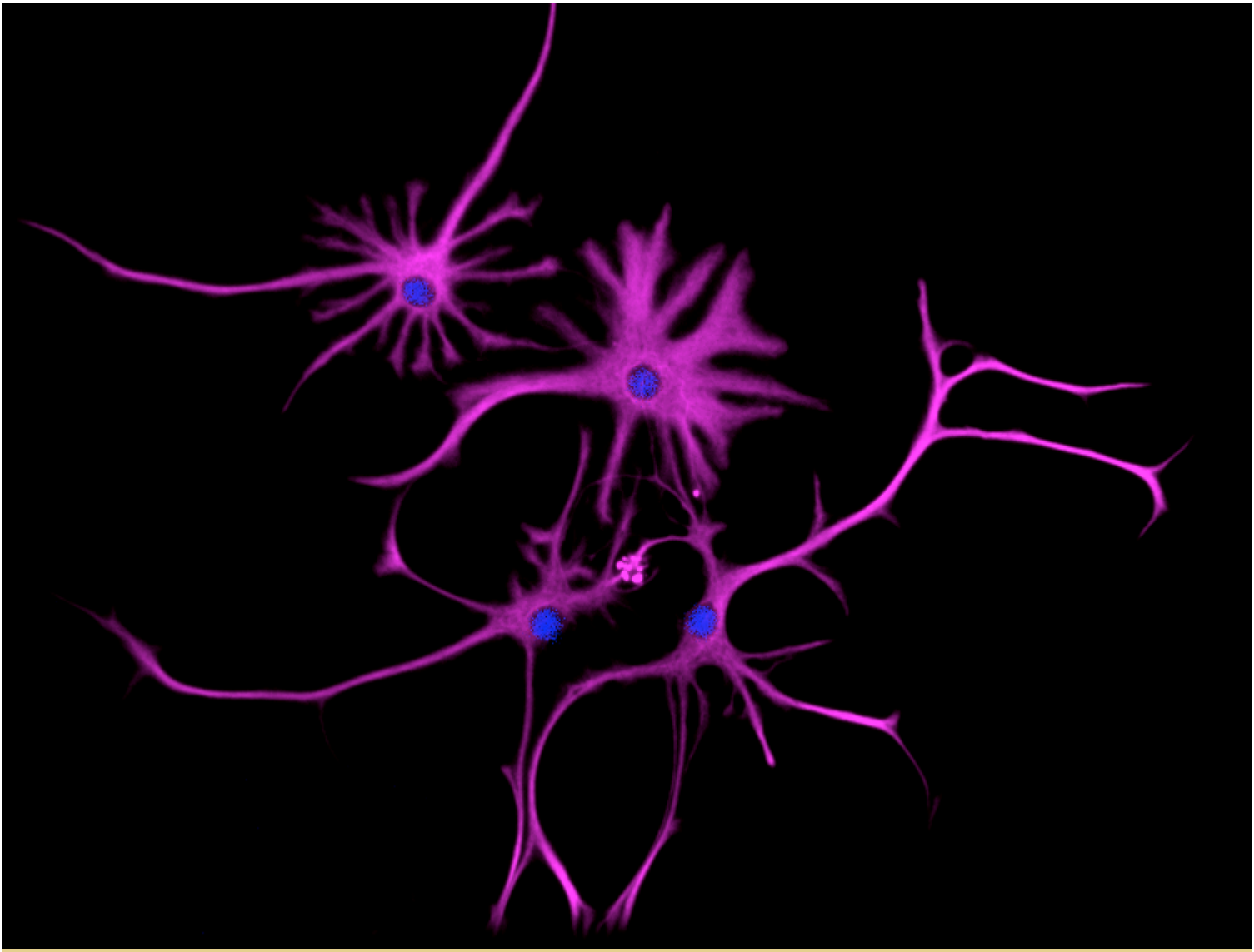

Vanessa Jorge Henriques

ASTROCYTIC A_{2A} RECEPTORS: NOVEL TARGETS TO MANAGE BRAIN DISORDERS

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica sob orientação científica do Doutor Nélio da Mota Gonçalves e do Professor Doutor Rodrigo Pinto dos Santos Antunes da Cunha e apresentada à Faculdade de Medicina da Universidade de Coimbra.

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Universidade de Coimbra

Astrocyte A_{2A} Receptors: Novel targets to manage brain disorders

Vanessa Jorge Henriques

Dissertation for the attribution of the Masters degree in the specialty field of Biomedical Research, submitted to the Faculty of Medicine of the University of Coimbra, Portugal. The research work presented in this dissertation was performed at the Center for Neuroscience and Cell Biology (CNC), University of Coimbra, Portugal, under the supervision of Doctor Nélio da Mota Gonçalves and Doctor Rodrigo Pinto dos Santos Antunes da Cunha.

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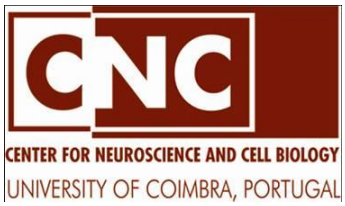

Front cover:

Primary astrocyte cell culture stained with glial fibrillary acidic protein (GFAP, magenta) and Diamidino-2-phenylindole (DAPI, blue).

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Abbreviations

AAV	Adeno-associated virus
Ab	Antibody
AD	Alzheimer's disease
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
AGO2	Endonuclease agronaute 2
ARs	Adenosine receptors
A_1R	Adenosine A_1 receptor
$A_{2A}R$	Adenosine A_{2A} receptor
Aβ	β-amyloid peptide
BBB	Blood-brain barrier
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3',5'-monophosphate
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
DAPI	Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DPCPX	1,3-dipropyl-8-cyclopenty lxanthine
DsRNA	Double stranded RNA
DNAse	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
GFAP	Glial fibrillary acidic protein
G-proteins	GTP-binding proteins
GPCRs	G protein-coupled receptors
HEK	Human embryonic kidney
HEPES	hydroxyethyl piperazineethanesulfonic acid
HBSS	Hanks' balanced salt solution
HIV-1	Human immunodeficiency virus
IF	Intermediate filament

IFN	Interferon
LB	Luria-Bertani
LTR	long terminal repeat
LVs	Lentiviral vectors
miRNA	microRNA
mRNA	Messenger RNA
Mok-G	Mokola *Lyssavirus* G glycoprotein
MSNs	Medium spiny neurons
NeuN	Neuronal nuclei protein
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
ON	Overnight
P_1	Purinergic receptor family 1
P_2	Purinergic receptor family 2
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PGK	Phosphoglycerate kinase
PFA	Paraformaldehyde
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPM	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
shA_{2A}R	Short hairpin against adenosine A_{2A} receptor
shCTR	Short hairpin control
shRNA	Short hairpin RNA
SIN	Self-inactivating sequence
siRNA	Small interfering RNA
TE	Tris-EDTA Buffer
VSV-G	Vesicular stomatitis virus G glycoprotein

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Abstract

Adenosine is a prototypic modulator of synaptic transmission in the CNS; it mainly controls excitatory transmission through a coordinated action of inhibitory A1 receptors and facilitatory adenosine A_{2A} receptors (A_1R, $A_{2A}R$) and, albeit their physiological and pathological role have mainly been assumed to result from their direct action on neurons, they are also present in astrocytes where they control astrogliosis, proliferation, cell death, and the release of neurotrophic factors and interleukins.

Astrocytes are the dominant subclass of non-neuronal glial cells of the brain; they are physically associated with synapses prompting the tripartite synapse concept to highlight their relevance in integrating neuronal networks by reciprocal chemical signaling. Astrocytes format synaptic plasticity since they are responsible for clearance of extracellular glutamate through glutamate transporters. This highlights the crucial involvement of astrocytes on the abnormal glutamate over-excitation implicated in both acute CNS injuries and diverse chronic neurodegenerative disorders.

The group of Purines at CNC recently showed that astrocytic glutamate uptake is diminished upon activation of astrocytic $A_{2A}R$, both under physiological and pathological conditions, which supports the ability of astrocytic $A_{2A}R$ to control pathophysiological processes involving the activity of glutamate transporters. Additionally, $A_{2A}R$ are aberrantly up-regulated upon different brain insults. Therefore, we now prompted the hypothesis of blocking astrocytic $A_{2A}R$ as a novel and promising strategy to prevent abnormal glutamate over-excitation, thus preventing biochemical-, functional- and behavioural-associated modifications. Hence, we sought to validate novel tools for selectively and region-specific down-regulate astrocytic $A_{2A}R$ to further probe their efficacy under physiological and pathological conditions.

This work was organized in 2 main steps: i) we first incorporated into a lentivector coated with Mokola Lyssavirus G glycoprotein (Mok-G) an RNA interference strategy to down-regulate $A_{2A}R$ also carrying a reporter gene, enhanced green fluorescent protein (EGFP) to allow cells to be identifiable,; ii) we then tested whether Mok-G coated lentivirus were able to selectively and efficiently transduce astrocytes *in vitro* (primary astrocyte cell cultures) and *in vivo* (into selected brain regions such as prefrontal cortex, striatum and hippocampus) for delivering $shA_{2A}R$ constructs to downregulate $A_{2A}R$ expression and density. We evaluated viral spreading and cell-type transduction through

immunofluorescent co-localization of the reporter gene EGFP with glial (GFAP and vimentin) and neuronal (NeuN) markers.

The present study showed that Mokola-G-coated lentivirus encoding $shA_{2A}R$ successfully infects astrocytes and down-regulate astrocytic A_{2A} receptors expression and density, at least *in vitro*. Major concerns should be considered when it comes to their *in vivo* application, especially since there are different transduction efficiencies as well as selectivity of astrocytic targeting in the adult rodent brain with further implications for therapeutic gene transfer.

Keywords: Purinergic receptors, Adenosine A_{2A} receptors, Astrocytes, lentiviral vectors, Mokola *Lyssavirus* G glycoprotein, short hairpin RNA.

Resumo

Adenosina é um modulador típico da transmissão sináptica no sistema nervoso central (SNC); controla maioritariamente a transmissão excitatória através da acção coordenada dos receptores inibitórios A_1 e excitatórios A_{2A} e, apesar do seu papel fisiológico e patológico resultar da sua ação preponderantemente em neurónios, estão também presentes em astrócitos onde controlam a astrogliose, a proliferação, a morte celular, e a libertação de factores neurotróficos e interleucinas.

Os astrócitos são as células da glia não neuronais mais prevalentes no cérebro e estão fisicamente associados às sinapses onde a reciprocidade da sinalização química permite o funcionamento íntegro dos circuitos neuronais, o que originou o conceito de sinapse tripartida. Os astrócitos são importantes mediadores da plasticidade sináptica uma vez que eliminam o glutamato extracelular através de transportadores de glutamato. Este facto reveste-se de particular relevo em situações de sobre-excitação anormal pelo glutamato caraterístico de lesões agudas no SNC bem como em diversas doenças neurodegenerativas.

O grupo de Purinas demonstrou recentemente que a activação dos receptores A_{2A} astrocitários diminui a captação de glutamato em condições fisiológicas e patológicas o que atesta capacidade dos receptores A_{2A} astrocitários em controlar processos patológicos através da actividade dos transportadores de glutamato. Uma vez que a densidade de receptores A_{2A} ($A_{2A}R$) está anormalmente aumentada em diversas situações de dano cerebral, propomos agora a hipótese de que o bloqueio dos receptores A_{2A} astrocitários se constitua como uma nova estratégia promissora para prevenir a sobre-excitação anormal do glutamato prevenindo assim as modificações bioquímicas, funcionais e comportamentais associadas. Por essa razão, validámos novas ferramentas para diminuir local e seletivamente a expressão de receptores A_{2A} astrocitários e posteriormente comprovar a sua eficácia em condições fisiológicas e patológicas.

Este trabalho foi executado em duas etapas: i) incorporou-se em um vector viral revestido com a glicoproteína G do vírus Mokola um RNA de interferência (shRNA) para diminuir a expressão astrocitária de $A_{2A}R$ e também um gene repórter, a proteína fluorescente verde (EGFP) para permitir a identificação das células infectadas; ii) testou-se a eficiência de infeção do lentivírus e a sua selectividade para astrócitos quer *in vitro* (em culturas primárias) quer *in vivo* (por administração dos lentivírus em regiões particulares do cérebro, tais como o córtex pré-frontal, o estriado e o hipocampo) e avaliou-se a diminuição da densidade dos receptores A_{2A} astrocitários mediada pelos shRNAs. Avaliou-se a difusão do vírus bem como a transdução celular através da co-localização por imunofluorescência do EGFP com marcadores de astrócitos (GFAP e vimentina) e de neurónios (NeuN).

Este estudo demonstrou que o lentivirus revestido com a glicoproteína G do vírus Mokola contendo sh$A_{2A}R$ é eficiente na infeção de astrócitos bem como na diminuição da densidade de $A_{2A}R$ *in vitro*. Uma vez que a eficiência de transdução bem como a seletividade para astrócitos destes vírus parece ser distinta em diferentes regiões do cérebro de roedor com implicações para a sua aplicabilidade terapêutica, a utilização desta estratégia deve ser ponderada.

Palavras-chave: Receptores purinérgicos, receptores de adenosina A_{2A}, vectores lentivirais, glicoproteína G do vírus Mokola, RNA de interferência.

Chapter 1

Introduction

1. Introduction

1.1. Purinergic system

The purinergic signaling is one of the most ancient systems known to exert essential functions in the brain, namely on synaptic activity and on neurotransmission; this mainly involves the activity of adenosine 5'-triphosphate (ATP), which stores chemical energy for cells metabolism and adenosine, which is involved in key pathways such as nucleic acid synthesis, amino acid metabolism and modulation of cellular status. Major attention was drawn on ATP and adenosine as a class of neuro-active substances with neurotransmitter properties, which regulate the flow rather than mediate information processing between neurons (Cunha and Ribeiro, 2000; Oliveira et al., 2016).

In 1978, Burnstock suggested the first division of purinergic receptors into two distinct classes: receptors sensitive to adenosine (P1 receptors) and receptors sensitive to nucleotides (P2 receptors). Both P1 and P2 receptors are present in all tissues of mammalian organisms; they have been argued to play a role in the modulation of several physiological processes such as immune cell recruitment, inflammation, neurotransmission, regulation of vascular and muscular tone and nociception (Abbracchio and Ceruti, 2007; Trautmann, 2009; Burnstock and Verkhratsky, 2010; Tsuda et al., 2010). Their ligands, adenosine and ATP, act as neurotransmitters and neuromodulators regulating microglial and astrocytic functions, controlling bi-directional neuron-to-glia cell communication and also long-term mechanisms during neurodevelopment (Krugel, 2016). This supports the increased interest on the therapeutic potential of targeting purinergic receptors to manage neuroinflammation, neurodegeneration and brain disorders (reviewed in Weisman et al., 2012; Burnstock, 2016).

Adenosine is an endogenous nucleoside modulator that is released from the majority of cells and is produced by the breakdown of ATP through several ectoenzymes

in the extracellular space. The concentration of extracellular adenosine is kept in equilibrium through the action of specific transporters and its effects are mediated through activation of a family of four G-protein-coupled receptors, namely A_1, A_{2A}, A_{2B}, and A_3 receptors. These receptors differ in their affinity for adenosine, in the type of G proteins recruited and in the downstream signaling pathways that are activated in the target cells (Fredholm et al., 2001). Several pathophysiological conditions are thought to be associated with changes of adenosine levels (Gessi et al., 2011), which are increased under metabolically unfavorable conditions..Nevertheless, the main assignment of adenosine is the reduction and repair of tissue injury through different receptor-mediated mechanisms, which may subsequently increase oxygen supply or demand ratio, anti-inflammatory actions and stimulation of angiogenesis (Linden, 2005).

Under physiological conditions, both nerve terminals and astrocytes release ATP into the extracellular space whose degradation by ecto-nucleotidases will give rise to the major source of adenosine modulating synaptic transmission (Cunha et al., 1996; Pascual et al., 2005). Additionally, adenosine can modulate astrocyte functions by controlling their reactivity, named astrogliosis, the release of neurotrophic factors and interleukins (Ciccarelli et al., 2001), and cell proliferation and death (Fredholm., 2007).

Purinergic signaling is crucial for the modulation of the glial response to center nervous system (CNS) injury. Astrocytes respond to extracellular ATP and ADP (Adenosine 5'-diphosphate) by activation of P2X and P2Y receptors subtypes and ultimately to gene expression modulation: in fact, ATP is the major endogenous mediator that determine reactive astrogliosis in acute brain injury (Heine et al., 2015).

Astrocytes are one of the main sources of extracellular adenine-based purines (Ciccarelli et al., 1999) and express a density of adenosine receptors different from neurons (Matos et al., 2012). Once released from astrocytes, adenosine can contact with its different receptors and may be taken up through nucleoside transporters present in astrocytes (Ciccarelli et al., 2001) and in neurons (Pinto-Duarte et al., 2005).

1.1.1. Adenosine and its receptors

Unlike ATP, adenosine does not act as a classical neurotransmitter, since it is not stored in vesicles, it is not released by exocytosis, it does not transfer unidirectional information from presynaptic to postsynaptic components, and it does not act only mainly in synapses (Cunha, 2001). It can be formed from the extracellular catabolism of released ATP (Cunha, 2008) or it can be released from the cytoplasm to the extracellular space through nucleoside transporters (Sebastião and Ribeiro, 2009) and acts as a homeostatic modulator (Gomes et al., 2011) where it fulfills a double role acting both as a homeostatic transcellular messenger and as a neuromodulator controlling neurotransmitter release and neuronal excitability (Cunha, 2001).

Adenosine exerts its effects via four cloned and pharmacologically characterized receptors: A_1, A_{2A}, A_{2B} and A_3 receptors, all of which couple to G proteins (Dare et al., 2007). Adenosine is a well-known modulator of synaptic transmission in the CNS as it controls excitatory transmission through a coordinated action of inhibitory and facilitatory A_1 and A_{2A} receptors ($A_{2A}R$), respectively (Fredholm et al., 2005) exerting neuroprotective effects under pathophysiological conditions (Cunha, 2001; Fredholm et al., 2005). A_1, A_{2A}, A_{2B} and A_3 receptors are activated by different ranges of endogenous adenosine concentrations, which differently control physiological responses (Fredholm et al., 1994). A_1R tend to suppress neuronal activity by a predominant presynaptic action, whereas $A_{2A}R$ promote transmitter release and postsynaptic depolarization (Stone et al., 2009). On one hand, A_1R inhibits the release of a variety of neurotransmitters by reducing calcium influx in response to action potential invasion of terminals (Wu and Saggau, 1997). On the other hand, activation of $A_{2A}R$ stimulates the release of neurotransmitters (Stone et al., 2009) through an increase in presynaptic calcium influx (Gonçalves et al., 1997). Little is known about A_{2B} and A_3 receptors, especially since they are present in low densities in brain tissue (Fredholm et al., 2005). A_{2B} receptors are expressed at low levels in almost all tissues, including brain and spinal cord, while A_3 receptors are most abundantly located in peripheral tissues (Stone et al., 2009).

Selective agonists and antagonists are available for all four adenosine subtypes and allowed the recent body of evidences regarding the adenosine receptors functions in health and disease conditions (Gessi et al., 2011). As an example, using selective A_1 receptors antagonists such as the 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) it was possible to unmask a tonic A_1 receptor-mediated adenosinergic tonus. Likewise with selective $A_{2A}R$ antagonists like SCH-58621 or ZM-241385 that could be used to access its functions (Sebastião and Ribeiro, 2009).

A lot of work has been devoted to understand the role of $A_{2A}R$ in the brain. Adenosine $A_{2A}R$ are mostly present in nerve terminals (Rebola et al., 2005); they are mainly located post-synaptically in GABAergic medium spiny neurons (MSN) (Schiffmann et al., 2007) and pre-synaptically in hippocampal and cortical neurons, where $A_{2A}R$ mostly control the release of different neurotransmitters such as glutamate (Lopes et al., 2002), GABA (Cunha and Ribeiro, 2000), acetylcholine (Rebola et al., 2002) and serotonin (Okada et al., 2001). $A_{2A}R$ have received special attention due to the neuroprotective effects mediated by their blockade in different chronic noxious brain conditions (Gomes et al., 2011): recent reports showed that the pharmacological and genetic deletion of $A_{2A}R$ diminishes neurodegeneration and brain dysfunction in diverse animal models of brain diseases, namely in Alzheimer's (Canas et al., 2009), Parkinson's (Schwarzschild et al., 2006), epilepsy (Cognato et al., 2010), Machado-Joseph disease (Gonçalves et al., 2013), and chronic stress (Batalha et al., 2013); Kaster et al., 2015), which paves the way for the translation of $A_{2A}R$ antagonists to manage brain disorders, especially since their safety profile has already been established during their establishment as novel anti-Parkinsonian drugs (Chen et al., 2014).

The observation that $A_{2A}R$ are mainly located in synapses, selectively controlling N-methyl-D-aspartate (NMDA) receptor (Rebola et al., 2008) and synaptic plasticity phenomena (d'Alcantara et al., 2001) and the deletion of neuronal $A_{2A}R$ is sufficient to afford neuroprotection (Kaster et al., 2015) , leads the hypothesis that the control of synaptotoxicity is a critical event in $A_{2A}R$ neuroprotection (Cunha and Agostinho, 2010). Yet, the possible role of $A_{2A}R$ in astrocytes still remains to be determined, especially since $A_{2A}R$ undergo a marked up-regulation in glial cells in neurodegenerative disorders (Rebola et al., 2011; Matos et al, 2012; Cunha et al., 2006; Gonçalves et al., 2013).

1.1.2. A_{2A} receptor blockade *vs* neuroprotection

A remarkable convergence of epidemiological and animal studies defines an inverse relation between caffeine consumption and the incidence of AD-like features (Maia and de Mendonça, 2002). It was shown that the chronic consumption of caffeine at mid-life inversely correlates with the incidence of dementia and its neuropathology (Eskelinen et al., 2009; Santos et al., 2010; Gelber et al., 2011), in agreement with similar conclusions obtained in different animal models (Dall'Igna et al., 2007; Arendash et al., 2009); this allowed proposing that the putative molecular target of caffeine, the adenosine A_{2A} receptors, are a key neuroprotective system in AD (Dall'Igna et al., 2007; Canas et al., 2009). The findings that caffeine and A_{2A}R blockade prevent memory impairment triggered by different noxious stimuli (reviewed in (Takahashi et al., 2008; Cunha and Agostinho, 2010)) strongly suggests that A_{2A}R can control essential mechanisms leading to neurodegeneration (Gomes et al., 2011). Glutamatergic transmission is one candidate mechanism by which A_{2A}R may control neurodegeneration. In fact A_{2A}R are enriched in glutamatergic synapses (Rebola et al., 2005), where they control synaptic plasticity and NMDA receptor activation (Rebola et al., 2008; Yu et al., 2008).

Since GLT activity is central for physiological and pathological conditions and astrocytic A_{2A}R afford a sustained control of GLT (Matos et al., 2012), astrocytic A_{2A}R might constitute an emerging candidate target, which will allow better understanding the neuroprotection afforded by A_{2A}R blockade on different brain insults (Gomes et al., 2011).

1.2. Astrocytes

Astrocytes are the dominant subclass of non-neuronal glial cells of the brain that have been shown to exert key roles in the regulation of synaptic transmission. In contrast to neurons, astrocytes have a smaller soma, numerous highly branched fine processes that extend for long distances making contact with neuronal processes at synapses (Fellin et al., 2006). Astrocytes were first studied by Cajal and colleagues from the structural standpoint. They proposed that astrocytes control sleep and waking states through electrical insulators that act as circuit breakers to facilitate sleep when extended between neurons, and facilitate wakefulness allowing neuronal circuits to communicate when retracted (Garcia-Marin et al., 2007). More than a century after Cajal's proposal, evidences were gathered supporting that astrocytes have a structural and functional association with neurons and are able to modulate behaviors (Halassa et al., 2009). As shown in figure 1, astrocytes occupy non-overlapping spatial territories in which a single astrocyte contacts hundreds of neuronal processes and multiple neuronal cell bodies (Bushong et al., 2002), and they can also identify a single synapse as an active glutamate releasing synapse (Bushong et al., 2002).

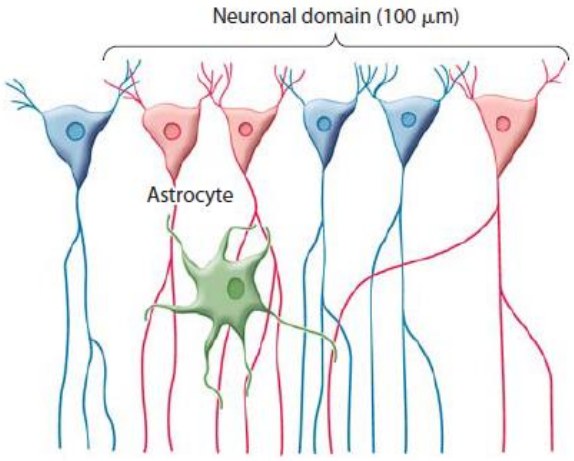


Figure 1 | Schematic representation of neuron-astrocyte signaling.

The release of glutamate from a single astrocyte (green cell) onto several dendrites can lead to the synchronization of groups of neurons (red cells) located within 100 μm of each other (adapted from Fellin et al, 2006).

Neuron-glia signaling is crucial for brain function. Since astrocytes express membrane receptors for almost all neurotransmitters, they can detect neuronal activity and increase the metabolic support of neurons via the release of a variety of chemical transmitters onto several dendrites (Figure 2) including glutamate, ATP (the main physiologically released source of ATP in the CNS (Anderson et al., 2004)), nitric oxide, peptides, chemokines and cytokines among others through different mechanisms (Halassa and Haydon, 2010). All these signaling molecules modulate neuronal excitability (Auld and Robitaille, 2003; Fiacco and McCarthy, 2004) and therefore control the neuronal network giving rise to the tripartite synapse concept where the astrocytic process enwrapping neuronal structures, namely the presynaptic neuronal terminal, the postsynaptic membranes of dendrites and neuronal somata (Heine et al., 2015). There is an interplay between glutamate and ATP involving their astrocytic re-uptake, which leads to a balanced excitation and inhibition to neuronal networks in the hippocampus (Fellin et al, 2006): when glutamate is released from nerve terminals, astrocytes immediately re-uptake glutamate through the activity of glutamate transporters. In opposition, released ATP is not taken by transporters but hydrolyzed in the extracellular space to adenosine. The release of ATP from astrocytes is not sufficient to have direct actions on neurons, but the delayed accumulation of extracellular adenosine has potent actions on A_1R, causing a delayed inhibition of the neuronal activity (Figure 2). Withal, the timing of these two gliotransmitters can afford balanced excitation and inhibition to neuronal network (Fellin et al., 2006).

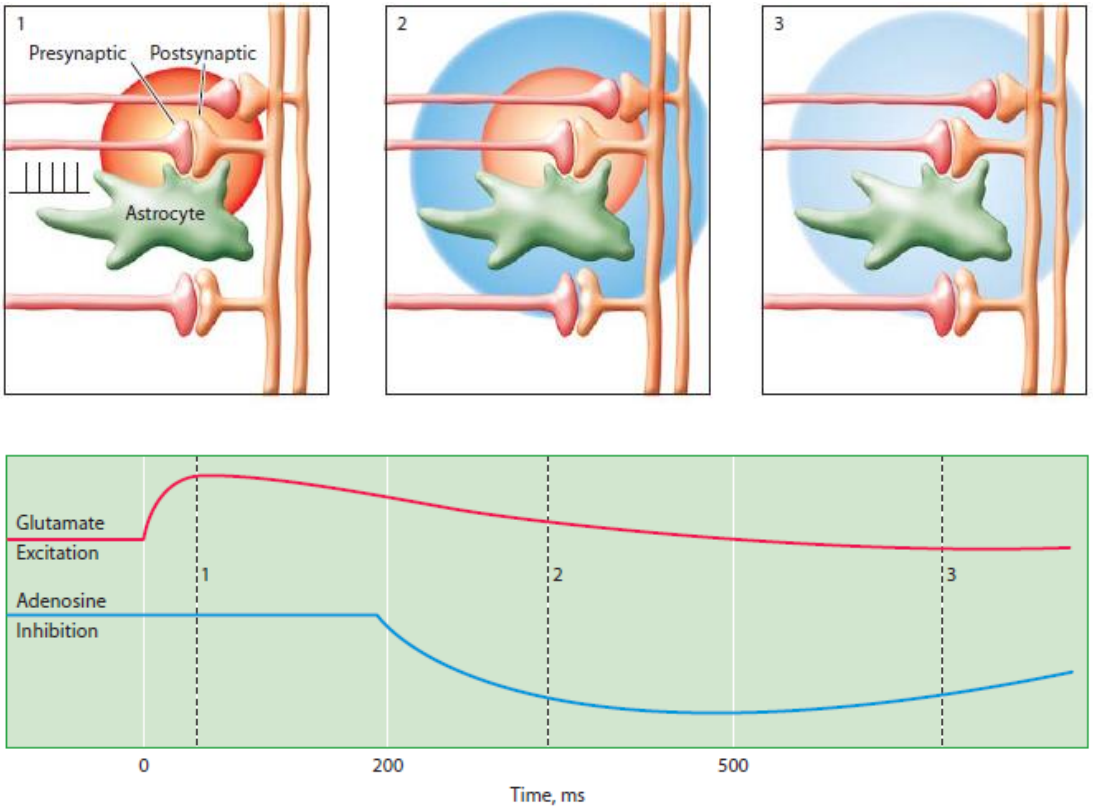


Figure 2 | Model of gliotransmission mediated by glutamate and ATP.

Activation of an astrocyte by synaptic activity leads to the release of two gliotransmitters: glutamate and ATP. The release of glutamate leads to a fast activation of NMDA receptors that depolarize close neurons (time point 1). In contrast, the release of ATP does not reach a concentration sufficient to trigger immediate neuronal effects through P_2 receptors. As an alternative, purinergic action has a temporal delay requiring the extracellular hydrolysis of ATP to adenosine (~200 ms). As a consequence of this delay, adenosine can diffuse to distant sites of action (time point 2) where it can suppress the excitation of synaptic transmission. After the clearance of glutamate from the extracellular space, adenosine-mediated synaptic suppression perseveres until it slowly re-equilibrates with cytosolic adenosine (time point 3) through the action of nucleoside transporters (adapted from Fellin et al, 2006).

Astrocytes are strategically positioned between synapses and blood vessels allowing the neurovascular coupling, the process by which neuronal activity is coupled to the cerebral blood flow (Takano et al., 2006). This is accomplished by astrocytic processes that form endfeet covering the entire cerebral vascular surface (Mathiisen et al., 2010). This endfeet helps maintaining the expression of endothelial tight junction proteins, transporters, and enzymes, in conjunction with pericytes (Winkler et al., 2011), which together comprise the blood-brain barrier (BBB) (Wolburg et al., 2009). An interesting relation exits between astroglial differentiation and BBB maturation.

Astroglial differentiation can be morphologically recognized as the polarization of astrocytes, which arises in parallel with the maturation of the BBB (Brillault et al., 2002).

1.2.1. Astrocyte reactivity and reactive astrogliosis

Reactive astrogliosis, a response of astrocytes observed in multiple neurological disorders, is a constitutive, graded, multistage, and evolutionary conserved defensive astroglial reaction (Pekny and Pekna, 2014). Astrocyte reactivity and reactive astrogliosis occur in many pathological situations such as trauma, ischemic damage, neuroinflammation and neurodegeneration (Pekny and Pekna, 2014). Reactive astrocytes exhibit altered gene expression and distinct morphological and functional features (Eddleston and Mucke, 1993; Eng., 1994; Pekny and Nilsson, 2005; Sofroniew, 2009) that are subtly regulated by a complex inter- and intra-cellular signaling (Sofroniew, 2009). Although Eddleston and Mucke (1993) defined reactive astrocytes as an increase in the number and size of cells expressing glial fibrillary acidic protein (GFAP), the major intermediate filament protein of mature astrocytes that serves as a cell-specific marker distinguishing differentiated astrocytes from other glial cells (Gomes et al., 1999), this concept and its molecular and cellular definition is still not clear. Increased GFAP-positive cells *in vivo* reveals predominantly phenotypic changes of resident astroglia rather than migration or proliferation. The changes experienced by reactive astrocytes differ with the nature and severity of the brain insult along a graduated continuum of progressive alterations in progressive cellular hypertrophy, in molecular expression and, in severe cases, proliferation and scar formation (Sofroniew, 2009). A massive amount of information indicates that reactive astrocytes provide beneficial effects in the CNS (Sofroniew, 2009). Studies using transgenic and experimental animal models provide evidences that reactive astrocytes protect brain cells in several ways that involves a variety of different mechanisms such as: (i) uptake of potentially excitotoxic glutamate (Rothstein et al., 1996), (ii) neuroprotection via adenosine release (Lin et al., 2008), (iii) neuroprotection by degradation of amyloid-beta (Aβ) peptides (Koistinaho et al., 2004) (iv) facilitation of blood brain barrier repair (Bush et al., 1999), among others. However, it is also known that reactive astrocytes may also play harmful roles during injury or disease through the production of reactive oxygen species (ROS) or specific

inflammatory cytokines (Swanson et al., 2004; Sofroniew, 2009). Overall, reactive astrocytes have the potential to influence brain injury outcomes both positively and negatively, and the overall effects are determined by specific signaling events and molecular effector mechanisms (Sofroniew, 2009).

There are several molecular features of reactive astrocytes and reactive astrogliosis that are distinct from the features of astrocytes in the non-injured brain As shown in figure 3, the morphology of reactive astrocytes in response to injury changes (the main cellular processes get thicker, and thus visible over a greater distance), and a significant number of reactive astrocytes proliferate and migrate to the lesion site (Sofroniew, 2009; Buffo et al., 2010). Since the key role and mechanisms by which reactive astrogliosis protect tissue and formation of neural cells and restrict the spread of inflammation and infection still remains elusive, further studies better dissecting the molecular changes of reactive astrogliosis can lead to design novel potential therapeutic targets that could be of interest in a wide range of neurological disorders (Michael, 2009).

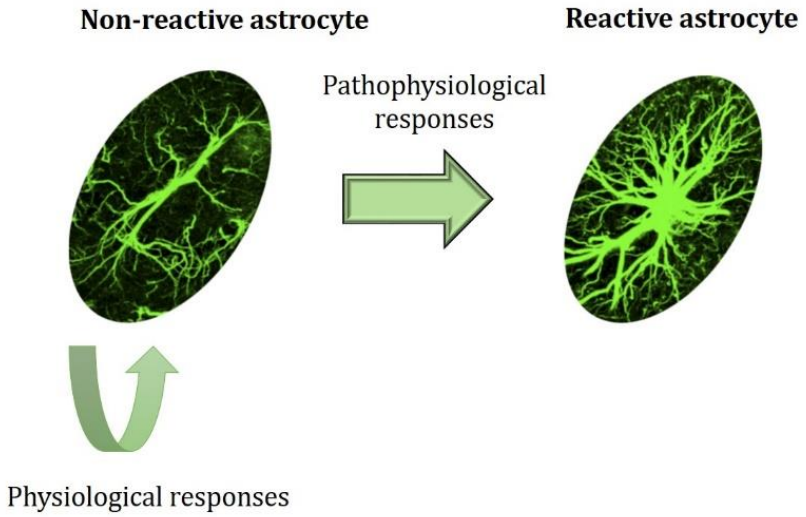


Figure 3 | Reactive astrogliosis

Reactive astrogliosis is a response of activated astrocytes after a brain injury or in neurodegenerative diseases. In most cases, it can be viewed as a defensive reaction counteracting acute stress, restoring the CNS homeostasis and limiting the tissue damage (adapted from Pekny and Pekna, 2014).

1.2.2. Astrocytic glutamate

Glutamate is the major excitatory neurotransmitter in the CNS; however its excessive extracellular accumulation contributes for the evolution of most neurodegenerative disorders (Benarroch, 2010). It was also the first gliotransmitter shown to be released by astrocytes, which are the main cell type responsible for glutamate uptake (Boison et al., 2010). Astrocytes is a significant source of extracellular glutamate, which can be released by a variety of mechanisms, particularly through the expression of plasma-membrane transporters for glutamate, via Na^+ and K^+ gradients (Bergles, 1998) and through the elevations of Ca^{2+} in astrocytes that induce an exocytotic release of glutamate from these cells (Bezzi et al., 2004). Because astrocytes express membrane receptors for almost all neurotransmitters, which are connected to the release of Ca^{2+} from intracellular stores, the astrocytic process can receive sufficient information (i.e., neurotransmitter released from synapses) leading to the activation of astrocytes due to the mobilization of their intracellular Ca^{2+} (Newman, 2003; Volterra and Steinhauser, 2004; Fellin et al., 2006). Activated astrocytes can release a diversity of neuroactive molecules including glutamate and ATP, but also nitric oxide, prostaglandins and D-serine (Fellin et al., 2004), which in turn influences neuronal excitability (Figure 4).

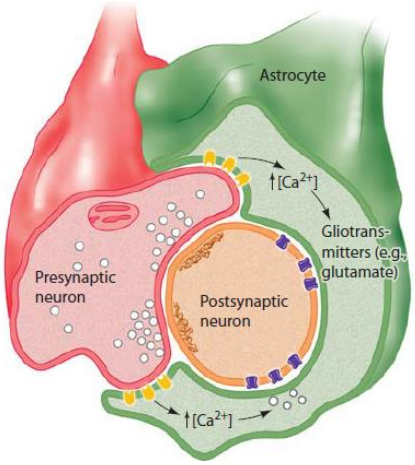


Figure 4 | Glutamate release from astrocytes and neuronal activation

Glutamate receptors are activated in the postsynaptic terminals and then released from the presynaptic Schaffer collateral terminals activating metabotropic receptors in the plasma membrane of the nearby

astrocytic processes. Activation of these receptors results in intercellular Ca^{2+} elevations in the astrocyte, which will lead to glutamate release from these glial cells (adapted from Fellin et al., 2006).

As glutamate is not metabolized in the extracellular environment, the maintenance of normal glutamatergic neurotransmission and the prevention of excitotoxicity depends on the reuptake of glutamate, essentially through astrocytic glutamate transporters (GLT) (Dunlop, 2006). Glutamate transporters are expressed by many cell types in the CNS, for instance in astrocytes, neurons, oligodendrocytes, microglia and endothelia, but astrocytes are the most important cells for maintaining normal extracellular glutamate concentrations. Astrocyte uptake can also contribute to shape the kinetics of glutamatergic synaptic activity (Anderson et al., 2004). The relevance and importance of GLT activity are underlined by the impact of their activity in the control of synaptic plasticity as well as in the demise of neurodegeneration (Tzingounis and Wadiche, 2007). There are planting reasons to postulate that astrocytes may succeed over neurons as synaptic glutamate sinks (Anderson and Swanson, 2004). Firstly, astrocyte membrane potentials are more stable than excitable neuronal membranes (Hertz et al., 1999). Secondly, astrocytes are better able to maintain physiological Na^+ and K^+ gradients during ATP depletion (Anderson and Swanson, 2000), which make them suitable to maintain low extracellular glutamate levels via ATP-dependent uptake mechanisms (Anderson and Swanson, 2000). And third, it is reasonable that neurons maintain high cytoplasmic glutamate concentrations to drive low-affinity vesicular uptake systems, whereas glutamate concentration in astrocytes is lower due to the rapid conversion to glutamine and 2-oxoglutarate (Hertz et al., 1999). Bergles et al (1999) suggested that this would create a transmembrane glutamate gradient that more strongly favor glutamate uptake by astrocytes (Anderson and Swanson, 2000).

The focus on the role of glutamate transporters as regulators of synaptic glutamate homeostasis has increased over the years. Several studies have shown a significant decrease of GLT activity in the cortex, platelets, fibroblasts and Alzheimer's disease (AD) patients (Scott et al., 1995; Masliah et al., 1996; Ferrarese et al., 2000; Zoia et al., 2005). It is also known that the AD-related peptide, Aβ can directly or indirectly influence the function of many related proteins, like ion-motive ATPases and calcium channels, as well as transporter systems (e.g. glucose transporters), which gives rise to the possibility that GLT may also be affected (Parpura-Gill et al., 1997; Mattson and

Chan, 2003; Sultana and Butterfield, 2008). Notwithstanding, no changes in GLT have been reported in aging and disease.

Regardless of the crucial role of astrocytes in the maintenance of the extracellular levels of glutamate, the regulation of astroglial transporters is still poorly understood. Data gathered by the group of Purines at CNC showed impaired activity of GLT in AD conditions (Matos et al., 2008) and the uptake activity is explicitly decreased by Aβ peptide (Parpura-Gill et al., 1997; Mattson and Chan, 2003; Fernandez-Tome et al., 2004).

1.2.3. Astrocytic A_{2A} receptors

Astrocytes have an essential role in controlling the extracellular levels of adenosine since, in adult neurons, adenosine kinase is mainly expressed in astrocytes (Studer et al., 2006). Moreover, glial cells are a source of extracellular purines, which in turn modulate glial activity (Fields and Burnstock, 2006). Actually, adenosine or ATP, acting through several subtypes of receptors that are located in the astrocytic membrane, can control glutamate and GABA uptake, astrocyte proliferation, as well as regulate the synthesis and release of several neuroprotective molecules and purine nucleotides (Pinto-Duarte et al., 2005; Schousboe and Waagepetersen, 2005; Matos et al., 2012; Burnstock, 2013). It is known that adenosine and guanosine can activate glial cells in order to respond to CNS injury, leading to astrogliosis, which includes its proliferation and hypertrophy (Muller et al., 1995; Rathbone et al., 1998; Ciccarelli et al., 2001). Some of these actions of purines on astrocytes have an impact in inflammation itself, having a dual and time-dependent actions in neuroprotection and neurodegeneration (Ribeiro et al., 2016).

Immediately after the observation that astrocytes can release glutamate and ATP in culture, , several studies showed that increased levels of intracellular Ca^{2+} within one astrocyte can lead to a Ca^{2+} wave that spreads through the coupled glial network, and that ATP is the signal mediating the Ca^{2+} wave. Indeed, ATP is a significant extracellular signaling molecule that astrocytes use to signal with one another as well as with neurons (Halassa et al., 2007).

Since excitatory transmission and excitotoxicity might be controlled by $A_{2A}R$ and modulated through astrocytic functions, further detailed studies are needed to dissect the precise mechanisms through which this is effective.

Astroglial reactions are critical for the regeneration of neuronal circuits following acute injury or neurological disorders where they determine the progression and outcome of the neuropathologies (Myer et al., 2006). Depending on the kind of injury, glial cells and neurons undergo cell death in consequence of tissue damage and ATP is released from damaged cells (Heine et al., 2015).

Astrocytes usually respond to CNS pathologies with their own activation, i.e., astrogliosis, and also through $A_{2A}R$ activation by extracellular adenosine, and migrate towards the central region of the lesion site and initiate the repair of the injured tissue. Activated astrocytes promote the reestablishment of CNS ionic homeostasis and the reorganization of essential barriers like the blood brain barrier. However, detrimental effects can be caused by the excessive secretion of signaling substances by these astrocytes (Heine et al., 2015), which will give rise to the formation of a tissue scar. On one hand, the formation of glial scar is beneficial since it isolates the injury from the healthy tissue preventing uncontrolled tissue damage and stabilizing fragile tissue. On the other hand, the glial scar can delay and restrict axonal regeneration, suspending the functional recovery.

Following injury to the nervous system or in neurodegenerative conditions such as Parkinson's disease and Alzheimer's disease, the astrocytes structure and protein expression are altered (Halassa et al., 2007). In fact, the group of Purines at CNC has recently reported changed expression of astrocytic GLT in an Alzheimer's disease animal model also showing increased astrocytic $A_{2A}R$ density; furthermore,it was shown that the blockade of $A_{2A}R$ abolished the impact of $A\beta$ on glutamate uptake. Therefore, this thesis work intends to design, produce and further explore the role of astrocytic $A_{2A}R$ in physiological and pathological conditions.

1.3. Viral-mediated gene transfer approaches

Astrocytes are involved in key physiological brain processes, which are usually altered in neurodegenerative diseases. To dissect the relative contribution of these cells in physiological conditions and further develop new therapeutic strategies for pathological conditions where astrocytes may exert crucial involvement in disease triggering or progression, novel challenging and useful strategies are needed. Gene transfer approaches are amongst the most used tools in research and clinical applications involving the CNS. Therapeutic genes can either be delivered to a selective target organ and defective genes can be replaced in a widespread manner in diseased animal models. Retrovirus are naturally occurring RNA viruses packaged into a capsid and a membranous envelope. Upon infection of a target cell, the RNA is reverse transcribed and integrates randomly into the host genome providing stable gene transfer (Blesch, 2004).

Lentivirus (LV) and adeno-associated virus (AAV) quickly became the most desired and used gene delivery tools (Kantor et al., 2014), being the most promising for long-term high-level transgene expression in the CNS. These vectors are essentially non-toxic, even when injected in high titers, and they induce at most limited inflammation or cellular immune responses in the brain (Bjorlund et al., 2000). However, there are some limiting factors regarding AAV vectors that could compromise their use in gene delivery, such as, the small DNA packaging capacity (less than 5 kb), which limit the size of the gene constructs that can be delivered with the AAV system, and the time required for transgene expression, which can take up to several days (Monahan and Samulski, 2000). On the other hand, lentiviruses are members of the *Retroviridae* family of viruses, named as such due to the reverse transcription of viral RNA genomes to DNA required before integration into the host genome; the Human Immunodeficiency Virus (HIV)-1 based lentiviral vectors are among the most widely studied vectors used for virus-mediated gene transfer since they have an efficient capacity for gene delivery, having a large cloning capacity (at least 9 kb), and are rather favorable for long-term expression of transgenes (Buchschacher and Wong-Staal, 2000). In addition, lentivirus mediate efficient gene transfer to non-dividing cells by virtue of the entry mechanism through the intact host

nuclear envelope, including neurons and glia; furthermore, they have the capacity to integrate the recombinant lentiviral construct into the host genome for permanent expression (Parr-Brownlie et al., 2015).

Lentiviral genomes are single-stranded RNA with genes encoding protein components of the capsid, enzymes such as reverse transcriptase - protease and integrase-, and envelope glycoproteins (*gag, pol*, and *env,* respectively) (Figure 5). A recombinant lentiviral vector genome requires long terminal repeats (LTRs) for genome replication integration, and an exogenous promoter used to express a transgene that enables identification of subpopulations of cells, overexpression or downregulation of genes or to target cells with a drug-or light-inducible protein to analyze cell function. The viral particles are packaged in human cell lines (HEK293 derivatives) to allow the generation of lentiviral particles by co-transfection of helper plasmids encoding the required genes and the genome capacity is 8-10 kb for maximal packaging efficiency (Dull et al., 1998).

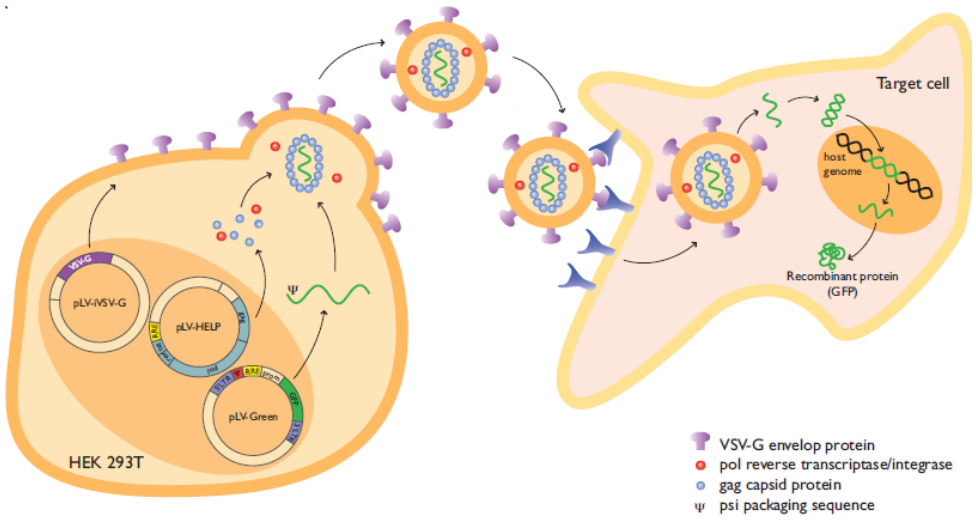


Figure 5 | Lentiviral vector production and cell transduction

Lentiviral vectors are expressed by several plasmids: (I) a lentiviral plasmid expression, for example pLV-Green, that contains the *psi* (¥) packaging sequence and the transgene gene inserted between the lentiviral LTRs allow target cell integration; (II) a packaging plasmid, such as pLV-HELP, encoding *pol, gag, rev* and *tat* viral genes and containing the rev-response element (RRE); (III) a pseudo-typing plasmid, pLV-VSV-G, for example, encoding the G protein of the Vesicular Stomatitis Virus envelope gene. Two days after transfection of HEK293T cells, the cell supernantant contains recombinant lentiviral vectors, which can be used to transduce the target cells. Once the lentiviral vector is in the target cell, the viral RNA is reverse-transcribed and stably integrated into the host genome (adapted from InvivoGen's products).

The expression profile of membrane proteins that serve as viral receptors is an important determinant of vector tropism. A large diversity of envelope glycoproteins derived from different viruses can be used for lentivirus to modulate the tropism. Selective gene delivery into the cell type requires receptor-targeted viral vectors, which can be generated by engineering glycoproteins incorporated into lentivirus particles. Selectivity of the resulting vector particles for the target cell type is mainly determined by the affinity and selectivity of the targeting ligand and also by the conditions of the selection process (Buchholz et al., 2015). Tropism can be modified by pseudotyping. The most commonly used lentivirus is pseudotyped with the Vesicular *Stomatitis* virus glycoprotein G (VSV-G). The VSV-G pseudotyped particles have a wide tropism through the use of low density lipoprotein receptors to enter cells, including both glia and neurons (Finkelshtein et al., 2013) and a strong body of evidences show that brain administration of VSV-G pseudotyped lentivirus infects mainly neurons. Recent studies have shown that new lentiviral vectors pseudotyped with various envelopes infect both neurons and glial cells at similar ranges. Colin and colleagues (2009) developed a Mokola-pseudotyped lentiviral vector to selectively target astrocytes. Astroglial cell-specific promoters have been suggested as an income of further restricting transgene expression. Nonetheless, this approach is currently limited due to the low level of transcriptional activity, the small number of characterized promoters and the difficulty of maintaining tissue-specific expression when genes are integrated into the viral vectors (Xu et al., 2001).

Another advantage of using lentiviral vectors is that they infect both differentiated and non-differentiated as well as dividing and non-dividing cells. Lentiviral vectors offer one of the most attractive options for shRNAs delivery to the CNS. Stable gene silencing *in vitro* and *in vivo* through the use of lentiviral vector-based RNAi are powerful tools for developing effective gene silencing therapeutics for many brain disorders (Porras and Bezard, 2008).

1.4. RNA interference strategies

Therapeutic approaches based on small interfering RNAs (siRNAs) and microRNAs (miRNAs) have dedicated major attention due to their key role in gene regulation, which makes them desirable targets for drug discovery and development. Small interfering RNA involve the introduction of a synthetic siRNA into the target cells to elicit RNA interference (RNAi), thus inhibiting the expression of a specific messenger RNA (mRNA) to produce a gene silencing effect (Behlke, 2006). In contrast, therapeutics based on miRNA include two approaches: miRNA inhibition and miRNA replacement (van Rooij et al., 2012). Both siRNAs and miRNAs are short RNA duplexes that target mRNA(s) to produce a gene silencing effect, however their mechanism of action are distinct (Deng et al., 2014).

RNA interference is a natural cellular process that silences gene expression by promoting mRNA degradation. According to Fire and Mello observations, awarded with a Nobel Prize, long double stranded RNA (dsRNA) mediates potent and selective silencing of homologous genes. This short dsRNA molecule is known as the siRNA, which has 21-23 nucleotides with 3' two-nucleotides overhangs.

Since the discovery of RNAi, dsRNA has been used as research tools to study gene functions of different type of cells. Yet, the delivery of exogenous long dsRNA, in mammalian organisms, is associated with the activation of the interferon (IFN) pathway, which will lead to the defense mechanism activation against the viral infection (Barik, 2005). Therefore, special care must be taken when designing siRNA therapeutics. Otherwise, short hairpin RNAs (shRNAs) can be used as an alternative to achieve a specific gene silencing effect via the RNAi mechanism (Rao et al., 2009). shRNAs are stem-loop RNAs, which are expressed in the nucleus, typically through the delivery of viral vectors. When expressed, they are transported to the cytoplasm for further processing, and subsequently loaded into the RISC (RNA-induced silencing complex) (Figure 6) for specific gene silencing activity (Lam et al., 2015).

Like miRNAs, siRNAs also inhibit gene expression in a post-transcriptional manner although the effects on gene silencing are different. siRNAs inhibit the expression of one specific target mRNA while miRNA regulate expression of multiple mRNAs (Davidson

and McCray, 2011). Viral vectors encoding shRNA or miRNA have been used to trigger RNAi and gene silencing effects (Rao et al., 2009).

There are different types of promoters that have been used for shRNA as well as siRNA expression in cells. Recent reports took advantage of the strength of RNA polymerase III promoters, principally those derived from the U6 small nuclear RNA and H1 RNA genes (Rossi, 2008). These promoters have a relatively simple structure, a well-defined transcription start-site, and they naturally drive the transcription of small RNAs. The first essential step for successful siRNA therapy is the design of a siRNA sequence that is effective and specific to the intended mRNA to minimize any off-target effect, and some vectors encode green fluorescent protein (GFP) as a marker for infectivity and to track the RNAi knockdown cells (Dreyer, 2011). The promoter controlling gene expression provides a further level of restriction in the lentiviral construct. Phosphoglycerate kinase (PGK) is one of the ubiquitous promoters mostly used to drive the transgene expression both in neurons and astrocytes (Buchholz et al., 2015).

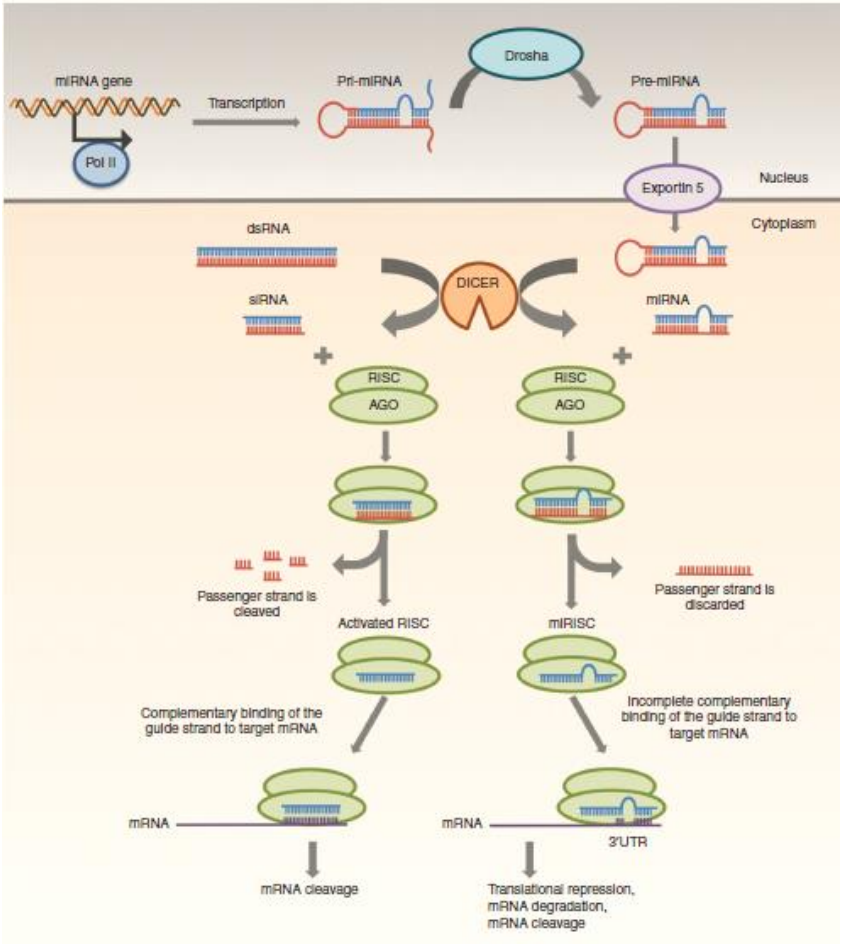


Figure 6 | Gene silencing mechanisms of siRNA and miRNA

siRNA: dsRNA is processed by Dicer (RNase III-like enzyme) into siRNA which is loaded by RISC. AGO2 (endonuclease agronaute 2), which is a component of RISC, cleaves siRNA passenger strand. The cleavage of mRNA is guide by the complementary binding of siRNA and the target mRNA.

miRNA: miRNA gene transcription is carried out by RNA polymerase II in the nucleus to give pri-mRNA, which is then cleaved by Drosha (microprocessor complex) to originate pre-miRNA. Then, the pre-miRNA is transported to the cytoplasm by Exportin 5, and is processed by Dicer into miRNA. The miRNA is loaded into the RISC where the passenger strand is eliminated and the miRISC is guided by the remaining guide to the target mRNA through partially complementary binding. The final mRNA could be inhibited via translational repression, degradation or cleavage (adapted from Lam et al., 2015).

Aims

It is known that astrocytes have several functions that have becoming increasingly recognized as being of highest importance in the maintenance of the neuronal environment, such as regulation of cerebral blood flow, maintenance of synaptic function and of the extracellular environment and stabilization of cell-cell communication in the CNS (Maragakis and Rothstein, 2006). In addition to, astrocytes respond to traumatic and biochemical brain injury with morphological and biochemical changes, in which adenosine plays a crucial role in controlling the metabolism of astrogliosis and the release of neuroactive substances (Dare et al., 2007). Withal, astroglial reactions are fundamental for the regeneration of neuronal circuits following brain injury or neurological disorders where they regulate and are determinant in the progression of neuropathologies (Myer et al., 2006), and the activation of A_{2A} receptors will increase astrocyte proliferation and activation, it becomes important to look forward whether astrocytes contribute to synaptic abnormalities and thus to neurodegenerative disorders.

The main goals of this work are: (i) Design novel lentiviral-mediated tools to manipulate adenosine A_{2A} receptors in astrocytes; (ii) Test in primary astrocytic cell cultures the lentiviral infection and further the downregulation of A_{2A} expression and density; and (iii) Stereotaxically administration in rodents of the newly developed lentivectors in selected brain regions and evaluate their spreading and cell-type transduction.

To accomplish our goals, in particular to achieve the validation of the novel molecular tool, we used primary astrocytic cell cultures and performed stereotaxically surgeries in rodents. The molecular tool was achieved by firstly incorporating an RNA interference strategy to down-regulate astrocytic $A_{2A}R$ in a lentiviral backbone also carrying a reporter gene (EGFP) under the control of the internal mouse phosphoglycerate kinase I (PGK) promoter, to allow cells to be identifiable. Afterwards, the lentiviral vectors were produced in 293T cells and then transfected in astrocytes and stereotaxically administrated in rodents.

Chapter 2

Material & Methods

2. Material & Methods

2.1 Animals

Young adult male C57BL/6 mice (Charles River, France) were kept under a conventional 12-hour light/dark cycle in a temperature-controlled room with food and water provided *ad libitum* and used at 8 weeks of age. All procedures were carried out in accordance with the European Community Directive 2010/63/EU, and approved by the Ethical Committee of the Faculty of Medicine/ Center for Neuroscience and Cell Biology of the University of Coimbra (ORBEA-86-2014/03032014).

2.2 Plasmid DNAs

Plasmids encoding EGFP either alone or with shRNAs for $A_{2A}R$ ($shA_{2A}R$) or control (shCTR) (kindly provided by Dr. Luis Pereira de Almeida, Department of New Preventive and Therapeutic Strategies, CNC, University of Coimbra, Portugal), shown in figure 7, were transformed in *Escherichia coli* competent cells and grown overnight (ON) in Luria-Bertani (LB) medium (Sigma). The isolation and purification of plasmids were performed using the JetStar™ 2.0 Plasmid Purification Kit (Genomed), as described in figure 8. Briefly, *Escherichia coli* cells were harvested by centrifugation at 12000 *g* for 10 min at 4°C (Beckman Coulter J-26 XPI) of the overnight LB-culture. The pellet was re-suspended with Cell Ressuspending Buffer (E1: 50 mM Tris-HCl, 10 mM EDTA, pH 8.0), enriched with 100 μg/mL RNase A, lysed with Lysis Buffer (E2: 200 mM NaOH, 1% SDS), precipitated with Precipitation Buffer (E3: 3.1 Potassium acetate, pH 5.5) and clarified by centrifugation at 12000 *g* for 10 min at room temperature (RT). The cleared lysate was added to a pre-packed anion exchange column (JetStar™ Maxi Columns), and then washed with Wash Buffer (E5: 100 mM sodium acetate, 800 mM sodium chloride, pH 5.0) and eluted with Elution Buffer (E6: 100 mM Tris-HCl, 1.25 M NaCl, pH 8.5).

Finally, the eluted plasmids were concentrated with isopropanol and washed with 70% ethanol. The final DNA pellets were re-suspended in a suitable volume of TE buffer (89 mM Tris-HCl, 2 mM EDTA, pH 8.0).

All purified plasmids were evaluated for their integrity through both restriction digestion and sequencing (GATC Biotech®, Germany). Briefly, each purified plasmid DNA was single digested with two restriction enzymes, both selected using the SECentral® software (Scientific & Educational Software, USA), giving rise to distinct and easily identifiable DNA band patterns in a horizontal agarose gel electrophoresis system. The plasmid encoding for EGFP only (pEGFP) was cut by HindIII and plasmids encoding for both shRNAs (pEGFPshRNA) were cut by BamHI and SspI. The lentiviral packaging plasmids were pREV and pGag/Pol, and the envelope plasmids pVSVG and pMokolaG (#15811, Addgene, USA; a gift from Miguel Sena-Esteves). All plasmids were sequenced by GATC Biotech (Germany) with the following home-designed primers (NZYTech, Portugal): RRE 3' Fwd ATC CTG GCT GTG GAA AGA T, for pEGFP; WPRE 3' Fwd CGG CTG TTG GGC ACT GAC AA, for EGFPshRNA plasmids; LNCX Fwd AGC TCG TTT AGT GAA CCG TCA GATC, for pREV; CMV 3' Fwd GGT CTA TAT AAG CAG AGC TGG T, for pGag/Pol and pVSVG; and b-globin-intron Fwd CTG GTC ATC ATC CTG CCT TT for pMokolaG; and used for viral production (see procedure below).

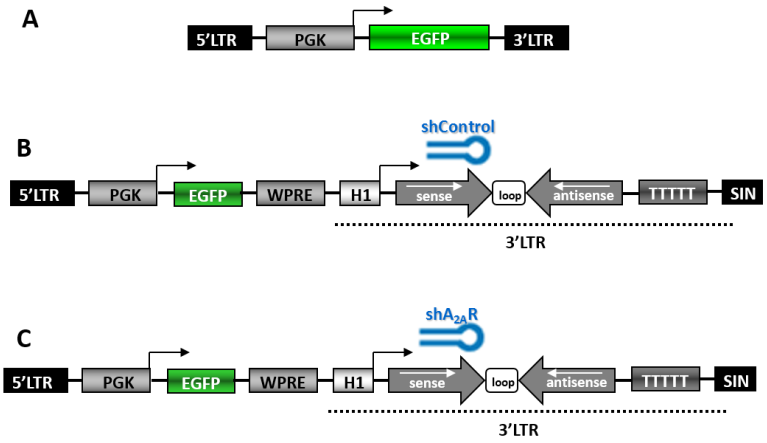


Figure 7 | Schematic representation of the transfer vectors pEGFP (A), pEGFPshControl (B) and pEGFPshA_{2A}R (C) constructs.

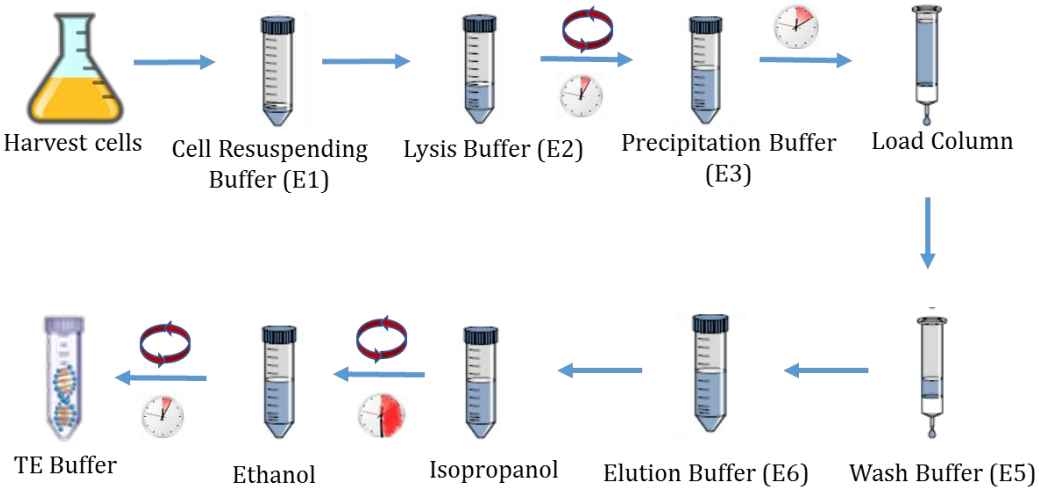


Figure 8 | Flow chart for purifying plasmid DNA using the JetStar™ 2.0 Plasmid Purification Kits.

2.3 Lentiviral vectors production

Lentiviral vectors encoding EGFP either alone or with shRNAs were produced in HEK293T cells with a four-plasmid system (Gonçalves *et al.*, 2013) and pseudotyped with either Mokola *Lyssavirus* G glycoprotein (Mok-G) or *Vesicular Stomatitis Virus* glycoprotein (VSV-G). Briefly, HEK293T cells were plated at day 1 at a density of 3.5 x10^6 cells in 10-cm^2 dishes and grown in Dulbecco's Modified Eagle's medium (DMEM, D5648, Sigma) supplemented with 10% Fetal Bovine Serum (FBS, Sigma) and 1% penicillin/streptomycin (Pen-strep, Sigma). At 60-80% confluence (day 2), cells were transfected through Calcium Phosphate method ($CaCl_2$ 1 M + HBS [50 mM HEPES, 280 mM NaCl, 12 mM Dextrose (glucose), 10 mM KCl, 1,5 mM $Na_2HPO_4.2H_2O$]) using the transfer vector (pEGFP and pEGFPshRNA), pREV, pGag/Pol and either pVSVG or pMokolaG plasmids at the proportion of 4.3:1:4.3:1.25, respectively. At day 4, the supernatant was collected, filtered through a 0.45 µm membrane (Millipore) and harvested by ultracentrifugation twice at 19000 rpm for 1:30 h at 4°C, then re-suspended in 1% bovine serum albumin (BSA, Sigma) in phosphate-buffered saline (PBS) and aliquoted and stored at –80 °C until use. The viral particle contents were determined

by HIV-1 p^{24} antigen ELISA (RETROtek, Gentaur, France), which is an enzyme linked immunoassay used to detect Human Immunodeficiency Virus Type 1 p^{24} antigen in cell culture medium. Microwells are coated with a monoclonal antibody that is specific for the p^{24} gene product of HIV-1. The viral antigen is specifically captured onto the immobilized antibody during incubation. The captured antibody is then reacted with Streptavidin-Peroxidase solution, developing a blue color (Figure 9) as the bound enzymes reacts with the substrate. At last, to stop the reaction, the stop solution was added to the samples and a yellow color develops. Resultant optical density is proportional to the amount of HIV-1 p^{24} present in the sample. Titers concentrations were obtained by interpolation from the standard curve. Viral stocks were stored at -80°C until use.

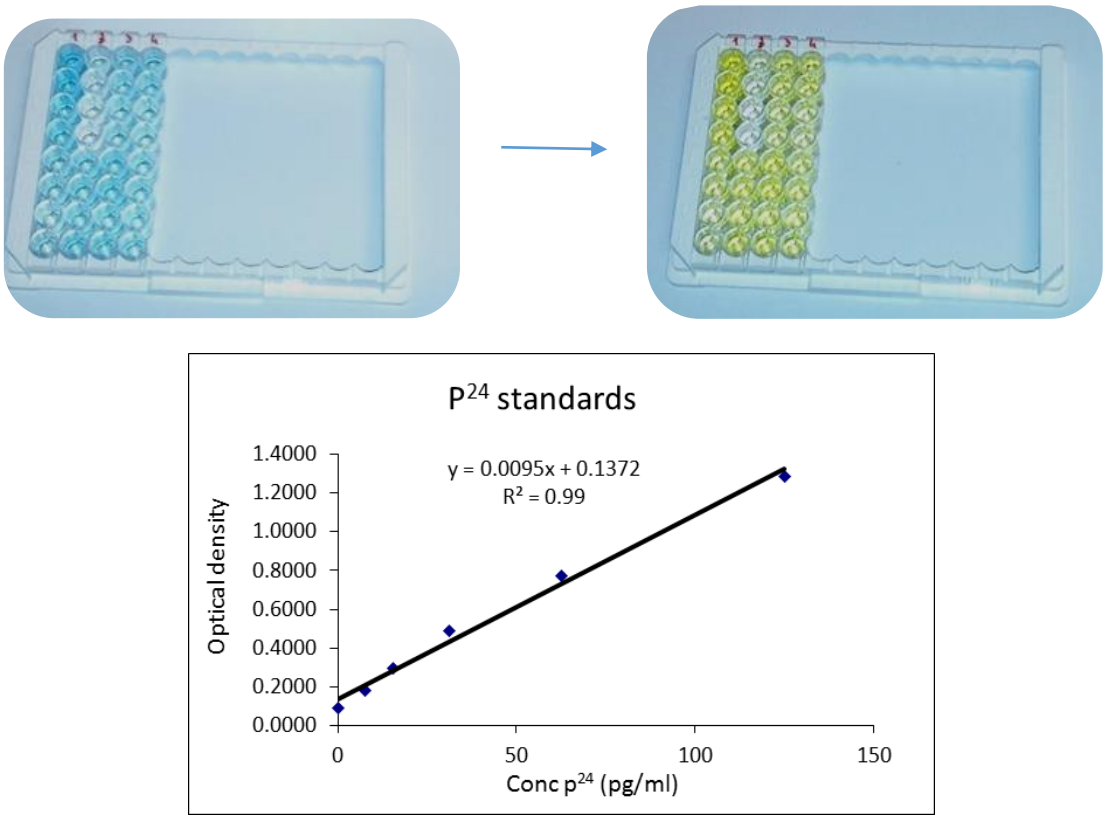


Figure 9 | Titration of lentivirus through HIV-1 p^{24} Antigen.

Concentration of HIV-1 p^{24} antigen standards: 125.0 pg/mL; 62.5 pg/mL; 31.3 pg/mL; 15.6 pg/mL; 7.80 pg/mL; 0 pg/mL (two first columns from each image). Blue color develops as the bound enzymes reacts with the substrate; after adding the stop solution, a yellow color develops and optical density is read at 450 nm using a microplate reader (SPECTRA max Plus, Molecular Devices®). The concentration of HIV-1 p^{24} antigen samples (ng/mL) were determined by interpolation from the standard curve (p^{24} standards graph).

2.4 Primary astrocyte cell culture

Rodent astrocytic primary cultures were prepared either from neocortex of E17 embryos or from cerebral cortices 5 days postnatal. Briefly, brains were dissected and cerebral cortices (left and right) were removed and isolated in ice-cold Hanks' balanced salt solution (HBSS, in mM: 5.36 KCl, 0.44 KH_2PO_4, 137 NaCl, 137 $NaHCO_3$, 0.34 $Na_2HPO_4.H_2O$, 5 Glucose, 10 Hepes, pH 7.2). The meninges were removed and the cortices were digested for 15 min with 0,125 % trypsin (type II-S, from porcine pancreas, Sigma- Aldrich) and 50 µg/mL deoxyribonuclease (DNAse, Sigma-Aldrich). Then, the enzymatic digestion was stopped with DMEM supplemented with 10% FBS, 1% Pen-strep and 3.7 g/L sodium bicarbonate ($NaHCO_3$). The final cell suspension was supplemented with DNAse and centrifuged for 5 min at 1000 rpm and the supernatant discarded. The obtained pellet was resuspended in DMEM and the number cells were counted in a hemocytometer. Afterwards, the cells were plated onto poly-D-lysine coated 100 mm Petri dishes at a density of 3 x10^6 cells/mL and maintained at 37°C in a 5% CO_2/ 95% room-air humidified incubator. The cell medium was replaced at the 7^{th} day. In order to separate microglial cells from the astrocytes monolayer, the mixed glial-cultures were shaken at 180 *g* for 5 min in an orbital shaker and the medium with detached microglial cells was discarded. Astrocytes were maintained in the petri dishes, washed with PBS and detached with 0.25% Trypsin to further plate in multiwells with cover slips and coated with poly-D-lysine at a density of 2 x 10^5 cells/mL. These cultures were maintained in culture for 5 days before the viral infections.

2.5 *In vitro* and *in vivo* lentiviral infections

Primary astrocytic cell cultures were infected with 50 ng of p24/µL of Mokola-G encoding EGFP (Mokola-G_EGFP) for analysis of infection efficiency, and later infected with 50 ng of p24/µL of Mokola pseudotyped lentiviral vectors encoding shCTR and shA_{2A}R for analysis of the efficiency to downregulate A_{2A}R. Briefly, half of the astrocyte culture medium was removed and kept at 4°C. Each well of the astrocytic culture plate

was infected with 1µl of each virus for 24 h, and the medium was then replaced with ½ conditioned and ½ fresh culture medium. Cultures were maintained at 37°C in a 5% CO_2/ 95% room-air humidified incubator for at least 25 days.

For *in vivo* transduction of lentiviral vectors, concentrated viral stocks were thawed on ice. Mice were anesthetized with Avertin® 2.0% (Sigma) (240 µg/g, intraperitoneally [i.p.]) and placed in a mouse stereotaxic frame (see figure 10). The head was placed in a stereotaxic apparatus for rodents and held in place by ear bars and a nose cone. A midline incision was made on the top of the skull and the skin retracted. The underlying fascia was then pushed away and a hole was drilled through the skull surface in specific coordinates of the brain: hippocampus (CA1 subregion), medial prefrontal cortex (mPFC) and dorsomedial striatum (see Table 1). A 26-gauge stainless steel needle connected to a Hamilton syringe was inserted into the brain hole and mice were administered with 2 µL of Mokola-G_EGFP and VSV-G_EGFP (300 ng of p24/µL) each site in the right and left hemispheres, respectively. After surgeries, mice were sutured and kept in their home cages for 4 weeks before being killed for immunohistological processing to further probe for morphological and neurochemical changes.

Figure 10 | Mouse stereotaxic frame for viral vector injections.

Table I - Coordinates used for viral stereotaxic injections

		Coordinates	
		A	B
Hippocampus	Antero-posterior	-1,94 mm	-2,18 mm
	Lateral	+/-1,60 mm	+/-1,90 mm
	Ventral	-1,50 mm	-1,60 mm
mPFC	Antero-posterior	+0,80 mm	
	Lateral	+/-1,25 mm	
	Ventral	-3,00 mm	
Dorsomedial striatum	Antero-posterior	+1,85 mm	
	Lateral	+/-0,35 mm	
	Ventral	-2,00 mm	

2.6 Immunochemistry

For *in vitro* analysis of lentiviral infection and $A_{2A}R$ silencing efficiencies, 28 DIV astrocytic cultures were fixed, permeabilized and blocked with 3% BSA in PBS, and then incubated ON at 4°C with the primary antibodies against Glial Fibrillary Acidic Protein (GFAP 1:100, Millipore, catalog: AB5804), cluster of differentiation molecule (Cd11b: 1:100, AbDSerotec, catalog: MCA275R Vimentin (Vimentin 1:300, Dako, catalog: M0725), neuron-specific nuclear protein (NeuN 1:400, Millipore, catalog: MAB377) and adenosine A_{2A} receptors ($A_{2A}R$ 1:500, Frontier Institute, catalog: AF1000) diluted in PBS supplemented with 3% BSA. After 24 h, cells were washed three times with PBS with 3% BSA and then incubated for 2 h RT with Alexa Fluor 647 conjugated goat anti-rabbit (1:500, Invitrogen, catalog: A21245), Alexa Fluor 555 conjugated goat anti-guinea pig (1:500, Life Technologies, catalog: A21435) and Alexa Fluor 568 conjugated donkey anti-mouse (1:500, Life Technologies, catalog: A10037). After incubation with the secondary antibody, cells were incubated with 4'6-diamidino-2-phenylindole (DAPI) (1:5000) and

washed three times with PBS and left to dry for 3 h. The preparations were visualized by fluorescent microscopy (Zeiss Axio Observer Z1, 10x objective).

For *in vivo* analysis of lentiviral selective targeting of astrocytes, mice were anesthetized 4 weeks post-surgeries with an overdose of Avertin® (2.5 x 240 µg/g, i.p.) and transcardiac perfused with PBS followed by fixation with 4% paraformaldehyde (PFA, Sigma). Brains were then removed and post-fixed in 4% PFA for 24h and cryoprotected by incubation in 25% sucrose for 48 h. The brains were frozen and then 25 µm coronal sections were cut using a cryostat (LEICA CM3050 S, Heidelberg, Germany) at -21°C. Slices were collected in anatomical series and stored in 48-well trays as free-floating sections in PBS supplemented with 0.02 µM sodium azide (Sigma). The trays were stored at 4°C until immunohistochemical processing. Slices were firstly washed with PBS three times and then processed with a permeabilizing and blocking solution composed of 0.3% Triton (Sigma), 1% donkey serum (Invitrogen) and 1% BSA for 1 h at RT in order to permeabilize and block non-specific antigen-antibody bindings, and then incubated with primary antibodies against the vertebrate neuron-specific nuclear protein (NeuN 1:400, Millipore, catalog: MAB377) and against the glial fibrillary acidic protein (GFAP 1:100, Millipore, catalog: AB5804) diluted in 0.1% Triton, 1% donkey serum and 1% BSA over two nights at 4°C. Slices were then washed three times with PBS and immunolabeled with two Alexa Fluor conjugates, either 594-conjugated donkey anti-mouse (1:200, Invitrogen, catalog: A21203) or 594-conjugated donkey anti-rabbit (1:200, Invitrogen, catalog: A21207) for 2 h at RT. After washed with PBS, slices were incubated with DAPI (1:5000) for 10 min. Finally, slices were mounted and covered with Mowiol® 4-88 medium (Sigma) on microscope slides and left to dry for 2 h. Preparations were visualized by confocal microscopy (ZEISS LSM 710).

2.7 Image acquisition

For data acquisition, images of astrocytes labeled with GFAP and Vimentin were acquired with 10x objective on a Zeiss Axio Observer Z1 microscope and images of astrocytes labeled with $A_{2A}R$ were acquired with 20x objective on a Zeiss Axio Imager Z2. Images of the brain sections labeled with GFAP and NeuN were acquired with an 40x objective on a ZEISS LSM 710 confocal microscope with ZEN image acquisition software (blue edition).

Chapter 3

Results

3. Results

According to the tripartite synapse model, pre-synaptic and post-synaptic neuronal elements are enclosed and regulated by astrocytes in the central nervous system. Astrocytes are thus key players in synaptic function and network by integrating synaptic information through bidirectional communication with neuronal elements rather than merely supporting neurons, which is of major relevance when considering the mechanisms by which occur many neurological disorders. Therefore, it is of utmost importance that new tools targeting astrocytes become available. This can be performed using the advantage of lentiviral vectors and its specific glycoprotein-dependent cell type tropisms in CNS for long-term transgene expression and gene silencing for many brain disorders. Aiming to dissect the role of astrocytic A_{2A} receptors under physiological conditions through a lentiviral-mediated genetic elimination of astrocytic A_{2A} receptors, we took advantage of an available RNA interference strategy for $A_{2A}R$ recently developed by Purines at CNC lab (Silva et al., 2016; Simões et al., 2016) to down-regulate $A_{2A}R$ but now inserted in a Mokola *Lyssavirus* G glycoprotein (Mok-G)- pseutotyped lentiviral vector, which is expected to selectively or at least mainly target astroglial cells (Colin et al., 2009).

3.1 Evaluation of the integrity of the plasmids encoding EGFP either alone or with shA_{2A}R or shCTR

Restriction enzyme digestion is a commonly used technique for molecular cloning as well as to quickly assess the integrity of a plasmid. There are hundreds of different restriction enzymes that are isolated from *Escherichia coli* to recognize specific DNA sequences but generally only 1 to 3 different enzymes are used to confirm the presence and orientation of the gene of interest in a particular plasmid backbone.

As shown in figure 11, all purified plasmids used for viral production were evaluated through restriction enzyme digestion and the resulted DNA band patterns were qualitatively analyzed in an agarose gel. The lentiviral packaging plasmids, namely, pRev was cut by EcoRI and by PvuII which gave rise to two fragments each (size

fragments: 3865 base pairs (bp), 309 bp; and 2840 bp, 1334 bp, respectively), and pGag/Pol plasmid was already confirmed by the group. The plasmid encoding MokolaG envelope was cut by PvuII (2514 bp, 2016 bp, 1936 bp) and HindIII (5462 bp, 1004 bp), and pVSVG was also already confirmed by the group. The plasmid encoding EGFP only (pEGFP) was cut by HindIII (3235 bp, 2606 bp, 951 bp, 845 bp and 584 bp), and the plasmids encoding for shRNAs (pEGFPshControl and pEGFPshA_{2A}R) were cut by BamHI (7891 bp and 2274 bp) and SspI (5214 bp, 3700 bp and 1098 bp).

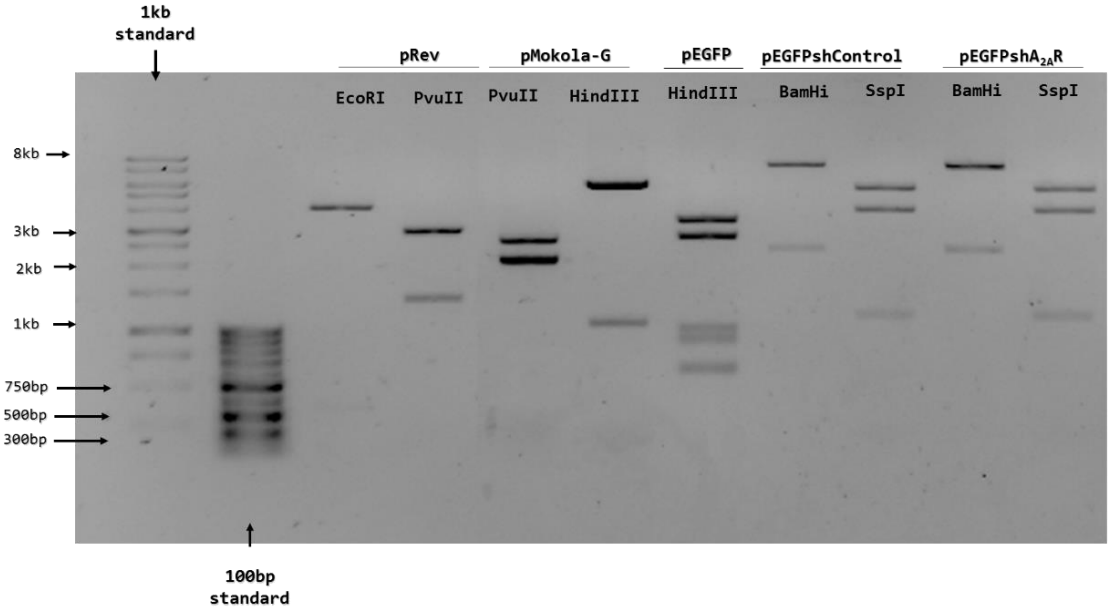


Figure 11| Plasmid DNA digestion through enzymatic restriction for plasmid integrity analysis.

Plasmid DNAs for lentiviral packaging (pRev and pMokola-G) and expression (pEGFP, pEGFPshControl and pEGFPshA_{2A}R) were digested with restriction enzymes to ensure their integrity for viral production.

3.2 Generation of a lentiviral vector pseudotyped with Mokola *Lyssavirus* G glycoprotein

Viral vectors allow long-term gene expression and larger diffusion in the brain than non-viral delivery systems (Delzor et al., 2013), which makes them desirable tools for studying gene functions and design gene therapy approaches. Since lentiviral vectors allow integration of a transgene into the genome they became exceptional tools to persistently overexpress or knockdown specific proteins of interest (Dreyer, 2011).

Vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped lentiviral vectors have been widely used for protein expression selectively in neurons (de Almeida et al., 2001). We now produced Mok-G coated lentiviral vectors for astrocytic targeting (Colin et al., 2009) encoding both EGFP to evaluate *in vitro* astrocyte infection efficiency and *in vivo* selective targeting of astrocytes, and short hairpin RNAs encoding a control sequence (EGFPshControl) and a sequence to downregulate adenosine A_{2A} receptors (EGFPshA_{2A}R), both with EGFP as a reporter gene, to further study the role of A_{2A}R in physiological and pathophysiological conditions. Calcium phosphate transfection of HEK293T cells with the transgene plasmids and lentiviral encoding protein plasmids of the capsid, enzymes reverse transcriptase (protease and integrase) and envelope glycoproteins is an easy, fast and cost-effective way of efficiently producing lentiviral vectors, as shown by the green fluorescence of the reporter gene EGFP upon transfection in figure 12, resulting from both transfection and further infection within 48h.

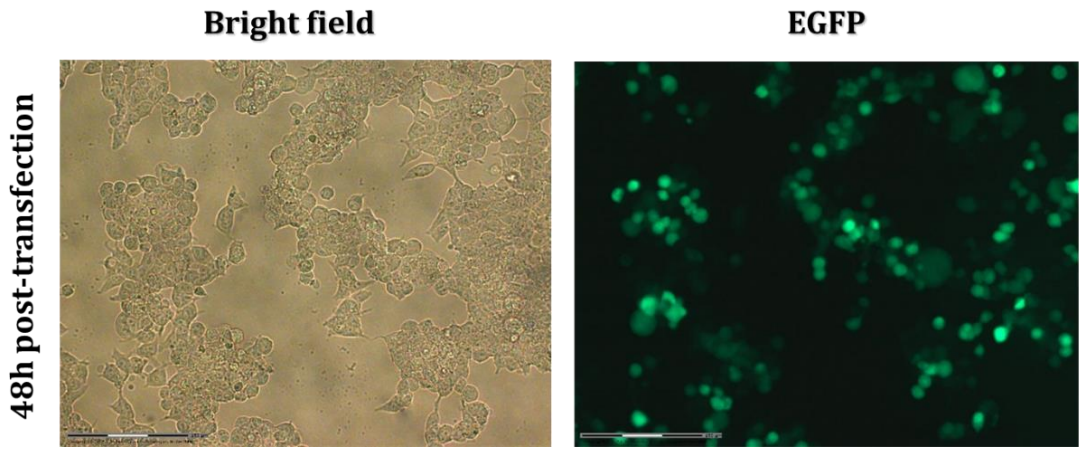


Figure 12| Transfection of HEK293T cells with pEGFP and lentiviral encoding proteins

Lentiviral vectors were produced in HEK293T cells coated with Mok-G. Forty-eight hours post-transfection of EGFP encoding plasmids allow a robust expression and density of enhanced green fluorescent protein meaning a successful transfection procedure and allowing the anticipation of a high tittered lentivirus for in vitro and in vivo applications.

3.3 Characterization of the primary astrocyte cell culture

A primary astrocyte cell culture is expected to be enriched in astrocytes. However, such cultures seldom have 100% of astrocytes and other cell types are also present, mostly microglia and oligodendrocytes, and also neurons, ependymal cells, fibroblasts or endothelial cells (Hansson, 1984). Therefore, we first characterized the purity of the astrocyte culture through an immunocytochemical analysis of different cell markers such as the glial fibrillary acidic protein (GFAP) and vimentin, which are the two main intermediate filament (IF) proteins expressed by astrocytes. Vimentin is the only IF type found in astrocytes, fibroblasts, endothelial cells, macrophages and lymphocytes (Evans, 1998) and GFAP is the major IF protein of mature astrocytes (Eng et al., 1971). A representative diagram of the number of different cell-types present in our primary astrocyte culture is shown in figure 13. Notably, only a few GFAP-positive cells were detected (7%, n=34) when compared to the number of vimentin-positive (47%, n=234) and vimentin- and GFAP-positive cells (31%, n=156) in a total of 499 counted nuclei

labelled with DAPI. Interestingly, 75 other cells (15%) were also present which were not labelled neither with the neuronal marker NeuN nor with CD11b.

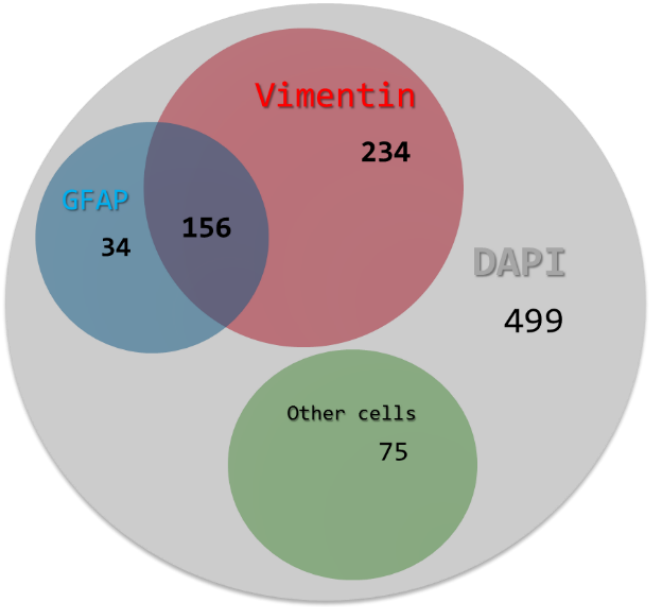


Figure 13| Characterization of the primary cell culture.

Representative diagram with the number of different cell-types in culture. DAPI (nuclear marker) labels all the population of cells. Vimentin (glial marker) represent the highest cell population and GFAP (mature astrocyte marker) a small number of astrocytes present in culture. The other remaining cells were neither neurons nor microglia cells.

3.4 Validation of Mok-G coated lentiviral vectors

3.4.1 Validation *in vitro*

To evaluate the efficiency of infection of astrocytes by Mok-G coated lentiviral vectors, we incubated the primary astrocyte culture with the Mok-G lentivirus encoding EGFP or vehicle (Figure 14). Twenty-five days after infection, we observed that 68% of astrocytes were infected, evaluated as the number of EGFP-positive *vs* the number of DAPI-positive cells.

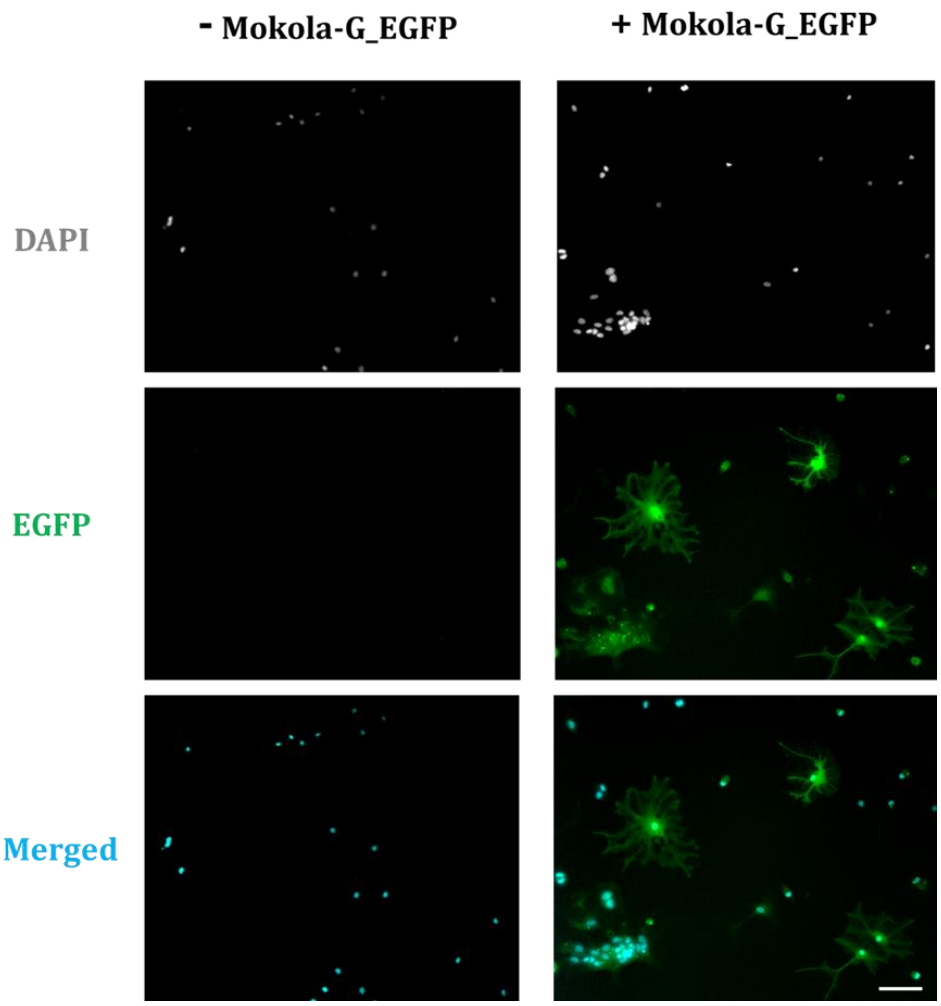
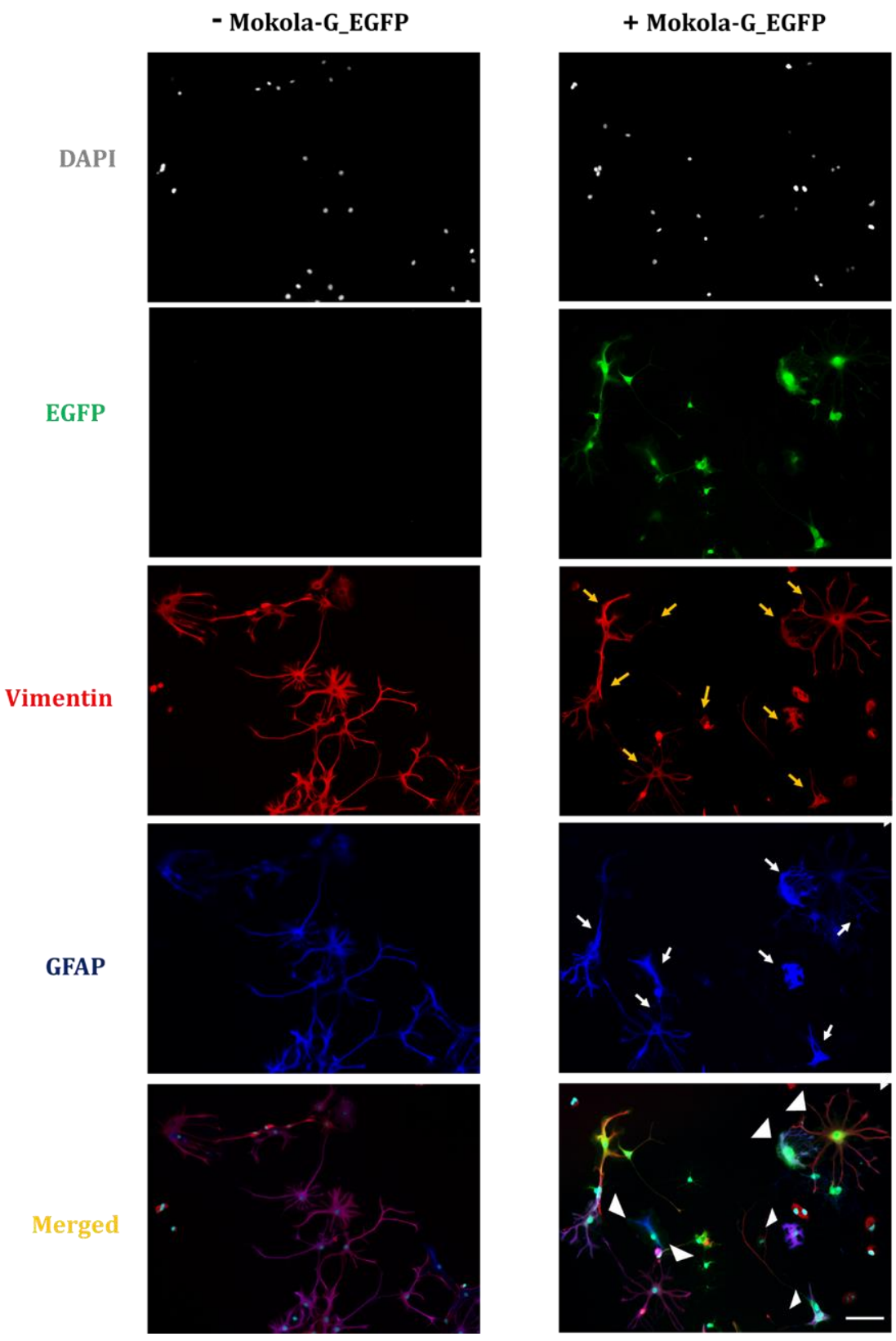


Figure 14| Infection of a primary astrocyte culture with Mok-G coated lentiviral vector encoding EGFP.

Immunostaining with DAPI (nuclei, grey) and fluorescence of EGFP (green) 25 days after infection. Scale bar: 100 µm.

We then evaluated whether the infected cells were indeed astrocytes by co-immunolabeling EGFP-positive cells with anti-GFAP and anti-vimentin antibodies. The transition between astrocytes expressing vimentin exclusively or both with GFAP to a GFAP-only expression pattern is considered a maturation marker for astrocytes (Bramanti et al., 2010). Immature astrocytes express mainly vimentin, which is transiently co-expressed with GFAP (Galou et al., 1996) and progressively replaced by GFAP in differentiated astroglial cells. Accordingly, there are cells expressing vimentin (Figure 15A, yellow arrows) only and GFAP (white arrows) only or both (arrowheads) in our primary astrocyte culture. Regarding the efficiency of MokolaG_EGFP virus for different cell-types, we observed that 100% of GFAP-positive cells co-localized with EGFP as well as 86% cells expressing vimentin only and 47% expressing both vimentin and GFAP, as quantified in figure 15B. Just a few non-identified cells (3%) were infected with MokolaG_EGFP virus.

(A)



(B)

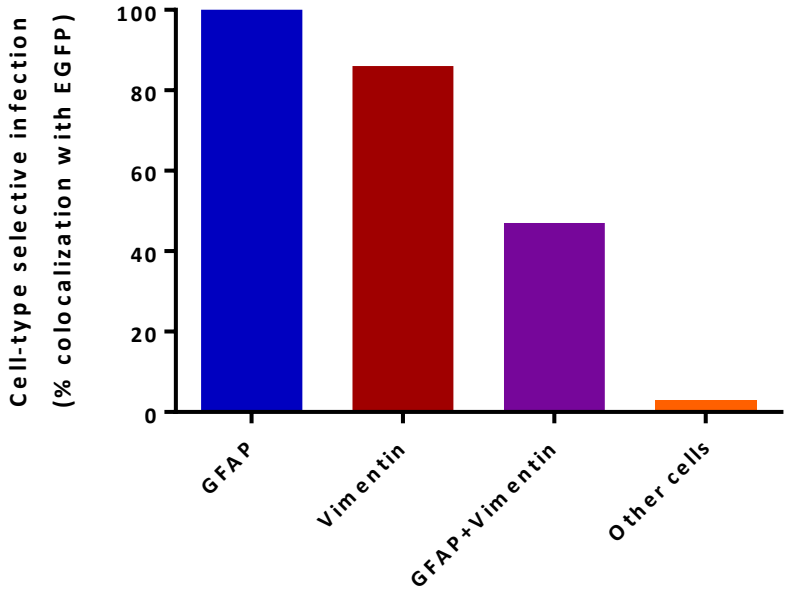


Figure 15| Efficiency and cell-type selective infection of Mokola-G_EGFP virus.

EGFP colocalization with GFAP (white arrows) and Vimentin (yellow arrows). **(A)** Cells were stained with GFAP (blue, mature astrocytes), Vimentin (red, immature astrocytes and other glial cells) and DAPI (grey, nuclei). Scale Bar: 100 μm. **(B)** Efficiency of EGFP infection of different types of cells. The results refer to a single culture.

3.4.1.1 Downregulation of astrocytic A_{2A} receptors

Genetic global elimination of adenosine A_{2A} receptors ($A_{2A}R$) was shown to produce robust neuroprotection in different brain disorders such as Alzheimer's and Parkinson's diseases (Canas et al., 2009, Schwarzschild et al., 2006). Since $A_{2A}R$ are mostly localized in synapses (Rebola et al., 2005a) and astrocytes are crucial in controlling the extracellular levels of adenosine (Studer et al., 2006) thus regulating synaptic transmission (Fellin et al., 2006), we now took advantage of the Mok-G lentiviral vectors encoding shRNAs to selectively downregulate $A_{2A}R$ ($shA_{2A}R$) in astrocyte primary cultures and further analyzed their efficiency to allow considering their use for *in vivo* applications. Primary astrocyte cultures infected with control Mok-G lentivirus, i.e, encoding a control short hairpin sequence (shCTR) display a robust immunoreactivity for $A_{2A}R$ in astrocytes (Figure 16, yellow arrows), which was highly diminished when $shA_{2A}R$ carrying lentivirus were used (white arrows).

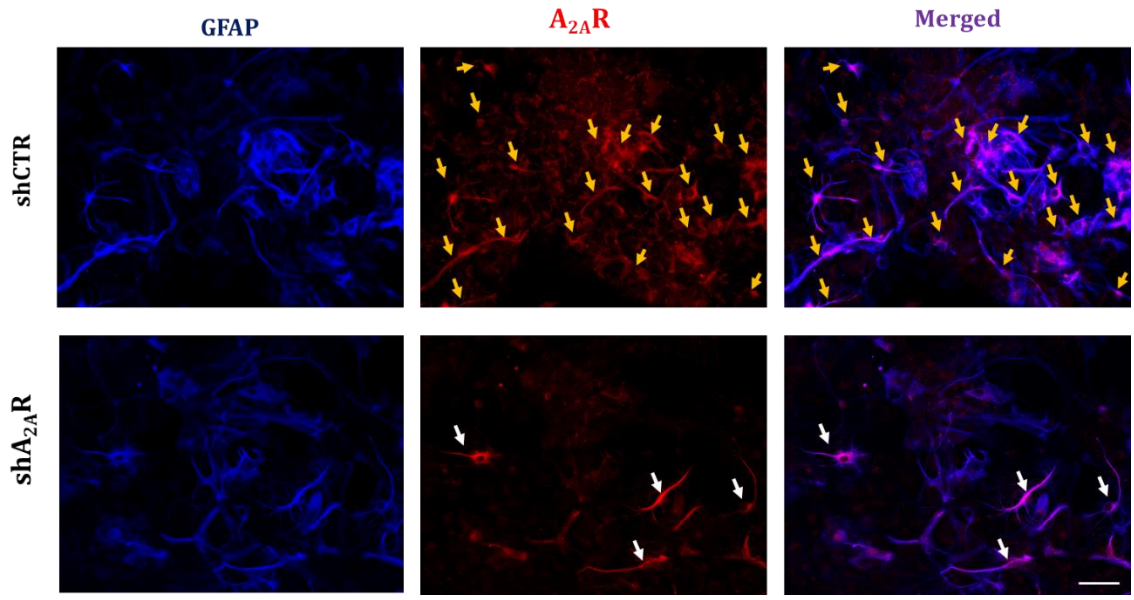


Figure 16| Downregulation of astrocytic A_{2A} receptors.

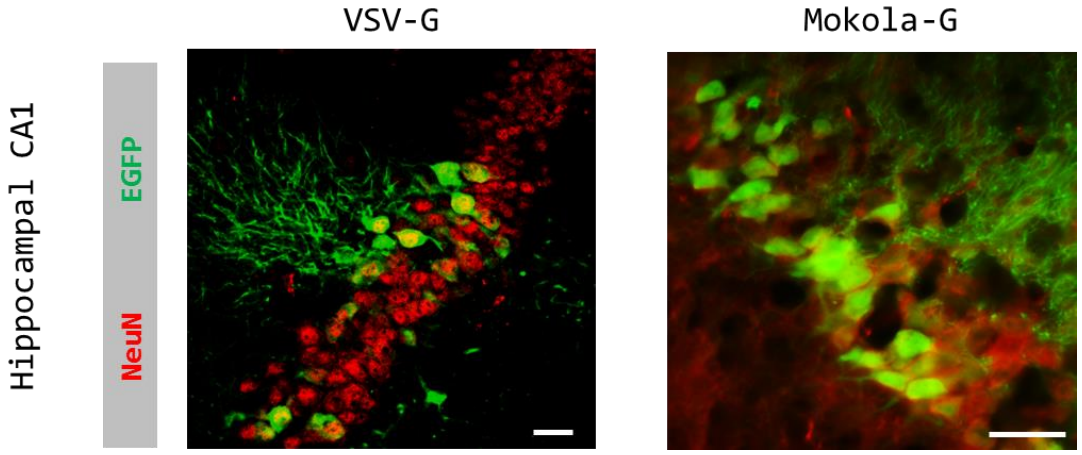
A_{2A} receptors are present in cultured astrocytes and are colocalizd with GFAP. Astrocytes were immunostained with GFAP (blue) and A_{2A} receptors (red) antibodies. Downregulation of $A_{2A}R$ in astrocytes (center image, white arrows). Scale Bar: 50 µm. The results refer to a single culture.

3.4.2 Validation *in vivo*

The intracerebral administration of lentiviral vectors (LV) provides an efficient and cost effective gene-transfer method allowing studying the role of particular proteins through over- and down-regulation. *In vivo*, LV particles pseudotyped with VSV-G efficiently transduce neurons (Naldini et al., 1996) while those pseudotyped with Mok-G transduce both neurons and astrocytes (Watson et al., 2002) but mostly the later (Colin et al, 2009). To test the efficiency of LV-mediated expression of EGFP in brain astrocytes, we administered the Mok-G and VSV-G (as control) pseudotyped LVs both encoding EGFP in ipsi- and contra-lateral brain hemispheres in the medial prefrontal cortex, dorsomedial striatum and hippocampus (CA1 sub-region).

As expected, four weeks post-injection, VSV-G-coated lentivirus did not colocalize with GFAP marker for astrocytes (Figure 17B, left panel) in any of the injected brain regions as they are known to only infect neurons (de Almeida et al., 2001); accordingly, EGFP expressed from VSV-G-coated lentivirus colocalized with NeuN marker (figure 17A), whereas the expression of EGFP through Mokola-G pseudotyped lentivirus was observed mainly in astrocytes, especially in the dorsomedial striatum (arrows, figure 17B) and in the hippocampus (arrowheads) and to a lower extent in the mPFC (right panel) and in a small set of neurons (Figure 17A).

(A)



(B)

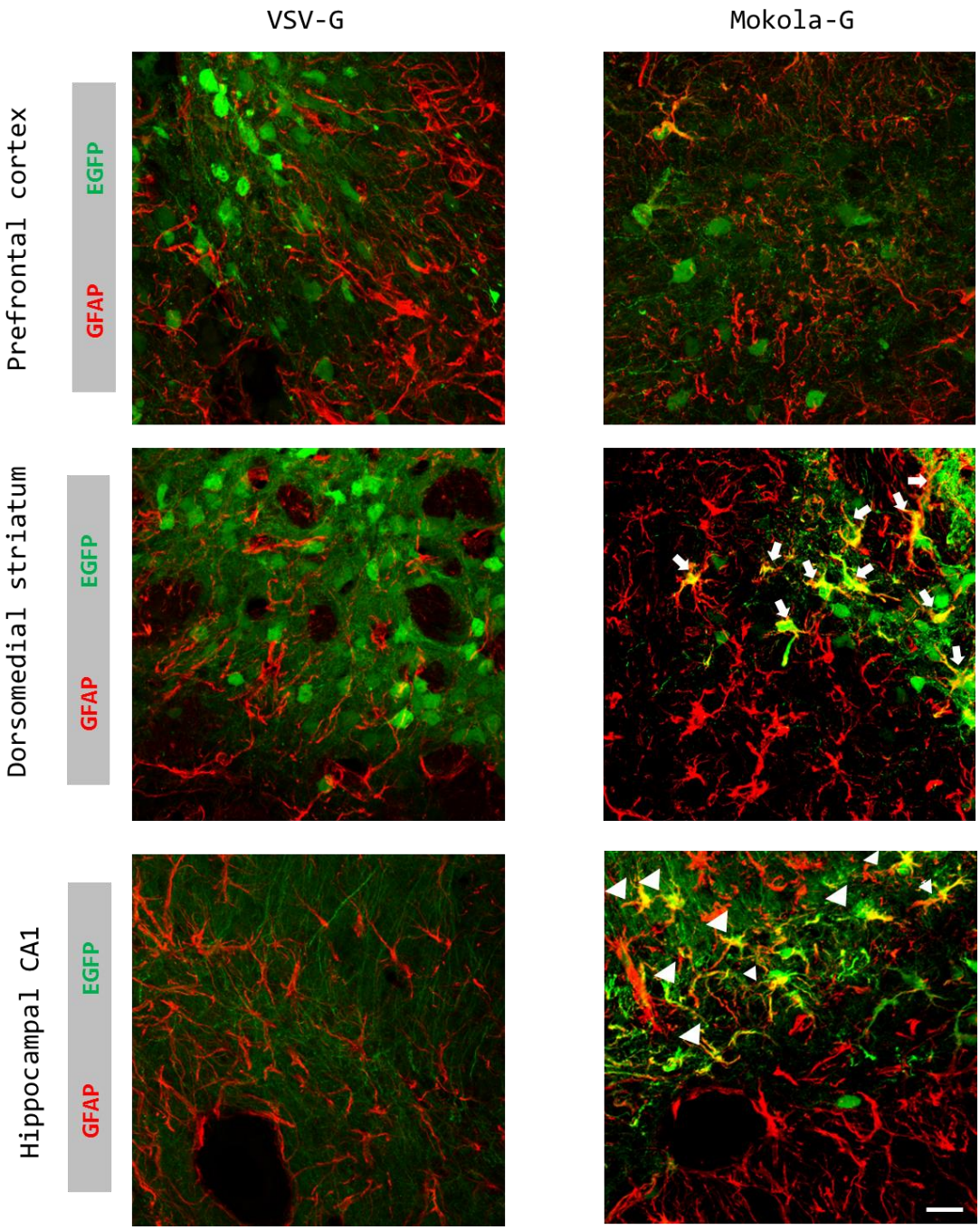
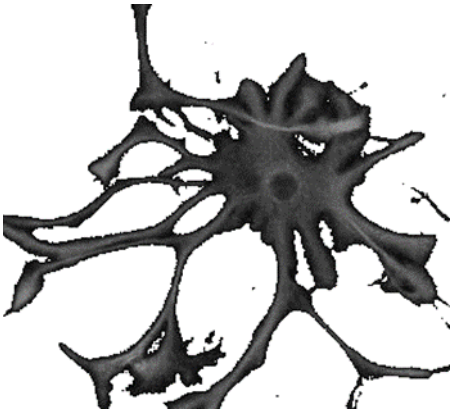


Figure 17| Colocalization of GFAP and NeuN with EGFP transduced through VSV-G and Mok-G coated lentiviral vectors in different brain regions.

Representative pictures of n=4 injected animals in the medial prefrontal cortex, dorsomedial striatum and hippocampus with VSV-G and Mokola-G coated lentivirus encoding EGFP in the left and right hemispheres, respectively. **(A)** Sections expressing EGFP (green) were stained with anti-NeuN (red) antibody. **(B)** Sections expressing EGFP (green) were stained with anti-GFAP (red) antibody Scale bar: 20µm.



Chapter 4

Discussion

4. Discussion

Cell-type-specific gene targeting has been increasingly used for studying the therapeutic benefits of selectively modifying gene expression in the brain, which shows a strong cellular heterogeneity (Merienne et al., 2015). Molecular engineering of lentiviral vectors has been used to express genes of interest in neurons and astrocytes. Nevertheless, delivery of shRNAs into astrocytes remains challenging because of the complexity of the tissue and the low levels of transcriptional activity (Xu et al., 2001).

Since selective lentiviral envelopes allow defining a specific cell tropism, we now developed a lentiviral vector (LV) that selectively or at least mostly infects astrocytes to attempt a selective down-regulation of astrocytic $A_{2A}R$. These LVs carried a reporter gene, enhanced green fluorescent protein (EGFP) to allow the cells to be identified. All the plasmids used for the viral production, including the plasmids of interest (pEGFP, pEGFPshA_{2A}R and pEGFPshCTR), and those for lentiviral packaging (pREV and pGagPol) and envelope (pVSV-G and pMokola-G) were successfully digested by enzymatic restriction giving the information that they were suitable for viral production. The LV pseudotyped with Mok-G was successfully generated and infected a primary astrocyte culture with a 68% infection efficiency. Since the primary astrocyte culture was not enriched in mature astrocytes (see review in Saura 2007), we then characterized the culture to better interpret the cell-type specificity of infection of the Mok-G coated virus. Our data showed that the culture was mainly composed by immature astrocytes (vimentin-positive cells), which were yet efficiently infected, as observed by the 86% cells expressing EGFP. Cells co-expressing GFAP and vimentin represented 31% of the total and only 47% of those also expressed EGFP, meaning that 1/3 of the total number of cells were in a transient change of maturation, and only circa ½ were successfully infected, i.e., expressing EGFP. Although just a few cells were mature astrocytes, all those were successfully infected since they were endowed with EGFP, which means that the Mok-G_EGFP lentiviral vectors efficiently infected mature astrocytes.

Adenosine receptors (AR) are present in astrocytes where they have an important role in controlling the metabolism of glucose, astrogliosis, cell proliferation,

cell volume changes, cell death, and the release of neurotrophic factors (review in Boison et al., 2009; Daré et al., 2007). $A_{2A}R$ play an important role in astrocyte endfeet controlling microcirculation in the brain, as adenosine is an endproduct generated by astrocytes to couple an increased neuronal activity with vasodilation (Philis, 2004). Since AR control mainly excitatory transmission through $A_{2A}R$ that also modulate astrocytic function, it becomes important to understand the different functions of $A_{2A}R$ in synaptic transmission, gliotransmission and astrocytic modulation. We produced LV pseudotyped with Mok-G encoding EGFPshControl or EGFPsh$A_{2A}R$ to dissect the efficiency of the genetic elimination of astrocytic $A_{2A}R$ and further evaluate their role under physiological conditions. Matos *et al* (2015) demonstrated that a transgenic mouse with selective elimination of $A_{2A}R$ in astrocytes displayed motor and memory dysfunctions relevant for schizophrenia. In this work, we validated a lentiviral-mediated strategy to target astrocytic $A_{2A}R$, as shown by the successful downregulation of $A_{2A}R$ in a primary astrocyte culture through the Mok-G-coated LVs encoding sh$A_{2A}R$.

Our approach was well succeeded *in vitro* but hardly confirmed *in vivo* since the experimental tools for selective manipulation of astrocytes *in vivo* tend to fail. In our study, we developed a Mokola *Lyssavirus* G glycoprotein enabling local, cell-type-specific and tightly controlled silencing *in vivo*. However, further improvements are needed to reduce the other cell type mediated expression of shRNAs, as we still observed a residual neuronal expression *in vivo*. Importantly, we observed a dramatic increase in the proportion of astrocytes transduced by Mok-G compared to VSV-G pseudotyped LVs: whereas there was hardly any expression of VSV-G LV in astrocytes, we observed that the Mok-G LV was expressed at least in two of the three brain regions transduced, namely in dorsomedial striatum and hippocampus. Although our novel Mok-G coated LV encoding sh$A_{2A}R$ are still not suitable for *in vivo* applications, they are reliable for *in vitro* studies of the role of astrocytic $A_{2A}R$ in astrocytic cultures.

Chapter 5

Concluding and future perspectives

5. Concluding remarks and future perspectives

The present study allowed us to conclude that the Mokola-G-coated lentivirus encoding shA_{2A}R were able to infect a primary astrocyte culture and further down-regulate astrocytic A_{2A} receptors expression and density. Regarding the lentiviral-mediated expression of EGFP *in vitro*, it is worth noting that EGFP was expressed not only in mature astrocytes but also in immature astrocytes and in a transient maturity state, i.e., in cells expressing both vimentin and GFAP.

Additionally, the administration of both Mok-G and VSV-G pseudotyped LVs (the later as internal control) in the medial prefrontal cortex, dorsomedial striatum and hippocampus of the same animal (different hemispheres) both encoding EGFP showed different transduction efficiencies in astrocytes, which highlights a special concern when considering the astrocytic selectivity of the virus for different brain region with further implications for therapeutic gene transfer. This particular issue is hard to address since no tissue-specific promoters for short hairpin RNAs expression were found so far which lead us to suggest seeking for specific microRNAs that can regulate cellular expression in order to increase expression of shRNAs in astrocytes simultaneously decreasing its expression in other cells types, in a way similar as it was recently reported by Merienne and colleagues (2015).

Although these Mokola-G pseudotyped lentivirus system for targeting astrocytes in adult animals still needs further improvements, it should be noted that this tool for downregulating astrocytic A_{2A}R is enough to proceed with *in vitro* studies to confirm the control of glutamate uptake and related mechanisms by astrocytic A_{2A}R to provide new insights about the putative astrocytic control of brain neurological disorders.

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