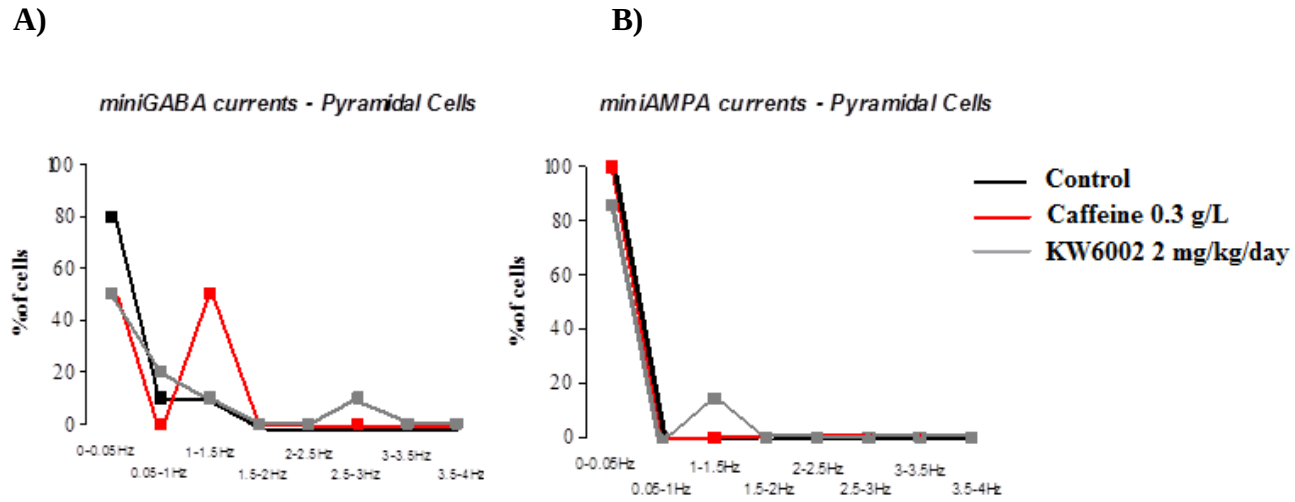


Fig. 4.7 – Effect of caffeine or KW6002 treatments during gestation and beginning of lactation in the frequency of miniGABA and mEPSCs received by pyramidal cells of CA3 from PD 5 to PD 7 pups. The distribution of frequencies of mIPSCs (**A**) and mEPSCs (**B**) recorded in pyramidal cells of pups born from caffeine (0.3 g/L) and KW6002 (2 mg/kg/day) show that both treatments had a more marked impact upon mIPSCs. The proportion of cells showing lower frequencies (lower than 0.05Hz) was decreased in pyramidal cells from treated animals. 5 to 7 days-old male pups were chosen to this analysis. 10 cells were used in this analysis.

Fig. 4.8

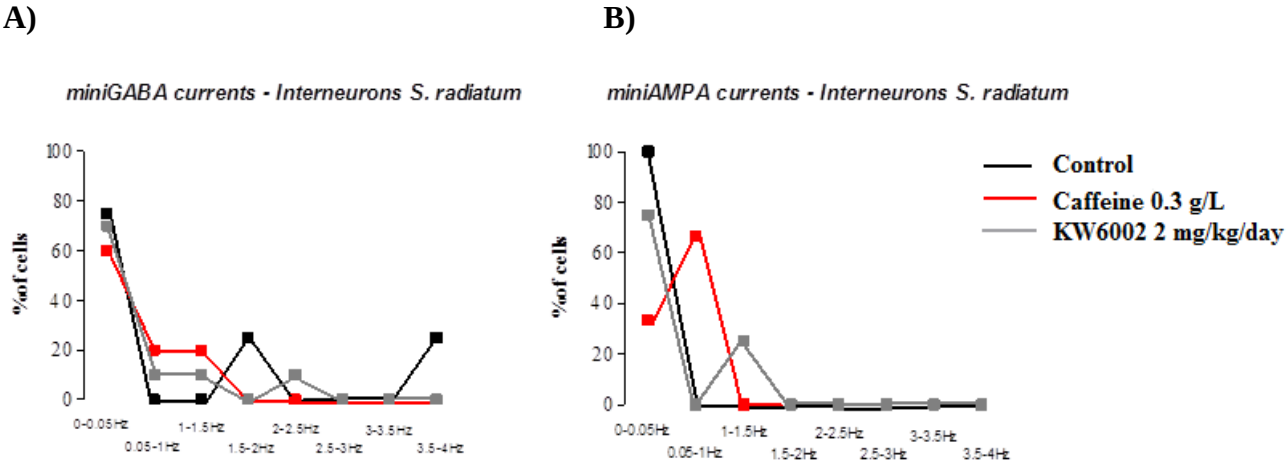


Fig. 4.8 – Effect of caffeine KW6002 treatments during gestation and beginning of lactation in the frequency of miniGABA and mEPSCs received by interneurons from the *S. radiatum* of CA3 from PD 5 to PD 7 pups. The distribution of frequencies of mIPSCs (**A**) and mEPSCs (**B**) recorded in interneurons from the *S. radiatum* of CA3 of 5-7 days-old male pups show a stronger effect of the treatments with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) administered to females before and during pregnancy and during lactation upon mIPSCs and especially upon mEPSCs. 10 cells were used in this analysis.

Another parameter analyzed was the charge transfer ratio in the three groups of animals, calculated by the product of frequency and area of GDPs (Brickley *et al.*, 1996) recorded at + 10mV (GABAergic events) and at – 60mV (AMPA/kainate receptor- mediated events) in pyramidal cells or in interneurons from the *Stratum radiatum* (**Fig. 4.10)**. Since it was observed that an acute application of either A_1 receptor or A_{2A} receptor antagonists changed the stability of AMPA and $GABA_A$ receptors (see **Chapter 3**), it seemed relevant to compare the amount of charge received by these two populations of cells through these two types of receptors in an attempt to evaluate the long-term impact of the antagonism of the adenosine receptors in the glutamatergic or GABAergic systems. This evaluation was carried out through the analysis of GDPs.

The average value of the ratio between the charge transfer carried out by GDPs recorded at + 10 mV and the charge transfer carried out by GDPs at – 60 mV in pyramidal cells recorded in slices from 5 to 7 days-old pups pups born from control females was 2.8 +/- 0.2 (n=4) and in pyramidal cells from pups born from caffeine-treated animals was 0.8 +/- 0.2 (n=7) and in pyramidal cells recorded in from pups belonging to KW6002-treated females was 1.1 +/- 0.4 (n=7). The comparison was made between the means of the groups that received the treatments (caffeine or KW6002) *via* the mother and the control group (which also includes cells from vehicle group), using a two-tailed *t*-test. Significant difference was observed between control and caffeine groups ($p < 0.0001$) and between control and KW6002 groups ($p < 0.05$); this means that the GABAergic drive into these cells is reduced in treated animals and pyramidal cells from these animals receive proportionally more glutamatergic than GABAergic charge (**Fig. 4.9A**).

In the case of interneurons (**Fig. 4.9B**), only the treatment with KW6002 seemed to have an effect and this effect seemed to be qualitatively opposite to that observed for pyramidal cells. Interneurons from KW6002-treated animals showed a higher ratio between $GABA_A$-mediated charge transfer and the charge transferred through ionotropic glutamate receptors which means that they receive proportionally more amount of charge from $GABA_A$ receptors than from AMPA/kainate receptors. As the ratio is calculated using the frequency and surface of the events, this increase may be related to the high frequency of GDPs observed in slices from KW6002-treated animals (**Fig. 4.4**). The mean value of $GABA_A$/AMPA+kainate charge transfer ratio in control interneurons was 2.0 +/- 0.6 (n=15) and it was larger in KW6002-treated animals - 5.7 +/- 1.9, $p<0.05$ (n=7). The $GABA_A$/AMPA+kainate charge transfer ratio in caffeine-treated interneurons was 2.3 +/- 0.9 (n=11) however, no difference ($p>0.05$) was observed between this group and control animals.

Fig. 4.9

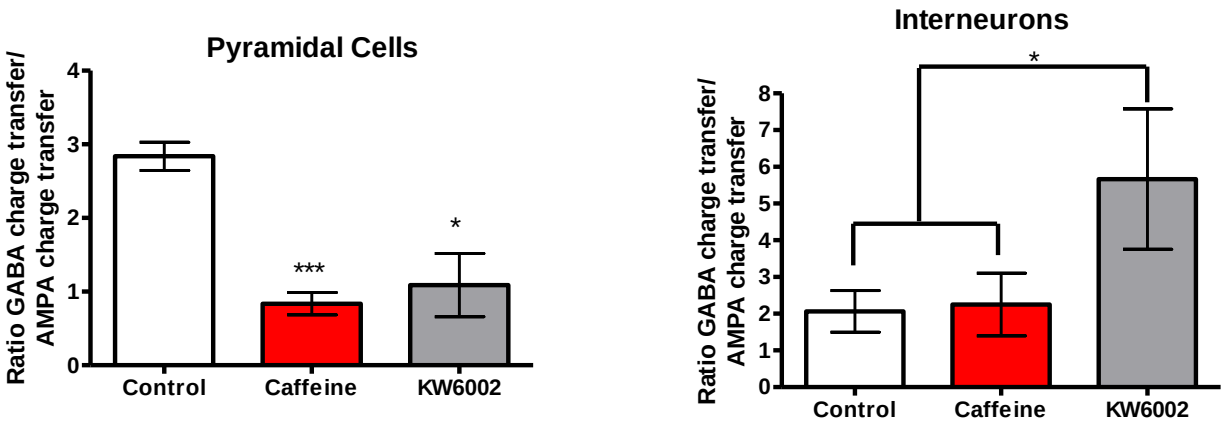


Fig. 4.9 – Effect of the chronic treatment of caffeine or KW6002 to the mothers during gestation and laction upon the ratio $GABA_A$/AMPA receptor-mediated charge transfer, in cells from pups belonging to these females. Principal cells (**A**) seem to receive at about three times more AMPA/kainate receptor-mediated charge than $GABA_A$ receptor-mediated charge. Interneurons from the *S. radiatum* (**B**) from pups whose mothers were treated during gestation and lactation with KW6002 (2 mg/kg/day), receive more GABAergic charge than pups born from control and caffeine (0.3 g/L)-treated mothers, suggesting differences in the rate of maturation or impairment in the functioning of GABAergic and/or glutamatergic systems. Results are mean +/-SEM of 4-7 pyramidal cells and 7-15 interneurons. Statistical significance was investigated using two-tailed *t*-test for comparison between means of control and treated groups and it is indicated in the figure as *$p<0.05$ and ***$p<0.0001$.

It has previously been suggested that the ratio AMPA/GABA charge transfer could be used as a marker for neuronal development (Akerman and Cline, 2007). Immature cells receive relatively weak excitatory glutamatergic input which increases with development. In the case of pyramidal cells belonging to caffeine- and KW6002-treated animals the ratio between GABA and AMPA transmission are decreased which means that in these cells, the AMPA-mediated transmission is more relevant by comparison to GABAergic transmission. It can be interpreted as a sign of a differential maturity or due to an imbalance between both types of neurotransmission. A glutamatergic excitation not compensated by GABAergic signaling leads to hyperexcitability and excitotoxicity (Ben-Ari *et al.*, 2007). A higher GABAergic drive will also lead to an impairment of developmental processes (Ben-Ari *et al.*, 2007). During development GABA has a complex role exciting immature neurons up to a certain level after which the effect becomes inhibitory due to

shunting mechanisms due to increase in membrane conductance through $GABA_A$ receptors and inactivation of voltage gated K^+ and Na^+ channel inactivation (Staley and Mody, 1992). In face of the obtained data and hypothesizing that there is a difference in the rate of maturation of the two populations of cells (more evident in KW6002-treated animals), it seems that there is an imbalance in the rate of maturation of interneurons and pyramidal cells. This homeostasis seems to be relevant for fundamental processes that can exclude neurons from being integrated in neuronal networks. For example, a signature of hypoactivity/hyperactivity resultant from an imbalance between excitation and inhibition (*e.g.* abnormal frequencies of Ca^{2+} waves and depolarization) received by neurons was shown to decrease the mRNA and protein density of neurotransmitters and their synthetic enzymes (Akerman and Cline, 2007). Thus, we investigated if the chronic treatments with caffeine and KW6002 might have modified the pattern of expression of glutamate decarboxylase 65 (GAD 65) and glutamate decarboxylase 67 (GAD 67), two enzymes involved in GABA synthesis and somatostatin, an inhibitor peptide in the hippocampus. GAD 65 and GAD 67 expression was shown to be regulated by activity (Patz *et al.*, 2003); thus, decreased activity decreased the number of GABA and GAD-immunoreactive neurons in the visual cortex of young adult monkeys. Also, somatostatin was shown to increase upon biccuculline (blocks GABAergic transmission) treatment and to decrease with CNQX (blocks glutamatergic transmission) treatment in postnatal rat slice cultures (Marty and Onteniente, 1997). The possibility that the chronic treatment with adenosine receptor antagonists during development may disrupt homeostasis of GABAergic and glutamatergic transmission in the hippocampus was already prompted by the previously reported electrophysiological data, albeit these results could not provide a direct causal relation.

Fig. 4.10 shows that, in the hippocampus of caffeine-treated animals *via* the mother, the labeling of GAD 67 was less defined, with less puncta visible around pyramidal cells. Also the cell body of the interneurons endowed with this enzyme also displayed a lower immunoreactivity, indicating a decreased density of this protein. The modifications induced by the treatments upon the expression of the other isoform, GAD 65 do not suggest an up- or down-regulation but simply a different organization of the protein. Looking at the **Fig. 4.11**, it is visible that immunoreactivity of GAD 65 assumes an an aberrant pattern in the *S. radiatum* and in the pyramidal cell layer. The treatment of the dams with caffeine or KW6002 seems to induce clusterization of GAD 65 in beaded-like structures. The origin of such structures of clusters of protein is unknown but it was observed with other markers of the GABAergic system, such as gephyrin, $\alpha 1$ and $\gamma 2$ subunits of $GABA_A$ receptor, as presented in **Fig. 4.12**, **4.13** and **4.14**, respectively, increasing the evidence that link the

vulnerability of the GABAergic system to the effect of the treatments with the adenosine receptor antagonists. Interestingly labeling with markers for glutamatergic synapses, such as VGLUT1 (**Fig. 4.15**), GluR1 (**Fig. 4.16**), GluR2 and GluR3 (**Fig.4.17**), NR1 (**Fig. 4.18**) and NR2 (**Fig. 4.19**) do not form such pattern of aggregation, suggesting that these varicosity-like structures are associated exclusively with the GABAergic compartment.

Fig. 4.10

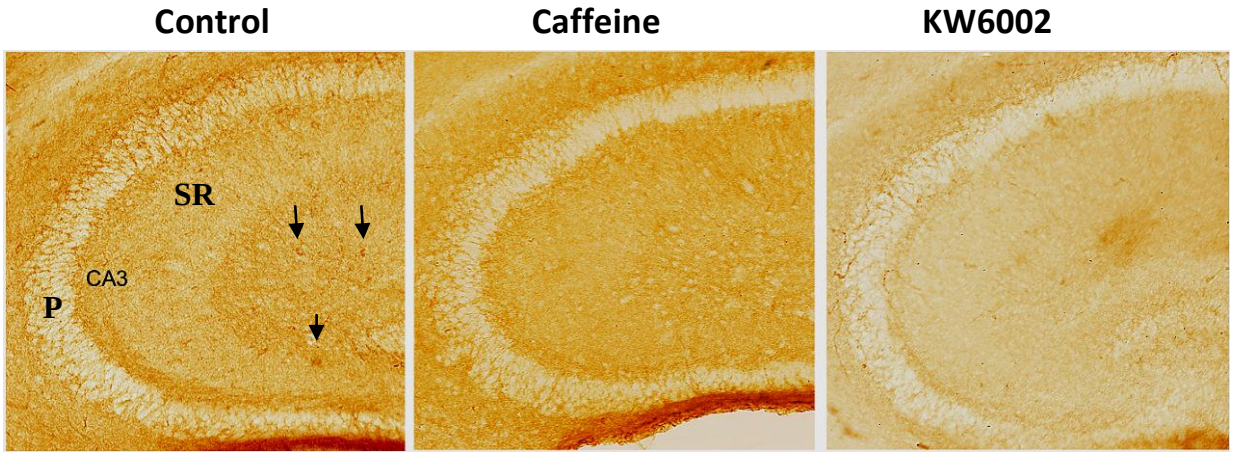


Fig. 4.10 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of GAD 67 in the hippocampus of 6 days-old pups. The chronic treatment with both adenosine receptor antagonists resulted in a lower immunoreactivity of GAD 67. In treated animals, it is less visible the number of puncta around pyramidal cells (P) and the cell bodies of interneurons from *Stratum radiatum* (SR), well visible in slices of control animals and indicated with an arrow (→), are less visible in the same regions of the hippocampus in slices from pups whose mothers were treated with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day). Magnification: 20×. More marked modifications were found in slices from KW6002-treated pups. The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

GluR1 subunit seems to be expressed in all layers of the hippocampus at PD 6 (see **Fig. 4.16**). It has a prominent expression in basal and apical dendrites from pyramidal cells, being mostly absent from cell bodies. Granular layer of the DG also show an intense labeling. The treatments of dams with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) did not change the pattern of GluR1 immunoreactivity. Acute exposure to A_1 or A_{2A} receptor antagonists induced an internalization of GluR1-containing AMPA receptors (see **Chapter 3.3**), however, the chronic treatment with adenosine receptor antagonists does not seem to result in modification in the pattern of location of GluR1-containing AMPA receptors in the hippocampus of chronically treated pups, maybe due to

compensatory mechanisms (*e.g.* increase in the rate of expression of GluR1 by the cells from pups born from mothers treated with adenosine A_1 and A_{2A} receptor antagonists).

GluR2 and GluR3 are two subunits that show dynamic movements in the plasma membrane that are dependent on synaptic activity (Ehlers, 2000). If the treatments with adenosine receptor antagonists indeed induce an abnormal synaptic activity, this should be reflected in the localization and immunoreactiviy of these two AMPA receptor subunits. This does not seem to happen as concluded from the evaluation of the **Fig. 4.17**. There is no evident difference in the pattern of immunoreactivity of GluR2 or GluR3 in pups derived from dams chronically treated with either caffeine or KW6002, during gestation and lactation. GluR2 and GluR3 are located in the cell bodies of pyramidal cells cells of the cornu Ammonis and of granular cells of the DG, interneurons from *S. oriens, S.radiatum* and *S. lacunosum moleculare* as well as interneurons and mossy cells present in the hilus, in both control and treated animals.

NMDA receptors had a role in the SCH58261-induced increase of frequency of GDPs (see **Chapter 3.1**). The immunoreactivity/localization of NR1 receptor subunit was found in the same regions and followed the same pattern of localization of GluR2 and GluR3 subunits (see **Fig. 4.18)**. The intensity of the labeling was higher in pups from caffeine- and in KW6002-treated dams, indicating an up-regulation of NR1 subunit. On the third horizontal panel of **Fig. 4.19**, a more punctiform labeling of NR2 subunit around pyramidal cells was found, the same occurring for granular cells (not shown in the figure). Also, interneurons and mossy cells from the hilus also seem to be endowed with more NR2 subunit immunoreactivity, indicating a putative up-regulation of NR2 subunit. It can also be interpreted as a higher clustering of NR2-containing receptors of the plasma membrane.

Taking into account the data presented previously, it seems that chronic treatment of caffeine and KW6002 does not generate a massive reorganization of the glutamatergic system at both pre (as shown by VGLUT1 labeling) and postsynaptic levels (evaluated by the labeling with GluR1, GluR2 and GluR3). However, the immunoreactivity for two subunits of NMDA receptors, NR1 and NR2, was increased in the hippocampus of treated animals, indicating a putative up-regulation of these proteins. An increased function of NMDA receptor may contribute to an increased excitability in the hippocampal slice, supporting the electrophysiological data described early in this chapter and in **Chapter 3.1**.

The immunoreactivity of somatostatin was also evaluated in the three experimental conditions (in the hippocampus of pups born from control, caffeine- and KW6002-treated dams). It was analyzed if the treatments with the adenosine receptor antagonists would have induced any modification in the population of cells of somatostatin interneurons in the hippocampus of 6 days-old pups. **Fig. 4.20** shows an immunohistochemistry image of coronal hippocampal slices from pups of control/vehicle dams and caffeine (0.3 g/L)/KW6002 (2 mg/kg/day) treated dams where this population of interneurons was labeled. Pups from KW6002-treated dams showed a lower number of somatostatin-labeled interneurons in the hippocampus which was not observed in pups from caffeine-treated dams. Several hypothesis may be proposed to justify this decreased number of interneurons in the hippocampus of KW6002-treated animals, namely: **a)** there is a delay in the maturation of this subtype of neurons; **b)** the interneurons were eliminated due to excitotoxicity; **c)** the migration of these cells was arrested; **d)** the density of somatostatin had a correlation with the activity, present in the tissue since it is regulated by activity as referred previously.

The fact that NR1 and NR2 density seemed to be up-regulated in treated animals and the fact that the frequency of GDPs was also increased in slices from these animals, suggests an increase in the excitability of this tissue; this hypothesis was reinforced by the observation of beaded-like structures in dendrites from several subtypes of interneurons (see **Fig. 4.21**). Similar structures have been reported in other studies and their appearance was shown to be related to mechanisms of excitotoxicity observed *in vivo* (Hori and Carpenter, 1994; Matesic and Lin, 1994) and *in vitro* (Bindokas and Miller, 1995; Al-Noori and Swann, 2000). These beaded-like structures are observed normally in dendrites that are major sites of excitatory synaptic input between neurons and where there is a high amount of receptors for excitatory amino acids (Racca *et al.*, 2000). Park and collaborators (Park *et al.*, 1996) indicated that these varicosities may form after exposure to brief and sublethal excitotoxic exposure to NMDA receptor agonists and are not attenuated using Ca^{2+}-free solutions (Hasbani *et al.*, 1998). Oliva and colaborators (Oliva *et al.*, 2002) reported a dendrotoxicity characterized by dendritic swelling and appearance of beads; this was observed in culture models where glutamate receptor agonists were applied and washout was sufficient to make them disappear (Hasbani *et al.*, 1998, 2001; Al-Noori and Swann, 2000). It was shown using cerebellar cultures that extracellular Ca^{2+} was involved in beaded-like structures formation (Bindokas and Miller, 1995). The formation of varicosities was reported to be rapid (after exposure to NMDA) and reversible and was accompanied by AMPA receptor internalization (Ikegaya *et al.*, 2001). Consequently, a rapid and long-lasting depression of synaptic transmission was observed, which was interpreted as an early and

self-protective response against excitotoxicity. The formation of these structures seems to be related with Na^+ influx through NMDA channels; Na^+ seems to be able to activate Na^+-dependent proteases (Yu and Salter, 1998) and, accordingly, proteases inhibitors diminished the NMDA-induced formation of varicosities. It was also found that preventing the formation of varicosities (in this case lowering the Na^+ concentration in the bathing medium) caused a significantly higher cellular damage (Yu and Salter, 1998). In our experimental model of chronic caffeine and KW6002 administration, the formation of varicosities was restricted to the population of interneurons.

There is evidence in the mature hippocampus that pyramidal cells are less resistant than interneurons to excitotoxic insults and it is independent on intracellular Ca^{2+} concentration (Avignone *et al.*, 2005). Perhaps, the formation of these beaded-like structures in interneurons is a feature increasing their resistance to excitotoxic insults. Varicosity-like structures seem to be induced by A_{2A} receptor blockade in interneurons since: **a)** A_{2A} receptors are abundant in dendrites from interneurons recorded in slices of pups from dams treated chronically with selective and non-selective A_{2A} receptor antagonists (**Fig. 4.21**); **b)** acute exposure to SCH58261 seems to induce the formation of these structures on dendrites of interneurons, mimicking the chronic effect of caffeine or KW6002 treatment; **c)** the blockade of A_1 receptors antagonism does not seem to be able to induce the formation of such structures (observed after an acute application of DPCPX, n=10).

A_{2A} receptors are mainly located in the dendritic layers of pyramidal cells (*Stratum radiatum* and *Stratum oriens*) and in the DG of the hippocampus at PD 6 (see **Chapter 1.1**). The absence of these beads in dendrites from pyramidal cells suggests that A_{2A} receptor signaling events are distinct between these two populations of cells. If it is true that these structures are related with mechanisms of excitotoxicity, the population of somatostatin-expressing neurons which is most sensitive to excitotoxic insults (Sloviter, 1987, 1991) would be expected to die. It would consequently decrease the number of somatostatin-expressing interneurons in the adult hippocampus. **Fig. 4.22**, shows two immunohistochemistry pictures of hippocampal sections in which somatostatin-expressing interneurons were labeled. Although in slices from PD 6 mice pups a marked difference in the number of these neurons was noticed, such a big difference was not noticed in adult (90 days-old) animals (although a quantification of the number of these interneurons was not performed).

Fig. 4.11

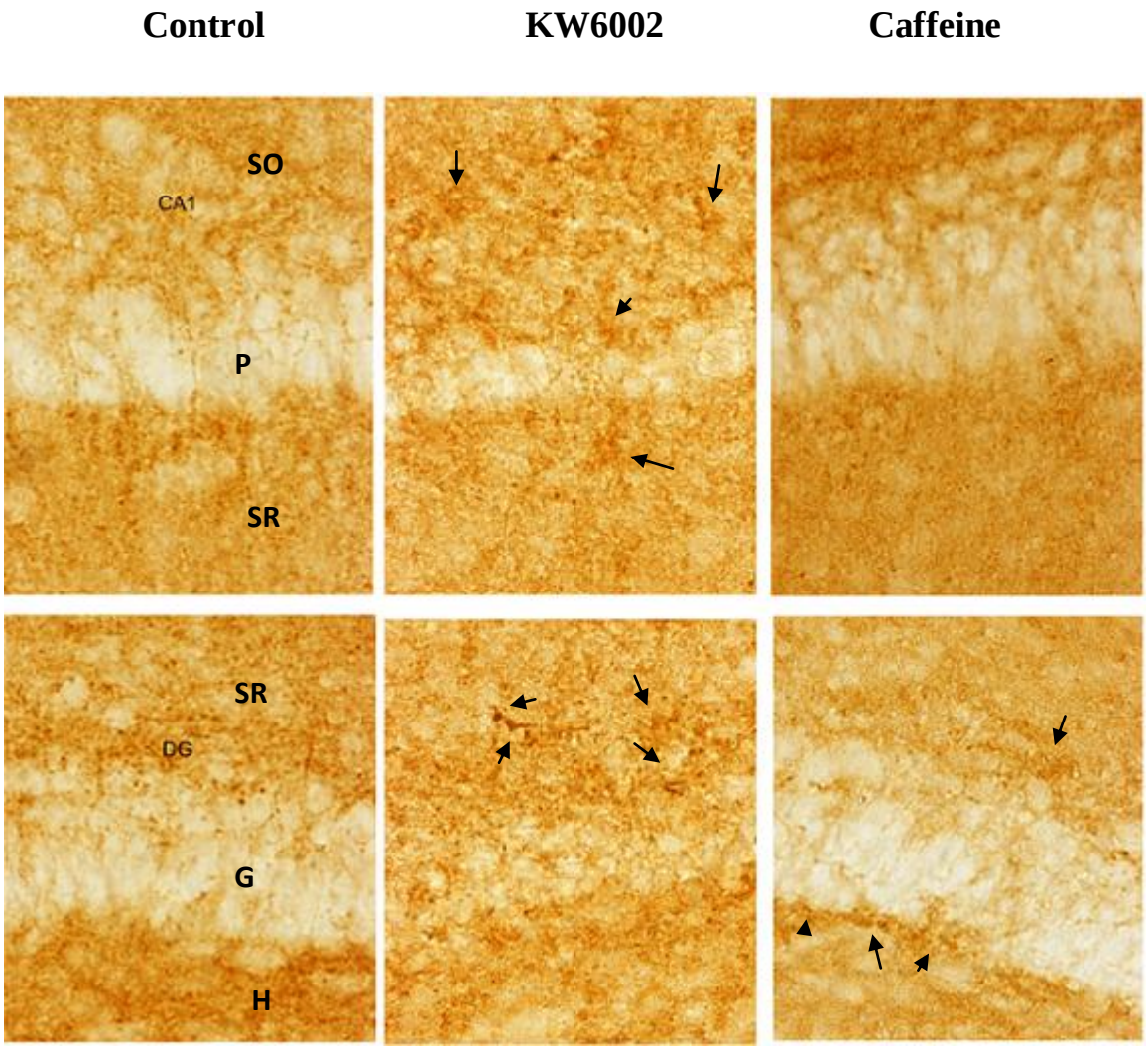


Fig. 4.11 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of GAD 65 in the hippocampus of 6 days-old pups. Sections of the hippocampus of a 6 days-old control, a caffeine- and a KW6002-treated pup, showing the labeling of GAD 65 in the *Stratum oriens* (SO), *Stratum radiatum* (SR) and pyramidal cell layer of CA1 region, as well as in the granule cell layer (G) and hilus (H) of the dentate gyrus (DG). In slices from animals that received KW6002, GAD 65 seems to assume an aberrant organization in clusters indicated by the black arrows (⟶).This aberrant organization makes the pyramidal and granular layers less visible. These clusters were also observed in slices from caffeine-treated pups, however, they do not "mask" the pyramidal and granular cell layers, as observed in KW6002-treated pups. Magnification: 40×. The images are representative of independent analysis of 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

Fig. 4.12

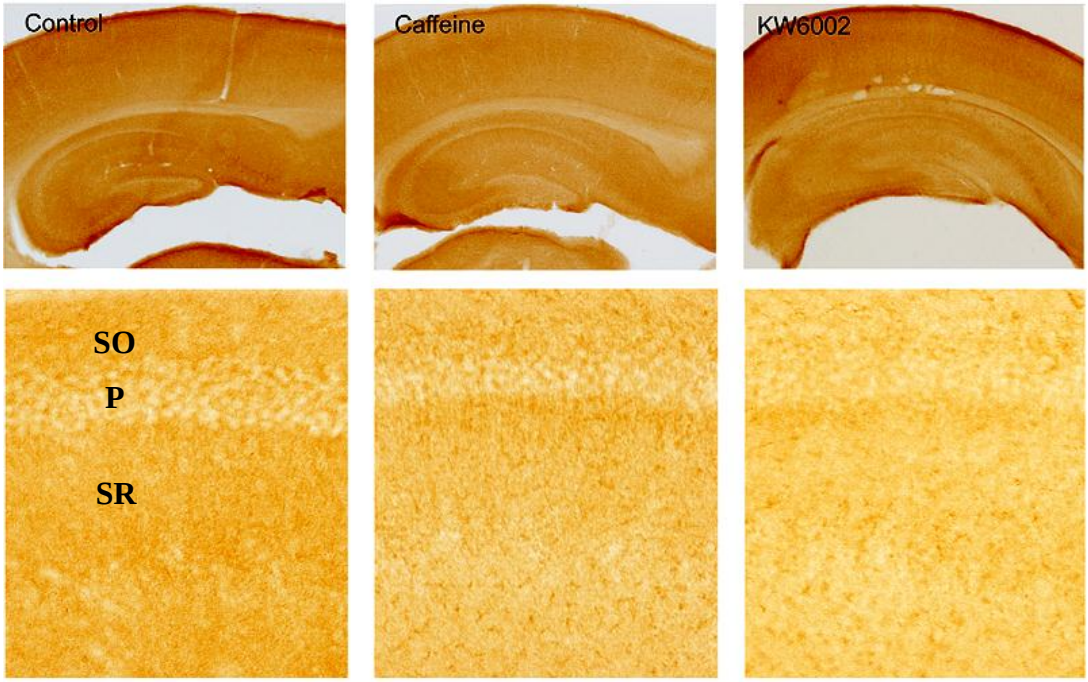


Fig. 4.12 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of gephyrin in the hippocampus of 6 days-old pups. Similarly to what was observed for GAD 65, a pattern of aggregation of gephyrin was observed, labeling with a selective anti-gephyrin antibody the hippocampal slices from 6 days-old pups born from mothers treated with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) before gestation, all the gestational period and the first 6 postnatal days. These pattern of aggregation was observed in all layers of the hippocampus, *Stratum oriens* (SO), *Stratum pyramidale* (P) and *Stratum radiatum* (SR). Magnification - Top horizontal panel (general view of the hippocampus): 2×, Bottom horizontal panel: 40×. The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

It is not know if the effect of the chronic treatments administered to dams upon somatostatin-expressing interneurons of 6 days-old pups is also visible in other populations of interneurons. The observation of brain sections from adult animals (3 months-old) does not seem to show any evident cell loss of somatostatin interneurons in the hippocampus (although a stereological analysis must be carried out in order to detect smaller differences in the number of cells). **Fig. 4.23** shows brain sections from pups from control, caffeine or KW6002-treated dams showing a classical cresyl violet staining to gauge the overall hippocampal morphology. None of the treatments did induce any massive reorganization of the hippocampus or any massive cell loss.

Fig. 4.13

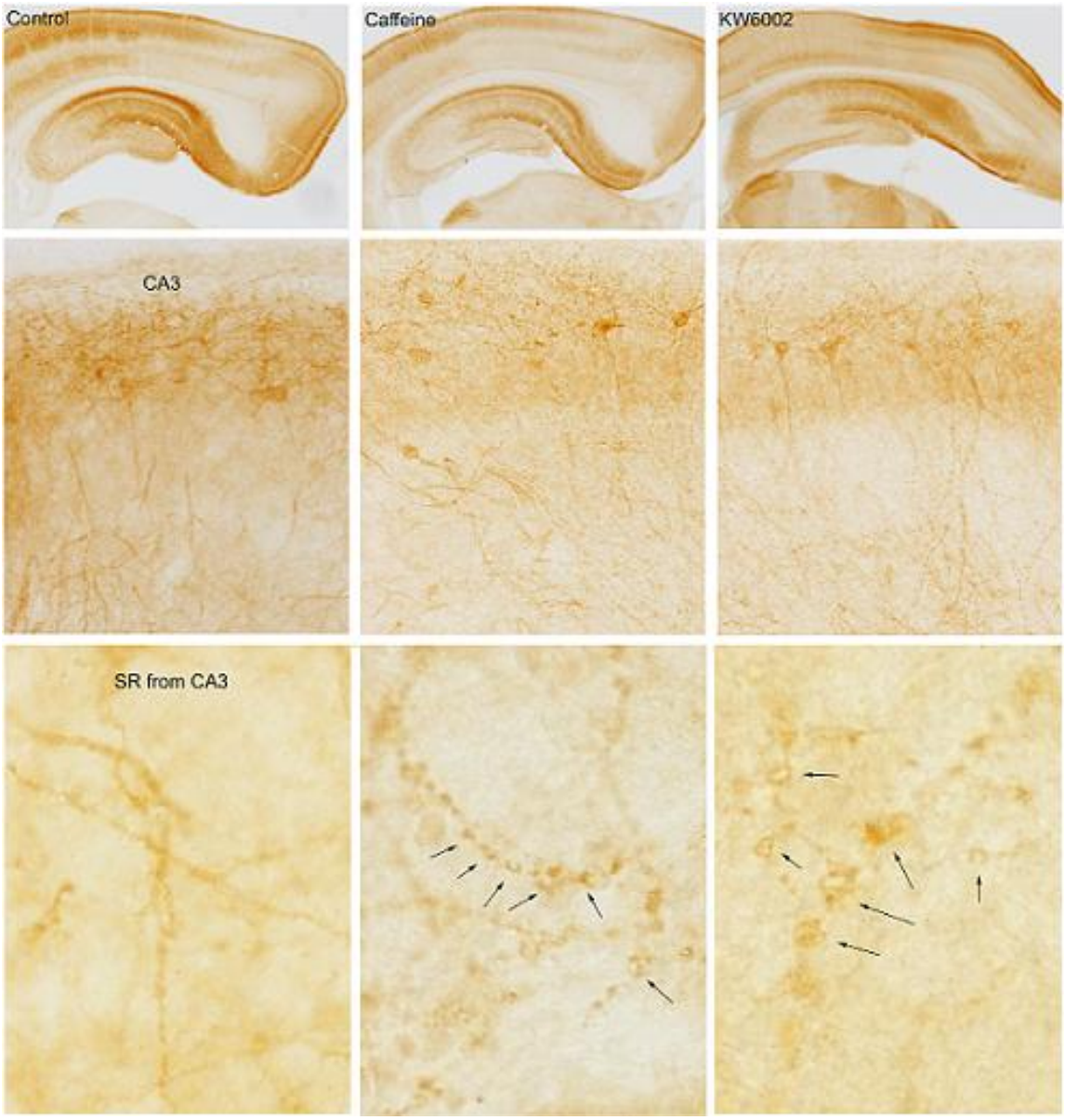


Fig. 4.13 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of α1 subunit of $GABA_A$ receptor in the hippocampus of 6 days-old pups. Top horizontal panel, low magnification (2×) and general view of the hippocampus of pups from control, caffeine (0.3 g/L) – and KW6002 (2 mg/kg/day) - treated dams, labeled with anti-α1 antibody. Middle horizontal panel: CA3 pyramidal cell layer and *Stratum radiatum* showing the general pattern of expression of this $GABA_A$ receptor subunit in the cell body and dendrites of interneurons (20×). Bottom horizontal panel: high magnification (60×) of dendrites from interneurons of the *Stratum radiatum*, showing dendritic beads indicated with black arrows (→), in interneurons of pups born from caffeine- or KW6002-treated animals. The exposure of pups to adenosine receptor antagonists during all gestational period and the first 6 postnatal days leads to a cellular "stress" specifically observed in interneurons. The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

Fig. 4.14

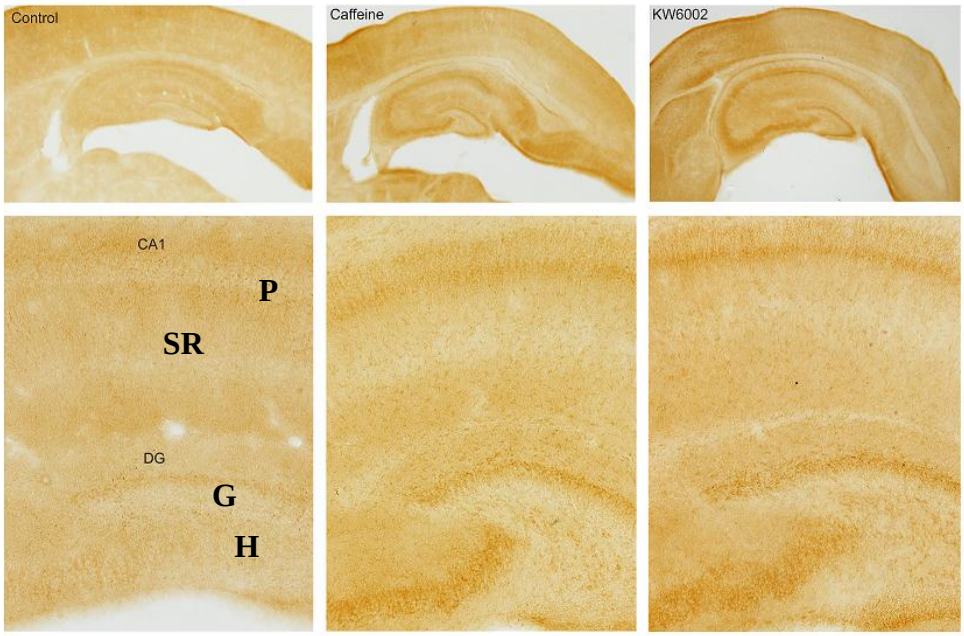


Fig. 4.14 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of γ2 subunit of $GABA_A$ receptor in the hippocampus of 6 days-old pups. General pattern of expression of γ2 subunit of $GABA_A$ receptors in the hippocampus of control, caffeine (0.3 g/L)- and KW6002 (2 mg/kg/day) -treated animals *via* the mother. The subunit also appears in beaded-like structures especially evident in the *Stratum radiatum* (SR) of these animals. Chronic treatments with adenosine receptor antagonists seem also lead to an apparent up-regulation of the subunit, evident in granule (G) and pyramidal (P) cell layers as well as in the hilus (H) of the DG. The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

Fig. 4.15

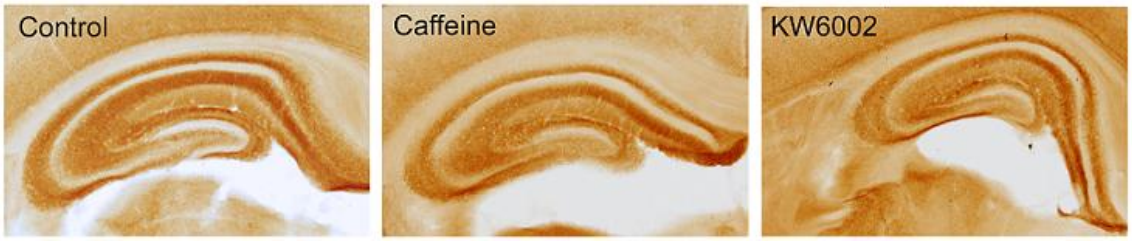


Fig. 4.15 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of VGLUT1 in the hippocampus of 6 days-old pups. Coronal sections of the mouse hippocampus from PD 6 pups labeled with the antibody anti-VGLUT1. No remarkable differences were observed in the pattern of immunoreactivity of VGLUT1, indicating that chronic treatments with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) to pregnant and lactating females did not perturbed the presynaptic components of glutamatergic synapses in the hippocampus of their pups. Magnification 2×. The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

Fig. 4.16

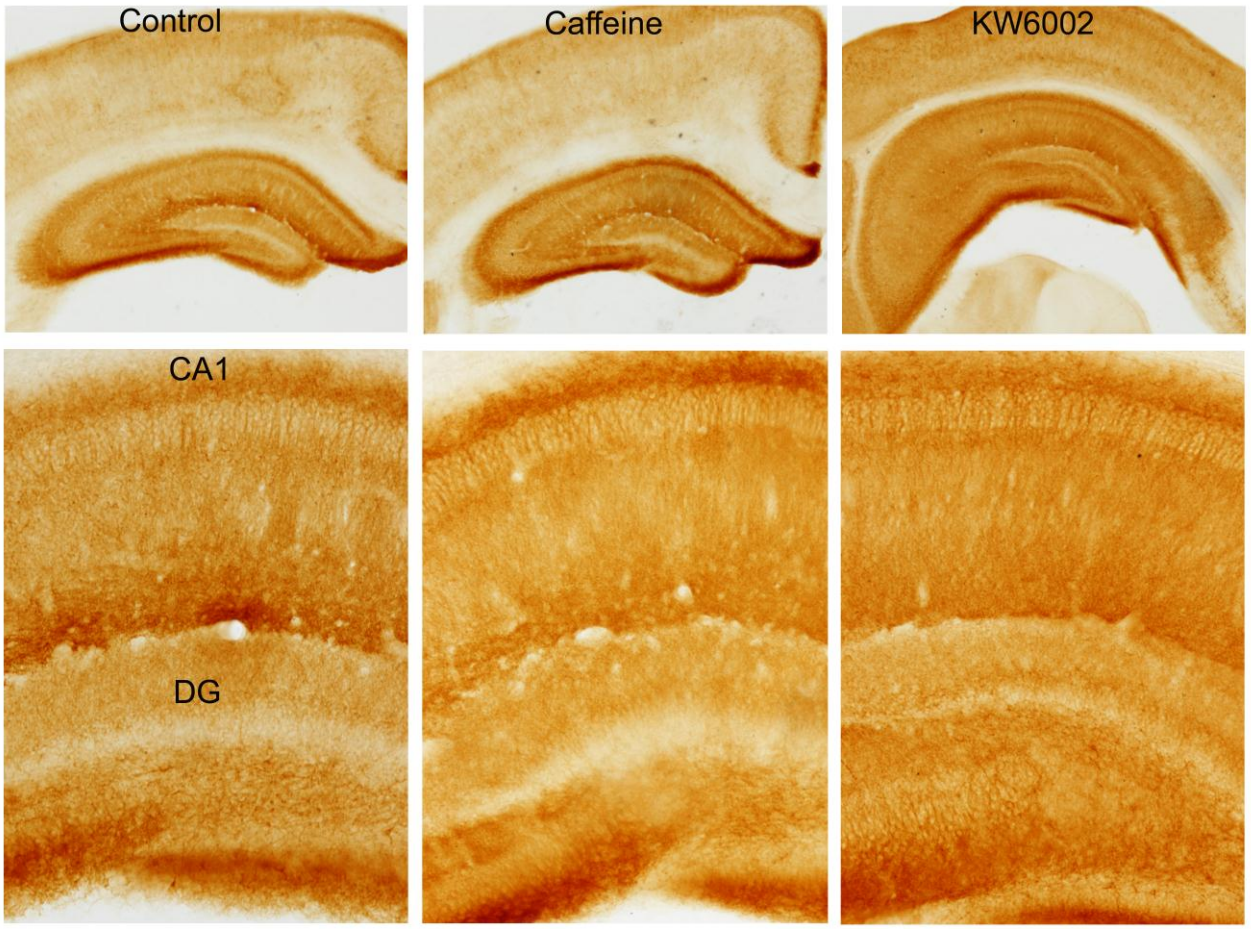


Fig. 4.16 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of GLUR1 in the hippocampus of 6 days-old pups. Coronal sections of mouse pup (PD 6) labeled with and antibody anti-GluR1 subunit. The pattern of expression of this subunit in the hippocampus of 6 days-old mice was not significantly changed by the treatments administered to the mothers (caffeine 0.3g/L or KW6002 2 mg/kd/day) throughout the embryonic and postnatal development (up to PD 6). Hippocampi from pups belonging to all experimental groups (control, caffeine and KW6002 treatment) present a robust expression of this AMPA receptor subunit in dendritic layers and in the molecular layer of the dentate gyrus (DG). Top horizontal panel: Magnification 2× and bottom horizontal panel: Magnification 10×. The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

Fig. 4.17

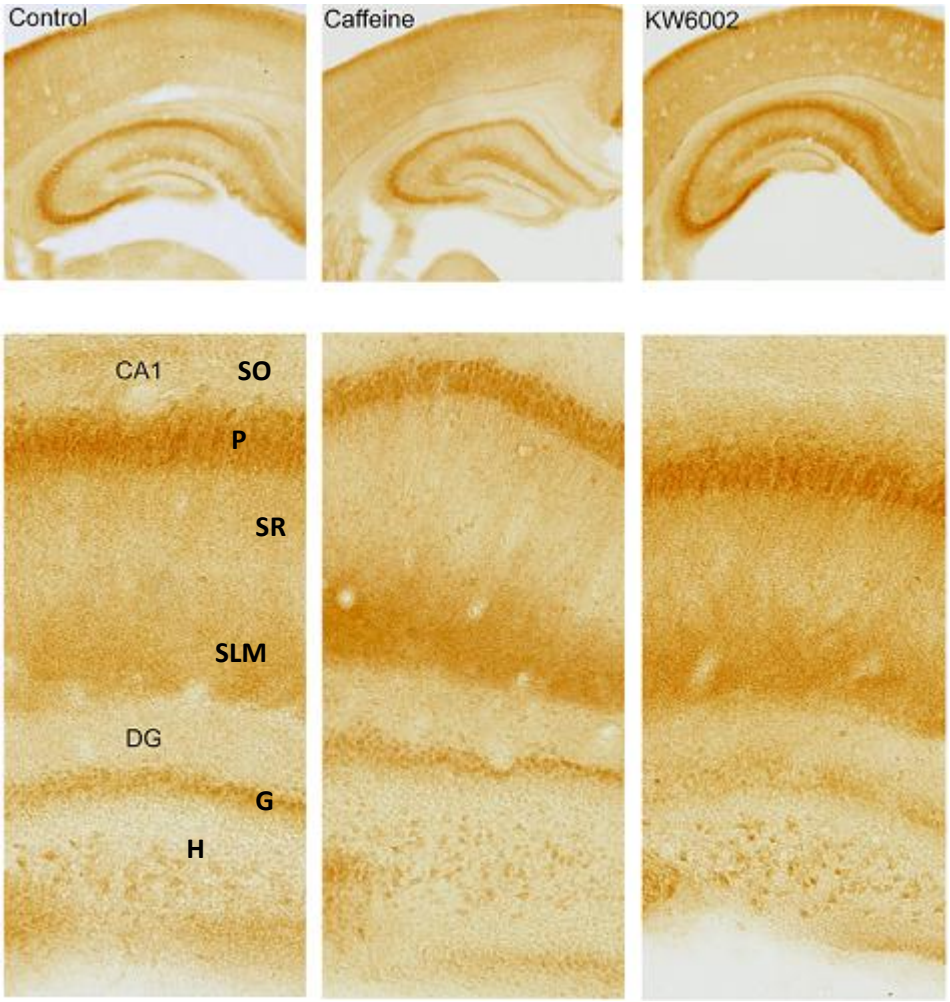


Fig. 4.17 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of GLUR1 in the hippocampus of 6 days-old pups. Coronal sections of 6 days-old pups born from control, caffeine (0.3 g/L) - and KW6002 (2 mg/kg/day) -treated dams, labeled with anti-GluR2,3 antibody. It was not observed a reorganization of the labeling of GluR2 and GluR3 in the hippocampus of pups from treated mothers. The subunits seem to be expressed in cells bodies of cells from the hilus of the DG, in granule cells and in cell bodies of pyramidal cells. Top horizontal panel (presenting the general aspect of the hippocampus of the pups belonging to control, caffeine or KW6002 treatment) : Magnification 2× and Bottom horizontal panel: magnification 10×, showing a more detailed aspect of the labeling with anti-GluR2,3 in all the layers of the hippocampus, *S. oriens* (SO), pyramidal cell layer (P), *S. radiatum* (SR), *S. lacunosum-moleculare* (SL-M), granule cell layer (G) and hilus (H). The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

Fig. 4.18

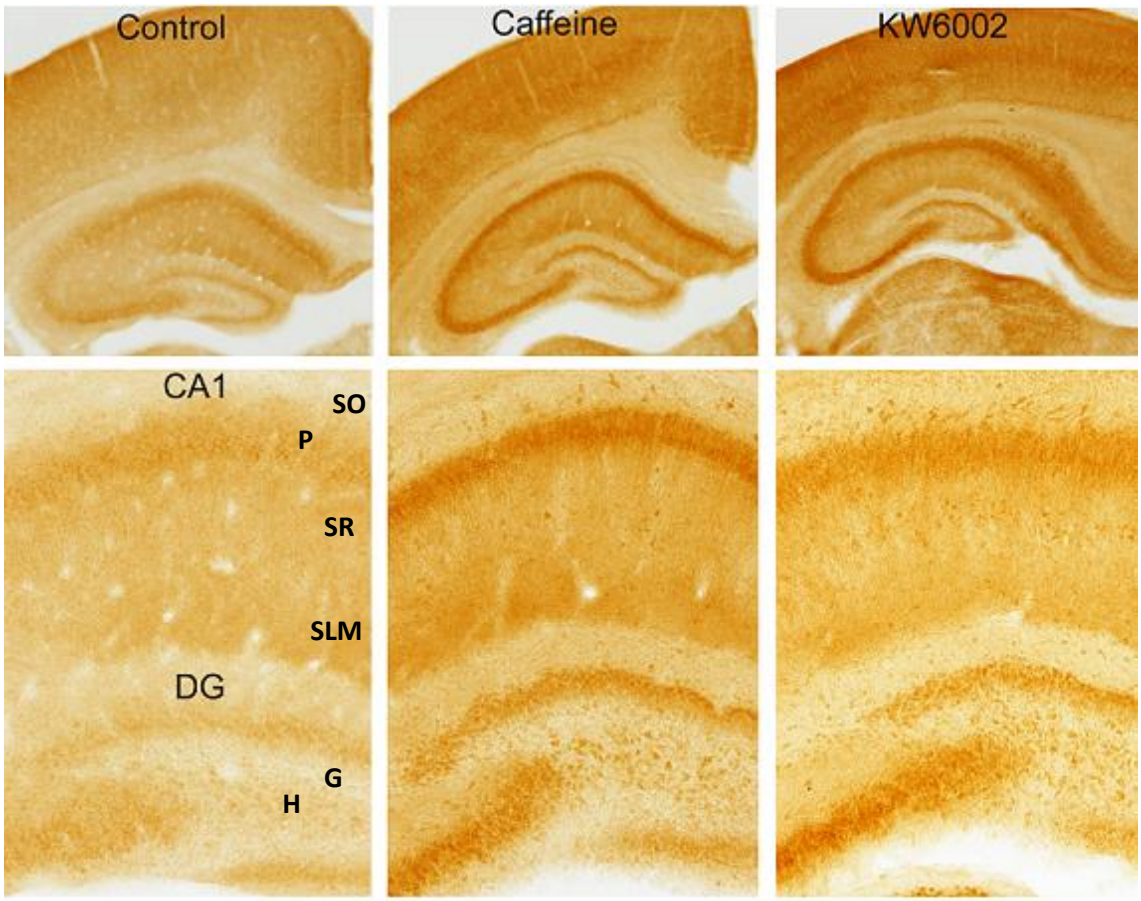


Fig. 4.18 - Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of NR1 in the hippocampus of 6 days-old pups. NR1 subunit of NMDA receptors is located in cell bodies of pyramidal cells, granular cell layer and hilus of the dentate gyrus (DG) also present in the cell body of interneurons and seemd to be up-regulated in the tissue of pups born from caffeine (0.3 g/L)- and KW6002 (2 mg/kg/day)-treated dams. Top horizontal panel: 2× (showing the general pattern of organization of the hippocampus labeled with anti-NR1); Bottom horizontal panel: 10× (showing the detail of the labeling in the hippocampal layers – *S. oriens* (SO), pyramidal cell layer (P), *S. radiatum* (SR), *S. Lacunosum moleculare* (SL-M), granule cell layer (G) and hilus (H) of the dentate gyrus (DG). The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity.

Fig. 4.19

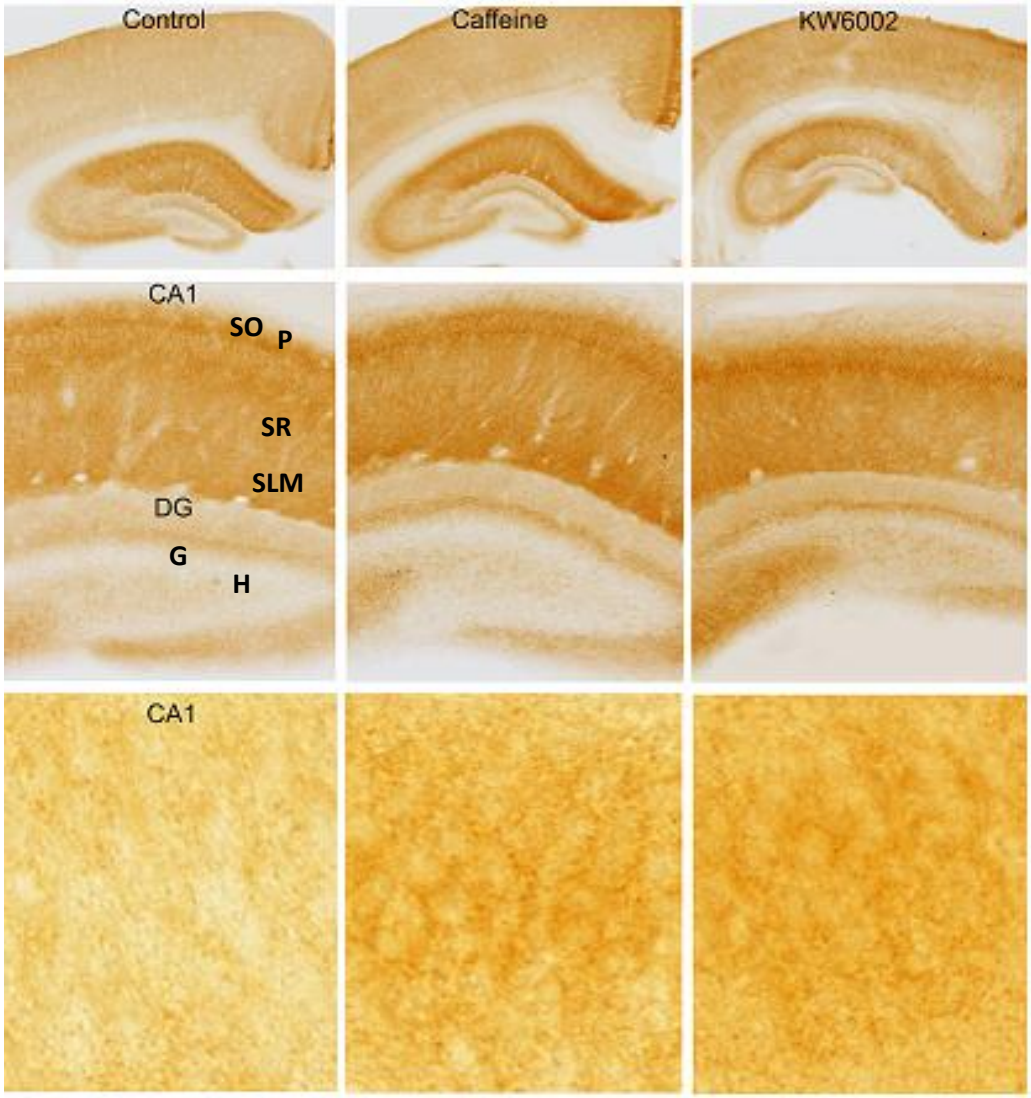


Fig. 4.19 - Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of NR2 in the hippocampus of 6 days-old pups. Coronal sections of the hippocampus from 6 days-old pups from control, caffeine (0.3 g/L)- and KW6002 (2 mg/kg/day)-treated mothers, showing the labeling with the antibody anti-NR2. The immunoreactivity is apparently stronger in cell bodies of pyramidal cells (and also around granular cells – not shown) of pups born from caffeine- and KW6002 treated mothers (see the third horizontal panel showing the organization in CA1 region). First horizontal panel: magnification 2×; second horizontal panel: magnification 10× and third horizontal panel: magnification 40×. The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

Fig. 4.20

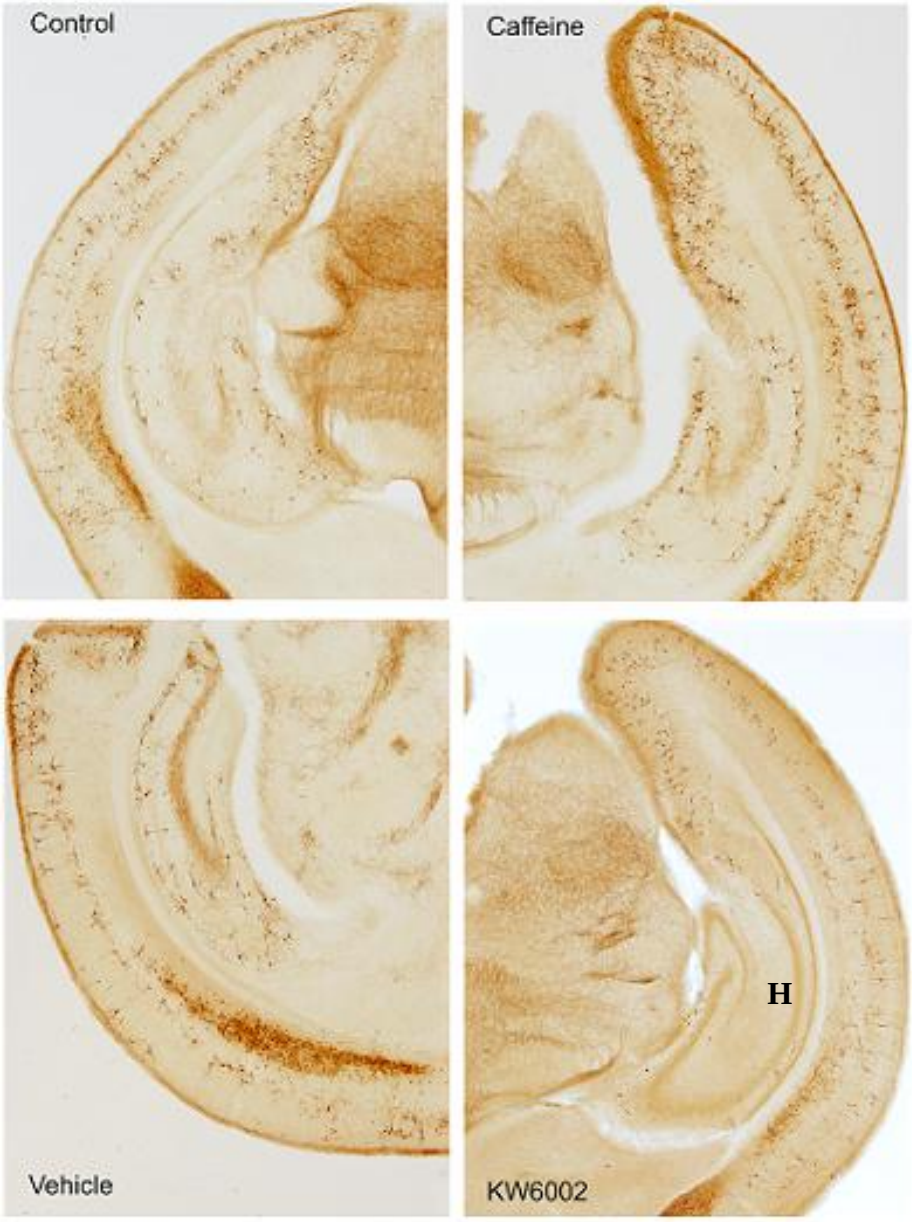


Fig. 4.20 – **Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of somatostatin in the hippocampus of 6 days-old pups.** In the image is shown the immunoreactivity of somatostatin in coronal brain slices of 6 days-old pups born from control, vehicle, caffeine (0.3 g/L)- and KW6002 (2 mg/kg/day)- treated dams. KW6002-treated animals seem to show a decreased immunoreactivity (and a lower number of interneurons) in the hippocampus (**H**). Interestingly, the labeling of somatostatin-positive cells in slices of pups from chronically caffeine-treated dams revealed interneurons possessing more developed dendrites and an apparent higher number of somatostatin-positive interneurons in the hippocampus (although a quantification using stereology was not performed). The images are representative of independent analysis to 4 animals *per* group. Magnification: 2×. It was not performed a quantification of the immunoreactivity.

Fig. 4.21

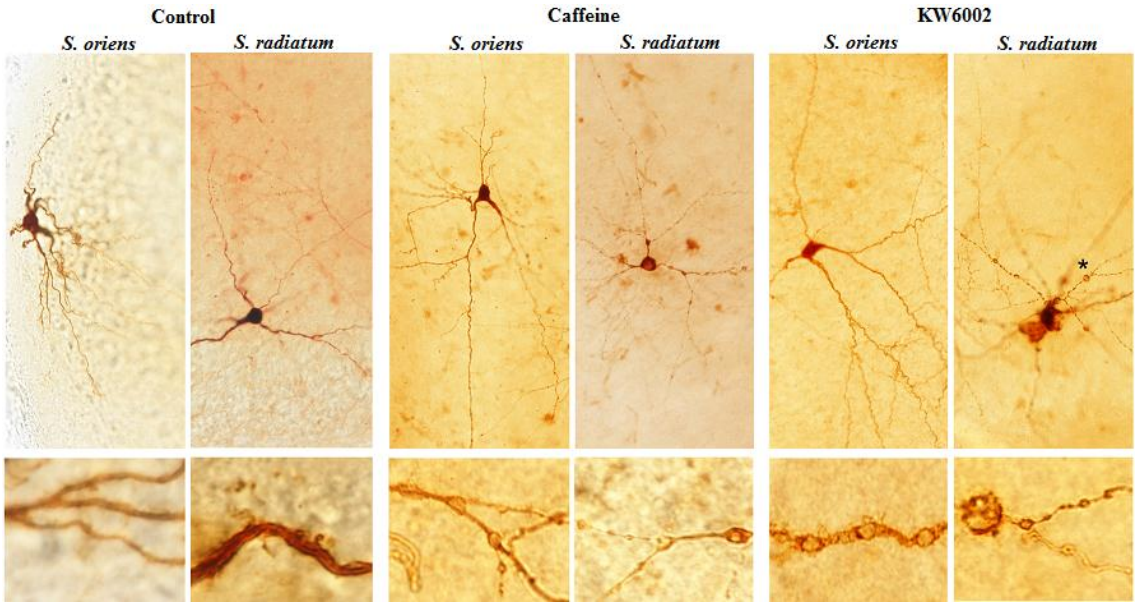


Fig. 4.21 – Effect of the chronic treatment with caffeine or KW6002 *via* the mother, in the pattern of immunoreactivity of somatostatin in the hippocampus of 6 days-old pups. Images from interneurons from the *Stratum radiatum* and *Stratum oriens* from 6 days-old pups from control, caffeine (0.3 g/L)- or KW6002 (2 mg/kg/day)- treated mothers. Cells were labeled with biocytin contained in the patch pipette solution. In the bottom horizontal panel it is visible a detail (magnification 100×) of dendrites of these interneurons exhibiting varicosity-like structures in slices from treated animals. This type of structure is not selective of a specific subtype of interneuron since it is found in interneurons from the *Stratum radiatum* and *Stratum oriens*. This observation was made in about 20 cells from at 5 pups.

Fig. 4.22

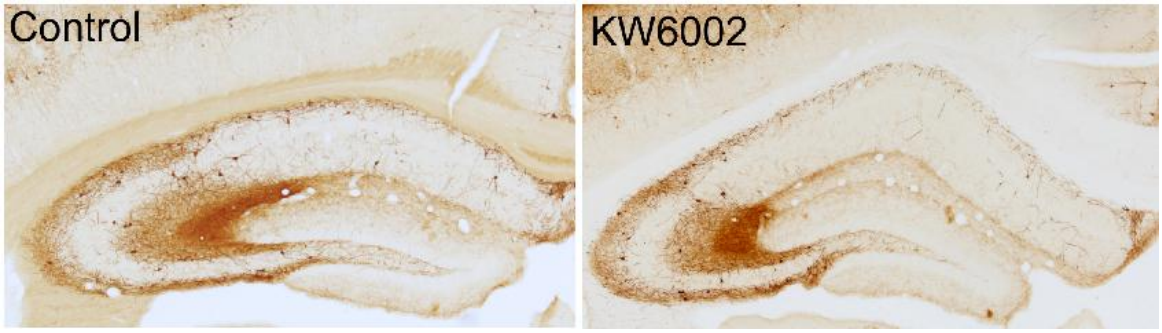


Fig. 4.22 – Chronic treatment with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) given to the dams during gestation and lactation does not seem to decrease permanentely the number of somatostatin-positive interneurons in the hippocampus of adult mice. Brain sections of the hippocampus from a control (left) and a KW6002-treated (right) animal of 90 days-old where the population of somatostatin -positive interneurons was labeled. Although the number of interneurons was markedly reduced in slices belonging to 6 days-old pups, such a difference does not seem to be found in adult animals (although a quantification of the number of somatostatin-positive interneurons using stereologic methods need to be done), suggesting that there is a recovery. Magnification: 2×.

Fig. 4.23

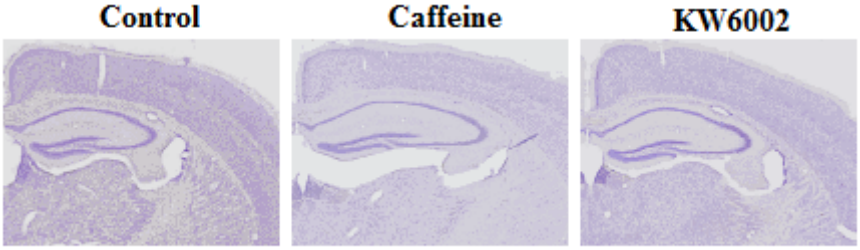


Fig. 4.23 – Chronic treatment with caffeine or KW6002 given to the dams during gestation and lactation does not seem to change the general organization of the hippocampus. Cresyl violet staining of sections of hippocampal slice showing the general organization of the hippocampus from control, caffeine (0.3 g/L)- and KW6002 (2 mg/kg/day)-treated animal. The treatments do not seem to have induced a massive reorganization of the hippocampus or a significant cell death. Magnification: 2×.

An arrest in the migration of these somatostatin interneurons in the brain of 6 days-old pups belonging to dams treated with adenosine receptor antagonists is also possible. However pups from caffeine-treated dams seem to show a normal number of well developed somatostatin-positive interneurons (see **Fig. 4.20**). If it is true that there is an imbalance of $GABA_A$- and glutamate receptor-mediated signaling in treated animals, it is plausible that the migration of the population of somatostatin interneurons would be affected. As reffered previously in the **Introduction** section, high and low concentrations of GABA may have antagonic effects in cell migration.

The cellular stress observed in interneurons in the first stages of development could leave long-lasting effects in adult animals. In part, chronic stress during early life has been shown to be potentially injurious in several animal models (Sánchez *et al.*, 2001; Brunson *et al.*, 2005); it can result in deficits in hippocampus-dependent learning and memory (Luine *et al.*, 1994) and perturb gene expression through epigenetic factors (Ravindran and Ticku, 2004; Veldic *et al.*, 2005).

To evaluate the long-term impact of the chronic treatments with caffeine (0.3 g/L) and KW6002 (2 mg/kg/day), physiological, morphological and behavioral analysis were carried out in adult mice. For the physiological analysis, we chose the population of pyramidal cells belonging to CA1 region because they seem to be a more homogeneous population of cells. AMPA and GABA as well as miniature glutamatergic and GABAergic currents were recorded. Cumulative frequency distributions and respective average values of amplitude, area, rise and decay time constants of both mEPSC and mIPSCs are presented in the **Fig. 4.24** and **Fig. 4.25**.

The amplitude of mEPSCs recorded in pyramidal cells from 90 days-old mice were 21.0 +/- 0.9 pA, 22.8 +/- 0.7 pA in slices from 90 days-old caffeine-treated animals and 20.7 +/- 0.6 pA in slices from 90 days-old mice treated with KW6002 during development (n=12 for each groups). The comparison between groups using the Kolmogorov-Smirnov test showed no significantly differences between the cumulative distributions of control and caffeine-treated conditions ($p > 0.05$) and between the cumulative distributions of control and KW6002 treated cells ($p > 0.05$).

The mean area of mEPSCs from control pyramidal cells was 240.6 +/- 14.8 pA×ms (n=12) and it was statistically different ($p<0.0001$) from areas of mEPSCs recorded in caffeine-treated (149.8 +/- 9.2 pA×ms, n=12) and KW6002-treated (103.3 +/- 15.7 pA×ms, n=12) pyramidal cells. This difference was supported by the statistical analysis using the Kolmogorov-Smirnov test for comparison between cumulative probability distributions ($p<0.0001$ between control and caffeine-treated groups and $p<0.0001$ between control and KW6002-treated groups). Modification of the area of mEPSCs was related with changes in the kinetic properties of the currents. The mean values for both rise time and decay time were different between control and treated-animals. The rise time from mEPSC currents recorded in pyramidal cells was 3.8 +/- 0.3 ms (n=12), in the control group, 1.3 +/- 0.1 ms in caffeine-exposed animals and and 1.6 +/- 0.2 ms in KW6002-exposed animals (n=12 for each group). $p<0.0001$, using a two-tailed *t*-test for comparison between means (control *versus* caffeine and control *versus* KW6002). The decay time constant was also different in the treated groups. The average decay time constant was 9.7 +/- 0.6 ms, in control conditions, which was significantly higher than in caffeine-treated animals (6.0 +/- 0.3 ms) and in KW6002-treated animals (5.5 +/- 0.3 ms), n=12 for each group, $p<0.0001$, using a two-tailed *t*-test for comparison between mean of control and treated groups. The difference was confirmed by the comparison of the probability distributions in different groups, ($p <0.0001$ between control and caffeine distributions and between control and KW6002-treated animals).

The kinetic properties and ion permeability of AMPA receptor-mediated currents is highly dependent on the subunit composition of AMPA receptors present on the postsynaptic membrane. GluR1-GluR4 subunits combine in tetramers in different stoichiometries to define the properties of the receptor (Ozawa *et al.*, 1998). In the hippocampus, GluR1, GluR2 and GluR3 subunits seem to be highly expressed in pyramidal cells. AMPA receptor subunits can also be expressed in two splice variants, flip and flop (Sommer *et al.*, 1990) and, in the adult, excitatory neurotransmission is mainly mediated by channels carrying the flop variant; however, exceptions were found in population of pyramidal cells of CA3 region (Monyer *et al.*, 1991), which express only flip variant in the mature

hippocampus. The channels assembled with the flop variant show a faster desensitization time constant, compared with flip variants (Mosbacher *et al.*, 1994). Flip and flop splice variants differ in their functional properties. One exception is GluR1 subunit which flip and flop variants have similar rates of desensitization (Pei *et al.*, 2009). RNA editing can also determine the rate of desensitization of AMPA receptor subunits. GluR1, GluR3 and GluR4 subunits contain glutamine (Q) in the M2 pore-loop segment. Editing at the R/G site reduce the amplitude and accelerate recovery from desensitization. Phosphorylation (Santos *et al.*,2009) and the geometry of synapses as well as the rate of clearance of the agonist can also have an impact in the properties of EPSCs.

Albeit the genetic composition of AMPA receptors was not analyzed, immunohistochemistry labeling of GluR1 subunit showed that pyramidal cells from treated animals have a stronger labeling around the cell body (see **Fig. 4.26A**). GluR1 subunit is the most permeable to Ca^{2+} (Kim *et al.*, 2005). The pattern of expression of GluR2 and GluR3 does not seem to be changed (**Fig. 4.26B**). A possible interpretation for this increase and correlation with activity for this higher concentration of GluR1 receptor around cell bodies of pyramidal cells will be discussed later in this chapter.

As AMPA receptors are the principal transducers of excitatory neurotransmission in the mammalian brain, an alteration in the subunit configuration of AMPA receptors is likely to lead to long-standing physiological changes at the synapse. LTP and LTD are ideal paradigms to investigate a possible consequence of alteration in AMPA receptor composition and its impact in the physiology of the synapses. The labeling found for GluR1 subunit is not punctiform (labeling the receptors in the membrane surface) but instead diffuse, which suggests the labeling is mainly intracellular (see **Fig. 4.26A**). It is plausible to think that the intracellular pool of GluR1-containing receptors is increased by comparison with the pool present at the plasma membrane in the hippocampus of pups from dams treated with caffeine and KW6002. Whether this observation can have an impact in LTP/LTD induction was not yet investigated, although this is certainly the next step to be done. In our model, no differences in the pattern of labeling of anti-GluR2/3 subunits was observed in CA1 pyramidal cells in the different groups (see **Fig. 4.26B)**, as also observed by Cornejo *et al.*, 2007.

Fig. 4.24

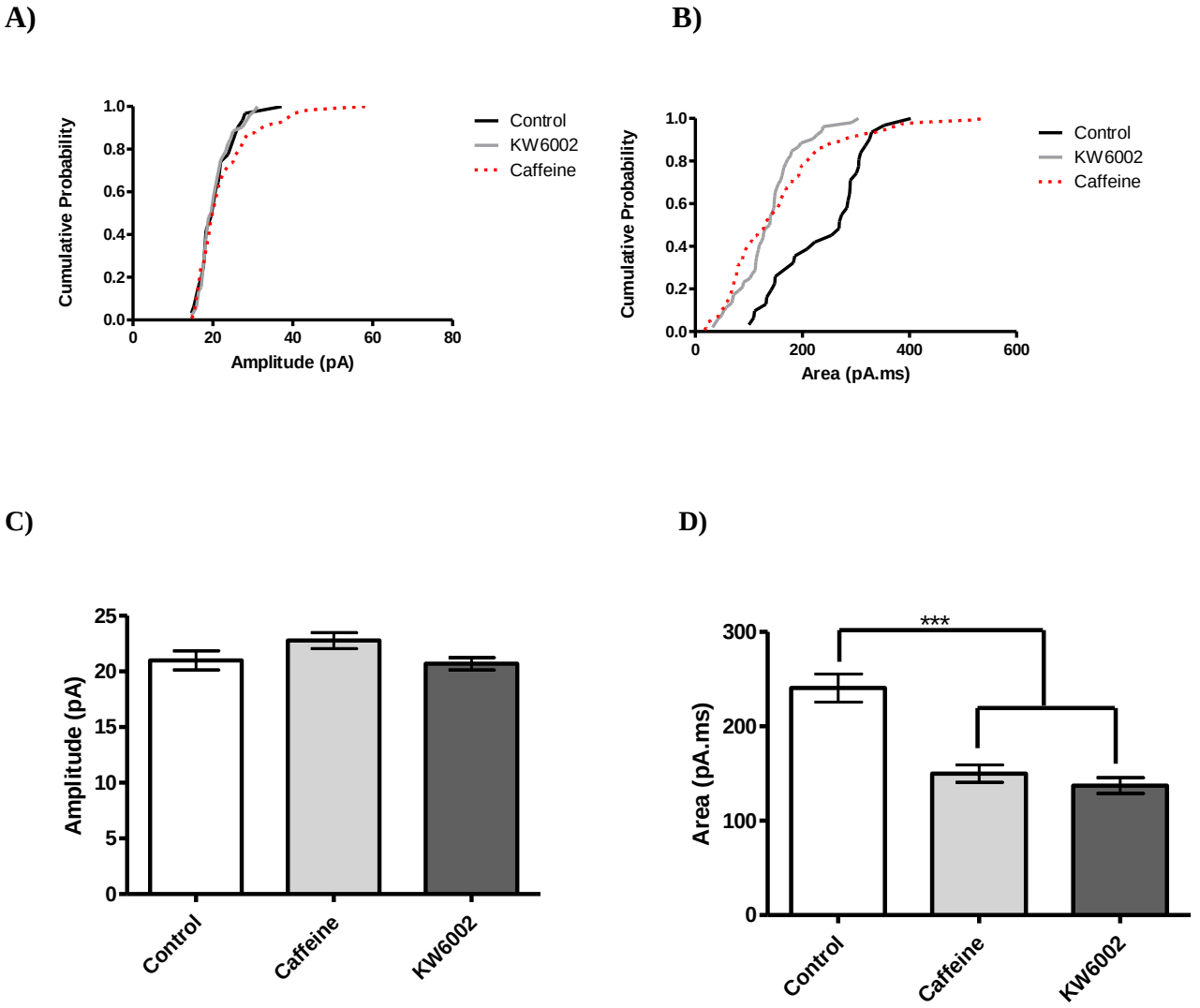


Fig. 4.24 – Properties of mEPSCs recorded in CA1 region of the hippocampus from slices of 90 days-old animals born from caffeine or KW6002 exposure during gestation and lactation. Cumulative probability distributions for the amplitude (**A**) and area (**B**) of mEPSC received by pyramidal cells from CA1 region of slices from adult mice (PD 90). The amplitude of the currents was not changed in treated animals (C) however a significant effect was observed upon their area in treated animals (**D**). Chronic administration of caffeine (0.3 g/L) and KW6002 (2 mg/kg/day) resulted in a decrease in the area of mEPSC currents. Results are mean +/- SEM of at least 100 analyzed currents from 12 independent pyramidal cells and the average data was compared using a two-tailed *t*-test. Cumulative probability distributions were analyzed using Kolmogorov-Smirnov test. Statistical significance is indicated in the figure as ***p<0.0001.

Fig. 4.25

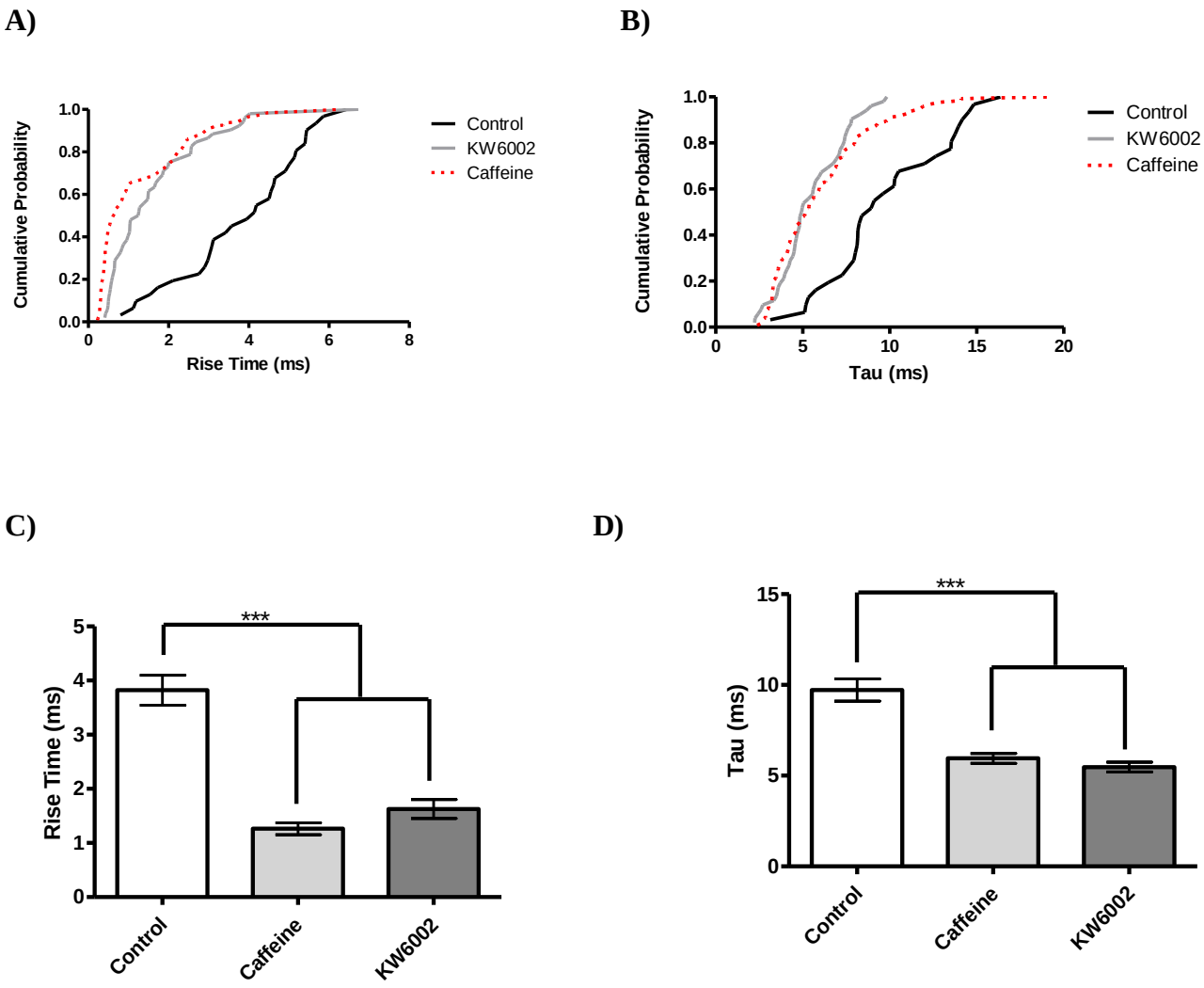
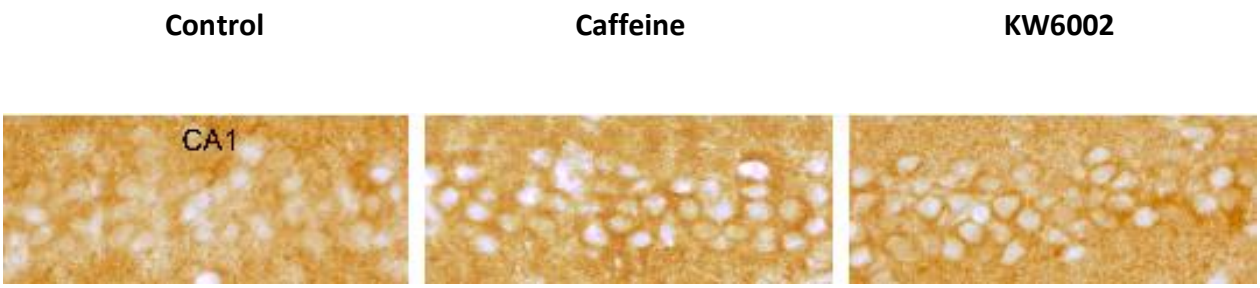


Fig. 4.25 – Kinetic properties of mEPSCs recorded in CA1 region of the hippocampus from slices of 90 days-old animals born from caffeine or KW6002 exposure during gestation and lactation. Cumulative probability distributions for kinetic parameters of mEPSCs, rise time (**A**) and tau (**B**). Glutamatergic miniature currents have lower rise times and decay time constants as indicated also in **(C**) and (**D**). Chronic administration of caffeine (0.3 g/L) and KW6002 (2 mg/kg/day) resulted in a decrease in both rise time and decay time constants of mEPSC currents. Results are mean +/- SEM of at least 100 analyzed currents from 12 independent pyramidal cells. Comparison between means was made using a two-tailed *t*-test and cumulative probability distributions were analyzed using Kolmogorov-Smirnov test. Statistical significance is indicated in the graph as ***p<0.0001.

Fig. 4.26

A)



B)

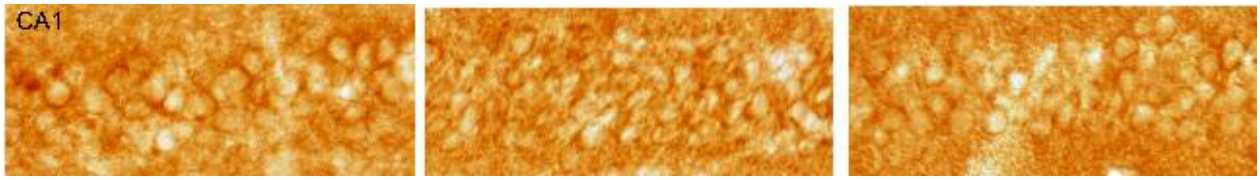


Fig. 4.26 – Pattern of distribution of GluR1, and GluR2/GluR3 in pyramidal cells from CA1 region of the hippocampus in slices from adult animals from mothers consuming caffeine and KW6002 during gestation and lactation. Immunohistochemistry of CA1 pyramidal cell region showing a more intense labeling of GluR1 (**A**) around the cell bodies of pyramidal cells in treated animals with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) during the developmental period. No changing in the labeling of GluR2/3 subunit was detected (**B**). The images are representative of independent analysis to 4 animals *per* group. It was not performed a quantification of the immunoreactivity in the different groups of animals.

Another modification observed by Cornejo and colleagues (Cornejo *et al.*, 2007) was an increased in the expression of NR2B receptor. They found a reduction of total NR2A immunoreactivity in animals that had experienced a single exposure to kainic acid at PD 7. No differences in the intracellular/membrane amount of receptors were found in these animals. Thus the pattern of expression of NR1 and NR2 subunits in control and caffeine- and KW6002-treated animals in the hippocampus was also investigated by immunohistochemistry. The results are presented in the **Fig. 4.27** and **Fig. 4.28**. In our experimental conditions, it was observed an apparent increase in the targeting of NR1-containing receptors to the plasma membrane of pyramidal cells as shown in **Fig. 4.27.** In control animals a higher amount of receptors containing NR1 seems to be located intracellularly (concluded by the diffuse labeling around cell bodies of pyramidal cells) and chronic treatment with either caffeine or KW6002 resulted in a stronger punctiform labeling at the surface of

cell bodies of pyramidal cells, indicating a targeting of the subunit to the plasma membrane from intracellular pools.

Fig. 4.27

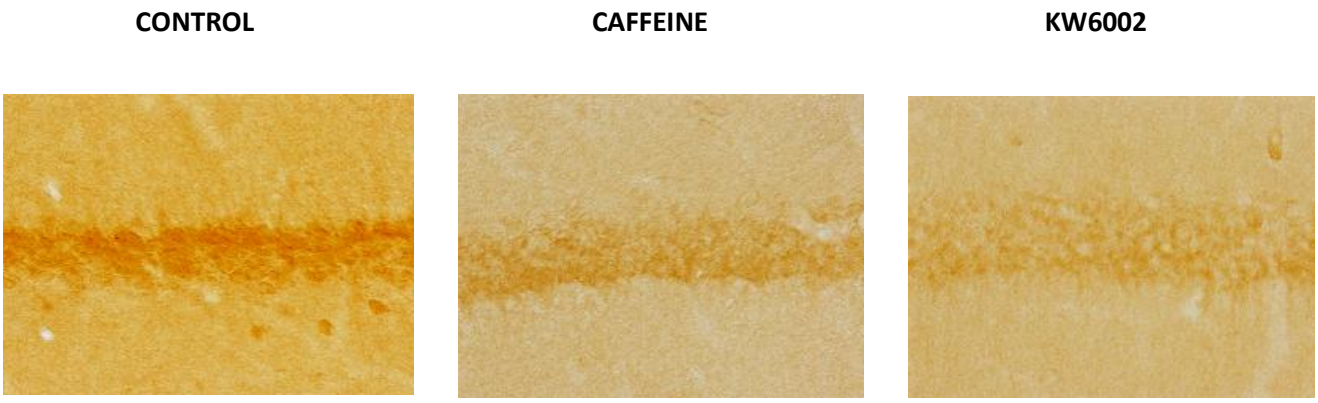


Fig.4.26 – Pattern of expression of NR1 in pyramidal cells from CA1 region of the hippocampus of adult animals born from mothers that consumed caffeine and KW6002 during gestation and lactation. Adult (90 days-old) animals exposed to caffeine and KW6002 *via* the mother, during gestation and lactation, present a more punctiform expression of NR1 subunit of NMDA receptor in cell bodies of pyramidal cells than control animals, suggesting that in control animals a bigger fraction of the NR1-containing receptors is present in the intracellular pool. The same type of modification was found in CA3 region and at the dentate gyrus (DG). The images are representative of 4 independent eperiments. Magnification: 40×. It was not performed a quantification of the immunoreactivity.

In the case of NR2 subunits, it was not observed any evident difference between the pattern of location of this protein in control and treated animals (see **Fig. 4.28**). This observation suggests that the treatments selectively modified the density and trafficking and/or localization of some NMDA receptor subunits.

NMDA receptors consist of two obligatory NR1 subunits and two regulatory subunits that can be NR2A-D and NR3A-B and the combination of NMDA receptor subunits will determine the functional properties of the NMDA receptor channels (CullCandy and Leszkiewicz, 2004). This heterogeneity can be further increased through alternative splicing (CullCandy *et al.*, 2001). NR2A/NR2B ratio seems to be activity-dependent modulated (Bellone and Nicoll, 2007; Sobczyk and Svoboda, 2007) and seems to optimize the threshold for inducing synaptic plasticity. NR2B-containing NMDA receptors carry more Ca^{2+} per unit of current (Sobczyk *et al.*, 2005) and interact preferentially with CaMKII (Strack and Colbran, 1998), compared with NR2A-containing receptors.

Several reports have supported a particular association of NR2B subunits with the induction of LTP (Barria and Malinow, 2005).

Fig. 4.28

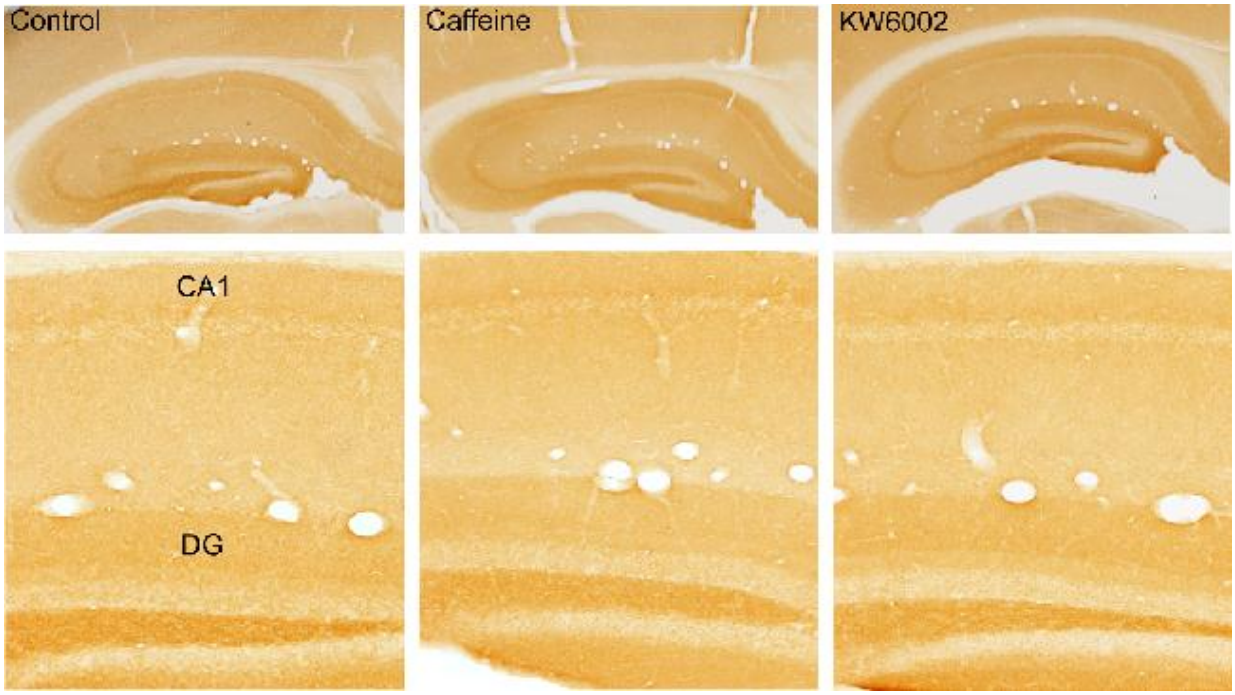


Fig. 4.28– Pattern of immunoreactivity of NR2 in pyramidal cells from CA1 region the hippocampus of adult animals whose mothers consumed caffeine or KW6002 during gestation and lactation. Immunohistochemistry showing sections of the hippocampus of 90 days-old mice labeled with the anti-NR2 antibody. It was not noticed any evident differences in the pattern of distribution immunoreactivity for this subunit in the hippocampus of control, caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) treated animals. Top panel – magnification: 2×; Botton panel – magnification 10×. Results are representative of at 4 independent experiments. It was not performed a quantitative analysis of the immunoreactivity.

Several studies indicated that NR2B-containing receptors are not required for induction of LTD (Hendricson *et al.*, 2002; Liu *et al.*, 2004; Morishita *et al.*, 2006). Making a general assumption, synapses that have a high NR2A/NR2B ratio favor induction of LTD by limiting Ca^{2+} entry. If the ratio NR2A/NR2B is elevated, a stronger stimulation is required for induction of LTP since it limits activation of Ca^{2+} entry and CaMKII activation. The antibody used recognized both NR2 subunit subtypes which did not allow distinguish between them. Neverthless, the future investigation of the properties of synaptic plasticity in control and treated adult mice will shed some light on the

anticipate modifications of NMDA receptor upon treatment with adenosine receptor antagonists during development.

It was next investigated if such an evident modification of the glutamatergic system was followed by reorganization of GABAergic synapses. In 6 days-old pups, the GABAergic system exhibited several traits of cellular stress. Interestingly, in the hippocampus of 90 days-old mice, the GABAergic system does not seem to suffer modifications as robust as in the glutamatergic system.The same analysis of GABAergic synaptic currents was performed in pyramidal cells from CA1 region and the results are presented in the **Fig. 4.29** and **Fig. 4.30**.

The amplitude of mIPSCs recorded in pyramidal cells from pups obtained from KW6002-treated dams displayed mIPSCs with higher amplitudes (24.0 +/- 0.8 pA, n=12, $p<0.05$, two-tailed *t*-test) comparatively to the currents recorded in control (20.36pA +/- 0.3875pA, n=12) and caffeine-treated (20.42pA +/- 0.3941pA, n=12) animals (see **Fig. 4.28A**), using the Kolmogorov-Smirnov test. However, the area of the currents does not seem to be changed ($p>0.05$). The mean values of the areas are 199.2 +/- 7.5 pA×ms for currents recorded from slices belonging to control animals (n=12), 206.4 +/- 7.5 pA×ms for caffeine-treated animals (n=12) and 206.0 +/- 10.3 pA×ms for KW6002-treated animals. This lack of change in the area of the mIPSCs is in accordance with the absence of effect in the kinetic parameters of these currents. The mean value of rise time was 1.9 +/- 0.07 ms in control condition, 1.4 +/- 0.07 ms in caffeine treated conditions and 1.5 +/- 0.08ms in KW6002 treated condition (n=12 for each condition). Both mean values of rise time from currents recorded in treated animals were significantly different from the mean value of rise time recorded in pyramidal cells from control animals ($p<0.001$). In control animals the decay time constant tau was 8.8 +/- 0.3ms, it was 9.4 +/- 0.3 ms in caffeine-treated condition and 7.9 +/- 0.3ms in KW600-treated mice (n=12). The cumulative probability distributions for the decay time constants did not showalso any statistical between the groups ($p> 0.05$, for the comparison between control and caffeine treatment distributions and $p > 0.05$, for the comparison between control and KW6002 treatment distributions).

The only significant difference at the morphological level was related to α3 subunit of $GABA_A$ (**Fig. 4.31)**. Apical dendrites from pyramidal cells seem to be endowed with a higher amount of α3 subunit, in adult animals (90 days-old) born from mothers that consumed caffeine (0.3 g/L) and KW6002 (2 mg/kg/day) during gestation and lactation. An alteration in subunit composition can occur in some pathological conditions (Fritschy, 2008), however, the impact the the up-regulation of this subunit of $GABA_A$ receptors on the function of the circuitry is still unknown. Maybe it is

involved in the control of pyramidal cell excitability. Stelzer and collaborators (Stelzer *et al.*, 1994) found that CA1-pyramidal-cell LTP was accompanied by an impairment of $GABA_A$ receptor function. Increased density of $GABA_A$ receptors in dendrites of pyramidal cells may contribute to LTP impairement. Whether this is the case in animals treated chronically with caffeine or KW6002 remains to be determined.

Dendrites from pyramidal cells are involved in amplification and integration (Papp *et al.*, 2001). Local GABAergic interneurons control the firing rate of pyramidal cells and modulate their spike timing, synchronizing their activity (Klausberger, 2009). This is of particular importance during manifestation of hippocampal oscilations. Apical dendrites in the *Stratum lacunosum moleculare* receive projections from the entorhinal cortex and thalamus. It was proposed that reduction of GABAergic input in the *Stratum lacunosum-moleculare* allows backpropagation of action potentials (Klausberger, 2009). This would potentiate entorhinal cortex synapses during ripple oscillations. This type of oscillation (100-250 Hz) occurs during slow-wave sleep and behavioral immobility and is though to represent stored information that is transferred to the neocortex during memory consolidation (*e.g.* Ramadan *et al.*, 2009). Also, dendritic input in pyramidal cells modulates excitatory input when pyramidal cells are most active during theta oscilations (Freund *et al.*, 2003; Klausberger *et al.*, 2005).

In summary, the chronic treatments with either caffeine or KW6002 triggered a long-lasting modification of the properties of miniature glutamatergic currents, through mechanisms that are still unknown. AMPA receptors from treated animals transport less charge displaying faster decay time constants. It can be interpreted as a signature of an abnormal developmental program. As discussed previously, this change in synaptic composition of AMPA receptor subunits could have consequences in the physiology of CA1 pyramidal functioning, which would reflect in the hippocampal function. A molecular imprinting caused by the treatments was also found in the GABAergic synapse. A relevant modification was the amount of α3-containing receptors in apical dendrites of pyramidal cells of CA1 region. As referred previously, it may also impact in hippocampal function.

Fig. 4.29

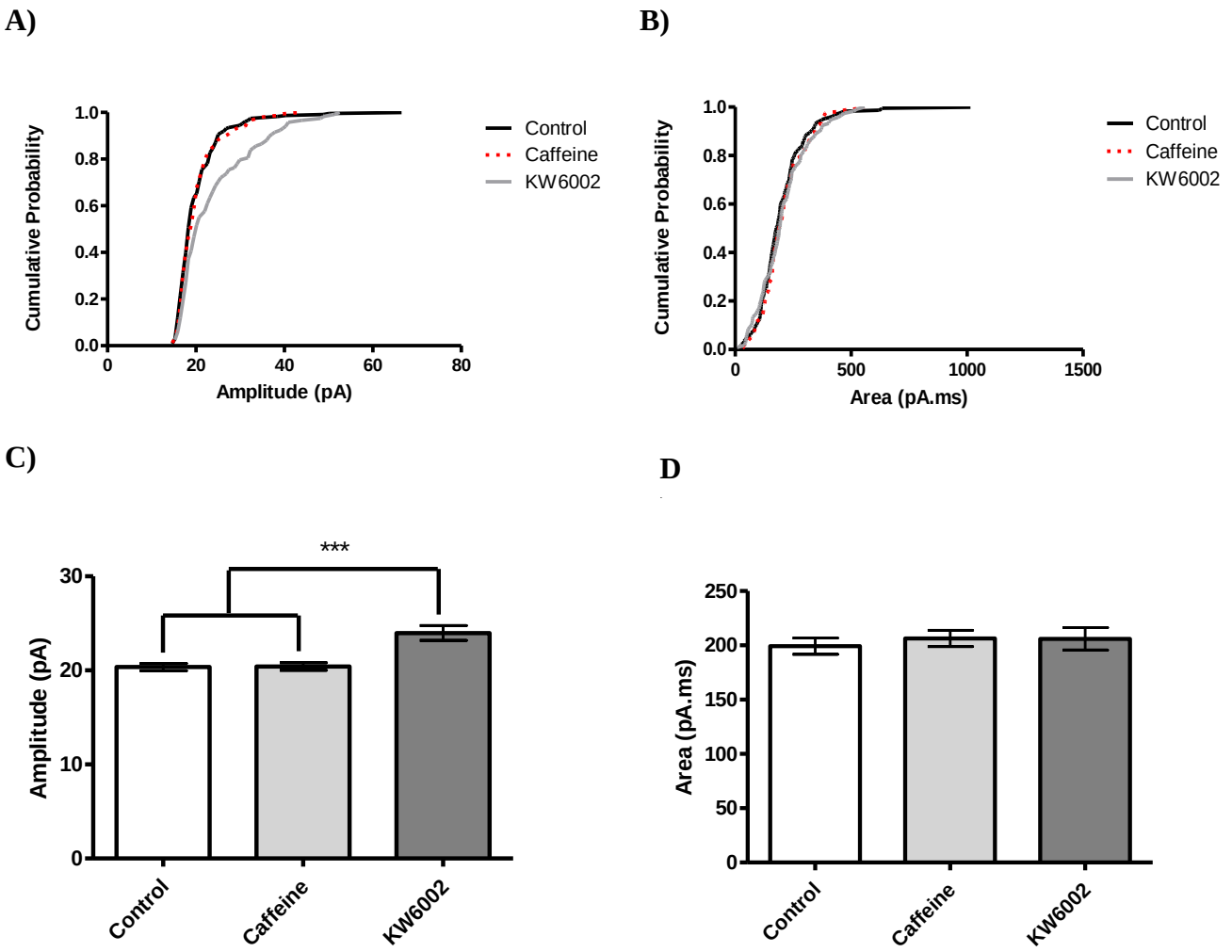


Fig. 4.29 – **Properties of mIPSCs from hippocampal pyramidal cells of 90 days-old animals born from caffeine and KW6002 exposure during gestation and lactation.** Cumulative probability distributions for the amplitude (**A**) and area (**B**) of mIPSCs recorded in pyramidal cells in CA1 region. In **C**) is shown that pyramidal cells from KW6002-treated animals display mIPSCs with higher amplitudes than pyramidal cells than control or caffeine-treated animals. The surface of these currents was not changed in treated animals (**D**). Results are mean +/- SEM of at least 100 analyzed currents from 12 indepependent experiments compared using two-tailed *t*-test. Cumulative probability distributions were analyzed using Kolmogorov-Smirnov test. Statistical significance is indicated in the figure as ***p<0.0001.

Fig. 4.30

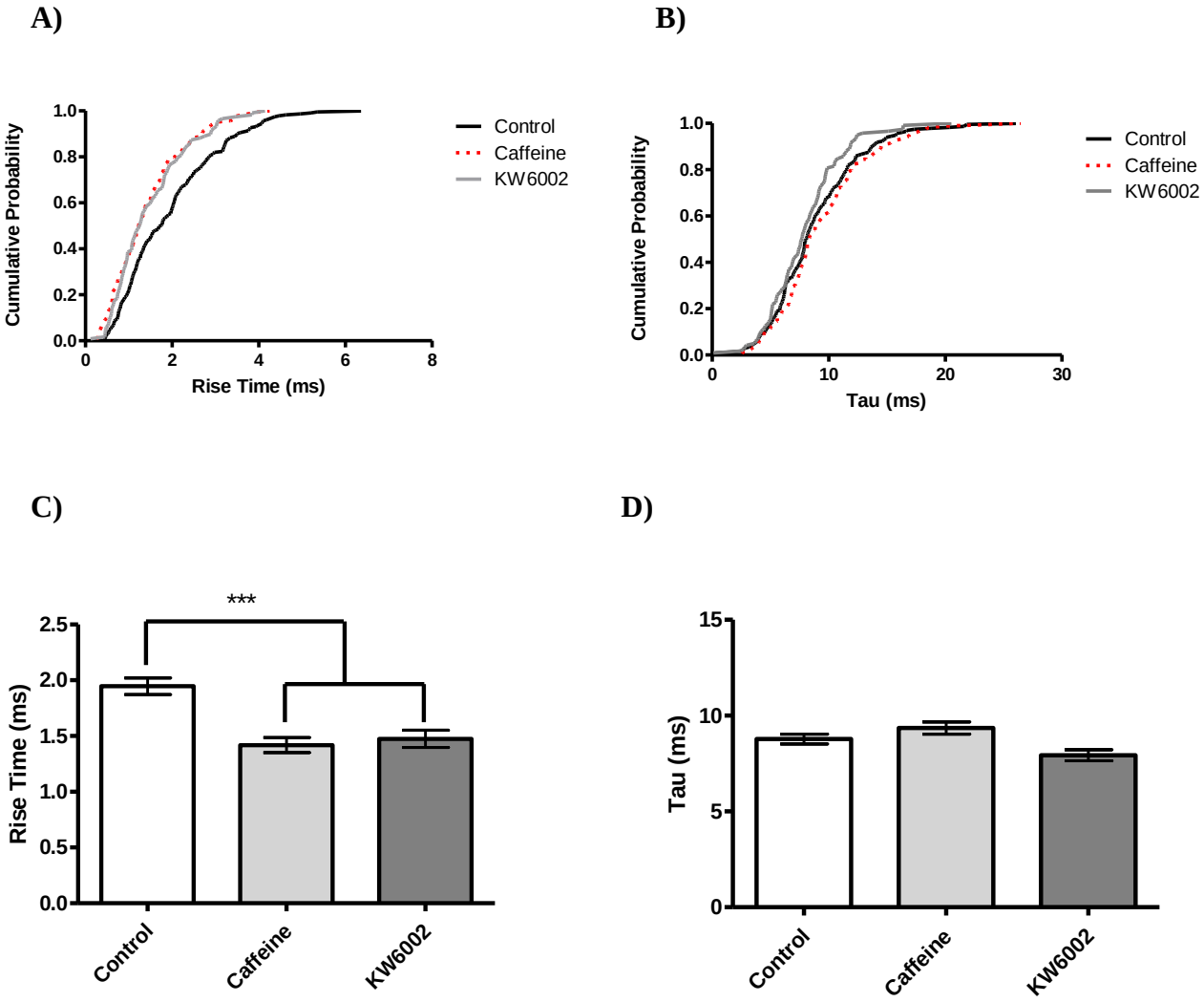


Fig. 4.30 – Kinetic properties of mEPSCs recorded in pyramidal cells from 90 days-old animals born from caffeine and KW6002 exposure during gestation and lactation. Cumulative probability distributions for the amplitude (**A**) and area (**B**) of mIPSCs recorded in the pyramidal cell layer of CA1 region. The small effects observed upon the kinetic parameters have no impact in mIPSCs surface (**C**) and (**D**). Results are mean +/- SEM of at least 100 analyzed currents from 12 independent experiments compared using *t*-test. Cumulative probability distributions were analyzed using Kolmogorov-Smirnov test. Statistical significance is indicated in the graphic as ***p<0.0001.

Fig. 4.31

CONTROL **CAFFEINE** **KW6002**

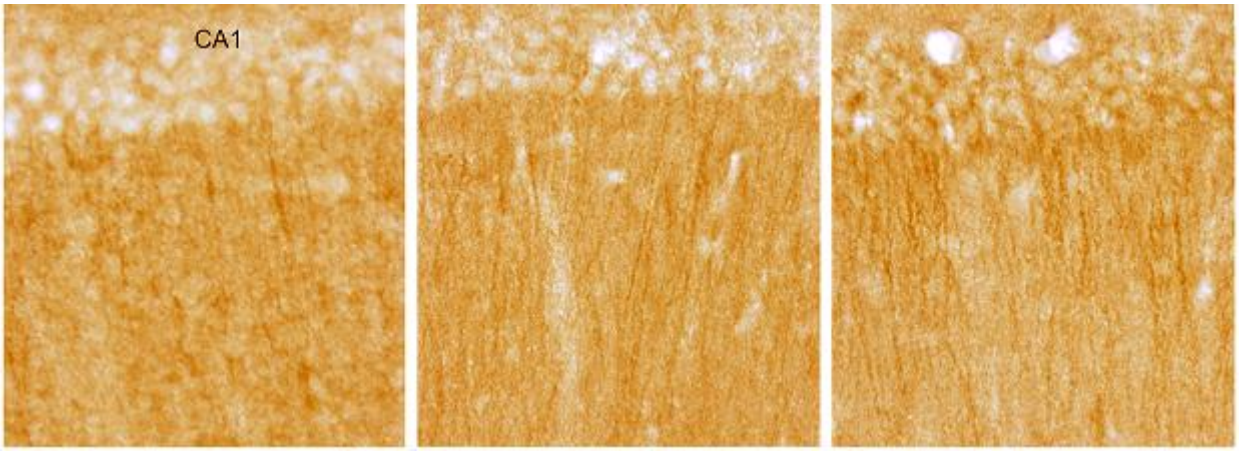


Fig. 4.31 – Pattern of expression of α3 subunit of $GABA_A$ receptors in pyramidal cells from CA1 region of adult animals from mothers consuming caffeine or KW6002 during gestation and lactation. Immunohistochemistry showing the labeling of α3 subunit of $GABA_A$ receptor in the CA1 region of the hippocampus in a 90 days-old mice born from control, caffeine (0.3 g/L)- and KW6002 (2 mg/kg/day)-treated dams during gestation and lactation. The dendrites from pyramidal cells of treated animals show a more intense labeling than cell belonging to control animals. In animals exposed to KW6002 during developmental period (gestation and 15 postnatal days), the receptor subunit seems also to be highly concentrated around cell bodies of pyramidal cells. Magnification: 40×. The figure is representative of 4 independent experiments. The immunoreactivity was not quantified.

Naïve and treated animals were also subjected to behavioral tests to determine if these molecular modifications were implicated in the construction of an abnormal brain circuitry. The locomotion of the animals belonging to the control, caffeine, KW6002 and vehicle was evaluated in an open field arena. In the second day of test, it was not found a difference in the locomotion of the animals evaluated by the number of crossings performed by the animals in the open field arena (n=7-10, $p>0.05$, two-tailed *t*-test - see **Fig. 4.32**).

Y maze and elevated plus maze were designed to evaluate non-spatial (Ennaceur and Delaccour, 1988; Bizot *et al.*, 2005) and spatial memory, respectively. **Fig. 4.33A)** presents the results obtained in the object recognition test. Both groups of treated animals showed differences in the object recognition test. Caffeine-treated animals (n=7) exhibited a lower value of recognition index, 0.30 +/- 0.06 ($p<0.05$, using a two-tailed *t*-student test), compared to control animals (n=8), 0.50 +/- 0.04. The same tendency was observed for KW6002-treated animals (n=7) comparing with vehicle

animals (n=10). Recognition index for vehicle animals was 0.40 +/- 0.03 and for animals treated with the selective A_{2A} receptor antagonist was 0.10 +/- 0.08 ($p<0.05$ comparing the means with a two-tailed *t*-student test).

Fig. 4.32

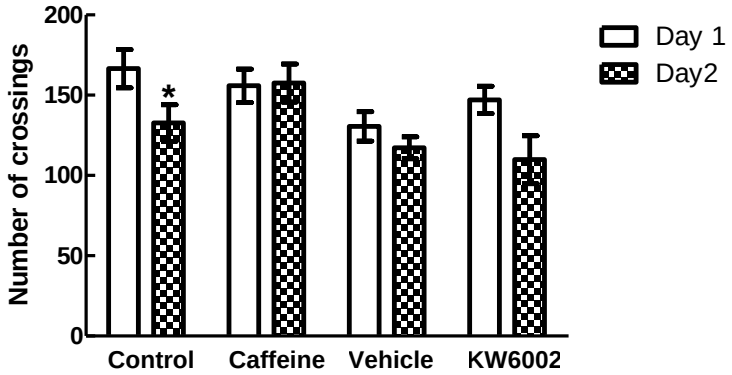


Fig. 4.32 - Locomotion of 90 days-old mice born from caffeine and KW6002 treated mothers using a behavioral test that assess spatial and non-spatial memory. The bar graphs show the locomotion of control, vehicle and caffeine (0.3g/L) or KW6002 (2 mg/kg/day) chronically treated animals during development. The treatments did not seem to modify the locomotion of the animals. Results are means +/- SEM and a two-tailed *t*-test was used to make comparisons between the means of the number of crossings performed by the animals in the ope field arena in the two experimental days. Control animals perform less crossings in the second day of test (n=7-10, $p<0.05$).

The Y-maze is a hippocampal-dependent memory performance test and measures spontaneous alternation performance (Hughes, 2004) which is dependent on spatial memory capacity (Lalonde and Chaudhuri, 2002). The animals were subjected to an 8-min sessions (trial and test) separated by a 2-h interval. Treated animals seemed to explore less the novel arm of the maze, indicating a cognitive deficit. Caffeine-treated animals (n=10) spent 35.5 +/- 1.7% of the time exploring this arm, a lower percentage of time when the comparison is made with control animals (41.1 +/- 1.5%, n=8, $p<0.05$) using a two-tailed *t*-student test for comparison between the means. The percentage of time spent in the novel arm by KW6002-treated animals (n=7) was also inferior, 36.8 +/- 2.3% by comparison with the time spent by vehicle animals (n=10) that was 43.4 +/- 2.3% ($p<0.05$, using a two-tailed *t*-student test for comparison between means (see **Fig. 4.32B**).

Fig. 4.33

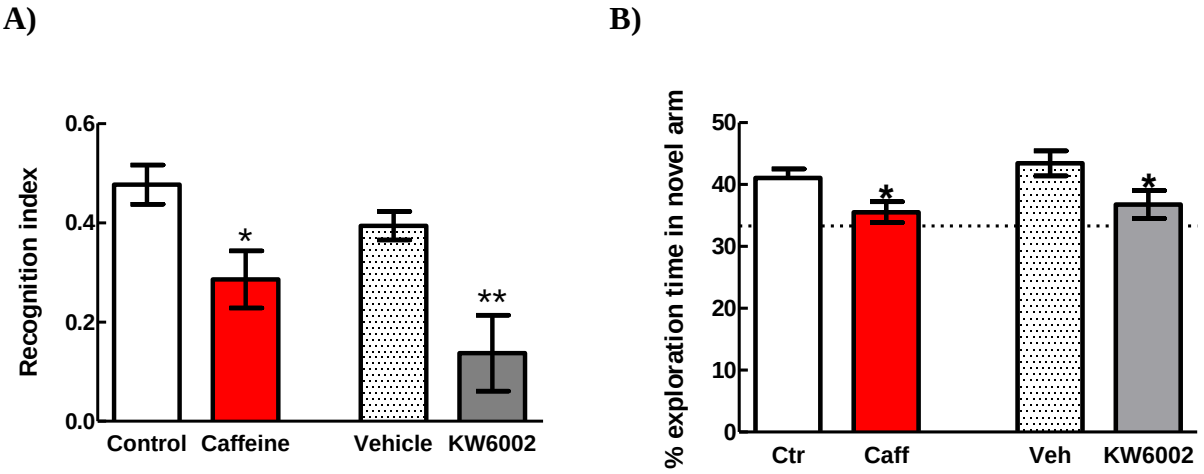


Fig. 4.33 – **Performance of 90 days-old mice born from caffeine and KW6002 treated mothers using a behavioral test that assess spatial and non-spatial memory.** The bar graphs show the effect of the long-term resulting from the chronic treatments with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) submited *via* the mother during developmental period, upon the recognition index measured, evaluated by the object recognition test (**A**) and the percentage of time spent in the exploration of the novel arm of Y-maze (**B**). Both treatments decreased the ability of the animals to recognize the new object placed in the open field arena, and the time spent by these animals exploring the novel arm of the Y-maze was lower, indicating a cognitive deficit of these animals when performing tasks that required non-spatial and spatial memory, respectively. Results are means +/- SEM and a two-tailed *t*-student test was used to make comparisons between the means (control *versus* caffeine and vehicle *versus* KW6002). Statistical significance is indicated in the graphic as *p<0.05 and **p<0.001.

The elevated plus maze, designed to measure the anxiety of the animals did not show significant differences among the groups of animals whose mothers were treated with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day). Animals belonging to the different groups spent the same amount of time in the open arms, 28.9 +/- 4.7% for control animals (n=9), 27.0 +/- 3.8% for caffeine group (n=9), 28.0 +/- 4.4% for vehicle group (n=9) and 23.6 +/- 3.2% for KW6002 group (n=7). In terms of percentage of entries in the novel arm the values were 28.9 +/- 4.7%, 27.0 +/- 3.8%, 28.0 +/- 4.4% and 23.5 +/- 3.2% in control, caffeine, vehicle and KW6002 groups, respectively. Performing a two-tailed *t*-student test to compare control *versus* caffeine and vehicle *versus* KW6002 groups, significant differences were not found between the groups (p>0.05).

Fig. 4.34

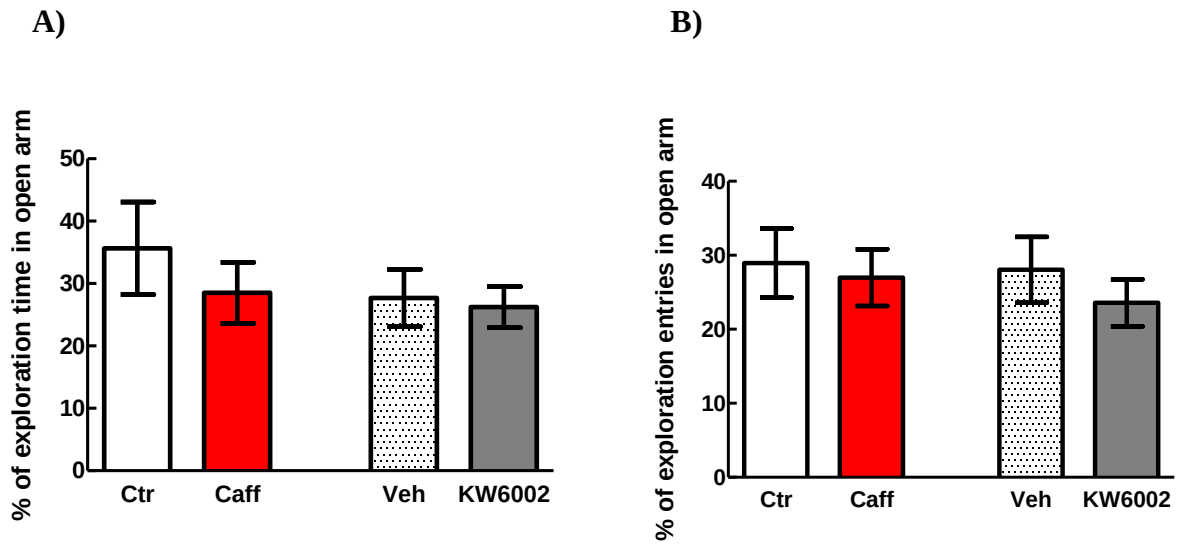


Fig. 4.34 – Evaluation of the anxiety of 90 days-old mice born from mothers treated with caffeine or KW6002 during gestation and lactation. The bar graphis show the effect of the chronic treatments of caffeine (0.3 g/L) or KW6002 (2 mg/kg/day) in the percentage of time spent by the different groups of animals (Control, Caffeine, Vehicle and KW6002) exploring the open arm of elevated plus maze (**A**) as well as the percentage of entries in this arm (**B**). Treatments with the drugs did not seem to induce any effect in the performance of the animals (n=7 *per* group), suggesting that they do not increase anxiety.

The observation that either caffeine or KW6002 treatments administered to the mothers causes an impairment of learning and memory performance of adult mice, suggests that the blockade of adenosine receptors with caffeine or KW6002 during development (using the mother as the vehicle to delivery of the drugs to the embryos and pups) resulted in a long-lasting effect in the brain circuitry involving hippocampal formation (in the case of spatial memory) but also extra-hippocampal circuits, since non-spatial memory was also affected.

Learning and memory deficits are generaly associated with pathological situations. Several modifications were described in the developing brain of pups treated with caffeine or KW6002. Some of them fit with scenarios of seizures, such as dendritic beads, NMDA receptor subunit up-regulation, increased frequency of glutamatergic and GABAergic currents as well as of GDPs (see Holopainen, 2008). Seizures during development induce cognitive deficits and make the brain more prone to seizures later on. Two morphologic alterations commonly associated to a hippocampus generating seizures is the sprouting of excitatory axons (Sutula, 2002) and the loss of specific populations of GABAergic interneurons (Cossart *et al.*, 2005). Somatostatin-expressing interneurons

are an extremely sensitive population of interneurons to excitotoxic insults (Oliva *et al.*, 2002). Although a quantification of the number of interneurons present in the hippocampus of treated animals was not done, the observation of the **Fig. 4.22** does not suggest a massive loss of this vulnerable population of interneurons. However, other modifications ascribed to the GABAergic system were observed, such as the modification of the density of $GABA_A$ receptors and of enzymes involved in the synthesis of GABA. GAD 65 and GAD 67 also seem to be up-regulated in neurons from animals injected with pilocarpine (Esclapez and Houser, 1999).

It was investigated the pattern of expression of VGLUT1 and GAD 65 and GAD 67 in the hippocampus of treated animals. Results are represented in **Fig. 4.35**, **Fig.4.36** and **Fig.4.37**. There is no massive reorganization of the glutamatergic compartment at the presynaptic level (see **Fig. 4.35**), However, the transport of GAD 65 from the cell body to axon terminals seems to be increased in treated animals as revealed by GAD 65 immunohistochemistry in brain slices in the absence presence of detergent. In the absence of the detergent the labeling of anti-GAD 65 is strong in cell bodies of GAD 65 expressing cells. In the presence of the detergent, the labeling is stronger in GABAergic synaptic terminals. It may indicate a more pronounced activity of GABAergic terminals from treated animals which can be putatively attributed to an increased and generalized excitability in the hippocampus of these animals (see **Fig. 4.36**). These results are corroborated with the intensity of labeling with the antibody against GAD 67 in several regions of the hippocampus. Cell bodies of interneurons located in the *Stratum radiatum* and *Stratum lacunosum moleculare* are more strongly labeled with the antibody and the hilar region of the GD is where the most remarkable difference of immunoreactivity was observed (see **Fig.4.37)**.

These results should be carefully interpreted because no quantitative measure of the immunoreactivity was made as well as confirmation with *in situ* hybridization to assess the rate of gene transcription. Also, whether or not these changes are associated to a pattern of abnormal excitability is not known given that since the expression of GAD is activity-regulated.

Fig. 4.35

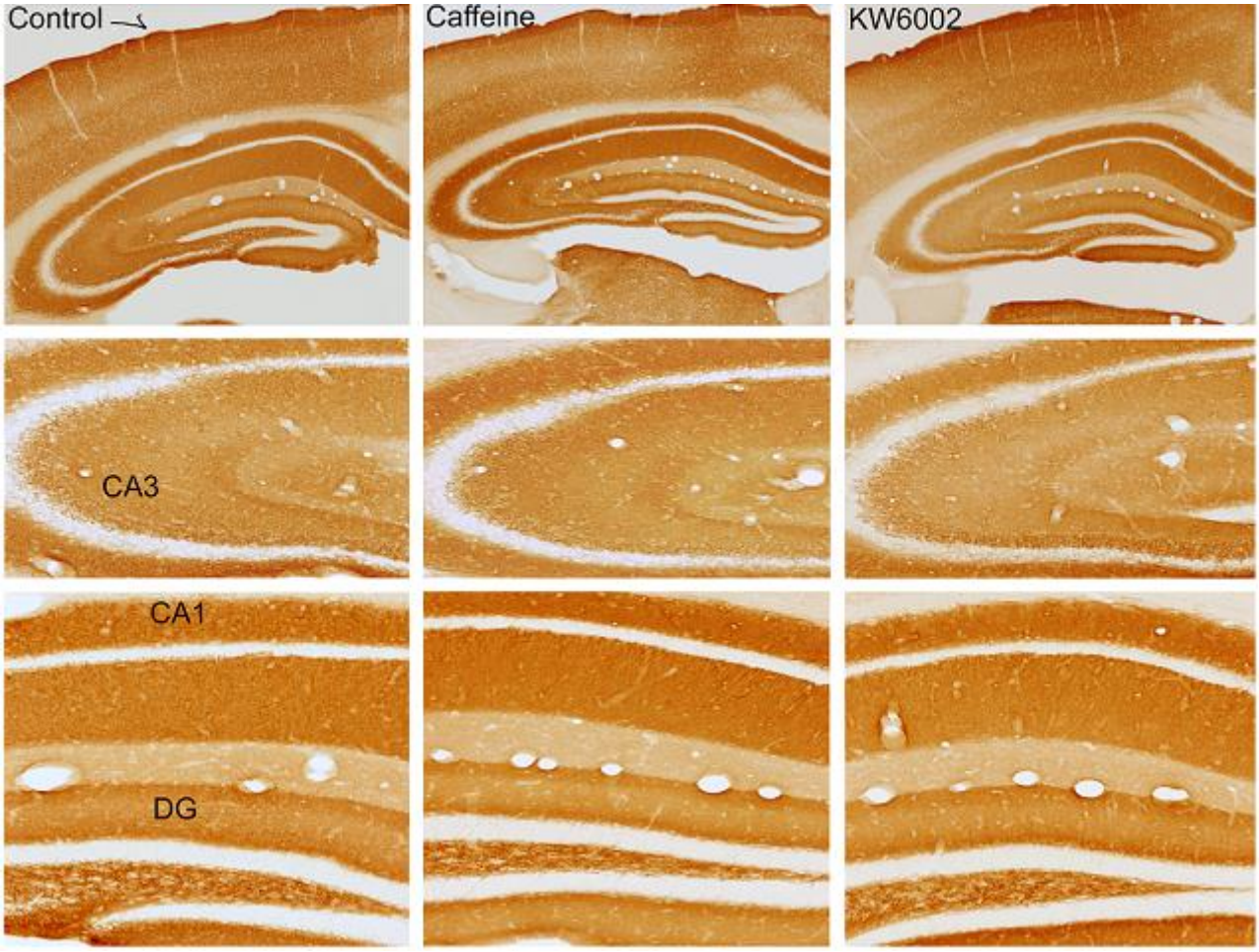


Fig. 4.35 – Effect of caffeine or KW6002 treatments to pregnant females during gestation and lactation on the pattern of immunoreactivity of VGLUT1 in the hippocampus of the first generation of adult mice. Immunohistochemistry showing that adult (90 days-old) treated animals born from females treated with caffeine (0.3 g/L) and KW6002 (2 mg/kg/day) during gestation and lactation do not present a massive reorganization of the glutamatergic compartment in the hippocampus in several compartments (CA3, CA1 and DG). The protein does not seem to be up- or down-regulated since the apparent intensity of the labeling is equivalent in slices belonging to different groups of animals. It was not observed any sprouting of axons in the CA3 region of the hippocampus which is considered a characteristic of an epileptic brain. Top panel - magnification 2× (general view of the hippocampus); Middle panel and bottom panels – magnification: 10× (showing a detail of CA3 and CA1 and dentate gyrus (DG) respectively. The figures are representative 4 independent experiments. It was not performed a quantitative measure of the immunoreactivity of VGLUT1.

Fig. 4.36

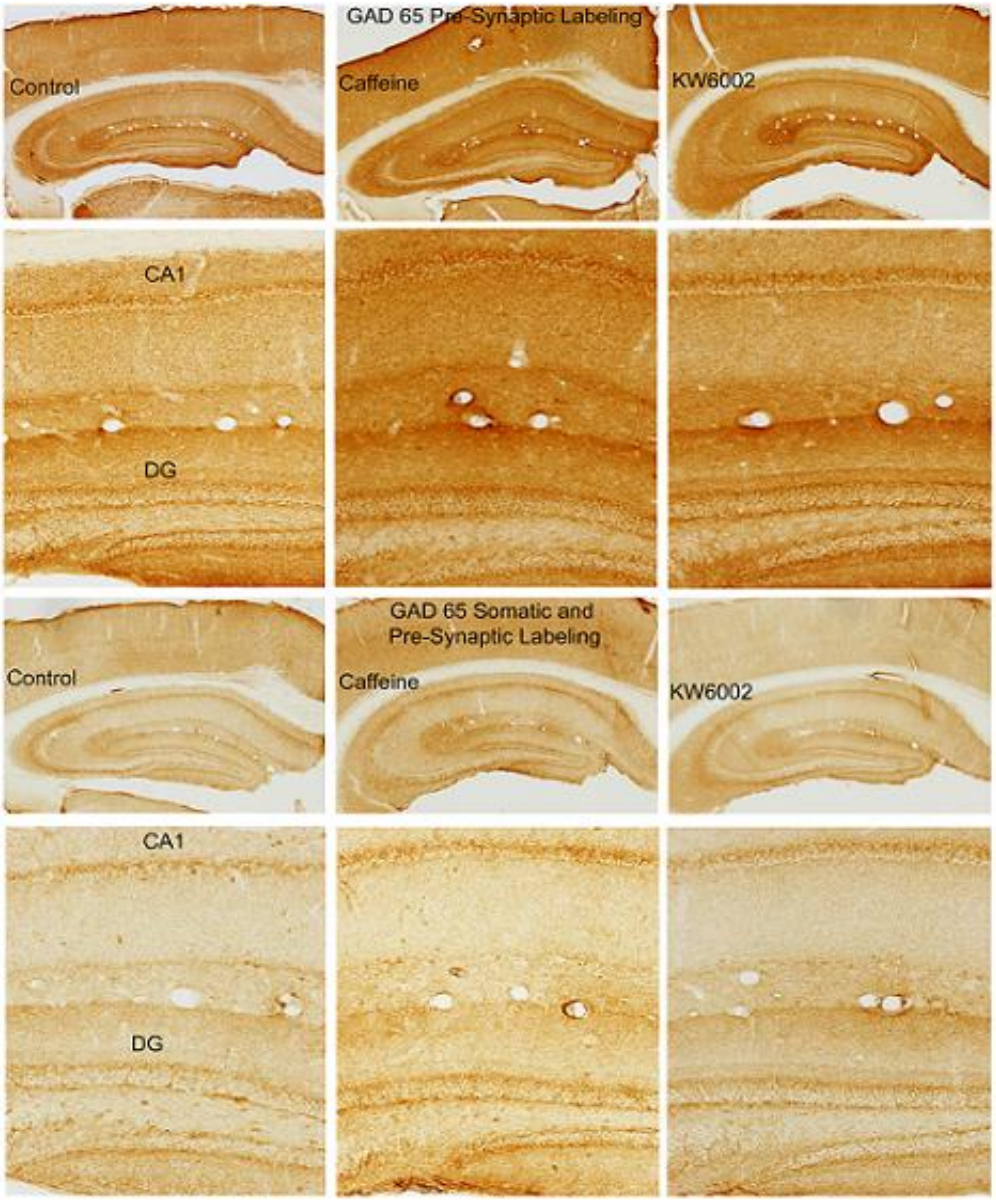


Fig. 4.36 - Effect of caffeine or KW6002 treatments to pregnant females during gestation and lactation on the pattern of immunoreactivity of GAD 65 in the hippocampus of the first generation of adult mice. Immunohistochemistry showing a general view (First and third rows of panels: magnification 2×) and the detail (Second and fourth row of panels: 10×) of the hippocampus from 90 days-old mice born from control or treated chronically during development with caffeine (0.3 g/L) or KW6002 (2 mg/kg/day), labeled with anti-GAD 65. When detergent was not used in the pretreatment of slices and incubation with the antibodies, GAD 65 labeling in cell bodies of interneurons is facilitated, giving a somatic and axonal pattern which correspond to the two third and fourth panels. When detergents are used, incubation of slices with anti-GAD 65 gives a more punctiform labeling corresponds to GABAergic terminals (First and second panels). Animals born from treated mothers apparently have a stronger immunoreactivity for GAD 65, labeling in presynaptic GABAergic terminals, however, the intensity of labeling in cell bodies of GAD 65-expressing cells is lower in treated animals (although a quantitative measure of immunoreactivity must be performed). It perhaps represents an increase in the targeting of GAD 65 from the soma to axon terminals where it seems to concentrate. The images are representative of 4 independent experiments.

Fig. 4.37

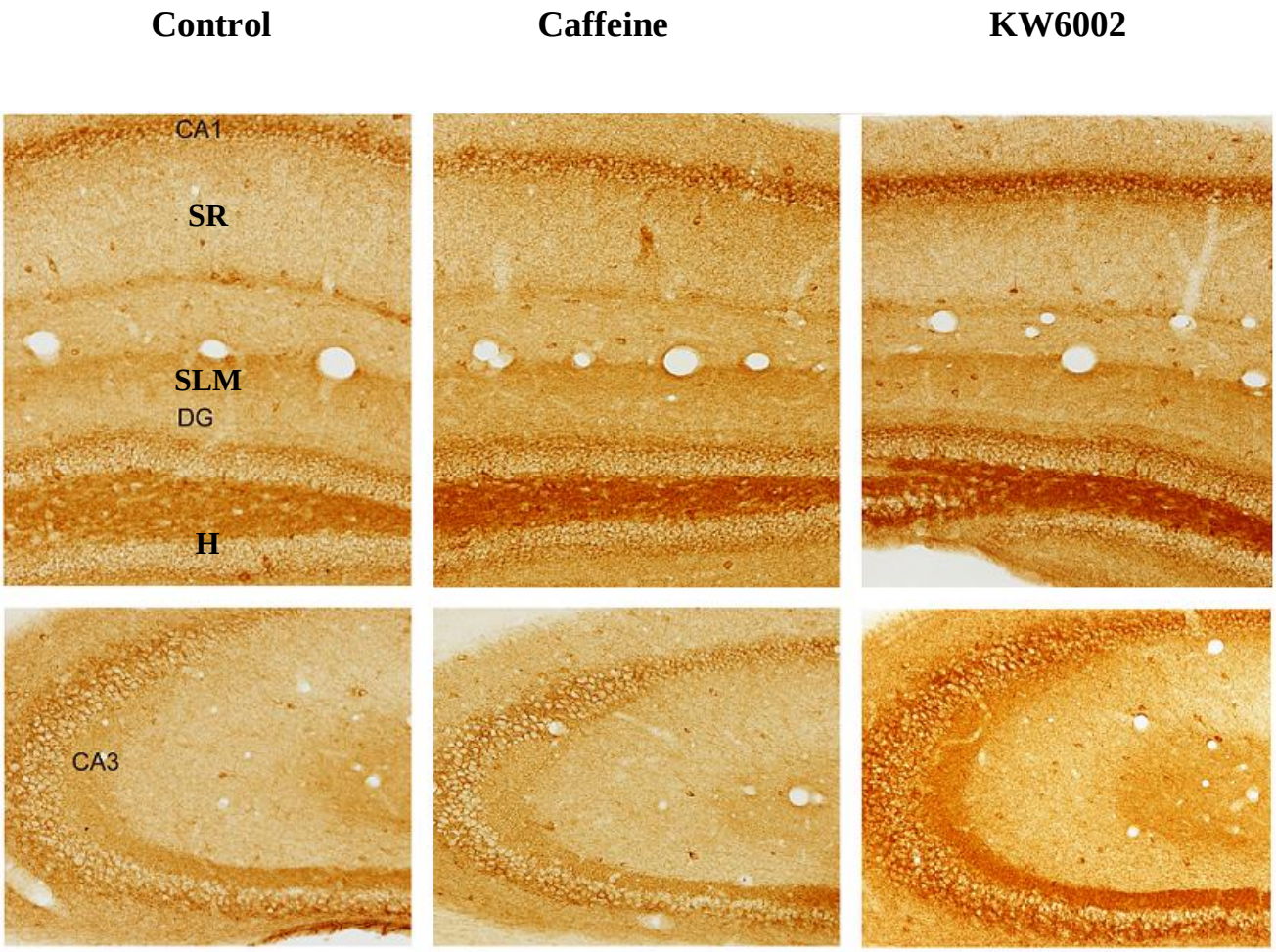


Fig. 4.37 - Effect of caffeine or KW6002 treatments to pregnant females during gestation and lactation on the pattern of immunoreactivity of GAD 67 in the hippocampus of the first generation of adult mice. Immunohistochemistry showing brain slices and the detail of the hippocampus from 90 days-old mice control or treated chronically during development with caffeine or KW6002, labeled with anti GAD 67 antibody. Treatments seem to increase the immunoreactivity for GAD 67 which can be visible in cell bodies of interneurons from the *Stratum radiatum* (SR) and *Stratum lacunosum moleculare* (SLM) of CA1 and especially CA3 regions, in pyramidal cells layer in the CA1 region and granular cells from the DG. The hilus (H) is the structure where the difference is more pronounced especially in KW6002-treated animals. The images are representative from 4 independent experiments.

Chapter 4 – Conclusions

This chapter reports the impact of the chronic administration of adenosine receptor antagonists during a developmental period where synaptogenesis, cell death and important steps of formation of the circuitry in the hippocampus are taking place (until PD 15). In **Chapters 2** and **3**, A_{2A} receptors are shown to modulate the process of cell death, preventing it, and in the **Chapter 3** a novel role for A_1 and A_{2A} receptors in the stabilization of synaptic sites was presented. This effect had an impact in the physiology of the hippocampal spontaneous activity in the hippocampal slice (GDPs expression and synaptic currents). As shown in the **Introduction**, GABA and glutamate are key neurotransmitters during the developmental period, exerting trophic actions and shaping brain circuits. Therefore it is anticipated that the modifications for the action of these neurotransmitters changing the course of a developmental program must result in adverse consequences in adulthood.

The GABAergic system seems to be extremely sensitive to minor perturbations during postnatal development. For example, two episodes of maternal separation were sufficient to induce permanent changes in hippocampal $GABA_A$ receptors (Hsu *et al.*, 2003). Our model was expected to generate even more massive modifications since, as shown in the **Chapter 3**, it was shown that A_1 and A_{2A} receptors were able to interfere with glutamatergic and GABAergic neurotransmission (generally depressing it). All events such as proliferation and differenciation of neuronal presursors, migration, maturation of glutamatergic synapses during the first postnatal week and generation of the first patterns of activity in the hippocampal slice during the first postnatal week are expected to be modified. Certainly compensatory mechanisms would develop; however, this model of intermittent exposure to adenosine receptor antagonists would minimize the development of compensatory mechanisms that generaly are observed by the use of knockout animals for adenosine receptors.

For a long time, caffeine consumption was considered non-harmful both for human (although the heterogeneity of the population analyzed generated some conflicting results) and animals (Áden, 2011). In animal models, only high doses of caffeine resulted in malformations of the fetus as well as behavioral modifications (Nehlig and Debry, 1994). More recent data also complicated the interpretation of these results since it was argued that it is the mother's genotype that is critical for behavioral modifications in the adult offspring and these effects were propagated to the second generation (Bjorklund *et al.*, 2008).

The precise cause(s) that triggered the modifications observed in our models of caffeine or KW6002 administration is (are) still unidentified. The utilization of the mother as a vehicle for the drugs has advantages and disadvantages when the origin of such modifications is of interest. For example, vasoconstriction of the placenta was hypothetized as a cause of spontaneous abortions (Godel *et al.*, 1992; Klebanoff *et al.*, 1999; Cnattingius *et al.*, 2000; Wen *et al.*, 2001), intrauterine growth restriction (Bracken *et al.*, 2003; Klebanoff *et al.*, 2002; Vlajinac *et al.*, 1997) in women that had a high consumption of caffeine (more than 300 mg/day). Also, episodes of apnea can be at the origin of seizures, however it was just observed when caffeine was withdrawn at birth in animal models of caffeine administration in the drinking water (Bodineau *et al.*, 2006). An altered cardiovascular function can also contribute to pathological effects in brain patterns of activity. Although some short-term effects were observed in animals treated with caffeine during development (Momoi *et al.*, 2008).

The treatment of animals with KW6002 gave some evidence that the effect can be triggered by A_{2A} receptor although if the physiological changes are related to direct effects in the brain or in the peripheric system is still undetermined.

In this chapter it was shown that moderate caffeine administration or selective blockade of A_{2A} receptor generate permanent modifications in the hippocampus functioning, probably related with cellular stress and an impaired developmental program.

General Conclusions and Future Perspectives

Brain development takes place pre and postnatally. Components of the purinergic system were found at the end of the first postnatal week. A_{2A} receptors seem to be present in progenitor and migrating cells suggesting new roles and function for this adenosine receptor subtype.

In the second week of gestation in rodents, and the first month of gestation in humans, a sequence of developmental processes including proliferation, migration, differentiation, synaptogenesis, apoptosis and myelination occur. In this time window, dynamic variations in the density of proteins belonging to the purinergic system were found (Chapter 1). A_{2A} receptors appear as a highly dynamic receptors whose expression seems to be tightly regulated. Progressive steps of down-regulation observed in the cortex and hippocampus make the receptor display the pattern observed in the adult. A_1 receptors appear as a more stable protein throughout the developmental period. The signaling mediated by ATP may also change. ATP receptors change in density in hippocampal membranes maybe reflecting a different relevance for this type of cell communication with age. For example, $P2X_1$, $P2X_4$, $P2Y_1$ and $P2Y_2$ suffer a strong up-regulation which occurs between PD 10 and PD 25. The peak of density of ecto-5'-nucleotidase was found at PD 20 in hippocampal membranes. This enzyme plays a role in the last enzymatic step for convertion of ATP into adenosine. In synapses, where both ATP and adenosine are present, the abundance of this enzyme may determine which signaling, "adenosinergic" or "ATPergic" will prevail. Nucleotide transporters were also found to change from location, being abundant in glial membranes during development, maybe in an attempt of serving a different role which is needed during brain development.

A future direction would be to determine, using subsynaptic preparations of membranes, where would be located the different components of the purinergic system and the relative amount of the proteins found in neuronal and glial membranes. Coupling this study with immunohistochemistry, a more clear picture of the function of these components and sites of action would be possible.

In Chapter 2, it was showed that A_{2A} receptors were able to avoid cell death by apoptosis in young neurons. Cell death is a necessary step for proper brain development. Blocking cell death may have a negative impact upon the organization of brain circuits. The activity of excitatory aminoacids is enhanced in the immature brain and this may confer to it a higher vulnerability to pathological excitation as observed during neonatal hypoxia-ischemia (Hattori and Wasterlain, 1990). The

modulation afforded by adenosine acting upon the A_{2A} receptors may be viewed as a point where is possible to develop a strategy of neuroprotection for the immature brain. The control of cell death seems to be mediated by the control of mitochondrial function. As a future perspective, the intracellular signaling pathways involved should be investigated.

In the Chapter 3, it was investigated the modulatory role of adenosine A_1 and A_{2A} receptors in the spontaneous activity recorded in the hippocampal slice from PD 5 to PD 7 mice pups. A control over the function of NMDA, AMPA and $GABA_A$ receptors seems to occur. The blockade of both subtypes of receptors seems to increase the frequency of GDPs and it seems to be related with an increase in the function of NMDA receptors. A depression of synaptic responses however was observed and it seems to be related with a control over AMPA and $GABA_A$ receptor function. Evidence was found relating A_1 and A_{2A} receptor-mediated signaling with the stability of AMPA and $GABA_A$ receptors in the plasma membrane of synaptic sites. An hypothesis explaining all the dynamics involving glutamate and GABA receptors by adenosine receptors would be a control in the trafficking of these ionotropic receptors. The regulation of neurotransmitter receptor transport and targeting is fundamental for maintenance of synaptic strength and synaptic plasticity (Collingridge *et al.*, 2004). A possible effect upon the cytoskeleton and motor proteins from synaptic sites may be involved in the elimination of receptors from synapses. For example, depolymerization of actin filaments leads to the removal of AMPA receptors from synapses (Kim and Lisman, 1999). A control over the cytoskeleton could explain the dynamic movement of receptors to and from the synapses observed for example for $GABA_A$ receptors and AMPA receptors after exposure to A_1 and A_{2A} receptor antagonist. In the glutamatergic synapse, for example, myosin VI interacts with SAP95 (Wu *et al.*, 2002), driving their internalization. The cytoskeleton is at the same time is the bound that links transmembrane AMPA receptor regulatory proteins to the arrival of AMPA receptors to the cell surface (Esteban, 2008). Thevananther *et al.*, 2001 showed evidence linking A_1 receptors to modulation of cytoskeletal dynamics through Rho kinase. A possible mechanism could be involved in the increase/decrease of glutamatergic and GABAergic receptors during synaptogenesis. Although the effect of A_1 and A_{2A} receptor blockade results in similar effects upon the stability of AMPA and $GABA_A$ receptors in the plasma membrane, the underlying signaling pathways leading to it may be different. For example, A_1 and A_{2A} receptor antagonists may mediate a decrease in the number of $GABA_A$ receptors in synaptic sites acting by different signaling pathways. DPCPX had a strong effect upon the kinetic properties of mIPSCs, suggesting a modulation of the properties of the channel and it does not seem to happen with SCH58261 that may decrease the frequency and amplitude of mIPSCs, having a subtle effect upon the kinetic properties of the channel. Gephyrin

would be a potential target of A_{2A} receptors. Recentely it was shown that gephyrin phosphorylation modulates GABAergic neurotransmission (Tyagarajan *et al.*, 2010). GSK3β, wich is a protein activated by adenosine receptors seems to phosphorylate gephyrin which resulted in an increase in the amplitude of miniIPSCS (Tyagarajan *et al.*, 2010). An increase in the levels of Ca^{2+}seems to be involved in activation of calpain-1 which would lead to proteolysis of gephyrin. Interestingly, the authors found that the changes observed in the GABAergic synapse had a parallel in glutamatergic synapse perhaps in an attempt to maintain the homeostasis. GSK3β would act in concert with PSD-95 changing simultaneously the number of glutamatergic and GABAergic receptors in a synapse to control excitability (Tyagarajan *et al.*, 2010).

A future project aiming the identification of the molecular signaling pathways involved in the endocytosis/exocytosis of glutamatergic and GABAergic receptors would be of interest.

In Chapter 4 it was found that interfering putatively with the purinergic system results in long-term deleterious effects. Although drugs used are supposed to act on brain adenosine receptors, direct brain effects cannot be established because there are too many variables involved (*e.g.*, the mother, the metabolisms of drugs and the dynamicas of the concentrations of drugs *in vivo*). The observation that KW6002 triggered almost the same effects as caffeine in adult animals born from treated mothers, strenghthens the hypothesis adenosine receptors are indeed involved in the short- and long-term modifications observed in the hippocamppus of mice born from treated mothers. Caffeine, KW6002 and respective metabolites are supposed to act on both brain and peripheric system of embryos/pups. To which extent an effect in the peripheric system exerted by the tested drugs triggers secondary/toxic effects was not determined. The analysis of the physiology/behavior of knockout animals for adenosine A_1 and A_{2A} receptors did not show the long-term cognitive deficits detected using Y-maze and Object Recognition tests (Fredholm *et al.*, 2005. Fredholm and collaborators summarize the physiological/behavioral characteristics of the knockout animals for adenosine receptors and the characteristics found in adult animals and global A_1 and A_{2A} receptor knockouts are not equivalent. The use of heterozygotes would have some advantages over the analysis of knockouts because it would mimick better the treatment of animals with the adenosine receptor antagonists (*e.g.* heterozigotes have half of the receptors functional and it is more similar to what happens when treatments with drugs are made). The major limitations of the knockouts, the developmental effect, tissue specificity are also limitations found in the protocol of treatment with adenosine receptor antagonists, and involvement of the mother to delivery of drugs to pups. Other alternatives such as the LoxP strategy for depletion of adenosine receptors from the brain (Scammel *et al.*, 2003) or viral vectors can be viewed as a future perspective in an attempt to elucidate in a more undoubted way the

intervention of adenosine in the shaping of brain circuits and the impact of the lack of adenosine-mediated signaling in a time-window corresponding to hippocampus development.

A quantitative detailed study would be necessary to evaluate the modifications in the hippocampus of mice pups in an attempt to understand its impact upon the long-term consequences observed in the adults. For example, the identification of an abnormal pattern of migration of certain types of interneurons or the analysis using morphological methods of the cellular modifications found in the neurons from treated pups (*via* the mother) would help to understand how the normal developmental program was interrupted to generate an hippocampus whose function is abnormal. Another possibility is that gene transcription was permanentely altered. Epigenetic modifications are a possible explanation to justify the long-term consequences observed in mice exposed to a "developmental stress" and that will remain as as imprinting of an initial damage. An adverse intrauterine environment and the violation of the rules governing the developmental program could compromise the normal functioning of brain in the adult. Epigenetic mechanisms in the normal CNS development suggest that defects in these factors could contribute to abnormal CNS function (Haycock, 2009).

The results measured in this thesis reinforce the potential of studying the purinergic system during brain development. Purines can control signaling pathways involved in fundamental stages of neural development and exert a powerful control over glutamatergic and GABAergic synaptic transmission in the immature hippocampus, necessary for proper migration and shaping of the hippocampal circuits. An interference of purine-mediated signaling in any of these stages of neural development may leave a permanent imprinting characteristic of an abnormal development with an adverse outcome.

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