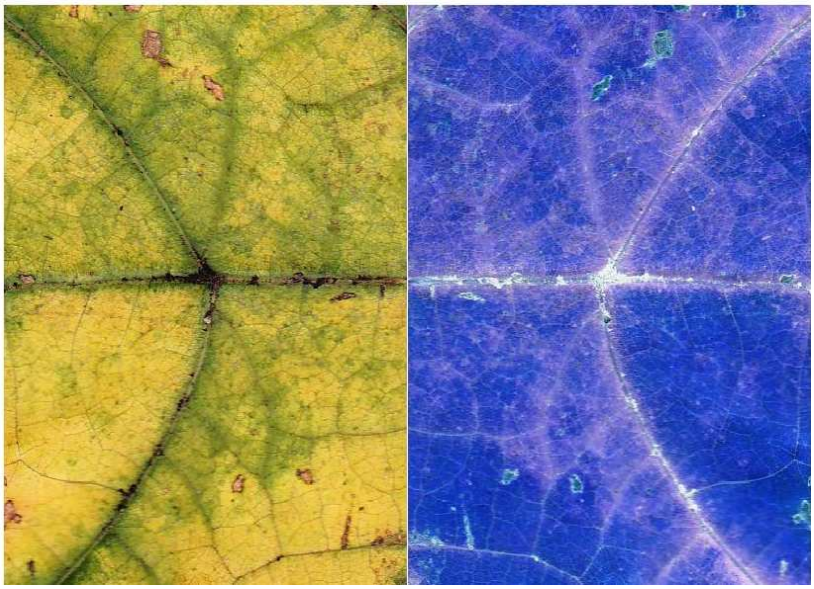
UNIVERSIDADE DE LISBOA

Instituto de Farmacologia e Neurociências, Faculdade de Medicina,

e Unidade de Neurociências, Instituto de Medicina Molecular

Modulation of glutamate AMPA receptors by adenosine, in physiological and hypoxic/ischemic conditions



Raquel Alice da Silva Baptista Dias

Doutoramento em Ciências Biomédicas

Especialidade em Neurociências

Lisboa, 2011

\\UNIVERSIDADE DE LISBOA

Instituto de Farmacologia e Neurociências, Faculdade de Medicina,

e Unidade de Neurociências, Instituto de Medicina Molecular

Modulation of glutamate AMPA receptors by adenosine, in physiological and hypoxic/ischemic conditions

Raquel Alice da Silva Baptista Dias

Tese Orientada pela Professora Doutora Ana Maria Sebastião

Doutoramento em Ciências Biomédicas

Especialidade em Neurociências

Lisboa, 2011

A impressão desta dissertação foi aprovada pelo Concelho Científico da Faculdade de Medicina de Lisboa em reunião de 19 de Abril de 2011.

The experimental work contained in this thesis was performed at the Institute of Pharmacology and Neuroscience, Faculty of Medicine and Unit of Neurosciences, Institute of Molecular Medicine, under the supervision of Professor Ana Maria Ferreira de Sousa Sebastião.

O trabalho experimental constante da presente tese foi realizado no Instituto de Farmacologia e Neurociências, Faculdade de Medicina e Unidade de Neurociências, Instituto de Medicina Molecular, sob orientação da Professora Doutora Ana Maria Ferreira de Sousa Sebastião.

Para o meu avô António.

Pelas tardes de Verão a fazer bolos de serradura e a martelar pregos tortos, no sótão.

Publications

The scientific content described in the present thesis has been the subject of two original articles, listed below. Regarding the second manuscript, only the experiments performed by the author were included in the corresponding results chapter, although reference to complementary results is made in their discussion.

- **Dias RB**, Ribeiro JA, Sebastião AM. Enhancement of AMPA currents and GluR1 membrane expression through PKA-coupled adenosine A_{2A} receptors. *Hippocampus, 2010*, epub ahead of print, PMID: 21080412

- Moidunny S, **Dias RB**, Wesseling E, Sekino Y, Boddeke HW, Sebastião AM, Biber K. Interleukin-6-type cytokines in neuroprotection and neuromodulation: oncostatin M, but not leukemia inhibitory factor, requires neuronal adenosine A1 receptor function. *Journal of Neurochemistry, 2010,* **114**:1667-77

Table of Contents

Figure index

List of abbreviations

ABP: AMPA receptor binding protein

AC: adenylate cyclase

aCSF: artificial cerebrospinal fluid

ADAC: adenosine amine congener

AIDA: 1-Amino-2,3-dihydro-1H-indene-1,5-dicarboxylic acid

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

AMPAR: AMPA receptor

AMP: adenosine 5´-monophosphate

ANOVA: analysis of variance

AP: alkaline phosphatase

ATP: adenosine 5´-triphosphate

BDNF: brain-derived neurotrophic factor

Bicc: bicuculline

BSA: bovine serum albumin

CA: cornu ammonis

CaMKII: calcium/calmodulin-dependent protein kinase

cAMP: 3´, 5´ -cyclic AMP; adenosine 3´, 5´ -cyclophosphate

CGRP: calcitonin gene-related peptide

CGS 21680: 2-[4-(2-p-carboxyethyl)phenylamino]-50-N-ethylcarboxamidoadenosine

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CNTF: ciliary neurotrophic factor

CPA: N6-cyclopentyladenosine

CPPG: (RS)-α-Cyclopropyl-4-phosphonophenylglycine

CREB: cAMP response element-binding protein

CT-1: cardiotrophin 1

DIC: differential infra-red interference contrast

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

DMSO: dimethylsulfoxide

DNA: deoxyribonucleic acid

DPCPX: 8-cyclopentyl-1,3-dipropylxanthine

DTT: dithiothreitol

EAAT: excitatory amino acid transporter

Ecto-5′-NT: Ecto-5′-nucleotidase

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene glycol tetraacetic acid

E-NPP: ectonucleotide pyrophosphatase/phosphodiesterase

ENT: equilibrative nucleoside transporter

E-NTPDase: ectonucleoside triphosphate diphosphohydrolase

EPSC: excitatory postsynaptic current

GABA: γ-aminobutyric acid

GAT: GABA transporter

GF109203X: bisindolylmaleimide I

GIRK: G-protein dependent inwardly rectifying K^+ channel

GRIP: glutamate receptor interacting protein

GTP: guanosine 5'-triphosphate

H-89: N-[2-((p-bromocinnamyl)amino)ethyl]5-isoquinolinesulfonamide

IL-6: interleukin-6

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HRP: horseradish peroxidase

JAK-STAT: Janus-activated kinase–signal transducer and activator of transcription

Kd: equilibrium dissociation constant

L-AP4: 2-amino-4-phosphonobutyrate

LIF: Leukemia inhibitory factor

LIFr: LIF receptor

LTD: long term depression

LTP: long term potentiation

LY 354740: (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid

MAPK: mitogen-activated protein kinase

MCA: middle cerebral artery

mEPSC: miniature excitatory postsynaptic current

NBQX: 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide

NHS: n-hydroxysuccinimide

NKCC: sodium/potassium/chloride co-transporters

NMDA: *N*-methyl-D-aspartate

NMDAR: NMDA receptor

NO: nitric oxide

NSF: *N*-ethylmaleimide-sensitive fusion protein

OSM: oncostatin M

OSMr: OSM receptor

PACAP: pituitary adenylate cyclase-activating polypeptide

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PDZ: post-synaptic density 95-discs large-zona occludens 1

PICK1: protein interacting with C kinase-1

PKA: protein kinase A

PKC: protein kinase C

PPF: paired-pulse facilitation

PSC: postsynaptic current

PSD-95: post-synaptic density 95

PTP: post-tetanic potentiation

PVDF: polyvinylidene fluoride

RIPA: radio-immunoprecipitation assay

RNA: ribonucleic acid

(RS)-APICA: (RS)-1-amino-5-phosphonoindan-1-carboxylic acid

RT-PCR: reverse transcription-polymerase chain reaction

SEM: standard error of the mean

SCH 58261: 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine

SDS: sodium dodecyl sulfate

SDS-PAGE: SDS polyacrylamide gel electrophoresis

Ser: Serine

SNAP: soluble NSF attachment protein

TARP: transmembrane AMPA receptor regulatory protein

TEA: tetraethylammonium

TEMED: 1,2-bis(dimethylamino)ethane

TBS: tris-buffered saline

TNF-α: tumor necrosis factor-α

Tris: tris-hydroxymethyl-aminomethane

Trk: tropomyosin-related kinase

TTX: tetrodotoxin

VIP: vasoactive intestinal peptide

Abstract

Most of the fast excitatory transmission in the brain is conveyed by ionotropic glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, formed by tetrameric assemblies of different subunit (GluR1-GluR4) composition. Modulation of AMPA receptors enables profound changes in synaptic efficiency, underlying the maturation of neuronal networks throughout development and plasticity, but also glutamate-mediated excitotoxicity. Accurate tuning of AMPA function can be attained by subunit phosphorylation, affecting channel properties and receptor trafficking rates. Accordingly, activation of noradrenergic and dopaminergic metabotropic receptors positively modulates AMPA receptor function through increased PKA activity and GluR1 phosphorylation, an effect restricted to brain areas targeted by pathways relying on these neurotransmitters. In contrast, adenosine is ubiquitously present throughout the nervous system, being released by glia and neurons or derived from the extracellular catabolism of adenine nucleotides. The present work thus aimed at evaluating the modulation of postsynaptic AMPA receptors by high-affinity, G-coupled A_1 and A_{2A} adenosine receptors and its implications for long term potentiation (LTP), widely perceived as the cellular correlate for memory formation (chapter 5.1). The involvement of A_{2A} (chapter 5.2) and A_1 (chapter 5.3) receptor-mediated tuning of glutamatergic transmission was further addressed in excitotoxicity conditions. Exogenous activation of A_{2A} receptors by 2-[4-(2-p-carboxyethyl)phenylamino]-50-N-ethylcarboxamidoadenosine (CGS21680) was found to significantly facilitate AMPA-evoked currents in CA1 pyramidal neurons, by a postsynaptic PKA-dependent mechanism leading to increased GluR1 membrane expression. The

functional impact of this modulation was evidenced by LTP facilitation at the CA3-CA1 synapse, following brief CGS21680 application. Moreover, endogenous A_{2A} receptor activation was required for ischemia-induced facilitation of glutamatergic transmission, revealing a conserved regulatory mechanism between both forms of plasticity, which may be of interest for functional recovery (through circuit rewiring) from stroke. Additionally, results suggests that some (OSM), but not all (LIF) immunoregulatory cytokines of the IL-6 family can exert neuroprotection from hypoxia through upregulation of A_1 receptors, to tone down synaptic transmission and consequently, energy expenditure.

Resumo

Os receptores ionotrópicos para o glutamato do tipo AMPA são responsáveis por mediar grande parte da transmissão excitatória rápida que tem lugar no sistema nervoso central. Estes receptores são constituídos por tetrâmeros, contendo diferentes combinações de subunidades (GluR1-GluR4) e a sua regulação é responsável por consideráveis alterações na eficiência da transmissão sináptica, inerentes à maturação de redes neuronais ao longo do desenvolvimento bem como a fenómenos de plasticidade. Contudo, os receptores AMPA são também moléculas-chave em situações de excitotoxicidade mediada por glutamato. Tanto a alteração da composição do tetrâmero, como variações no estado de fosforilação das suas subunidades, constituem mecanismos que permitem uma regulação eficaz da função destes receptores. De facto, há evidência de que à activação de receptores metabotrópicos para a dopamina ou noradrenalina se associa um aumento de função AMPA, através de um aumento na actividade da PKA e consequente exarcebamento do estado de fosforilação de subunidades GluR1. No entanto, este tipo de regulação da função AMPA encontra-se necessariamente restrito às áreas cerebrais que recebem inervação dopaminérgica ou noradrenérgica. O mesmo não acontece com a modulação pela adenosina, cuja presença é ubíqua no sistema nervoso, uma vez que pode ser libertada por células da glia e neurónios, através de locus pré-, pós- e não- sinápticos. Além disso, o catabolismo de nucleótidos de adenina, co-libertados com diferentes neurotransmissores, representa uma fonte de adenosina extracelular adicional. O trabalho experimental de que trata a presente tese teve assim como objectivo investigar uma possível modulação da componente AMPA pós-sináptica, através da activação dos receptores

de alta afinidade para a adenosina, do tipo A_1 e A_{2A}. Foram ainda abordadas as implicações deste mecanismo de regulação para o estabelecimento de potenciação a longo prazo (LTP) da eficiência sináptica, a qual é encarada como o substrato celular para a formação de novas memórias (capítulo 5.1). A ocorrência de uma possível modulação da transmissão glutamatérgica pelos receptores A_{2A} (capítulo 5.2) e A_1 (capítulo 5.3) em situações de excitotoxicidade foi estudada através da aplicação de modelos de isquémia e hipóxia. A grande maioria do trabalho experimental foi realizado em células piramidais da área CA1 do hipocampo, que representa uma população neuronal particularmente propensa a expressar alterações de eficiência sináptica de acordo com os níveis de actividade, mas que é também especialmente susceptível a morte por excitotoxicidade, após isquémia.

As observações mais relevantes descritas na presente tese dizem respeito à facilitação significativa de correntes evocadas por ejecção de AMPA (correntes AMPA), observada após activação dos receptores A_{2A} com um agonista selectivo (CGS 21680). Esta facilitação teve expressão por meio de um mecanismo dependente de PKA, mas não de síntese proteica, tendo sido reproduzida pela perfusão de um activador da adenilato ciclase (forskolina). Além disso, as acções do agonista A_{2A} não foram afectadas aquando do bloqueio simultâneo dos receptores $GABA_A$, NMDA e canais de sódio dependentes da voltagem, pelo que nenhum dos últimos parece desempenhar um papel na mesma. É ainda possível concluir que o efeito do agonista A_{2A} terá um locus de expressão pós-sináptico, na medida em que se manteve, mesmo em condições de bloqueio do disparo de potenciais de acção. Efectivamente, observou-se que a perfusão de CGS 21680 conduziu a

um aumento na amplitude de correntes pós-sinápticas excitatórias espontâneas, o que sugere um acréscimo na capacidade de resposta (pós-sináptica) ao glutamato libertado. É sabido que a fosforilação pela PKA de subunidades GluR1 aumenta a probabilidade de abertura de receptores AMPA, além do que leva a um aumento do seu tráfego (em receptores que contenham subunidades GluR1) para porções extra-sinápticas da membrana. Desta forma, quando se avaliou a expressão destas subunidades ao nível da membrana, verificou-se que tanto a expressão total, como a de subunidades fosforiladas se encontrava aumentada após tratamento com o agonista A_{2A}. A relevância deste acréscimo na expressão membranar de receptores AMPA traduziu-se numa facilitação da LTP entre neurónios das zonas CA3 e CA1, após um curto tratamento com CGS 21680. Além disso, a modulação endógena da componente AMPA pós-sináptica revelou-se necessária à expressão de LTP, já que a última se encontrou francamente diminuída em situações de bloqueio dos receptores A_{2A}. Face aos resultados obtidos na primeira parte deste trabalho, é pois possível inferir que, em condições de aumento súbito da adenosina extracelular proporcionadas por um exarcebamento da actividade neuronal, a activação de receptores A_{2A}, facilita a fosforilação pela PKA de subunidades GluR1 – aumentando a disponibilidade de receptores nas reservas membranares a partir das quais estes são recrutados para a sinapse, permitindo reforçar a transmissão sináptica.

A exposição a períodos transitórios de hipóxia ou isquémia é suficiente para induzir um aumento mantido na eficiência sináptica que partilha muitos dos mecanismos inerentes à expressão de LTP, incluindo um ganho de função pela componente AMPA pós-sináptica.

Com o objectivo de investigar a contribuição por uma possível modulação pelos receptores A_{2A} da transmissão glutamatérgica para esta forma de plasticidade, compararam-se os efeitos de breves insultos isquémicos, na presença e na ausência (controlo) de um antagonista selectivo dos receptores A_{2A} da adenosina (SCH 58261). A sujeição ao episódio isquémico transitório começou por anular a transmissão sináptica, a qual recuperou após reoxigenação, progressivamente, até valores acima dos valores iniciais de referência. Estas observações validam portanto o recurso a este tipo de insulto isquémico transitório para o estudo de formas de plasticidade induzidas por isquémia. Para além disso, observou-se que esta facilitação da transmissão glutamatérgica era completamente perdida, aquando da inclusão de espermina na solução intracelular (com acesso ao compartimento citosólico), o que sugere que requererá a activação de receptores AMPA permeáveis ao cálcio (bloqueados por espermina intracelular). Estes resultados revelam assim mais um mecanismo comum à chamada "LTP induzida por isquémia" e à sua correspondente fisiológica (LTP), a qual depende também da inserção sináptica, transitória, de receptores AMPA permeáveis ao cálcio. Quando o insulto isquémico foi realizado na presença de um antagonista A_{2A}, tão pouco se observou uma facilitação da transmissão glutamatérgica após isquémia. Estes dados são consistentes com um envolvimento endógeno dos receptores A_{2A} nesta forma de plasticidade, à semelhança da contribuição que têm para a expressão de LTP. A recuperação da transmissão sináptica em condições de bloqueio dos receptores A_{2A} manteve-se inalterada na presença de espermina intracelular, sugerindo o recrutamento de um mecanismo comum, em ambos os casos (bloqueio dos receptores AMPA permeáveis ao cálcio e bloqueio dos receptores A_{2A}). Para uma

melhor compreensão dos mecanismos inerentes a este tipo de plasticidade, será necessária uma avaliação do intervalo de tempo pelo qual os receptores AMPA permeáveis ao cálcio permanecem na sinapse, bem como testar directamente a hipótese de que os receptores A_{2A} modulem a inserção de receptores AMPA permeáveis ao cálcio. Ainda assim, esta segunda parte do presente trabalho tem o mérito de revelar um (novo) mecanismo comum a duas formas de plasticidade consideradas completamente distintas até recentemente, colocando em evidência um envolvimento relevante dos receptores A_{2A} nas alterações da eficiência sináptica desencadeadas por isquémia.

É desde há muito reconhecido o potencial neuroprotector que a activação de receptores A_1 da adenosina oferece contra insultos de hipóxia e/ou isquémia, ainda que a aplicação terapêutica de agonistas A_1 se encontre muito dificultada pela ocorrência de graves efeitos secundários. É plausível que a administração de moléculas capazes de modular a função destes receptores possa oferecer uma alternativa viável ao uso de agonistas A_1. De facto, esta aplicação foi recentemente sugerida para a interleucina 6 (IL-6), capaz de proteger neurónios de dano por excitotoxicidade, através de um aumento nos níveis de receptores A_1 e um consequente aumento da sua sensibilidade ao tónus adenosinérgico. Com o objectivo de avaliar se esta acção seria extensível a outros membros da mesma família de interleucinas, analisaram-se os efeitos do tratamento com oncostatina (OSM) e factor inibidor de leucemia (LIF) sobre a modulação da transmissão glutamatérgica dependente de receptores A_1. Após tratamento com OSM, mas não com LIF, observou-se uma potenciação da inbição das respostas sinápticas glutamatérgicas induzida por perfusão de um

agonista selectivo de receptores A_1 (CPA), o que é consistente com um ganho de função destes receptores. A relevância desta interacção para uma situação de modulação da transmissão sináptica aquando de um insulto excitotóxico, reflectiu-se numa maior inibição da transmissão glutamatérgica, dependente da activação de receptores A_1, após sujeição a um episódio hipóxico transitório. Estes dados funcionais corroboram os resultados de colaboradores, que mostram que apenas o tratamento com OSM consegue aumentar os níveis de proteína e mRNA correspondentes ao receptor A_1. No seu conjunto, os resultados sugerem que o acréscimo de função dos receptores A_1 da adenosina é necessário à neuroprotecção exercida pelas citocinas IL-6 e OSM, mas não para a protecção mediada por LIF. No entanto, a pleiotropia de efeitos conhecida para esta família de interleucinas, bem como o tipo de protocolo usado para testar o seu envolvimento na neuroprotecção contra hipóxia (pré-tratamento), tornam prematura qualquer sugestão do seu uso como potenciais agentes terapêuticos.

Do presente trabalho emerge desta forma a noção da modulação de receptores A_1 como mecanismo redundante capaz de conferir propriedades de neuroprotecção a citocinas imunorreguladoras. Por outro lado e através da activação de receptors A_{2A}, com consequente ganho de função AMPA, este trabalho mostrou como a adenosina é capaz de modular a plasticidade sináptica, o que se poderá revelar de particular interesse no estabelecimento de novos contactos sinápticos que ocorre em resposta a insultos isquémicos.

1 Introduction

In the beginning of the 20th century, it was commonly accepted by the scientific community that synaptic communication relied upon bio-electricity. One of the first clues that this might not be the case emerged from Ramón y Cajal´s cutting edge discoveries, revealing distinguishable gaps between communicating neurons, which questioned the possibility of electrical transmission through them. In 1921, the seminal work by Otto Loewi with *ex-vivo* vagus nerve preparations came to confirm the notion that neurons could indeed communicate by releasing unknown chemical substances (Loewi, 1921). Loewi dissected two beating hearts out of frogs, maintained them in saline solution and severed the autonomous innervation to one of them. By electrical stimulation of the vagus, the first heart was made to beat slower and when the liquid bathing it was applied to the second heart, a comparable decrease in heart rate was observed. This experiment elegantly showed that there had to be some soluble chemical, released upon vagus nerve stimulation, which was controlling the heart rate. Loewi´s *Vagusstoff* was later found to be acetylcholine, which was thus the first identified neurotransmitter. As for glutamate, its role as the principal excitatory neurotransmitter in the mammalian brain would slowly become established over the course of more than twenty years, perhaps because it seemed unlikely that this non-essential, highly abundant amino-acid, central to energy metabolism, could in addition function as a tightly regulated neurotransmitter. In 1954, Hayashi observed that glutamate injection into the brain or carotid arteries produced convulsions, leading him to

hypothesize it might also work as a transmitter molecule (Hayashi, 1954). On the following years, Watkins and his co-workers further explored the actions of glutamate and related amino-acids upon populations of neurons (Curtis and Watkins, 1959; Curtis et al., 1960; Curtis and Watkins, 1961), which prompted them to propose the existence of different types of glutamate receptors, according to ligand selectivity. Interestingly, the realization that glutamate and other excitatory amino-acids exerted their excitatory actions via multiple receptors preceded their establishment as synaptic neurotransmitter receptors. Indeed, glutamate receptors were only definitely shown to be synaptic receptors in the late 1970´s, by the sensitivity of certain excitatory pathways in the spinal cord to specific NMDA receptor antagonists (Biscoe et al., 1977). By the early 1980´s, glutamate was commonly accepted to exert postsynaptic actions upon three families of ionotropic receptors which were named after their preferred agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. All of these constitute intrinsic cation permeable channels, albeit with variable permeabilities to sodium and calcium ions, depending on the type and subunit composition of the receptor (reviewed in Meldrum, 2000). Metabotropic, G-protein coupled, glutamate receptors (mGluRs) were not described until 1987, having been cloned in the early 1990´s (Schoepp and Conn, 1993) (Table 1).

Table 1 – Glutamate Receptor Classification, Subunit Composition and Pharmacology

	Receptor family	Subunit Nomenclature (e.g., Watkins and Jane, 2006)	Subunit Nomenclature (see Collingridge et al., 2009)	Agonists	Antagonists
Ionotropic receptors	AMPA	GluR1-4	GluA1-4	Glutamate AMPA Kainate L-quisqualate	CNQX NBQX
	NMDA	NR1 NR2A-D NR3A,B	GluN1 GluN2A-D GluN3A/B	Glutamate NMDA	DL-APV
	Kainate	GluR5-7 KA1,2	GluK1-5	Glutamate Kainate Domoate	CNQX Topiramate
Metabotropic receptors	Metabotropic group I	mGluR1 mGluR5		Glutamate L-quisqualate	AIDA Fenobam
	Metabotropic group II	mGluR2,3		Glutamate LY 354740	*(RS)*-APICA
	Metabotropic group III	mGluR4 mGluR6-8		Glutamate L-AP4	CPPG

Despite this wide variability of glutamate receptors, most of the fast excitatory transmission that takes place in the mammalian central nervous system is conveyed by AMPA receptors (Figure 1.1). Therefore, the presence and function of these receptors at synapses must be carefully regulated in order to ensure not only correct neuronal communication**,** as also the reinforcement of synaptic efficacy that is thought to underlie the formation of new memories (Bliss and Collingridge, 1993; Martin et al., 2000).

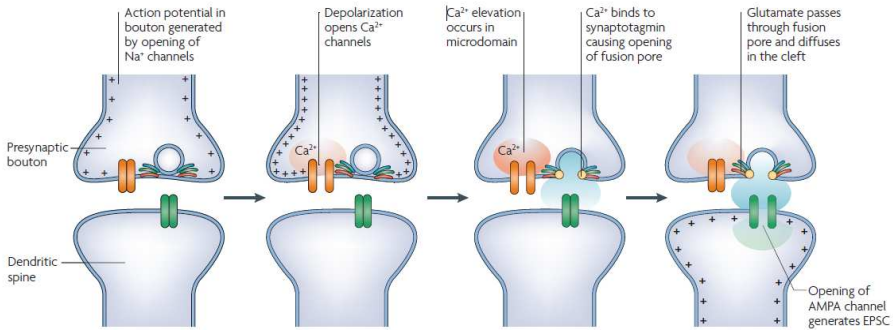


Figure 1.1.Steps in the process of chemical synaptic transmission. These steps occur in both vertebrates and invertebrates, during chemical neurotransmission at the neuromuscular junction as well as in central synapses. The example given applies to fast synaptic glutamatergic transmission, but also to that mediated by other neurotransmitters and corresponding postsynaptic ionotropic receptors. Adapted from Lisman et al., 2007.

1.1 AMPA receptors

AMPA receptors are ionotropic, ligand-gated receptors which undergo conformational changes upon glutamate binding, rendering them transiently permeable to cation influx and causing a net depolarization of the postsynaptic membrane that is responsible for propagating information at excitatory synapses throughout the brain. They are tetramers composed of four subunits (GluR1-GluR4), which combine in different stoichiometries to form ion channels with distinct functional properties (Hollmann and Heinemann, 1994) (see Figure 1.1.1). Accordingly, expression of receptor subunits is developmentally regulated and is brain region specific (reviewed in Shepherd and Huganir, 2007). Furthermore, receptor subunit composition determines not only channel properties (single-channel conductance, desensitization kinetics), but also stoichiometric association with transmembrane AMPA receptor regulatory proteins (TARPs), which regulate receptor maturation and trafficking (Gereau and Swanson,

2008). Aside from that introduced from different subunit combinations, AMPA receptor diversity is also generated within each subunit, by variations arising from alternative splicing, RNA editing and post-transcriptional modifications, such as phosphorylation and/or palmitoylation. Each AMPA receptor subunit comprises four hydrophobic domains, three of which traverse the membrane (TM1, TM3 and TM4), while one (M2) forms a re-entrant loop that faces the cytoplasm and contributes to the assembly of the cation permeable channel pore (reviewed by Santos et al., 2009) (Figure 1.1). Reflecting their most likely evolutionary origin, the structure of AMPA receptors is in accordance with the fusion of three gene segments that were once individual bacterial proteins. As such, the amino terminal is homologous to the bacterial leucine-isoleucine-valine–binding protein while the ligand-binding domain resembles a bacterial lysine-arginine-orthinine binding protein (Gereau and Swanson, 2008). In turn, the two segments (S1 and S2) of the ligand-binding domain are interrupted by the ion channel pore, whose structure is similar to that of bacterial K^+ channels. Both the extracellular and transmembrane regions share a high degree of homology between different AMPAR subunits (Gereau and Swanson, 2008). The same is not true for their carboxyl terminal (C terminal), which is the most variable region among different subunits and the site of subunit-governed protein interactions. Within the C terminal, different phosphorylation sites can also be found, which has implications for receptor trafficking and synaptic delivery (Esteban, 2003).

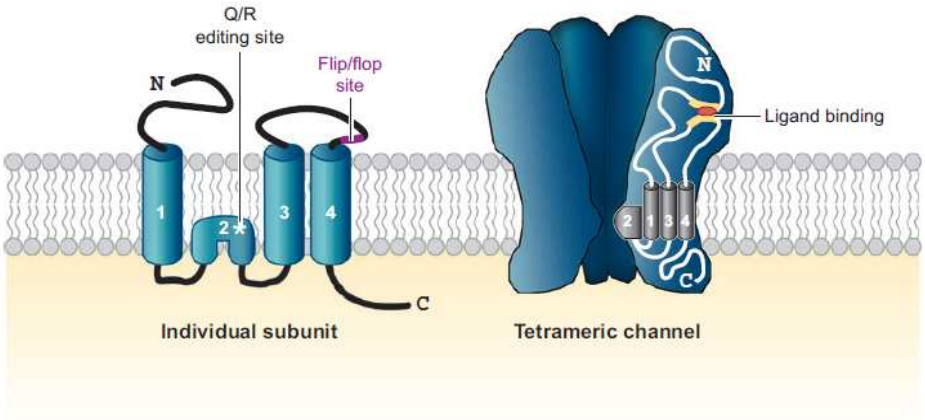


Figure 1.2. Structure and composition of AMPA receptors. Structure of AMPAR subunits and the tetrameric channel they form. Individual subunits are composed of four transmembrane domains, and the channel consists of four subunits, which are usually two dimers. In the endoplasmic reticulum, two subunits combine to form an initial dimer, which requires interactions between amino terminals; this is followed by a second dimerization process relying upon association of membrane-spanning and extracellular loop regions (e.g., Zipp, 2007). Adapted from Shepherd and Huganir, 2007.

1.1.1 Alternative splicing of AMPA receptor subunits

Different post-transcriptional modifications can amplify the functional diversity of AMPA receptors, by generating multiple subunit isoforms. For instance, the mRNA for each AMPA receptor subunit can be alternatively spliced to produce either a flip or a flop isoform, a process which introduces variation within the extracellular portion of the protein, close to the final transmembrane domain (TM4). Although flip and flop subunit forms only differ in a few aminoacid residues, their incorporation affects receptor desensitization, as well as export from the endoplasmic reticulum (reviewed by Santos et al., 2009) and possibly even receptor formation and stoichiometry (Brorson et al., 2004). Accordingly, the expression of flip and forms varies throughout development and across different neuronal types. The flop versions, which predominate in the adult brain, generally desensitize much more

rapidly than the flip forms in response to glutamate (Sommer et al., 1990). In addition, alternative splicing affects the pharmacologic properties of the AMPA receptor channel, conditioning its differential sensitivity to allosteric modulators (Monyer et al., 1991).

The intracellular, C terminal domains of GluR2 and GluR4 subunits may also undergo alternative splicing (Gallo et al., 1997). As a consequence, GluR2 and GluR4 can be expressed as both short- and long- tailed proteins. As such, GluR1, the predominant splice form of GluR4 and an alternative splice form of GluR2 (GluR2L) have longer cytoplasmic tails than GluR3, the predominant splice form of GluR2 and the alternative splice form of GluR4 (GluR4c) (Santos et al., 2009) (see Figure 1.1.1.1). Importantly, receptors which comprise only short-tailed subunits (e.g., GluR2/GluR3) are subject to constitutive trafficking to and from the synaptic membrane, unlike what occurs with those containing long-tailed subunits (e.g., GluR1/GluR2), whose synaptic targeting occurs in an activity-dependent way (Esteban et al., 2003). Finally, alternative splicing of the C terminal regulates AMPA receptor function by conditioning binding to specific interacting proteins, as well as regulation by protein phosphorylation (Song and Huganir, 2002).

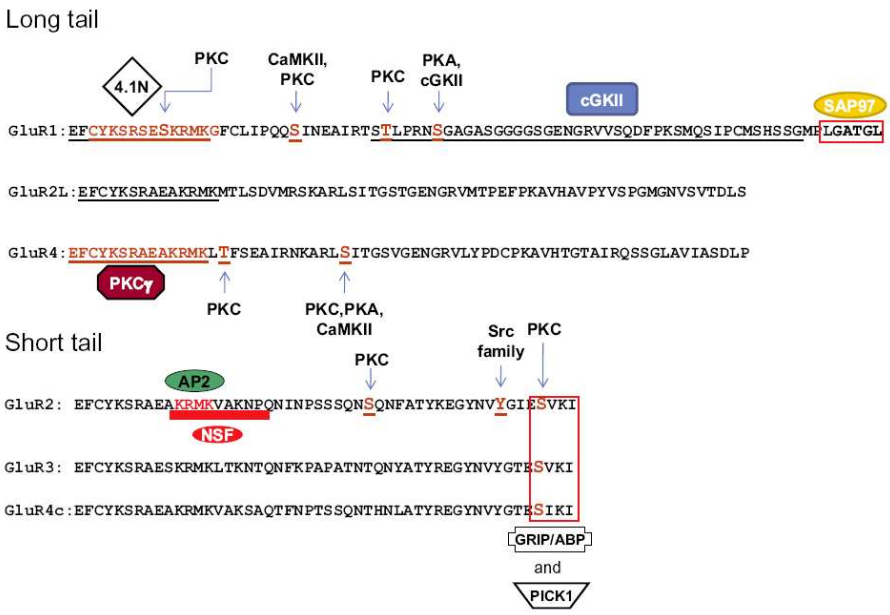


Figure 1.1.1.1. Localization of protein binding and phosphorylation sites in the C terminal of AMPA receptor subunits. In addition to the predominanf subunit forms expressed, alternatively spliced forms of GluR2 (Glu2L) and GluR4 (GluR4c) are also considered. Protein binding sites are indicated by boxes, adjacent to proteins with which they have been described to interact. Phosphorylation sites are underlined and evidenced by a larger font size. Adapted from Santos et al., 2009.

1.1.2 RNA Editing of AMPA Receptor subunits

Additional AMPA receptor diversity is generated by RNA editing, a process involving enzymatic deamination of ribonucleotides (adenosine residues) in pre-spliced mRNA encoding glutamate receptor subunits (Bass, 2002). This mechanism brings about the replacement of a gene-encoded aminoacid by a different, non-coded one, thus allowing the assembly of receptors with novel physiological properties. In AMPA receptors, RNA editing mostly concerns the GluR2 subunit, where it mediates the conversion of a glutamine (CAG) to an arginine (CGG) codon in the ion channel pore region. As a result, edited GluR2 subunits possess an arginine (R) in the M2 membrane spanning

segment at position 586, whereas GluR1, GluR3 and GluR4 subunits have a glutamine (Q) in the homologous position (Hammond, 2008). This process affects retention of GluR2 subunits at the endoplasmic reticulum and also the properties of the AMPA receptors they come to integrate. In fact, Q/R editing constitutes a major quality control checkpoint, detaining GluR2 subunits in the endoplasmic reticulum and diminishing the formation of GluR2 homomeric tetramers (see Santos et al., 2009). RNA editing is also a widespread mechanism for regulating both the calcium permeability and channel rectification properties of the ion channel. AMPA receptors containing Q/R edited GluR2 subunits display low single channel conductance and little permeability to calcium ions, dictated by the size and positive charge of the substitute arginine residue (Burnashev et al., 1992; Jonas and Burnashev, 1995). Most GluR2 subunits in the adult brain exist in their edited form and evidence suggests that deficient RNA editing of this subunit correlates with neuronal death in amyotrophic lateral sclerosis (Kwak and Kawahara, 2005), as well as with dysfunctional AMPA function after ischemia (Peng et al., 2006). In contrast, the remaining subunits mostly exist in their non-edited forms (Burnashev et al., 1992), which, when assembled into GluR2-free homomers or heteromers, display permeability to calcium and inward-rectifying properties due to the glutamine present in the pore-forming region. Calcium permeability is thus governed by the relative expression of GluR2 subunits, and the (GluR1+GluR3)/GluR2 ratio has been used as a predictor of the assembly of calcium-permeable AMPA receptors (see Pelligrini-Gianpetro et al., 1997). Calcium permeable, GluR2-lacking AMPA receptors are also susceptible to blockade by endogenous, intracellular polyamines, which mediate channel blockade in a voltage-dependent

manner (Donevan and Rogawski., 1995; Washburn et al., 1997) (see Figure 1.1.2.1). In agreement with its elemental contribution for overall AMPA receptor function, the relative abundance of GluR2 at synaptic AMPARs has been shown to increase with development, in cultured CA1 neurons (Pickard et al., 2000). Indeed, in the adult hippocampus, the AMPA receptor pool is mostly composed of GluR2/GluR3 and GluR1/GluR2 complexes (Wenthold et al., 1996), causing approximately linear current-voltage relationships, with a mean reversal potential of 0 mV (Jonas and Sakmann, 1992).

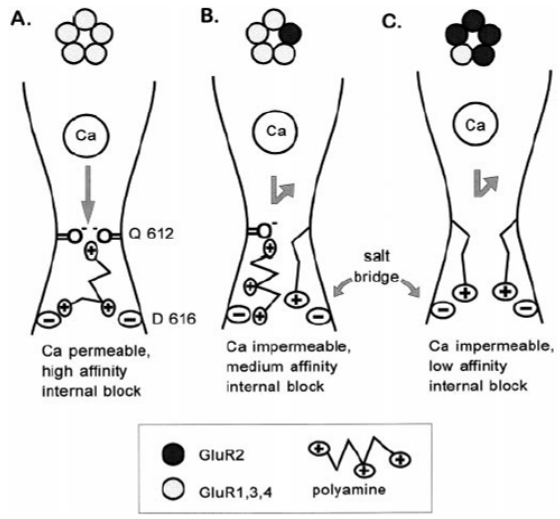


Figure 1.1.2.1. Model accounting for the differential dependence of calcium permeability and inward rectification on GluR2 abundance. The essence of the model is that the ring of carbonyl oxygens in GluR2-lacking AMPA receptors (A) contributes to or forms a binding site for permeating divalent cations. Internal polyamines also interact with this ring of polar residues. Incorporation of a single positively charged arginine into the Q/R site (B) disrupts the ring of carbonyl groups, which is postulated to eliminate the divalent ion binding site. This arginine also neutralizes a negative charge at the internal channel mouth by formation of a salt bridge between its guanidinium group and the carboxyl group of the aspartate, which reduces the number of anionic binding sites for internal polyamines and thus decreases their affinity for the channel. As more arginines are incorporated into the Q/R site (C), the number of negative charges at the channel mouth decreases, and hence polyamine affinity for the internal blocking site, is progressively reduced. Adapted from Washburn et al., 1997.

1.1.3 AMPA receptor subunit phosphorylation

After synthesis, AMPAR subunits are subject to post-translational modifications such as glycosylation, phosphorylation and palmitoylation. Each AMPA receptor subunit can be *N*-glycosylated at 4-6 different sites of its extracellular domain, a modification which protects from proteolytic clivage but that is not required for ligand recognition and does not seem to significantly affect receptor assembly or trafficking (Jiang, 2006). Although it has been proposed that this sort of protein modification may be involved in receptor maturation and transport, its functional repercussions are still unclear. AMPA receptor subunits can also undergo palmitoylation of cysteine residues in their C terminal and M2 domain, with consequences for postsynaptic receptor trafficking and membrane expression (see Santos et al., 2009). Phosphorylation of AMPA receptor subunit residues can occur under resting conditions or as a reponse to changes in synaptic activity. With the exception of GluR3, all AMPAR subunits have been reported to undergo phosphorylation on several amino acid residues of their C terminal, by a variety of kinases (Jiang et al., 2006). For instance, phosphorylation of GluR2 subunits at Serine 880 by PKC has been implicated in AMPA receptor internalization during NMDA-independent Long Term Depression (LTD) of synaptic transmission in the cerebellum (Chung et al., 2003). Interestingly, phosphorylation of Tyrosine 876 (Tyr 876) by Src family tyrosine kinases can also facilitate rapid internalization of GluR2-containing membrane AMPA receptors (Hayashi and Huganir, 2004). In both cases, subunit phosphorylation modulates the receptor´s ability to bind regulatory proteins that assist in its trafficking to and from the membrane. The

function of a third potential PKC phosphorylation site (Ser 863) identified on the C-terminal of the GluR2 subunit (Hirai et al., 2000) remains unknown. GluR4 subunits comprise two phosphorylation sites in their intracellular C terminals. One of them, Serine 842, can be phosphorylated in vitro by PKA, PKC and CaMKII (Carvalho et al., 1999) and its phosphorylation by PKA has been shown to be both required and sufficient for synaptic delivery of homomeric receptors (Esteban et al., 2003). Phosphorylation of Ser 842 by PKC can also increase calcium influx through activated AMPA receptors, in cultured retinal neurons (Carvalho et al., 1998). In addition, threonine 830 represents a potential phosphorylation site for PKC (Carvalho et al., 1999). As for GluR1 subunit phosphorylation, it has been the subject of intense research, prompted by the pivotal role that GluR1-containing receptors have been shown to play in hippocampal plasticity (Zamanillo et al., 1999; Shi et al., 1999). As a result of such research, four phosphorylation sites have been described to occur in the C-terminal of GluR1 subunits. These are serine 831 (Ser 831), which can be phosphorylated by both PKC (Roche et al., 1996) and CaMKII (Mammen et al., 1997); serine 845 (Ser 845), a PKA phosphorylation site (Roche et al., 1996); serine 818 (Ser 818, Boehm et al., 2006) and threonine 840 (Thr 840, Munton et al., 2007; Lee et al., 2007), which are two additional PKC phosphorylation sites that were recently discovered. Phosphorylation of Ser 845 by PKA has been shown to facilitate receptor peak open probability (Banke et al., 2000) and lead to long term potentiation (LTP) of naïve synapses (Lee et al., 2000), while its dephosphorylation associates with decreased synaptic strength underlying LTD (Ehlers, 2000). Phosphorylation of Ser 831 by CamKII enhances single channel conductance (Derkach et al., 1999) and

intervenes in the recruitment of silent synapses, as well as in synaptic transmission facilitation during LTP (reviewed by Palmer et al., 2005). Of note is the fact that although both Serine 831 and Serine 845 are required for AMPAR delivery into synapses, transgenic mice with mutations in both phosphorylation sites still display reduced LTP (Lee et al., 2003a). Recently, a third regulatory phosphorylation site has been implicated in AMPA receptor trafficking underlying LTP. Phosphorylation of Serine 818 (by PKC) was shown to increase after LTP and pharmacological induction of its phosphorylation could enable synaptic incorporation of GluR1-containing AMPA receptors, possibly through modified interaction with candidate proteins capable of modulating receptor trafficking (Boehm et al., 2006). Although it is still not clear how do the above identified phosphorylation events on GluR1 subunits interact to produce synaptic potentiation, it is possible that a conjunctive phosphorylation by Ser 845, Ser 831 and Ser 818 may be required (Boehm et al., 2006). Finally, LTP induction has no effect on GluR1 phosphorylation at Threonine 840 (Delgado et al., 2007). In turn, dephosphorylation of this site, secondary to NMDA receptor activation and protein phosphatase 1/2A activity, has been shown to correlate with changes in synaptic strength during hippocampal LTD (Delgado et al., 2007). As these examples illustrate, different phosphorylation sites are associated with distinct alterations in AMPA receptor properties and/or trafficking pattern. However, in what concerns the role played by phosphorylation sites in the C terminal of GluR1 subunits, all of them have been shown to affect some form of synaptic plasticity (Lee et al., 2000; Esteban, 2003; Boehm et al., 2006; Delgado et al., 2007).

1.1.4 AMPA receptor trafficking

Most AMPARs are thought to be synthesized in the neuronal cell body, far away from the dendritic spines whose receptor population they ultimately integrate. Before reaching this synaptic target, they must thus accomplish a series of trafficking steps, which include regulated exit from the endoplasmic reticulum and active transport through the microtubular cytoskeleton. All of these processes involve intricate networks of protein-protein interactions, between subunit domains and different sets of anchoring and regulatory proteins. The impact that interaction with these regulatory proteins had for AMPA receptor trafficking became clear with the study of the stargazer mouse, a spontaneous mutant that displays head tossing and ataxic gate and absence epilepsy (Ziff, 2007). In these animals, lack of functional stargazin compromises surface delivery of functional AMPA receptors in cerebellar granule cells, a phenotype that could be rescued by expressing the wild-type protein. Interestingly, when using a version of stargazing that lacked a binding site for post-synaptic density 95 (PSD-95), only AMPA receptor delivery to non-synaptic sites was recovered, demonstrating that stargazin´s ability to restore receptor delivery to synapses requires a secondary interaction with this scaffolding protein (Chen et al., 2000). Besides stargazin (or γ–2), seven other related proteins (γ–1, γ–3 to 8) constitute the transmembrane AMPA receptor regulatory protein (TARP) family (see Ziff, 2007). TARPs belong to the group of interacting proteins that lack a PDZ (post-synaptic density 95-discs large-zona occludens 1) domain, as well as NSF (*N*-ethylmaleimide-sensitive fusion protein) or the 4.1 protein. The second major group of proteins that govern AMPA receptor surface expression

is constituted by proteins with PDZ domains. PDZ domains are one of the best characterized protein interaction modules, responsible for mediating protein interactions by recognizing and binding peptide epitopes within their interacting partners. Widely expressed, PDZ domains were first recognized as sequence repeats in the primary structures of the PSD-95, disk-large and zona occludens-1 proteins and were named accordingly (Kurakin et al., 2007). Each PDZ doman binds only one ligand and binding selectivity is thought to result from variations in the size and geometry of the peptide-binding region (Palmer et al., 2005). Well known examples of PDZ-domain containing proteins are GRIP/ABP (glutamate receptor interacting protein/AMPA receptor binding protein) and PICK1 (protein interacting with C kinase-1). Frequently, PDZ-domain containing proteins function as scaffolds at the post-synaptic density, where they mediate organization and maintenance of large macromolecular complexes (see Figure 1.1.4.1).

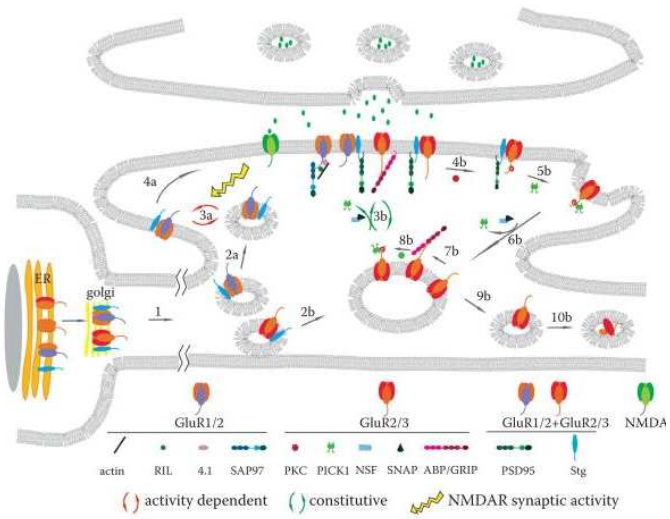


Figure 1.1.4.1. Schematic representation of the role played by different interacting proteins upon AMPAR trafficking. Pathway (a) represents GluR1 trafficking and pathway (b) represents GluR2 trafficking. AMPAR complexes exit from the ER/Golgi associated with

stargazin (1). GluR1-containing AMPARs are inserted at extrasynaptic sites through an activity-dependent mechanism (3a). Stargazin binds PSD95, which localizes the complex at the synapse where the receptor can bind other scaffolding proteins such as 4.1N (4a). Stargazin can then be released. GluR2-containing AMPAR are inserted at synaptic sites through a constitutive mechanism (3b). PICK1 facilitates the transport of the receptor possibly both to and from the plasma membrane but is removed from the AMPAR by the NSF/SNAP (SNAP, soluble NSF attachment protein) when the receptor reaches the plasma membrane. NSF/SNAP/PICK1 form a transient complex with GluR2-containing AMPAR (not shown on this schematic). AMPA receptors are stabilized at the membrane by scaffolding proteins. Phosphorylation by PKC prevents interaction between ABP/GRIP and GluR2 (4b) and favors the PICK1/GluR2 interaction (5b). AMPA receptors are then internalized following a coupling with PICK1, and AMPAR/PICK1 complex are destabilized by NSF/SNAP when the receptor reaches its destination (6b). GluR2-containing AMPARs can then interact with an intracellular pool of ABP/GRIP, possibly in the endosome (7b), and get recycled to the membrane through interaction with PICK1 (8b) or targeted to lysosomes (9b) and degraded (10b). Adapted from Kittler and Moss, 2006.

As illustrated in Figure 1.1.4.1, local receptor insertion and removal from synapses is a dynamic process. In fact, AMPA receptors are continuously being delivered and removed in and out of synapses in response to neuronal activity, adjusting synaptic strength during brain development and experience-dependent plasticity (reviewed by Esteban, 2003). Interestingly, receptor cycling dynamics between intracellular receptor stores and the cell surface is largely dependent upon receptor subunit composition. In fact, while GluR2–GluR3 oligomers are continuously being shipped to synapses in a manner independent of neuronal activity *(*Shi et al., 2001*)*, synaptic delivery of GluR1–GluR2 (Esteban et al., 2003*)* and GluR4-containing receptors (Zhu et al., 2000) requires increased synaptic activity and consequent NMDA receptor activation. The first trafficking pathway thus allows the number of AMPARs at synapses to be preserved despite protein turnover (constitutive pathway), whereas the second (regulated pathway) enables activity-dependent regulated synaptic delivery of

GluR1–GluR2 receptors to synapses, during plasticity (Malinow et al., 2000) (Figure 1.1.4.2).

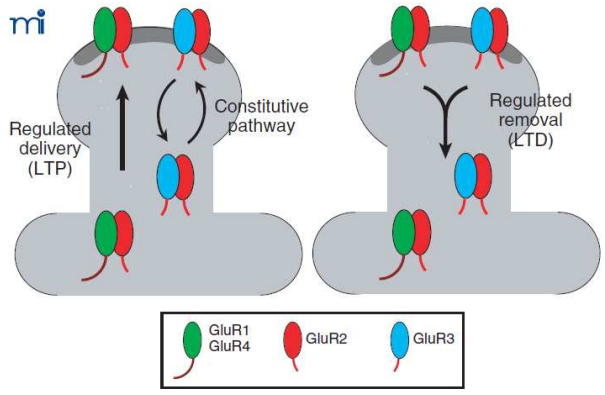


Figure 1.1.4.2. Constitutive and regulated trafficking of AMPARs at synapses. Left. AMPAR oligomers containing GluR1 or GluR4 subunits are added into synapses in an activity-dependent manner (regulated delivery) during long-term potentiation (LTP). GluR2–GluR3 oligomers are continuously cycling (constitutive pathway) in and out of synapses. Right. The activity-dependent (regulated) removal of AMPARs from synapses (LTD) is likely to affect all receptor populations. Adapted from Esteban, 2003.

There is also a marked discrepancy between the trafficking profile of extrasynaptic and synaptic receptors. Indeed, if extrasynaptic receptors constitute a highly mobile population, synaptic AMPA receptors behave as a rather immobile pool under basal conditions. This notion can be derived from elegant experiments using focal glutamate uncaging and irreversible AMPAR blockade, coupled to patch-clamp recordings, which have shown that rapid delivery of AMPARs from internal stores is restricted to nonsynaptic sites (Adesnik and Nicoll, 2005). In these experiments, the authors used a photoreactive AMPA receptor antagonist which, when irradiated with UV light, promptly and irreversibly blocks surface receptors. Therefore, the unsilencing of AMPA receptor-mediated responses can only occur when these are

replaced by spare AMPA receptors, present at intracellular stores. Hence, by using patch-clamp recordings to follow the 'recovery' of AMPA receptor-mediated currents immediately after a global or focal photoinactivation of surface receptors, a direct and quantitative measurement of the exocytosis and lateral diffusion of native AMPA receptors could be attained. When applying this method to evaluate delivery of internal AMPA receptors to synapses, recovery of sucrose-evoked synaptic AMPA currents was found not to occur until several hours after the insult. Furthermore, this timecourse was independent of neuronal activity, since it was unchanged in the presence of tetrodotoxin. Interestingly, when measuring the recovery of AMPA-mediated currents from extrasynaptic membrane patches (pulled from the soma), a ~ 40% recovery of the initial current value was observed within 30 min. Subsequenty, it was shown that recovery of extrasynaptic currents could also occur by lateral diffusion, in the second timescale, as was observed when blocking receptors from only a small patch of membrane and measuring recovery of responses to focal glutamate release in whole-cell configuration (previously shown to impair receptor delivery from internal pools). Comparably fast lateral diffusion has also been shown by others to occur in dendrites (Tardin et al., 2003). Overall, the work of Adesnik and Nicoll (2005) helps devise a model in which diffusion along the surface is a primary route for targeting AMPA receptors to the synapse, with which receptors from internal stores cycle on a much slower timescale, possibly because of the closely packed structure of the postsynaptic density (see Figure 1.1.4.3). This trafficking pattern has most obvious implications for LTP, where regulated delivery of receptors is crucial for synaptic reinforcement (Triller and Choquet, 2005).

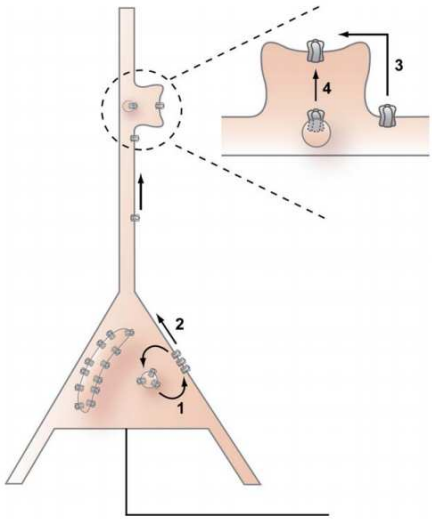


Figure 1.1.4.3. A Model of Basal AMPA Receptor Trafficking. A large intracellular pool of AMPA receptors exchanges rapidly (1) with extrasynaptic somatic AMPA receptors, and these newly inserted AMPA receptors then travel laterally (2) out to dendrites to reside stably at synapses. The lateral diffusion of perisynaptic receptors into the synapse may be regulated by accessory synaptic proteins (3). The exchange of intracellular receptors with synaptic receptors is slow (4). Adapted from Adesnik and Nicoll, 2005.

In CA1 pyramidal neurons, the extrasynaptic pool of AMPARs is almost exclusively composed of GluR1-containing receptors and knock-out animals for this subunit display significant plasticity impairments (Andràsfalvy et al., 2003). One may hypothesize that any mechanism that brings about increased delivery of AMPARs to the extrasynaptic contingent can potentially enhance the ability of that synapse to be reinforced, by increasing the number of receptors available for synaptic tagging. Accordingly, D_1 dopamine receptor activation has been shown to significantly promote LTP in cultured hippocampal neurons, by increasing the size of the GluR1 extrasynaptic pool in a PKA-dependent way (Gao et al., 2006). Likewise, exposure to noradrenaline and emotional stress can drive GluR1 phosphorylation at Ser 845 and Ser 831, leading to synaptic delivery of GluR1-containing AMPA receptors, thus lowering the threshold for LTP (Hu et al., 2007). PKA phosphorylation of GluR1 subunits has been shown to cause AMPA receptor externalization from internal stores to membrane extrasynaptic pools (Oh et al., 2006). In fact, a two-step model for

delivery of GluR1-containing AMPARs to synapses during activity-dependent LTP proposes that phosphorylation of the Ser 845 residue by PKA can traffic AMPARs to extrasynaptic sites, making them available for subsequent delivery to synapses during activity-dependent plasticity, by lateral diffusion (see Figure 1.1.4.4). In turn, there is ample proof that synaptic insertion of GluR1-containing AMPARs contributes to the synaptic strengthening observed during LTP induction (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Malenka and Bear, 2004).

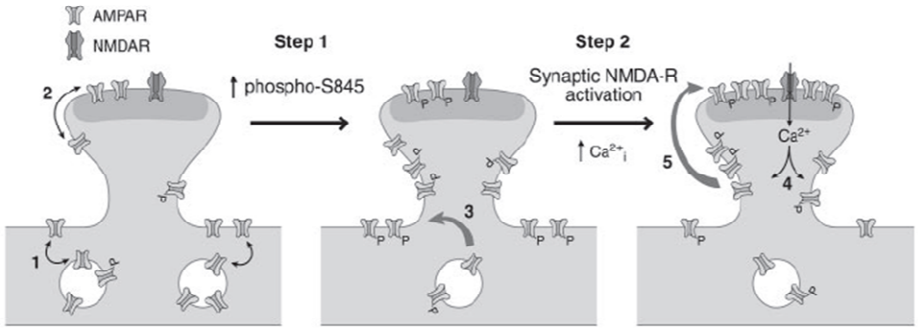


Figure 1.1.4.4. Two-step model for synaptic delivery of AMPARs during LTP. Under basal conditions (left panel), GluR1 has a low phosphorylation at Ser-845. Constitutive recycling occurs between the surface and internal pools (1) and the synaptic and extrasynaptic pools (2) of AMPARs. Increasing Ser-845 phosphorylation (Step 1) stimulates trafficking of internal GluR1-containing AMPARs to extrasynaptic sites on the surface membrane, which primes AMPARs for synaptic incorporation (3). During strong synaptic activation (Step 2), synaptic NMDARs are activated, resulting in increased intracellular calcium (4). Calcium triggers the activation of signaling cascades, which drives GluR1-containing AMPARs to synapses from extrasynaptic sites by lateral diffusion (5). Thus, the two-step model for synaptic delivery of AMPARs consists of delivery of GluR1-containing AMPARs to extrasynaptic sites in a phospho-Ser-845- dependent manner (Step 1, the priming step), followed by synaptic incorporation of AMPARs, which requires synaptic NMDAR activation and calcium (Step 2). Adapted from Oh et al., 2006.

It should be noted that the elemental role played by the trafficking of GluR1-containing receptors in adult animals is, at earlier stages,

attributable to GluR4-containing ones (Esteban, 2003). Both subunits are composed of long intracellular C terminals, which share considerable aminoacid homology and a conserved PKA phosphorylation site (Ser 845 for GluR1 and Ser 842 for GluR4; Esteban et al., 2003), consistent with the similar trafficking behavior they follow. Thus, early in the postnatal development of the hippocampus, regulated delivery of AMPARs involves GluR4-containing receptors and PKA-mediated phosphorylation of GluR4 subunits is necessary and sufficient for triggering receptor delivery to synapses (Esteban et al., 2003). As GluR4 subunit expression declines and that of GluR1 increases with age (Zhu et al., 2000), regulated delivery of AMPARs then requires GluR1 phosphorylation by PKA, but at this stage, activation of a second signaling cascade (involving CaMKII activity) is further required for receptor delivery (Esteban et al., 2003). The greater elaboration of cellular processes and transduction pathways involved in AMPAR delivery underlying plasticity that is observed in adult animals is in agreement with the empirical observation that it is more difficult to induce and express synaptic plasticity, later in life (Rosenzweig and Barnes, 2003).

1.1.5 Synaptic Plasticity

In 1949, Donald Hebb proposed that “When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased” (Hebb, 1949). To this day, Hebb´s law stands for the paradigm of information storage in the brain being encoded by changes

in the strength of synaptic transmission between communicating neurons. At the time, Hebb´s postulate did not receive much consideration from neurophysiologists in the field, such as Ben Burns or John Eccles, for whom the "Hebb synapse" was little more than a self-evident conceptual embodiment of the post-tetanic potentiation (PTP) they had already observed in spinal neurons (reviewed in Bliss, 2003). As the name implies, PTP corresponds to a large enhancement of synaptic efficacy observed after brief periods of high frequency synaptic activity. Too brief to possibly account for the neural basis of memory, PTP led Burns´ student Tim Bliss away from the spinal cord pathways and into the cortical networks that were presumably the neural seat of memory (Eccles, 1966). In fact, it seemed plausible that the spine apparatuses, which were much more prevalent on neocortical and hippocampal pyramidal cells, formed part of the cellular machinery underlying memory (Hamlyn, 1963). Discouraged by contradictory results observed from working with highly complex cortical networks, Bliss turned to the hippocampus for need of simplification. Soon after and together with Terje Lömo, Bliss delivered a single tetanus to the perforant path in the intact rabbit hippocampus, obtaining a huge potentiation of the evoked synaptic response in the dentate gyrus that persisted for hours (Bliss and Lömo, 1973). That they grasped the potential significance of these early findings could be read from the cautious suggestion that such activity-dependent, sustained change in synaptic efficiency might be "potentially useful for information storage" (Bliss and Lömo, 1973). Meanwhile, long term potentiation (LTP) has been observed in a variety of other neural structures, such as the cerebellum (Jörntell and Hansel, 2006), the amygdala or the corpus striatum (Chapman et al., 2003), among others. Some suggest LTP may

even take place at all excitatory synapses in the mammalian brain (Malenka and Bear, 2004). In addition to its longevity, LTP has other characteristics that make it an attractive candidate mechanism for the storage of information in the brain. First of all, it is an input-specific process, since a single pathway can be potentiated without affecting inactive neighbouring inputs to the same cell (Andersen et al., 1980). Secondly, LTP abides stimulus cooperativity, the synaptic property by which many concurrent weak stimulations of converging afferent fibres can cooperate to induce LTP in the postsynaptic cell. Finally, the property of stimulus associativity can be derived from the fact that mild afferent stimulation that is subthreshold for inducing LTP can lead to LTP at a given synapse, provided it is paired with a strong LTP-inducing stimulation at a neighboring synapse (Kemp and Manahan-Vaughan, 2007). The latter feature is particularly interesting when memory formation is conceived as the means by which one can associate events or entities in the outside world. Interestingly, the same properties that argue for LTP as being the cellular correlate of memory formation have also been found to apply for long term depression (LTD) of synaptic transmission (see Kemp and Manahan-Vaughan, 2007) that ensues prolonged low frequency (0.5-3 Hz) afferent stimulation (Malenka and Bear, 2004). Like LTP, LTD has been shown to occur in many brain areas, including the cerebellar cortex, neocortex, hippocampus, striatum and nucleus accumbens (Artola and Singer, 1993). It is thought that LTD results from a subtreshold calcium entry into the postsynaptic cell, which is lower than that necessary to induce LTP and it has been proposed that, by counterbalancing LTP, LTD might serve to create a higher signal-to-noise ratio that would maximally sharpen the ability of synapses to store frequency-based

information (Stanton, 1996). This view is challenged by evidence that LTD and LTP encode separate aspects of declarative memory in the hippocampus and that LTD may play a fundamental role in spatial learning (Kemp and Manahan-Vaughan, 2007). Noteworthy, in addition to undergoing sustained changes in efficiency, synapses can also express short-term forms of plasticity, such as the aforementioned post-tetanic potentiation (PTP) and paired-pulse facilitation (PPF). In PPF, the postsynaptic response to a second stimulus is enhanced relative to the first, provided that the interstimulus interval is brief enough (around 50-60 ms; see Thomson, 2000) for residual calcium from the first stimulus to augment presynaptic transmitter release evoked by the second stimulus. Still, long term potentiation and long term depression remain the most widely studied physiological models of memory formation in the mammalian brain. Recently, evidence emerged that in vivo experience can indeed generate LTD (of responses in the visual cortex, by brief monocular deprivation; Rittenhouse et al., 1999) or LTP (in the ventral tegmental area, by cocaine injection; Ungless et al., 2001) in specific synapses of the brain. In particular, the work by Gruart and colleagues (2006) has shown that it is possible to observe LTP in the hippocampus of mice in the process of learning something, providing a definite relationship between activity-dependent synaptic plasticity and associative learning in behaving animals. These studies highlight the importance of continuing to pursue the mechanisms underlying such modifications in synaptic strength.

1.1.5.1 AMPA receptors in Synaptic Plasticity: NMDA receptor-dependent LTP in the CA1 area

In line with its initial discovery in the hippocampus, NMDA receptor-dependent LTP in the CA1 region of the hippocampus represents the most extensively studied form of synaptic plasticity. With regards to the cellular events that underlie this form of LTP, considerable evidence points towards changes in presynaptic transmitter release, postsynaptic responses and changes in synapse structure. The contribution made by each side of the synapse (pre- or post- synaptic) remains a highly debated subject, but there is now little doubt as to the importance of postsynaptic modifications in AMPA receptor function and localization, during LTP (Malenka and Nicoll, 1999). Lingering doubts on the subject were definitely cast away with the discovery that some excitatory synaptic contacts are actually "silent" synapses, containing only NMDA receptors at the postsynaptic density level. Since depolarization is required to relieve the Mg^{2+}-mediated blockade of NMDA receptors that occurs at resting potentials, these NMDA receptor–only synapses are functionally silent at hyperpolarized membrane potentials. Thus, even when transmitter is released, they are not be able to yield a measurable response, unless recordings were to be attained at positive membrane voltages. The "unsilencing" of such synapses has been shown to follow LTP induction, upon which AMPA receptor–mediated currents can then be detected (Liao et al., 1995). Since their original discovery in the hippocampus, silent synapses have now been described throughout the central nervous system. In most locations, silent synapse prevalence is high in the first postnatal days and gradually declines towards the second postnatal week, as synaptic

connections mature (see Kerchner and Nicoll, 2008). These entities may therefore constitute the immature stage of a synaptic contact which will, or will not, be reinforced throughout development. It should be noted that compelling evidence also points to a presynaptic component in the expression of early LTP, when using confocal microscopy and calcium-sensitive dyes to study LTP at individual visualized CA1 synapses (Emptage et al., 2003). In fact, it has been proposed that presynaptic microstructural changes in the early stages of long-lasting plasticity occur in a coordinated fashion to postsynaptic alterations (Antonova et al., 2001), which might be accomplished by a retrograde messenger communicating from the postsynaptic neuron back to the presynaptic terminal (Malenka and Bear, 2004). Indeed, current models accept that postsynaptic LTP is triggered postsynaptically, by activation of NMDA receptors prompting a rise in intracellular calcium, to which ensues local activation of signaling pathways that ultimately bring about changes in the number, function and sub-cellular localization of AMPA receptors. By depending on both activation by glutamate and membrane voltage, the NMDA receptor thus functions as a coincidence detector, providing a molecular basis for Hebbian LTP. In fact, only when there is coincident pre- and post- synaptic activity, will calcium entry be allowed to trigger plasticity (Malenka and Nicoll, 1999). Consistent with this view of a regulatory role for NMDARs in plasticity, are the early findings that NMDA receptor antagonists can completely block the generation of LTP, despite having minimal effects on basal synaptic transmission (Collingridge et al., 1983). In addition to prompting kinase activation and AMPAR phosphorylation with consequences for AMPA receptor trafficking and function, activation of NMDA receptors is upstream of changes in gene transcription and

protein synthesis, required to stabilize synaptic potentiation over time (West et al., 2002). Involved pathways include the mitogen-activated protein kinase (MAPK) cascade, leading to regulation by phosphorylation of specific transcription factors such as cAMP response element-binding protein (CREB) (Wang et al., 2007) and activation of gene expression programs behind synapse formation and restructuring (Rao and Finkbeiner, 2007). Interestingly, evidence has emerged that AMPA receptors are also able to induce nuclear gene expression in an activity-dependent manner (Rao and Finkbeiner, 2007) (Figure 1.1.5.1).

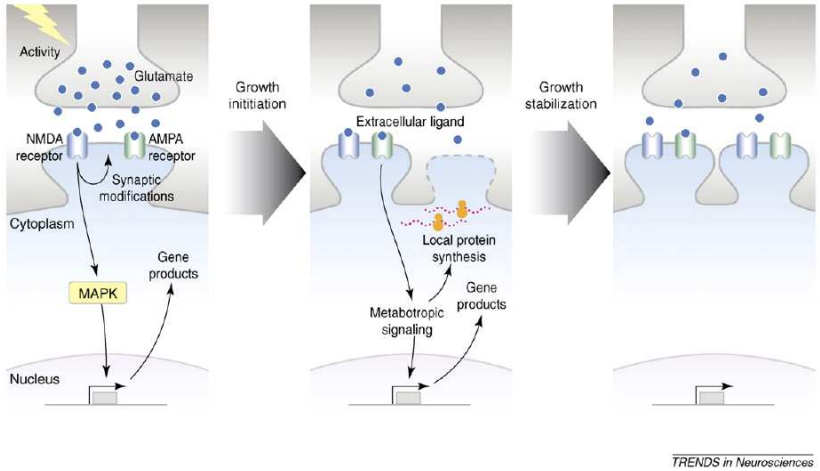


Figure 1.1.5.1.1. NMDA and AMPA receptors regulate synapse formation, growth and stabilization. During bouts of synaptic activity, NMDA receptors initiate signaling cascades that lead to modifications of the activated synapse and activation of MAPK and nuclear gene expression. Nascent gene products promote synapse formation, represented as a second dendritic spine apposed to the presynaptic terminal. In this model, metabotropic signaling by synaptic AMPA receptors regulates transcription and dendritic mRNA translation, thereby promoting the growth and stabilization of the newly formed spine. Adapted from Rao and Finkbeiner, 2007.

Classical LTP models assigning regulatory roles to NMDA receptors and effector functions to AMPA receptors have been further questioned

by recent data showing that, for a short period after LTP is triggered, calcium-permeable AMPA receptors are targeted to the synapse, where they remain until their gradual replacement by GluR2-containing AMPARs, over a period of about 25 minutes (Figure 1.1.5.2). Furthermore, calcium signaling through these transiently synaptic calcium-permeable AMPA receptors seems to be required for solidifying and maintaining the increase in synaptic strength that follows LTP, in CA1 hippocampal pyramidal neurons (Plant et al., 2006). It thus becomes apparent that, in addition to materializing the sustained increase in synaptic strength that occurs during LTP, AMPA receptors are also able to regulate both LTP induction and expression profile changes that stabilize synaptic changes over time and may even exert influences opposed to those of NMDA receptors, thus expanding the complexity of adaptive responses of neurons to synaptic activity (Rao and Finkbeiner, 2007).

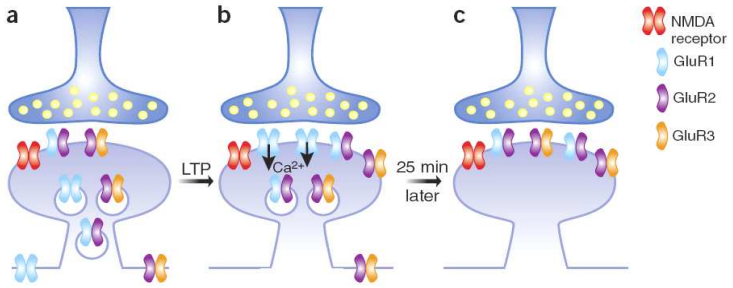


Figure 1.1.5.1.2. Changes in the subunit composition of AMPARs during LTP in CA1 pyramidal neurons. (a) Normally, synapses contain heteromeric AMPARs consisting of GluR1-GluR2 and GluR2-GluR3. Pools of spare AMPARs, including GluR1 homomers, are shown in the spine cytoplasm and in the extrasynaptic membrane. (b) Immediately after LTP, GluR1 homomers are inserted into the synapse and thus provide a new source of Ca^{2+} that seems to be required for maintaining the increase in synaptic strength. (c) About 25 minutes after LTP triggering, GluR1 homomers have been replaced by GluR2-containing receptors. Adapted from Kauer and Malenka, 2006.

Still, it is becoming increasingly clear that this prototypical form of NMDA receptor-dependent LTP in the CA1 area shares only some of the properties and mechanisms of other, different forms of LTP that characterize particular synapses throughout the brain. For instance, LTP at mossy fiber synapses does not require NMDA receptor activation (Harris and Cotman, 1986) and seems to involve increased presynaptic PKA activity (Villacres et al., 1998), leading to sustained modulation of neurotransmitter release machinery. Similarly, LTP in corticothalamic synapses has been shown to be reversible, of presynaptic expression and to require PKA activity, but not NMDA receptor activation (Castro-Alamancos and Calcagnotto, 1999). Even in the CA1 region, non-hebbian plasticity can be induced by repetitive postsynaptic depolarization (in the absence of coincident presynaptic and postsynaptic activity) and trigger a form of LTP that is occluded by induction of “conventional” NMDA receptor-dependent LTP (Kato et al., 2009). What is more, the latter is well known to have different molecular requirements, across development (Esteban et al., 2003). This prompts an absolute need to clarify at which synapses are plasticity phenomena being studied, during which developmental stage and the conditions in which they are triggered. LTP studies must thus specify the stimulation protocol used, the age of experimental animals used, as well as which receptors and pathways are targeted by the induction protocol. Throughout the present work, data specifically concern NMDA receptor-dependent LTP in the CA1 region of the hippocampus, in young animals.

The various forms that LTP may assume emphasize the importance of ensuring that neurons have the ability to express long-lasting activity-dependent synaptic modifications, as one of the key mechanisms by which experience modifies neural circuit behavior. The redundancy of plasticity processes used by neurons to adapt to changes in synaptic activity is illustrated by the fact that many of the cellular and molecular mechanisms that ensure the plastic remodeling of synaptic circuits after ischemia are common to those underlying NMDA receptor-dependent CA1 LTP (Di Filippo et al., 2008).

1.2 Excitotoxicity: AMPA receptors in ischemia

The neurotoxic potential of glutamate is known since the 1950s, when Hayashi reported that injecting glutamate into the brain produced convulsions (Hayashi, 1954), a couple of years before Lucas and Newhouse found that injection of L-glutamate into mice retinas would destroy its inner neural layers (Lucas and Newhouse, 1957). Over a decade later, Olney observed that subcutaneous injections of monosodium glutamate elicited acute neuronal necrosis in several regions of the developing brain, including the hypothalamus (Olney, 1969). He would subsequently be responsible for coining the term excitotoxicity, to describe the process by which excitatory amino acids elicit cell death (Olney, 1986). Neuronal injury by excitotoxicity is most frequently caused by excessive activation of glutamate receptors and it underlies cell death after epileptic convulsions, stroke, spinal cord trauma or head injury (Faden et al., 1989; Dirnagl et al., 1999; Vincent and Mulle, 2009). Excitotoxicity is in addition a hallmark of several neurodegenerative disorders, such as Parkinson’s disease,

Alzheimer's disease, Huntington's disease, amyotrophic lateral sclerosis and multiple sclerosis (reviewed by Dong et al., 2009) and has even been proposed to facilitate tumor growth (Rothstein and Brem, 2001). Considerable interest has thus focused on the cellular and molecular mechanisms that underlie glutamate receptor-mediated neuronal death. In what concerns hypoxia and ischemia specifically, excitotoxicity is triggered by shortage of substrate delivery (oxygen, glucose) secondary to some sort of cerebral blood flow restriction. In humans, such a scenario is most frequently associated with ischemic stroke. When deprived of oxygen and glucose, ischemic neurons increase their consumption of ATP, which decreases energy stores and compromises ATP-dependent maintenance of the resting potential by the sodium/potassium pump. As a result, anoxic depolarization spreads through neuronal and glial cells alike (Somjen, 2001). In neurons, depolarization triggers activation of presynaptic voltage-dependent calcium channels and glutamate release, while in astrocytes, depolarization causes glutamate transporter reversal (reviewed by Dirnagl et al., 1999). The resultant build-up of extracellular glutamate brings about overactivation of ionotropic AMPA, NMDA and kainate glutamate receptors, with a consequent increase in the influx of sodium and calcium ions (see Figure 1.2.1). Excessive calcium influx, is not only directly caused by NMDA receptor activation, as it also occurs secondarily to an increase in the levels of intracellular sodium, through the sodium/calcium exchanger. Activation of voltage-gated calcium channels constitutes a third source of calcium entry into the postsynaptic neuron (reviewed by Lee et al., 1999).

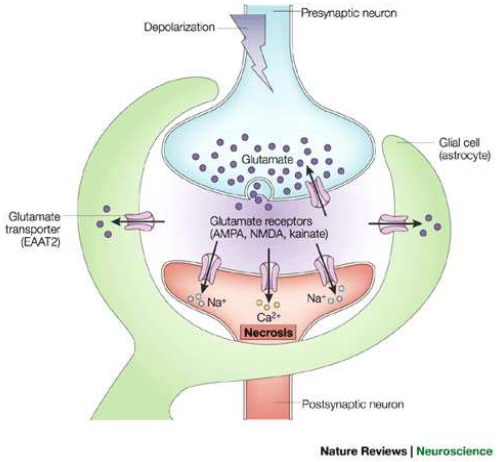


Figure 1.2.1. Excitotoxic cell death. Glutamate binds to and opens specific ionotropic receptor channels on postsynaptic neurons. Gating of these channels provokes an influx of calcium ions inside the cell either directly (through glutamate receptors that conduct both calcium and sodium) or indirectly (through the secondary activation of voltage-gated calcium channels). The sharp increase of intracellular calcium concentration is a principal death-signalling event that is involved in both necrosis and apoptosis. EAAT2, excitatory amino acid transporter 2. Adapted from Syntichaki and Tavernarakis, 2003.

Prompted by excessive activation of glutamate receptors, there is a rise in the levels of free intracellular calcium, that unleashes the activation of several intracellular pathways and enzymes (proteases, phospholipases, endonucleases) as well as increased generation of free radicals that overwhelms scavenging mechanisms leading to membrane damage by lipid peroxidation (Choi, 1999). In turn, ischemia-induced oxidative stress leads to the phosphorylation and membrane translocation of sodium/potassium/chloride co-transporters (NKCC), which mediate electroneutral ion influx (1 Na^+:1 K^+:2 Cl^-) into astrocytes (see Jayakumar and Norenberg, 2010). Passive entry of water molecules then causes cell swelling leading to edema, which can directly damage endangered neurons by further restricting blood flow.

Indirectly, cell edema raises the intracranial pressure and this can complicate most gravely if herniation occurs and the brain stem is compressed, in which case the functions of crucial regulatory centers, such as those controlling breathing and heart rate, are lost (Snell, 2009). In the ischemic territory, the core of brain tissue that is exposed to the most severe blood flow restriction rapidly succumbs to necrotic death. The area surrounding this central ischemic territory, where blood flow is reduced but some energy metabolism remains, is often called the grey area (or ischemic penumbra), the at-risk ring of cells that may or may not undergo apoptosis on the course of several hours to days (reviewed by Broughton et al., 2009). Interestingly, the generalized increase in neuronal excitability that occurs in this area may be useful for establishing new synaptic contacts between surviving neurons which may recover, at least partly, some of the functions formerly ensured by the ischemic core population. The process of structural remodeling, which shares many of the mechanisms involved in physiological plasticity (Di Filippo et al., 2008) is believed to underlie spontaneous functional recovery from stroke (Murphy and Corbett, 2009). Reducing cell death by delayed excitotoxicity and positively modulating plasticity phenomena in the ischemic penumbra thus represent the main goals for therapeutic intervention in stroke patients. In what concerns delayed cell death by apoptosis, two general pathways are considered. Organized cell death can either be initiated by internal events, such as mitochondria disruption and release of cytochrome c with downstream caspase activation (intrinsic pathway); or it can result from activation of “death receptors” by their specific ligands (extrinsic pathway). The intrinsic pathway depends upon a calcium influx trigger, which can have several sources: (1) activation of group 1 metabotropic

glutamate receptors, (2) activation of NMDA receptors, (3) activation of voltage-sensitive calcium channels, (4) de-activation of extrusion and/or sequestration systems and (5) activation of calcium-permeable AMPARs (Tanaka et al., 2000). In fact, it has long been known that glutamate receptor antagonists can reduce injury derived from hypoxic and ischemic insults, both *in vitro* (Rothman, 1984) and *in vivo* (Simon et al., 1984). Curiously, post-ischemic blockade of AMPA, but not that of NMDA receptors, protects against ischemia-induced delayed cell death of CA1 pyramidal cells, even when antagonists are given as late as 24h after the insult (Nellgard and Wieloch, 1992; Sheardown et al., 1993). In addition, when compared to NMDA receptor antagonists, treatment with AMPAR antagonists seems to afford wider therapeutic time windows, suggesting the latter receptors may remain active for a longer timescale (Turski et al., 1998). Both findings argue for an important role played by AMPAR in delayed cell death (Weiss and Sensi, 2000). And indeed, compelling evidence suggests that specific neurological insults (such as ischemia) can drive a selective decrease in GluR2 subunit expression, prompting formation of calcium-permeable AMPA receptors which exacerbate glutamate toxicity (Pellegrini-Giampetro et al., 1997). According to this concept (also known as the GluR2 hypothesis), insulted neurons which normally express calcium-impermeable AMPARs, are not able to adapt to the acute increase in permeability to calcium that is due to newly synthesized GluR2-lacking AMPA receptors. This hypothesis fits nicely with data from transient forebrain ischemia models in rodents, where GluR2 mRNA expression becomes markedly reduced in the CA1 pyramidal area, a region most vulnerable to ischemia (Choi, 1995). The fact that there are viable neuronal populations in the hippocampus which already comprise a

considerable proportion of GluR2-lacking receptors, such as GABAergic interneurons (McBain and Dingledine, 1993), does not necessarily contradict the GluR2 hypothesis. In fact, the sustained expression of calcium-permeable AMPARs may be compensated for, by a higher ability for Ca^{2+} buffering and extrusion or by altered desensitization of AMPA currents (reviewed by Pellegrini-Giampetro et al., 1997). Furthermore, it seems this mechanism is also extendable to other neuropsychiatric conditions and disorders (see Table 3).

Table 3 - Studies supporting the central role played by GluR2 subunits in different models of neuronal injury (Pellegrini-Giampetro et al., 1997)

Condition	Cell type	Reference
GluR2 expression is preferentially reduced prior to cell death		
Transient global ischemia	CA1 pyramidal cells	Pellegrini-Giampetro et al., 1992
Status epilepticus	CA3 pyramidal cells	Friedman et al., 1994
Mutant spastic rats	Cerebellar purkinje cells	Margulies et al., 1993
Schizophrenia	Parahippocampal pyramidal cells	Eastwood et al., 1995
Amyiotrophic lateral sclerosis	Spinal motor neurons	Virgo et al., 1996
Ca^{2+}-permeable AMPARs are formed prior to cell death		
Developing brain	Retinal ganglion cells	Rörig and Grantyn, 1993
Transient global ischemia	CA1 pyramidal cells	Gorter et al., 1997
Editing-deficient GluR2 mice	CA3 pyramidal cells	Brusa et al., 1995
Activation of Ca^{2+}-permeable AMPARs is neurotoxic		
Primary cultures	Cerebellar Purkinje cells	Brorson et al., 1994
Primary cultures	Neocortical neurons	Turetsky et al., 1994
Cell line	Oligodendroglial lineage	Yoshioka et al., 1995

Aside from calcium-mediated toxicity, GluR2-lacking AMPARs additionally allow the influx of Zn^{2+} ions, released during excitatory synaptic transmission. Once in the cytosol, Zn^{2+} interferes with mitochondrial metabolism, disrupting the mitochondrial membrane potential and affecting reactive oxygen species formation, further aggravating neuronal damage (reviewed by Weiss and Sensi, 2000).

Still, regardless of all that is already known about the mechanisms underlying ischemia-induced neuronal death, therapeutic approaches in stroke patients are currently limited to early treatment with tissue plasminogen activator, used as a clot lytic (Del Zoppo et al., 2009). For instance, in what concerns therapeutic strategies related to glutamate receptors, it has not been easy to extrapolate the neuroprotective potential of AMPA receptor blockade from animal models to clinical use in stroke patients (Besancon et al., 2008). While some suggest new therapeutic targets other than glutamate receptor antagonists should be considered (Besancon et al., 2008), it is possible that the failure to translate results from the bench to the bedside is due to weaknesses in experimental stroke study design that can potentially be corrected should specific recommendations be followed (Dirnagl, 2006). A more serious problem concerns the significant fraction of stroke patients that developed severe side-effects of reduced consciousness ranging from stupor to coma, when treated with AMPA receptor antagonists (Walters et al., 2005). Considering that excitatory synaptic transmission throughout the nervous system crucially depends on maintaining physiological AMPA receptor function, these side-effects may prove difficult to overcome.

In alternative, it is possible that pharmacological modulation of AMPA receptor function could represent a viable option to the use of antagonists. Candidate molecules would be expected to be able to endogenously affect synaptic transmission, either through regulation of channel properties or receptor delivery rates to the membrane, both of which can be regulated by phosphorylation. Activation of signaling pathways targeting the phosphorylation of AMPA receptor subunits

would therefore constitute a pre-requisite for such a candidate molecule. A widespread expression pattern, matching that of AMPA receptors themselves, would constitute a second major pre-requisite. Adenosine, an ubiquitous molecule involved in homeostatic coordination of brain function that is intensely released following neuronal damage, fulfills both requirements.

1.3 Neuromodulation by adenosine

Purines and purine nucleotides are essential components of all living cells. Indeed, ATP is used as the general currency in energy conversions by organisms ranging from bacteria to mammals, while adenosine serves as precursor to nucleic acid synthesis. Similarly to what occurs with glutamate, to the central function that ATP and adenosine play in metabolism, adds an elemental role in both intracellular and extracellular signaling, made possible by their ubiquitous expression across animal evolution. Yet, while ATP may function as a neurotransmitter in some brain areas (Burnstock, 2007), adenosine is neither stored nor released as a classical neurotransmitter. Instead, it reaches the extracellular space by several non-exocytotic mechanisms, where it functions as an ubiquitous signaling substance capable of influencing synaptic transmission and passive membrane properties, ultimately ensuring energy homeostasis maintenance (Sebastião and Ribeiro, 2009), much as it does in other excitable tissues, such as the heart, where its actions were first described (Drury and Szent-Györgyi, 1929). In the heart as in the brain, by decreasing cellular activity and enhancing the delivery of metabolic substrates through arteriolar dilatation, adenosine enables the maintenance of an

efficient ratio of energy expenditure to energy supply (Ribeiro et al., 2003). In what concerns synaptic transmission, rather than exerting direct actions, adenosine tunes neuronal communication by influencing neurotransmitter release, as well as the actions of neurotransmitters and other neuromodulators, thus setting the tone in several physiological (sleep, learning, memory) and pathological processes, such as epilepsy, stroke or addiction (Sebastião and Ribeiro, 2009). Its actions are mediated by the activation of G protein –coupled seven transmembrane domain receptors, which are widely expressed throughout the mammalian nervous system, by both neurons and glial cells (Fields and Burnstock, 2006).

1.3.1 Adenosine receptors

Early evidence for the occurrence of adenosine receptors first arose from the work of Satin and Rall (1970) showing that the ability of adenosine to increase cAMP levels in the brain could be blocked by theophylline. At that time, theophylline was only known as a phosphodiesterase inhibitor and its treatment was expected to promote cAMP accumulation in brain slices. Not only did it not affect cAMP levels, theophylline actually prevented the 20-30 fold increase in cAMP that was caused by adenosine (Satin and Rall, 1970). This data led the authors to question the site of action targeted by both compounds and made them raise the hypothesis that they might interact with extracellular structures. Similar results were then observed at the neuromuscular junction, where theophylline was found to prevent the decrease in neurotransmitter release induced by adenosine (Ginsborg and Hirst, 1972). In this study, adenosine was used as a tool to enhance

the levels of cAMP, as a means to test the involvement of cAMP in transmitter release. The fact that adenosine decreased, rather than increased, transmitter release came as the first baffling result. The second was that theophylline´s effect could not be explained by a direct action of theophylline upon neurotransmitter release towards increased quantal content, since the inhibitory effect of adenosine did not depend on the initial quantum content values (Ginsborg and Hirst, 1972). If anything, treatment with theophylline should increase assay sensitivity to detect inhibitory effects by adenosine. Both studies therefore suggested that adenosine could be acting through a membrane receptor, susceptible to extracellular blockade by theophylline. Meanwhile, the ability of adenosine to modulate neurosecretory mechanisms, soon shown to be mimicked by co-released ATP (Ribeiro and Walker, 1973; 1975), drove fruitful research in the field. Subsequently, Geoffrey Burnstock came up with the first nomenclature for purinergic receptors (Burnstock, 1976). Acknowledging the occurrence of distinct types of receptors for adenosine in neurons, van Calker and co-workers (1979) further proposed their subdivision into either inhibitory A_1 or excitatory A_2 receptors. Nearly at the same time, Londos and colleagues (1980) came to a similar proposal, subdividing adenosine receptors as either Ra (stimulatory) or Ri (inhibitory), but this nomenclature would not prevail. Several years later, receptor cloning studies would come to reveal the expression of four types of adenosine receptors - A_1, A_{2A}, A_{2B} and A_3 - in a variety of species, including man (see Dunwiddie and Masino, 2001). Importantly, while A_1 and A_{2A} receptors display high affinity for adenosine and are expected to exert important regulatory functions in physiological conditions, A_{2B} and A_3 receptors are low-affinity receptors most likely to play a role in pathological conditions

featuring increased extracellular concentrations. Noteworthy, A_3 receptors display high affinity for adenosine in humans, unlike what occurs in the rat (see Dunwiddie and Masino, 2001). In addition, receptor subtypes that most frequently couple to Gs proteins (A_{2A} and A_{2B}) mostly impart excitatory actions as opposed to those preferably coupled to Gi/Go proteins (A_1 and A_3 receptors). Indeed, in the central nervous system, as well as in the periphery, activation of A_1 receptors exerts inhibitory effects upon synaptic transmission and neuronal excitability, while A_{2A} and A_{2B} receptor activation is responsible for excitatory actions (Sebastião and Ribeiro, 1996). These actions can be ascribed, at least in part, to the modulation of K^+ and Ca^{2+} channels that has been shown to follow adenosine receptor activation (see Figure 1.3.1.1.). Activation of adenosine receptors can also trigger a broad range of signaling cascades, in a way which is attributable, at least in part, to their G protein specificity (Fredholm et al., 2001). For instance, A_{2A} receptors are most frequently coupled to adenylate cyclase activation and consequent cAMP formation leading to PKA activation, but they can also couple to different G proteins (Fredholm et al., 2001). Which signal transducing pathway is operated, may depend on both the nature of the effector system and the availability of G proteins and kinases in the receptor´s vicinity. Accordingly, presynaptic PKA- (Cristóvão-Ferreira et al., 2009) and PKC- mediated (Lopes et al., 2002; Pinto-Duarte et al., 2005) A_{2A} receptor actions have been identified in the rat hippocampus.

	A_1	A_{2A}	A_{2B}	A_3
Adenosine Affinity	~70 nM	~ 150 nM	~ 5100 nM	~ 6500 nM
G-protein coupling	$G_{i/o}$	G_s	G_s G_q	$G_{i/o}$ G_q
Effects of G-protein coupling	↓ cAMP ↑ IP_3 ↑ K^+ ↓ Ca^{2+} currents ↑ MAPK	↑ cAMP ↑ MAPK	↑ cAMP ↑ IP_3 ↑/↓ MAPK	↓ cAMP ↑ IP_3 ↑ MAPK

Figure 1.3.1.1. Adenosine receptors can couple to several G proteins. The possible effects of G-protein coupling for each adenosine receptor subtype are also listed. Adapted from Dunwiddie and Masino, 2001 and Rees et al., 2003.

Adenosine receptors can also influence the action of other neuromodulators, by regulating the activation of receptors for neuropeptides, such as calcitonin gene-related peptide (CGRP) and vasoactive intestinal peptide (VIP). Other targets for regulation by adenosine receptor activation include nicotinic acetylcholine receptors, as well as NMDA and metabotropic glutamate receptors (see Sebastião and Ribeiro, 2000). In accordance with the plethora of functions that is tuned by the adenosinergic tonus, adenosine receptor expression is brain-region specific (Ribeiro et al., 2002) and varies through development (e.g., Shaw et al., 1986). In what concerns receptor distribution, the A_1 receptor is highly expressed in the cerebral cortex, hippocampus and cerebellum, while the A_{2A} receptor is strongly expressed by striatopallidal GABAergic neurons and is found at much lower levels in other brain regions (Figure 1.3.1.2). A_3 receptors (which are high-affinity adenosine receptors in the human brain), are expressed at an intermediate level in the human cerebellum and hippocampus (see Ribeiro et al., 2003).

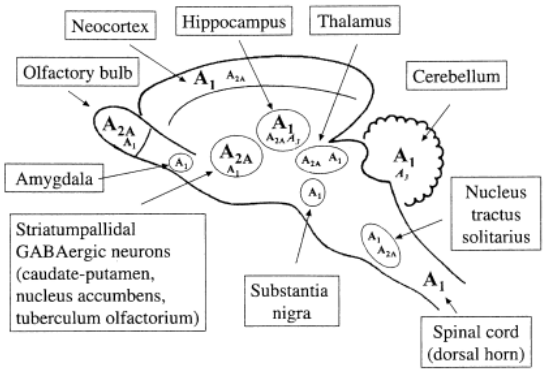


Figure 1.3.1.2. Distribution of high affinity adenosine receptors (A1, A2A and human A3) in the rat brain. Relative adenosine receptor expression is shown for the main regions of the central nervous system where adenosine has been proposed to interfere with brain dysfunction and disease. Higher levels of expression are indicated by larger alphabets. Adapted from Ribeiro et al., 2002.

1.3.2 Regulation of extracellular adenosine levels

Appropriate regulation of extracellular adenosine abundance in the brain is crucial for neural processing, since small changes in adenosine levels can profoundly affect the degree of synaptic inhibition mediated by A_1 receptors. In the hippocampal slice preparation, endogenous extracellular adenosine levels have been estimated to fall within a 140-200 nM range (Dunwiddie and Masino, 2001), concentrations which suffice for activation of high-affinity adenosine receptors only. Two major sources of adenosine in the extracellular compartment can be considered. On the one hand, conditions of increased presynaptic stimulation favor ATP release and adenosine formation results from ATP catabolism by a cascade of ectoenzymes, the ecto-5-nucleotidases family (Figure 1.3.2.1). This pathway of adenosine formation leads to preferential activation of A_{2A} receptors (Correia-de-Sá et al., 1996). On the other hand, at rest or upon low frequency stimulation, A_1 receptor activation is prompted by adenosine released through equilibrative

nucleoside transporters (ENTs) (Correia-de-Sá et al., 1996). Indeed, ENTs keep adenosine levels in balance between the extracellular and intracellular compartment, by either favoring adenosine influx or efflux according to its gradient across the cell membrane. Nonetheless, enhancement of extracellular adenosine levels can be achieved by transporter inhibitors such as dipyridamole and nitrobenzylthioinosine or by targeting enzymes involved in the intracellular adenosine metabolism, such as adenosine kinase, which phosphorylates adenosine into AMP (Sebastião and Ribeiro, 2009). As such, adenosine kinase inhibition by iodotubercidin markedly enhances extracellular adenosine levels, with consequences for hippocampal synaptic transmission (Diógenes et al., 2004). Interestingly, it was recently reported that empirical symptomatic treatment for Parkinson´s disease, by deep brain stimulation, also relies on an increase in the extracellular adenosine concentration (Bekar et al., 2008).

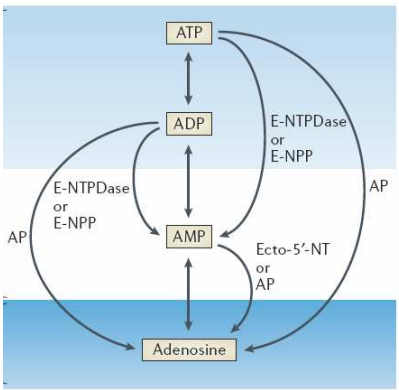


Figure 1.3.2.1. The metabolism of extracellular ATP is regulated by several ectonucleotidases. These include members of the E-NTPDase (ectonucleoside triphosphate diphosphohydrolase) family and the E-NPP (ectonucleotide pyrophosphatase/phosphodiesterase) family. Ecto-5′-nucleotidase (Ecto-5′-NT) and alkaline phosphatase (AP) catalyse the nucleotide degradation to adenosine. Adapted from Fields and Burnstock, 2006.

The extracellular adenosine concentration is also known to greatly increase under conditions of enhanced energy requirements and consequent depletion of the intracellular ATP stores. Such scenarios are commonly associated with exacerbated neuronal activity during seizures and with shortage of metabolic substrates, owing to blood flow restriction, causing ischemia. When the intracellular concentration of adenosine rises enough to exceed extracellular levels, direct adenosine efflux is prompted by equilibrative transporters (Dunwiddie and Masino, 2001). In fact, after 5 min of severe ischemia, adenosine is released in large amounts reaching extracellular concentrations of 25–30 μM, as measured from hippocampal slices (Pearson et al., 2006). Once in the extracellular space, adenosine dampens neuronal activity by at least four different cellular mechanisms: 1) pre-synaptic inhibition of neurotransmitter release, 2) synaptic inhibition of calcium influx through voltage dependent calcium channels, 3) inhibition of NMDA receptors, and 4) activation of G-protein dependent inwardly rectifying K^+ channels (GIRKs) that mediate membrane hyperpolarization (De Mendonça et al., 2000). These modulatory actions suppress neuronal activity, preserve ATP stores and protect neurons from excitotoxicity (Dunwiddie and Hoffer, 1980) and are mainly attributable to A_1 receptors (de Mendonça et al., 2000). A_1 adenosine receptors have also been implicated in preconditioning, the process by which exposure to a brief episode of mild hypoxia or ischemia affords protection from subsequent insults of greater severity. In the brain, preconditioning involves adenosine release, activation of A_1 receptors and that of ATP-sensitive K^+ channels (Heurteaux et al., 1995) and possibly downregulation of pro-apoptotic factors (Ordonez et al., 2010). A central role for A_1 adenosine receptors in adenosine-mediated

neuroprotection is also consistent with several reports of A_1 receptor agonists being able to protect from neuronal damage, in both *ex vivo* and *in vivo* animal models of excitotoxicity (de Mendonça et al., 2000). Furthermore, administration of adenosine amine congener (ADAC, an A_1 receptor agonist) can diminish neuronal damage after global forebrain ischemia, even when given a few hours after the initial insult (Bischofberger et al., 1997). Unfortunately, therapeutic usage of A_1 receptor agonists as possible neuroprotective agents in ischemia has been hindered by severe peripheral side effects, which include sedation, bradycardia and hypotension (White et al., 1996). Also, ischemia itself can induce changes in the adenosinergic system, by triggering downregulation of A_1 receptor levels in insulted areas (reviewed by de Mendonça et al., 2000). Alternative therapeutic approaches, aiming at upregulating A_1 receptor numbers in excitotoxicity conditions, could hopefully prove useful to enhance protection by adenosine. Accordingly, the pleiotropic cytokine interleukin 6 (IL-6), which is not neuroprotective by itself, has been shown to enable neuronal rescue from glutamate-induced death, by enhancing adenosine A_1 receptor expression (both mRNA and protein) in mouse cortical neurons (Biber et al., 2008). Interestingly, neuroprotection may be a common feature of other members of the IL-6 cytokine family, such as leukemia inhibitory factor (LIF) and oncostatin M (OSM), since all IL-6-type cytokines rely upon gp130 receptor subunits for signaling, which leads to many shared redundant functions (Heinrich et al., 2003; Kamimura et al., 2003) (see Figure 1.3.2.2).

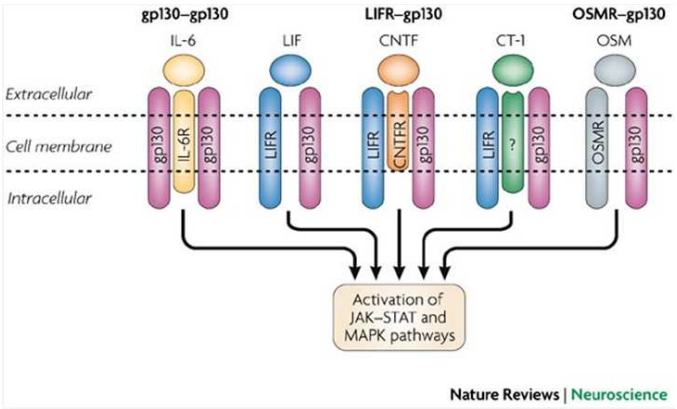


Figure 1.3.2.2. Receptor complexes activated by different members of the IL-6 cytokine family. gp130 homodimers associate with specific interleukin receptors such as the IL-6 receptor to mediate the actions of IL-6. Leukemia inhibitory factor (LIF) binds to heterodimers of LIF receptor (LIFr) and gp130. LIFr–gp130 heterodimers can also associate with other receptor subunits to bind ciliary neurotrophic factor (CNTF) and cardiotrophin 1 (CT-1). The oncostatin M receptor (OSMr) forms heterodimers with gp130 to bind oncostatin M (OSM). The signal-transducing subunit gp130 is found in all complexes, and is responsible for the intracellular activation of the Janus-activated kinase–signal transducer and activator of transcription (JAK–STAT) and the mitogen-activated protein kinase (MAPK) pathways. Adapted from Bauer et al., 2007.

It is not known, however, whether neuroprotection by LIF or OSM can also be indirectly exerted, through an increase in A_1 receptor levels.

There is much less consensus as to the role played by adenosine A_{2A} receptors in ischemia (e.g. de Mendonça et al., 2000), but several studies suggest their activation may exert deleterious effects (Chen et al., 2007). Accordingly, A_{2A} receptor antagonists have been shown to be neuroprotective in *in vivo* models of cerebral ischemia (Gao and Phillis, 1994; Melani et al., 1996) and A_{2A} knockout mice are less sensitive to injury, as observed in a transient focal ischemia model (Chen et al., 1999a). Still, unlike what happens with A_1 receptors, not much is known concerning the mechanism(s) through which adenosine A_{2A} receptors may modulate ischemia-induced cellular damage.

Recently, A_{2A} receptor blockade was shown to delay the occurrence of anoxic depolarization brought about by severe ischemia in the CA1 area, with beneficial consequences for neuronal survival (Pugliese et al., 2009). Although the underlying cellular mechanisms were not pursued, the authors noted that the time window of A_{2A} receptor-mediated protective effects was comparable to that previously observed when treating hippocampal slices with glutamate receptor antagonists (Tanaka et al., 1997). It was thus proposed that the protection afforded by A_{2A} antagonists might involve an impairment in A_{2A} receptor-mediated potentiation of glutamatergic transmission at pre- and/or postsynaptic sites (Pugliese et al., 2009).

As depicted above, regulation of glutamate receptor function and expression, in particular that of AMPA receptors, is crucial for sustained changes in synaptic transmission efficiency, underlying plasticity. In fact, long-term potentiation of synapses greatly relies upon tuning both AMPA receptor trafficking and subunit composition, ultimately setting the amount of signal generated by a postsynaptic neuron, in response to a given presynaptic stimulus. Many of these changes in AMPA receptor function are also observed in ischemia conditions, when extracellular glutamate build-up unleashes overactivation of postsynaptic neurons, with deleterious consequences. Bearing in mind that pathology most frequently arises when physiological processes escape regulational mechanisms, it is reasonable to expect that key mediators in LTP expression also prove to be elemental in excitotoxicity establishment. Any such candidate molecule targeting AMPA receptor function is likely to be of

therapeutic interest. Therefore, a main objective of the present work was to understand how adenosine, acting through high-affinity A_1 and A_{2A} receptors, might affect AMPA receptor-mediated excitatory synaptic transmission.

2 Aim

The experimental work described in this thesis was designed to address a putative modulation by adenosine of AMPA receptor-mediated excitatory synaptic transmission in the hippocampus. In order to accomplish this general aim, three specific objctives were pursued:

I. To assess a putative modulation of postsynaptic AMPA receptor-mediated responses by A_{2A} adenosine receptors in CA1 pyramidal cells, as well as transduction pathways involved and implications for synaptic plasticity.

II. To grasp the consequences of A_{2A} receptor-induced modulation of hippocampal glutamatergic synaptic responses for ischemia-induced changes in synaptic transmission.

III. To clarify whether modulation of A_1 receptor function constitutes a common intermediary strategy for neuroprotection afforded by different IL-6 type cytokines.

3 Techniques

3.1 Patch-clamp Recordings

The first known electrophysiology experiments date back to the 1660´s, when the Dutch microscopist Jan Swammerdam developed a neuromuscular preparation of the frog leg inducing muscle contraction upon “irritation” (Stillings, 1975). In fact, by using a silver wire to deliver nerve “irritation”, Swammerdam actually came close to discovering the nature of signal propagation between nerves and muscles. Yet, it would not be until 1791 that experimental support for the electric nature of the nerve impulse would emerge from Luigi Galvani´s fundamental work “*De viribus electricitatis in motu musculari commentarius*”. Using the inferior limbs and crural nerves of the frog and inserting a metal wire across the exposed spinal cord, Galvani recognized the electrical excitation of the nerve-muscle unit, as well as the relationship between stimulus intensity and muscle contraction. His continuing studies culminated in the first demonstration of a propagating action potential (Galvani, 1841; Piccolino, 1998) and the elaboration of Galvani´s theory on animal electricity, according to which biological tissues are kept in a state of “disequilibrium” that enables their response to external stimuli. Even more impressive were his prescient thoughts on animal electricity resulting from the accumulation of positive and negative charges on the external and internal surfaces of the muscle (or nerve fiber) and the possibility that water-filled channels might be responsible for mediating the current flow required for tissue excitation (reviewed in

Piccolino, 1998). As it was, Galvani´s pioneering experiments marked the beginning of an "instrumental period" during the course of which the full potential of electrophysiology would progressively be revealed. Some of the major instrumental breakthroughs concern the early use of electromagnetic galvanometers, which enabled Hermann von Helmoltz to estimate the speed of nerve impulse propagation (Helmholtz, 1850) and Émile du Bois-Reymond to measure the drop in potential difference between cut and intact tissue surfaces that accompanies the excitation of nerve and muscle (du Bois-Reymond, 1884). By developing the differential rheotome, Julius Bernstein introduced accurate temporal resolution and made the first true recordings of resting and action potentials; he also confirmed Helmoltz data of nerve conduction velocity being approximately 25-30 m/s. Bernstein further reported that the whole "negative fluctuation" (action potential) lasted 0.8 to 0.9 ms and that it could even lead to "sign reversal" (action potential overshoot), a finding he unfortunately failed to pursue (reviewed by Nilius, 2003). Interestingly, Bernstein explained intracellular negativity at rest (around –60mV) as resulting from the membrane being selectively permeable to K^+, in these conditions. According to his theory, a nerve impulse would originate from a sudden, non-selective, increase in membrane permeability to all ions; this "membrane breakdown" would be responsible for bringing the potential difference to zero (Bernstein, 1902). Charles Overton, on the other hand, hypothesized Bernstein´s "negative fluctuation" was dependent upon selective Na^+ and K^+ exchange between the extracellular and intracellular compartments (Overton, 1902). The question was definitely settled by Alan Hodkin and Andrew Huxley, who uncovered the ionic basis of the action potential in the giant squid

axon and proved the latter hypothesis right. Initially, Hodgkin and Huxley took advantage of intracellular electrode technology to measure the potential inside a squid nerve at rest (~ -45 mV) and at the peak of an action potential (~ +40 mV), showing that polarity could indeed reverse in its course (Hodgkin and Huxley, 1939). At the time, they had no idea of what caused the overshoot (Huxley, 2002). So, after the War had ended, they pursued the subject and found that the charge carried outward by K^+ efflux was enough to restore the resting potential after a spike, which was compatible with active extrusion of sodium or some other internal cation during the rising phase of the action potential (Hodgkin and Huxley, 1947). In order to study how an action potential might be generated by ionic currents under the influence of membrane potential changes, they employed the voltage-clamp technique, which had just been developed by Cole (1949). By performing simple voltage steps under different extracellular ionic compositions, Hodgkin and Huxley were able to divide the recorded current into components carried by Na^+ and K^+, which were fitted according to their time and voltage dependence, thus enabling a reconstruction of the action potential (Hodgkin and Huxley, 1952). Their pioneering work emphasized the importance that activation and deactivation of sodium and potassium channels had for action potential generation, prompting a quest to measure ion currents through single ion channels. Sakmann and Neher made the first recordings of single-channel currents, from denervated frog muscle fibers, by using glass pipettes of narrow tip (Neher and Sakmann, 1976). The high resistance that developed between the pipette tip and the patch of cell membrane to which it was pressed, in the order of 50 MΩ, was called a “seal” and consequently, “patch-clamp” was born. The technique would soon be revolutionized

by the discovery that application of gentle suction enabled the formation of a giga-seal, a tight and stable mechanical contact with a resistance of 10-100 GΩ (hence, giga-seal), which considerably reduced background noise and increased time resolution (Sigworth and Neher, 1980). Furthermore, this "improved" patch-clamp technique (Hamill et al., 1981) enabled the formation of excised membrane patches and also allowed access to the cytosol and the study of signal-transduction mechanisms, when performed in the whole-cell configuration (Figure 3.1.1). Even though much work in single-channel currents is done in the cell-attached mode, this configuration can not provide any information on the resting potential of the cell, nor are the extra- or intra –cellular solutions changed easily. Excised membrane patches, either inside-out or outside-out patches, may provide a suitable option, in which one can manipulate the intracellular or extracellular solutions, respectively. Still, whole-cell recording is by far the most widely configuration, for by establishing electrical continuity between the pipette solution and the cell interior, it enables small, mammalian cells, to be voltage or current-clamped. Using this configuration, macroscopic currents flowing through the "whole" membrane are recorded. In contrast, when working with excised patches or in the cell-attached configuration, only the current passing through the channels contained within the tip of the recording electrode can be recorded. It is interesting how the whole-cell recording configuration, initially thought of as a by-product of the patch-clamp technique, grew to become one of the most crucial and widespread techniques to approach the function and regulation of excitable cells. Even more curious is the fact that whole-cell recording does not even fit into the strict definition of

"patch-clamp", as in it, the clamp is applied to a whole cell and not a small patch of membrane.

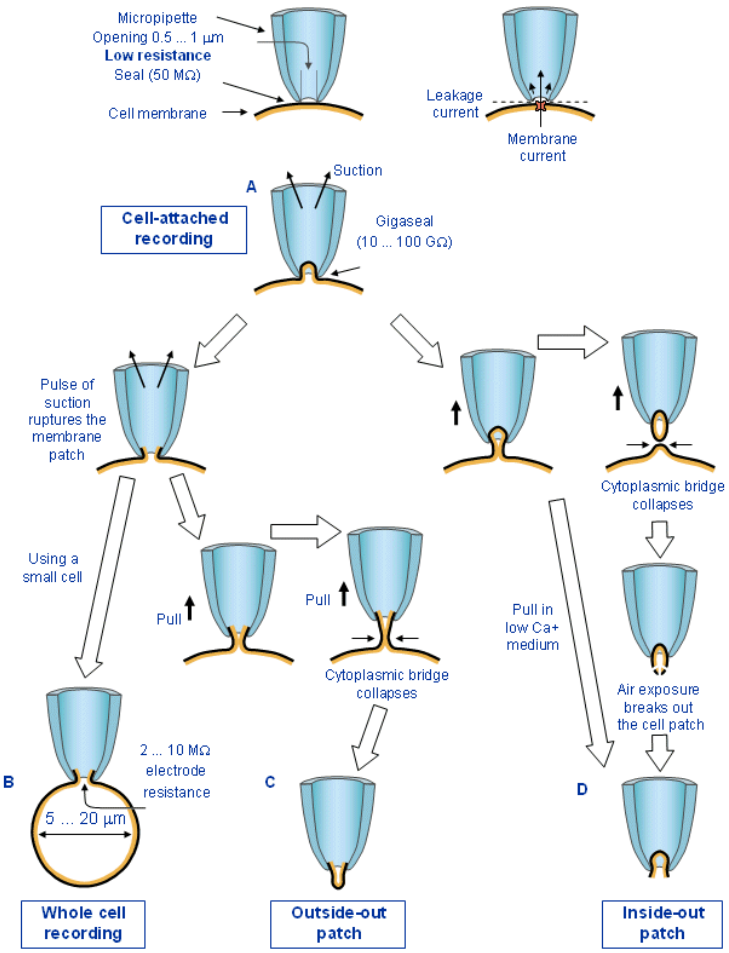


Figure 3.1.1. Patch-clamp recordings can be performed under four different configurations. When the pipette is sealed to the cell membrane, single channel currents can be recorded in cell-attached patch mode. The seal is so stable that the patch can even pulled off the cell and dipped in variety of tested solutions (inside-out or excised-patch mode). If the cell-attached patch is deliberately ruptured by suction, the whole-cell configuration is achieved; pulling the pipette away from the cell in the whole-cell configuration results in the formation of an outside-out patch. A major advantage of the whole-cell configuration is the possibility of introducing specific inhibitors or even fluorescent dyes, selectively into the recorded cell. Patch-clamp recordings typically use glass micropipettes with open tip diameters (~1 µm) much wider than those used to impale cells in traditional intracellular recordings ("sharp microelectrodes"). Image source: www.bem.fi (modified from Hamill et al., 1981).

3.1.1 Applications and technical pitfalls of the patch-clamp technique

The advent of patch-clamp was first crucial for providing insight into unitary conductance and kinetic behavior of ion channels already partly investigated by classical voltage-clamp experiments in the giant squid axon. For instance, it was possible to separate the processes of ion permeation through a single channel from those regulating its opening and closure ("channel gating"). Indeed, while the amplitude of the single channel current gives the experimenter a measure of ion permeation through a particular channel type, gating kinetics can be estimated from the intervals between transitions (conformational changes enabling the channel to switch between closed and open states). In turn, open channel probability is measured by the fraction of time the channel stays open, which will determine the amplitude of the current that would be measured in whole-cell configuration, given by: single channel current times the open probability times the number of channels (Ogden et al., 1994). Secondly, patch-clamp recordings in the whole-cell configuration allowed a shift in preferred cell models for electrophysiology (from large muscle fibers and giant axons to small round mammalian cells), by enabling the application of voltage-clamping to cells that were too small for two electrode voltage-clamp.

Simply put, the voltage-clamp method allows ion flow across a cell membrane to be measured as electric current, whilst the membrane voltage is held under experimental control with a feedback amplifier. The usefulness of this technique comes first from allowing the experimenter to distinguish between membrane ionic and capacitive currents; but also because it is much easier to interpret membrane

currents flowing through membrane areas kept under uniform, controlled voltage. The latter aspect is particularly relevant considering that the gating of most ionic channels is controlled by membrane voltage. Initially, voltage-clamp experiments used a two electrode negative feedback circuit, one to record voltage and the other to pass current needed to maintain the command membrane potential (Figure 3.1.2). Briefly, the voltage electrode is connected to a pre-amplifier that feeds the signal to the clamping amplifier, which also receives an input from the signal generator that determines the command potential. The clamping amplifier then subtracts the recorded membrane potential from the command potential and sends an output through the current electrode. Whenever the cell deviates from the holding voltage, the clamping amplifier generates a signal corresponding to the difference between the command potential and the actual voltage of the cell, producing a current that is equal and of opposite polarity to that flowing through the cell membrane. Continuous monitoring of current injection thus gives the experimenter an accurate reproduction of the currents flowing across the membrane. Two electrode clamps can be applied to cells that are large and robust enough to allow insertion of two electrodes without causing damage (Meech and Standen, 1975), such as snail and molluscan giant neurons.

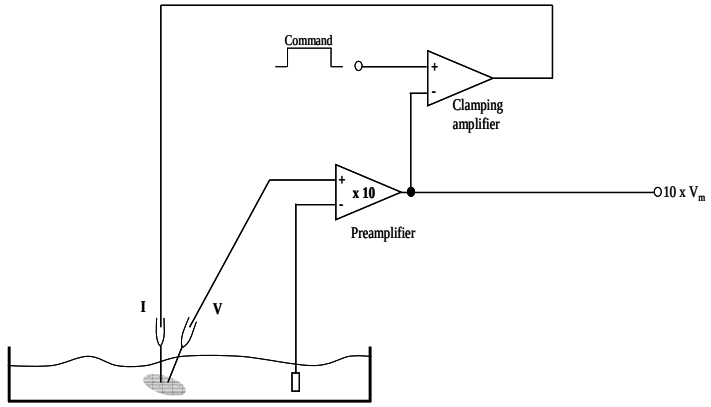


Figure 3.1.2. Two-electrode voltage-clamp schematic circuit. The cell (usually an oocyte or a molluscan giant neuron) is impaled with two microelectrodes, one to record voltage and the other to pass current. A preamplifier records membrane potential and the clamping (or feedback) amplifier passes current to control this potential.

While two-electrode voltage clamp amplifiers traditionally employ voltage followers, the patch clamp amplifier is a sensitive current-to-voltage converter, with a high gain set by the large feedback resistor, R_f (Figure 3.1.3) (Ogden et al., 1994). The high gain patch-clamp operational amplifier is connected on the circuit so that the current flowing through the membrane at a given moment is measured as a voltage drop across the feedback resistor. Indeed, a patch-clamp amplifier containing a feedback resistor of 50 GΩ allows minute currents (10^{-12} A) to be measured. For instance, a 1 pA current flowing through a single channel will, according to Ohm´s law, produce a voltage drop across R_f given by $V = 50 \cdot 10^9 \cdot 1 \cdot 10^{-12}$ V $= 50 \cdot 10^{-3}$ V$= 50$ mV.

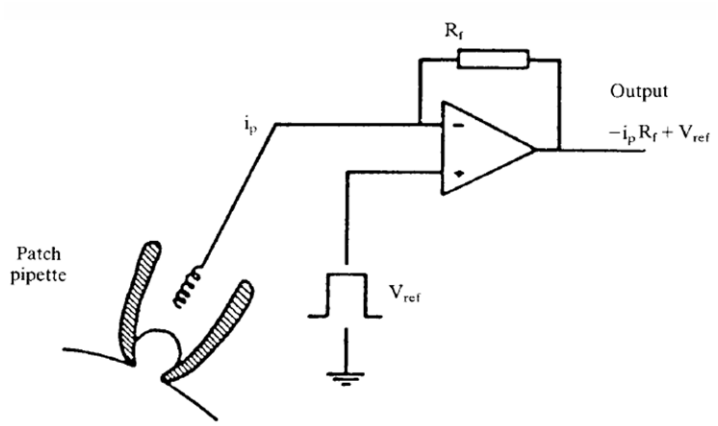


Figure 3.1.3. Schematic diagram of the headstage current/voltage amplifier. The gain (V_o/i_p, in mV/pA) is set by the feedback resistor (R_f), according to $V_o = -R_f \cdot i_p + V_{ref}$. The potential inside the pipette can be held at a steady state level, or it can be changed in a step-wise manner (V_{ref}). The current/voltage converter, as any other operational amplifier, is composed of an array of transistors and other components fabricated on a single chip of semi-conductor material. Its behavior follows 4 basic rules. First, the open-loop voltage gain (before any negative feedback is applied) is very high, typically 10^6. The input resistance of the amplifier can be considered infinite, so current flow into the inputs is negligible. In turn, the amplifier´s output resistance is considered to be null, meaning that there will be very little output voltage change when the output loads current changes. Finally, the output voltage can go positive or negative with respect to ground, but its amplitude is limited to +/- the voltage supply to the amplifier. Introduction of a feedback circuit ensures that the inverting input of the amplifier (-) is always at the command voltage (V_{ref}). As the inputs draw no current, any current entering the inverting input (from the cell) must be removed via the feedback resistor. Adapted from Ogden et al., 1994.

However, delivery of fast changing commands (such as the leading edges of rectangular pulses used to monitor formation of a giga-seal) will originate large currents, due to charging capacitance associated with the pipette walls and the cell membrane. Because these may saturate the amplifier for large voltage steps, compensation circuits are usually employed to offset both the “fast” (mainly due to charging the pipette) and “slow” (attributable to the cell) components of the capacitive transients. The usual procedure consists of cancelling the fast capacitive transients once a giga-seal is attained (in cell-attached

mode), as these tend to change little throughout a patch-clamp recording. Slow capacitive transients will only become an issue once the whole-cell configuration is established and access is gained to the behavior of the “total” neuronal membrane as a capacitor.

As illustrated in Figure 3.1.4, a common procedure is to use a 5 mV square test pulse to monitor the establishment of a giga-seal, which is always the starting point for any patch-clamp recording. Briefly, with the recording electrode in the bath and filled with intracellular solution, one measures the current passed by the electrode in response to the 5 mV pulse (Figure 3.1.4A). Acording to Ohm´s law, the size of this test pulse current gives the experimenter a measure of the electrode´s tip resistance. Subsequently, changes in the size of the test pulse current will account for changes in the electrode´s resistance. As the electrode tip touches the cell surface, there is a slight decrease in the size of the test pulse current that mirrors the increase in resistance to current flow (Figure 3.1.4B). This can be used as a signal to release the positive pressure (continuously injected through the pipette tip to keep it free of matrix and cell debris) and apply gentle suction. In alternative, one can use differential infra-red interference (DIC) optics to guide the electrode´s approach and contact to the target cell. In this case, the appearance of a dark dimple in the site of contact with the membrane constitutes the signal to release the positive pressure and apply gentle suction. Usually, together with gentle suction, delivery of a negative command potential (as the experimenter switches the recording mode to voltage-clamp) aids in the formation of a seal. As the experimenter succeeds in pulling a small patch of membrane into the pipette tip and as the size of the test pulse current decreases until virtually no current

flows between electrode tip and cell surface, a giga-seal is formed (R > 1 GΩ) (Figure 3.1.4C). After nulling the fast capacitive transients (Figure 3.1.4D) break-in is achieved by applying strong, consecutive pulses of suction. As the whole-cell configuration is attained, the resistance to current flow falls and the slow capacitive transients (caused by charging of the cell´s membrane) become evident (Figure 3.1.4E).

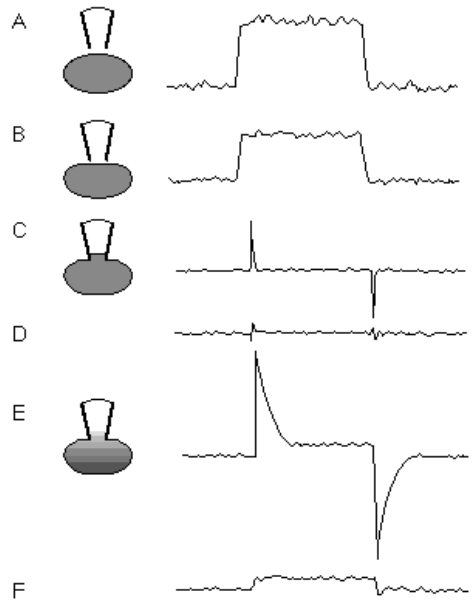


Figure 3.1.4. Test pulses produce different current responses as one proceeds through the establishment of a whole-cell voltage clamp recording. The physical relationship between the patch electrode and the cell is illustrated schematically on the left. The size of the current change produced by the test pulse goes down as the resistance across the patch electrode tip goes up. Thus, a reduction in test-pulse current indicates closer contact between the electrode tip and the cell. (A) The electrode is just above the cell, not in direct contact, so the resistance is low (1 to 10 MΩ) and the test pulse current is large. (B) The electrode touches the cell surface, the resistance goes up slightly, and the test pulse current gets smaller. (C) A giga-seal has formed as the result of gentle suction, which pulls a small patch of membrane up into the electrode tip. The resistance is high (>1 GΩ), so except for the transients, the test pulse current is virtually flat. (D) The electrode capacitance transient is nulled. (E) Break-in is achieved by strong suction that removes the patch of membrane in the electrode tip, but leaves the seal and cell intact. The resistance goes down and large capacitance transients are seen. Perfusion of the cell interior begins. (F) The whole-cell capacitance transient is nulled. Since steps D and F are

purely electrical adjustments, the diagram of the cell and patch pipet is the same as in C and E, respectively. Adapted from: www.currentprotocols.com

Once in whole-cell recording configuration, another important source of error concerns the access resistance between the cell interior and the amplifier, which emerges from the restriction in current flow that occurs across the tip of the recording electrode (see Figure 3.1.5). Thus, the simple action of measuring membrane voltage through a recording electrode introduces an artifact, according to which current passing from the cell to the amplifier will cause a voltage drop across the electrode resistance. This has two immediate consequences for voltage-clamp efficiency: first, it prevents any voltage step applied through the recording electrode from reaching the full command amplitude and secondly, it leads to an underestimation of the transmembrane currents that are "seen" by the amplifier. Also, once the whole-cell configuration is established, the series resistance (also called access resistance) becomes in series with the cell membrane, which behaves as a capacitor, thus combining with it to form a low-pass RC filter. Again, this will introduce artifacts both in signals coming from, as in those going to, the voltage-clamped cell. In fact, in response to a square voltage step delivered through the recording electrode, there will be a slow exponential charging of the cell membrane potential characterized by the time constant derived from $\tau=R_sC_m$ (Figure 3.1.5). For a series resistance of 10 MΩ and a cell capacity of 12 pF, settling of the clamp follows τ=120 μs. Conversely, from the recording electrode perspective, current injection at the beginning of the square step will describe slow capacitive transients, also characterized by $\tau=R_sC_m$ (Figure 3.1.5). In fact, because a capacitor requires current flow to

change its voltage, it is first necessary to charge the parasitic membrane capacitance, in order to measure a change in membrane potential at the amplifier. Although these transients only change what is recorded and not the cell behavior itself (contrary to the voltage change applied to the membrane), they do introduce a low pass filtering effect that may distort events with durations close to τ, such as the rising phase of an action potential or even the rise time of synaptic currents.

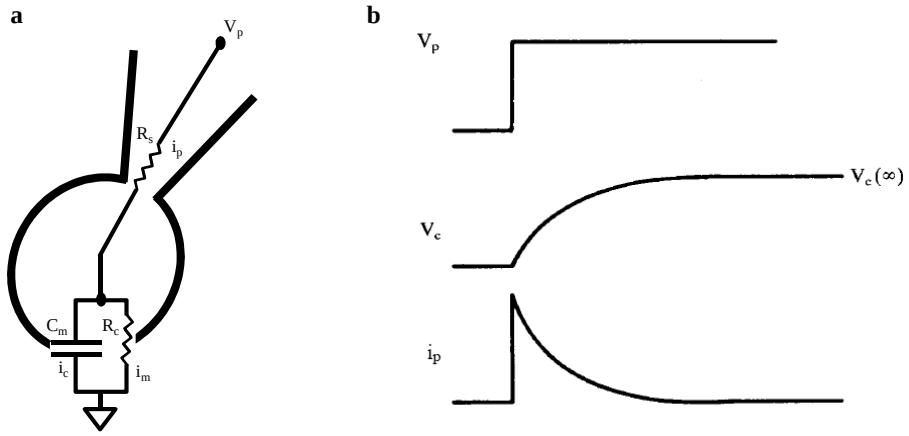


Figure 3.1.5. Equivalent circuit of whole-cell recording. (a) Equivalent circuit of whole cell recording. Current i_m flows in the cell resistance R_c and i_c in the capacitance. Pipette current, $i_p=i_m+i_c$, flows in the series resistance R_s between pipette and cell and produces a voltage error $V_p-V_c=i_pR_s$. (b) Time course of changes of V_c and i_p following a step of V_p. Modified from Ogden et al., 1994.

To compensate for these errors, which assume particular relevance when studying the activation of voltage-gated channels or the rise time of fast synaptic currents, feedback circuitry can be used to nullify the "slow" capacity transients, by injecting a symmetrical, matching, amount of current into the recording pipette. However, as noted above, capacity compensation will only subtract capacitive currents from the

amplifier output; it will not compensate for the slow change in cell membrane potential that arises from the combination of C_m with R_s. The only solution to obtain a better voltage clamp is to use separate resistance compensation based on positive feedback circuitry, which adds a signal proportional to the current extracted from the output signal, to the command potential. This positive feedback will increase the pipette voltage when current flows and the voltage error thus tends to be greatest, functioning as though the recording electrode had a smaller resistance. The caveat of this procedure is that R_s frequently fluctuates during long recordings (either as the membrane tip gets clogged or as the ruptured membrane patch reseals), compromising accurate compensation. Also, if the frequency with which R_s compensation is added to the command potential is too high, the underlying circuit reverberates, which can lead to electroporation of the neuronal membrane. For these reasons, an alternative strategy to diminish the errors introduced by series resistance is simply to use as low as possible resistance pipettes and to choose small cells, which will contribute with a smaller C_m.

Finally, patch-clamp recordings in the whole-cell configuration have the disadvantage of prompting cell perfusion with the pipette solution, since its volume exceeds by far that of the cytosolic compartment. This is associated with a rundown of responses relying upon second messenger activation, such as intracellular calcium release. Rundown of responses can be avoided by performing patch-clamp recordings using antibiotic-containing pipette solution (Horn and Marty, 1988), in what is known as the perforated patch technique. When the pore-forming antibiotic proteins assemble in the membrane patch contained

within the pipette tip, access to the cell interior is attained without cytosol dialysis – but at the expense of even higher series resistance and lower recording stability than that typically obtained in whole-cell configuration.

If one is aware of the technique´s pitfalls and tries to minimize the errors introduced (e.g. by keeping Rs constant throughout the experiment), then whole-cell patch-clamp recordings constitute an excellent tool to address a number of processes underlying neuronal communication. The signals amenable to recording can be divided into those corresponding to passive cell properties (input resistance, membrane time constant) and those ascribed to active cell properties. The latter include a range of signals (from action potentials to macroscopic currents mediated by voltage-dependent ion channels) that can further be categorized into synaptic and non-synaptic responses. Non-synaptic responses can be evoked by local agonist application and are most frequently composed of mixed post - and non –synaptic components (e.g AMPA-evoked currents). Recording of synaptic responses can be used to study spontaneous events (miniature excitatory or inhibitory postsynaptic currents, recorded after blockade of activity-dependent release) or afferent-evoked postsynaptic currents, upon electrical stimulation of presynaptic fibers projecting to the recorded neuron. In the latter case, brain slice preparations are most frequently used, since these preserve, to some extent, physiological circuit anatomy.

3.2 Acute brain slice preparations

By definition, electrophysiology studies the electrical properties of biological cells and tissues, through measurements of voltage changes or electrical current flows, on a wide variety of scales - from single ion channel proteins to complex field potentials measured in whole tissues like the heart. In practical terms, this requires the placing of a recording electrode on a preparation of biological material, which can range from living organisms (*in vivo* recordings) down to dissociated cells from excised tissue. Early patch-clamp recordings elected large muscle fibres (Neher and Sakmann, 1976), giant axons or isolated mammalian cells (Maruyama and Petersen, 1982) as preferred preparations, which proved quite useful to the evaluation of single-channel currents. But the essence of crucial subjects, such as the connectivity between different neuronal populations, their intrinsic properties and morphologies, or the occurrence of various forms of synaptic plasticity, would only become attainable with the development and establishment of the brain slice preparation. In the early 1950´s, Professor Henry McIlwain was actively engaged in quantitative studies of the energy metabolism in the brain. Having identified the need for brain tissue preparations, he gradually devised several apparatuses for obtaining brain slices, which within a few years led to the creation of mechanical choppers allowing mass production of slices. What is more, he applied electrophysiological techniques to confirm that his slices were metabolically active and contained neurons with healthy resting membrane potentials (Li and McIlwain, 1957; Yamamoto and McIlwain, 1966). These early reports elegantly demonstrated the groundbreaking notion that brain tissue could actually be kept alive and healthy outside the body. This idea, which had a strong impact on the

scientific community, resulted in an explosion of research performed on central nervous system physiology and pharmacology. Nowadays, the brain slice preparation remains a powerful tool for electrophysiologists, enabling them to accurately determine the environment of the slice; have visual control of where recording and stimulating electrodes are placed; study the effects of drugs applied in know concentrations – either to the entire slice or just some portions of it - and also the possibility of preserving the tissue for later biochemical or anatomical analysis. Together with the development of cutting solutions with compositions that minimized excitotoxic damage (e.g. with decreased calcium and increased magnesium concentation) during slice preparation, introduction of vibrating microtomes for tissue processing greatly increased the success of patch-clamp recordings from acutely prepared brain slices (see Aitken et al., 1995). The brain slice preparation has been particularly exploited in hippocampal research.

3.2.1 The hippocampal slice model

The hippocampus lies within the medial temporal human lobe and its distinctive, curved shape has fascinated early anatomists, who have named it after the seahorse monster of Phoenician and Greek mythology, as well as after the ram's horns of the Egyptian god Ammon. Its general layout holds across the full range of mammalian species, although its general morphology does vary. For instance, in the primate brain, but not in that of rodents, the anterior end of the hippocampus is expanded to form the pes hippocampus.

The hippocampal formation has traditionally been included in the limbic system, a loose classification term that included a group of structures lying in the border zone between the cerebral cortex and the hypothalamus – forming a unified “limbic lobe” (Broca, 1878).

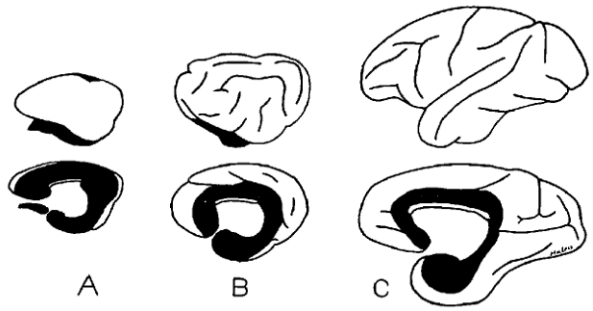


Figure 3.2.1.1. The great limbic lobe of Broca. In the above drawings where the lateral and medial surfaces of the brains of rabbit (A), cat (B), and monkey (C) are drawn roughly to scale, the limbic lobe is represented in black. The figure illustrates that the limbic lobe, as Broca pointed out, forms a common denominator in the brains of all mammals. Note how the lobe surrounds the brain stem, a situation that suggested Broca's use of the term "limbic." Modified from McLean, 1954.

It is now known that the limbic system actually involves many other structures beyond this border zone, which cooperate for the control of emotion, behavior and drive, as well as towards a reference role in memory (Snell, 2009). As such, the limbic system is now considered to comprise the subcallosal, cingulated and parahippocampaly gyri, the hippocampal formation, amygdaloid nucleus, mamillary bodies and the anterior thalamic nucleus. In particular, the hippocampal formation is composed of the hippocampus, the dentate gyrus and the parahippocampal gyrus.

Macroscopically, the hippocampal formation is formed of two C-shaped interlocking cell layers: the granular cell layer of the dentate gyrus and the pyramidal cell layer of the Ammon´s horn (hippocampus

proper) and the subiculum (which, together with the entorrhinal cortex, is a component of the parahippocampal gyrus) (Lopes da Silva et al., 1990).

The hippocampus proper is composed of multiple subfields. Though terminology varies among authors, the terms most frequently used are dentate gyrus and the cornu ammonis (or Ammon´s horn). The cornu ammonis (CA) is usually differentiated into the CA1, CA2, and CA3 areas and it can be structured depthwise, according to seven clearly defined strata (or layers). The alveus is the most superficial layer of the hippocampus and it contains the axons from CA1 pyramidal neurons, which will subsequently constitute the fimbria and the crus of the fornix, forming one of the major outputs of the hippocampus, directed to the mammilary bodies. Below the alveus, the stratum oriens contains the cell bodies of inhibitory basket cells and horizontal trilaminar cells. The stratum oriens also contains the basal dendrites of pyramidal neurons, onto which synapse efferent fibers from other pyramidal cells, septal fibers and commissural fibers from the contralateral hippocampus. Deep to the stratum oriens, there is the stratum pyramidale, where the cell bodies of the pyramidal neurons can be found. This stratum also contains the soma of many interneurons, including axo-axonic (or chandelier) cells, bistratified cells, and radial trilaminar cells. Still, the predominant structure in this layer corresponds to the cell bodies of pyramidal neurons, which are exclusively found here. Below the stratum pyramidale and in the CA3 area only, one can find the thin stratum lucidum (or mossy fiber layer), through which mossy fibers from the dentate gyrus granule cells course towards CA3 pyramidal neurons. The stratum radiatum contains septal

and commissural fibers as well as the Schaffer collateral fibers that project from CA3 onto CA1 pyramidal neurons. A considerable proportion of dendritic inputs to pyramidal neurons therefore locates to this stratum (see Figure 3.2.1.2). Some interneurons that can be found in more superficial layers can also be found in the stratum radiatum, including basket cells, bistratified cells, and radial trilaminar cells. The underlying stratum lacunosum still contains Schaffer collateral fibers, as well as perforant pathway fibers, which project from the superficial layers of the entorhinal cortex onto all the fields of the hippocampal formation (Witter et al., 2000). Because of its small size, this thin stratum can be grouped with the subjacent stratum moleculare, into a single stratum called stratum lacunosum-moleculare (Lopes da Silva et al., 1990). The stratum moleculare is the deepest stratum of the hippocampus and it contains the synaptic connections between perforant pathway fibers and the distal, apical dendrites of pyramidal cells. Below the stratum moleculare, the hippocampal sulcus (or fissure) separates the CA1 field from the dentate gyrus.

In the dentate gyrus, similar strata can also be considered. These are the polymorphic layer (or CA4 area or hilar region), the stratum granulosum and the stratum moleculare. The polymorphic layer is the most superficial layer of the dentate gyrus and it contains many interneurons, as well as efferent fibers from the dentate gyrus granule cells onto the CA3 pyramidal cell layer. The stratum granulosum contains the cell bodies of the granule cells. In the inner third of the stratum moleculare, commissural fibers from the contralateral dentate gyrus, as well as inputs from the medial septum, form synapses with the proximal dendrites of granule cells. The external two thirds of the

stratum moleculare constitute the deepest strata and they contain the perforant pathway fibers that will synapse onto the distal apical dendrites of granule cells.

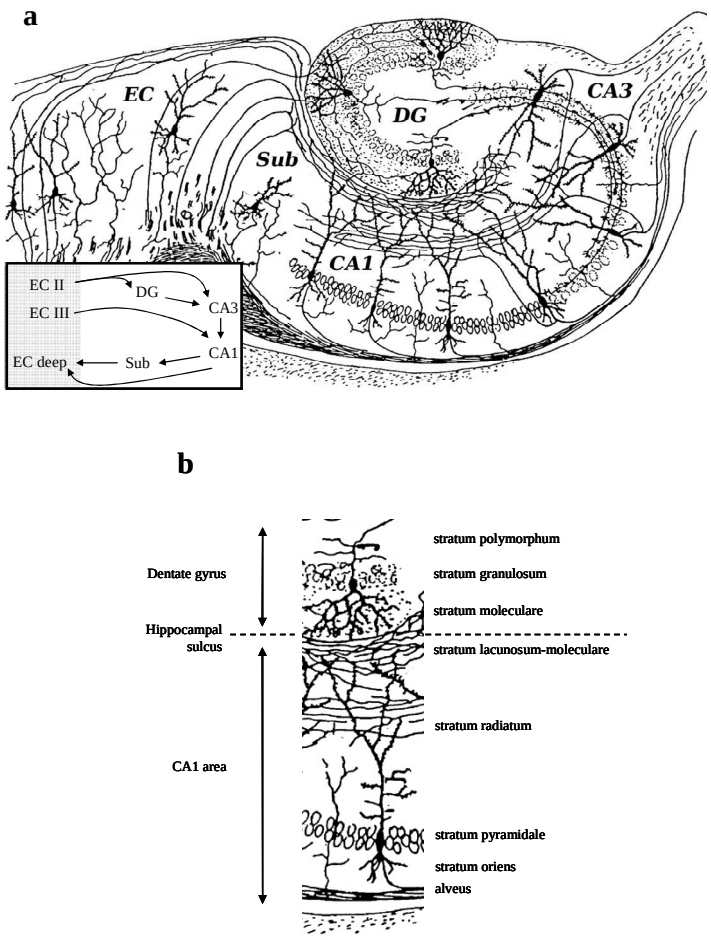


Figure 3.2.1.2. Diagram of a transverse hippocampal slice. (a) The drawing contemplates the discrete strata that comprise the hippocampal formation, as well as the cell types between which the main synaptic connections are established. These are also summarized in the lower inset. DG: dentate gyrus. Sub: subiculum. EC: entorhinal cortex. (b) Hippocampal slice section depicting the different strata contained in the CA1 and dentate gyrus areas. The drawing in (a) is modified from Rámon y Cajal, 1909.

As evidenced by the early drawings of Rámon y Cajal, the major pahways of signal flow through the hippocampal formation constitute a loop, in which most external input originates from (and ultimately comes back to) the entorhinal cortex. In fact, the fibers of the perforant pathway, which arise from the layers II and III of the entorhinal cortex, profusely project onto the dentate gyrus and CA3 area, or to the CA1 area, respectively (reviewed by Witter and Amaral, 2004). From the dentate gyrus granule cells, efferent fibers send information onto CA3 pyramidal cells, which in turn send their axons to CA1 pyramidal neurons through a set of fibers known as the Schaffer collaterals. From the CA1 area, axons project mainly onto to the entorrhinal cortex, but can also project to subicular neurons which in turn, send their afferents onto the entorrhinal cortex. Per Andersen called this major pathway of information (dentate gyrus-CA3-CA1) the trysynaptic circuit (Andersen et al., 1969; 1971) (see Figure 3.2.1.2, lower inset). He also noted that all these connections were preserved when the hippocampus was cut transversally to its long axis (Andersen et al., 1969). Not only was this concept the basis of Andersen´s lamellar hypothesis (according to which the hippocampus was composed of a series of parallel strips operating independently), as it additionally found expression in the introduction of the hippocampal slice preparation by Skrede and Westgaard (1971). Aside from preserving the main physiological circuits intact, 400–500 μm thick transverse slices were subsequently shown to remain alive for several hours, when kept in an oxygenated bath of artificial cerebrospinal fluid. This slice preparation thus enabled access to different hippocampal areas by experimental drugs that could easily be washed out of the slice, together with easy placement of recording and stimulating electrodes. Also, by belonging to the three-

layered arquicortex, the hippocampus provided a relatively simplified version of the remaining cerebral cortex, therefore constituting an appealing option for early plasticity studies – a trend that persists to these days (reviewed by Cooke and Bliss, 2006).

Finally, other than the essential trisynaptic glutamatergic circuit initially described by Andersen and colleagues (1969; 1971), the hippocampus proper comprises also several local inhibitory circuits, mediated by different kinds of GABAergic interneurons – which, despite representing less than 10% of the neuronal hippocampal population, exert a prominent regulation of overall excitability (see Freund and Buzsáki, 1996). It should also be noted that several subcortical structures impinge their outputs onto the hippocampus, as is the case of the amygdala, the thalamus, the hypothalamus, the ventral tegmental area, the medial septal nucleus or the reticular formation (Nieuwenhuys et al., 2008). As a consequence, a wide variety of neurotransmitters subserve neuronal communication in the hippocampus (Lopes da Silva et al., 1990).

In order to better address the complexity underlying hippocampal function, one can use a multidisciplinary approach by combining functional evaluation of hippocampal transmission (e.g. by performing electrophysiological recordings of afferent-evoked responses) to analytical techniques applied to hippocampal samples.

3.3 Western Blot analysis

The basic principle behind electrophoresis and western blot analysis dates back to Faraday's law of electrolysis, according to which the mass

of a substance altered at an electrode during electrolysis is directly proportional to the quantity of electricity transferred at that electrode (Faraday, 1834). Arne Tiselius used it to develop the Tiselius apparatus, which first allowed electrophoretic separation of colloidal solutions (Tiselius, 1937). This device was composed of a U-shaped cell filled with buffer solution and electrodes immersed at its ends. On applying voltage, the compounds of a given mixture of charged components, for instance sample proteins, migrated to the anode or cathode depending on their charges. However, this initial apparatus was not able to accurately differentiate electrophoretically similar compounds, for their mobility through solutions. The limitation was addressed by new electrophoresis methods, which used solid or gel matrices to separate compounds into discrete and stable bands (zones), in what was defined as zone electrophoresis. Indeed, the introduction of starch (and later polyacrylamide) gels enabled the efficient separation of proteins, making it possible with relatively simple technology to analyze complex protein mixtures (reviewed by Vesterberg, 1989). A further breakthrough occurred when Towbin and colleagues (1979) used electrophoresis to transfer sample proteins from a polyacrylamide gel to a sheet of nitrocellulose - much like had previously been done with DNA samples, by virtue of the Southern Blot technique. In the nitrocellulose membrane, immobilized proteins kept the original gel pattern and became easily detectable by immunological procedures (Towbin et al., 1979). The foundations were laid for the Western Blot technique (Burnette, 1981) as we now know it. The technique remains to this day widely used to study the presence, abundance, relative molecular mass and post-translational modifications of specific

proteins; as well as a valuable immunodiagnostic research tool in medicine.

Application of a Western Blot protocol encompasses several steps, divided into three fundamental work stages: separation of a protein mixture by means of gel electrophoresis; protein transfer onto a membrane (actual blot); and detection of target proteins. Preceding these steps is tissue preparation, which involves mechanical processing of solid tissues (using a homogenizer or sonication methods) and treatment with different detergents to enhance cell lysis and protein solubilization. In order to minimize protein digestion, protease and phosphatase inhibitors are usually added to the medium and mechanical processing is performed at low temperatures, typically 0-4ºC. After discarding the undigested material, tissue homogenates are frozen and stored. Prior to analysis by Western Blot, protein denaturation is achieved by mixing the sample protein mixture with SDS-containing Laemmli buffer (Laemmli, 1970) and exposing it to 95ºC for 5 min. Each component of the Laemmli buffer serves a specific purpose in protein sample preparation. For instance, beta 2-mercaptoethanol reduces intra and inter-molecular disulfide bonds in the proteins, facilitating their segregation according to overall size but not shape; whilst sodium dodecyl sulfate (SDS detergent) imposes to each protein the same overall negative charge and affects hydrophobic bonds within the protein, compromising protein structure (Figure 3.3.1). Again, this allows proteins to be separated according to size (molecular weight) and not by charge. Other components include glycerol, which increases the density of the sample, easing its application to the gel; and bromophenol blue, a dye which serves as a migration indicator.

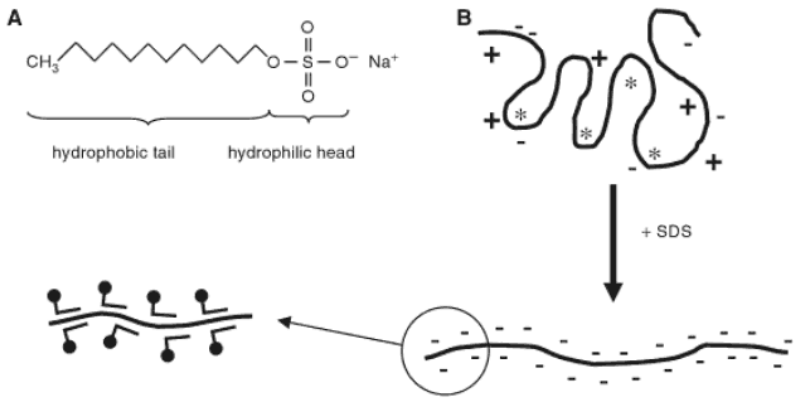


Figure 3.3.1. Effect of the anionic detergent SDS on proteins. (A) Structural formula of SDS (sodium dodecyl sulfate). The molecule can be divided into a hydrophobic and a hydrophilic area. (B) The form-giving hydrophobic areas (*) within the protein are dissolved, so that it is only present in stretched linear form. Through this, the strongly charged SDS overlaps the self-charge of the proteins. Adapted from Luttman et al., 2006.

3.3.1 Gel Electrophoresis

According to the sample and the nature of the gel used, protein separation can be made by isoelectric point, protein shape, or molecular weight. Most commonly, separation of protein mixtures is attained using SDS polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular mass (measured in Kdaltons). As referred, SDS confers homogeneous negative charge to sample proteins and destabilizes bonds relying upon hydrophobic interactions. As higher protein structures are lost, proteins assume a linearized form. Combined with previous reduction of disulphide bridges by β-mercaptoethanol in the sample Laemmli buffer, SDS enables complete denaturation of sample proteins, as well as allows migration of negatively charged proteins onto the positively charged electrode through the polyacrylamide mesh of the gel. Indeed, the gel is formed by a polymer of acrylamide monomers, organized into a 3D

network, by means of a cross-linking agent 1,2-Bis(dimethylamino)ethane (TEMED). Because of the tight meshes formed, smaller protein molecules move faster upon charge application compared to larger ones, resulting in clearly separated protein bands according to overall size. Depending on the molecular mass of the protein mixture to be separated, the acrylamide content of the gel is adjusted so as to enable optimal band resolution. As such, the lower the acrylamide concentration, the better the resolution of lower molecular weight proteins. In the discontinuous version of the SDS-PAGE, proteins first migrate through a stacking gel, which concentrates them in a sharp band before they can access the actual separation gel (Figure 3.3.1.1). It should be noted that the acrylamide concentration is not the only determinant of protein detection sensitivity, in SDS-PAGE. In fact, the thickness of the gel and the amount of protein that is loaded also importantly impact how sensitive the method is. Indeed, when gel thickness exceeds 2 mm, protein transfer onto membranes becomes less effective.

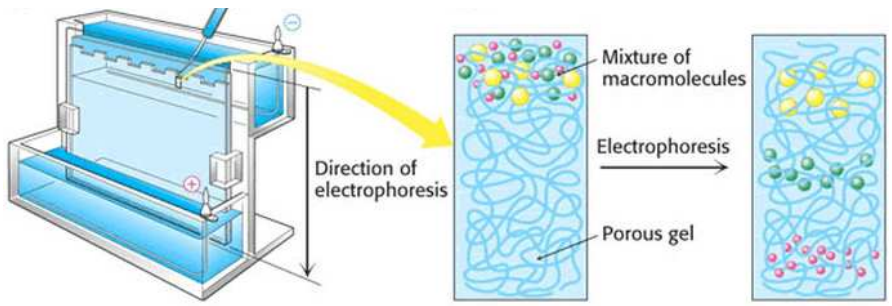


Figure 3.3.1.1. Discontinuous SDS-PAGE electrophoresis procedure. Samples are first loaded into wells in the gel, with one lane being usually reserved for a marker or ladder (commercially available mixture of proteins of defined molecular weights, stained to form visible, coloured bands). When voltage is applied, proteins migrate into it at different speeds, according to molecular weight, causing smaller proteins to progress further along the gel. Image source: imb-jena.de.

3.3.2 Protein Transfer to a membrane (blot)

Upon protein separation into discrete bands, different methods are available for transferring proteins onto membranes, such as simple diffusion (Kurien and Scofield, 1997), electrophoresis (Towbin, 1979) or vacuum-assisted solvent flow (Peferoen et al., 1982). Of these, electrophoresis is most commonly performed; either in a suitable tank (wet blot) or according to the semi-dry method (Luttman et al., 2006). Wet blots have the advantage of applying less heat to the preparation and favour protein integrity, but they require larger quantities of transfer buffer. The transfer buffer itself provides electrical continuity between the electrodes and must therefore be conductive; typical transfer buffers contain Tris base, glycine, methanol and SDS. It also provides a chemical environment that preserves protein solubility without impairing the protein adsorption to the membrane, during transfer. Indeed, by relaxing protein binding to SDS, methanol further improves protein transfer, being also important for stabilizing gel dimension. However, most buffers undergo Joule heating during transfer, which may prompt the need to use cooled transfer buffer or for surrounding the transfer tank with ice. The choice of an appropriate blotting membrane also greatly affects protein transfer. Nitrocellulose and polyvinylidene fluoride (PVDF) membranes are the types most frequently used and pore size is adjusted to the molecular weight of the protein to be blotted, since transfer efficacy for small proteins decreases with increasing pore size. In a wet blot, membranes are first soaked in transfer buffer and then are placed above the gel and jammed between layers of porous foam sponge sheets and filter papers, in a gel cassette (Figure 3.3.2.1.).The whole structure is placed inside a transfer tank,

completely submerged in cold transfer buffer and is then connected to a power source, allowing protein blotting towards the cathode, to which the membrane is oriented. Current application and transfer time are balanced to enable optimal transfer; long transfer times are usually better suited for tank systems, although they require cooling of the unit and/or internal recirculation of the transfer buffer. This is preferable to using large currents, which may cause proteins to migrate rapidly through the membrane but without being adsorbed.

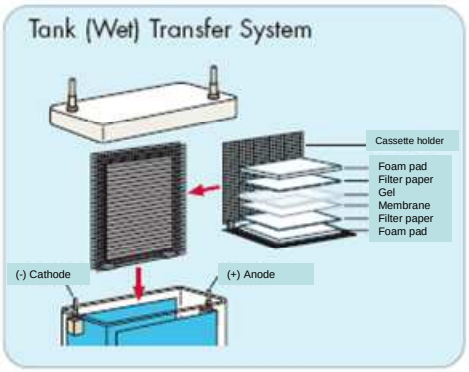


Figure 3.3.2.1. Protein Blotting procedure. In a typical wet blot, a sandwich structure is placed in a tank filled with transfer buffer. Proteins migrate from the negative (gel) to the positive (membrane) pole. Picture source: komabiotech.co.kr.

By the end of the blotting process, Ponceau S dye can be used to provide visual proof of a successful transfer, after which it can easily be washed off the membrane. Blotted proteins are then amenable to detection.

3.3.3 Protein Detection

During the detection process, the membrane is "probed" for a protein of interest, in a two-step process that involves the binding of a primary antibody raised against that given protein, whose signal is amplified by binding of secondary antibody molecules linked to a reporter enzyme, which when exposed to an appropriate substrate prompts a visible reaction. But before blotted proteins can actually be detected, it is first necessary to minimize nonspecific binding of exogenous protein onto the membrane. This is attained by incubating the membrane with a given protein (e.g., 5% non fat milk powder in TBS solution, for 1 h) that will cover nonspecific binding sites, thus diminishing the background signal upon protein detection and increasing its sensitivity. After blocking, the membrane is incubated in a dilute solution of primary antibody (few μg/mL), under gentle agitation. The primary antibody is most frequently diluted in powdered milk or bovine serum albumin (BSA), whichever is previously used to block unspecific binding. Incubation time and temperature influence antibody binding efficiency. Indeed, incubation at room temperature enables stronger binding, not only specific (target protein) but also non-specific binding (background noise) and this may be counterbalanced by shorter incubation times. The membrane is then rinsed of unbound primary antibody, after which it is then exposed to a secondary antibody, directed at a species-specific portion of the primary antibody. Because several secondary antibody molecules bind each primary antibody available, the signal is amplified and protein sensitivity detection increased. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish

peroxidase. In the latter case, protein-bound antibodies become detectable by cleavage of a chemiluminescent agent, with formation of a luminescent reaction product. The luminescent signal, proportional to the amount of target protein blotted, can then be used to impress photographic film placed upon the membrane. It is also possible to use radioactive labeled secondary antibodies, which has the advantage of increasing detection sensitivity. Data analysis includes protein size estimation, which is attained by comparing stained bands to those corresponding to the molecular weight marker loaded onto the gel. The amount of target protein detected is scaled to the expression of a structural protein (such as actin or tubulin), in control and test lanes. This correction controls for possible differences in the amount of protein loaded into each well or inconsistent blotting efficiency. Protein levels are then evaluated through densitometric analysis of protein bands, using imaging software.

3.4 Protein Biotinylation

Combination of SDS-PAGE with protein tagging can be used as a way to reduce sample complexity and enhance the ability to discriminate changes at the level of a subpopulation of proteins within a complex cell lysate. One such strategy relies upon protein biotinylation, which may be used to selectively tag surface proteins in a given biological preparation. In fact, cell surface biotinylation has emerged as an important tool for studying the expression and regulation of membrane receptors (Huh and Wenthold, 1999) and transporters specifically expressed at the plasma membrane, differentiating them from the protein population localized to organelle membranes.

Proteins can be biotinylated either chemically or enzimatically. Enzymatic biotinylation targets specific lysine residues within a certain protein sequence. Indeed, biotin functions as a co-enzyme in several carboxylase-mediated reactions, such as those underlying fatty acid synthesis, branched-chain amino acid catabolism and gluconeogenesis. In these reactions, biotin covalently binds to specific lysine residues in carboxylases, in a process (biotinylation) that requires ATP and is catalyzed by holocarboxylase synthetase (Zempleni et al., 2009). As a consequence of its small size (244 Da), biotin can bind proteins without significantly affecting their biological activity.

By contrast, in chemical biotinylation, different reactive groups can be incorporated into the valeric acid side chain of commercially available biotin molecules, which enables unspecific biotinylation of amine residues, carbohydrates or sulfhydryl groups, according to the reactive group in question. A most frequently used form, sulfo-N-hydroxysuccinimide esters of biotin (sulfo-NHS-biotin), can establish stable amide bonds with primary amine groups (present on lysine side chains), therefore functioning as a non-specific protein labeling agent (Figure 3.4.1). As such, this method introduces a tag into any polypeptide bearing an exposed lysine residue. Furthermore, since sulfo-NHS-biotin molecules readily dissolve in polar solutions and are charged by the sulfonate group on the succinimidyl ring, they cannot permeate the cell membrane. This makes them into a suitable tool for selective “tagging” of surface proteins.

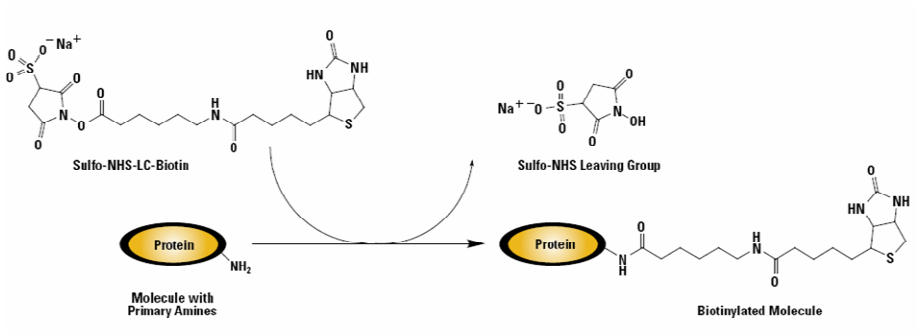


Figure 3.4.1. Reaction of Sulfo-NHS-LC-biotin with a primary amine. Amine reactive Sulfo-NHS ester reacts rapidly with any primary amine-containing molecule to attach the biotin label via a stable amide bond. In turn, the sulfonate group attached to the biotinylation reagent increases its water solubility, which renders the compound cell-impermeable and also allows biotin labeling to be done under more physiologic conditions. The Sulfo-NHS reactive group is attached to the valeric acid side chain of biotin by a linker of variable length. The purpose of this linker or "spacer arm" is to make biotin more accessible for streptavidin binding. Image source: www.piercenet.com.

Following cell surface biotinylation, tagged proteins need to be isolated from the original sample, which is accomplished by exploring the highly-stable interaction of biotin with two related proteins, avidin and streptavidin. Indeed, biotin can bind very tightly to the tetrameric protein avidin, with an equilibrium dissociation constant (Kd) in the order of 10^{-15}, close to the strength of a covalent bond (Green, 1963). Most frequently, however, biotin-based techniques rely on conjugation with streptavidin, a bacterial protein which displays less non-specific binding than avidin, while also binding biotin with comparable affinity (Chaiet and Wolf, 1964). Each avidin/streptavidin molecule has four binding sites for biotin.

Surface biotinylation studies have mostly been performed on either cell lines or dissociated neuronal primary cultures, ever since their initial description in the epithelial polarity field (Sargiacomo et al., 1989).

Application of the technique was recently extended to more physiological preparations, namely, to acute hippocampal slices (Thomas-Crussels et al., 2003). Since then, several studies have used it to measure alterations of the surface protein pool, as a way to investigate net effects of receptor trafficking mechanisms (e.g., Oh et al., 2006; Rial Verde et al., 2006). Slices can be prepared as for electrophysiology experiments and after recovery, are placed in adequate incubation chambers, in the presence of excess biotinylation reagent. In these conditions, an incubation period of 45 min can ensure penetration of biotin throughout 350 μm slices (Thomas-Crussels et al., 2003). In order to block all reactive biotin in excess, slices are then exposed to buffers containing amines (lysine or glycine), which will compete with the biotinylation reaction. In between treatments, slices are washed with phosphate buffered saline (PBS) or another buffer solution. Mechanical tissue disruption is performed on ice and in the presence of protease inhibitors to guard against proteolysis. Generally, cells are lysed and membrane-solubilized in a buffer containing a non-ionic detergent. Undigested material and extra debris are then removed from the homogenate sample, by centrifugation. After discarding the precipitated, undigested material, the soluble fraction can then be incubated with streptavidin agarose beads. Usually, precipitation of biotinilated proteins with streptavidin is performed over night and under gentle agitation, so as to maximize recovery of tagged polypeptides. The whole-cell fraction can then be separated from streptavidin-biotin complexes by centrifugation. After washing away unbound beads, biotinilated proteins can be eluted by resuspending the bead pellet in a small volume of Laemmli buffer, containing a percentage of SDS. Further dissociation of tagged polypeptides from

streptavidin is attained by heating the samples to 95-100 ºC, followed by centrifugation to precipitate the beads away from the eluted proteins (supernatant). Proteins tagged in this way, corresponding to the surface component of the cellular population contained in the hippocampal slice, can then be loaded to a gel and separated by electrophoresis.

4 Materials and Methods

Experiments were conducted using acute hippocampal slices prepared either from young (3-4 weeks old) male Wistar rats, or from adult C57Bl6 male mice, with at least 10 weeks of age (Harlan Iberica, Spain). The animals were kept under standardized temperature, humidity and lighting conditions, and had access to water and food *ad libitum*. All animal procedures were carried out according to the European Community Guidelines for Animal Care (European Communities Council Directive - 86/609/EEC).

4.1 Tissue Preparation

The animals were sacrificed by decapitation under deep halothane or isofluorane anaesthesia. The brain was quickly removed, hemisected and one hippocampus used to obtain transverse slices (300 μm-thick), cut on a vibratome (VT 1000 S; Leica, Nussloch, Germany) in ice-cold dissecting solution containing (in mM): sucrose 110; KCl 2.5; $CaCl_2$ 0.5; $MgCl_2$ 7; $NaHCO_3$ 25; NaH_2PO_4 1.25; glucose 7, oxygenated with 95% O_2 and 5% CO_2, pH 7.4. Slices were first incubated for 30 min at 35°C in artificial cerebrospinal fluid (aCSF), containing (in mM): NaCl 124; KCl 3; NaH_2PO_4 1.25; $NaHCO_3$ 26; $MgSO_4$ 1; $CaCl_2$ 2; and glucose 10, pH 7.4, gassed with 95% O_2 and 5% CO_2, and used after recovering for at least 1 hour at room temperature.

4.2 Patch-clamp recordings

Individual slices were fixed with a grid in a recording chamber (1 ml plus 200 µl dead volume) and were continuously superfused at 2-3 ml/min with aCSF. Unless stated otherwise, drugs were added to this superfusion solution and reached the recording chamber within approximately 1 min.

Patch pipettes had resistance of 4–7 MΩ when filled with an internal solution containing (in mM): K-gluconate 125; KCl 11; $CaCl_2$ 0.1; $MgCl_2$ 2; EGTA 1; HEPES 10; MgATP 2; NaGTP 0.3 and phosphocreatine 10, pH 7.3, adjusted with NaOH (1 M), 280–290 Osm. In some experiments, and where specified, H-89 (1 µM), GF109203X (1 µM), cycloheximide (10 µM) or spermine (500 µM) were added to the pipette solution, so as to impair PKA or PKC activity, postsynaptic protein synthesis or calcium-permeable AMPA receptors, respectively. In these experiments, a 30 min period, prior to drug application, was allowed for diffusion of the inhibitor into the intracellular milieu of recorded cells.

Whole-cell patch-clamp recordings were obtained from CA1 pyramidal cells or stratum radiatum interneurons, which were visualized with an upright microscope (Zeiss Axioskop 2FS) equipped with infrared video microscopy and differential interference contrast optics. Recordings were performed at room temperature (22−24°C). Pyramidal cells were functionally distinguished from interneurons by their slower firing frequencies, longer action potentials and for featuring spike-frequency adaptation (Madison and Nicoll, 1984; Schwartzkroin, 1975) during a 500 ms step depolarization up to -40 mV. Current recordings were

performed in voltage-clamp mode (Vh = -70 mV) with either an EPC-7 (List Biologic, Campbell, CA) or an Axopatch 200B (Axon Instruments) amplifier. The junction potential was not compensated for and offset potentials were nulled before giga-seal formation. Small voltage steps (5 mV, 50 msec) were delivered before current recordings to monitor the access resistance. The holding current was also constantly monitored throughout the experiment and when any of these parameters varied by more than 20%, the experiment was rejected. The current signal was filtered using built in 2 or 3 and 10 kHz three-pole Bessel filters and data were digitized at 2, 5 or 10 kHz, under the control of either the LTP (Anderson and Collingridge, 2001), winLTP 0.94 (Anderson, 1991-2006) or pClamp 10 (Molecular Devices) software programs. Data were analysed using either the off-line reanalysis version of winLTP or Clampfit 10.2 software.

4.2.1 AMPA-evoked postsynaptic currents

Postsynaptic currents (PSCs) were elicited once every 2 min by pressure ejection (5-10 psi, 4-20ms, pneumatic picopump, PV 820 WPI Instruments, or Toohey Spritzer) of AMPA (60-120 μM) through a micropipette positioned near the soma of the recorded cell.

4.2.2 mEPSC Recordings

Miniature excitatory postsynaptic currents (mEPSCS) were recorded in aCSF supplemented with tetrodotoxin (TTX, 0.5 μM) and bicuculline (bicc, 20 μM), except where otherwise indicated. Analysis of miniature events was performed with the Clampfit 10.2 software, by scanning

gap-free recordings for asymmetric events with the rise time shorter than the decay time and amplitudes > background, using template-based event search (Clements and Bekkers, 1997). mEPSC data were sampled at 10 KHz and an offline low-pass Gaussian filter (400 Hz with a -3dB cut-off) was used. mEPSC frequency and amplitudes were established by analyzing two 10 min periods, one immediately before addition of the test drug to the superfusion medium and the other of the final 10 min period recorded in its presence. mEPSC frequency ranged between 0.16 and 2.5 Hz and the average number of events analyzed in each recording was 418±59 (mean±SEM from 18 experiments). To address the possibility of rundown or loss of sensitivity of AMPA receptors during whole-cell recordings of spontaneous miniature excitatory currents (Wang et al., 1991), test drugs were applied at least 30 minutes after establishment of the whole-cell configuration, a time at which the decline of miniature events has been shown to be minimum (Sokolova et al., 2006).

4.2.3 EPSC recordings and LTP

Afferent-evoked excitatory postsynaptic currents (EPSCs) were elicited by 0.2 ms rectangular pulses, delivered once every 30 s through a concentric electrode (Harvard) placed in the Schaffer Collaterals/comisural afferents of the CA1 area. Averages of four consecutive individual recordings were used for analysis, so as to match the time course of experiments with AMPA-mediated PSCs. EPSCs were recorded from CA1 pyramidal neurons at Vh=-70 mV, in aCSF containing bicuculline (20 μM, to prevent activation of postsynaptic $GABA_A$ receptors and therefore minimize the influence of

GABAergic transmission) and with an external Mg^{2+} concentration of 1 mM, so that most of the EPSC response corresponded to the AMPA component of glutamatergic transmission. Long term potentiation (LTP) of EPSCs was induced by coupling depolarization to 0 mV for 15s with the delivery of four brief high-frequency tetani (50 pulses at 50 Hz) spaced by 4s intervals (Chen et al., 1999b), in aCSF containing an external Ca^{2+} concentration of 4 mM and no bicuculline, except otherwise indicated. Data were not included for analysis when pairing failed to result in a ≥40% potentiation in the first two minutes after paired stimuli delivery.

4.2.4 Hypoxia induction

Hypoxia was induced by substituting the artificial cerebrospinal fluid (aCSF) by an identical aCSF pre-equilibrated with 95% N_2 / 5% CO_2 for 4 min. This manipulation reduces bath oxygen tension in the recording chamber from ≈ 600 mmHg to ≈ 250 mmHg (Sebastião et al., 2001). Each slice was subjected to a single period of hypoxia, since the effects of hypoxia may be modified by subsequent episodes in the same slice (Schurr et al., 1986; Pérez-Pinzón, 1999).

4.2.5 Oxygen/glucose deprivation

In vitro ischemia was induced by replacing 10 mM glucose-containing aCSF with that containing 7mM sucrose (3mM glucose), gassed with 95% N_2 / 5% CO_2, for 10 min (Rossi et al., 2000).

4.3 Protein biotinylation

Whole-cell and membrane protein extracts were prepared from control and test slices, which were incubated under oxygenation, for 40 minutes in the presence or absence of a test drug. Two slices per group, per experiment, were used. Slices were then washed three times with ice-cold PBS/Ca^{2+}/Mg^{2+} buffer composed of (in mM): 136.9 NaCl, 2.7 KCl, 4.3 $NaH_2PO_4 \cdot 2(H_2O)$, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$, and incubated with EZ-Link sulfo-NHS-LC-biotin (1mg/mL, Pierce) for 1 hour, at 4ºC using gentle agitation, which has been shown to ensure a complete biotinylation in hippocampal slices even thicker (400 μm) (Thomas-Crussels et al., 2003) than those presently used (300 μm). Biotin was dissolved in a biotinylation buffer composed of (in mM): 10 TEA, 2 $CaCl_2$, 150 NaCl, pH 7.4. Slices were washed three times with cold PBS/Ca^{2+}/Mg^{2+} buffer and then incubated with 100 mM glycine in PBS/Ca^{2+}/Mg^{2+} buffer, for 30 minutes at 4ºC, so as to quench free biotin. After washing again three times with cold PBS/Ca^{2+}/Mg^{2+} buffer, slices were lysed on ice, by mechanical homogenization in RIPA (lysis buffer composed of 50 mM Tris base pH8, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% NP40), supplemented with protease inhibitor cocktail tablets (Roche). Samples were centrifuged at 13000 rpm (16060 G; Biofuge Fresco, Heraeus, UK) for 10 minutes (at 4ºC) and the supernatant was separated from the pellet (discarded as undigested material) and further processed. The protein content of each sample was then determined, using the Bradford method (Bradford, 1976); average protein concentration was 1.34± 0.20 mg/mL for control samples and 1.36 ± 0.26 mg/mL for those treated with the A_{2A} receptor agonist. Samples were left to incubate with streptavidin (Sigma, 2 μL/10 μg of sample protein) overnight at 4ºC, in orbital agitation.

Samples were centrifuged at 14000 rpm for 10 minutes and the supernatant (whole-cell fraction of both control and test samples) was stored in the freezer for further immunoblot analysis. The pellet (biotinilated proteins conjugated with streptavidin - surface membrane fraction) was then washed 3 times with RIPA, by 10 min centrifugations at 13000 rpm, after which 70 μL of Laemli buffer (350 mM Tris-HCl pH 6.8; 600 mM DTT; 30% glycerol; 10% SDS and 0.012% Bromophenol Blue) were added to the sample, before a 5 min incubation at 95ºC. After a 10 min centrifugation at 13000 rpm, the supernatant was collected (containing membrane proteins free of streptavidin-biotin conjugates) and stored for immunoblot analysis.

4.4 Immunoblot analysis

Samples processed as above were run on standard 8% sodium dodecyl sulphate poliacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham). After blocking for 1 hr with a 5% milk solution, membranes were then probed using anti-phospho-Ser-845-GluR1 (1:1500 or 1:2000, Chemicon), anti-GluR1 (1:4500, Upstate) or anti-ß-actin (1:5000, Abcam) primary antibodies, with which they were left to incubate overnight, at 4ºC. Incubation with anti-rabbit IgG-HRP conjugated secondary antibodies (1:7500, Biorad) was performed at room temperature, for 1 hour. Development of signal intensity was done using the ECL Plus Western Blotting Detection System (Amersham) and quantifications were attained by densitometric scanning of the films, performed with the Image J software. β-actin density was used as a loading control.

4.5 Drugs

CPA (N6-cyclopentyladenosine, Tocris), CGS 21680 (2-[4-(2-*p*-carboxyethyl)phenylamino]-5'-N-ethylcarboxamidoadenosine, Sigma), SCH 58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine, Sigma-Aldrich) and H-89 (N-[2-((p-bromocinnamyl)amino)ethyl]5-isoquinolinesulfonamide, Sigma-Aldrich) were made up in 5 mM stock solutions in dimethylsulfoxide (DMSO). DPCPX (8-cyclopentyl-1,3-dipropylxanthine, Sigma-Aldrich) was also prepared as a 5 mM stock solution, in DMSO. TTX (Tetrodotoxin, Tocris), AMPA (RS-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, Sigma-Aldrich) and DL-APV (2-amino-5-phosphonovaleric acid, Tocris) were made up in water, in 1, 10 and 25 mM stock solutions, respectively. Cycloheximide (Tocris) was also prepared in water, in a 10 mM stock solution. GF109203X (bisindolylmaleimide I, Tocris) and bicuculline (Sigma-Aldrich) were made up in DMSO, in 1 and 100 mM stock solutions, respectively. CNQX was either prepared as a 100 mM stock solution in DMSO (6-cyano-7-nitroquinoxaline-2,3-dione, Tocris), or as a 10 mM stock solution in water (CNQX -disodium salt, Ascent). Forskolin (Sigma-Aldrich) was prepared in DMSO, in a 10 mM stock solution. Aliquots of all stock solutions were kept at -20ºC until use. The maximum DMSO concentration used in the perfusion solution (0.0026%, v/v) was devoid of effects on AMPA-PSCs amplitude.

4.6 Preparation of recombinant cytokine samples

Mouse recombinant Oncostatin M (OSM, Sigma) was prepared in PBS containing 0.1% BSA (stock concentration: 25 μg/mL) and applied to

acute slices at a final concentration of 10 ng/mL. Human recombinant Leukemia factor (LIF) was provided at a stock concentration of 10 µg/ml in PBS (Millipore) and used at a final concentration of 10 ng/mL.

4.7 Statistical Analysis

Results are expressed as the mean±SEM of n experiments. Statistical significance was either assessed by two-tailed Student's t test; or by performing one-way ANOVA followed by Dunnett´s post hoc test for comparison between multiple experimental groups. A p value of less than 0.05 was considered to account for significant differences. Analyses were conducted with the GraphPad Software.

5 Results

5.1 Activation of A_{2A} Adenosine Receptors Facilitates AMPA receptor-mediated responses in CA1 Pyramidal Neurons with consequences for synaptic plasticity

Rationale:

Most of the fast excitatory transmission that takes place in the central nervous system is mediated by ionotropic glutamate AMPA receptors and their modulation is thus accountable for profound changes in synaptic efficiency. Accordingly, an increase in the number of AMPARs available at synaptic membranes, via activity-driven changes in AMPAR trafficking, is widely accepted as a major mechanism for long term potentiation (LTP) of excitatory transmission (Malenka and Bear, 2004). Activity-dependent release of adenosine and of its precursor, ATP, into the synaptic cleft, is well established to bring about fine-tuning of synaptic transmission (Sebastião and Ribeiro, 2000). Indeed, through activation of high affinity A_1 receptors, adenosine depresses the release of excitatory neurotransmitters, being part of a negative feedback loop capable of refraining neuronal excitability. As for A_{2A} receptors, after first evidence that they could enhance synaptic transmission (Sebastião and Ribeiro, 1992), the underlying mechanism has been proposed to rely upon attenuation of A_1 receptor-mediated inhibition of excitatory transmission (Cunha et al., 1994). Since then, attention has mainly focused on A_{2A} receptor-

mediated presynaptic actions. These include facilitation of glutamate release by restraining the inhibitory actions of A_1 receptors (Lopes et al., 2002), with which they colocalize in subsets of glutamatergic nerve terminals (Rebola et al., 2005a). Reports on postsynaptic actions of A_{2A} receptors in the hippocampus comprise depolarization associated with changes in input resistance, compatible with potassium-channel inhibition (Li and Henry, 1998) and attenuation of A_1 receptor-mediated postsynaptic inhibition of cell firing (O'Kane and Stone, 1998). Also, there is indirect evidence for a nonpresynaptically mediated facilitation of the early phase of afferent evoked synaptic potentials, which was interpreted as a postsynaptic facilitation of AMPA receptor function, dependent on activation of AC-coupled A_{2B} receptors (Kessey and Mogul, 1997). However, the low affinity of A_{2B} receptors for adenosine makes them less likely to play a relevant role at synapses under physiological conditions (Ribeiro et al., 2002). Strikingly, no study has directly evaluated whether postsynaptically located A_{2A} receptors could influence AMPA receptor functioning, even though their high affinity for adenosine prompts activation under non-pathological conditions. Moreover, A_{2A} receptors are positively coupled to adenylate cyclase (Furlong et al., 1992; Fredholm et al., 2001) and AMPA receptor subunits are substrate for PKA dependent phosphorylation (Banke et al., 2000) with consequences for receptor cycling (Ehlers, 2000) and membrane delivery (Oh et al., 2006), therefore to plasticity phenomena (Oh et al., 2006; Esteban et al., 2003). In what concerns their distribution in the hippocampus, A_{2A} receptors are expressed in presynaptic terminals, in the postynaptic density and in the cell body of pyramidal neurons, as indicated by immunocytochemistry (Rebola et al., 2005b) and in situ hybridization

(Lee et al., 2003b) studies. They thus occur in proximity of postsynaptic AMPA receptors. The work described in this chapter was designed to address a putative modulation by A_{2A} receptors of AMPA receptor functioning, as well as consequences for synaptic transmission and plasticity phenomena.

Exogenous activation of A_{2A} adenosine receptors facilitates AMPA-evoked currents in CA1 pyramidal neurons but not in stratum radiatum interneurons

In order to elucidate whether modulation of synaptic transmission by adenosine in the hippocampus might include A_{2A} receptor-mediated regulation of postsynaptic AMPA receptor-mediated responses, whole-cell recordings of agonist-evoked AMPA currents or electrically-evoked excitatory postsynaptic currents (EPSCs) were combined with exogenous application of A_{2A} receptor ligands. AMPA-evoked currents were recorded from either CA1 pyramidal cells or stratum radiatum interneurons (Figure 5.1.1).

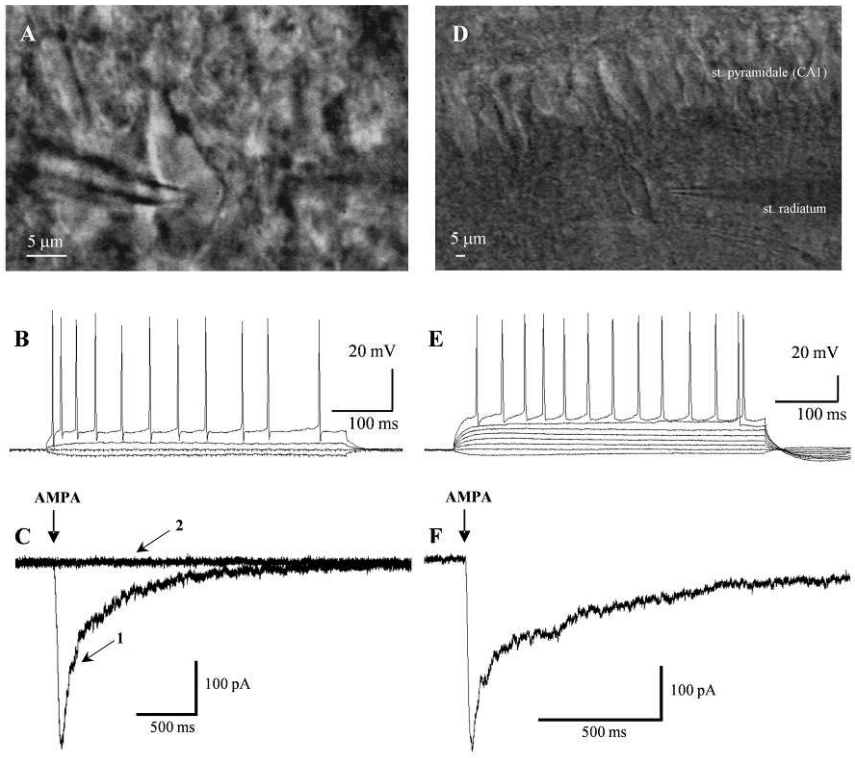


Figure 5.1.1. Patch-clamp recordings of AMPAR-mediated currents. AMPA receptor-mediated currents were recorded from CA1 pyramidal cells (A-C) or stratum radiatum

interneurons (D-F), from acutely prepared hippocampal slices. Cells were visually identified by their characteristic morphological features (A,D) and functionally, by firing patterns obtained in response to current injection through the recording electrode (B,E), as described in Methods; note accommodation of firing frequency in pyramidal cells (B) but not in interneurons (E). Stratum radiatum interneurons were further identified by their position relative to the CA1 pyramidal layer, as evidenced by a magnification (D) 1/4X from that used to establish patch-clamp recordings (A). Local pressure application (5-10 psi; 4-20 ms) of AMPA (60-120 μM) onto the soma of recorded cells was used to elicit macroscopic postsynaptic excitatory currents (C1,F) mediated by AMPA-type glutamate receptors, which were completely abolished by superfusion of the selective AMPA receptor antagonist CNQX (50 μM, C2).

Whole-cell recordings of agonist-evoked currents were first employed to address a putative modulation of postsynaptic AMPA currents by addition of a selective A_{2A} receptor agonist (CGS 21680) to the superfusion medium, in CA1 pyramidal cells. In these conditions, application to the hippocampal slice of CGS 21680 (30 nM; Jarvis et al., 1989) significantly enhanced the peak amplitude of AMPA-evoked currents recorded from CA1 pyramidal neurons. Recorded currents started to increase only about 10–15 min after starting CGS 21680 perfusion, with the maximum effect being observed at the end of about 50 min (Figure 5.1.2A). This enhancement was not reversible at least within 30 min after starting drug removal from the bath. Current amplitude enhancement by CGS 21680 (30 nM) was observed in 8 out of 11 cells (73%). Absence of response to CGS 21680 also occurred in subsequent sets of experiments, with the percentage of non-responding cells usually being less than 1/3 of total cells tested. Data from all cells was, however, pooled together when calculating averaged effects of the A_{2A} receptor agonist. This method was preferred because it avoids bias while calculating effects, though it may lead to underestimation of CGS 21680 actions in responding cells. Accordingly, to keep information on the effects obtained in individual cells available, statistical panels

shown throughout the chapter report pooled averaged results as well as effects in individual cells. An average increase of 26.9±6.9% (n=11, $P<0.05$, as compared with baseline values) in the peak amplitude of AMPA currents was attained 36–40 min after adding CGS 21680 (30 nM) to the superfusion medium. However, as shown in Figure 5.1.2A, AMPA currents continued to undergo an enhancement beyond this time point so that 54–60 min after addition of CGS 21680, current amplitude enhancement reached 45.2±5.9% (n=8). In most of the experiments described in the present chapter, CGS 21680 was applied for 40–50 min; therefore, to allow comparison between CGS 21680 effects in different drug conditions, and unless otherwise stated, the effect of CGS 21680 was quantified in each experiment by taking the current amplitude values recorded during the last three time points of a 40-min perfusion.

As expected, AMPA current facilitation by CGS 21680 was fully prevented when a selective A_{2A} antagonist, SCH 58261 (100 nM; Zocchi et al., 1996), was added to the superfusion medium for at least 30 min prior to CGS 21680 application (n=5, Figure 5.1.2B). In some of the experiments and to save recording time, SCH 58261 was added to the superfusion immediately after establishing whole-cell configuration. However, in a subset of experiments, responses were allowed to stabilize before addition of the A_{2A} antagonist and in no case did its perfusion for 40 min appreciably affect the amplitude of AMPA currents (n=3, Figure 5.1.2C).

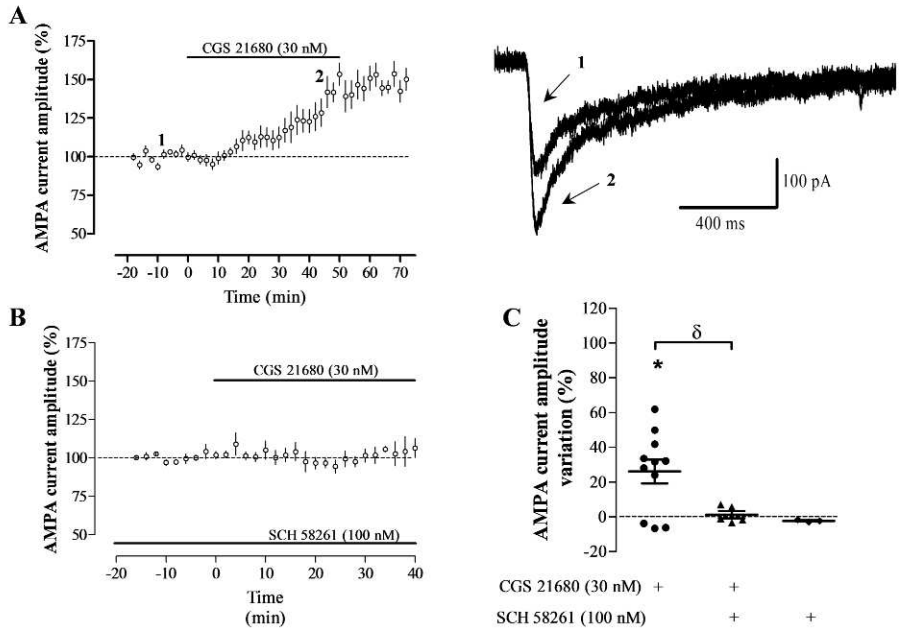


Figure 5.1.2. Activation of A_{2A} adenosine receptors potentiates the amplitude of AMPAR-mediated currents recorded from CA1 pyramidal cells. (A) Whole-cell voltage-clamp recordings of AMPA currents (right) and averaged time-course changes in AMPA current peak amplitude (left) caused by superfusion of the selective A_{2A} agonist CGS 21680 (30 nM). Superimposed traces in the right panel represent AMPA-evoked currents obtained in one representative cell under control (1) and test (CGS 21680) conditions (2). In each time-course panel, (A,B,D,E), each point represents the average of individual macroscopic responses to focal pressure application of AMPA, elicited once every 2 minutes. Horizontal bars on the time-course panels indicate the time at which tested drugs were applied to the superfusion medium; 100% corresponds to the averaged amplitude calculated for the 5-10 AMPA currents recorded immediately before drug application. (B) Averaged time-course changes in AMPA current peak amplitude caused by CGS 21680 (30 nM), when added to superfusion medium containing a selective antagonist of A_{2A} receptors (SCH 58261, 100 nM). (C) Individual (dots) and average (bars) changes in current amplitude induced by the A_{2A} receptor ligands, as indicated below each data set. Values are mean ± SEM. To allow comparison between all data sets at the same time points, values correspond to percentage changes recorded 34-40 min after initiating CGS 21680 superfusion. * $p<0.05$ (two-tailed paired Student's t test, compared with baseline, using absolute current values); δ $p<0.05$ (one-way ANOVA followed by Dunnett´s multiple comparison test).

The apparent irreversibility of the increase in AMPA current amplitude that ensued CGS 21680 superfusion, raised the hypothesis that the A_{2A}

receptor agonist would only need to be present for a short time, in order to trigger a response. Indeed, when CGS 21680 was superfused by only a brief, 10 min period, a significant facilitation of AMPA-evoked currents, by 21.3±6.4% (n=7, $P<0.05$ as compared with baseline, Figure 5.1.3A), was still observed. The facilitation induced by a short time application of CGS 21680 was slightly smaller, but not significantly different from that observed when using longer (40 min) CGS 21680 superfusion times ($P>0.05$, Figure 5.1.3D). The smaller effect of CGS 21680 after a short time application could be expected on the basis of the long period needed for this ligand to reach the equilibrium with its receptor (Jarvis et al., 1989). Still, these results clearly show that a continuous activation of A_{2A} receptors is not necessary for the increase in AMPA current amplitude to occur, suggesting that activation of A_{2A} receptors is only required to trigger the activation of a transduction pathway, and from this point onwards the presence of the A_{2A} receptor agonist is no longer a necessary step.

A_{2A} receptor-induced Potentiation of AMPA currents does not require NMDA or $GABA_A$ receptor activation, nor does it depend on synaptic activity.

Since excitatory synaptic transmission in the hippocampus is under GABAergic control (Lopes da Silva et al., 1990) and GABA release from hippocampal nerve terminals is enhanced by A_{2A} receptor activation (Cunha et al., 2000), a set of experiments was performed in the presence of a selective $GABA_A$ receptor antagonist (bicuculline, 20 μM) to evaluate if the observed A_{2A} receptor mediated facilitatory effect could result from any interference with GABAergic transmission. Also, because A_{2A} receptors have been shown to directly affect NMDA

receptor function (Nikbakht and Stone, 2001; Rebola et al., 2008), a selective NMDA receptor antagonist (APV, 10 μM) was concurrently added to the superfusion medium in these experiments. The sodium channel blocker, tetrodotoxin (TTX, 0.5 μM) was applied as well, so as to prevent action potential generation, and therefore, neuronal communication. Under these conditions, activation of A_{2A} receptors caused a 29.7±10.4% facilitation of AMPA-evoked currents (n=8, $P<0.05$, Figure 5.1.3B,D), measured 34–40 min after CGS 21680 application, thus dismissing the possibility that the effect of the A_{2A} receptor agonist is due to modified NMDA and/or $GABA_A$ receptor-mediated transmission or to any other mechanisms requiring action potential dependent neuronal communication.

A_{2A} receptor-induced potentiation of AMPA-evoked currents is concentration-dependent.

When using lower concentrations of CGS 21680 (1–10 nM), smaller but significant facilitations of AMPA-evoked currents were attained, except for the lowest concentration tested (Figure 5.1.3C). However, no significant effect was observed when CGS 21680 was applied at a concentration of 100 nM (n=4, $P>0.05$, Figure 5.1.3C). The absence of effect observed for the highest concentration could be due to desensitization of A_{2A} receptors, or to A_1 receptor activation due to loss of selectivity of the agonist at high concentration. Indeed, it has been reported that CGS 21680 at concentrations higher than 100 nM failed to increase cAMP levels at hippocampal nerve terminals (Lopes et al., 2002) and that at micromolar concentrations it even inhibited hippocampal synaptic transmission through an A_1 receptor-related mechanism (Lupica et al., 1990).

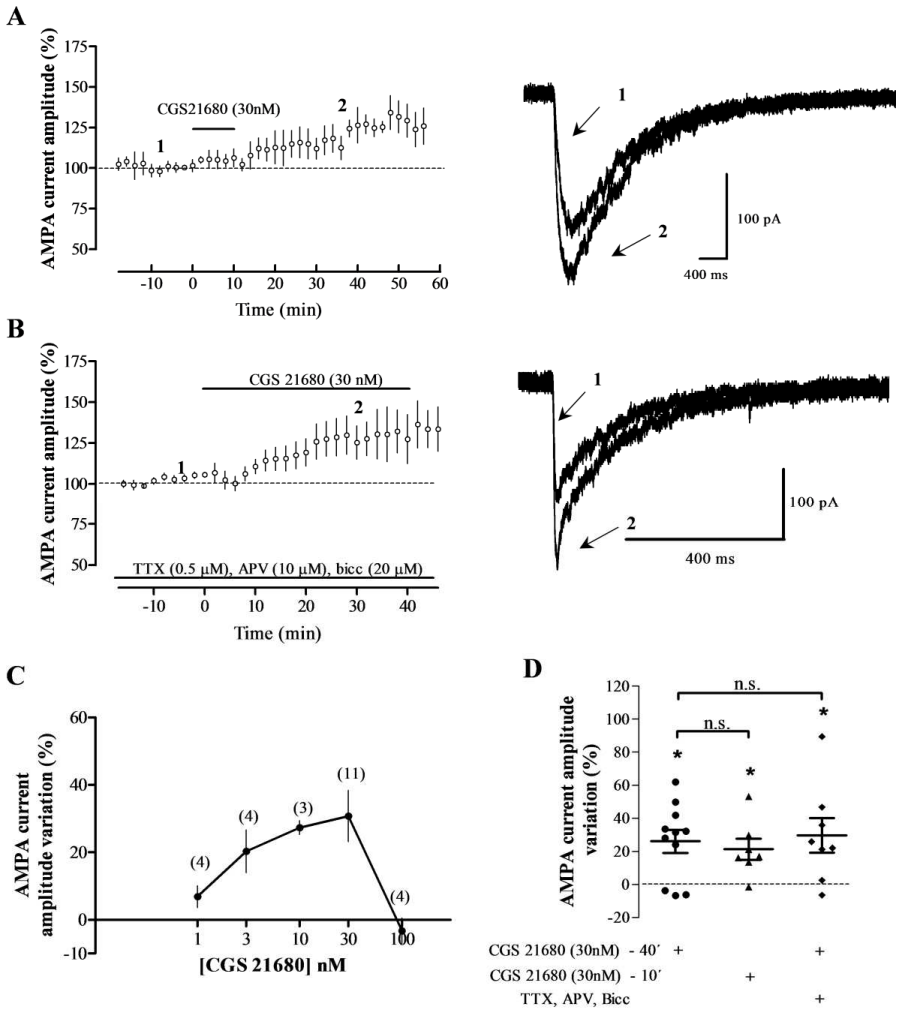


Figure 5.1.3. A_{2A} receptor- induced potentiation of AMPA currents does not depend on NMDA or $GABA_A$ receptor activation, nor does it depend on synaptic activity. (A) Time-course changes (left) and recordings (right) of AMPA-evoked currents in experiments where CGS 21680 (30 nM) was added to the superfusion medium for only a 10 min period. Note that under these conditions, CGS 21680 triggered a facilitation of AMPA currents that was not statistically different (D) from that caused by longer applications of CGS 21680. (B) Averaged time-course panel and recordings (right) of current amplitude changes caused by 30 nM CGS 21680 when applied in the presence of a selective $GABA_A$ receptor antagonist bicuculline (Bicc, 20 µM), the sodium channel blocker tetrodotoxin (TTX, 0.5 µM) and the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid (APV, 10 µM). Average facilitation of AMPA-evoked currents in these conditions was not significantly different from that measured in the absence of the blockers (D). (C) Concentration-response curve for the effect of the A_{2A} receptor agonist. Facilitation of AMPA current peak amplitude was significant for all concentrations tested ($p<0.05$, two-tailed paired t test, compared with baseline), except for the lowest (1 nM) and the highest (100 nM). The number of experiments for each concentration is

indicated between parentheses. Each point is the averaged effect attained 40 min after initiating CGS 21680 superfusion. (D) Values are mean ± SEM. To allow comparison between all data sets at the same time points, values correspond to percentage changes recorded 34-40 min after initiating CGS 21680 superfusion. * $p<0.05$ (two-tailed paired Student's t test, compared with baseline, using absolute current values); n.s. $p>0.05$ (one-way ANOVA followed by Dunnett´s multiple comparison test).

Activation of A_1 adenosine receptors inhibits the amplitude of AMPA-evoked postsynaptic currents in CA1 pyramidal cells.

Although the focus of the work described in this chapter concerned A_{2A} receptor-mediated modulation of AMPA currents, adenosine A_1 receptors are also expressed in the hippocampus and frequently operate to counteract A_{2A} actions, as they are able to inhibit AC/PKA dependent mechanisms (van Calker et al., 1979).

I therefore decided to evaluate a putative modulation of postsynaptic AMPA currents by A_1 receptors and observed that superfusion of a selective A_1 receptor agonist, N6-cyclopentyladenosine (CPA, 100 nM; Williams et al., 1986) caused a decrease in peak current amplitude (Figure 5.1.4A). Since A_1 and A_{2A} receptor agonists are both able to influence AMPA currents recorded from CA1 pyramidal cells, and given previous reports on A_{2A} receptor-mediated actions in the hippocampus requiring tonic A_1 receptor activation (Lopes et al., 2002), it was considered relevant to evaluate if A_1 receptor activation is necessary for CGS 21680-induced potentiation of AMPA responses. Should this hypothesis apply, then an A_1 receptor antagonist would be expected to block the effect of CGS 21680. A selective A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), was therefore used at a concentration (100 nM) nearly 200 times its K_i value

for A_1 receptors at the hippocampus (Sebastião et al., 1990). At this concentration, DPCPX had no appreciable effect upon AMPA current amplitude (Figure 5.1.4B) and did not prevent CGS 21680-induced facilitation of AMPA currents (Figure 5.1.4C), precluding the possibility that the now reported neuromodulatory action of A_{2A} receptors results from an interaction with A_1 receptors.

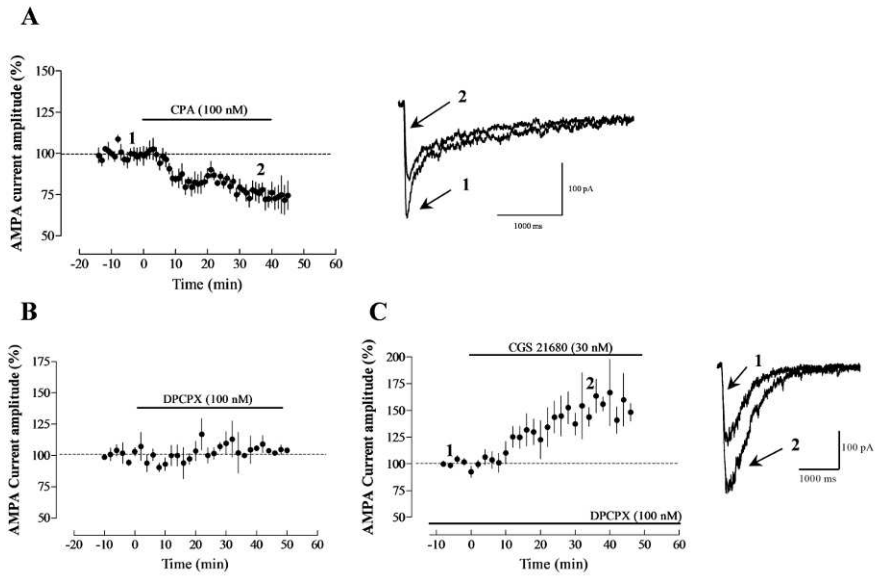


Figure 5.1.4. Activation of A_1 adenosine receptors inhibits the amplitude of AMPA-evoked postsynaptic currents in CA1 pyramidal cells. (A) Average time-course changes in AMPA current amplitude caused by superfusion of the selective A_1 adenosine receptor agonist N6-cyclopentyladenosine (CPA, 100 nM, n=6). Each point represents the average of individual macroscopic responses to focal pressure application of AMPA, elicited once every minute. The right panel shows superimposed tracings from a representative cell, illustrating an AMPA-evoked current recorded in the control period and one obtained 30 min upon adding CPA to the superfusion solution. (B) Superfusion of the A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 100 nM) did not significantly affect AMPA-evoked currents (n=3), and in its presence, the ability of the A_{2A} receptor agonist to facilitate current amplitude was preserved (n=4) (C). In the right panel are shown representative current tracings recorded in the control period (1) and 30-40 min after initiating CGS 21680 superfusion. For B and C, each point represents the average of individual macroscopic responses to focal pressure application of AMPA, elicited once every 2 minutes.

A_{2A} receptor-induced potentiation of AMPA-evoked currents does not occur in stratum radiatum interneurons.

Pyramidal neurons of the CA1 hippocampal area are responsible for the excitatory output of the hippocampus and are under control of inhibitory GABAergic interneurons. Interneurons at the stratum radiatum receive glutamatergic inputs from CA3 pyramidal cells and impinge their inhibitory output to other interneurons and to CA1 pyramidal cells (Lopes da Silva et al., 1990). Therefore, changes in the responsiveness of interneurons to excitatory inputs may have profound and diverse influence on hippocampal output signaling. We thus evaluated whether A_{2A} receptors could also modulate AMPA currents at stratum radiatum interneurons. Experiments were carried out in as in pyramidal cells except that the electrodes were placed over a stratum radiatum interneuron. CGS 21680 (30 nM) was tested in seven cells from slices prepared from five different animals, and in no case did its perfusion for 40 min appreciably affect the amplitude of recorded AMPA-evoked currents (n=7, Figure 5.1.5A-B). Similarly, when evaluating the effect of the A_{2A} receptor agonist upon AMPA-evoked currents recorded from stratum oriens interneurons, no significant effect was observed either (n=8; 7 animals; Figure 5.1.5C-D).

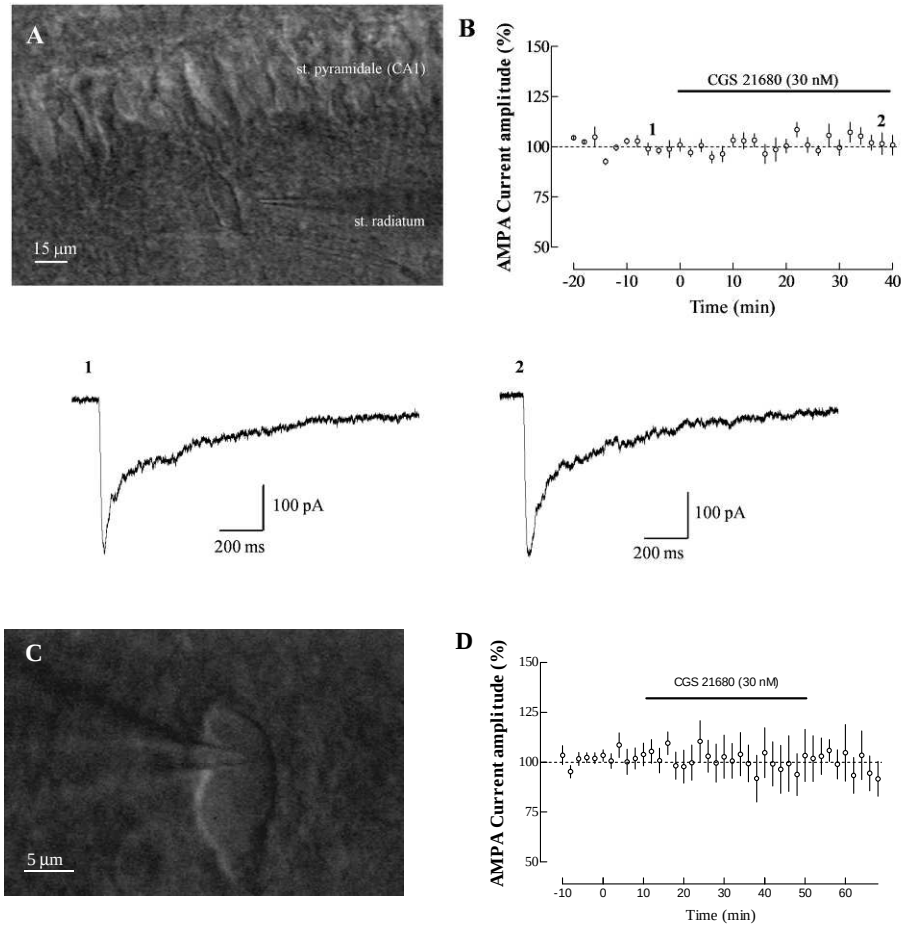


Figure 5.1.5. Activation of A_{2A} adenosine receptors does not affect the amplitude of AMPA-evoked postsynaptic currents recorded from *stratum radiatum* or *stratum oriens* interneurons. (A) Morphological features and location of a stratum radiatum interneuron below the pyramidal cell layer, in the hippocampal slice. (B) Averaged time-course changes in AMPA-evoked current amplitude caused by superfusion of CGS 21680 (30 nM, n=7). Current tracings (lower panel) of AMPA-PSCs recorded from a representative cell before (1) and 40 min after (2) starting superfusion of the A_{2A} receptor agonist. (C) Morphological features of a stratum oriens interneuron. (D) Averaged time-course changes in AMPA-evoked current amplitude caused by superfusion of CGS 21680 (30 nM), from 8 stratum oriens interneurons.

Having established that A_{2A} receptor-mediated facilitation of postsynaptic AMPA receptors was not extended to other cell types in the hippocampus, such as stratum radiatum (Figure 5.1.5A-B) or

stratum oriens interneurons (Figure 5.1.5C-D), experiments were designed to ascertain the mechanisms underlying the effects observed in CA1 pyramidal cells.

Activation of A_{2A} Receptors increases the amplitude, but not the frequency, of spontaneous miniature Excitatory Miniature Currents (mEPSCs)

Results reported above (Figure 5.1.3B) showing that facilitation of AMPA-evoked currents was preserved in conditions of impaired action potential-dependent neuronal communication, strongly indicated that it was brought about by changes in the postsynaptic cell. To further test whether A_{2A} receptor-mediated facilitation of AMPA-evoked currents was due to a postsynaptic mechanism, I evaluated changes in the frequency and amplitude of miniature excitatory postsynaptic currents caused by superfusion of the A_{2A} receptor agonist. These experiments were performed in aCSF supplemented with TTX (0.5 μM) and bicuculline (20 μM) to prevent both spontaneous firing of neurons and contribution of GABAergic transmission. Superfusion of CGS 21680 (30 nM) resulted in an increase in the amplitude of TTX-resistant mEPSCs by 10.2±3.3% (n=11, $P<0.05$ when compared with absolute baseline values, Figure 5.1.6), measured 30–40 min after starting CGS 21680 superfusion, as compared to mEPSCs recorded in the 10 min previous to it. In absolute values, mEPSC amplitude was 9.9±0.9 pA in the baseline period and 11.0±1.1 pA, 30–40 min after addition of the A_{2A} receptor agonist to the superfusion. mEPSC frequency was not affected by CGS 21680 superfusion, as it was 100.1±8.0% of baseline

values (0.44±0.07 Hz before and 0.43±0.08 Hz after CGS 21680; n=11, Figure 5.1.6). To control for the possibility that this change in mEPSC amplitude could result from time-dependent changes rather than CGS 21680 application, we repeated the experimental design (bicuculline present), but omitting the addition of the A_{2A} receptor agonist to the bath solution. A comparison of the changes observed in mEPSC amplitude and frequency in these control experiments (n=7) with those observed at the same time point (40 min), but in the presence of CGS 21680, is shown in Figure 5.1.6. Amplitude variation, but not frequency, was significantly different in both groups (Figure 5.1.6B).

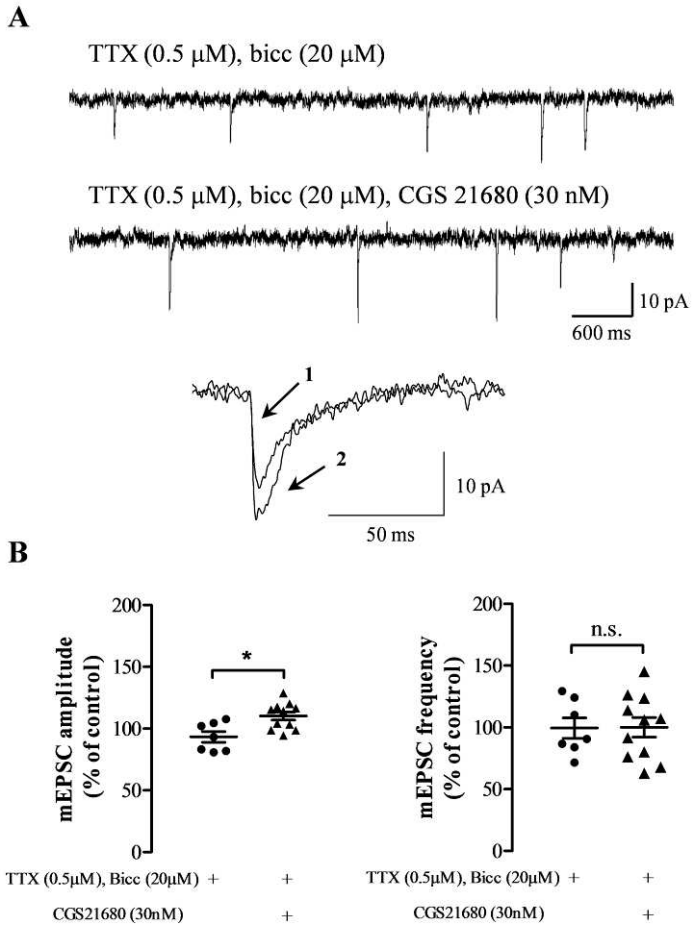


Figure 5.1.6. Activation of A_{2A} receptors increases the amplitude, but not the frequency, of spontaneous miniature excitatory postsynaptic currents (mEPSCs). (A) In the upper panel are shown representative tracings of mEPSCs recorded in whole-cell configuration from a CA1 pyramidal cell, in the absence (upper trace) and presence (lower trace) of the A_{2A} receptor agonist CGS 21680 (30 nM). The two superimposed events depicted in the lower panel illustrate one mEPSC recorded in control conditions (1) and one recorded in the presence of the A_{2A} agonist (2), from the same cell as in the upper panel. The sodium channel blocker, tetrodotoxin (TTX, 0.5 μM) and the $GABA_A$ receptor antagonist bicuculline (bicc, 20 μM) were present throughout recordings of spontaneous events. mEPSC frequency and amplitude changes were quantified by analyzing two 10 min periods, one immediately before addition of the A_{2A} receptor agonist and the other corresponding to the final 10 min period recorded in its presence. These variations were compared with those measured in the absence of the A_{2A}R agonist (control experiments), in the same time points of recording. (B) Values are shown as mean ± SEM, as well as individual data obtained in the absence (n=7) and presence (n=11) of CGS 21680, as indicated below each data set. Each point represents the averaged mEPSC amplitude (left) or frequency (right) recorded from each cell 30-40 min after adding CGS 21680 or after the same time of recording, but in the absence of CGS 21680, as indicated below

each data set; 100% corresponds to data recorded before adding/not adding CGS 21680. Individual run down for each cell and in each condition can be evaluated by the deviation of each point from 100%. n.s. $p>0.05$ and * $p<0.05$ (two-tailed unpaired Student´s *t*-test, compared with control experiments).

Altogether, these data provide a further indication that A_{2A} receptor activation postsynaptically enhances AMPA receptor-mediated events. However, considering that neither the expression of A_{2A} receptors in hippocampal neurons (Lee et al., 2003b), nor that of AMPA receptors (e.g., Shi et al., 1999) is restricted to a synaptic level, it would be highly unlikely that changes in mEPSC amplitude alone could fully account for the observed facilitation of AMPA-evoked currents by A_{2A} receptor activation. Indeed, the difference of magnitude between the effects observed in mEPSC amplitude and those obtained when measuring AMPA-evoked currents suggests the involvement of changes not only in the synaptic AMPA receptor pool, as also in the peri-synaptic and extrasynaptic receptor populations.

Intracellular Blockade of PKA Activity Prevents AMPA Current Potentiation

An enhancement of AMPA receptor function as a result of Ser 831 and/or Ser 845 phosphorylation has been reported (Banke et al., 2000; Derkach et al., 1999). Ser 831 can be phosphorylated by both PKC and CaMKII, resulting in increased AMPA receptor single-channel conductance, while Ser 845 phosphorylation by PKA increases open-channel probability (Banke et al., 2000) and enhances delivery of GluR1-containing AMPA receptors to extrasynaptic sites (Oh et al., 2006). Also, several early studies reported increases in the amplitude of

AMPA receptor-mediated responses in cultured hippocampal neurons, upon activation of PKA (Greengard et al., 1991; Wang et al., 1991; Rosenmund et al., 1994). Interestingly, it was recently found that the neuropeptide PACAP can enhance postsynaptic AMPA receptor function in the hippocampus, in a PKA-dependent way (Costa et al., 2009). Considering that A_{2A} receptors are also coupled to excitatory G proteins which most frequently leads to a raise in intracellular cyclic AMP levels (Fredholm et al., 2001), we hypothesized that the A_{2A} receptor agonist could be potentiating AMPA receptor function by means of a PKA-dependent mechanism. To address this possibility, cells were loaded with protein kinase inhibitors through the patch pipette solution. When a selective PKC inhibitor (GF109203X, 1 μM; Toullec et al., 1991) was present in the pipette filling solution, CGS 21680 significantly potentiated AMPA current amplitude by 21.0±5.9%, after 34–40 min of agonist superfusion ($n=8$, $P<0.05$, Figure 5.1.7A). This facilitation is slightly smaller but not significantly different from that observed in the absence of the inhibitor after the same time of perfusion ($P>0.05$, Figure 5.1.7C). When PKA activity was impaired by addition of the PKA inhibitor, H-89 (1 μM; Chijiwa et al., 1990), to the pipette filling solution, facilitation of AMPA-evoked currents was completely abolished ($n=6$, Figure 5.1.7B,C). These data strongly indicated that PKA activity was required for the facilitation of AMPA-evoked current amplitude observed after addition of the A_{2A}R agonist to the superfusion medium. However, they did not support that activation of the PKA pathway alone was sufficient to trigger an increase in AMPA-mediated responses, recorded in our experimental conditions. For this reason, we tested the effect of an adenylate cyclase activator, forskolin, in AMPA-evoked currents (Figure 5.1.7).

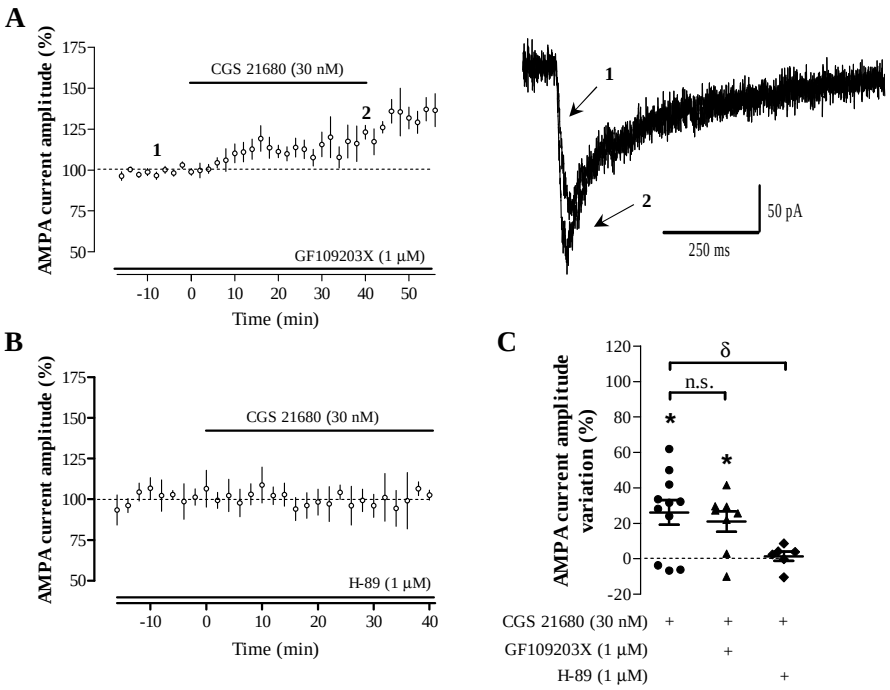


Figure 5.1.7. Facilitation of AMPA-evoked currents by A_{2A} receptor activation is dependent on postsynaptic PKA, but not PKC, activity. In A and B are shown averaged time-courses of current amplitude changes caused by 30 nM CGS 21680 after loading of recorded cells with either a selective PKC inhibitor (GF109203X, 1 µM) (A) or a selective PKA inhibitor (H-89, 1 µM) (B). In panel A (right) are also shown superimposed current tracings of AMPA-evoked currents obtained from a representative GF109203X-loaded cell, in the absence (1) and presence (2) of the A_{2A} receptor agonist. In all experiments, a 30 min period prior to CGS 21680 application was allowed for diffusion of the inhibitor into the intracellular milieu of recorded cells. In each experiment, the involvement of PKA and of PKC activity was tested in different slices taken from the same hippocampus; a positive control for a GF109203X-loaded cell was a pre-requisite to pursue with testing the influence of H-89 in another cell. (C) Averaged and individual effects of CGS 21680 when applied alone, or in the presence of protein kinase inhibitors, as indicated below each data set. Effects were quantified at 34-40 min after CGS 21680 addition. Values are mean ± SEM. * $p<0.05$ (two-tailed paired t-test, compared with baseline, using absolute current values); n.s. $p>0.05$ and δ $p<0.05$ (one-way ANOVA followed by Dunnet´s multiple comparison test).

Bath application of forskolin (1 µM; Seamon et al., 1981) caused a facilitation of AMPA current amplitude by 33.7±8.7% when applied for 40–50 min (n=10, P<0.05, Figure 5.1.8A) and by 25.4±7.2%, when

applied for only 10 min (n=6, Figure 5.1.8B), as measured 34–40 min after adding forskolin to the aCSF. Therefore, PKA is required for the enhancement of AMPA currents by the A_{2A} receptor agonist, and adenylate cyclase activation mimics A_{2A} receptor activation. In contrast, PKC activity does not seem to be required for A_{2A} receptor-induced enhancement of AMPA currents, with the slightly smaller CGS 21680 effect observed upon PKC activity inhibition being probably due to interaction of the two transduction pathways, as has been described for the regulation of Na(v)1.2 sodium channels (Cantrell et al., 2002) and GAT-1 GABA transporters (Cristóvão-Ferreira et al., 2009). Altogether, data support the idea that A_{2A} receptors facilitate AMPA currents through a mechanism that involves adenylate cyclase activation, cyclic AMP formation and PKA activation.

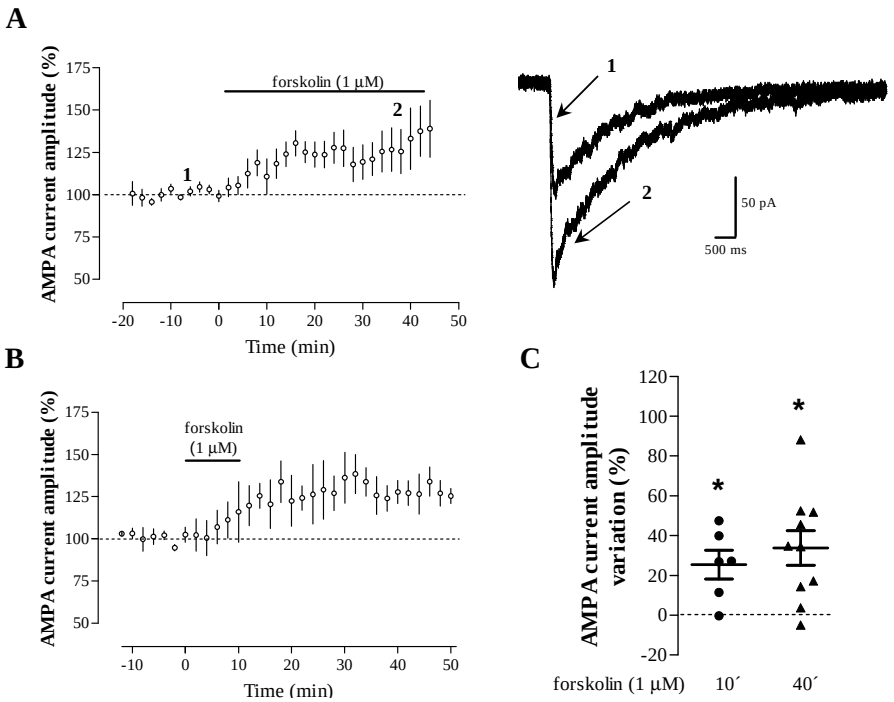


Figure 5.1.8. AMPA-evoked currents are potentiated by superfusion of an adenylate cyclase activator. Superfusion of an adenylate cyclase activator (forskolin, 1 µM) for 40 min (A) or 10 min (B) significantly increased the amplitude of postsynaptic AMPA currents, when compared with baseline. (C) Values are mean ± SEM of the effects observed following forskolin application by the period indicated below each data set, measured 34-40 min after starting its perfusion. * $p<0.05$ (two-tailed paired t-test, compared with baseline, using absolute current values.

These results raised the hypothesis that enhancement of AMPA currents induced by A_{2A} receptor activation could result from PKA-induced phosphorylation of GluR1 AMPA subunits with subsequent increase in surface expression of GluR1 subunits (Oh et al., 2006).

The A_{2A} Receptor Agonist CGS 21680 Increases GluR1 Surface Expression

GluR1 recycling between the plasma membrane and endossomal compartments has been shown to be controlled by PKA phosphorylation in dissociated hippocampal neurons (Ehlers, 2000). Even more relevant to this work is the finding that GluR1 phosphorylation by PKA activation in intact hippocampal slices correlates with increased expression of GluR1-containing AMPA receptors in extrasynaptic sites (Oh et al., 2006). To directly test the hypothesis that A_{2A} receptor activation could lead to an enhancement of the surface expression GluR1 receptor subunits, slices were incubated with or without CGS 21680 (30 nM) for 40 min, biotinylated samples were prepared and run on 8% SDS-PAGEs. Expression of the GluR1 AMPA receptor subunit was analyzed by western blot, as were the levels of GluR1 phosphorylated at Ser 845, known as the PKA phosphorylation site (Roche et al., 1996), by using an antibody that specifically recognizes that subunit form (Mammen et al., 1997). In slices incubated with the A_{2A} receptor agonist, the surface expression of GluR1 phosphorylated at Ser 845 was increased by 18.8±4.0% when compared with control slices ($n=5$, $P<0.05$, Figure 5.1.9A). This increase occurred in the biotinilated fraction, but not in the whole-cell fraction (% change: -2.3±4.4%). Furthermore, when the biotinylated fractions were tested for total GluR1 immunoreactivity, those that had been prepared from slices exposed to the A_{2A} receptor agonist revealed a 17.5±2.1% increase in the membrane expression of GluR1 when compared to control slices ($n=5$, $P<0.05$, Figure 5.1.9B). These findings suggest an association between A_{2A} receptor activation, GluR1

phosphorylation at the Ser 845 residue and an increase in the membrane expression of GluR1-containing AMPA receptors.

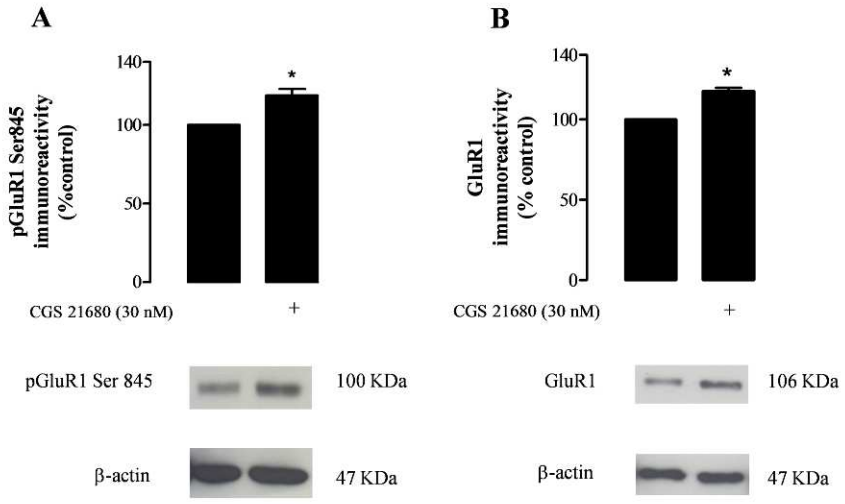


Figure 5.1.9. Activation of A_{2A} receptors enhances membrane expression of phospho Ser845 GluR1. Expression of (A) GluR1 subunits phospshorylated at Ser 845 (pGluR1 Ser845, n=5) or (B) total GluR1 in biotinilated fractions (n=5) isolated from slices that had been incubated for 40 min in the absence or in the presence of the A_{2A} receptor agonist, CGS 21680 (30 nM), as indicated below the columns. The same membrane extracts were used for analysis in (A) an (B). Lower panels in both (A) and (B) show representative Western Blots obtained from control slices (left lane) and from slices treated with CGS 21680 (30 nM) for 40 min (right lane). β-actin was used as a loading control (bottom lanes). Values are average pGluR1 Ser 845 (A) or GluR1 (B) immunoreactivity, normalized to β-actin. * $p<0.05$; two-tailed paired Student´s t-test.

A_{2A} Receptor-Induced Potentiation of AMPA Currents Does Not Depend on Postsynaptic Protein Synthesis

The insertion of newly synthesized glutamate receptors in the postsynaptic membrane can occur within a few minutes (Huber et al., 2000). This prompted us to check whether de novo synthesis of AMPA receptor subunits was required for the observed A_{2A} receptor-induced enhancement of AMPA-evoked currents. Recorded cells were loaded

with the protein synthesis inhibitor cycloheximide, which was applied through the patch pipette filling solution in order to inhibit protein synthesis in the recorded cell only, without affecting the remaining cells in the slice. This method of cycloheximide application has been previously shown to efficiently inhibit protein synthesis with consequences for LTP and spine expansion (Yang et al., 2008a). We used a 10 μM cycloheximide concentration because application of even a lower cycloheximide concentration (3.5 μM) for 30 min proved to efficiently inhibit protein synthesis in hippocampal slices, as measured by ^{3}H-valine incorporation (Stanton and Sarvey, 1984). A minimum time of 30 min between establishing whole cell configuration and application of CGS 21680 was used to allow diffusion of the drug into the cell (Lüscher et al., 1999). Under such conditions, CGS 21680 (30 nM) caused a significant facilitation of evoked AMPA current amplitude (27±5.2%, measured 34–40 min after application of the A_{2A} agonist, n=13, $P<0.05$, Figure 5.1.10A), which was not significantly different from that obtained after similar exposure time in cells not loaded with the protein synthesis inhibitor ($P>0.05$, Figure 5.1.10B). This indicates that, at least within a time frame of 40 min, A_{2A} receptor-mediated facilitation of AMPA function does not require de novo synthesis of AMPA receptor subunits and, therefore, suggests that the increase in surface GluR1 expression observed upon A_{2A} receptor activation results from the externalization of pre-existing AMPA receptors and/or prevention of their internalization. Interestingly, increased availability of membrane AMPA receptors, brought about by changes in receptor trafficking dynamics, has been shown to positively modulate LTP expression in the hippocampus (Gao et al., 2006; Oh et al., 2006). In order to ascertain whether A_{2A} receptors might affect

synaptic transmission with implications for plasticity phenomena, subsequent experiments employed electrical stimulation of the CA1 afferent fibers and synaptically evoked excitatory postsynaptic currents (EPSCs) were recorded from CA1 pyramidal neurons.

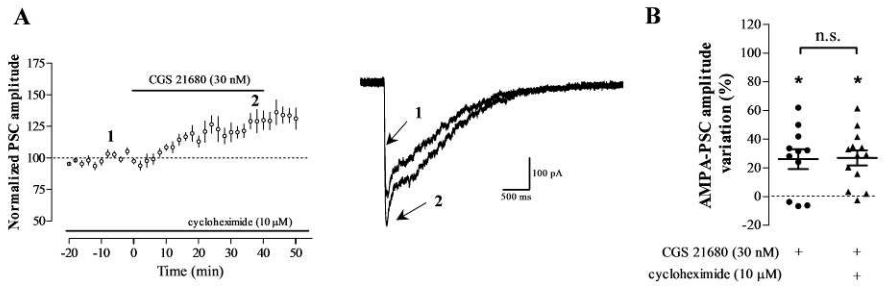


Figure 5.1.10. Facilitation of AMPA-evoked currents by A_{2A} receptor activation is not dependent on postsynaptic protein synthesis. (A) Averaged time-course of current amplitude changes caused by 30 nM CGS 21680 upon loading of recorded cells with a protein synthesis inhibitor (cycloheximide, 10 μM). The right panel in (A) shows superimposed current tracings of AMPA-evoked currents recorded in the absence (1) and presence (2) of CGS 21680, from a representative cycloheximide-loaded cell. In all experiments, a 30 min period prior to CGS 21680 application was allowed for diffusion of cycloheximide into the intracellular milieu of recorded cells. (B) Averaged and individual effects of CGS 21680 when applied in the absence or in the presence of cycloheximide, as indicated below each data set. Effects were quantified at 34-40 min after CGS 21680 addition. Values are mean ± SEM. n.s. $p>0.05$, two-tailed unpaired t-test; * $p<0.05$ (two-tailed paired t-test, compared to baseline, using absolute current values).

A_{2A} receptors modulate basal synaptic transmission and LTP expression

When recording excitatory synaptic responses from CA1 pyramidal cells, superfusion of the A_{2A} receptor agonist was found to cause a small, but significant, potentiation of EPSC peak amplitude by 9.7±3.6%, at the end of 34–40 min (n=11, $P<0.05$, Figure 5.1.11A). This facilitation of afferent-evoked EPSCs by CGS 21680 was about the same as the A_{2A} agonist-induced enhancement of mEPSC amplitude

(Figure 5.1.6B), but significantly smaller than that measured in chemically evoked AMPA currents ($p<0.05$, Figure 5.1.11C). This difference in the magnitude of effects observed in both mEPSCs and EPSC recordings, when compared to those observed in AMPA-evoked currents, suggests distinct contributions by post- and extrasynaptic A_{2A} receptors may converge to regulate AMPA receptor function in the postsynaptic neuron (see Discussion).

To evaluate consequences for synaptic plasticity, we induced LTP by using a protocol (see Methods) that couples bursts of high frequency stimulation with postsynaptic depolarization of the recorded neuron (Chen et al., 1999b). To minimize failures in LTP induction, these experiments were performed in aCSF containing 4 mM Ca^{2+}, based on early evidence that inputs tetanized in high (4 mM) Ca^{2+} aCSF displayed greater amounts of potentiation than those tetanized in control (2 mM Ca^{2+}) solution (Huang et al., 1988). Makhinson et al. (1999) reported that a 10 min superfusion of aCSF with high (5 and 10 mM) Ca^{2+} -containing aCSF induced a small and transient potentiation of synaptic transmission that was converted to a lasting, LTP-like potentiation when the slice had been previously exposed to the AC activator forskolin. Importantly, superfusion of aCSF with high calcium by itself did not induce sustained LTP (Huang et al., 1988; Makhinson et al., 1999). Accordingly, in control slices, this pairing protocol induced a 13.1±4.7% increase in EPSC peak amplitude (n=6, Figure 5.1.11B), measured 40–50 min after delivery of the LTP-inducing stimuli. However, in slices taken from the same hippocampus but that had been briefly (10 min superfusion) exposed to the A_{2A} receptor agonist (30 nM CGS 21680, 20–30 min before LTP induction), the

peak EPSC amplitude was increased by 52.1±12%, 40–50 min after LTP induction (n=6, $P<0.05$ compared with baseline; $P<0.05$ as compared with absence of CGS 21680, Figure 5.1.11B, D). These data, taken together with evidence that CGS 21680 leads to increased surface expression of the GluR1 subunit, strongly suggest that A_{2A} receptor activation is important for the maintenance of LTP in the hippocampus, perhaps by regulating the extrasynaptic contingent of GluR1-containing AMPA receptors, which are continuously recycling with intracellular pools (Adesnik et al., 2005; Oh et al., 2006).

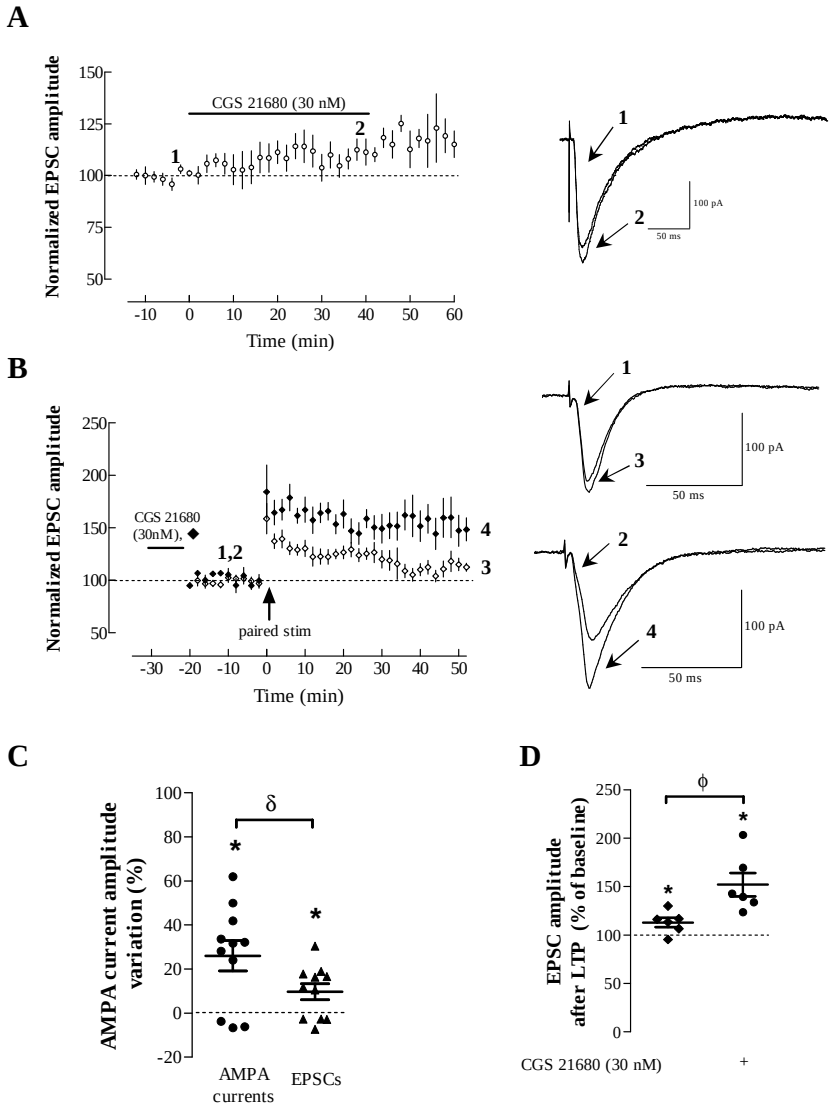


Figure 5.1.11. Activation of A_{2A} receptors facilitates afferent-evoked EPSCs and Long Term Potentiation (LTP). (A) Averaged time-course of afferent evoked excitatory postsynaptic current (EPSC) peak amplitude changes caused by CGS 21680 (30 nM). In the right panel are shown superimposed current tracings of EPSCs recorded at the time-points indicated in A, for a representative cell. Current facilitation by CGS 21680 was significantly smaller (C) when recording afferent-evoked EPSCs (right columns) than that attained when recording AMPA-PSCs (left columns). (B) Averaged time-course of EPSC peak amplitude changes induced by a pairing LTP protocol in the absence (◊) or after (♦) a brief exposure to CGS 21680 (30 nM), 20-30 min before LTP induction; in each experiment, LTP with and without brief exposure to CGS 21680 was tested in two slices from the same hippocampus. In some experiments LTP was elicited first in the test slice (exposed to CGS 21680) while in others it was first induced in control slices, with similar results. To save recording time,

exposure to CGS 21680 for 10 min started immediately after going to whole cell configuration; the agonist was then removed from the bath and LTP induced 20-30 min after. Amplitude EPSC values recorded for 10 min before LTP induction were normalized to 100% and were 98.4±8.6 pA in cells that had been pre-exposed to CGS 21680, and 111±16.5 pA in control ones. In the right panel are shown superimposed current tracings of EPSCs recorded before (1,2) and 40-50 min after induction of LTP (3,4), from control (1,3; ◊) and CGS 21680-exposed cells (2,4; ♦). Note that the magnitude of LTP was significantly higher (D) when a 10 min superfusion of CGS 21680 preceded delivery of the LTP-inducing protocol. Values are mean ± SEM. * $p<0.05$ (two-tailed paired *t* test, compared with baseline); δ $p<0.05$ (two-tailed unpaired *t* test, compared with CGS 21680 effect upon AMPA-PSCs); ϕ $p<0.05$ (two-tailed paired *t* test, compared with control LTP).

Given that high-frequency neuronal stimulation leads to synaptic release of the adenosine precursor, ATP (Wieraszko et al., 1989), which leads to extracellular accumulation of adenosine favoring A_{2A} receptor activation (Cunha et al., 1996; Correia-de-Sá et al., 1996), we further hypothesized that an endogenous A_{2A} receptor-mediated potentiation of AMPA function could impact upon LTP expression. We therefore evaluated the influence of the selective A_{2A} receptor antagonist, SCH 58261 (100 nM) upon LTP. These experiments were conducted in the presence of a $GABA_A$ receptor blocker (bicuculline, 20 μM) which, by precluding inhibition of pyramidal cells by GABAergic neurons, allowed the expression of a more robust LTP, being therefore a suitable protocol to evaluate a putative tonic LTP inhibition. LTP was induced as above (Figure 5.1.11). Under these conditions, an 87.2±19% increase in EPSC amplitude was observed in control slices 40–50 min after stimulus delivery (n=4, Figure 5.1.12), a facilitation that was significantly different from that observed in the absence of bicuculline (Figure 5.1.12B). In contrast, in conditions of continuous superfusion of A_{2A} receptor antagonist (SCH 58261, 100 nM), the magnitude of LTP was found to be significantly lower than

that observed in control conditions (21.8±6.4% increase, 40-50 min after induction; n=4, Figure 5.1.12A,C).

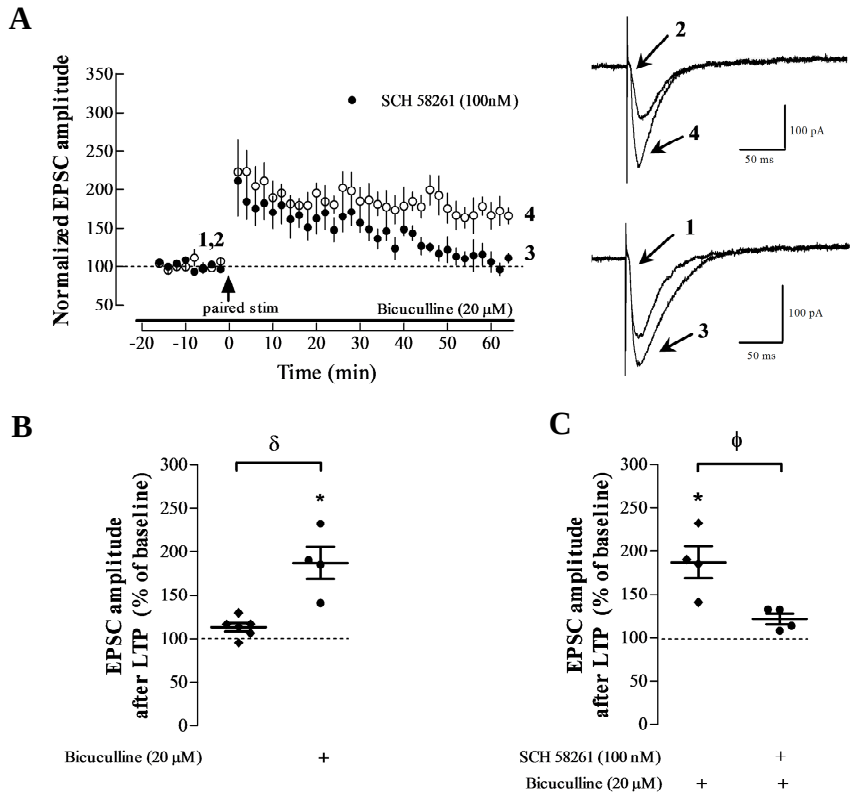


Figure 5.1.12 Endogenous modulation of LTP expression by A_{2A} receptor activation. (A) Averaged time-course and current tracings of EPSC amplitude changes induced by the same pairing LTP protocol in the presence of bicuculline (20 µM), from control slices (○) and those continuously exposed to a selective A_{2A} antagonist, SCH 58261 (●). Baseline amplitude EPSC values were 132±25.9 pA in cells exposed to SCH 58261, and 123±29.6 pA in control ones. For each n, LTP in the presence or absence of the A_{2A} receptor antagonist was tested in two slices from the same hippocampus; in some experiments LTP was elicited first in the test slice (exposed to SCH 58261) while in others it was first induced in control slices, with similar results. (B) LTP elicited in the presence of bicuculline was significantly higher than that obtained by the same protocol, in its absence. (C) A_{2A} receptor blockade significantly diminishes LTP expression, in the presence of bicuculline. Values are mean ± SEM. * $p<0.05$ (two-tailed paired *t* test, compared with baseline, using absolute current values); δ $p<0.05$ (two-tailed unpaired *t* test, compared with LTP elicited in the absence of bicuclline); ϕ $p<0.05$ (two-tailed paired *t* test, compared with control LTP).

Taken together, these results show that not only can an exogenously applied A_{2A} receptor agonist enhance LTP, but also and more importantly, its expression is considerably diminished by A_{2A} receptor blockade, highlighting an important endogenous regulatory role of adenosine A_{2A} receptors upon LTP. In addition, the fact that A_{2A} receptor-mediated changes in LTP expression were observed in the presence of a $GABA_A$ receptor blocker, allows us to exclude the possibility that these changes might arise from modifications in the GABAergic circuits of the hippocampus.

Discussion

The main findings that arose from the work described in this chapter were that activation of adenosine A_{2A} receptors postsynaptically enhances AMPA receptor mediated responses in the hippocampus, by means of a PKA-dependent mechanism, and that this correlates with increases in the surface expression of GluR1 subunits phosphorylated at the Ser 845 residue, as well as with enhancements in synaptic transmission and plasticity. After first evidence that adenosine A_{2A} receptors at the hippocampus can enhance synaptic transmission (Sebastião and Ribeiro, 1992), subsequent studies mostly assume that excitatory actions following A_{2A} receptor activation derive from an attenuation of A_1 receptor-mediated inhibition of excitatory transmission (Cunha et al., 1994). Indeed, this kind of interaction is known to underlie A_{2A} receptor-mediated facilitation of glutamate release (Lopes et al., 2002). In order to investigate whether such an interaction would also apply for adenosine receptor-mediated tuning of the postsynaptic responsiveness to glutamate release (which is, at the resting potential, mostly mediated by AMPA receptors), patch-clamp recordings of AMPA-evoked currents or afferent-evoked excitatory synaptic currents (EPSCs) were performed and its modulation by A_{2A} receptors, analyzed.

Our results show that adenosine A_{2A} receptors postsynaptically facilitate AMPA receptor functioning and phosphorylation, an action that does not require de novo protein synthesis, thus suggestive of enhanced surface expression of already existing receptors. The PKA dependency of these effects could be concluded from the loss of effect of the A_{2A} receptor agonist in cells loaded with a PKA inhibitor, as well

as from the CGS 21680-induced increase in the expression of membrane GluR1 subunits phosphorylated at the PKA site. Furthermore, postsynaptic PKC inhibition did not significantly affect the facilitation of AMPA currents by CGS 21680. However, A_{2A} receptors can couple to different G proteins (Fredholm et al., 2001) and the signal transducing pathway operated may depend on both the nature of the effector system and the availability of G proteins and kinases in the receptor's vicinity. Accordingly, presynaptic PKA- (Cristóvão-Ferreira et al., 2009) and PKC- mediated (Lopes et al., 2002; Pinto-Duarte et al., 2005) A_{2A} receptor actions have been identified in the hippocampus.

Blockade of A_{2A} receptors prevented the facilitatory action of CGS 21680 on AMPA currents but was devoid of effect when added in its absence. This may imply that (1) under the superfusion conditions used, endogenous extracellular adenosine, through A_{2A} receptor activation, was not tonically modulating AMPA receptors or (2) any pre-existing modulation by endogenous adenosine is hard to revert by later addition of the antagonist. The second possibility seems plausible since the consequences of A_{2A} receptor activation with the exogenous agonist, CGS 21680, were hardly reversible. Furthermore, even a brief exposure to the agonist was enough to trigger a sustained facilitation of AMPA receptor-mediated responses. This long lasting modulation of AMPA currents might be particularly relevant in conditions of coincident transient increases in the extracellular levels of adenosine and glutamate, such as occurs during high-frequency neuronal firing, when A_{2A} receptors could provide a positive feedback loop to reinforce glutamate-induced plasticity.

Previous evidence for an enhancement of LTP in the CA1 area after A_{2A} receptor activation has already been reported (de Mendonça and Ribeiro, 1994), as adenosine deaminase (that degrades endogenous extracellular adenosine into inosine) failed to affect LTP, which was facilitated by A_1 receptor blockade. Similarly, Forghani and Krnjevic (1995) found that a mixed A_1/A_2 adenosine receptor antagonist (8(p-sulfophenyl)theophylline) did not facilitate LTP, despite the fact that perfusion of a selective A_1 receptor antagonist did enhance it. It was, therefore, proposed that A_2 receptor activation by endogenous adenosine was counteracting tonic A_1 receptor mediated inhibition of LTP. Others reported that A_2 receptor antagonists can depress CA1 hippocampal LTP of fEPSPs (Sekino et al., 1991; Fujii et al., 2000), suggestive of a tonic facilitatory action by endogenous adenosine. Accordingly, in the nucleus accumbens, A_{2A} receptor blockade and receptor deletion have been shown to impair LTP (D'Alcantara et al., 2001). A_{2A} receptors are also essential for high-frequency induced LTP of NMDA EPSCs at the CA3 hippocampal area (Rebola et al., 2008). Being established that A_{2A} receptor activation facilitates plasticity phenomena, our goal with the experiments now described was not to exhaustively evaluate this process but, instead, to know if conditions that lead to an A_{2A} receptor-mediated increase in the membrane expression of GluR1 subunits and postsynaptic AMPA receptor function in the CA1 area, also caused LTP facilitation. Most noticeable, we could observe LTP potentiation even after a brief exposure to the A_{2A} receptor agonist prior to LTP induction, and the effect was particularly evident at late time points of recording, consistent with the relevance of AMPA receptor membrane insertion for LTP expression and consolidation (Chowdhury et al., 2006; Yang et al., 2008b). A

relevant contribution of endogenous A_{2A} receptor activation can be concluded from the present findings that A_{2A} receptor blockade leads to an attenuation of LTP, which was also more evident at late time points of recording, again indicating that the role of A_{2A} receptors is more related to facilitation of LTP expression and consolidation than to LTP induction. Changes in LTP might reflect in learning. Interestingly, blockade of A_{2A} receptors in mice leads to associative learning impairment (Fontinha et al., 2008).

Receptor delivery to extrasynaptic sites, followed by lateral diffusion towards synaptic localizations, is a crucial step for synaptic reinforcement and plasticity (Triller and Choquet, 2005). Strikingly, the extrasynaptic pool of AMPA receptors in CA1 pyramidal neurons is almost exclusively composed of GluR1-containing receptors, which are also a significant part of the synaptic pool, thus playing a major role in synaptic strength regulation (Andrasfalvy et al., 2003). The finding that CGS 21680 caused a more pronounced facilitation of AMPA-evoked currents (accounting for synaptic, perisynaptic and extrasynaptic AMPA currents) than afferent-evoked EPSCs (mostly synaptic currents) is suggestive of a facilitation of both perisomatic and extrasynaptic AMPA receptors by A_{2A} receptors, which have been shown to be expressed in these fractions (Lee et al., 2003b; Rebola et al., 2005b). Furthermore, phosphorylation of GluR1 subunits at Ser 845 significantly correlates with selective delivery of GluR1-containing AMPA receptors to extrasynaptic sites (Oh et al., 2006). In light of this, our data suggests that A_{2A} receptors, through PKA activation and subsequent GluR1 phosphorylation, may facilitate AMPA receptor delivery to extrasynaptic sites, therefore priming a step that reinforces LTP.

Being clear from the present results that A_{2A} receptors promote facilitation of AMPA currents, enhance surface expression of GluR1 containing AMPA receptors and facilitate LTP, it has to be pointed out that enhanced surface expression of AMPA receptors might not be the sole mechanism by which A_{2A} receptors affect LTP. Indeed, CA1 LTP is a complex phenomenon that involves pre- and postsynaptic processes, requiring activation of NMDA receptors followed by increased cytoplasmatic calcium levels and subsequent activation of different transducing systems, including PKA and CamKII (Esteban et al., 2003); ultimately leading to changes in AMPA receptor expression at synaptic sites (Malenka and Nicoll, 1999). So, any influence of A_{2A} receptors in these steps might result in changes in LTP. Direct presynaptic mechanisms are unlikely since the A_{2A} receptor agonist did not affect mEPSC frequency, which agrees with previous reports that in very young animals A_{2A} receptors do not directly influence glutamate release (Lopes et al., 2002).

Modulation of NMDA receptor function by A_{2A} receptors in CA3 pyramidal neurons, with consequences for plasticity induced by mossy fiber stimulation, has been reported (Rebola et al., 2008). This LTP of NMDA-EPSCs also requires activation of mGluR5 receptors, with which A_{2A} receptors colocalize and functionally interact, by playing a permissive role in mGluR5 receptor-mediated potentiation of NMDA effects in the hippocampus (Tebano et al., 2005). Additionally, endogenous A_{2A} receptor activation, through a PKA-dependent process, triggers Brain-derived neurotrophic factor (BDNF) facilitatory influences upon CA1 LTP (Fontinha et al., 2008; Assaife-Lopes et al., 2010), being well established that hippocampal LTP is strongly impaired in both BDNF (Patterson et al., 1996) and TrkB (Minichiello

et al., 1999) knockout mice. While it is not possible, with the work herein described, to ascertain the extent to which any of these mechanisms (and possibly others) contributes to the A_{2A} receptor-induced modulation of LTP, it seems highly unlikely that it should be unrelated to the PKA-dependent enhancement of extrasynaptic AMPA receptor reserve and GluR1 externalization.

Gao et al. (2006) found that D1 dopamine receptor activation increases the size of the GluR1 extrasynaptic pool in a PKA-dependent way, which significantly promoted LTP in cultured hippocampal neurons. D1 dopamine receptors, like adenosine A_{2A} receptors, are positively coupled to adenylate cyclase. However, dopaminergic inputs to the hippocampus are scarce (Lopes da Silva et al., 1990), while adenosine is present and released by glia and neurons, from pre-, post-, and nonsynaptic sites. Furthermore, extracellular adenosine can also be formed from the catabolism of adenine nucleotides, known to be released together with neurotransmitters and in particular during high-frequency neuronal firing (see Sebastião and Ribeiro, 2009). Therefore, the present findings that A_{2A} receptor activation enhances AMPA evoked currents, GluR1 membrane expression and LTP, allow the identification of a modulator of AMPA receptor function that is ubiquitous at the extracellular space, in particular at synapses firing at high frequency, under conditions particularly prone for synaptic reinforcement. One can therefore propose that adenosine is one of the endogenous substances responsible for regulation of GluR1 Ser-845 phosphorylation tonus and hence, for the reserve of GluR1-containing AMPA receptors at extrasynaptic pools, priming them for synaptic insertion and reinforcement of synaptic strength.

5.2 A_{2A} Adenosine receptor activation Modulates Ischemia-induced Plasticity in the CA1 area

Rationale:

Adenosine, through activation of A_{2A} receptors, can affect delivery of AMPA receptors to the membrane, with impact for plasticity phenomena (chapter 5.1). Initially, the enhancement in glutamate and ATP release that characterizes the onset of an ischemic insult is not fundamentally different from the increased synaptic activity conditions that trigger physiological LTP. Accordingly, many of the cellular scaffolds of LTP expression, such as changes in the synaptic AMPA receptor population (Malenka and Nicoll., 1999), have also been found to occur after ischemic insults (Quintana et al., 2006; Dixon et al., 2009) and have even been proposed to be key determinants for neuronal fate (Pellegrini-Giampetro et al., 1997). Further, ischemic events are known to influence the biochemical pathways that are required for translating transient calcium signals into persistent increases in synaptic strength (Di Filippo et al., 2008). In line with this, there is ample evidence that oxygen/glucose deprivation exerts long-term effects on the efficacy of synaptic transmission, by triggering a post-ischemic long-term potentiation phenomenon (i-LTP), which may serve to shape redundant connectivity into new functional and structural circuits, capable of recovering specific functions (Murphy and Corbett, 2009). Perilesional areas, where blood flow is less severely impaired as a result of an ischemic stroke, are thought to be

particularly suitable for recovery through plastic rewiring (Di Filippo et al., 2008; Murphy and Corbett, 2009). In the context of middle cerebral artery (MCA) occlusion (the most frequent cause for ischemic stroke in humans), such perilesional areas correspond to structures which, although not directly supplied by the occluded vessel, are nonetheless affected by blood flow restriction, in a delayed manner. In accordance with this, experimental MCA occlusion most severely affects the temporal and parietal cortices, the basal ganglia and the internal capsule, but also and less severely, nearby structures such as the hippocampus (Popp et al., 2009). Such perilesional areas have been the focus of neuroprotective approaches aiming at reducing delayed cell death in the aftermath of an ischemic stroke. Recently, it has been proposed that neuroregenerative strategies, focusing on rehabilitative physical therapy, post-injury enhancement of neurogenesis and pharmacological modulation of ischemia-induced plasticity, may constitute a viable option to neuroprotective approaches (Murphy and Corbett, 2009). This prompts the need to better characterize the extent to which ischemia-induced plasticity relies upon the cellular mechanisms underlying physiological LTP, which can hopefully serve to identify candidate regulatory molecules of therapeutic interest.

Bearing in mind that ischemia triggers the release of high amounts of adenosine (Dunwiddie and Masino, 2001), as well as plastic remodeling of excitatory synaptic contacts (Di Filippo et al., 2008), I hypothesized that neuromodulation by A_{2A} receptors might impose changes in synaptic transmission after transient oxygen and glucose deprivation. Furthermore, the experimental work described in this chapter pursued the requirement of alterations in postsynaptic AMPA

receptor functioning for ischemia-induced tuning of synaptic transmission efficiency. Experiments were therefore performed on acute hippocampal slices, subjected to brief oxygen and glucose deprivation insults, while continuously recording AMPA receptor-mediated afferent evoked currents (EPSCs) from CA1 pyramidal neurons.

Transient oxygen-glucose deprivation triggers changes in afferent-evoked excitatory transmission

The effects of a transient ischemic episode on excitatory synaptic transmission were studied in CA1 pyramidal cells, by switching the extracellular, superfusion solution, to one that mimicked ischemia conditions, for a 10 min period (protocol adapted from Rossi et al., 2000). After allowing afferent-evoked responses to stabilize, application of such a transient ischemic insult caused a marked reduction in EPSC amplitude, which reached a 85±3.7% decrease from baseline values within 10 min ($n=14$, $P<0.05$, Figure 5.2.1C). Upon re-oxygenation, there was a gradual recovery of synaptic responses such that 50-60 min after intiating the ischemic insult, a rebounding excitation led average EPSC amplitudes to be 157±17.4% of baseline values ($n=7$, $P<0.05$, Figure 5.2.1D). These findings are in line with those previously observed in organotypic hippocampal slice cultures, where administration of a similar in vitro ischemia protocol resulted in a comparable lasting enhancement of excitatory transmission (Quintana et al., 2006).

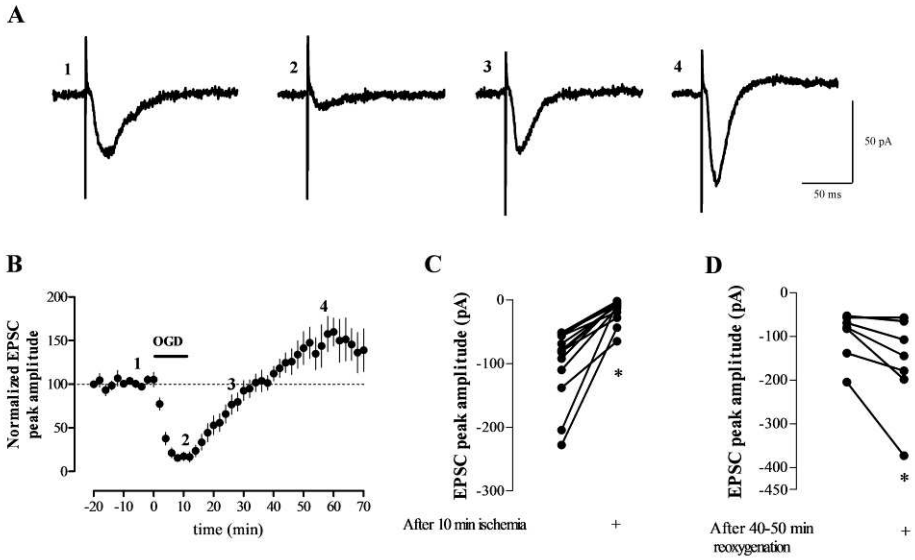


Figure 5.2.1. Transient in vitro ischemia causes a significant increase in afferent-evoked Excitatory Postsynaptic Current (EPSC) amplitude. (A) EPSC current tracings from a representative CA1 pyramidal cell illustrate the inhibition of baseline synaptic responses (1) after delivery of a transient ischemic episode (2), as well as signal recovery upon reoxygenation (3) which even leads to an increase in EPSC amplitude (4), when compared to baseline values. (B) Averaged time-course of EPSC peak amplitude changes caused by brief ischemia (induced by replacing 10 mM glucose-containing aCSF with that containing 7mM sucrose plus 3mM glucose, gassed with 95%N_2/5% CO_2, for 10 min). The time-points depicted refer to those illustrated by representative tracings in (A). Inhibition of synaptic responses was measured in 14 cells and was found to be significant, when compared to absolute baseline EPSC values - calculated from the 10-20 min period recorded before ischemia delivery (C). Initial experiments performed to characterize the ischemia protocol did not extend until 50-60 min of recording, but for the 7 cells allowed to proceed thus far and which remained stable until this time-point, the increase in EPSC amplitude was found to be significant, when compared to absolute baseline values (D). Values on the left columns (C,D) represent individual EPSC peak amplitude values recorded in the baseline period, whereas those on the right refer to individual current values recorded either after 10 min ischemia (C) or after 40-50 min reoxygenation (50-60 min period in (B)). * $p<0.05$ (two-tailed paired *t* test, compared with absolute baseline values).

Furthermore, when routinely superfusing a selective AMPA receptor blocker (CNQX, 50 μM) at the end of the recording period, virtually all of the recorded synaptic response was abolished (Figure 5.2.2). This indicates that for these experimental conditions (holding potential of -

70 mV and in the presence of 1mM external Mg^{2+}), most of the EPSC response corresponds to the AMPA-mediated postsynaptic component of synaptic transmission.

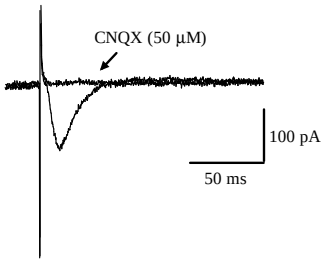


Figure 5.2.2. Afferent-evoked EPSCs mainly comprise a postsynaptic AMPA component. EPSC current tracings from a representative CA1 pyramidal cell illustrate how superfusion of a selective AMPA receptor antagonist (CNQX, 50 μM) completely abolishes evoked synaptic responses.

Ischemia-induced facilitation of afferent-evoked synaptic responses requires calcium-permeable AMPA receptors.

In CA1 pyramidal cells, GluR2-lacking, calcium permeable AMPA receptors have been show to be transiently incorporated into synapses upon LTP induction and their function has been proposed to be required for LTP consolidation (Plant et al., 2006; Guire et al., 2008). Reports of a similar switch in synaptic AMPA receptor subunit composition after in vitro ischemia have recently emerged (Dixon et al., 2009), although its significance for ischemia-induced plastic changes was not pursued. To test whether the increase in EPSC amplitude which was elicited by a brief ischemic insult in our experimental conditions might be dependent upon GluR2-lacking AMPA receptors, experiments in which the pipette solution contained spermine were performed. Positively charged spermine (and to a lesser extent, other poylamines such as spermidine) selectively blocks GluR2-lacking receptors, because it is attracted by the negatively charged ring of carbonyl-oxygen groups present in the glutamine residues of GluR1,

GluR3 and GluR4 subunits. In contrast, spermine is repelled by the positively charged arginine residue in GluR2 subunits and will thus not bind GluR2-containing receptors (reviewed by Pellegrini-Giampetro, 2003). In whole-cell configuration, dialysis of endogenous polyamines occurs within the first 5-10 minutes of recording, thereby reducing their ability to inhibit calcium-permeableAMPA receptors (Kamboj et al., 1995). A strategy that allows circumventing this problem is to include exogenous spermine in the pipette solution (e.g., Donevan and Rogawski, 1995). Indeed, 100 μM of intracellular spermine have been reported to block GluR2-lacking AMPA receptor channels by 18–36% at resting potentials, with the proportion of blocked channels increasing with membrane depolarization (Bowie and Mayer, 1995). When spermine (500 μM) was added to the intracellular solution, transient ischemia caused an average 82±3.9% depression of EPSC amplitude (n=9, Figure 5.2.3B), a similar value to that observed in the absence of spermine (Figure 5.2.1B). However, even though synaptic responses gradually recovered toward baseline values, no facilitation of afferent-evoked responses was observed upon 40-50 min of reoxygenation, unlike what occurred in the absence of spermine (n=6, $P<0.05$, Figure 5.2.3C).

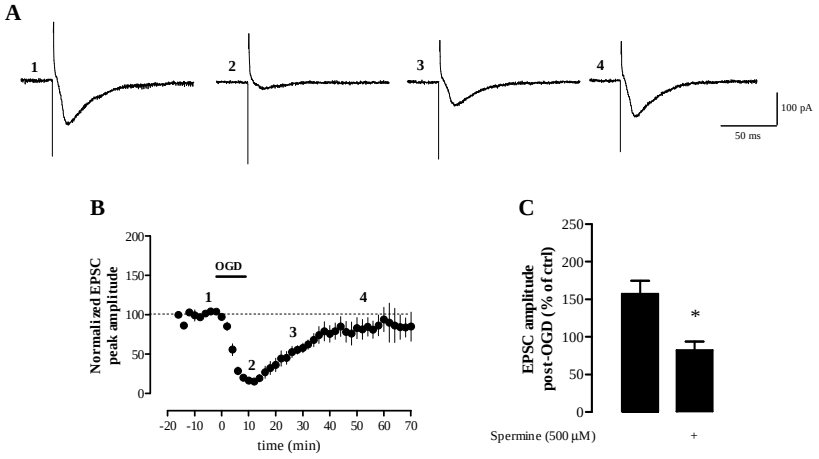


Figure 5.2.3. Intracellular spermine prevents ischemia-induced increase in afferent evoked EPSCs. (A) EPSC current tracings from a representative CA1 pyramidal cell illustrate the inhibition of baseline synaptic responses (1) after delivery of a transient ischemic episode (2), as well as signal recovery upon reoxygenation (3,4), in the presence of internal spermine (500 µM). (B) Averaged time-course of EPSC peak amplitude changes caused by brief ischemia, induced as before. The time-points depicted refer to those illustrated by representative tracings in (A). Average EPSC values measured after 40-50 min reoxygenation in the presence of internal spermine, were significantly smaller than those obtained in control conditions (C). Values are mean±SEM of EPSC amplitudes recorded after 40-50 min of reoxygenation. * $P<0.05$ (two-tailed unpaired t-test; compared to EPSC recovery measured with a control pipette solution).

These findings support the notion that activation of GluR2-lacking, calcium permeable AMPA receptors is required for ischemia-induced facilitation of excitatory synaptic transmission in the hippocampus, suggesting this is another cellular mechanism utilized by both forms of plasticity. Taking into account the role of A_{2A} receptor activation for surface expression of AMPA receptors and its influence upon LTP (chapter 5.1) and that the impairment in energy metabolism triggered by ischemia leads to increased extracellular adenosine levels, I next evaluated whether activation of A_{2A} adenosine receptors could account

for the enhancement of excitatory synaptic transmission observed after brief ischemia.

Blockade of A_{2A} receptors prevents ischemia-induced facilitation of afferent evoked synaptic responses.

When a selective A_{2A} receptor antagonist was added at least 20 min before inducing ischemia and was present throughout the remaining experiment, transient ischemia caused an average 85±3.3% depression of EPSC amplitude (n=7, Figure 5.2.4B), a similar value to that observed in control conditions (Figure 5.2.1B). However, and as it was observed in the presence of internal spermine, no facilitation of afferent-evoked responses was obtained upon 40-50 min of reoxygenation (n=6, P<0.05, Figure 5.2.4C).

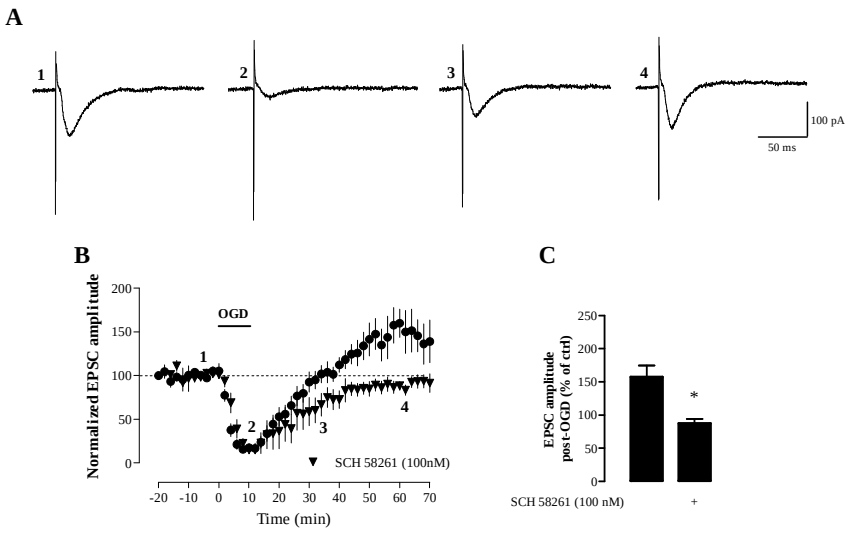


Figure 5.2.4. A_{2A} receptor blockade prevents ischemia-induced increase in afferent evoked EPSCs. (A) EPSC current tracings from a representative CA1 pyramidal cell illustrate the inhibition of baseline synaptic responses (1) after delivery of a transient ischemic episode (2), as well as signal recovery upon reoxygenation (3,4), in the presence of a selective A_{2A} receptor antagonist (SCH 58261, 100 nM). (B) Averaged time-course of EPSC peak amplitude changes caused by brief ischemia in the presence (▼) or absence (●) of SCH 58261 (100 nM). The

time-points depicted refer to those illustrated by representative tracings in (A). Average EPSC values measured after 40-50 min reoxygenation in the presence of the A_{2A} receptor antagonist were significantly smaller than those obtained in control conditions (C). Values are mean±SEM of EPSC amplitudes recorded after 40-50 min of reoxygenation. * P<0.05 (two-tailed unpaired t-test, compared to EPSC recovery measured in the absence of the A_{2A} receptor antagonist).

In the same experimental conditions (SCH 58261 applied throughout the experiment) but in the concurrent presence of internal spermine, EPSC recovery upon 40-50 min of reoxygenation was not significantly different from that obtained in the absence of internal polyamines (n=4; P>0.05, Figure 5.2.5C).

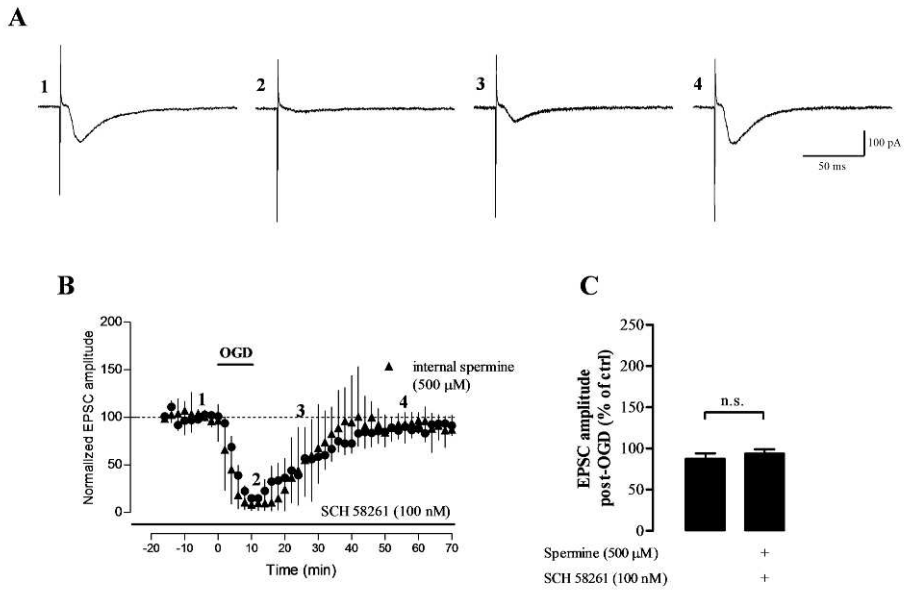


Figure 5.2.5. Prevention of ischemia-induced facilitation of EPSC amplitude by A_{2A} receptor blockade is preserved in the presence of internal spermine. (A) EPSC current tracings from a representative CA1 pyramidal cell illustrate the inhibition of baseline synaptic responses (1) after delivery of a transient ischemic episode (2), as well as signal recovery upon reoxygenation (3,4), in the presence of a selective A_{2A} receptor antagonist (100 nM) and in the concurrent presence of internal spermine (500 μM). (B) Averaged time-course of EPSC peak amplitude changes caused by brief ischemia in the presence of SCH 58261 and in the presence (▲) or absence (●) of internal spermine. The time-points depicted refer to those illustrated by representative tracings in (A). EPSC recovery after 40-50 min of reoxygenation was not

significantly different between experimental conditions (C). Values are mean±SEM of EPSC amplitudes recorded after 40-50 min of reoxygenation. n.s. P>0.05 (two-tailed unpaired t-test, compared to EPSC recovery in the presence of SCH 58261 but in the absence of internal spermine).

It thus seems that either blocking calcium-permeable AMPA receptors with internal spermine, as well as preventing activation of adenosine A_{2A} receptors during and after ischemia has the same effect upon EPSC recovery as does simultaneously blockade of both kinds of receptors. These findings suggest that either 1) A_{2A} receptor blockade and internal spermine block ischemia-induced facilitation of EPSC amplitude by acting upon the **same** cellular process or 2) blockade of one kind of receptor maximally impairs ischemia-induced EPSC facilitation, such that further blockade of the second receptor type exerts a **redundant** effect, even though it may act through an independent mechanism. Sequential effects in consecutive steps, with the same final functional consequences, may also occur. For instance, the above results would be compatible with a sequential interference of the A_{2A} receptor antagonist and internal spermine in the externalization, synaptic delivery and activation of calcium-permeable AMPA receptors. According to this hypothesis, A_{2A} receptor activation would first regulate delivery of GluR1-containing AMPA receptors to extrasynaptic membrane pools, which, upon recruitment to the postsynaptic density, would then be amenable to blockade by internal spermine.

Discussion

The main findings that arose from the work described in the present chapter are that activation of calcium-permeable AMPA receptors is required for ischemia-induced facilitation of excitatory synaptic transmission in the hippocampus, which also depends upon adenosine A_{2A} receptor activation.

It has long since been known that global ischemia, leading to delayed cell death in the CA1 area, can suppress GluR2 subunit mRNA and protein expression, thus increasing AMPA receptor-mediated Ca^{2+} influx into vulnerable neurons (Liu and Zukin, 2007). Furthermore, experimental downregulation of GluR2 expression (by knockdown experiments) is sufficient to induce delayed death of CA1 neurons (Oguro et al., 1999). However, although there is considerable evidence for alterations in AMPAR subunit composition and function from hours or even days after injury, receptor changes at early times after ischemia remain unclear. Indeed, little is known about changes in the AMPA receptor synaptic population that may underlie the plasticity phenomena triggered by ischemia, although strong evidence supports that i-LTP shares quite a few other hallmarks of physiological LTP (Di Filippo et al., 2008).

Recently, Guire and colleagues have proposed that in the CA1 area, a transient addition of calcium-permeable AMPA (homomeric GluR1) receptors to the synaptic population, even when only accounting for ~5% of the existing synaptic population, is enough to produce the potentiation observed after LTP (Guire et al., 2008). In fact, GluR2-lacking AMPA receptors have significantly higher channel conductance

than do GluR2-containing receptors (Swanson et al., 1997) and such changes in channel properties are one of the mechanisms by which synaptic AMPA receptor-mediated responses can be potentiated (Derkach et al., 2007). It is also well established that CA1 pyramidal neurons do not contain calcium-permeable AMPA receptors in basal conditions (Monyer et al., 1991; Wenthold et al., 1996) and their rapid insertion in the synapse during LTP most likely relies upon prompt lateral diffusion of GluR1-containing calcium-permeable receptors from extrasynaptic pools (Guire et al., 2008). The present finding that ischemia-induced enhancement of excitatory synaptic transmission is abolished by internal spermine (loaded into the postsynaptic neuron at a concentration capable of blocking a significant proportion of calcium-permeable AMPA receptors) constitutes, to the best of my knowledge, the first evidence of a very early common feature between i-LTP and its physiological counterpart (i.e., synaptic activation of GluR2-lacking AMPA receptors). These results also highlight an important postsynaptic component in ischemia-induced facilitation of synaptic transmission, since it was impaired by loading of spermine onto the postsynaptic neuron alone.

Noteworthy, although the present results implicate activation of calcium-permeable AMPA receptors in ischemia-induced facilitation of excitatory transmission, they give no detailed information about the time-course according to which synaptic recruitment of these receptors may occur. This question could be addressed by using patch-clamp recordings of spontaneous miniature excitatory postsynaptic currents (mEPSCs), to follow changes in synaptic calcium-permeable AMPA receptor content over time, after ischemia. In fact, because calcium-

permeable AMPA receptors exhibit more rapid deactivation kinetics than GluR2-containing ones (Grosskreutz et al., 2003), a decrease in average mEPSC decay time (dependent on the deactivation kinetics of the synaptic AMPA receptor population) can be taken to account for an increase in the number of calcium-permeable AMPA receptors present at synapses (Guire et al., 2008). The data to be obtained with such experiments may help to elucidate whether ischemic insults of a greater severity can trigger the sustained recruitment of calcium-permeable AMPA receptors, in contrast to their transient incorporation in physiological LTP-inducing conditions or in contrast to what may happen when ischemia is less severe. If confirmed, this would explain why in some conditions transient ischemia unleashes delayed death by excitotoxicity, while in others it triggers plastic changes compatible with a neuroregenerative rewiring of redundant circuitry, enabling recovery of lost synaptic contacts (Murphy and Corbett, 2009). It is known that in physiological conditions, neuronal activity regulates the subunit composition of synaptic AMPA receptors by a homeostatic plasticity process, according to which repetitive activity or sensory experience favors synaptic incorporation of calcium-impermeable AMPA receptors (downregulating synaptic strength), while activity blockade leads to incorporation of calcium-permeable receptors (thus scaling up synaptic transmission). In line with this, synaptic insertion of GluR1 homomeric AMPA receptors has been proposed to be required for subsequent synaptic recruitment of GluR2-containing AMPA receptors, so as to counteract increased calcium permeability (see Pozo and Goda, 2010). It is reasonable to expect that failure of this auto-regulatory mechanism can lead to sustained changes in synaptic AMPA receptor composition after severe ischemia, which would then render

neurons more susceptible to death by excitotoxicity (Pellegrini-Giampetro et al., 1997).

It should be noted that endogenous polyamines can also modulate other types of cation channels, such as inwardly rectifying potassium channels and NMDA receptors (reviewed by Williams, 1997). In fact, intracellular spermine contributes to intrinsic gating and rectification properties of strong inward rectifier K^+ channels and its addition to the pipette solution can be used to maintain the rectifying character of these currents (Ficker et al., 1994). Also, spermine can potentiate NMDA receptor activity, by acting on at least two extracellular polyamine binding sites (Williams et al., 1997). Still, since changes in inward rectifying potassium channels are mostly expected to affect the resting potential of the neuron, any putative resulting effect of internal spermine at this level should be controlled by having performed all ischemia experiments under voltage-clamp mode. In what concerns interactions with NMDA receptors, although primarily active at extracellular sites, high concentrations of intracellular spermine (1-10 mM) can also reduce unitary conductance through recombinant NMDA receptors at positive potentials (Araneda et al., 1999). However, even the highest concentration used (10 mM) by others brought about only a minimal reduction in current measured at negative membrane potentials (Araneda et al., 1999).

The role of A_{2A} receptors in ischemia remains a controversial one (Jacobson and Gao, 2006; de Mendonça et al., 2000), but inequivocal evidence for their deleterious role in cerebral ischemia arose from the observation that both cerebral infarction and neurological deficits were attenuated in A_{2A} receptor knock-out mice mice subjected to temporary

middle cerebral artery occlusion, when compared with wild-type littermates (Chen et al., 1999b). In what concerns the mechanisms underlying protection from ischemia-induced damage in the presence of A_{2A} receptor antagonists, it has been proposed that they may rely upon an impairment of glutamate release facilitation (Simpson et al., 1992), but also in modulation of postsynaptic glutamate receptor function (Pugliese et al., 2009). Loading of the postsynaptic neuron with spermine (which blocks GluR2-lacking, calcium-permeable AMPA receptors) had the same effect upon EPSC recovery and facilitation after ischemia, as did A_{2A} receptor blockade with a selective antagonist. As mentioned, this could be explained by A_{2A} receptor-mediated regulation of GluR1-containing AMPA receptor delivery to extrasynaptic membrane pools, which, upon recruitment to the postsynaptic density, would subsequently be amenable to blockade by internal spermine. While it would be tempting to speculate that prevention of ischemia-induced facilitation of EPSCs may rely upon activation of PKA signaling with consequences for membrane insertion of GluR1-containing AMPA receptors, such a claim warrants further experiments, resourcing first to postsynaptic loading of PKA inhibitors. Secondly, a direct assessment of A_{2A} receptor-mediated regulation of the calcium-permeable AMPA receptor pool available for synaptic recruitment could be achieved by comparing ischemia-induced changes in mEPSC decay time, in the presence and absence of selective A_{2A} receptor antagonists.

From the present results, it is not possible to conclude whether A_{2A} receptor activation plays a deleterious or a protective role from the ischemic insult used. It would first be necessary to elucidate whether or

not the observed ischemia-induced increase in excitatory transmission efficiency is detrimental, by following neuron outcome upon the initial insult, for a longer timescale. Modulation of neuronal viability by A_{2A} receptors could then be pursued by performing the same ischemia protocol, in the presence or absence of selective antagonists. These experiments could be performed on organotypical slices or cultured hippocampal cells, but with caution directed toward its limitations. Indeed, if dissociate neuronal cultures lack network architecture typical of the intact brain and a physiological balance between excitatory and inhibitory transmission (Pozo and Goda, 2010), organotypic slices gradually adapt their circuitry to the absence of physiological input and output structures, which originates abnormal reverberating circuits. In fact, plastic compensatory plasticity can itself become detrimental and even represent a cause for acquired epilepsy. A well documented example refers to injury-induced axonal sprouting in hippocampal dentate granule cells and the consequent formation of recurrent excitatory synapses, which is thought to convert them into an epileptogenic population of neurons, capable of promoting seizure initiation and/or propagation through the hippocampal pathway (reviewed by McNamara, 1999).

Despite all the important questions that wait to be answered, the herein presented results strongly support a major role for A_{2A} receptor activation in ischemia-induced facilitation of synaptic excitatory responses, highlighting their role in i-LTP, which adds to the one they play in physiological LTP (chapter 5.1).

5.3 Crosstalk between immunoregulatory cytokines of the Interleukin-6 family and neuronal adenosine A_1 receptor function: implications for synaptic transmission regulation and neuroprotection from excitotoxic damage

Rationale:

Depression of synaptic transmission during hypoxic/ischemic insults is at least in part mediated by a marked increase in the extracellular adenosine concentration and consequent activation of A_1 adenosine receptors (Fowler, 1989). Extracellular adenosine can tune down neuronal activity by at least three cellular mechanisms: presynaptic inhibition of neurotransmitter release; postsynaptic inhibition of calcium influx (through NMDA receptors and voltage-dependent calcium channels) and activation of G protein-dependent inwardly rectifying K^+ channels that mediate postsynaptic membrane hyperpolarization (De Mendonça et al., 2000; Dunwiddie and Masino, 2001). By reducing metabolic demand, adenosine thus preserves ATP stores, while also supressing glutamatergic transmission, ultimately protecting neurons from excitotoxicity. Extracellular adenosine is thought to exert these acute neuroprotective actions mainly through activation of high-affinity A_1 receptors. Indeed, A_1 receptor blockade has been shown to reduce depression of synaptic transmission during prolonged hypoxia and to prevent transmission recovery upon reoxygenation (Sebastião et al., 2001). However, as mentioned before (Introduction, subchapter 1.3.2), clinical translation of the consistent neuroprotection afforded by A_1 receptor agonists in animal models of

ischemia has been made difficult by severe peripheral side effects and/or receptor desensitization (De Mendonça et al., 2000).

Aside from adenosine, other neuroprotective factors include members of the IL-6 cytokine family, such as IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor, novel neurotrophin-1 and cardiotropin-1, which have been reported to exert neuroprotective actions in various neuronal subpopulations (Holtmann et al., 2005; Wen et al., 2005; Weiss et al., 2006; Gurfein et al., 2009; Suzuki et al., 2009). Still, the mechanisms by which different IL-6 family members afford neuroprotection are not well understood.

Cytokines of the IL-6 family exert their effects through activation of three different receptor complexes. In fact, IL-6 and IL-11 are the only IL-6 type cytokines that signal via gp130 homodimers, while the remaining members activate heterodimers of either gp130 and LIFr (LIF, ciliary neurotrophic factor, cardiotropin-1 and novel neurotrophin-1) or gp130 and OSMr (OSM) (Bauer et al., 2007). It has been proposed that the shared activation of gp130 receptor subunit-mediated signalling may account for many redundant properties among the members of the IL-6 family. Evidence that treatment with IL-6 can afford neuroprotection by enhancing the expression and function of neuronal A_1 receptors (Biber et al., 2008) therefore prompted the hypothesis that IL-6 type cytokines in general might be able to exert their neuroprotective properties via facilitation of neuronal A_1R function. To address this hypothesis, a collaboration with Knut Biber´s lab was initiated, in which I compared the influence that treatment with OSM and LIF exerted upon A_1 receptor-dependent depression of synaptic transmission, in mouse hippocampal slices. Indeed, work done

by the other collaboration partner has provided evidence for fundamental differences in the mechanisms underlying neuroprotection conferred by either LIF or OSM, against glutamate-induced toxicity, in cultured cortical neurons (Moidunny et al., 2010).

These cytokines were chosen because, given the three possible receptor complexes, by addressing neuroprotection by LIF and OSM and crossing information with what is already known for IL-6, one can investigate every receptor/signaling combination known to be activated by IL-6 family cytokines. Furthermore, even though LIF expression is very low in the central nervous system under physiological conditions, it is rapidly increased upon exposure to ischemic insults (Suzuki et al., 2000). Data obtained from an animal model of epilepsy, which also leads to neuronal death by excitotoxicity, has also revealed that after a single, prolonged seizure, there was a marked increase in LIF and OSM expression which was most noticeable in the hippocampus (Jankowsky et al., 1999). These results provide extra reason for studying the mechanisms underlying LIF- and OSM- mediated neuroprotection and to elucidate whether their actions involve facilitation of A_1 receptor-mediated responses.

I therefore next investigated whether sustained exposure of hippocampal slices to OSM or LIF could modify the neuromodulatory actions of A_1 receptors on synaptic transmission, by performing whole-cell recordings of afferent-evoked EPSCs from CA1 pyramidal cells.

Modulation of A_1 receptor-mediated actions on synaptic transmission by OSM, but not LIF

Activation of A_1 receptors is well established to decrease synaptic transmission in the hippocampus (Sebastião et al., 1990), which has been attributed to inhibition of N-type calcium channels leading to presynaptic glutamate release inhibition (Manita et al., 2004). It is also known that this A_1 receptor-mediated inhibition of synaptic transmission protects synapses against excitotoxic insults (Sebastião et al., 2001).

In the present work, and to evaluate if OSM and/or LIF could enhance synaptic transmission inhibition by adenosine A_1 receptors, experimental activation of A_1 receptors was induced by superfusion of a selective A_1 receptor agonist (CPA, 30 nM). To prevent pre-conditioned responses, the effects of CPA were investigated in only one neuron per slice, from control and OSM or LIF (10 ng/mL, for 4h) treated slices, prepared from the same hippocampus. Addition of CPA (30 nM) to the superfusion medium inhibited EPSC peak amplitude by 48±7.7% (n=4), in control slices (Figure 5.3.1A). When recording from slices that had been pre-incubated with OSM, average EPSC inhibition by CPA was significantly increased to 73±6.7% (n=4, $p<0.05$, Figure 5.3.1C). In contrast, average EPSC inhibition caused by superfusion of the A_1 receptor agonist was not significantly affected ($P>0.05$, Figure 5.3.2C) by pre-exposure to LIF (n=4, Figure 5.3.2A).

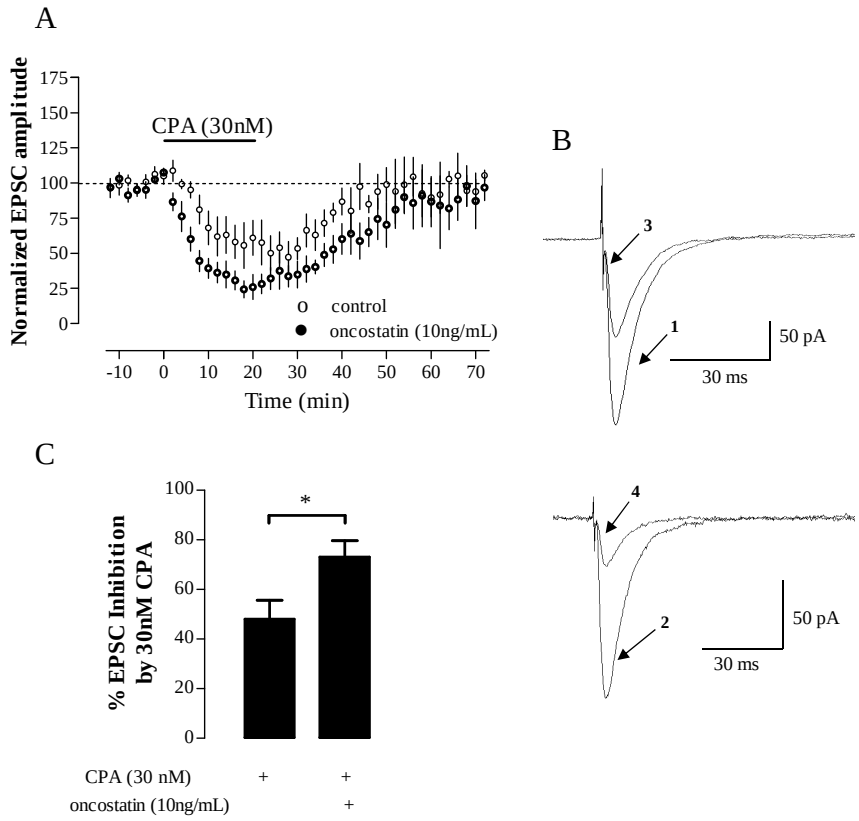


Figure 5.3.1 OSM potentiates inhibition of synaptic transmission caused by A_1 receptor activation. (A) Averaged time-course of afferent evoked EPSC peak amplitude changes caused by a 20 min application of the A_1 receptor agonist cyclopentyladenosine (CPA, 30nM), in control (○) versus test (●) slices from the same hippocampus (n=4). Test slices were incubated with 10 ng/mL oncostatin, for at least 4h. In the right panel (B) are shown superimposed current tracings of EPSCs recorded before (1,2) and 20-30 min after (3,4) addition of CPA to the superfusion medium, in representative control (1,3) and test (2,4) cells. (C) Inhibition of synaptic transmission caused by activation of adenosine A_1 receptors was significantly higher when recording from slices that had been previsouly exposed to oncostatin (10 ng/mL, at least 4 hours) compared with naïve ones. The percentage of EPSC inhibition corresponds to the average EPSC decrease measured 20-30 minutes after starting CPA application.* $P<0.05$ (paired t-test, compared with CPA-induced inhibition observed in the absence of OSM).

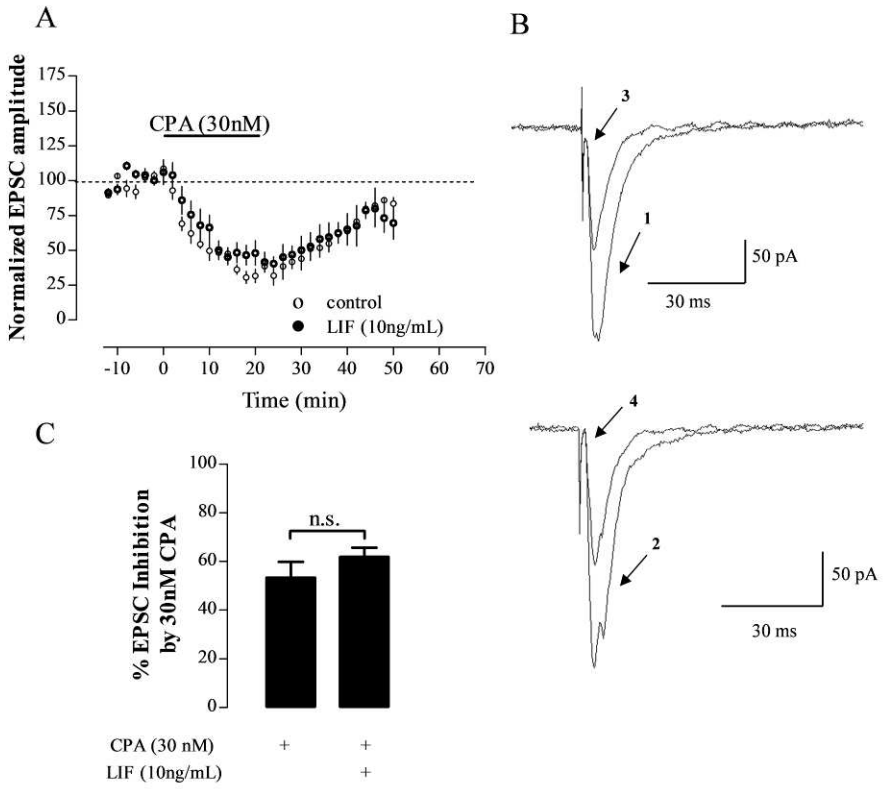


Figure 5.3.2. LIF does not alter inhibition of synaptic transmission caused by A1 receptor activation. (A) Averaged time-course of afferent evoked EPSC peak amplitude changes caused by a 20 min application of CPA (30 nM), in control (○) versus test (●) slices. Test slices were incubated for at least 4 hours with LIF (10 ng/mL). In the right panel are shown superimposed current tracings of EPSCs recorded before (1,2) and 20-30 min after (3,4) introduction of CPA in the superfusion medium, in representative control (1,3) and LIF-treated, test (2,4) cells. (C) Average inhibition of synaptic transmission caused by activation of adenosine A_1 receptors was not significantly different between naïve and LIF-treated slices. The percentage of EPSC inhibition was calculated as before; comparisons were made between control and test slices, taken from the same hippocampus (n=4). n.s. P>0.05 (paired t-test, compared with CPA-induced inhibition of EPSC amplitude observed in control conditions).

These results are in agreement with data obtained from RT-PCR and western blot analysis, according to which treatment with OSM, but not LIF, can selectively upregulate A_1 receptor mRNA and protein levels (Moidunny et al,. 2010). Furthermore, the experiments with LIF

incubation served to control for the possibility that prolonged pre-incubation time (4h) per se might be responsible for the potentiation of CPA-induced inhibition of EPSC amplitude, since this faciliation was observed in slices incubated with OSM but not in slices that had been incubated with LIF for similar time periods.

OSM potentiates A_1 receptor-mediated depression of synaptic transmission during hypoxia

Modulation by OSM of A_1 receptor-mediated fine-tuning actions, if functionally relevant, is expected to be able to also affect the decrease in excitatory transmission that follows the onset of a brief hypoxic episode. Indeed, the consistent increase in the extracellular concentration of endogenous adenosine that follows hypoxia and the subsequent A_1 receptor-dependent inhibition of synaptic transmission (Sebastião et al., 2001) is thought to be a major neuroprotective mechanism against hypoxia- and ischemia-induced brain damage (Rudolphi et al., 1992).

In order to investigate whether OSM-induced modulation of A_1 receptor function had any functional impact in these conditions, we used an hypoxia protocol of similar duration to one previously shown to reversibly inhibit synaptic transmission in the CA1 area, in a A_1 receptor-dependent way (Brust et al., 2006).

In control slices, such a brief (4 min) hypoxic insult caused a 42±4.8% maximum inhibition of EPSC amplitude (n=7). However, in hippocampal slices that had previously been exposed to OSM (10 ng/mL, for 4h), the same hypoxic insult led to a 69±3.4% inhibition of excitatory synpatic responses (Figure 5.3.3A), which was significantly

higher than that observed in control slices prepared from the same hippocampus (n=7, P<0.05, Figure 5.3.3C).

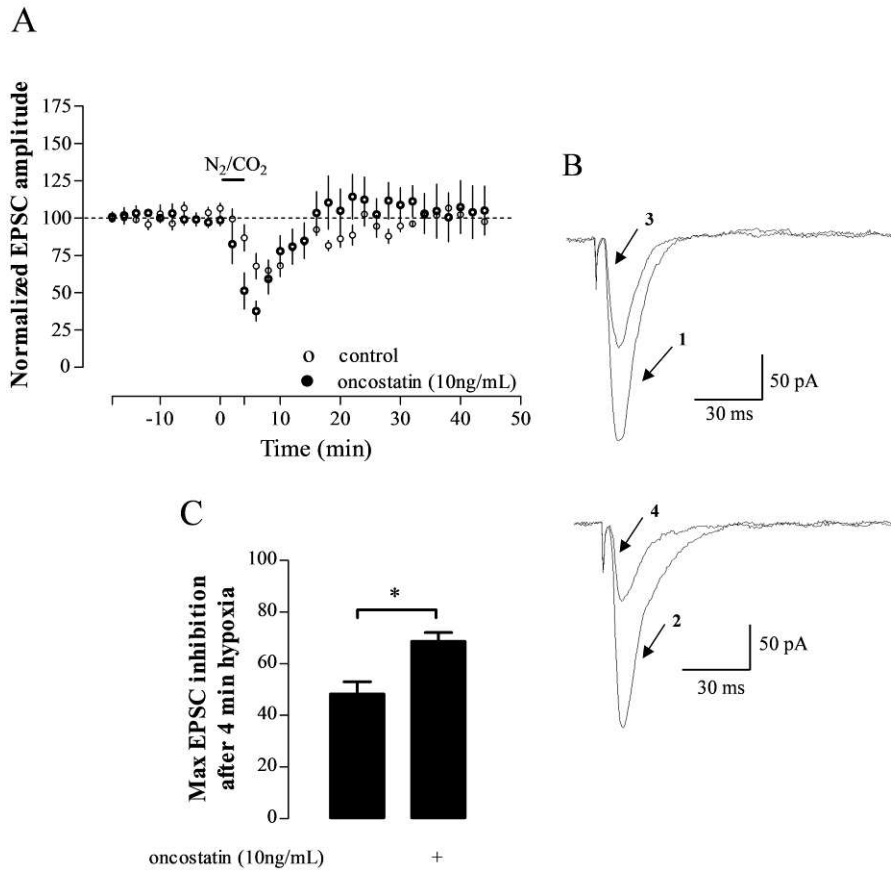


Figure 5.3.3. Oncostatin M potentiates hypoxia-induced inhibition of synaptic transmission. (A) Averaged time-course of afferent evoked EPSC peak amplitude changes caused by a 4 min hypoxic insult, recorded from control (aCSF-treated) and test slices (incubated for at least 4 hours with 10ng/mL oncostatin M) prepared from the same hippocampus. Each point represents the average amplitude of 4 EPSCs, evoked once every 30s by electrical stimulation of the Schaffer collaterals; 100% corresponds to the averaged amplitude calculated for the 5-10 EPSCs recorded immediately before hypoxia. The hypoxic insult consisted of replacing oxygenated aCSF ($95\%O_2/5\%CO_2$) by aCSF saturated with $95\%N_2/5\%CO_2$ for 4 min. Maximum inhibition was determined as the lowest EPSC amplitude recorded for each experiment, at either 6 or 8 min after hypoxia onset. In the right panel (B) are shown superimposed current tracings of EPSCs recorded before (1,2) and 8 min after hypoxia (3,4) was induced, in two representative control (1,3) and test (2,4) cells. (C) Maximum inhibition of synaptic transmission by hypoxia was significantly higher when recording from slices that had been exposed to oncostatin (10ng/mL, n=7). * $P < 0.05$, paired t-test (compared with EPSC inhibition observed in control conditions, in the absence of incubation with OSM).

To further ensure that the hypoxia-induced inhibition of excitatory synaptic responses was dependent upon activation of A_1 adenosine receptors, delivery of the hyposic insult was performed in the presence of a selective A_1 receptor antagonist (DPCPX, 50 nM, applied 30 min before hypoxia induction). In these conditions, the hypoxia-induced depression of EPSC amplitude was greatly reduced, or even abolished, both in control and oncostatin-treated slices (Figure 5.3.4A).

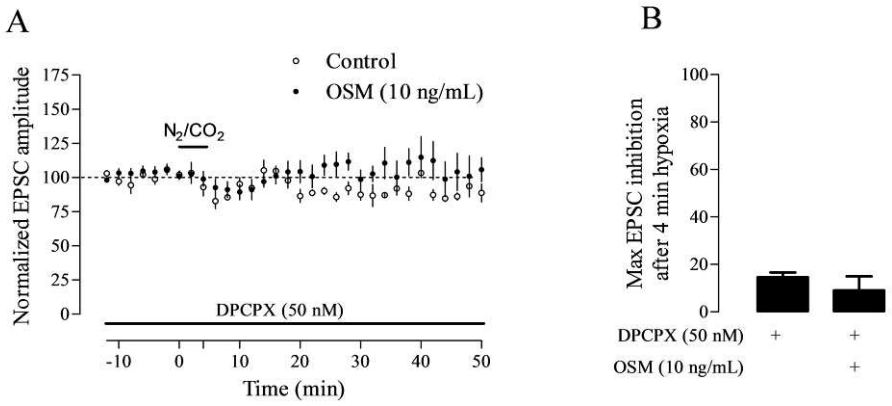


Figure 5.3.4. Hypoxia-induced inhibition of synaptic transmission is dependent on adenosine A_1 receptor activation. (A) Averaged time-course of afferent-evoked EPSC peak amplitude changes caused by a 4 min hypoxic insult, delivered under conditions of A_1 receptor blockade by the selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 50 nM), recorded from control (A) and oncostatin-treated (B) slices (n=5). Maximum inhibition corresponds to the lowest EPSC amplitude recorded, 8 min after hypoxia onset. (C) In either condition, maximum inhibition of synaptic transmission in response to hypoxia was greatly reduced when compared to that observed in the absence of the adenosine A_1 receptor antagonist (see Figure 5.3.3 for comparison).

Discussion

In addition to a well-documented role in infection, inflammation, bone, muscle and cardiovascular function, cytokines of the IL-6 family are also accountable for signaling functions in the developing and adult brain, both in physiological conditions as also in response to brain injury and disease (Bauer et al., 2007). In fact, neuroprotective properties are a feature common to different members of the IL-6 cytokine family, that may be due to the shared usage of gp130 receptor subunits in their signaling cascade (Taga and Kishimoto, 1997). However, support for this hypothesis was lacking and the cellular mechanisms operated by different IL-6 type cytokines are still largely unknown.

A recent study has provided a possible explanation for the mechanisms underlying IL-6-mediated neuroprotection. Indeed, treatment with IL-6, prompting gp130 homodimer activity, was found to cause an up-regulation of neuronal A_1 receptor expression and function both in vitro and in vivo, which was mandatory for IL-6-dependent neuroprotection (Biber et al., 2008). The work described in this chapter aimed at investigating a putative involvement of A_1 receptor function in neuroprotection and neuromodulation by either treatment with OSM (OSMr/gp130 heterodimer activation) or LIF (LIFr/gp130 heterodimer activation). Such questions were addressed in acute hippocampal slices (present chapter) and in a collaboration work performed on cultured neurons from wild-type and A_1 receptor knockout mice (Moidunny et al., 2010).

Pre-treatment for 24 hours with either OSM or LIF attenuates excitotoxicity in cultured neurons from cortex and hippocampus

(Moidunny et al., 2010), which reinforces the idea of a principal neuroprotective effect of IL-6-type cytokines (Ransohoff et al., 2002). Given that both cytokines display a comparable efficacy and furthermore share part of their receptor complexes, similar mechanisms of action might be expected. However, while blockade of neuronal A_1 receptor function with a selective antagonist completely abolished the neuroprotective effects of OSM, LIF-induced neuroprotection was left unaffected. Furthermore, while the neuroprotective effect of LIF is preserved in A_1 receptor knockout animals, pre-treatment with OSM failed to affect neuronal survival after glutamate toxicity in the absence of functional adenosine A_1 receptors (Moidunny et al., 2010).

When evaluating consequences for synaptic transmission, a 4h pre-treatment with IL-6 was previously found to potentiate the A_1 receptor-mediated inhibition of synaptic transmission in hippocampal slices, an effect that was of particular importance during short periods of hypoxia (Biber et al. 2008). Accordingly and as described in this chapter, pre-treatment with OSM, but not LIF, significantly increased A_1 receptor-mediated inhibition of afferent-evoked synaptic responses in the CA1 area of the hippocampus. Such a modulation of A_1 receptor function was also observed in response to a 4 min hypoxic insult, presumably by increasing the A_1 receptor-mediated inhibition of synaptic responses that takes place in such conditions (Brust et al., 2006). Thus, OSM, but not LIF, sensitizes neuronal A_1 receptor-mediated responses, similarly to what was previously observed for pre-treatment with IL-6 (Biber et al., 2008). Consistent with this data, real-time PCR and western blot analysis revealed that both A_1 receptor mRNA and protein expression levels are increased in OSM-treated, but not in LIF-treated neurons

(Moidunny et al., 2010). Taken together, these findings strongly suggest that two members of the IL-6-type cytokine family (IL-6 and OSM) depend on A_1 receptor function for their neuroprotective properties, whereas LIF induces neuroprotection via a different, unknown, mechanism. However, since both cytokines are neuroprotective in vitro and may reduce ischemic damage in vivo (Suzuki et al., 2009), a further understanding of operated signaling pathways and mechanisms of action may prove useful for their establishment as possible therapeutic candidate molecules. It should also be noted that the role of "pro-inflammatory" cytokines in stroke is still a controversial subject. For instance, increased formation of TNF-α can aggravate excitotoxicity by inhibiting glutamate uptake, but it may also favor recovery from stroke by enhancing the production of neurotrophic factors, such as BDNF (see Ceulemans et al., 2010). The same duality of functions has also been described for other acute "inflammatory" mediators like NO and IL-6, reflecting the complex role of brain inflammation after injury (e.g., Ekdahl et al., 2009). Future therapeutic approaches must thus always take into account the balance between inflammatory versus protective features of different IL-6-type cytokine family members and hopefully, appropriate cytokine combinations may provide the most efficient alternative.

Although LIF and OSM are highly related members of the IL-6 type cytokine family (Jeffery et al., 1993; Nicola et al., 1993) sharing most properties, distinct effects upon activation of LIF and OSM receptor complexes have been reported before. For example, only OSMr/gp130 heterodimer activation is able to promote osteoblast differentiation, whereas activation of both OSMr/gp130 and LIFr/gp130 heterodimers

in these cells inhibited the expression of osteocalcin, a protein required for bone-building (Malaval et al., 2005). Also, evidence for selective roles of OSM and LIF during haematopoiesis and their effects upon the regulation of certain target genes has been provided (Tanaka et al., 2003; Weiss et al., 2005), reinforcing the idea that different IL-6-type cytokine receptor complexes may activate specific signaling cascades.

It is at the moment unclear which signaling pathways are important for the neuroprotective effects of treatment with LIF, OSM and IL-6. Still, it has recently been shown that the basal as well as induced expression of A_1 receptors is regulated by the nuclear transcription factor - kappa B (NFkB) (Jhaveri et al., 2007). Noteworthy, OSM can regulate protein synthesis, through NFkB activation, in smooth muscle cells (Nishibe et al., 2001). When subjected to oxidative stress, these cells display an increase in A_1 receptor mRNA levels that is prevented by NFkB inhibitors (Nie et al., 1998). IL-6 has also been shown to activate NFkB in intestinal cells (Wang et al., 2003). Therefore, it seems likely that increased expression of A_1 receptors by OSM and IL-6 is also, at least partially, regulated by NFkB activation.

Regardless of which signaling pathways may be underlying differential effects of the three possible receptor complexes for IL-6 type cytokines, it is irrefutable that adenosine A_1 receptors arise as key players in neuroprotection against excitotoxicity induced by both OSM and IL-6. It has long been known that neuronal A_1 receptor activation can suppress neuronal activity and glutamatergic transmission, reduce oxidative stress, minimize metabolic demand and hence preserve ATP stores, protecting neurons from excitotoxicity (Schubert et al., 1997). Importantly, the data obtained raises the question of whether

shortcomes in prevention of ischemia-induced neuronal damage by treatment with A_1 receptor agonists may be potentially overcome by using strategies that indirectly enhance the expression of these receptors, such as treatment with IL-6 or OSM. Therefore, it is very important to further explore the acute effects of these cytokines upon the progression of neuronal viability, when the exposure is made posteriorly to the initial ischemic or excitotoxic insult. Finally, given the pleiotropy of these cytokines, before they can be regarded as therapeutic options, appropriate doses need to be accurately established, so as to avoid detrimental inflammatory responses.

6 General Conclusions

On the course of little more than two decades, research in neuroscience has catapulted glutamatergic transmission, and in particular AMPA receptors, to the spotlight. Because they carry the bulk of excitatory transmission throughout the central nervous system, their modulation profoundly affects neuronal signalling, as well as the changes it undergoes under physiological (LTP) and pathological (ischemia) conditions.

The work contained in this thesis first pursued the hypothesis that adenosine, an ubiquituous modulator of neuronal activity and communication, might directly affect AMPA receptor function, in the hippocampus. The results obtained unequivocally show that exogenous activation of A_{2A} receptors modulates AMPA receptor function in CA1 pyramidal cells, by a postsynaptic mechanism dependent on PKA activity, but that most probably does not require protein synthesis. Exogenous activation of A_{2A} receptors was furthermore found to correlate with increased expression of total and phosphorylated GluR1 subunits, at the membrane level. Finally, the physiological relevance of this A_{2A} receptor-mediated modulation of the postsynaptic AMPA component was highlighted by the significant inhibition of LTP expression that ocurred in conditions of A_{2A} receptor blockade. These findings thus enabled the identification of a novel modulator of AMPA receptor function that is ubiquituous in the extracellular space – particularly in synapses firing at high frequency, prone to undergo

activity-dependent reinforcement. Regulation of the GluR1 Ser-845 phosphorylation tonus controlling the reserve of GluR1-containing AMPA receptors at extrasynaptic pools (required for synaptic strengthening) may therefore constitute a new mechanism by which adenosine A_{2A} receptors fine-tune synaptic transmission and even the ability that a particular synapse has of being reinforced.

A_{2A} receptors were subsequently shown to be key players in ischemia-induced facilitation of hippocampal excitatory synaptic transmission, since this form of plasticity was completely abolished in the presence of a selective A_{2A} receptor antagonist. Interestingly, this effect was mimicked by internal spermine and consequent blockade of calcium-permeable AMPA receptors; but it was not altered when both drugs were applied simultaneously. It is therefore possible that A_{2A} and calcium-permeable AMPA receptor blockade are operating through a common pathway. Furthermore, these results unequivocally show that again, changes at the AMPA receptor level as well as modulatory effects by A_{2A} receptors are both fundamental for a form of synaptic plasticity.

Finally and since the A_1 receptor, the other high-affinity adenosine receptor subtype, is highly expressed in the hippocampus and constitutes a main target for adenosine released during hypoxic/ischemic insults, I looked at its role in the neuroprotective actions of two immunoregulatory cytokines. Evidence was provided for the ability of one of these immunoregulatory agents to exert neuroprotection indirectly, by causing an upregulation of adenosine A_1 receptors. Indeed, treatment with OSM, but not LIF, was clearly shown to enhance the A_1 receptor-dependent inhibition of synaptic

transmission after transient hypoxia – a major mechanism by which adenosine is known to confer protection from excitotoxicity-induced damage. Although very far from providing a clinical alternative to A_1 receptor–based therapies, the latter findings show that the curtains have not yet closed on the A_1 receptor field of neuroprotection.

7 Future Perspectives

A central assumption in neurobiology states that changes in behavior are underlied by dynamic regulation of the strength of individual synapses. In turn, extensive research has made clear that such plastic changes in synaptic efficiency greatly rely upon activity-dependent modulation of postsynaptic AMPA receptor function. The present work allowed the identification of a mechanism by which adenosine, through A_{2A} receptor activation, may facilitate an enhancement of the postsynaptic AMPA component, in conditions of increased neuronal activity - such as those prompting LTP induction.

Energy imbalance in excitotoxic conditions can also trigger considerable adenosine release, which affords neuroprotection via activation of A_1 receptors and prompt EPSC inhibition – in a way amenable to regulation by cytokines of the IL-6 family. In turn, and as shown here, activation of A_{2A} receptors was shown to be necessary for ischemia-induced facilitation of synaptic transmission. It would therefore be particularly interesting to investigate whether A_{2A} receptors are also able to tune AMPA receptor-mediated responses in excitotoxic conditions. For this purpose, patch-clamp recordings of AMPA-evoked currents (as in chapter 5.1) could be performed using the same ischemia protocol (as in chapter 5.2), in the presence or absence of a selective A_{2A} receptor antagonist. Furthermore, monitoring of receptor trafficking, using cells transfected with fluorescent GluR1 or GluR2 subunits, could be used to address whether or not A_{2A}

receptors can affect AMPA receptor membrane delivery from internal pools. Such experiments would additionally provide a way to elucidate the extent to which plasticity induced by ischemia obeys the same paradigms widely described for physiological LTP.

Indeed, strong evidence has already established several links between physiological LTP and its ischemia-induced counterpart. Noteworthy, the work contained in this thesis identifies a novel common feature between both forms of plasticity: the requirement for calcium-permeable AMPA receptor signaling. Further experimental work may help cast light on whether or not excitotoxicity is the price to pay for plasticity. It seems an unlikely coincidence that a brain area where researchers have always found plasticity so easy to induce, should also constitute a most vulnerable region to excitotoxicity. Bearing in mind that pathology seldomly invents new mechanisms, but rather subverts existing ones, it may be that a continuum from physiological to pathological activity-induced tuning of synaptic transmission, is yet to emerge. This hypothesis would fit nicely with contradictory results on the detrimental versus beneficial nature of ischemia-induced facilitation of synaptic transmission.

In fact, this form of plasticity has recently been proposed to help the brain cope with neuronal loss after stroke, by sculpting diffuse, redundant connectivity into new functional and structural circuits that may recover specific functions. Therefore, considerable therapeutic benefit may arise from the administration of pharmacological modulators of LTP, during the several hours to days after the initial incident during which regenerative approaches are hypothesized to enhance spontaneous functional recovery. Before attempts to identify

appropriate modulators are made, it is vital to elucidate when and why do plastic changes become a no-return path. Considering that the non-competitive AMPA receptor antagonist, talampanel, was recently proposed for phase III clinical trials in patients with amyotrophic lateral sclerosis (a well known model of excitotoxicity), AMPA receptors and their modulator molecules are likely to prove key players for determining cell fate. Extensive experimental work is therefore required to begin completing this puzzle and enable an accurate understanding of the pathophysiological events that may constitute future therapeutic targets in stroke.

8 Acknowledgements

Agradeço aos meus pais os primeiros passos no mundo da ciência. Empoleirada num banco, a desenhar princesas nos quadros dos laboratórios de química orgânica do ISEL, ou a correr pelo Complexo com a Rutinha. Obrigada por me darem a conhecer o mundo, por sempre me terem apoiado nas grandes decisões e por serem exemplos de vida únicos. Pai, muito obrigada pela capa, bem sei que não te pedi uma tarefa fácil! Mum, não tenho como agradecer toda a preciosa ajuda desde que entrei nestas andanças..

À minha tia Bequinhas, por teres ajudado a acabar de definir o esboço de pessoa com que te viste a cargo. Pela alegria que me ensinaste a retirar das pequenas coisas. Porque sem ti nunca teria aprendido a apreciar a suavidade fragrante das primeiras noites de Primavera.

Ao Gamboa, pela amizade que vem de há já uns bons anos, que tanto prezo. E por me teres dado a conhecer o Alexandre, claro.

A quem não tenho como agradecer. Por tudo. Pelos muitos jantares que fizeste, em noites de véspera de exame. Por teres aturado os serões a trabalhar ou a estudar, os dias de experiência que nunca acabaram à hora suposta e pela formatação da tese. Por me fazeres feliz...

A toda a minha restante família, de raiz e adquirida, obrigada pelo apoio e pela paciência, pela estrutura que nos ampara. Desculpem estar sempre a ler qualquer coisa mesmo quando estou convosco. Obrigada também à Vanessa e à Ana Pires, pelos jantares em que voltamos todas

aos tempos (descansados) do Camões. E à Irina, pela paciência com que toleras os silêncios de meses, que me permite manter a minha amiga de sempre. À amizade da Taiadjana, Ritinha, Liliana, Sílvia e Filipa, que apesar de se terem ocupado de ramos tão diferentes da Biologia e de estarem espalhadas pelo mundo, estão sempre tão próximas. Ao Miguel Jacob, ao Switch e a todos os amigos dos patins, pelas aventuras partilhadas entre campeonatos e viagens que definiram uma dimensão paralela da minha vida que sempre revisitarei com carinho.

Gostaria de agradecer em seguida aos meus orientadores, Professor Alexandre Ribeiro e Professora Ana Maria Sebastião, os principais responsáveis pela minha formação científica.

Ao Professor, um muito sincero obrigada por me ter aceite no seu laboratório e pela confiança que sempre depositou em mim. Pelas oportunidades que me proporcionou e pela experiência de vida singular através da qual modela a forma dos seus alunos viverem o exercício da actividade científica; em que consiste, como e para quê se fazem experiências no santuário da medicina. Ainda hoje tento não o desapontar.

À Professora, ser-me-á porventura mais difícil ainda expressar adequada gratidão. Obrigada pelo seu entusiasmo contagiante pela ciência e pela electrofisiologia em particular; pelo gosto que tem em discutir novas ideias e experiências; por ver sempre as pessoas à frente de tudo o resto. E acima de tudo, obrigada por arranjar sempre tempo para as coisas com que lhe aparecemos à porta, por muito cansada ou ocupada que esteja ou pequenas que estas sejam. Pelas asas que nos dá.

Sophie, ma petite, muito muito obrigada. Acima de tudo pela amizade, que começou pela companhia nas experiências “impossíveis” de biotinilação. E pela proposta com que te saíste numa bela tarde, que, bem, a modos que te sentenciou a aturares-me por mais uns bons anos. Pelas intermitências de ciência e às vezes, de direito constitucional, que alegram tardes intermináveis de marranço. E obrigada à Pipinhas, que nos vai alimentando.

Não posso também deixar de expressar a minha gratidão a quem me introduziu ao dia-a-dia do laboratório e ao patch-clamp. Muito obrigada ao António, à Catarina e à Diana toda a ajuda e paciência, ao longo dos primeiros meses no lab.

Ao Brunito e à Carina, por terem partilhado o entusiasmo da primeira SPF e o canudo com os primeiros posters.. e à Natália e ao Vasco, pela amizade e os almoços no sushi que passaram a dar todo um novo significado ao Samurai. À Sandrita, pela cor que dás à existência dos que te são queridos.. obrigada.

Ao Diogo, que foi a primeira pessoa que “apresentei” ao patch e que deixa uns sapatos impossíveis de encher. Que foi talhado para a ciência e que me vai deixar estupidamente orgulhosa com o futuro brilhante que aí vem. Obrigada pela discussão de ideias e por partilhares comigo os dias em que nada funciona, como só alguém que pena à mercê das vontades do set-up e das variações de resistência em série pode compreender.

Ao Jorge, à Vânia, Filipa, Daniela, ao Armando, Joaquim, à Mariana, à Andreia, à Catarina, Joaquim e também ao André e Rita, que pela primeira vez me fizeram sentir velha no laboratório! Agora

compreendo como sabe bem ver o vosso entusiasmo com as primeiras experiências e primeiras reuniões científicas. E à Mariana, que veio trazer música e boa disposição ao nosso cantito e que ajudou a salvar o set-up no dia da inundação!!..

À Ritinha, pela amizade, por estar sempre disponível para ajudar os outros, incluindo a mim e à Sofia. À Júlia, pela epopeia das exps da memantina e à Mizé, pelo carinho enquanto nossa Professora. E ao Professor Alexandre de Mendonça, pela perspectiva única de clínico e cientista com que enriquece os projectos do laboratório.

À Elvira e ao Senhor João, por toda a ajuda – mesmo quando, no último caso, a contrapartida é sobreviver ao dia a seguir aos jogos do Benfica com o Sporting. À Alexandra, que se desdobra nas mais variadas tarefas com que a sobrecarregamos, e à Luísa, Paula e Paula pelo companheirismo. À Cristina, que me ajudou e à Sofia com o que podia e não podia, muito muito obrigada.

À Professora Ana Luísa Carvalho, pelos anticorpos anti -GluR1 e -pSer845 and to Eric Guire, for his help with the slice biotinylation protocol. Without them the biotinylation assays would never have worked as fast as they did. To Sham and Knut Biber, i´m grateful for the opportunity to explore the world of cytokines and the plane in which it intersects the realm of adenosine. Ao Pedro Lima, pela ajuda (mesmo durante as férias) com a candidatura e financiamento para o Microelectrode Course em Plymouth. E finalmente, expresso a minha gratidão à Fundação para a Ciência e a Tecnologia, pelo financiamento (SFRH/BD/27761/2006) que me permitiu concretizar este trabalho.

9 References

Abrahamsson T, Gustafsson B, Hanse E (2008) AMPA silencing is a prerequisite for developmental long-term potentiation in the hippocampal CA1 region. *J Neurophysiol* **100**: 2605-2614

Adesnik H, Nicoll RA, England PM (2005) Photoinactivation of native AMPA receptors reveals their real-time trafficking. *Neuron* **48**: 977-985

Aitken PG, Breese GR, Dudek FF, Edwards F, Espanol MT, Larkman PM, Lipton P, Newman GC, Nowak TS Jr, Panizzon KL, et al (1995) Preparative methods for brain slices: a discussion. *J Neurosci Methods* **59**: 139-149

Andersen P, Bliss TV, Lomo T, Olsen LI, Skrede KK (1969) Lamellar organization of hippocampal excitatory pathways. *Acta Physiol Scand* **76**: 4A-5A

Andersen P, Bliss TVP, Skrede KK (1971) Lamellar organization of hippocampal excitatory pathways. *Exp Brain Res* **13**: 222-238

Andersen P, Sundberg SH, Sveen O, Swann JW, Wigström H (1980) Possible mechanisms for long-lasting potentiation of synaptic transmission in hippocampal slices from guinea-pigs. *J Physiol* **302**: 463–482

Andràsfalvy BK, Smith MA, Borchardt, Sprengel R, Magee JC (2003) Impaired regulation of synaptic strength in hippocampal neurons from GluR1-deficient mice. *J Physiol* **552**: 35-45

Antonova I, Arancio O, Trillat AC, Wang HG, Zablow L, Udo H, Kandel ER, Hawkins RD (2001) Rapid increase in clusters of presynaptic proteins at onset of long-lasting potentiation. *Science* **294**:1547-50

Araneda RC, Lan JY, Zheng X, Zukin RS, Bennett MV (1999) Spermine and arcaine block and permeate N-methyl-D-aspartate receptor channels. *Biophys J* **76**: 2899-2911

Artola A, Singer W (1993) Long-term depression of excitatory synaptic transmission and its relationship to long-term potentiation. *Trends Neurosci* 16: 480-487

Assaife-Lopes N, Sousa VC, Pereira DB, Ribeiro JA, Chao MV, Sebastião AM (2010) Activation of adenosine A2A receptors induces TrkB translocation and increases BDNF-mediated phospho-TrkB localization in lipid rafts: Implications for neuromodulation. *J Neurosci* **30**: 8468–8480

Banke TG, Bowie D, Lee H, Huganir RL, Schousboe A, Traynelis SF (2000) Control of GluR1 AMPA receptor function by cAMP-dependent Protein Kinase. *J Neurosci* **20:** 89-102

Bass BL (2002) RNA editing by adenosine deaminases that act on RNA. *Annu. Rev. Biochem.* **71:** 817–846

Bauer S, Kerr BJ, Patterson PH (2007) The neuropoietic cytokine family in development, plasticity, disease and injury. *Nat Rev Neurosci* **8:** 221-232

Bekar L, Libionka W, Tian GF, Xu Q, Torres A, Wang X, Lovatt D, Williams E, Takano T, Schnermann J, Bakos R, Nedergaard M (2008) Adenosine is crucial for deep brain stimulation-mediated attenuation of tremor. *Nat Med* **14**: 75-80

Bernstein J (1902) Untersuchungen zur Thermodynamik der bioelektrischen Ströme I. *Pflügers Arch Gesamte Physiol Menschen Tiere* **92:** 521–562

Besancon E, Guo S, Lok J, Tymianski M, Lo EH (2008) Beyond NMDA and AMPA glutamate receptors: emerging mechanisms for ionic imbalance and cell death in stroke. *Trends Pharmacol Sci* **29**: 268-275

Biber K, Pinto-Duarte A, Wittendorp MC, Dolga AM, Fernandes CC, Von Frijtag Drabbe Künzel J, Keijser JN, de Vries R, Ijzerman AP, Ribeiro JA, Eisel U, Sebastião AM, and Boddeke HW (2008) Interleukin-6 Upregulates Neuronal Adenosine A1

Receptors: Implications for Neuromodulation and Neuroprotection. *Neuropsychopharmacology* **33**: 2237-2250

Bischofberger N, Jacobson KA, von Lubitz DKJE (1997) Adenosine A1 receptor agonists as clinically viable agents for treatment of ischemic brain damage. *Ann NY Acad Sci* **825**: 23–29

Biscoe TJ, Evans RH, Francis AA, Martin MR, Watkins JC, Davies J, Dray A (1977) D-α-aminoadipate as a selective antagonist of amino acid-induced and synaptic excitation of mammalian spinal neurones. *Nature* **270**: 743–745

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J Physiol* **232**: 331-356

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**: 31-39

Bliss TV (2003) A journey from neocortex to hippocampus. *Philos Trans R Soc Lond B Biol Sci* **358**: 621-623

Boehm J, Kang MG, Johnson RC, Esteban J, Huganir RL, Malinow R (2006) Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. *Neuron* **51**: 213-225

Bowie, D. andMayer,M. (1995) Inward rectification of both AMPA and kainate subtype glutamate receptors generated by polyamine-mediated ion channel block. *Neuron* **15**: 453–462

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254

Bredt DS, Nicoll RA (2003) AMPA receptor trafficking at excitatory synapses. *Neuron* **40**: 361-379

Broca P (1878) Anatomie comparee des circonvolutions cerebrales: Le grand lobe limbique et la scissure limbique dans la serie des mammiferes. Rev anthrop **1**:385, ser. 2

Brorson JR, Manzolillo PA, Miller RJ (1994) Ca2+ entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells. *J Neurosci* **14**: 187-197

Brorson JR, Li D, Suzuki T (2004) Selective expression of heteromeric AMPA receptors driven by flip-flop differences. *J Neurosci* **24**: 3461–3470

Broughton BR, Reutens DC, Sobey CG (2009) Apoptotic mechanisms after cerebral ischemia. *Stroke* **40**: e331-339

Brusa R, Zimmermann F, Koh DS, Feldmeyer D, Gass P, Seeburg PH, Sprengel R (1995) Early-onset epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. *Science* **270**: 1677-1680

Burnashev N, Monyer H, Seeburg PH, Sakmann B (1992) Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. *Neuron* **8**: 189–198

Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* **112**: 195-203

Burnstock G (1976) Purinergic receptors. *J Theor Biol* **62**: 491–503

Burnstock G (2007) Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev* **87**: 659–797

Brust TB, Cayabyab FS, Zhou N, MacVicar BA (2006) p38 Mitogen-Activated Protein Kinase Contributes to Adenosine A1 Receptor-Mediated Synaptic Depression in Area CA1 of the Rat Hippocampus. *J Neurosci* **26**: 12427-12438

Cajal SR (1909) Histologie du système nerveux central de l´homme et des vertébrés. Maloine Editeurs, Paris.

Cantrell AR, Tibbs VC, Yu FH, Murphy BJ, Sharp EM, Qu Y, Catterall WA, Scheuer T (2002) Molecular mechanism of convergent regulation of brain Na(1) channels by protein kinase C, protein kinase A anchored to AKAP-15. *Mol Cell Neurosci* **21**: 63–80

Carew TJ, Hawkins RD, Abrams TW, Kandel E (1984) A test of Hebb's postulate at identified synapses which mediate classical conditioning in Aplysia. *J Neurosci* **4** 1217-1224

Carvalho AL, Duarte CB, Faro CJ, Carvalho AP, Pires EV (1998) Calcium influx through AMPA receptors and through calcium channels is regulated by protein kinase C in cultured retina amacrine-like cells. *J Neurochem* **70**: 2112-2119

Carvalho AL, Kameyama K, Huganir RL (1999) Characterization of phosphorylation sites on the GluR4 subunit of the alpha-amino-3-hidroxy-5-methylisoxazole-4-propionic acid receptor. *J Neurosci* **19**: 4748-4754

Castro-Alamancos MA, Calcagnotto ME (1999) Presynaptic long-term potentiation in corticothalamic synapses. *J Neurosci* **19:** 9090-9097

Chaiet L, Wolf FJ (1964) The properties of streptavidin, a biotin-binding protein produced by Streptomycetes. *Arch Biochem Biophys* **106**: 1-5

Ceulemans AG, Zgavc T, Kooijman R, Hachimi-Idrissi S, Sarre S, Michotte Y (2010) The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia. *J Neuroinflammation* **7**: 74

Chapman PF, Ramsay MF, Krezel W, Knevett SG (2003) Synaptic plasticity in the amygdala: comparisons with hippocampus. *Ann N Y Acad Sci* **985**:114-124~

Chen H-X, Otmakhov N, Lisman J (1999a) Requirements for LTP induction by pairing in hippocampal CA1 pyramidal cells. *J Neurophysiol* **82**: 526–532

Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA (1999b) A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J Neurosci* **19**: 9192–9200

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* **408**: 936-43

Chen JF, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, Geiger J, Lopes LV, de Mendonça A (2007) Adenosine A2A receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and 'fine tuning' modulation. *Prog Neurobiol* **83**: 310–331

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H (1990) Inhibition of forskolin induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *J Biol Chem* **265**: 5267–5272

Choi DW (1990) Cerebral hypoxia: some new approaches and unanswered questions. *J Neurosci* **10**: 2493-2501

Choi DW (1995) Calcium: still center-stage in hypoxic-ischemic neuronal death. *Trends Neurosci* **18**: 58-60

Chowdhury S, Shepherd JD, Okuno H, Lyford G, Petralia RS, Plath N, Huganir RL, Worley PF (2006) Arc interacts with the endocytic machinery to regulate AMPA Receptor Trafficking. *Neuron* **52**: 445–459

Chung HJ, Steinberg JP, Huganir RL, Linden DJ (2003) Requirement of AMPA receptor GluR2 phosphorylation for cerebellar long-term depression. *Science* **300:** 1751–1755

Cole KS (1949) Dynamic electrical characteristics of the squid axon membrane. *Arch Sci Physiol* **3**: 253–258

Collingridge GL, Kehl SJ, McLennan H (1983) Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. *J Physiol* **334**: 33-46

Collingridge GL, Olsen RW, Peters J, Spedding M (2009) A nomenclature for ligand-gated ion channels. *Neuropharmacology* 56: 2-5

Cooke SF, Bliss TV (2006) Plasticity in the human central nervous system. *Brain* **129**: 1659-1673

Correia-de-Sá P, Timóteo MA, Ribeiro JA (1996) Presynaptic A1 inhibitory/A2A facilitatory adenosine receptor activation balance depends on motor nerve stimulation paradigm at the rat hemidiaphragm. *J Neurophysiol* **76**: 3910-3919

Costa L, Santangelo F, Li Volsi G, Ciranna L (2009) Modulation of AMPA receptor-mediated ion current by pituitary adenylate cyclase-activating polypeptide (PACAP) in CA1 pyramidal neurons from rat hippocampus. *Hippocampus* **19**: 99–109

Cristóvão-Ferreira S, Vaz SH, Ribeiro JA, Sebastião AM (2009) Adenosine A_{2A} receptors enhance GABA transport into nerve terminals by restraining PKC inhibition of GAT-1. *J Neurochem* **109**: 336-347

Cunha RA, Johansonn B, van der Ploeg I, Sebastião AM, Ribeiro JA, Fredholm BB (1994) Evidence for functionally important adenosine A2A receptors in the rat hippocampus. *Brain Res* **649**:208–216

Cunha RA, Vizi ES, Ribeiro JA, Sebastião AM (1996) Preferential release of ATP, its extracellular catabolism as a source of adenosine upon high- but not low-frequency stimulation of rat hippocampal slices. *J Neurochem* **67**: 2180–2187

Cunha RA, Ribeiro JA (2000) Purinergic modulation of [(3)H]GABA release from rat hippocampal nerve terminals. *Neuropharmacology* **39**: 1156–1167

Curtis DR, Phillis JW, Watkins JC (1959) Chemical excitation of spinal neurones. *Nature* **183**: 611-612

Curtis DR, Watkins JC (1961) Analogues of glutamic and gamma-amino-n-butyric acids having potent actions on mammalian neurones. *Nature* **191**:1010-1011

Delgado JY, Coba M, Anderson CN, Thompson KR, Gray EE, Heusner CL, Martin KC, Grant SG, O'Dell TJ (2007) NMDA receptor activation dephosphorylates AMPA receptor glutamate receptor 1 subunits at threonine 840. *J Neurosci* **27**: 13210-13221

Del Zoppo GJ, Saver JL, Jauch EC, Adams HP Jr (2009) Expansion of the time window for treatment of acute ischemic stroke with intravenous tissue plasminogen activator: a science advisory from the American Heart Association/American Stroke Association. *Stroke* **40**: 2945-2948

D'Alcantara P, Ledent C, Swillens S, Schiffmann SN (2001) Inactivation of adenosine A2A receptors impairs long term potentiation in the nucleus accumbens without altering basal synaptic transmission. *Neuroscience* **107**: 455–464

De Mendonça A, Ribeiro JA (1994) Endogenous adenosine modulates long-term potentiation in the hippocampus. *Neuroscience* **62**: 385–390

De Mendonca A, Sebastiao AM, and Ribeiro JA (2000) Adenosine: does it have a neuroprotective role after all? *Brain Res Brain Res Rev* **33**: 258-274

Derkach V, Barria A, Soderling TR (1999) Ca^{2+}/calmodulin-dependent protein kinase II enhances channel conductance of α-amino-3-hydroxy-5-methylisoxazole-4-propionate type glutamate receptor. *Proc Natl Acad Sci* **96:** 3269-3274

Derkach VA, Oh MC, Guire ES, Soderling TR (2007) Regulatory mechanisms of AMPA receptors in synaptic plasticity. *Nat Rev Neurosci* **8**:101–113

Di Filippo M, Tozzi A, Costa C, Belcastro V, Tantucci M, Picconi B, Calabresi P (2008) Plasticity and repair in the post-ischemic brain. *Neuropharmacology* **55**: 353-362

Diógenes MJ, Fernandes CC, Sebastião AM, Ribeiro JA (2004) Activation of adenosine A_{2A} receptor facilitates brain-derived neurotrophic factor modulation of synaptic transmission in hippocampal slices. *J Neurosci* **24**: 2905-2913

Dirnagl U, Iadecola C, Moskowitz MA (1999) Pathobiology of ischemic stroke: an integrated review. *Trends Neurosci* **22**: 391–397

Dirnagl U (2006) Bench to bedside: the quest for quality in experimental stroke research. *J Cereb Blood Flow Metab* **26**:1465-1478

Dixon RM, Mellor JR, Hanley JG (2009) PICK1-mediated glutamate receptor subunit 2 (GluR2) trafficking contributes to cell death in oxygen/glucose-deprived hippocampal neurons. *J Biol Chem* **22:** 14230-14235

Donevan SD, Rogawaski MA (1995) Intracellular polyamines mediate inward rectification of Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Proc Natl Acad Sci USA* **92:** 9298-9302

Dong XX, Wang Y, Qin ZH (2009) Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Acta Pharmacol Sin* **30**: 379–387

Drury AN, Szent-Györgyi A (1929) The physiological activity of adenine compounds with special reference to their action upon the mammalian heart. *J Physiol* **68**: 213–237

du Bois-Reymond E (1884) Untersuchungen über thierische elektricität, 1848–1884 (2 bande). Reimer, Berlin

Dunwiddie TV, Hoffer BJ (1980) Adenine nucleotides and synaptic transmission in the in vitro rat hippocampus. *Br J Pharmacol* **69**: 59-68

Dunwiddie TV, Masino SA (2001) The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci* **24**: 31-55

Eastwood SL, McDonald B, Burnet PW, Beckwith JP, Kerwin RW, Harrison PJ (1995) Decreased expression of mRNAs encoding non-NMDA glutamate receptors GluR1 and GluR2 in medial temporal lobe neurons in schizophrenia. *Brain Res Mol Brain Res* **29**: 211-223

Eccles JC (1966) Conscious experience and memory. In *Brain and conscious experience* (ed J. C. Eccles), pp. 314-344. New York: Springer

Ehlers MD (2000) Reinsertion or degradation of AMPA receptors determined by activity-dependent endocytic sorting. *Neuron* **28**: 511–525

Ekdahl CT, Kokaia Z, Lindvall O (2009) Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience* **158**: 1021-1029

Emptage NJ, Reid CA, Fine A, Bliss TV (2003) Optical quantal analysis reveals a presynaptic component of LTP at hippocampal Schaffer-associational synapses. *Neuron* **38**:797-804

Esteban JA (2003) AMPA Receptor Trafficking: A Road Map for Synaptic Plasticity. *Mol Interv* **3**: 375-385

Esteban JA, Shi SH, Wilson C, Nuriya M, Huganir RL, Malinow R (2003) PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. *Nat Neurosci* **6:** 136–143

Faden AI, Demediuk P, Panter SS, Vink R (1989) The role of excitatory amino acids and NMDA receptors in traumatic brain injury. *Science* **244**: 798–800

Faraday M (1834) On Electrical Decomposition. *Philosophical Transactions of the Royal Society* **124**: 79

Ficker E, Taglialatela M, Wible BA, Henley CM, Brown AM (1994) Spermine and spermidine as gating molecules for inward rectifier K+ channels. *Science* **266**: 1068 – 1072

Fields RD, Burnstock G (2006) Purinergic signalling in neuron-glia interactions. *Nat Rev Neurosci* **7**: 423-436

Fontinha BM, Dio´genes MJ, Ribeiro JA, Sebastião AM (2008) Enhancement of long-term potentiation by brain-derived neurotrophic factor requires adenosine A2A receptor activation by endogenous adenosine. *Neuropharmacology* **54**: 924–933

Forghani R, Krnjevic K (1995) Adenosine antagonists have differential effects on induction of long-term potentiation in hippocampal slices. *Hippocampus* **5**: 71–77

Fowler JC (1989) Adenosine antagonists delay hypoxia-induced depression of neuronal activity in hippocampal brain slice. *Brain Res* **490**: 378-384

Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J (2001) Nomenclature and classification of adenosine receptors. *Pharmacol Rev* **53**: 527–552

Freund TE, Buzsáki G (1996) Intemeurons of the hippocampus. *Hippocampus* **6:** 347-470

Friedman LK, Pellegrini-Giampietro DE, Sperber EF, Bennett MV, Moshé SL, Zukin RS (1994) Kainate-induced status epilepticus alters glutamate and GABAA receptor gene expression in adult rat hippocampus: an in situ hybridization study. *J Neurosci* **14**: 2697-2707

Fujii S, Kato H, Ito K, Itoh S, Yamazaki Y, Sasaki H, Kuroda Y (2000) Effects of A1 and A2 adenosine receptor antagonists on the induction and reversal of long-term potentiation in guinea pig hippocampal slices of CA1 neurons. *Cell Mol Neurobiol* **20**: 331–350

Furlong TJ, Pierce KD, Selbie LA, Shine J (1992) Molecular characterization of a human hrain adenosine A2 receptor. *Mol Brain Res* **15**: 62–66

Gallo V, Upson LM, Hayes WP, Vyklicky L, Winters CA, Buonanno A (1992) Molecular cloning and developmental analysis of a new glutamate receptor subunit isoform in cerebellum. *J Neurosci* **12:** 1010 –1023

Gao Y, Phillis JW (1994) CGS 15943, an adenosine A2 receptor antagonist, reduces cerebral ischemic injury in the Mongolian gerbil. *Life Sci* **55**: L61–L65

Gao C, Sun X, Wolf ME (2006) Activation of D_1 receptors increases surface expression of AMPA receptors and facilitates their synaptic incorporation in cultured hippocampal receptors. *J Neurochem* **98**: 1664-1677

Galvani L (1791) De viribus electricitatis in motu musculari commentarius. *Bon Sci Art Inst Acad Comm* **7**: 363–418

Galvani L (1841) Opere edite ed inedite del Professore Luigi Galvani raccolte e pubblicate dall´Accademia delle Science dell´Istituto di Bologna. Dall´Olmo, Bologna

Gereau RW, Swanson GT (ed) (2008) The Glutamate Receptors. Totawa, NJ: Humana Press

Ginsborg BL, Hirst GD (1972) The effect of adenosine on the release of the transmitter from the phrenic nerve of the rat. *J Physiol* **224**: 629-645

Gorter JA, Petrozzino JJ, Aronica EM, Rosenbaum DM, Opitz T, Bennett MV, Connor JA, Zukin RS (1997) Global ischemia induces downregulation of Glur2 mRNA and increases AMPA receptor-mediated Ca2+ influx in hippocampal CA1 neurons of gerbil. *J Neurosci* **17**: 6179-6188

Green NM (1963) Avidin. 1. The use of (14C)biotin for kinetic studies and for assay. *Biochem J* **89**: 585-591

Greengard P, Jen J, Nairn AC, Stevens CF (1991) Enhancement of the glutamate response by cAMP-dependent protein kinase in hippocampal neurons. *Science* **253**: 1135-1138

Grosskreutz J, Zoerner A, Schlesinger F, Krampfl K, Dengler R, Bufler J (2003) Kinetic properties of human AMPA-type glutamate receptors expressed in HEK293 cells. *Eur J Neurosci* **17**: 1173–1178

Gruart A, Muñoz MD, Delgado-García JM (2006) Involvement of the CA3–CA1 Synapse in the Acquisition of Associative Learning in Behaving Mice. *J Neurosci* **26**:1077-87

Guire ES, Oh MC, Soderling TR, Derkach VA (2008) Recruitment of Calcium-Permeable AMPA Receptors during Synaptic Potentiation Is Regulated by CaM-Kinase I. *J Neurosci* **28**: 6000–6009

Gurfein BT, Zhang Y, López CB, Argaw AT, Zameer A, Moran TM, John GR (2009) IL-11 regulates autoimmune demyelination. *J Immunol* **183**: 4229–4240

Hammond C (2008) Cellular and molecular neurophysiology (3rd edition). Amsterdam: Elsevier

Hamlyn LH (1963) An electron microscope study of pyramidal neurons in the Ammon's horn of the rabbit. *J Anat* **97**: 189-201

Harris EW, Cotman CW (1986) Long-term potentiation of guinea pig mossy fiber responses is not blocked by N-methyl D-aspartate antagonists. *Neurosci Lett* **70**: 132-137

Hayashi T (1954) Effects of sodium glutamate on the nervous system. *Keio J Med* **3:** 183-192

Hayashi T, Huganir RL (2004) Tyrosine phosphorylation and regulation of the AMPA receptor by SRC family tyrosine kinases. *J Neurosci* **24:** 6152–6160

Hebb DO (1949) The Organization of Behavior: A Neuropsychological Theory, John Wiley & Sons, New York

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Mueller-Newen G, Schaper F (2003) Principles of interleukin (IL)-6-type cytokines signalling and its regulation. *Biochem J* **374**: 1–20

Helmholtz H (1850) Note sur la vitesse de propagation de l'agent nerveux dans les nerfs rachidiens. *C R Acad Sci (Paris)* **30**: 204–206

Heurteaux C, Lauritzen I, Widmann C, Lazdunski M (1995) Essential role of adenosine, adenosine A1 receptors, and ATP-sensitive KC channels in cerebral ischemic preconditioning. *Proc Natl Acad Sci USA* **92**: 4666–4670

Hirai H, Yoshioka K, and Yamada K (2000) A simple method using 31P-NMR spectroscopy for the study of protein phosphorylation. *Brain Res Brain Res Protoc* **5**: 182-189

Hodgkin AL, Huxley AF (1939) Action potentials recorded from inside a nerve fibre. *Nature* **144**: 710–711

Hodgkin AL, Huxley AF (1947) Potassium leakage from an active nerve fibre. *J Physiol* **106**: 341-367

Hodgkin AL, Huxley AF (1952) A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol* **117**: 500–544

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. *Annu Rev Neurosci* **17**: 31-108

Hollmann M, Maron C, Heinemann S (1994) N-glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1. *Neuron* **13**: 1331–1343

Holtmann B, Wiese S, Samsam M, Grohmann K, Pennica D, Martini R, Sendtner M (2005) Triple knock-out of CNTF, LIF, and CT-1 defines cooperative and distinct roles of these neurotrophic factors for motoneuron maintenance and function. *J Neurosci* **25**: 1778–1787

Horn R, Marty A (1988) Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J Gen Physiol* **92**:145-159

Hu H, Real E, Takamiya K, Kang MG, Ledoux J, Huganir RL, Malinow R (2007) Emotion enhances learning via norepinephrine regulation of AMPA-receptor trafficking. *Cell* **131**: 160-173

Huang YY, Gustafsson B, Wigström H (1988) Facilitation of hippocampal long-term potentiation in slices perfused with high concentrations of calcium. *Brain Res* **456**: 88–94

Huber KM, Kayser MS, Bear MF (2000) Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science* **288**: 1254–1257

Huh KH, Wenthold RJ (1999) Turnover analysis of glutamate receptors identifies a rapidly degraded pool of the N-methyl-D-aspartate receptor subunit, NR1, in cultured cerebellar granule cells. *J Biol Chem* **274**:151-157

Huxley A (2002) From overshoot to voltage clamp. *Trends Neurosci* **25**: 553-558

Isa T, Iino M, Itazawa S, Ozawa S (1995) Spermine mediates inward rectification of Ca^{2+}-permeable AMPA receptor channels. *Neuroreport* **6:** 2045-2048

Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* **5**: 247–264

Jayakumar AR, Norenberg MD (2010) The Na-K-Cl Co-transporter in astrocyte swelling. *Metab Brain Dis* **25**: 31-38

Jankowsky JL, Patterson PH (1999) Differential regulation of cytokine expression following pilocarpine-induced seizure. *Exp Neurol* **159**: 333–346

Jarvis MF, Schulz R, Hutchison AJ, Do UH, Sills MA, Williams M (1989) [3H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. *J Pharmacol Exp Ther* **251**: 888–893

Jeffery E, Price V, Gearing DP (1993) Close proximity of the genes for leukemia inhibitory factor and oncostatin M. *Cytokine* **5**: 107–111

Jhaveri KA, Reichensperger J, Toth LA, Sekino Y, Ramkumar V (2007) Reduced basal and lipopolysaccharide-stimulated adenosine A1 receptor expression in the brain of nuclear factor-kB p50-/- mice. *Neuroscience* **146:** 415–426

Jiang J, Suppiramaniam V, Wooten MW (2006) Posttranslational modifications and receptor-associated proteins in AMPA receptor trafficking and synaptic plasticity. *Neurosignals* **15**: 266-282

Jonas P, Burnashev N (1995) Molecular mechanisms controlling calcium entry through AMPA-type glutamate receptor channels. *Neuron* **15:** 987-990

Jonas P, Sakmann B (1991) Glutamate receptor channels in isolated patches from CA1 and CA3 pyramidal cells of rat hippocampal slices. *J Physiol* **455:** 143-171

Jörntell H, Hansel C (2006) Synaptic memories upside down: bidirectional plasticity at cerebellar parallel fiber-Purkinje cell synapses. *Neuron* **52**: 227-238

Kamboj SK, Swanson GT, Cull-Candy SG (1995) Intracellular spermine confers rectification on rat calcium-permeable AMPA and kainate receptors. *J Physiol* **486**: 297–303

Kamimura D, Ihsihara K, Hirano T (2003) IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol* **149**: 1–38

Kato HK, Watabe AM, Manabe T (2009) Non-Hebbian synaptic plasticity induced by repetitive postsynaptic action potentials. *J Neurosci* **29**: 11153-11160

Kauer JA, Malenka RC (2006) LTP: AMPA receptors trading places. *Nat Neurosci* **9:** 593-594

Kemp A, Manahan-Vaughan D (2007) Hippocampal long-term depression: master or minion in declarative memory processes? *Trends Neurosci* **30**: 111-118

Kerchner GA, Nicoll RA (2008) Silent synapses and the emergence of a postsynaptic mechanism for LTP. *Nat Rev Neurosci* **9:** 813-825

Kessey K, Mogul DJ (1997) NMDA-independent LTP by adenosine A2 receptor-mediated postsynaptic AMPA potentiation in the hippocampus. *J Neurophysiol* **78**: 1965–1972

Kittler JT, Moss SJ (2006) The Dynamic Synapse: Molecular Methods in Ionotropic Receptor Biology. Boca Raton (FL): CRC Press

Kullmann DM, Asztely F, Walker MC (2000) The role of mammalian ionotropic receptors in synaptic plasticity: LTP, LTD and epilepsy. *Cell Mol Life Sci* **57**: 1551–1561

Kumar SS, Bacci A, Kharazia V, Huguenard JR (2002) A developmental switch of AMPA receptor subunits in neocortical pyramidal neurons. *J Neurosci* **22:** 3005-3015

Kurakin A, Swistowski A, Wu SC, Bredesen DE (2007) The PDZ domain as a complex adaptive system. *PLoS One* **2**: e953

Kurien BT, Scofield RH (1997) Multiple immunoblots after non-electrophoretic bidirectional transfer of a single SDS-PAGE gel with multiple antigens. *J Immunol Methods* 205: 91-94

Kwak S, Kawahara Y (2005) Deficient RNA editing of GluR2 and neuronal death in amyotropic lateral sclerosis. *J Mol Med* **83**: 110-120

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685

Lee JM, Zipfel GJ, Choi DW (1999) The changing landscape of ischaemic brain injury mechanisms. *Nature* **399**: A7–A14

Lee HK, Barbarosie M, Kameyama K, Bear MF, Huganir RL (2000) Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature* **405**: 955-999

Lee HK, Takamiya K, Han JS, Man H, Kim CH, Rumbaugh G, Yu S, Ding L, He C, Petralia RS, Wenthold RJ, Gallagher M, Huganir RL (2003a) Phosphorylation of AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. *Cell* **112**: 631–643

Lee YC, Chien CL, Sun CN, Huang CL, Huang NK, Chiang MC, Lai HL, Lin YS, Chou SY, Wang CK, Tai MH, Liao WL, Lin TN, Liu FC, Chern Y (2003b) Characterization of the rat A2A adenosine receptor gene: A 4.8-kb promoter-proximal DNA fragment confers selective expression in the central nervous system. *Eur J Neurosci* **18**: 1786–1796

Lee HK, Takamiya K, Kameyama K, He K, Yu S, Rossetti L, Wilen D, Huganir RL (2007) Identification and characterization of a novel phosphorylation site on the GluR1 subunit of AMPA receptors. *Mol Cell Neurosci* **36**: 86-94

Li CL, McIlwain H (1957) Maintenance of resting membrane potentials in slices of mammalian cerebral cortex and other tissues in vitro. *J Physiol* **139**: 178-190

Li H, Henry JL (1998) Adenosine A2 receptor mediation of pre- and postsynaptic excitatory effects of adenosine in rat hippocampus in vitro. *Eur J Pharmacol* **347**: 173–182

Liao D, Hessler NA, Malinow R (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* **375**: 400-404

Lisman JE,Raghavachari S,Tsien RW (2007) The sequence of events that underlie quantal transmission at central glutamatergic synapses. *Nat Rev Neurosci* **8**: 597-609

Liu SJ, Zukin RS (2007) Ca2+-permeable AMPA receptors in synaptic plasticity and neuronal death. *Trends Neurosci* **30**: 126-134

Loewi O (1921) "Über humorale Übertragbarkeit der Herznervenwirkung. I."*Pflügers Archiv* **189:** 239-242

Londos C, Cooper DM, Wolff J (1980) Subclasses of external adenosine receptors. *Proc Natl Acad Sci U S A* **77**: 2551-2554

Lopes da Silva FH, Witter MP, Boeijinga PH, Lohman AH (1990) Anatomic Organization and Physiology of the limbic cortex. *Physiol Rev* **70**: 453–511

Lopes LV, Cunha RA, Kull B, Fredholm B, Ribeiro JA (2002) Adenosine A_{2A} receptor facilitation of hippocampal synaptic transmission is dependent on tonic A_1 receptor inhibition. *Neuroscience* **112**: 319-329

Lucas DR, Newhouse JP (1957) The toxic effect of sodium L-glutamate on the inner layers of the retina, *Arch Ophthalmol* **58:** 193–201

Lupica CR, Cass WA, Zahniser NR, Dunwiddie TV (1990) Effects of the selective adenosine A2 receptor agonist CGS 21680 on in vitro electrophysiology, cAMP formation and dopamine release in rat hippocampus and striatum. *J Pharmacol Exp Ther* **252**: 1134–1141

Lüscher C, Xia H, Beattie EC, Carroll RC, von Zastrow M, Malenka RC, Nicoll RA (1999) Role of AMPA receptor cycling in synaptic transmission and plasticity. *Neuron* **24**: 649–658

Luttman W, Bratke K, Küpper M (2006) Immunology. The experimenter series. Oxford, Academic Press.

MacLean PD (1954) "Studies on Limbic System ('Visceral Brain') and Their Bearing on Psychosomatic Problems." In Wittkower E and Cleghorn R (Eds): Recent Developments in Psychosomatic Medicine. London, Pitman

McNamara JO (1999) Emerging insights into the genesis of epilepsy. *Nature* **399**: A15–A22

Madison DV, Nicoll RA (1984) Control of the repetitive discharge of rat CA1 pyramidal neurons in vitro. *J Physiol* **354**: 319-331

Makhinson M, Chotiner JK, Watson JB, O'Dell TJ (1999) Adenylyl cyclase activation modulates activity-dependent changes in synaptic strength and Ca21/calmodulin-dependent kinase II autophosphorylation. *J Neurosci* **19**: 2500–2510

Malaval L, Liu F, Vernallis AB, Aubin JE (2005) GP130/OSMR is the only LIF/IL-6 family receptor complex to promote osteoblast differentiation of calvaria progenitors. *J Cell Physiol* **204**: 585–593

Malenka RC, Nicoll RA (1999) Long-term potentiation—a decade of progress? *Science* **285**: 1870–1874

Malenka R, Bear M (2004) LTP and LTD: an embarrassment of riches. *Neuron* **44**: 5–21

Malinow, R, Malenka, RC (2002) AMPA receptor trafficking and synaptic plasticity. *Annu Rev Neurosci* **25**: 103–126

Malinow R, Mainen ZF, and Hayashi Y (2000) LTP mechanisms: From silence to four-lane traffic. *Curr Opin Neurobiol* **10:** 352–357

Mammen AL, Kameyama K, Roche KW, Huganir RL (1997) Phosphorylation of the α -amino- 3-hydroxy-5-methylisoxazole-4-propionic acid receptor GluR1 subunit by calcium/ calmodulin-dependent kinase II. *J Biol Chem* **272**: 32528–32533

Manita S, Kawamura Y, Sato K, Inoue M, Kudo Y, Miyakawa H (2004) Adenosine A(1)-receptor-mediated tonic inhibition of glutamate release at rat hippocampal CA3-CA1 synapses is primarily due to inhibition of N-type Ca2+ channels. *Eur J Pharmacol* **499**: 265–274

Margulies JE, Cohen RW, Levine MS, Watson JB (1993) Decreased GluR2(B) receptor subunit mRNA expression in cerebellar neurons at risk for degeneration. *Dev Neurosci* **15**: 110-120

Martin SJ, Grimwood PD, Morris RG (2000) Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu Rev Neurosci* **23**: 649-711

Maruyama Y, Petersen OH (1982) Cholecystokinin activation of single-channel currents is mediated by internal messenger in pancreatic acinar cells. *Nature* **300**: 61–63

Mayer ML (2006) Glutamate receptors at atomic resolution. *Nature* **440**: 456–462

McBain CJ, Dingledine R (1993) Heterogeneity of synaptic glutamate receptors on CA3 stratum radiatum interneurones of rat hippocampus. *J Physiol* **462**: 373-392

Meech RW, Standen NB (1975) Potassium activation in Helix aspersa neurones under voltage clamp: a component mediated by calcium influx. *J Physiol* **249**: 211-239

Melani A, Pantoni L, Bordoni F, Gianfriddo M, Bianchi L, Vannucchi MG, Bertorelli R, Monopoli A, Pedata F (2003) The selective A2A receptor antagonist SCH 58261 reduces striatal transmitter outflow, turning behavior and ischemic brain damage induced by permanent focal ischemia in the rat. *Brain Res* **959**: 243–250

Meldrum BS (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *J Nutr* **130**: 1007S-10015S

Minichiello L, Korte M, Wolfer D, Kuhn R, Unsicker K, Cestari V, Rossi-Arnaud C, Lipp HP, Bonhoeffer T, Klein R (1999) Essential role for TrkB receptors in hippocampus-mediated learning. *Neuron* **24**: 401–414

Moidunny S, Dias RB, Wesseling E, Sekino Y, Boddeke HW, Sebastião AM, Biber K (2010) Interleukin-6-type cytokines in neuroprotection and neuromodulation: oncostatin M, but not leukemia inhibitory factor, requires neuronal adenosine A1 receptor function. *J Neurochem* **114**: 1667-1677

Monyer H, Seeburg PH, Wisden W (1991) Glutamate-operated channels: developmentally early and mature forms arise by alternative splicing. *Neuron* **6**: 799–810

Munton RP, Tweedie-Cullen R, Livingstone-Zatchej M, Weinandy F, Waidlich

M, Longo D, Gehrig P, Potthast F, Rutishauser D, Gerrits B, Panse C, Schlapback R, Mansuy IM (2007) Qualitative and quantitative analyses of protein phosphorylation in naïve and stimulated mouse synaptosomal preparations. *Mol Cell Proteomics* **6**:283–293

Murphy TH, Corbett D (2009) Plasticity during stroke recovery: from synapse to behaviour. *Nat Rev Neurosci* **10**: 861-872

Neher E, Sakmann B (1976) Single-channel currents recorded from membrane of denervated frog muscle fibres. *Nature* **260**: 799–802

Nieuwenhuys R, Voogd J, van Huijzen C, van Huijzen C (2008) The human central nervous system. (4^{th} edition) Springer, Berlin.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* **391**: 85-100

Nellgard B, Wieloch T (1992) Postischemic blockade of AMPA but not NMDA receptors mitigates neuronal damage in the rat brain following transient severe cerebral ischemia. *J Cereb Blood Flow Metab* **12**: 2-11

Nicola NA, Cross B, Simpson RJ (1993) The disulfide bond arrangement of leukemia inhibitory factor: homology to oncostatin M and structural implications. Biochem. Biophys. *Res Commun* **190**: 20–26

Nie Z, Mei Y, Ford M, Rybak L, Marcuzzi A, Ren H, Stiles GL, Ramkumar V (1998) Oxidative stress increases A1 adenosine receptor expression by activating nuclear factor kappa B. *Mol Pharmacol* **53**: 663–669

Nikbakht MR, Stone TW (2001) Suppression of presynaptic responses to adenosine by activation of NMDA receptors. *Eur J Pharmacol* **427**: 13–25

Nilius B (2003) Pflugers Archiv and the advent of modern electrophysiology. From the first action potential to patch clamp. *Pflugers Arch* **447**: 267–271

Nishibe T, Parry G, Ishida A, Aziz S, Murray J, Patel Y, Rahman S, Strand K, Saito K, Saito Y, Hammond WP, Savidge GF, Mackman N, Wijelath ES (2001) Oncostatin M promotes biphasic tissue factor expression in smooth muscle cells: evidence for Erk-1/2 activation. *Blood* **97**: 692–699

Oh MC, Derkach VA, Guire ES, Soderling TR (2006) Extrasynaptic membrane trafficking regulated by GluR1 serine 845 phosphorylation primes AMPA receptors for long-term potentiation. *J Biol Chem* **281**: 752-758

Ogden D (1994) Microelectrode Techniques. The Plymouth Workshop Handbook (2nd edition). Cambridge: The Company of Biologists Ltd

Oguro K, Oguro N, Kojima T, Grooms SY, Calderone A, Zheng X, Bennett MV, Zukin RS (1999) Knockdown of AMPA Receptor GluR2 Expression Causes Delayed Neurodegeneration and Increases Damage by Sublethal Ischemia in Hippocampal CA1 and CA3 Neurons. *J Neurosci* **19**: 9218-9227

O'Kane EM, Stone TW (1998) Interaction between adenosine A1 and A2 receptor-mediated responses in the rat hippocampus in vitro. *Eur J Pharmacol* **362**: 17–25

Olney JW (1969) Brain Lesions, Obesity, and other disturbances in mice treated with monosodium glutamate. *Science* **164**: 719–721

Olney JW (1986) Inciting excitotoxic cytocide among central neurons. *Adv Exp Med Biol* **203**: 631–645

Ordonez AN, Jessick VJ, Clayton CE, Ashley MD, Thompson SJ, Simon RP, Meller R (2010) Rapid ischemic tolerance induced by adenosine preconditioning results in Bcl-2 interacting mediator of cell death (Bim) degradation by the proteasome. *Int J Physiol Pathophysiol Pharmacol* 2: 36-44

Overton E (1902) Beiträge zur allgemeinen Muskel- und Nervenphysiologie. II. Mittheilung. Ueber die Unentbehrlichkeit von Natrium- (oder Litium-) Ionen für den Contractionsact des Muskels. *Pflügers Arch* **92**:346–380

Palmer CL, Cotton L, Henley JM (2005) The molecular pharmacology and cell biology of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Pharmacol Rev* **57**: 253-277

Patterson SL, Abel T, Deuel TA, Martin KC, Rose JC, Kandel ER (1996) Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* **16**: 1137–1145

Pearson T, Damian K, Lynas RE, Frenguelli BG (2006) Sustained elevation of extracellular adenosine and activation of A1 receptors underlie the post-ischaemic inhibition of neuronal function in rat hippocampus in vitro. *J Neurochem* **97**: 1357–1368

Peferoen, M, Huybrechts R, De Loof A (1982) "Vacuum-blotting: a new simple and efficient transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose". *FEBS Letters* **145**: 369–372

Pellegrini-Giampietro DE, Zukin RS, Bennett MV, Cho S, Pulsinelli WA (1992) Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proc Natl Acad Sci USA* **89**: 10499-10503

Pellegrini-Giampietro DE, Gorter JA, Bennett MV, Zukin RS (1997) The GluR2 (GluR-B) hypothesis: Ca(2+)-permeable AMPA receptors in neurological disorders. *Trends Neurosci* **20**: 464-470

Pellegrini-Giampietro DE (2003) An activity-dependent spermine-mediated mechanism that modulates glutamate transmission. *Trends Neurosci* **26**: 9-11

Peng PL, Zhong X, Tu W, Soundarapandian MM, Molner P, Zhu D, Lau L, Liu S, Liu F, Lu Y (2006) ADAR2-dependent RNA editing of AMPA receptor subunit GluR2 determines vulnerability of neurons in forebrain ischemia. *Neuron* **49**: 719-733

Pérez-Pinzón MA (1999) Excitatory and inhibitory pathways for anoxic preconditioning neuroprotection in hippocampal slices. *Adv Exp Med Biol* **471**:165-173

Piccolino M (1998) Animal electricity and the birth of electrophysiology: the legacy of Luigi Galvani. *Brain Res Bull* **46:** 381-407

Pickard L, Noel J, Henley JM, Collingridge GL, Molnar E (2000) Developmental changes in synaptic AMPA and NMDA receptor distribution and AMPA receptor composition in living hippocampal neurons. *J Neurosci* **20:** 7922-7931

Pinto-Duarte A, Coelho JE, Cunha RA, Ribeiro JA, Sebastião AM (2005) Adenosine A_{2A} receptors control the extracellular levels of adenosine through modulation of nucleoside transporters activity in the rat hippocampus. *J Neurochem* **93**: 595-604

Plant K, Pelkey KA, Bortolotto ZA, Morita D, Terashima A, McBain CJ, Collingridge GL, Isaac JT (2006) Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. *Nat Neurosci* **9**: 602-604

Popp A, Jaenisch N, Witte OW, Frahm C (2009) Identification of ischemic regions in a rat model of stroke. *PLoS One* **4**: e4764

Pozo K, Goda Y (2010) Unraveling mechanisms of homeostatic synaptic plasticity. *Neuron* **66**: 337-351

Pugliese AM, Traini C, Cipriani S, Gianfriddo M, Mello T, Giovannini MG, Galli A, Pedata F (2009) The adenosine A2A receptor antagonist ZM241385 enhances neuronal survival after oxygen-glucose deprivation in rat CA1 hippocampal slices. *Br J Pharmacol* **157**: 818–830

Quintana P, Alberi S, Hakkoum D, Muller D (2006) Glutamate receptor changes associated with transient anoxia/hypoglycaemia in hippocampal slice cultures. *Eur J Neurosci* **23**: 975-983

Ransohoff RM, Howe CL, Rodriguez M (2002) Growth factor treatment of demyelinating disease: at last, a leap into the light. *Trends Immunol* **23**: 512–516

Rao VR, Finkbeiner S (2007) NMDA and AMPA receptors: old channels, new tricks. *Trends Neurosci* **30**: 284-291

Rebola N, Rodrigues RJ, Lopes LV, Richardson PJ, Oliveira CR, Cunha RA (2005a) Adenosine A1 and A2A receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. *Neuroscience* **133**: 79–83

Rebola N, Canas PM, Oliveira CR, Cunha RA (2005b) Different synaptic and subsynaptic localization of adenosine A2A receptors in the hippocampus and striatum of the rat. *Neuroscience* **132**: 893–903

Rebola N, Lujan R, Cunha RA, Mulle C (2008) Adenosine A2A receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. *Neuron* **57**: 121–134

Rees DA, Scanlon MF, Ham J (2003) Adenosine signalling pathways in the pituitary gland: one ligand, multiple receptors. *J Endocrinol* **177**: 357-364

Rial Verde EM, Lee-Osbourne J, Worley PF, Malinow R, Cline HT (2006) Increased expression of the immediate-early gene arc/arg3.1 reduces AMPA receptor-mediated synaptic transmission. *Neuron* **52**: 461-474

Ribeiro JA, Walker J (1973) Action of adenosine triphosphate on endplate potentials recorded from muscle fibres of the rat diaphragm and frog sartorius. *Br J Pharmacol* **49:** 724-725

Ribeiro JA, Walker J (1975) The effects of adenosine triphosphate and adenosine diphosphate on transmission at the rat and frog neuromuscular junctions. *Br J Pharmacol* **54:** 213-218

Ribeiro JA, Sebastião AM, de Mendonça A (2002) Adenosine receptors in the nervous system: Pathophysiological implications. *Prog Neurobiol* **68**: 377–392

Ribeiro JA, Sebastião AM, de Mendonça A (2003) Participation of Adenosine receptors in neuroprotection. *Drug News Perspect* **16**: 80-86

Rittenhouse CD, Shouval HZ, Paradiso MA, Bear MF (1999) Monocular deprivation induces homosynaptic long-term depression in visual cortex. *Nature* **397**: 347-350

Roche KW, O'Brien RJ, Mammen AL, Bernhardt J, Huganir RL (1996) Characterization of multiple phosphorylation sites on the AMPA receptor GluR1 subunit. *Neuron* **16**: 1179–1188

Rörig B, Grantyn R (1993) Rat retinal ganglion cells express Ca(2+)-permeable non-NMDA glutamate receptors during the period of histogenetic cell death. *Neurosci Lett* **153**: 32-36

Rosenmund C, Carr DW, Bergeson SE, Nilaver G, Scott JD, Westbrook GL (1994) Anchoring of proteinkinase A is required for modulationof AMPA/kainate receptors on hippocampal neurons. *Nature* **368**: 853–856

Rosenzweig ES, Barnes CA (2003) Impact of aging on hippocampal function: Plasticity, network dynamics, and cognition. *Prog Neurobiol* **69:** 143–179

Rossi DJ, Oshima T, Attwell D (2000) Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* **403**: 316-321

Rothman SM (1984) Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death. *J Neurosci* **4**: 1884-1891

Rothstein JD, Brem H (2001) Excitotoxic destruction facilitates brain tumor growth. Nat Med 7: 994-995

Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB (1992) Neuroprotective role of adenosine in cerebral ischaemia. *Trends Pharmacol Sci* **13**: 439–445

Sargiacomo M, Lisanti M, Graeve L, Le Bivic A, Rodriguez-Boulan E (1989) Integral and peripheral protein composition of the apical and basolateral membrane domains in MDCK cells. *J Membr Biol* **107**: 277-286

Sattin A, Rall TW (1970) The effect of adenosine and adenine nucleotides on the cyclic adenosine 3', 5'-phosphate content of guinea pig cerebral cortex slices. *Mol Pharmacol* **6**: 13-23

Seamon KB, Padgett W, Daly JW (1981) Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc Natl Acad Sci USA* **78**: 3363–3367

Sebastião AM, Stone TW, Ribeiro JA (1990) On the inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: antagonism by 1,3,8-substituted xanthines. *Br J Pharmacol* **101**: 453–459

Sebastião AM, Ribeiro JA (1992) Evidence for the presence of excitatory A2 adenosine receptors in the rat hippocampus. *Neurosci Lett* **138**: 41–44

Sebastião AM, Ribeiro JA (1996) Adenosine A2 receptor-mediated excitatory actions on the nervous system. *Prog Neurobiol* **48**: 167-189

Sebastião AM, Ribeiro JA (2000) Fine-tuning neuromodulation by adenosine. *Trends Pharmacol Sci* **21**: 341-634

Sebastião AM, de Mendonca A, Moreira T, Ribeiro JA (2001) Activation of synaptic NMDA receptors by action potential-dependent release of transmitter during hypoxia impairs recovery of synaptic transmission on reoxygenation. *J Neurosci* **21**: 8564-8571

Sebastião AM, Ribeiro JA (2009) Adenosine Receptors and the Central Nervous System. In: Handbook of Experimental Pharmacology, 193 (Wilson CN, Mustafa SJ, ed). Springer-Verlag, Berlin Heidelberg. p 471-534

Sekino Y, Ito K, Miyakawa H, Kato H, Kuroda Y (1991) Adenosine (A2) antagonist inhibits induction of long-term potentiation of evoked synaptic potentials but not of the population spike in hippocampal CA1 neurons. *Biochem Biophys Res Commun* **181**: 1010–1014

Schwartzkroin PA (1975) Characteristics of CA1 neurons recorded intracellularly in the hippocampal in vitro slice preparation. *Brain Res.* **85(3)**: 423-436

Schoepp DD, Conn PJ (1993) Metabotropic glutamate receptors in brain function and pathology. *Trends Pharmacol Sci* **14:** 13-20

Schubert P, Ogata T, Marchini C, Ferroni S, Rudolphi K (1997) Protective mechanisms of adenosine in neurons and glial cells. *Ann NY Acad Sci* **825**: 1–10

Schurr A, Reid KH, Tseng MT, West C, Rigor BM (1986) Adaptation of adult brain tissue to anoxia and hypoxia in vitro. *Brain Res* **374**: 244-248

Shaw C, Hall SE, Cynader M (1986) Characterization, distribution, and ontogenesis of adenosine binding sites in cat visual cortex. *J Neurosci* **6**: 3218-3228

Sheardown MJ, Suzdak PD, Nordholm L (1993) AMPA, but not NMDA, receptor antagonism is neuroprotective in gerbil global ischaemia, even when delayed 24 h. *Eur J Pharmacol* **236**: 347-353

Sheng M, Lee SH (2001) AMPA Receptor Trafficking and the Control of Synaptic Transmission. *Cell* **105**: 825–828

Shepherd JD, Huganir RL (2007) The Cell Biology of Synaptic Plasticity: AMPA Receptor Trafficking. *Annu Rev Cell Dev Biol* **23**: 613–643

Shi SH, Hayashi Y, Petralia RS, Zaman SH, Wenthold RJ, Svoboda K, Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. *Science* **284**:1811-1816

Shi S, Hayashi Y, Esteban JA, Malinow R (2001) Subunit-specific rules governing AMPA receptor trafficking to synapses in hippocampal pyramidal neurons. *Cell* **105**: 331–343

Sigworth FJ, Neher E (1980) Single Na+ channel currents observed in cultured rat muscle cells. *Nature* **287**: 447-449

Simon RP, Swan JH, Griffiths T, Meldrum BS (1984) Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. *Science* **226**: 850-852

Simpson RE, O'Regan MH, Perkins LM, Phillis JW (1992) Excitatory transmitter amino acid release from the ischemic rat cerebral cortex: effects of adenosine receptor agonists and antagonists. *J Neurochem* **58**: 1683-1690

Skrede KK, Weestgaard RH (1971) The transverse hipocampal slice: a well-defined cortical structure maintained in vitro. *Brain Res* **35**: 589-593

Soderling TR, Derkach VA (2000) Postsynaptic protein phosphorylation and LTP. *Trends Neurosci* **23**: 75–80

Sokolova IV, Lester AL, Davidson N (2006) Postsynaptic mechanisms are essential for forskolin-induced potentiation of synaptic transmission. *J Neurophysiol* **95**: 2570–2579

Somjen GG (2001) Mechanisms of spreading depression and hypoxic spreading depression-like depolarization. *Physiol Rev* **81**: 1065–1096

Sommer B, Keinanen K, Verdoorn TA, Wisden W, Burnashev N, et al. 1990. Flip and flop: a cell-specific functional switch in glutamate-operated channels of the CNS. *Science* **249:** 1580–1585

Song I, Huganir RL. 2002. Regulation of AMPA receptors during synaptic plasticity. *Trends Neurosci* **25:** 578–588

Snell RS (2009) Clinical Neuroanatomy (7th edition). Lippincott Williams & Wilkins

Srivastava S, Osten P, Vilim FS, Khatri L, Inman G, States B, Daly C, DeSouza S, Abagyan R, Valtschanoff JG, Weinberg RJ, Ziff EB (1998) Novel anchorage of GluR2/3 to the postsynaptic density by the AMPA receptor–binding protein ABP. *Neuron* **21**: 581–591

Stanton PK, Sarvey JM (1984) Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. *J Neurosci* **4**: 3080–3088

Stanton PK (1996) LTD, LTP, and the sliding threshold for long term synaptic pasticity. *Hippocampus* **6**: 35-42

Stillings D (1975) Did Jan Swammerdam beat Galvani by 134 years? *Med Instrum* **9**:226

Suzuki S, Tanaka K, Nogawa S, Ito D, Dembo T, Kosakai A, Fukuuchi Y (2000) Immunohistochemical detection of leukemia inhibitory factor after focal cerebral ischemia in rats. *J Cereb Blood Flow Metab* **20**: 661-668

Suzuki S, Tanaka K, Suzuki N (2009) Ambivalent aspects of interleukin-6 in cerebral ischemia: inflammatory versus neurotrophic Aspects. *J Cereb Blood Flow Metab* **29**: 464–479

Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. *J Neurosci* **17**: 58–69

Syntichaki P, Tavernarakis N (2003) The biochemistry of neuronal necrosis: rogue biology? *Nat Rev Neurosci* **4**: 672-684~

Taga T, Kishimoto T (1997) Gp130 and the interleukin-6 family of cytokines. *Annu Rev Immunol* **15**: 797–819

Tanaka E, Yamamoto S, Kudo Y, Mihara S, Higashi H (1997) Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro. *J Neurophysiol* **78**: 891–902

Tanaka H, Grooms SY, Bennett MV, Zukin RS (2000) The AMPAR subunit GluR2: still front and center-stage. *Brain Res* **886**: 190-207

Tanaka M, Hirabayashi Y, Sekiguchi T, Inoue T, Katsuki M, Miyajima A (2003) Targeted disruption of oncostatin M receptor results in altered hematopoiesis. *Blood* **102**: 3154–3162

Tardin C, Cognet L, Bats C, Lounis B, Choquet D (2003) Direct imaging of lateral movements of AMPA receptors inside synapses. *EMBO J* **22:** 4656–4665

Thomas-Crusells J, Vieira A, Saarma M, Rivera C (2003) A novel method for monitoring surface membrane trafficking on hippocampal acute slice preparation *J Neurosci Methods* **125:** 159–166

Thomson AM (2000) Facilitation, augmentation and potentiation at central synapses. *Trends Neurosci* **23**: 305-312

Tiselius A (1937) A new apparatus for electrophoretic analysis of colloidal mixtures" *Transactions of the Faraday Society* 33: 524

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J (1991) The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* **266**: 15771–15781

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76: 4350-4354

Triller A, Choquet D (2005) Surface trafficking of receptors between synaptic and extrasynaptic membranes: and yet they do move! *Trends Neurosci* **28**: 133-139

Turetsky DM, Canzoniero LM, Sensi SL, Weiss JH, Goldberg MP, Choi DW (1994) Cortical neurones exhibiting kainate-activated Co2+ uptake are selectively vulnerable to AMPA/kainate receptor-mediated toxicity. *Neurobiol Dis* **1**: 101-110

Turski L, Huth A, Sheardown M, McDonald F, Neuhaus R, Schneider HH, Dirnagl U, Wiegand F, Jacobsen P, Ottow E (1998) ZK200775: a phosphonate quinoxalinedione AMPA antagonist for neuroprotection in stroke and trauma. *Proc Natl Acad Sci USA* **95**: 10960-10965

Ungless MA, Whistler JL, Malenka RC, Bonci A (2001) Single cocaine exposure in vivo induces long-term potentiation in dopamine neurons. *Nature* **411**: 583–587

van Calker D, Müller M, Hamprecht B (1979) Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem* **33**: 999–1005

Vesterberg O (1989) History of Electrophoretic Methods. *J Chromat*, 480: 3-19

Villacres EC, Wong ST, Chavkin C, Storm DR (1998) Type I adenylyl cyclase mutant mice have impaired mossy fiber long-term potentiation. *J Neurosci* **18**: 3186-3194

Vincent P, Mulle C (2009) Kainate receptors in epilepsy and excitotoxicity. *Neuroscience* **158**: 309–323

Virgo L, Samarasinghe S, de Belleroche J (1996) Analysis of AMPA receptor subunit mRNA expression in control and ALS spinal cord. *Neuroreport* **7**: 2507-2511

Walters MR, Kaste M, Lees KR, Diener HC, Hommel M, De Keyser J, Steiner H, Versavel M (2005) The AMPA antagonist ZK 200775 in patients with acute ischaemic stroke: a double-blind, multicentre, placebo-controlled safety and tolerability study. *Cerebrovasc Dis* **20**: 304-309

Wang L-Y, Salter MW, MacDonald JF (1991) Regulation of kainate receptors by cAMP-dependent protein kinase and phosphatases. *Science* **253**: 1132–1135

Wang L, Walia B, Evans J, Gewirtz AT, Merlin D, Sitaraman SV (2003) IL-6 induces NF-kappa B activation in the intestinal epithelia. *J Immunol* **171**: 3194–3201

Wang JQ, Fibuch EE, Mao L (2007) Regulation of mitogen-activated protein kinases by glutamate receptors. *J Neurochem* **100**: 1-11

Washburn MS, Numberger M, Zhang S, Dingledine R (1997) Differential dependence on GluR2 expression of three characteristic features of AMPA receptors. *J Neurosci* **17**: 9393-9406

Weiss JH, Sensi SL (2000) Ca^{2+}-Zn^{2+} permeable AMPA or kainate receptors: possible key factors in selective neurodegeneration. *Trends Neurosci* **23**: 365-371

Weiss TW, Kvakan H, Kaun C, Zorn G, Speidl WS, Pfaffenberger S, Maurer G, Huber K, Wojta J (2005) The gp130 ligand oncostatin M regulates tissue inhibitor of metalloproteinases-1 through ERK1/2 and p38 in human adult cardiac myocytes and in human adult cardiac fibroblasts: a possible role for the gp130/gp130 ligand system in the modulation of extracellular matrix degradation in the human heart. J Mol Cell Cardiol **39**: 545–551

Weiss TW, Samson AL, Niego B, Daniel PB, Medcalf RL (2006) Oncostatin M is a neuroprotective cytokine that inhibits excitotoxic injury in vitro and in vivo. *FASEB J* **20:** 2369–2371

Wen TC, Rogido MR, Moore JE, Genetta T, Peng H, Sola A (2005) Cardiotrophin-1 protects cortical neuronal cells against free radical-induced injuries in vitro. *Neurosci Lett* **387**: 38–42

Wenthold RJ, Petralia RS, Blahos J II, Niedzielski AS (1996) Evidence for multiple AMPA receptor complexes in hippocampal CA1/CA2 neurons. *J Neurosci* **16**: 1982-1989

West AE, Griffith EC, Greenberg ME (2002) Regulation of transcription factors by neuronal activity. *Nat Rev Neurosci* **3**: 921-931

White PJ, Rose'Meyer RB, HopeW (1996) Functional characterization of adenosine receptors in the nucleus tractus solitarius mediating hypotensive responses in the rat. *Br J Pharmacol* **117**: 305–308

Wieraszko A, Goldsmith G, Seyfried TN (1989) Stimulation-dependent release of adenosine triphosphate from hippocampal slices. *Brain Res* **485**: 244–250

Williams M, Braunswalder A, Erickson TJ (1986) Evaluation of the binding of the A1 selective adenosine radioligand, cyclopentyladenosine (CPA), to rat brain tissue. *Naunyn-Schmiedeberg's Arch Pharmacol* **332**: 179–183

Williams K (1997) Modulation and block of ion channels: a new biology of polyamines. *Cell Signal* **9**: 1-13

Witter MP, Naber PA, van Haeften T, Machielsen WC, Rombouts SA, Barkhof F, Scheltens P, Lopes da Silva FH (2000) Cortico-hippocampal communication by way of parallel parahippocampal-subicular pathway. *Hippocampus* **10**: 398-410

Witter MP, Amaral DG (2004) Hippocampal region. In: Paxinos G, editor. The Rat Nervous System, 3rd ed. New York: Academic Press

Yamamoto C, McIlwain H (1966) Electrical activities in thin sections from the mammalian brain maintained in chemically-defined media in vitro. *J Neurochem* **13**: 1333-1343

Yang Y, Wang XB, Frerking M, Zhou Q (2008a) Spine expansion and stabilization associated with long-term potentiation. *J Neurosci* **28**: 5740–5751

Yang Y, Wang X-B, Frerking M, Zhou Q (2008b) Delivery of AMPA receptors to perisynaptic sites precedes the full expression of long term potentiation. *Proc Natl Acad Sci USA* **105**: 11388–11393

Yoshioka A, Hardy M, Younkin DP, Grinspan JB, Stern JL, Pleasure D (1995) Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors mediate excitotoxicity in the oligodendroglial lineage. *J Neurochem* **64**: 2442-2448

Zamanillo D, Sprengel R, Hvalby O, Jensen V, Burnashev N, Rozov A, Kaiser KM, Koster HJ, Borchardt T, Worley P, et al. (1999) Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. *Science* **284:** 1805–1811

Zempleni J, Wijeratne SS, Hassan YI (2009) Biotin. *Biofactors* **35**: 36-46

Zhu JJ, Esteban JA, Hayashi Y, Malinow R (2000) Postnatal synaptic potentiation: delivery of GluR4- containing AMPA receptors by spontaneous activity. *Nat Neurosci* **3**: 1098–1106

Ziff EB (2007) TARPs and the AMPA receptor trafficking paradox. *Neuron* **53**: 627-633

Zocchi C, Ongini E, Conti A, Monopoli A, Negretti A, Baraldi PG, Dionisotti S (1996) The non-xhantine heterocyclic compound [3H]-SCH58261 is a new potent and selective A2A adenosine receptor antagonist. *J Pharmacol Exp Ther* **276**: 398–404