

**UNIVERSITÀ
DI PAVIA**

**Dipartimento di Biologia e Biotecnologie
“Lazzaro Spallanzani”**

Laurea Magistralis in Molecular Biology and Genetics

**The GR–RACK1 Axis as a Molecular Link Between Chronic
Mild Stress and Neurodegenerative Susceptibility**

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Academic Year 2025/2026

Abstract (English)

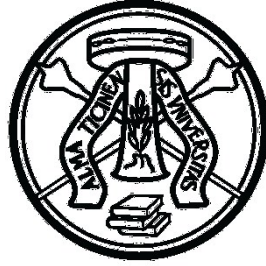
Chronic stress and major depressive disorder are associated with dysregulation of the hypothalamic-pituitary-adrenal axis, prolonged glucocorticoid exposure and impaired neuroplasticity. These alterations have also been implicated in the increased vulnerability to cognitive decline and Alzheimer's disease. Among the molecular mechanisms potentially linking stress-related disorders to neurodegeneration, the glucocorticoid receptor/RACK1/BDNF axis has emerged as a relevant pathway. RACK1 is a multifunctional scaffold and ribosome-associated protein involved in signal transduction, translational regulation and neuroplasticity-related processes. Previous evidence indicates that RACK1 expression is regulated by glucocorticoids through glucocorticoid receptor-dependent mechanisms and that RACK1 may contribute to BDNF promoter regulation.

This thesis investigated whether alterations in glucocorticoid receptor alternative splicing, particularly through the SRSF3/GR α pathway, may contribute to RACK1 dysregulation in stress-related and Alzheimer's disease-associated conditions. To address this aim, molecular alterations were analysed in a chronic mild stress model, in differentiated SH-SY5Y cells exposed to cortisol or amyloid- β peptides, in the cortex of Tg2576 mice, and in human Alzheimer's disease cortical samples and transcriptomic datasets.

In the chronic mild stress model, vulnerable animals showed increased circulating corticosterone levels, increased SRSF3 expression and reduced RACK1 protein levels, whereas resilient animals displayed increased SRSF9 and GR β expression together with preserved or increased RACK1 levels. In differentiated SH-SY5Y cells, cortisol increased SRSF3 expression, favoured GR α -dependent signalling and reduced RACK1 expression at both mRNA and protein levels. Silencing of SRSF3 or GR α , as well as pharmacological inhibition of GR, prevented cortisol-induced RACK1 downregulation, supporting a functional role for the SRSF3/GR α axis in this mechanism. In Alzheimer's disease-related models, amyloid-associated pathology was accompanied by increased SRSF3 and GR α expression and reduced RACK1 protein levels. Importantly, RACK1 protein was also significantly reduced in the cortex of Alzheimer's disease patients.

However, analysis of human cortical transcriptomic datasets did not show a corresponding decrease in RACK1 mRNA levels, indicating that the in vitro model does not fully recapitulate the complexity of human Alzheimer's disease. This discrepancy suggests that RACK1

dysregulation in Alzheimer's disease may involve additional post-transcriptional, translational or protein stability mechanisms. Overall, these findings support the hypothesis that SRSF3 and GR α may act as molecular mediators of an unfavourable cellular environment that ultimately affects RACK1 protein expression. Further studies using more complex Alzheimer's disease models will be required to better dissect this pathway and clarify its relevance in neurodegenerative vulnerability.



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Anno Accademico 2025/2026

Abstract (Italiano)

Lo stress cronico e il disturbo depressivo maggiore sono associati a una disregolazione dell'asse ipotalamo-ipofisi-surrene, a una prolungata esposizione ai glucocorticoidi e a un'alterazione della neuroplasticità. Questi cambiamenti sono stati inoltre implicati in una maggiore vulnerabilità al declino cognitivo e alla malattia di Alzheimer. Tra i meccanismi molecolari potenzialmente coinvolti nel collegamento tra disturbi legati allo stress e neurodegenerazione, l'asse recettore dei glucocorticoidi/RACK1/BDNF è emerso come una via particolarmente rilevante. RACK1 è una proteina multifunzionale con ruolo di scaffold e componente associata al ribosoma, coinvolta nella trasduzione del segnale, nella regolazione traduzionale e in processi legati alla neuroplasticità. Evidenze precedenti indicano che l'espressione di RACK1 è regolata dai glucocorticoidi attraverso meccanismi dipendenti dal recettore dei glucocorticoidi e che RACK1 può contribuire alla regolazione del promotore di BDNF.

Questa tesi ha investigato se alterazioni nello splicing alternativo del recettore dei glucocorticoidi, in particolare attraverso la via SRSF3/GR α , possano contribuire alla disregolazione di RACK1 in condizioni associate allo stress e alla malattia di Alzheimer. A questo scopo, sono state analizzate alterazioni molecolari in un modello di stress cronico lieve, in cellule SH-SY5Y differenziate esposte a cortisolo o peptidi amiloide- β , nella corteccia di topi Tg2576 e in campioni corticali umani di pazienti con malattia di Alzheimer, insieme all'analisi di dataset trascrittomici umani.

Nel modello di stress cronico lieve, gli animali vulnerabili hanno mostrato un aumento dei livelli circolanti di corticosterone, un incremento dell'espressione di SRSF3 e una riduzione dei livelli proteici di RACK1, mentre gli animali resilienti hanno mostrato un aumento di SRSF9 e GR β , insieme a livelli di RACK1 preservati o aumentati. Nelle cellule SH-SY5Y differenziate, il trattamento con cortisolo ha aumentato l'espressione di SRSF3, favorito una segnalazione dipendente da GR α e ridotto l'espressione di RACK1 sia a livello di mRNA sia a livello proteico. Il silenziamento di SRSF3 o GR α , così come l'inibizione farmacologica del recettore dei glucocorticoidi, ha prevenuto la riduzione di RACK1 indotta dal cortisolo, supportando un ruolo funzionale dell'asse SRSF3/GR α in questo meccanismo. Nei modelli associati alla malattia di Alzheimer, la patologia correlata all'amiloide è risultata accompagnata da un aumento di SRSF3 e GR α e da una riduzione dei livelli proteici di RACK1. In modo

rilevante, anche nella corteccia di pazienti con malattia di Alzheimer è stata osservata una significativa riduzione della proteina RACK1.

Tuttavia, l'analisi dei dataset trascrittomici corticali umani non ha mostrato una corrispondente riduzione dei livelli di mRNA di RACK1, indicando che il modello in vitro non ricapitola completamente la complessità della malattia di Alzheimer nell'uomo. Questa discrepanza suggerisce che la disregolazione di RACK1 nella malattia di Alzheimer possa coinvolgere ulteriori meccanismi post-trascrizionali, traduzionali o legati alla stabilità proteica. Nel complesso, questi risultati supportano l'ipotesi che SRSF3 e GR α possano agire come mediatori molecolari di un ambiente cellulare sfavorevole che, in ultima analisi, influenza l'espressione proteica di RACK1. Ulteriori studi, basati su modelli più complessi di malattia di Alzheimer, saranno necessari per caratterizzare meglio questa via e chiarirne la rilevanza nella vulnerabilità neurodegenerativa.

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1. Introduction

1.1 RACK1 structural features and interaction sites

The Receptor for Activated C Kinase 1 (RACK1) is a 36 kDa highly conserved intracellular scaffolding protein encoded by the *RACK1* gene located on chromosome 5q35.3. RACK1 was originally cloned from a chicken liver cDNA library and human B-lymphoblastoid cell line and was isolated from a rat brain cDNA library as a specific partner of the activated protein kinase C beta II (PKC β II) (1, 2). However, RACK1 is also able to interact with other PKC isoforms, shuttling these proteins to their sites of action, facilitating the cross-talk among distinct signalling pathways or recruiting other signalling proteins (3). RACK1 is now recognized as a multifunctional signaling hub for different kinases and membrane receptors as well as involved in a wide range of cellular processes, including signal transduction, cell growth, migration, apoptosis, stress responses, and translational regulation (3). Through its ability to simultaneously interact with multiple protein partners, RACK1 acts as a key mediator of diverse biological events like development, brain activity, immune response and cancer, coordinating different signaling pathways and contributing to the maintenance of cellular homeostasis. Crystallographic analyses identified RACK1 as a member of the tryptophan-aspartate repeat (WD repeat) protein family and shares a high degree of homology with the β subunit of G-proteins. The WD40 repeat (also known as β -transducin repeat) is a short structural motif characterized by the presence of about 40 amino acids with the Glycine-Histidine (G-H) dipeptide at the N-terminus and the Tryptophan-Aspartic acid (W-D) dipeptide at the C-terminus (3) (Figure 1).

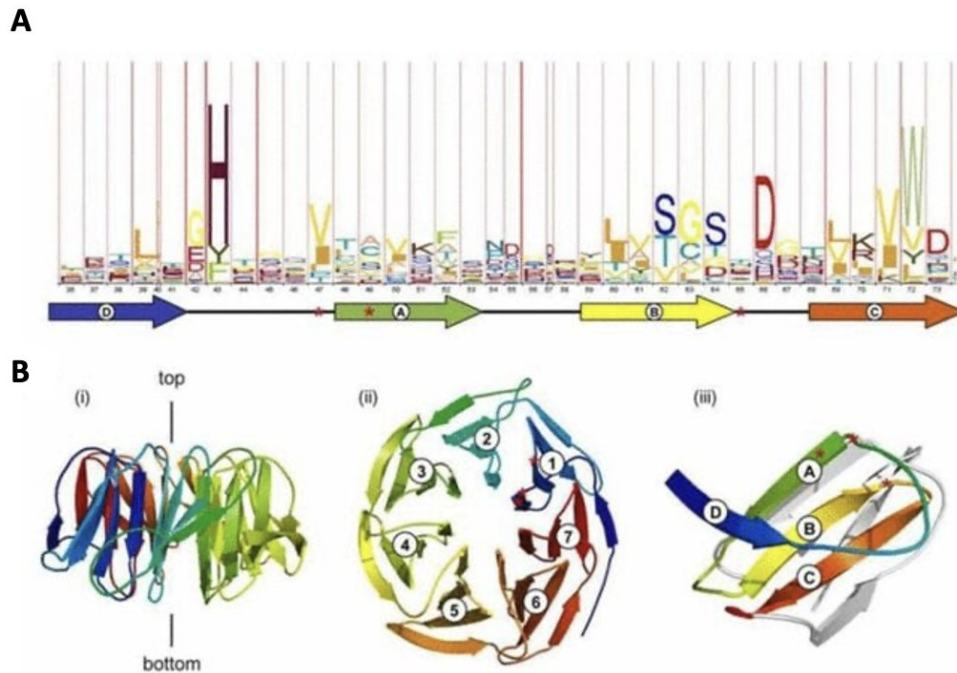


Figure 1. Sequence and structural features of WD40 domains, which contain several copies of the 44–60 residues WD40 repeat. WD40 repeats typically fold into seven-bladed β -propellers with each blade comprising a four-stranded anti-parallel β -sheet. **(A)** Sequence logo for WD40 repeats. The letter plots represent amino acid conservation at each position. The corresponding β -strands within a repeat are depicted below. Each WD40 repeat typically contains a variable region of 11–24 residues followed by key signature sequence features: a glycine–histidine (GH) dipeptide at the end of strand D, three small amino acids (serine, glycine etc.) at the C-terminal end of strand B and a tryptophan–aspartate (WD) dipeptide at the end of strand C. **(B)** WD40 domains often fold into seven-bladed β -propellers with a funnel-like shape; the narrower part of the funnel containing the DA and BC loops is defined as the top domain and the wider part as the bottom of the propeller (i). The WD40 sequence repeat corresponds to strand D of one blade followed by strands A, B and C of the next blade (iii). The WD40 sequence repeat therefore is not equivalent to a single propeller blade. The main chain is coloured using a gradient from red at the N-terminus to blue at the C-terminus (ii, iii). Residues often involved in peptide interactions on the top surface are highlighted with red asterisks. Adapted from (4).

Tandem pairs of these repeats form a solenoid structure called the WD domain. While lacking enzymatic activity, the peculiarity of proteins belonging to the WD family usually lies in their ability to act as scaffolds for other proteins and complexes, thus constituting fundamental platforms for the formation of protein complexes involved in the regulation of different cellular pathways, essential for the maintenance of homeostasis (Figure 2).

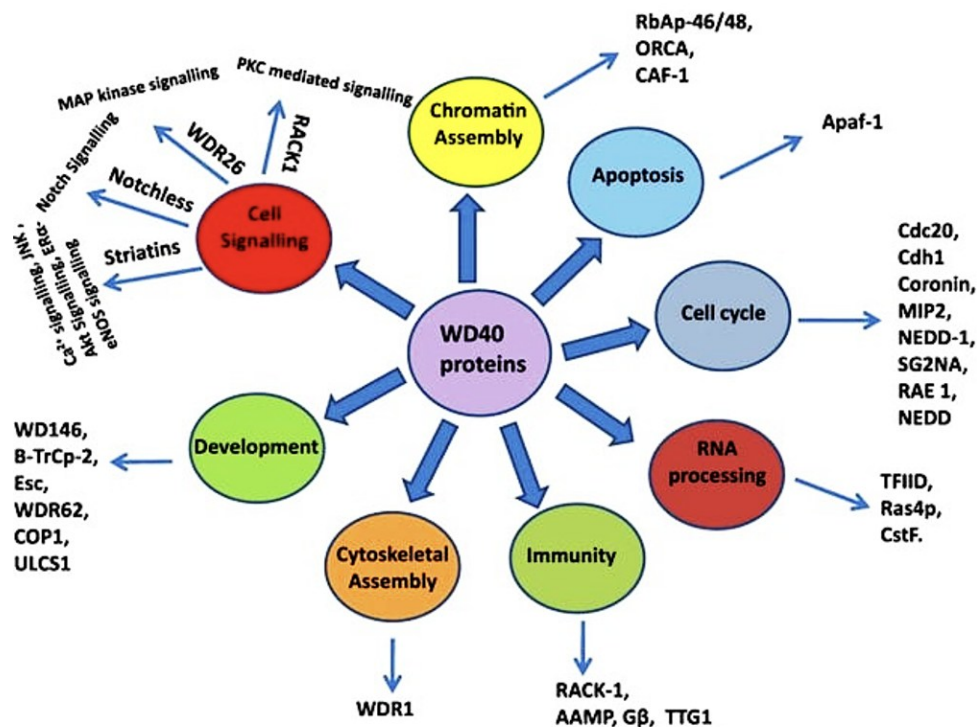


Figure 2. Molecular mechanisms in which WD 40 proteins are involved. The figure shows the most important molecular mechanisms where WD 40 proteins are involved, including RACK1 protein in immunity and cell signalling pathways. Adapted from (4).

The first identified and most studied WD40 protein is the β subunit of the heterotrimeric G proteins ($G\beta$) which, in its inactive state, is found in association with the γ and α subunits forming the heterotrimeric G-protein associated with the class of receptors defined as G-protein coupled receptors (GPCRs). By sharing a 41% structural homology with $G\beta$, RACK1 is one of the best studied scaffold proteins featuring 7 WD 40 domains that are arranged around a central axis, forming a propeller structure where the blades are connected to each other by means of loops of different lengths (Figure 3) (5, 6). Compared to other WD40 family proteins, RACK1 contains a longer and more flexible loop between domain 6 and 7, which is the main responsible for protein-protein interactions. Following phosphorylation on specific tyrosine residues present in domains 1, 4, 5 and/or 6, RACK1 undergoes conformational changes which allow the exposure of other anchor sites, favouring interaction with other proteins (3). It was also observed that there are three possible interaction surfaces: the apex, the base, and the lateral part of the structure, to allow the formation of multiple bonds, justifying the implication of RACK1 in a wide variety of molecular pathways (2, 7).

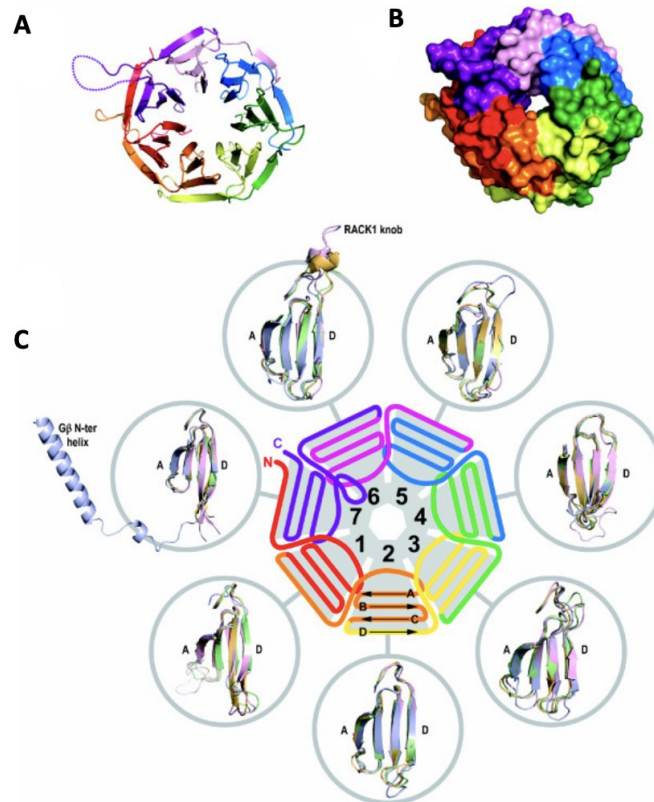


Figure 3. RACK1 structural details. RACK1 crystal structure (A) and surface rendition (B) top view illustrating the seven-bladed β -propeller structure. (C) Schematic representation of tertiary RACK1 structure and organisation of WD-repeats, with the representation of the 7 domains folded into 4 β -sheets to form the β -propeller structure; peripheral circles display the superimposition of individual propeller blades for G β (in blue) and for different RACK1 orthologues. Adapted from (3).

Despite being originally described to act as a shuttling protein for the activated PKC β II, RACK1 was also demonstrated to bind a plethora of other partners including MAPK, Jun N-terminal kinase (JNK), and the cAMP specific phosphodiesterase PDE4D5 (8, 9). Experimental evidence also identified other RACK1-interacting partners, including the tyrosine kinase oncoprotein SRC (10, 11), the protein serine phosphatase PP2A (12, 13), and the focal adhesion kinase (FAK) (14, 15). Moreover, RACK1 can interact with the cytoplasmic tail of various receptors including the insulin-like growth factor receptor I (IGF-IR), the NMDA- receptor, the β -integrin receptor, the common β -chain of the IL-5, IL-3/GM-CSF receptor, different ion channels as well as androgen receptor, acting as a scaffolding partner for different signalling cascades (3, 16).

1.2 Protein domains interacting with RACK1

The seven-bladed β -propeller structure typical of the WD protein family provides multiple exposed surfaces for interaction and acts as docking site for its binding partners, allowing the formation of homo- and heterodimers (17). In this regard, RACK1 seven-bladed β -propeller architecture allows the binding of its WD domains with multiple protein domains, such as Src-homolog 2 domain (SH2) (18, 19), C2 domains (20), pleckstrin-homology domains (PH), naturally unfolded domains (NUF) and other WD40 domains (3) (Figure 4). Because of its ability to interact with a large spectrum of protein partners, RACK1 is functionally flexible and can modulate function and assembly of multiple signalling complexes. These interactions enable RACK1 to modulate protein activity, stability, intracellular localisation, and protein–protein interactions to coordinate multiple signalling pathways in the cell, with important implications both in physiological and pathological conditions (3, 16, 21). The C2 domain is one of the two domains involved in the interaction between PKC β II and RACK1, but is also present in other several proteins are with in functions ranging from signal transduction to vesicular transport, including other PKCs like the novel PKC (nPKC) family member PKC ϵ as well as synaptogamine and phospholipase C (PLC) (2, 22). Indeed, the interaction with the C2-domain containing proteins is especially important for the PKC-mediated signalling, as RACK1 acts as a scaffold to facilitate the association of the PKC isoforms with the downstream effector PLC and modulates the signal transduction pathways (16, 20). In this regard, since PLC activity is regulated by the phosphorylation of a tyrosine residue, a positive feedback mechanism has been hypothesized, where PKC β II, in proximity to PLC thanks to RACK1, is responsible for PLC phosphorylation, generating diacylglycerol amounts localized close to PKC β II for its functions (22). The same C2 domain present in the different PKCs has also been studied in the p120GAP enzyme (23).

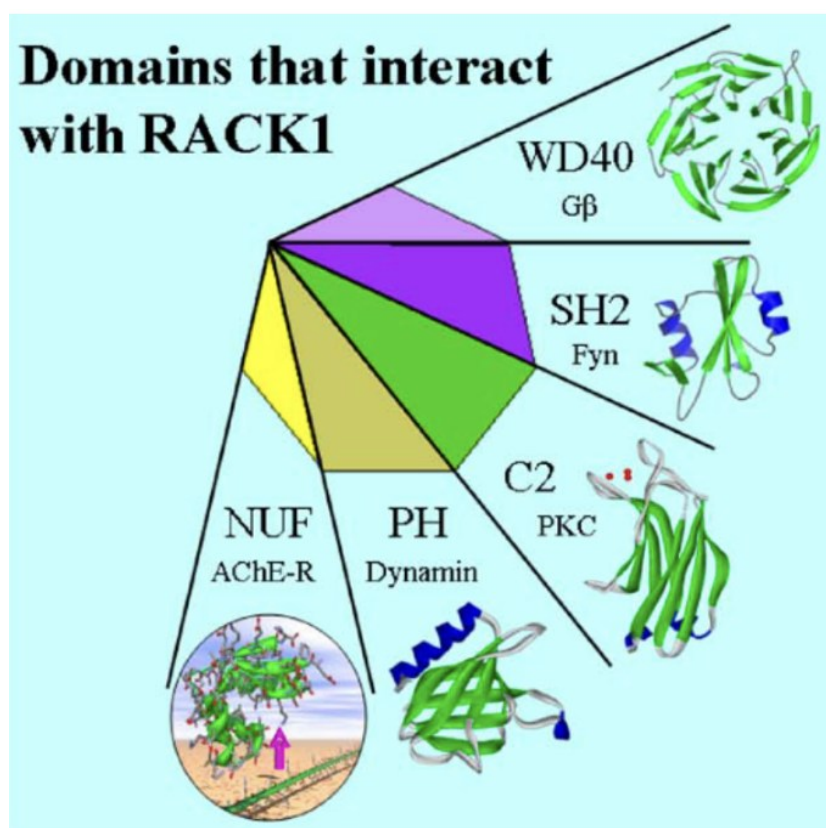


Figure 4. RACK1 interacts with a variety of proteins with distinct folds. The structurally distinct folds that can interact with RACK1 are depicted in the image. With a conserved pocket that recognises pY and a more variable pocket that binds three to six residues C-terminal to the pY, SH2 domains have a core anti-parallel β -sheet encircled by two α -helices that bind to particular phospho (pY)-containing peptide motifs. The C2 domains are made up of two, four-stranded β -sheets that form three loops at the top of the domain and four at the bottom. They contain 130 residues necessary for phospholipid binding in a Ca^{2+} -dependent or independent way. PH domains have a conserved shape with a C-terminal amphipathic α -helix and a β -barrel made up of two perpendicular, anti-parallel β -sheets. NUF domains are renowned for their ability to undergo conformational changes and structural flexibility. Adapted from (24).

RACK1 can also interact with proteins containing the PH domain, consisting of 120 amino acids, like Dynamin-1, a master regulator of endocytosis and vesicle trafficking (23). It has been shown that the formation of the RACK1–PKC β II–Dynamin-1 complex promotes the phosphorylation and activity of Dynamin-1 (7), supporting a role for RACK1 in membrane trafficking and intracellular transport processes (16, 25). Literature data show that RACK1 can also interact with proteins containing the SH2 domain, consisting of 100 amino acids characterized by the presence of a non-variable structure pocket and a variable pocket that confers binding specificity. Among SH2-containing proteins able to interact with RACK1 there

are the GTPase activating protein (GAP) and members of the Src family kinases like Fyn and Src, which are involved in many signalling pathways regulating cell proliferation, differentiation and migration. In particular, Fyn is known for its ability to phosphorylate the NR2B subunit of the NMDA receptor, while its binding to RACK1 reduces its receptor activity (26, 27). Moreover, RACK1 can also interact with other WD40 domain-containing proteins originating heterodimers or other RACK1 proteins resulting in homodimers (24), broadening its potential to build multiprotein complexes and coordinate diverse cellular functions. (18) (17, 19). Altogether, the different interactions established by RACK1 with a plethora of molecular partners indicates that its intracellular role spans from shuttling signalling proteins to their sites, thus facilitating the cross-talk between signalling pathways, to its specific scaffold function. This identifies a complex network of signalling elements that defines the physiological and pathological role of RACK1. In fact, the correlation between RACK1 levels and physiological and pathological conditions related to their changes are extremely important. Because of this importance, it is paramount to dissect the transcriptional mechanisms regulating the expression of RACK1 (28).

1.3 RACK1 as ribosomal protein and its role in translational control

Besides its role as a scaffold for signalling molecules, RACK1 has also been identified as a structural component of the eukaryotic 40S ribosomal subunit. This finding greatly increased the biological relevance of RACK1 and uncovered its involvement in translational regulation and protein synthesis (3, 29, 30). Structurally, ribosomal RACK1 is located in the head region of the 40S subunit, exposed to the cytoplasm and in a position to contact translation factors, signalling molecules and RNA-binding proteins (Figure 5). Such localisation suggests a possible role of RACK1 as a link between extracellular signalling pathways and the translational machinery, coordinating cellular responses to environmental stimuli (29-31).

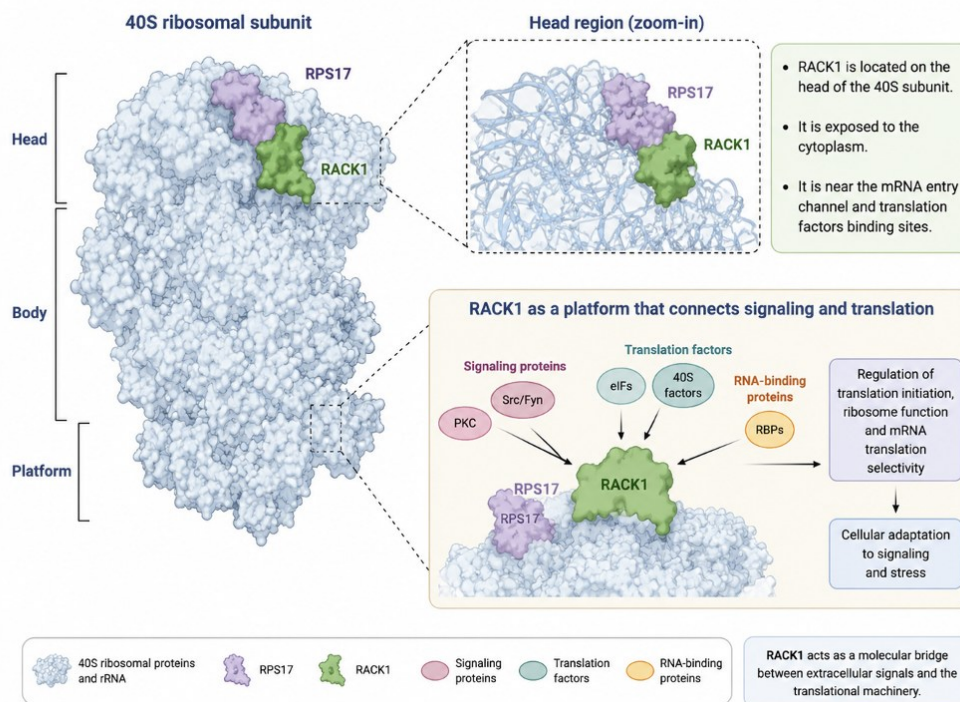


Figure 5. RACK1 as a ribosomal scaffold linking signal transduction and translational regulation.

Positioned atop the head portion of the 40S ribosomal subunit, RACK1 interacts with ribosomal proteins, translation factors, signalling molecules, and RNA-binding proteins while remaining accessible to the cytoplasm. Because of its strategic location, RACK1 serves as a molecular platform that combines translational regulation and extracellular signalling pathways, supporting ribosome function, mRNA translation, and cellular stress adaptability. Created with BioRender. Adapted from (3, 30, 31).

Indeed, RACK1 association to the ribosome has important implications for translational regulation. RACK1 regulates mRNA translation efficiency and selective protein synthesis by acting as a platform for the recruitment of signalling proteins and translation-associated factors. In particular, RACK1 has been shown to be involved in the regulation of translation initiation and ribosome quality control pathways, enabling cells to rapidly adjust protein synthesis in response to physiological and environmental cues (30-32). In addition to its role in protein synthesis, ribosome-associated RACK1 is involved in the maintenance of cellular proteostasis. Increasing evidence supports a role for RACK1 in coordinating translational responses during cellular stress and modulates pathways involved in protein quality control. Its strategic position on the ribosome allows RACK1 to combine signalling events and translational regulation, and thus modulate cellular adaptation to stress conditions. These functions have attracted much attention in the context of neurodegenerative disorders, in which changes in proteostasis, translational control and stress responses are key pathological mechanisms (3, 31, 33). Despite

not being required for ribosome assembly, but several studies have shown that RACK1 functions as a scaffold for the recruitment of signalling molecules and translation-associated proteins to actively translating ribosomes (3, 34), thus regulating translation initiation and translational efficiency (32). One of the best characterised RACK1-dependent mechanisms is the recruitment of PKC β II to the ribosome. Ribosome-associated PKC β II promotes translation initiation through promoting eIF6 phosphorylation, a factor involved in the control of 60S-40S subunit joining (34, 35). In agreement with these observations, disruption of RACK1-PKC β II interaction results in impaired translational activity (34). In addition to its role in global protein synthesis, RACK1 is involved in the regulation of selective mRNA translation by interacting with RNA-binding proteins and messenger ribonucleoprotein complexes (30, 32). These observations suggest a role of RACK1 in the fine regulation of gene expression at post-transcriptional level. These observations indicate that RACK1 can be considered as a hub for signalling pathways, mRNA metabolism and protein synthesis, making it a key regulator of cellular responses to environmental stimuli and stress conditions (3, 32). Therefore, the fine-tuned control of RACK1 transcriptional regulation is of fundamental interest to understand its role in health and diseases due to the multifaceted nature of its different molecular interactions and mechanisms.

1.4 RACK1 gene structure and promoter elements

The first studies regarding RACK1 gene organization were conducted on the porcine *Rack1* (36). Different putative transcriptional factor binding sites were identified in the 5'-flanking region, such as E2F, Activator Protein 1 (AP1), transcription factor (SP1), c-Myc, Nuclear Factor 1 (NF1), Yin Yang 1 (YY1), Nuclear Factor-kB (NF-kB/c-rel) and a serum response element (SRE) (Figure 6A).

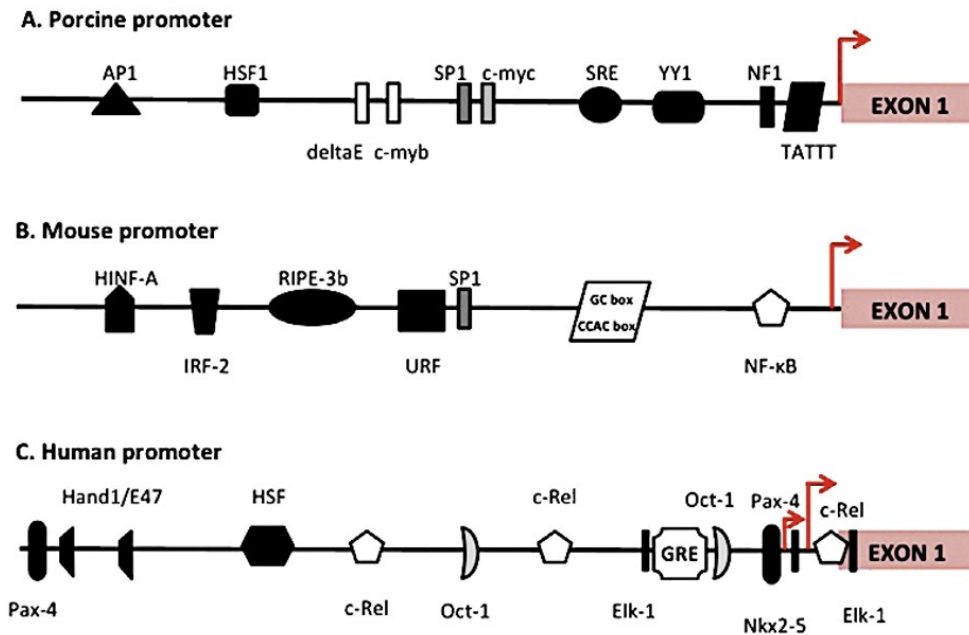


Figure 6. Bioinformatic analysis of the promoter region of genes encoding RACK1. The figure shows potential binding sites for the transcriptional factors in porcine promoter (A), in mouse promoter (B) and in human promoter (C). Adapted from (28).

Further studies were focused on the characterization of the murine *Rack1* gene structure, identified in a 14-kb region of mouse genomic DNA and found to contain 8 exons and 7 introns. Different putative cis-acting elements in 1.5-kb of proximal 5'-flanking sequence were recognized, including a 5'-CCAC-3' box and sites for histone nuclear factor-A (HINF-A), interferon regularity factor-2 (IRF-2), footprint II factor, rat insulin promoter element 3b (RIPE-3b), SP1, urokinase transcription factor (URF) and NF-kB transcription factors. Two different transcription start sites at 265 and 285 nucleotides upstream of the translation initiation site. Additional functional characterization of the murine *Rack1* gene promoter suggested that the NF-kB binding site is an important positive regulatory element (Figure 6B). Finally, the promoter region of human *RACK1* gene (formerly known as guanine nucleotide binding protein beta polypeptide 2-like 1, *GNB2L1*), was studied by cloning a 2-kb region 5' of the *RACK1* human gene to observe differences from the other species. The human gene has 8 exons and 7 introns, spanning about 7-kb. *RACK1* is mapped to chromosome 5q35.3 in proximity to the telomere region (37). The human *RACK1* gene has two main transcription start sites and a bioinformatic analysis revealed several putative cis- acting elements, including binding sites for several transcriptional factors belonging to a smooth muscle/cardiomyocyte specific family were recognized. Among these, Hand1/E47 is implicated in cardiomyocytes

differentiation and the smooth muscle specific factors Ets like protein-1 (Elk-1) and Nkx2-5 (found in the 3' of the second transcription start site) are cardiac specific homeobox. Further, myogenin/NF1 factor can also be considered of this group. Moreover, four binding sites for c-Rel (a member of NF-kB transcription factor family), octamer transcription factor-1 (Oct-1) and three paired homeodomain transcription factor-4 (Pax-4) binding sites (one of which is contained in the 3' of the first mapped transcription start) were also found. Finally, a consensus sequence for the binding of glucocorticoid receptor (GR) was identified (Figure 6C).

1.5 The GRE site and glucocorticoid-mediated RACK1 regulation

The bioinformatic analysis of human *RACK1* promoter region revealed the presence of a Glucocorticoid Response Element (GRE) consensus sequence (5'-AGAACACCTCCGGAAAGCACA-3'), identified at the nucleotide position -186/-165 relative to the transcription start site (37). The glucocorticoid receptor (GR) can regulate both transcriptional activation and repression of different genes. GR-dependent transcriptional activation requires DNA-mediated receptor dimerization through positive GREs (38) (Figure 7B).

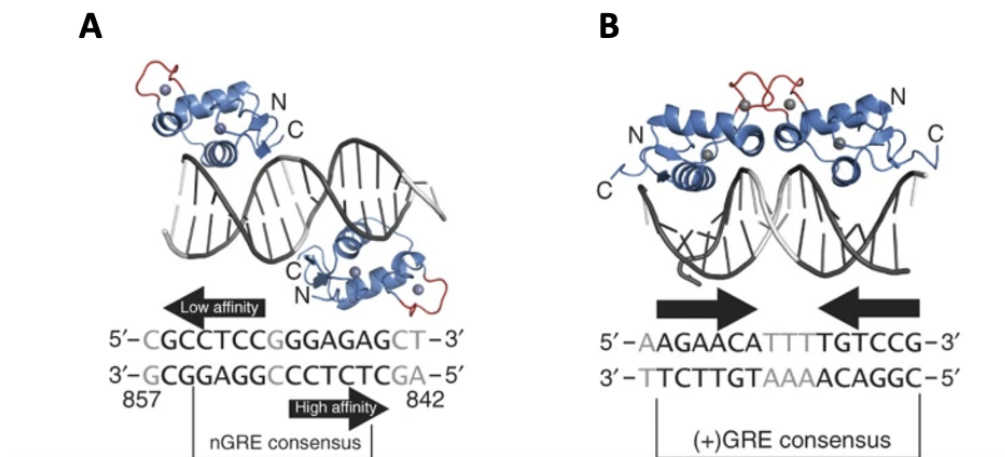
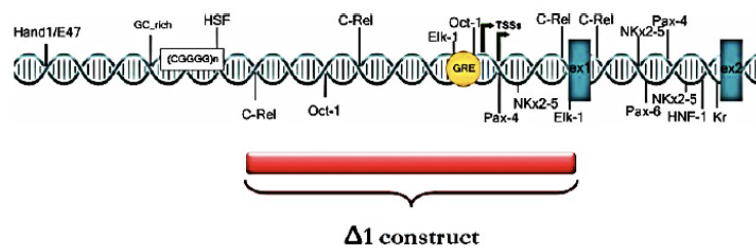


Figure 7. GR interacts with nGREs in a unique orientation, preventing receptor dimerization. (A) Overall structure of GR DNA-Binding Domain (DBD) (in blue) in complex with the thymic stromal lymphopoietin nGRE (in gray). Bases containing the GR binding sites are displayed in black, while Zn²⁺ are illustrated as grey spheres. The GR dimerization interface (in red) of one GR monomer is orientated away from the second monomer, in an everted repeat conformation. **(B)** GR binds to a (+)GRE as a homodimer in an inverted repeat conformation to enable a contact between dimerization loops. Adapted from (38).

These elements contain two inverted repeats of the 5'-AGAACA-3' sequence separated by three nucleotides (Figure 7B). On the other hand, the negative GRE (nGRE) consensus sequence 5'-CTCC(N)0-2GGAGA-3' is structurally different compared to activating sequences and mediates DNA-dependent trans-repression. In this regard, it has been demonstrated that two GR monomers can bind an nGRE in a reverted repeat orientation with strong negative cooperativity (Figure 7A). This different interaction mechanism of GR-DNA binding represents a key paradigm for GR-mediated trans-repression (38), which is central to understand *RACK1* transcriptional regulation. Indeed, the discovery of a putative nGRE consensus sequence in human *RACK1* gene promoter prompted to study the role of glucocorticoids (GCs) in the regulation of *RACK1* expression. A major analysis of the role of the GRE element in *RACK1* transcriptional regulation was conducted employing three different luciferase reporter constructs, namely $\Delta 1$, $\Delta 6$ and $\Delta 9$ (39) (Figure 8).

A



B

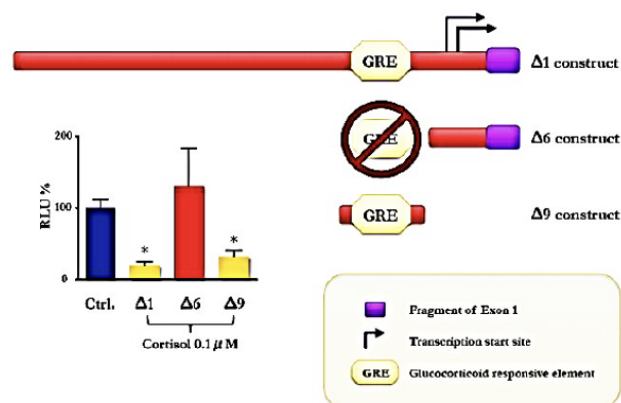


Figure 8. Deletion fragments produced to investigate the role of the GRE sequence in the RACK1 promoter.

(A) The red bar indicates the major luciferase deletion fragment of the RACK1 promoter region ($\Delta 1$) isolated and studied by (37). (B) The $\Delta 1$ luciferase reporter construct represents the entire promoter region described in (37), including the two major transcriptional start sites and part of exon 1. The construct $\Delta 6$ represent a promoter fragment which does not include the GRE sequence while the $\Delta 9$ constructs includes only the GRE sequence. The inset graph represents a sample of the data described by (39). THP-1 cells were transfected with the reporter constructs and treated with cortisol. It has been observed that the constructs bearing the GRE site show reduced luciferase activity while the $\Delta 6$ construct does not respond to cortisol suggesting a specific role for the GRE element as a negative regulator of RACK1 gene promoter activity.

The $\Delta 1$ luciferase reporter construct contains the entire 2-kb region upstream the human *RACK1* gene (37) (Figure 8A), $\Delta 6$ construct is a promoter fragment lacking the GRE sequence and $\Delta 9$ includes only the GRE sequence (Figure 8B). A more detailed analysis was performed in acute monocytic leukaemia cells THP-1, employing the aforementioned reporter constructs and cortisol treatments (0.1 and 0,5 μ M). These results demonstrated that, in the presence of promoter constructs bearing the putative nGRE, cortisol induced a significant downregulation of luciferase activity (Figure 8B). Cortisol was also able to reduce RACK1 expression at both the mRNA and protein levels (40). On the other hand, mifepristone, a potent GR antagonist, prevented cortisol effects (41). Additional evidence suggests that other corticosteroids, such as budesonide, methylprednisolone, betamethasone, prednisone, and prednisolone, can affect RACK1 expression through GR-dependent transcriptional mechanisms (Figure 9). All these compounds, acting at the transcriptional level, can modulate the RACK1 mRNA and protein expression, supporting the notion that RACK1 is an important target of corticosteroid-induced anti-inflammatory effects (41) (Figure 9).

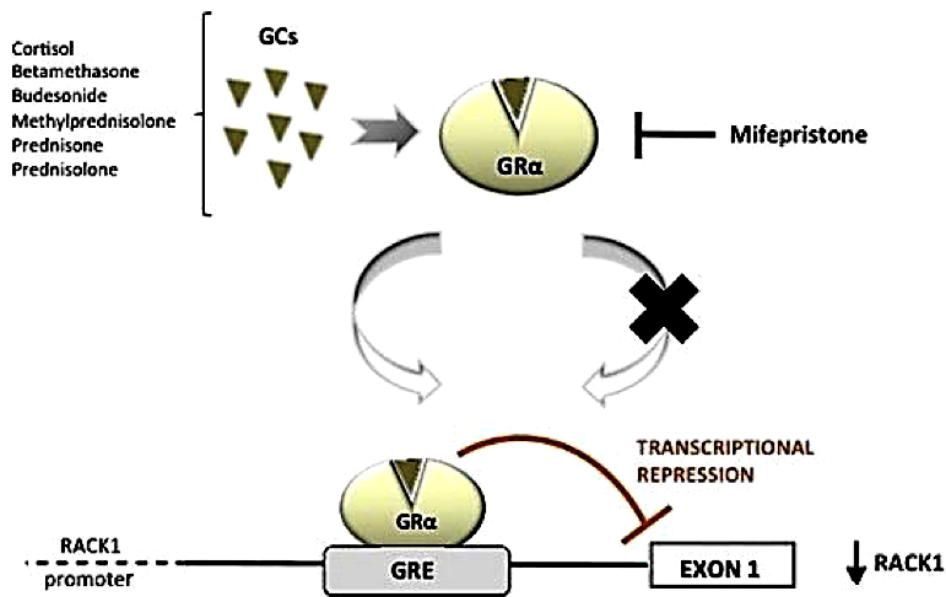


Figure 9. GR-mediated transcriptional regulation of RACK1. By binding to GR α , glucocorticoids promote its nuclear translocation and receptor activation. Glucocorticoid response elements (GREs) in the RACK1 promoter interact with activated GR α , causing transcriptional repression and decreased RACK1 expression. Modified from (28).

RACK1 transcriptional regulation studies began with the observation that reduced secretion of dehydroepiandrosterone (DHEA) during aging was associated to the decrease of RACK1 expression and PKC-dependent functions. Moreover, *in vitro* and *in vivo* administration of DHEA was able to restore RACK1 expression to normal levels in cells from aged animals and human (42-44). It has been experimentally confirmed that DHEA exerts anti-GC properties in the regulation of different processes involved in the immune response and the evidence concerning the effect on RACK1 is consistent with this antagonistic paradigm (28). In this context, it was then demonstrated that DHEA counteracts cortisol effects on RACK1 expression through the interference with GR alternative splicing (45).

1.6 The DHEA-cortisol balance in the regulation of GR alternative splicing

GR, a ligand-activated transcription factor encoded by the *NR3C1* gene, is primarily responsible for the biological effects of GCs (46). Without ligand, GR is mainly in the cytoplasm as part of a multiprotein complex containing heat shock proteins and immunophilins. GC binding causes conformational changes in the receptor, enabling its dissociation from the

chaperones and its translocation into the nucleus, where it regulates the expression of a variety of target genes (47) (Figure 10). As a consequence, GR regulates a number of biological processes, e.g. inflammation, metabolism, cell survival, neuronal plasticity and stress adaptation thanks to its transcriptional activity (46).

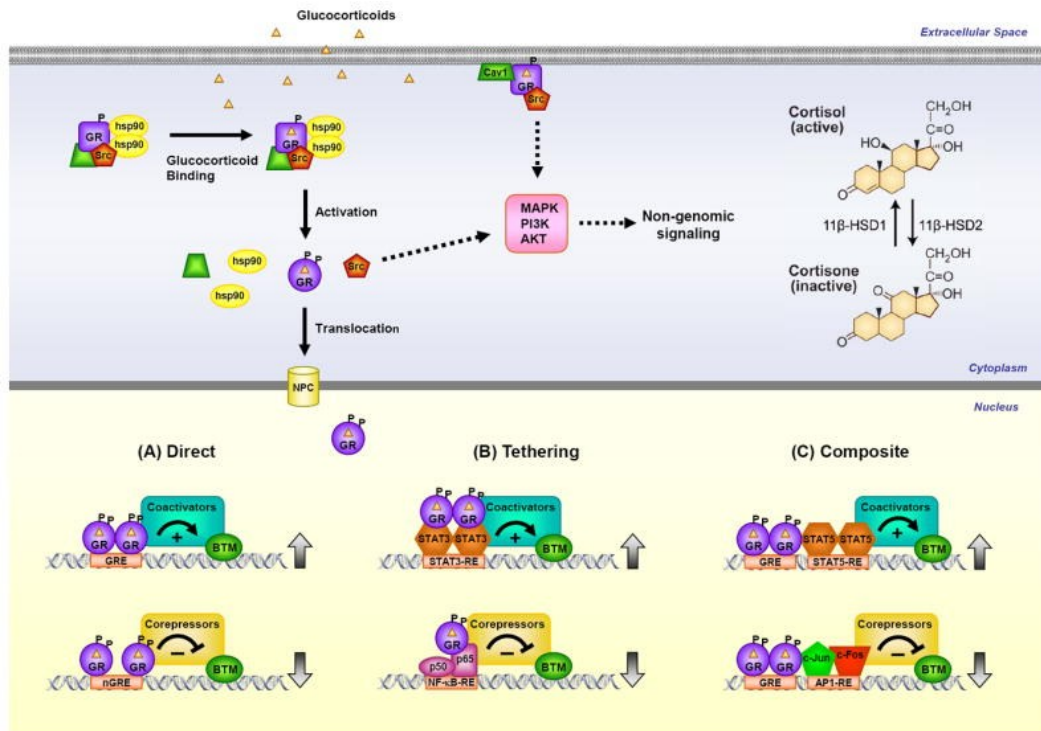


Figure 10. Canonical and non-canonical glucocorticoid receptor signaling pathways. After binding to glucocorticoids, the glucocorticoid receptor (GR) separates from its cytoplasmic chaperone complex and moves to the nucleus, where it uses composite DNA-binding mechanisms, tethering interactions with other transcription factors, or direct binding to glucocorticoid response elements (GREs) to control gene expression. Rapid non-genomic signalling pathways comprising MAPK, PI3K, and AKT cascades can also be triggered concurrently by GR. Adapted from (46).

In humans, GR is encoded by the *NR3C1* gene, which is extensively alternatively spliced, creating multiple receptor isoforms with different biological functions. The two major isoforms of these variants are GR α and GR β , which differ in the C-terminal region as a result of alternative splicing of exon 9. GR α is considered the classical GC-responsive receptor and mediates most of the induced transcriptional effects by glucocorticoids. Conversely, since it lacks part of the ligand binding domain, GR β does not bind GCs (46, 48) and is usually considered to act as dominant-negative regulator for GR α (49, 50). Regarding *NR3C1* gene, exon 9 alternative splicing is regulated by specific serine/arginine-rich splicing factors (SRSFs), such as SRSF3 and SRSF9 (Figure 11). These factors determine the relative

production of GR α and GR β isoforms and, thus, not only cell sensitivity to GCs, but also on RACK1 expression levels.

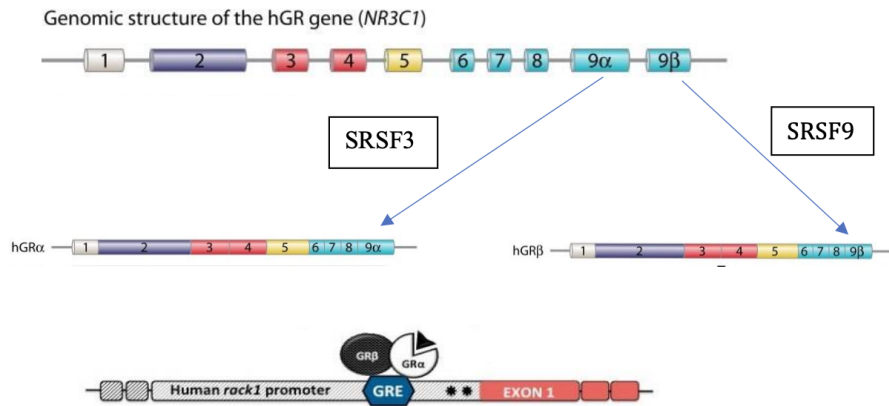


Figure 11. Alternative splicing of *NR3C1* generates GR α and GR β isoforms. Alternative splicing in *NR3C1* exon 9 generates two highly homologous isoforms, GR α and GR β . The two kinds of receptors share the first 727 amino acids at their N terminus coded by first eight exons (NTD, N-terminal domain, DBD, DNA binding domain and H, hinge region), and differ only at their carboxyl-terminus (LBD, ligand binding domain): GR α has an additional 50 amino acids coded by exon 9 α but GR β has only another 15 amino acids coded by exon 9 β . As a result of these differences, GR β is unable to bind GC and cannot trans-activate GC-sensitive genes. Adapted from (40, 51).

In this regard, after GC binding, the activated GR α translocates to the nucleus and binds GRE sequences in the *RACK1* promoter. This interaction leads to transcriptional repression and downregulation of RACK1 expression. Pharmacological inhibition of GR activity prevents this effect, indicating that the GC-induced downregulation of RACK1 is mediated through a GR-dependent mechanism. These results identify RACK1 as a direct target of GC signalling and suggest that changes in GR activity may have a profound impact on RACK1-dependent cellular functions (28, 39-41, 45). Noteworthy, the presence of the GR β / α inactivating complex on a GRE site was demonstrated to influence RACK1 expression (45). In this regard, it has been demonstrated that DHEA induces an increase of GR β / α complex by upregulating GR β , thus counteracting the cortisol-induced binding of GR α to the *RACK1* promoter region. Further studies on its mechanism of action showed that DHEA induced the up-regulation of total GR mRNA, directed to the GR β isoform by concurrently increasing the expression of SRSF9. In contrast, cortisol exerted an opposite shift in GR expression pattern, promoting the splicing of the GR α isoform at the expense of GR β by up-regulating SRSF3 and the concurrent down-regulation of SRSF9. These data suggest that cortisol and DHEA can directly influence GR

alternative splicing and revealed a critical balance between the SRSFs in the control of exon inclusion and exclusion in GR mRNA (28, 52) (Figure 12).

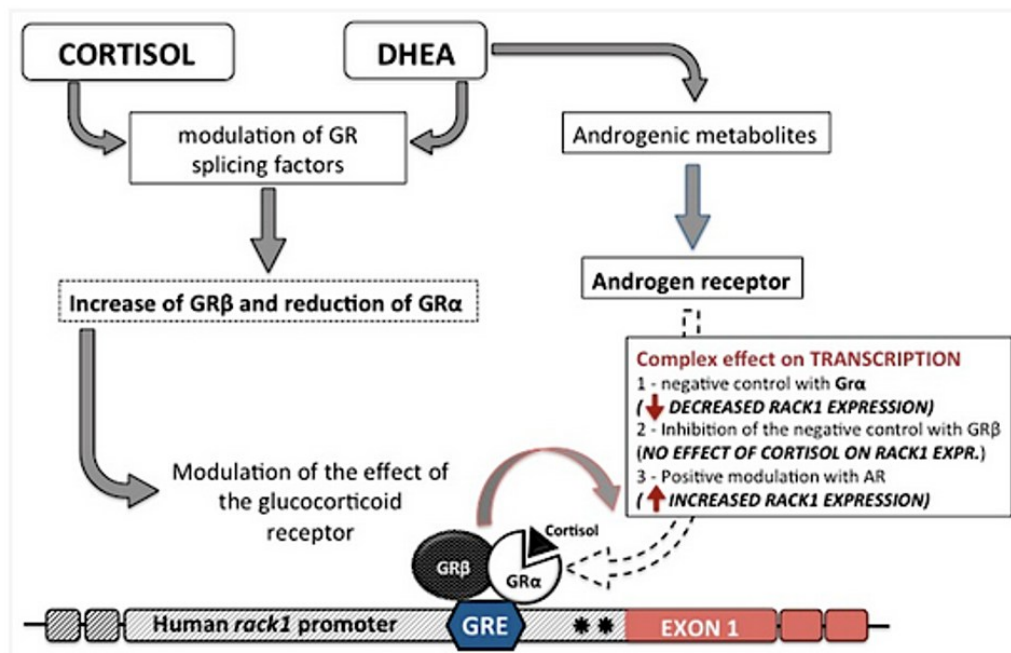


Figure 12. Schematic representation of the complex hormonal balance that controls RACK1 expression. Cortisol and DHEA can influence GR alternative splicing by controlling the inclusion/exclusion level of exon 9 in GR mRNA. Therefore, the anti-glucocorticoid effect of DHEA is due, at least in part, to the modulation of the expression of splicing factors involved in GR mRNA splicing. In addition, DHEA exerts its effect on RACK1 expression also through its transformation into active androgen steroids. Adapted from (40).

In this regard, changes in SRSF3 and SRSF9 expression have been linked to altered GR signalling, which in turn has been associated with stress-related disorders and impaired neuroplasticity (40, 53). Moreover, while acute GC release generally exerts a positive effect by allowing energy mobilisation and adaptation to stress, chronic exposure to high GC levels may have negative effects on multiple tissues, particularly in the central nervous system (54). In this context, chronic GC signalling has been associated with impaired synaptic plasticity, reduced neurogenesis, cognitive dysfunction and increased susceptibility to neuropsychiatric and neurodegenerative disorders (55). Considering that chronic stress is associated with prolonged activation of the HPA axis and elevated GC exposure as well as RACK1 key roles in neuronal plasticity, synaptic function and stress adaptation mechanisms, the dysregulation of the GR-RACK1 pathway has been proposed as a molecular mechanism mediating the effects of chronic

stress on neuronal dysfunction and increased vulnerability to psychiatric and neurodegenerative diseases (56, 57).

1.7 The hypothalamic-pituitary-adrenal axis, glucocorticoid signalling and neurostress

The HPA axis is the main neuroendocrine signalling that mediates the physiological stress response. Activation of the hypothalamic neurons leads to release of the corticotropin releasing hormone (CRH), which stimulates the secretion of the adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. In turn, ACTH stimulates the adrenal cortex to produce GCs to induce systemic adaptations that allow coping with stress and maintenance of homeostasis (54, 58). GCs are steroid hormones released from the adrenal cortex upon activation of the HPA axis and represent the main endocrine mediators of the physiological stress response (59, 60). Under physiological conditions, GCs contribute to the maintenance of homeostasis by regulating metabolism, immune function, neuronal activity, behavioural adaptation to environmental challenges (60). This response is tightly controlled by GC-mediated negative feedback mechanisms mainly through GRs expressed in the hypothalamus, the pituitary gland and several brain areas involved in stress regulation (e.g. hippocampus, prefrontal cortex and amygdala) to limit HPA axis over-activation. However, persistent stress can interfere with this feedback system, resulting in sustained GC exposure and chronic activation of stress-related signalling pathways (56, 58). Increasing evidence indicates a dysregulation of the HPA axis in a variety of stress-related psychiatric disorders, especially major depressive disorder (MDD), a severe and extremely diffuse disease which affects more than 10% of population and it is estimated becoming one of the main causes of disability and the contributor to disease charge (61-64). MDD has been characterized as a recurrent disorder, with about 50% of patients showing relapse (62). Many of the remitted patients show episodes of depression in the first appearance of the disease with a persistence of residual symptoms (63, 64). HPA axis hyperactivation and increased circulating cortisol levels have been consistently reported in subsets of MDD patients and are associated with alterations in cognition, mood regulation and neuronal plasticity (65, 66). In this regard, a correlation between increased cortisol levels, HPA axis hyperactivation and depression has been thoroughly investigated in the past (67, 68). Consistently with this paradigm, treatment with the GC corticosterone in rodents resulted in behavioural modifications similar to those observed in humans (69). According to the corticosteroid receptor hypothesis for depression, stress signals that over-activate the HPA axis have an important function in the induction of psycho-emotional disorders. GCs, as the final products of this axis, are involved in different physiological and behavioural responses to

stress. In this regard, stress has been described as an environmental factor capable to induce psychopathologies and depressive phenotype (70) and has been recognized as a dangerous risk factor for the development of psychiatric disorders. Noteworthy, the effect of stress may depend on the duration and the intensity of the experience and on the period of life when it first happens (61). Environmental stressors (e.g. such as trauma, conflicts, or childhood maltreatment) were found to be contributors to the development and progression of mental disease, including MDD (71). Furthermore, depression and environmental stress were demonstrated to be highly connected (71, 72). In this regard, multiple lines of evidence suggest that epigenetic changes are an important mechanism through which stress leads to the development of depressive symptoms. Stressors resulting in DNA epigenetic changes, altering its chromatin organization and its expression (e.g. DNA methylation and histone acetylation), could be involved in pathological behaviours observed in depressed patients (Figure 13).

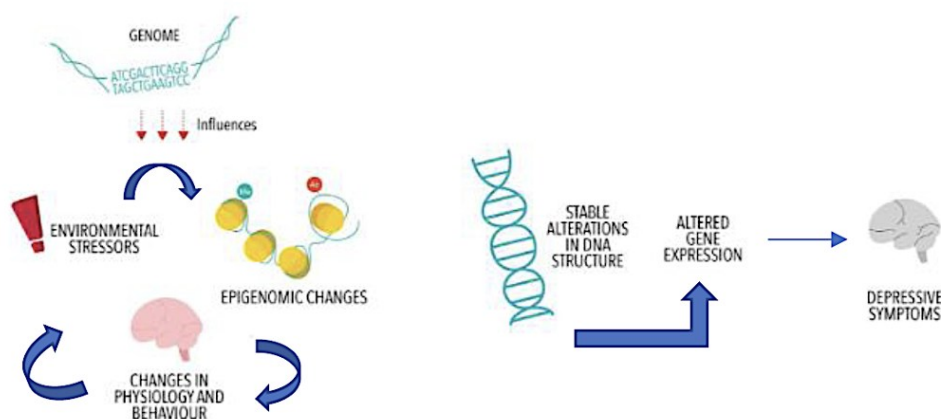


Figure 13. Conceptual pathway connecting environmental stress to depression. Schematic representation of the proposed link between environmental stress, epigenetic modifications, altered gene expression, and the development of depressive symptoms. Adapted from (73).

In DNA methylation, methyl groups are added to the DNA bases cytosine or adenine (creating 5-methylcytosine and N6-methyladenine respectively), modifying its expression pattern by altering the access to transcriptional factors without altering the DNA sequence. When positioned in a gene promoter, DNA methylation acts to repress gene transcription. In histone acetylation, acetyl groups are added to lysine residues within its N-terminal tail protruding from the histone (the core proteins which organize DNA into structural units) core of the nucleosome, with the effect to increase transcriptional activity of acetylated segment (74, 75).

Evidence from preclinical and clinical observation have demonstrated associations between stress and depression (71, 72), between stress and epigenetic alterations and between depression and epigenetic alterations (74, 75). Both stress and depression have been associated with epigenetic alterations in genes involved in mediating resilience and vulnerability to stress (75) including stress response-related genes involved in neurotransmission and neurotrophic signalling such as brain-derived neurotrophic factor (BDNF) (76-79). However, clinical studies reporting a correlation among all the three components are still limited. Studies on different animal models demonstrated that stress is connected to changes in the epigenome and in the development of depressive behavioural (80, 81). Furthermore, chronic stress has been reported to alter histone acetylation in rodents' forebrain, with a consequent increase of anxiety behaviour and major vulnerability to stress. It has been also demonstrated that adult rats subjected to stress since a young age (for example, pups separated from the mothers at the birth) show more stress-induced depressive behaviours and major sensibility to stress through epigenetic changes in the hippocampal BDNF (81).

1.8 The brain-derived neurotrophic factor (BDNF)

BDNF is a neurotrophic factor involved different signalling pathways important for neuronal proliferation, migration, differentiation, survival and for processes like synaptic plasticity, memory, and learning (82). BDNF main function in the adult brain is synaptic regulation and its different effects on brain synapses derive from its complex signalling cascades. The interactions between neuronal activity and synaptic plasticity through BDNF make it an essential regulator of cellular processes that underlie cognition and other complex behaviours. Indeed, numerous studies confirmed that BDNF plays an important role in hippocampal long-term potentiation (LTP), a long-term improvement of synaptic efficacy thought for learning and memory (Figure 14).

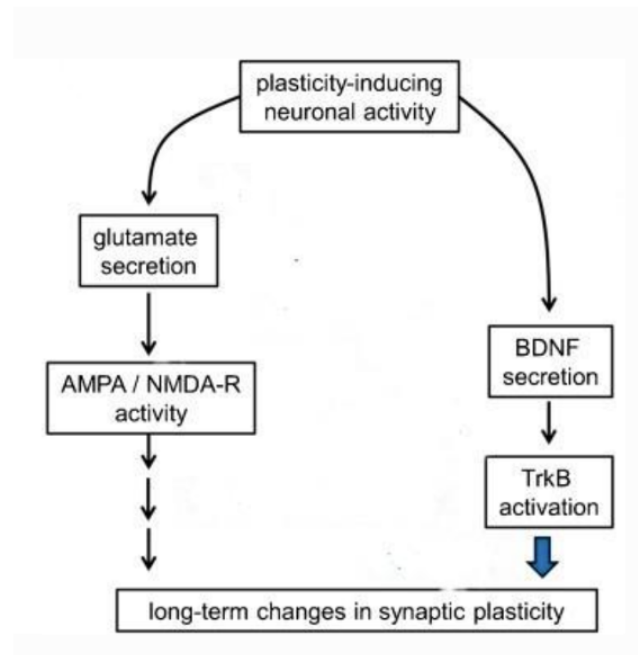


Figure 14. Overview of BDNF signalling. BDNF is produced at synapses during neural activity and binds to TrkB to control modulatory signalling cascades. BDNF-TrkB increases the release of neurotransmitters in pre-synapses and increases neuronal excitation in post-synapses. Adapted from (83).

Deficits in BDNF signalling contribute to the pathogenesis of several psychiatric disorders, including MDD and Alzheimer's disease (AD) (84). Signalling cascades regulated by BDNF and its receptor, the receptor tyrosine kinase TrkB, connect neuronal growth and differentiation with synaptic plasticity. Recent studies have observed a complexity in the synaptic communication of mature BDNF and its precursor pro BDNF, not only between pre- and postsynaptic neuronal targets but also with participation of glial cells (83). While mainly involved in programmed cell death in the peripheral nervous system, in the brain BDNF rather mediates region-specific effects on synaptic function and neuronal morphology. Three different bioactive proteins originate from the *BDNF* gene, namely the precursor pro-BDNF, mature BDNF, and the pro domain of BDNF (Figure 15).

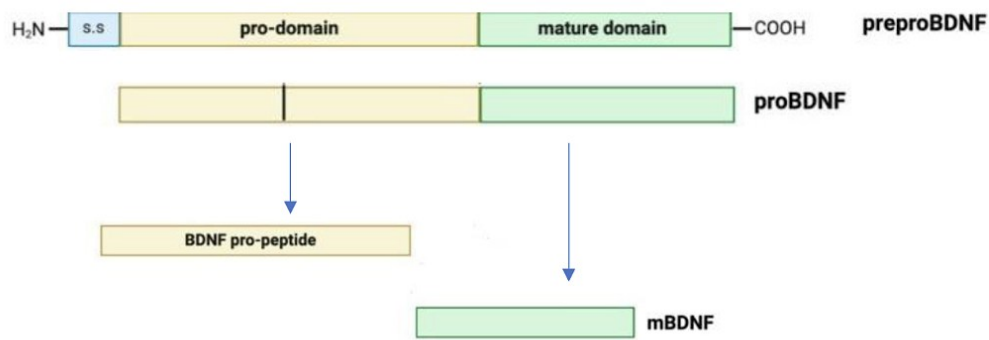


Figure 15. Structure of the different BDNF forms. Pre-pro BDNF is mainly divided into three parts: the signal sequence (SS), comprising the first 18 amino acids at the N-terminus and acting as a zip code to direct the protein into the secretory pathway before being cleaved off; the pro-domain, and the mature domain near the C-terminal end. Cleavage of pre-pro BDNF generates two separate peptides, BDNF pro-peptide and mature BDNF, which act via different, specific receptors. Adapted from (85).

However, there is an in-progress debate about the cellular locus where the conversion of pro BDNF to BDNF takes place, and how much and where pro BDNF is secreted by neurons (86). BDNF carries a translocation signal peptide (pre-pro BDNF), which is removed during import into the secretory protein transport pathway. The resulting pro BDNF is processed, and the pro peptide is finally cleaved off to generate the mature neurotrophin form of about 13 kDa. Mature BDNF forms homodimers that are secreted in both constitutive and regulated pathways. Following secretion, pro BDNF can be cleaved by extracellular proteases, leading to the formation of mature BDNF. There is an evidence that pro BDNF and BDNF utilize distinct receptors to mediate neuronal actions from neuronal remodelling to synaptic communication, and plasticity. While BDNF can increase neuronal excitability and synaptic strength pro BDNF can reduce neuronal excitability, decrease synaptic efficiency, and facilitate synaptic depression (83). Since depression and stress are involved in BDNF expression and signalling, a role for RACK1 in stress-induced depressive-like behaviour has been recently investigated due to its involvement in BDNF epigenetic regulation and expression.

1.9 RACK1-mediated BDNF epigenetic regulation and the GR-RACK1-BDNF axis in neurostress

As discussed in the previous sections, BDNF is a key neurotrophin involved in neuronal survival, synaptic plasticity, learning and memory. Its expression is tightly regulated by neuronal activity and by epigenetic mechanisms that control the accessibility of specific BDNF

promoters. Among these, promoter IV is particularly relevant in the nervous system because it is highly responsive to neuronal activity and participates in the regulation of activity-dependent BDNF transcription. In this context, RACK1 has emerged not only as a scaffolding protein involved in intracellular signalling, but also as a regulator of BDNF expression through mechanisms involving chromatin remodelling. The first evidence linking RACK1 to BDNF transcription derived from studies showing that RACK1 is required for the induction of BDNF expression following activation of the cAMP pathway (87, 88). In SH-SY5Y cells and in primary hippocampal neurons, stimulation with forskolin, an activator of adenylyl cyclase, increases BDNF expression. However, RACK1 silencing strongly reduces this effect, indicating that RACK1 is necessary for cAMP-dependent BDNF transcription. In particular, RACK1 appears to exert a selective effect on BDNF exon IV expression, since RACK1 knockdown impairs the forskolin-induced activation of exon IV more markedly than other BDNF exons. Conversely, the intracellular delivery of recombinant Tat-RACK1 increases BDNF exon IV expression (88), further supporting a direct role for RACK1 in promoter IV activation. Although RACK1 does not contain a classical DNA-binding domain, it can translocate to the nucleus and associate with the chromatin complex. Following cAMP pathway activation, RACK1 interacts with histones H3 and H4 and becomes associated with BDNF promoter IV. This association appears to be promoter-specific, since RACK1 binding was detected at promoter IV but not at promoter VI. These observations suggest that RACK1 acts as a chromatin-associated scaffold, allowing intracellular signalling pathways to converge on selected transcriptional regulatory regions of the BDNF gene (82). A key element in this mechanism is methyl-CpG-binding protein 2 (MeCP2), a transcriptional regulator that binds methylated DNA and is involved in chromatin compaction and transcriptional repression. At the BDNF promoter IV, MeCP2 acts as a repressor under basal conditions. Upon neuronal activation, MeCP2 is phosphorylated and released from promoter IV, allowing chromatin relaxation and transcriptional activation. RACK1 participates in this process by promoting the dissociation of MeCP2 from BDNF promoter IV. Indeed, forskolin treatment induces MeCP2 release from promoter IV, whereas RACK1 silencing prevents this dissociation. Therefore, RACK1 is required for the removal of MeCP2-mediated repression at BDNF promoter IV. The release of MeCP2 is functionally linked to histone modifications. MeCP2 can recruit histone deacetylases to chromatin, thereby maintaining a transcriptionally repressed state. RACK1-mediated dissociation of MeCP2 favours histone acetylation at BDNF promoter IV, particularly acetylation of histone H4. This chromatin remodelling event increases promoter accessibility and supports the transcription of BDNF exon IV. Thus, RACK1 contributes to

BDNF expression not only by coordinating cytoplasmic signalling cascades, but also by acting at the nuclear level as an epigenetic regulator of promoter-specific transcription (82, 89) (Figure 16).

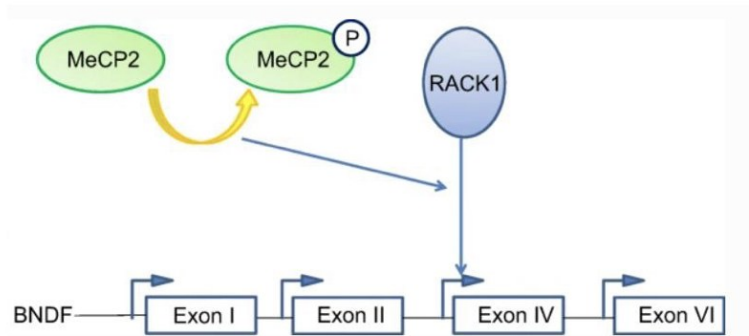


Figure 16. Interaction between RACK1 and BDNF exon IV through the increase of the extent of MeCP2 phosphorylation. RACK1-mediated epigenetic regulation of BDNF exon IV. RACK1 promotes MeCP2 phosphorylation and its release from BDNF promoter IV, facilitating chromatin relaxation and transcriptional activation of BDNF exon IV. Adapted from (89).

This mechanism is particularly relevant in the context of stress, because BDNF expression is highly sensitive to GC signalling. Chronic stress and prolonged GC exposure are generally associated with reduced BDNF levels and impaired neuroplasticity, especially in brain areas involved in mood regulation and cognitive function, such as the hippocampus and prefrontal cortex. Since RACK1 is required for BDNF promoter IV activation, the GC-dependent regulation of RACK1 represents a possible mechanism through which stress may impair BDNF.

1.10 Depression as a risk factor for Alzheimer’s disease: BDNF as molecular link through altered glucocorticoid/GR signalling

AD is the most common cause of dementia worldwide and represents one of the major causes of disability and loss of independence in the elderly population (90, 91). Clinically, AD is characterised by a progressive decline of cognitive functions, initially involving episodic memory, and subsequently affecting language, executive functions, visuospatial abilities, and daily living activities with disease progression (91). From a neuropathological point of view, AD is associated with extracellular amyloid- β (A β) deposition, intracellular accumulation of

hyperphosphorylated tau neurofibrillary tangles, synaptic dysfunction, neuroinflammation and progressive neuronal loss (33). Despite substantial advances in understanding the molecular mechanisms underlying AD pathology, effective disease-modifying therapies remain limited, making the identification of modifiable risk factors and early molecular alterations a major priority (91, 92). Although age remains the major risk factor for AD, increasing attention has been devoted to modifiable or partially modifiable conditions that may increase susceptibility to neurodegeneration. Among these, depression has emerged as a relevant clinical and biological risk factor. Depression is one of the most common psychiatric disorders worldwide and is associated with significant functional impairment, morbidity, and reduced quality of life (93). Depressive symptoms are frequently observed in AD patients, but several clinical and epidemiological studies suggest that depression may not only represent a neuropsychiatric manifestation of dementia (94, 95). A history of depression, especially when recurrent or occurring in midlife or late life, has been associated with increased risk of cognitive decline and dementia later in life (96, 97). This association has been observed in various populations and age groups, supporting the hypothesis that depression could serve as an independent risk factor for neurodegeneration (92, 96). However, the exact nature of this association remains debated, as depression may also be an early prodromal sign of AD-related neuropathological events (97, 98). Notably, there is growing evidence that depression may not only be a neuropsychiatric manifestation of AD, but also a factor in the onset and progression of the disease (92, 97). Therefore, depression may act as an independent risk factor, a prodromal manifestation, a comorbidity, or a condition that accelerates ongoing neurodegenerative processes. These possibilities are not mutually exclusive and may converge on common molecular pathways linking affective disorders and AD. Evidence for alterations in stress-related pathways, neuroinflammatory processes, synaptic plasticity, neurotrophic signalling and GC regulation in both depressive disorder and AD support the existence of shared mechanisms between depression and AD (57, 97, 99). Clinical evidence indicates that chronic depressive states are often associated with impaired memory, attention, executive function and information processing speed, which points to the involvement of the brain areas also affected during the AD progression (100, 101). In this regard, neuroimaging studies reported structural changes in the hippocampus and prefrontal cortex in patients with recurrent depression. These findings suggest that a long-term depressive disorder may promote neurobiological changes relevant to neurodegeneration (57, 102). There is also experimental evidence accounting for a direct contribution of chronic stress and depressive disorders to AD-related pathology. Animal studies have found that prolonged stress exposure is associated with memory deficits and

increased risk of neurodegenerative processes due to changes in neuronal plasticity and stress-responsive signalling cascades (57, 103). Furthermore, stress-induced behavioural changes are often associated with molecular alterations similar to those observed in the early stages of AD development (104). Interestingly, post-mortem and clinical studies have reported an increased accumulation of amyloid- β peptides and neurofibrillary pathology in patients with both AD and depressive symptoms compared to patients with AD only (105, 106). These findings suggest that depression may enhance pathological mechanisms involved in AD development, and thereby promote disease progression. The molecular bases for the association of MDD with AD are still not completely understood, although increasing evidence of dysregulation of stress-related neuroendocrine pathways as a common pathogenic mechanism has been reported (96, 97). Among the mechanisms proposed to explain this association, HPA axis dysregulation and altered GCs signalling have received particular attention (57, 60). As described above, chronic psychological stress and recurrent depressive episodes can disrupt the physiological negative feedback of the HPA axis, leading to prolonged GC exposure and altered GR signalling (56). Increased cortisol levels and impaired GR responsiveness have been reported in subsets of MDD patients, where they are associated with altered mood regulation, cognitive dysfunction and impaired neuronal plasticity (57, 66). Importantly, HPA axis dysregulation has also been described in AD. Elevated cortisol levels have been reported in plasma and cerebrospinal fluid of AD patients and have been associated with disease severity and cognitive decline (107, 108). Furthermore, a dysfunctional GC signalling has been associated with hippocampal atrophy, memory impairment, and increased neurodegenerative processes, suggesting that chronic stress exposure may directly contribute to AD pathogenesis (103, 104) (Figure 17).

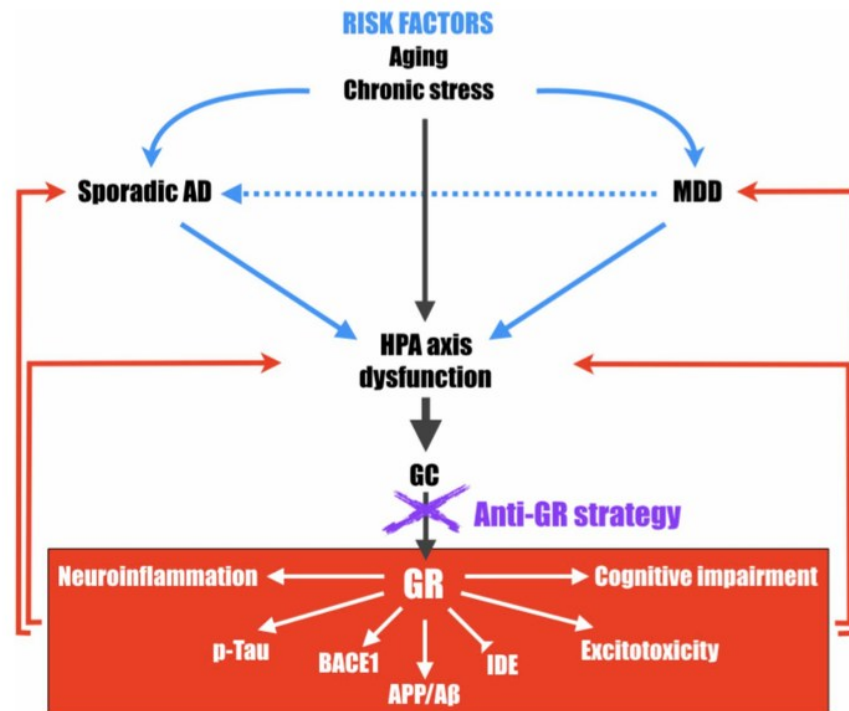


Figure 17. Central role of the HPA axis dysfunction, GC and GR in the development of MDD and AD. Ageing and conditions associated with disruption of the HPA axis can be regarded as significant risk factors for AD. Long-term stress or stress-related diseases can cause neuroinflammatory processes, excitotoxicity, Tau hyperphosphorylation, amyloid- β ($A\beta$) buildup, and cognitive impairment, all of which can lead to AD. Adapted from (109).

Excessive GC exposure may affect brain regions that are particularly vulnerable in both depression and AD, including the hippocampus, prefrontal cortex and amygdala. These areas are essential for memory, emotional regulation and adaptive responses to stress, and are highly sensitive to the detrimental effects of prolonged GC signalling. In this regard, experimental evidence has shown that chronic GC exposure impairs dendritic complexity, decreases neurogenesis and disrupts synaptic plasticity leading to cognitive deficits similar to those observed in early AD (57, 59, 103). These changes resemble many of the structural and functional abnormalities seen in early AD. Chronic GC exposure not only induces brain structural alterations, but has been also shown to affect a variety of molecular pathways involved in neuronal survival and relevant to AD pathology. At the molecular level, chronic GC exposure may contribute to AD-related pathology through multiple converging mechanisms. Experimental evidence indicates that high GC levels can promote amyloidogenic processing of amyloid precursor protein (APP), increase amyloid- β ($A\beta$) accumulation and

favour tau hyperphosphorylation (110, 111). In addition, sustained GR activation can enhance oxidative stress, mitochondrial dysfunction and alterations in calcium homeostasis, thereby increasing neuronal vulnerability (104, 112). The GR-RACK1-BDNF axis provides a mechanistic framework to connect these observations. Previous studies demonstrated that RACK1 is negatively regulated by cortisol through GR-dependent mechanisms and that RACK1 is required for BDNF promoter IV activation (20, 21, 87). In particular, RACK1 contributes to BDNF transcription by promoting chromatin remodelling and the release of MeCP2-mediated repression at BDNF promoter IV. Therefore, alterations in GC signalling may affect BDNF-dependent neuroplasticity through the modulation of RACK1 expression. Since GR alternative splicing determines the relative abundance of functionally distinct GR isoforms, particularly GR α and GR β , an imbalance in this process may influence the transcriptional effects of GCs on RACK1 and downstream BDNF regulation. This mechanism may be particularly relevant in AD-related conditions, where chronic stress, altered GC signalling, reduced neurotrophic support and neurodegenerative vulnerability converge (32, 48, 67, 68). Taken together, these observations support the hypothesis that altered GR alternative splicing may contribute to changes in RACK1 expression and BDNF signalling, thereby linking depression-related neuroendocrine dysfunction to AD-associated molecular alterations.

2. Aim of the thesis

Chronic stress and depression-related glucocorticoid dysregulation are major risk factors for cognitive decline and Alzheimer's disease (AD), yet the molecular mechanisms linking stress susceptibility to neurodegeneration remain incompletely understood. The overarching goal of this thesis is to determine whether differential regulation of glucocorticoid receptor (GR) alternative splicing by the splicing factors SRSF3 and SRSF9 represents a molecular mechanism linking chronic stress vulnerability with AD-related pathology. Specifically, this work investigates whether an imbalance favoring the SRSF3/GR α axis over the protective SRSF9/GR β pathway disrupts RACK1 homeostasis, with potential downstream consequences for neurotrophic signaling.

The first objective of this thesis is to characterize the molecular mechanisms underlying resilience and vulnerability to chronic stress *in vivo*. To this end, we investigate the differential regulation of the SRSF3/SRSF9-GR α /GR β -RACK1 signaling axis in animals displaying either resilient or vulnerable phenotypes following a Chronic Mild Stress (CMS) protocol. Furthermore, we aim to establish the relationship between prolonged glucocorticoid exposure, alterations in SRSF3 and SRSF9 expression, GR alternative splicing, and RACK1 regulation using differentiated neuronal cells exposed to cortisol in combination with gene-silencing approaches.

The second objective is to determine whether these molecular pathways is also altered in Alzheimer's disease-related conditions. To strengthen the translational relevance of these findings, we further examine the transcriptional and protein expression profiles of this signaling pathway in post-mortem human AD cortical tissue, with particular attention to potential post-transcriptional mechanisms contributing to RACK1 dysregulation.

3. Materials and Methods

3.1 Reagents, culture media and antibodies

Hydrocortisone/cortisol (Cat. No. H0888-1G) and mifepristone/RU486 (PubChem CID: 55245) were purchased from Sigma-Aldrich. Cortisol was prepared as a 1 mM stock solution in DMSO, whereas mifepristone was prepared as a 10 mM stock solution in DMSO. Stock aliquots were stored at -20°C and diluted in complete SH-SY5Y culture medium at the time of use to reach the desired final concentrations. The final concentration of DMSO in the culture medium was kept below 0.1%. Anti-RACK1 (sc-17754), anti-GAPDH (sc-365062), anti-GR α (clone G-5, sc-393232), and anti-SRSF9 (sc-134036) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-SRSF3 rabbit polyclonal antibody (Cat. No. A6067) was purchased from ABclonal (Woburn, MA, USA). Anti- β -tubulin antibody was purchased from Sigma-Aldrich/Merck (Cat. No. T0198-25UL). Electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA). Primers and probes for GR α , GR β , RACK1, SRSF3, SRSF9, and 18S rRNA were purchased from Eurofins Genomics.

3.2 Cell culture, differentiation and treatments

Human neuroblastoma SH-SY5Y cells from the European Collection of Cell Cultures (ECACC No. 94030304) were cultured in a medium with equal amounts of Eagle's minimum essential medium (Cat. No. M2279-500ML) and Nutrient Mixture Ham's F-12 (Cat. No. HAM-12-A), supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 0.1 mg/ml streptomycin 100 IU/ml penicillin was purchased from Sigma-Aldrich/Merck (Cat. No. P0781-100ML), and 1X MEM non-essential amino acid solution (M7145, Merck) at 37°C in 5 % CO_2 -containing, and 95 % air atmosphere. SH-SY5Y cells were treated with different concentrations of cortisol (1 and 5 μM) according to previous data (37, 39). SH-SY5Y cells have a stable karyotype consisting of 47 chromosomes and can be differentiated from a neuroblast-like state into mature human neurons through a different mechanism including the vitamin A derivative retinoic acid was purchased from Sigma-Aldrich/Merck (Cat. No. R2625-50MG). Therefore, SH-SY5Y cells can be considered a validate model for a multitude of neurobiology experiments (113-115).

Neuronal differentiation was induced using retinoic acid (RA). Cells were seeded at approximately 40–50% confluence and, after 24 hours, the medium was replaced with

differentiation medium consisting of MEM/F12 supplemented with 1% FBS, L-glutamine, and antibiotics. RA was added at concentrations ranging from 10 μ M depending on the experimental set. Cells were maintained under differentiation conditions for approximately 6 days, with medium replacement every 2–3 days. Morphological changes, including neurite outgrowth, were monitored during the differentiation period.

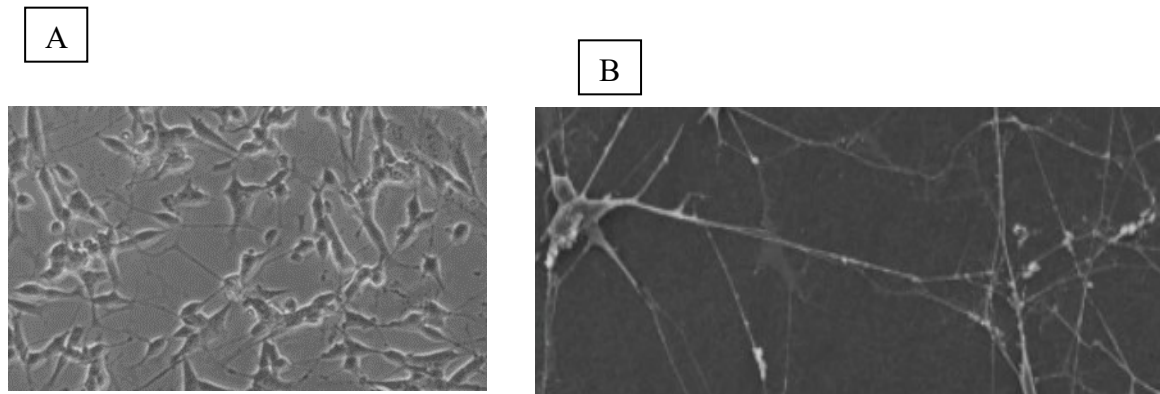


Figure 18. SH-SY5Y neuroblastoma cells. The figure A represents undifferentiated cells; the figure B represents differentiated SH-SY5Y (adapted from ATCC® CRL- 2266).

Cells were detached from the vessel using Trypsin 1% in PBS 1X (Phosphate-Buffered Saline) followed by viable cell counting using the Trypan Blue assay (116), then plated according to the requirements of each experimental assay upon reaching approximately 70–80% confluence. To demonstrate the role of the glucocorticoid receptor, cells were first incubated in charcoal-stripped FBS (DCC) (Cat. No. F6765-100ML) medium for 24 hours to remove endogenous steroid hormones, and then treated with cortisol at final concentrations of 1 or 5 μ M for 24 or 48 h, according to the experimental design. For amyloid- β treatments, differentiated SH-SY5Y cells were exposed to A β 1–40 or A β 1–42 for 48 h to model amyloid-related cellular stress. At the end of the treatment, cells were collected for Western blot analysis of SRSF3, SRSF9, GR α and RACK1 protein expression.

3.3 siRNA-mediated silencing of SRSF3 and GR α

Transfection consists in the introduction of exogenous genetic material into cells. To evaluate whether cortisol effect was GR α -mediated, induced RNA interference on GR α was assessed using the commercially available *GR siRNA mix (MISSION® esiRNA)* from *Sigma-Aldrich*. esiRNA transfection was performed using *Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher)*, it generates liposomes aggregating with esiRNAs that merge with the cell membrane releasing the genetic material inside the cell. Cells were seeded in complete

medium, and the day after the transfection mix was prepared and administrated following the provider indication. Silencing was performed in 6 multi-wells according to manufacturer's instructions.

Briefly, SH-SY5Y cells were transfected with SRSF3-specific *siRNA* or GR α *siRNA* using *Lipofectamine RNAiMAX* Transfection Reagent, according to the manufacturer's instructions. For each well, two separate mixtures were prepared in DMEM medium without FBS and antibiotics: MIX1 containing SRSF3 *siRNA* or GR α *siRNA* diluted in DMEM, and MIX2 containing *Lipofectamine RNAiMAX* diluted in DMEM. The mixtures were incubated separately for 5 min at room temperature, then combined and incubated for 20 min to allow siRNA–lipid complex formation. The transfection mixture was added to each well, and cells were incubated at 37°C in 5% CO₂. 48 hours after transfection, cells were treated for 24 h with 1 μ M cortisol and then collected for immunoblotting analysis.

3.4 Plasmid DNA preparation, transient transfection and luciferase assay

The Δ 1 reporter plasmid constructs were purified with the HiSpeed® Plasmid Midi Kit (Qiagen, Valencia, CA, United States) and then DNA was quantified and assayed for purity using Quantus™ fluorometer (Promega, Madison, WI, United States). Plasmids transient transfection was carried out using Lipofectamine® 2000 (Invitrogen Carlsbad, CA, United States), following the manufacturer's instructions. Each luciferase reporter construct plasmid DNA was co-transfected with the pRL-TK renilla luciferase expressing vector to measure the transfection efficiency (Promega, Madison, WI, United States). Differentiated SH-SY5Y cells were treated as described in figure legends and then lysed with Passive Lysis Buffer 1X, provided by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, United States), following the manufacturer specifications. Luminescence was measured with a 20/20n Luminometer (Turner Bio-Systems, Sunnyvale, CA, United States), with 10s integration time (37).

3.5 Animals and experimental models

Adult male (post natal day 70) Wistar rats (Charles River, Germany) were adapted, housed in groups of 10, to the animal facilities one month before the start of the experiment. During the whole experiment animals were singly housed in standard conditions (12-h light/dark, constant temperature (22 $\pm$ 2 °C) and humidity (50 $\pm$ 5 %)). All procedures used in this study have

conformed to the rules and principles of the 86/609/ EEC Directive and have been approved by the Local Bioethical Committee at the Institute of Pharmacology, Polish Academy of Sciences, Krakow, Poland. All efforts were made to minimize animal suffering, to reduce the number of animals used and the animal studies comply with the ARRIVE guidelines.

Four-month-old Tg2576 mice and age-matched WT controls were used. Cerebral cortex samples were collected and processed for Western blot analysis. Samples were obtained through collaboration with Università Piemonte Orientale.

3.6 Chronic mild stress procedure and sucrose consumption test

Before starting of the experiment, rats were trained to consume 1 % sucrose solution as previously described (117) and sucrose consumption test (SCT) (one bottle test) was performed at weekly intervals throughout the duration of the study. Sucrose intake was calculated by measuring pre-weighted bottles filled with the 1 % sucrose solution. The cut off to discriminate among vulnerable and resilient animals was set at 50 %: vulnerable rats consumed approximately 50 % less sucrose solution in comparison to the baseline (training values), whereas resilient animals showed similar levels of sucrose intake to their baseline, despite the CMS procedure. Animals were randomly divided into two groups: one group of animals was unstressed (No stress group) and the other one was exposed to the CMS procedure for a period of 2 consecutive weeks. The protocol consists of periods of: food or water deprivation, 45-degree cage tilt, intermittent illumination, soiled cage, paired housing, low intensity stroboscopic illumination, periods of no stress (118). Based on the result of the sucrose consumption test carried out following the first 2 weeks of stress, two groups of animals were identified: those showing a significant reduction of sucrose intake in comparison to the control group, ie. developing an anhedonic-like behavior, (CMS vulnerable) and those that did not develop anhedonia (CMS resilient). 24 h after the final stressor animals were decapitated (between 9:10 and 12:25 a.m.), the trunk blood was collected in tubes with ethylenediaminetetraacetic acid and the prefrontal cortex was dissected from the whole brain according to the plates 34–43 of the atlas of (119). and stored at 80 °C.

3.7 Measurement of plasma corticosterone levels

After collection, blood specimens were centrifuged for 20 min at 3000 g at 4 °C for the separation of plasma. Corticosterone plasma levels were assessed with the IBL international enzyme linked immunosorbent assay according to manufacturers' instructions.

3.8 Human AD cortex samples

Human cortical samples from control subjects and AD patients were provided through a collaboration with Prof. Wolozin's laboratory in Boston and were used for Western blot analysis of RACK1 protein expression.

3.9 RNA extraction

Total RNA was extracted from tissue samples using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy), according to the manufacturer's instructions. RNA concentration and purity were assessed by spectrophotometric analysis, as previously described (61). To avoid genomic DNA contamination, RNA samples were treated with DNase (Thermo Fisher Scientific, Italy).

For SH-SY5Y cell samples, total RNA was isolated from cell pellets using the RNeasy Plus Mini Kit (Qiagen), following the manufacturer's instructions. RNA concentration was quantified using the Quantus™ Fluorometer (Promega, Madison, WI, United States). Extracted RNA samples were stored at -80°C until further analysis.

3.10 Protein extraction and Western blot analysis

Organ Tissue

Brain cortical tissues/prefrontal cortex samples were homogenized using a glass-glass potter in the same sucrose homogenization buffer. The total tissue homogenates were centrifuged at $1,000 \times g$ for 10 minutes at 4°C to obtain a pellet enriched in nuclear components. This pellet was resuspended in a specific nuclear buffer (20 mM HEPES, 0.1 mM dithiothreitol (DTT), 0.1 mM EGTA) supplemented with protease and phosphatase inhibitors. The remaining supernatant was further centrifuged at $10,000 \times g$ for 15 minutes at 4°C to isolate

the membrane fraction pellet, which was then resuspended in the same nuclear buffer. The purity of these tissue-derived fractions was verified as previously detailed (120).

SH-SY5Y cells, treated with cortisol, were collected by scraping in cool PBS 1X, and centrifuged at 550 x g at 4°C for 5 min. Cells were lysed by adding from 50 µL up to 100 µL of Lysis Buffer + protease/phosphatase inhibitor cocktail to each cell pellet, depending on its size. After the lysis process, cells are left in ice for 20 minutes and then are sonicated 2-3 times for 20 second at 20Hz to disrupt cell membranes.

The protein content of total cell lysates, cerebral tissue fractions, were determined using the Bradford assay. Bovine Serum Albumin (BSA) as standard was used for calibration curve, and using an INNO-S microplate reader (LTEK/Aurogene) to evaluate absorbance at 595 nm. Sample protein quantification was performed in 96 multi-well plate by adding 18µL of deionized water, 2 µL of sample and 180 µL of Bradford 1X for each well. The data were analysed through *Microsoft Excel* to obtain samples loading volumes. Samples were resuspended in Laemmli Buffer 4X (Bio-Rad Laboratories, Cat. No. 1610747) and boiled at 95°C in thermoblock for 5 minutes to denature proteins.

3.11 SDS-PAGE and Immunoblotting

Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) is a technique that can be used to separate proteins based on their molecular weight. Equivalent amounts of extracted proteins (10 µg up to 30 µg) were loaded in the electrophoresis gel. The protein separation was performed using an electrophoresis camera (BioRad) and soaking gels in the cooled Running Buffer 1X. Gels were run at 120 V. Gels were prepared in our laboratory trough acrylamide polymerization between two glass spacers and adding sample comb during the polymerization of the stacking gel. Gels were composed by: Acrylamide (Acrilogel 2,6 Solution 40% was purchased from Sigma-Aldrich/Merck (Cat. No. A7168-100ML), deionized water, SDS 10%, TRIS HCl 1,5 M pH 8.8 for running gel, TRIS HCl 0,5 M pH 6.8 for stacking gel, ammonium persulfate 10% and TEMED (N,N,N',N'-Tetramethylethylenediamine) as catalysts. Stacking gel was prepared with 5% of polyacrylamide, whereas running gels percentage of polyacrylamide depend on the targeted proteins weigh and the sample concentrations (Table 1).

Table 1. To prepare the gel, the following protocol was used:

Protein	Gel % of polyacrylamide	Gel thickness
RACK1 (36 kDa)	10%	1.5 mm
B- Tubulin (55 kDa)	10%	1.5 mm
GR α (97 kDa)	8%	1.5 mm
GAPDH (37 kDa)	10%	1.5 mm
SRSF3 (20 kDa)	10%	1.5 mm
SRSF9 (26 kDa)	10%	1.5 mm

Protein transfer was performed using the Trans-Blot Turbo system and Trans-Blot Turbo Transfer Packs containing 0.2 μ m nitrocellulose membranes (Bio-Rad Laboratories, Cat. No. 1704158), according to the manufacturer's instructions. Membranes were coloured with red Ponceau S (*Sigma*) and then were wet in 5% BSA solution in TBS 1 X - 0,1 % Tween at room temperature for about 1 hour to block non-specific binding sites. At the end, membranes were incubated overnight at 4°C with protein- specific antibody in tilting agitation. Antibodies were diluted in 5% BSA in TBS 1 X - 0,1 % Tween.

The day after, membranes were washed for 3 times each 10 minutes in TBS 1 X - 0,1 % Tween and were incubated for 1 hour at room temperature in tilting agitation with the secondary antibody, IgG peroxidase anti-mouse (Table 2).

Table 2. Primary and secondary antibody for protein detection in Western Blot analysis:

Primary antibody	Primary antibody dilution	Secondary antibody dilution
Mouse monoclonal antibody anti- RACK1 (sc-17754) (Santa Cruz Biotechnology)	1:1000	1:4000
Mouse monoclonal antibody anti- Tubulin (sc-T0198) (Sigma)	1:1000	1:5000
Mouse monoclonal antibody GAPDH (sc-365062) (Santa Cruz Biotechnology)	1:1000	1:2000
Mouse monoclonal antibody GR α (sc-393232) (Santa Cruz Biotechnology)	1:1000	1:2000
Rabbit polyclonal anti-SRSF3 (ABclonal, Cat. No. A6067)	1:1000	1:4000
Mouse monoclonal anti-SRSF9 (Santa Cruz Biotechnology, sc-134036)	1:1000	1:2000

Immunodetection was performed using chemiluminescence reaction *Western Bright ECL (Enhanced Chemiluminescence)* kit. For signal detection was used ChemiDoc™ MP Imaging System (Bio-Rad Laboratories) and finally the results of the membranes images were analysed by using *ImageJ (NIH)*.

3.12 RT-qPCR

RT-qPCR was performed to evaluate the mRNA expression levels of genes involved in the GR/RACK1 pathway, according to the experimental model analysed.

For tissue samples, gene expression analysis was performed using the iScript™ one-step RT-PCR kit for probes (Bio-Rad Laboratories, Italy) on a CFX384 real-time PCR detection system (Bio-Rad Laboratories, Italy). Samples were run in 384-well plates in triplicate as multiplexed reactions, using 18S rRNA as the endogenous reference gene. Primers and probes for GR α , GR β , RACK1, SRSF3, SRSF9 and 18S rRNA were purchased from Eurofins Genomics. qPCR analyses for human RACK1, SRSF3, SRSF9 and 18S rRNA were performed as previously described (28, 121).

For SH-SY5Y cell samples, RNA was reverse-transcribed using the QuantiTect Reverse Transcription Kit (*Qiagen*), according to the manufacturer's instructions. Briefly, RNA samples were incubated in a thermocycler for 15 min at 42°C, followed by 3 min at 90°C. Real-time PCR was then performed using the QuantiTect SYBR Green PCR Kit (*Qiagen*). RACK1 and 18S primers were used as previously described (28). 18S rRNA was used as the endogenous reference gene.

Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (122) and expressed relative to the corresponding control group.

3.13 Statistical analysis

Each experimental group consists of 4–8 rats. In vitro data consist of at least three independent experiments. Data are presented as means $\pm$ SEM. Statistical analyses were conducted with GraphPad Prism version 7 (*GraphPad Software, San Diego, CA, USA*). Statistical differences were determined by analysis of variance (*ANOVA*) followed, when significant, by an appropriate post hoc test, as indicated in the figure legends. In all reported statistical analyses, effects were designated as significant if the p value was < 0.05 .

3.14 Buffer's composition

Running Buffer 10X pH 8.3

- 30g Tris Base (Trizma)
- 144g Glycine
- 10g SDS 1%
- 1L Water

Running Buffer 1X (500 mL)

- 50 mL Running Buffer 10X
- 450 mL Water

TBS 10X pH 7.5

- 61g Tris Base (Trizma)
- 88g NaCl 1,5 M
- 1L Water

TBS-T 1X

- 100 mL TBT-T 10X
- 900 mL Water
- 1 mL Tween

BSA 5%

- 50 mL TBS-T 1X
- 2,5 g BSA

Lysis Buffer (100mL)

- 0,788 g Tris/HCl 50 mM pH 7.4
- 0,877 g NaCl 150 mM
- 0,186 g EDTA 5 mM
- 1 mL Triton X-100 1%
- + Phosphatase/protease inhibitor cocktail 1:100

Tris HCl 1,5 M pH 8.8

- 18,15 g Tris Base (Trizma)
- 100 mL Water

Tris HCl 0,5 M pH 6.8

- 6,01 g Tris Base (Trizma)
- 100 mL Water

4. Results

4.1 Anhedonic-like behavior induced by chronic mild stress is associated with increased circulating corticosterone levels

The Sucrose Consumption Test (SCT) was used to identify rats exhibiting vulnerability or resilience in response to chronic stress exposure. One-way ANOVA revealed a significant effect of stress on sucrose intake (Figure 19A). A subset of stressed animals, hereafter referred to as vulnerable (CMS vul), exhibited an anhedonic-like phenotype, as evidenced by a significant decrease in sucrose consumption compared to unstressed animals. Conversely, another subset of rats, referred to as resilient (CMS res), showed a sucrose intake comparable to that of unstressed controls, which was significantly higher than that of vulnerable animals. Similarly, one-way ANOVA showed a significant effect of stress on plasma corticosterone levels (Figure 19B). Specifically, the CMS vul group displayed higher circulating corticosterone levels than the No stress group.

Finally, we evaluated the correlation between plasma corticosterone levels and sucrose intake across the stressed subpopulations. As shown in (Figure 19C), this analysis revealed a significant negative correlation. Overall, these data confirm the behavioral stratification of stressed animals into vulnerable and resilient subgroups and indicate that vulnerability to CMS is associated with increased circulating corticosterone levels and reduced sucrose intake (118).

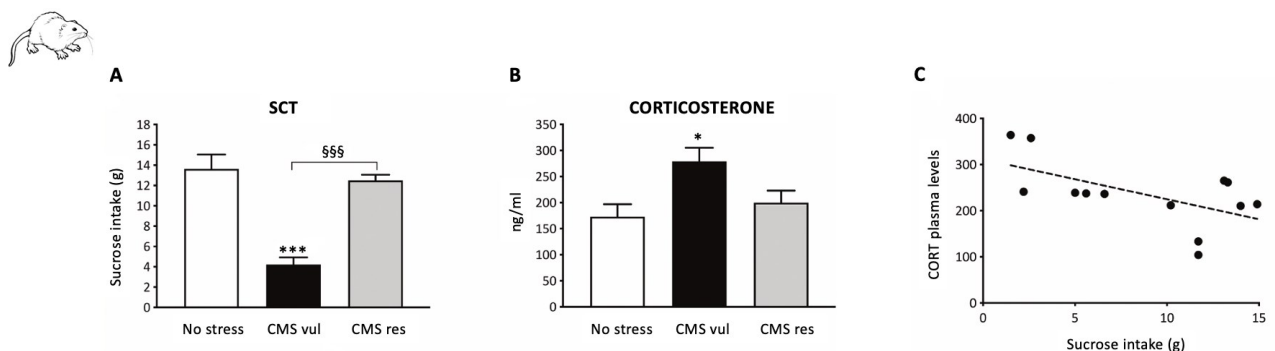


Figure 19. Chronic mild stress induces anhedonic-like behavior and alters circulating corticosterone levels.

(A) Sucrose intake measured by the sucrose consumption test in non-stressed animals, CMS-vulnerable animals, and CMS-resilient animals after two weeks of chronic mild stress. (B) Plasma corticosterone levels measured in the same experimental groups. (C) Pearson's correlation analysis between sucrose intake and plasma corticosterone levels in CMS-vulnerable and CMS-resilient animals. Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$, *** $p < 0.001$ versus No stress; §§§ $p < 0.001$ versus CMS vulnerable animals.

4.2 Chronic mild stress differentially modulates the GR splicing/RACK1 pathway in vulnerable and resilient animals

Previous evidence has shown that chronic stress leads to elevated levels of circulating corticosterone, suggesting a stress-mediated alteration in the functionality of the HPA axis, which is fundamental to the proper maintenance of brain processes. The HPA axis is related to the functionality of the α and β isoforms of the GR, which is the primary regulator. Alterations in the GR splicing factors SRSF3 and SRSF9 appear to regulate the relative production of the two GR isoforms, resulting in distinct isoform-specific effects on glucocorticoid signaling (48, 123, 124).

As illustrated in (Figure 20A), chronic stress did not affect GR α gene expression whereas a significant increase of GR β mRNA levels were significantly increased in resilient animals compared with both unstressed controls and vulnerable rats (Figure 20B). Consequently, the GR β /GR α ratio was significantly altered, showing a specific elevation in the CMS res group (Figure 20C). Additionally, stress significantly influenced the protein expression of SRSF9 (also known as SRp30c), the SR splicing factor that selectively mediates the production of the GR β isoform. Specifically, resilient animals displayed a marked upregulation of SRSF9 protein levels compared to both unstressed controls and vulnerable animals (Figure 20D), a finding consistent with the elevated mRNA levels of GR β observed in this group (Figure 20B). This concomitant increase in GR β and SRSF9 suggests that resilience to CMS is associated with a distinct GR splicing profile, characterized by enhanced GR β -related signaling. Furthermore, consistent with the elevated circulating corticosterone levels (Figure 19B) and the role of SRSF3 in promoting the GR α isoform, which is linked to active glucocorticoid signaling, vulnerable animals showed a significant increase in SRSF3 expression compared to both unstressed controls and resilient rats (Figure 20E). In this context, we specifically examined RACK1 expression, given that it is a GR-regulated gene (125). RACK1 protein expression was significantly reduced in CMS vulnerable animals, whereas CMS resilient animals showed increased RACK1 levels compared with both CMS vulnerable and unstressed animals (Figure 20F).

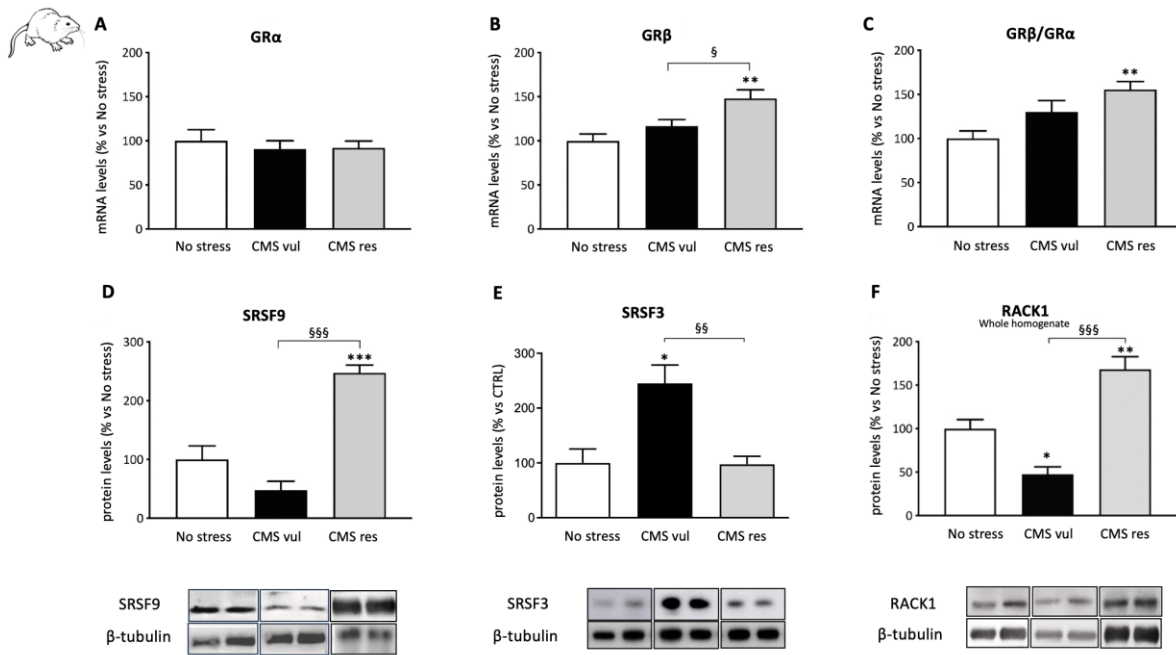


Figure 20. Chronic mild stress differentially modulates glucocorticoid receptor splicing-related factors in vulnerable and resilient animals. (A) GR α mRNA levels, (B) GR β mRNA levels, and (C) GR β /GR α ratio were measured in the prefrontal cortex of non-stressed (No stress), chronic mild stress-vulnerable (CMS vul), and chronic mild stress-resilient (CMS res) rats by quantitative real-time PCR. Gene expression values were normalized to the housekeeping gene and expressed as percentage of the No stress group (set at 100%). (D) SRSF9 protein expression, (E) SRSF3 protein expression, and (F) RACK1 protein levels in whole homogenates were evaluated by Western blot analysis. Representative immunoblots are shown below each graph. Protein levels were normalized to β -tubulin and expressed as percentage of the No stress group (set at 100%). Data are presented as mean $\pm$ SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus No stress; § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ versus CMS vul.

4.3 Cortisol recapitulates stress-associated SRSF3/SRSF9 imbalance in differentiated SH-SY5Y cells

To determine whether glucocorticoid exposure could directly modulate the splicing factors altered in the CMS model, differentiated SH-SY5Y cells were treated with 1 or 5 μ M cortisol for 24 or 48 h as an *in vitro* model of prolonged glucocorticoid exposure (125-127). Similarly, our human neuronal *in vitro* model of chronic glucocorticoid stress showed cortisol treatment increased SRSF3 expression and reduced SRSF9 expression at both mRNA and protein levels (Figure 21A–D). Consequently, a significant decrease in RACK1 mRNA and protein expression was also observed (Figure 21E, F).

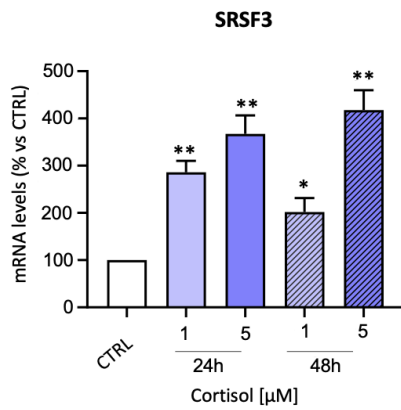
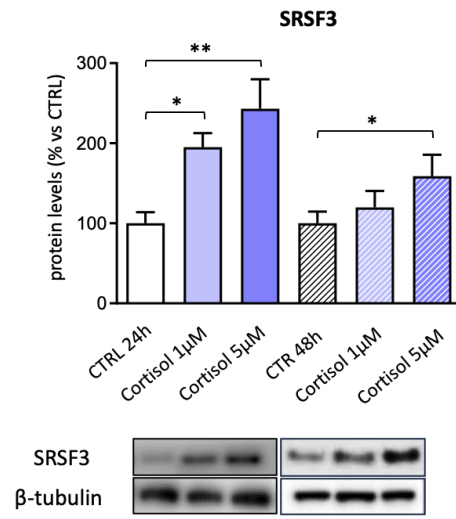
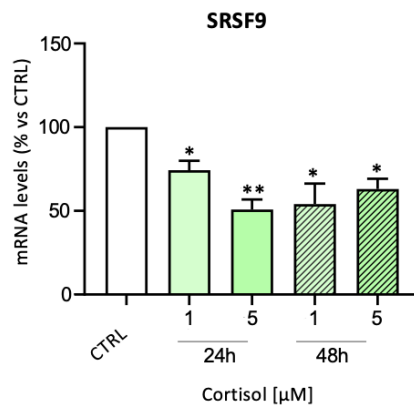
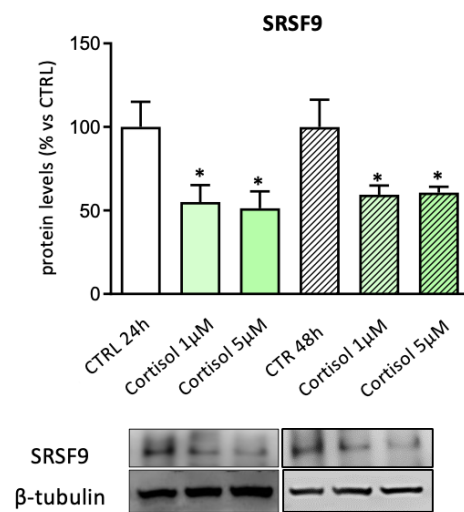
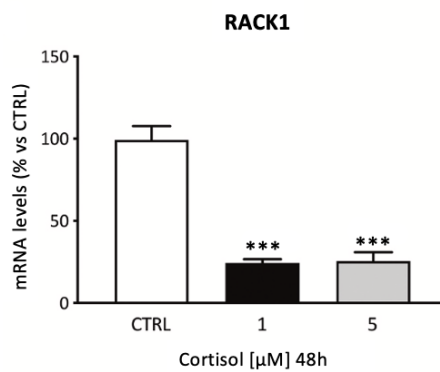
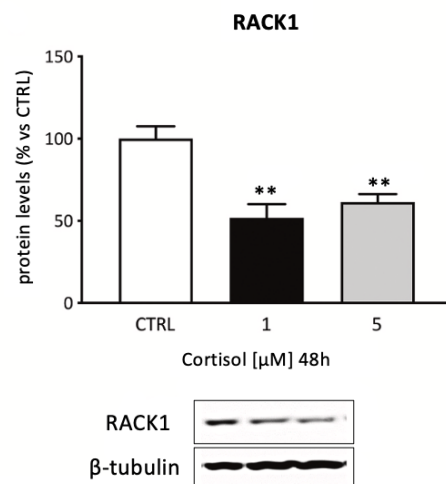
**A****B****C****D****E****F**

Figure 21. Cortisol differentially regulates SRSF3, SRSF9, and RACK1 expression in differentiated SH-SY5Y cells. (A) SRSF3 mRNA levels and (B) SRSF3 protein levels were evaluated in differentiated SH-SY5Y cells treated with cortisol at 1 or 5 μ M for 24 or 48 h. Cortisol treatment significantly increased SRSF3 expression at both the transcriptional and protein levels. (C) SRSF9 mRNA levels and (D) SRSF9 protein levels were assessed under the same experimental conditions. In contrast to SRSF3, cortisol treatment significantly reduced SRSF9 expression at both mRNA and protein levels. (E) RACK1 mRNA levels and (F) RACK1 protein levels were evaluated in differentiated SH-SY5Y cells treated with cortisol at 1 or 5 μ M for 48 h. Cortisol treatment significantly reduced RACK1 expression at both the transcriptional and protein levels compared with control cells. Representative Western blots are shown below the corresponding protein quantifications. Gene expression values were determined by qRT-PCR and normalized to the appropriate housekeeping gene. Protein levels were normalized to β -tubulin and expressed as percentage of control cells (CTRL, set at 100%). Data are presented as mean $\pm$ SEM from at least three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus CTRL.

4.4 SRSF3 and GR α mediate cortisol-induced RACK1 downregulation

Given the cortisol-induced upregulation of SRSF3 and the concomitant reduction in RACK1 expression, we next investigated whether SRSF3 contributes to this effect by modulating GR α expression, a well-established target of SRSF3-dependent alternative splicing. SRSF3 silencing efficiently reduced SRSF3 protein expression under both basal and cortisol-treated conditions (Figure 22A, B). Consistent with previous reports (28, 52), this cortisol-mediated upregulation of SRSF3 was associated with a significant increase in GR α levels (Figure 22A, C). Silencing of SRSF3 abolished this response (Figure 22A, C), further supporting the notion that glucocorticoids sustain their signaling activity by regulating GR splicing in favor of GR α , a mechanism that is also operative in this experimental context. Notably, SRSF3 silencing also prevented the cortisol-induced downregulation of RACK1, which remained comparable to control levels despite cortisol treatment (Figure 22A, D), indicating that SRSF3 is required for the suppressive effect of cortisol on RACK1 expression.

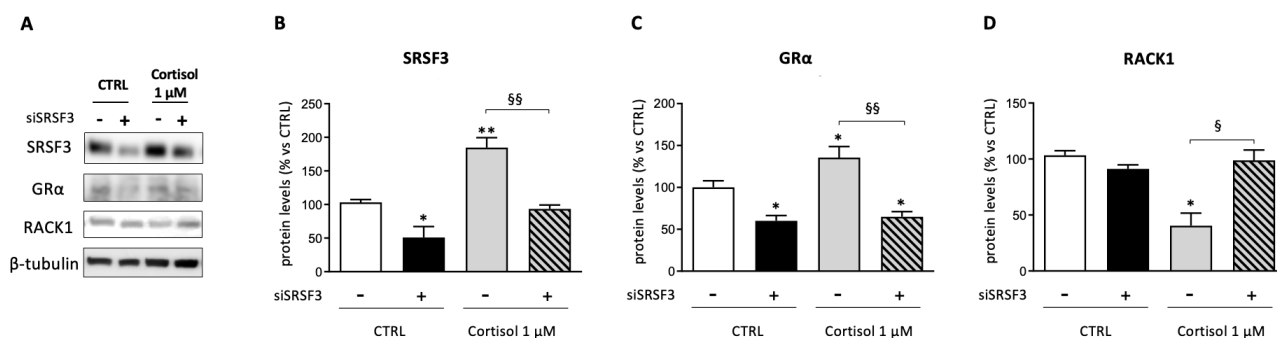


Figure 22. SRSF3 silencing prevents cortisol-induced GR α upregulation and RACK1 downregulation in differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were transfected with siSRSF3 or control siRNA and treated with cortisol 1 μ M. (A) Representative Western blots showing SRSF3, GR α , RACK1, and β -tubulin protein levels. (B–D) Quantification of SRSF3 (B), GR α (C), and RACK1 (D) protein expression. Protein levels were normalized to β -tubulin and expressed as percentage of control cells. Data are presented as mean $\pm$ SEM from independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. * p < 0.05, ** p < 0.01 versus corresponding control; § p < 0.05, §§ p < 0.01 versus cortisol-treated cells.

To further test the involvement of GR α in cortisol-mediated RACK1 regulation, GR α was silenced in differentiated SH-SY5Y cells. GR α silencing prevented the cortisol-induced reduction of RACK1 protein levels, indicating that GR α is required for this response (Figure 23A–C). Consistently, pharmacological inhibition of GR with mifepristone (RU486) also prevented cortisol-induced RACK1 downregulation (Figure 23D). Finally, luciferase reporter analysis showed reduced activity of the Δ 1 RACK1 promoter construct following cortisol treatment, supporting promoter-level regulation of RACK1 by glucocorticoid signaling (Figure 23E). Together, these results support a model in which cortisol-induced SRSF3 upregulation favors GR α -dependent repression of RACK1 expression.

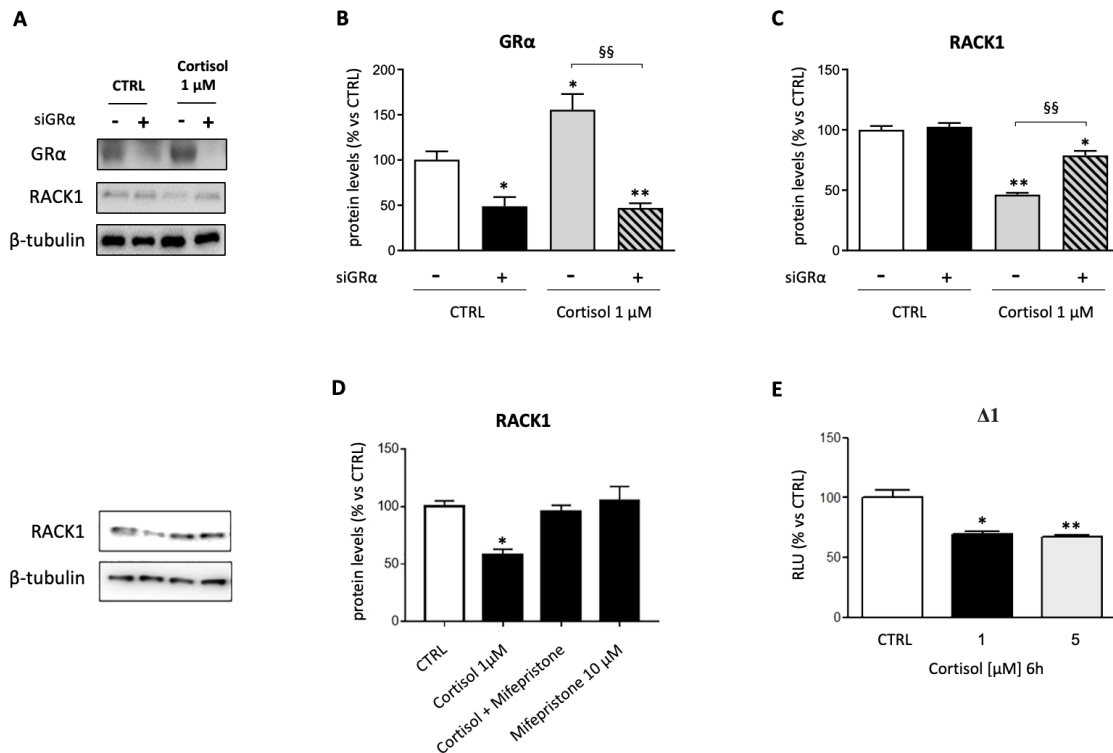


Figure 23. GR α mediates cortisol-induced regulation of RACK1 expression. (A) Representative Western blots showing GR α and RACK1 protein levels following siGR α transfection and cortisol treatment (1 μ M). (B–C) Quantification of GR α (B) and RACK1 (C) protein levels following GR α silencing in control and cortisol-treated cells. (D) Effect of the GR antagonist mifepristone (10 μ M) on cortisol-induced RACK1 protein expression. Representative immunoblots are shown below the corresponding graph. (E) Luciferase reporter assay showing the activity of the Δ 1 RACK1 promoter construct after 6 h exposure to cortisol (1 or 5 μ M). Protein levels were normalized to β -tubulin and expressed as percentage of CTRL cells (set at 100%). Luciferase activity is expressed as relative luciferase units (RLU) normalized to CTRL. Data are presented as mean $\pm$ SEM from three independent experiments (n = 3). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test. *p < 0.05, **p < 0.01 vs corresponding CTRL group; §§p < 0.01 vs cortisol-treated cells.

4.5 Amyloid- β triggers SRSF3-dependent splicing of the GR and RACK1 downregulation: evidence from *in vitro* and *in vivo* models

To determine whether the GR splicing mechanism identified under glucocorticoid stress also operates in the context of amyloid pathology, we next examined SRSF3-GR signaling in experimental models of AD. A dysregulation of the HPA axis has been associated with elevated GC levels in AD (128), and increased cortisol levels have also been reported in patients with mild cognitive impairment (MCI). Consistent with this evidence, stress or the chronic administration of GCs increase the expression of amyloid precursor protein (APP) and the

enzymes responsible for amyloidogenic processing, specifically β -secretase 1 (BACE1) and presenilin 1 (PSEN1) (129). Indeed, different studies have demonstrated that cortisol increases the production of A β by enhancing the levels of the enzyme BACE1 through genomic processes involving GR binding to the BACE1 gene promoter region, thereby enhancing transcription (129). In accordance with these findings, subchronic GR inhibition with mifepristone (RU486), has been shown to rescue early episodic memory and synaptic plasticity deficits in four-month-old Tg2576 mice, a model of AD characterized by overexpressing a mutant form of APP (isoform 695) with the Swedish mutation (KM670/671NL), resulting in elevated levels of A β and ultimately amyloid plaques. Indeed, research has demonstrated that this model exhibits features reminiscent of early human AD pathology, including disruptions to the HPA axis, circadian rhythm, feedback mechanisms, and episodic memory (130). These findings suggest the potential for soluble A β species, particularly A β_{1-42} , to modulate GR isoform expression and thereby influence downstream GR-dependent signaling pathways. Indeed, in the cortex of four-month-old Tg2576 mice, an over-expression of SRSF3 was detected (Figure 24A), correlating with an increase in the GR α isoform, which ultimately led to RACK1 downregulation (Figure 24B, C). Similar results were obtained in differentiated SH-SY5Y exposed to low nanomolar concentrations of A β_{1-40} or A β_{1-42} for 48 h (Figure 24D, E). This paradigm is commonly used to mimic the chronic, sublethal, amyloid-induced cellular stress that is typical of the early, symptomatic, pre-plaque stage observed in Tg2576 mice. These findings are in line with previous evidence showing that SH-SY5Y cells treated with A β_{1-42} fibrils promote SRSF3 overexpression, which has been shown to be responsible for the alternative splicing of tropomyosin receptor kinase B (TrkB) pre-mRNA, leading to the generation of TrkB-Shc. This is a truncated version of the TrkB receptor that, while still able to bind BDNF, lacks the intracellular kinase domain and, therefore, cannot activate downstream pathways essential for neuronal survival, growth, differentiation, and plasticity (131). Together with the previously described RACK1-BDNF axis these findings further support a central role for SRSF3 in orchestrating neurotrophic dysregulation under conditions of chronic stress and amyloid exposure.

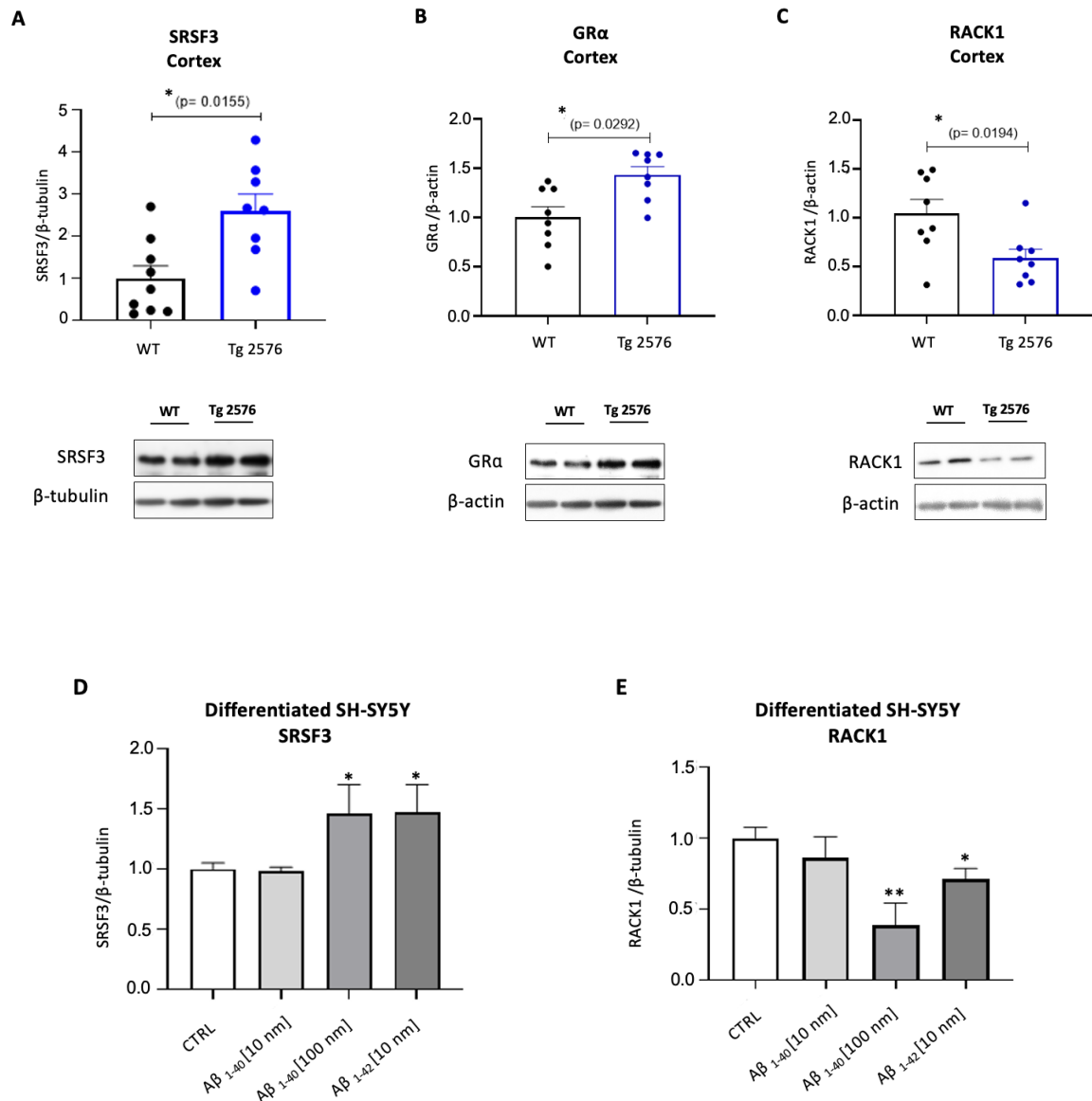


Figure 24. Amyloid-related pathology is associated with SRSF3/GRα upregulation and RACK1 downregulation in AD-related models. (A) SRSF3 protein levels, (B) GRα protein levels, and (C) RACK1 protein levels were evaluated by Western blot in the cerebral cortex of four-month-old WT and Tg2576 mice. Representative immunoblots are shown below the corresponding quantifications. (D) SRSF3 and (E) RACK1 protein levels were assessed in differentiated SH-SY5Y cells exposed to Aβ₁₋₄₀ at 10 or 100 nM, or Aβ₁₋₄₂ at 10 nM, for 48 h. Protein levels were normalized to the corresponding loading control and expressed relative to the control group. Data are presented as mean ± SEM. Statistical analysis was performed using unpaired Student's t-test for WT versus Tg2576 comparisons and one-way ANOVA followed by Tukey's multiple comparison test for in vitro experiments. *p < 0.05, **p < 0.01 versus the corresponding control group.

4.6 Analysis of SRSF3/GR/RACK1 expression in human AD cortex

Since RACK1 exerts its biological functions primarily at the protein level as a scaffold and ribosome-associated protein, we next evaluated RACK1 protein expression in human AD cortex samples. Western blot analysis showed a significant reduction of RACK1 protein levels in AD cortex compared with control samples (Figure 25). Thus, RACK1 showed divergent regulation at the transcript and protein levels in AD-related human datasets and tissue samples, suggesting that its expression in AD may be influenced by regulatory mechanisms beyond transcript abundance.

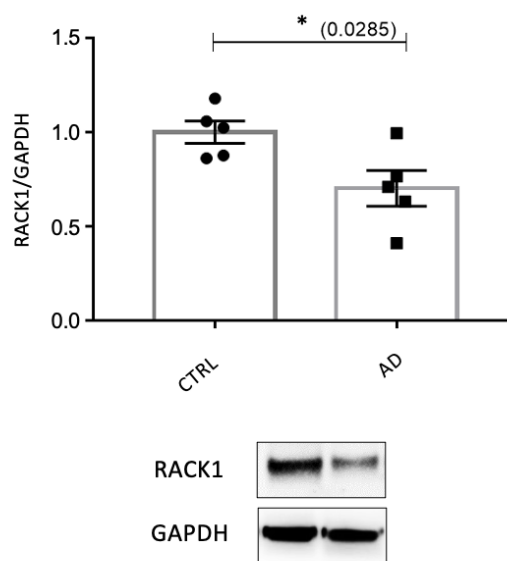


Figure 25. RACK1 protein expression is reduced in the cerebral cortex of Alzheimer's disease patients. RACK1 protein levels were analyzed by Western blot in cerebral cortex samples from control (CTRL) and Alzheimer's disease (AD) patients. Representative immunoblots for RACK1 and GAPDH are shown below the graph. Protein expression was normalized to GAPDH and is presented as the RACK1/GAPDH ratio. Data are shown as mean $\pm$ SEM, with individual biological samples represented by dots. Statistical significance was determined using an unpaired Student's *t*-test. The exact *p* value is indicated above the comparison (*p* = 0.0285).

To complement the protein analysis, we next explored whether the expression of genes belonging to this pathway was altered in the cerebral cortex of AD patients. Analysis of two independent human cortical transcriptomic datasets revealed a trend toward dysregulation of several components of the SRSF3/GR/RACK1 pathway in AD samples. SRSF3 expression was increased in AD cortex, while NR3C1 showed a similar pattern. SRSF9 did not show major

changes. RACK1 transcript levels were also increased in AD cortical datasets, suggesting transcriptional alteration of this pathway in AD cortex (Figure 26A–H).

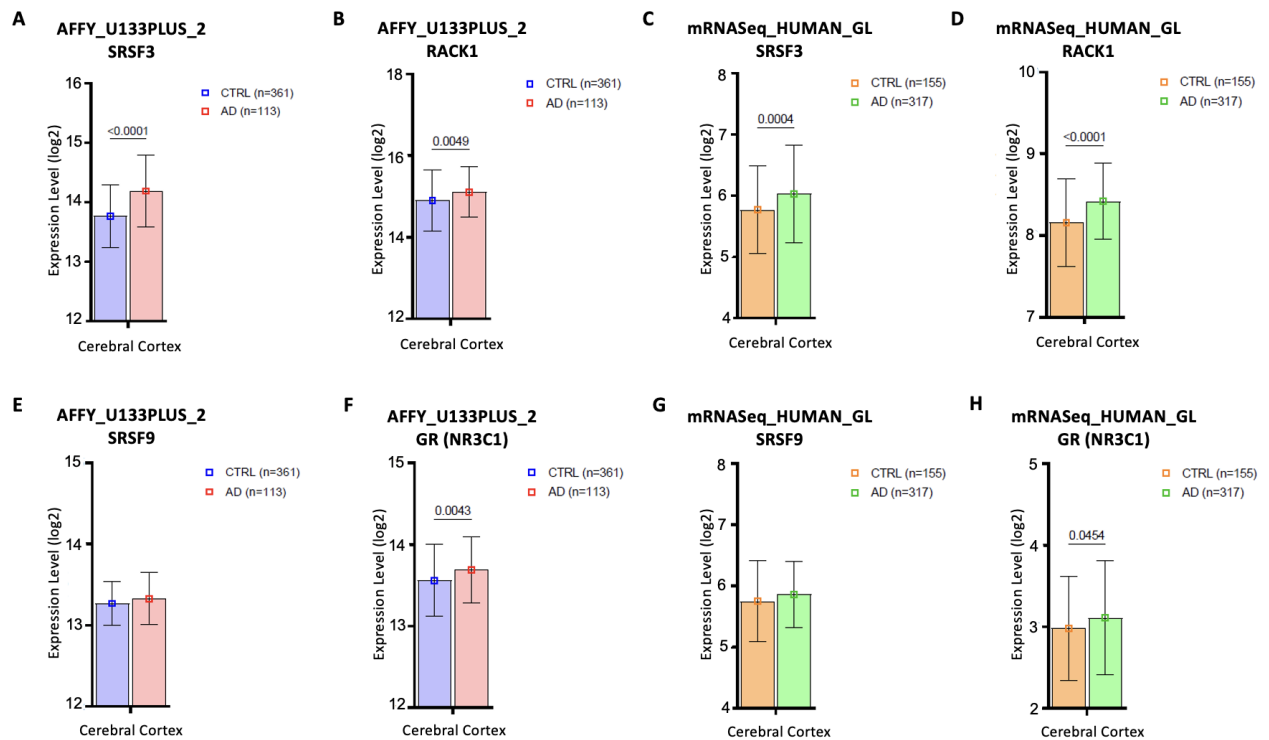


Figure 26. Dysregulation of the SRSF3/GR/RACK1 pathway in the cerebral cortex of Alzheimer's disease patients. Gene expression levels of SRSF3 (A, C), RACK1 (B, D), SRSF9 (E, G), and GR (NR3C1) (F, H) were analyzed in cerebral cortex samples from control (CTRL) and Alzheimer's disease (AD) patients using two independent human transcriptomic datasets: AFFY_U133PLUS_2 (A, B, E, F) and mRNASeq_HUMAN_GL (C, D, G, H). Expression values are reported as log₂-transformed normalized expression levels. Data are presented as mean ± SD. Statistical significance was determined using an unpaired Student's *t*-test. Exact *p* values are indicated above the corresponding comparisons. Sample sizes are reported in each panel. Data source name (AFFY_U133PLUS_2) and (mRNASeq_HUMAN_GL).

5. Discussion and Conclusions

The present study demonstrates that susceptibility or resilience to chronic mild stress (CMS) is determined by a distinct and divergent regulation of the glucocorticoid receptor (GR) signaling pathway within the prefrontal cortex. Central to this mechanism is the balance between GR alternative splice variants, which is differentially controlled by the splicing factors SRSF3 and SRSF9 and ultimately dictates the expression of the scaffold protein RACK1.

Resilient animals exhibited a selective increase in SRSF9 expression accompanied by enhanced levels of the GR β isoform, resulting in a marked elevation of the GR β /GR α ratio. Since GR β is widely recognized as a dominant-negative regulator of GR α -mediated transcriptional activity (50), this molecular profile likely represents an adaptive mechanism that limits excessive glucocorticoid signaling, thereby protecting neuronal tissue from corticosterone-induced toxicity. In contrast, vulnerable animals showed no changes in GR β expression but displayed a significant increase in SRSF3, a splicing factor that preferentially promotes the generation of the transcriptionally active GR α isoform. This shift reinforces glucocorticoid signaling, leading to persistent activation of GR α and subsequent downregulation of RACK1, a scaffold protein whose expression is negatively regulated by chronic GR activation (39-41).

Our *in vitro* experiments using differentiated SH-SY5Y neuronal cells provide direct mechanistic support for the observations obtained in vulnerable animals. Prolonged cortisol exposure, employed as a cellular model of chronic stress, faithfully reproduced the vulnerable phenotype by inducing a dose-dependent increase in SRSF3 together with a concomitant reduction in SRSF9. This imbalance promoted GR α upregulation and consequent repression of RACK1 expression. Importantly, silencing SRSF3 completely abolished both cortisol-induced GR α upregulation and RACK1 downregulation, demonstrating that SRSF3 acts as an essential upstream regulator of this pathological cascade. Collectively, these findings indicate that resilience is not merely the absence of pathological responses but rather an active molecular adaptation mediated by the SRSF9/GR β axis, which functions as a transcriptional safeguard against excessive glucocorticoid signaling. Conversely, vulnerability is driven by activation of the SRSF3/GR α pathway, which amplifies stress-induced transcriptional responses and ultimately disrupts RACK1 homeostasis.

To extend the translational significance of these findings, we investigated whether the SRSF3/GR/RACK1 signaling axis identified under chronic glucocorticoid stress is also

involved in Alzheimer's disease (AD), a neurodegenerative disorder strongly associated with hypothalamic-pituitary-adrenal (HPA) axis dysregulation and chronic hypercortisolemia. Sustained glucocorticoid exposure has been shown to accelerate amyloidogenesis through GR-dependent transcriptional activation of APP, BACE1, and PSEN1, suggesting that dysregulated GR signaling may contribute directly to AD pathogenesis.

We first explored this hypothesis *in vivo* using 4-month-old Tg2576 mice, an early-stage model of amyloid pathology. These animals exhibited a significant cortical increase in SRSF3 and the transcriptionally active GR α isoform, accompanied by a marked reduction in RACK1 protein levels. This molecular signature closely mirrors the vulnerable phenotype observed in the CMS model. Considering previous evidence demonstrating that pharmacological GR inhibition with mifepristone (RU486) ameliorates cognitive deficits in Tg2576 mice, our findings suggest that early amyloid accumulation exploits the SRSF3/GR α signaling pathway to potentiate glucocorticoid-dependent neurotoxicity, thereby promoting the initial loss of RACK1.

Consistent with the *in vivo* findings, treatment of differentiated SH-SY5Y cells with nanomolar concentrations of soluble A β 1–40 was sufficient to induce SRSF3 upregulation. These data provide direct mechanistic evidence that soluble amyloid species can independently activate the initial steps of this neurotoxic pathway, establishing a feed-forward mechanism in which amyloid pathology and GR dysregulation mutually reinforce one another.

To validate the translational relevance of these observations, we analyzed publicly available human AD transcriptomic datasets together with post-mortem cortical samples. Both approaches consistently revealed increased expression of SRSF3 and NR3C1, the gene encoding GR, in agreement with our experimental models. Interestingly, however, RACK1 expression displayed a striking discrepancy between transcript and protein levels. While transcriptomic analyses showed increased RACK1 mRNA expression, Western blot analysis of human AD cortical tissue demonstrated a significant reduction in RACK1 protein abundance.

This apparent discordance strongly suggests that RACK1 expression becomes uncoupled from its transcriptional regulation during advanced stages of AD and is instead predominantly controlled by post-transcriptional mechanisms. At least two non-mutually exclusive processes may explain this phenomenon. First, the increased RACK1 mRNA levels may reflect a compensatory transcriptional response aimed at restoring protein levels in the face of progressive neuronal dysfunction. However, this homeostatic attempt appears insufficient to

recover functional RACK1 expression. Second, the highly toxic cellular environment characteristic of the AD brain may profoundly impair RACK1 protein synthesis and stability through mechanisms such as microRNA-mediated translational repression, defective ribosome activity, and enhanced ubiquitin-proteasome-dependent degradation (33, 132). Together, these alterations would effectively override transcriptional upregulation, resulting in a substantial depletion of functional RACK1 protein.

Importantly, the apparent differences between the human AD samples and the Tg2576 model should also be interpreted in the context of disease stage. Human post-mortem tissues represent advanced, late-stage neurodegeneration occurring in aged individuals, whereas the Tg2576 mice analyzed in this study correspond to an early phase of amyloid pathology. Although this model does not fully reproduce the complexity of late-stage Alzheimer's disease, it provides valuable insight into the earliest molecular events preceding extensive neurodegeneration. Our findings therefore support a model in which early activation of the SRSF3/GR α pathway represents a molecular priming event that establishes a persistent state of glucocorticoid hypersensitivity. As disease progresses, this chronic imbalance may progressively compromise RACK1 homeostasis, shifting from an initial transcriptionally driven dysregulation to profound translational and post-translational impairment, ultimately contributing to neuronal dysfunction and disease progression.

Overall, the present study identifies the SRSF3/SRSF9-dependent regulation of GR alternative splicing as a previously unrecognized molecular switch governing susceptibility or resilience to chronic stress. Moreover, it demonstrates that the same signaling axis is aberrantly activated during the early stages of Alzheimer's disease, linking glucocorticoid dysregulation with amyloid pathology through a common mechanism centered on RACK1 loss. Overall, the present study identifies the SRSF3/SRSF9-dependent regulation of GR alternative splicing as a previously unrecognized molecular switch governing susceptibility or resilience to chronic stress. Moreover, our findings suggest that dysregulation of this signaling axis may also contribute to the early molecular events associated with Alzheimer's disease, linking glucocorticoid signaling and amyloid pathology through alterations in RACK1 homeostasis. Further studies in additional experimental models and human systems will be required to determine the extent to which modulation of the SRSF3/GR α /RACK1 and SRSF9/GR β pathways contributes to disease progression and to evaluate their potential as targets for therapeutic intervention.

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