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Hematopoiesis in Transgenic Rats Overexpressing Neuronal Adenosine A_{2A} Receptors

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RESUMO

A hematopoiese é um processo contínuo pelo qual são geradas as células do sangue, a partir das células estaminais hematopoiéticas. Nos adultos, ocorre na medula óssea e a sua regulação envolve vários factores e múltiplas vias de sinalização. Duas grandes linhagens derivam das células estaminais hematopoiéticas: a linhagem mielóide, onde se inserem os monócitos/macrófagos, plaquetas, glóbulos vermelhos e granulócitos (eosinófilos, basófilos e neutrófilos), e a linhagem linfóide, composta pelas células B, células T e células NK.

Os glucocorticóides são uma classe de hormonas esteróides responsáveis por um vasto leque de acções no organismo, entre as quais proliferação celular, resposta a stress e processos cognitivos. Têm um efeito evidente nas várias células hematopoiéticas, afectando a sua proliferação, diferenciação e migração entre a medula óssea e a circulação sanguínea. De forma geral, os glucocorticóides promovem a proliferação de células mielóides e diminuem o número de linfócitos, em circulação.

Exposição a glucocorticóides promove um aumento dos níveis de neutrófilos em circulação e atrasa a sua apoptose, promovendo ainda um aumento de células eritróides. Tratamento com glucocorticóides está também correlacionado com um aumento na função fagocitária dos macrófagos. Por outro lado, a linhagem linfóide é negativamente influenciada pelos glucocorticóides, sendo que exposição a estas hormonas promove uma redução no número de células T e B em circulação.

Os níveis de glucocorticóides em circulação estão sujeitos a oscilações diárias, que se traduzem num robusto ritmo circadiano. Estas oscilações influenciam vários factores envolvidos na regulação das células estaminais hematopoiéticas. Pensa-se que altos níveis de corticosterona, o principal glucocorticóide em roedores, induzem apoptose de células estaminais hematopoiéticas e reduzem a sua capacidade de repopulação da medula óssea, enquanto baixos níveis de corticosterona induzem a proliferação das mesmas células. Factores como CXCL12 e Notch1 estão envolvidos na migração e diferenciação das células estaminais hematopoiéticas, respectivamente, e ambos encontram-se sob influência do ritmo circadiano dos glucocorticóides. Concentrações anormais destes factores podem comprometer a hematopoiese, sugerindo que as oscilações diárias de glucocorticóides desempenham um papel importante na regulação das células hematopoiéticas.

Recentemente, foi demonstrado que a sobreexpressão neuronal de receptores A_{2A} de adenosina, uma característica de envelhecimento cerebral e neurodegeneração, é suficiente para gerar alterações no eixo hipotálamo-hipófise-adrenais, levando a uma disfunção no ritmo circadiano dos níveis plasmáticos de corticosterona, em ratos.

Este receptor está envolvido em funções motoras, desordens de ansiedade, stress, envelhecimento e doenças degenerativas, como o Alzheimer. Durante o normal envelhecimento do cérebro há um aumento dos receptores A_{2A} no hipocampo. A mesma tendência é encontrada nos pacientes de Alzheimer e pensa-se que este receptor é necessário para a neurotoxicidade das placas de amilóide beta, uma característica desta doença.

As consequências da sobreexpressão neuronal de $A_{2A}R$ no cérebro e em processos neurológicos têm vindo a ser estudadas. Contudo, as consequências desta sobreexpressão para todo o organismo, mais precisamente para o sistema hematopoiético, são actualmente desconhecidas. Usando um modelo transgénico, Tg(CaMKII-h$A_{2A}R$), foi possível estudar o papel da sobreexpressão neuronal de $A_{2A}R$ na hematopoiese e na regulação das células hematopoiéticas, através da acção de glucocorticóides. Os ratos transgénicos apresentam uma sobreexpressão neuronal de $A_{2A}R$, o que induz, entre outros efeitos, uma disrupção no ritmo circadiano dos níveis de corticosterona.

Ao longo do estudo, vários parâmetros hematopoiéticos foram abordados. Usando um ensaio de formação de colónias em meio semi-sólido, avaliámos tanto o número como a capacidade de diferenciação das células hematopoiéticas progenitoras de ratos transgénicos, em comparação com ratos wild-type. Diferenças a nível das várias populações de células hematopoiéticas foram avaliadas recorrendo a citometria de fluxo. Foram ainda exploradas alterações a nível do microambiente vascular da medula óssea, quer por observação de vasos sanguíneos quer por quantificação génica de diversos factores, recorrendo a técnicas de imuno-histoquímica, imunofluorescência e RT-PCR.

Ratos transgénicos apresentam um aumento nos números de células totais da medula óssea femoral. Este aumento celular pode ser resultado de uma alteração na composição celular da medula óssea, uma vez que foi observado um aumento dos números de células mielóides. A sobreexpressão neuronal de $A_{2A}R$ parece beneficiar a linhagem mielóide e modular o microambiente vascular da medula. Apesar de não existir uma diferença nos seus números totais, as células hematopoiéticas progenitoras de ratos transgénicos geraram mais colónias percursoras de granulócitos e de granulócitos/monócitos, revelando uma preferência pela linhagem mielóide. Os resultados de citometria de fluxo acompanharam esta tendência, revelando um aumento do número de células $CD11b^+$, um marcador de células mielóides. Também os resultados de RT-PCR revelaram um aumento da expressão relativa de *Csf1,* um factor estimulante de colónias de macrófagos.

A nível do microambiente vascular da medula óssea, foi observado um aumento dos vasos sanguíneos positivos para CD105, um marcador endotelial, apesar da expressão de factores envolvidos em angiogénese, se encontrar diminuída nos ratos transgénicos.

A sobreexpressão neuronal de $A_{2A}R$ parece não ter nenhuma influência sobre o número de megacariócitos, uma vez que a marcação com vWF e a expressão relativa de trombopoietina não revelaram diferenças entre os ratos transgénicos e wild-type.

Tanto as disfunções na via de sinalização dos glucocorticóides e a sobreexpressão neuronal de $A_{2A}R$ estão relacionadas com processos neurodegenerativos e envelhecimento cerebral. Para além deste facto, são usados frequentemente glucocorticóides sintéticos na clínica, como tratamento de várias doenças, tornando importante o estudo destes receptores e das suas acções em todo o organismo. O nosso trabalho sugere que a sobreexpressão neuronal de $A_{2A}R$ tem um papel na hematopoiese, através dos efeitos de glucocorticóides, promovendo uma alteração na composição celular da medula óssea e no seu microambiente vascular.

Em conjunto, os resultados apresentados contribuem para um maior conhecimento sobre o papel da sobreexpressão neuronal de $A_{2A}R$ e dos glucocorticóides na regulação hematopoiética.

Palavras-Chave: hematopoiese; $A_{2A}R$; glucocorticóides; ratos transgénicos

ABSTRACT

Hematopoiesis is the continuous process of blood cells generation from hematopoietic stem cells. Its regulation involves multiple pathways and regulatory signals. Glucocorticoids, a class of steroid hormones responsible for a wide range of effects in the organism, are known to have well-defined effects in hematopoietic cells, affecting their proliferation, differentiation and function.

It was recently shown that neuronal overexpression of the adenosine A_{2A} receptor – a characteristic of aging and neurodegeneration - induces impairments in glucocorticoid receptor actions. Ultimately, this leads to dysfunctions in the hypothalamic-pituitary-adrenal axis, caused by obliteration of the circadian rhythm of plasma glucocorticoids in rats. However, the consequence of this disruption to the whole organism, more precisely to the hematopoietic system, remains unknown. Using transgenic rats with neuronal overexpression of $A_{2A}R$ it was possible to explore the role of this overexpression in hematopoiesis and in hematopoietic cells regulation, via glucocorticoid actions.

We evaluated several hematopoietic parameters, such as hematopoietic progenitor cells' differentiation capacity, differences in hematopoietic cells and in the bone marrow vascular niche. Our main finding is that neuronal overexpression of $A_{2A}R$ triggers a shift in the bone marrow cellular composition, by favouring myeloid cell differentiation. Furthermore, neuronal overexpression of $A_{2A}R$ seems to promote changes in the bone marrow vascular niche, increasing the number of $CD105^+$ blood vessels and modulating the expression of angiocrine factors.

Together, these results contribute to unravel the role of neuronal overexpression of $A_{2A}R$ and glucocorticoids in hematopoiesis.

Keywords: hematopoiesis; $A_{2A}R$; glucocorticoids; transgenic rats

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ABBREVIATIONS LIST

$A_{2A}R$ – adenosine A_2A receptor

ACTH - adrenocorticotropic hormone

ANGPT1 – angiopoietin 1

APC – allophycocyanin

BFU-E – burst-forming unit – erythrocytes

BSA – bovine serum albumin

CaMKIIα – calcium/calmodulin-dependent protein kinase II

cAMP – cyclic adenosine monophosphate

cDNA – complementary deoxyribonucleic acid

CFU – colony-forming unit

CFU-G - colony-forming unit - granulocytes

CFU-GM - colony-forming unit – granulocytes/monocytes

CFU-M - colony-forming unit – monocytes

CRH – corticotropin-releasing hormone

CSF1 – colony stimulating factor 1

CXCL12 – c-x-c motif chemokine 12

CXCR4 – c-x-c chemokine receptor 4

DAPI – 4',6-diamidino-2-phenylindole

DNA – deoxyribonucleic acid

EDTA – ethylenediaminetetraacetic acid

FACS – fluorescence-activated cell sorting

FITC – fluorescein isothiocyanate

GC – glucocorticoid

GR – glucocorticoid receptor

HIER – heat induced epitope retrieval

HPA – hypothalamic-pituitary-adrenal

HPRT1 – hypoxanthine-guanine phosphoribosyltransferase

HSC – hematopoietic stem cell

IF – immunofluorescence

IHC – immunohistochemistry

PBS – phosphate-buffered saline

PIER – proteolytic induced epitope retrieval

RCB – red blood cell

RNA –ribonucleic acid

RT-PCR – real time-polymerase chain reaction

SCF – stem cell factor

SEM – standard error of mean

TBS – tris-buffered saline

Tg – transgenic

THPO – thrombopoietin

VEGF – vascular endothelial growth factor

vWF – Von Willebrand Factor

WT – wild type

1. INTRODUCTION

1.1. Hematopoiesis and the Bone Marrow Microenvironment

Hematopoiesis in mammals is the continuous process by which all blood cells are generated from hematopoietic stem cells (HSCs), in the bone marrow of adults (Ernst, 2009). One of the first evidence for the existence of HSCs appeared in 1963, when Till and McCulloch showed that a specific class of hematopoietic cells could proliferate and form different types of cell colonies when transplanted into the bone marrow of irradiated mice (Becker et al., 1963). This finding led to additional studies in order to better understand and characterize HSCs.

Hematopoietic stem cells are rare cells with the potential for self-renewal and differentiation into multilineage progenitors. HSCs follow a hierarchy of progenitor cells that become progressively restricted to a specific lineage, producing mature blood cells, such as red blood cells, white blood cells and platelets (Orkin and Zon, 2008). Two lineages are thought to arise from hematopoietic stem cells, the myeloid and the lymphocyte lineage, depending if the HSC generates a common myeloid precursor cell or a common lymphocyte precursor cell, respectively (**Figure 1.1**). The myeloid lineage includes megakaryocytes, erythrocytes, granulocytes (neutrophils, basophils and eosinophils) and monocytes while natural killer cells, T and B cells belong to the lymphocyte lineage (Kondo, 2010).

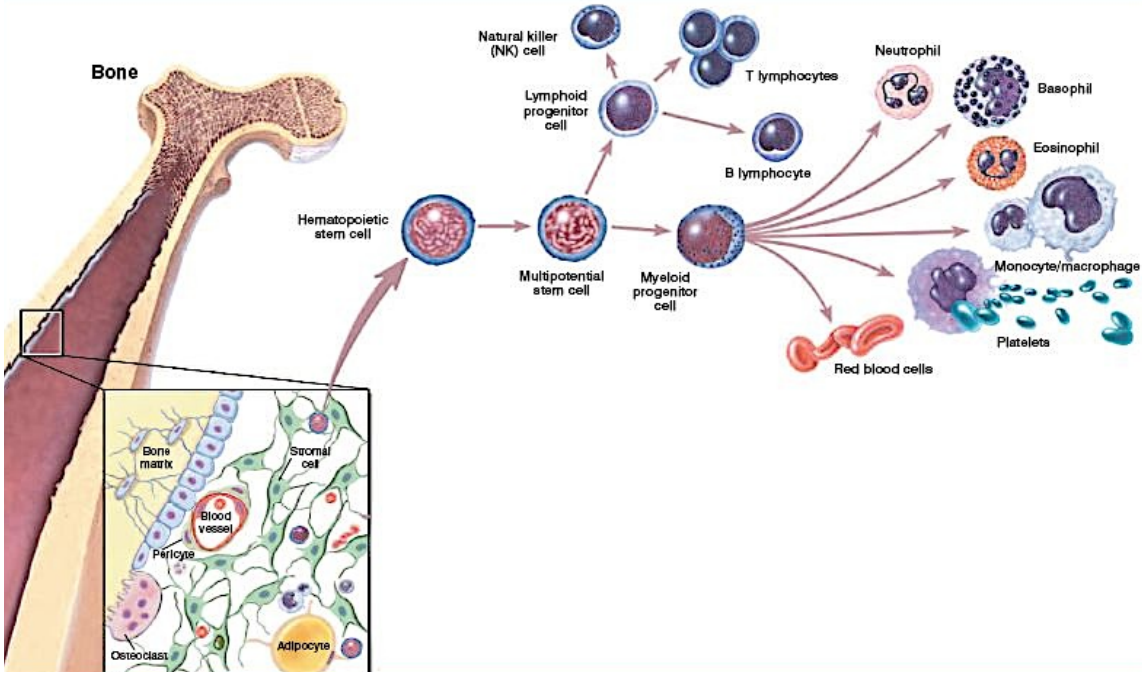

Figure 1.1 – The Bone Marrow Microenvironment. The bone marrow and its different cell types act as a microenvironment for hematopoietic stem cells. Two lineages are thought to arise from HSCs, the myeloid lineage and the lymphocyte lineage (adapted from Winslow and Kibiuk, 2001).

Regulation of hematopoiesis and HSCs fate involves multiple pathways and regulatory signals. The bone marrow provides a microenvironment that regulates the quiescence, self-renewal and differentiation of hematopoietic stem cells, through interaction with several cell types, soluble factors and even physical cues (Mendelson and Frenette, 2014).

A subset of HSCs is believed to be located tangent to the bone, near blood vessels, suggesting that HSCs interact with cells existing in this niche (Anthony and Link, 2014; Mendelson and Frenette, 2014). The bone marrow microenvironment contains stromal cells, a diverse cell population that includes endothelial cells, osteoblasts, mesenchymal stem cells, macrophages, perivascular cells, adipocytes and neuronal cells (Anthony and Link, 2014).

Endothelial cells line the surface of blood vessels. HSCs are frequently adjacent to fenestrated sinusoids, a type of bone marrow blood vessels with a single layer of endothelial cells, which allow cells to pass from the bone marrow to the bloodstream (Morrison and Scadden, 2014). Endothelial cells regulate HSCs proliferation through the expression of essential factors, including CXCL12 and stem cell factor (SCF). CXCL12 is believed to retain HSCs in the bone marrow and SCF promotes HSCs self-renewal (Anthony and Link, 2014). Specific perivascular cells express high amounts of CXCL12, being called CXCL12-abundant reticular (CAR) cells. Similar to endothelial cells, CAR cells regulate HSC self-renewal, proliferation and egress into circulation (Mendelson and Frenette, 2014). In contrast, adipocytes seem to play an inhibitory role in HSCs maintenance, as adipocyte-rich bone marrow shows a decrease in HSC numbers compared with normal or adipocyte-poor bone marrow (Anthony and Link, 2014).

HSCs are also regulated by signals from the sympathetic nervous system, via regulation of the daily fluctuations of CXCL12 (Kollet et al., 2013; Mendelson and Frenette, 2014). The central nervous system controls circulating HSC numbers, believed to peak during the resting phase, which can be at night for diurnal species or during the day for nocturnal species (Méndez-Ferrer et al., 2008).

Differentiation and mobilization of other hematopoietic cells also follows a circadian rhythm, evidenced by circadian variations in cytokines and hormones which are involved in hematopoiesis (Scheiermann et al., 2013). In humans, B and T cells show an increase in their circulating numbers at night and a decrease in the morning. The reverse happens in nocturnal animals, such as rodents (Kawate et al., 1981). Migration of B and T cells from the bloodstream to tissues occurs during the active phase. Recruitment of lymphocytes to tissues during the most physical and active period of the day helps to heal any injury and maintain the immunesurveillance of the organism (Scheiermann et al., 2013). Neutrophils

follow a pattern that is similar to the circadian oscillations of HSCs and lymphocytes (Casanova-Acebes et al., 2013; Scheiermann et al., 2013).

1.2. Role of Glucocorticoids in Hematopoietic Cells

Glucocorticoids are a class of steroid hormones responsible for a wide range of effects in the organism (Kadmiel and Cidlowski, 2013). They regulate a broad spectrum of essential physiological functions, such as growth, reproduction, cognition, behaviour, cell proliferation and survival, as well as having immunosuppressive and anti-inflammatory actions (Jiang et al., 2014; Nicolaides et al., 2014).

Several studies show a reverse correlation between the daily oscillations in the number of hematopoietic cells and the circadian rhythm of glucocorticoids (Abo et al., 1981; Kawate et al., 1981; Kollet et al., 2013). Glucocorticoids (GCs) follow a very robust circadian rhythm, but in contrast with hematopoietic cells, GC circulating levels peak during the active period and decrease during the resting phase (Nicolaides et al., 2014). The glucocorticoid receptor (GR) is expressed in almost every cell type, including hematopoietic cells, suggesting that glucocorticoids might have a role in hematopoiesis and blood cells regulation (Kadmiel and Cidlowski, 2013**)**.

As mentioned, GCs follow a circadian rhythm and their synthesis and release into circulation is under control of the hypothalamic-pituitary-adrenal (HPA) axis regulation (Tsigos and Chrousos, 2002). The HPA axis receives input from the suprachiasmatic nucleus, the master circadian clock of the organism (Dickmeis, 2009), which leads to the secretion of corticotropin-releasing hormone (CRH) from secretory neurons of the hypothalamic paraventricular nucleus (**Figure 1.2**). Secretion of CRH by the hypothalamus stimulates the release of adrenocorticotropic hormone (ACTH) from the

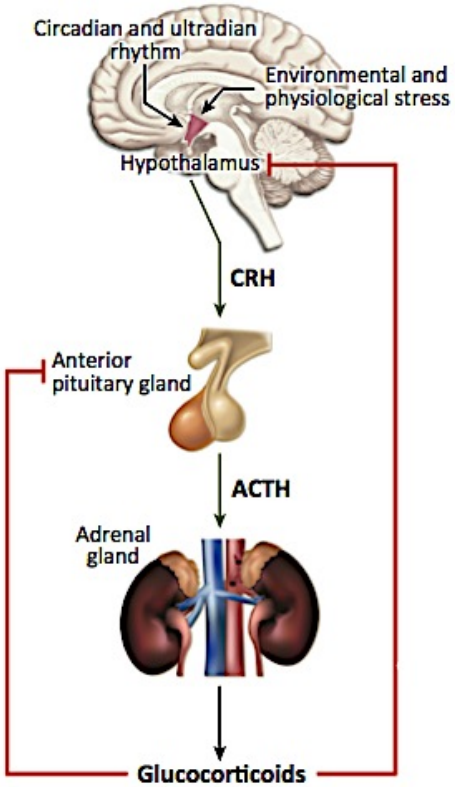

Figure 1.2 – Schematic representation of the HPA axis regulation. CRH is secreted from specific hypothalamic neurons upon stress stimuli and induces ACTH secretion by the anterior pituitary gland. In turn, ACTH stimulates GC synthesis in the adrenal gland cortex. An adequate function of the HPA axis is maintained by a negative-feedback loop (adapted from Kadmiel and Cidlowski, 2013).

anterior pituitary gland, which in turn stimulates the synthesis and secretion of glucocorticoids (cortisol in humans, corticosterone in rodents) by the adrenal gland cortex. A negative-feedback loop maintains the homeostasis of glucocorticoid levels by supressing ACTH release in the pituitary gland and CRH in the hypothalamus (Bellavance and Rivest, 2014; Newton, 2000; Tsigos and Chrousos, 2002).

Glucocorticoids have very diverse effects and modulate the function of different cell types by binding to their evolutionary conserved nuclear receptor. The GR is an intracellular receptor that under basal conditions is present in the cytoplasm and translocates to the cell nucleus after binding to its ligand (Schoneveld et al., 2004; Uchoa et al., 2014). Glucocorticoids bind to GR, inducing a conformational change in the receptor, which enables the ligand-receptor complex to translocate to the nucleus. Once within the nucleus, the activated GR acts on gene expression, inducing specific genes transactivation or transrepression (Henneicke et al., 2014; Schaaf and Cidlowski, 2003). Plasma membrane bound GRs mediate the rapid, non-genomic actions of glucocorticoids, which occur mainly in cells from the immune system (Jiang et al., 2014). GRs are widely expressed in nearly all tissue types and organs systems, having two main roles: maintaining glucose homeostasis and coordinating stress and immune responses (Kadmiel and Cidlowski, 2013).

Relevant to the present study, glucocorticoids exert different effects in each hematopoietic cell type. In general, addition of small amounts of glucocorticoids to bone marrow cultures promotes an accumulation of myeloid cells and a decrease in lymphocytes (Trottier et al., 2008).

Exposure to exogenous GCs promotes an increase in circulating neutrophil numbers and in their life span, probably caused by a delay in neutrophil apoptosis (Bellavance and Rivest, 2014; Liles et al., 1995). In contrast, patients treated with GCs show a decrease in circulating eosinophil numbers. These cells are involved in allergic reactions and chronic allergic diseases, such as asthma. In fact, asthmatic patients treated with GCs show a decline in eosinophil numbers in their airways (Schleimer and Bochner, 1994). Monocytes are phagocytic cells, crucial for the immune system, and exposure to glucocorticoids increases their phagocytic function (Bellavance and Rivest, 2014). Regarding erythrocytes, it was shown that glucocorticoids promote (*in vitro*) the differentiation of erythroid colonies and the proliferation of erythroid cells (Bauer et al., 1999).

The lymphocyte lineage is negatively affected by glucocorticoids, supporting the notion of GCs' immunosuppressive role. Migration of T cells from the bloodstream back to the bone marrow is enhanced after GC treatment, resulting in decreased T cell numbers in circulation (Bellavance and Rivest, 2014; Kadmiel and Cidlowski, 2013). B cells capacity to produce antibodies is supressed upon glucocorticoid exposure, as well as their proliferation

and differentiation, resulting in decreased circulating B cell numbers (Kadmiel and Cidlowski, 2013).

Glucocorticoids also seem to influence more undifferentiated cells. Oscillations of GC levels result into alterations in their circadian rhythm, which in turn regulates the levels of factors involved in HSCs regulation. High concentrations of corticosterone were shown to induce HSCs apoptosis and to reduce bone marrow repopulation in mice, while low levels induced HSCs proliferation. Daily fluctuations of CXCL12 regulate HSC egress from the bone marrow to the peripheral blood and Notch1 (an important receptor involved in hematopoietic differentiation) oscillations are associated with HSC differentiation and self-renewal. Both factors are believed to be influenced by alterations in corticosterone levels and abnormal concentrations of either factor can compromise the normal functioning of the bone marrow compartment and hematopoiesis as a whole, suggesting that daily physiological GC oscillations are needed to a balanced hematopoietic stem cell proliferation, differentiation and migration (Kollet et al., 2013).

1.3. A_{2A} Receptors and Glucocorticoid Oscillations

There is growing evidence that disruption of the glucocorticoid circadian rhythm promotes alterations in the carbohydrate and lipid metabolism, immune response, mood and cognitive functions, leading to the development of many human diseases. For example, Cushing Syndrome is a clinical syndrome with a high prevalence among humans and it is associated with a disturbed cortisol circadian rhythm. Cushing's patients have elevated levels of endogenous cortisol, which can cause severe long-term consequences, such as diabetes mellitus, osteoporosis, bone fractures hypertension, recurrent infections and sleep disorders (Chung et al., 2011). In contrast, Addison's disease is an autoimmune disorder that causes degeneration of the adrenal cortex, leading to low levels of cortisol in humans. Deficiency in cortisol is associated with impaired stress responses, weight loss, general weakness and low blood pressure (Rose and Herzig, 2013).

Glucocorticoids and HPA axis dysfunctions are also involved in cognitive impairments and brain aging. As GRs are highly expressed in the hippocampus, many experimental studies have focused on how glucocorticoids affect its functions (Vyas and Maatouk, 2013). Chronic exposure to GCs has been associated with neurophysiological and neuroanatomical changes, including hippocampal atrophy (Belanoff et al., 2001).

Several reports found high cortisol levels in the plasma, saliva and cerebral spinal fluid of Alzheimer's Disease (AD) patients. AD is a slow and progressive dementia that mainly damages the hippocampus, by deposition of extracellular plaques containing amyloid beta around neurons. Increased cortisol levels were shown to be correlated with poor

memory scores, more amyloid beta deposits and a faster disease progression in general (Vyas and Maatouk, 2013).

Increased plasma glucocorticoid levels and disruption of GC daily oscillations also occur during the normal aging process. Long-term studies showed that more pronounced changes in cortisol levels over time, when compared with basal levels, were a good predictor of cognitive deficits in healthy elderly individuals. In addition, the highest cortisol concentrations were found in patients with poor memory and attention performance (Belanoff et al., 2001).

In recent years, an association between stress response and adenosine modulation has been suggested. In fact, there is a striking parallel between the outcome of a specific adenosine receptor activation and GR signaling. The A_{2A} receptor ($A_{2A}R$) is one of several adenosine receptors and its over-activation mimics the impaired effects of glucocorticoids (Batalha et al., 2014).

The purine nucleoside adenosine and its receptors are involved in the modulation of different central nervous system activities, more precisely, synaptic transmission, neuronal excitability and neurotransmitters release. All four existing adenosine receptors (A_1, A_{2A}, A_{2B}, A_3) are G protein-coupled receptors and the neuromodulatory role of adenosine is mediated by the balance between A_1, responsible for inhibitory actions, and A_{2A}, involved in excitatory actions. These receptors exert their actions by stimulating adenylyl cyclase through G protein coupling activation. Adenylyl cyclase then converts ATP into cAMP, which acts as a second messenger that modulates different signaling cascades (Batalha, 2009; Moreau and Huber, 1999).

In contrast with A_1 receptors, which are widely expressed in the brain, A_{2A} receptors have a more restricted expression. High levels of $A_{2A}R$ mRNA can be found in the striatum, nucleus accumbens and olfactory tubercle, but a low expression has been detected in almost all other brain regions. In non-neuronal tissues, $A_{2A}R$ expression can be found in immune cells, where they control inappropriate inflammatory responses. This receptor plays a role in motor functions, anxiety disorders and in the sleep-wake cycle. Like glucocorticoids, $A_{2A}R$ is also involved in stress response, aging and human diseases, such as Alzheimer (Moreau and Huber, 1999).

In the aging brain, there is an increase in $A_{2A}R$ levels in the hippocampus while A_1 receptors decrease, a pattern that also occurs in Alzheimer patients (Moreau and Huber, 1999). Higher $A_{2A}R$ levels were found in the hippocampus of AD patients and there is evidence that their presence is necessary for the neurotoxicity of amyloid beta deposits. Blockade of this receptor, using antagonists, was shown to provide neuroprotection and increase the resistance of neuronal cells to insults (Rahman, 2009).

Considering the similarities between glucocorticoid impaired actions and $A_{2A}R$ over-activation, several studies were conducted to explore their interaction using stress and transgenic animal models.

In 2009, the effect of chronic stress in $A_{2A}R$ and GR levels was evaluated using the maternal separation method, a neonatal stress model capable of inducing permanent changes in stress response and brain function. One of the main results showed a lasting increase in $A_{2A}R$ levels in the hippocampus (**Figure 1.3a**) and a decrease in GR levels (**Figure 1.3b**) in pups separated from their mother after birth. Generally, there is a decrease in GR expression when plasma glucocorticoid levels are temporally high, in order to limit GC actions. But in animals subjected to maternal separation, a long-term increase in circulating corticosterone levels was observed (**Figure 1.3c**), which can suggest a faulty HPA axis regulation (Batalha, 2009). Administration of KW6002, a selective A_{2A} receptor antagonist, to maternal separated animals was capable of reverting the long-lasting effects of the $A_{2A}R$ up-regulation and restoring the normal HPA axis activity, including corticosterone levels (Batalha et al., 2013).

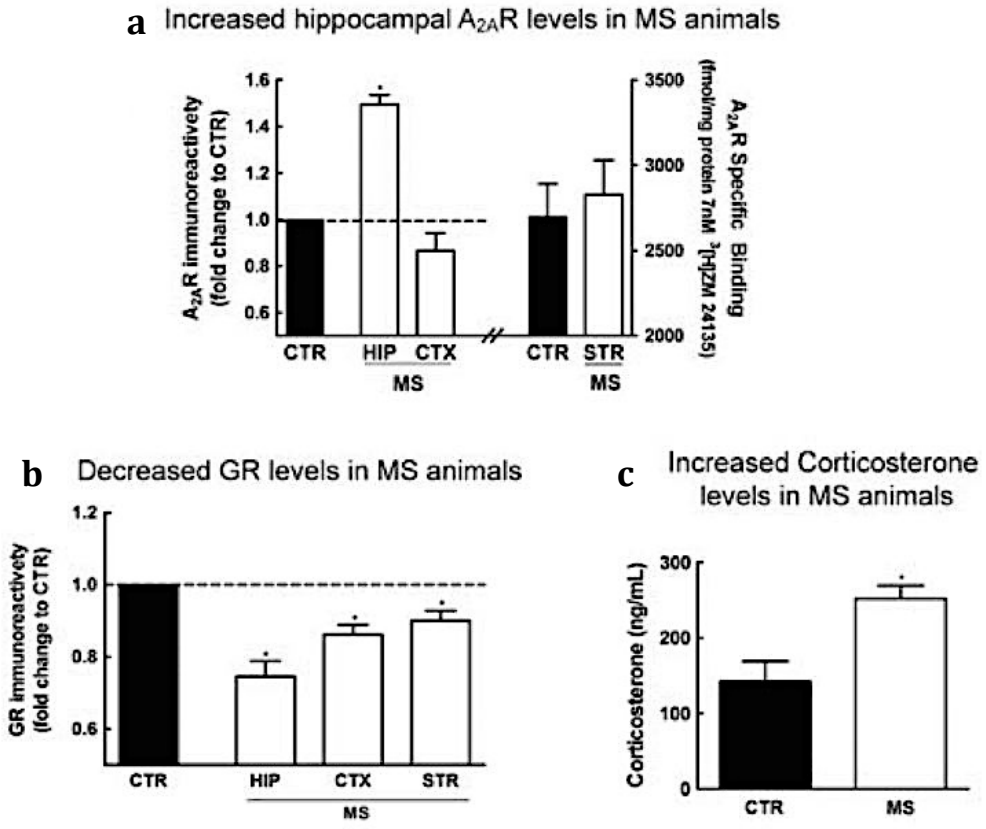

Figure 1.3 – Region-specific effects of maternal separation. Chronic stress induced by maternal separation (MS) is capable of inducing alterations in **(a)** A_{2A} receptor levels in the hippocampus (HIP) but not in the cortex (CTX) and striatum (STR); **(b)** glucocorticoid levels; **(c)** plasmatic corticosterone levels measured at 8 am. Results are the mean ± SEM of 3 to 9 experiments; **p*<0.05, comparing with the control group (CTR) and analysed with an unpaired Student *t*-test (adapted form Batalha et al., 2013).

While results showed an association between GR signaling impairment and $A_{2A}R$ overexpression, there was no evidence of how the two receptors interacted with each other. Using a transgenic rat model that overexpresses $A_{2A}R$, it was possible to evaluate the impact of $A_{2A}R$ in the HPA axis function and GR-related actions.

Transgenic rats were generated by microinjection of a linearized DNA construct into the male pronucleus of Sprague-Dawley rat zygotes. The DNA construct consisted in a full-length human $A_{2A}R$ cDNA, a CaMKIIα promoter and a polyadenylation cassette of bovine growth hormone. The use of the CaMKIIα promoter restricted the overexpression of $A_{2A}R$ to the hippocampus, avoiding striatal overexpression (Coelho et al., 2014).

After several behavioural tests, it was shown that Tg(CaMKII-h$A_{2A}R$) rats displayed depressive-like behaviour and increased locomotor activity (Coelho et al., 2014).

Regarding the interaction with glucocorticoids, overexpression of $A_{2A}R$ was sufficient to promote a decrease in GR levels in the hippocampus and disrupt the normal circadian rhythm of corticosterone. In addition, neuronal cell cultures treated with $A_{2A}R$ antagonists showed a decline in the transcriptional activity of GR and in GR translocation to the nucleus, suggesting that $A_{2A}R$ can directly modulate GR actions. The opposite occurred when cells were treated with $A_{2A}R$ agonists: there was an increase in GR transcriptional activity and in its translocation from cytoplasm to the nucleus. By decreasing GR levels in the hippocampus but increasing GR translocation, $A_{2A}R$ is able to potentiate GR activation and increase stress susceptibility. However, the specific mechanism by which $A_{2A}R$ can trigger GR transcriptional activity has not been clarified (Batalha et al., 2014).

Similar to the maternal separation model, circulating corticosterone levels were measured in Tg(CaMKII-h$A_{2A}R$) rats. Transgenic animals had higher levels of plasma corticosterone in the morning compared with wild-type (WT) and had lost their normal circadian rhythm, reinforcing the notion that $A_{2A}R$ overexpression leads to HPA axis impairments (Batalha et al., 2014).

2. AIMS

It was recently shown that neuronal overexpression of adenosine A_{2A} receptor induces impairments in glucocorticoid receptor actions. Ultimately, this leads to HPA axis dysfunctions, caused by obliteration of the circadian rhythm of plasma corticosterone in rats. Both $A_{2A}R$ overexpression and GR signaling impairments are involved in neurodegenerative diseases, namely Alzheimer's Disease, and brain aging. But the consequences of this disruption to the whole organism, more precisely to the hematopoietic system, are still unknown.

This project **aimed to test if neuronal $A_{2A}R$ overexpression had a role in hematopoiesis via glucocorticoid actions**, as it is known that GCs have well-defined effects in hematopoietic cells, affecting either their proliferation, differentiation or function. Using Tg(CaMKII-h$A_{2A}R$) rats, it was possible to study how impairments in the normal function of the HPA axis affect several types of hematopoietic cells and the differentiation capacity of hematopoietic progenitor cells. In addition, other features of the hematopoietic microenvironmet, such as the bone marrow vasculature, were explored (**Figure 2.1**).

Our working hypothesis was that Tg(CaMKII-h$A_{2A}R$) rats would show similar results to those reported in previous studies that approached glucocorticoid contributions in hematopoiesis. However, given the conflicting data generated in different studies, namely due to distinct experimental setups and the use of different synthetic GCs, our study aimed to clarify the importance of GCs in hematopoiesis, by using a transgenic model with endogenous variations in corticosterone levels. This model allowed a better understanding of the role of glucocorticoids and neuronal overexpression of $A_{2A}R$ in hematopoiesis.

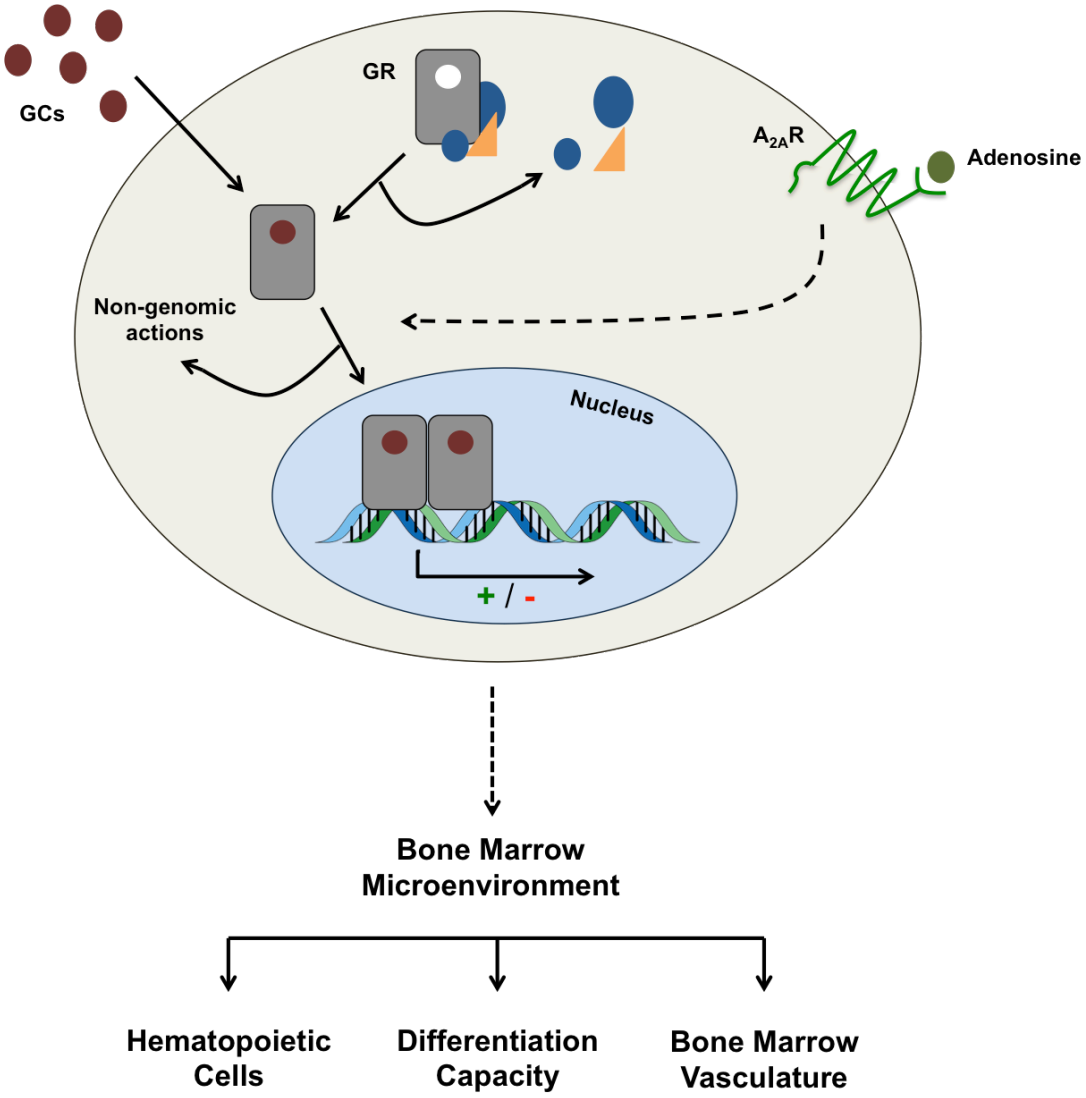

Figure 2.1 – Schematic representation of glucocorticoid actions modulation by $A_{2A}R$. Adenosine A_{2A} receptor can regulate glucocorticoid receptor transcriptional activity and translocation to the nucleus. The precise mechanism underlining GR modulation by $A_{2A}R$ is still unclear (dashed line). Overexpression of $A_{2A}R$ induces dysfunctions in the daily oscillations of glucocorticoids, which in turn can promote alterations in several physiological mechanisms and systems. GCs can exert their actions in the hematopoietic compartment, possibly affecting hematopoietic cells, hematopoietic progenitor cells differentiation capacity and bone marrow vasculature.

3. METHODS

3.1. Animals

Transgenic rats overexpressing the human adenosine A_{2A} receptor, Tg(CaMKII-hA_{2A}R), were provided by Luísa Lopes' lab at IMM. Male and female rats with ages from 8 to 17 weeks were used in all experiments. Littermates' wild type (WT) rats were used as controls. Environmental conditions were kept constant during the whole experiment: food and water ad lib, 21±0,5ºC, 60±10% relative humidity and 12 hours light/dark cycles.

All animal procedures were performed in accordance with the guidelines of the European Community (Directive 2010/63/EU) and approved by the Instituto de Medicina Molecular Internal Committee and the Portuguese Animal Ethics Committee.

Drug Treatment: Transgenic rats were divided in two experimental groups, Tg(CaMKII-hA_{2A}R) and KW. Vehicle (0.025% methylcellulose) or KW6002, a selective antagonist for A_{2A} (3mg kg^{-1} per day, 0.025% methylcellulose), were orally administered for at least one month in the drinking water of Tg(CaMKII-hA_{2A}R) and KW groups, respectively.

3.2. Bone Marrow Cells Isolation

Rats were anesthetized under isoflorane atmosphere before being killed by decapitation, always during the morning. Bone marrow cells were flushed from femurs with a syringe and a 21-gauge needle filled with sterile PBS-EDTA. Red blood cells were incubated for 15 minutes in the dark with 5 ml of RBC Lysis Buffer 1x (Santa Cruz Biotechnology). After red blood cell lysis, the remaining cells were passed through a cell strainer and washed with sterile PBS. After washing, cells were centrifuged at 1200 rpm for 5 minutes and resuspended in Iscove's Modified Dulbecco's Medium (IMDM GlutaMAX™ Supplement; Life Technologies) with 2% Fetal Bovine Serum.

Enrichment Step: To increase progenitor cell numbers, an enrichment step was performed. Bone marrow cells isolated from femurs were incubated at 37ºC and 5% of CO_2 in a plastic petri dish. After two hours, floating cells were collected and adherent cells were discarded. Cell counts were performed in a hemocytometer and cell viability was determined by trypan blue exclusion.

3.3. Peripheral Circulating Blood Cells Analysis

Trunk blood resulting from decapitation was collected into EDTA-coated tubes. Whole blood counts were automatically performed using the pochH-100i Automated Hematology Analyzer (Sysmex).

3.4. Colony-Formating Unit Assay

A Colony-Forming Unit Assay in a semi-solid matrix was performed to test the presence of progenitor cells and their differentiation capacity. After the enrichment step, isolated bone marrow cells (10^5) were plated in duplicate with 400 µl of methylcellulose (Methocult; Stem Cell Technologies) and 100 µl of IMDM 2% FBS, in a 24-well plate. Cells were incubated for 7 to 10 days at 37ºC and 5% of CO_2. After incubation, the number of colony-forming units (CFUs) was counted and colonies were characterized according to established morphology criteria as CFU-GM (colony-forming unit – Granulocytes/Monocytes), CFU-G (colony-forming unit – Granulocytes), CFU-M (colony-forming unit – Monocytes) and BFU-E (burst-forming unit – Erythrocytes) (StemCell Technologies, 2005). Representative images of each colony type were taken using an inverted microscope (Zeiss Primovert).

3.5. Flow Cytometry Analysis

Isolated bone marrow cells were incubated over-night in a plastic petri dish at 37ºC and 5% CO_2. Cells were stained with a mouse anti-rat CD90-FITC and mouse anti-rat CD11b-APC antibody. Cells (10^6) were incubated in 100 µl PBS 1% BSA and the chosen antibody for at least 30 minutes at 4ºC and washed three times with PBS 1% BSA afterwards. Data acquisition was performed on a FACSCalibour and analyzed with FACSDiva software (BD Bioscience).

Apoptosis Assay: Isolated bone marrow cells were washed with sterile PBS. 10^6 cells were incubated in the dark with 100 µL of 1x Binding Buffer and stained with Annexin V-FITC for 20 minutes at room temperature. Data acquisition was performed on a FACSCalibour and analyzed with FACSDiva software (BD Bioscience).

3.6. Immunostaining

Rat's femurs were collected, fixed in 4% paraformaldehyde and paraffin-embedded according to standard histological protocols. Three to four µm sections were cut and used in immunostaining protocols. All used antibodies are described in **Table 1.1**.

Antigen Retrieval: Antigen recovery methods are used to reveal the epitopes and facilitate antibody binding. Slides were primarily treated following the proteolytic induced epitope retrieval (PIER) or the heat induced epitope retrieval (HIER) method. In the PIER method, slides were first deparaffinized and then incubated in a humidified chamber with pepsin (pH=1.7) at 37ºC for 20 minutes. In the HIER method, slides were immersed in a high pH buffer and incubated at 95ºC for at least one hour, in a PT Link Pre-Treatment Module (DAKO).

Immunofluorescence Assay: After deparaffinization, slides were pretreated following the PIER method. After being outlined with a hydrophobic pen, samples were blocked using PBS 0.01% Tween 1% BSA for 30 to 60 minutes at room temperature. Next, sections were incubated over-night at 4ºC with a rabbit anti-human vWf antibody. After washing three times with PBS, a polyclonal anti-rabbit Alexa 594 was used as a secondary antibody. Sections were incubated for one hour at room temperature. Nuclei were counterstained with DAPI. Images were acquired using a Leica DM5000b wild field fluorescence microscope (Leica Microsystems) and analyzed using ImageJ software. Megakaryocytes were quantified by counting ten representative images of each individual.

Immunohistochemistry Assay: Slides were pretreated following the HIER method described above. Afterwards, samples were incubated in the dark with hydrogen peroxidase 3%, in order to block endogenous peroxidase activity, and next with normal horse serum, to reduce non-specific staining, both during 30 minutes and at room temperature. Slides were then incubated over-night at 4ºC with a goat anti-mouse CD105 antibody. After washing three times with distilled water and TBS, samples were incubated for one hour at room temperature with a ready-to-use anti-goat secondary antibody. The secondary antibody was coupled with horseradish peroxidase, an enzyme that catalyses DAB chromogen into a brown coloured product. Sections were incubated with DAB for 2 to 10 minutes and counterstained with hematoxylin, according with standard histological protocols. Ten representative images of each femur were taken using a Leica DM2500 brightfield microscope (Leica Microsystems) and analyzed using ImageJ software.

Proliferation Assay: Slides were subjected to the HIER method and blocked with hydrogen peroxidase 3% and normal horse serum, as described above. Samples were incubated overnight at 4ºC with a rabbit anti-Ki67 antibody. After washing three times with distilled water and TBS, slides were incubated with a ready-to-use anti-rabbit secondary

antibody for one hour at room temperature. DAB revelation and counterstaining were performed as previous slides. Ten representative images of each femur were taken using a Leica DM2500 brightfield microscope (Leica Microsystems). Proliferating cells were quantified using ImmunoRatio, an online application for automated image analysis (Institute of Biomedical Technology).

3.7. RNA extraction and RT-PCR

Isolated bone marrow cells were centrifuged at 1200 rpm for 5 minutes, collected to 1 ml of TRIzol Reagent (Invitrogen) and RNA was extracted according to manufacturer's instructions. Samples were homogenizing using TRIzol and then incubated with chloroform and centrifuged at 14000 rpm for 20 minutes at 4ºC to separate RNA from DNA and the organic phase. The aqueous phase, which contains the RNA, was transferred into a new eppendorf and stored overnight at -20ºC. Then, samples were washed using ethanol 80% and resuspended in DEPC H_2O. Extracted RNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Reverse transcription was performed with SuperScript II (Invitrogen), according to the manufacturer's protocol. Quantitative PCR was performed with Power SYBR Green PCR Master Mix (Roche), according with manufacturer's protocol. A total volume of 8.3 µl of mix (primers, water, BSA and SYBR Green) and 2.0 µl of cDNA was added to each well of a PCR plate. The used primers are described in **Table 1.2**. Amplification of *Hrpt1* was used for sample normalization. RT-PCR was performed on a ViiA^{TM} 7 Real-Time PCR System (Life Technologies).

3.8. Data Analysis

Prism (GraphPad Software) was used for statistic analysis. Data presented are the mean value and error bars represent SEM of *n* experiments. The non-parametric Mann-Whitney *U* test was used to compare differences between WT, Tg(CaMKII-hA_{2A}R) and KW experimental groups. Values of $p<0.05$ were consider statistically significant. Results with only two or less animals per experimental group were not statistically analysed.

Table 1.1 – List of Antibodies used in Flow Cytometry and Immunostaining

Antibody	Use	Antigen Recovery	Dilution	Brand
Annexin V-FITC	FACS	-	1:200	BD Bioscience 556420
CD90-FITC	FACS	-	1:200	BD Bioscience 554894
CD11b -APC	FACS	-	1:200	BD Bioscience 562102
vWF	IF	PIER	1:300	DAKO A0082
CD105	IHC	HIER	1:100	R&D AF1320
Ki67	IHC	HIER	1:100	Abcam 16667
Anti-rabbit	IF	-	1:500	Thermo Scientific A11012
Anti-rabbit	IHC	-	Ready to use	DAKO K4011
Anti-goat	IHC	-	Ready to use	Vector Laboratories MP-7405

Table 1.2 – Amplified Genes and Primers used in RT-PCR

Name of Gene	Primer	Sequence
Hypoxanthine phosphoribosyltransferase 1	r HPTR F	GACCGCTTTTCCCGCGAGCC
	r HPTR R	TCACGACGCTGGGACTGAGGG
Thrombopoietin	r Thrombopoietin F	TGTCCCCACCCCACTCTGTGC
	r Thrombopoietin R	GTGTGGGGCCTCTCCCCTGA
Colony stimulating factor 1 (macrophage)	r CSF1 F	GCCACCGAGAGGCTACAGGAA
	r CSF1 R	TTTGGACACAGGCCTCGTTCTGTT
Angiopoietin 1	r Angiopoietin-1 F	TGATGCCTGTGGCCCTTCCA
	r Angiopoietin-1 R	CATGGTTTTGCCCCGCAGTGT
Chemokine (C-X-C motif) ligand 12	r SDF1 F	GCATCAGTGACGGTAAGCCA
	r SDF1 R	TCTCAAAGAATCGGCAGGGG
Chemokine (C-X-C motif) receptor 4	r CXCR4 F	TCTCAAAGAATCGGCAGGGG
	r CXCR4 R	TGGAGAGCGAGCATTGCC
Vascular endothelial growth factor A	r VEGF-A F	GCACTGGACCCTGGCTTTAC
	r VEGF-A R	TCTGCTCCCCTTCTGTCGT

4. RESULTS

4.1. Increased Bone Marrow Cell Counts in Transgenic Rats

In order to understand if $A_{2A}R$ up-regulation has a role in hematopoiesis, via glucocorticoid actions, we evaluated several hematopoietic parameters in rats with neuronal $A_{2A}R$ overexpression and aberrant corticosterone production.

Femoral bone marrow cells of WT, Tg(CaMKII-h$A_{2A}R$) and KW rats were counted after an enrichment step. There was a significant increase in transgenic rats (6.34 ± 0.54 $\times 10^6$; n=4) bone marrow cell counts compared with WT animals (3.35 ± 0.13 $\times 10^6$; n=6; *p*=0.014) (**Figure 3.1a**). KW6002-treated rats (5.70 ± 1.17 $\times 10^6$; n=4) also obtained higher bone marrow cell counts compared with WT (*p*=0.042) but did not show a significant improvement compared with non-treated transgenic rats (*p*=0.886).

Since Tg(CaMKII-h $A_{2A}R$) rats showed higher bone marrow cell counts, we assessed if this increase was related to alterations in proliferation and apoptosis rates. For the proliferation assay, we used the nuclear marker Ki67 and proliferation rates were calculated using an automated image analysis program. There was no difference between transgenic (20.24 ± 1.34%; n=2) and WT (20.80 ± 1.65%; n=2) proliferating rates (**Figure 3.1b**).

Apoptosis rates were assessed by Annexin V staining, using flow cytometry. Cells undergoing apoptosis lose their plasma membrane and expose phospholipid phosphatidylserine to the extracellular environment. Annexin V binds to this component, allowing us to identify apoptotic cells. Transgenic rats (14.9%; n=1) showed an increase of 3.2% of apoptotic cells compared with WT (11.7%; n=1; **Figure 3.1c**). The results were not elucidatory, given that only one individual per experimental group was analyzed.

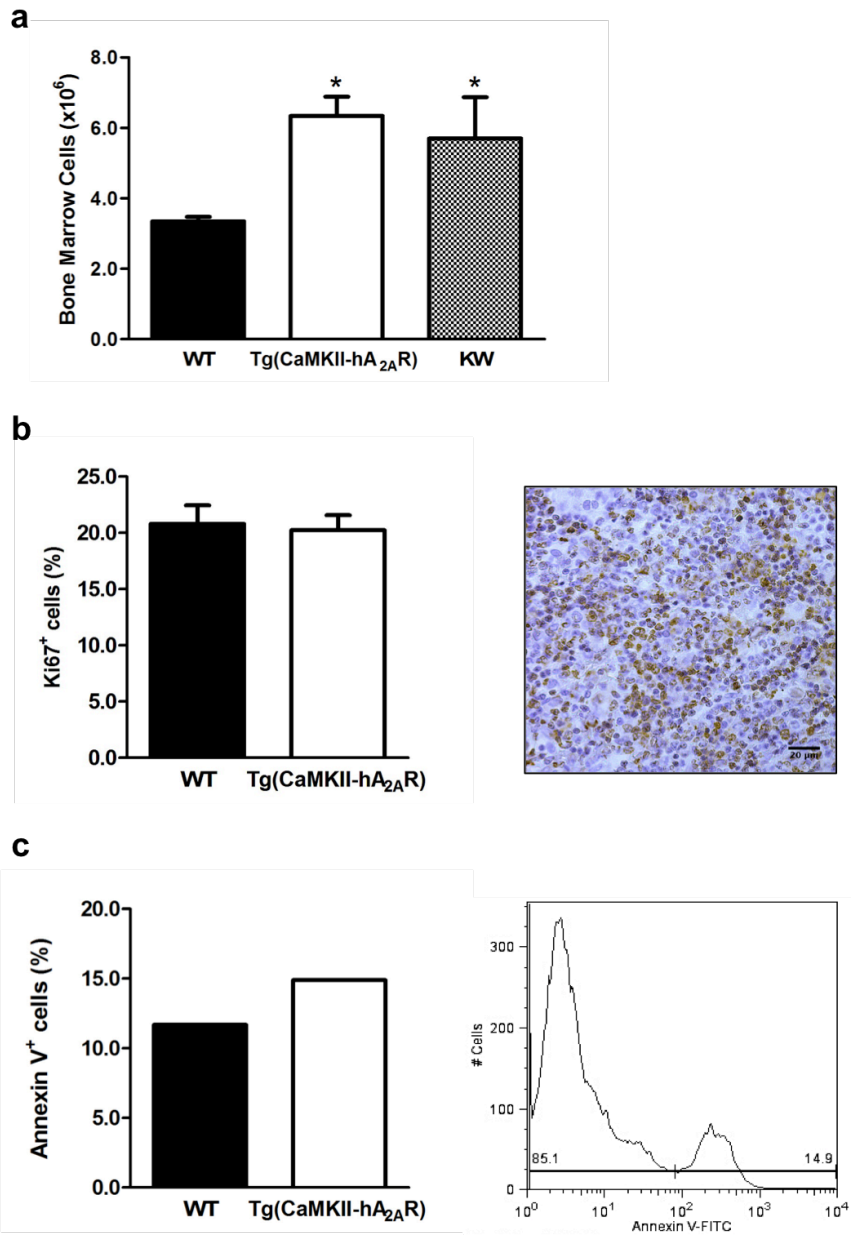

Figure 3.1 – Rats with neuronal $A_{2A}R$ overexpression have higher bone marrow cells counts. (a) Transgenic rats have more cells in their femoral bone marrow when compared with their littermate's WT rats, without showing differences in **(b)** proliferation rates. Representative image of Ki67 staining (40x objective); **(c)** Apoptosis results were not elucidatory, though transgenic rats show a slight increase compared with WT, as assessed by flow cytometry. Representative histogram for Annexin V staining. Data presented are the mean value ± SEM of *n* (1-6) experiments; **p*<0.05 compared with WT (Mann-Whitney *U* test).

4.2. Differentiation of CFU-G and CFU-GM

To test the presence of progenitor cells and their differentiation capacity, we performed a Colony-Forming Unit Assay in a semi-solid matrix, methylcellulose. Colonies were quantified and characterized according with established criteria as CFU-Monocytes, CFU-Granulocytes, CFU-Granulocytes/Monocytes and BFU-Erythrocytes. There was no difference in the total number of colonies between transgenic (18.25 ± 2.06 colonies; n=4), WT (16.50 ± 2.84 colonies; n=3) and KW6002-treated (18.75 ± 1.46 colonies; n=4) rats (**Figure 3.2a**), neither in CFU-M (**Figure 3.2b**) and BFU-E (**Figure 3.2c**).

However, a tendency for higher colony numbers of CFU-G (**Figure 3.2d**) and CFU-GM (**Figure 3.2e**) was found in transgenic rats. WT progenitor cells generated, in average, 0.83 ± 0.60 CFU-G and 1.83 ± 1.09 CFU-GM, while transgenic rats' progenitor cells generated 1.88 ± 0.47 CFU-G and 3.75 ± 1.27 CFU-GM. Rats treated with KW6002 showed similar colony numbers as WT, with the exception of BFU-E, in which KW6002-treated animals displayed a tendency for higher colony numbers.

4.3. Overexpression of $A_{2A}R$ Benefits Myeloid Cells

Next, we explored the expression of different hematopoietic markers by flow cytometry. CD11b is a surface marker expressed in myeloid cells and its expression is rapidly up-regulated upon neutrophil activation. Two subsets of cells were found within the CD11b positive cells (**Figure 3.3a**). Only cells with high CD11b expression were quantified, in all experimental groups.

Transgenic rats (22.50 ± 1.05%; n=3) showed an increase of 6.58 ± 1.41% in $CD11^+$ cells compared with WT animals (15.92 ± 0.94%; n=5; p=0.036). Treatment with KW6002 had no effect in CD11b expression, as KW rats (24.00 ± 3.90%; n=2) also showed an increase in $CD11b^+$ cells compared with WT individuals.

Surface marker CD90 is expressed in different bone marrow cells populations, such as hematopoietic stem cells, early myeloid and erythroid cells, and immature B lymphocytes. CD90 negative cells are mainly neutrophils and mature erythrocytes. $CD90^+$ cell percentages were significantly decreased in transgenic rats (18.93 ± 0.32%; n=4) compared with WT (32.10 ± 2.92%; n=4; p=0.029). In similarity with CD11b results, treatment with KW6002 (21.40 ± 2.70%; n=2) did not affect CD90 expression in transgenic animals (**Figure 3.3b**).

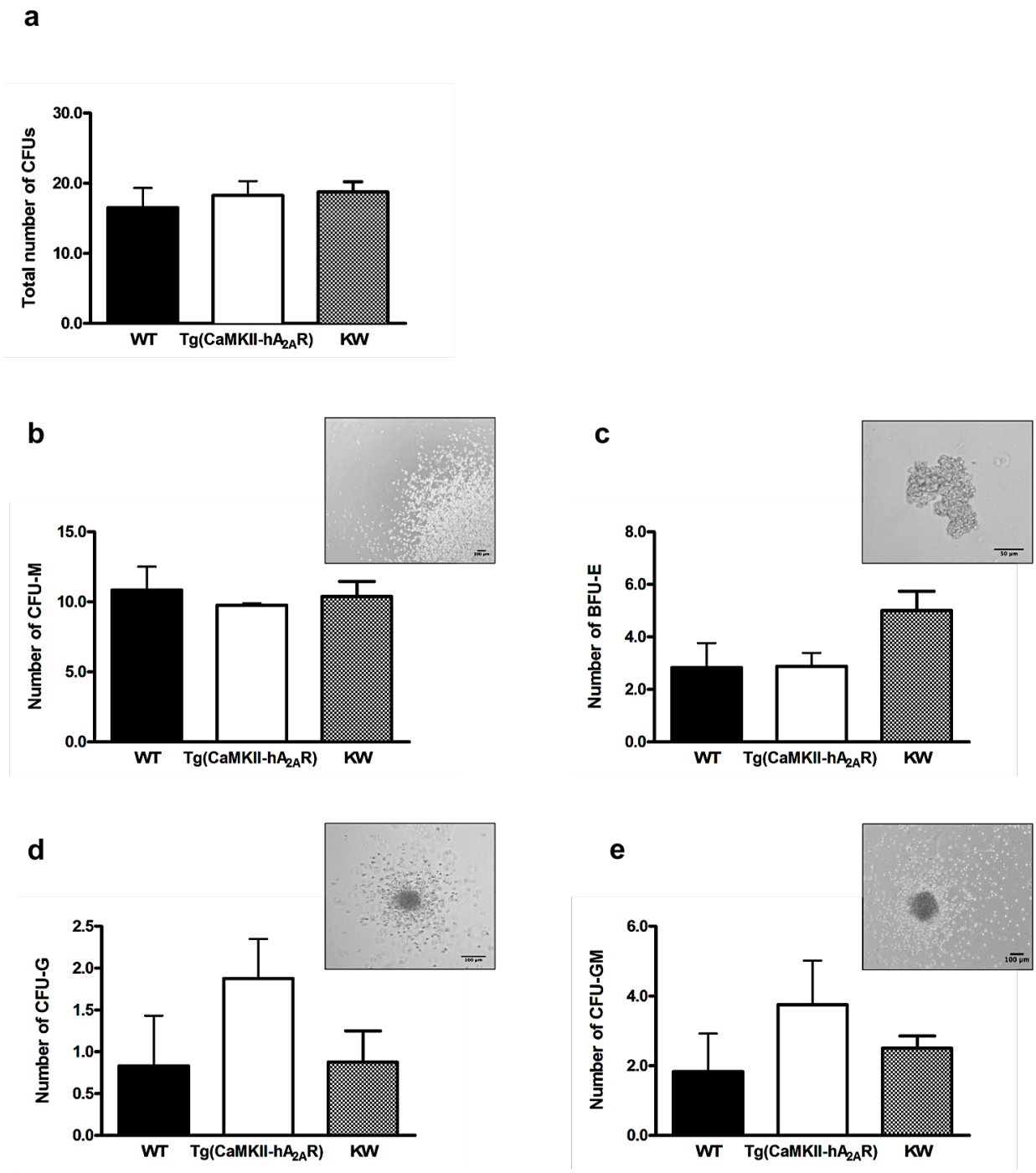

Figure 3.2 – Transgenic rats' progenitor cells generate more CFU-G and CFU-GM. Transgenic and WT rats obtained similar colony counts for **(a)** total CFUs, **(b)** CFU-Monocytes and **(c)** BFU- Erythrocytes, but transgenic animals showed higher numbers of **(d)** CFU-Granulocytes and **(e)** CFU-Granulocytes/Monocytes. KW rats showed similar results as WT, except for BFU-E. Representative images of each colony type. Data presented are the mean value ± SEM of *n* (3-4) experiments.

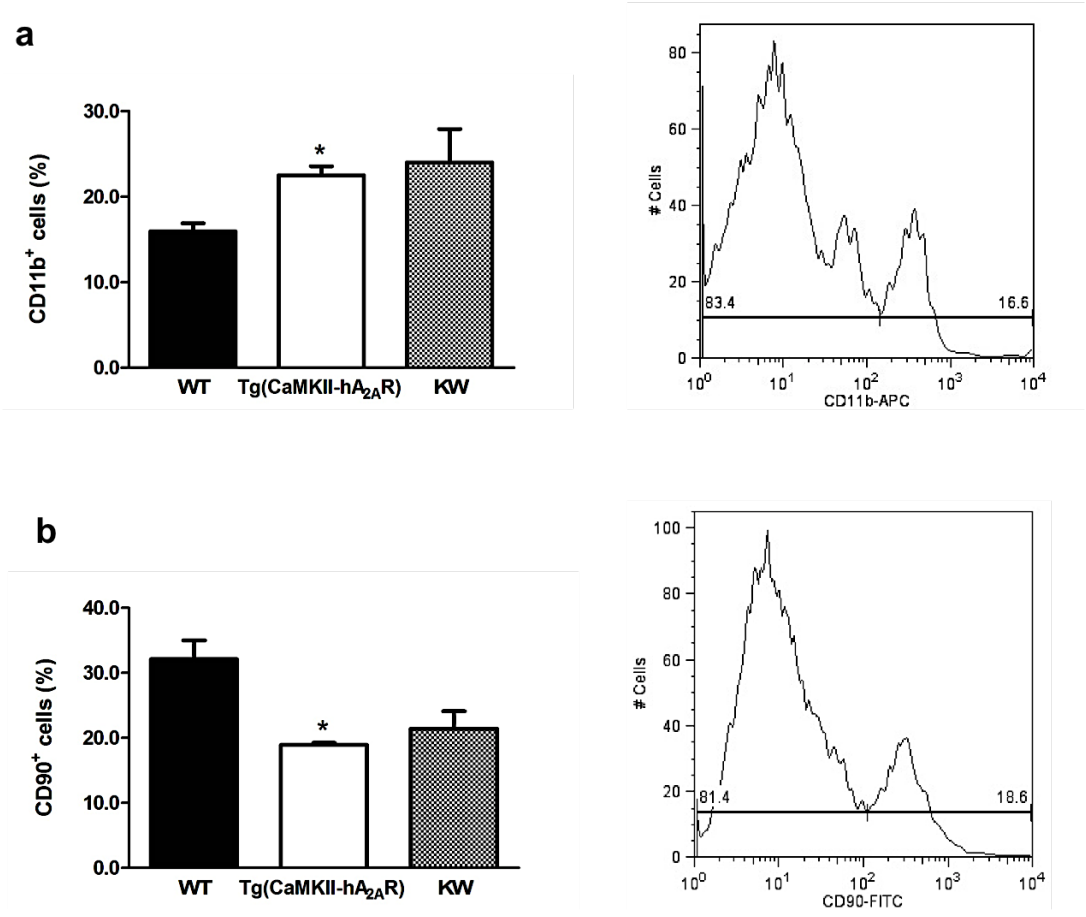

Figure 3.3 – Transgenic rats produce more myeloid cells. Transgenic rats showed increased numbers of **(a)** $CD11b^+$ cells and decreased numbers of **(b)** $CD90^+$ cells compared with WT rats, as assessed by flow cytometry. In both cases, KW6002 treatment did not seem to revert the effect. Representative histograms of CD11b and CD90 staining. Data presented are the mean value ± SEM of *n* (2-5) experiments; **p*<0.05 compared with WT (Mann-Whitney *U* test).

4.4. Modulation of the Bone Marrow Vascular Niche

The bone marrow provides a microenvironment capable of regulating and interacting with hematopoietic cells. We explored differences in the bone marrow vascular niche, in order to evaluate if $A_{2A}R$ overexpression had any effect in the hematopoietic microenvironment.

CD105 is a surface marker highly expressed in proliferating vascular endothelial cells, allowing us to identify bone marrow blood vessels. After CD105 staining, transgenic rats (12.75 ± 0.57 vessels per field; n=3) showed higher numbers of $CD105^+$ blood vessels per field when compared with WT (10.56 ± 0.71 vessels per field; n=3; *p*=0.023) (**Figure 3.4**).

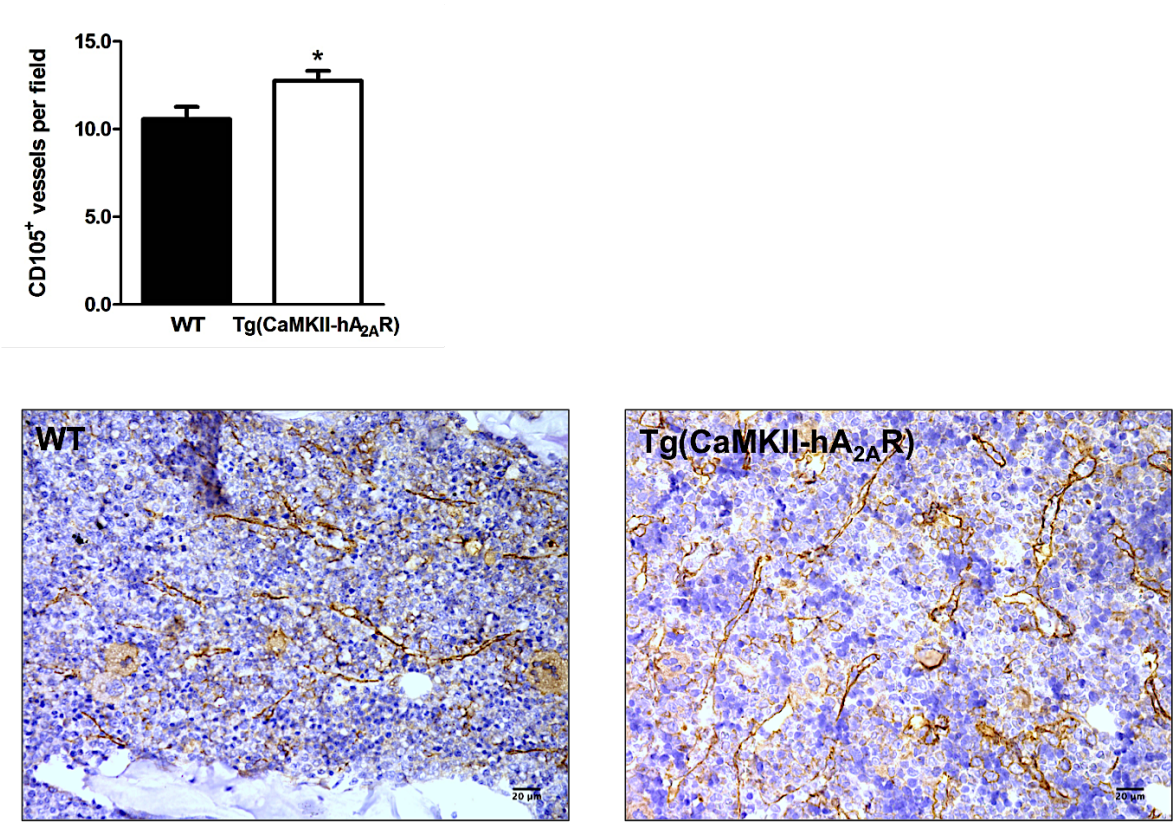

Figure 3.4 – Transgenic rats have more CD105$^+$ blood vessels. Transgenic rats showed a significant increase in bone marrow CD105$^+$ blood vessels per field when compared with WT individuals. Representative images of CD105 staining in femoral bone marrow (40x objective). Data presented are the mean value ± SEM of *n* (2-3) experiments; **p*<0.05 compared with WT (Mann-Whitney *U* test).

We extracted mRNA from isolated bone marrow cells and quantified the relative expression of several genes involved in the regulation of hematopoietic cells and blood vessels formation, by RT-PCR. Only one individual per experimental group was analyzed, making it difficult to evaluate the effect of $A_{2A}R$ overexpression in gene expression. However, the transgenic rat showed a increase in *Csf1* levels and a slight decrease in *Angpt1* and *Vegfa* relative expression when compared with WT (**Figure 3.5**). *Csf1* modulates monocyte differentiation, proliferation and survival while *Angpt1* and *Vegfa* are involved in blood vessels formation.

We also quantified megakaryocytes using the Von Willebrand Factor (vWF) marker, a glycoprotein found in the blood plasma, endothelial cells and megakaryocytes. No significant difference was found in vWF$^+$ megakaryocyte numbers between WT (26.53 ± 0.95 megakaryocytes per field; n=3) and transgenic (29.10 ± 1.079 megakaryocytes per field; n=3; *p*=0.119) rats (**Figure 3.6**).

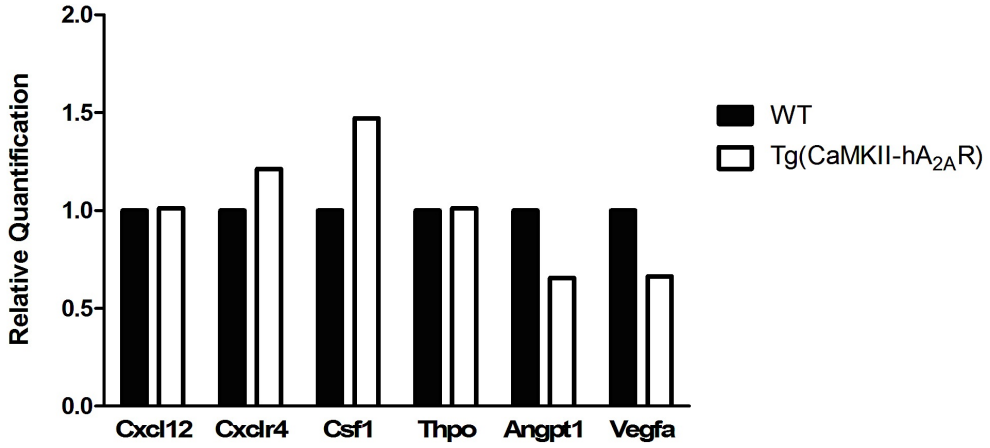

Figure 3.5 – Overexpression of $A_{2A}R$ modulates gene expression. Relative quantification of mRNA extracted from isolated bone marrow cells was assessed by RT-PCR. The transgenic rat showed an increase in the relative expression of *Csf1* and decreased levels of *Angpt1* and *Vegfa* compared with WT. Data presented are the fold change of mRNA levels compared with WT of 1 experiment.

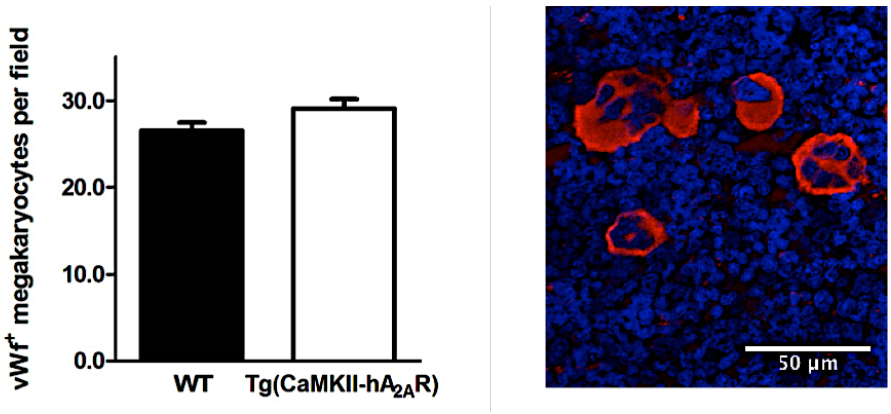

Figure 3.6 – vWF^+ megakaryocytes do not show alterations in transgenic rats. WT and transgenic rats showed similar numbers of vWF^+ megakaryocytes per field. Representative image of vWF staining (red) counterstained with DAPI (blue) (40x objective). Data presented are the mean value ± SEM of 3 experiments.

4.5. Variations in Peripheral Blood Cell Counts

Trunk blood was collected after rats' decapitation into EDTA-coated tubes and blood cells were automatically counted using a Hematology Analyser. White blood cells were automatically separated according with their size. Small white blood cells seem to represent lymphocytes while large white blood cells seem to represent neutrophils. No significant result was found in any parameter but minor differences were found in all cell types.

Regarding white blood cells (WBC), transgenic rats showed a slight increase in total WBC (**Figure 3.7a**), small white blood cells (W-SCC) (**Figure 3.7b**), and a more pronounced increase in large white blood cells (W-LCC) (**Figure 3.7c**) when compared with WT cell counts. KW6002-treated animals also showed increased WBC counts, either in total WBC, small or large white blood cells.

The same pattern was found in platelets (**Figure 3.7d**) and red blood cells (RBC) (**Figure 3.7e**) numbers. Transgenic and KW animals showed higher cell numbers in both parameters when compared with WT rats.

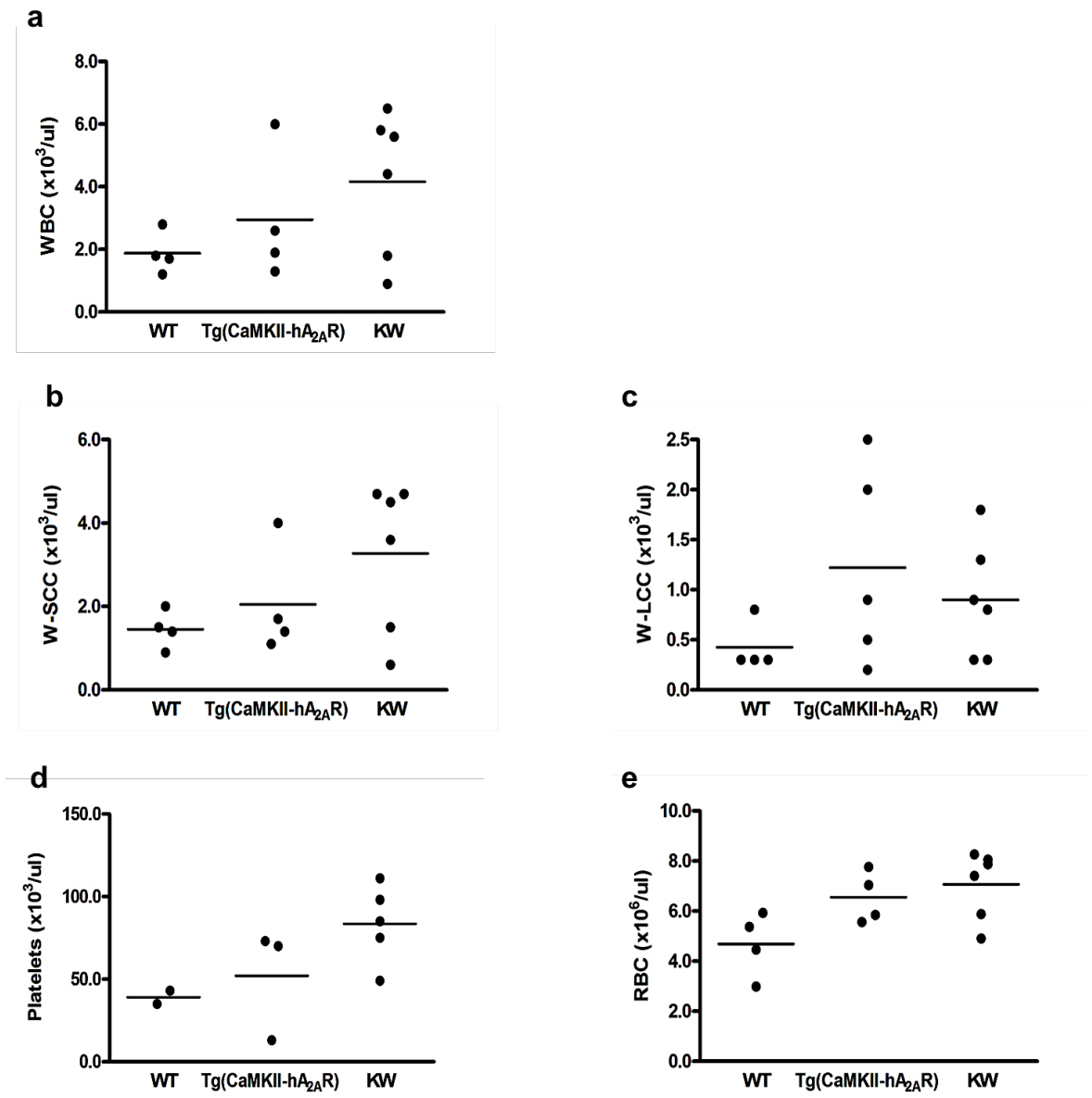

Figure 3.7 – Peripheral blood cell counts alterations in transgenic rats. Transgenic rats showed an increase in **(a)** white blood cells, **(b)** small white blood cells, **(c)** large blood cells, **(d)** platelets and **(e)** red blood cells. Treatment with KW6002 did not seem to revert the effect. Each point represents one individual. Data presented are the absolute value of each individual and the mean value *n* experiments.

5. DISCUSSION

Neuronal overexpression of the adenosine A_{2A} receptor induces dysfunctions in the daily oscillations of glucocorticoids, which in turn leads to alterations in several physiological processes. It is known that GCs have well-defined effects in hematopoietic cells, affecting their proliferation, differentiation and function.

Using Tg(CaMKII-hA_{2A}R) rats, it was possible to explore the consequences of A_{2A}R overexpression and GR signaling impairments in the hematopoietic compartment and hematopoiesis. Several hematopoietic parameters were explored, such as bone marrow cell counts, presence of hematopoietic progenitor cells and their differentiation capacity, as well as the bone marrow vascular niche.

Our main finding is that neuronal overexpression of A_{2A}R triggers a shift in the bone marrow cellular composition, by skewing hematopoietic progenitor cells towards the myeloid lineage, which is accompanied by alterations in the bone marrow vascular niche.

5.1. Changes in Bone Marrow Cellular Composition

Femoral bone marrow cell counts were higher in transgenic rats compared with their wild type littermates. To determine the cause of this effect, we performed a proliferation assay. No difference was found in proliferation rates between the two experimental groups, which led us to believe that transgenic rats have high bone marrow cell counts since birth. To support this hypothesis, we would need to explore changes in bone marrow cell counts at different ages, as well as proliferation and apoptosis rates.

Changes in the bone marrow cellular composition can be a result of alterations in one or more hematopoietic lineages (Travlos, 2006). Our results suggest that the higher numbers we observed in bone marrow cell counts are related to the increase in myeloid cells numbers.

In the Colony-Forming Unit Assay, transgenic and WT rats' hematopoietic progenitor cells generated similar numbers of total CFUs. However, transgenic rats' progenitor cells generated more CFU-G and CFU-GM, precursors of granulocytes and monocytes. Flow cytometry analysis showed that transgenic animals had more $CD11b^+$ cells compared with WT. CD11b is a myeloid marker that specifically binds to the α-subunit of Mac-1, a cell surface receptor involved in phagocytic, adhesion, chemotaxis and migration processes (Spriel et al, 2000). Both monocytes and neutrophils express CD11b and its expression is rapidly up-regulated upon neutrophil activation (Tamatani et al., 1993). Together, these two

results suggest that overexpression of $A_{2A}R$ benefits myeloid cells, more specifically monocytes and granulocytes.

In fact, glucocorticoids seem to promote the generation of myeloid cells, increasing monocyte and neutrophil numbers in both bone marrow and bloodstream (Trottier et al., 2008). Neutrophils are the most abundant type of granulocytes. They play an important role in innate immunity, being the first line of defence against invading organisms (Hirsch et al., 2012). Mature neutrophils have a short life span and it is believed that glucocorticoids can delay their apoptosis, resulting in increased levels of circulating neutrophils. Additionally, GCs promote neutrophil release from the bone marrow into the bloodstream and delay their migration into tissues (Liles et al., 1995).

The exact mechanism by which glucocorticoids exert their actions in myeloid cells, in particular neutrophils, remains unclear. Modulation of several apoptotic factors might account for the inhibitory effects of GCs on neutrophil apoptosis. For example, the Fas receptor, present in neutrophils, is involved in apoptosis and it has been shown that synthetic glucocorticoids are able to regulate its signaling pathway (Liles et al., 1995).

Whole blood cell counts seemed to follow the same tendency found in the CFU Assay. Transgenic rats had, on average, more circulating white blood cells in circulation compared with WT rats. However, results were very inconstant for the three experimental groups, probably because of the blood sampling method used. Blood was collected from the site of decapitation, which led to a mix of both arterial and venous blood and a wide range of values. A different blood sampling method should be used for a more accurate analysis and accuracy of results.

The reason behind GC-mediated myeloid expansion remains unknown. However, it seems that glucocorticoids promote the innate immune system, which depends on the action of different cells, such as macrophages and granulocytes. While myeloid cells proliferation is promoted by glucocorticoids, the lymphoid lineage is down-regulated, as GC exposure leads to decreased lymphocyte levels, in both bone marrow and circulation (Trottier et al., 2008). Furthermore, glucocorticoids are able to induce the expression of genes involved in the activation of innate immunity, such as the Toll-like receptor 2 gene (Kadmiel and Cidlowski, 2013). This way, the first line of immune defence is strengthened, in order to protect the organism against as many infections and invading organism as possible, specifically during stress conditions (Laakko and Fraker, 2002; Trottier et al., 2008).

In agreement with this hypothesis, it is possible that Tg(CaMKII-h$A_{2A}R$) animals also display a decrease in lymphocyte numbers, as assessed by CD90 staining. This marker, also called Thy-1, is a membrane glycoprotein involved in signal transduction and it is expressed by pluripotent hematopoietic stem cells, B cells, and early myeloid and erythroid precursors, in the rat's bone marrow (Hermans and Opstelten, 1991).

Regarding B cells, the effects of glucocorticoids have not been extensively studied, but a correlation between treatment with synthetic GCs and a reduction of the circulation B cell activating factor, and consequently decreased B cell numbers, proliferation and antibody production, has been described (Kadmiel and Cidlowski, 2013; Zhen et al., 2011).

Hematopoietic stem and progenitor cells proliferation and differentiation are also affected by glucocorticoids. As mentioned before, CXCL12 is believed to retain HSCs in the bone marrow and a disruption of the circadian rhythm of corticosterone leads to alterations in its daily fluctuations, damaging the normal migration of HSCs. It was also reported that exposure to high levels of corticosterone reduced *Notch1* transcription, a receptor associated with HSC proliferation, and promoted hematopoietic progenitor cells apoptosis (Kollet et al., 2013).

Transgenic rats showed a significant reduction in $CD90^+$ cells compared with WT, but since Thy-1 is expressed in different cell types, the majority of them being influenced by GC levels, we cannot identify the exact cell population that is being supress without using more specific markers (Kadmiel and Cidlowski, 2013; Kollet et al., 2013).

Treatment with the adenosine A_{2A} receptor antagonist, KW6002, displayed variable results. KW6002-treated animals showed no improvement regarding femoral bone marrow cell counts, CD11b and CD90 staining, having similar results to non-treated transgenic rats. However, in the colony-forming unit assay, KW6002-treated animals showed similar results to WT rats, with the exception of BFU-E. This discrepancy in results can be explained by the duration of the KW6002 treatment. The drug was administered in the animals' drinking water for a month, which might not be sufficient to revert the effects of $A_{2A}R$ overexpression. In order to evaluate the treatment efficiency, we should quantify GC levels of treated animals and KW6002 capacity to restore the normal circadian rhythm of corticosterone. We also do not know which A_{2A} receptors are being blocked by KW6002, since $A_{2A}R$ is expressed in several brain regions and non-neuronal tissues, such as immune cells. This way, KW6002 might be blocking receptors in different sites of the organism, besides the A_{2A} receptors overexpressed in the forebrain, leading to unwanted effects.

5.2. Alterations in the Bone Marrow Vascular Niche

The bone marrow provides a microenvironment capable of regulating hematopoietic stem cells, through their interaction with several cell types and soluble factors (Mendelson and Frenette, 2014). Given the proximity of HSCs to blood vessels, it is believed that endothelial cells might modulate their homeostasis. In fact, the vascular niche is an important component of the bone marrow microenvironment and contributes to the proliferation and differentiation of HSCs, through angiocrine factors. These paracrine growth factors are

released by endothelial cells and are responsible for regenerating damaged tissues, by promoting tissue-specific stem and progenitor cells self-renewal (Butler et al., 2010; Nolan et al., 2014).

Using the CD105 marker we quantified bone marrow blood vessels, in order to explore the effects of $A_{2A}R$ overexpression in the vascular niche. Transgenic rats showed more $CD105^{+}$ vessels in their bone marrow compared with WT animals. This change in the bone marrow vasculature might represent an additional mechanism by which corticosterone is able to modulate hematopoiesis, in rats.

However, this increase in blood vessels numbers was not supported by RT-PCR results. The transgenic rat analysed showed a decrease in both *Angpt1* and *Vegfa* when compared with WT. Both factors promote endothelial survival, proliferation and migration. ANGPT1 is responsible for blood vessel stability and prevents them from leaking while VEGF increases the permeability of the vascular wall (Gavard et al., 2008; Kim et al., 2008). While these results seem contradictory, endothelial cells are not the only producers of ANGPT1 and VEGF and the observed decrease in the relative expression of both factors might be a consequence from GC actions in other cell types, not explored during this study.

Regarding glucocorticoid actions on endothelial cells, it is believed that GCs inhibit angiogenesis, by promoting alterations in cell morphology and reducing cell-to-cell contact rather than inhibition of endothelial cells proliferation or viability (Logie et al., 2010).

Contrary to what was found in previous studies (Kollet et al., 2013), CXCL12 and its receptor CXLR4 did not showed relevant differences in their relative expression, but more individuals per experimental group should be analysed for more elucidatory results.

According with previous results, *Thpo* levels did not show any difference between transgenic and WT rats. Thrombopoietin is the primary regulator of megakaryocyte progenitors and stimulates megakaryocyte differentiation (Hitchcock and Kaushansky, 2014). As *Thpo* levels, megakaryocyte numbers per field did not show any difference between the two experimental groups, as assessed by vWF staining, suggesting that neuronal overexpression of $A_{2A}R$ does not affect megakaryocytes.

Csf1 expression levels further support the notion that neuronal overexpression of $A_{2A}R$ benefits the myeloid lineage. CSF1 is a macrophage colony stimulating factor and acts on myeloid progenitor cells, promoting monocyte/macrophage differentiation (Rathinam et al., 2011). The transgenic rat showed an increase in *Csf1* levels compared with WT, a result that follows the tendency found in flow cytometry analysis and the colony-forming unit assay. To further support this hypothesis, other myeloid genes expression should be quantified, such as granulocyte (Csf3) and granulocyte-macrophage (Csf2) colony stimulating factors.

5.3. Final Remarks

To further explore the role of neuronal $A_{2A}R$ overexpression in hematopoiesis, via glucocorticoid actions, it would be interesting to perform the same procedures at a different time point of the corticosterone daily rhythm. This way, it would be possible to expand our knowledge about the function of glucocorticoids levels and their circadian rhythms in hematopoiesis. Additionally, irradiated animals should be used, in order to evaluate their recovery capacity and emphasize the differences we observed in non-irradiated animals during this study.

Preliminary results suggest a similarity between the effects of neuronal $A_{2A}R$ overexpression and the normal aging process, in hematopoiesis. Both transgenic animals and normal aged WT rats showed increased numbers of myeloid cells and granulocyte precursors colonies and decreased numbers of $CD90^+$ cells (**Supplementary Figure 1.1 and 1.2**). To further explore this apparent similarity, more wild-type rats with different ages should be evaluated.

Given that $A_{2A}R$ overexpression and glucocorticoid receptor impairments are both involved in neurodegenerative and aging processes, and that synthetic glucocorticoids are widely prescribed in clinical treatments, it is important to study the roles of both receptors in different systems of the organism. Our work allowed us to explore the role of glucocorticoids and neuronal overexpression of $A_{2A}R$ in hematopoiesis.

In conclusion, our results suggest that $A_{2A}R$ overexpression promotes alterations in the bone marrow vascular niche and triggers a shift in the bone marrow cellular composition (**Figure 4.1**), favouring myeloid cells differentiation and decreasing the numbers of a diverse cell population, consisting of B cells and hematopoietic progenitor cells.

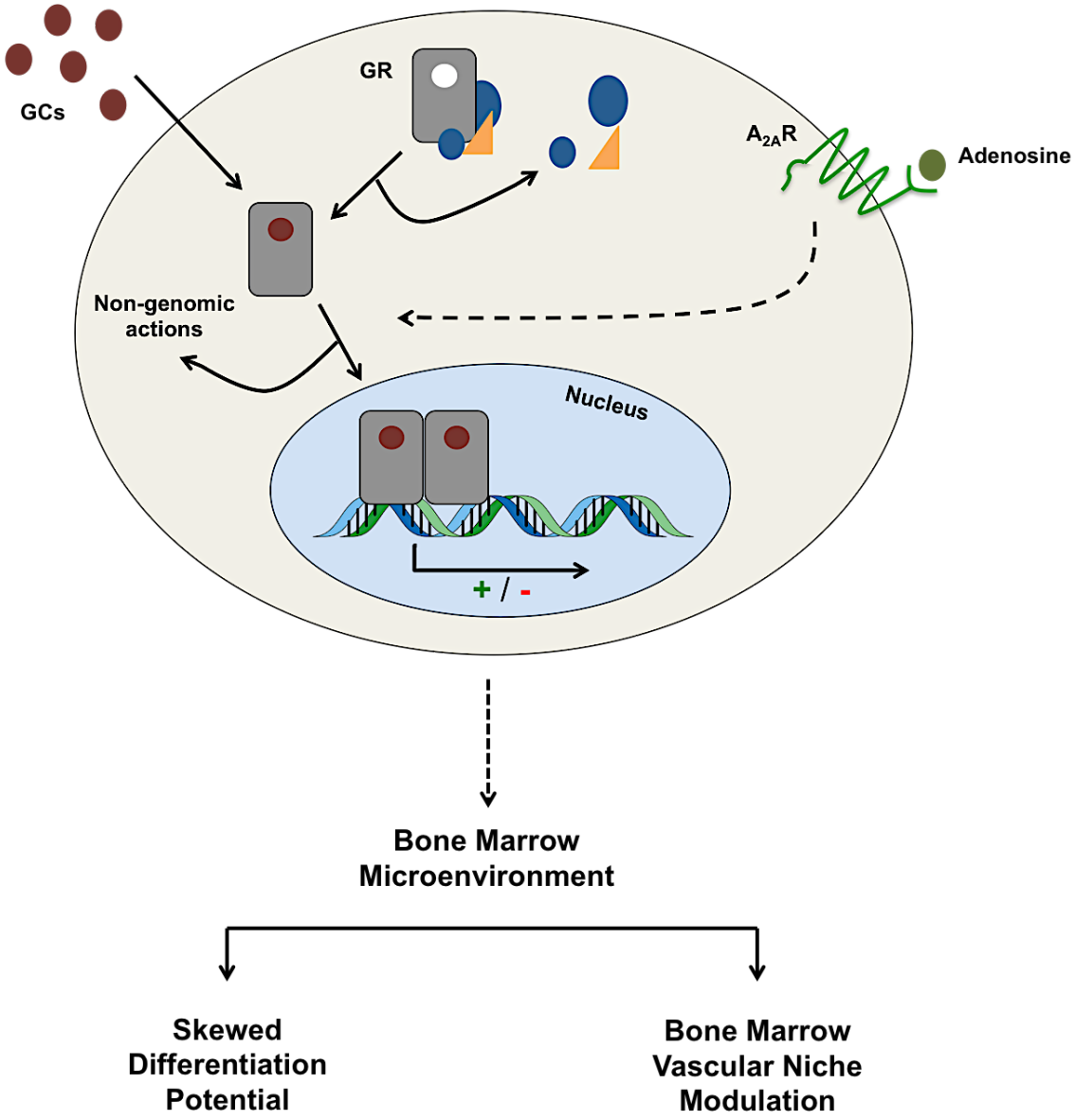

Figure 4.1 – Schematic representation of glucocorticoid actions modulation by $A_{2A}R$, in hematopoiesis. Overexpression of $A_{2A}R$ induces dysfunctions in the daily oscillations of glucocorticoids, which in turn can promote alterations in several physiological mechanisms and systems. GCs exert their actions in the hematopoietic compartment, leading to changes in hematopoietic cells, hematopoietic progenitor cells differentiation capacity and bone marrow vasculature. Our results suggest that overexpression of $A_{2A}R$ benefits myeloid cells differentiation and modulates the bone marrow vascular niche.

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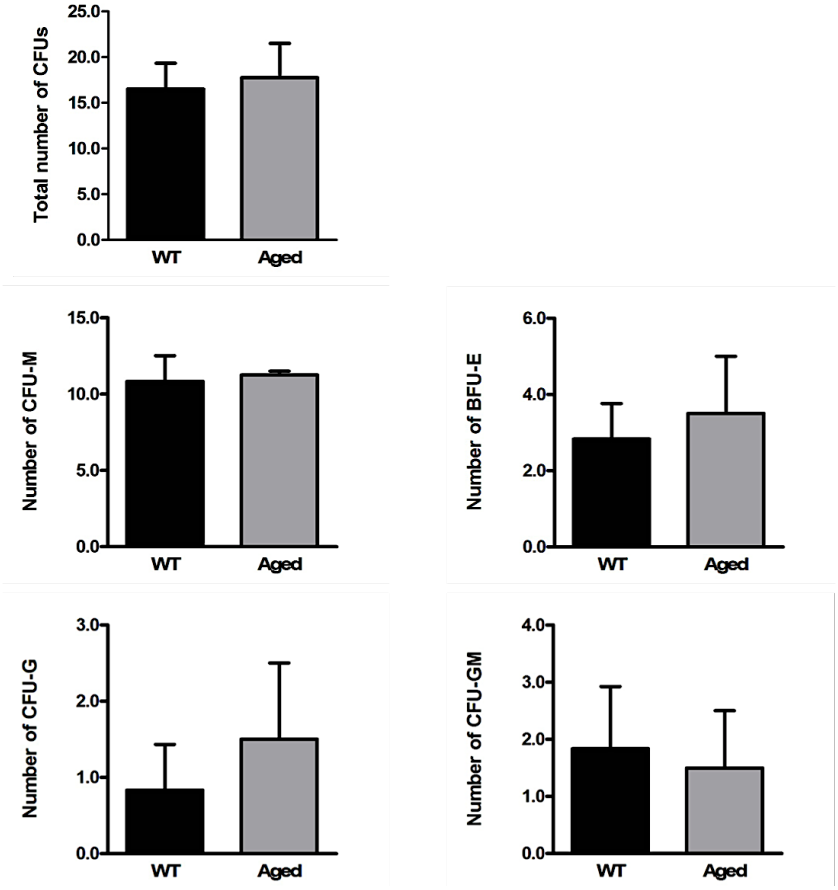
7. SUPPLEMENTARY DATA

Transgenic rats with neuronal overexpression of $A_{2A}R$ have a specific phenotype that resembles the normal brain aging process. We used normally aged WT rats with 40 weeks (Aged; n=2) to explore if transgenic rats had a similar aging phenotype in the bone marrow. The previously described procedures were performed in aged rats and results were compared with young adult WT rats.

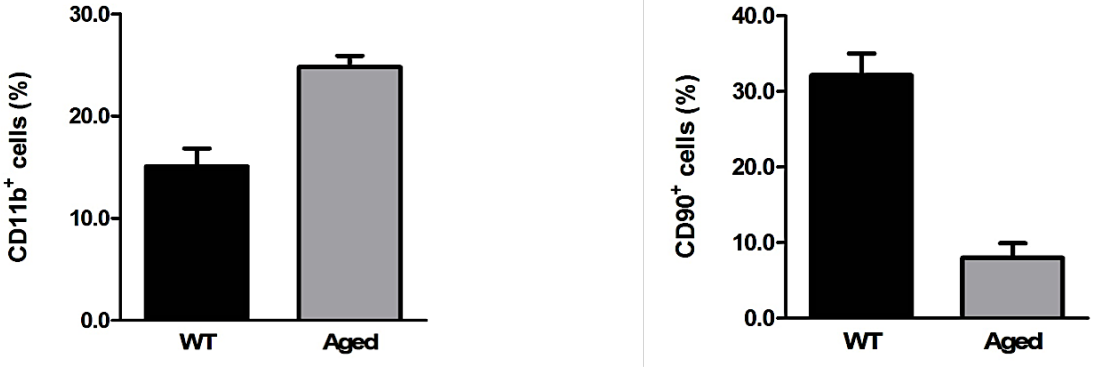
Aged rats showed higher bone marrow cell counts compared with young animals (n=6) but this effect is not an outcome of aging. Older rats have increased body weight compared with young animals, which leads to higher bone marrow cell counts. If normalized for each animal body weight, bone marrow cell counts would be similar in both aged and young animals.

In the Colony-Forming Unit Assay, aged and young WT (n=3) rats obtained similar CFU numbers (**Supplementary Figure 1.1**). However, aged animals showed higher numbers of CFU-G compared with young individuals.



Supplementary Figure 1.1 – Aged rats' progenitor cells produce more CFU-G. Absolute numbers of total CFUs, CFU-Monocytes, BFU- Erythrocytes, CFU-Granulocytes and CFU-Granulocytes/Monocytes. Aged rats showed similar results as WT, with the exception of CFU-G numbers. Data presented are the mean value ± SEM of *n* (2-3) experiments.

Regarding flow cytometry analysis, older WT rats showed an increase in $CD11b^+$ cells and a decrease in $CD90^+$ cell compared with young WT animals (n=5; 4) (**Supplementary Figure 1.2**).



Supplementary Figure 1.2 – Aged rats produce more myeloid cells. Older rats showed increased numbers of $CD11b^+$ cells and decreased numbers of $CD90^+$ cells when compared with WT animals. Data presented are the mean value ± SEM of *n* (2-5) experiments.