Stress, depression and adrenal gland: an insight into the adrenal medullary catecholaminergic system.

Stress, depressão e supra-renal: compreender o sistema catecolaminérgico da medula da supra-renal.

Magda Matos Santana

Faculdade de Farmácia
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COVER: Image of differentiated chromaffin progenitor cell immunostained for phenylethanolamine-*N*-methyltransferase (PNMT, green) and β-III-tubulin (red). Nuclei were visualized with Hoechst 33342 (blue).

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List of abbreviations

AADC	Aromatic L-amino acid decarboxylase
Acc.SD	Accumulated standard deviation
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
Ascl1	Achaete-scute complex homolog 1 (also known as Mash1 or Hash1)
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BH_4	Tetrahydrobiopterin
BMPs	Bone morphogenic proteins
BrdU	5-Bromo-2'-deoxyuridine
BSA	Bovine serum albumine
cDNA	Complementary DNA
CgA	Chromogranin A
CGH	Comparative genomic hybridization
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CRF	Corticotropin-releasing factor
Ct	Cycle threshold
Cx36	Connexin 36
Cx43	Connexin 43
DβH	Dopamine-β-hydroxylase
DHEA	Dehydroepiandrosterone
DMEM-F12	Dulbecco’s modified Eagle’s medium/F-12
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence
EGF	Endothelial growth factor
EP	Epinephrine
ESCs	Embryonic stem cells
FGF-2	Fibroblast growth factor-2
FST	Forced swimming test
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA2	GATA binding protein 2

GATA3	GATA binding protein 3
GDNF	Glial cell-derived neurotrophic factor
GDP	Guanosine diphosphate
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate
Hand2	Heart- and neural crest derivatives-expressed protein 2
Hash1	Achaete-scute complex homolog 1 (also known as Ascl1 or Mash1)
$HClO_4$	Perchloric Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPA	Hypothalamus-pituitary-adrenal
HPLC-ED	High performance liquid chromatography with electrochemical detection
HPRT-1	Hypoxanthine phosphoribosyltransferase 1
IR	Infrared
L-DOPA	L-3,4-dihydroxyphenylalanine
MAO	Monoamine oxidase
Mash1	Achaete-scute complex homolog 1 (also known as Ascl1 or Hash1)
MC2R	Melanocortin 2 receptor
NE	Norepinephrine
NET	Norepinephrine transporter
NGFR	Nerve growth factor receptor
NSCs	Neural stem cells
NSF	N-ethylmaleimide sensitive fusion
NPY	Neuropeptide Y
OCT	Optimal cutting temperature
OD	Optical density
PACAP	Pituitary adenylate cyclase-activating polypeptide
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD	Parkinson’s disease
Phox2a	Paired-like homeobox 2a
Phox2b	Paired-like homeobox 2b
PLL	Poly-L-lysin
PMSF	Phenylmethylsulphonylfluoride
PNMT	Phenylethanolamine-*N*-methyltransferase
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-PCR
RT-qPCR	Quantitative RT-PCR
SA	Sympathoadrenal

SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Sf-1	Steroidogenic factor-1
SGC	Small granule containing
SIF	Small intensely fluorescent
SNARE	Soluble NSF attachment receptor
SNAP25	Synaptosomal-associated protein 25
SNS	Sympathetic nervous system
Sox9	Sex determining region Y-box 9
Sox10	Sex determining region Y-box 10
SoxE	Sex determining region Y-box of group E
TBS-T	Tris-buffered saline with tween 20
TGF-β	Transforming growth factor-β
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor-α
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
Tris-HCl	Tris hydrochloride
UCS	Unpredictable chronic stress
UDP	Uridine diphosphate
UTP	Uridine triphosphate
VAMP2	Vesicle-associated membrane protein 2 or synaptobrevin 2
VIP	Vasoactive intestinal polypeptide
VMAT-1	Vesicular monoamine transporter isoform 1
VMAT-2	Vesicular monoamine transporter isoform 2
VMATs	Vesicular monoamine transporters
ZF	*Zona fasciculata*
ZG	*Zona glomerulosa*
ZR	*Zona reticularis*
ZO-1	Zonula occudens-1

Resumo

A glândula supra-renal é o principal órgão periférico envolvido na resposta ao stresse e as catecolaminas, libertadas pela medula supra-renal, são os principais mediadores de várias alterações fisiológicas necessárias para manter a homeostasia. No entanto, quando o stresse é prolongado e/ou repetitivo ocorrerão respostas adaptativas, incluindo na medula da glândula supra-renal, que podem contribuir para o desenvolvimento de doenças, tais como a depressão e a doença cardiovascular. Com o objetivo de compreender a fisiopatologia das disfunções relacionadas com o stresse, e particularmente os mecanismos de ligação entre a depressão e as doenças cardiovasculares que podem estar subjacentes à elevada co-morbilidade destas duas doenças, foi estudado o sistema catecolaminérgico da medula da glândula supra-renal num modelo animal de depressão induzida por exposição crónica a situações de stresse de forma imprevisível. Para isso, murganhos foram submetidos a 7 e 21 dias de stresse crónico imprevisível (UCS) e o conteúdo dos grânulos cromafins [neuropeptídeo Y (NPY) e cromogranina A (CgA)], as enzimas de síntese de catecolaminas [hidroxilase da tirosine (TH), β hidroxilase da dopamine (DβH) e metiltransferase da feniletanolamina (PNMT)], os transportadores de catecolaminas [transportador da noradrenalina (NET), transportador vesicular de monoaminas 1 e 2 (VMAT1 and VMAT2)], a maquinaria de exocitose [proteína associada ao sinaptossoma 25 (SNAP25), Sintaxina1A, proteína 2 associada à membrana da vesicula (VAMP2) e sinaptofisina] e a comunicação entre as células através de junções comunicantes [conexina 36 (Cx36), conexina (Cx43) e zonula oclusiva 1 (ZO-1)] foram avaliadas na medula da glândula supra-renal. Após 7 dias, foi observado um aumento dos niveis de mRNA da TH, DβH, PNMT, NPY, VMAT2 e SNAP25 na medula da glândula supra-renal de murganho, enquanto os níveis de mRNA da ZO-1 diminuíram comparativamente aos controlos. Em oposição, após 21 dias, o UCS induziu uma redução dos níveis de mRNA da PNMT, NET, VMAT1, sintaxina1A, SNAP-25, VAMP2 e ZO-1, mas um aumento dos níveis de mRNA da Cx43 na medula da glândula supra-renal. Além disso, 21 dias de UCS induziram uma diminuição dos níveis de proteína das enzimas de síntese das catecholaminas TH, DβH e PNMT. Também foi observado um menor conteúdo de adrenalina (EP) e noradrenalina (NE) por proteína na medula da glândula supra-renal e menores níveis de EP no soro dos murganhos submetidos a 21 dias de UCS. Tendo em conta estes resultados, este trabalho sugere que, apesar de haver um aumento inicial da expressão de marcadores

catecolaminérgicos na medula da glândula supra-renal, há um prejuízo da função da medula da supra-renal em murganhos submetidos a 21 dias de UCS.

A presença de células progenitoras cromafins com potencial proliferativo foi demonstrada na medula da supra-renal de bovinos, porém, a função fisiológica destas células continua por elucidar. Assim, de modo a perceber se as células progenitoras cromafins podem ter um papel nas respostas adaptativas induzidas pelo stresse, tais como as observadas no modelo animal de depressão descrito anteriormente, determinou-se o tamanho da glândula supra-renal e a expressão de marcadores de células progenitoras cromafins (Sox9, Notch1, Sox10, nestin, Mash1, Phox2b) na medula da glândula supra-renal de murganhos submetidos a UCS. Os resultados mostram que o UCS induz um aumento no peso e no volume da glândula-supra renal. Tanto o volume do córtex como da medula da supra-renal estavam aumentados, mas não foram observadas alterações da razão entre o volume do córtex e o volume da medula. Após 7 dias de UCS, a medula tinha níveis menores de mRNA da Sox9 e Notch1, enquanto os níveis de mRNA de Mash1 e Phox2b estavam aumentados comparativamente aos controlos. A expressão de antígeno nuclear de proliferação celular (PCNA), um marcador de proliferação celular, também estava diminuída na medula da glândula supra-renal de murganhos após 7 dias de UCS. Em oposição, após 21 dias de UCS não foram observadas diferenças nos níveis de mRNA da Sox9, Notch1, Sox10, nestina, Mash1, Phox2b e PCNA. Portanto, após 7 dias de exposição a condições de stress imprevisíveis ocorre uma diminuição dos reguladores necessários para a manutenção das células progenitoras cromafins num estado indiferenciado (Sox9, Notch1 and PCNA), com concomitante aumento de fatores de transcrição requeridos para a diferenciação das células cromafins. Estas evidências sugerem que a diferenciação das células progenitoras cromafins induzida pelo stresse pode ter um papel na melhoria da função da supra-renal durante as primeiras exposições a estímulos de stresse imprevisíveis.

Células progenitoras cromafins foram já isoladas da medula da glândula supra-renal de bovinos sobre a forma de esferas em suspensão, denominadas "cromosferas". Estas cromosferas podem representar um interessante modelo *in vitro* para estudar os mecanismos subjacentes à adaptação da medula da glândula supra-renal ao stresse. O último objetivo deste trabalho foi o isolamento, a caracterização e a diferenciação das células progenitoras cromafins obtidas de glândulas supra-renais adultas humanas. As células progenitoras cromafins humanas foram

mantidas em cultura sobre a forma de esferas em suspensão, durante 10-12 dias, em condições de baixa aderência e na presença de fator de crescimento do fibroblasto-2 (FGF-2) e fator de crescimento do entotélio (EGF). Estas culturas primárias de cromosferas humanas foram caracterizadas pela expressão de vários marcadores de células progenitoras, incluindo nestina, CD133, Notch1, receptor do fator de crescimento neural (NGFR), Snai2, Sox9, Sox10, Phox2b, and Ascl1. Em oposição, o marcador de células cromafins diferenciadas, PNMT, diminuiu significativamente após os 12 dias de cultura das células. Além disso, as células progenitoras cromafins humanas quando colocadas em lamelas revestidas com poli-L-lisina/laminina e na presença de FGF-2 diferenciaram-se em dois tipos de células "neuron-like", as células TH^+/β-III-tubulinas$^+$ e as TH^-/β-III-tubulinas$^+$, e em células cromafins (TH^+/$PNMT^+$). Estes resultados demonstram que as células progenitoras cromafins estão presentes na medula da glândula supra-renal humana e podem ser isoladas e diferenciadas *in vitro*. As culturas de cromosferas humanas podem assim ser utilizadas como modelo *in vitro* para o estudo do desenvolvimento da medula da glândula supra-renal e das suas respostas adaptativas ao stresse. Além disso, constituem uma potencial nova fonte de células para uso em transplantes celulares e medicina regenerativa, especialmente no tratamento de doenças neuroendócrinas e neurodegenerativas.

Em sumário, este trabalho contribui para melhor compreender as disfunções da medula da supra-renal induzidas pelo stresse crónico e permite o estabelecimento de um novo modelo *in vitro* para identificar os mecanismos envolvidos nas respostas adaptativas da medula da supra-renal ao stress.

Abstract

The adrenal gland is the primary peripheral organ involved in stress response and catecholamines, released from the adrenal medulla, are major mediators of several physiological changes necessary to maintain homeostasis. However, prolonged and/or repetitive stress could lead to adaptive responses, including in the adrenal medulla, which may contribute to the development of diseases, such depression and cardiovascular disease. To understand the pathophysiology of stress-related disorders, and particularly the linking mechanisms between depression and cardiovascular disease that might underlie the elevated co-morbidity of these two diseases, the adrenal medullary catecholaminergic system was studied in a mouse model of depression induced by unpredictable chronic stressor exposures. Mice were submitted to 7 or 21 days of unpredictable chronic stress (UCS) and the chromaffin granule content [neuropeptide Y (NPY) and chromogranin A (CgA)], catecholamine biosynthetic enzymes [tyrosine hydroxylase (TH) dopamine-β-hydroxylase (DβH) and phenylethanolamine-N-methyltransferase (PNMT)], catecholamine transporters [norepinephrine transporter (NET), vesicular monoamine transporter 1 and 2 (VMAT1 and VMAT2)], exocytotic machinery [synaptosomal-associated protein 25, (SNAP25), Syntaxin1A, vesicle-associated membrane protein 2 (VAMP2) and synaptophysin) and gap junctional communication [connexin 36 (Cx36), connexin 43 (Cx43) and zonula occudens-1 (ZO-1)] were evaluated in adrenal medulla. After 7 days, increased TH, DβH, PNMT, NPY, VMAT2 and SNAP25 mRNA levels were observed in mouse adrenal medulla, whereas ZO-1 mRNA levels decreased compared to controls. In contrast, after 21 days, UCS had induced a reduction of PNMT, NET, VMAT1, synthaxin1A, SNAP-25, VAMP2 and ZO-1 mRNA levels, but an increase of Cx43 mRNA levels, in the adrenal medulla. In addition, 21 days of UCS induced a decrease in protein levels of TH, DβH and PNMT. Lower epinephrine (EP) and norepinephrine (NE) content *per* protein in adrenal medulla and lower levels of EP in serum were also observed in mice submitted to 21 days of UCS. Regarding these results, this work suggests that, although there is an initial increase in the expression of catecholaminergic markers in the adrenal medulla, there is an impairment of adrenal medulla function in mice submitted to 21 days of stress exposures.

The presence of chromaffin progenitor cells with proliferative potential was recently demonstrated in bovine adult adrenal medulla, but the physiological function of these cells remains to be

elucidated. Therefore, to unravel whether chromaffin progenitor cells could play a role in adaptative responses to stressors, such like those observed in the previously referred mouse model of depression, the size of adrenal gland and the expression of chromaffin progenitor cell markers (Sox9, Notch1, Sox10, nestin, Mash1, Phox2b) in adrenal medulla were determined in mice submitted to UCS. It was observed that UCS induces an increase in adrenal gland weight and volume. Both adrenal cortex and medulla volume increased, but no alterations were observed in adrenal cortex/medulla volume ratio. After 7 days of UCS, adrenal medulla had lower mRNA levels of Sox9 and Notch1, whereas the mRNA levels of Mash1 and Phox2b were increased compared to controls. The expression of proliferating cell nuclear antigen (PCNA), a maker of cell proliferation, was also decreased in adrenal medulla of mice after 7 days of UCS. In contrast, after 21 days of UCS no differences were observed in Sox9, Notch1, Sox10, nestin, Mash1, Phox2b and PCNA mRNA levels. Thus, 7 days of unpredictable stressor exposure induced a downregulation of regulators required for the maintenance of chromaffin progenitor cells at an undifferentiated state (Sox9, Notch1 and PCNA), with concomitant upregulation of transcriptional factors required for chromaffin cell differentiation [Mash1 (Ascl1) and Phox2b]. This evidence suggests that chromaffin progenitor cell differentiation induced by stress might play a role in the enhancement of adrenal medulla function during the first exposures to unpredictable stressors.

Chromaffin progenitor cells were previously isolated from bovine adult adrenal medulla as free-floating spheres, named chromospheres. These chromospheres might represent an interesting *in vitro* model to study the mechanisms behind adrenal medulla adaptation to stress. Nevertheless, it is unknown whether chromaffin progenitor cells could also be present and isolated from human adult adrenal medulla. Therefore, the last aim of this work was the isolation, characterization and differentiation of chromaffin progenitor cells from adult human adrenal glands. Human chromaffin progenitor cells were cultured in low-attachment conditions for 10–12 days as free-floating spheres in the presence of fibroblast growth factor-2 (FGF-2) and endothelial growth factor (EGF). These primary human chromosphere cultures were characterized by the expression of several progenitor markers, including nestin, CD133, Notch1, nerve growth factor receptor (NGFR), Snai2, Sox9, Sox10, Phox2b and Ascl1. In opposition, PNMT, a marker for differentiated chromaffin cells, significantly decreased after 12 days in culture. Moreover, when plated on poly-L-lysine/laminin-coated slides in the presence of FGF-2, human chromaffin progenitor cells were

able to differentiate into two distinct neuron-like cell types, TH^+/β-III-tubulin$^+$ cells and TH^-/β-III-tubulin$^+$ cells, and into chromaffin cells (TH^+/$PNMT^+$). These last findings from this work demonstrate that chromaffin progenitor cells are present in the human adrenal medulla and can be isolated and differentiated *in vitro*. Chromospheres cultures might therefore be a used as an *in vitro* model to study adrenal medulla development and adaptative responses to stress. These cultures are also a potential new cell source for cell transplantation and regenerative medicine, especially in the treatment of neuroendocrine and neurodegenerative diseases.

In summary, this work contributes to a better understanding of adrenal medulla dysfunctions induced by chronic stress and allowed the establishment of a new *in vitro* model to identify the mechanism involved in adrenal medulla adaptive responses do stressors.

CHAPTER 1

General introduction

1.1 The adrenal gland

1.1.1 Overview of the anatomy, morphology and histology of adrenal gland

The adrenal glands are small endocrine organs embedded in adipose tissue and located in the retroperioneum, above each kidney (Fig. 1.1). The morphorlogy of adrenal glands differ between species. The mouse adrenal glands are ovoid in shape and weight about 3 mg in male and 7 mg in female [35,107]. In humans, the right adrenal gland is triangular shaped while the left adrenal gland is semilunar shaped. Human normal adult adrenals weight 6 to 10 g and have a length, width and thickness of about 5 cm, 3 cm and 1 cm, respectively [269,284].

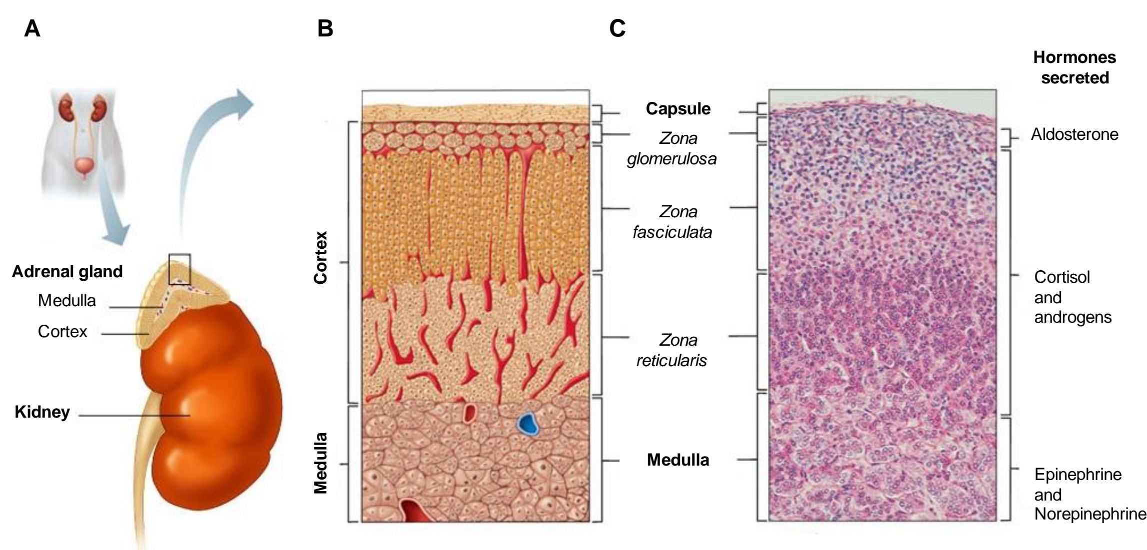

Figure 1.1 The human adrenal gland.
The adrenal glands are located in the retroperioneum, above each kidney (**A**). Each adrenal gland is enclosed by a fibrous capsule and consists of two distinct zones: the outer adrenal cortex and the inner adrenal medulla (**B**). The adrenal cortex is in turn divided into three zones: *zona glomerulosa*, *zona fasciculata* and *zona reticularis*, which produces a different functional class of steroid hormones (aldosterone, cortisol and androgens). The adrenal medulla mainly secretes catecholamines, epinephrine and norepinephrine (**C**). Adapted from http://antranik.org/the-endocrine-system.

Each adrenal gland is enclosed by a fibrous capsule and consists of two distinct zones: the outer adrenal cortex and the inner adrenal medulla (see sections 1.1.2 and 1.1.3, respectively). In mammals, the adrenal cortex and medulla were traditionally viewed as two clearly separated endocrine tissues, but currently it is widely assumed that medullary cells can be found in all zones of adult adrenal cortex and in the subcapsular region. Conversely, cortical cells are also present in adrenal medulla, where they form islets either surrounded by medullary tissue or preserving

some contact to the rest of the cortex [37,39]. In young mice, the adrenal gland exhibits a unique zone, the X-zone, which is formed by basophilic cells that surround the medulla. The X-zone appears at approximately 10 days of age and disappears at sexual maturity in male mice or first pregnancy in female mice [35,107,134,174,192].

The adrenal glands are supplied by the superior, middle and inferior suprarenal arteries, which arise from the inferior phrenic artery, abdominal aorta and renal artery, respectively. Once they reach the outer surface of the gland the superior and middle suprarenal arteries divide to form a capsular plexus of sinusoid capilares that supply the adrenal cortex, whereas the inferior suprarenal artery crosses the cortex without branching to supply the adrenal medulla. After the adrenal glands have been supplied with blood from these arteries, the blood drains through the central suprarenal vein to the left renal vein or directly to the inferior vena cava on the right side [131,132,284].

The adrenal glands receive both intrinsic and extrinsic innervations. Its capsule contains a nerve plexus of fibres arising from the splanchnic nerve and associated plexuses of the sympathetic nervous system (SNS), combined with parasympathetic contributions from the phrenic and vagal nerves. From these plexuses, nerve fibers extend to the adrenal cortex and medulla to form further networks surrounding the cells present in these zones [294].

1.1.2 The adrenal cortex

The adrenal cortex derives from mesoderm and consists of three distinct zones that differ from each other in morphological features and in the steroid hormones they produce [199]. The outermost *zona glomerulosa (ZG)* is very thin, with small unpigmented cells arranged in clusters that mainly produce aldosterone. The *zona fasciculata (ZF)* is the middle and largest zone of the adrenal cortex and is the main place of glucocorticoids production (mostly cortisol, in humans, and corticosterone, in rodents). It consists of columnar cells that form long cords rich in cytoplasmatic lipid vacuoles. The inner *zona reticularis (ZR)* is adjacent to the adrenal medulla and formed by networks of interconnected small pigmented cells that produce glucocorticoids and androgens, such as dehydroepiandrosterone (DHEA) [264,284].

Cortisol, aldosterone and DHEA are hormones from the adrenal cortex regulating the response to stress (see section 1.2), water balance and blood pressure, and sexual maturation, respectively [264,284].

1.1.3 The adrenal medulla

The adrenal medulla has its origin in the ectoderm and is mainly formed by neuroendocrine chromaffin cells [180]. The adrenal medulla has a proportion ranging from 10 to 30% of total adrenal gland [39] and itself essentially represents an enlarged and specialized sympathetic glanglia. Chromaffin cells are considered modified postganglionic sympathetic neurons that, instead of synapsing with an effector organ or tissue, release catecholamines directly into the bloodstream, functioning as an endocrine organ rather than nerve cells [284]. In addition to chromaffin cells, small clusters of cortical cells, small intensely fluorescent (SIF) cells (also called small granule containing (SGC) cells), Schwann cells and fibroblasts are also found in adrenal medulla [161]. The adrenal medulla plays a pivotal role in the response to stress (see section 1.2).

1.1.3.1 The chromaffin cell

Chromaffin cells are neuroendocrine cells, mainly found in adrenal medulla, highly specialized in the synthesis, storage and release of catecholamines. The term “chromaffin” was introduced by Alfred Kohn (1867–1959) as a result of the typical histochemical reaction of these cells with chromium salts that leads to a yellow-brown coloration after staining due to the catecholamine oxidation (“chromaffin reaction”) [213]. The chromaffin cells are polyhedral and arranged in clusters surrounded by thin layers of connective tissue with numerous nerves and blood vessels. The most remarkable morphological feature of chromaffin cells is the presence of catecholamine-containing chromaffin granules in their cytoplasm [80].

1.1.3.1.1 Catecholamine biochemistry and biosynthesis

The term catecholamine refers, in general, to all organic compounds that have a catechol nucleus (benzene ring with two adjacent hydroxyl substituents) and an amine group. Dopamine (DA), norepinephrine (NE) and epinephrine (EP) are catecholamines that act as neurotransmitters in

the central nervous system (CNS) and SNS [54,395,396]. NE and EP are also hormones from the adrenomedullary system [2,55,284]. EP was the first biological catecholamine, and also hormone, being discovered. It was identified by John Abel and Albert Crawford in 1897 [2] and thereafter isolated in crystalline form by Jokishi Takamine [363].

Catecholamines are synthetized from the amino acid L-tyrosine. L-tyrosine is typically acquired from diet or can be obtained through the hydroxylation of the amino acid phenylalanine in the liver. The catecholamine biosynthetic pathway begins with the conversion of L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA) by the rate limiting enzyme tyrosine hydroxylase (TH). TH is a substrate-stereospecific cytoplasmatic enzyme that requires molecular oxygen, Fe^{2+} and tetrahydrobiopterin (BH_4) as co-factors. This enzyme is a specific phenotypic marker of catecholamine producing cells. L-DOPA is subsequently decarboxylated into DA by the nonspecific enzyme, aromatic L-amino acid decarboxylase (AADC), whose activity depends on the levels of its cofactor pyridoxal-phosphate (vitamin B6). DA is then transported from the cytoplasm to the chromaffin granules by the vesicular monoamine transporter 1 (VMAT1) and converted into NE by the enzyme dopamine-β-hydroxylase (DβH). DβH is present in chromaffin granules in both soluble and membrane-bound form and requires molecular oxygen, Cu^{2+} and ascorbic acid for activity. Finally, NE is converted into EP by the soluble enzyme phenylethanolamine N-methyltransferase (PNMT), which uses S-adenosylmethionine as a methyl donor. This reaction occurs in the cytoplasm and thus is dependent on NE leakage from chromaffin granules. EP is further transported and stored in the chromaffin granules [125] (see sections 1.1.3.1.2 and 1.1.3.1.4; Fig. 1.2).

1.1.3.1.2 Catecholamine storage in chromaffin granules

Chromaffin granules are membrane-bound, electron-dense secretory organelles with 150-350nm of diameter in which catecholamines are concentrated and stored [167,168,220]. Each chromaffin cell contains about 10,000 granules, which on average account for 14% of the cytoplasmic volume of the chromaffin cell [16,220,303,393]. By electron microscopic it was identified two morphological different chromaffin granules in adrenal medulla: one with the storage of EP, the other with storage of NE.

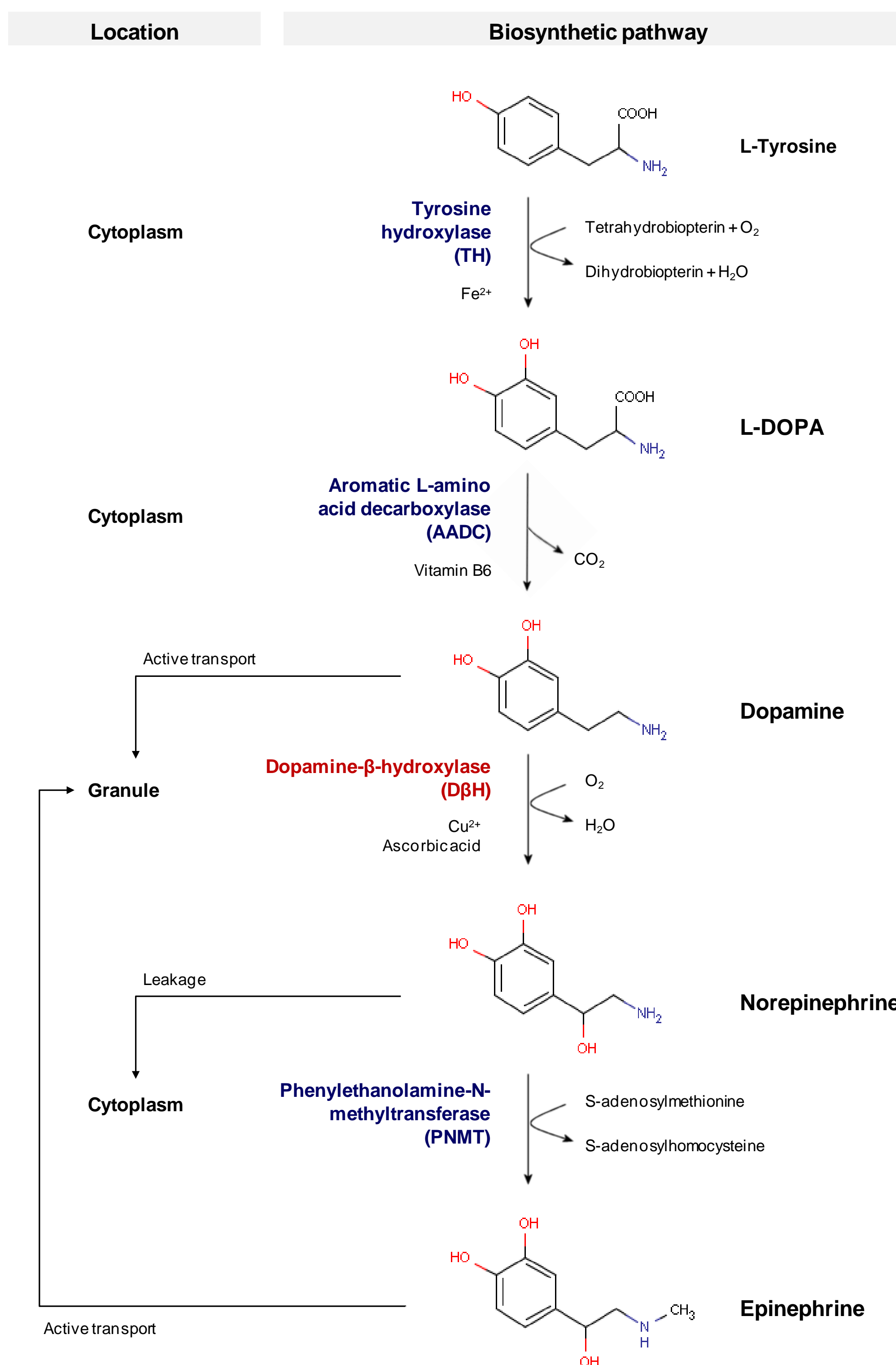

Figure 1.2 Catecholamine biosynthetic pathway.
The amino acid L- tyrosine is converted to dihydroxyphenylacetic acid (L-DOPA) by the enzyme tyrosine hydroxylase (TH). L-DOPA is then decarboxylated by aromatic L-amino acid decarboxylase (AADC) to the neurotransmitter dopamine (DA). Dopamine β-hydroxylase (DβH) generates norepinephrine from DA, and finally, norepinephrine (NE) is converted to epinephrine (EP) by the enzyme phenylethanolamine N-methyltransferase (PNMT). The co-factors required for the activity of catecholamine biosynthetic enzymes and the location of each reaction within the cell are indicated in the figure (see text for details).

EP-containing granules are characterized by a round shape and moderately electron-dense content, whereas NE-containing granules are irregular and highly electron-dense. This allowed the identification of two distinct chromaffin cell populations: the adrenergic- and the noradrenergic-chromaffin cells, respectively [80,81].

The relative proportion of adrenergic to noradrenergic chromaffin cell populations is specie-specific, such as the proportions of NE and EP in adrenal medulla. In some species, like the rat, cat, dog and horse, the adrenal medulla consists of 10%–20% noradrenergic cells, contrasting with rabbits and chipmunks in which the adrenal medulla is almost exclusively composed of adrenergic cells [361]. Based on immunohistochemical data, it was suggested that human adrenal medulla is entirely composed by adrenergic chromaffin cells [75]. Despite the interspecies variations, EP is the mainly synthesized catecholamine by adrenal medulla among all [17,308].

Catecholamine co-storage with other biological active substances

Although catecholamines represent the large proportion of chromaffin granule content, a variety of other bioactive substances are co-stored and co-released with catecholamines in chromaffin granules (table 1.1). Chromogranin A (CgA) and neuropeptide Y (NPY) are highly abundant in chromaffin granules and have being considered important regulators of adrenal medullary catecholaminergic system. Chromogranin A is a highly acidic glycoprotein that belongs to the family of granins, which accounts for more than 80% of the soluble proteins present in chromaffin granules [288]. CgA plays an important role in granule biogenesis and hormone/transmitter sorting to the regulated secretory pathway in endocrine cells [118]; it is also a precursor for several bioactive peptides with autocrine, paracrine, and endocrine functions, such vasostatins I and II, catestatin, cateslytin and chromacin [164,214].

NPY is a 36-amino acid peptide that acts through the activation of G-protein-coupled receptors (Y_1, Y_2, Y_3, Y_4, Y_5), which belongs to the pancreatic polypeptide family. NPY is one of the most abundant peptides in mammals, being highly expressed in central and peripheral nervous system [337]. In adrenal medulla, NPY is stored in chromaffin granules in concentrations highly variable between species [123,249]. NPY has a stimulatory effect on catecholamine release from mouse and human chromaffin cells, playing an important role in the autocrine/paracrine modulation of chromaffin cell secretion [58,59,314,316].

Table 1.1 Composition of chromaffin granules.

Chromaffin granule components	References
Monoamines	
Epinephrine, Norepinephrine, Dopamine, Serotonin.	[47,175,187,397]
Granins	
Chromogranin A and B; Secretogranins II, III, V and VI	[114,163,182,345]
Granin-derived bioactive peptides	
Vasostatins I and II; Catestatin; Cateslytin; Chromacin; secretolytin WE-14; EM66.	[248,270,355,367]
Glycoproteins	
Dopamine β hydroxylase; H^+-ATPase; Glycoprteins II, III and V.	[310,360]
Prohormone processing enzymes and protease inhibitors	
Cathepsin L; Aminopeptidase B; prohormone processing enzymes PC1/3 and PC2; carboxypeptidase E; Aspartic proteinase; Tissue-type plasminogen activator; Endopin 1 and 2.	[18,19,177, 183-186]
Transmitter peptides	
Neuropeptide Y; opioid peptides (enkephalins, endorphins and dynorphins); Substance P; vasoactive intestinal polypeptide (VIP); neurotensin; secretoneurin; adrenomedullin; calcitonin gene-related peptide (CGRP); bombesin; ubiquitin: ubifungin; $ubiquitin_{1-34}$; arginin-vasotocin; guanylin; galanin.	[20,173,206,211, 212,222,234,238, 241,249,277,285,3 51,381]
Growth and neurothrophic factors	
Fibroblast growth factor-2 (FGF-2); transforming growth factor-β (TGF-β); glial cell line derived neurotrophic factor (GDNF); nerve growth factor (NGF); brain-derived neurotrophic factor (BDNF); Neurotrophin-3.	[34,70,149,162,21 7,232]
Cytokines	
Interleukine-1β; interleukine-6; tumor necrosis factor-α (TNF-α).	[130,142,329]
Nucleotides	
Adenosine triphosphate (ATP); adenosine diphosphate (ADP); adenosine monophosphate (AMP); guanosine triphosphate (GTP); guanosine diphosphate (GDP); uridine triphosphate (UTP); uridine diphosphate (UDP).	[3,298]
Ions	
Ca^{2+}; Mg^{2+}; K^+; Cl^-.	[415,416]
Others	
Alzheimer amyloid precursors; ascorbic acid; coenzyme A glutathione disulphide; mucopolyssacharides.	[86]

For review see references [86,99,386,415,416].

1.1.3.1.3 Catecholamine release from chromaffin cells

Catecholamine secretion by chromaffin cells occurs by exocytosis [50,271] and is mainly controlled by the synaptic release of acetylcholine (ACh) from splanchnic nerve terminals. Once released, ACh binds to nicotinic cholinergic receptors present in chromaffin cell, leading to an increase in membrane permeability to Na^+. This results in a large depolarization of cell membrane with consequent opening of various types of voltage-sensitive Ca^{2+} channels. The rise in intracellular Ca^{2+} triggers the release of exocytotic granules [16,17,93,100,399].

Functional communication between chromaffin cells, with particular involvement of gap junction-mediated coupling, has been suggested as a complement to synaptic transmission to amplify catecholamine release from chromaffin cells after synaptic stimulation [77,79,257]. Gap junctions are specialized regions of the plasma membrane in which proteins, named connexins, assembly to form hemichannels that connect across the intercellular space of adjacent cells. This allows the exchange of ions and small molecules between coupled cells and consequently the propagation of electrical signal, leading to simultaneous multicellular increases of intracellular Ca^{2+} (Fig. 1.3) [262,323,387].

Various biologically active substances, such as pituitary adenylate cyclase-activating polypeptide (PACAP), VIP and substance P, can also act as modulators of catecholamine release. They are co-released with ACh from sympathetic preganglionic nerve terminals or released from other neuronal elements, including sensory and parasympathetic neurons and ganglionic cells [215,223,246,425]. Additionally, endocrine or paracrine/autocrine regulators, such glucocorticoids, angiotensin II, histamine, NPY and cytokines, also have been demonstrated to play a key role in the modulation of catecholamine secretion from chromaffin cells [41,48,99,156,286,307,408,419,423,429].

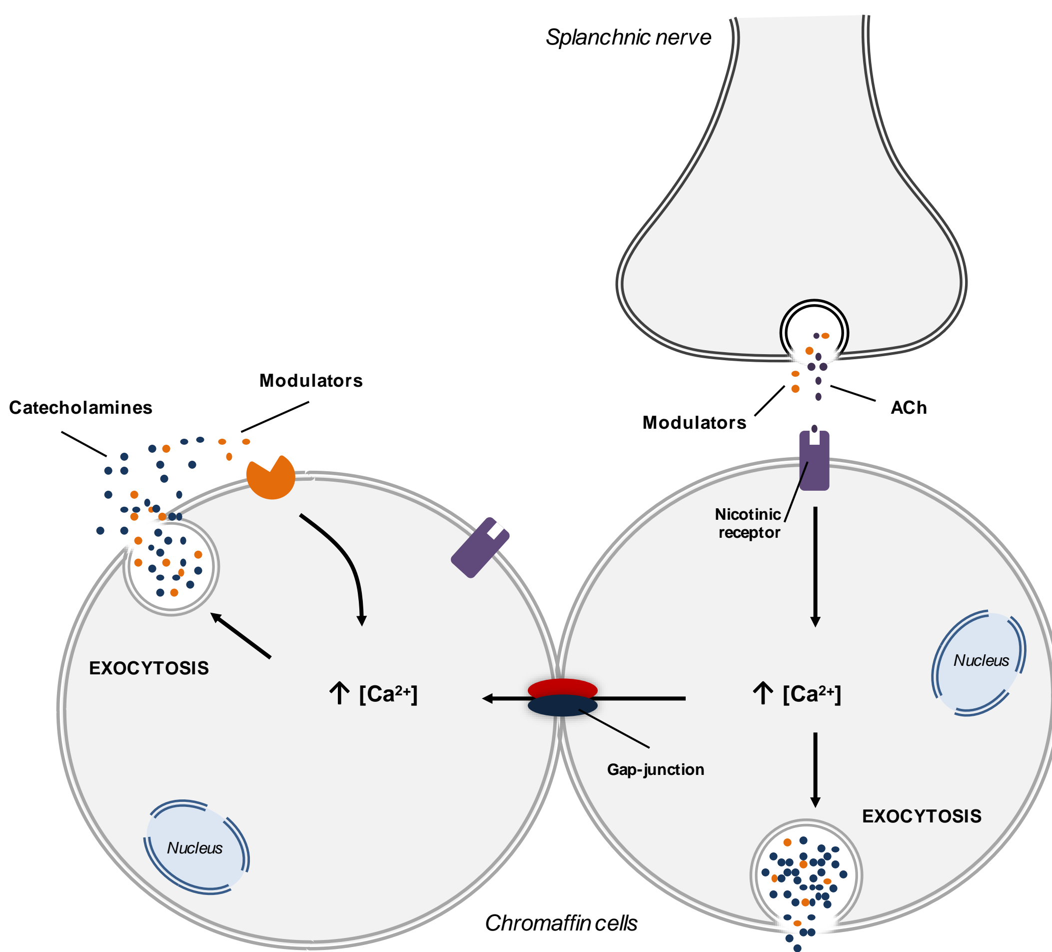

Figure 1.3 Regulation of chromaffin cell exocytosis.
Acethylcholine (ACh), released from splanchnic nerve terminals, binds to nicotinic receptors present in chromaffin cell membranes, leading to an increase in intracellular calcium ([Ca^{2+}]). This results into the release of chromaffin granule content by exocytosis. Transmitter potentials propagated through gap junctional coupling are sufficient to stimulate exocytosis in adjacent chromaffin cells. In addition to ACh, exocytosis in chromaffin cells is regulated by other paracrine/autocrine modulators. Adapted from [14].

The exocytotic pathway

Exocytosis occurs through a pathway consisting of multiple functionally definable stages (Fig.1.4). The first step consists in the recruitment of granules to the subplasmalemmal area, which requires disassembly of a cortical actin barrier. This is an ATP- and Ca^{2+}-dependent step, although it can also be activated by alternative Ca^{2+}-independent pathways [63,374]. The following stages involve the close association of the granules with the plasma membrane ("docking") and the ATP-dependent preparatory reactions ("priming") that precede fusion [52,357].

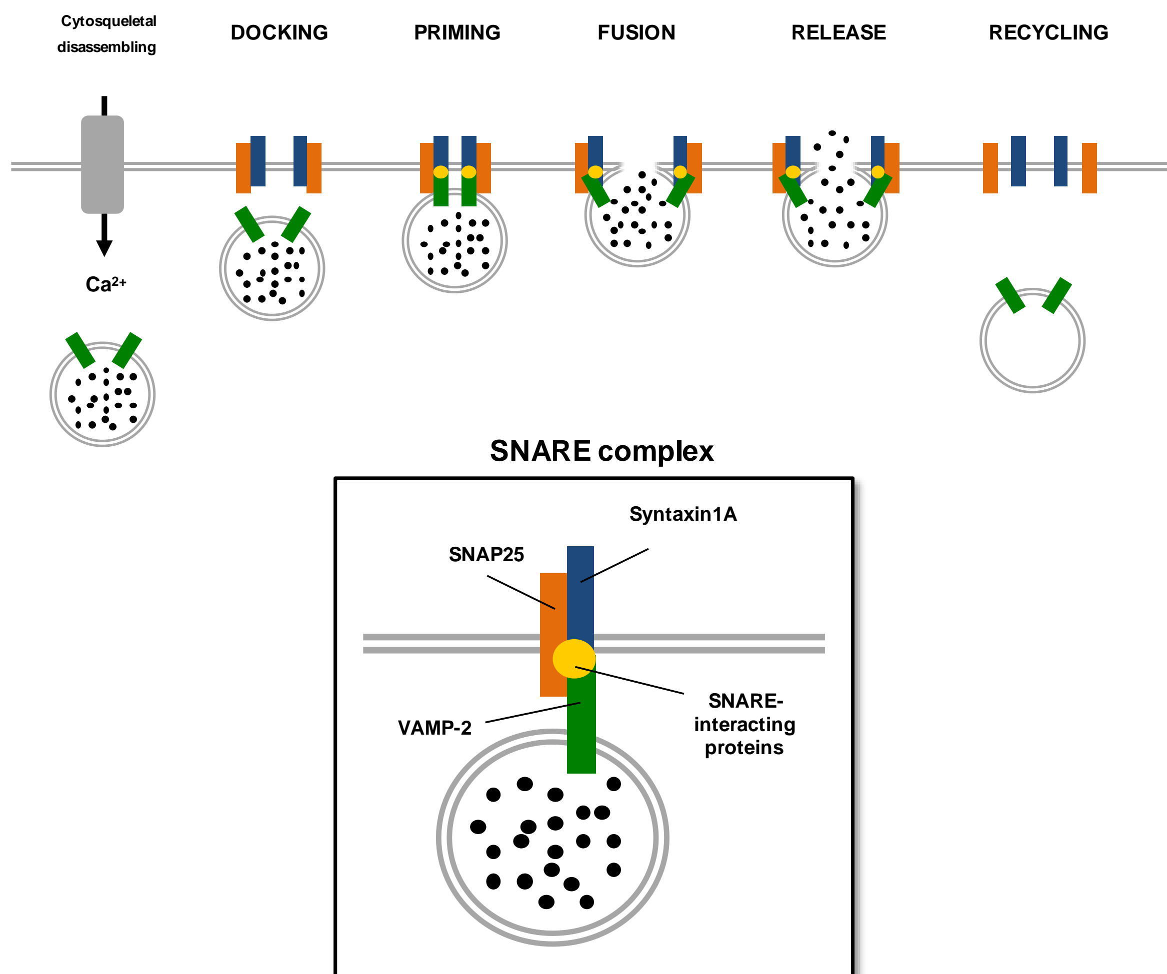

Figure 1.4 Exocytotic pathway.
Calcium (Ca^{2+}) entry into the cell leads to disassembly of the cortical actin cytoskeleton, allowing granule recruitment and docking at the plasma membrane. A priming process prepares membranes for fusion and subsequent release of granule content. The SNARE complex plays a key role during exocytosis by providing a bridge between the granule and the plasma membrane and driving the membrane fusion. Adapted from [51].

Although details are still controversial, it is widely accepted that docking and fusion are catalyzed by the synaptic SNARE proteins, particularly the vesicle-associated membrane protein-2 (VAMP-2), syntaxin-1A and synaptosomal-associated protein-25 (SNAP-25). When in contact, these SNAREs form a helical complex (SNARE complex) that pulls the membranes together and drives membrane fusion. SNARE complex is regulated by a number of other proteins, which some of them are referred in Table 1.2. After fusion, SNARE complex is disassembled and individual SNAREs are recycled [49,51,341,358].

Table 1.2 Key proteins that regulate chromaffin granule exocytosis.

Protein	Function
Essential proteins of SNARE complex	
VAMP-2 Syntaxin1A SNAP-25	Combine to form a helical complex, which drives the membrane fusion.
Other regulatory proteins	
NSF and α-SNAP	Disassemble the SNARE complex; allow individual SNAREs to be recycled.
Synapsin I-III	Tether the vesicles to the cell membrane cytoskeletal network, controlling the number of vesicles available for release.
Synaptotagmin I-III	Ca^{2+} binding proteins; calcium sensors during exocytosis; accelerates fusion.
Synaptophysin	Control of the quantal size and the duration of the exocytotic events.
Munc18	Regulates the availability of syntaxin1A for SNARE complex formation at the fusion site; role during vesicle docking.

References [49,51,384] [389] [253] [105,143,341,358]

Mechanisms of exocytosis

Exocytosis in chromaffin cells can occur by different mechanisms (Fig. 1.5). In "full fusion" exocytosis the chromaffin granules dock at the cell membrane and then entirely fuse with it, discharging the whole granule components into the extracellular space. This is a rapid mechanism of granule discharge which is physiologically relevant during sympathetic activation in stressful situations [129,364,394].In opposition, in "kiss and run" exocytosis the granule membrane does not completely merge with the cell membrane, but instead forms a transient fusion pore, which rapidly closes pinching the granule back off from the cell membrane. This implies that only part of the granule content is released during this process. The physiological significance of ''kiss-and-run'' exocytosis remains controversial, but it might be possible that the transient pore acts as a molecular sieve discriminating the released molecules according to their size, solubility and electrical charge [8,292,378]. Another alternative mechanism of exocytosis is the "piecemeal degranulation", whereby discrete vesicles loaded with granule content detach from

the granule membrane, move through the cytoplasm and then fuse with the plasma membrane, leading to content discharge. This mechanism provides a tightly governed mechanism of catecholamine release, particularly during resting conditions [85,87,88]. Finally, another mechanism of exocytosis, named "sequential exocytosis", has been described [76,282]. In "sequential exocytosis" omega (Ω)-shaped profiles of vesicles persist at the site of exocytosis after fusion and become targets for exocytosis of vesicles in the deeper layers of the cytoplasm. Sequential exocytosis in chromaffin cells is infrequent during weaker stimulation and only occurs at high levels of stimulation. Therefore, this mechanism might represent a remarkable strategy to increase catecholamine release during stress [209,210].

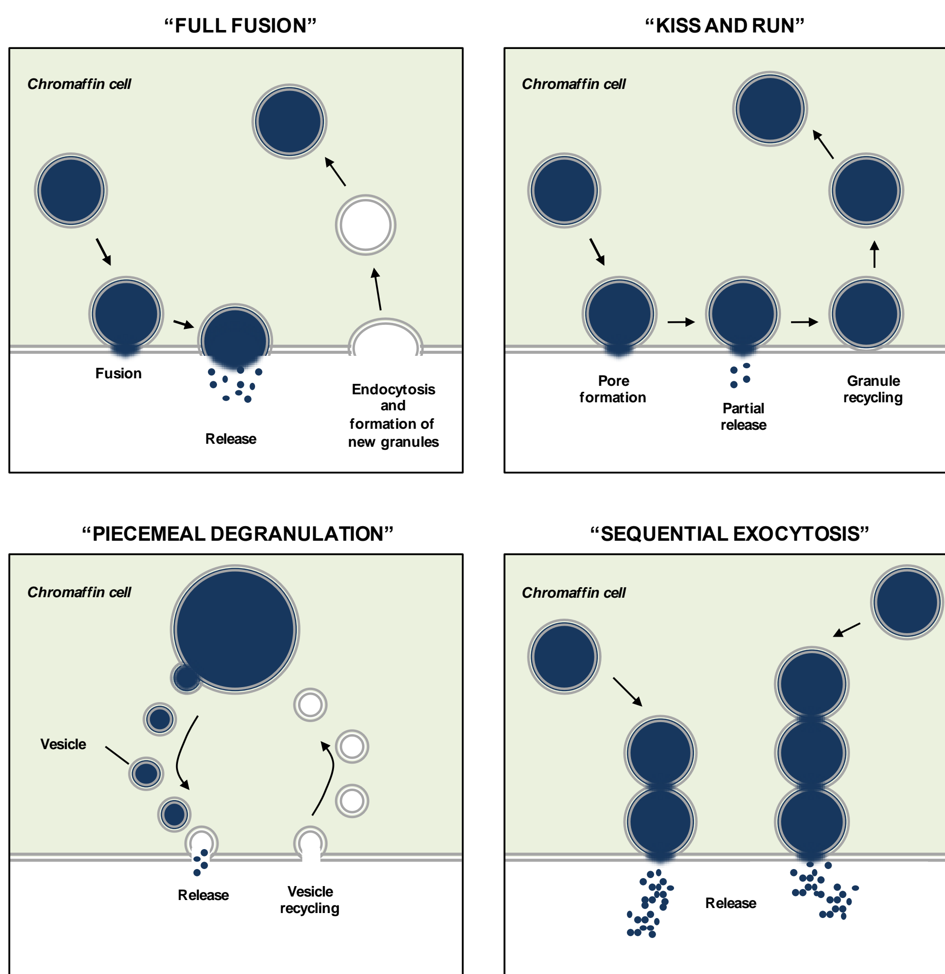

Figure 1.5 Mechanisms of exocytosis.
In "full fusion" exocytosis the chromaffin granules discharge the whole granule components into the extracellular space. In "kiss and run" exocytosis the granule membrane forms a transient fusion pore that only allows the release of part of the granule content. During "piecemeal degranulation" discrete vesicles loaded with granule content detach from the granule membrane and then fuse with the plasma membrane, leading to content discharge. In "sequential exocytosis" vesicles persist at the site of exocytosis after fusion and become targets for exocytosis of vesicles in the deeper layers of the cytoplasm. Adapted from [87].

1.1.3.1.4 Catecholamine re-uptake and metabolism

Following release, catecholamine clearance from the extracellular space occurs via two main pathways: 1) catecholamine dilution into the bloodstream; 2) catecholamine re-uptake into the cells.

Catecholamine reuptake through cell membrane occurs via an active transport mediated by the NE transporter (NET). This transport is a Na^+- and temperature-dependent process that displays high affinity, but low capacity, to catecholamines. NE is translocated by NET about 2-fold more effectively than EP [115], but until now no specific transporter has yet been identified in mammals. In rat adrenal medulla it was shown that NET is mostly, if not exclusively, present in adrenergic chromaffin cells and the adrenergic cells from the human adrenal medulla also express this transporter [75,203,302]. Therefore, it is likely that NET is responsible for the uptake of both NE and EP. The regulation of NET transporter is dependent on catecholamine levels and it can occur divergently or in parallel with changes in catecholamine synthesis [83,154,237,406].

Once inside the chromaffin cell, catecholamines can be recycled by active transport into the chromaffin granules or metabolized. The catecholamine transport to chromaffin granules is mediated by the vesicular monoamine transporters (VMATs), which use energy provided by the proton gradient established by the H^+ transporter [165,191,194]. Cloning studies revealed the existence of two VMATs isoforms (VMAT1 and VMAT2) that are encoded by two separate genes and displayed different cellular distributions, pharmacological properties and substrate affinity [120,244]. In rat adrenal medulla, VMAT2 is expressed in the noradrenergic cells, while VMAT1 is expressed widely in both types of chromaffin cells [369]. VMAT2 has a general higher affinity for monoamines than VMAT1 [121,300].

The metabolism of recaptured catecholamines occurs through oxidative deamination catalyzed by the enzyme monoamine oxidase (MAO) or by O-methylation mediated by catechol-O-methyltransferase (COMT) and essentially takes place in the same cells where monoamines are produced. Moreover, most of the metabolism occurs independently of exocytotic release and only a small fraction of catecholamine metabolites is formed from circulating catecholamines. In fact, catecholamines stored in chromaffin granules exist in a dynamic equilibrium with catecholamines in the surrounding cytoplasm, where passive outward leakage is counterbalanced by inward active transport controlled by VMATs. Although only a small fraction of the catecholamines in the

cytoplasm escapes sequestration by VMATs, this fraction represents a major source of catecholamine metabolites [116].

1.1.3.1.5 Steady-state catecholamine levels in chromaffin cells

Catecholamines exert their effects in many tissues contributing to the regulation of different physiological systems. Therefore, in order to sustain appropriate levels of catecholamines in both intra- and extracellular locations, a strict regulation and coordination of catecholamine synthesis, storage, release, re-uptake and metabolism occurs in chromaffin cells (Fig. 1.6). Disruptions of these mechanisms can result in significant changes in adrenal medulla function that might contribute to the development of diseases (see section 1.2).

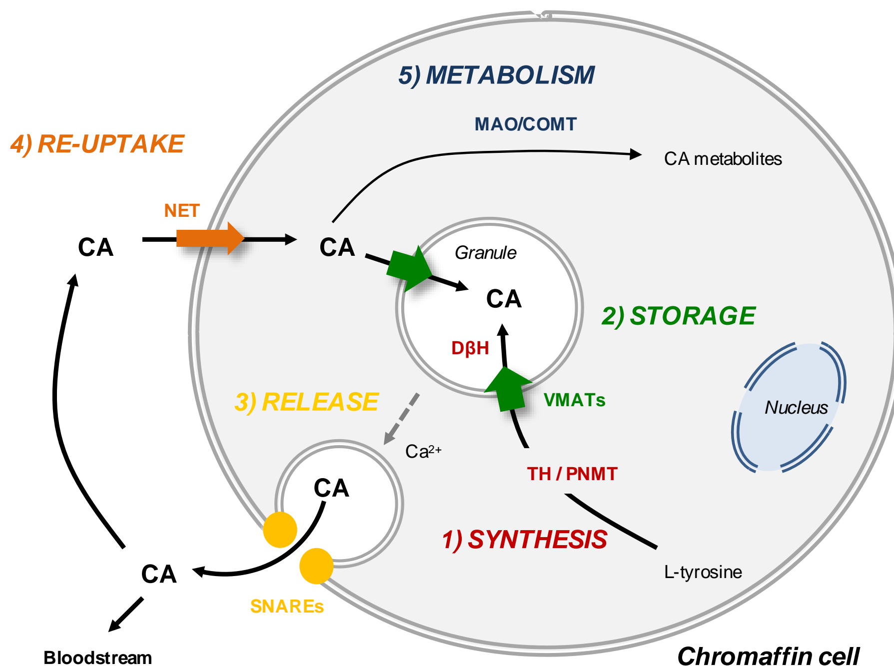

Figure 1.6 Steady-state catecholamine levels in chromaffin cells.
Catecholamine (CA) levels in both intra- and extracellular locations are regulated by a rigorous coordination of their synthesis, storage, release, re-uptake and metabolism. CA are synthesized from the amino acid L-tyrosine (1) and stored in chromaffin granules due to an active transport mediated by vesicular monoamine transporters, VMATs (2). CA exocytosis is induced by calcium (Ca^{2+}) and driven by SNARE proteins (3). CA are released to the bloodstream or can be re-uptaken into the chromaffin cell (4). CA are then recycled and stored in chromaffin granules or metabolized by the enzymes monoamine oxidase (MAO) and/or catechol-O-methyltransferase (COMT). Adapted from [116].

1.1.3.2 The adrenal medulla development

The adrenal medulla develops from the neural crest. The neural crest is a group of transient and multipotent ectodermal cells that emerge from the dorsal surface of neural tube and migrate along defined routes during development giving rise to diverse cell types, including adrenomedullary chromaffin cells [43,84]. According to traditional view, chromaffin cells, sympathetic neurons and SIF cells have their origin in neural crest cells that aggregated at the dorsal aorta and in response to extrinsic environmental factors, such bone morphogenic proteins (BMPs), acquire catecholaminergic properties giving rise to fate-restricted sympathoadrenal (SA) progenitor cells (Fig. 1.7) [178,180,385]. These SA progenitor cells subsequently take a second migratory route from the dorsal aorta to sympathetic ganglia and adrenal glands, where they finally differentiate into sympathetic neurons and chromaffin cells, respectively. In the light of more recent studies, evidence suggests that specification of neuronal and neuroendocrine phenotypes might occur earlier [180,385]. Indeed, observations in mouse embryos indicate that progenitors of chromaffin cells may migrate to the adrenal gland as “undifferentiated” neural crest cells, expressing general neural crest cell markers such the transcription factors from SoxE family, but still lacking SA markers [153,312].

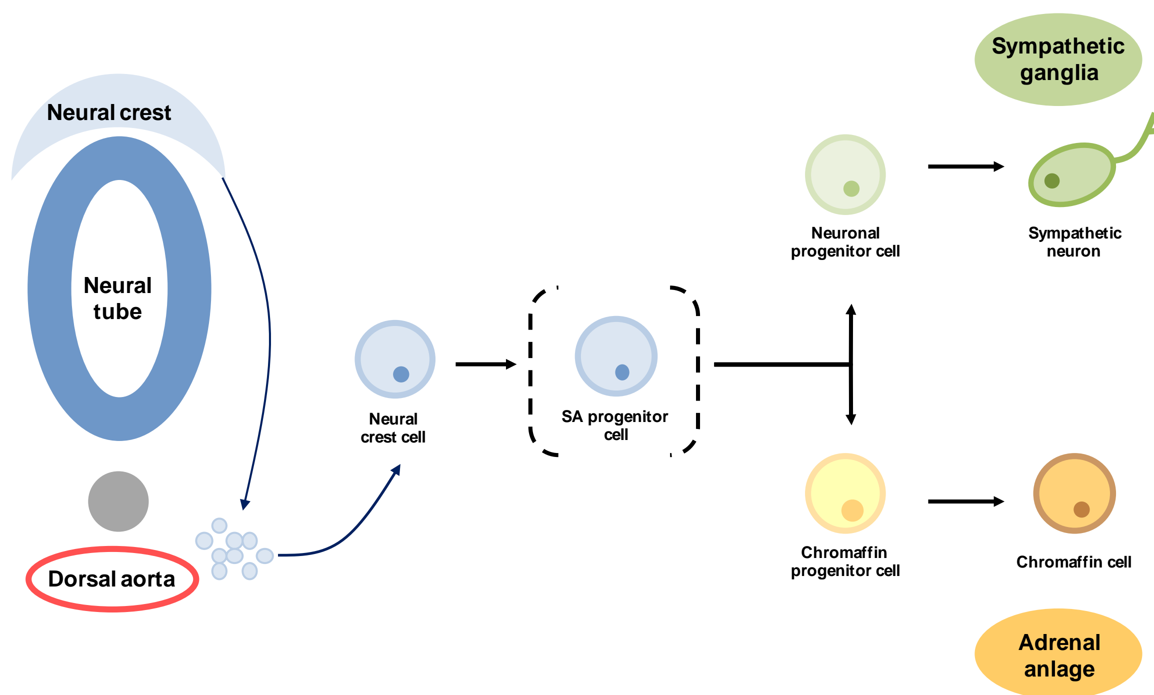

Figure 1.7 Sympathoadrenal lineage development.
Accordingly to the traditional view, chromaffin cells and sympathetic neurons derive from a common sympathoadrenal (SA) progenitor cell which develops from neural crest cell that have aggregated at dorsal aorta. SA progenitor cells then migrate to the sympathetic ganglia and adrenal anlage where they undergo final differentiation in response to local environment. However, more recent studies suggest that both chromaffin cells and sympathetic neurons develop independently from neural crest cells, following different maturation schedules. Adapted from [180].

The further differentiation of neural-crest derived progenitor cells into sympathetic neurons and chromaffin cells is promoted by a complex network of transcription factors, which are required to induce the expression of the enzymes essential for NE synthesis, TH and DβH (Fig. 1.8). Some of the identified transcription factors include Ascl1 or Mash1, Phox2a/b, Hand2 and GATA 2/3 [180,272]. The transcription factor Phox2b is considered the most important factor for the development of both chromaffin cells and sympathetic neurons *in vivo* [45]. Indeed, in Phox2Bb$^{LacZ/LacZ}$ mouse embryos the neural crest derived progenitor cells fail to develop a centrally localized adrenal medulla and do not express any markers and transcription factors that suggest their differentiation further than the neural crest cell stage [181]. Phox2b is required for the specification of nearly all SA specific attributes as well as for the induction of other components of the transcriptional network, except Mash1 [181,296,377]. However, maintenance of Mash1 expression in sympathetic neurons and chromaffin cells also depends on Phox2b [181,296]. Expression of Mash1 starts immediately once neural crest cells accumulate at the

dorsal aorta, but is transient in both chromaffin and sympathetic neuronal derivatives. Despite the early occurrence of Mash1, loss of Mash1 does not cause a complete abolishment of chromaffin cell and sympathetic neuron development [151,152,171,179,340], as observed in Phox2b mutant mouse embryos. However, in Mash1 null mice, Phox2b failed to initiate expression of TH and DβH and the maiority of cells in adrenal medulla presented an immature character, lacking ultrastructural features of typical chromaffin cells [179].

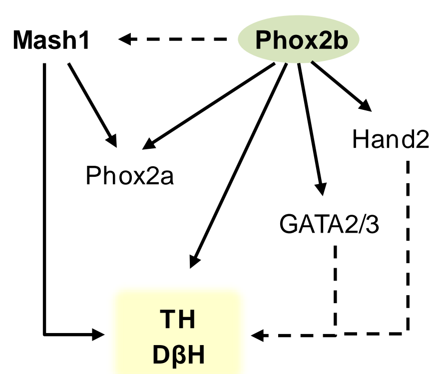

Figure 1.8 Transcriptional network regulating sympathoadrenal cell development.
Differentiation of SA cells and induction of the catecholamine biosynthetic enzymes tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DβH) is promoted by the interaction of the transcription factors Phox2b, Mash1, Hand2, Phox2a and GATA2/3.The transcription factor Phox2b plays the most fundamental role for the development of both chromaffin cells and sympathetic neurons, being required for the earliest events of sympathoadrenal (SA) differentiation. Phox2b is essential for the specification of nearly all SA specific attributes as well as for the induction of the other components of the transcriptional network, such Phox2a, Gata2/3 and Hand2. Mash1 expression induction is independent of Phox2b, occurs immediately after neural crest cells aggregated at dorsal aorta and is transient in both chromaffin and sympathetic neuronal derivatives. Phox2a expression is not indispensable for SA differentiation. Dashed lines indicate a requirement for maintenance. Adapted from [178].

The postmigratory environment of chromaffin cells also plays an important role in the specification of the endocrine characteristics of chromaffin cells. For long it was assumed that glucocorticoids derived from adrenal cortex were essential to block neuronal differentiation of SA progenitors and promote chromaffin cells differentiation by downregulation of neuronal markers and induction of PNMT [9,10,36,101,265]. However, study of glucocorticoid receptor (GR) knockout mice revealed that glucocorticoids are dispensable for most aspects of chromaffin cells differentiation and even in mice deficient for steroidogenic factor-1 (Sf1), which do not develop an adrenal cortex, chromaffin cells accumulate and differentiate at the exact site where the adrenal anlage develops in wildtype mice [250]. Even so, it is unquestionably that adrenal cortex regulates some aspects of

chromaffin cell development, including PNMT expression and its postnatal survival [9,153,265,295].

1.1.3.3 The chromaffin progenitor cell

The adrenal medulla contains a small number of neural-crest derived progenitor cells. The presence of these chromaffin progenitor cells in adult adrenal medulla was suggested by accumulated evidences: 1) chromaffin cell dynamic turnover and the chromaffin cells's ability to proliferate throughout life [370-373,390]; 2) the expression of receptor tyrosine kinase RET, which is only expressed during development, in pheochromocytomas, suggesting that these tumors could develop from persisting SA progenitors in adult adrenal medulla [128,216]; 3) the presence of neural crest-derived progenitor cells in diverse adult tissues [42,92,155,276,293,411] and 4) the regeneration of adrenal medulla after injury [64,65].

Chromaffin progenitor cells were isolated from bovine adrenal medulla as free floating spherical colonies, named “chromospheres” [74,325], which are morphologically analogous to neurospheres obtained from brain. Bovine chromospheres expressed different neural and neural crest stem cell markers (nestin, vimentin, Sox1, Sox9, Musashi1 and nerve growth factor receptor, NGFR), which are not expressed (or are expressed at lower levels) in freshly isolated chromaffin cells, but lack the expression of PNMT, a maker of differentiated chromaffin cells [74]. Moreover, bovine chromospheres are able to differentiate *in vitro* into both neuronal and neuroendocrine phenotypes [73,74,398]. *In vitro* experimental conditions to differentiate chromaffin progenitor cells into dopaminergic neurons have also been established [398] with the aim to use these cells in cell-based therapies for the treatment of neurodegenerative diseases (see section 1.1.3.4). Nonetheless, the presence of these cells in human adrenal medulla was not yet described.

1.1.3.4 Clinical application of adrenal medullary grafts and chromaffin cells

The unique features of chromaffin cells, particularly their close relation to sympathetic neurons, plasticity and ability to secrete a variety of neuroactive substances, soon promoted their use for cell transplantation in patients with neurodegenerative diseases.

Between 1985 and 1999, a significant number of Parkinson patients received adrenal medullary grafts or chromaffin cell transplants, showing some improvements of clinical symptoms that unfortunately disappeared 1-2 years after transplantation (Table 1.3).

The transient improvement has been attributed to trophic effects induced by the grafts, since the poor survival of chromaffin cells in brain and their low production of dopamine has suggested that their contribution is negligible [7,299]. Therefore, it remains a priority to enhance the survival rate of cells after transplantation and increase dopaminergic release in order to replace dopamine depletion observed in the striatum of these patients. In this context, different grafting strategies and other cell sources have been explored to improve transplantation efficacy. Neural crest derived chromaffin progenitor cells isolated from adult adrenal medulla represent a potential strategy for cell transplantation in patients with Parkinson's disease (PD) [7,111,113].

Based on previous pre-clinical studies using animal models, human medullary tissue and chromaffin cells have also been used for transplantation in patients with chronic pain (Table 1.4). This approach has provided more promising and consistent results, attributed to the secretion of catecholamines and opioids from chromaffin cells.

In addition, adrenal medullary and chromaffin cell grafts have been tested for antidepressant activity after transplantation into the rat frontal neocortex and lumbar subarachnoid space [342-344,368]. Transplants into the frontal neocortex proved to be effective in the reduction of immobility time in the forced swimming test (FST). The effects observed were attributed to catecholamine secretion from grafts [342-344]. Similarly, rats with adrenal medullary transplants into the lumbar subarachnoid space were less susceptible to become helpless after administration of inescapable shocks [368].

Table 1.3 Trials of adrenal chromaffin cell grafts in Parkinson's disease (PD) patients.

Cell source	Patients/Graft site	Effects	Ref.
AM	Patient 1: 55-year old woman Patient 2: 46-year old woman /right caudate nucleus	Patient 1 (evaluation 20 months post-graft): no change. Patient 2 (evaluation 6 months post-graft): moderate decrease of rigidity and akinesia 25% reduction of L-dopa.	[22]
AM	2 PD patients/putamen	Temporary improvements (weeks) assessed with quantitative tests.	[242]
AM	2 PD patients/ventricular wall	Long-term improvements (developing over months) assessed with subjective ratings.	[251]
AM	11 PD patients/ventricular wall	Long-termimprovements (developing over months) assessed with electromyograms and photographic recordings of movements.	[103]
AM	10 PD patients/caudate nucleus	Long-term improvements assessed with rating scales.	[190]
AM	7 PD patients/ventricular wall	Long-term improvements assessed with neurophysiological tests.	[291]
AM	17 PD patients/ventricular wall	Slight improvements assessed with Columbia rating scales.	[6]
AM	19 PD patients/ventricular wall	Mixed long-termimprovements assessedwith rating scales and self-assessments.	[136,137]
AM	3 PD patients/ventricular wall	Modest improvements assessed with rating scales.	[188]
AM	7 PD patients/ventricular wall	Slight improvements assessed with rating scales.	[200]
AM	5 PD patients/caudate nucleus	Evaluation at 3 years post-graft: sustained bilateral amelioration in 4 of 5 patients, levodopa of 2 patients reduced, neurophysiological and electrophysiological improvements.	[252]
AM + transected peripheral nerve	55-year old woman Stage IV (Died after 1 year)/caudate nuclei (bilateral)	Evaluation at 3 months post-graft: improvement of gait during "on" periods, decreased "off" period from 76% to 53%, UPDRS-on: decreased from 65 to 43, UPDRS-off: decreased from 151 to 120, reduction of L-Dopa, improvement of akinesia, dense network of TH-ir fibers around the graft site.	[91]
Autologous AM + intercostal nerve	5 PD patients Stage IV-V/right caudate and putamen	Evaluated at 24 months post-graft: UPDRS "off" improved 32.9% (ns), UPDRS "on": improved from 16.1 to 14.1, time motor test scores —"off period" improved 34.9%.	[403]
Human ACC (cultured with ELF-MF)	59 year-old woman PD stage III/caudate nucleus	Evaluation at 1–7 months post-graft: decreased 70% L-Dopa dosage. increased 15.8% D2DA receptors. increased 20.45% glucose metabolism.	[104]

Abbreviations: AM, adrenal medulla; ELF-MF, extremely low frequency magnetic fields; DA, dopamine. UPDRS, Unified Parkinson's Disease Rating Scale. Adapted from [7].

Table 1.4 Trials of adrenal chromaffin cell grafts in chronic pain patients.

Cell source	Patients/Graft site	Pain etiology	Effects	Ref.
Human adrenal medullary tissue	5 patients/ subarachnoid	Cancer pain	4/5 patients decrease pain scores 4/5 increased concentration of met-enkephalin and catecholamines in CSF.	[417]
Human chromaffin cells	8 patients/ subarachnoid	Cancer pain	Reduction pain, high levels of met-enkephalin CSF.	[235]
Encapsulated bovine chromaffin cells	7 patients/ subarachnoid	6 cancer pain/1 neurogenic pain	Pain reduction, survival and biochemical function of the encapsulated cells. Reduction morphine intake.	[46]
Human adrenal medullary tissue	11 PD patients/ventricular wall	Cancer pain	Reduction pain score. Reduction morphine intake Increase CSF met-enkephalin levels.	[33]
Human adrenal medullary tissue	15 patients/ subarachnoid	Cancer pain	Reduction morphine intake 5 patients no longer required morphine Increase CSF met-enkephalin levels.	[236]

Abbreviations: CSF, cerebrospinal fluid. Adapted from [7].

1.2 Stress and adrenal gland

1.2.1 The adrenal gland response to stress

The principal physiological responses to stress are mediated by the SA system, which includes the SNS and the adrenal medulla, and the hypothalamic-pituitary-adrenocortical (HPA) axis. The adrenal gland, as part of both systems, plays a pivotal role in the maintenance of homeostasis, through the secretion of glucocorticoids and catecholamines (Fig. 1.9) [61,138,313].

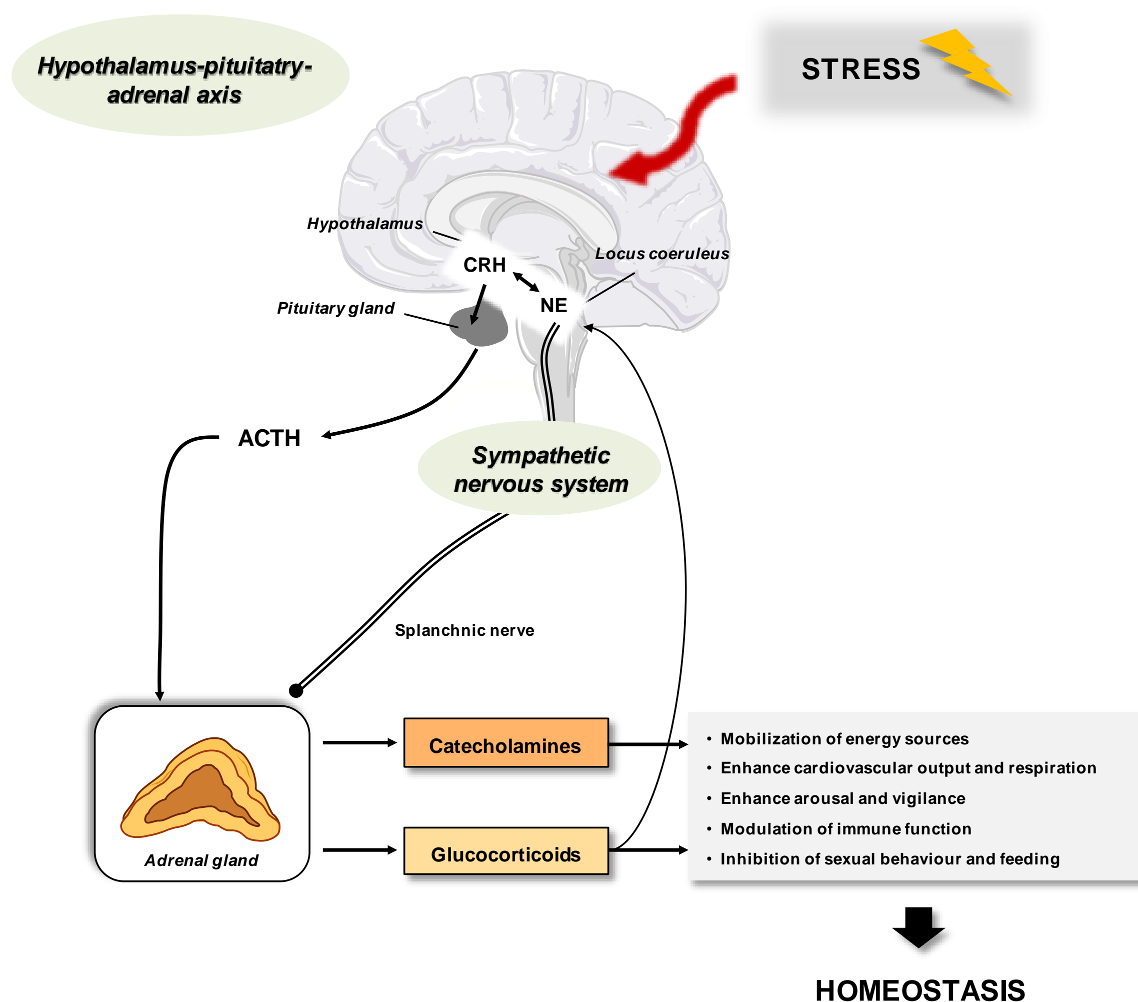

Figure 1.9 Integrated responses to stress
Stress perception induces activation of both hypothalamus-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS). Corticotrophin releasing-factor (CRF) released from the hypothalamus stimulates adrenocorticotropic hormone (ACTH) secretion by the anterior pituitary gland. ACTH reaches the adrenal gland and induces glucocorticoid synthesis and secretion from adrenal cortex. Simultaneously, norepinephrine (NE) is released from the neuronal network localized in the locus coeruleus (LC), leading to catecholamine release from sympathetic nerve terminals and adrenal medulla. Glucocorticoids and catecholamines have complementary action throughout the body that are responsible for homeostasis maintenance. Cortisol also acts centrally exerting a negative feedback, preventing a hormonal overproduction.

1.2.1.1 The adrenal cortex and HPA axis

Stress perception induces the release of corticotrophin-releasing factor (CRF) from hypothalamic neurons, which in turn stimulates the synthesis and secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland. When in blood circulation, ACTH rapidly reaches the adrenal glands and binds to ACTH receptors present in adrenocortical cell membrane, leading to an increase in cAMP and subsequent induction of glucocorticoid secretion. An increase in the plasma levels of glucocorticoids is detected within minutes, but the glucocorticoid peak only appears between 30 min and 1 h after the onset of the stressor [56,61,313].

Glucocorticoids are the final effectors of the HPA axis and act by binding to intracellular receptors present in most tissues, leading to regulation of energy metabolism, immune response and brain function (see table 1.5 in section 1.4.3) [264,284]. Regulation of the HPA system activity is modulated by a negative feedback loop exerted by glucocorticoids in hypothalamus and pituitary gland, preventing a hormonal overproduction by adrenal cortex [94,95].

1.2.1.2 The adrenal medulla and sympathoadrenal system

Stress stimuli induce central activation of the locus coeruleus-NE (LC-NE) sympathetic system. Through the SNS efferent pre-ganglionic fibers of the splanchnic nerve, whose cell bodies lie in the intermediolateral column of the spinal cord, catecholamine secretion is evoked from chromaffin cells of adrenal medulla, seconds after stress exposure [140,228]. Catecholamines in blood rapidly reach the various tissues, bind to adrenergic receptors and exert their multiple actions throughout the body, particularly on the cardiovascular and metabolic systems, with consequent adjustments of blood pressure, cardiac rhythm and energy resources (see table 1.5 in section 1.4.3) [264,284,313]. This suite of responses mediated by catecholamines comprise the so-called “fight or flight” response introduced by Cannon in the early 1900s [53].

1.2.2 Effects of stress on adrenal gland function

Stress triggers important adaptative responses in the adrenal gland that are essential to improve adrenal medulla function and consequently to sustain the capacity of the organism to cope with a changing environment [228,320]. However, while initially beneficial, when stress is recurrent the

response may become maladaptive and then promote the development of a variety of disorders (see section 1.2.3). The effects of stress on both adrenal medulla and cortex have been widely investigated in order to understand how the beneficial responses to acute stress are modified by repeated stress, leading to detrimental consequences.

1.2.2.1 Effects of stress on adrenal cortex

The acute effects of stress on adrenal cortex are related to the induction of glucocorticoid biosynthesis from adrenocortical cells by ACTH and are essential to support appropriate hormone release required to maintain homeostasis [13,254,348]. On the other hand, under certain conditions of repeated stress, the adrenal cortex is able to maintain high levels of glucocorticoid secretion, independently of ACTH stimulation. Indeed, discrepancies between glucocorticoids and ACTH plasma concentrations are often reported in different chronic stress models [109,427]. The exact mechanisms responsible for this effect are not completely understood, but evidence suggests that the regulation of adrenocortical secretion during stress is dependent on other factors, such as the paracrine/autocrine actions of adrenal released factors and adrenal cortex innervation [38,110,112,189,382] Moreover, an enlargement of adrenal cortex in animals subjected to chronic stress is often reported [144,327,383,418] and has also been associated with the increase in glucocorticoids secretion [256].

1.2.2.2 Effects of stress on adrenal medulla

Over the last decades many different stressors have been used to study the effects of stress on adrenal medulla function, particularly those effects related to the catecholamine biosynthesis (Table 1.5). A single short episode of stress showed to be sufficient to trigger changes in the expression of catecholamine biosynthetic enzymes, although these changes were transient and no significant alterations in enzyme activities and protein levels were detected [225,261,280,392]. On the other hand, long time and/or repeated stress exposures induced robust and sustained activation of TH, DβH and PNMT mRNA levels, with concomitant increases in protein levels and enzyme activity, which remain for a long time even after the termination of the stress [172,224,229,230,280].

Table 1.5 Effects of stress on adrenal medulla catecholamine biosynthetic enzymes.

Types of stress		Catecholamine biosynthetic enzymes	References
Immobilization	Single (1 episode)	↑ TH (mRNA, phosphorylation Ser31) ↑ DβH (mRNA) ↑ PNMT (mRNA)	[225,261,280,392]
	Repeated (> 1 episode)	↑ TH (mRNA, protein, activity) ↑ DβH (mRNA, activity) ↑ PNMT (mRNA, protein, activity)	
Cold	Single (1 episode)	↑ TH (mRNA, activity) ↑ PNMT (mRNA, protein)	[27,28,126, 127,227,349, 407,424]
	Repeated (> 1 episode)	↑ TH (mRNA, protein, activity)	
Glucoprivation (Insulin or 2-Deoxy-D-Glucose)	Single (1 administration)	↑ TH (mRNA, activity, phosphorylation Ser40 and Ser31)	[227,319,336,391]
	Repeated (6/7 administrations)	↑ TH (mRNA, protein, activity)	
Psychosocial	Isolation	↑ TH (mRNA, protein) or ↓ TH (mRNA) ↑ DβH (mRNA, protein) ↑ PNMT (mRNA, protein)	[133,274,289,304]
	Social defeat	↑ TH (mRNA, phosphorylation) ↑ DβH (mRNA) ↑ PNMT (mRNA)	
Footshock	Single (1 session)	↑ TH (phosphorylation Ser31) ↓ TH (phosphorylation Ser19)	[290,301,352]
	Repeated (> 1 session)	↑ TH (activity)	
Exercise	Run	↑ TH (mRNA) – 5 days/4 weeks ↓ TH (mRNA, activity) – 10 weeks	[102,379,380]

From these studies it was concluded that adrenal medulla responses to stress are highly dependent on duration and repetition of stress and mainly involve increases in expression, synthesis and activity of catecholamine biosynthetic enzymes. In addition, stressor specificity on the activation of adrenal medullary catecholaminergic system was also observed [226,321]. For example, cold stress induced an increase of TH mRNA, protein and enzyme activity that returned

to baseline after 28 days of exposure to the same stressor [28]. This effect was not observed in rats under immobilization, in which TH mRNA, protein and activity remainded elevated even after 41 stressor exposures [280]. Different changes in catecholamine biosynthetic enzymes were also observed when chronically stressed animals were exposed to the same (homotypic) or a novel stressor (heterotypic) [321].

In addition to enhanced catecholamine synthesis and storage, an increase in the size of the adrenal medulla is often observed in animals exposed to repeated stress [144,327,383,418]. Other adrenal medulla adaptive responses to stress are related to changes in synaptic transmission, cell-cell communication between medullary chromaffin cells, catecholamine transport and chromaffin granule content (summarized in Table 1.6) [78,79]. Moreover, immobilization stress showed to induce an up-regulation of Mash-1 [243], a transcription factor essential in chromaffin cell maturation that is expressed in chromaffin progenitor cells [179], suggesting a putative role of chromaffin progenitor cells on adrenal medulla response to stress. Nevertheless, the effects of stress on chromaffin progenitor cells and its contribution to adrenal medulla adaptative responses remain to be elucidated.

Table 1.6 Other effects of stress on adrenal medulla.

Types of stress	Effects on adrenal medulla	References
Immobilization	↑ NPY (mRNA) ↑ CgA and CgB (mRNA) ↑ VMAT-2 (mRNA, protein) ↑ BDNF (mRNA) ↑ Mash1 (mRNA)	[170,243,322,369]
Cold	↑ NPY (mRNA) ↑ α-9 nicotinic receptor subunit (mRNA, protein) ↑ Connexin 43 and 36 (mRNA, protein) ↑ZO-1 (protein)	[78,79,170]
Glucoprivation (Insulin or 2-Deoxy-G-Glucose)	↑ NPY (mRNA) ↑ proenkephalin (mRNA)	[124,231]
Exercise	↑ NPY (mRNA)	[239]

1.2.3 Adrenal gland and stress-related disorders

The previously described changes in the adrenal gland induced by chronic stress might promote an enhanced susceptibility to the development of a variety of diseases. Indeed, the pathogenesis of stress-related disorders has been associated with sustained and excessive secretion of stress hormones, in particular glucocorticoids and catecholamine, which influence the activities of multiple homeostatic systems [71,72,139,353]. The physiological effects and pathologies that result from dysregulated glucocorticoid and catecholamine secretion are summarized in Table 1.7.

Table 1.7 Effects of adrenal gland hormones in both physiological and pathological conditions.

<table>
<tr><th colspan="2">Physiological effects of adrenal stress hormones</th><th>Stress-related disorders</th></tr>
<tr><td>CATECHOLAMINES</td><td>GLUCOCORTICOIDS</td><td></td></tr>
<tr><td>Cardiovascular system
↑ Cardiac contractility
↑ Cardiac conduction velocity
↑ Heart rate
Vasoconstriction / vasodilatation
Platelet aggregation</td><td>Cardiovascular system
Potentiation of induced vasoconstriction

Hydro-electrolytic system
↑ Na^+ and water absorption</td><td rowspan="2">Cardiovascular disease
Hypertension
Atherosclerosis
Dyslipidemia
Diabetes
Obesity
Metabolic syndrome</td></tr>
<tr><td colspan="2">Endocrine system and metabolism
↑ Gluconeogenesis
↑ Glycogenolysis
↑ Lipolysis
↑ β-oxidation
↑ Glucagon
↓ Glycogenesis
↓ Glucose uptake
↓ Insulin</td></tr>
<tr><td colspan="2">Immune systems
↑ Humoral immunity
↑ Anti-inflammatory cytokines
↓ Cellular immunity
↓ Pro-inflammatory cytokines</td><td>Inflammation
Allergy
Autoimmunity diseases
Infections</td></tr>
<tr><td></td><td>Central nervous system
Regulates memory, mood, cognition, sleep, food intake</td><td>Depression and anxiety
Sleep disturbances
Neurodegenerative diseases</td></tr>
<tr><td>Other systems
Bronchodilatation
Relaxation of gastrointestinal and urinary muscle
Thermogenesis</td><td>Other systems
↓ Calcium intestinal absorption
↓ Bone formation</td><td></td></tr>
</table>

References: [11,71,117,157,264,400]

1.3 Depression

1.3.1 Epidemiology, diagnostic and treatment of depression

Depression is a common and disabling psychiatric disorder with a lifetime prevalence of about 20% [205]. According to the World Health Organization, depression is among the five leading causes of global disease, including developing countries, and estimations predict that by the year of 2030 depression will be the second leading cause of disease burden worldwide [409].

Depression is more common in women than in men and it is estimated that 5.8% of men and 9.5% of women will experience a depressive episode in a 12-month period. Moreover, depressive episodes tend to be recurrent in most patients; the risk of a second episode after the first is about 50%. People who have a second episode have a further relapse risk of 70% and after the third episode the risk of relapse increases to 90%. The condition is chronic for about 1 in 5 people [410].

Diagnosis of depression is performed according to the criteria indicated in Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), which characterizes a major depressive episode by at least five of the symptoms described in Table 1.8, where at least one is a core symptom. The symptoms must be evident almost daily for at least 2 weeks [15]. Although the definition of symptoms followed rigorous statistical validating criteria, they are more subjective–qualitative rather than objective–quantitative. Inclusion of biological diagnostic criteria is urgent but will require extensive investigation on the biological correlates and mechanisms of depression.

Table 1.8 Symptoms of depression according DMS-IV.

Symptoms of depression
Core symptoms
• Depressed mood most of the day;
• Diminished interest or pleasure in all or most activities.
Other symptoms
• Significant unintentional weight loss or gain;
• Insomnia or sleeping too much;
• Agitation or psychomotor retardation noticed by others;
• Fatigue or loss of energy;
• Feelings of worthlessness or excessive guilt;
• Diminished ability to think or concentrate, or indecisiveness;
• Recurrent thoughts of death.

Reference: [15]

Depression is recognized as a significant international public health issue that causes personal suffering, increased morbidity and impaired social and work functioning. Depressive disorders significantly influence the outcome of other comorbid medical illnesses, such as cardiovascular disease (see section 1.2), cancer and diabetes, resulting in increased health care costs and mortality [29,57,122]. The public health impact of depression is, in part, due to the fact that pharmacological therapy for depression is limited. The available antidepressant drugs act essentially through the inhibition of the reuptake or degradation of monoamine neurotransmitters [273], but only 50-70% of the patients exhibit satisfactory responses to treatment. For patients that do respond, therapeutic effects develop slowly, usually over several weeks of drug administration, which frequently lead to discontinuation of treatment. Nevertheless, 25-35% of the patients remain resistant to the treatment even after 6 weeks of therapy. In addition to monoamines, many other targets have been analyzed, including glucocorticoid receptor and CRF receptor antagonists and histone deacetylase inhibitors, but until now none of these studies has resulted in a new adequate treatment of the disease [32,82,145]. The improvement of therapeutic options will benefit from the identification of the underlying mechanistic pathways involved in the development of the disease.

1.3.2 Etiology and neurobiology of depression

Despite the high prevalence and considerable impact of the disease, the underlying causes of depression are far from being completely understood. Family, twin and adoption studies provide evidence of the heritability of depression and estimates that genetic risk to develop depression is likely to be in the range of 40-50% [240,359]. The remaining variability is attributed to the environment. Stress has been considered determinant in the etiology of depression and stressful life events, including those that occur in early life, have a causal relationship with the onset of depressive episodes and are important predictors of disease development [159,202,258,297,405]. Moreover, there is wide consensus and support from many different studies that long-term exposures to uncontrollable and unpredictable life stressors play a key role in the development of disease. Indeed, chronic stress seems to have a stronger association with depressive episodes

than acute stress. In addition, chronic stress predicts higher levels and amplifies the impact of acute events [160,204,260,297].

Evidence from prospective studies shows that the association between stressful life events and depression depends on prior characteristics of the individuals exposed to the events. Even though stressful life events increase the risk of depression, there are significant differences in how individuals respond to the same stressful event, and these differences may be explained in part by genetic factors [159,204]. The current view of the etiology of depression recognizes that a complex interplay of genetic and environmental factors might contribute to the vulnerability of an individual to depression and the now accepted theories for the neurobiological pathways implicated in the development of depression are largely based on genetic-environmental interactions that underlie the dysregulation of brain monoamine systems, neurotrophic signaling and neuroendocrine-neuroimmune interactions (reviewed in [1,218,326] and summarized in Figure 1.10).

1.3.3 Animal models of depression

The elucidation of the neurobiology and pathophysiology of depression and discovery of new and improved antidepressants takes advantage of the use of valid and reliable pre-clinical models of disease. In this context, animal models are indispensable research tools that offer the greatest translational potential and allow a mechanistic and systematic investigation by producing defined and quantifiable correlates to human disease.

Although a full consensus regarding the prerequisites of a valid animal model of depression is still lacking in the scientific community, there are three minimal requirements widely accepted: 1) predictive validity (produce behavioral changes that are reversed by treatment modalities effective in humans); 2) face validity (reproduce in animals aspects of the illness in humans); and 3) construct or etiologic validity (reproduce the true disease process or its etiology in humans). Additionally, the model should have a good reproducibility between investigators [108].

The complex and multifactorial patterns of depression together with subjectivity associated with the diagnosis highlights the difficulties to accurately mimic this disease in an animal model [283].

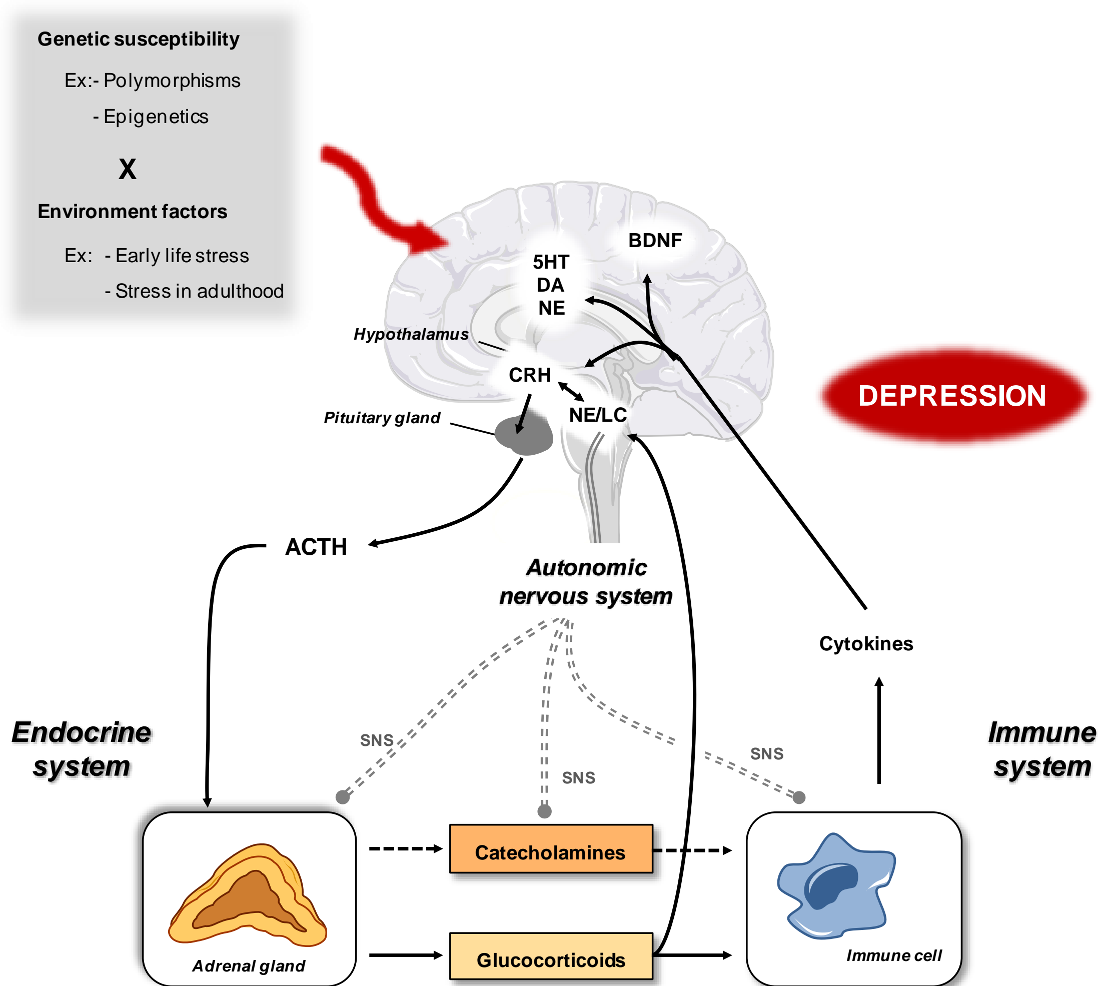

Figure 1.10 Etiology and neurobiology of depression
According to the current view, depression results from a complex interaction between genes and environment that leads to the dysregulation of: 1) brain noradrenergic, dopaminergic and serotoninergic systems, 2) neurotrophic signaling and neurogenesis and 3) neuroendocrine-neuroimmune interactions. It is suggested that exposure to environmental stressors in susceptible individuals might lead to altered monoamine systems and neurogenesis in brain and hyperactivation of stress systems. HPA hyperactivity induces increased glucocorticoid secretion from adrenal cortex that directly and indirectly contributes to the development of depression due its effect on brain and immune system, respectively. Sympathoadrenal dysfunction has also been implicated, but its role in the neurobiology of disease remains largely unquantified.

Even so, a variety of different animal models of depression have been developed and are currently being used. A brief description of each model is shown in Table 1.9. Most animal models of depression are either based on environmental challenges or on manipulation of sensory and integrative functions of the brain. In addition, genetic manipulation has also been widely applied to generate depression models. There is considerable variability in the extent to which the different animal models reproduce features that resemble a depressive-like state and although none of the presently available animal models is able to reproduce all aspects of depression and very few

satisfy all three validating criteria, existing paradigms have proven extremely useful not only in the identification and improvement of antidepressant substances, but also in the validation of neurobiological concepts [97,412,413].

Table 1.9 Animal models of depression.

Models		**Comments**
Lesion-based	Olfactory bulbectomy	• Changes in all major neurotransmitter systems; • Mimics the slow onset of antidepressant action reported in clinical studies; • Does not reproduce etiology of disease; • Uncertain mechanisms of action.
Pharmacological	Reserpine	• Sensitive to antidepressants; • Mood-lowering effects is unclear; • Nonselective for all amines; • Does not reproduce etiology of disease.
	Tryptophane	• Sensitive to antidepressants; • Does not reproduce depressive signs or symptoms; • Does not reproduce etiology of disease.
	Psychostimulant withdrawal	• Causes depression-like symptoms; • Sensitive to antidepressants; • Does not reproduce etiology of disease.
Genetic	Genetic engineering in mice	• Includes forward and reverse genetics; • Modulates the expression of genes involved in the neurobiology of disease; • Rather represent models of predisposition to disease than valid genetic models;
	Selective breeding	• Highlights individual differences in susceptibility to depression.
Environmental (stress-based) Stress in adulthood	Social stress	• Sensitive to chronic antidepressants; • Rather induces “social anxiety” than depression • Poor reproducibility.
	Unpredictable chronic stress	•Sensitive to chronic antidepressants; • Reproduce etiology of disease; • Induce neurobiological and behavioural changes similar to those observed in depression; • Poor reproducibility.
Early life stress	Maternal deprivation	• Induce neuroendocrine and behavioural changes that persist into adulthood; • Effects of antidepressants are uncertain.

Adapted from [97].

1.3.3.1 Unpredictable chronic stress model

The unpredictable chronic stress (UCS) paradigm is a widely used rodent model of depression, originally described by Katz and Hersh (1981) [198] and further developed by Willner et al (1987) [414], which emphasizes the role of stress in the etiology of depression. In this model, animals gradually develop a chronic depressive-like state due to sequential and unpredictable exposures to different stressors, over a sustained period of time (ranging from 10 days to 8 weeks). UCS induces long lasting changes of behavioral and neurobiological parameters resembling dysfunctions observed in depressed patients (Table 1.10). Moreover, the effects induced by this paradigm are reversed by chronic, but not acute, treatment with antidepressants [267]. Therefore, UCS paradigm is a model of depression with good construct, face and predictive validity [166,412,413]. Although reproducibility of UCS had been questioned, it has been significantly improved and UCS paradigms have demonstrated to be a suitable tool to investigate the neurobiology of depression, and thus contribute to the development of novel targets for the treatment of depressed patients.

1.3.4 Co-morbidity with cardiovascular disease

Over the last years, several epidemiological studies demonstrated that there is an association between depression and cardiovascular pathophysiology, such as coronary artery disease (CAD), myocardial infarction (MI) and congestive heart failure (CHF).

Patients with depression have increased risk of developing cardiovascular disease as well as an increased risk of mortality after experiencing a cardiac event [44,122,193]. The association between mood disorders and cardiovascular disease is independent of other cardiovascular risk factors, such as hypertension, high cholesterol, increased body mass index, history of cardiac related problems and disease severity, and it has been demonstrated in both individuals with or without established cardiovascular pathophysiology [146].

Given that depression and cardiovascular disease are two of the leading causes of disability worldwide, the co-morbidity of depression and cardiovascular disease is an important public health concern and the knowledge of the mechanisms of this interaction would be of particular

importance to improve the treatment of depressed patients that suffer from cardiovascular pathology.

Table 1.10 Unpredictable chronic stress in rodents correlates with depression in humans.

	Unpredictable chronic stress (rodents)	**Ref.**	**Depression (humans)**	**Ref.**
Behavioral effects	↓ Sucrose intake/preference; ↓ Reactivity to reward; ↓ Palatable/sweet food intake; ↑ Immobility time in forced swimming test/learned helplessness; ↓ Male sexual and aggressive behavior; ↓ Self-care and grooming; ↑ REM sleep latency.	[412]	Markedly diminished interest/pleasure (“anhedonia”); Psychomotor agitation or retardation; Fatigue or loss of energy; Insomnia or hypersomnia.	[15,413]
Neurobiological effects	***Monoamine systems*** ↓ 5HT in brain; ↑ $5HT_{2A}$ receptor in brain; ↓ $5HT_{1A}$ receptor in brain; ↑ β-adrenergic receptor in brain; ↑ MAO-A in brain.	[166]	***Monoamine systems*** ↓ 5HT metabolite in CSF; ↑ $5HT_2$ receptor in blood and brain; ↓ 5HT transporter density in platelets and brain; ↓ L-tryptophan in plasma; ↑ β-adrenergic receptor in brain; ↑ MAO-A in brain.	[326]
	Neuroimmune system ↑ TNFα, IL-1β, IL-6 in blood and brain.	[147,275]	***Neuroimmune systems*** ↑ IL-1β, IL-6, INFs in blood; ↑ prostanglandins in blood; ↓ NK cell activity; ↑ C-reactive protein (CRP).	[106,309]
	Neuroendocrine system ↑ Corticosterone in blood; ↓ Glucocorticoid receptor in brain.	[21,147,166, 197,255,311]	***Neuroendocrine system*** ↑ Cortisol in blood; ↑ ACTH in blood; ↑ CRF in CSF; Impaired glucocorticoid negative feedback.	[146,219]
	Other systems ↓ BDNF in brain.	[166]	***Other systems*** ↓ BDNF in brain.	[196]

Stress has been proposed as an underlying trigger leading to the development of both depression and cardiovascular disease [193] and therefore dysfunction of stress systems and its associated pathophysiological changes are potential mechanisms to explain the link between depression and

cardiovascular disease. Indeed, a hyperactivity of HPA axis has been consistently demonstrated in depressed patients, reflected by alterations of CRF, increases in circulating ACTH and cortisol and impaired feedback regulation of HPA [146]. In addition, several other abnormalities often associated with depression, such as insulin resistance, abdominal obesity and increased inflammatory cytokine production, might also be a consequence of HPA dysregulation [40]. Thus, HPA dysfunction in depressed patients can contribute to the development of cardiovascular disease through direct effects of cortisol on heart and blood vessels and/or indirect effects on metabolic and immune systems, which leads to consequent disruption of neuroendocrine-neuroimmune systems and/or autonomic function (figure 1.11) [193,219,278,400].

Moreover, sympathoadrenal hyperactivation in depression has also been suggested by some studies that reported elevated plasma levels and urinary excretion of NE and its metabolites in depressed patients [306]. However, the examination of indices of sympathetic nervous function in patients with depression has given rise to inconsistent results and some other works described a tendency to diminished urinary excretion of NE and its metabolites. In addition, a bimodal distribution of sympathetic activity in depressed patients, with patients showing high and others low values of sympathetic activity, was also reported [26,44].Therefore, although sympathoadrenal system hyperactivation has often been suggested to contribute for the development of cardiovascular pathophysiology in depressed patients, through the effects of catecholamines on cardiovascular and endocrine systems [208,375], the SNS and adrenal medullary function in depression remains poorly investigated and understood.

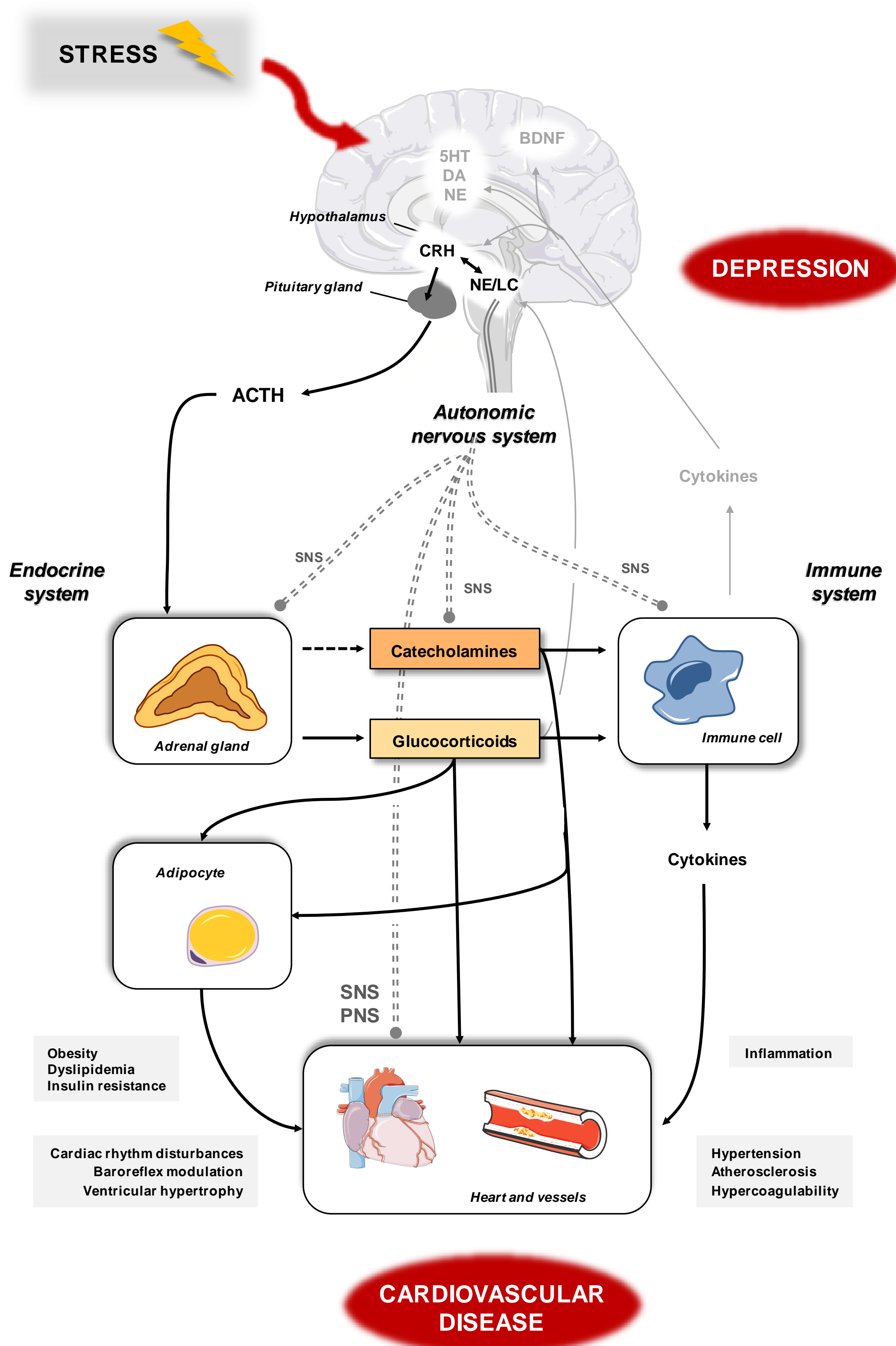

Figure 1.11 Role of stress on the association between depression and cardiovascular disease. Abnormal prolonged activation of HPA axis in depression leads to increased glucocorticoid secretion from adrenal cortex. Glucocorticoids might contribute to the development of both depression and cardiovascular disease thought direct effects on brain, heart and blood vessels and/or indirect effects on adipose tissue and immune cells. Sympathoadrenal dysfunction might also play a role in the disruption of autonomic, endocrine and immune systems in depressed patients, but these interactions remain largely unquantified.

CHAPTER 2

Objectives

2.1 Main objectives

Stress is considered a key determinant in the development of both depression and cardiovascular disease. The adrenal medulla plays a key role in the response to stress by releasing catecholamines, which are major mediators of several physiological changes necessary to maintain homeostasis. Adrenal medulla adaptative changes induced by stress are essential in enhancing the organisms to cope with a changing environment. However, when stress becomes recurrent the alterations induced in the adrenal medullary catecholaminergic system may be deleterious leading to the development of disease. Adrenal medullary dysfunction in depressed patients, as a result of maladaptation to long term exposures to environmental stressors, might be a potential mechanism that linking depression to cardiovascular disease and thus contributing to the higher co-morbidity of these two pathologies. However, function of the adrenal medulla in depression has been poorly investigated. In this context, the first aim of this work was to study the adrenal medullary catecholaminergic system in a model of depression induced by repeated exposures to unpredictable stressors.

The adult adrenal medulla contains a small number of neural-crest derived chromaffin progenitor cells; the physiological role of these cells, however, remains to be elucidated. Chromaffin progenitor cells might be involved in adrenal medulla adaptative responses to stressors and thus pathological changes of chromaffin progenitor cells could contribute to the development of stress-related disorders, such as depression and cardiovascular disease. Therefore, the second aim of this work was to explore the role of chromaffin progenitor cells in adrenal medulla response/adaptation to stress in the animal model referred above.

Chromaffin progenitor cells were already isolated from bovine adult adrenal medulla as free-floating spheres, named chromospheres. Bovine chromospheres express different neural and neural crest stem cell markers and are able to differentiate, *in vitro,* into both neuronal and neuroendocrine phenotypes. Nevertheless, it is unknown whether chromaffin progenitor cells could also be present in human adrenal gland and, similarly to bovine, be isolated from human adult adrenal medulla. Chromospheres might represent an interesting *in vitro* model to study the mechanisms behind adrenal medulla adaptation to stress, contributing to a better understanding of adrenal medulla dysfunctions in stress related-disorders. Moreover, due to its properties, chromaffin progenitor cells were suggested as a potential new cell source for cell transplantation

in the treatment of neuroendocrine and neurodegenerative diseases. Taking this into account, the last aim of this work was to isolate, characterize and differentiate chromaffin progenitor cells from human adult adrenal medulla.

In summary, the purpose of this work is to contribute to a better understanding of adrenal medulla dysfunctions induced by chronic stress and establish a new human *in vitro* model to identify the mechanism involved in adrenal medulla adaptive responses do stressors. Moreover, this study also aims to open new perspectives in the field of regenerative medicine, regarding the potential use of chromaffin progenitor cells in the treatment of neurodegenerative and neuroendocrine diseases.

CHAPTER 3

The adrenal medullary function in depression induced by chronic stress

Chapter submitted for publication
Santana MM, Rosmaninho-Salgado J, Cortez V, Kaster MP, Aveleira CA, Cavadas C. *Impact of unpredictable chronic stress model of depression on adrenal medulla: is adrenal medulla a link between depression and cardiovascular disease? (Neurobiology of disease)*

3.1 Abstract

Depression and cardiovascular diseases are two of the major causes of disability worldwide and epidemiological studies have demonstrated that there is a link between depression and cardiovascular pathology. Dysfunction of the sympathoadrenal system has been proposed to explain cardiovascular system dysfunction in depressed patients. However, the adrenal medullary function in depression has been poorly investigated. Therefore, the aim of this work was to study the adrenal medullary catecholaminergic system in a depression model induced by chronic stress. Mice were submitted to 7 and 21 days of unpredictable chronic stress (UCS) and the chromaffin vesicle content, catecholamine biosynthetic enzymes, catecholamine transporters, exocytotic machinery and gap junctional communication were evaluated in adrenal medulla. At day 7, UCS mice had increased mRNA levels of TH, DβH, PNMT, NPY, VMAT2 and SNAP-2, whereas the mRNA levels of ZO-1 were decreased compared to controls. No differences between groups were observed in the mRNA levels of CgA, NET, VMAT1, synthaxin1A, VAMP2, synaptophysin, Cx43 and Cx36. In contrast, after 21 days, UCS mice showed reduced mRNA levels of PNMT, NET, VMAT1, synthaxin1A, SNAP-25, VAMP2 and ZO-1, but increased mRNA levels of Cx43. No differences were observed in the mRNA levels of TH, DβH, CgA, NPY, VMAT2, synaptophysin and Cx36. In addition, 21 days of UCS induced a decrease in protein levels of TH, DβH and PNMT. Lower EP and NE content per protein in adrenal medulla was also observed after 21 days in UCS mice. No differences in NE serum levels were observed between groups, but EP serum levels were lower in the UCS group. In conclusion, this study suggests that, despite the initial increase in the expression of catecholaminergic adrenal medulla markers, an impairment of adrenal medullary function is observed when mice are exposed to unpredictable stressors for a period of 21 days.

3.2 Introduction

Depression and cardiovascular diseases are two of the major causes of disability worldwide. Projections from the World Health Organization indicates that depression and ischemic heart disease will be the two leading causes of burdens of disease in 2030 [409]. Moreover, both experimental and epidemiological studies have demonstrated an extensive co-morbidity and an association between depression and cardiovascular diseases [44,146,148,193,278]. Although the mechanisms underlying the link between depression and cardiovascular disease remain poorly understood, it is known that environmental stressors are responsible for the development of both cardiovascular diseases and mood disorders [12,23,62,146,268,330,350,388]. Therefore, the neuroendocrine changes triggered by stress have been proposed as putative mechanisms to explain the link between cardiovascular disease and depression [278,350].

The adrenal gland is the most important peripheral organ involved in stress response and catecholamines, released from the adrenal medulla, are the primary mediators for many of the physiological changes necessary to maintain the body's homeostasis, particularly those related to the cardiovascular and metabolic systems [93]. Catecholamines, particularly epinephrine (EP) and norepinephrine (NE), are synthesized, stored and secreted by the neuroendocrine chromaffin cells present in adrenal medulla. The main pathway of catecholamine biosynthesis begins with the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH), which is the rate-limiting enzyme. The synthesis of NE from DA is catalyzed by dopamine-β-hydroxylase (DβH), while finally phenylethanolamine-N-methyltransferase (PNMT) converts NE to EP. Catecholamines are stored in chromaffin granules, together with other proteins and neuropeptides, such chromogranin A (CgA) and neuropeptide Y (NPY), that are co-released with catecholamines by exocytosis upon sympathetic activation induced by a stressful stimulus [86]. Anchorage of chromaffin vesicles at the chromaffin cell membrane is mediated by soluble NSF attachment receptor (SNARE) proteins that form a complex during membrane fusion, thus allowing the release of the chromaffin granule content to the extracellular space. Once released, catecholamines reach the bloodstream or can be re-uptaken into the chromaffin cells by the norepinephrine transporter (NET). Catecholamines, either recaptured or *de novo* synthesized are then stored in chromaffin granules by an active transport system mediated by vesicular monoamine transporters (VMATs) [17,49,86,115,125,165].

Stress is known to increase adrenal medulla size and induce significant changes in the adrenal medullary catecholaminergic system, which contributes to enhanced adrenal medullary function. The corresponding effects include alterations in catecholamine biosynthetic enzymes, catecholamine transporters, gap junctional communication and chromaffin vesicles content [79,170,228,320,322,369,401]. With time these alterations become maladaptive and are associated with increased susceptibility to a number of disorders, such as those related to cardiovascular system. Therefore, dysfunction of the sympathoadrenal (SA) system induced by stress has been implicated in the dysfunction of the cardiovascular system in depressed patients [193,278]. However, adrenal medullary function in depression has been poorly investigated. Thus, the aim of this work was to study the adrenal medullary catecholaminergic system in the unpredictable chronic stress (UCS) model of depression by evaluating the catecholamines, chromaffin granule constituents, catecholamine biosynthetic enzymes, catecholamine transporters, exocytotic machinery and gap junctional communication. UCS induces a chronic depressive-like state that develops gradually over time, due to exposure of animals to a series of unpredictable stressors, and is characterized by behavioral and neuroendocrine changes similar to those observed in depressed patients [166,412,413], and also by dysfunction of the cardiovascular system [146]. Therefore, the knowledge about the function of the adrenal medulla in this model will provide a better understanding of the pathophysiology of depression that might be relevant to unravel the mechanisms that links depression to cardiovascular disease.

3.3 Material and methods

3.3.1 Animals

Male, 9-weeks old C57/BL6 mice (Charles River, Barcelona) were individually housed under a 12h light/dark cycle in a humidity/temperature controlled room, with *ad libitum* access to a standard chow diet and water, except when food and water deprivation was specified by the stress protocol. Animals were allowed 5 days to acclimatize to the surroundings before each UCS protocol. All experimental procedures were performed in accordance with the European Union Directive 86/609/EEC for the care and use of laboratory animals. All people working with animals have received appropriate education (FELASA course) as required by the Portuguese authorities. In addition, animals are housed in our licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006). The present study and the animal experimentation described were included in a project approved and financed by the Portuguese Science Foundation. Center for Neuroscience and Cell Biology (CNC) animal experimentation board also approved the utilization of animals for this project (reference PTDC/SAU-FCF/108110/2008).

3.3.2 Unpredictable chronic stress protocol

The UCS protocol was performed as previously described [414] with some modifications. For 21 days the mice were exposed to the following stressors: two periods of damp bedding (24h), two periods of paired housing (1h), three periods of restraint stress (2h, 3h and 4h), two periods of cold bath (15ºC, 20 min), two periods of inescapable shock (0.7mA for 3s, five times with one minute interval between shock), two periods of exposure to the shock apparatus without shock (1h) after the inescapable shock, two periods of inversion of the light/dark cycle, two periods of cage tilt (24h), two periods of food and water deprivation (24h) and two exposures to an empty water bottle (1h) after water food and water deprivation. This paradigm was designed to maximize unpredictability and therefore each stressor was randomly applied to the experimental group once a day at different times. The control mice were, in turn, daily handled in a gentle and consistent way to minimize stress induced by manipulation. Animals were used for behavioural tests or sacrificed for tissue collection 24h after the last stressor.

3.3.3 Forced swimming test

The forced swimming test (FST) was performed to evaluate depressive behavior of mice [89,247,338,354]. Mice were dropped individually into glass cylinders (height: 25 cm, internal diameter: 10 cm) containing 15 cm water, maintained at 23-25ºC, and the immobility time (ceased struggling and remained floating motionless in the water) for a 6-min period was measured. An increase in immobility over time is considered to be associated with behavioural despair, which is a feature of a depressive state [89,338].

3.3.4 Serum and adrenal medulla collection

Mice were anesthetized and killed by decapitation. Trunk blood was collected and serum was separated by centrifuging (2000xg for 15 minutes at 4ºC) and kept at -80ºC. Adrenal glands were removed and adrenal medullae were carefully dissected from adrenal cortex. Samples were immediately frozen on dry ice (protein extraction and catecholamine quantification) or protected from degradation with RNAlater (Qiagen, Germany), according to the manufacturer's instructions (RNA extraction).

3.3.5 Catecholamine quantification

For adrenal medulla catecholamine content quantification, the adrenal medullae were sonicated in Krebs buffer (111 mmol/L NaCl, 2.5 mmol/L $CaCl_2$, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, 24.8 mmol/L $NaHCO_3$, 11.1 mmol/L glucose, and 15 mmol/L HEPES, pH 7.4) and half of the supernatant was immediately acidified with $HClO_4$ to a final concentration of 0.4M and kept at -80ºC until use. The other half was used for protein quantification to normalize the results. Catecholamine concentrations in supernatant and serum were determined by HPLC with electrochemical detection (HPLC-ED). Catecholamines from serum samples were extracted with alumina before injection, as described previously [58].

3.3.6 RNA extraction and cDNA synthesis

Total RNA was extracted from the tissue using the RNeasy Midi Kit (Qiagen, Germany), following the manufacturer's instructions. Two animals (four medullae) were used per individual sample (n). During RNA extraction, all samples were treated with DNAse (Qiagen, Germany) to eliminate any contamination with genomic DNA. The total amount of RNA was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific, USA) and RNA purity was evaluated by measuring the ratio of OD at 260 and 280 nm. cDNA was obtained from the conversion of 0.5μg of total RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, USA), according to the manufacturer's instructions, and stored at -20ºC until use.

3.3.7 Quantitative RT-PCR

Quantitative RT-PCR (RT-qPCR) was performed in the StepOnePlus™ Real-Time PCR thermocycler (Applied Biosystems, Life Technologies Corporation, USA) using 96-well microtitre plates and iQ Syber Green Supermix (Bio-Rad, USA). Primers for the target and reference genes were pre-designed or ordered from Qiagen (Table 3.1). A master mix was prepared for each primer set containing the appropriate volume of 2x iQ Syber Green Supermix (Bio-Rad, USA) and 10x QuantiTect Primer or 0,125μM of the designed primers. For each reaction, 8 μl of master mix were added to 2 μl of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 10 μl per well. The reactions were performed according to the manufacturer's recommendations: 95°C for 3 min followed by 45 cycles at 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. The melting curve protocol started immediately after amplification. Additionally, the PCR products were run on a 1% agarose gel to confirm their size. Normfinder analysis was performed to choose the best reference genes for normalization. The reference genes were β-2-microglobulin and HPRT-1 (Acc.SD= 0.23) for qPCR performed with the samples collected at day 7 and HPRT-1 (SD= 0.15) for the samples collected at day 21. Relative mRNA quantification was performed using the ΔCt method for genes with the same amplification efficiency [245].

Table 3.1 Primer sequences

Gene	Primer sequence (5'-3')
DβH	F: TGGGTGCCAAGGCATTTTAC R: TCCGTGGGTTGTGGTAATGA
CgA	F: CCAAGGTGATGAAGTGCGTC R: GGTGTCGCAGGATAGAGAGGA
VMAT-1	F: GCCATGGGGACTCCACTTTT R: AGCGTGGGCTCTAATATGGC
VMAT-2	F: GGACCACAACTGCCCCATTA R: CGTTAGAGGGGCTCAGTCAC
NET	F: AGGGCCACCATTTTCTCGTT R: CTCATTCGGCTAGCACCACA
Syntaxin1A	F: GGAAGGTCTGAACCGTCCAT R: TTTGCAGCGTTCTCGGTAGT
SNAP25	F: TGATGCCCGGGAAAATGAGA R: ATCCTGTCGATCTGGCGATT
VAMP2	F: GCCGACCACAATCTGGTTCT R: GCAAGGATGAGGCCAGAGAC
Synaptophysin	F: TCAGTGAAGCCCACGAAGAC R: GGTTGAGGGGTGGAGACCTA
HPRT-1	F: GTTGAAGATATAATTGACACTGG R: CAAGGGCATATCCAACAAC
β-2-microglobulin	F: GGTCGCTTCAGTCGTCAG R: CATACAGGCCGGTCAGTG
TH	Ordered from Qiagen (QuantiTect Primer assay cat no. QT00101962)
PNMT	Ordered from Qiagen (QuantiTect Primer assay cat no. QT01053556)
Cx36	Ordered from Qiagen (QuantiTect Primer assay cat no. QT00165711)
Cx43	Ordered from Qiagen (QuantiTect Primer assay cat no. QT00173635)
ZO-1	Ordered from Qiagen (QuantiTect Primer assay cat no. QT00493899)

3.3.8 Protein quantification and sample preparation

The adrenal medullae were sonicated at 4°C in RIPA buffer [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.5% deoxycholate; 0.1% sodium dodecyl sulphate (SDS); 200 µM phenylmethylsulphonylfluoride (PMSF); 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4; 10 mM

NaF)], supplemented with mini protease inhibitor cocktail tablet (Roche, Germany). Protein obtained in the resulting homogenate was quantified using an infrared (IR)-based spectrometry system, Direct Detect (Merck Millipore, Germany) and denatured with the SDS buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue). After heating for 5 min at 95ºC, the samples were frozen at -20ºC until use.

3.3.9 Western blot analysis

Samples were separated in a 4-10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrophoretic transfer onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in TBS-T (137 mM NaCl, 20 mM Tris, 0.1% Tween 20, pH 7.6) and incubated overnight at 4ºC with mouse anti-TH (1:1,000, Merck Milipore, Germany), sheep anti-DβH (1:2,500, Thermo scientific, USA) or rabbit anti-PNMT (1:1,000, Enzo Life Sciences, USA) diluted in blocking solution. After three washes with TBS-T, the membranes were incubated for 1 hour, at room temperature, with an alkaline phosphatase-linked secondary antibody, specific to rabbit or mouse immunoglobulin G (1:20,000, Amersham Biosciences, GE Healthcare, UK). Immunoreactive bands were visualized using enhanced chemifluorescence (ECF) substrate in the Versa-Doc 3000 imaging system (Bio-Rad, USA). Densitometry of the bands was quantified using Quantity One Software (Bio-Rad, USA).

3.3.10 Statistical methods

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using two way analysis of variance (ANOVA) or Student's unpaired *t* test with two-tailed p value, as indicated in figure legends. A value of $p \leq 0.05$ was considered significant. Prism 5.0 (GraphPad Software) was used for all statistical analysis.

3.4 Results

3.4.1 Effect of UCS on body weight, forced swimming test and corticosterone levels

Mice body weight was determined before starting the stressor exposures (Day 0) and once a week until the end of the UCS protocol (Day 7, Day 14 and Day 21). An effect of time on body weight was observed [F(3,24)=33.86, P< 0.0001, two-way ANOVA test]. The control and UCS mice showed 3.4±0.7g and 3.1±0.6g (n=5) weight gain, respectively, at day 21. However, no significant differences were observed in body weight between control and UCS over time (Fig. 3.1A).

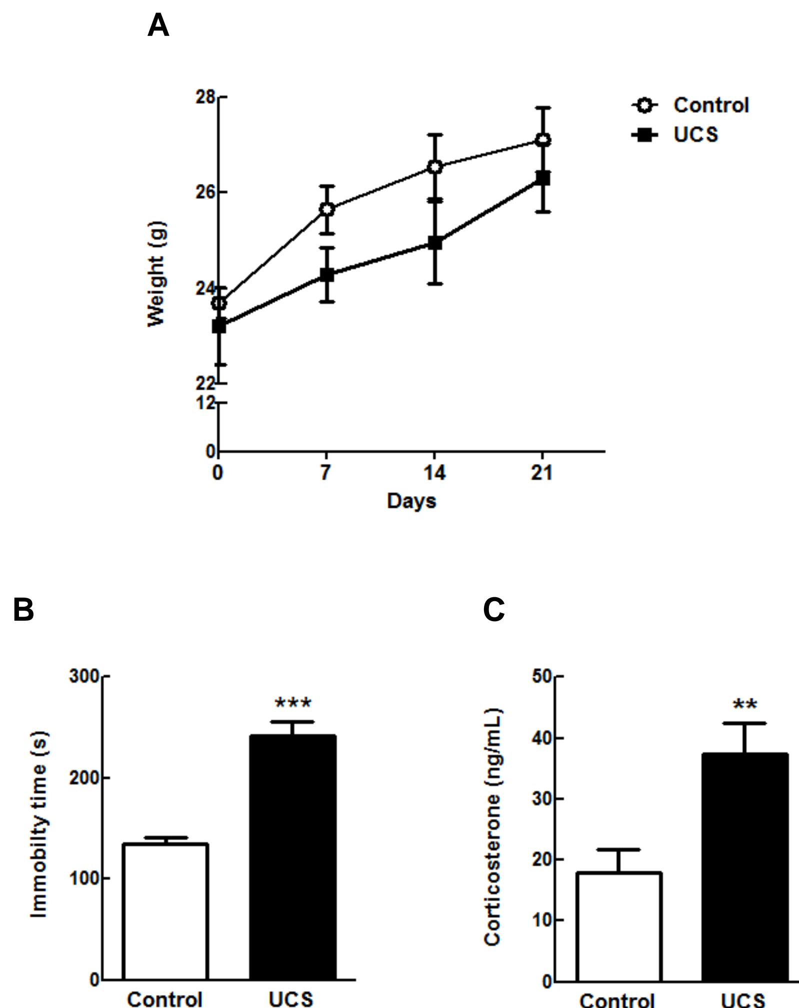

Figure 3.1 Effect of UCS on body weight, forced swimming test (FST) and corticosterone levels
Mice body weight was determined before starting the stressor exposures (Day 0) and once a week (Day 7, Day 14 and Day 21) until the end of the UCS protocol (n=5) (**A**). The FST was performed after 21 days of UCS to evaluate the depressive behavior of mice (n=5) (**B**). Corticosterone levels were also measured after 21 days of UCS (n=6) (**C**). Results are expressed as mean±SEM. Student's *t* test. ***p*≤0.01, ****p*≤0.001, compared to control.

The FST was performed after 21 days of unpredictable exposures to different stressors in order to evaluate the mice depressive like-behavior. Stressed mice showed higher immobility time (241.2±14.1s, n=6) compared with the control group (135.2±6.4s, n=6, Fig. 3.1B). In addition, serum corticosterone levels of mice submitted to 21 days of UCS were higher than the controls (control=13.16±2.31ng/mL; UCS=31.11±5.30ng/mL; mean±SEM; Fig. 3.1C).

3.4.2 Effect of UCS catecholamine biosynthetic enzymes and catecholamine levels in adrenal medulla

Changes in adrenal medulla catecholamine biosynthesis during stress are frequently associated with alterations in the expression of the catecholamine biosynthetic enzymes: TH, DβH and PNMT. These alterations depend on the type, duration and frequency of stress exposures [228,320]. Therefore, TH, DβH and PNMT mRNA levels were evaluated by RT-qPCR after 7 and 21 days of UCS.

As shown in Fig. 3.2 (A-C), mice submitted to 7 days of UCS had a 60%, 141% and 103% increase in TH, DβH and PNMT mRNA levels, respectively. In contrast, after 21 days of UCS no differences were observed in TH and DβH mRNA levels, whereas PNMT mRNA levels decreased 38% in stressed mice compared to controls (Fig. 3.2A-C).

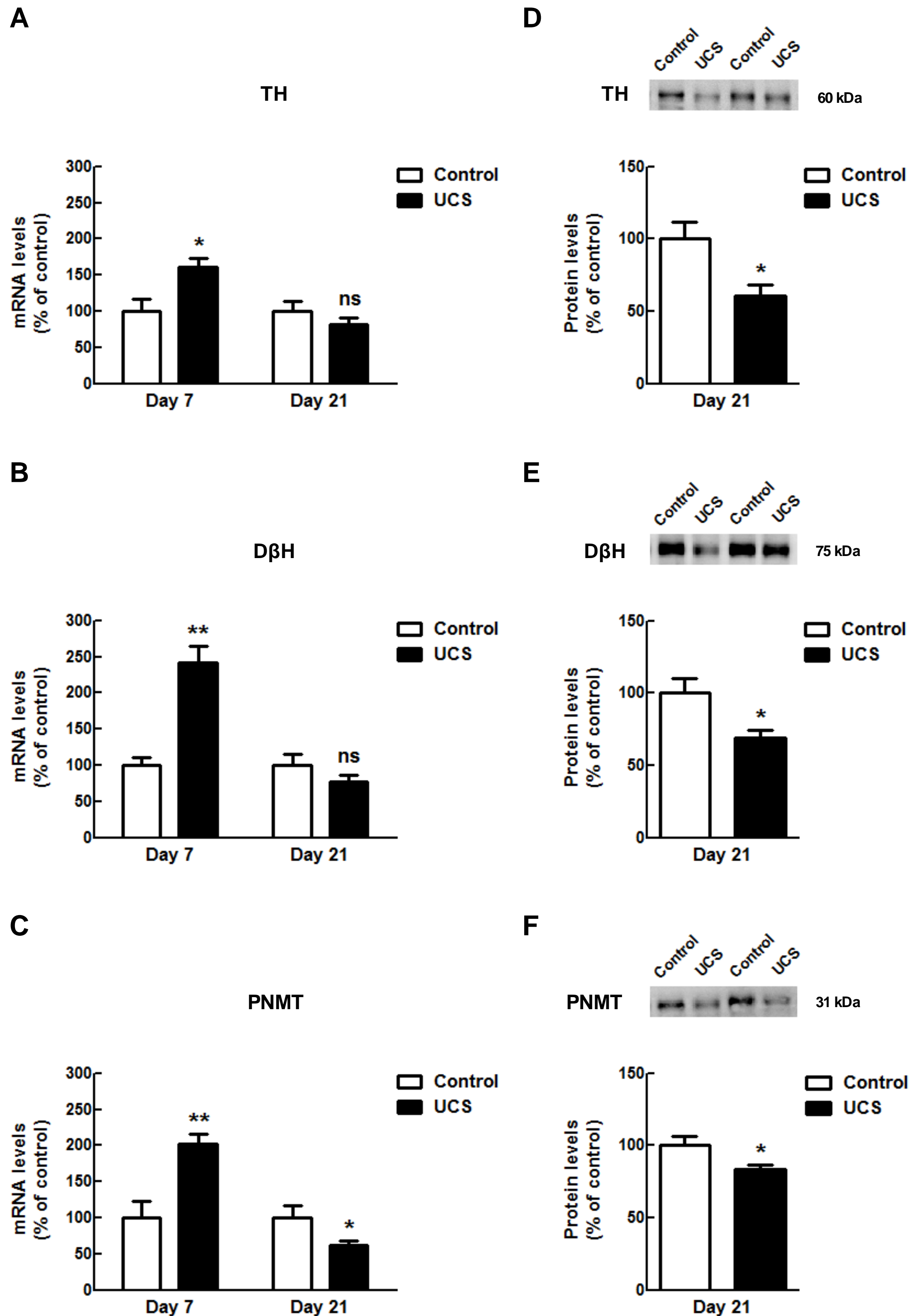

Figure 3.2 Effect of UCS on catecholamine biosynthetic enzymes in adrenal medulla.
Mice were submitted to 7 or 21 days of UCS, sacrificed 24h after the last stressor and adrenal medullae were isolated for analysis. Total mRNA was extracted and the transcript levels of TH, DβH and PNMT were evaluated by RT-qPCR after 7 and 21days of UCS. Results are expressed as % of control and presented as mean±SEM. n=4-6. Student's *t* test. ns=not statistically significant, $^{*}p \leq 0.05$, compared to control (**A**-**C**). TH, DβH and PNMT immunoreactivity was evaluated by Western Blotting in total protein extracts. The intensity of the bands was determined by quantitative densitometric analysis. Representative figures of two animals per group (n=2) are presented above the graphs. Results are expressed as % of control and presented as mean±SEM. n=5-6. Student's *t* test. $^{*}p \leq 0.05$ compared to control (**D-F**).

To determine whether alterations in the mRNA levels could be associated with similar changes in proteins, TH, DβH and PNMT protein content was evaluated by Western Blot at the end of the UCS protocol (21 days). UCS induced a 39%, 31% and 17% decrease in adrenal medulla TH, DβH PNMT protein levels, respectively, when compared to the control group (Fig. 3.2D-F).

In addition, the catecholamine levels were quantified in both serum and adrenal medulla of mice submitted to 21 days of UCS and respective controls. As shown in Fig. 3.3A, stressed mice had lower EP levels in serum than control mice (control=39.4±4.8 pmol/mL; UCS=27.2±2.7 pmol/mL), whereas no significant changes were observed in serum NE levels (control=27.4±2.2 pmol/mL; UCS=31.6±2.7 pmol/mL). In adrenal medulla, UCS induced a decrease in NE and EP content per protein (control=93.6±14.7 pmol NE/µg and 183.6±19.8 pmol EP/µg; UCS= 54.0±9.4 pmol NE/µg and 113.2±14.7 pmol EP/µg; mean±SEM; Fig. 3.3B).

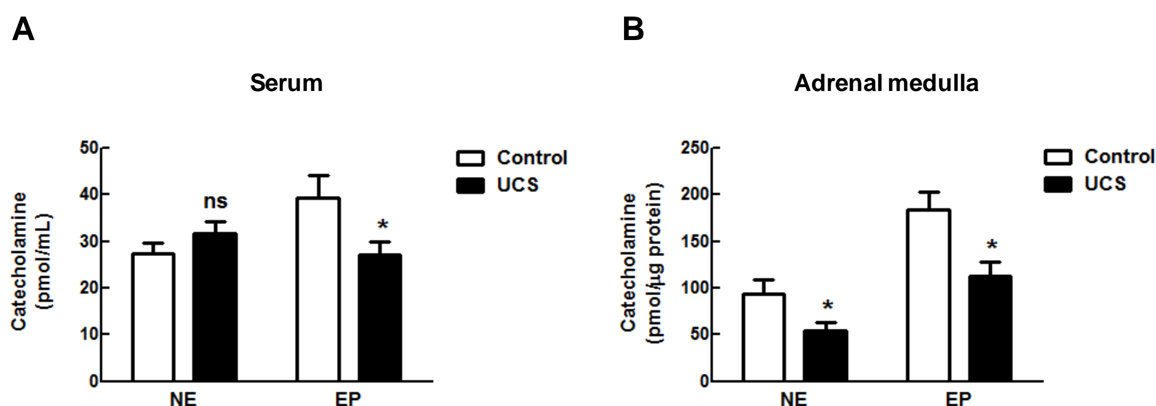

Figure 3.3 Effect of UCS on adrenal medulla and serum catecholamine levels.
Mice were submitted 21 days of UCS and sacrificed 24h after the last stressor. NE and EP levels were determined by HPLC-ED in both serum (n=24-31) (**A**) and adrenal medulla (n=3-4) (**B**). Results are presented as mean±SEM.. Student's *t* test. ns=not statistically significant, *$p \leq 0.05$ compared to control.

3.4.3 Effect of UCS on chromaffin granules constituents: NPY and CgA

Catecholamines are co-stored in chromaffin secretory granules with a variety of other proteins and neuropeptides, including CgA and NPY [86]. It is known that cold and immobilization stress modulate the expression levels of both CgA and NPY in rat adrenal gland [170,322,401]. However, the effect of UCS on these chromaffin granules constituents has not been previously investigated. Therefore, the effect of UCS on CgA and NPY mRNA levels was evaluated by RT-qPCR after 7 and 21 days of UCS. At day 7, no significant changes were observed between CgA mRNA levels in adrenal medulla of UCS and control mice (Fig. 3.4A). However, UCS mice had a 76% increase in the NPY mRNA levels, compared to control mice (Fig. 3.4B). No differences in the expression of both mRNA levels of CgA and NPY were observed after 21 days of stress exposures (Fig. 3.4A-B).

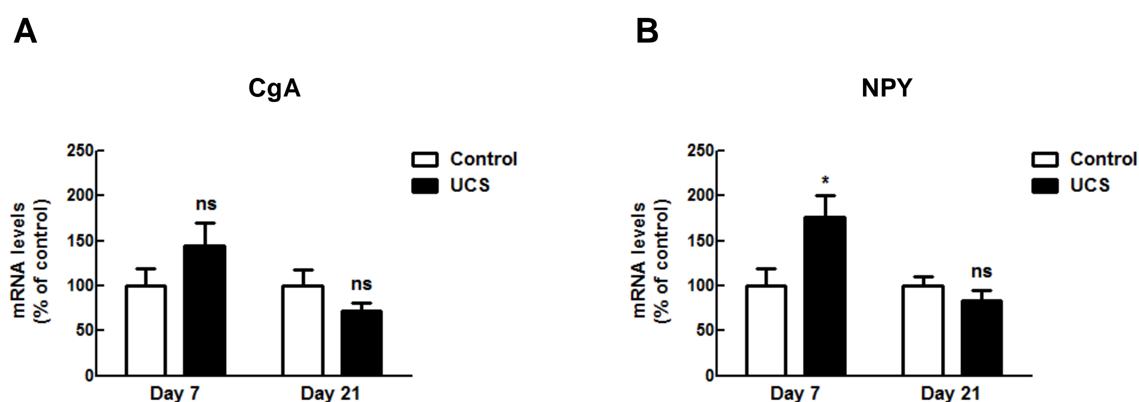

Figure 3.4 Effect of UCS on the content of NPY and CgA in chromaffin granules.
Mice were submitted to 7 or 21 days of UCS and sacrificed 24h after the last stressor. Total mRNA was extracted from adrenal medullae and the transcript levels of CgA (**A**) and NPY (**B**) were evaluated by RT-qPCR. Results are expressed as % of control and presented as mean±SEM. n=4-6. Student's *t* test. ns=not statistically significant, $^{*}p \leq 0.05$, compared to control.

3.4.4 Effect of UCS on catecholamine transporters in adrenal medulla

After exocytosis, the intracellular stores of catecholamines are replenished through re-uptake by an active transport system that, together with *de novo* synthesis, contributes to sustain a cellular steady-state level of catecholamine. This transport is mediated by NET, a monoamine transporter present in chromaffin cell membrane and by the vesicular transporters, VMAT-1 and VMAT-2, which are responsible for the uptake of catecholamines into the chromaffin granules [115,165].

Alterations in catecholamine transporters are also a mechanism by which chromaffin cells can adapt and properly respond to stress [322,369]. The expression levels of NET, VMAT-1 and VMAT-2 were, therefore, determined after 7 and 21 days of UCS by RT-qPCR (Fig. 3.7). At day 7, only the mRNA levels of VMAT-2 were significantly elevated in adrenal medulla of UCS mice (64% increased compared to control; Fig. 3.5B), whereas no differences in the mRNA levels of VMAT-1 and NET were observed between groups (Fig. 3.5A, C). In contrast, after 21 days, UCS mice had a 35% and 53% decrease in VMAT-1 and NET mRNA levels (Fig. 3.5A, C), respectively, but no significant differences were observed in the expression of VMAT-2 between the control and UCS groups (Fig. 3.5B).

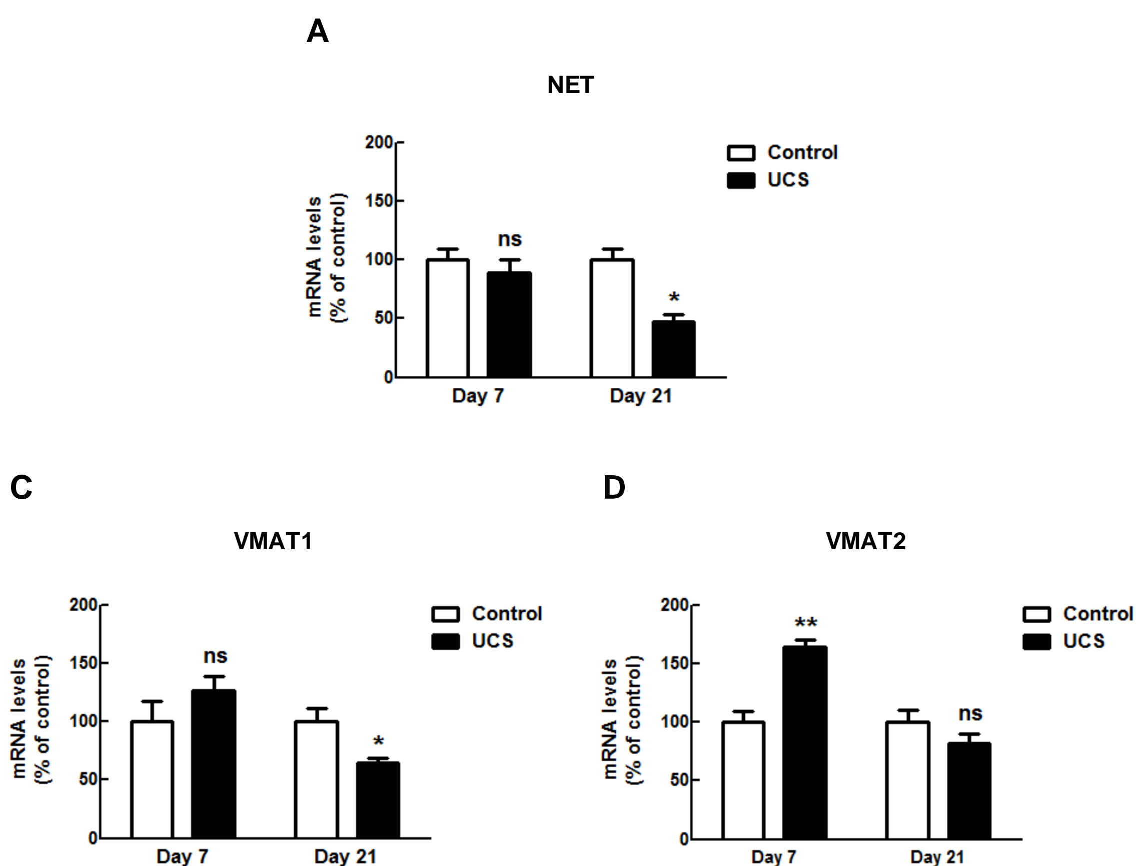

Figure 3.5 Effect of UCS on catecholamine transporters in adrenal medulla.
Mice were submitted to 7 or 21 days of UCS and sacrificed 24h after the last stressor. Total mRNA was extracted from adrenal medulla and the transcript levels of NET (**A**), VMAT-1 (**B**) and VMAT-2 (**C**) were evaluated by RT-qPCR. Results are expressed as % of control and presented as mean±SEM. n=4-6. Student's *t* test. ns=not statistically significant, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, compared to control.

3.4.5 Effect of UCS on exocytotic machinery in adrenal medulla

The chromaffin granule content is released to the extracellular space by exocytosis. Exocytosis is mediated by the SNARE complex, in which the membrane-associated SNAP25 and syntaxin1A interact with proteins from the chromaffin vesicles, such VAMP2 and synaptophysin to create a stable complex. This allows the anchorage of the vesicle to the plasma membrane, driving the membrane fusion, as required. Although the effects of stress on the expression of these proteins in chromaffin cells are not understood, it is known that they play a fundamental role in catecholamine release from chromaffin cells [17,49]. To evaluate the effects of UCS on Syntaxin1A, SNAP25, VAMP2 and synaptophysin in adrenal medulla, the transcript levels of these proteins were quantified by RT-qPCR at days 7 and 21 (Fig. 3.6).

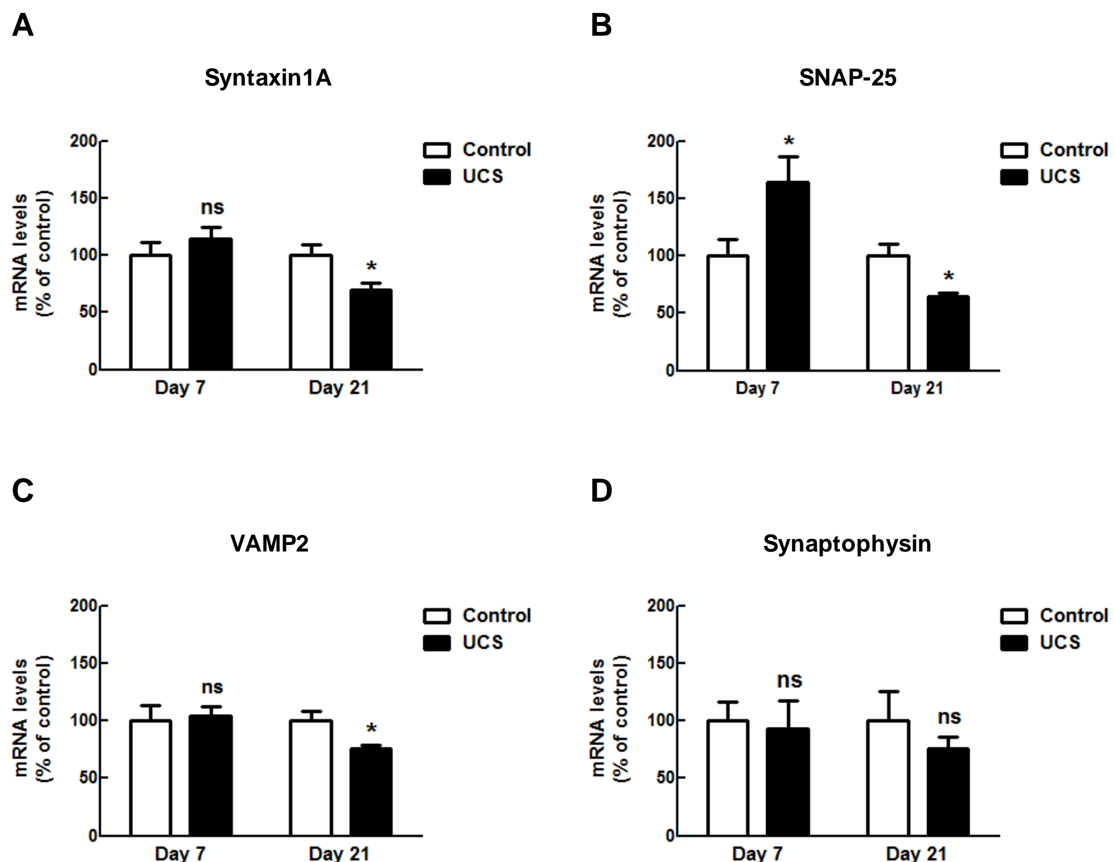

Figure 3.6 Effect of UCS on exocytotic machinery in adrenal medulla.
Mice were submitted to 7 and 21 days of UCS and sacrificed 24h after the last stressor. Total mRNA was extracted from adrenal medulla and the transcript levels of Syntaxin1A (**A**), SNAP-25 (**B**), VAMP2 (**C**) and synaptophysin (**D**) were evaluated by RT-qPCR. Results are expressed as % of control and presented as mean±SEM. n=4-6. Student's *t* test. ns=not statistically significant, $^{*}p\leq0.05$, compared to control.

Mice submitted to 7 days of UCS showed no differences, compared to control, in the expression levels of Syntaxin1A, VAMP2 and synaptophysin (Fig. 3.6A, C and D). On day 7, a significant increase (65%) in SNAP-25 mRNA levels was observed in stressed mice when compared to controls (Fig. 3.6B). However, after 21 days, stressed mice had a 30%, 36% and 24% decrease in Syntaxin1A, SNAP-25 and VAMP2 mRNA levels, respectively, compared to controls (Fig. 3.6A-C). No differences were observed in synaptophysin mRNA levels between control and stressed groups after 21 days of stress (Fig. 3.6C).

3.4.6 Effect of UCS on gap junction-mediated intercellular communication in adrenal medulla

It was shown that gap junction-mediated intercellular communication between chromaffin cells also contributes to catecholamine secretion and constitutes an efficient complementary process to synaptic neurotransmission to amplify catecholamine release [257]. Gap-junction channels are formed by connexons that consist of complex of proteins denominated connexins. Many different connexins are present in adrenal medulla [77], but connexin 43 (Cx43) and connexin 36 (Cx36) have been identified as important regulators of catecholamine release during cold stress [79]. ZO-1 is a scaffolding protein that interacts with Cx36 and Cx43 at the plasma membrane contributing to the assembly and/or stability of gap junctions. Levels of ZO-1 are also modulated by cold stress [79]. To evaluate the effects of UCS on gap-junctional chromaffin cell communication, Cx43 or Cx36 mRNA levels were investigated in adrenal medulla at day 7 and at the end of the UCS protocol (day 21). As shown in Fig. 3.9 A and B, after 7 days of UCS no differences in the expression of either Cx43 or Cx36 were observed between UCS and control groups. However, after 21days, Cx43 mRNA levels increased by 48% when compared to controls, while no changes were observed in Cx36 levels between groups (Fig. 3.7A-B). ZO-1 mRNA levels were decreased by 55% and 36% in stressed mice compared with controls, after 7 and 21 days of UCS, respectively (Fig. 3.7C).

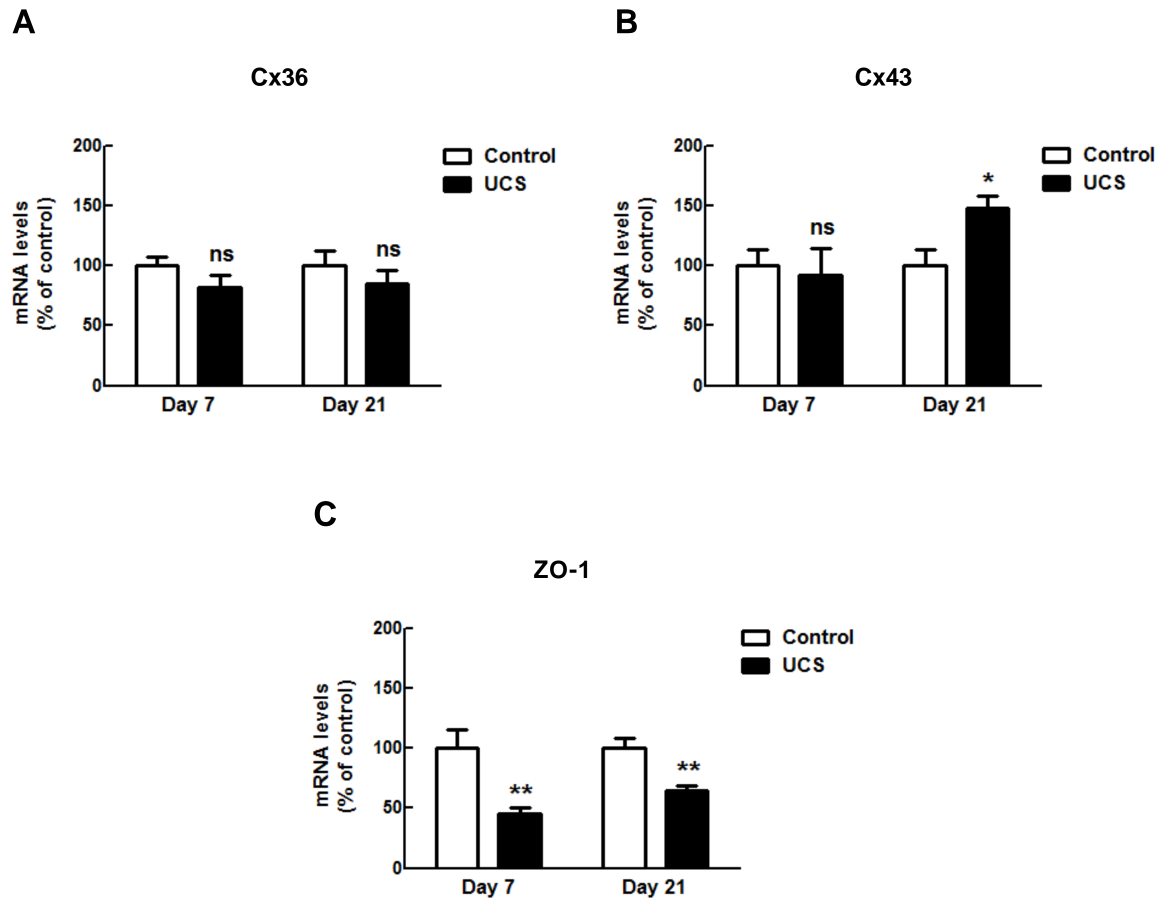

Figure 3.7 Effect of UCS on gap junction-mediated intercellular communication in adrenal medulla.
Mice were submitted to 7 or 21 days of UCS and sacrificed 24h after the last stressor. Total mRNA was extracted from adrenal medulla and the transcript levels of Cx43 (**A**), Cx36 (**B**) and ZO-1 (**C**) were evaluated by RT-qPCR. Results are expressed as % of control and presented as mean±SEM. n=4-6. Student's *t* test. ns=not statistically significant, $^{*}p \leq 0.05$, compared to control.

3.5. Discussion

In the present study, the changes in the adrenal medulla catecholaminergic system in a depression model induced by chronic stress were evaluated. The UCS model of depression was originally performed by Katz and Hersh (1981) [198] and further developed by Willner et al (1987) to induce a chronic depressive-like state in rodents, as a consequence of unpredictable and sequentially exposures to a variety of stressors over a sustained period of time [413,414]. Long-term exposure to uncontrollable life stressors can precipitate the development of depressive disorders in humans [62,268]; thus, UCS paradigms have been considered models with good etiologic validity as they mimic the effect of stress on the etiology of depression [97,166,412,413].

In this work, mice submitted to 21 days of UCS showed a depressive-like behavior and hyperactivity of the HPA axis, as it was demonstrated by the increased immobility time during FST and by the higher serum corticosterone levels in UCS mice. The increased immobility following UCS was seen in animals showing decreased reactivity to rewards and has been associated with an anhedonic state [339,354,366]. Increased corticosterone levels have also been often described in animals following unpredictable stressor exposures [21,197,255,311]. These behavioral and neuroendocrine changes parallels with the melancholic symptoms and HPA hyperactivation observed in depressed patients [146,166,219,412,413].

Studies with rodents showed that stressful conditions, such as immobilization and cold, induced changes in adrenal medulla transcriptome that are relevant for adrenal medulla adaptation (or maladaptation) to stress [79,170,243,320-322,369,401] and are associated with increased susceptibility to disorders, such as cardiovascular disease [4,71,139,141]. Since these changes are highly dependent on the type and duration of stress stimuli [228,321] it is unknown whether unpredictable and sequentially exposures to a variety of stressors, which leads to depression, could also induce significant transcriptional changes in the adrenal medulla. Therefore, the effect of UCS on the transcript levels of important players in the regulation of catecholamine synthesis and release from adrenal chromaffin cells were evaluated. The mRNA levels were quantified at two different time points: seven days after the beginning of the experimental protocol (day 7) and at the end of the protocol (day 21).

After 7 days of exposures to unpredictable stressors, it was observed an increase in the expression of catecholamine biosynthetic enzymes (TH, DβH and PNMT), suggesting an

enhancement of catecholamine synthesis in adrenal medulla. Increased mRNA levels of catecholamine biosynthetic enzymes in adrenal gland during stress are associated with increased catecholamine biosynthesis [125,228,320,420]. It was also shown that stress not only promotes catecholamine synthesis but also increases the capacity of catecholamine storage, through changes on VMATs [322,369]. Of the two VMATs isoforms responsible for catecholamine unptake into secretory granules under basal conditions, VMAT-1 is the most expressed and is present in both rat noradrenergic and adrenergic chromaffin cells [322,369]. VMAT-2 is expressed predominantly in the rat noradrenergic chromaffin cells, but not in the adrenergic chromaffin cells [322,369]. In the present study, after 7 days of UCS, mRNA levels of VMAT-2 were increased. These results are in agreement with previous works where it was demonstrated that exposure of rats to repeated immobilization stress leads to a selective increase in gene expression of VMAT-2, including in adrenergic chromaffin cells [369]. Higher levels of VMAT-2 after 7 days of UCS suggest that adrenal chromaffin cells might be able to provide a better response to the increased requirement of catecholamines during the first week of stress exposures.

The exocytotic machinery was evaluated to investigate whether exocytosis is also a mechanism by which chromaffin cells regulates catecholamine release during UCS. It was observed that the adrenal medulla of 7 days stressed mice had elevated mRNA levels of membrane-associated SNAP25 from the SNARE complex, whereas no changes were observed in the levels of syntaxin1A, VAMP2 and synaptophysin. SNAP25 is a fusion protein that appears to be necessary for sequential exocytosis, which only occurs at high levels of stimulation in adrenal chromaffin cells, such those observed during stress stimulus [209,210]. The higher levels of SNAP-25 in adrenal medulla after 7 days of UCS suggest that sequential exocytosis might occur as an additional response of mouse chromaffin cells to increase catecholamine release.

At day 7 of UCS, stressed mice exhibit elevated expression levels of NPY in adrenal medulla. NPY acts as a local hormone that modulates catecholamine release from human and mouse chromaffin cells and its increase during stress might contribute to the amplification of catecholamine secretion by adrenal medulla [58,59,314-316].

Taken together, the alterations in the adrenal medulla transcriptome observed after 7 days of daily exposures to unpredictable stressors suggest that adrenal medulla responds by enhancing the capacity of chromaffin cells to synthesize, store and release catecholamines. However, it was

observed that with repeated exposures to different unpredictable stressors occurs a change in transcriptional regulation; the genes upregulated at 7 days of UCS returned to levels comparable to the controls or were downregulated after 21 days of UCS.

Regarding the catecholamine biosynthetic enzymes, TH and DβH mRNA levels in adrenal medulla of UCS mice returned to levels similar to those from the control mice, whereas there was a decrease in PNMT mRNA levels. In addition, a significant decrease in the protein levels of the three catecholamine biosynthetic enzymes (TH, DβH and PNMT) was observed, suggesting a decrease in catecholamine synthesis. The discrepancies between mRNA and protein levels of TH and DβH suggest that post-transcriptional mechanisms might be involved in the regulation of these enzymes, as has previously been proposed [365,420,421]. In addition, the downregulation of the catecholamine transporters (NET and VMAT1) observed after 21 days of UCS suggest a reduction of catecholamine recycling upon exocytosis and catecholamine storage in chromaffin granules, which might be a consequence of lower catecholamine biosynthetic activity and catecholamine levels. Indeed, previous studies demonstrated that NET regulation is dependent on catecholamine synthesis and levels [154,406]. In mice exposed to 21 days of UCS, chromaffin cells also seem to have a dysregulation of catecholamine release, as suggested not only by the reduced mRNA levels of synthaxin 1A, SNAP25 and VAMP2, but also by the changes in the expression of Cx43 and ZO-1. Two crucial steps in the regulation of exocytosis are the docking and fusion of chromaffin granules with the plasma membrane, which is mediated by the SNARE complex [51,389]. The SNARE proteins syntaxin1A, SNAP25 and VAMP2 have been considered the minimal machinery required for SNARE complex formation [105,404,422]. Chromaffin cell membranes are highly enriched with syntaxin1A, SNAP25 and VAMP2 [60] and therefore the downregulation of these proteins, after 21 days of UCS, might compromise exocytosis.

Previous studies have also shown that, in chromaffin cells, gap-junctions are responsible for the propagation of the electrical signal leading to Ca^{2+} increase in adjacent cells and the consequent catecholamine release by exocytosis [257]. In this study, 21 days of UCS stress induced an increase in Cx43 mRNA levels, but also a significant reduction in the levels of ZO-1. Although the interaction between Cx43 and ZO-1 is not well characterized and no studies were performed in chromaffin cells, evidence suggests that ZO-1 might mediate the delivery of Cx43 to the gap junctional plaques, and may, therefore, play a critical role for gap junction formation [135,233]. As

such, the lower levels of ZO-1 induced by UCS might induce a reduction of gap junction formation, even though the Cx43 levels were elevated, which may trigger a decrease in potential transmission between chromaffin cells and consequent reduction of exocytosis.

The results presented in this work suggest that mice submitted to 21 days of UCS have decreased catecholamine synthesis, re-uptake, storage and release, suggesting an impairment of adrenal medulla function. In fact, after 21 days, stressed mice had decreased levels of NE and EP per protein in adrenal medulla and lower EP levels in serum compared to controls. It is important to notice that in the present study catecholamines were measured in blood collected from trunk after decapitation. Decapitation is associated with an increase in the circulating catecholamines due to a stimulation of secretion by the adrenal medulla [30,150,305] and therefore the serum levels mesuared might mimic, at least in part, a situation of stress exposure rather than the basal levels of catecholamines. In this context, this study is in agreement with a previous report where rats submitted to 4 weeks of UCS had a lower increase in catecholamine levels compared to controls, when submitted to an acute episode of immobilization stress [347]. Non-psychotic depressed patients also showed significantly lower levels of EP than control patients after dexamethasone administration, which mimics HPA activation during stress [318].

Dysfunction of adrenal medulla in depression, compromising EP secretion, might have implications in the development of cardiovascular alterations observed in depressed patients. In fact, the lack of EP secretion from adrenal medulla during stress gives rise to an abnormal response from cardiovascular system, as it was demonstrated by alterations of blood pressure, cardiac output and heart rate in PNMT knockout mice submitted to restraint stress and treadmill exercise [24,25]. Interestingly, EP secretion from the adrenal medulla is markedly reduced with advancing age in both resting and stressful conditions [332], although the risk of developing cardiovascular disease in elderly is higher. In contrast, old people have increased sympathetic nervous system and HPA axis activity [98,332,362]. Therefore, although the exact mechanisms remain to be clarified, it is possible that an impairment of adrenal medulla function in depression induced by chronic stress might contribute to the development of cardiovascular dysfunction, perhaps through a hyperactivation of other stress systems. The idea that failure of one physiological stress system could induce dysregulated compensatory mechanisms in other stress systems was already discussed by McEwen et al (1998) [259]. Further studies will be required to

clarify this hypothesis. To understand the specific mechanisms that underlie depression and cardiovascular pathology is of particular relevance and importance for the development of appropriate strategies for the treatment of patients with depression and cardiovascular disease.

CHAPTER 4

The chromaffin progenitor cell and its role in adrenal medulla response/adaptation to stress

Chapter in preparation for publication in peer-reviewed international journal

4.1 Abstract

Chronic stress is often linked to adrenal gland enlargement and to enhanced hormone secretion by chromaffin cells of adrenal medulla. Recently, it was demonstrated that neural crest derived chromaffin progenitor cells, with proliferative potential, persist in adult adrenal medulla in an undifferentiated state. However, the physiological function of chromaffin progenitor cells and their putative role in adrenal medulla adaptation to stress remains to be elucidated. Therefore, the aim of this work was to study the role of chromaffin progenitor cells in adrenal medulla response/adaptation to stress, using an animal model of unpredictable chronic stress (UCS). Mice were submitted to 7 or 21 days of UCS, the adrenal glands were collected and weighted. The volume of adrenal medulla and cortex was estimated by using the Cavalieri method. After 21 days of UCS, an increase in adrenal gland weight and volume was observed. Both adrenal cortex and medulla volume were increased, with no alterations in adrenal cortex/medulla volume ratio. The mRNA levels of chromaffin progenitor cell markers (Sox9, Notch1, Sox10, nestin, Mash1 and Phox2b) and PCNA, a cell proliferation marker, were evaluated after 7 or 21 days of UCS by RT-qPCR. After 7 days of UCS, adrenal medulla had lower mRNA levels of Sox9, Notch1 and PCNA, whereas the mRNA levels of Mash1 and Phox2b were increased compared to controls, suggesting an induction of differentiation into chromaffin cell. No differences between groups were observed in the mRNA levels Sox10 and nestin. After 21 days, no differences were observed in the mRNA levels of Sox9, Notch1, Sox10, nestin, Mash1, Phox2b and PCNA. In conclusion, our study shows that chromaffin progenitor cell differentiation might contribute to the enhancement of adrenal medulla function during stress. This study highlights a putative role of chromaffin progenitor cells in adrenal medulla function and suggests a more complex regulation of adrenal medulla catecholaminergic system during stress.

4.2 Introduction

The adrenal gland is the most important peripheral endocrine organ of the organism involved in the control of the physiological response to stress. Activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathoadrenal system trigger the secretion of glucocorticoids and catecholamines from adrenal cortex and adrenal medulla, respectively, into the blood circulation [72,284,353]. These hormones exert multiples complementary actions throughout the body, particularly on the cardiovascular and metabolic systems, which are essential for the maintenance of homeostasis [284]. Exposure to stressful stimuli induces significant alterations in the adrenal gland that are important to enhance hormone secretion and improve the capacity of the organism to cope with a changing environment [228,320]. However, prolonged and/or repetitive stress could lead to adaptative responses that may contribute to the development of a variety of endocrine, immune and psychiatric diseases [71]. The pathogenesis of these stress-related disorders has been associated to a sustained and excessive secretion of stress hormones by the adrenal gland. In fact, chronic stress is often linked to adrenal gland enlargement and to enhanced hormone release, namely catecholamines, synthesized by chromaffin cells of the adrenal medulla [144,228,266,317,327,383,418]. Moreover, the increased catecholamine release, which occurs in response to repeated exposure to stressors, is associated, in many different animal models of stress, with elevated mRNA, protein and/or enzymatic activity of catecholamine synthesizing enzymes and other important players for maintaining the correct functioning of catecholaminergic system [79,228,320-322,369,420].

Recently, it was demonstrated that neural crest derived chromaffin progenitor cells, with proliferative potential, persist in adult adrenal medulla in an undifferentiated state [74,111,113]. These chromaffin progenitor cells were already isolated from bovine adrenals as free floating colonies, named chromospheres [74]. *In vitro,* these cells have a higher expression of different neural crest/sympathoadrenal markers as compared to mature chromaffin cells and the ability to differentiate into mature chromaffin cells expressing the catecholamine biosynthetic enzymes, tyrosine hydroxylase (TH) and phenylethanolamine-N-methyltransferase (PNMT) [73,74,398]. However, the physiological function of chromaffin progenitor cells and their putative role in adrenal medulla adaptation to stress, namely the development of adrenal hyperplasia and the increase of hormone synthesis capacity by adrenal medulla, remains to be elucidated. Therefore,

the aim of this work was to study the role of chromaffin progenitor cells on adrenal medulla response/adaptation to stress, using a mouse model of unpredictable chronic stress (UCS).

4.3 Material and methods

4.3.1 Animals

Male C57/BL6 mice (Charles River, Barcelona) with 9 weeks old were individually housed under a 12h light/dark cycle in a humidity/temperature controlled room, with *ad libitum* access to a standard chow diet and water, except when food and water deprivation was specified by the stress protocol. Animals were allowed 5 days to acclimate to the surroundings before beginning any experimentation. All experimental procedures were performed in accordance with the European Union Directive 86/609/EEC for the care and use of laboratory animals. Moreover, all the people working with animals have received appropriate education (FELASA course) as required by the Portuguese authorities. In addition, animals are housed in our licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006). The present study and the animal experimentation described were included in a project approved and financed by the Portuguese Science Foundation. Center for Neuroscience and Cell Biology (CNC) animal experimentation board also approved the utilization of animals for this project (reference PTDC/SAU-FCF/108110/2008).

4.3.2 Unpredictable chronic stress protocol

The UCS protocol was performed as previously described [414] with some modifications. For 21 days the mice were exposed to the following stressors: two periods of damp bedding (24h), two periods of paired housing (1h), three periods of restraint stress (2h, 3h and 4h), two periods of cold bath (15ºC, 20 min), two periods of inescapable shock (0.7mA for 3s, five times with one minute interval between shock), two periods of exposure to the shock apparatus without shock (1h) after the inescapable shock, two periods of inversion of the light/dark cycle, two periods of cage tilt (24h), two periods of food and water deprivation (24h) and two exposures to an empty water bottle (1h) after water food and water deprivation. This paradigm was designed to maximize unpredictability and therefore each stressor was randomly applied to the experimental group once a day at different times. The control mice were, in turn, daily handled in a gentle and consistent way to minimize stress induced by manipulation. Animals were sacrificed for tissue collection 24h after the last stressor.

4.3.3 Estimation of adrenal gland volume

The volume of the adrenal gland and its subregions (cortex and medulla) was estimated using the Cavalieri method [287]. For that purpose, adrenal glands were collected, fixed with 4% paraformaldehyde and embedded in optimal cutting temperature (OCT) medium before being frozen at -80ºC. Adrenal gland sections of 15μm thickness were cut using a cryostat (Leica, Germany) and collected in super frost plus slides (Thermo scientific, USA). For analysis with the Cavalieri estimator probe of the Stereo Investigator Software, the sections chosen were sampled in order to obtain 10-15 sections covering the adrenal medulla subregions. Thus, a total of 18-21 sections were used covering the whole adrenal gland. The first section for analysis was selected using the random number generator. The orientation of the grid was also randomly chosen by the software and the size of the grid was selected such that, on average, 200-250 points would be counted per adrenal medulla subregion. Adrenal medulla subregion was identified according to its localization and morphology.

4.3.4 RNA extraction and cDNA synthesis

Mice were sacrificed and adrenal medullae were carefully dissected from adrenal cortex and immediately protected from degradation with RNA later (Qiagen, Germany). Total RNA was extracted from adrenal medullae using the RNeasy Midi Kit (Qiagen, Germany), according to the manufacturer's instructions. Two animals (four medullae) were used *per* individual sample. During RNA extraction, all samples were treated with DNAse (Qiagen, Germany) to eliminate any contamination with genomic DNA. Total amount of RNA was quantified by optical density (OD) measurements using a ND-1000 Nanodrop Spectrophotometer (Thermo Scientific, USA) and RNA purity was evaluated by measuring the ratio of OD at 260 and 280 nm. The cDNA was obtained from the conversion of 0.5μg of total RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, USA), according to the manufacturer's instructions, and stored at -20ºC until use.

4.3.5 Quantitative RT-PCR

Quantitative RT-PCR (RT-qPCR) was performed in the StepOnePlus™ Real-Time PCR thermocycler (Applied biosystems, Life Technologies Corporation, USA) using 96-well microtitre plates and iQ Syber Green Supermix (Bio-Rad, USA). Primers for the target and reference genes were pre-designed or ordered from Qiagen (Table 4.1). A master mix was prepared for each primer set containing the appropriate volume of 2x iQ Syber Green Supermix (Bio-Rad, USA) and 10x QuantiTect Primer or 0,125µM of the designed primers. For each reaction, 8 µl of master mix were added to 2 µl of template cDNA. All reactions were performed in duplicate (two cDNA reactions per RNA sample) at a final volume of 10 µl per well. The reactions were performed according to the manufacturer's recommendations: 95°C for 3 min followed by 45 cycles at 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. The melting curve protocol started immediately after amplification. Additionally, the PCR products were run on a 1% agarose gel to confirm their size. Normfinder analysis was performed to choose the best reference genes for normalization. The reference genes used were β-2-microglobulin and HPRT-1 (Acc.SD= 0.23) for qPCR performed with the samples collected at day 7 and HPRT-1 (SD= 0.15) for the samples collected at day 21. Relative mRNA quantification was performed using the ΔCt method for genes with the same amplification efficiency [245].

4.3.6 Statistical methods

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using Student's unpaired *t* test with two-tailed p value, as indicated in figure legends. A value of $p \leq 0.05$ was considered significant. Prism 5.0 (GraphPad Software) was used for all statistical analysis.

Table 4.1 Primer sequences

Gene	Primer sequence (5'-3')
Sox9	F: AACTTCTGTGGGAGCGACAA R: GAGGAGGGAGGGAAAACAGAG
Nestin	F: GCCACTCCCTTCTCTAGTGC R: CGTCGATTGAGCTCCCACAT
Notch1	F: ACGTAGTCCCACCTGCCTAT R: ACAGGTGCCCTGATTGTAGC
PCNA	F: GCACGTATATGCCGAGACCT R: TTGCCAAGCTCTCCACTTGC
HPRT-1	F: GTTGAAGATATAATTGACACTGG R: CAAGGGCATATCCAACAAC
β-2-microglobulin	F: GGTCGCTTCAGTCGTCAG R: CATACAGGCCGGTCAGTG
Sox10	Ordered from Qiagen (QuantiTect Primer assay cat no. QT01046451)
Mash1	Ordered from Qiagen (QuantiTect Primer assay cat no. QT01760696)
Phox2b	Ordered from Qiagen (QuantiTect Primer assay cat no. QT00131747)

4.4 Results

4.4.1 Effect of UCS on adrenal gland size

At the end of the UCS protocol (day 21), adrenal gland size was determined by the measurement of weight and volume (Fig. 4.1A-B). The stressed mice had an adrenal gland weight significantly higher than the control mice (control mice: $weight_{adrenal\ gland}$=2.17±0.06mg; UCS mice: $weight_{adrenal\ gland}$=3.15±0.21mg; Fig. 4.1A). In part, the weight increase of the adrenal gland induced by UCS was due to an increase in adrenal medulla weight (control mice: $weight_{adrenal\ medulla}$=0.86±0.11mg; UCS mice: $weight_{adrenal\ medulla}$=1.18±0.10mg; Fig. 4.1A).

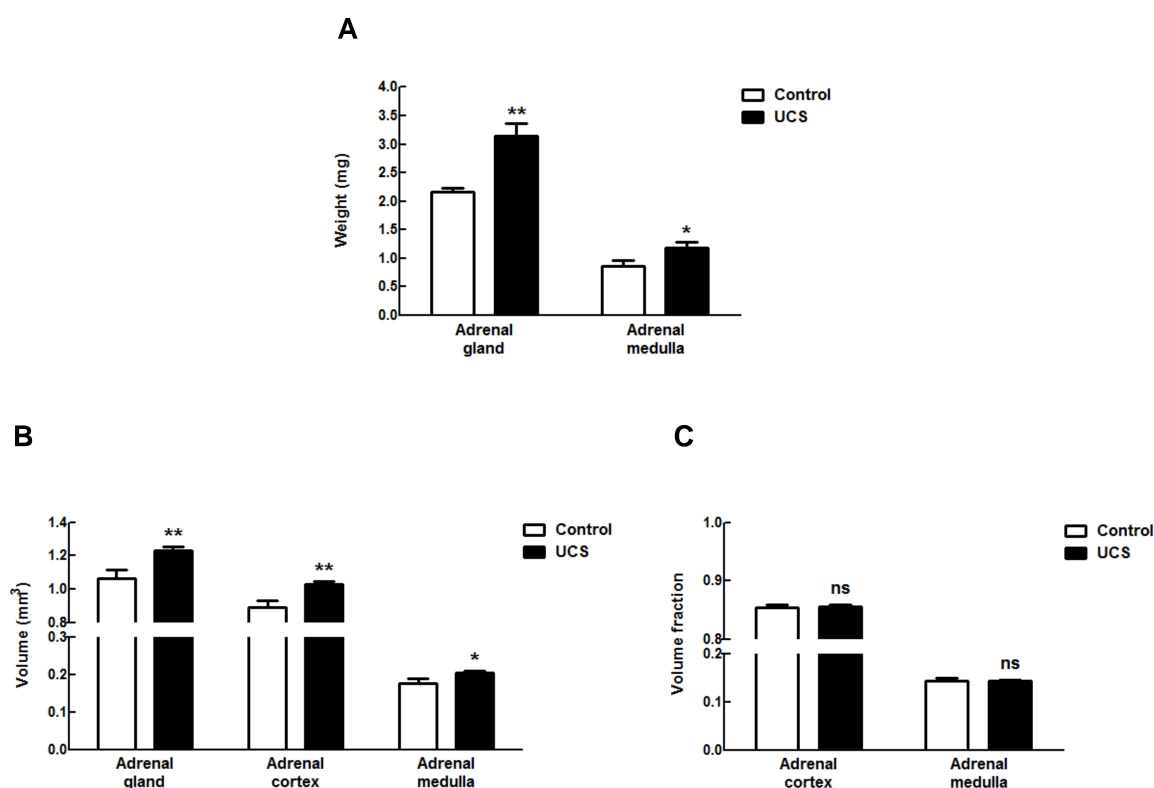

Figure 4.1 Effect of UCS on adrenal gland size.
Mice were submitted to 21 days of UCS. ***A)*** Adrenal gland and adrenal medulla weight was measured. Results are presented as mean±SEM. n=5-6. Student's *t* test. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ compared to control. ***B)*** The volume of adrenal gland, adrenal cortex and adrenal medulla was determined by the Cavalieri method. Results are presented as mean±SEM. n=4-6. Student's *t* test. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ compared to control. ***C)*** The volume fraction corresponding to adrenal cortex and adrenal medulla was determined. Results presented as mean±SEM. n=4-6. Student' *t* test. ns=not statistically significant.

In agreement with the weight increase, the volume of adrenal glands from the UCS group was higher than the volume of adrenals from the control group, as a consequence of an increase in both adrenal cortex and adrenal medulla volume (control mice: $volume_{adrenal\ gland}$=1.07±0.05mm^3, $volume_{adrenal\ cortex}$=0.89±0.04mm^3, $volume_{adrenal\ medulla}$=0.18±0.01$mm^3$; UCS mice: $volume_{adrenal\ glang}$=1.23±0.02mm^3, $volume_{adrenal\ cortex}$=1.03±0.02mm^3, $volume_{adrenal\ medulla}$=0.20±0.01$mm^3$; Fig. 4.1B).

To determine whether the increase in adrenal cortex and medulla was proportional, the adrenal cortex and medulla fractions were determined. As showed in Fig. 4.1C, no differences were observed in the adrenal cortex and adrenal medulla fractions between control (cortical fraction=0.855±0.005; medullar fraction=0.145±0.005) and UCS mice (cortical fraction=0.856±0.003; medullar fraction=0.144±0.003).

4.4.2 Effect of UCS on adrenal medulla progenitor cells markers

Bovine chromaffin progenitor cells express several progenitor cell markers [74,325]. In the present study, mRNA of chromaffin progenitor cells markers (Sox9, Notch1, Sox10, Nestin, Mash1 and Phox2b) was also detected in mouse adrenal medulla (Fig. 4.2).

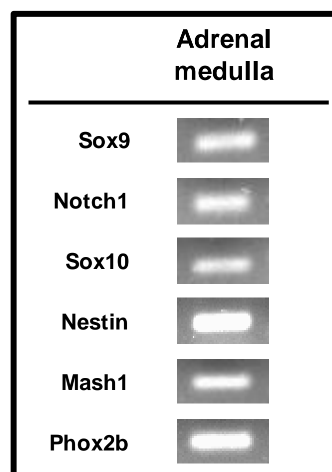

Figure 4.2 Chromaffin progenitor cell markers expression in mouse adrenal medulla.
Total mRNA was extracted from adrenal medulla of 12 weeks old mice. The expression of Sox9, Sox10, Notch1, Nestin, Mash1 and Phox2b was detected by RT-qPCR. PCR products were run on a 1% agarose gel containing ethidium bromide.

The effect of UCS on the expression of these markers was determined by RT-qPCR at two different time points: after 7 days of stressor exposures (day 7) and at the end of the protocol (day 21). As showed in Fig. 4.3A and B, after 7 days of UCS, the adrenal medulla of stressed mice showed a 51% and 39% decrease in Sox 9 and Notch1 mRNA levels, respectively, compared to adrenal medulla of control mice.

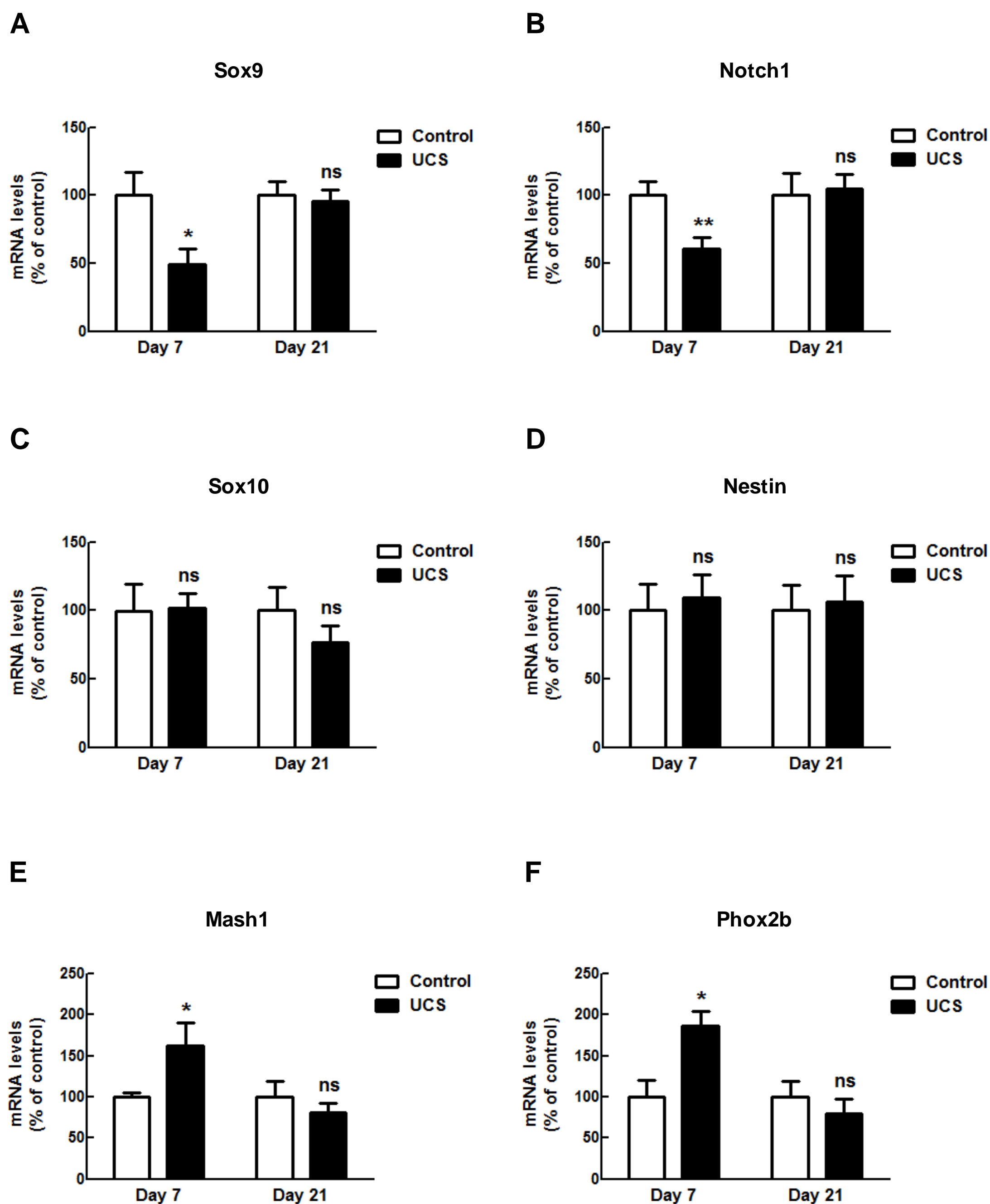

Figure 4.3 Effect of UCS on chromaffin progenitor cell markers.
Mice were submitted to 7 or 21 days of UCS and sacrificed 24hours after the last stressor. Total mRNA was extracted from adrenal medulla and the transcript levels of Sox9 (**A**), Notch1 (**B**), Sox10 (**C**), nestin (**D**), Mash1 (**E**) and Phox2b (**F**) were evaluated by RT-qPCR. Results are expressed as % of control and presented as mean±SEM. n=4-6. Student's *t* test. ns=not statistically significant, $^{*}p\leq0.05$, compared to control.

In opposition, Mash1 and Phox2b mRNA levels were increased by 63% and 86%, respectively, in adrenal medulla of stressed mice after 7 days of UCS (Fig. 3E-F). No significant differences were observed regarding the mRNA levels of Sox10 or nestin (Fig. 3C and D). On the other hand, no significant changes were observed in the expression of progenitor cell markers after 21 days of UCS (Fig. 4.3).

The mRNA levels of proliferating cell nuclear antigen (PCNA), a marker of cell proliferation [356], were also evaluated. As showed in Fig. 4.4, after 7 days there was a 22% decrease in PCNA mRNA levels in adrenal medulla of UCS mice when compared to controls. At day 21, no significant differences between groups were observed in PCNA mRNA levels.

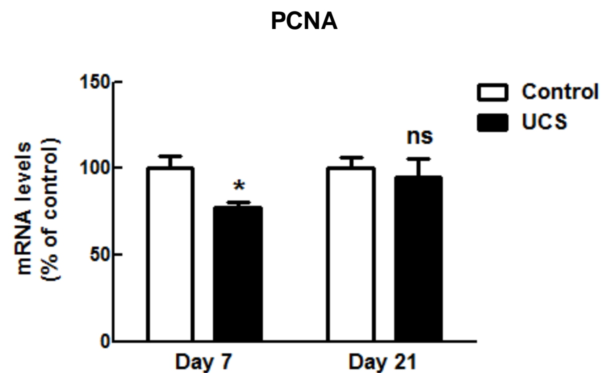

Figure 4.4 Effect of UCS on the cell proliferation marker PCNA.
Mice were submitted to 7 or 21 days of UCS and sacrificed 24hours after the last stressor. Total mRNA was extracted from adrenal medulla and the transcript levels of PCNA, a marker of cell proliferation, were evaluated by RT-qPCR. Results are expressed as % of control and presented as mean±SEM. n=4-6. Student's *t* test. ns=not statistically significant, $^{*}p \leq 0.05$, compared to control.

4.5 Discussion

In the present study, it was showed that UCS induced an enlargement of adrenal gland, as a consequence of proportional increase in both adrenal cortex and adrenal medulla. Others also described an increase of adrenal gland size in different rodent models of chronic stress, including a similar model of UCS [144,317,327,383]. The increase in adrenal medullary function induced by stress has been shown to be a result of increased capacity of catecholamine synthesis, storage and release from adrenal medullary chromaffin cells [228,320,322,369].

Recently, the presence and isolation of neural crest derived progenitor cells in adult bovine adrenal medulla was reported [74,325]. Although not yet isolated, the presence of chromaffin progenitor cells in adult rodent adrenal medulla was suggested by: 1) BrdU incorporation, which indicates proliferation, in mouse and rat adrenal medulla [370-372]; 2) immunoreactivity for nestin, a neural stem cell maker, observed in rat adrenal medulla [31]; 3) electron microscopic observation of undifferentiated cells with primitive appearance in salinomycin-injured rat adrenal medulla [31,64,65]. In the present study, the detection of mRNA for different progenitor cells makers (Sox9, Sox10, Notch1, Nestin, Mash1 and Phox2b) in total mRNA extracts from adrenal medullary tissue also suggests the presence of chromaffin progenitor cells in mouse adrenal medulla. Therefore, given the fact that chromaffin progenitor cells are present in adult adrenal medulla, have proliferative potential and are able to differentiate into mature chromaffin cells, it was hypothesized that chromaffin progenitor cells could be related to the increase in adrenal medulla size and/or the enhancement of adrenal medulla function observed during stress. To answer the question, the effects of UCS on mRNA levels of different chromaffin progenitor markers (Sox9, Sox10, Notch1, Nestin, Mash1 and Phox2b) were investigated. After 7 days of UCS, it was observed a significant decrease of Sox9 and Notch1 mRNA levels in adrenal medulla of stressed mice. Sox9 and Notch1 are important to maintain the cells in an undifferentiated state [331,334], and their downregulation induce cell differentiation into different neural crest cell derivatives [68,281,402]. Although the pathways implicated in chromaffin cell differentiation remains poorly understood, some evidence suggests a role of Notch signaling pathway during sympathoadrenal differentiation [385,398]. Preliminary data reported by Unsicker et al (2012) revealed a downregulation of Notch1 during migration or initial colonization of neural crest derived progenitors to adrenal anlage. Moreover, *in vitro* data showed that neuronal differentiation of

bovine chromaffin progenitor cells is accompanied by a downregulation of the Notch signaling pathway [398]. Therefore, the decreased Notch1 mRNA levels observed in adrenal medulla of mice submitted to 7 days of UCS suggests that chromaffin progenitor cells differentiation into mature chromaffin cells might be occurring at that time point. Interestingly, recent studies proposed that Sox9 is as a primary target gene for Notch1 [66,158,263]. Therefore, the simultaneous downregulation of Notch1 and Sox9, observed in the present study, suggests that Nocth1 signaling might regulate chromaffin cell differentiation through the modulation of the transcription factor Sox9.

Moreover, it has also been shown that inhibition of Notch signaling induces the expression of various basic helix-loop-helix (bHLH) transcription factors, such Mash1 and Phox2b, which have relevant roles in neuronal determination and differentiation [279,335,426]. Mash1 and Phox2b are also crucial for the induction of catecholamine biosynthetic enzymes during chromaffin cell. These transcription factors interact with other important transcription factors, such as Phox2a, Hand2 and Gata2/3, leading to the induction of the expression of TH and DβH, the first catecholamine biosynthetic enzymes being expressed during chromaffin cell development. [179-181,385]. In this study, after 7 days of UCS, it was observed an increase in the expression of both Mash1 and Phox2b. Moreover, adrenal medulla of mice submitted to UCS also had higher mRNA levels of TH, DβH and PNMT (see chapter 3), the enzymes required for catecholamine synthesis and present in mature chromaffin cells. Thus, downregulation of Sox9 and Notch1 with concomitantly upregulation of Mash1, Phox2b, TH, DBH and PNMT, observed after 7 days of stressor exposures, suggest an induction of chromaffin cell differentiation. The lower PCNA mRNA levels present in adrenal medullae of mice after 7 days of UCS indicates a decrease in cell mitotic activity, which is in accordance with the arrest of progenitor cell proliferation associated with the induction of cell differentiation [119,324,333]

It is important to emphasize that an increased expression of catecholamine biosynthetic enzymes might also be a consequence of transcriptional regulation in adult chromaffin cells. Therefore, induction of chromaffin progenitor cell differentiation, resulting in increased chromaffin cell number, and transcriptional regulation of catecholamine biosynthesis in mature chromaffin cells might be complementary mechanisms to enhance adrenal medulla function during stress.

After 21 days of UCS, the expression levels of Sox9, Notch1, Mash1, Phox2b and PCNA in adrenal medulla returned to levels comparable to controls, suggesting that chromaffin cell differentiation induced by unpredictable stressors was no longer occurring. In conclusion, this study shows that chromaffin progenitor cell differentiation might contribute to the enhancement of adrenal medulla function during stress, highlighing a putative role of chromaffin progenitor cells in adrenal medulla function. Moreover, this work also suggests that a more complex regulation of adrenal medulla catecholaminergic system occurs during stress.

CHAPTER 5

Chromaffin progenitor cells from human adult adrenal medulla

Chapter adapted from the publication:
Santana MM, Chung KF, Vukicevic V, Rosmaninho-Salgado J, Kanczkowski W, Cortez V, Hackmann K, Bastos CA, Mota A, Schrock E, Bornstein SR, Cavadas C, Ehrhart-Bornstein M, *Isolation, characterization, and differentiation of progenitor cells from human adult adrenal medulla*. Stem Cells Transl Med, 2012 Nov. 1(11):783-91.

5.1 Abstract

Chromaffin cells, sympathetic neurons of the dorsal ganglia, and the intermediate small intensely fluorescent cells derive from a common neural crest progenitor cell. Contrary to the closely related sympathetic nervous system, within the adult adrenal medulla a subpopulation of undifferentiated progenitor cells persists, and recently, a method to isolate and differentiate these progenitor cells from adult bovine adrenals was established. However, no studies have elucidated the existence of adrenal progenitor cells within the human adrenal medulla. In this study, the isolation, characterization and differentiation of chromaffin progenitor cells obtained from adult human adrenals is described. Human chromaffin progenitor cells were cultured in low-attachment conditions for 10–12 days as free-floating spheres in the presence of fibroblast growth factor-2 (FGF-2) and epidermal growth factor. These primary human chromosphere cultures were characterized by the expression of several progenitor markers, including nestin, CD133, Notch1, nerve growth factor receptor, Snai2, Sox9, Sox10, Phox2b, and Ascl1 on the molecular level and of Sox9 on the immunohistochemical level. In opposition, phenylethanolamine*N*-methyltransferase (PNMT), a marker for differentiated chromaffin cells, significantly decreased after 12 days in culture. Moreover, when plated on poly-L-lysine/laminin-coated slides in the presence of FGF-2, human chromaffin progenitor cells were able to differentiate into two distinct neuron-like cell types, tyrosine hydroxylase $(TH)^+$/β-3-tubulin$^+$ cells and TH^-/β-3-tubulin$^+$ cells, and into chromaffin cells (TH^+/$PNMT^+$). This study demonstrates that chromaffin progenitor cells are present in the human adrenal medulla and can be isolated and differentiated *in vitro*. Chromospheres cultures might therefore be a used as an *in vitro* model for the study of adrenal medulla development and adaptative responses to stress and, moreover, might be a potential new cell source for cell transplantation and regenerative medicine, especially in the treatment of neuroendocrine and neurodegenerative diseases.

5.2 Introduction

Chromaffin cells, sympathetic neurons of the dorsal ganglia, and the intermediate small intensely fluorescent cells develop from neural crest-derived sympathoadrenal (SA) progenitor cell that aggregate at the dorsal aorta. SA progenitors then migrate to the secondary sympathetic ganglia and the adrenal medulla, where they differentiate into mature sympathetic neurons and chromaffin cells, respectively [180]. Accumulated evidence suggests the persistence of a subpopulation of these neural crest-derived progenitor cells in adult adrenal medulla [113]. In fact, chromaffin progenitor cells were isolated from bovine adult adrenal medulla as free floating spherical colonies, named "chromospheres" [74]. Bovine chromospheres expressed different neural and neural crest stem cell markers and were able to differentiate, *in vitro,* into both neuronal and neuroendocrine phenotypes, being suggested as a potential new cell source for cell transplantation in the treatment of neurodegenerative and neuroendocrine diseases [73,74,398]. Physiologically, chromaffin progenitor cells seem to be implicated in the enhancement of adrenal medulla function during stress (Santana et al., unpublished data; see chapter 4) and on the ability of adrenal medulla regeneration after injury [64,65]. Nevertheless, the existence of chromaffin progenitor cells in adult human adrenal medulla was not yet investigated. The isolation of chromaffin progenitor cells from human adult adrenal medulla will allow the establishment of an interesting *in vitro* model to study the mechanisms behind adrenal medulla regeneration and adaptation to stress, contributing to a better understanding of adrenal medulla dysfunctions in stress related-disorders. In addition, the characterization of chromaffin progenitor cells from adult human adrenal glands would be a prerequisite for its future use in regenerative medicine. In this context, the aim of this study was the isolation, characterization and differentiation of chromaffin progenitor cells from adult human adrenal glands.

5.3 Material and methods

5.3.1 Cell culture of human adrenal medulla progenitor cells

The ethical committees of the University of Dresden and the University of Coimbra approved this study. Human adrenal glands were obtained from kidney transplant donors (Coimbra) and from patients undergoing nephrectomy where the ipsilateral adrenal was removed together with the kidney (Dresden). A cell digest from the medullary tissue was obtained as previously described [316]. Briefly, the glands were cleaned of fat tissue and opened, and the medullae were separated from the majority of the cortex by scraping off the brown interdigited islets of chromaffin cells with a scalpel. Medullary tissue was collected in 0.2% collagenase Type H solution (Sigma-Aldrich, USA) and incubated for 45 minutes at 37°C. The digested tissue was washed twice with culture medium and filtered through a 100µm strainer. For chromosphere culture, the cell digest was resuspended in Dulbecco's modified Eagle's medium/F-12 (DMEM-F12) with GlutaMAX, 100 U/ml penicillin, and 100 µg/ml streptomycin, and supplemented with 1% B27, 10ng/ml fibroblast growth factor-2 (FGF-2), 10 ng/ml endothelial growth factor (EGF; all from Invitrogen, USA), and 5 µg/ml heparin (Sigma-Aldrich, USA). Chromospheres were allowed to grow for 10–12 days in low attachment flasks at 37°C in a 5% CO_2 atmosphere. The medium was changed every 4th day.

5.3.2 RNA extraction and RT-PCR

Cells were collected and disrupted in RNeasy lysis buffer with 1% β-mercaptoethanol and total RNA was extracted using the RNeasy Plus Mini kit (Qiagen, Germany) according to the manufacturer's instructions. MMLV reverse transcriptase, 5x reverse transcriptase buffer, oligo(dT) 15 primer, and RNase inhibitor (Promega, USA) were used for the reverse transcription (RT) of 1 µg of total RNA. cDNA amplifications were done using specific primer pairs (Table 5.1) in NH_4 reaction buffer with 1.5 mM MgCl2, 250 µM dNTPs, 0.5 µM of each primer, and 2.5U/20µl Taq DNA polymerase (Stratec, Germany).

Table 5.1 Primer sequences

Gene	Primer sequence (5'-3')	Annealing temperature (ºC)
Nestin	F: ACCTCAAGATGTCCCTCAGC R: TCAGGACTGGGAGCAAAGAT	60
CD133	F: TCGACAATGTAACTCAGCGTCTTCC R: GCCACACAGTAAGCCCAGGTAGTAA	60
Notch1	F: CTCACGCTGACGGAGTACAAGTG R: CTTCTGGCCACACTCGTTGACAT	60
NGFR	F: TGCTGCTGTTGCTGCTTCTG R: GTTCCACCTCTTGAAGGCTATG	60
Snai2	F: GCGATGCCCAGTCTAGAAAA R: GCAGTGAGGGCAAGAAAAAG	58
Sox9	F: AGTACCCGCACTTGCACAAC R: CGTTCTTCACCGACTTCCTC	60
Sox10	F: GCAAGGCAGACCCGAAGC R: GTCCAACTCAGCCACATCAAAG	60
Phox2b	F: AACCAGAATAACGACCGCGA R: AAAAAGGTGAACCGACTCGG	60
Ascl1	F: CTCGACTTCACCAACTGGTTCTGAG R: GGGTTGGTTGACTGTTTTCGTTTTT	58
TH	F: AACCAGAATAACGACCGCGA R: AAAAAGGTGAACCGACTCGG	60
PNMT	F: GCCTACCTCCGCAACAACTA R: TGGTGATGTCCTCAAAGTGG	60
MC2R	F: CCCAGAAAGTTCCTGCTTCA R: TGCTGTGTTGTTGATGTTTTCA	60
GAPDH	F: GAAGGTGAAGGTCGGAGTCA R: GACAAGCTTCCCGTTCTCAG	60
β-actin	F: GCCGTCTTCCCCTCCATCGTG R: GGAGCCACACGCAGCTCATTGTAGA	65

Thermal cycler conditions were as follows: denaturation at 94°C for 5 minutes, followed by 40 cycles at 94°C for 15 seconds, optimal annealing temperature for 15 seconds, and 72°C for 15 seconds, with a final extension step at 72°C for 1 minute. The housekeeping gene GAPDH was amplified from each cDNA sample. PCR amplification products were analyzed on 1% agarose gel containing ethidium bromide.

5.3.3 Quantitative RT-PCR

Expression levels of nestin, phenylethanolamine *N*-methyltransferase (PNMT), and melanocortin receptor 2 (MC2R) were measured using SYBR Green master mix (Qiagen, Germany) in a light cycler (Light Cycler 1.5; Roche Applied Science, Switzerland), as previously described [73,74]. To generate standard curves for quantitative RT-PCR (RT-qPCR), DNA fragments were amplified, cloned into plasmid vectors (pCRII-TOPO), and transformed into competent *Escherichia coli* using the TOPO TA Cloning kit (Invitrogen, USA). Plasmids were purified (Maxi Plasmid kit; Qiagen) and their concentration was measured by spectroscopy. A serial dilution of plasmid was used to generate a linear regression standard curve. PCRs were performed in capillaries with initial denaturation at 94°C for 4 minutes; 45–50 cycles of amplification at 94°C for 15 seconds, annealing for 20 seconds, and elongation at 72°C for 15 seconds; and a final extension at 72°C for 4 minutes. The expression level of each gene was calculated by relative quantification to the expression of the reference gene β-actin.

5.3.4 Cell differentiation

Chromospheres were mechanically dissociated, as described previously for neurosphere cultures [376], plated on slides coated with 50µg/ml poly-L-lysine (PLL) and 5µg/ml laminin (both from Sigma-Aldrich, USA), and cultured for 1 additional day to allow cell attachment and expansion. Removing EGF from the medium and increasing the FGF-2 concentration to 20 ng/ml induced cell differentiation. Cells were allowed to differentiate for 6 days at 37°C in 5% CO2 atmosphere. Half of the medium was replaced by fresh medium every 2nd day.

5.3.5 Immunofluorescence staining

Chromospheres embedded in optimal cutting temperature (OCT) medium (Tissue-Tek, USA) were cut into 7µm cryosections using a cryostat (Leica, Germany) and collected to Super Frost Plus glass slides (R. Langenbrinck, Germany). For immunostaining, cryosections were fixed in 4% paraformaldehyde for 20 minutes at room temperature and incubated in 0.1 M glycine solution for 30 minutes at room temperature. Cells were permeabilized with 1% Triton X-100 for 5 minutes,

and nonspecific binding was prevented by incubating cells with blocking solution [3% (wt/vol) fatty acid-free bovine serum albumin (BSA) supplemented with 0.2% Tween 20] for 1 hour at room temperature. Cryosections were then incubated with the primary antibody rabbit anti-SOX9 (1:200; Abcam, UK) overnight at 4°C. Cells were washed three times with phosphate-buffered saline (PBS) and incubated for 1 hour at room temperature with the secondary antibody donkey anti-rabbit Cy5 (1:200; Jackson Immunoresearch Laboratories, USA). Nuclei were stained with Hoechst (1 µg/ml; Sigma-Aldrich) for 5 minutes. All antibody solutions were prepared in blocking solution. A negative control, without primary antibody, was performed to check for nonspecific binding and identify background due to cell autofluorescence. Cells were visualized with a fluorescent microscope (PALM Laser microdissection; Carl Zeiss, Germany), coupled to an AxioCam HRc camera (Carl Zeiss, Germany). Differentiated cells were stained using a similar protocol. The primary antibodies and secondary antibodies used were the following: mouse anti-β-3-tubulin (1:500; Covance, USA), rabbit anti-tyrosine hydroxylase (TH) (1:1,000; Chemicon, USA) and rabbit anti-PNMT (1:250; Enzo Life Sciences Inc., USA), anti-mouse Alexa Fluor 594 (1:200; Invitrogen, USA), goat anti-rabbit Alexa Fluor 488 (1:200; Invitrogen), and donkey anti-rabbit Cy5 (1:200; Jackson Immunoresearch Laboratories, USA). Differentiated cells were visualized using a laser scanning confocal microscope, LSM 510 META (Carl Zeiss, Germany).

5.4 Results

5.4.1 Free-floating chromospheres obtained from human adult adrenal medulla

Recently, chromaffin progenitor cells from bovine adrenal medulla were isolated and their culture as free-floating spherical colonies, named chromospheres, was established [74]. To investigate whether chromaffin progenitor cells could also be isolated from human adrenal medulla we prepared a cell digest from medullary tissue of human adrenal glands. The digested cells were further cultured in low-attachment conditions, to avoid adherence of the cells to the surface, and in the presence of growth factors (EGF and FGF-2, 10 ng/ml). Under these conditions some of the cells were maintained in suspension, and after 10–12 days free floating spheres, morphologically similar to bovine chromospheres, could be observed with an average diameter of 42.2±1.1 µm (minimum = 15.8µm and maximum = 86.9µm; mean±SD of 130 spheres from three independent cell culture) (Fig. 5.1).

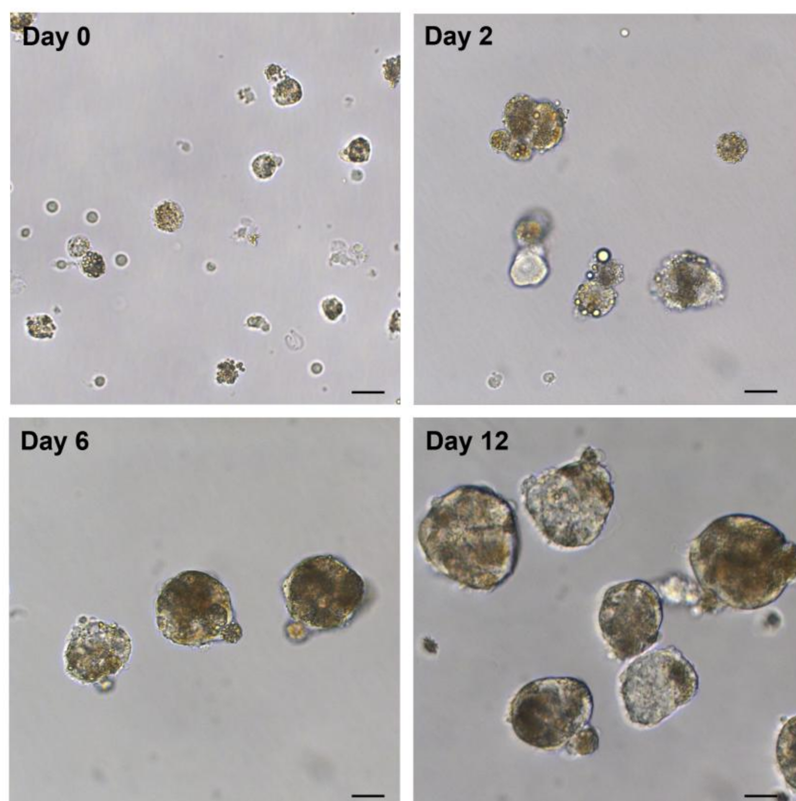

Figure 5.1 Morphology of human chromospheres.
Cells from human adrenal medulla were isolated and cultured in low-attachment conditions in the presence of growth factors (epidermal growth factor and fibroblast growth factor-2; 10 ng/ml). Free-floating spheres were observed after 12 days in culture. Phase-contrast images. Scale bars = 20 µm.

The number of isolated cells differed among the three glands investigated. At day 12, the total number of spheres obtained was 26,577 per 1,912,000 total isolated chromaffin cells from gland 1; 284,850 per 26,375,000 total isolated chromaffin cells from gland 2; and 88,281 per 7,062,500 total chromaffin cells from gland 3. This corresponds to 1.24±0.155 spheres (day 12) per 100 chromaffin cells (day 0) (mean±SD from three independent cell culture).

5.4.2 Human chromospheres express different progenitor cell markers

RT-PCR analysis of mRNA expression revealed the expression of several progenitor markers in human chromospheres after 12 days in culture that were not detectable or were present at low levels in primary chromaffin cells (day 0). These included nestin, CD133 (prominin-1), notch1, nerve growth factor receptor (NGFR), Snai2, Sox9, Sox10, Phox2b, and Ascl1 (Hash1). Primary chromaffin cells expressed the chromaffin cell marker PNMT, the enzyme catalyzing the final step of epinephrine synthesis (Fig. 5.2A). Quantitative real-time PCR revealed a significant effect of sphere formation on PNMT and nestin expression. Expression of PNMT drastically decreased after 12 days in culture; in parallel, nestin expression increased threefold over the time of culture (Fig. 5.2B). Primary cultures of human chromaffin cells also contain adrenocortical cells as revealed by the expression of MC2R, which was significantly reduced during sphere culture for 12 days (Fig. 5.2B). Immunofluorescent staining revealed nuclear staining for Sox9 in chromosphere cells, with 46±7% Sox9-positive cells (mean±SD of 50 spheres from two independent cell cultures); the negative control, where the first antibody was omitted, showed no unspecific staining (Fig. 5.2C).

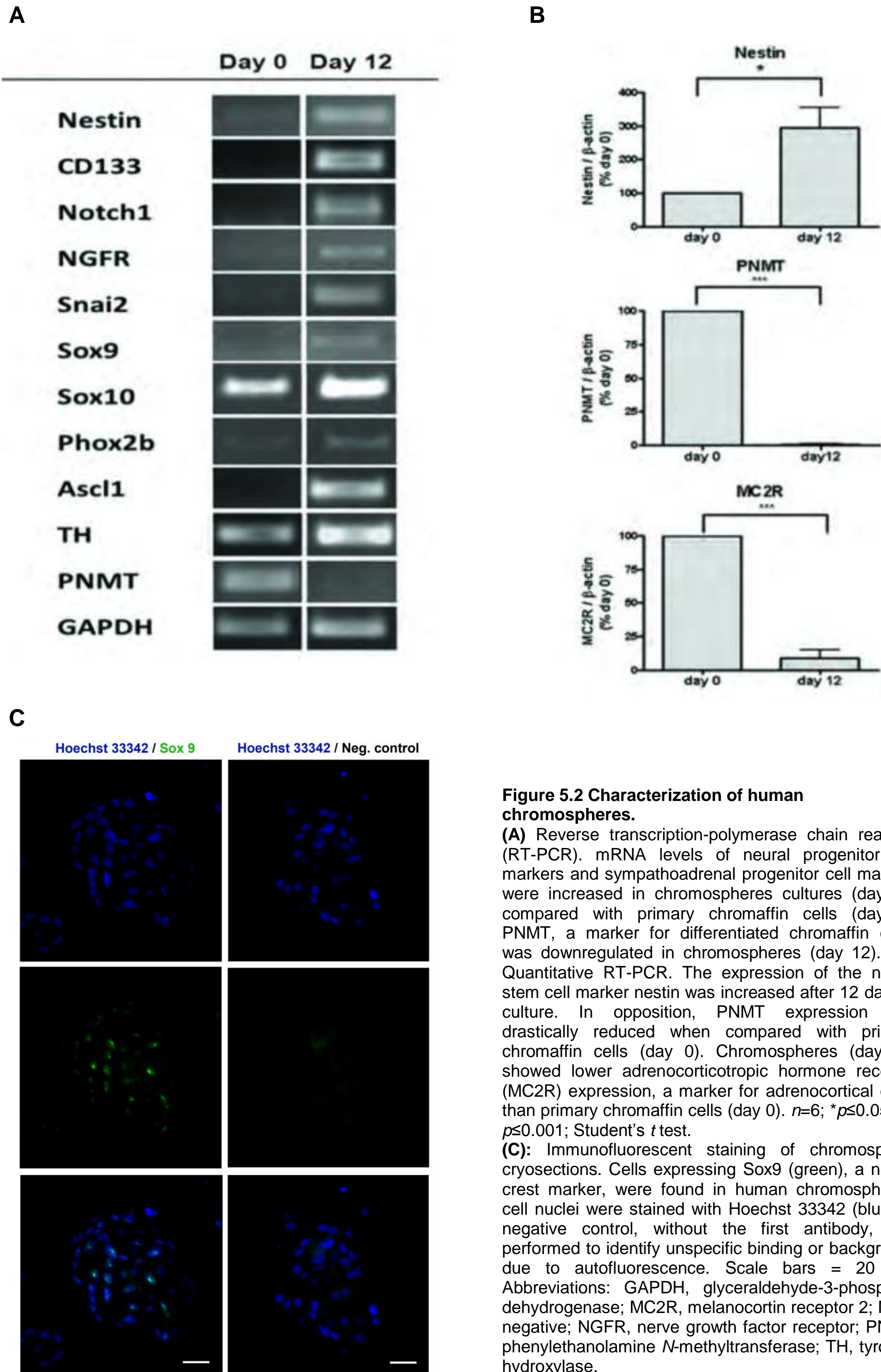

Figure 5.2 Characterization of human chromospheres.
(A) Reverse transcription-polymerase chain reaction (RT-PCR). mRNA levels of neural progenitor cell markers and sympathoadrenal progenitor cell markers were increased in chromospheres cultures (day 12) compared with primary chromaffin cells (day 0). PNMT, a marker for differentiated chromaffin cells, was downregulated in chromospheres (day 12). **(B):** Quantitative RT-PCR. The expression of the neural stem cell marker nestin was increased after 12 days in culture. In opposition, PNMT expression was drastically reduced when compared with primary chromaffin cells (day 0). Chromospheres (day 12) showed lower adrenocorticotropic hormone receptor (MC2R) expression, a marker for adrenocortical cells, than primary chromaffin cells (day 0). n=6; *p≤0.05; *** p≤0.001; Student's t test.
(C): Immunofluorescent staining of chromosphere cryosections. Cells expressing Sox9 (green), a neural crest marker, were found in human chromospheres; cell nuclei were stained with Hoechst 33342 (blue). A negative control, without the first antibody, was performed to identify unspecific binding or background due to autofluorescence. Scale bars = 20 µm. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MC2R, melanocortin receptor 2; Neg., negative; NGFR, nerve growth factor receptor; PNMT, phenylethanolamine N-methyltransferase; TH, tyrosine hydroxylase.

5.4.3 Human chromaffin progenitor cells differentiate into neuron-like cells

To characterize the differentiation potential of isolated human chromaffin progenitor cells, chromospheres were dissociated and cells were plated onto PLL/laminin-coated slides. Over the following 24 hours the cells adhered to the surface; then EGF was removed from the medium and the FGF-2 concentration was increased to 20 ng/ml, inducing cell differentiation. After 2 days, the development of neurites was observed; 6 days after plating some cells revealed long neurites (Fig. 5.3C). The differentiating cells were characterized by immunofluorescent staining for the early neuronal marker β-3-tubulin and the catecholaminergic marker TH. Double staining revealed the presence of neuron-like cells with a catecholaminergic phenotype, β-3-tubulin^{+}/TH^{+} (Fig. 5.3B–4D). Noncatecholaminergic neuronal cells, β-3-tubulin^{+}/TH^{-}, could also be observed (Fig. 5.3A). As expected, differentiated chromaffin cells were also obtained in culture, as shown by the immunoreactivity to PNMT and TH (Fig. 5.3E).

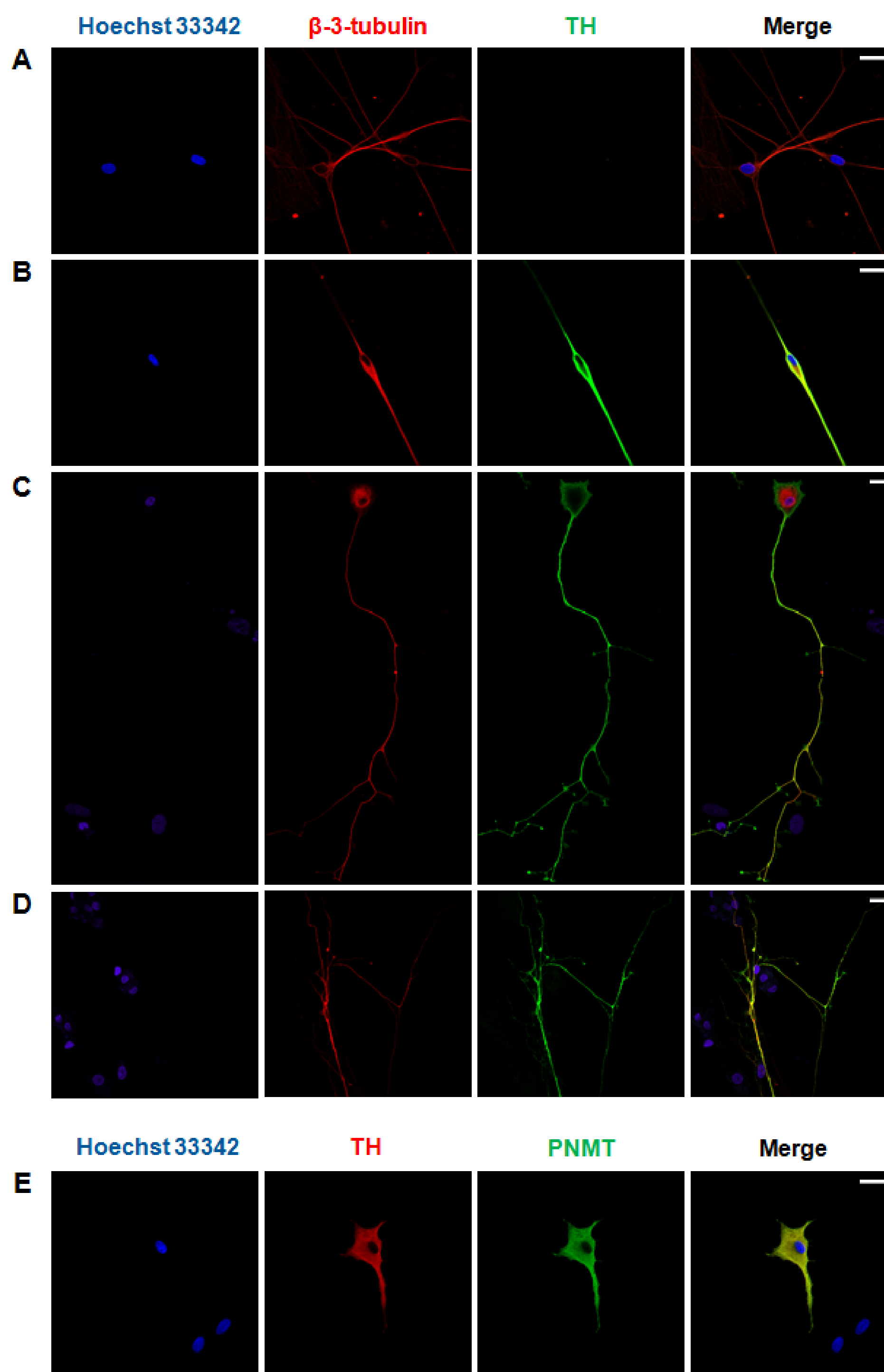

Figure 5.3 Immunofluorescent staining of differentiated chromosphere cells.
Different types of neuron-like cells were obtained after plating the dissociated chromosphere cells on poly-L-lysine/laminin-coated slides in a medium supplemented with fibroblast growth factor-2 (20 ng/ml): TH^-/β-3-$tubulin^+$ cells **(A)** and TH^+/β-3-$tubulin^+$ cells **(B–D)**. Differentiated chromaffin cells (TH^+/$PNMT^+$) were also present in culture **(E)**. Nuclei: Hoechst 33342 (blue). Scale bars=20μm. Abbreviations: PNMT, phenylethanolamine *N*-methyltransferase; TH, tyrosine hydroxylase.

5.5 Discussion

In the present study, human chromaffin progenitor cells were isolated from adult human adrenals by adapting a method previously established for bovine adrenals [74]. Similar to chromaffin progenitor cells from bovine [73,74,398], free-floating spherical colonies (chromospheres) were enriched in human chromaffin progenitor cells when cultured in low-attachment conditions and in the presence of EGF and FGF-2. During chromosphere formation, levels of nestin mRNA, a marker for neural progenitor cells, were significantly increased. In contrast, the selective culture conditions led to a dramatic reduction of the expression of PNMT, a marker of mature chromaffin cells. This drastic decrease of PNMT expression after 12 days suggests a selective enrichment of the progenitor cell population existing within human adrenals. In fact, the majority of cells obtained from primary cultures of adrenal medullae are differentiated chromaffin cells, which express PNMT and require adherent conditions to survive. Chromospheres from adult human adrenals show similarities to PNMT-negative progenitor cell-enriched spheres previously observed in primary cultures of human fetal chromaffin cells [428]. The "stemness" of human chromosphere cells is indicated by the expression of several progenitor markers. In contrast to primary chromaffin cells, chromosphere cells expressed genetic markers for neural stem cells (nestin, CD133, NGFR, and Snai2). These cells also expressed Sox9, a member of the SoxE subgroup with an important role in embryonic migration and differentiation of neural crest derivatives [67,176]. In addition, Sox9 has been identified as a common marker for multiple tissue-specific progenitors [5] and neural stem cells in the brain [346]. Sox9 also induces the expression of Sox10 in neural crest cells, which in turn is required for the specifications and survival of chromaffin precursors [195,312]. Sox10 expression is downregulated in adult adrenal medulla, and its importance is restricted to the early stages of adrenal medulla development [96,201,207,221]. The increase in Sox10 expression in human chromospheres is therefore in agreement with an enrichment in chromaffin progenitor cells. The sympathoadrenal development is regulated by a complex network of interacting transcription factors that are activated to specify both neural and chromaffin cell lineages. The pro-neural gene *Mash1*, or *Hash1* in humans, is the mammalian homolog of the *Drosophila* achaete-scute complex encoding the helix-loop-helix-type transcription factor Acsl1, which is expressed in the majority of sympathoadrenal progenitors during embryogenesis and is a key factor in the development of chromaffin cells [152,179]. In

rodents, Mash1 expression depends on the expression of Phox2b, another transcription factor expressed in all central and peripheral noradrenergic neurons, which is essential for SA development and for the very early steps of chromaffin cell and sympathetic neurons differentiation [178,179,181,272]. In accordance, both Acsl1 and Phox2b were upregulated in human chromospheres, highlighting the SA progenitor features of this culture.

Thereafter, with the aim to evaluate the potentiality of human chromaffin progenitor cells to differentiate *in vitro* we induced the differentiation of chromaffin progenitor cells by promoting cell adherence in the presence of FGF-2. In these conditions, chromaffin progenitor cells developed long neurites and differentiated into two different neuronal phenotypes: catecholaminergic neuron-like cells, characterized by the presence of the early neuronal marker β-3-tubulin and the catecholaminergic marker TH, and noncatecholaminergic neuron-like cells, which were immunoreactive for β-3-tubulin but not for TH. Moreover, and as expected, these progenitor cells also differentiated into chromaffin cells, which were characterized by the presence of the enzymes TH and PNMT. Thus, in the present study it was showed that chromaffin progenitor cells can be successfully isolated from human adrenals and, like bovine chromaffin progenitors, are able to differentiate into both neuron-like and chromaffin cells. Chromospheres suspensions and their further differentiation in monolayer cultures might represent a new *in vitro* developmental model and excellent tool to identify the mechanisms and signal transduction pathways involved in chromaffin cell differentiation. To study at the cellular level the effects of stress hormones on chromaffin progenitor cells will also contribute to a better understanding of adrenal medulla adaptative responses to stress and find putative strategies for the treatment of stress-related disorders and adrenal medulla dysfunctions. Furthermore, modifications of this differentiation protocol by change or add other factors, such as NGF, bone morphogenic proteins, leukemia inhibitory factor or retinoic acid, could lead to increased yield and survival of neuron-like progenitor cells [69,73,328,398]. The establishment of appropriate *in vitro* culture conditions for human chromaffin progenitor cell neuronal differentiation will be an important advance for its further use in cell transplantation, namely in the treatment of neurodegenerative diseases. Unfortunately, the human adrenal gland is characterized by intense intermingling of medullary and cortical cells [112], making the separation of the two tissues a difficult task. Although adrenocortical cells were clearly reduced during sphere culture, as shown by the decrease in the

expression of the adrenocortical cell marker MC2R, protocols also need to be established to entirely remove these cells from progenitor cultures for their therapeutic use in the future. Despite this, chromaffin progenitor cells seem to be a promising cell source due to the potential use in autologous transplantations avoiding immune rejection and the ethical and political controversies related to the use of other cell sources, such as ESCs or NSCs [90,111,113,169].

In conclusion, this study proves the existence of chromaffin progenitor cells in the human adrenal medulla and demonstrates that they can be isolated from adult adrenal glands. These cells open new perspectives and challenges in the field of regenerative medicine, especially regarding their potential use in the treatment of neurodegenerative and neuroendocrine diseases. Furthermore, chromospheres cultures can be used as an *in vitro* model for the study of adrenal medulla development and adaptative responses to stress.

CHAPTER 6

Concluding remarks

6. Concluding remarks

Stress has been proposed as an underlying trigger that causes both depression and cardiovascular disease contributing for the co-morbidity of these two pathologies [193]. Therefore, to understand the role of sympathoadrenal system in the link between depression and cardiovascular disease, in the present work the adrenal medullary catecholaminergic system was studied in an animal model of depression. The results obtained strongly suggest that in depression induced by chronic stress there is an impairment adrenal medulla function. This evidence argues against the most common theories that implicate the development of cardiovascular diseases in depressed patients to a hyperactivity of sympathoadrenal system [193,278,350]. In fact, and according to other stress models, it was observed that the initial response of adrenal medulla to stressors goes towards the improvement of catecholaminergic function by transcriptional changes that promotes a enhancement of catecholamine synthesis, storage and release from chromaffin cells [228]. Additionally, the present work suggests that an increase in the number of mature chromaffin cells, due to chromaffin progenitor cell differentiation, might also be a mechanism to improve adrenal medullary function. Nevertheless, with continued exposures to different unpredictable stressors the adaptive response resulted into diminished adrenal medullary function. Attenuated adrenal medullary response to stressors in depressed patients might lead to the development of cardiovascular disease, perhaps due to deregulated compensatory mechanisms in other stress systems. Further studies will be required to unravel the complexity of relationships that occurs between adrenal medulla and other stress systems in depression. In this context, the use of isolated chromaffin progenitor cells, particularly from human adrenals, and its further differentiation *in vitro,* might represent a new excellent tool to study the effects of many different stress hormones on chromaffin progenitor cells, contributing to a better understanding of the cellular pathways involved adrenal medulla adaptive responses to stress and its interactions with other stress systems. The knowledge of the specific mechanism behind the pathophysiology of stress-related disorders, such depression and cardiovascular disease, would be of particular relevance for the management, improvement and development of appropriate therapeutic options. Finally, it is important to highlight that additionally to the contribution in the understanding of adrenal medulla dysfunctions, the human chromaffin progenitor cells might also represent a potential new cell source for cell transplantation in

regenerative medicine, especially in the treatment of neuroendocrine and neurodegenerative diseases

CHAPTER 7

References

7. References

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