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Role of adenosine A_{2A} receptors in the neuroprotective effect of corticotrophin releasing hormone (CRH)

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RESUMO

O factor de libertação da corticotrofina (*corticotrophin releasing factor*, CRF) é um componente essencial na regulação do stresse. No eixo hipotálamo-hipofisário (*hypothalamic-pituitary-adrenal*, HPA) é libertado do hipotálamo, activando os receptores de subtipo CRF_1R e CRF_2R, que regulam a libertação de corticotrofina pela hipófise. Esta, por sua vez, induz a libertação de glucocorticóides das glândulas supra-renais. Findo o período de stresse, os glucocorticóides regulam negativamente a libertação de CRF e diminuem a activação do eixo HPA, restabelecendo a homeostasia inicial do sistema. Recentemente, para além da sua acção no eixo HPA, a presença dos receptores de CRF foi observada em neurónios provenientes de outras áreas cerebrais como o córtex ou o hipocampo. O CRF tem funções neuroprotectoras perante vários tipos de insulto, como a excitotoxicidade induzida por glutamato, a agregação da proteína β-amilóide ou a indução de stress oxidativo. Curiosamente, a adenosina, um neuromodulador, exerce o mesmo tipo de função neuroprotectora em áreas cerebrais semelhantes, por activação dos seus receptores. Enquanto a activação dos receptores de adenosina de subtipo A_1 (A_1R) induz um decréscimo da excitabilidade neuronal, a activação dos receptores de subtipo A_{2A} ($A_{2A}R$), na generalidade, aumenta-a. Estes receptores possuem grande afinidade para a adenosina e estão presentes na maioria das áreas cerebrais, embora com diferentes níveis de expressão. Assim, para modular a excitabilidade neuronal existem estratégias farmacológicas que consistem quer na activação dos A_1R quer no bloqueio dos $A_{2A}R$. No entanto, a última abordagem tem sido recentemente mais investigada e é largamente usada na prevenção de morte celular tanto em modelos *in vitro* como *in vivo*.

Em situações de isquémia, observa-se tanto um aumento dos níveis de CRF como de adenosina no cérebro de rato. Este aumento sugere uma acção relevante destes mediadores quer na manutenção da viabilidade neuronal quer na regulação da expressão de diversos genes importantes para o desenvolvimento e diferenciação neuronal. Por outro lado, sugere uma possível interacção entre os receptores do CRF e da adenosina. Esta hipótese tornou-se ainda mais provável após a observação, no nosso laboratório, que a administração oral de antagonistas dos receptores A_{2A} reverte os efeitos do stresse no hipocampo, induzidos por separação maternal em ratos. Esta reversão pode ser consequência de regulação dos constituintes do eixo HPA, como os glucocorticóides ou o CRF. Adicionalmente, as vias de sinalização intracelular activadas pela acção do CRF (através dos receptores CRF_1R e CRF_2R) e da adenosina

(através dos $A_{2A}R$) são semelhantes. Envolvem activação de proteínas G_s com consequente aumento de actividade de cinases de proteínas, como a cinase A (PKA), a cinase C (PKC) e a *mitogen-activated protein kinase* (MAPK).

O objectivo deste trabalho foi estudar a acção do CRF na viabilidade celular em condições de excitoxicidade induzida por glutamato e avaliar a possível interacção com os receptores de adenosina do subtipo A_{2A}.

Foram usadas culturas primárias de neurónios de córtex de embriões de rato com 18 dias de gestação. Ao oitavo dia, as culturas foram tratadas durante 24 horas com glutamato, nas concentrações de 20 a 1000 µM, em condições de bloqueio ou activação dos receptores A_{2A} e do CRF, através da aplicação de agonistas e antagonistas dos respectivos receptores. O glutamato é um aminoácido excitatório e um dos responsáveis pela excitotoxicidade induzida por eventos isquémicos no sistema nervoso central. Ao actuar nos receptores ionotropicos de glutamato induz a entrada de cálcio e sódio extracelulares assim como a libertação de cálcio do reticulo endoplasmático. O aumento de cálcio e sódio intracelulares, pode levar à morte celular quer por apoptose quer por necrose. A técnica de marcação simultânea com as sondas nucleares iodeto de propidio (PI) e *Syto-13* foi usada com o objectivo de caracterizar a viabilidade celular nas diferentes condições.

Inicialmente, foi investigada a concentração de glutamato (de 20 a 1000 µM) a ser aplicada às células em cultura com o objectivo de reproduzir as consequências de um insulto neuronal *in vivo*. Simultaneamente aos ensaios de viabilidade celular, foram determinados os níveis de um mediador de morte por apoptose, a caspase-3, pela técnica de *Western blotting*. Este insulto provocou um decréscimo na viabilidade celular que é dependente da concentração de glutamato usada, sendo superior para concentrações mais altas. Por seu lado, a activação da via apoptótica ocorre preferencialmente para baixas concentrações de glutamato (até 50 µM). Para o desenvolvimento do restante trabalho a concentração seleccionada foi a de 100 µM, que causou uma diminuição para 76,4±1,63% (n=4) da viabilidade celular, visto originar tanto morte por apoptose como por necrose neste modelo.

A função neuroprotectora do CRF na gama de concentrações de glutamato anteriormente descrita foi observada através da aplicação de urocortina (10 pM), um composto da família do CRF com semelhante afinidade para os dois receptores de CRF. Embora a urocortina aparente ter funções protectoras em todas as concentrações de glutamato usadas, o aumento mais significativo na viabilidade neuronal foi observado na presen-

ça de glutamato 100 µM (de 76,4±1,63% para 90,5±2,23%, P<0,001, n=3). Esta variação na sobrevivência neuronal, perante um insulto neurotóxico, não apresenta o mesmo padrão nos níveis de caspase-3. Para baixas concentrações de glutamato a urocortina aumenta os níveis de caspase-3, enquanto para concentrações de glutamato elevadas, os níveis deste marcador apoptótico parecem diminuir. Esta proteína, característica da indução de morte celular por apoptose exclui o dano causado por necrose. Durante esta primeira etapa do trabalho conclui-se que a urocortina exerce funções protectoras sobretudo na prevenção do processo necrótico induzido pelo glutamato.

Numa etapa seguinte foram diferenciados os efeitos dos dois tipos de receptores do CRF na neuroprotecção. Assim, usando dois antagonistas selectivos, antalarmina (10nM) para CRF_1R e anti-Sauvagina-30 (10nM) para CRF_2R, foi observado que o bloqueio de cada um dos receptores de forma independente leva à perda da função da urocortina (73,9±3,53% bloqueando os CRF_1R, P<0,01 n=4, e 76,0±2,80% ao bloquear os CRF_2R, P<0,01, n=4). Conclui-se que a activação simultânea dos dois receptores pela urocortina é necessária para que ela exerça o seu efeito neuroprotector perante um insulto de glutamato (100 µM). Durante este processo de bloqueio dos receptores de CRF, observou-se uma interacção directa entre os dois fármacos usados, antalarmina e anti-Sauvagina-30, que os impede de bloquear eficientemente os receptores de CRF, quando usados em simultâneo. Esta interacção foi avaliada através de técnicas de fluorescência que permitem observar a diminuição do sinal produzido pelo aminoácido fenilalanina, presente na anti-Sauvagina-30, com a adição de concentrações crescentes de antalarmina. Assim, quando introduzidos simultaneamente no meio celular, são ineficazes a bloquear o efeito da urocortina.

Após a observação da função da urocortina na protecção da morte celular induzida por glutamato, estudou-se a modulação exercida pelos $A_{2A}R$ nesse efeito. Confirmou-se que o bloqueio dos $A_{2A}R$, através do antagonista SCH 58261 (50 nM) é neuroprotector. Por outro lado, a activação deste subtipo de receptores está descrita como nociva para este tipo de células. Contudo, a activação directa destes receptores pelo agonista selectivo, CGS 21680 (30nM), não alterou a viabilidade celular, provavelmente devido à elevada concentração de adenosina no meio extracelular.

A modulação simultânea dos receptores A_{2A} e de CRF revelou a existência de uma interacção entre os mesmos. A neuroprotecção conferida pelo bloqueio dos $A_{2A}R$ é dependente da activação dos receptores de CRF do tipo 2 mas independente dos do tipo 1, facto que foi observado pelo bloqueio selectivo dos receptores de CRF em condições de antagonismo dos $A_{2A}R$. A protecção concedida pelo antagonista SCH 58261

é suprimida quando os CRF_2R se encontram bloqueados (de 88,3±1,53% para 74,8±4,91%, P<0,01, n=5) revelando-se independente do bloqueio dos CRF_1R (87,7±3,48%, n=4).

Simultaneamente, em células PC12 diferenciadas com *Nerve Growth Factor* (NGF), observou-se a modulação dos níveis dos $A_{2A}R$ pelos seus ligandos. A activação dos $A_{2A}R$ diminui os seus níveis (48,9±5,5%, P<0,001, n=4), enquanto o bloqueio os aumenta (144±15,2%, P<0,01, n=5). Os ligandos dos receptores de CRF não alteram os níveis dos $A_{2A}R$ em condições basais. Porém, quando aplicados em simultâneo com os ligandos dos $A_{2A}R$ o mesmo não se observa. Concretamente, ocorre uma redução nos níveis dos $A_{2A}R$ na presença do seu antagonista quando os receptores CRF_1R se encontram bloqueados e os CRF_2R activados (39,4±14,4% do controlo, P<0,01, n=4).

Em suma, este trabalho permitiu observar a função neuroprotectora dos receptores do CRF em neurónios em cultura perante um insulto de glutamato, quer pela acção directa nos seus receptores quer pela modulação dos receptores de adenosina do subtipo A_{2A}. O modelo usado permite diferenciar as alterações na viabilidade das células em cultura por duas vias de promoção da morte celular, apoptose e necrose, respectivamente para baixas e elevadas concentrações de glutamato. A neuroprotecção conferida pelo CRF ocorre sobretudo na prevenção da morte celular por necrose e é dependente da activação simultânea dos dois tipos de receptores, CRF_1R e CRF_2R. Para além dos efeitos directos através dos seus receptores, o CRF exerce uma modulação dos $A_{2A}R$. Estes receptores apresentam expressão diminuída em condições de activação dos CRF_2R e bloqueio dos $A_{2A}R$. Este fenómeno de regulação dos $A_{2A}R$ por parte dos CRF_2R constitui uma alternativa à protecção por CRF atrás descrita. Novas abordagens terapêuticas, que incluam a modulação destes dois tipos de receptores podem ser futuramente testadas com o objectivo de diminuir a morte neuronal provocada por insultos excitotóxicos em eventos isquémicos.

ABSTRACT

In hypoxia, glutamate excitotoxicity induces neuronal death. Simultaneously, adenosine is released and is accompanied by an increase of the stress mediator, corticotrophin-releasing factor (CRF). *In vivo* modulation of adenosine A_{2A} receptors ($A_{2A}R$) reverses hippocampal stress-induced effects. This raises the question whether $A_{2A}R$ regulate CRF actions. We now evaluated the interaction between the blockade of $A_{2A}R$ and the activation of CRF receptors (CRFR), upon glutamate insult.

Primary rat cortical neuronal cultures (9 days *in vitro*) were challenged with glutamate (20-1000µM, 24 hours). The effects of the CRFR and $A_{2A}R$ ligands on cell viability were measured using propidium iodide and Syto-13 fluorescence staining. Pro-caspase-3 fragmentation was used as an apoptotic marker. $A_{2A}R$ levels were quantified in NGF-differentiated PC12 cells by Western blotting.

Glutamate decreased cell viability in a concentration-dependent manner. At 100 µM we observed a reduction of viability to 76.4±1.63% of control ($P<0.001$, n=6). Urocortin (10pM), a CRFR agonist, increased cell survival to 90.5±2.23% ($P<0.001$ compared to glutamate, n=3). This effect was abolished by blocking either CRF_1R or CRF_2R with antalarmin (10nM) or anti-Sauvagine-30 (10nM), respectively. Activation of $A_{2A}R$ did not affect cell death induced by glutamate. However, $A_{2A}R$ blockade with a selective antagonist SCH 58261 (50nM) improved cell viability against the glutamate insult. This effect was dependent on CRF_2R but not on CRF_1R activation. The $A_{2A}R$ levels measured in PC12 cells were modulated by CRF_2R. The $A_{2A}R$ upregulation induced by SCH 58261 (144±15.2%, $P<0.01$ n=5; 50 nM) was abolished by CRF_2R ($P<0.01$, n=4) but not by CRF_1R activation.

Overall these data show a protective role of CRF in cortical neurons, against glutamate-induced death, either directly by CRFR activation or by modulating $A_{2A}R$ actions. The neuroprotection achieved by $A_{2A}R$ blockade requires CRF_2R activation, which might result from CRF_2R modulation of $A_{2A}R$ levels. The interaction between these receptors may point toward novel pharmacological approaches based on common molecular pathways.

Keywords: Adenosine; A_{2A} receptors; Corticotrophin Releasing Factor (CRF); CRF_1R; CRF_2R; Neuroprotection.

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ABBREVIATION LIST

Aβ	amiloid-β peptide
A_1R	adenosine A_1 receptor subtype
$A_{2A}R$	adenosine A_{2A} receptor subtype
a-Sau	anti-Sauvagine-30
ACTH	adrenocorticotrophin hormone
AMPA	L-α-amino-3-hydroxy-5-methylisoxazole-4-propionate
Ant	antalarmin
BDNF	brain-derived neurotrophic factor
cAMP	cyclic adenosine monophosphate
CeA	central nucleus of the amygdala
CNS	central nervous system
CREB	cAMP response element-binding
CRF	corticotrophin releasing factor (formerly known by CRH for corticotrophin releasing hormone)
CRF_1R	CRF receptor of subtype 1
CRF_2R	CRF receptor of subtype 2
CRFR	CRF receptors
CTR	control
DAPI	4',6-diamidino-2-phenylindole
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
Glu	L-glutamic acid
GSK3β	glycogen synthase kinase 3β
HBSS	Hanks' balanced salt solution
HPA axis	hypothalamic-pituitary-adrenal axis
IP3	inositol trisphosphate
LTD	long-term depression

LTP	long-term potentiation
MAP2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor-κB
NGF	nerve growth factor
NMDA	N-methyl-D-aspartic acid
PFA	paraformaldahyde
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
PI	propidium iodide
Urc	urocortin
Urc2	urocortin 2
Urc3	urocortin 3
TrkB	tropomiosine related kinase B receptor

1. BACKGROUND

Adenosine is a modulator in the brain that binds to several G-protein coupled receptors (reviewed by Sebastião and Ribeiro, 2000). Among these, the adenosine receptors of A_{2A} subtype ($A_{2A}R$) are captivating targets to pharmacologic modulation. It has been shown that their blockade is useful in stroke and stress related episodes (Cunha et al., 2006). The mechanism by which the blockade of $A_{2A}R$ is able to reverse stress effects remains unknown. They could, presumably, interfere either in the regulation of the action of the glucocorticoids or alternatively, in the effects of the corticotrophin releasing factor (CRF) that are regulators of the Hypothalamic-Pituitary-Adrenal (HPA) axis.

CRF was described in extrahypothalamic sites (Swanson et al., 1983), where it was shown to have a neuroprotective role against several brain insults, as excitatory amino acids, hypoxia or amyloid-β_{25-35} peptide (Aβ) (Fox et al., 1993; Chalmers et al., 1995; Pedersen et al., 2001). Curiously, $A_{2A}R$ antagonists have the same properties in neuronal cells (reviewed by Chen et al., 2007). We therefore hypothesised that the established neuroprotective effect achieved by blocking A_{2A} receptors upon stress conditions could be linked to an action on CRF mediated effects.

1.1. FUNCTION OF CORTICOTROPHIN RELEASING FACTOR

Corticotrophin Releasing Factor (CRF, formerly abbreviated CRH for Corticotrophin Releasing Hormone), a 41 amino acid peptide, is an important signalling molecule released from paraventricular nucleus of hypothalamus (Vale et al., 1981). Together with adrenocorticotrophin hormone (ACTH) and glucocorticoids has an important role in HPA axis regulation upon stressful events (Vale et al., 1981). Interestingly, CRF is also implicated in modulation of anxiety, depression, food intake control, learning and memory (reviewed by Sarnyai et al., 2001), which can be due to its expression in extrahypothalamic sites, as presented in Figure 1.1.A, like amygdala (central nucleus) and hippocampus (GABAergic interneurons, Chalmers et al., 1995). Its basal physiological concentration is around 38 pg/mL (approximately 8 pM) in adult human cerebrospinal fluid (Kling et al., 1991).

CRF binds to two families of G-protein-coupled receptors, subtype 1 and 2 (CRF_1R and CRF_2R), which have more than 70% of amino acid sequence similarity and are present both in neuronal and glial cells (Kapcala and Dicke, 1992; Lovenberg et al., 1995; Hauger et al., 2003). CRF binding activates several excitatory and inhibitory G-proteins with an order of potency: $G_s \geq G_o > G_{q/11} > G_i > G_z$ (Hillhouse and Grammatopoulos, 2006)

which results in an increase or decrease, respectively, of intracellular cyclic-AMP (cAMP) and inositol trisphosphate (IP_3) levels (Grammatopoulos et al., 2001). These molecules have different downstream effects as the increase of activation of cAMP-dependent protein kinase A (PKA), protein kinase C (PKC) or mitogen-activated protein kinase (MAPK, Rossant et al., 1999; Elliott-Hunt et al., 2002; Blank et al., 2003). The activation of these pathways increases the levels of glycogen synthase kinase 3β (GSK3β) and cAMP response element-binding protein (CREB), as well as its phosphorylation (Bayatti et al., 2003) while nuclear factor-κB (NF-κB) is repressed (Lezoualc'h et al., 2000). As a consequence, the pattern of gene expression is altered (Rossant et al., 1999) which includes the upregulation of the transcription of CRF and of brain-derived neurotrophic factor (BDNF) genes (Spengler et al., 1992; West et al., 2001; Bayatti et al., 2005).

CRF receptors (CRFR) are widely distributed in the central nervous system (CNS, De Souza et al., 1985). CRF_1R has an abundant yet selective expression in rat brain as described in Figure 1.1.B (Chen et al., 2000). It expression is broadly complementary to the distribution of CRF binding sites, with higher expression in cortex, cerebellum, amygdala, hippocampus, and olfactory bulb (Potter et al., 1994). CRF_2R receptor family is composed by three splicing variants, $CRF_{2(a)}R$, $CRF_{2(b)}R$ and $CRF_{2(c)}R$ (reviewed in Dautzenberg et al., 2001) that are expressed in more restricted brain areas compared to CRF_1R. CRF_2R is confined to subcortical structures, with higher levels in the lateral septal nucleus and the hypothalamus but also in lower levels in olfactory bulb, amygdala and hippocampus (Chalmers et al., 1995; Van Pett et al., 2000).

The different CRF receptors have a complementary action in the regulation of HPA axis activity. While CRF_1R triggers its activation by increasing ACTH release from pituitary, CRF_2R is required to its gradual attenuation by restablishing the corticosterone levels after a stressful situation (reviewed by Reul and Holsboer, 2002). CRF_1R is located postsynapticaly in dendritic spines, close to excitatory synapses (Chen et al., 2004b; Chen et al., 2010) whereas CRF_2R is located both in pre- and postsynaptic terminals of the central nucleus of the amygdala (CeA) (Liu et al., 2004). In CeA, activation of CRF_1R depresses while activation of CRF_2R facilitate glutamatergic transmission (Liu et al., 2004). These opposed actions and localizations suggest different roles of these receptors in CRF signalling in the CNS (Chalmers et al., 1995).

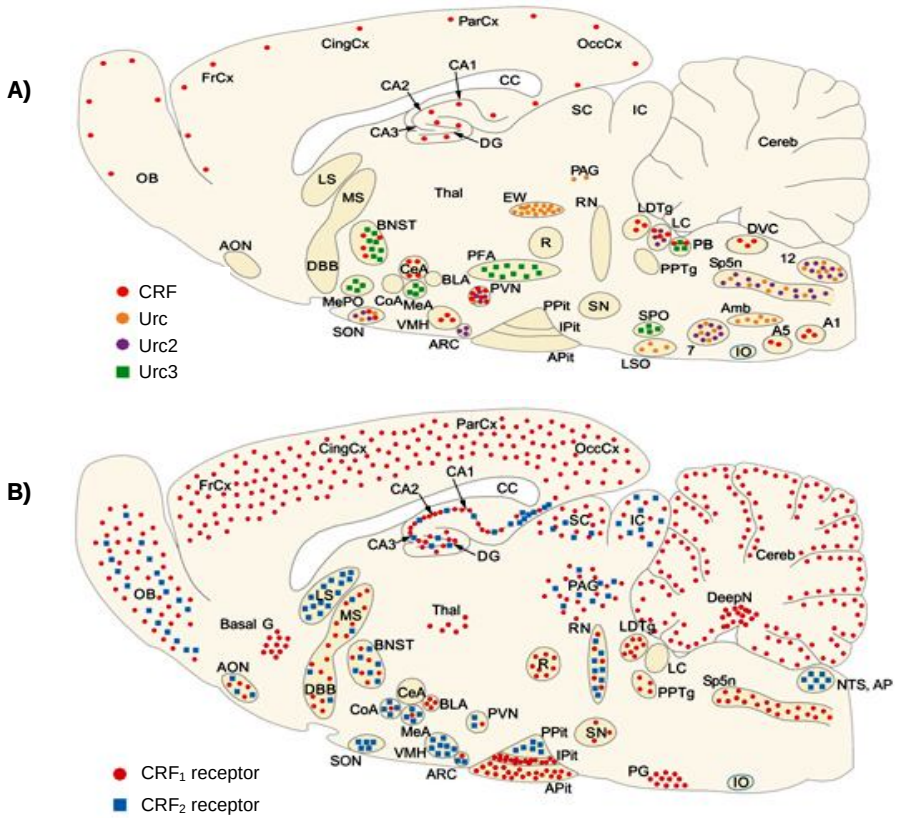

Figure 1.1 - **A)** Distribution of mRNA encoding for the CRF and related peptides in rat brain. Urocortin 1 (Urc), Urocortin 2 (Urc2) and Urocortin 3 (Urc3) are recently discovered peptides that bind to CRF receptors with different selectivity. **B)** Distribution of mRNA encoding for the CRF receptors in rat brain (CRF_1R in red and CRF_2R in blue). 7, facial nucleus; 12, hypoglossal nucleus; Amb, ambiguus nucleus; AON, anterior olfactory nucleus; AP, area postrema; Apit, anterior pituitary; ARC, arcuate nucleus; Basal G, basal ganglia; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CA1-3, fields CA1-3 of Ammon's horn; CC, corpus callosum; CeA, central nucleus of the amygdala; Cereb, cerebellum; CingCx, cingulate cortex; CoA, cortical nucleus of the amygdala; DBB, diagonal band of Broca; Deep N, deep nuclei; DG, dentate gyrus; FrCx, frontal cortex; IC, inferior colliculi; IO, inferior olive; IPit, intermediate pituitary; LC, locus coeruleus; LDTg, laterodorsal tegmental nucleus; LS, lateral septal nucleus; LSO, lateral superior olive; MeA, medial nucleus of the amygdala; MePO, median preoptic area; MS, medial septum; NTS, nucleus tractus solitarii; OB, olfactory bulb; OccCx, occipital cortex; PAG, periaquaductal gray; ParCx, parietal cortex; PFA, perifornical area; PG, pontine gray, PPit, posterior pituitary; PPTg, pedunculopontine tegmental nucleus; PVN, paraventricular nucleus of hypothalamus; R, red nucleus; RN, raphe nuclei; SC, superior colliculi; SN, substantia nigra; SON, supraoptic nucleus; SP5n, spinal trigeminus nucleus; SPO, superior paraolivary nucleus; Thal, thalamus; VMH, ventromedial nucleus of hypothalamus. Adapted from Reul *et al.* (2002)**.**

1.1.1. EXTRAHYPOTHALAMIC ROLE OF CORTICOTROPHIN RELEASING FACTOR

The hippocampus is a brain area crucial for learning and memory and particularly susceptible to stress effects (Foy et al., 1987). In hippocampus, CRF has modulatory actions, particularly excitatory. CRF increases the frequency of spontaneous discharges of hippocampal neurons (Aldenhoff et al., 1983). It also promotes a long-lasting enhancement in synaptic efficacy given by an increase of amplitude and slope of popula-

tion excitatory postsynaptic potentials (Wang et al., 1998). CRF is also involved in neuronal plasticity both in long-term potentiation (LTP) and long term depression (LTD) (Wang et al., 1998; Miyata et al., 1999). However, the CRF receptor subtype involved in this hippocampal plasticity is still unknown.

These synaptic actions reveal both an immediate as well as a delayed gene dependent effect of CRF in the brain.

CRF is released during an hypoxic or ischemic event and acts as a neuroprotective factor at nanomolar or even picomolar concentrations, in hippocampal slice or using hippocampal or cortical primary cultures, respectively (Fox et al., 1993; Pedersen et al., 2001). In primary culture neuronal cells, death induced by neurotoxic insults, as $FeSO_4$, 4-hydroxynonenal (HNE), amyloid-β_{25-35} peptide (Aβ) or glutamate, can be almost completely reverted with exogenous CRF application (Pedersen et al., 2001; Elliott-Hunt et al., 2002). This effect is consistent in several brain areas (cerebral cortex, hippocampus and cerebellum, Bayatti et al., 2003). CRF also provides moderate protection against an hypoxia insult in a brain slices preparation (Fox et al., 1993). It is speculated that this neuroprotection is due to an increased expression of BDNF, or other neurotrophins, triggered by CRFR activation (Bayatti et al., 2005; Hauger et al., 2009). CRF neuroprotective actions can still be observed during or a few hours after a glutamate insult (Elliott-Hunt et al., 2002), which could have therapeutic implications in patients with cerebral ischemia.

1.2. ADENOSINE INFLUENCE ON NEUROPROTECTION: MODULATION BY ADENOSINE RECEPTORS

Adenosine receptors are another pharmacological target to achieve neuroprotection in the brain. Adenosine is present in all cells as an important molecule in cellular metabolism (Levene and Tipson, 1931). However, the roles of adenosine are beyond it, acting in brain cells as a neuromodulator, either directly by modulating postsynaptic responses or by modulating the response of other receptors (Sebastião and Ribeiro, 2009).

The biological role of adenosine is carried by widespread and high affinity receptors, A_1R and $A_{2A}R$, and low affinity receptors, $A_{2B}R$ and A_3R (Fredholm et al., 2001). These are G-protein coupled receptors that decrease or increase intracellular cAMP levels, respectively if adenosine is bound to either to A_1R or $A_{2A}R$. The major G-proteins involved in this signal transduction are the inhibitory G_i and G_o and the excitatory G_s and G_{olf} (Linden, 2001). Intracellular signalling is mediated by PKA, PKC and MAPK path-

ways with consequent activation of CREB (Cunha and Ribeiro, 2000). Different types of K^+, Na^+ and Ca^{2+} channels are also regulated by adenosine receptors (for review see Ribeiro et al., 2003b).

The neuronal modulation by adenosine depends on a balance between A_1R and $A_{2A}R$ activation. Since extracellular adenosine is enough to tonically activate both receptors, their selective activation is directly related to their levels in the different brain areas. A_1R that are pre-, post- and non-synaptic (Schubert et al., 1994) have a widespread distribution, with higher levels on hippocampus, cerebral cortex and cerebellum (Reppert et al., 1991). In the hippocampus, A_1R act either pre-synaptically by inhibiting glutamate release from nerve terminals (Dunwiddie and Haas, 1985) and post-synaptically through NMDA receptors inhibition (de Mendonca et al., 1995; Rebola et al., 2003).

In an opposed pattern, as showed in Figure 1.2, $A_{2A}R$ are less expressed in those areas but with abundant distribution in striatum and olfactory blub (Jarvis and Williams, 1989; Ribeiro et al., 2003b). $A_{2A}R$ are located both pre- and post-synaptically within these brain areas but also in cortex and hippocampus (Li and Henry, 1998; Hettinger et al., 2001).

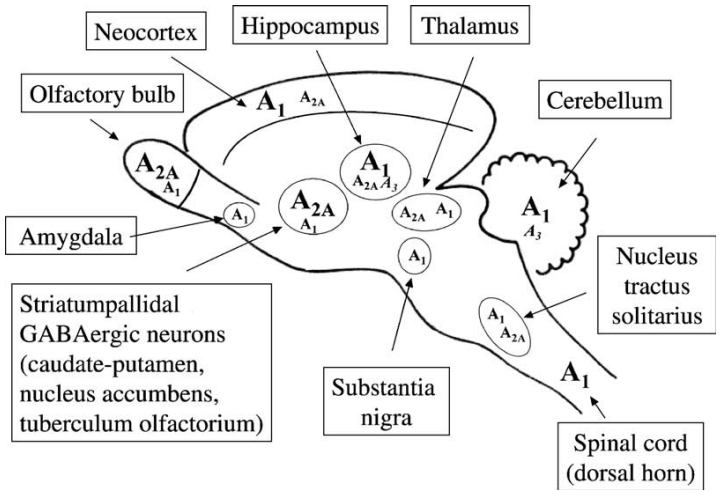

Figure 1.2 - Distribution of adenosine receptors in the main regions of the rat central nervous system. Higher levels of expression are indicated by bigger font sizes (from Ribeiro et al., 2003b)**.**

Adenosine is continuously formed in intracellular and extracellular medium. When produced intracellularly, adenosine is exported by specific transporters, reaching nanomolar values in normal conditions (40 to 110 nM, Ballarin et al., 1991). Besides this continuous release, extracellular adenosine levels are increased by specific neurotransmitters action (Carswell et al., 1997; Delaney and Geiger, 1998). As an example, adeno-

sine nucleotides are coreleased with glutamate to extracellular medium (Schousboe et al., 1989) and consequently hydrolysed to adenosine by extracellular nucleotidases (reviewed by Zimmermann, 1992). During excitotoxic events, as ischaemia, excitatory amino acids are over released and extracellular adenosine levels rises more than 20 fold (Andine et al., 1990). The extracellular concentration of adenosine also affects its modulatory function. In cortex or hippocampus, if adenosine reaches elevated levels, like in ischemic insult, it would desensitize the inhibitory A_1R while the excitatory $A_{2A}R$ remain active (Fernandez et al., 1996; Fredholm, 1997), leading to an exacerbated cell death. Additionally, in *in vitro* experiments, glutamate insult to cultured cortical neurons increases both A_1R and $A_{2A}R$ levels (Castillo et al., 2010), leading to a new different ratio between these two receptors that was already shown to alter receptor signalling pathways (Lopes et al., 1999a, 1999b).

Pharmacologic modulation of adenosine receptors is being pointed as neuroprotection in stroke, several degenerative diseases, epilepsy and multiple sclerosis (reviewed by Ribeiro et al., 2003a). A notable neuroprotection was acquired by A_1R activation through a reduction of neuronal excitability but it was dismissed by unwanted peripheral effects including sedation, bradycardia and hypotension (reviewed by Fredholm et al., 2005). In absence of A_1R pharmacological modulation, $A_{2A}R$ antagonists are used to control the disproportionate release of excitatory amino acids, which is involved in neuronal toxicity (reviewed by Chen et al., 2007). This neuroprotective effect of A_1R activation or $A_{2A}R$ blockade was observed both *in vitro* and *in vivo* (Gao and Phillis, 1994; Popoli et al., 2002; Dall'Igna et al., 2003; Castillo et al., 2010).

2. AIM

Glutamate excitotoxicity is responsible for neuronal death in hypoxia. The release of adenosine that occurs as a consequence of hypoxic events is accompanied by an increase in the levels of the stress regulator, corticotrophin releasing factor (CRF) in the brain. In addition, the *in vivo* modulation of adenosine receptor of A_{2A} subtype ($A_{2A}R$) is responsible for the reversion of stress-induced effects in the hippocampus (Batalha et al., 2010). This raises the question whether $A_{2A}R$ regulate the main stress mediators, either CRF or glucocorticoids. We now intended to disclose a possible pharmacological synergy between the neuroprotective effects of $A_{2A}R$ blockade and the activation of CRF receptors (CRFR), under stress conditions as glutamate insult.

To achieve that purpose, four major tasks were designed:

1st - Optimize an *in vitro* glutamate insult model using primary neuronal cultures from rat brain cortex.

2nd - Evaluate the effect of the CRF receptors activation in glutamate-induced cell death. Discriminate the CRF receptor subtype involved by selectively blocking the CRF_1R or the CRF_2R.

3rd - Disclose the functional interaction between A_{2A} and CRF receptors in the previously established model, by pharmacologic modulation of these receptors.

4th - Evaluate the ability of CRF receptor activation in modulating the $A_{2A}R$ levels, using NGF-differentiated PC12 cells.

3. METHODS

3.1. PRIMARY RAT CORTICAL NEURONAL CULTURES

Cortical neurons were cultured from 18 to 19 days Sprague Dawley rat embryos (E18-E19, adapted from Brewer, 1997; Castro et al., 2004). The pregnant rat was anesthetized with Halothane (Sigma, Spain) and decapitated. The embryos were collected in HBSS (Hanks' Balanced Salt Solution, Gibco, UK) medium and rapidly decapitated. Meninges and white mater were removed and cortices were incubated 15 minutes in HBSS (now with Calcium 1mM and Magnesium 1mM, Gibco/Invitrogen, UK) and 0.025% trypsine. Cells were centrifuged 3 times and washed with HBSS (with Calcium 1mM and Magnesium 1mM, supplemented with 10% FBS, Gibco) and resuspended in Neurobasal Medium (Gibco/Invitrogen, UK). After counted, cells were plated on poly-L-lysine-coated 24-well or 6-well plates at densities of $8x10^4$ cells/coverslip (cell viability and immunocytochemistry assays) or $1.2x10^6$ cells/well (Western blotting). Neurons were grown for 8-9 days at 37°C in a 5% CO_2 humidified atmosphere in Neurobasal medium with 2% B-27 supplement (Gibco/Invitrogen, UK), glutamate 25 µM (Sigma, Spain), glutamine 0.5 mM (Gibco/Invitrogen, UK), and 2 U/mL Pen/Strep (Sigma, Spain). Medium was totally replaced by day 4 (without glutamate) and 60 minutes before drug treatment (without glutamate and B-27 supplement).

3.2. PC12 CELLS

PC12 cells, first described by Greene and Tischler (1976), are derived from a spontaneous rat pheochromocytoma. These cells were purchased from Sigma (Spain) and used to observe variations in protein levels by Western blotting technique. To minimize the risk of mutations, passages between 10 and 20 were used in all procedures.

Cells were grown at 37°C in a 5% CO_2 humidified atmosphere in DMEM (Dulbecco's modified Eagle's medium, Invitrogen 41966-029) supplemented with essential amino-acids (Sigma M7145, UK), 2 U/mL Pen/Strep (Sigma, Spain), glutamine 0.5mM (Gibco/Invitrogen, UK) and 10% of Fetal Bovine Serum (FBS). Cells were splitted each 14 days and cell medium changed weekly. A new set of cells was prepared by lifting the cells by trypsine catalysis (0.025% for 5 minutes at 37ºC). Cells were counted and inserted in 6-well plates (150 000 cells/well, in 2mL of DMEM with 10% FBS). Cell medium was changed in day 4 and 60 minutes prior to cell treatment.

3.2.1. PC12 DIFFERENTIATION WITH NGF

PC12 cells can differentiate in a more neuronal phenotype upon treatment with nerve growth factor (NGF) 50 ng/mL for 7 days (Greene and Tischler, 1976). Cells were plated at 100 000 cells/mL confluency in previously described medium supplemented with 50ng/mL of NGF 7S for 7 days. After that period, visual detection of branching was confirmed.

3.3. GLUTAMATE INSULT

In primary cultured neuronal cells with 9 days *in vitro*, L-glutamic acid (or glutamate) was used as neurotoxic insult in 20 to 1000 µM range for 24 hours (Tamura et al., 1993). Other neurotoxic insults like Kainate 100 µM, Kainate 100 µM + Cyclothiazide 30 µM (Rebola et al., 2005) and Staurosporine 1 µM (Pike et al., 1998) were used in this model but no increase in an apoptotic marker (pro-caspase-3 cleavage to caspase-3 on western blotting technique) was observed. Same glutamate treatment was tried in PC12 cells but cell viability was preserved. Amiloid-β_{25-35} peptide (Aβ, 25 µM) was used as positive control for apoptosis (Estus et al., 1997).

3.4. PRE-INCUBATION WITH AGONISTS AND ANTAGONISTS OF CRF AND ADENOSINE A_{2A} RECEPTORS

Receptors' antagonists were applied 15 minutes before cell insult, while their agonists were placed in cell medium right before glutamate or Aβ treatment. Urocortin was preferred to CRF because it binds to CRF_1R and CRF_2R with similar affinity, while CRF binds with higher affinity (20 fold) to CRF_1R than CRF_2R (reviewed in Dautzenberg et al., 2001). Antalarmin is a specific CRF_1R non-peptide antagonist (K_i=1nM) that has almost no affinity to CRF_2R (Chen et al., 1996; Webster et al., 1996). anti-Sauvagine-30 (a-Sau) blocks CRF_2R (K_i=1.4nM) and CRF_1R (K_i=154 nM) differently (Ruhmann et al., 1998).

As reviewed by Klotz (2000), $A_{2A}R$ agonist CGS 21680 is highly specific for this subtype of receptors (K_i=27nM, versus 290nM, 89µM and 67nM of A_1, A_{2B} and A_3 receptors). SCH 58261 has a high affinity to $A_{2A}R$ (K_i=0.6nM, while A_1, A_{2B} and A_3 receptors have higher values of K_i).

$A_{2A}R$ binding molecules were used as Rebola et al. (2005), while CRFR agonist and antagonists' use was based on Pedersen et al. (2002) and Elliott-Hunt et al. (2002) reports. Due to its toxic effects on cells, DMSO concentration in cell medium was maintained below 0.001%. In general, the tasks were based on the protocol presented in Figure 3.1.

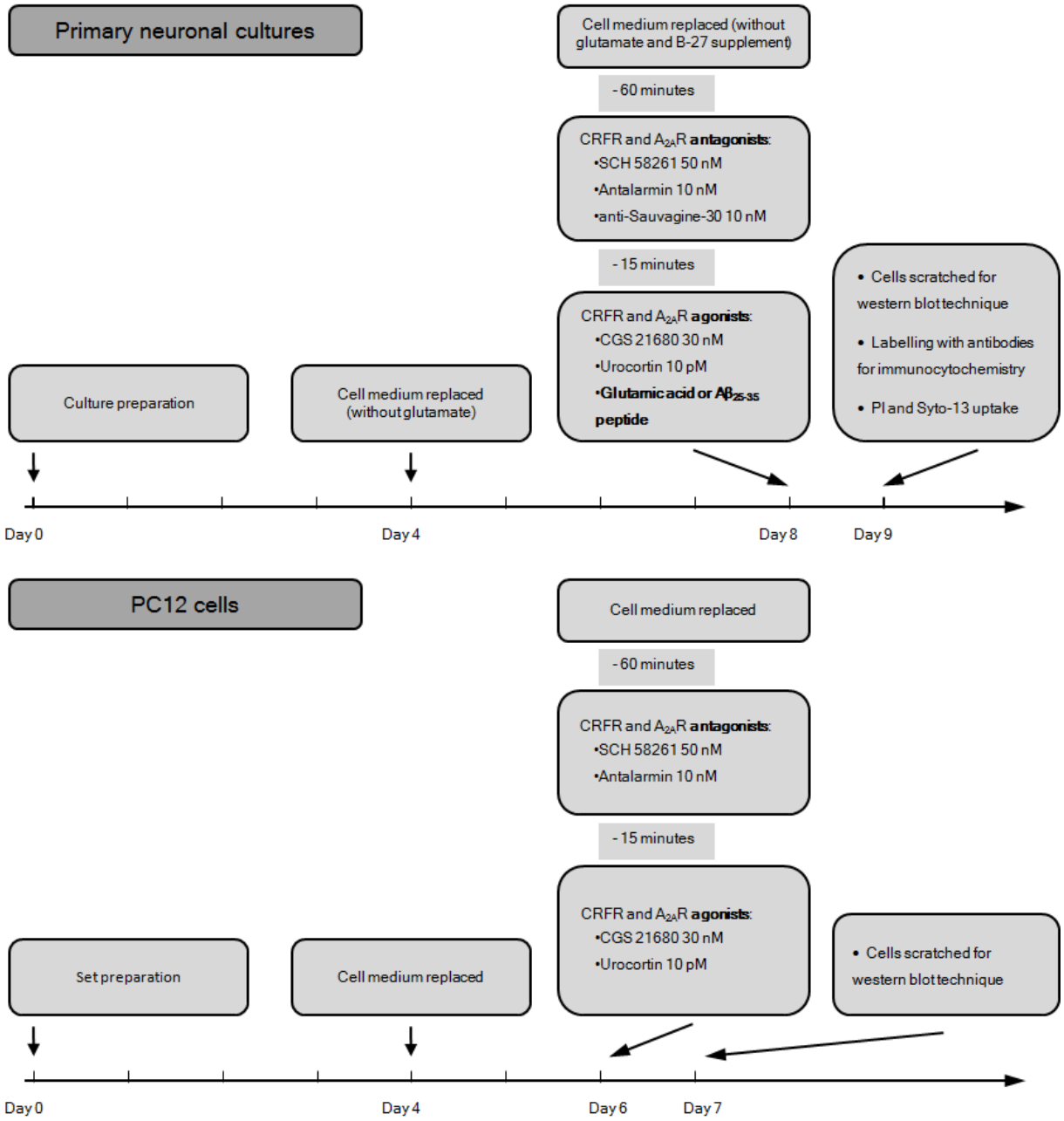

Figure 3.1 - Summary of treatment protocols of primary neuronal cultured cells and PC12 cells.

3.5. PROPIDIUM IODIDE AND SYTO-13 UPTAKE ASSAY

To distinguish the mechanism involved in cell death, cells were incubated simultaneously with two fluorescent nucleic acid stains (Jones and Senft, 1985): Syto-13 (Invitrogen, USA), capable of enter on living cells and emits at 509 nm when excited at 488 nm, and Propidium Iodide (PI, Sigma, Spain), which only enters cells through a disrupted membrane, absorbing preferentially at 535 nm and emitting at 617 nm.

Cells previously growth in a coverslip were removed from the incubator and washed with KREBS-HEPES (117 mM NaCl, 3 mM KCl, 10 mM Glucose, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 10 mM HEPES, 2mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4). Cells were incu-

bated with Syto-13 (4 µM) and PI (5 µg/mL) for 3 minutes at room temperature, followed by direct observation on Axiovert 200 fluorescence microscope using filter sets 10 (excitation 450-490nm and emission 515-565nm) and 15 (excitation 534-558nm and emission above 590nm). Three to four arbitrary photographs from each coverslip were shot and an average of 1600 cells was counted per condition in each experiment. As presented in Figure 3.2, viable cells are presented with homogeneous cell body labelled with Syto-13, whereas primary and secondary apoptotic cells show fragmented or condensed nucleus (respectively labelled with Syto-13 or PI). Necrotic cells are presented as diffuse blots, emitting in PI range (Canas et al., 2009). This method has the disadvantage of exclude cells that detached the coverslip, by random events but also by the drug treatment of each coverslip.

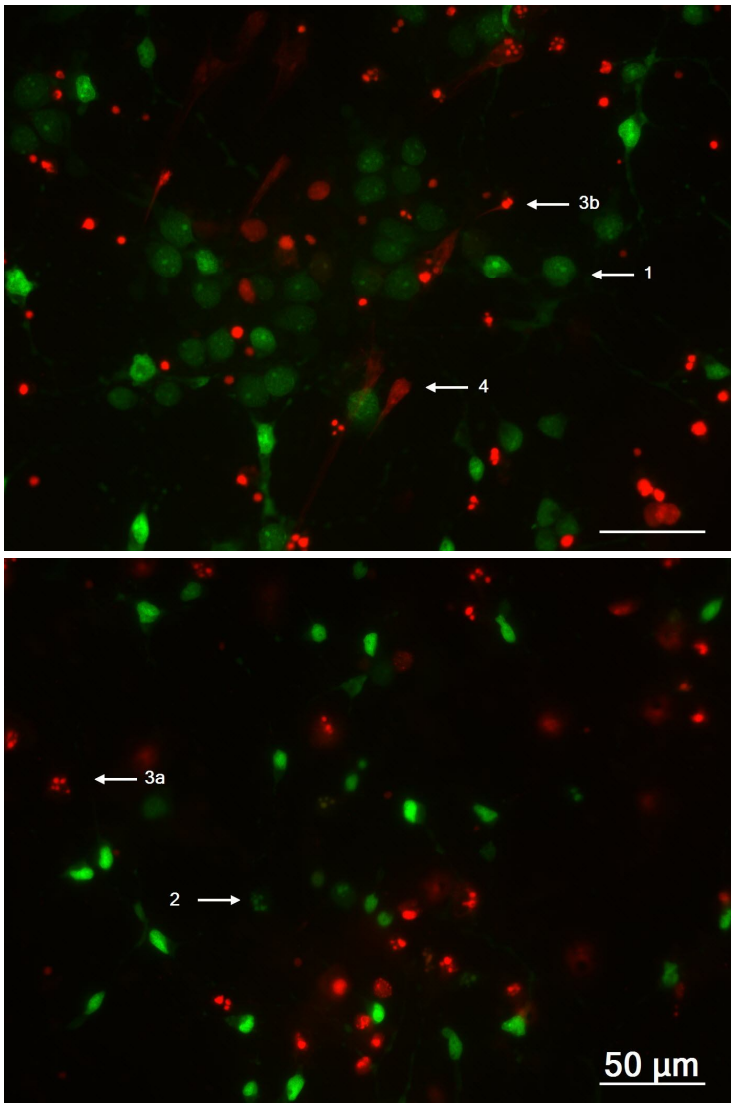

Figure 3.2 - Representative images of cultured cells labelled with PI and Syto-13 using 400x magnification. Cells were classified in 4 classes: **1)** living cells, which emits green radiation and presents a homogeneous cell body; **2)** primary apoptotic cells, green cells with fragmented or condensed nucleus; **3)** secondary apoptotic cells that emit in red band of the spectra and presents fragmented (3a) or condensed (3b) cell nucleus; and **4)** necrotic cells, which are presented as diffuse red blots. Scale bar represents 50 µm.

After cell counting, the formula used to obtain cell viability was:

$$\%\ viability = \frac{number\ of\ living\ cells}{number\ of\ total\ cells}$$

Equation 1 - Formula to obtain cell viability trough PI and Syto-13 labelling technique. The number of living cells corresponds to the ones that do not present any apoptotic or necrotic marker (represented as number 1 in Figure 3.2), whether total cells include the living plus the dying cells obtained from the sum of the cells that present apoptotic (2 and 3 from Figure 3.2) or necrotic markers (4 from Figure 3.2).

3.6. IMMUNOCYTOCHEMISTRY

This technique, firstly discovered by Coons et al. (1942), was used to characterize primary cortical neuronal cultures with 9 days *in vitro*. After cell medium removed, cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4 and 10 mM Na_2HPO_4, pH 7.4) and fixed for 10 minutes at room temperature with 4% PFA in PBS. After PBS washes cells were permeabilized with 0.1% Triton-X in PBS 0.1% gelatine. Cells were incubated for 1 hour at room temperature with primary antibodies diluted in PBS 0.1% gelatine (mouse anti-MAP2 1:200, Millipore MAB3418, and rabbit anti-GFAP 1:100, Sigma G9269). After washes (0.05% Tween-20 in PBS) cells were incubated with secondary antibodies diluted in PBS 0.1% gelatine (anti-mouse Alexa Fluor 568 and anti-rabbit Alexa Fluor 488, both 1:400, from Invitrogen). DAPI (70 µg/mL, Sigma) was used to label cell nucleus. Coverslips were mounted with MOWIOL (Sigma) and cells were observed in Axiovert 200 fluorescence microscope.

3.7. PREPARATION OF TOTAL PROTEIN EXTRACTS

Cells from primary cultures with 9 days *in vitro* and PC12 cells with 7 days of NGF differentiation were washed with cold PBS. Using NP-40 lysis buffer pH 8.0 (1% Nonidet P40, 150 mM NaCl, 50 mM Tris-base, 1 mM EDTA, 5 mM DTT, proteases inhibitors - Complete, EDTA-free Protease Inhibitor cocktail tablets, Roche) cells were mechanically scratched. The resulting solution was centrifuged at 13 000 rpm during 10 minutes at 4^{o}C and pellet (composed by cell nucleuses, intact cells and cell's residues) was discarded while the supernatant, composed by cellular proteins, was used in western blotting technique.

3.8. WESTERN BLOTTING

The protein concentration was achieved using the BioRad DC Protein assay Kit based on Lowry (1951) due to the high levels of detergents in the sample. The appropriate volume of each sample was diluted with water and sample buffer (350 mM Tris pH 6.8,

30% glycerol, 10% SDS, 600 mM DTT and 0.012% Bromophenol blue). The samples were denatured either at 60-70°C for 15-20 minutes in the particular case of A_{2A} receptor or at 95°C for 5 minutes for caspase-3.

Based on protocol of Towbin et al. (1979), the samples, the molecular weight markers and positive control (rat striatum homogenate for $A_{2A}R$) were separated by SDS-PAGE (10% or 12% according to the protein molecular weight and a 5% stacking) in denaturing conditions and electro-transferred to nitrocellulose membranes (GE Healthcare) or PVDF membranes (Millipore). The percentage of resolving gels and protein loading amounts are summarized in Table 1. Membranes were blocked with 5% non-fat dry milk for 1 hour, washed with TBS-T 0.1% (Tris Buffer Saline with 0.1% Tween-20 solution, 200 nM Tris, 1.5 M NaCl,) and incubated with primary antibody (diluted in TBS-T, 3% Bovine Serum Albumin and 0.1% NaN_3) overnight at 4ºC. After washing again for 30 minutes, the membranes were incubated with horseradish peroxidise (HRP, EC 1.11.1.7) conjugated secondary antibody (in 5% non-fat dry milk) for 1 hour at room temperature (primary and secondary antibody dilutions are in Table 1). After 40 minutes of washing with TBS-T followed by 20 minutes in TBS (same as TBS-T without 0.1% Tween-20), chemoluminescent detection was performed with ECL-PLUS western blotting detection reagent (GE Healthcare) using X-Ray films (Fujifilm). Optical density was determined with Image-J software and normalized to the respective α-tubulin or pro-caspase-3 band density.

Table 1 - Primary and secondary antibodies used in western blotting technique.

	Protein loading (µg)	Resolving gel %	Primary antibody	Animal	Dilution	Secondary antibody	Dilution
$A_{2A}R$	80	10	Upstate (sc-13937)	Mouse	1:4000	Santa Cruz Biotechnology (goat anti-rabbit, sc-2004; goat anti-mouse, sc-2005)	1:7500
Caspase-3	30	12	Santa Cruz Biotechnology (sc-7148)	Rabbit	1:1000		1:15000
α-Tubulin	-	-	Abcam (ab4074)	Rabbit	1:2000		1:15000

3.9. PHARMACOLOGICAL AGENTS

Urocortin and **antalarmin** were purchased from Sigma-Aldrich (Spain). **Staurosporine** was from Roche (Germany). **NGF 7S** was from Invitrogen (UK). 4-[2-[[6-Amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl] benzene propanoic acid (**CGS 21680**), 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo [4,3-e] [1,2,4] triazolo [1,5-c] pyrimidin-5-amine (**SCH 58261**), **L-Glutamic Acid** and **anti-Sauvagin-30** were pur-

chased from Tocris (UK). **$A\beta_{25-35}$ peptide** was from Bachem (Switzerland). These drugs were diluted in the assay solution from sock aliquots made in water or DMSO stored at -20^{o}C. All other reagents used were of the highest purity available and proper for cell cultures.

3.10. STATISTICS

The values presented are mean ± SEM of *n* independent experiments. In primary neuronal cell culture, each *n* is related to results obtained from embryos of different pregnant rats. In PC12 cells, each *n* represents one different passage of cells of a new thawed set. In statistical tests between three or more conditions, a one way ANOVA was used, followed by a Bonferroni's Multiple Comparison post hoc test. Values of $P<0.05$ were considered to be statistically significant. In each comparison, the following code was used: * $P<0.05$; ** $P<0.01$; *** $P<0.001$ to compare one condition to the control. # $P<0.05$; ## $P<0.01$; ### $P<0.001$ to compare one condition to glutamate 100µM. ‡ $P<0.05$; ‡‡ $P<0.01$; ‡‡‡ $P<0.001$ to compare with other condition rather than control or glutamate 100µM.

4. RESULTS

4.1. CHARACTERIZATION OF THE PRIMARY NEURONAL CELL CULTURES

Primary cortical neuronal cultures with 9 days *in vitro* (DIV) were used during the first part of this work. The cell preparation was labelled with anti-MAP2 (microtubule-associated protein 2 - neuronal marker) and anti-GFAP (glial fibrillary acidic protein - astrocyte marker) antibodies. Approximately 50% of cells were found to be red labelled, related to anti-MAP2 expression (Figure 4.1). The contamination with astrocytes was less than 20%, as shown by the cells positive for anti-GFAP green labelling. The remaining cells, labelled with DAPI, did express neither MAP-2 nor GFAP.

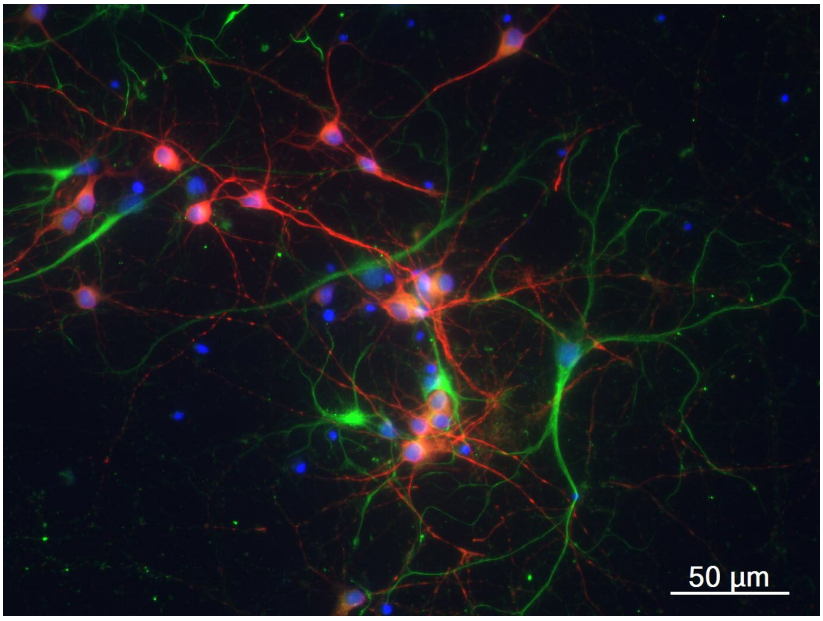

Figure 4.1 - Primary cortical neuronal culture with 9 days *in vitro* in control condition. Neurons are labelled with anti-MAP2 antibody that emits red radiation, whereas astrocytes present a green colour by staining with anti-GFAP antibody. Cell nucleus is marked with DAPI, which emits blue radiation. The photograph is from one representative cell culture preparation in the control condition.

4.2. CELL VIABILITY UPON GLUTAMATE INSULT

Glutamate exerts neurotoxic damages to cortical cultured cells through caspase-3 pathway activation (Du et al., 1997; Castro et al., 2004). This excitatory amino acid activates NMDA receptors leading to an increase of intracellular Ca^{2+} and Na^{+} (Schramm et al., 1990) and consequently to necrosis and apoptosis (Ankarcrona et al., 1995). Neuronal cultures were treated for 24 hours with five different glutamate concentrations (20, 50, 100, 500 and 1000 µM) that intended to represent several degrees of cell injury either by apoptosis or necrosis (Bonfoco et al., 1995). Amiloid-β_{25-35} peptide (Aβ, 25 µM) increases cell death by apoptosis (Estus et al., 1997) and was used as positive

control in cell viability assessment. Cell survival upon glutamate insult was obtained by labelling with propidium iodide (PI) which is incorporated by dying or death cells emitting in red band of spectrum and with Syto-13 which stains with green living cells.

Control (CTR) cells with 9 days *in vitro* presented approximately 64.4 ± 0.834% (n=6) of viability that correspond to the percentage of cells that do not present any apoptotic or necrotic markers, i.e. condensed or fragmented apoptotic nucleus, either green or red, or characteristic red blots of necrotic cells. As presented in Figure 4.2, preincubation with glutamate resulted in a reduction of cell viability, in a concentration-dependent manner, reaching the minimum viability of 66.2 ± 5.97% (normalized to the control, P<0.001, n=3) for glutamate 1000 µM.

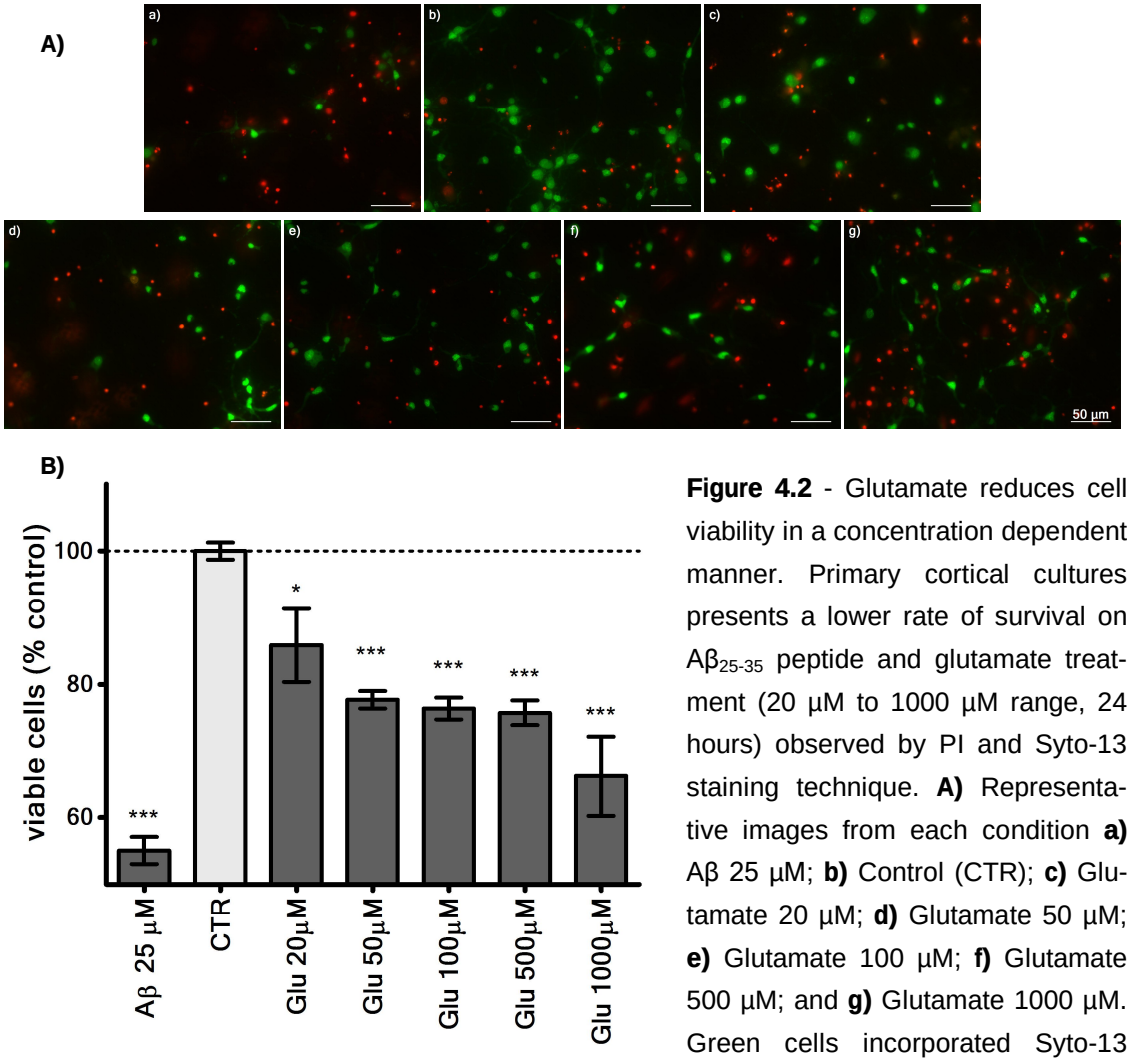

Figure 4.2 - Glutamate reduces cell viability in a concentration dependent manner. Primary cortical cultures presents a lower rate of survival on $A\beta_{25-35}$ peptide and glutamate treatment (20 µM to 1000 µM range, 24 hours) observed by PI and Syto-13 staining technique. **A)** Representative images from each condition **a)** Aβ 25 µM; **b)** Control (CTR); **c)** Glutamate 20 µM; **d)** Glutamate 50 µM; **e)** Glutamate 100 µM; **f)** Glutamate 500 µM; and **g)** Glutamate 1000 µM. Green cells incorporated Syto-13 while red cells are labelled with PI. Scale bar represents 50 µm. **B)** Percentage of cell viability in previously described conditions, compared to control. During all experiments control has approximately 65% of viable cells (all but apoptotic and necrotic cells). Each bar is the mean ± SEM of three to six experiments. * P<0.05 and *** P<0.001 compared to control, calculated using a one way ANOVA test plus a Bonferroni post hoc test.

Neuronal apoptosis induced by glutamate insult is mediated by caspase-3 (Du et al., 1997). Caspase-3 is an effector caspase responsible for the late apoptotic process stages that leads to controlled cell death (Porter and Janicke, 1999; Zeiss, 2003). Caspase-3 fragment formed by hydrolysis from pro-caspase-3 was quantified by *Western blotting* technique as an apoptotic marker.

Caspase-3 levels were increased in glutamate and $A\beta_{25-35}$ peptide treated cells, as shown in Figure 4.3. The caspase-3 levels were gradually increased through glutamate concentration reaching a maximum level at 50 µM (468 ± 20.3% of control, P<0.001, n=3). For higher concentrations of glutamate, caspase-3 levels slightly decreased in relation to the maximum level. Staurosporine, an apoptosis inducer, failed to increase caspase-3 levels (185±83.0% of control, non significative from control, n=3, data not shown).

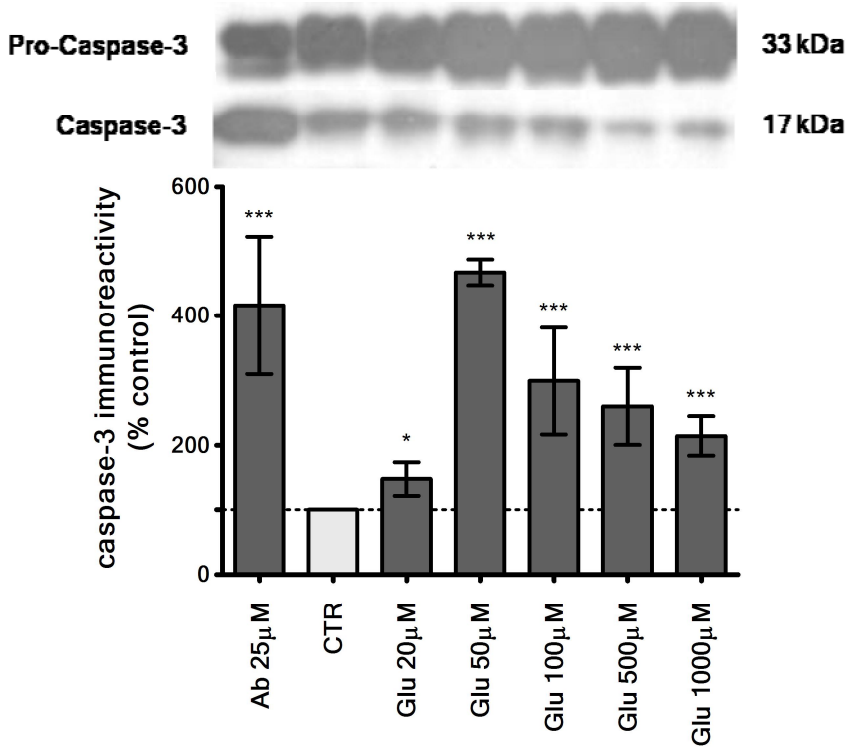

Figure 4.3 - Glutamate insult (20 to 1000 µM) increases caspase-3 levels in primary cortical cells homogenates. Using Western blotting, the specific caspase-3 immunoreactivity was normalized to pro-caspase-3 in each condition. $A\beta_{25-35}$ peptide 25 µM was used as positive control in pro-caspase-3 fragmentation. Each bar is the mean ± SEM of three to four experiments. * P<0.05; *** P<0.001 compared to control calculated using a one way ANOVA test, followed by a Bonferroni post hoc test. At the top a representative image of the western blot is presented.

4.3. EFFECT OF CORTICOTROPHIN RELEASING FACTOR ON GLUTAMATE NEUROTOXICITY

Urocortin, a CRF family peptide, activates both subtype 1 and 2 of CRF receptors, CRF_1R and CRF_2R (Vaughan et al., 1995). Urocortin 10pM was applied to cell medium immediately before glutamate (20 to 1000 µM range). In Figure 4.4 it is possible to ob-

serve that urocortin increased cell survival in the presence of glutamate, reaching statistical significance for 50 µM (from 77.7±1.31% to 88.6±1.36%, P<0.01, n=3) and for 100 µM (from 76.4±1.63% to 90.5±2.23%, P<0.001, n=3). For higher concentrations of glutamate (500 and 1000 µM) urocortin did not change cell viability as compared to glutamate alone. Urocortin by itself did not alter cell viability (95.3±2.44% compared to control 100±1.29%, n=3).

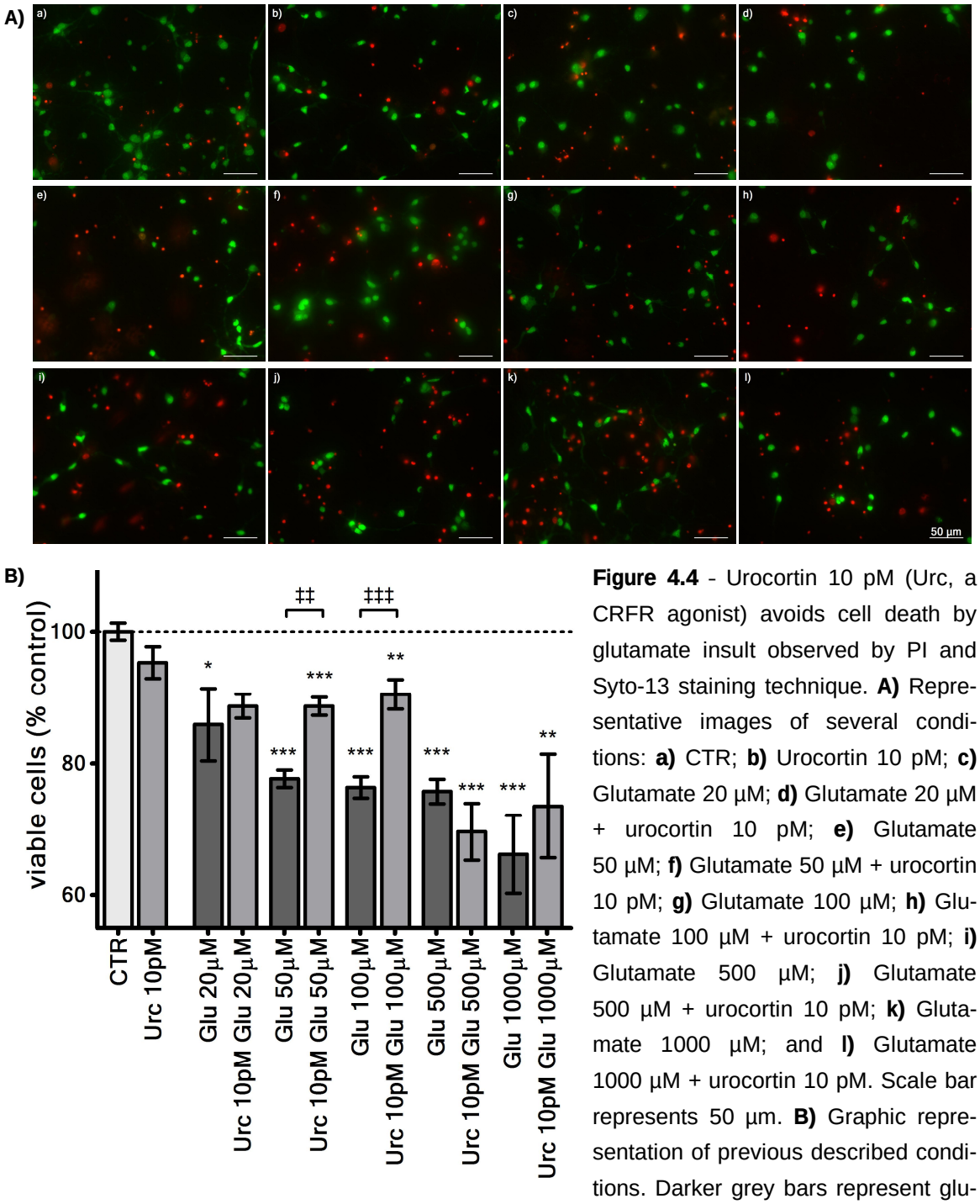

Figure 4.4 - Urocortin 10 pM (Urc, a CRFR agonist) avoids cell death by glutamate insult observed by PI and Syto-13 staining technique. **A)** Representative images of several conditions: **a)** CTR; **b)** Urocortin 10 pM; **c)** Glutamate 20 µM; **d)** Glutamate 20 µM + urocortin 10 pM; **e)** Glutamate 50 µM; **f)** Glutamate 50 µM + urocortin 10 pM; **g)** Glutamate 100 µM; **h)** Glutamate 100 µM + urocortin 10 pM; **i)** Glutamate 500 µM; **j)** Glutamate 500 µM + urocortin 10 pM; **k)** Glutamate 1000 µM; and **l)** Glutamate 1000 µM + urocortin 10 pM. Scale bar represents 50 µm. **B)** Graphic representation of previous described conditions. Darker grey bars represent glutamate concentrations in absence of urocortin, whereas brighter grey bars show the values of glutamate with urocortin 10 pM. Results are mean ± SEM of three experiments. * P<0.05, ** P<0.01 and *** P<0.001 compared to control; ‡‡ P<0.01, ‡‡‡ P<0.001, compared the two selected conditions, calculated using a one way ANOVA test plus a Bonferroni post hoc test.

In parallel to cell viability assays, caspase-3 levels were measured by Western blotting. The results obtained, represented in Figure 4.5, present the role of urocortin in caspase-3 formation. Compared to the same glutamate concentration, urocortin had a tendency to increase caspase-3 in the first segment of the curve (below glutamate 50 µM) and to decrease it in the second segment (above glutamate 100 µM). Urocortin alone did not change this terminal apoptotic marker (108±9.13%, non significative differences compared to control, n=3).

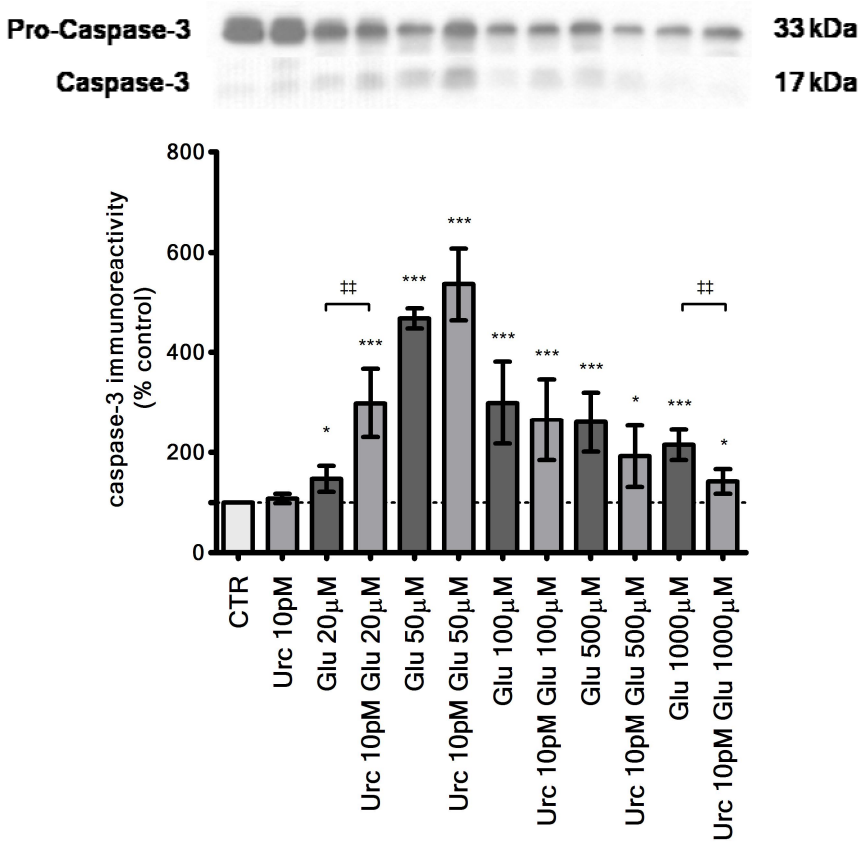

Figure 4.5 - Caspase-3 immunoreactivity observed by western blotting technique. Caspase-3 immunoreactivity for each condition was normalized to the one of pro-caspase-3. Dark grey bars represent the values of pro-caspase-3 fragmentation in previously described glutamate concentrations. In brighter grey are shown the same glutamate concentration with urocortin 10 pM. Results are mean ± SEM of three to four experiments. * P<0.05 and *** P<0.001 compared to control, ‡‡ comparing the two shown conditions, calculated using a one way ANOVA test followed by a Bonferroni post hoc test. At the top a representative image of the western blot is presented.

Glutamate 100 µM was the concentration selected for the following experiments. At this concentration, glutamate causes mild apoptosis that is characterized by condensed nucleus without DNA fragmentation (see Figure 4.4.e for an example), in opposite to lower glutamate concentrations where apoptosis is characterized by nucleus fragmentation, as glutamate 20 µM (see Figure 4.4.c). Glutamate 100 µM is enough to induce measurable cell death and simultaneously avoid cell detachment caused by cell necrosis (Ankarcrona et al., 1995). Higher concentrations of glutamate are associated to cell necrosis (with consequent plate detaching) while lower concentrations resulted in an insignificant amount of cell death as observed in this work and in previous ones (Ankarcrona et al., 1995).

We now intended to describe the subtype of CRFR that is responsible for this protection by using selective CRF_1R and CRF_2R antagonists, respectively antalarmin 10nM (Ant, firstly discovered by Chen et al., 1996) and anti-Sauvagine-30 10nM (a-Sau, presented by Ruhmann et al., 1998).

According to Figure 4.6, the previously observed protection by urocortin 10pM against cell death was lost by blocking CRF_1R or CRF_2R independently, by antalarmin (decreasing from 90.5± 2.23% to 73.9± 3.53%, $P<0.01$, n=4) or anti-Sauvagine-30 (from 90.5± 2.23% to 76.0±2.80%, $P<0.01$, n=4). Curiously, when the two antagonists were applied simultaneously, the effect of urocortin was not prevented. The effect of the two antagonists in the absence of urocortin did not affect cell death induced by glutamate 100 µM (76.4±1.63%, with glutamate 100 µM, and 70.4±3.97%, glutamate 100 µM plus both antagonists, n=4). In addition, the two antagonists applied together did not affect cell viability by themselves, in the absence of the glutamate insult (99.0±3.88%, Figure 4.7, 6^{th} bar).

The effect of the different drugs alone on cell viability was controlled (Figure 4.7). Glutamate 100µM reduced cell viability by 23.6±1.63% ($P<0.001$, n=4), whereas neither CRFR nor $A_{2A}R$ ligands affected cell viability assessed by PI and Syto-13 labelling.

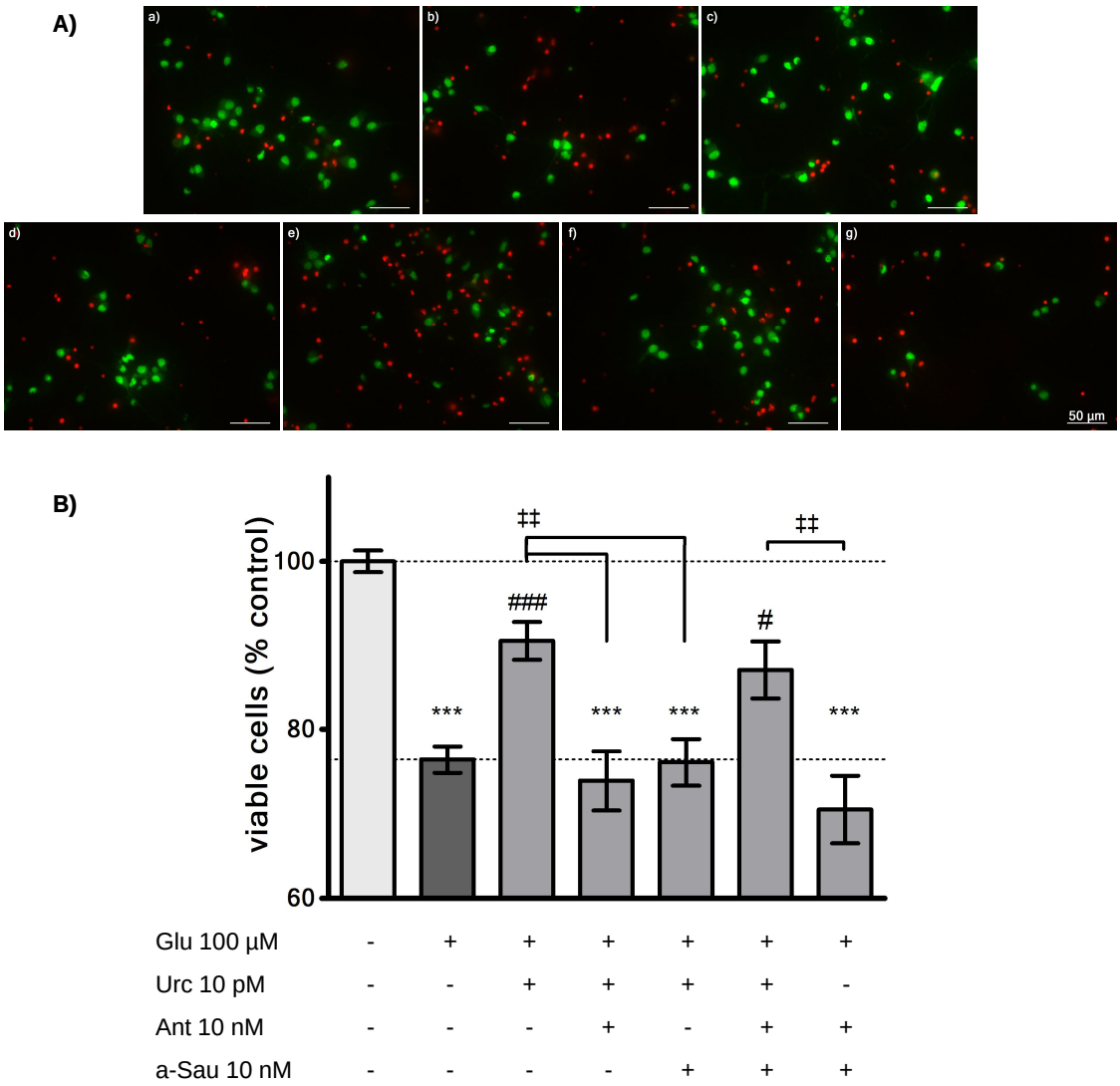

Figure 4.6 - Assessment of cell viability by PI and Syto-13 labelling method in presence of glutamate 100 µM and CRFR agonist (Urc 10 pM) and antagonists (Ant 10 nM and a-Sau 10nM). **A)** Representative images of each condition are presented: **a)** CTR; **b)** Glutamate 100 µM; **c)** Glutamate 100 µM + urocortin 10 pM; **d)** Glutamate 100 µM + urocortin 10 pM + antalarmin 10 nM; **e)** Glutamate 100 µM + urocortin 10 pM + anti-Sauvagine-30 10 nM; **f)** Glutamate 100 µM + urocortin 10 pM + antalarmin 10 nM + anti-Sauvagine-30 10 nM; and **g)** Glutamate 100 µM + antalarmin 10 nM + anti-Sauvagine-30 10 nM. Scale bar represents 50 µm. **B)** Graphic summary of previous conditions. Lines at 100% and near 80% represent control and glutamate 100 µM. Each value is mean ± SEM of three to four experiments. * P<0.05 and *** P<0.001 compared to control; ### P<0.001 compared to glutamate 100 µM; ‡‡ P<0.01; ‡‡‡ P<0.001 comparing the selected conditions, calculated using a one way ANOVA test followed by a Bonferroni post hoc test.

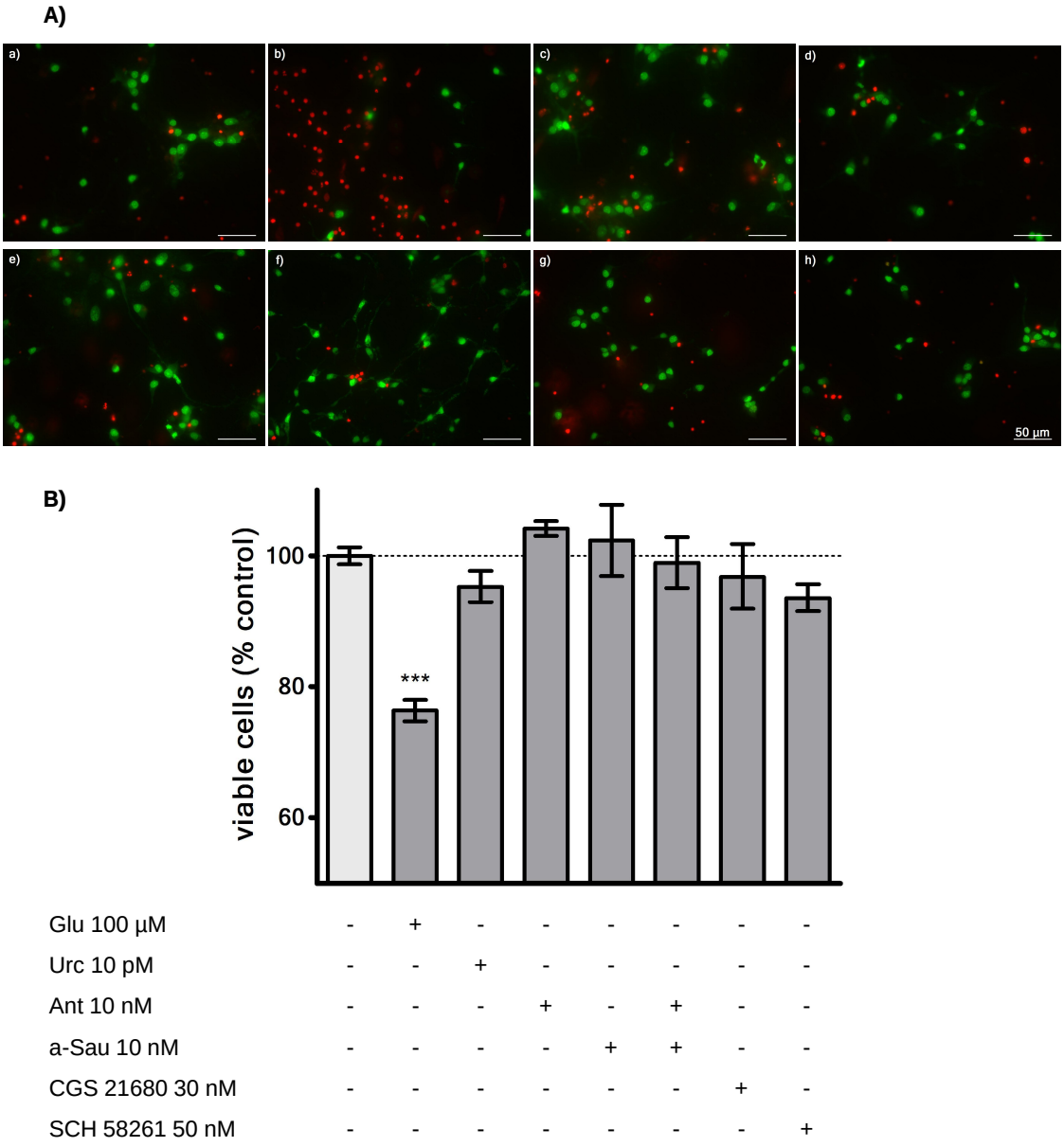

Figure 4.7 - Effects of each drug on cell viability by PI and Syto-13 labelling. **A)** Representative images of each condition are presented: **a)** CTR; **b)** Glutamate 100 µM; **c)** Urocortin 10 pM; **d)** Antalarmin 10 nM; **e)** anti-Sauvagine-30 10 nM; **f)** Antalarmin 10 nM + anti-Sauvagine-30 10 nM **g)** CGS 21680 30 nM; **h)** SCH 58261 50 nM. Scale bar represents 50 µm. **B)** Graphic summary of previous conditions. Lines at 100% represent control. Each value is mean ± SEM of three to five experiments. *** P<0.001 compared to control, calculated using a one way ANOVA test followed by a Bonferroni post hoc test.

4.4. Involvement of A_{2A} receptors on CRF neuroprotection

It was previously described that activation of adenosine A_{2A} receptors ($A_{2A}R$) leads to an exacerbated cell death. In an opposite way, blockade of $A_{2A}R$ reverses glutamate induced cell death (Castillo et al., 2010). We now studied the possible interaction between $A_{2A}R$ and CRFR effects. By blocking $A_{2A}R$, with a selective antagonist (SCH 58261 50nM), or activating, with a selective agonist (CGS 21680 30nM), we evaluated the cell viability in presence of CRFR agonist, urocortin 10pM, and antagonists, antalarmin 10nM and anti-Sauvagine-30 10nM.

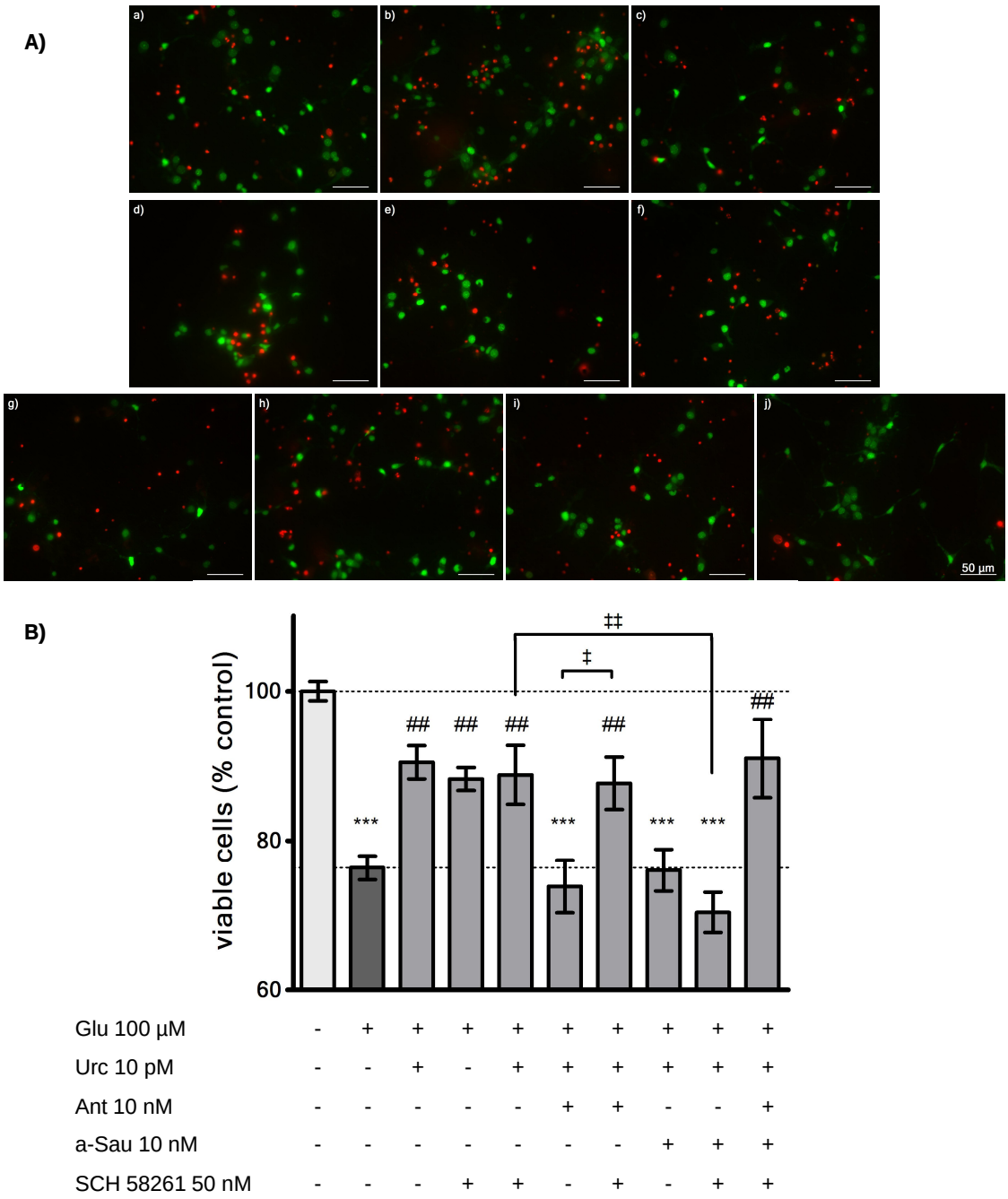

Figure 4.8 - $A_{2A}R$ blockade changes cell viability obtained by PI and Syto-13 labelling method. **A)** Representative images of different conditions: **a)** CTR; **b)** Glutamate 100 µM; **c)** Glutamate 100 µM + urocortin 10 pM; **d)** Glutamate 100 µM + SCH 58261 50nM; **e)** Glutamate 100 µM + urocortin 10pM + SCH 58261 50nM; **f)** Glutamate 100 µM + urocortin 10pM + antalarmin 10nM; **g)** Glutamate 100 µM + urocortin 10pM + antalarmin 10nM + SCH 58261 50nM; **h)** Glutamate 100 µM + urocortin 10pM + anti-Sauvagine-30 10nM; **i)** Glutamate 100 µM + urocortin 10pM + anti-Sauvagine-30 10nM + SCH 58261 50nM; and **j)** Glutamate 100 µM + urocortin 10pM + antalarmin 10nM + anti-Sauvagine-30 10nM + SCH 58261 50nM. Scale bar represents 50 µm. **B)** Graphical representation of previous conditions. Lines at 100% and near 80% represent control and glutamate 100 µM. Each value is mean ± SEM of three to five experiments. *** P<0.001 compared to control; ‡ P<0.05 and ‡‡ P<0.01 between the two shown conditions; ## P<0.01 compared to glutamate 100 µM, calculated using a one way ANOVA test followed by a Bonferroni post hoc test.

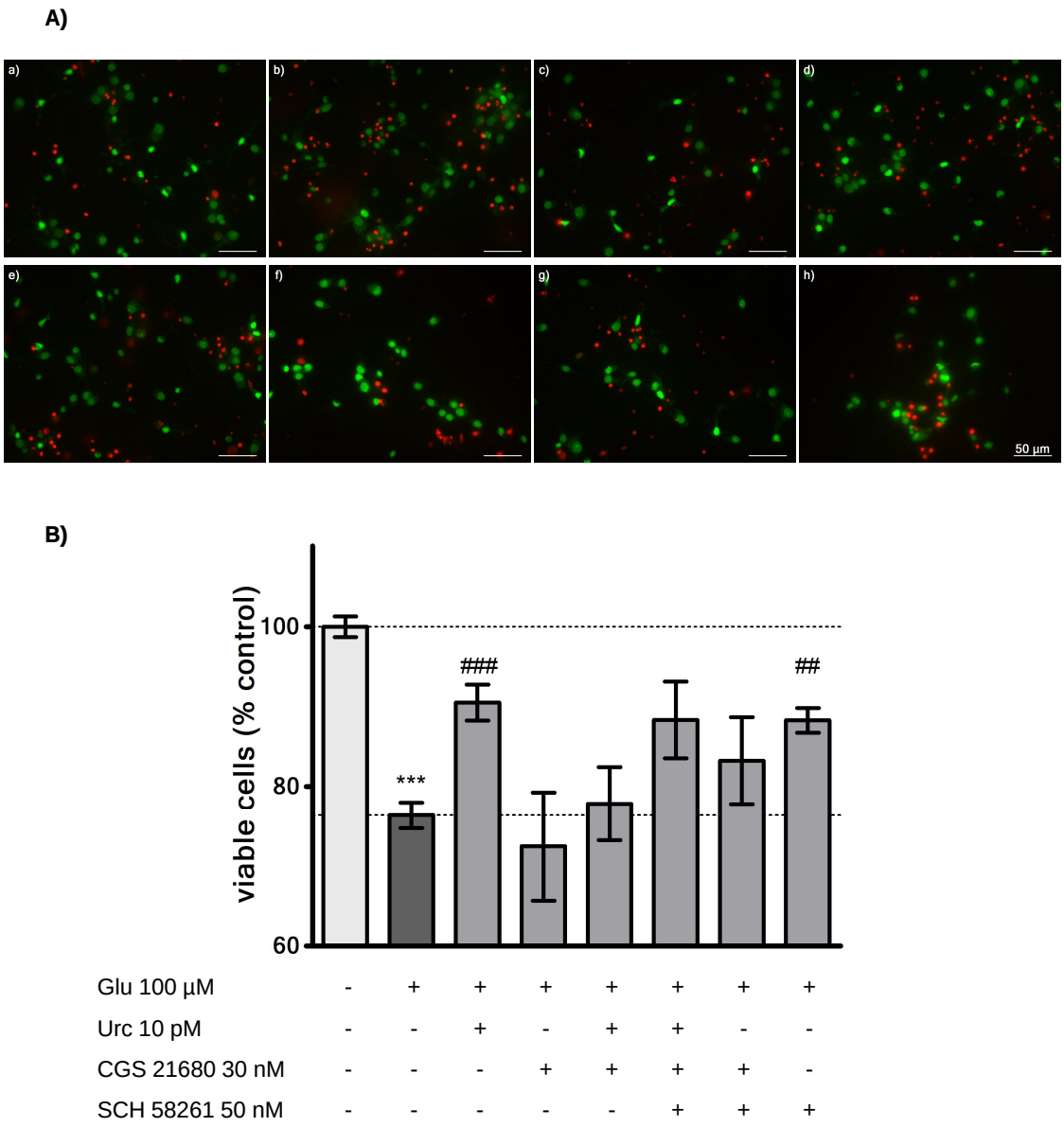

Figure 4.9 - Activation of $A_{2A}R$ by CGS 21680 30nM in neuronal viability upon a glutamate insult. The ratio of cell death was achieved by PI and Syto-13 technique. **A)** On Top, representative images of following conditions: **a)** CTR; **b)** Glutamate 100 µM; **c)** Glutamate 100 µM + urocortin 10 pM; **d)** Glutamate 100 µM + CGS 21680 30nM; **e)** Glutamate 100 µM + urocortin 10 pM + CGS 21680 30nM; **f)** Glutamate 100 µM + urocortin 10 pM + CGS 21680 30nM + SCH 58261 50nM; **g)** Glutamate 100 µM + CGS 21680 30nM + SCH 58261 50nM; and **h)** Glutamate 100 µM + SCH 58261 50nM. **B)** Schematic representation of previously described conditions. Lines at 100% and near 80% represent control and glutamate 100 µM. The results (mean ± SEM) were obtained from three experiments. *** P<0.001 compared to control; ## P<0.01, ### P<0.001 compared to glutamate 100 µM, calculated using a one way ANOVA test followed by a Bonferroni post hoc test.

As presented in Figure 4.8, the blockade of $A_{2A}R$ by its selective antagonist, SCH 58261 (50 nM) did not change the neuroprotection induced by Urc. However, SCH 58261 (50 nM) alone prevented the cell death induced by glutamate (76.4±1.63% to 88.3±1.53%, P<0.01, n=3). This improvement of cell viability due to SCH 58261 was

maintained with selective blockade of CRF_1R (87.6±3.48%, n=4) but not with CRF_2R blockade (74.8±4.91%, P<0.01 compared with SCH 58261 and glutamate 100 µM, n=5). Curiously, the ability of SCH 58261 to increase cell viability relative to glutamate 100 µM was maintained even when both CRFR agonist and antagonists are present (91.0±5.23% compared to 76.4±1.63%, P<0.01, n=3).

On contrast, as shown in Figure 4.9, $A_{2A}R$ activation with CGS 21680 30nM did not altered cell death in presence of glutamate 100 µM (72.4±6.79%, not reaching statistical significance from glutamate 100 µM, n=4), but seems to avoid urocortin neuroprotection previously presented (from 90.5±2.23% to 77.8±4.59% with CGS 21680, n=3). However, SCH 58261, an $A_{2A}R$ antagonist had a tendency to revert CGS 21680 effects in glutamate toxicity, ensuring that the selective $A_{2A}R$ agonist, CGS 21680, was acting mostly in this subtype of receptors.

4.5. MODULATION OF A_{2A} RECEPTOR LEVELS BY CRF

In order to further understand the neuroprotective effects of CRF and its interaction with $A_{2A}R$, we evaluated whether the levels of expression of these receptors are affected by crossactivation. The cortical expression of A_{2A} receptors is very low (Lopes et al., 1999) and is technically difficult to detect the expression levels of this receptor subtype. So, we used nerve growth factor (NGF)-differentiated PC12 cells that proved to be a valid model to study changes in $A_{2A}R$ since these receptors are highly expressed in these cells (Arslan et al., 1997) which display a neuronal phenotype in these conditions (Greene and Tischler, 1976). In this task the levels of $A_{2A}R$ will be measure in homeostatic conditions, i.e. no excitotoxic insult was applied to the cells.

We then intended to observe variations in $A_{2A}R$ in the presence of its ligands as well as CRFR agonist (urocortin) and antagonist (antalarmin). The results presented in the Figure 4.10 showed that $A_{2A}R$ levels are regulated by their own ligands but are not affected by CRFR ones. While the $A_{2A}R$ agonist, CGS 21680, downregulated (48.9±5.5%, P<0.001 compared to control, n=4) the antagonist, SCH 58261, upregulated $A_{2A}R$ levels (144±15.2%, P<0.01 compared to control, n=5). Neither CRF agonist, urocortin (88.3±9.4% of control, n=4), nor antagonist, antalarmin (80.7±14.5% in relation to control, n=3), significantly changed $A_{2A}R$ levels in NGF-differentiated PC12 cells.

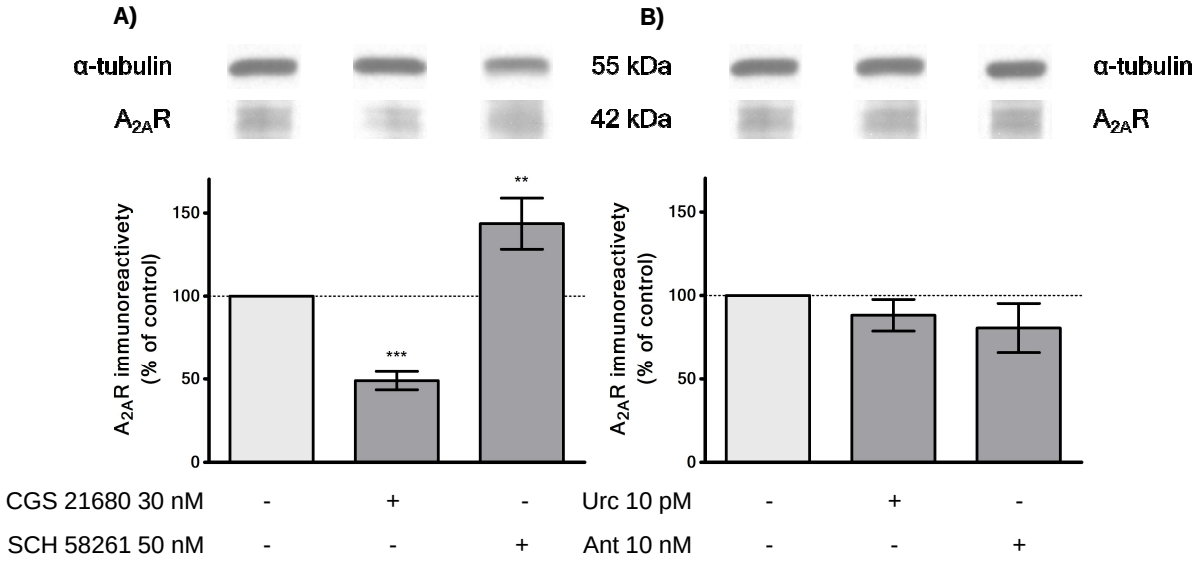

Figure 4.10 - **A)** Modulation of $A_{2A}R$ levels by its agonist, CGS 21680 30 nM, and antagonist, SCH 58261 50 nM, in NGF-differentiated PC12 cells. **B)** $A_{2A}R$ levels are not changed with CRFR agonist, urocortin 10 pM, or antagonist, antalarmin 10 nM. $A_{2A}R$ immunoreactivity was normalized to the correspondent α-tubulin immunoreactivity. Results are mean ± SEM of three to five experiments. ** P<0.01; *** P<0.001 compared to control, calculated using a one way ANOVA test followed by a Bonferroni post hoc test. At the top a representative image of the western blot is presented.

As presented in Figure 4.11, CRFR ligands did not change the previously described regulation of $A_{2A}R$ by CGS 21680 (49.9±9.5% for urocortin in presence of CGS 21680, n=5 and 42.5±7.26% when CRF_1R are blocked in presence of CGS 21680, n=6). In an opposite direction, CRFR binding molecules altered $A_{2A}R$ levels when applied simultaneously with SCH 58261. In Figure 4.12, while urocortin seems to be irrelevant to modify the upregulation of $A_{2A}R$ by SCH 58261 (115±29.9%, n=5), antalarmin decreased $A_{2A}R$ levels, either in presence of urocortin (84.5±13.9%, P<0.05 compared with SCH 58261, n=5) or alone when $A_{2A}R$ are blocked (39.4±14.4%, P<0.01 compared to SCH 58261 alone or control conditions, n=4).

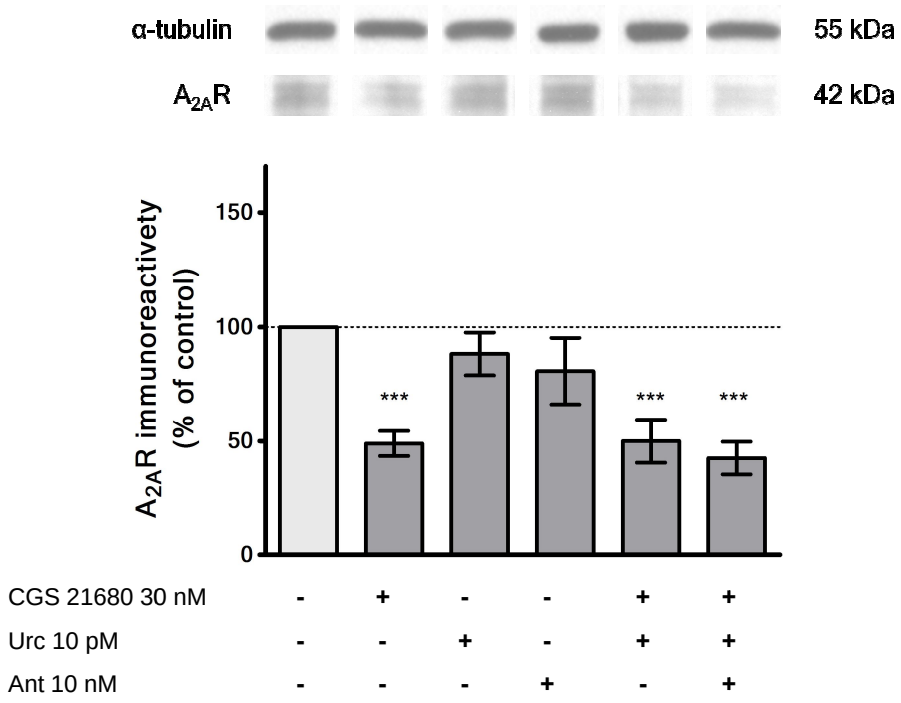

Figure 4.11 - CGS 21680 downregulation of $A_{2A}R$ levels in differentiated PC12 is not changed by CRFR ligands (urocortin 10 pM and antalarmin 10 nM). $A_{2A}R$ immunoreactivity was normalized to α-tubulin. Results are mean ± SEM of three to five experiments. *** P<0.001 compared to control, calculated using a one way ANOVA test followed by a Bonferroni post hoc test. At the top a representative image of the western blot is presented.

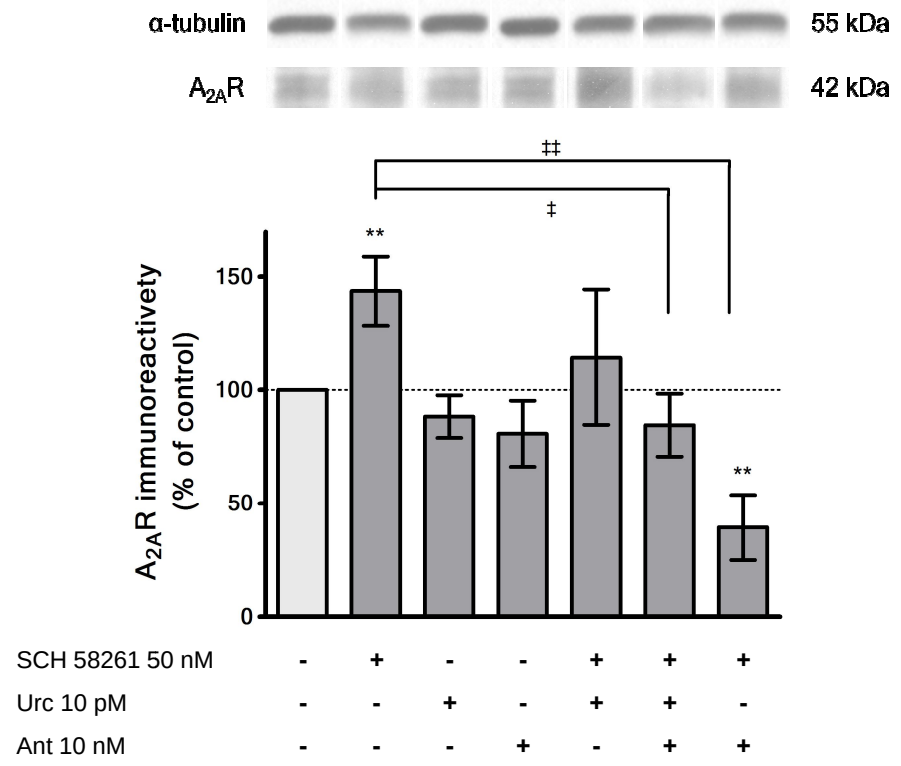

Figure 4.12 - SCH 58261 upregulation of $A_{2A}R$ levels in differentiated PC12 is reversed by CRFR antagonist, antalarmin 10nM. $A_{2A}R$ signal was normalized to α-tubulin. Results are mean ± SEM of three to five experiments. ** P<0.001 compared to control, ‡ P<0.05, ‡‡ P<0.001 compared to the shown condition, calculated using a one way ANOVA test followed by a Bonferroni post hoc test. At the top a representative image of the western blot is presented.

5. DISCUSSION

The release of adenosine that occurs as a consequence of hypoxic events in the brain (Andine et al., 1990) is accompanied by an increase in the levels of the stress regulator, corticotrophin-releasing factor (CRF, Chen et al., 2004a). In addition, the *in vivo* modulation of adenosine A_{2A} receptors ($A_{2A}R$) is responsible for reversion of stress-induced effects in the hippocampus (Batalha et al., 2010). This raises the question whether $A_{2A}R$ regulate the levels or function of the main stress mediators, either CRF or glucocorticoids. We now intended to relate the neuroprotective effects of $A_{2A}R$ blockade with the activation of CRF receptors (CRFR), under stress conditions (glutamate insult), in an attempt to disclose a possible pharmacological synergy.

Two basic approaches were used. On the initial tasks, primary rat neuronal cortical cultures were used to evaluate cell death through a glutamate insult protocol. This model was abundantly used in the past to observe neuroprotective effects of several drugs and their target receptors on cell death, either by apoptosis or necrosis (Tamura et al., 1993). On a second step, the regulation of $A_{2A}R$ levels was studied in NGF-differentiated PC12 cells.

The major findings of this work were 1) a protective role of CRF in cortical neurons against glutamate insult that is dependent on both CRF receptor subtypes, CRF_1R and CRF_2R; 2) that the CRF-induced neuroprotection was provided either directly by CRFR activation or by modulating $A_{2A}R$ actions, through a downregulation of $A_{2A}R$ mediated by CRF_2R activation.

5.1. ESTABLISHMENT OF A NEURONAL MODEL THAT RESPONDS TO EXCITOTOXIC INSULTS

Primary rat cortical neuronal cultures are mainly composed by neuronal cells. Although these cells are the core of central nervous system function, astrocytes hold an important role in brain homeostasis, either by regulating neuronal metabolism or by removing signalling molecules from synaptic cleft, as neurotransmitters. Due to this symbiotic behaviour, the cultures used in this work present a low percentage of astrocytes (less than 20%). In addition, neuroprotective effects of adenosine analogues against excitotoxic mediators were previously related to glial cells (reviewed by Fields and Burnstock, 2006).

During glutamate insult protocols, cell viability was measured by propidium iodide (PI) and Syto-13 uptake technique. Cultured cells with 9 days *in vitro* present a viability of

approximately 65% which is in accordance to previous published works (Tamura et al., 1993; Pedersen et al., 2001; Rebola et al., 2005). Glutamate increase cell death in *in vitro* neurons in a concentration dependent form, reaching significant differences from control with a minimum of 20 µM which is in accordance with previous results (Choi et al., 1987). With the lowest glutamate concentrations, cells present a high rate of pro-caspase-3 fragmentation, an apoptotic mechanism characteristic of glutamate insult in this type of cells (Du et al., 1997). However, for higher glutamate concentrations (above 100 µM) cell death is not caused mostly by apoptosis. For these glutamate concentrations cells preferentially die by necrosis, which is a caspase-3 independent process. These observations were confirmed by a concentration dependent increase in cell death by PI and Syto-13 method without the correspondent raise of pro-caspase-3 fragmentation. Presumably rapid necrosis is preferred at high glutamate concentrations due to a robust activation of NMDA and AMPA receptors that drastically increases intracellular calcium and sodium and disrupts the mitochondrial membrane potential and leads to cellular swelling by osmosis (reviewed by Greenwood and Connolly, 2007). This necrotic process is characterized by an early membrane rupture caused by an acute but severe stressful event. On other hand, apoptosis is a form of controlled cell death that is characterized by the presence of a signalling cascade, in which caspases have a crucial role. Similarly to necrosis, apoptosis is triggered by mitochondrial swelling and cytochrome *c* releasing. At low concentrations of glutamate (below 100 µM) necrosis is replaced by a delayed apoptosis (Ankarcrona et al., 1995; Bonfoco et al., 1995). In our experimental conditions, although AMPA receptors desensitize through time, NMDA do not (Fedele and Raiteri, 1996; Nahum-Levy et al., 2001). This leads to a continuous incorporation of calcium in intracellular medium, which will induce apoptosis rather than necrosis at low concentrations of glutamate, as observed by Bonfoco et al. (1995).

Although PI and Syto-13 labelling provides quick and visual perceptive results it is inadequate to measure death by necrosis. Cells are attached to poli-L-lysine but when a necrotic stimulus takes place cell membrane ruptures and all intracellular components are released to extracellular medium, which includes DNA. As this method uses DNA probes, it is difficult to observe all the necrotic cells after an extreme toxic insult (Ankarcrona et al., 1995). As a consequence, although the number of total cells in each coverslip did not decrease with glutamate concentration (data not shown), the values obtained for cell mortality with higher glutamate concentrations can be underestimated. To avoid this technical limitation, a commercial LDH assay kit was used to measure the amount of lactate dehydrogenase (LDH, E.C. 1.1.1.27), a cytoplasmatic enzyme that is

released to cell medium as a consequence of the necrotic process. However, this method did not show any relevant changes even with an extreme concentration of glutamate, 10 mM (data not shown).

5.2. UROCORTIN PREVENTS GLUTAMATE INDUCED CELL DEATH

Primary cortical neuronal cultured cells used in this work have been described to possess 9.8 fmol/mg protein of CRFR (Kapcala and Dicke, 1992), with a higher preponderance of CRF_1R compared to CRF_2R in cerebral cortex (Reul and Holsboer, 2002). However, CRF_2R are also present in our working model since brain areas where they are mostly expressed (as the hippocampus) were not removed during the process. Urocortin (a peptide from CRF family that has equivalent affinity to CRF_1R and CRF_2R) decreased cell death by glutamate insult in all concentrations except glutamate 500 µM, reaching a statistical significance with its maximum effect in glutamate 100 µM insulted cells. This concentration of glutamate enhances cell death both by apoptosis and necrosis. For higher concentrations, severe cell necrosis occurs and urocortin seems to be inefficient in reverting cell death. On the other hand, at lower glutamate concentrations, the evoked cell death may not reach enough magnitude to allow a significant and measurable decrease of cell death with urocortin.

This ability of urocortin to prevent glutamate-induced cell death is not mimicked by the levels of caspase-3, a final apoptotic marker, instead of viability. Actually, these results suggest that urocortin may, in fact, contribute to apoptosis for lower glutamate concentrations (below 100 µM) and decrease it for higher glutamate concentrations. Having in mind that apoptosis is the primary process below 100 µM of glutamate and necrosis is preferred above that concentration, probably the protection said from cell death that we see by urocortin is rather on necrosis than apoptosis. As presented before, for lower concentrations of glutamate, caspase-3 levels even increase further with urocortin treatment. We assume that this is caused due to elevation of intracellular calcium by activation of CRFR, involving PKC and PKA pathways (Blank et al., 2003), which will release calcium from endoplasmic reticulum but also increase calcium transport from extracellular medium (Dermitzaki et al., 2004). This increase in cytoplasmatic calcium concentration will affect mitochondria membrane potential and initiate apoptotic cascade through caspase-3 signalling. However, this effect on caspase-3 has no repercussion on overall cell viability possibly due to an amplified expression of pro-survival factors like BDNF caused by a delayed action of CRFR on gene expression (Bayatti et al., 2005; Hauger et al., 2009). CRF was also found to potentiate BDNF effects through

TrKB activation, a process that promotes cell survival (reviewed by Hauger et al., 2009).

Pedersen and colleagues (2001) had observed neuroprotective effects of urocortin in hippocampal and cortical neurons against 20 µM of glutamate, a very low concentration. However, they only assessed viability with no distinction of the cell death process occurring. With that glutamate concentration, the apoptosis process is favoured in relation to necrosis (Ankarcrona et al., 1995). Therefore, to mimic the evoked cell death caused by excessive excitatory amino acids in ischemia, which is a mixed process of apoptosis and necrosis (Martin et al., 1998), as we have now shown, care must be taken and higher concentrations of glutamate should be used instead.

5.3. CRF_1R AND CRF_2R ACTIVATIONS HAVE A NEUROPROTECTIVE ROLE IN GLUTAMATE EXCITOTOXICITY

CRF receptors subtypes have different functions either in HPA axis or in cell neuroprotection (Pedersen et al., 2002; Reul and Holsboer, 2002). We intended to observe which CRF receptor subtype is responsible for urocortin-induced neuroprotection against glutamate insults (100 µM). By using selective antagonists of each CRFR (antalarmin or anti-Sauvagine-30) we were able to observe a loss of effect of urocortin in glutamate evoked cell death, either by blocking CRF_1R or CRF_2R. CRF_1R activation was previously described to afford neuroprotection (Pedersen et al., 2002) and our work is in accordance with those studies. In previous reports, CRF_2R was discarded from any function in neuroprotection against oxidative stress (Pedersen et al., 2002), but there is no data available for the role of CRF_2R activation in glutamate-induced neurotoxicity. Urocortin 2, a CRF_2R agonist, is not able to revert cell death caused by radical oxygen species, whereas Urocortin, a CRFR nonspecific agonist is able to protect neuronal cells from equivalent insult, by activating CRF_1R (Pedersen et al., 2002).

Here we observe a neuroprotective effect by activating CRF_2R during glutamate insults in cortical neurons in culture. Despite reports that high concentrations of anti-Sauvagine-30 are also capable of blocking CRF_1R, K_D=154 nM to CRF_1R and K_D=1.4 nM to CRF_2R (Ruhmann et al., 1998), this does not seem to be the case, since the concentration used (10 nM) is ten times lower than the K_D for the CRF_1R receptor. Other important question related to the involvement of CRF_2R in neuroprotection is the ratio between the two receptors. While CRF_1R levels are elevated, CRF_2R are less expressed on cultured neurons (Reul and Holsboer, 2002), which could lead to unspecific blockade of CRF_1R by CRF_2R antagonists (as anti-Sauvagine-30). However this does not seem to be the case, since CRF_2R are present in primary cultures used and anti-

Sauvagine-30 concentration is insufficient to block completely CRF_1R. In conclusion, CRFR activation by urocortin is neuroprotective against glutamate insults only when both CRF_1R and CRF_2R are active, since blockade of each one selectively was enough to prevent the neuroprotective effect of urocortin.

Curiously, when applied together, the two CRFR antagonists failed to block the urocortin effect against cell death induced by glutamate. This result was unexpected due to previous data that suggest the involvement of both receptors in neuroprotection. We therefore tried to distinguish: 1) an unspecific action of urocortin in a receptor-independent manner (an effect never observed in previously published works) or 2) an interaction between the two molecules which could lead to the loss of antagonistic effect to either CRF_1R or CRF_2R. We tested the latter hypothesis, by searching for a direct interaction between antalarmin and anti-Sauvagine-30. As presented in appendix 6.1, antalarmin decreases the fluorescence emission of the amino acid phenylalanine present in anti-Sauvagine-30, in a concentration-dependent manner, suggesting a possible physical interaction between these two CRFR antagonists. Thus, using them together could lead to an ineffective blockade of both CRFR subtypes.

5.4. CRF_2R, BUT NOT CRF_1R, IS ESSENTIAL TO NEUROPROTECTION BY $A_{2A}R$ BLOCKADE

Adenosine A_{2A} receptors are modulatory targets against neurologic insults (reviewed by de Mendonca et al., 2000) and share functional similarities with CRF receptors, as neuroprotection (Fox et al., 1993). Therefore, we explored a possible interaction between these two receptors either at the protein expression levels or studying their functional role in the prevention of cell death by glutamate insult.

Using PC12 cells, we were able to assess the $A_{2A}R$ level which is technically difficult using neurons, since the expression levels in cortex and hippocampus are below the limit of detection of the technique (Lopes et al., 1999b). This cell line has an abundant expression of both $A_{2A}R$ and CRFR (Arslan et al., 1997; Dermitzaki et al., 2007). Using nerve growth factor (NGF) for 7 days, cells were differentiated to acquire a neuronal phenotype (Greene and Tischler, 1976). After these differentiation, PC12 cells present a neuronal like morphology (neurite like fibers growth) and synthesize and store dopamine and norepinephrine that are released by calcium dependent processes (Greene and Tischler, 1976). The $A_{2A}R$ concentration in differentiated cells is of 472 fmol/mg of protein (Arslan et al., 1997). Comparing to neuron cells (193±18 fmol/mg protein in cortex of young rats, Lopes et al., 1999b), this amplified biological model is more useful to observe differences in $A_{2A}R$ levels. However, this cell line lacks the two ionotropic

receptors for glutamate, NMDA and AMPA receptors (Sucher et al., 1993; Sudo et al., 1997). No excitotoxic glutamate effects on cell viability were observed either by pro-caspase-3 fragmentation on western blotting or by the trypan blue exclusion protocol (that enters death cells and renders them blue labelled, while living ones remain colourless).

In this work we observed that $A_{2A}R$ levels are modulated by their agonists and antagonists, as expected. While the $A_{2A}R$ agonist, CGS 21680 30 nM, decreases $A_{2A}R$ levels, their antagonist, SCH 58261 50 nM, has the opposite effect. This upregulation of $A_{2A}R$ by their antagonism was expected and is in accordance with previous results from our laboratory that show an increase of these receptors after oral administration of the $A_{2A}R$ antagonist, KW 6002 (Batalha et al., 2010). In addition, the reduced levels of the excitatory $A_{2A}R$ by their activation with CGS 21680 can provide a regulatory mechanism in prevention of extreme neuronal excitability. The CRF_1R antagonist, antalarmin, applied alone does not change $A_{2A}R$ levels. However, if applied in combination with the $A_{2A}R$ antagonist, SCH 58261, leads to a decrease in $A_{2A}R$ levels. There seems to be an interaction between this two neuroprotective targets in brain, CRF and adenosine (through $A_{2A}R$), although the mechanism involved is still unknown. To answer this question, a possibility would be study the intracellular pathways activated by these receptors, as PKA, PKC or MAPK. G-protein desensitisation and the binding to multiple G-proteins is also an hypothesis for this interaction to occur.

Besides this direct interaction in the receptors levels, CRF and A_{2A} receptors can be interacting in their neuroprotective function. Therefore, we assess cell viability by PI and Syto-13 staining technique in the presence of agonists and antagonists of the receptors. Blockade of $A_{2A}R$ by SCH 58261 reverted the cell death induced by 100 mM of glutamate, as previously observed in neurons (reviewed by Cunha, 2005). We then tested the combined actions of CRFR activation and $A_{2A}R$ blockade upon a glutamate insult. No addictive neuroprotection to that afforded by urocortin was observed by blocking $A_{2A}R$. Further studies are needed to explore if this is due to a common path shared by both receptor subtypes in rescuing neuronal cells from evoked death.

In presence of urocortin, SCH 58261 neuronal protective effects were abolished by CRF_2R but not by CRF_1R blockade, suggesting that the $A_{2A}R$ role in neuroprotection is dependent on CRF_2R, but independent of CRF_1R. This might occur as a result of the downregulation of $A_{2A}R$ previously observed in PC12 cells with simultaneous use of SCH 58261, urocortin and antalarmin (i.e. CRF_2R activation and $A_{2A}R$ blockade). CRF_2R and $A_{2A}R$ are metabotropic receptors located both in pre- and post-synaptically

(Li and Henry, 1998; Liu et al., 2004). Downregulation of $A_{2A}R$ achieved by CGS 21680 occurs by the continuous binding to the receptors, and consequent activation of intracellular pathways, promoted by G_s proteins. The receptor levels decrease to downregulate the signalling through that receptor (Shankaran et al., 2007). SCH 58261 upregulation is a consequence of the opposite phenomenon, an insufficient activation of the $A_{2A}R$.

While the majority of reports points to $A_{2A}R$ activation as being harmful to cells (Gao and Phillis, 1994; Popoli et al., 2002; Dall'Igna et al., 2003; Castillo et al., 2010), a minority of studies suggest that $A_{2A}R$ activation is neuroprotective upon a kainate insult (Jones et al., 1998; Rebola et al., 2005). In our work, we were not able to detect any significantly changes in cell viability upon application of a $A_{2A}R$ agonist, CGS 21680. This may occur due to an excessive activation of $A_{2A}R$ by endogenous adenosine, which is present in higher concentrations after cell death induced by glutamate (Dunwiddie and Masino, 2001). Under these conditions, an $A_{2A}R$ agonist applied exogenously has little or no effect, as observed in our results. One way to test this hypothesis would be to test the activation of $A_{2A}R$ in the presence of the enzyme adenosine deaminase (ADA, E.C. 3.5.4.4) which metabolises adenosine to its inactive metabolite inosine as it is released preventing its accumulation in the cell medium (Geiger and Nagy, 1986). Nevertheless, $A_{2A}R$ activation by CGS 21680 seems to avoid urocortin neuroprotection upon glutamate 100 µM stimulus.

The mechanisms underlying these neuroprotective effects still need to be explored. Both $A_{2A}R$ and CRFR are able to alter gene expression. Whereas blockade of $A_{2A}R$ is neuroprotective, by reducing PKA and PKC phosphorylation activity (Cunha, 2005; Fredholm et al., 2005), CRFR activation leads to increased PKA and PKC activity. PKA and PKC phosphorylation leads to insertion of NMDA receptors in the cell membrane (Leonard and Hell, 1997), contributing to the excitotoxicity induced by glutamate (Leveille et al., 2008). It seems that CRFR neuroprotection must be accomplished through some alternative mechanisms. One of the targets could be BDNF and its receptor TrKB, which were shown to be regulated by CRF (Bayatti et al., 2005; Hauger et al., 2009). These receptors, which have pro-survival effects, also enhance their activity in the presence of $A_{2A}R$ agonists (Diogenes et al., 2004), linking once more CRF and adenosine receptors.

In contrast with previous reports, that described urocortin neuroprotection throughout time upon glutamate insult (Elliott-Hunt et al., 2002), we used an unique time point, 24 hours. Since neuronal death and protein levels were evaluated in this discrete time

point, we cannot speculate about the kinetics of the observed effects or the long-term consequences for cell survival. It would be interesting to explore if CRF_2R activation is needed previously or only during $A_{2A}R$ blockade to afford neuroprotection.

Overall these data show a new role of CRF against glutamate-induced neuronal death, either by direct activation of CRF receptors or by modulating $A_{2A}R$ actions. The observed neuroprotection by $A_{2A}R$ blockade requires CRF_2R activation which might result of the ability of CRF_2R to modulate the levels of $A_{2A}R$ directly. The interaction between the two receptors can point towards new pharmacological approaches exploring putative common molecular pathways.

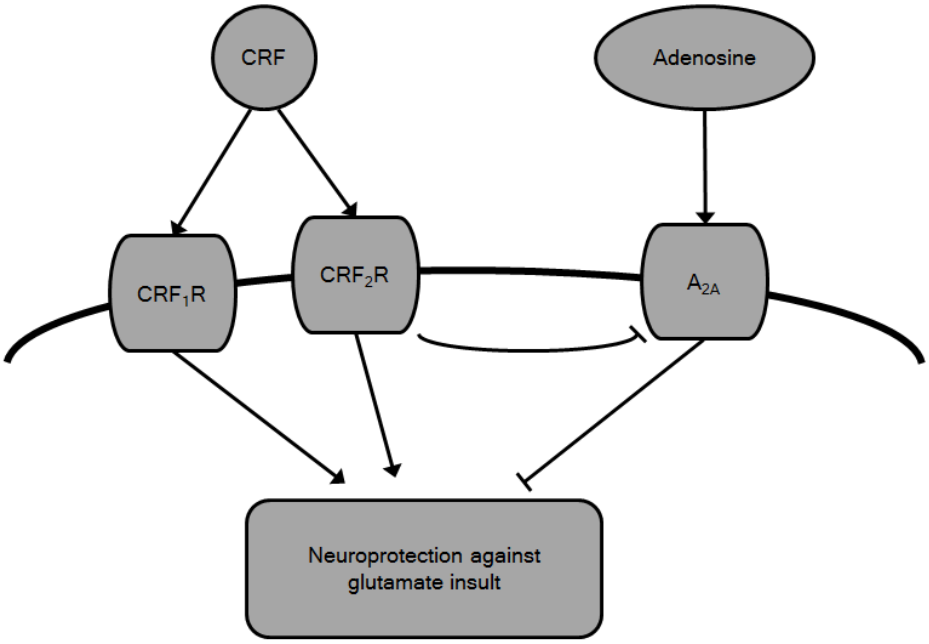

Figure 5.1 - Schematic summary of the results obtained in this work. CRF activation of both CRFR affords neuroprotection against glutamate insult. The proposed mechanism presents CRF_2R as a negative modulator of $A_{2A}R$ and thus preventing $A_{2A}R$ noxious actions in neurons, when activated by adenosine.

6. APPENDIX

6.1. PHYSICAL INTERACTION BETWEEN ANTALARMIN AND ANTI-SAUVAGINE-30

Antalarmin and anti-Sauvagine-30, the two selective antagonists used in this work were never used simultaneously in the past. We, therefore, searched for interactions of these molecules that could lead to an aggregation and consequently to an inhibition of their functional role. anti-Sauvagine-30 is a CRF_2R selective antagonist peptide that contains one Phenylalanine in its composition. This amino acid has fluorescent proprieties with maximum of absorbance at 260 nm and a band of emission between 260 and 320 nm (Teale and Weber, 1957).

In Figure 6.1 are presented fluorescence spectra for different solutions of anti-Sauvagine-30 and antalarmin. Fluorescence emitted by phenylalanine (present in anti-Sauvagine-30) decreases inversely with antalarmin concentration, i.e. antalarmin is a quencher of the phenylalanine, which presents a possible physical interaction between these two CRFR antagonists.

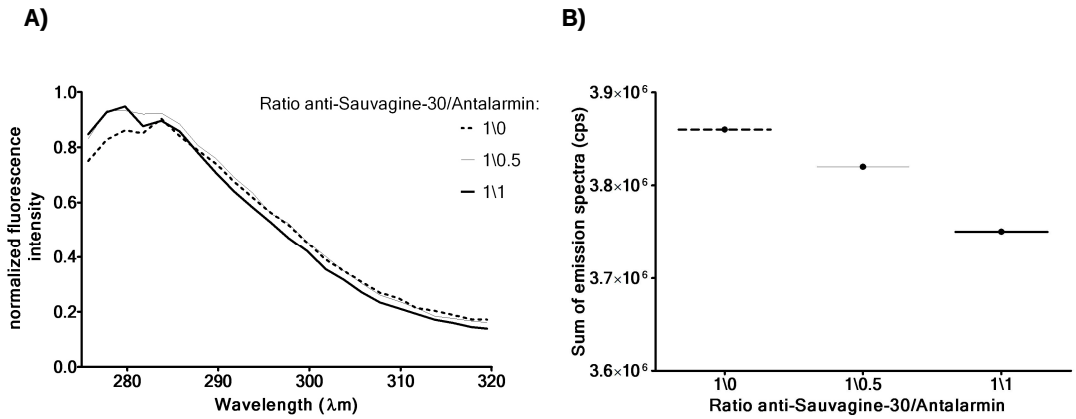

Figure 6.1 - Anti-Sauvagine-30 and antalarmin could physically interact. **A)** Fluorescence spectra of solutions of anti-Sauvagine-30 and antalarmin in different proportions (1\0; 1\0.5 and 1\1) when excited at 260 nm. Fluorescence intensity at each wavelength was normalized to the one of same antalarmin concentration. **B)** Representative graph of the sum of each point (from 275 to 320 nm) from each spectrum presented in A).

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