DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Role of adenosine receptors in suicide

Ana Carolina Gonçalves de Almeida Xavier

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Role of adenosine receptors in suicide

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Paula Canas (Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e do Professor Doutor Ângelo Tomé (Departamento de Ciências da Vida, Universidade de Coimbra).

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Abbreviation list

5HT, serotonin
A_1R, adenosine A_1 receptor
A_{2A}R, adenosine A_{2A} receptor
A_{2B}R, adenosine A_{2B} receptor
A_3R, adenosine A_3 receptor
AC, adenylyl cyclase
ACC, anterior cingulate cortex
ADA, adenosine deaminase
ADK, adenosine kinase
AMP, adenosine monophosphate
AMPA, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid
ARs, adenosine receptors
ATP, adenosine triphosphate
ANOVA, analysis of variance
APS, ammonium persulfate
BA25, Brodmann's area 25
BCA, 2-(4-carboxyquinolin-2-yl)quinoline-4-carboxylic acid
BSA, bovine serum albumin
cAMP, cyclic adenosine monophosphate
CAPS, 3-(cyclohexylamino)propane-1-sulfonic acid
CBF, cerebral blood flow
CNS, central nervous system
CGS 21680, 3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxyoxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid
CLAP, cocktail of proteases inhibitors
CPA, (2R,3R,4S,5R)-2-[6-(cyclopentylamino)purin-9-yl]-5-(hydroxymethyl)oxolane-3,4-diol
CTR, control
DA, dopamine
DBS, deep brain stimulation
DMSO, methylsulfinylmethane
dpm, disintegrations per minute
DSM-5, Diagnostic Statistical Manual 5^{th} edition
DTT, 1,4-bis(sulfanyl)butane-2,3-diol
ECF, enhanced chemifluorescence
ECT, electroconvulsive therapy
EDTA, 2-[2-[bis(carboxylatomethyl)amino]ethyl-(carboxylatomethyl)amino]acetate
EPSCs, excitatory postsynaptic currents
EXTRA, extrasynaptic fraction
FR%, fractional release
GABA, γ-aminobutyric acid
GFAP, glial fibrillary acidic protein
Glu, glutamate
GPCR, G protein-coupled receptor family
HBM, HEPES buffered medium
HEK, human embryonic kidney (cells)
HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HPA, hypothalamic-pituitary-adrenal
IB, isolation buffer
INMLCF, Instituto Nacional de Medicina Legal e Ciências Forenses, I. P.
K^+, potassium
LTD, long-term depression
MAPK, mitogen-activated protein kinase

MC, medial caudate nucleus
MDD, major depressive disorder
mGluR, metabotropic glutamate receptor
mGluR5, metabotropic glutamate receptor subtype 5
MRI, magnetic resonance imaging
NE, norepinephrine
ND, not determined
NMDA, N-methyl-D-aspartate
NT, nerve terminals
OFC, orbital frontal cortex
PBS, phosphate buffered saline
PC, posterior caudate nucleus
PET, positron emission tomography
PFC, prefrontal cortex
PIP, pyridoxal phosphate
PKA, protein kinase A
PKC, protein kinase C
PLC, phospholipase C
PMI, postmortem interval
PMSF, phenylmethanesulfonyl fluoride
POST, postsynaptic density
PRE, presynaptic active zone
PSD-95, postsynaptic density protein 95
PVDF, polyvinylidene fluoride
RIPA, radioimmunoprecipitation assay
RNA, ribonucleic acid
RT, room temperature
S1, first stimulation period
S2, second stimulation period
SD, sleep deprivation
SDS, sodium dodecyl sulfate
SDS-PAGE, SDS-polyacrylamide gel electrophoresis
SEM, standard error of the mean
SNAP, synaptosomal-associated protein
SNAP-25, synaptosomal-associated protein 25
SNARE, SNAP receptor
SUI, suicide
SYNAP, initial synaptosomal fraction
TE, total extracts
TEMED, N,N,N',N'-tetramethylethane-1,2-diamine
TRIS,(hydroxymethyl)aminomethane
TBS, trizma buffered saline
TBS-T, TBS with tween 20
TMS, transcranial magnetic stimulation
VAMP, vesicle-associated membrane protein

Abstract

Major depressive disorder (MDD), the most prevalent mental illness, is a chronic and recurrent condition (Lucas *et al.,* 2011). MDD seems to be associated with abnormalities in regions that mediate emotional and stress responses (Manji *et al.,* 2001). A strong link between suicide and depression has been showed, with more than 86% of suicide victims having a depressive disorder, and committing suicide most often after a major depressive episode (Coryell & Young, 2005; Rihmer, 2007). So, it is possible to study the brain tissue from suicide completers, in order to understand the neurobiological basis of depression. Suicide has been identified as a serious public health problem. Risk factors, such as biological, psychiatric and cultural, interact in a complex manner to this pathology (Bertolote & Fleischmann, 2005).

Adenosine is an endogenous nucleoside that influences many functions in the central nervous system (CNS) (Fredholm *et al.,* 2005), acting as a homeostatic modulator and also as a neuromodulator at the synaptic level, where it modulates the release of neurotransmitters, the post-synaptic responsiveness and the action of other receptor systems (Cunha, 2001). Adenosine acts *via* activation of four G-protein coupled receptors (GPCRs) (Fredholm, 1997), in the brain, it acts especially through activation of two adenosine receptors (ARs), inhibitory A_1 (A_1R) and facilitatory A_{2A} ($A_{2A}R$) receptors (Cunha, 2005; Wei *et al.,* 2011).

The interest in the role of adenosine in mood disorders arised from previous studies that have recognized a relationship between caffeine intake, mood changes and specific psychiatric symptoms (Kawachi *et al.,* 1996; Lara, 2010; Lucas *et al.,* 2011; Lucas *et al.,* 2013), since caffeine has biological effects as competitive antagonist of A_1R and $A_{2A}R$ (Chen *et al.,* 2013; Cunha *et al.,* 2008; El Yacoubi *et al.,* 2003; Fredholm *et al.,* 2005; Fredholm, 2007). There is also evidence that different therapeutic strategies used to control mood disorders are related to the adenosine modulation system (Chen *et al.,* 2013; Cunha *et al.,* 2008; El Yacoubi *et al.,* 2001; Gomes *et al.,* 2011). In animal models of manipulation of ARs, there are modified behavioral responses considered relevant for mood in humans (Gomes *et al.,* 2011).

The main goal of this study was to understand the differences in the localization, density and function of both A_1R and $A_{2A}R$ between control subjects and suicide completers, in brain areas affected by depression. Also, we aimed to comprehend if these alterations are related with synaptic changes. For this purpose, a postmortem study was performed in male subjects who died by suicide, aged-matched with non-suicide controls who died by natural causes or accidents. Human brain samples were

obtained at autopsies performed in *Instituto Nacional de Medicina Legal e Ciências Forenses, I. P.* (INMLCF), Coimbra, Portugal. Several brain areas were studied: Brodmann area 25 (BA25); medial caudate nucleus (MC); posterior caudate nucleus (PC) and hippocampus.

To comprehend the normal localization of A_1R and $A_{2A}R$ we compared the relative abundance of A_1R and $A_{2A}R$ in total extracts (TE) and in nerve terminals (NT), isolated using an adapted discontinuous Percoll gradient protocol (Dunkley *et al.,* 1986, 2008), from human brain areas of the same sample. In all brain areas studied, both receptors were found enriched in NT. We then refined the information on ARs subsynaptic localization, using a fractionation method (Phillips *et al.,* 2001; Rebola *et al.,* 2005), and it was observed that $A_{2A}R$ are mainly located outside the active zone and A_1R are present in all synaptic fractions.

Synaptic changes present in the brain of suicide completers were then studied and it was observed a down-regulation of synaptosomal-associated protein 25 (SNAP-25) on BA25 and MC together with a decrease in postsynaptic density protein 95 (PSD-95) levels on PC and hippocampus. Astrocytic marker glial fibrillary acidic protein (GFAP) down-regulation was observed in BA25 and hippocampus.

Several alterations in the density of ARs, in the different brain regions, were observed in suicide completers when compared with age-matched controls. We described: an up-regulation of $A_{2A}R$ in TE, on BA25 and PC; an up-regulation of A_1R in TE, on MC; a down-regulation of A_1R in NT, on PC.

Due to their particular distribution in the brain and their functional properties, ARs constitute an attractive opportunity for developing innovative compounds for the treatment of specific neurodegenerative and psychiatric disorders, such as MDD. Our work has provided information about changes present in the brains of suicide completers and might contribute to better understand the modulatory role of ARs in depression. There are still numerous questions that demand careful attention to further explore this system and develop novel strategies to control mood disorders, particularly MDD.

Keywords: Postmortem human brain; Suicide; Major depressive disorder; Adenosine A_1 receptor; Adenosine A_{2A} receptor.

Resumo

A perturbação depressiva major (MDD), a mais predominante das doenças mentais, é uma condição crónica e recorrente (Lucas *et al.,* 2011). A MDD parece estar associada com alterações nas regiões que medeiam as respostas emocionais e de stress (Manji *et al.,* 2001). Uma forte ligação entre o suicídio e a depressão foi demonstrada, uma vez que mais de 86% das vítimas de suicídio apresentavam uma perturbação depressiva, na maioria dos casos, ocorrendo o suicídio depois de um episódio depressivo major (Coryell *et al.,* 2005; Rihmer, 2007). Assim, o tecido cerebral de vítimas de suicídio pode ser utilizado para estudar a neurobiologia da depressão. O suicídio foi identificado como um problema sério de saúde pública. Vários fatores de risco biológicos, psiquiátricos e culturais interagem de uma maneira complexa para esta patologia (Bertolote & Fleischmann, 2005).

A adenosina é um nucleósido endógeno que influencia muitas funções do sistema nervoso central (CNS), agindo como modulador homeostático e também como neuromodulador ao nível sináptico, onde modula a libertação de neurotransmissores, a capacidade de resposta pós-sináptica e a ação de outros sistemas receptores (CNS) (Cunha, 2001; Fredholm *et al.,* 2005). A adenosina actua em quatro receptores acoplados à proteína G (GPCRs) (Fredholm, 2007), no cérebro age especialmente através da activação de dois receptores de adenosina (ARs), inibitório A_1 (A_1R) e facilitatório A_{2A} ($A_{2A}R$) receptores (Cunha, 2005; Wei *et al.,* 2011).

O interesse do papel da adenosina nos distúrbios de humor surgiu de numerosos estudos que reconheceram uma relação entre o consumo de cafeína, alterações de humor e sintomas psiquiátricos específicos (Kawachi *et al.,* 1996; Lara, 2010; Lucas *et al.,* 2011; Lucas *et al.,* 2013), uma vez que a cafeína tem efeitos biológicos como antagonista competitivo dos A_1R e $A_{2A}R$ (Chen *et al.,* 2013; Cunha *et al.,* 2008; El Yacoubi *et al.,* 2003; Fredholm *et al.,* 2005; Fredholm, 2007). Também há evidências de que as estratégias terapêuticas utilizadas para controlar distúrbios de humor estão relacionados com o sistema modulatório de adenosina (Chen *et al.,* 2013; Cunha *et al.,* 2008; El Yacoubi *et al.,* 2001; Gomes *et al.,* 2011). Em modelos animais de manipulação de ARs há respostas comportamentais consideradas relevantes para o humor nos humanos (Gomes *et al.,* 2011).

O principal objectivo deste estudo era perceber as diferenças na localização, densidade e função dos A_1R e $A_{2A}R$, entre vítimas de suicídio e controlos, em áreas afectadas pela depressão. Além disso, tentámos compreender se essas alterações estão relacionadas com modificações sinápticas. Para este propósito foi realizado um

estudo postmortem em sujeitos do sexo masculino suicidas, pareados com controlos da mesma idade que morreram de causas naturais ou acidentes. As amostras de cérebro humano foram obtidas em autópsias realizadas no Instituto Nacional de Medicina Legal e Ciências Forenses, I. P. (INMLCF), Coimbra, Portugal. Foram estudadas diversas áreas cerebrais: área de Brodmann 25 (BA25); núcleo caudado médio (MC); núcleo caudado posterior (PC) e hipocampo.

Para compreender a localização normal dos A_1R e do $A_{2A}R$ comparámos a abundância relativa dos ARs em extratos totais (TE) e em terminais nervosos (NT), isolados usando um protocolo adaptado de gradiente de Percoll discontínuo (Dunkley *et al.,* 1986, 2008), das áreas cerebrais da mesma amostra. Em todas as áreas estudadas os dois receptores encontram-se enriquecidos nos NT. De seguida, refinámos a informação acerca da localização subsináptica dos ARs, usando um método de fracciomento (Phillips *et al.,* 2001; Rebola *et al.,* 2005) e foi observado que os $A_{2A}R$ estão maioritariamente localizados fora da zona activa da sinapse e os A_1R estão presentes em todas as fracções sinápticas.

Foram então estudadas alterações sinápticas presentes no cérebro de suicidas e foi observada uma diminuição de densidade da *synaptosomal-associated protein 25* (SNAP-25) na BA25 e no MC juntamente com um decréscimo nos níveis de *postsynaptic density protein 95* (PSD-95) no PC e no hipocampo. Foi observado uma diminuição da densidade do marcador astrocítico *glial fibrillary acidic protein* (GFAP) na BA25 e no hipocampo.

Várias alterações na densidade dos ARs, nas diferentes regiões do cérebro, foram observadas nas amostras de suicidas quando comparadas com os controlos. Nós descrevemos: aumento da densidade de $A_{2A}R$ em TE, na BA25 e no PC; aumento de densidade de A_1R em TE, no MC; diminuição de densidade de A_1R em NT, no PC.

Devido à sua particular distribuição no cérebro e as suas propriedades funcionais, os ARs constituem uma oportunidade atrativa para desenvolver compostos inovadores para o tratamento de distúrbios psiquiátricos e neurodegenerativos, como a MDD. O nosso trabalho forneceu informação sobre as mudanças presentes no cérebro de suicidas e pode contribuir para uma melhor compreensão do papel modulador dos ARs na depressão. Há ainda numerosas questões, que requerem atenção cuidadosa, para continuar a explorar este sistema e desenvolver novas estratégias para controlar os distúrbios de humor, particularmente, a MDD.

Palavras-chave: Cérebro humano postmortem; Suicídio; Perturbação depressiva major; Receptor de adenosina A_1; Receptor de adenosina A_{2A}.

Chapter 1. Introduction

1.1 Major depressive disorder

Major depressive disorder (MDD), the most prevalent mental illness, dramatically impairs the quality of life and represents a life-threatening condition (Manji *et al.,* 2001; Nemeroff & Owens, 2002; World Health Organization, 2011). It affects up to 20% of the global population (Berton & Nestler, 2006; Krishnan & Nestler, 2008; Nestler *et al.,* 2002), and by 2020, MDD will be the second leading cause of global disability burden (Murray & Lopez, 1997).

Psychiatric disorders are defined through recognized classifications such as the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) on the basis of behavioral modifications found in patients in comparison with a standardized population. Although ongoing studies, the neurobiological basis of depression is, so far, not well understood (Krishnan & Nestler, 2008).

MDD is a chronic and recurrent condition (Lucas *et al.,* 2011), characterized by one or more major depressive episodes, without history of maniac, mist or hippomaniac episodes (American Psychiatric Association, 2013). A major depressive episode is characterized by a period of at least two weeks during which the individual presents a depressive mood or a loss of interest in almost all activities, together with at least four additional symptoms of depression (American Psychiatric Association, 2013). Depressive mood can be described by: sadness, loss of interest or pleasure, feelings of guilt or low self-esteem, disturbed sleep or appetite, feelings of exhaustion, and reduced concentration, that can impair the ability to function at work and handle daily life (American Psychiatric Association, 2013). Disease phenotypes of MDD include not only episodic and persistent mood disturbances, but also a series of cognitive, motoric, autonomic, endocrine and sleep/wake abnormalities (Manji *et al.,* 2001). Approximately 15% of individuals with MDD can die by suicide (American Psychiatric Association 2013), and many other deleterious health-related effects have been documented. MDD, apart from being a disease with psychological manifestations, is a systemic disease with deleterious effects on multiple organ systems (Manji *et al.,* 2001).

Regardless of its prevalence and substantial impact, there is still a lot to know about its pathogenesis. One major difficulty in studying MDD is to develop an animal model that truly mimics the patient symptoms, as some of this disorder hallmarks, such as depressed mood, low self-esteem or suicidality, are hardly accessible in non-humans (Yan *et al.,* 2010).

As MDD can be hastened by stressful life events, the majority of animal models of depression are based on induced stress (Yan *et al.*, 2010). These include several models, such as, the learned helplessness (Chourbaji *et al.,* 2005), the chronic mild stress (Willner, 2005) and the social defeat stress (Golden *et al.,* 2011). Models of early life stress are also used, producing significant effects that last until adulthood (Ladd *et al.*, 2000). Despite the above mentioned models, other types are used, such as pharmacological, lesion and mutant models (Yan *et al.*, 2010).

As previously stated, the major obstacle of research on MDD pathogenesis is the fact that the currently available validated animal models represent only some of the MDD features, but not the pathogenesis (Yan *et al.,* 2010). However, almost all mental disorders can increase the risk for suicide (Bertolote & Fleischmann 2002), research has shown a strong link between suicide and depression, with, at least, 86% of the suicide victims having a depressive disorder, most often accomplished during or after a major depressive episode (Coryell & Young*,* 2005; Rihmer, 2007). So, the brain tissue from suicide completers can be studied in order to understand the neurobiological basis of depression.

Suicide is a serious public health problem, being among the 10 leading causes of death for most countries at all ages (Bertolote & Fleischmann, 2005). Around the world, in the year 2002, approximately 877,000 people committed suicide, which represents a global mortality rate of 16 per 100,000 or one death every 40 seconds (Bertolote & Fleischmann, 2005). This rate varies regarding different demographic groups and geographic regions. Furthermore, by 2020, it is estimated that approximately 1.53 million people will die from suicide (Bertolote & Fleischmann, 2005). In Portugal, suicide is the major cause of non-natural death (Freitas, 2010).

Positron emission tomography (PET) imaging studies, that evaluate cerebral blood flow (CBF) and glucose metabolism, showed multiple abnormalities in mood disorder patients, relative to healthy patients (Mayberg, 2003). Abnormalities of several brain structures were also demonstrated by magnetic resonance imaging (MRI) and postmortem investigations (Manji *et al.*, 2001). Findings, albeit not homogeneous, denote reductions in hippocampus volume (Bremner *et al.,* 2000; Caetano *et al.,* 2004; Frodl *et al.,* 2006; Neumeister *et al.,* 2005; Sheline *et al.,* 2003) with greater impact on more persistent forms of MDD (Lorenzetti *et al.*, 2009). Evidences further support volumetric alterations in the basal ganglia (Bonelli *et al.*, 2006; Hickie *et al.,* 2007; Pillay *et al.,* 1998), and it seems that the caudate nucleus, putamen and globus pallidus may

be impaired in more severe subtypes of depression (Lorenzetti *et al.,* 2009). Alterations in the anterior cingulate cortex (ACC), particularly a sub-region usually termed Brodmann area 25 (BA25), have been observed in functional neuroimaging studies of depressed patients (Gotlib *et al.,* 2005; Pizzagalli *et al.,* 2004).

These evidences indicated a dysregulation of limbic and cortical regions involved in emotional regulation and experience (Davidson *et al.,* 2002; Mayberg, 1997; Phillips *et al.,* 2003a, b), including hippocampus, amygdala, ACC, orbital frontal cortex (OFC) and basal ganglia (Lorenzetti *et al.,* 2009).

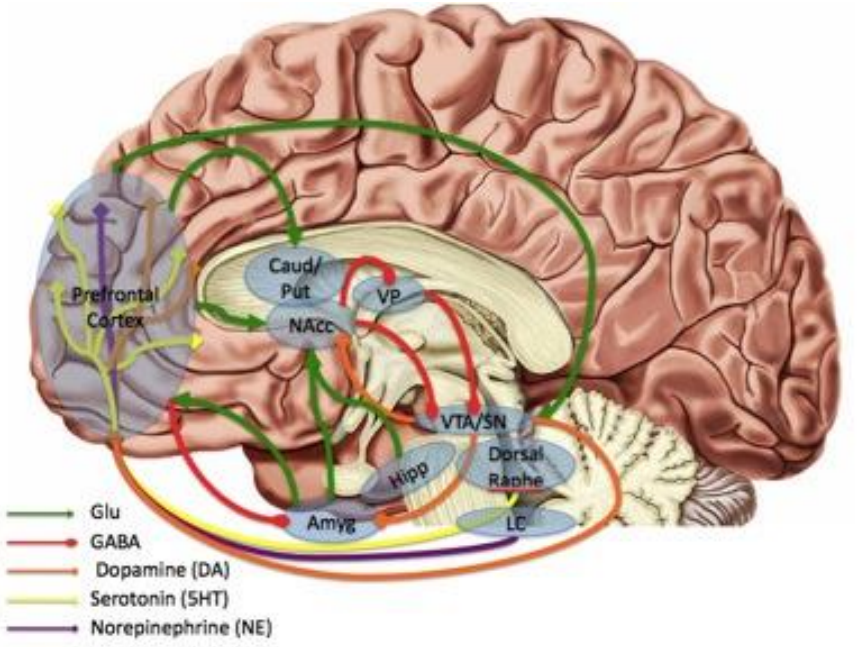

Figure 1. Regions, neurotransmitters and circuits implicated in the pathology of major depressive disorder (MDD) by human neuroimaging studies (adapted from Treadway & Pizzagalli, 2014).

BA25 is a “node” in a network connecting the cortex with limbic regions and brainstem (Pandya *et al.,* 1981).

The striatum, comprising the caudate nucleus and putamen, is the primary input structure to the basal ganglia circuitry, serving as an emotional-motor interface. Through the selective distribution of the axonal terminal arborizations of cortical sources it is relatively segregated to differentially support motor, executive, and affective or limbic processing (Furman *et al.,* 2011). Basal ganglia receives projections from the frontal cortex, comprising ACC and OFC, as well as by associative cortex and limbic regions, comprehending the hippocampus, amygdala and related regions. Output neurons project from the basal ganglia to the midbrain and thalamus, which

return to the frontal cortex, so that the information is processed and then relayed back to distinct cortical regions (Furman *et al.,* 2011).

The striatum and globus pallidus are organized in parallel to connect with cortical and limbic regions (Furman *et al.,* 2011). Afferent and efferent neurons of the hippocampus are bundled together in the same paths. The two major pathways are the fornix and entorhinal cortex, via the cingulate cortex (Sheline, 2003). The fornix connects to the septum, striatum and hypothalamus. In turn, the cingulate cortex, projects to the temporal lobe cortex, orbital cortex, and olfactory bulb (Sheline, 2003).

Anomalous connectivity of the frontostriatal linkages contributes to the anhedonic, ruminative and psychomotor dysfunctions characteristics of MDD (Furman *et al.*, 2011). Neocortex and hippocampus may mediate cognitive aspects of depression besides memory impairments, such as feelings of worthlessness, hopelessness, guilt, doom, and suicidality (Krishnan & Nestler, 2008). The striatum, amygdala and related brain areas, are important in emotional memory, and can mediate: increased anxiety; decreased initiative, reward for pleasant activities and motivation (Krishnan & Nestler, 2008). Lack or excess of sleep, appetite, and energy, as well as a loss of interest in sex and other pleasurable activities, implicates a role for the hypothalamus (Nestler *et al.,* 2002). The frontal cortex, besides being relevant for cognitive functions, is also important for modulation of basal ganglia and limbic regions activity (Sheline, 2003).

Many patients with MDD seem to display disruption of brain networks (Menon, 2011) and characteristics of a neuroprogressive illness (Moylan *et al.,* 2013). Numerous neurobiological pathways can interact simultaneously, contributing to this pathogenesis, including neurotransmitter systems with disruption of monoaminergic transmission, such as serotonergic, dopaminergic and noradrenergic (Moylan *et al.,* 2013). Evidences for other neurotransmission system disruption also exist, such as cholinergic, GABAergic and glutamatergic (Manji, 2001).

MDD may also be related to abnormalities in the circadian rhythm (Carlson, 2013) and the hypothalamic-pituitary-adrenal (HPA) axis, activated during the body's response to stressors, seems to be overactive in depressed patients (Pariante & Lightman, 2008).

As so, biological theories of depression must comprehend systems necessary for the processing and experience of emotions, involving autonomic, endocrine and cognitive states that couple the perception of stimuli to an adaptive behavioral response (Mayberg, 2000).

Table 1. Comparison of human postmortem studies in depression (adapted from Russo & Nestler, 2013).

Brain region	Human postmortem analysis
Hippocampus	↓ Synapses density ↓ Glial cell density
Basolateral amygdala	↓ Grey matter ↓ Glial cell density
Medial prefrontal cortex	↓ White matter ↓ Dendritic branching ↓ Glial cell density

At cellular level, depression can be characterized by a decreased neuronal soma size in cortical and hippocampal neurons and increased packing density in hippocampal neurons (Stockmeier & Rajkowska, 2004). Results from previous studies also indicate the presence of a synaptic pathology in the ACC in mood disorders, contributing to the dysfunction of cingulate neural circuits (Eastwodd & Harrison, 2001). Earlier findings suggest abnormalties of SNARE (SNAP receptor) mechanism proteins, a fundamental molecular component of neural connectivity which consists of three proteins, syntaxin, synaptosomal-associated protein 25 (SNAP-25) and vesicle-associated membrane protein (VAMP) in mental disorders (Hones *et al.,* 2002). Abnormalities in glutamatergic transmission in MDD have been reported, and the results revealed a reduction in PSD-95 expression levels (Feyissaa *et al.,* 2009). Additionally, MDD is linked to deregulation of neurotransmission, decreased neuronal synapses and failure in synaptic plasticity in areas that regulate mood. The alterations seem to particularly affect excitatory connections and may be progressive (Eastwodd & Harrison, 2001; Femenia *et al.,* 2012). MDD is also characterized by alterations in size and shape of glial cells, indicative of reduction in the number of normally functioning glial cells (Stockmeier & Rajkowska, 2004). Changes in astrocytes density and in the expression of their markers have been shown (Stockmeier & Rajkowska, 2004), along with microglial activation (Steiner *et al.,* 2011). Impairments in glia can lead to deficiency in neurotrophic and angiogenic factors and disturbed myelination that are likely to contribute to the pathophysiology of MDD (Rajkowska *et al.,* 2007). As glia, especially astrocytes, regulates the levels of extracellular glutamate, and thus protects neurons from cell death and provides them energy, astrocytic pathology may also promote glutamate-mediated neuronal excitotoxicity (Stockmeier & Rajkowska, 2004).

There are several treatments available that vary according to the severity of the disease. Current available therapeutics are antidepressants, certain forms of psychotherapy and electroconvulsive therapy (ECT) (Gomes *et al.,* 2011; Nemeroff & Owens, 2002). All approved classes of antidepressants act in blockade of presynaptic monoamine transporter proteins, inhibition of monoamine oxidase, or inhibition or excitation of pre- or postsynaptic receptors (Gomes *et al.,* 2011; Nemeroff & Owens, 2002). ECT antipsychotic effects are associated with normalization of BA25 theta hypoactivity (McCormick *et al.,* 2009). These treatments are safe, however, less than half of patients achieve complete remission, and others exhibit partial or intolerant responses to treatment (Gomes *et al.,* 2011). This highlights the inadequacy of currently available treatments and the need to discover new antidepressants (Gomes *et al.,* 2011; Nemeroff & Owens, 2002). New treatment techniques, such as deep brain stimulation (DBS) (Malone *et al,.* 2009; Mayberg *et al.,* 2005; Holtzheimer & Mayberg, 2010) and transcranial magnetic stimulation (TMS) (Carpenter *et al.,* 2012; Janicak *et al.,* 2013) are emerging, based on the differential neural responses in cortico-striato-limbic circuits of depressed patients (Treadway & Pizzagalli, 2014).

Ongoing research aims to define the cause for depression, finding new biomarkers, developing diagnostic tests and better treatments based on these brain alterations.

1.2 Adenosine

1.2.1 An endogenous neuromodulator

Physiological actions of adenosine exogenously administered were first described by Drury and Szent-Györgyi in 1929, who observed pronounced cardiovascular effects. The discovery made by Sattin and Rail in 1970 that, in brain cells, adenosine stimulates cyclic adenosine monophosphate (cAMP) formation, began a new stage of adenosine research, leading to the discovery of adenosine receptors (ARs) and their subclassification (Mueller & Scior, 1993).

Adenosine does not act as a neurotransmitter, once it is not stored in vesicles or released by exocytosis, it does not transfer information unidirectionally from presynaptic to postsynaptic components, or act only or predominantly in synapses (Fredholm *et al.,* 2005). Instead, the nucleoside adenosine exerts two modulatory influences in the Central Nervous System (CNS), acting as a homeostatic modulator and also as a neuromodulator at the synaptic level. As a neuromodulator: it controls the flow of information between neurons in the brain by fine tuning on-going synaptic transmission; as an upstream regulator of a wide-range of neurotransmitters, it also modulates post-synaptic responsiveness action of other receptor systems and signaling pathways that converge to contribute to the expression of an array of important brain functions (Cunha, 2001; Cunha, 2005; Cunha, 2008; Fredholm *et al.,* 2005; Prediger *et al.,* 2006; Svenningsson *et al.,* 1999; Wei *et al.,* 2011).

The concentration of adenosine in the extracellular compartment is the consequence of many biological processes, being formed inside or outside cells, it can appear in the extracellular milieu through different mechanisms (Chen *et al.,* 2013; Fredholm *et al.,* 2005; Fredholm, 2007). Adenosine can be released through nucleoside transporters, after an increase in the intracellular levels of adenosine or a reversal of the sodium gradient; can be formed in the extracellular space through the ectonucleotidase pathway on release of adenine nucleotides, especially adenosine triphosphate (ATP); or after release of cAMP, but this pathway has been found to be of minor importance when physiological parameters affected by adenosine are being studied (Cunha, 2008; Fredholm *et al.,* 2005; Fredholm, 2007).

Extracellular adenosine, which is relevant for modulation of synaptic transmission, might originate not only from nerve terminals but also from the activated postsynaptic component and from surrounding non neuronal cells, depending on the stimuli (Cunha 2008; Fredholm *et al.* 2005; Fredholm 2007).

Nonconcentrative nucleoside transporters and enzymes of adenosine metabolism regulate extracellular concentrations of adenosine. Adenosine deaminase (ADA) and adenosine kinase (ADK) generally reduce adenosine concentrations by forming inosine and AMP, respectively. ADK is the primary route of adenosine metabolism with ADA coming into play only when large amounts of adenosine have to be cleared (Fredholm *et al.,* 2005). During brain development there is a change in the location of ADK, with a transition from a predominantly neuronal expression to a predominantly astrocytic expression (Boison, 2006). ADA is more abundant in astrocytes, than in neurons, reflecting the importance of non neuronal uptake of adenosine (Fredholm *et al.,* 2005).

The activity of nucleoside transporters can be regulated by the activation of ARs through protein kinase pathways (Fredholm *et al.,* 2005).

1.2.2 Adenosine receptors

Adenosine is a purine nucleoside that exists in all cells, where it is a metabolite involved in key pathways of primary metabolism and a constituent of other bioactive molecules. The intra- and extracellular levels of adenosine equilibrate with each one, so, if the levels of intracellular adenosine rise in a particular cell, the extracellular adenosine levels will gradually increase in the surroundings of the cell. Extracellular adenosine is then capable to act on ARs located both in the cell membrane of neighbouring cells and in the cell that released adenosine in the first place (Cunha, 2005).

ARs are a family of cell surface receptors and so far there are four subtypes known and pharmacologically characterized: A_1, A_{2A}, A_{2B}, and A_3. The ARs are G protein-coupled receptors (GPCRs) and consist of a single polypeptide chain that transverses the membrane from the extracellular side beginning at the N terminus to form seven transmembrane helices. The A_1 and A_3 receptors preferentially couple to Gi protein, and the A_{2A} and A_{2B} receptors subtypes couple to Gs or Go (Chen *et al.,* 2013; Cunha, 2005; Fredholm, 2007; Moreau & Huber, 1999; Mueller *et al.,* 2011; Prediger *et al.,* 2006; Sciotti *et al.,* 1992), but it seems that all ARs have potential to couple to different G proteins and to different transducing systems, depending on their degree of activation and their particular cellular and sub-cellular localization (Cunha, 2005).

A_{2B} and A_3 receptors have low abundance in the brain, thus, the role of adenosine in the brain, to modulate neurotransmitter systems, neuronal excitability, and synaptic plasticity, is currently considered to be mediated by a balanced activation of A_1 and A_{2A} receptors (Cunha, 2005; Wei *et al.,* 2011). This balanced activation depends

not only on the transient levels of extracellular adenosine, but also on the direct interaction between both receptors. So, at low concentrations of extracellular adenosine, A_1 receptor-mediated tonic inhibition of neurotransmitter release predominates and, as the concentration of adenosine rises, A_{2A} receptor modulation prevails (Cunha, 2001; Cunha, 2005).

The different ARs subtypes differ with regard to control of their expression and their rate of desensitization/resensitization, and upon prolonged exposure to agonists all ARs subtypes desensitize (Fredholm *et al.,* 2005). The ARs have also been proposed to participate in both homo and heterodimerization or even oligomerization (Mueller *et al.,* 2011; Uriguen *et al.,* 2009).

Even though ARs are widespread in the brain, they are not homogeneously located in brain regions or cell types (Cunha, 2005; Cunha, 2008; Fredholm *et al.,* 2005; Svenningsson *et al.,* 1999; Uriguen *et al.,* 2009; Wei *et al.,* 2011).

1.2.2.1 A_1 receptor

The A_1 receptor is the most highly conserved ARs subtype between species and probably exhibits the greatest affinity for adenosine (Chen *et al.,* 2013; Cunha, 2005; Cunha, 2008; Fredholm *et al.,* 2005; Wei *et al.,* 2011).

Its activation inhibits adenylate cyclase (AC) activity, activates potassium channels (including KATP channels), blocks transient calcium channels and increases inositol-1,4,5-trisphosphate and intracellular calcium levels by activating phospholipase C (PLC), it modulates neuronal activity by blocking neurotransmitter release and reducing the firing rate (Chen *et al.,* 2013).

It is widely expressed throughout the body with the highest levels found in the brain, especially at excitatory nerve endings (Chen *et al.,* 2013; Cunha, 2005; Cunha, 2008; Fredholm *et al.,* 2005; Wei *et al.,* 2011) in neurons of cortex, hippocampus, and cerebellum and only moderately expressed in striatum, being also expressed at intermediate levels elsewhere in the brain (Chen *et al.,* 2013; Wei *et al.,* 2011).

From previous studies in rodents, we know that although especially found in neurons, they are also located in other cell types in the brain, such as astrocytes, microglia, and oligodendrocytes (Fredholm *et al.,* 2005). At sub-cellular level, A_1 receptors were mostly found in nerve terminals in the plasma membrane, at both pre-synaptic zone and postsynaptic density, in hippocampus (Rebola *et al.,* 2003).

1.2.2.2 A_{2A} receptor

A_{2A} receptor also exhibits a high affinity for adenosine and is widely expressed in different tissues, at varying levels (Cunha, 2005; Fredholm et al., 2005; Moreau & Huber, 1999; Schiffmann et al., 1991; Svenningsson et al., 1999).

In the brain, A_{2A} receptor activation stimulates the protein kinase A (PKA) pathway (Chen *et al.,* 2013; Fredholm *et al.* 2005; Wei *et al.,* 2011) and may also signal through a protein kinase C (PKC) pathway or even trigger alternative signaling pathways, by interacting with other receptors and signaling molecules (Wei *et al.,* 2011), regulating motor activity, psychiatric behaviors, the sleep/wake cycle and neuronal cell death (Chen *et al.,* 2013). In a G protein-independent way, A_{2A} receptors might mediate other signaling pathways responsible for regulation of its own expression and the activation of mitogen-activated protein kinase (MAPK) (Fredholm, 2007).

Within brain, A_{2A} receptors are concentrated in basal ganglia, predominantly expressed in striatum, but they are also present throughout the brain with a considerably lower density (Cunha, 2005; Fredholm *et al.,* 2005). In extrastriatal areas, distinct levels are found in the cortex, amygdala, olfactory tubercles, hippocampus, hypothalamus, thalamus and cerebellum (Cunha, 2005; Fredholm *et al.,* 2005; Moreau & Huber, 1999; Schiffmann *et al.,* 1991; Svenningsson *et al.,* 1999).

In previous studies, using rodent samples, A_{2A} receptors were present in neurons and also in astrocytes, microglia, and blood vessels throughout the brain (Fredholm *et al.,* 2005). At sub-cellular level, A_{2A} receptors were found in the active zone of the pre-synaptic sites nerve terminals of hippocampus, whereas in the striatum is predominantly located at post-synaptic density, while a minority is located in the pre-synaptic site in cortico-striatal terminals (Fredholm *et al.,* 2005; Rebola *et al.,* 2005; Wei *et al.,* 2011).

Although in rodents and humans the distribution of A_{2A} receptors is similar, the extrastriatal levels appear to be higher in humans than in rodents (Fredholm *et al.,* 2005).

1.3 Mood and adenosine

Adenosine and its analogues have been shown to produce depressant-like behavioral effects, relevant for human condition (Minor *et al.,* 1994; Woodson *et al.,* 1998).

Patients with MDD, in clinical studies, were found to have reduced serum ADA activity, with a consequent increase in adenosine tonus, which was inversely correlated with disease severity (Wei *et al.,* 2011).

Furthermore, animal models designed to reveal the neurobiological basis of depression point to a role for ARs, in the regulation of mood (Batalha *et al*., 2013; Gomes *et al.,* 2011; Wei *et al.,* 2011).

The strongest evidence supporting the relation between adenosine and depression in preclinical models came from manipulation of ARs, both selective A_{2A} receptor receptor blockade by antagonists, as well as global A_{2A} receptor genetic depletion were shown to reverse signs of behavioral despair in the tail suspension and forced swim tests, independently of its hyperlocomotor effect (El Yacoubi *et al.,* 2001; El Yacoubi *et al.,* 2003; Moreau & Huber, 1999; Wei *et al.,* 2011). In animal models, it is possible to implicate endogenous adenosine neuroregulation as a central compensatory mechanism in learned helplessness and conservation-withdrawal (Minor *et al.,* 1994; Woodson *et al.,* 1998).

Stress is a known risk factor for depression, so, the study of animal models of chronic stress can help to discover potential anti-depressant drugs. A_{2A} receptors seem to be involved in the stress-associated impairments, as the administration of a selective antagonist reverts the consequences of stress on spatial memory, synaptic plasticity and neuronal morphology in the hippocampus (Batalha *et al.,* 2013).

Several epidemiological studies have explored a relationship between caffeine intake, mood changes and specific psychiatric symptoms (Cunha *et al.,* 2008; Gomes *et al.,* 2011). These studies are important to understand the role of the adenosine modulation system in the control of mood, since methylxanthines, such as caffeine (1,3,7-trimethylxanthine), have profound biological effects as competitive antagonists of A_1 and A_{2A} receptors (Chen *et al.,* 2013; El Yacoubi *et al.,* 2003; Fredholm, 2007), and they are the only known molecular target of caffeine, at nontoxic doses (Cunha *et al.,* 2008; Fredholm *et al.,* 2005). Coffee consumption may bring benefits related to its psychostimulant effects, increasing alertness (Lara, 2010; Smith, 2002). Additionally coffee consumption is considered to improve psychomotor performance, cognition and elevate mood in humans (Cunha *et al.,* 2008; El Yacoubi *et al.,* 2003; Lara, 2010).

Furthermore, moderate coffee intake has been associated with reduced risk of depression in a dose-dependent manner (Lucas *et al.,* 2011; Lucas *et al.,* 2013). Prior observations also suggest that suicide risk is lower among persons with higher consumption of coffee (Kawachi *et al.,* 1996). Even though it has psychostimulant effects at low to moderate doses, they are dose dependent and typically biphasic, so, with higher doses, the reverse effect can be observed (Lucas *et al.,* 2011), triggering behavioral modifications, both anxiety disorders as well as depressive-like conditions. Similar effects to high doses of caffeine are reported during withdrawal (Juliano & Griffiths, 2004; Kruger, 1996; Stephenson, 1977). The threshold for the anxiogenic effect of caffeine seems to be influenced by a polymorphism of the A_{2A} receptor, being restricted to some individuals with at least a predisposition for specific anxiety disorders, and is influenced by genetic and ethnic factors (Lara, 2010).

Studies trying to explain the mechanisms underlying antidepressant action have shown effects related to the adenosine system (Gomes *et al.,* 2011), as many drugs clinically used to manage depressive disorders may exert their effects by altering extracellular adenosine concentrations and signaling (Chen *et al.,* 2013), binding to ARs and, in a dose-dependent manner, reducing the activity of ectonucleotidases. Also, antidepressants reverse the adenosine-induced immobility in animals submitted to inescapable shocks and forced swimming tests (Kulkarni & Mehta, 1985).

Additionally, strategies used to control mood disorders, as ECT and sleep deprivation (SD), cause an adaptation of the adenosine neuromodulator system, increasing adenosine concentration and A_1 receptor activation (Gomes *et al.,* 2011). For instance, antidepressive-like benefits of SD require astrocytic signaling through A_1 synaptic receptors (Hines *et al.,* 2013). Short-term adaptive neuronal response is triggered through inhibitory A_1 receptors, in slow wave sleep and cerebral metabolic activity; and long term adaptive changes through up-regulation of A_1 and probably of A_{2A} receptors (Gomes *et al.,* 2011).

Adenosine neuromodulation system actions in depression are complex, due to its ability to modulate several neurotransmission systems (dopaminergic, glutamatergic and serotonergic as well as the corticotrophin system) (Gomes *et al.,* 2011); glial metabolism and neuroinflammation (Cunha *et al.,* 2008; Uriguen *et al.,* 2009). And so far, it is unknown whether the manipulation of both A_1 and A_{2A} receptors, a neuroprotective strategy suggested for other disorders and pathologies, can be explored for MDD.

To sum up, since the discovery of its physiological actions, adenosine system is a target for drug development (Gomes *et al.,* 2011).

The main function of adenosine neuromodulation system is to maintain homeostasis and promote adaptation of neuronal systems, acting as a normalizing system by limiting edges of extreme functioning of biological systems, modulating anxiety, exploration, aggressive behaviors, depressive responses, and sensorimotor gating (Lopes *et al.,* 2011). Therefore, excess or reduced adenosine in a system, and subsequently too high or too low ARs activation or expression levels, will lead to a failure in adapting to environment and cause a predisposition to disease (Gomes *et al.,* 2011).

ARs distribution in the brain and their functional properties create an opportunity to develop new compounds for the treatment of neurodegenerative and psychiatric disorders, such as MDD. The adenosine system can be used to manipulate brain circuits to restore their proper function, and there seems to be a parallel benefit of manipulating both A_1 and A_{2A} receptors (Cunha, 2005).

There are still numerous questions that demand careful attention to further explore this system and develop novel strategies to control mood disorders, particularly MDD. An obvious need is to understand the importance and what are the alterations of ARs in brain areas affected by MDD, and what are the mechanisms behind those changes.

Chapter 2. Aims

Aims of this work:

- To understand the differences in the brain localization, density and function of adenosine A_1 and A_{2A} receptors, between control subjects and suicide completers;

- To evaluate if these modifications are accompanied by:

    - Synaptic imbalance, in brain areas affected by depression;

    - Astrocytic changes, in brain areas affected by depression.

Chapter 3. Materials and Methods

3.1 Materials / Reagents / Biological samples

3.1.1 Reagents

Table 2. Reagents and drugs used.

Reagent	Supplier
1,4-bis(sulfanyl)butane-2,3-diol (DTT)	Sigma-Aldrich (Portugal)
2-[2-[bis(carboxylatomethyl)amino]ethyl-(carboxylatomethyl)amino]acetate (EDTA) tetrasodium	Sigma-Aldrich (Portugal)
2-[2-[3,4-bis(2-hydroxyethoxy)oxolan-2-yl]-2-(2-hydroxyethoxy)ethoxy]ethyl dodecanoate (Tween 20)	Sigma-Aldrich (Portugal)
2-(4-carboxyquinolin-2-yl)quinoline-4-carboxylic acid (BCA) kit	Thermo scientific (USA)
2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES)	Sigma-Aldrich (Portugal)
2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol (Triton-X – 100)	Sigma-Aldrich (Portugal)
2,6-dibromo-4-[3-(3,5-dibromo-4-hydroxyphenyl)-1,1-dioxo-2,1λ6-benzoxathiol-3-yl]phenol (bromophenol blue)	Sigma-Aldrich (Portugal)
2-amino-2-(hydroxymethyl)propane-1,3-diol (Trizma base)	Sigma-Aldrich (Portugal)
2-aminooxyacetic acid	Sigma-Aldrich (Portugal)
(2R,3R,4S,5R)-2-[6-(cyclopentylamino)purin-9-yl]-5-(hydroxymethyl)oxolane-3,4-diol (CPA)	Sigma-Aldrich (Portugal)
(2R,3R,4S,5S,6R)-2-[(2S,3S,4S,5R)-3,4-dihydroxy-2,5-bis(hydroxymethyl)oxolan-2-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol (sucrose)	Sigma-Aldrich (Portugal)
30% acrylamide/Bis solution	Bio Rad (Portugal)
3-[4-[2-[[6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxyoxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid (CGS 21680)	Tocris – Biogen (Spain)
3-(cyclohexylamino)propane-1-sulfonic acid (CAPS)	Sigma-Aldrich (Portugal)

(3R,4S,5S,6R)-6-(hydroxymethyl)oxane-2,3,4,5-tetrol (glucose)	Sigma-Aldrich (Portugal)
[^{14}C]-U-glutamate	PerkinElmer (USA)
Ammonium persulfate (APS)	Sigma-Aldrich (Portugal)
Bovine serum albumin (BSA)	Sigma-Aldrich (Portugal)
Calcium chloride ($CaCl_2$)	Sigma-Aldrich (Portugal)
Cocktail of proteases inhibitors (CLAP)	Roche Diagnostics (Portugal)
ECF Western blotting reagent	GE Healthcare (United Kingdom)
Hydrochloric acid (HCl)	Sigma-Aldrich (Portugal)
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich (Portugal)
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich (Portugal)
Methanol	Sigma-Aldrich (Portugal)
Methylsulfinylmethane (DMSO)	Sigma-Aldrich (Portugal)
N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)	Sigma-Aldrich (Portugal)
Percoll	GE Healthcare (United Kingdom)
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich (Portugal)
Potassium chloride (KCl)	Sigma-Aldrich (Portugal)
Potassium dihydrogen phosphate (KH_2PO_4)	Sigma-Aldrich (Portugal)
Propane-1,2,3-triol (glycerol)	Sigma-Aldrich (Portugal)
RNeasy Mini kit	Qiagen – Izasa (Portugal)
Scintillation liquid	Zinsser Analytic (Germany)
Sodium dodecyl sulfate (SDS)	Bio Rad (Portugal)
Sodium azide	Sigma-Aldrich (Portugal)
Sodium chloride (NaCl)	Sigma-Aldrich (Portugal)
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma-Aldrich (Portugal)
Sodium hydrogen phosphate ($NaH_2PO_4 \cdot H_2O$)	Sigma-Aldrich (Portugal)

3.1.2 Antibodies

Table 3. Primary antibodies for Western blot.

Antibody	Supplier	Host	Dilution	Protein bands (kDa)
Adenosine A_1 receptor (A_1R)	Thermo Scientific #PA3-041A	Rabbit	1:1000	37
Adenosine A_{2A} receptor ($A_{2A}R$)	Santa Cruz #SC-13937	Rabbit	1:500	43
β-actin	Sigma #A5316	Mouse	1:20000	42-43
Glial fibrillary acidic protein (GFAP)	Millipore #AB5804	Rabbit	1:20000	50
Postsynaptic density-95 protein (PSD-95)	Sigma #P246	Mouse	1:20000	95
Synaptosomal-associated protein 25 (SNAP-25)	Sigma #S5187	Mouse	1:20000	25
Synaptophysin	Sigma #S5768	Mouse	1:20000	38-42
Syntaxin	Sigma #S0664	Mouse	1:5000	35

Table 4. Secondary antibodies for Western blot.

2nd Antibody (anti species)	Supplier	Dilution
Mouse	GE Healthcare #NIF 1316	1:20000
Rabbit	GE Healthcare #NIF 1317	1:20000

3.1.3 Human samples

Human brain samples were obtained at autopsies performed in the *Instituto Nacional de Medicina Legal e Ciências Forenses, I. P.* (INMLCF), in Coimbra. The tissue samples were collected by the pathologist Dr. Beatriz Silva with all procedures approved by the INMLCF and subject to the rules of the European Consortium of Nervous Tissues: BrainNet Europe II, to protect the identity of individual donors.

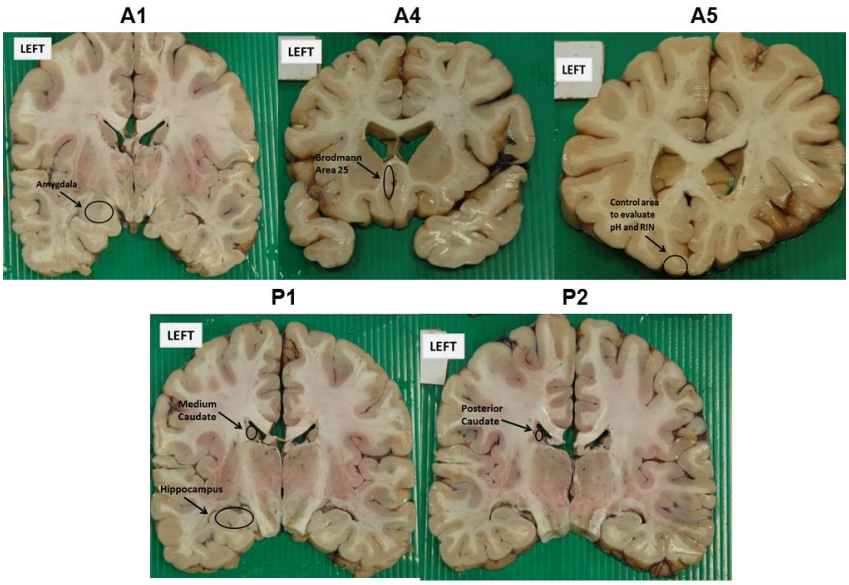

Figure 2. Coronal slices from human brain samples. As reference point they used the mammillary bodies. (A) – Anterior (Before mamillary bodies). (B) - Posterior (After mamillary bodies). Images gently provided by Dr. Beatriz Silva from INMLCF.

After being collected, the samples were placed in tubes, and were either rapidly frozen in liquid nitrogen and stored at -80ºC in a freezer, or they were placed on ice for functional assays.

The study was performed in male subjects who died by suicide and in age-matched non-suicide controls, who died by natural causes or accidents. To blind the individuals identification, a code-system of sequential numbers was created to match the brain samples collected to the individuals. Individuals excluded from the study were ones consuming medically-prescribed psychoactive drugs at the time of death; with neuro-pathological features of neurological disorders or psychiatric conditions such as addiction, eating disorders, schizophrenia or phobia; in coma or with artificial respiration before death.

An important aspect in conducting postmortem research is the quality of the tissue, because unlike animal samples, human tissue conditions at death can't be

controlled. Since the most sensitive indicator of tissue quality is ribonucleic acid (RNA) integrity number (RIN) and that there is a good correlation between RIN and the pH (Stan *et al.,* 2006), the integrity of the tissue was validated by measurement of brain pH and RIN. However, neither postmortem interval (PMI), pH, nor RIN impact on protein concentrations in the human tissue, since even when RNA is degraded, protein levels remain stable (Stan *et al.,* 2006).

Table 5. Demographic characteristics and cause of death of individual cases of suicide completers and their respective controls.

Number	Sex	Age	Cause of death	Postmortem Interval (hours)	pH	RIN	Control or Suicide
1	M	81	Hanging	39.6	6.55	6.7	Suicide
2	M	66	Hanging	43.8	6.6	6.6	Suicide
3	M	80	Hanging	45.8	6.49	5.5	Suicide
4	M	41	Pulmonar hemorrhage	36.1	6.43	6.7	Control
5	M	44	Diazinon organophosphate poisoning	20.9	6.3	5.7	Suicide
6	M	17	Hanging	48.0	6.69	6.9	Suicide
7	M	60	Hanging	28.1	6.48	6.8	Suicide
8	M	65	Cardiopathy	52.3	5.81	ND	Control
9	M	81	Cardiopathy	16.5	5.96	ND	Control
10	M	60	Cardiopathy	76.8	6.44	ND	Control
11	M	21	Car accident	31.3	6.59	5.5	Control
12	M	22	Car accident	32.7	6.64	6.3	Control
13	M	53	Hanging	25.3	6.44	ND	Suicide
14	M	72	Cardiopathy	149.9	6.06	ND	Control
15	M	58	Hanging	20.5	6.42	ND	Suicide

16	M	64	Burn	*	6.65	6.8	Control
17	M	60	Work accident	22.5	6.53	7.8	Control
18	M	83	Cardiopathy	79.0	6.69	6.4	Control
19	M	80	Accident	20.7	6.71	5.9	Control
20	M	22	Hanging	34.6	6.53	5.9	Suicide
21	M	32	Cardiopathy	29.8	6.69	7.8	Control
22	M	76	Car accident	44.5	6.49	3.8	Control
23	M	61	Accidental drowning	63.5	6.72	6.6	Control
24	M	66	Hanging	32.0	6.6	6.9	Suicide
25	M	54	Cardiopathy	11.1	6.38	4.3	Control
26	M	71	Accidental fall	54.7	5.98	2.5	Control
27	M	60	Hanging	46.7	6.71	5.9	Suicide
28	M	62	Hanging	44.7	6.55	6.3	Suicide
29	M	81	Car accident	32.9	6.2	6.1	Control
30	M	63	Work accident	24.9	6.46	5.4	Control
31	M	29	Hanging	27.35	6.6	6	Suicide
32	M	62	Burning Accident	40.5	6.22	5.4	Control
33	M	30	Hanging	68.5	6.63	6.2	Suicide

* The brain was frozen; ND - Not determined.

Table 6. Demographic characteristics of suicide completers and age-matched controls groups.

	Sex	**Age**	**Postmortem Interval (hours)**	**pH**	**RIN**
Suicide	M	51.9±7.0	34.6±3.6	6.5±0.04	6.5±0.2
Control	M	54.5±6.7	39.2±5.6	6.5±0.08	6.4±0.2

3.2 Methods

3.2.1 pH determination

Frozen brain tissue samples were homogenized manually by using a micropistill. The pH was measured with a pH electrode (Sentek), previously calibrated with 3 standards (pH= 4.00; pH = 7.00; pH = 10.00) at 4ºC (Monoramu *et al.,* 2009).

3.2.2 Total RNA extraction

Samples stored at -80ºC were thawed and extraction of total RNA was performed. For this purpose, we used the RNeasy® Mini Kit (Qiagen), following the instructions provided by the manufacturer, with minor adaptations.

Quantification of total RNA was performed at the end of extraction, as well as determination of RNA samples' concentration and purity,, using a spectrophotometer (NanoDrop®, Thermo Scientific) through the ratio between the absorvances at wavelengths 260 and 280 nm (A260/A280).

The samples were stored again in a freezer at -80 º C.

3.2.3 RNA analysis

The samples were run on an Agilent 2100 Bioanalyzer, which automatically calculates the sample RIN, through an algorithm based on the ribosomal peaks and the extent of RNA degradation products from the entire electropherogram and then assigns a score from 1 to 10 (Schroeder *et al.,* 2006). RNA analysis was performed in collaboration with Dr. Manuela Grazina.

3.2.4 Total extracts preparations

Human brain samples were homogenized at 800 r.p.m. for 10-15 strokes in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% IGEPA (NP-40), 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS), supplemented with CLAP, 0.1 mM DTT and 0.1 mM PMSF, and then sonicated for at least 15 min at 4ºC. The samples were stored at -20ºC until further use.

3.2.5. Human synaptosomes isolation

Synaptosomes are re-sealed, functional nerve terminals, which were separated from their axons and postsynaptic connections. A synaptosomal preparation encloses all common neuronal content, including cytoplasm, synaptic vesicles and mitochondria, with low presence of synaptic and glial plasma membranes and extrasynaptosomal mitochondria. Their function closely resembles nerve terminals *in vivo*, allowing the study of neurotransmitters release. (Dunkley *et al.,* 1986, 2008)

3.2.5.1 Human synaptosomes isolation in a discontinuous Percoll gradient

Using our group know-how in rodents (Rebola *et al.,* 2005; Canas *et al.,* 2009; Gomes *et al.,* 2009) we optimized the purification of nerve terminals from the human brain using an adapted discontinuous Percoll gradient protocol (Dunkley *et al.,* 1986, 2008).

Human fresh brain tissue, from a particular brain region, was homogenized in a sucrose solution (0.32 M sucrose, 1 mM EDTA and 10 mM Tris pH 7.4, at 4ºC) at 800 r.p.m. for 10-15 strokes.

The homogenates were centrifuged at 1000 *g* for 10 min at 4º C (Avanti J-26 XPI centrifuge, rotor JA-25-50), supernatants collected and further centrifuged at 20000 *g* for 20 min at 4ºC (Avanti J-26 XPI centrifuge, rotor JA-25-50). Supernatants were discarded and pellets resuspended in the sucrose solution. This suspension was slowly and carefully placed on top of a discontinuous Percoll gradient, previously prepared.

The gradient was built in a tube as follow, from bottom to top: 3 ml of a 23% (v/v) Percoll solution, 5 ml of a 10% (v/v) Percoll solution and 3 ml of a 3% (v/v) Percoll solution. Percoll solutions were previously prepared in the sucrose solution and maintained on ice until use.

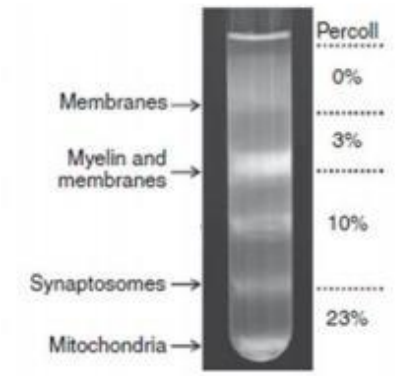

Figure 3. Representation of the discontinuous Percoll gradient (adapted from Dunkley *et al.,* 2008).

The gradients were centrifuged at 16000 *g* for 20 min at 4ºC, without deceleration (Avanti J-26 XPI centrifuge, rotor JA-25-50). Synaptosomes were collected between the 10% (v/v) and 23% (v/v) Percoll layers and diluted in 15 ml of HEPES buffered medium (HBM) without calcium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 10 mM Glucose and 10 mM HEPES, pH 7.4). After centrifugation at 22000 *g* for 11 min at 4º C, free-moving pellets were collected, resuspended in HBM and centrifuged at 11000 *g* for 11 min, at 4ºC. Finally, synaptosomal pellets were resuspended in RIPA buffer supplemented with CLAP, 0.1 mM DTT and 0.1 mM PMSF, and stored, at -20ºC until further use for protein quantification.

3.2.5.2 P2 crude synaptosomal fraction preparation

Human fresh brain tissue, from a particular brain region, was homogenized in a sucrose solution (0.32 M sucrose; 1 mM EDTA; 10 mM HEPES, pH 7.4 at 4ºC) at 800 r.p.m. for 10-15 strokes.

The homogenates were centrifuged at 5000 *g* for 5 min (eppendorf centrifuge). The supernatant was collected and centrifuged at 14000 *g* for 10 min (eppendorf centrifuge) to obtain the pellet that represents P2 crude synaptosomal fraction. This preparation was then used for [^{14}C]Glutamate release assay.

3.2.6 Subsynaptic fractionation

A fractionation method, previously described (Phillips *et al.,* 2001) and used by our group (Rebola *et al.,* 2005), was performed to allow the separation of extrasynaptic, presynaptic active zone and postsynaptic active zone fractions from synaptosomes. With this technique, the accessibility of antibodies to epitopes located at synapses is enhanced by the solubilization of different subsynaptic components, which disrupt the dense protein matrix (Phillips *et al.,* 2001; Rebola *et al.,* 2005).

The efficiency of separation is demonstrated by the capability to restore the immunoreactivity for the different markers in the several fractions: SNAP-25 in the presynaptic active zone, PSD-95 in the postsynaptic density and synaptophysin (vesicle protein not tightly linked to synaptic scaffold) outside the active zone (extrasynaptic fraction). The initial synaptosomal fraction (synap), from where fractionation began, was used as an internal control (Rebola *et al.,* 2005).

Brain samples, from the different regions, were homogenized in Isolation Buffer (IB, containing: 0.32 M Sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, CLAP and 0.1 mM PMSF). The homogenate was re-suspended in 2 M sucrose and 0.1 mM $CaCl_2$ and the obtained mixture (1.25 M sucrose) gently agitated. This suspension was separated into Ultraclear^{TM} tubes and 1 M sucrose solution, containing 0.1 mM $CaCl_2$, was carefully added in order to allow the formation of a gradient. The tubes were equilibrated with IB solution and centrifuged at 100000 *g* at 4º C, for 3 h (Beckman Coulter – Optima CL – 100XP DU ultracentrifuge, rotor SW41Ti).

The IB solution and the myelin layer, present at the interface IB/1 M sucrose, were removed and the synaptosomes were collected at the interface the 1 M and 1.25 M sucrose.

Synaptosomes fraction was diluted in 10 times its volume with IB and centrifuged at 15000 *g* for 30 min (Avanti J-26 XPI centrifuge, rotor JA-25-50).

The pellet was re-suspended with IB solution, and part of the obtained supernatant, correspondent to the total synaptosomal fraction (initial synaptosomal fraction from where the fractionation began) was centrifuged at maximum speed for 5 min (eppendorf centrifuge) and the obtained pellet re-suspended with 5% SDS and stored at -20ºC, to be used as an internal control of the fractionation.

The remaining suspension was diluted 10 times with pre-cooled 0.1 mM $CaCl_2$. An equal volume of solubilization buffer 2x (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension.

The mixture was incubated for 30 min on ice with mild agitation and then centrifuged at 40000 *g* for 30 min, at 4ºC (Avanti J-26 XPI centrifuge, rotor JA-25-50).

The pellet represents the synaptic junctions and the supernatant the extrasynaptic fraction. The extrasynaptic fraction was then decanted and proteins precipitated with six volumes acetone at -20º C, overnight.

The synaptic junctions pellet was washed with solubilization buffer 1x (20 mM Tris, 1% Triton X-100, pH 6.0) and re-suspended in 10 volumes of solubilization buffer 1x (20 mM Tris, 1% Triton X-100, pH 8.0).

This mixture was incubated for 30 min on ice, with mild agitation, and then centrifuged at 40000 *g* for 30 min at 4º C (Avanti J-26 XPI centrifuge, rotor JA-25-50).

The obtained supernatant corresponds to the presynaptic active zone and was processed as described for the extrasynaptic fraction. The insoluble pellet represents the postsynaptic density, which was resuspended in a minimal volume of 5% SDS with 0.1 mM PMSF and stored overnight at -20ºC.

The extra and presynaptic fractions were pelleted by centrifugation at 18000 *g* for 30 min at -15º C (Sorvall RC6, rotor SS34), solubilized in 5% SDS with 0.1 mM PMSF and then stored at -20º C.

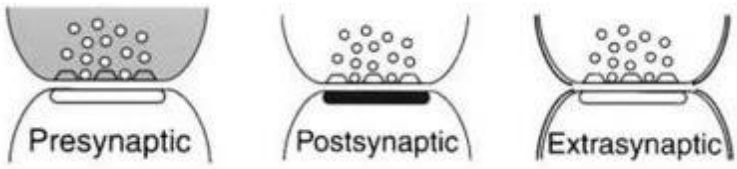

Figure 4. Schematic representation of the subsynaptic components that are expected to be enriched in each of the fractions isolated (adapted from Phillips *et al.,* 2001).

3.2.7 Protein quantification by the BCA method

Protein concentration determination was performed using the BCA protein assay kit (Pierce® BCA Protein Assay, Thermo Scientific) using bovine serum albumin (BSA) as a protein standard.

The absorbance was read at 570 nm in a spectrophotometer.

The standard curve was then used to calculate the protein concentration in µg/µL.

3.2.8 Western blot

After determination of protein concentration, samples protein content was denatured in 6x sample buffer (500 mM Tris/SDS buffer pH 6.8, 600 mM DTT, 10.3% SDS, 30% glycerol and 0.012% bromophenol blue) for 20 min at 70°C, and stored at −20°C until use.

Gel electrophoresis was carried out in a 10% polyacrylamide resolving gel with 4% polyacrylamide stacking gel and proteins were then transferred to previously activated polyvinylidene difluoride (PVDF) membranes for 2 h, at 4º C. The membranes were then blocked for 1 h, by immersing them in 5% milk in TBS-T (TBS 1x+ 0.1% Tween 20), on a shaker at room temperature, and then incubated in the diluted primary antibody, in 1% milk in TBS-T, at 4°C overnight.

After washing with TBS-T, the membranes were then incubated with the corresponding alkaline phosphatase-linked anti-species secondary antibody, diluted in 1% milk in TBS-T, for 2 h at room temperature. To perform immunodetection, membranes were washed in TBS-T, and revealed by an enhanced chemifluorescence (ECF) substrate (Amersham^{TM} ECF substrate for Western Blotting, GE Healthcare) and visualized in a VersaDoc system (Bio Rad).

Table 7. Polyacrylamide gel formulation.

Gel Formulation (1 Gel: 1.5 mm)	**10%**	**4%**
Tris-buffer, 1.5 M, pH 8.8 (Resolving gel)	2.5 ml	-
Tris-buffer, 0.5 M, pH 6.8 (Stacking gel)	-	2.5ml
Acrylamide 30 % (Bio-Rad)	3.3 ml	1.3 ml
Water	4.1 ml	6.1 ml
SDS 10% (Sigma-Aldrich)	100 µl	100 µl
TEMED (Sigma-Aldrich)	5 µl	10 µl
APS 20 % (freshly prepared, diluted in water)	100 µl	100 µl

3.2.9 Stripping and reprobing of membranes

To remove the ECF substrate, membranes were submerged in 40% methanol and were incubated at room temperature (RT) for 30 min with agitation. Afterwards, the membranes were rinsed twice in TBS-T for 10 min.

For antibodies removal, the membranes were submerged twice in stripping solution (15 g/L glycine, 10 mL/L Tween 20, 1 g/L SDS, pH 2.2) for 30 min, and washed twice in TBS-T for 10 min.

The membranes were then blocked by immersing them in 5% milk in TBS-T for 1 h at RT with agitation, and incubated overnight with primary antibody and proceeded as in the Western blot protocol.

3.2.10 [^{14}C]Glutamate release from synaptosomes

P2 crude synaptosomal fraction were diluted with Krebs' solution (113 mM NaCl; 3mM KCl; 1.2 mM $MgSO_4$; 1.2 mM KH_2PO_4; 2.5 mM $CaCl_2$; 10 mM glucose; 15 mM HEPES, pH 7.4), containing 100 µM aminooxyacetic acid. In order to prevent [^{14}C]glutamate metabolism, aminooxyacetic acid, a general inhibitor of pyridoxal phosphate (PlP)-dependent enzymes, including glutamate decarboxylase, was included in all solutions.

Synaptosomes were then incubated with 20 µM [^{14}C]-U-glutamate for 10 min, at 37ºC. A 116-micro volume chamber perfusion setup was filled with the preloaded synaptosomes which were trapped by one layer of Whatman GF/C filters (GE Healthcare). The filters were then superfused continuously at a rate of 0.7 mL/min until the end of the experiment, at 37ºC.

After the 10 min washout, 2 min content were collected in scintillation vials containing 2.5 mL of scintillation liquid, and the filters with the synaptosomes were also harvested to obtain the total radioactivity content.

Synaptosomes were stimulated twice (S1, first stimulation period; S2, second stimulation period) with 30 mM KCl within a 10 min interval.

100 µM of Adenosine A_1 receptor agonist CPA or 30 µM of Adenosine A_{2A} receptor agonist CGS 21680 were bath applied in the Krebs' solution before the S2 and superfused until the end of the experiment. DMSO (0.1% v/v) was used as control solution.

Vials content was assayed for the [^{14}C] content by a label protocol using a Tricarb β-counter (PerkinElmer), and disintegration/minute (dpm) values were expressed as a fractional release (FR%), i.e. the percent of actual content in the effluent as a function of the total synaptosomal content. For instance, if 10 units of radioactivity leave the chamber in a 2-min period and the filter retained 90 units, this calculates as 10 units left 90+10 original units, i.e. as 10 FR%.

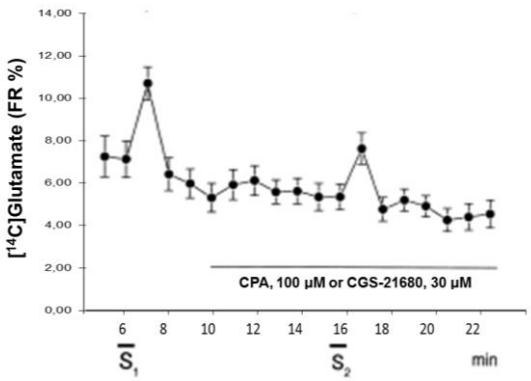

Figure 5. Representation of a release experiment.

3.3. Data presentation

Results are presented as mean ± SEM. Values of the number of experiments (n) is indicated in figure legends. Data is presented in percentage of total extracts or age--matched controls. Either a one sample t-test compared with the hypothetical value of 100 or a one-way analysis of variance (ANOVA) followed by post-hoc Newman-Keuls test, was used to define statistical differences between means, which were considered significant for a $p < 0.05$.

Chapter 4. Results

4.1 Brodmann area 25 (BA25)

4.1.1 Nerve terminals validation

To validate this nerve terminals (NT) preparation, the relative amount of synaptic and glial markers was compared in NT and in total extracts (TE) of the same sample, by Western blot analysis.

We observed an increase in the density of synaptosomal-associated protein 25 (SNAP-25) (134.0% ± 40.0%, n=5), synaptophysin (152.2% ± 41.1%, n=6) and syntaxin (87.2% ± 20.5%, n=6) in NT preparation compared to TE (Figure 6).

We also observed a decrease in the density of fibrillary acidic protein (GFAP) in NT (61.4% ± 3.7%, n=10) than in TE (Figure 6).

These results indicate that the synaptic markers SNAP-25, synaptophysin and syntaxin are enriched in BA25 nerve terminals as compared with total extracts, and as expected there is an enrichment of GFAP density in total extracts when compared with the nerve terminals preparation. These results validate human nerve terminals isolation in BA25.

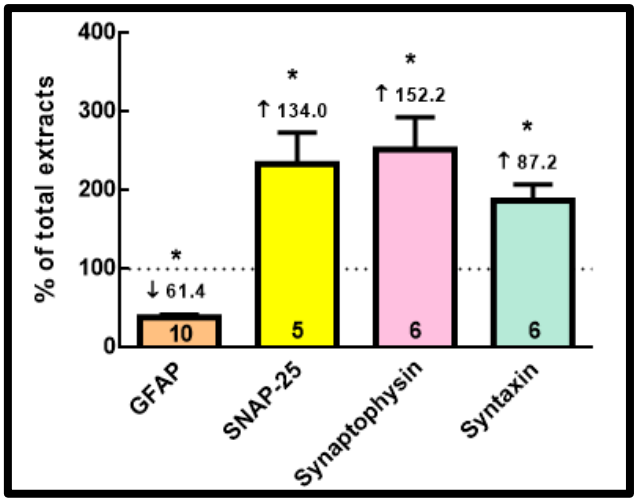

Figure 6. Validation of nerve terminals preparation from BA25 - using several synaptic markers (SNAP-25, synaptophysin and syntaxin) and a glial marker (GFAP). The data is presented in percentage of total extracts. Data are mean±SEM of n=5-10. * $p < 0.05$, one sample t-test compared with the hypothetical value of 100.

4.1.2 Adenosine receptors localization

To comprehend the normal localization of adenosine A_1 receptors (A_1R) and adenosine A_{2A} receptors ($A_{2A}R$) we used Western blot analysis, comparing the relative abundance of A_1R and $A_{2A}R$ in TE and in NT of BA25 of the same sample.

We observed an increase in the imumunoreactivity of both A_1R (42.8 % ± 9.5%, n=15) and $A_{2A}R$ (42.7 % ± 15.8%, n=6) in NT preparation compared to TE (Figure 7).

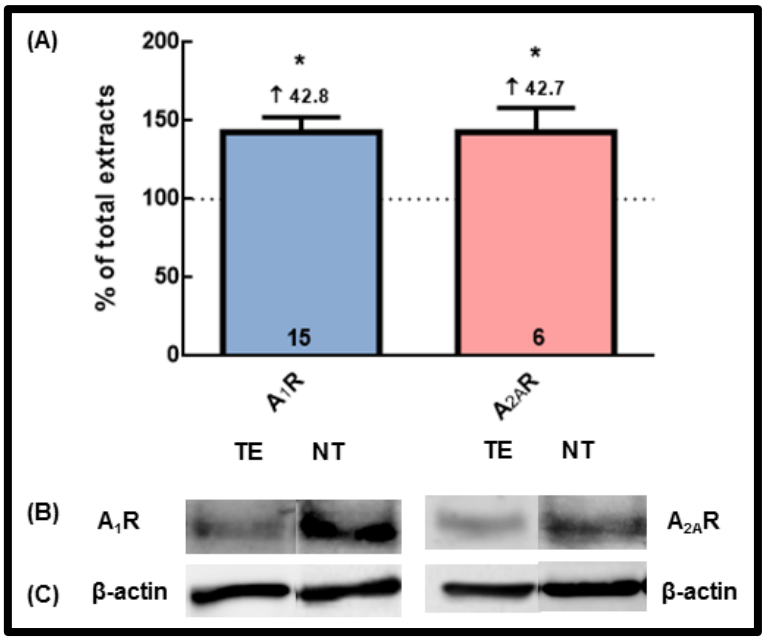

Figure 7. A_1R and $A_{2A}R$ enrichment in BA25 nerve terminals. (A) A_1R and $A_{2A}R$ are enriched in nerve terminals. (B) Representative Western blot of the A_1R and $A_{2A}R$ levels in both preparations. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of total extracts. Data are mean±SEM of n=6-15. * $p < 0.05$, one sample t-test compared with the hypothetical value of 100.

4.1.3 Adenosine receptors subsynaptic localization

After testing if there was an enrichment of A_1R and $A_{2A}R$ in NT, we went to refine the information about their subsynaptic localization. It was analyzed if A_1R and $A_{2A}R$ are differently distributed in the different subsynaptic fractions. Therefore, we used a fractionation procedure, previously validated by our group (Rebola *et al.,* 2005), which allows an effective separation of the non-active zone, presynaptic active zone and post-synaptic fractions (Rebola *et al.,* 2005; Phillips *et al.,* 2001).

4.1.3.1 A_1 receptor

The data presented in Figure 8 shows that A_1R is located in all fractions of synaptosomes.

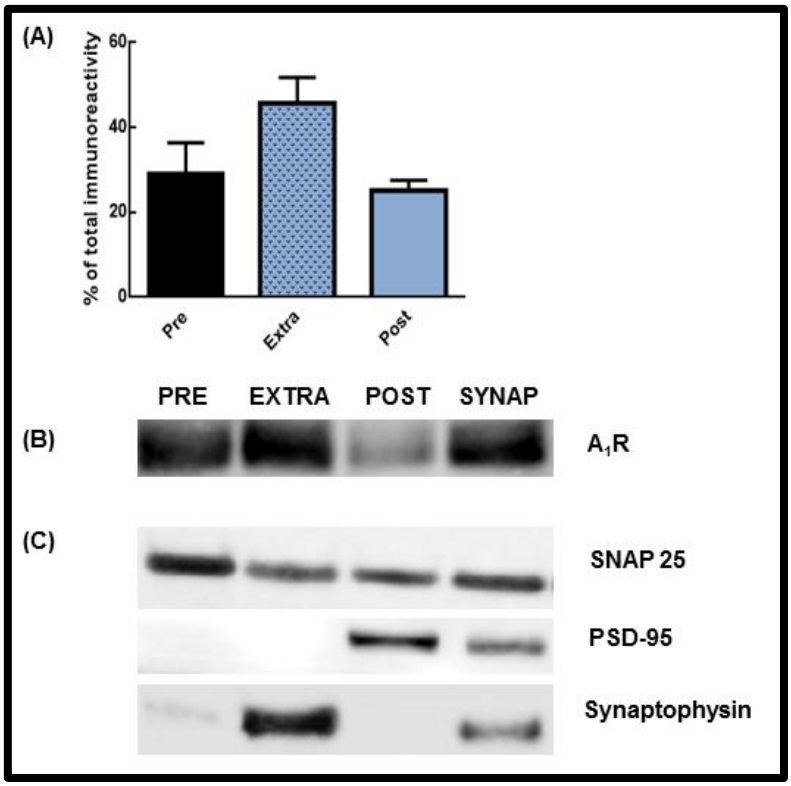

Figure 8. A_1R is present in all synaptic fractions of BA25. (A) BA25 subsynaptic levels of A_1R. (B) Representative Western blot of the subsynaptic distribution of A_1R. (C) The efficiency of the fractionation is illustrated by the ability to recover the immunoreactivity for SNAP-25 in the presynaptic active zone (PRE), PSD-95 in the postsynaptic density (POST) and synaptophysin in the extrasynaptic fraction (EXTRA). The data are present as mean±SEM of n=3, one-way ANOVA.

4.1.3.1 A_{2A} receptor

The data presented in Figure 9 shows that $A_{2A}R$ is mainly located in the extrasynaptic fraction of nerve terminals, although it is also present in the presynaptic density at a considerably lower level.

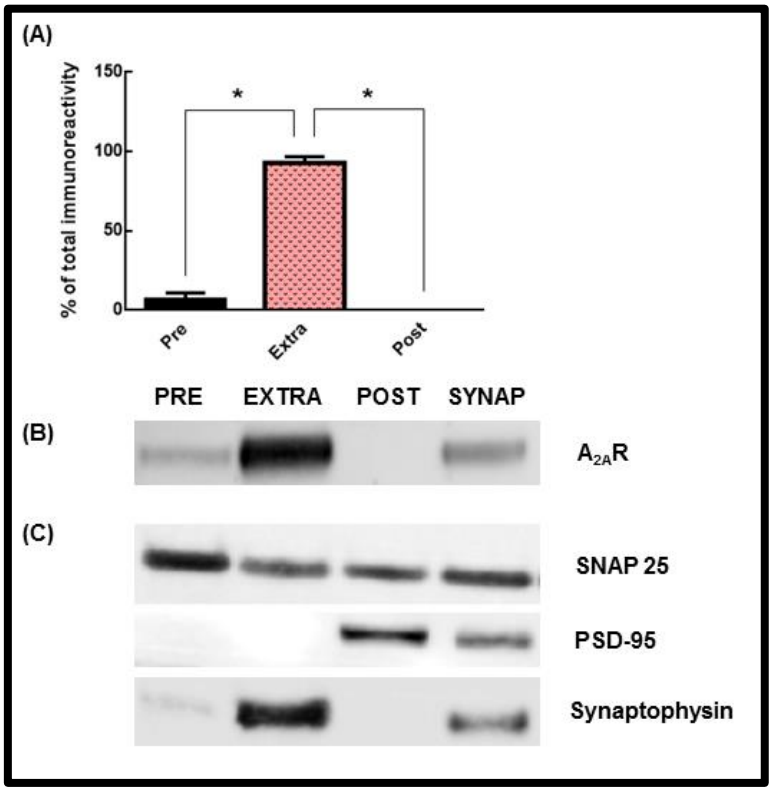

Figure 9. $A_{2A}R$ is mostly present in BA25 extrasynaptic fraction. (A) BA25 subsynaptic levels of $A_{2A}R$. (B) Representative Western blot of the subsynaptic distribution of $A_{2A}R$ (C) The efficiency of the fractionation is illustrated by the ability to recover the immunoreactivity for SNAP-25 in the presynaptic active zone (PRE), PSD-95 in the postsynaptic density (POST) and synaptophysin in the extrasynaptic fraction (EXTRA). Data are mean±SEM of n=3. * $p < 0.05$, one-way ANOVA followed by post-hoc Newman-Keuls test.

4.1.4 Synaptic markers in suicide

In this part of the study, we mainly focus on determining the changes of different synaptic markers in the BA25, namely SNAP-25, synaptophysin and syntaxin in suicide completers in comparison with age-matched controls, in NT. This was assessed by Western blot analysis, and the immunoreactivity of each band was normalized with β-actin.

In BA25 nerve terminals, there was a decrease in SNAP-25 density (43.9% ± 10.9%, n=10) with suicide (Figure 10).

No significant differences were found, in the immunoreactivity of synaptophysin (96.9% ± 11.6%, n=9) and syntaxin (93.4% ± 9.0%, n=9), in the suicide group compared with age-matched controls (Figure 10).

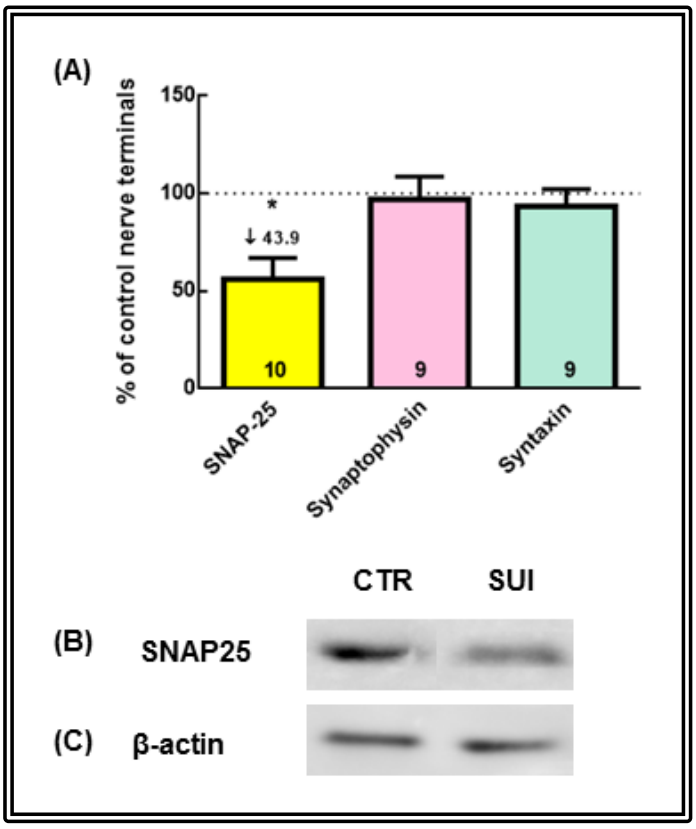

Figure 10. Down-regulation of SNAP-25 density with suicide in nerve terminals from BA25. (A) SNAP-25 density decreases with suicide in nerve terminals. (B) Representative Western blot of the SNAP-25 levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of control nerve terminals. Data are mean±SEM of n=9-10, * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.1.5 Astrocytes in suicide

Additionally, we also tested for astrocytic marker to gauge for changes of astrocytes in suicide completers, in TE. This was assessed by Western blot analysis, and the immunoreactivity of each band was normalized with β-actin.

In BA25, there was a decrease of GFAP density (39.2% ± 13.7%, n=5) in suicide completers, when compared with age-matched controls (Figure 11).

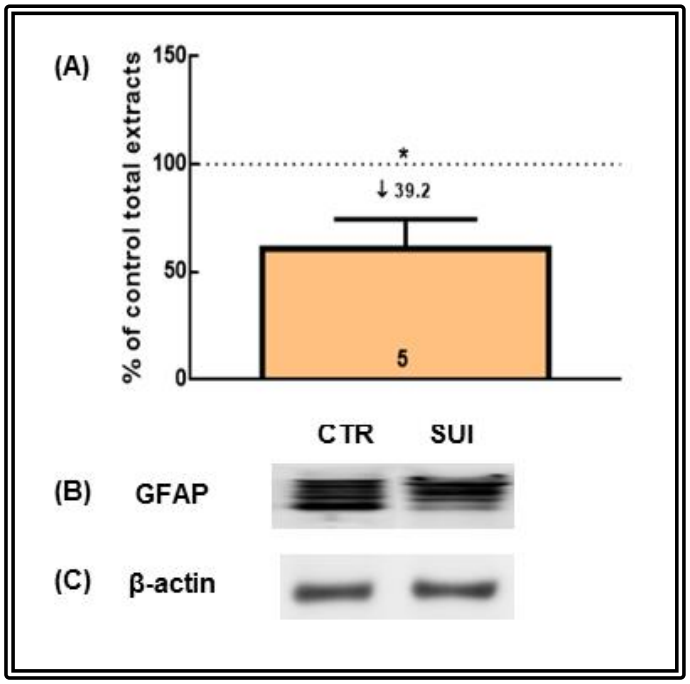

Figure 11. Down-regulation of GFAP density with suicide in TE from BA25. (A) GFAP density decreases with suicide in the total extracts from BA25. (B) Representative Western blot of the GFAP levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of control total extracts. Data are mean±SEM of n=5. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.1.6 Adenosine receptors in suicide

After mapping A_1R and $A_{2A}R$ localization and density in BA25, we went to try to understand if there were any changes of these receptors with suicide.

4.1.6.1 A_1 receptor

The relative abundance of A_1R in both TE and NT from suicide completers, in comparison to samples from age-matched controls, was assessed by Western blot analysis. The immunoreactivity of each band was normalized with β-actin.

No significant differences were found, in the BA25 A_1R immunoreactivity, in both TE (104.4% ± 6.5%, n=6) and NT (90.6% ± 17.6%, n=6) preparations, in the suicide group when compared with the control group (Figure 12).

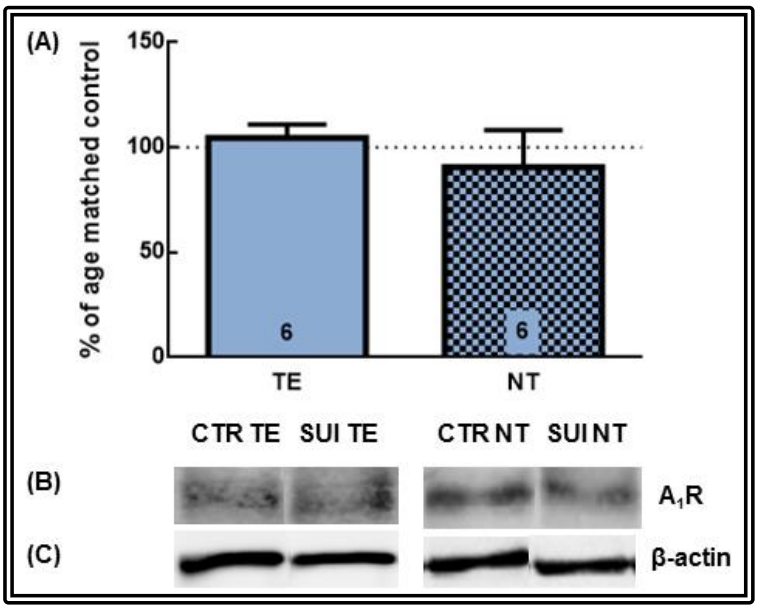

Figure 12. No changes of A_1R densities with suicide in BA25. (A) A_1R densities do not change with suicide in both preparations. (B) Representative Western blot of the A_1R levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of the respective age-matched control. Data are mean±SEM of n=6, one sample t-test compared with the hypothetical value of 100.

4.1.6.1 A_{2A} receptor

The relative abundance of $A_{2A}R$ in both TE and NT from suicide completers, in comparison to samples from age-matched controls, was assessed by Western blot analysis. The immunoreactivity of each band was normalized with β-actin.

In BA25, there was an up-regulation $A_{2A}R$ density in TE (48.4% ± 20.4%, n=9), with suicide (Figure 13).

No significant differences were found, in the BA25 $A_{2A}R$ density in NT (117.6% ± 13.8%, n=9) from suicide completers when compared with age-matched controls (Figure 13).

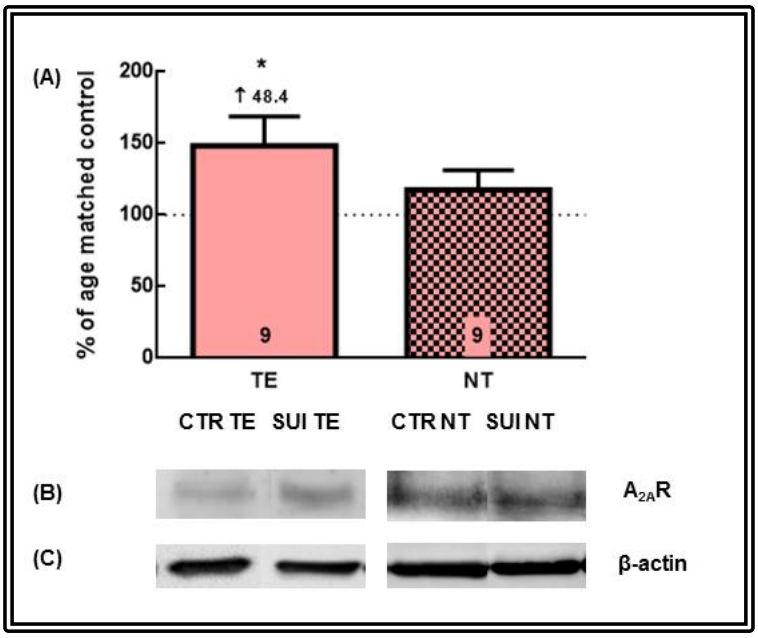

Figure 13. Up-regulation of $A_{2A}R$ density with suicide in total extracts from BA25. (A) $A_{2A}R$ density increase with suicide in the total extracts. $A_{2A}R$ density does not change with suicide in nerve terminals. (B) Representative Western blot of the $A_{2A}R$ levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of respective age-matched control. Data are mean±SEM of n=9. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.1.7 Conclusion

In conclusion, by comparing the levels of A_1R and $A_{2A}R$ in BA25 nerve terminals and total extracts, it was found that both receptors are enriched in BA25 nerve terminals. We then refined the information on ARs subsynaptic localization, using a fractionation method, and it was observed that $A_{2A}R$ are mainly located outside the active zone and A_1R are present in all synaptic fractions.

Synaptic and astrocytic changes present in BA25 of suicide completers were studied and was observed a down-regulation of both SNAP-25 and GFAP.

An up-regulation of $A_{2A}R$ density in total extracts was observed in suicide completers when compared with age-matched controls.

4.2 Medial caudate nucleus (MC)

4.2.1 Nerve terminals validation

As for the previously studied area, the relative amount of synaptic and glial markers was compared in TE and in NT of the same sample, by Western blot analysis, in order to validate NT preparation in medial caudate nucleus (MC).

We observed an increase in the density of SNAP-25 (39.0% ± 15.4%, n=8), synaptophysin (29.1% ± 5.8%, n=8) and postsynaptic density protein 95 (PSD-95) (21.8% ± 7.2%, n=10) in NT preparation compared to TE (Figure 14).

We also observed a decrease in the density of GFAP in NT (38.6% ± 11.6%, n=5) than in TE (Figure 14).

These results indicate that the synaptic markers SNAP-25, synaptophysin and PSD-95 are enriched in MC nerve terminals as compared with total extracts and, as expected, there is an enrichment of GFAP density in total extracts when compared with the nerve terminals preparation. These results then validate human nerve terminals isolation in MC.

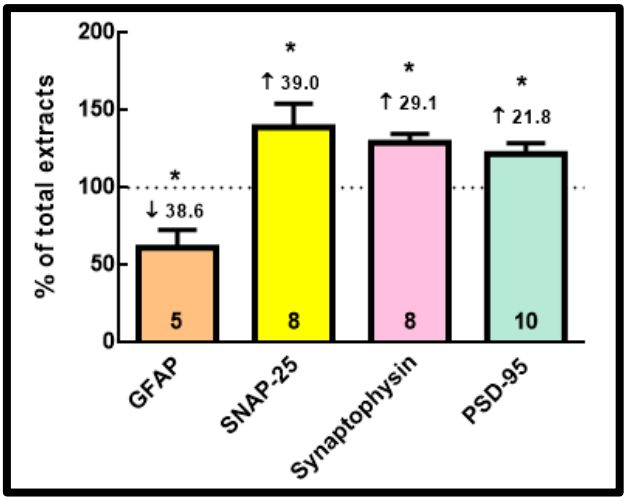

Figure 14. Validation of nerve terminals preparation from MC – using several synaptic markers (SNAP-25, synaptophysin and PSD-95) and a glial marker (GFAP). The data is presented in percentage of total extracts. Data are mean±SEM of n=5-10. * $p<0.05$ one sample t-test compared with the hypothetical value of 100.

4.2.2 Adenosine receptors localization

As in BA25, we first went to comprehend the normal localization of A_1R and $A_{2A}R$ receptors. Using Western blot analysis, the relative abundance of A_1R and $A_{2A}R$ was compared in TE and in NT of MC of the same sample.

We observed an increase in the immunoreactivity of both A_1R (27.1 % ± 10.5%, n=11) and $A_{2A}R$ (23.7% ± 7.1%, n=11) in NT preparation compared to TE (Figure 15).

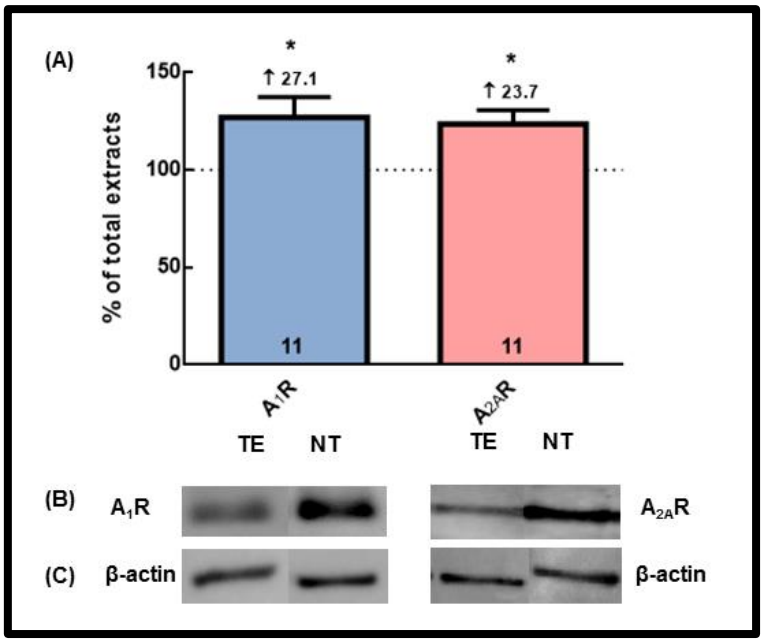

Figure 15. A_1R and $A_{2A}R$ enrichment in nerve terminals from MC. (A) A_1R and $A_{2A}R$ are enriched in nerve terminals. (B) Representative Western blot of the A_1R and $A_{2A}R$ levels in both preparations. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of total extracts. Data are mean±SEM of n=11. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.2.3 Adenosine receptors subsynaptic localization

After testing if there is a enrichment of A_1R and $A_{2A}R$ in NT, we went to refine the information on their subsynaptic localization. It was analyzed how A_1R and $A_{2A}R$ are distributed in the different subsynaptic fractions. Therefore, we used a fractionation procedure (Phillips *et al.*, 2001), previously validated by our group (Rebola *et al.,* 2005).

4.2.3.1 A_1 receptor

The results presented in Figure 16 are preliminary results (n=2), showing that A_1R is located in all fractions of synaptosomes.

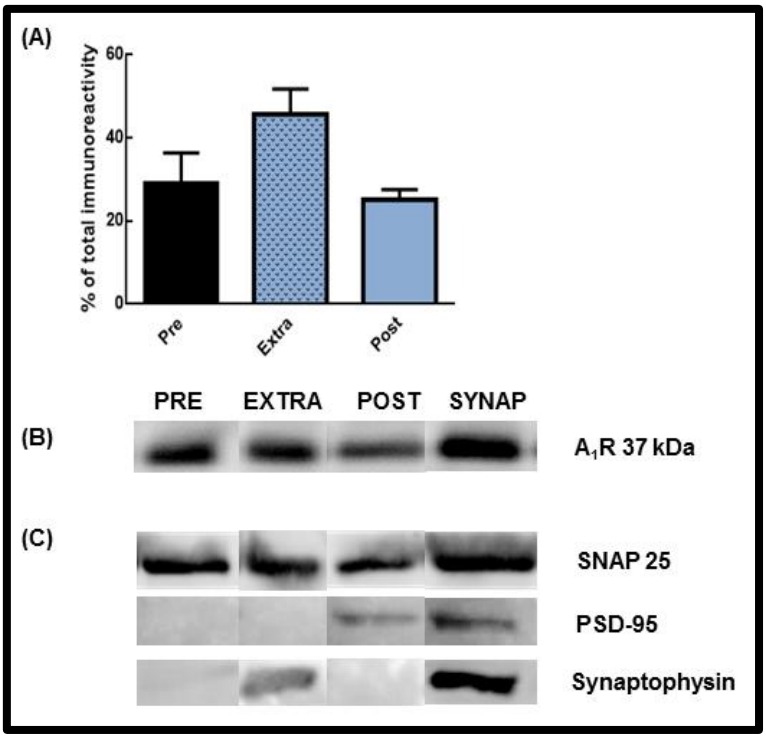

Figure 16. A_1R is present is in all synaptic fractions of MC. (A) MC subsynaptic levels of A1R. (B) Representative Western blot of the subsynaptic distribution of A1R. (C) The efficiency of the fractionation is illustrated by the ability to recover the immunoreactivity for SNAP-25 in the presynaptic active zone (PRE), PSD-95 in the postsynaptic density (POST) and synaptophysin in the extrasynaptic fraction (EXTRA). Data are preliminary results (n=2).

4.2.3.2 A_{2A} receptor

The data presented in Figure 17 shows that $A_{2A}R$ is mainly located in the extrasynaptic fraction of synaptosomes, although it is also present in the pre- and postsynaptic densities at a lower level.

The results presented are preliminary results (n=2).

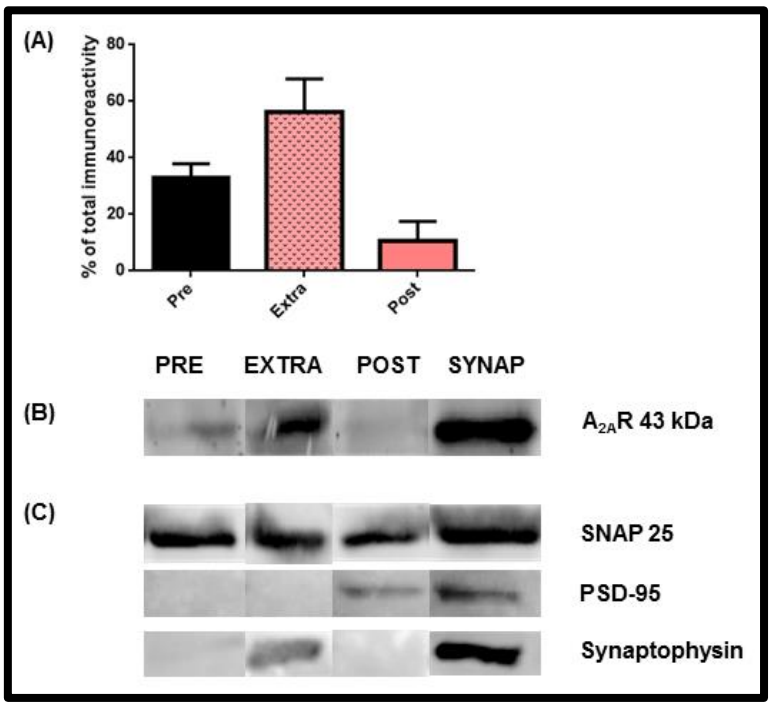

Figure 17. $A_{2A}R$ are mainly located outside the active zone in MC. (A) MC subsynaptic levels of $A_{2A}R$. (B) Representative Western blot of the subsynaptic distribution of $A_{2A}R$ (C) The efficiency of the fractionation is illustrated by the ability to recover the immunoreactivity for SNAP-25 in the presynaptic active zone (PRE), PSD-95 in the postsynaptic density (POST) and synaptophysin in the extrasynaptic fraction (EXTRA). Data are preliminary results (n=2).

4.2.4 Synaptic markers in suicide

We tried to determine the changes of different synaptic markers in the MC, namely SNAP-25, synaptophysin and a post-synaptic marker of glutamatergic synapses (PSD-95) in suicide completers in comparison with age-matched controls, in NT. This was assessed by Western blot analysis, and the immunoreactivity of each band was normalized with β-actin.

In MC nerve terminals, there was a decrease in SNAP-25 density (16.3% ± 6.3%, n=6) with suicide (Figure 18).

No significant differences were found, in the immunoreactivity of synaptophysin (99.9% ± 5.5%, n=5) and PSD-95 (99.5% ± 12.0%, n=6), in the suicide group when compared with non-suicide controls (Figure 18).

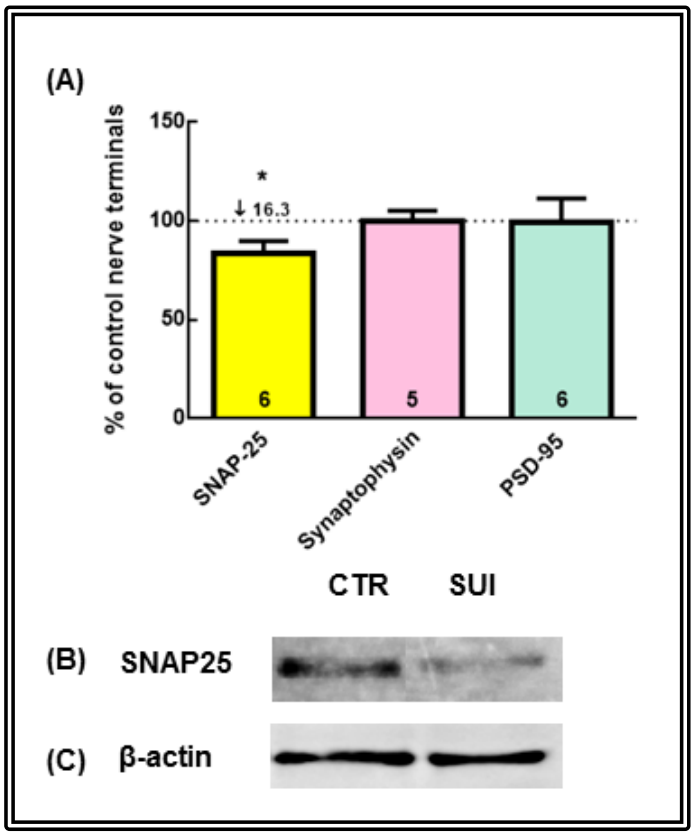

Figure 18. Down-regulation of SNAP-25 density with suicide in nerve terminals from MC. (A) SNAP-25 density decreases with suicide in nerve terminals. (B) Representative Western blot of the SNAP-25 levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of control nerve terminals. Data are mean±SEM of n=9-10. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.2.5 Astrocytes in suicide

We also tested for an astrocytic marker, in MC, to determine if there were any changes of astrocytes in suicide completers, in TE. This was assessed by Western blot analysis, and the immunoreactivity of each band was normalized with β-actin.

In MC, there were no significant differences in GFAP density (120.2% ± 17.0%, n=5) of suicide completers, when compared with age-matched controls (Figure 19).

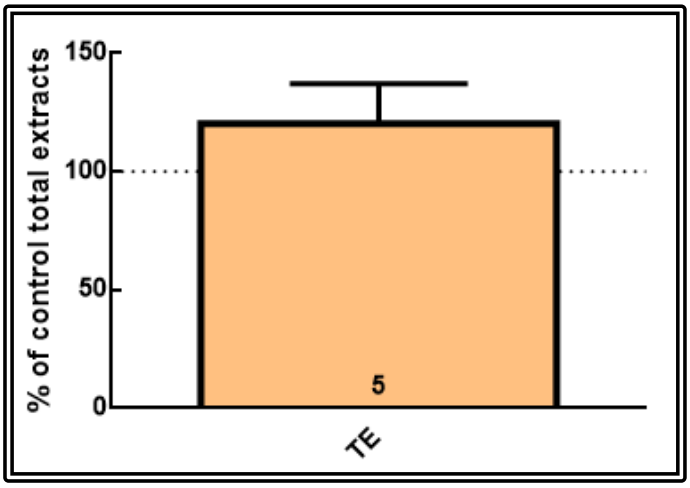

Figure 19. GFAP density levels do not change with suicide in MC total extracts. The data is presented in percentage of control total extracts. Data are mean±SEM of n=5, one sample t-test compared with the hypothetical value of 100.

4.2.6 Adenosine receptors in suicide

After mapping ARs in MC, we also went to try to understand if there are variations of both A_1R and $A_{2A}R$ with suicide.

4.2.6.1 A_1 receptor

The relative abundance of A_1R in both TE and NT from suicide completers, in comparison to samples from age-matched controls, was assessed by Western blot analysis. The immunoreactivity of each band was normalized with β-actin.

In MC, there was an up-regulation of A_1R density in TE (31.8% ± 12.0%, n=6), with suicide (Figure 20).

No significant differences were found, in the MC A_1R density in NT (108.1% ± 17.4%, n=6) from suicide completers when compared with age-matched controls (Figure 20).

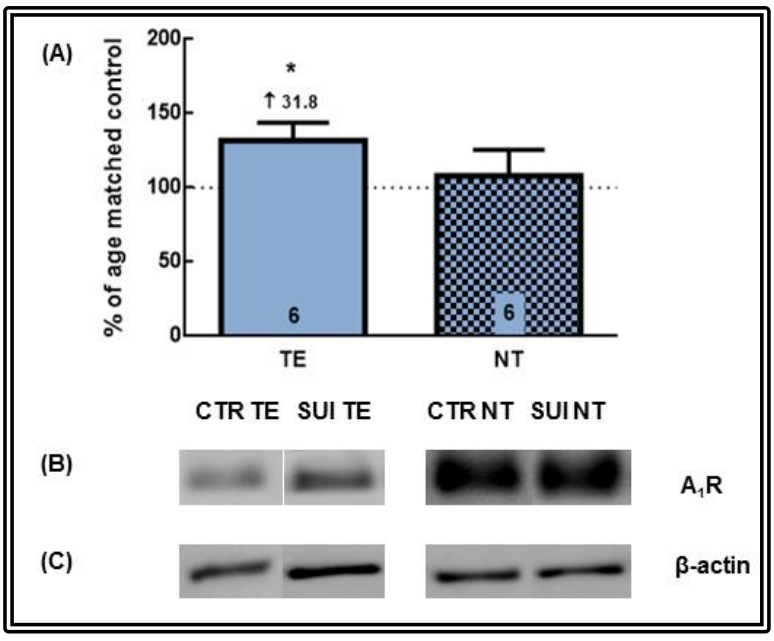

Figure 20. A_1R up-regulation with suicide in the TE from MC. (A) A_1R density increase with suicide in the TE from MC by Western blot. A_1R density does not change with suicide in the NT from MC. (B) Representative Western blot of the A_1R levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of the respective age-matched control. Data are mean±SEM of n=6. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.2.6.2 A_{2A} receptor

The relative abundance of $A_{2A}R$ in both TE and NT from suicide completers, in comparison to samples from age-matched controls, was assessed by Western blot analysis. The immunoreactivity of each band was normalized with β-actin.

No significant differences were found, in the MC $A_{2A}R$ immunoreactivity, in both TE (112.5% ± 8.2%, n=5) and NT (107.0% ± 5.2%, n=4) preparations, in the suicide group when compared with the control group (Figure 21).

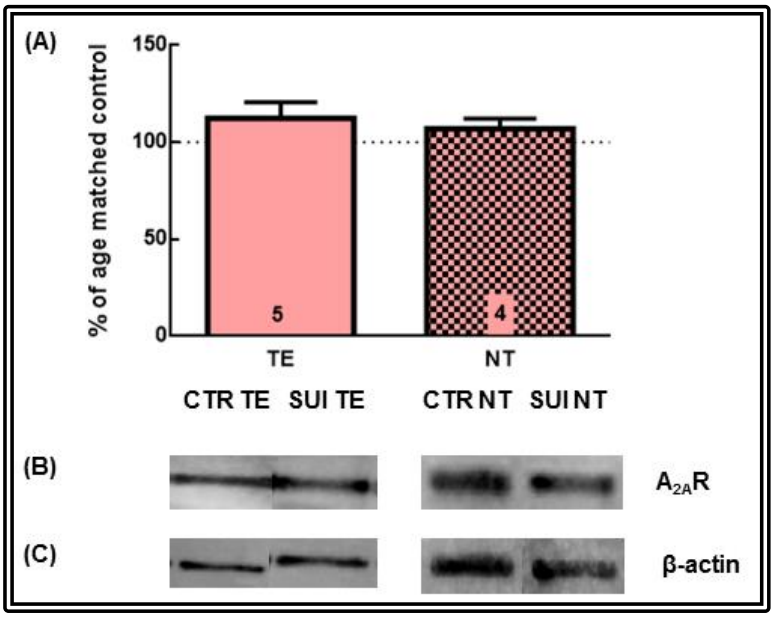

Figure 21. No changes in $A_{2A}R$ density with suicide in the nerve terminals and total extracts from MC. (A) $A_{2A}R$ densities do not change with suicide in both, nerve terminals and total extracts, preparations. (B) Representative Western blot of the $A_{2A}R$ levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of the respective age-matched controls. Data are mean±SEM of n=4-5, one sample t-test compared with the hypothetical value of 100.

4.2.7 Conclusion

So, we can conclude that, such as in BA25, both A_1R and $A_{2A}R$, in MC, are enriched in the nerve terminals. We refined the information on their subsynaptic localization, using a fractionation method, and it was observed that $A_{2A}R$ are mainly located outside the active zone and A_1R are present in all synaptic fractions.

We also provided the information about the synaptic changes present in MC of suicide completers, showing down-regulation of SNAP-25 density levels with suicide, similar to BA25.

An up-regulation of A_1R density in total extracts was observed in suicide completers when compared with age-matched controls.

4.3 Posterior caudate nucleus (PC)

4.3.1 Nerve terminals validation

To validate NT preparation from posterior caudate nucleus (PC), the relative amount of synaptic and glial markers was compared in TE and in NT of the same sample, by Western blot analysis.

We observed an increase in the density of SNAP-25 (35.2% ± 10.0%, n=10), synaptophysin (79.7% ± 25.9%, n=13) and PSD-95 (57.8% ± 20.4%, n=13) in NT preparation compared to TE (Figure 22).

We also observed a decrease in the density of GFAP in NT (41.0% ± 6.3%, n=10) compared with TE (Figure 22).

These results indicate that the synaptic markers SNAP-25, synaptophysin and PSD-95 are enriched in PC nerve terminals as compared with total extracts, and that the astrocytic marker GFAP is enriched in PC total extracts. These results validate human nerve terminals isolation in PC.

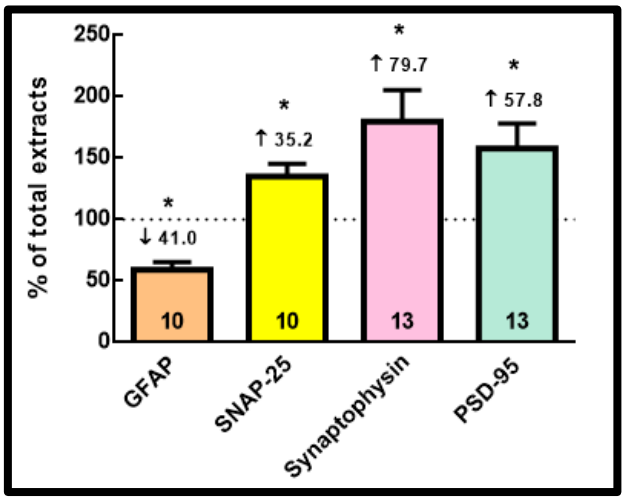

Figure 22. Validation of nerve terminals preparation from PC - using several synaptic markers (SNAP-25, synaptophysin and PSD-95) and a glial marker (GFAP). The data is presented in percentage of total extracts. Data are mean±SEM of n=10-13. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.3.2 Adenosine receptors localization

To comprehend the normal localization of A_1R and $A_{2A}R$ receptors we used Western blot analysis, comparing the relative abundance of A_1R and $A_{2A}R$ in TE and in NT of PC of the same sample.

We observed an increase in the imumunoreactivity of both A_1R (23.3% ± 8.9%, n=11) and $A_{2A}R$ (65.5% ± 18.9%, n=11) in NT preparation compared to TE (Figure 23).

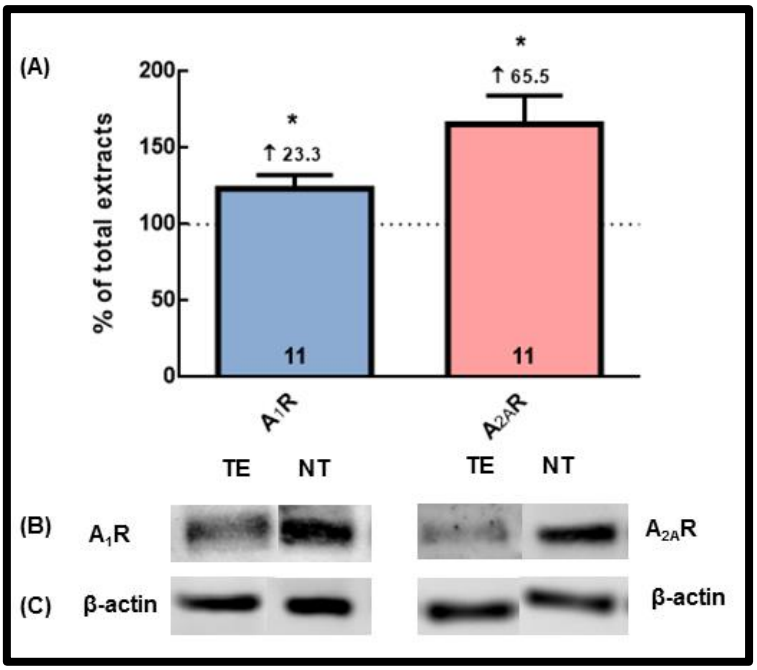

Figure 23. A_1R and $A_{2A}R$ enrichment in nerve terminals from PC. (A) A_1R and $A_{2A}R$ are enriched in nerve terminals. (B) Representative Western blot of the A_1R and $A_{2A}R$ levels in both preparations. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of total extracts. Data are mean±SEM of n=11. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.3.3 Synaptic markers in suicide

We then focused on determining the changes of different synaptic markers in the PC, namely SNAP-25, synaptophysin and PSD-95, in suicide completers in comparison with age-matched controls. This was assessed by Western blot analysis and the immunoreactivity of each band was normalized with β-actin.

No significant differences were found, in the immunoreactivity of SNAP-25 (79.4% ± 9.8%, n=5) and synaptophysin (99.7% ± 8.8%, n=5), in the suicide group when compared with age-matched controls (Figure 24).

In PC nerve terminals, there was an decrease in PSD-95 levels (35.6% ± 10.4%, n=6) in the suicide completers group when compared with age-matched controls (Figure 24).

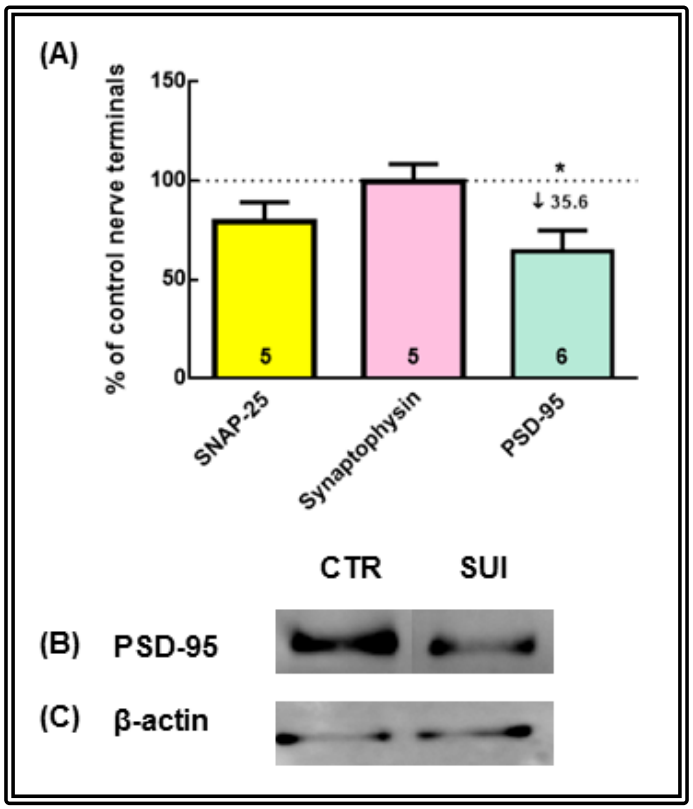

Figure 24. Down-regulation of PSD-95 density with suicide in nerve terminals from PC. (A) PSD-95 density decreases with suicide in nerve terminals. (B) Representative Western blot of the PSD-95 levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of control nerve terminals. Data are mean±SEM of n=5-6. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.3.4 Astrocytes in suicide

In this brain area, we also tested for astrocytic changes in suicide completers.

The relative abundance of GFAP in TE from suicide completers, in comparison to samples from age-matched controls was assessed by Western blot analysis. The immunoreactivity of each band was normalized with β-actin.

In PC, GFAP density do not change with suicide (73.7% ± 23.3%, n=6), inTE (Figure 25).

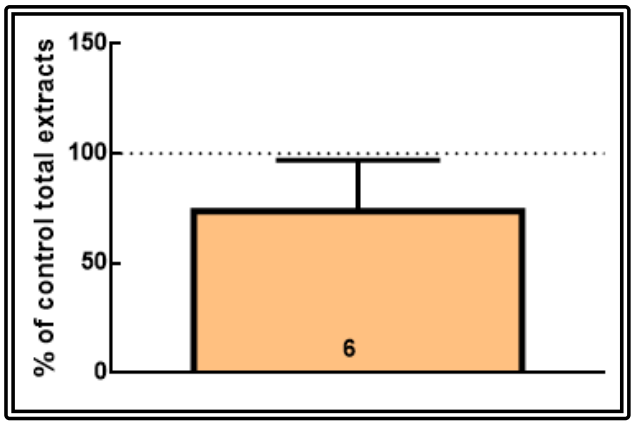

Figure 25. GFAP density levels do not change with suicide in total extracts of PC. The data is presented in percentage of control total extracts. Data are mean±SEM of n=5, one sample t-test compared with the hypothetical value of 100.

4.3.5 Adenosine receptors in suicide

After observing A_1R and $A_{2A}R$ localization in PC, we went to try to understand the differences between suicide completers and non-suicidal controls.

4.3.5.1 A_1 receptor

The relative abundance of A_1R in both nerve terminals and total extracts from suicide completers, in comparison to samples from age-matched controls was assessed by Western blot analysis. The immunoreactivity of each band was normalized with β-actin.

No significant differences were found, in the PC A_1R immunoreactivity in TE (96.7% ± 12.9%, n=6) preparations, in the suicide group compared with control group (Figure 26).

In PC, there was a down-regulation of A_1R in NT (35.0% ± 10.4%, n=5), with suicide (Figure 26).

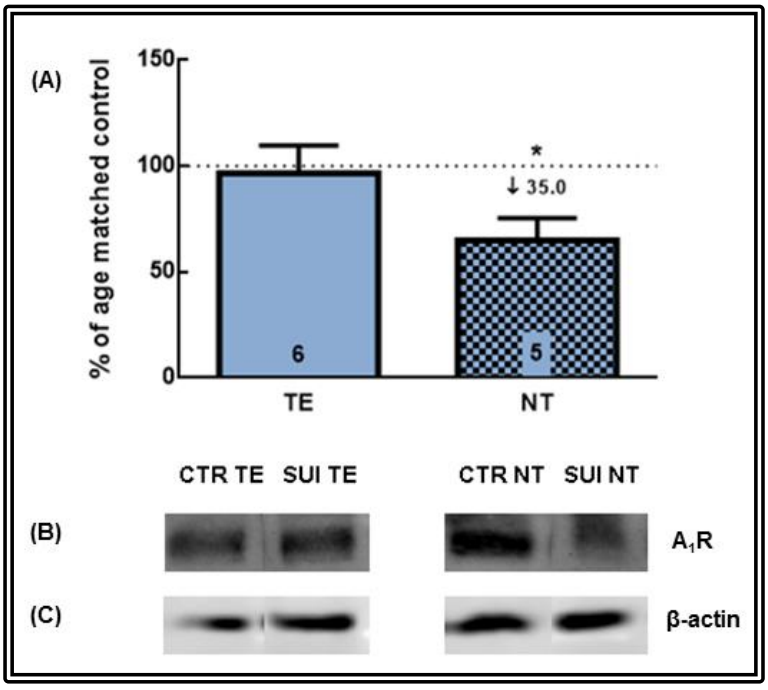

Figure 26. Down-regulation of A_1R density with suicide in nerve terminals from PC. (A) A_1R density do not change with suicide in total extracts and A_1R density decrease with suicide in nerve terminals. (B) Representative Western blot of the A_1R levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of the respective age-matched controls. Data are mean±SEM of n=5-6. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.3.5.2 A_{2A} receptor

The relative abundance of $A_{2A}R$ in both TE and NT from suicide completers, in comparison to samples from age-matched controls, was assessed by Western blot analysis. The immunoreactivity of each band was normalized with β-actin.

In PC, there was an up-regulation $A_{2A}R$ density in TE (29.9% ± 11.2%, n=6), with suicide (Figure 27).

No significant differences were found, in PC $A_{2A}R$ density in NT (92.1% ± 10.1%, n=5) from suicide completers when compared with age-matched controls (Figure 27).

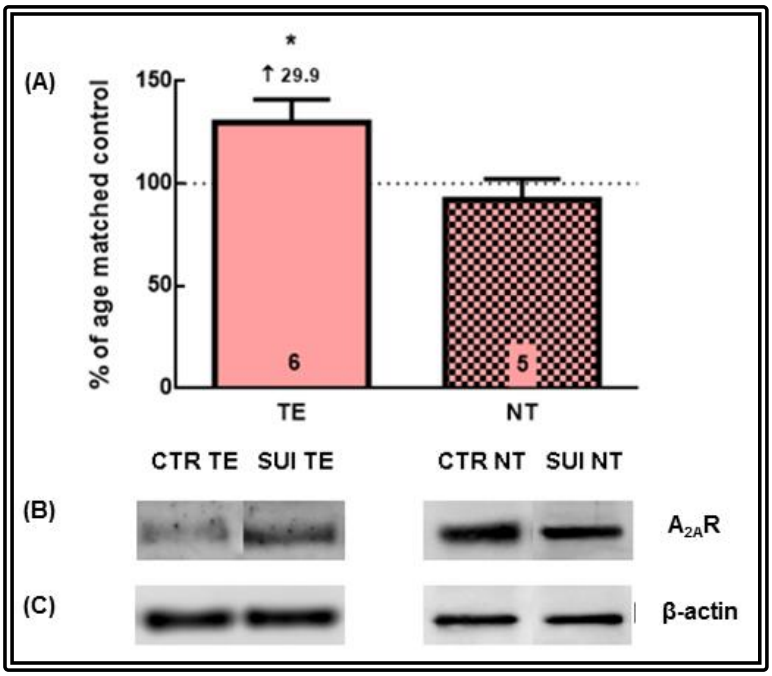

Figure 27. Up-regulation of $A_{2A}R$ with suicide in total extracts from PC. (A) $A_{2A}R$ density increases in total extracts and do not change in the nerve terminals with suicide. (B) Representative Western blot of the $A_{2A}R$ levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of the respective age-matched controls. Data are mean±SEM of n=5-6, * $p<0.05$ one sample t-test compared with the hypothetical value of 100.

4.3.6 Conclusion

Through comparison of the levels of A_1R and $A_{2A}R$ in PC TE and NT, it was found that both receptors are enriched in PC nerve terminals.

The synaptic changes present in PC of suicide completers were then studied, and it was observed a down-regulation of PSD-95 density levels with suicide.

A down-regulation of A_1R density in NT, as well as a increase in immunoreactivity levels of $A_{2A}R$ in TE, was observed in suicide completers when compared with age-matched controls.

4.4 Hippocampus

4.4.1 Nerve terminals validation

To validate hippocampus NT preparation, the relative amount of synaptic and glial markers was compared in TE and in NT of the same sample, by Western blot analysis, as for the other brain regions.

We observed an increase in the density of SNAP-25 (42.3% ± 7.3%, n=10), synaptophysin (63.5% ± 10.7%, n=9) and PSD-95 (94.5% ± 3.8%, n=6) in NT preparation compared to TE (Figure 28).

We also observed a decrease in the density of GFAP in NT (30.1% ± 6.5%, n=12) than in TE (Figure 28).

These results indicate that the synaptic markers SNAP-25, synaptophysin and PSD-95 are enriched in hippocampus nerve terminals as compared with total extracts, and as expected there is an enrichment of GFAP density in total extracts when compared with the nerve terminals preparation. Thus validating human nerve terminals isolation in hippocampus.

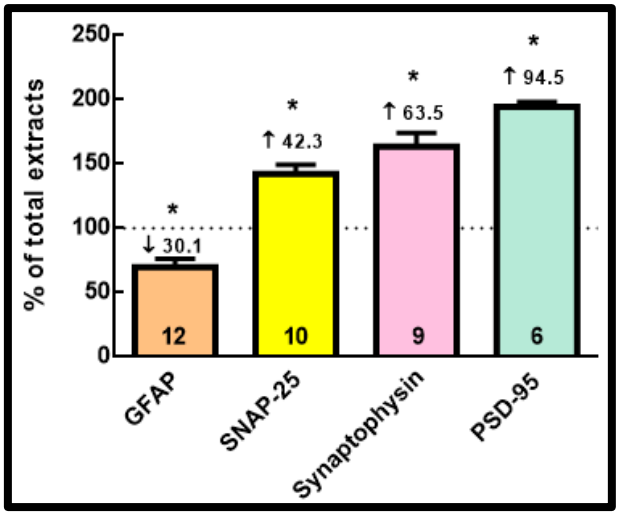

Figure 28. Validation of NT preparations from hippocampus - using several synaptic markers (SNAP-25, synaptophysin and PSD-95) and a glial marker (GFAP). The data is presented in percentage of total extracts. Data are mean±SEM of n=6-12, * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.4.2 Adenosine receptors localization

To comprehend the normal localization of A_1R, we used Western blot analysis, to compare the relative abundance of A_1R in TE and in NT of hippocampus of the same sample.

We observed an increase in the immunoreactivity of A_1R (62.2% ± 13.6%, n=6) in NT preparation compared to TE (Figure 29).

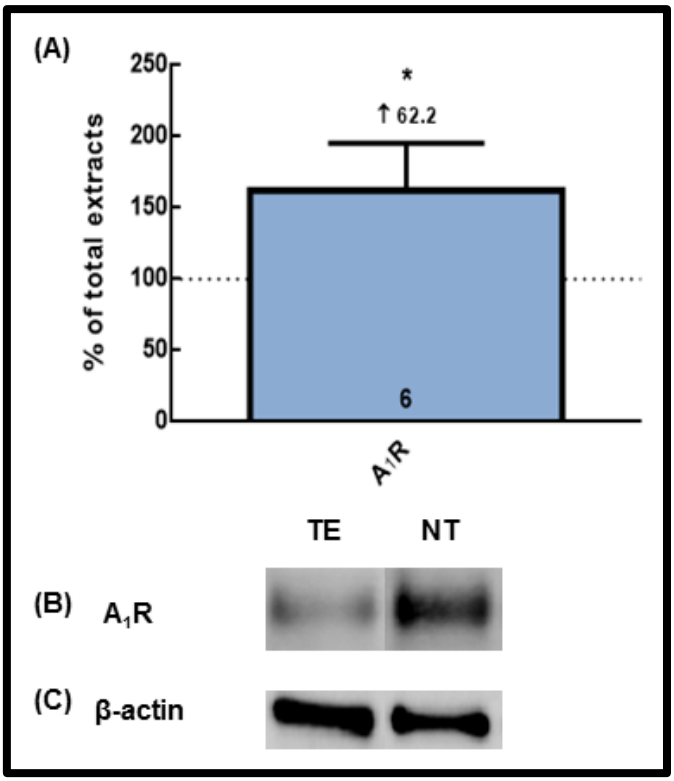

Figure 29. A_1R enrichment in nerve terminals from hippocampus. (A) A_1R are enriched in nerve terminals. (B) Representative Western blot of the A_1R levels in both preparations. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of total extracts. Data are mean±SEM of n=6. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.4.3 Adenosine receptors subsynaptic localization

After observing that there is an enrichment of A_1R in hippocampus NT, we went to refine the information on its subsynaptic localization. It was analyzed how A_1R is distributed in the different subsynaptic fractions, using a fractionation procedure (Phillips *et al.,* 2001), previously validated by our group (Rebola *et al.,* 2005).

4.4.3.1 A_1 receptor

The data presented in Figure 30 shows that A_1R is located in all fractions of synaptosomes.

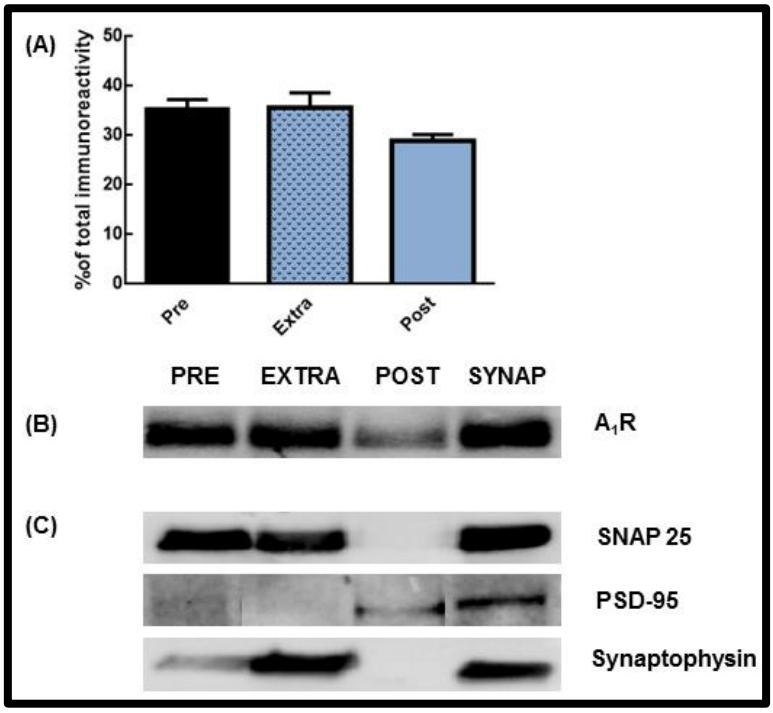

Figure 30. A_1R is present is in all synaptic fractions of hippocampus. (A) MC subsynaptic levels of A_1R. (B) Representative Western blot of the subsynaptic distribution of A_1R. (C) The efficiency of the fractionation is illustrated by the ability to recover the immunoreactivity for SNAP-25 in the presynaptic active zone (PRE), PSD-95 in the postsynaptic density (POST) and synaptophysin in the extrasynaptic fraction (EXTRA). The data are present as mean±SEM of n=4, one-way ANOVA.

4.4.4 Synaptic markers in suicide

Then we went to determine the changes of synaptic markers in hippocampus NT, namely SNAP-25, synaptophysin and PSD-95, with suicide. This was assessed by Western blot analysis, and the immunoreactivity of each band was normalized with β-actin.

In hippocampus NT, no significant differences were found, in the immunoreactivity of SNAP-25 (162.4% ± 41.3%, n=7) and synaptophysin (114.7% ± 14.5%, n=5), in the suicide group compared with non-suicide controls (Figure 31).

There was a decrease in PSD-95 density (29.3% ± 8.0%, n=4) with suicide (Figure 31).

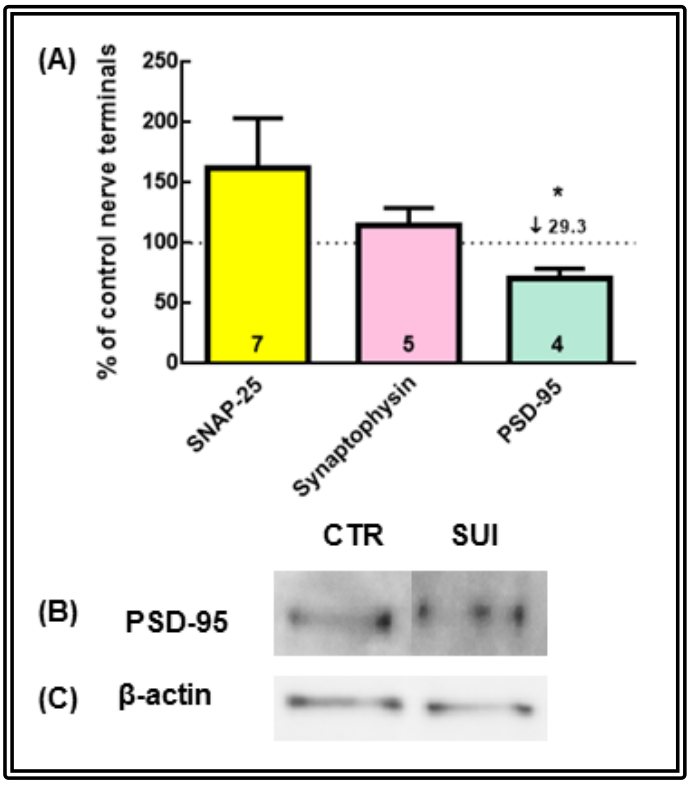

Figure 31. Down-regulation of PSD-95 density with suicide in nerve terminals from hippocampus. (A) PSD-95 density decreases with suicide in nerve terminals. (B) Representative Western blot of the PSD-95 levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of control nerve terminals. Data are mean±SEM of n=4-7. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.4.5 Astrocytes in suicide

Additionally, we also tested for an astrocytic marker to gauge for changes of astrocytes in the hippocampus of suicide completers. This was assessed by Western blot analysis, and the immunoreactivity of each band was normalized with β-actin.

In hippocampus, the GFAP density decreased (17.5% ± 4.1%, n=6), in TE with suicide (Figure 32).

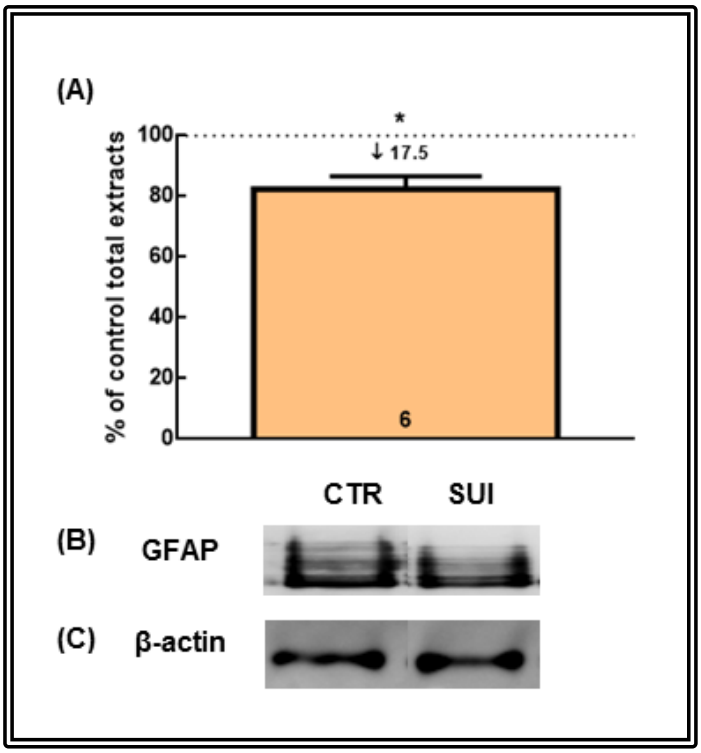

Figure 32. Down-regulation of GFAP density with suicide in total extracts from hippocampus. (A) GFAP density decreases with suicide in nerve terminals. (B) Representative Western blot of GFAP levels in the suicide and control samples. (C) Representative Western blot of the β-actin density (control for protein loading). The data is presented in percentage of control total extracts. Data are mean±SEM of n=6. * $p<0.05$, one sample t-test compared with the hypothetical value of 100.

4.4.6 Conclusion

In conclusion, by comparing the levels of A_1R in hippocampus TE and NT, it was found that, such as in all the other areas previously studied, A_1R are enriched in NT, and that they are present in all synaptic fractions.

We also showed a down-regulation of PSD-95 and GFAP density levels with suicide, providing information on synaptic and astrocytic changes present in hippocampus of suicide completers.

4.5 [^{14}C]Glutamate release from synaptosomes

We aimed to define if there is an deregulation of the function of synaptic ARs in individuals who committed suicide; for this purpose, we merged our expertise in following the chemically-evoked release of neurotransmitters such as glutamate from human NT (Katona *et al.,* 2000) with our pharmacological expertise to characterize $A_{2A}R$ controlling glutamate release from rodents (Lopes *et al.,* 2002) to compare the impact of A_1R and $A_{2A}R$ on the evoked glutamate release from human P2 crude synaptosomes from amygdala, BA25, MC, and hippocampus of controls, with a PMI of less than 24 h.

The evoked release of [^{14}C]glutamate from chemically-stimulated P2 crude synaptosomes, was carefully optimized in human tissue from the different brain areas.

These preliminary results confirmed that this protocol can be used for studying the neurotransmitters release from synaptosomes using human postmortem samples, which will allow further studies, to comprehend the changes in ARs modulatory function with suicide.

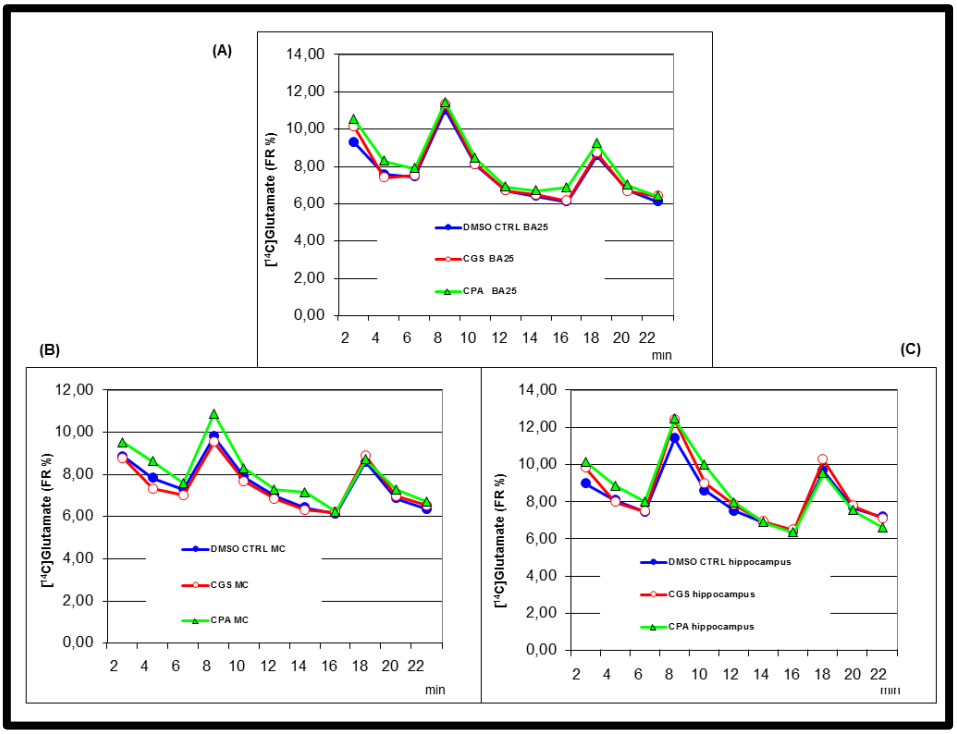

Figure 33. [^{14}C]Glutamate release from synaptosomes. (A) BA25. (B) MC. (C) Hippocampus.

Chapter 5. Discussion

5.1 Adenosine receptors localization

Purines and purine nucleotides are essential to all living cells (Arch & Newsholme, 1978; van den Berghe *et al.*, 1992), being released not only from neurons but also from other cells producing several effects on multiple organ systems by binding to purinergic receptors, widespread in the brain (Stone *et al.*, 2009). Adenosine is an important molecule in brain signalling, controling the flow of information so that synaptic transmission can occur in a harmonic way. Extracelular acummulation of adenosine is connected with neurotransmitters release and to neuronal firing frequency and intensity (Cunha, 2005; Dunwiddie & Haas *et al,.* 1985).

The knowledge about adenosine receptors (ARs) localization in the human brain, both celular and synaptic, provides an important insight on the adenosine modulatory role.

From our results, we can assume that both adenosine A_1 receptor (A_1R) and adenosine A_{2A} receptor ($A_{2A}R$) display a predominant synaptic localization, being also present, at substantially lower levels, in other neuronal locations and cell types.

Previous PET studies in human volunteers, and autoradiographic studies in postmortem human brain samples, have investigated the distribution of A_1R, using radioligands. A_1R wide distribution in the human brain was denoted and it was shown that they are not homogeneously located in all cortex regions, with strong labelling in pyramidal cells (Fukumitsu *et al.*, 2005; Schindler *et al.*, 2001).

Our results, on A_1R localization, are in agreement with previous studies, in which they were also found in all synaptic fractions (Fredholm *et al.*, 2005; Schubert *et al.,* 1994). So we can infer, from research done in rodents, an adenosine role in inhibiting neuronal excitability and synaptic transmission mediated through the activation of synaptic A_1R (Fredholm *et al.*, 2005; Greene & Haas, 1991; Phillis & Wu, 1981; Ribeiro *et al.,* 2002; Schubert *et al.,* 1994).

Presynaptic A_1R, in animal models, inhibit synaptic transmission, this may rely on the coupling of A_1R to the inhibition of N-type calcium channels (Lopes *et al.,* 2011; Wu & Saggau, 1994; Yawo & Chuhma, 1993) leading to a decreased stimulus-evoked release of glutamate (Proctor & Dunwiddie, 1987; Thompson *et al.,* 1992; Dunwiddie & Haas, 1985; Ambrósio *et al.*, 1997). They can also decrease miniature events in excitatory synapses, probably depending on presynaptic A_1R ability to down-regulate the sensitivity of the release apparatus (Rebola *et al*, 2003, 2005).

From studies performed in rodents, A_1R located in the postsynaptic density manage signal integration and can impact the responsiveness to excitatory stimuli controlling simultaneously N-type calcium channels and N-methyl-D-aspartate (NMDA) receptors, to survey neuronal firing burst, by leading to neuronal hyperpolarization (de Mendonça *et al.,* 1995; de Mendonça & Ribeiro, 1997; Klishin *et al.,* 1995).

Extrasynaptic A_1R may also be important to control potassium currents, inducing neuronal hyperpolarization, as it happened in animals (Greene & Haas, 1991).

As previously stated, although less abundant, A_1R also have non-neuronal localizations, which we know from rodents, it is important to modulate both astrocytic and microglia function in brain (van Calker & Biber, 2005).

$A_{2A}R$ display a widespread distribution in the brain (Fredholm *et al.,* 2005) more abundantly in several basal ganglia structures (Svenningsson *et al.,* 1997), where they control psychomotor behavior (Xu, 2005). Accordingly with our results, they are mostly located in synapses. $A_{2A}R$ play an essential role in synaptic plasticity control, rather than having an impact on the control of basal synaptic transmission (d'Alcantara *et al.,* 2001; Rebola *et al.,* 2008).

From studying their synaptic localization we observed that $A_{2A}R$ are mainly located outside the active zone, although they are also present in the presynaptic active zone and postsynaptic density, at a considerably lower level.

Their extrasynaptic location may have an important role to control extrasynaptic glutamate levels, extrapolating from studies in animals, $A_{2A}R$ might control glutamate excitotoxicity and the consequent trigger of neuronal dysfunction of brain circuits. In primates striatopallidal complex, metabotropic glutamate receptors (mGluR) showed a localization similar to that described for $A_{2A}R$ in rats (Hettinger *et al.*, 2001), being commonly found postsynaptically and perisynaptically to asymmetric synapses (Smith *et al.*, 2000). Previous studies have reported functional evidence for the possible existence of synergistic $A_{2A}R$/mGluR5 interactions modulating the function of the GABAergic striatopallidal neurons (Díaz-Cabiale *et al.,* 2002), in rat striatum and in membrane preparations from human embryonic kidney (HEK)-293 cells transiently cotransfected with both receptors. These results suggest that $A_{2A}R$ may have synergistic interactions implicated in neuronal function and dysfunction (Ferré *et al.,* 2002).

Accordingly to studies performed in rodents, in the pre-synaptic active zone, they can control the release of glutamate and the shutting down of the A_1R-mediated inhibition of synaptic transmission (Lopes *et al.*, 1999, Rebola *et al.*, 2005), which enhances synaptic efficiency upon increasing neuronal activity. $A_{2A}R$ appear to be

recruited upon high frequency stimulation triggering synaptic plasticity (Rebola *et al.*, 2008).

Postsynaptic $A_{2A}R$, can control NMDA receptors (Rebola *et al.,* 2008; Wirkner *et al.*, 2004), modulating AMPA mediated currents (Dias *et al.*, 2012), as seen in studies performed on rodents.

Although, their neuronal function, ARs are also located in other cell types in the brain, such as glial cells. So their activation should not be restricted to direct neuronal activity control, participating also in neuron-glia communication (Cunha, 2005; Fernandez *et al.*, 1996; Pinto-Duarte *et al.*, 2005). For instance, extrapolating from results in animals, astrocytic $A_{2A}R$ could control synaptic plasticity and neurodegeneration, by sensing synaptic transmission to adjust glutamate extracellular levels; through modulation of transporters activity (Matos *et al.,* 2012); or exocytotic glutamate release (Li *et al.,* 2001; Nishizaki *et al.,* 2002).

So, there are different populations of ARs located in different cellular and sub-cellular populations that may play different roles in the control of neuronal circuits function.

5.2 Alterations in suicide

Synaptic dysfunction is a key aspect of depression (Duman & Aghajanian, 2012). Furthermore ARs, as previously demonstrated in animal models (Rebola *et al.,* 2005), and now in human samples, are mainly synaptic receptors. Besides, we know that $A_{2A}R$ have an impact on cognitive dysfunction only in conditions involving synaptic deterioration, by way of controlling neurodegeneration (Cunha G *et al.,* 2008). So, it is possible that mood-related disorders trigger a synaptic dysfunction, accompanied by a deregulation of ARs. The performed evaluation of the loss of synaptic markers in suicide completers may then be extremely helpful in identifying the molecular mechanisms underlying the mood-associated impairments.

From our observations, we have denoted an altered density of synaptosomal-associated protein 25 (SNAP-25) with down-regulation in both, nerve terminals of cingulate cortex (BA25) and medial caudate nucleus (MC) of suicide completers. This may be in accordance with previous research, showing decreased volume of caudate nucleus and altered connectivity with cingulate cortex (Bluhm *et al.,* 2009; Pizzagalli *et al.,* 2009). In previous observations, in human frontal cortex, no changes, in SNAP-25 levels relative to controls, were observed in depressed suicide patients (Honer *et al.,* 2002).

Altered expression of SNARE proteins, particullarly SNAP-25, can contribute to impaired synaptic neurotransmission (Owe-Larsson *et al.,* 1999) and abnormal behavior, leading to suicidal behavior such as disinhibition, impulsivity and aggressiveness (Mann, 1998). These alterations may be involved in difficulty on initiating behavior and motor movements, as well as in learning, memory, pleasure, motivation and social behavior disability. So, impaired reward-processing due to, basal ganglia and cortical, dysfunctional cognitive and motor regulation, may also be responsible for the anhedonic symptoms of MDD (Bluhm *et al.,* 2009; Butters *et al.,* 2009; Parashos *et al.,* 1998). For instance, animals with reduction in SNAP-25 display behavioral and neurochemical abnormalities (Hess *et al.,* 1992, 1996; Heyser *et al.*, 1995), reduced glutamate content and impaired release mechanisms (Raber *et al.*, 1997).

At synaptic sites, postsynaptic density protein 95 (PSD-95) plays a key role in mediating trafficking, clustering, and downstream signaling events, following receptor activation (Feyissaa *et al.,* 2009). We have observed a loss of PSD-95 in the posterior caudate nucleus and hippocampus of suicide completers. This observation is in accordance to what was demonstrated in the literature, patients with depressive

disorders exhibit characteristics of synaptic dysfunction in hippocampus (Jorgensen & Riederer, 1985), and a study examining the expression of PSD-95 shows a decrease in protein expression levels in depressed subjects relative to controls, in prefrontal cortex (PFC) (Feyissaa *et al.,* 2009). That is in accordance with previous studies, performed in animal models, in which it was observed an impaired synaptic plasticity, a modified pattern of glutamate release and a loss of synaptic markers. This is consistent with the memory impairments associated with depression (Batalha *et al.,* 2013; Cunha *et al.,* 2006; Cunha & Agostinho, 2010), and may also be involved in anhedonia.

Several structural and cellular changes, including marked glial anomalies, have been observed in association with MDD (Sanacora & Banasr, 2013). Our results have shown a decreased level of the glial fibrillary acidic protein (GFAP) in both BA25 and hippocampus. This protein, although expressed by several cell types in the CNS, is considered an astrocytic marker (Sanacora & Banasr, 2013). Previous postmortem reports of mood disorder patients have shown evidences for glial cell pathology, in the PFC, ACC, amygdala, and hippocampus (Sanacora & Banasr, 2013). Notably, GFAP expression has been reported to decrease in frontal cortex of individuals with psychiatric conditions, such as schizophrenia, bipolar disorder and depression (Johnston-Wilson *et al.,* 2000).

Astrocytes communicate bidirectional with neurons at the synaptic level by responding to synaptic activity in order to modulate synaptic transmission (Haydon, 2001; Volterra & Meldolesi, 2005). Besides releasing glutamate, purines (ATP and adenosine), GABA, and D-serine (Halassa *et al.,* 2007; Haydon, 2001; Perea *et al.,* 2009) they can also influence synaptic function through the release of growth factors and related molecules (Barres, 2008).

As already explained, astrocytic pathology may impact brain function at the levels of synapses, circuits and behavior (Fiacco *et al.,* 2009), and glia, especially astrocytes, can regulate the levels of extracellular glutamate, and thus protecting neurons from cell death and provide them energy. So, astrocytic deregulation may promote glutamate-mediated neuronal excitotoxicity and lead to altered synaptic transmission (Stockmeier & Rajkowska, 2004), already associated with many other brain disorders (Sheldon and Robinson, 2007; Kim *et al.,* 2011).

Altered sleep homeostasis is one of MDD symptoms and, as previously mentioned, a night of total SD is one of instant relief therapies used for suicidal patients (Germain *et al.,* 2008; Hemmeter *et al.,* 1998). Adenosine-astrocytic signaling is implicated in the control of human sleep and gliotransmission seems to be altered in areas that contribute to depressive symptoms, such as frontal cortex and hippocampus

(Bachmann *et al.,* 2012; Mazzotti *et al.,* 2011). SD, in mice, elevates astrocyte-derived adenosine, and the elevation of extracelular adenosine levels activates A_1 synaptic receptors signaling, which promotes the antidepressants effects (Hines *et al.,* 2013). So, the decrease of astrocytes, in these brain regions, can also contribute to a reduction: in the astrocytic release of adenosine and in the consequent A_1 synaptic receptors activation and signaling, leading to disturbed sleep, a known depressive symptom.

From our results, posterior caudate nucleus A_1 synaptic receptors levels are decreased, which is accompained by a down-regulation of PSD-95, a post-synaptic density marker of excitatory nerve terminals. So, it is possible that there could be a loss of synaptic density in suicide (Feyissaa *et al.,* 2009), these synaptic alterations may be associated with the loss of A_1R present in the synapse.

This A_1R down-regulation role in depression is supported by the observations of anxiolytic actions of A_1R agonists administration in rodents (Florio *et al.,* 1998; Jain *et al.,* 1995). A loss of the synaptic A_1R, important in management of signal integration and neuronal hyperpolarization (de Mendonça *et al.,* 1995; de Mendonça & Ribeiro, 1997; Klishin *et al.,* 1995; Thompson *et al.,* 1992), can be responsible for a decrease in adenosine signaling. This can explain some depressive symptoms related to the emotional-motor circuitry, such as anhedonia and psychomotor dysfunctions (Furman *et al.*, 2011).

So, in cases such as depression, in which the cell is overstimulated, the A_1R activation leads to an inhibition of the cell activity. The observed up-regulation of A_1R in the caudate nucleus is probably a system attempt to control the overstimulation.

In noxious brain conditions, extracellular adenosine levels increase and ARs are activated. If this overexpression is prolonged ARs levels will suffer modifications, A_{2A}R will be activated and A_1R desensitized, that will contribute to hamper the neuroprotective effectiveness of the A_1R activation in chronic brain pathologies (Fredholm, 1997; Fernandez *et al.,* 1996). Accordingly, A_{2A}R levels are usually increased, whereas those of A_1R are decreased, in hippocampus (Cunha, 2005). Also in older animals, presenting an ARs imbalance (Canas *et al.*, 2009; Cunha *et al.,* 1995), altered transduction mechanisms associated to ARs were described (Lopes *et al.,* 1999), suggesting that A_1R and A_{2A}R balance is essential to adenosine role, as a normalizing device to promote the adequate response in neuronal circuits.

$A_{2A}R$, in total extracts from BA25 and posterior caudate nucleus, underwent an up-regulation with suicide, which can be associated with a gain of function, as $A_{2A}R$ overactivation may facilitate excitatory neurotransmission to the caudate nucleus.

This may be related with the already reported synaptic and astrocytic dysfunction that underlies both memory impairment and mood-related alterations.

This rationale is in agreement with studies performed on both animal models of memory dysfunction and of mood alterations showing synaptic changes (Honer *et al.,* 2002). Besides, blockade of $A_{2A}R$ has previously been reported to decrease cortical damage (Gao & Phillis, 1994; Chen *et al.,* 1999; Melani *et al.,* 2003; Behan & Stone, 2002). The evidence of deregulation of $A_{2A}R$ expression is in line with studies showing that $A_{2A}R$ have a central role in anxiety-related disorders under many different conditions (Shen & Chen, 2009). Also, $A_{2A}R$ null mice are a valuable model to study mood disorders (Deckert, 1998), as these mice display absence of an anxiogenic response (El Yacoubi *et al.,* 2000; Ledent *et al.,* 1997).

Additionally, $A_{2A}R$ are enriched in the caudate-putamen and nucleus accumbens, where they can interact physically with dopamine D_2R to regulate reward, habitual behavior and locomotor activity (Cunha *et al.,* 2008). it has previous been reported, in pathological situations (Agostinho *et al.,* 2000; Rebola *et al.,* 2003) and upon chronic stress, directed to the basal ganglia (Pinna *et al.,* 2002; Tomiyama *et al*., 2004), an increase in $A_{2A}R$ expression levels. Besides, activation of $A_{2A}R$, whose levels where found increased on noxious stimulli (Kobayashi & Millhorn, 1999; Kobayashi *et al.,* 2000), leads to enhanced glutamate extracelular levels (Marcoli *et al.,* 2003; Melani *et al.,* 2003; Popoli *et al.*, 2002; O'Reagan *et al.*, 1992).

The fact that their levels were only altered in total extracts, but not in nerve terminals, pointed that $A_{2A}R$ expressed in non-neuronal cells may be affected by this disorder. As $A_{2A}R$ are expressed in neurons, astrocytes and microglia, they have been implicated in controlling glial function and brain metabolic adaptation, processes that are deregulated in several psychiatric conditions (Lee *et al.,* 2007; Rajkowska & Miguel-Hidalgo, 2007). Thus $A_{2A}R$ up-regulation may be explained by their non-neuronal role in controlling aberrant synaptic plasticity, once we know that glutamate release from astrocytes (Li *et al.,* 2001; Nishiazaki *et al.,* 2002) is enhanced by $A_{2A}R$ in rodents. Furthermore, $A_{2A}R$ were found to control glutamate uptake by glial cells (Matos *et al.,* 2012), through inhibition of glutamate transport into astrocytes (Pintor *et al.,* 2004).

So, $A_{2A}R$ overactivation, may be a cause or an aggravating factor for disease state and may explain why neuroprotection afforded by $A_{2A}R$ blockade is evident in cortical (Cunha, 2005) and striatal (Popoli *et al.,* 2002) areas.

Chapter 6. Conclusions

The results presented in our work provide a comparative analysis of the synaptic and subsynaptic distribution of adenosine receptors on cingulated cortex, caudate nucleus and hippocampus tissue, in human postmortem samples.

Besides, we reported MDD pathology-related changes in the distribution of synaptic and astrocytic proteins, as well as of the relative abundance alterations of A_1R and $A_{2A}R$ with suicide. This might explain the relationship of caffeine consumption with a decrease incidence of depression and suicide, and thus providing possible molecular targets that are responsible for dysfunction in MDD.

Moreover we carefully optimized the evoked release of [^{14}C]glutamate from chemically-stimulated P2 crude synaptosomes protocol in human tissue, and confirmed that this protocol can be used for studying the neurotransmitters release from synaptosomes using human postmortem samples.

However, there are some questions that remain unanswered. One of them is if $A_{2A}Rs$ show the same localization pattern in hippocampus as in other brain regions, and if hippocampal ARs suffer changes with suicide. Localization of ARs in glial cells and their astrocytic function still remains to be explored. Also, we aim to define if there is an deregulation of the modulatory function of ARs in suicide completers. Additionally, we look forward to explore these alterations in amygdala.

Nevertheless, our work might contribute to better understand the modulatory role of ARs in depression. This will probably be a setting up point for further research, involving the development of more effective and neuroprotective therapeutical approaches, able to control the progression of mood-related disorders, particularly MDD.

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