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Adenosine A_{2A} receptors role in stress-induced neurobiological modifications

Sara Beatriz Gomes Fernandes

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Nélio da Mota Gonçalves (Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e do Professor Doutor Ângelo José Ribeiro Tomé (Faculdade de Ciências e Tecnologia, Universidade de Coimbra)

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ABBREVIATIONS

ANOVA	Analysis of variance
A_{2A}R	Adenosine A_{2A} receptor
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CTR	Control
EPM	Elevated plus maze
GABA	Gamma-amino butyric acid
MYM	Modified y-maze
HPA-axis	Hypothalamic-Pituitary-Adrenal axis system
OD	Object displacement
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl fluoride
PSD-95	Post-synaptic density 95
RNA	RNA Ribonucleic acid
RIPA	Radioimmunoprecipitation assay buffer
RST	Restraint stress
SEM	Standard error of the mean
shCTR	Short hairpin Control
shA_{2A}R	Short hairpin against A_{2A} Receptor
SNAP-25	Synaptosomal-assocated protein 25
SI	Social interaction
TBST	Tris fuffered saline and Tween 20
vGAT	Vesicular gabaergic transporter
vGluT1	Vesicular glutamatergic transporter 1
vGluT2	Vesicular glutamatergic transporter 2

SUMMARY

Chronic caffeine consumption inversely correlates with the incidence of depression and anxiety, which are disabling disorders that increasingly affect millions of people around the world, and prevents phenotypic changes caused by chronic stress in rodents through adenosine A_{2A} receptors ($A_{2A}R$) antagonism, since they are significantly increased in these situations. Notwithstanding, neither the neurobiology of depression nor the mechanisms by which the $A_{2A}R$ can control mood dysfunction are well understood.

In this study we characterized an animal model of stress, namely restraint, by subjecting rodents to different behavioural paradigms, namely reward-based, anxiety-based, memory performance and social interaction test, pertinent for mood-related disorders, and studied the impact of the $A_{2A}R$ located in amygdala neurons thereupon.

Our results show that selective $A_{2A}R$ genetic deletion diminished the anxiogenic and loss of memory performance induced by stress.These adaptations were accompanied by prevention in synaptic alterations also stress-induced, such vGluT1 and vGAT levels in synaptic terminals. These results are consistent with the hypothesis that A_{2A} receptors play an essential role in chronic stress deviations and is a potential target to backslide the consequences.

RESUMO

O stress é uma das principais causas de modificações de humor (depressão e ansiedade) na sociedade atual, afetando milhões de pessoas em todo o mundo. Como tal, e porque 1/3 dos fármacos disponíveis no mercado não surtem qualquer efeito, há uma necessidade eminente de encontrar novos alvos terapêuticos que permitam o seu tratamento eficaz. Estudos recentes demonstram que o consumo crónico de cafeina, que atua como antagonista não seletivo dos recetores $_{A2A}$, está inversamente correlacionado com a incidência de ansiedade e depressão, e que o seu bloqueio farmacológico seletivo ou deleção genética em roedores previne as modificações induzidas pelo stress crónico.

Este trabalho pretendeu caracterizar as alterações comportamentais e bioquímicas num modelo de stress de imobilização, pertinente para o estudo de modificações de humor, e avaliar o impacto dos recetores A_{2A} em particular da amígdala na ocorrência e prevenção dessas

modificações, uma vez que a sua densidade se encontra aumentada nesta região do cérebro que se julga ser determinante em modificações de humor.

Os nossos resultados demonstram que a deleção genética dos recetores A_{2A} da amígdala é eficaz na prevenção da ansiedade e perda de memória induzida pelo stress de imobilização repetido, e que estes efeitos parecem estar associados à prevenção de um potencial desequilíbrio da transmissão sináptica na amígdala.

Por fim, este trabalho evidencia que os recetores A_{2A} da amígdala são determinantes nas modificações comportamentais e bioquímicas induzidas pelo stress, e que estes constituem um potencial novo alvo terapêutico para o tratamento de doenças de humor.

KEYWORDS

Stress, behaviour, A_{2A} Receptors, amygdala,

INTRODUCTION

Depression and anxiety disorders are the leading causes of burden of disease worldwide (de Kloet et al 2005) affecting over 350 millions of people (Lucas et al 2011). These disorders are associated with significant decline in daily functioning and psychosocial impairments together with an increased risk for suicide. Hence, there is a major socio-economical pressure to prevent or alleviate its behavioural consequences. Albeit recent advances have been made towards understanding the neurobiology of depression, no therapeutic strategies achieve adequate clinical efficiency as 2/3 of depressive patients fail a sustained remission and 1/3 fail to respond to the currently available antidepressant treatments (Serafini et al., 2015); this highlights the need for novel and innovative therapeutics for depression.

Role of Adenosine and its receptors in the Central Nervous System

Adenosine is a homeostatic purine released from most cells and is present throughout the central nervous system; it acts as a fine-tuning modulator of neuronal activity by regulating the release of neurotransmitters, such as glutamate, GABA, acetylcholine and serotonin, and the action of neuromodulators, such as neuropeptides and neurotrophic factors (eg.: BDNF) through its G-protein-coupled purinergic receptors (A1, A_{2A}, A2B and A3), which confers them the ability to ultimately modulate animal behaviour. Since the adenosine receptors in the central nervous system, especially the inhibitory A_1R and facilitatory $A_{2A}R$, are located in all nervous system cells, neurons and glia (Biber et al., 1997; Dixon et al., 1996; Gebicke-Haerter et al., 1996), and adenosine is intensively released upon injuries, together they play a key role in the homeostatic coordination of the brain function controlling the efficiency and plasticity-regulated synaptic transmission. Whenever this homeostasis is disrupted, pathology may be installed and selective receptor antagonism or agonism required within a short time-frame to handle the outcome of the insult.

A_1R is widely distributed in the brain, being highly expressed in cortex, cerebellum and hippocampus (Dixon et al 1996, Reppert et al 1991), whereas $A_{2A}R$ is densely located in GABAergic medium spiny neurons (MSNs) of the indirect pathway and olfactory bulb (Jarvis & Williams 1989, Parkinson & Fredholm 1990) and at lower levels in cortex, cerebellum and hippocampus (Cunha et al 1995, Svenningsson et al 1997). Both adenosine receptors also exhibit

distinct subcellular localizations which may be related to each particular receptor function. A_1R is mostly found at the presynaptic active zone and also abundantly located in the postsynaptic density in rat hippocampus (Rebola et al 2003) where they inhibit synaptic transmission by reducing the release of glutamate among other neurotransmitters (Dunwiddie & Masino 2001). In turn, $A_{2A}R$ is mostly found in the postsynaptic active zone of striatal MSNs and in hippocampal presynaptic nerve terminals; a minority of striatal $A_{2A}R$ is also present in presynaptic corticostriatal glutamatergic afferents (Rebola et al 2005a). Since $A_{2A}R$ display different subcellular localizations, it also operates distinct functions: while postsynaptic $A_{2A}R$ control the signaling in MSNs (reviewed in (Fredholm & Svenningsson 2003), presynaptic $A_{2A}R$ mediates facilitation of the release of neurotransmitters, such as glutamate (Lopes et al 2002), GABA (Cunha & Ribeiro 2000), acetylcholine (Rebola et al 2002) and serotonin (Okada et al 2001). The facilitation of glutamate release may play a role in noxious conditions, as several $A_{2A}R$ antagonists have been shown to be protective by this mechanism (Melani et al 2003, Orru et al 2011, Popoli et al 2002).

The overall neuromodulatory role of adenosine in the CNS is thus a balance between A_1R and $A_{2A}R$ functions as they can be located at the same synapse (Rebola et al 2005c). Together they can modify cellular responses to conventional neurotransmitters or receptor ligands. A_1R tend to suppress neuronal activity by a predominant presynaptic action, while $A_{2A}R$ are more likely to promote transmitter release and postsynaptic depolarization.

Adenosine A_{2A} receptors in the context of brain insults

Adenosine receptors are modulators of neuronal function and synaptic plasticity and thus playing a crucial role within the occurrence, development of diverse neuropathological conditions. Based on their distribution and distinct roles, A_1R and $A_{2A}R$ oppositely contribute to cell alterations and damage upon a wide range of insults on distinct brain areas, wherein alterations in receptor number and/or properties may easily occur. In parallel, several adenosine receptors' ligands, agonists and antagonists acutely and chronically administered, have intriguingly demonstrated significantly different and diverse effects on damage outcomes in a plethora of brain disorders due to the nature of each pathogenesis at the corresponding brain areas. In fact, chronic noxious brain conditions exhibit an A_1R down-regulation in parallel with an $A_{2A}R$ up-regulation, typified by an increase in expression and density of $A_{2A}R$ found in diverse animal models, namely Parkinson's disease (PD), epilepsy and restraint stress (see Figure 1; (Cunha et al 2006, Pinna

et al 2002, Rebola et al 2005b, Tomiyama et al 2004), which prompts considering the manipulation of $A_{2A}R$ as a promising therapeutic target to manage adenosine signaling. Since the pharmacological blockade of $A_{2A}R$ prevented stress-induced functional and behavioural modifications (Kaster el al., 2015; Batalha et al., 2013), and the amygdala is argued to be responsible for depressive-mediated behaviours, we now decided to investigate whether the manipulation of $A_{2A}R$ selectively in the amygdala structure is able to prevent stress-induced alterations.

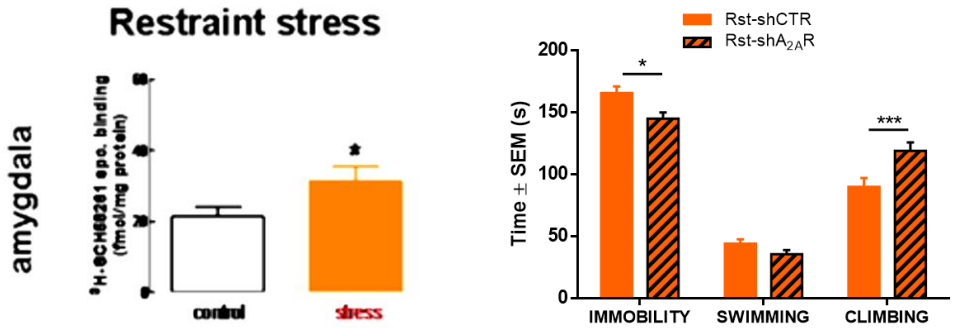

Figure 1 - Preliminary data showing enhanced density of $A_{2A}R$ in nerve terminal membranes of the amygdala of restrained animals, gauged by the enhanced binding density of the selective $A_{2A}R$ antagonist 3H-SCH58261 (left). The administration of $A_{2A}R$ silencing sequences in the amygdala of restrained animals displayed an antidepressant activity typified by the significant decrease (*$p < 0.05$) in the immobility time in the forced swimming test and significant increase (***$p < 0.001$) in their climbing behaviour.

Animal model of repeated stress: neurobiological modifications

There are several stressful insults able to trigger phenotypic and neurophysiological alterations in rodents resembling mood-related disorders and lead to complementary animal models, namely learned helplessness, physical stress, chronic unpredictable stress, fear conditioning, maternal deprivation, social defeat and restraint stress, all proving to be valuable tools to identify and characterize the brain circuits involved in stress response, notwithstanding none completely mimicking the human situation. Restraint stress is the most widely used method to induce stress in animals (reviewed in Buynitsky and Mostofsky, 2009); it is an immobilization procedure applied by several ways depending on the technique and/or material used, and results in physiological (Rybkin et al., 1997) and emotional (Wood et al., 2003) changes non-related to pain.

Subjecting rodents to repeated stress triggers a constellation of behavioural modifications resembling the human situation (Chiba et al., 2012), where the hippocampus, the amygdala, and the prefrontal cortex appear to be the most affected brain regions. Accordingly, previous studies have demonstrated an atrophy of dendrites, namely in length and number of branch points, in the hippocampal CA3 pyramidal neurons of rats subjected to repeated restraint stress, correlated with the observation of abnormal memory performance (Magarinos et al., 1997; McEwen and Magarinos, 1997; Vyas et al., 2002), in parallel with an increase in dendritic length and dendritic spines in the basolateral amygdala (Magarinos et al., 1997; Padival et al., 2013). Additionally, restraint stress also showed to alter the regulation of the microtubule networks in the axons and dendrites of neurons in the hippocampus and in the cortex, particularly of tau and microtubule-associated protein 2 (MAP2) (Yan et al., 2010), and also the expression of a large variety of synaptic proteins in hippocampal nerve terminals (Cunha et al., 2006), and in the prefrontal cortex (Muller et al., 2011), proteins directly involved in synaptic transmission and whose changes may contribute to the morphological, functional and behavioural changes observed in experimental models of stress and therefore playing a key role in the pathophysiology of stress-related disorders.

The group of Purines at CNC has previously demonstrated that animals, subjected to repeated restraint stress showed an imbalance in A_1 and A_{2A} adenosine receptors, with the $A_{2A}R$ being up-regulated which was found associated with a decrease in synaptic protein markers. (Cunha et al., 2006). Additionally, the group also showed that caffeine prevents some of the modification induced by stress in behavioural tasks and also at neurochemical level, pre and post-synaptically, reverting the effect of stress, by mimicking the global genetic depletion of A_{2A} receptors (Kaster et al 2015). Together, these evidences suggest that $A_{2A}R$ antagonism or deletion might be successfully applied in restraint stress models as a promising therapeutic approach to manage its consequences.

Gene transfer to CNS: research and therapeutic applications

RNA interference (RNAi) is a constitutive mechanism through which cells can control genetic expression inhibiting the formation of an mRNA product. This mechanism has been exponentially used in the last decade as a tool to induce the knockdown of genes in order to study their direct involvement in many physiological and pathological conditions (Jakobsson and

Lundberg, 2006; Tiscornia et al., 2003). Lentiviral vectors have been highly attractive gene delivery tools by their powerful capability of *in vivo* transduction in a variety of systems, in particular, the brain; lentivirus are also advantage since they transduce either dividing and non-dividing cells (Naldini, 1998). When combined to RNAi, it became a powerful tool allowing and improving RNAi treatment strategies.

Since we aimed to prevent the upregulation of $A_{2A}R$ in the amygdala upon a stress situation to probe for their direct role in stress-induced behavioural and synaptic modifications, we stereotaxically administered lentiviral vectors encoding a short hairpin for $A_{2A}R$ in the rodents' amygdala.

AIMS

Our main goal was to investigate the impact of manipulating a neuromodulation system operated by $A_{2A}R$ as an effective strategy to control stress-induced behavioural alterations and synaptic remodeling.

The specific aims of this thesis were as follows:

i) To behaviourally characterize an animal model of repeated stress pertinent to mood-related disorders;

ii) To dissect morphological modifications probing alterations of synaptic markers in the amygdala;

iii) To investigate whether the silencing of $A_{2A}R$ selectively in the amygdala is effective in controlling stress-induced behavioural alterations and synaptic remodeling**.**

METHODS

Animals. Male Wistar 8 weeks-old rats (Charles River, Barcelona, Spain) were housed and kept under a conventional 12-h light-dark cycle maintained on a temperature-controlled room with food and water provided *ad libitum* and used at 8 weeks of age. The experiments were carried out in accordance with the European Union legislation (2010/63/EU) for the care and use of laboratory animals.

Lentiviral vectors and in vivo manipulations. Viral vectors were produced as previously described (Gonçalves et al 2013). Concentrated viral stocks were thawed on ice. After IP anaesthesia of 32 rats with 14 mg/kg xylazine and 128 mg/kg ketamine, lentiviral vectors encoding short hairpin $A_{2A}R$ ($shA_{2A}R$) or Control sequence (shCTR) were stereotaxically injected into the amygdala (blAMY) in the following coordinates: antero-posterior (AP): -2.8mm; medial-lateral (ML): ±4.8mm; dorso-ventral (DV): -9.0mm. Rats received 1.5 µL injections of lentivirus (500'000 ng of p24/mL) in both hemispheres. Different groups of animals were kept in their home cages for 1 week before restraint stress procedure. The rats were killed by decapitation within 24-48h and the amygdala region dissected and frozen until use.

Restraint Stress Procedure. Rats in the control groups were not exposed to stress but handled once daily during the experiment. Rats in the stressed groups were restrained for 4 h per day for 14 days, followed by 2 days of behavioural tests.

BEHAVIOURAL ANALYSIS

Sucrose preference test. All the animals were tested for sucrose preference, over a six day period using a two-bottle choice test as previously described (Crawford et al 2013). On the first day, rats were singly housed and habituated to drinking from two bottles, with water. The bottles had similar characteristics and contained approximately the same volume of liquid. On the following four days, rats were trained on the sucrose choice procedure, in which one of the water bottles was replaced with a bottle containing a 0.8% or a 0.125% sucrose (S0389, Sigma, St. Louis, MO, USA) solution. Rats were allowed to drink freely from both bottles for 20h. The bottles were weighed and refilled each day 10A.M in the morning. The position of the bottles was

switched daily to avoid place preferences. At the end of the last habituation day, the bottles were removed and weighted and the animals were water deprived for 20 h and then given a 1 h preference test. Sucrose preference was measured by calculating the percentage of drank sucrose solution out of the total liquid consumed.

Splash test. The Splash test consists in squirting a 10% sucrose solution to the animal's dorsal coat, as previously described (Isingrini et al 2010). Since the solution is viscous to the animals and dirties their dorsal coat, the animals tend to remove it by initiating grooming behaviour. The time the animals spent grooming is a measure of their self-care and allows to see differences between depressed-like and non-depressed-like animals, once that a decrease in this behaviour correlates with symptoms of depression, such as apathy (Willner 2005). The animals were placed in individual boxes, squirted with the sucrose solution and their behaviour recorded with Windows Media Player Software, for ten minutes.

Open-field. Spontaneous locomotion was assessed by using a grey acrylic made open-field box (1 m wide × 1 m deep × 60 cm high). The animals were placed in the centre of the apparatus in a single 10 min session. The apparatus was cleaned before and after each trial with a 10% ethanol solution. ANY-maze software analysed data as measures the total the total distance travelled in the field.

Elevated plus maze. The apparatus consisted of a black cross-shaped Plexiglas placed 65 cm above the floor. The four arms were 45 cm long and 10 cm wide and connected by a central platform (10 x 10 cm). Two opposite arms were surrounded by gray walls (50 cm high, closed arms) while the two others were devoid of enclosing walls (open arms). Sessions were video-recorded with ANY-maze Video Tracking Software (Stoelting Europe, Dublin, Ireland). Entries (four-paw criterion) and time spent in enclosed and open arms were measured, together with open/total arm entry and duration ratios [(seconds in each arm/ total time exploring arms) × 100]. An entry occurred whenever the rat crossed from one arm to another with four paws. Rats were gently placed in the central square facing one of the closed arms. It was allowed to explore freely and undisturbed during a single 10 min session.

Modified Y-maze. This test was performed to assess spatial recognition memory and behaviour evaluation was based on the preference of the animals to explore new areas (Cognato et al 2010). The task consisted in a trial and test phase of 5 minutes, separated by an inter-trail interval (ITI) of 90 minutes. The Y-maze consisted in a three arms apparatus that were randomly designated: start arm, other arm and novel arm (blocked during trial phase). In the trial phase, the animals were placed in the start arm allowed to freely explore the apparatus, only with start and other arms opened. After the ITI, the novel arm was opened and animals were able to explore the entire apparatus (Cognato et al 2010, Soares et al 2013). The entire task was video-recorded with ANY-maze Video Tracking Software (Stoelting Europe, Dublin, Ireland). Automated criteria used: Entries and time spent each arm were measured. An entry occurred whenever the rat crossed from one arm to another with four paws.

Object Displacement. Memory performance was evaluated by using a grey acrylic made open-field box (1 m wide × 1 m deep × 60 cm high), in which two identical objects were displayed. The test was performed in three different phases: trial phase, in which the animal is free to explore the apparatus, for five minutes; sample phase, one hour after the trial phase, in which the objects are presented to the animal who is free to explore for five minutes; and a test phase, with an hour interval from the sample phase, in which one of the objects is moved (the one that the animal explored the most) (Hattiangady et al 2014). The animal was always placed in the center of the open-field box. The task was video-recorded with ANY-maze Video Tracking Software (Stoelting Europe, Dublin, Ireland). The animal was considered to be exploring the object when in the zone of the objects, defined in ANY-maze software.

Social interaction. The test was performed in the open-field apparatus, on the day following the last day of stress protocol. Forty animals were divided into five groups where 32 were singly housed for 5 days prior to testing, and 8 were kept paired and undisturbed until exposure to the unfamiliar peer All animal groups were subjected to a familiarization phase, for 7.5 minutes, in the test arena. Forty five minutes after the last familiarization phase, the tested rats were paired with an unfamiliar peer, placed in the center of the arena and tested for social interaction (Habr et al 2014). Experimental rats were intermixed, and the observations were made between 3:00PM and 7:00PM. Tree observers blinded to experimental conditions performed this test. Behavioural evaluation was performed as previously described: frequency of play behaviour (PB), social investigation (SI) and contacts (Varlinskaya et al 2010).

Statistical Analysis. Statistical comparisons were performed using either an unpaired Student's t test or one-sample t test. Results are expressed as mean ± standard error of the mean (SEM). Significance thresholds were set at $p < 0.05$, $p < 0.01$, or $p < 0.001$, as defined in the text.

HISTOLOGICAL ASSESSMENTS

Synaptosomes. The synaptosomes from amygdala were prepared as before (Rebola et al 2005a). The tissue was added to 8 mL of 0.32 M sucrose solution containing 1 mM EDTA, 1 mg/mL bovine serum albumin (BSA; Sigma) and 10 mM HEPES (pH 7.4) and homogenized in a Potter. The volume of the suspension was completed to 10 mL with sucrose solution and centrifuged at 3000g for 10 minutes at 4ºC. The supernatants were collected and centrifuged at 14000g for 12 minutes at 4ºC. The supernatants were discarded and pellets were resuspended in 1 mL of 45% v/v Percoll solution made in Krebs-Ringer solution (composition in mM: NaCl 140. KCl 5, HEPES 10. EDTA 1, Glucose 5, pH 7.4). After centrifugation at 14000g for 2 minutes (in an eppendorf centrifuge) the top layer was collected (synaptosomal fraction) and washed in Krebs-Ringer solution by ressuspension and subsequent centrifugation at 14000g for 2 minutes. The final pellets were resuspended in RIPA solution (lysis buffer) with PMSF at 1mM and stored until use.

Immunocytochemistry. Synaptosomes previously obtained were put in coverslips of 16mm, previously coated with poly L-Lysine, for 1 hour to adhere. Then were fixed with 4% paraformaldehyde for 15 minutes and incubated with phosphate buffered saline (PBS) with 0.2% Triton X-100 for 10 minutes, at room temperature (RT), in order to permeabilize. Blockage to prevent unspecific binding was performed with PBS with 3% BSA and 5% goat serum. Synaptosomes were then washed with PBS with 3% BSA and incubated with IgG primary antibodies againt synaptophysin (1:1000; Millipore), vGluT1 (1:1000; Synaptic System), vGluT2 (1:200; Synaptic System), vGAT (1:500; Synaptic System), diluted in PBS with 3% BSA, overnight at 4ºC. After wash three to five times with PBS with 3% BSA, synaptosomes were incubated with AlexaFluor-647 (far-red) labelled goat anti-rabbit (1:1000; Invitrogen) and AlexaFluor-594 (red) labelled goat anti-guinea pig (1:1000; Invitrogen) IgG secondary antibodies, for 1 hour, at RT. Coverslips were washed with PBS, dried with absorbent paper and mounted with Moviol reagent. Samples where then analyzed with a Confocal LSM 710 Zeiss Microscope.

Western Blotting. Synaptosomes previously obtained were tested for the total amount of protein using the bicinchoninic acid (BCA; Pierce) method. Samples were prepared according to the protein amounts previously determined through optimization with similar tissue and loaded onto gels with 10% of acrylamide (Biorad). Proteins were separated by electrophoresis under denaturing and reducing conditions (100 to 120V for 1 hour) and then transferred into polyvinylidenedifluoride PVDF (AplliChem Panread) membranes. Ponceau procedure was performed to visually guarantee that the transfer went well. Blots were then blocked in BSA 5% in Tris-Buffered Saline with 0.1% Tween (TBT-T 0.1%) for 1 hour at room temperature (RT) and finally incubated overnight, at 4ºC, with primary antibodies against SNAP-25 (1:15000; Sigma), PSD-95 (1:15000; Chemicon), vGluT1 (1:10000; Synaptic System), vGAT (1:2500; Synaptic System), diluted in TBST with 5% BSA. After washed three to five times with TBST with 0.5% BSA, membranes were incubated with IgG secondary antibodies Anti-Mouse (1:20000; Thermo Scientific); Anti guinea-pig (1:15000; Sigma); Anti-Rabbit (1:20000; St. Cruz Biotech), diluted in TBST with 5% BSA. The protein bands were visualized with VersaDoc, after the incubation of the membrane with chemofluorescence reagent (ECF; GE Heathcare). Membranes were then reprobed with GAPDH (1:3000; St. Cruz Biotech; Anti-Rabbit 1:10000) as a loading control.

RESULTS

TIME COURSE

Figure 2 - Time course protocol. All animals were stereotaxically injected in the amygdala and administered with lentivirus encoding a short hairpin for $A_{2A}R$ ($shA_{2A}R$) or control (shCTR). Seven days later and during 14 days, half of the animals were restrained. By the end, all animals were subjected to 2 days of behavioural testing pertinent to depression (hedonic sucrose and splash tests) and to probe for other depressive-related modifications, namely anxiety (elevated plus maze, open-field), memory (modified Y-maze, object displacement), and social avoidance (social interaction test).

We carried out a protocol which started by stereotaxically injecting lentivirus encoding a short hairpin against $A_{2A}R$ ($shA_{2A}R$) or control (shCTR), in amygdala of rat brains, followed by 7 days of recovery. Then, during 14 days, half of the animals were subjected to restraint stress and in the end, all animals were subjected to 2 days of behavioural testing to assess depressive-like behaviour (hedonic sucrose and splash tests) and to probe for other depressive-related modifications, such as anxiety (elevated plus maze, open-field), memory (modified Y-maze, object displacement), and social avoidance (social interaction test) (Fig.2).

HEDONIC PROFILE

One key biological alteration to allow depression diagnosis is anhedonia, i.e., the absence of pleasure and self-care. Therefore, to investigate whether restrained animals lost their appetite for a palatable solution, we performed the sucrose preference and splash tests. As expected, control animals (Ctr) injected with control lentivirus (shCTR) displayed high preference for sucrose (90.7 ± 8.7%, n=2). Although not expected, restrained animals (Rst) also displayed high sucrose

preference (91.6 ± 4.8%, n=2). Animals injected with shA$_{2A}$R revealed no sucrose preference-induced alterations (94.1 ± 5.2%, n=2 for Ctr; 50.5 ± 49.0%, n=2 for Rst); percentage values from

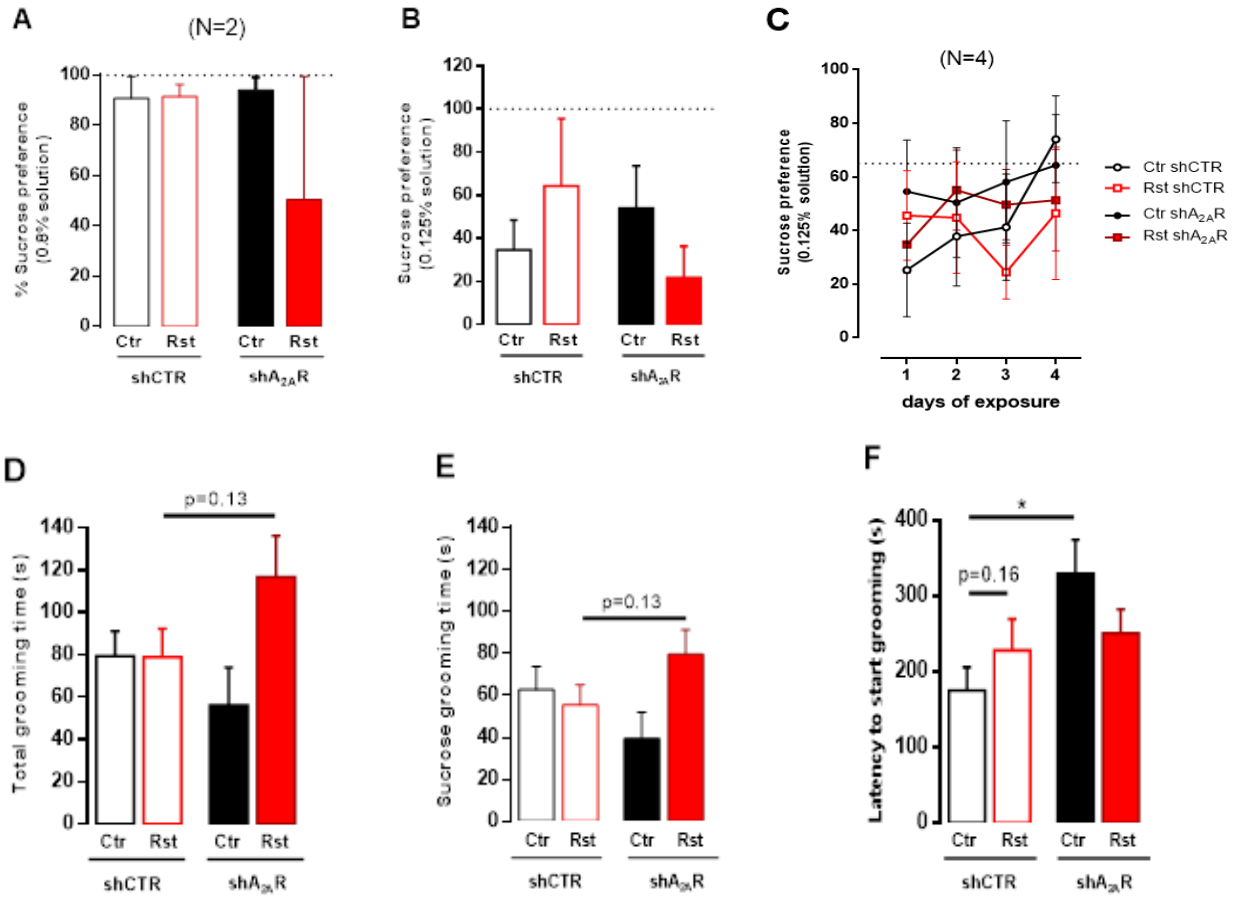

Figure 3 – Effect of A$_{2A}$R silencing in the amygdala n the hedonic profile of restrained animals. (A), (B) and (C) are data from sucrose preference test: (A) percentage of preference for a 0.8% sucrose solution, in an one hour test after 24 h of deprivation (n=2); (B) preference of a 0.125% sucrose solution in an one hour test after 24 h of deprivation and (C) preference for a 0.125% sucrose solution in a 5-day test (habituation). (D), (E) and (F) are data from the splash test: (D) total time that the animal spent grooming; (E) grooming for sucrose, and (F) is the latency which animals start grooming.

Rst shA$_{2A}$R group were due to clear place preference of the bottles (Fig 3A). Since these results were accompanied by increased total liquid consumption when compared to total volumes consumed when two bottles of water were made available to the animals (data not shown), we changed to a 0.125% sucrose solution to reduce the putative excessive palatability which would make the solution too much desirable either to restrained animals, and also to minimize the satiety variable. In fact, sucrose preference was highly reduced as control shCTR animals displayed 34.8 ± 13.7% (n=5) preference for sucrose (Fig 3B). Nevertheless, no significant changes ($p > 0.05$) were observed in any of the other experimental groups. Therefore we then analyzed whether the different groups behave differently during the 4 days of sucrose solution habituation (20h/day)

since liquid starvation (20h) was performed prior the 1h of testing which may have differently interfered each experimental group as regards their initiative to drink; additionally, the testing was performed at morning when animals are at their inactive period. In fact, Ctr shCTR animals displayed a robust increase in sucrose preference during habituation (Fig 3C: 25.3 ± 17.5% at day 1 to 74.1 ± 16.2% at day 4; $p = 0.08$, n=4) which was not observed in restrained animals (45.6 ± 16.7% at day 1 to 46.5 ± 24.7% at day 4; $p = 0.96$, n=4). On the other hand, animals injected with shA_{2A}R in the amygdala, both control and restrained, revealed a slight increase in their sucrose preference (Ctr shA_{2A}R: 54.6 ± 19.2% at day 1 to 64.4 ± 19.9% at day 4, $p = 0.60$. n=4; Rst shA_{2A}R: 34.8 ± 10.7% at day 1 to 51.3 ± 18.9% at day 4, $p = 0.55$, n=4).

We further performed an alternative behavioural assessment for the hedonic state of the animals, namely splash test, which also indirectly measures their self-care. Although no major alterations in total and sucrose grooming were observed between control and restrained animals injected with shCTR (Fig. 3D and E; total grooming: 79 ± 12 sec for Ctr, 79 ± 13 sec for Rst; sucrose grooming: 63 ± 11 sec for Ctr, 56 ± 10 sec for Rst;; $p > 0.05$, n=8), restrained animals displayed a clear increase, although not reaching statistically significance ($p = 0.16$), in the latency to start grooming when compared to control animals (Fig. 3F). Selective silencing of A_{2A}R in the amygdala induced a slight decrease in the total and sucrose grooming in a control situation (Fig. 3D and E: Ctr shA_{2A}R: $p = 0.30$ and $p = 0.18$, n=8, respectively) and a significant ($p < 0.05$) increase in the latency to start grooming (Fig. 3F). Appositively, shA_{2A}R in restrained animals resulted in an increase ($p = 0.13$) in both total and sucrose grooming (Fig. 3D and E) although not changing the latency to start grooming, as expected for an antidepressant drug (Fig. 3F).

ANXIETY PROFILE

Since repeated stress for at least 21 days induces anxiety in a wide range of stress insults (Kaster et al 2015), we further evaluated whether 14 days of restraint stress would also be sufficient to induce anxiety. Therefore, we subjected animals to an elevated plus maze (EPM) paradigm. Indeed, restrained animals displayed a significant reduction (7.0 ± 2.2%, $p < 0.05$) in the percentage of time exploring the open arms (Fig. 4A), thus meaning animals were more anxious than controls (16.1 ± 3.8%). Interestingly, genetic elimination of A_{2A}R in the amygdala prevented this behaviour in restrained animals (15.8 ± 4.1%) while it did not change controls (19.3 ± 4.9%). We also looked at the total distance travelled in both EPM and in an open arena (open field apparatus, OF), since the reduced time exploring the open arms by restrained animals could

be explained by a reduction in locomotion. Yet, no differences were found between groups in the EPM (Fig. 4B) and in the OF (Fig. 4C).

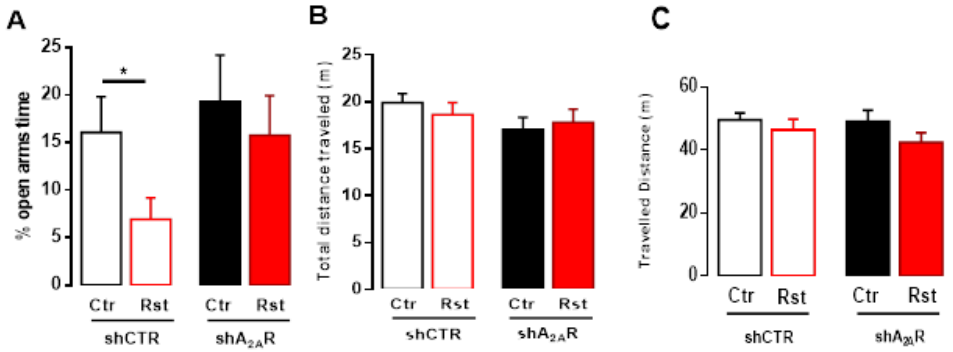

Figure 4 – Genetic blockade of $A_{2A}R$ in the amygdala prevented restraint stress-induces anxiety. Animals were tested in an elevated plus maze (EPM) and in an open field (OF) for evaluated for their anxiety and locomotion. (A) Percentage time animals spent exploring the open arms of the EPM. (B) Total distance travelled in the EPM apparatus. (C) Total distance travelled in the OF apparatus.

MEMORY PERFORMANCE

The loss of short-term memory in restrained animals was also confirmed in OD test since, in comparison to control shCTR-injected animals, the displacement index was not significantly different from 0.50 probability of exploring both objects (Fig. 6). Interestingly, silencing $A_{2A}R$ in the amygdala of restrained animals prevented this loss, while it turned decreased in control animals, probably due to increased error bars meaning high heterogeneity of the controls responding to the $A_{2A}R$ deletion.

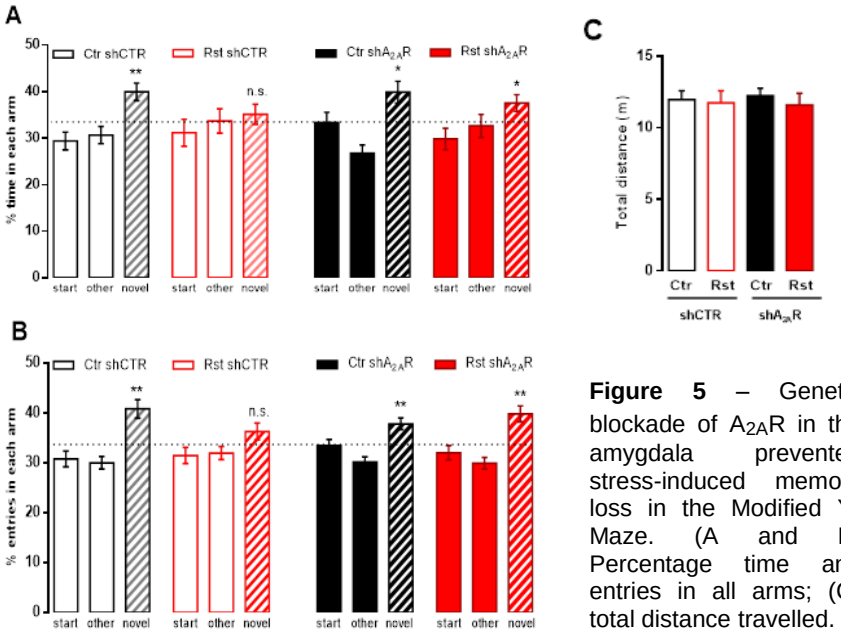

Figure 5 – Genetic blockade of $A_{2A}R$ in the amygdala prevented stress-induced memory loss in the Modified Y-Maze. (A and B) Percentage time and entries in all arms; (C) total distance travelled.

We have previously shown that repeated stress decreases memory performance in different paradigms, namely in the modified Y maze (MYM) and in the object displacement (OD) tests, upon 3 weeks exposure to chronic unpredictable stress (CUS) (Kaster et al., 2015). We now evaluated whether restraint stress was also able to induce memory deterioration in a temporally shorter stress protocol, i.e., of 14 days. The applied MYM protocol allows the evaluation of short-term spatial memory. As expected, control animals injected with shCTR in the amygdala behave normally, as they entered and spent more time exploring the novel arm (Fig. 5A and B; $p < 0.01$). By contrast, restrained animals injected with control sequence (shCTR) displayed no different percentages of entries and time spent in the novel arm when compared to 0.33 probability of exploring each arm ($p > 0.05$), which means restrained animals did not recognize the novel arm. No such loss of performance was observed in neither restrained nor in control animals injected with $A_{2A}R$ silencing sequence in the amygdala (Fig. 5A: $p < 0.05$; Fig. 5B: $p < 0.01$). Total travelled distances were also not different between each experimental group, which indicates all animals expressed equal locomotion profile exploring all arms (Fig. 5C).

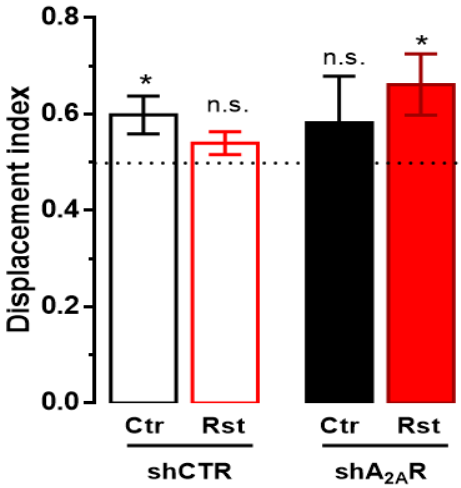

Figure 6 - Genetic blockade of $A_{2A}R$ in the amygdala prevented short-term memory loss induced by restraint stress in object displacement paradigm.

The loss of short-term memory in restrained animals was also confirmed in OD test since, in comparison to control shCTR-injected animals, the displacement index was not significantly different from 0.50 probability of exploring both objects (Fig. 6). Interestingly, silencing $A_{2A}R$ in the amygdala of restrained animals prevented this loss, while it turned decreased in control animals, probably due to increased error bars meaning high heterogeneity of the experimental group responding to the manipulation.

SOCIAL BEHAVIOUR

The Social interaction (SI) test consists in placing unknown con-specific animals together and monitoring social behaviour during 5-15 min (reviewed in Toth and Neumann, 2013). Although restraint stress is a non-social stress paradigm, our animals were socially deprived during 5 days prior behavioural testing due to technical issues involving the sucrose preference test procedure. In addition, there have been several reports showing impaired social interaction and increased or decreased social avoidance by isolated and restrained animals, respectively, also with changed anxiogenic profile. Therefore, we also subjected our animals to a SI paradigm to evaluate whether restraint stress also exerted an alteration in their social behaviour. We analyzed the frequencies of distinct social behaviours, such as play behaviour (pouncing + playful nape attack, chasing and pinning), social investigation (sniffing) and contacts (crawling over and under + social grooming) (van Kerkhof et al 2013) and no major differences were found between all experimental groups (Fig. 7). Nevertheless, since there were a number of animals displaying aggressive behaviour during SI testing, we further looked at their group ID and found they were restrained animals (injec

described previously (Caramaschi et al 2007) and in a near future in order to confirm this impression.

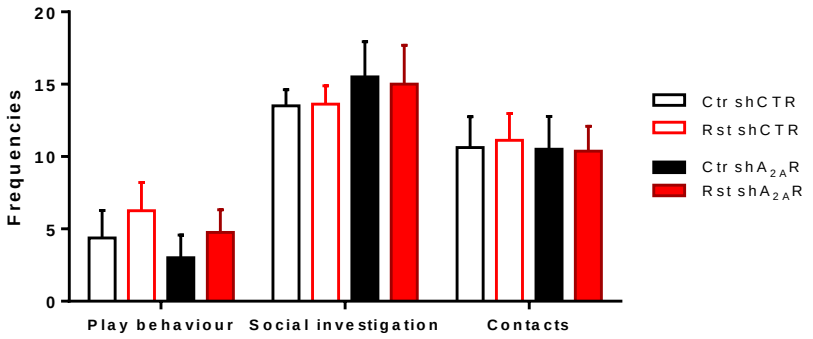

Figure 7 – Restraint stress as well as $A_{2A}R$ silencing in the amygdala did not significantly modify any of the analyzed social behaviours: Play behaviour, Social Investigation and Contacts.

SYNAPTIC REMODELING

We have previously reporter that synaptic adaptations occurred upon CUS (Kaster et al., 2015), namely reduction of synaptic markers, such as synaptosomal-associated protein 25 (SNAP-25) and markers of glutamatergic terminals (vGluT1), which correlated with the behavioural alterations. Therefore, we further analyzed if those alterations were also present upon repeated restraint stress and whether selective $A_{2A}R$ silencing in the amygdala was able to prevent it. We first optimized and validated the immunoblotting conditions (antibody dilutions, incubation times and protein quantities) for pre- and postsynaptic markers, such as SNAP-25 and postsynaptic density protein 95 (PSD-95), as well as markers of glutamatergic (vesicular glutamatergic transporter 1: vGluT1) and GABAergic (vesicular GABAergic transporter: vGAT) terminals in amygdala synaptossomal preparations of naïve animals (Fig. 8). The chosen protein quantities for each marker were: 5µg (SNAP-25), 30µg (PSD-95), and 10µg (vGluT1 and vGAT) (see Fig. 8).

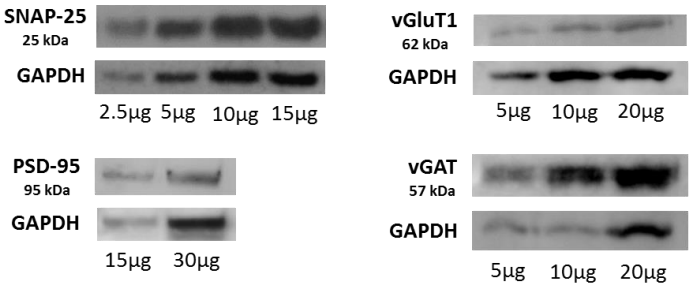

Figure 8 – Immunoblotting optimization for pre- and postsynaptic markers, namely SNAP-25 and PSD-95, respectively, as well as for glutamatergic (vGluT1) and GABAergic terminals (vGAT).

We thus similarly labelled the amygdala synaptossomes of each experimental group. Restraint stress induces a reduction although not significant of PSD-95 (Fig. 9A: 70 ± 5% Rst shCTR *vs* 100 ± 31% Ctr shCTR, n=3) while it did not change SNAP-25 marker (Fig. 9B), as well as a clear reduction in glutamatergic terminals (Fig. 9C: 34 ± 18% Rst shCTR *vs* 100 ± 41% Ctr shCTR, n=3) and slightly in GABAergic terminals (Fig. 9D: 75 ± 4% Rst shCTR *vs* 100 ± 42% Ctr shCTR, n=3). Interestingly, $A_{2A}R$ silencing in the amygdala almost completely prevented such alterations while not modifying control situation (Fig. 9A-D).

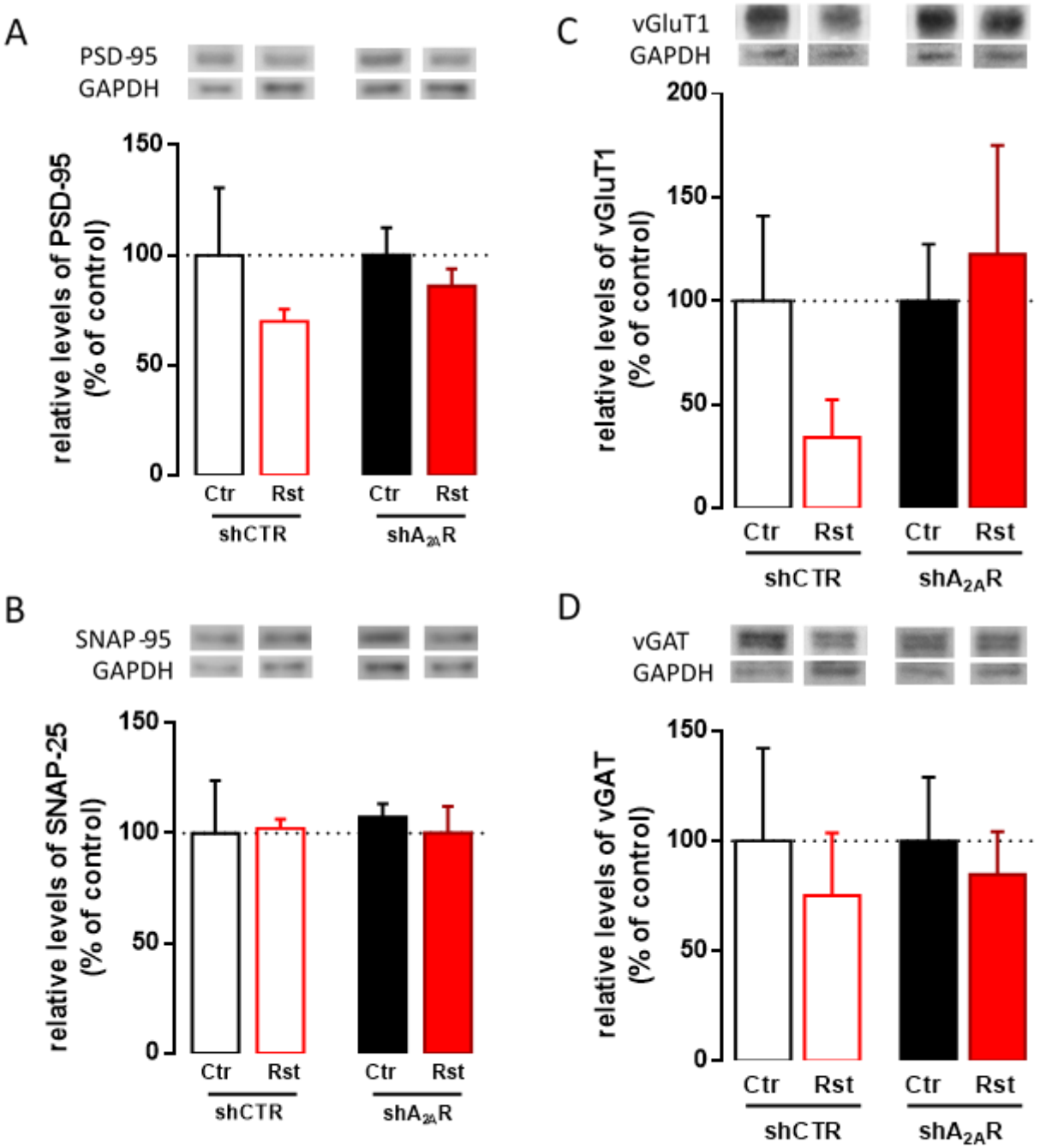

Figure 9 – Restraint stress induces a reduction although not significant of postsynaptic but not presynaptic markers, as well as a reduction in glutamatergic terminals and also of GABAergic terminals in amygdala synaptossomes. Immunoblotting for pre- and postsynaptic markers, namely SNAP-25 (B) and PSD-95 (A), respectively, as well as for glutamatergic (C, vGluT1) and GABAergic terminals (D, vGAT).

Since the $A_{2A}R$ are endowed in synapses and aberrantly increased in amygdala nerve terminals upon repeated stress (see Fig. 1 of preliminary data), and we are silencing $A_{2A}R$ mainly in amygdala neurons due to neurotropism of lentiviral vectors (de Almeida et al 2001) we then

intended to perform double-labeling immunocytochemical analysis of plated amygdala synaptossomes in order to dissect in which type of neurons: i) $A_{2A}R$ were indeed significantly increased upon stress induction; and ii) occurred lentiviral-mediated $A_{2A}R$ silencing which contributed to the prevention of behavioural modifications induced by stress. Unfortunately, unsolved problems with the $A_{2A}R$ antibody unrelated with our group (loss of quality of the company's antibody) made it impossible to carry out these confirmations on schedule. Although not sufficient, in order to help explaining the behavioural modifications induced by repeated restraint stress and the preventive actions exerted by $A_{2A}R$ silencing in the amygdala, we further dissected whether restraint stress induced an alteration in the relative percentages of glutamatergic (immunopositive for vGluT1) and GABAergic (immunopositive for vGAT) amygdalar inputs and also if those levels were prevented by $A_{2A}R$ silencing as observed by western blot analysis. As expected, amygdala is predominantly composed of glutamatergic nerve terminals, as observed in the control shCTR-injected animals (Fig. 10A: 84.0 ± 4.6%), rather than in GABAergic nerve terminals (Fig. 10B: 32.6 ± 4.5%) but, unlike the previous results, there were no significant changes in glutamatergic nerve terminals in the amygdala of restrained animals (Rst shCTR), which together with the previous data might be interpreted as a stress-induced specific loss of vGluT1, and not vGluT2, terminals only. Yet, as previously shown there is a consistent decrease, although not significant, in GABAergic nerve terminals in restrained animals. Silencing of $A_{2A}R$ in the amygdala did not change the percentage of glutamatergic nerve terminals (Fig. 10A) but, again contrary of previous western blot analysis, it decreased the percentage of the GABAergic nerve terminals, irrespective to the control or restraint group (Fig. 10B filled bars: 20.1 ± 2.1% of Ctr $shA_{2A}R$ and 21.1 ± 1.2% of Rst $shA_{2A}R$ *vs* 32.6 ± 4.5% of Ctr shCTR, n=2-3, $p > 0.05$). It is worth noting that although this immunocytochemistry approach is more sensitive to the desired quantification, it only allows the

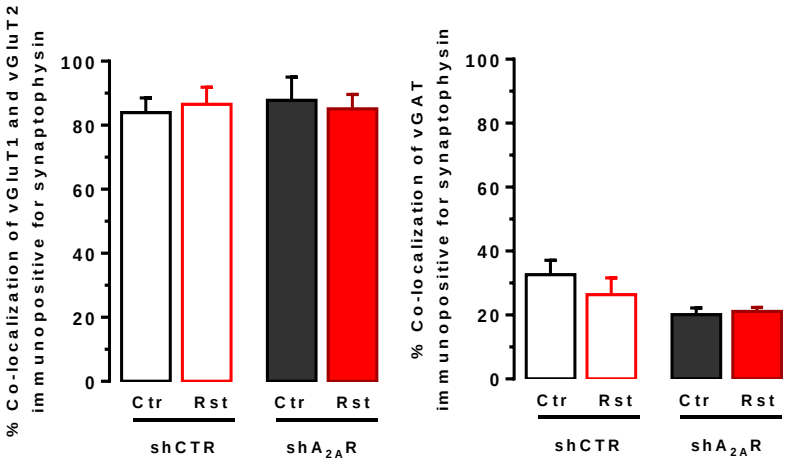

Figure 10 – Restraint stress did not alter the number of glutamatergic nerve terminals, but induced a slight decrease in the GABAergic nerve terminals (n=3). $A_{2A}R$ silencing exerted a decrease in GABAergic nerve terminals irrespective to the control or restraint group. Percentage of glutamatergic terminals (immunopositive for vGluT1 and vGluT2 and co-localized with synaptophysin) (A) and percentage of GABAergic nerve terminals (immunopositive for vGAT and co-localized with synaptophysin) in synaptosomal preparations of amygdala.

relative co-localization of epitopes to be quantified, irrespective of their absolute staining, which varies among groups of plated nerve terminals.

DISCUSSION / CONCLUSIONS

In the present study, we carried out a behavioural and biochemical characterization of an animal model of stress, pertinent for mood-related disorders, and tested the impact of selective $A_{2A}R$ genetic elimination from amygdala neurons thereupon. Our results show that restraint stress decreased reward-based depressive behaviour typified by no change in preference for a sucrose solution during time as well as loss of self-care once latency to start grooming a palatable solution was increased; it also induces anxiety and loss of memory performance with no changes in social behaviour. Although not significant, these behavioural alterations were accompanied by decreased postsynaptic markers (PSD-95) as well as decreased GABAergic (immunopositive for vGAT) and glutamatergic (immunopositive (Western blot) for vGluT1) nerve terminals. Nevertheless, selective $A_{2A}R$ silencing in the amygdala induced a slight although not significant increase in sucrose preference as well as an increase in the total and sucrose grooming in restrained animals in opposition of control non-stressed animals where $A_{2A}R$ silencing reduced total and sucrose grooming in parallel with increased latency to start grooming, as displayed by restrained animals. Additionally, it also prevented stress-induced anxiety and loss of memory performance while it did not change much control non-stressed animals in the same paradigms. These preventive effects were accompanied by a prevention in synaptic changes, namely of decreased PSD-95 and glutamatergic terminals and also of GABAergic terminals although inconsistently due probably to technical issues related to western blot and Immunocytochemistry assays.

Epidemiological studies show that caffeine consumption correlates inversely with the incidence of depression (Lucas et al 2011). Accordingly, several studies indicate that long term consumption of caffeine, which mainly acts through $A_{2A}R$ antagonism, effectively prevents memory deterioration in different animal models (Takahashi et al 2008). Notably, we found an enhanced $A_{2A}R$ density in the hippocampus, amygdala and striatum upon repeated stress and that chronic consumption of moderate doses of caffeine normalized mood (Cunha et al 2008). Additionally, a recent report showed that selective $A_{2A}R$ antagonism prevented the behavioural modifications resulting from maternal separation-induced stress (Batalha et al 2013). Yet, this pharmacological antagonism globally target $A_{2A}R$ not dissecting the relative contribution of their actions in selective brain regions, especially in the amygdala which play a key role in mood-related modifications. This was the rationale to undertake this study, i.e., to investigate if the long-term

selective deletion of $A_{2A}R$ in the amygdala neurons could effectively be sufficient to alleviate the negative impact of stress on brain function and thus on behaviour.

Regarding specific details of each behavioural testing, there were no differences between the retrained animals and the control group in any of the sucrose preference tests performed, initially with 0.8% sucrose solution and then 0.125% sucrose solution. This adjustment was due to the fact that all the animals groups were drinking approximately the same quantity of sucrose solution, contrarily to the expected (Rygula et al 2005), leading us to think whether the solution was too sweet that even the restrained animals would prefer it. Also, previous studies indicate that lower sucrose concentrations produce better results than the higher ones (Hollis et al 2011). Even so, changing to 0.125% did not change much the results (Goshen et al 2008, Willner 1996), with no significant differences between the animal groups. A possible explanation is the increase of corticosterone levels (Charmandari et al 2005) in stress situation that lead to an increase in blood sugar levels (Mason 1968) which may decrease the will and the need for the animals to consume sweet solutions. In modified Y maze paradigm, the restrained animals demonstrated an impairment in spatial memory. This is consistent with previous reports showing that chronically stressed animals spent less time exploring the novel arm when compared to controls (Wright & Conrad 2008). Herein, restrained animals with amygdala $A_{2A}R$ silencing showed no loss of memory performances, which might be explained by the fact that an increase in $A_{2A}R$ receptors in a stress situation would led to a loss in hippocampal neurons directly connected to memory formation (Magarinos et al 1997, McEwen & Magarinos 1997, Vyas et al 2002).

Finally, the compiled data in this thesis, by itself, is still insufficient to claim a robust preventive effect of selective $A_{2A}R$ in amygdala neurons in stress-induced conditions, pertinent to depression, for two main reasons: i) although preliminary data of the group demonstrated an antidepressant effect of $A_{2A}R$ silencing in a depressive despair-based behavioural paradigm, forced swimming test (data not shown since it is a matter of debate of a doctoral thesis), no depressive-like modification was observed when restrained animals were compared to control non-stressed animals; and ii) the alternative depressive reward- and self-care- based test, i.e., the splash of a palatable solution in the dorsal coat of the animal, also did not altered the animals' grooming upon stress induction. Together, these may be due to a shorter duration of stress induction (14 days only) when compared to other stress protocols of 21 days (or higher), which might be under the limit from which this behaviour becomes evident. Still, these data shows a pivotal role of amygdala $A_{2A}R$ in behavioural and biochemical modifications induced by stress and that their selective genetic elimination might constitute a novel target to manage mood-related disorders.

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