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Partners, targets and modulators of LSD1 in stress-response regulation

(Sector Bio / 13 - Applied Biology)

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INTRODUCTION

The ability to learn is essential for every form of life. In pluricellular organisms endowed with a nervous system, no matter how rudimental, such a cognitive function is undertaken by neurons. These excitable cells talk to each other at the level of synapses, where neurotransmitter release propagates an initial sensory stimulus to many postsynaptic neurons eventually mediating behavioral effects [1] [2]. The process of memorization takes place whenever a given stimulus successfully changes the electric properties of a competent synapse, allowing a neuron to respond differently when the same stimulus is perceived again [3] [4]. In other words, to some extent, to remember means to recognize something already seen.

Memories are morphologically, biochemically and electrically encoded at the level of dendritic spines (the postsynaptic side of the synapses) that appear, grow, shrink and disappear in a highly *plastic* fashion [1] [2]. We now know that these stimuli-induced changes of neuronal morphology are instrumental to remember [5].

Although short-term memory formation can be independent on protein synthesis, the process of gene expression is fundamental to memory consolidation. Newly synthesized receptors, scaffold and cytoskeleton proteins are indeed required to shape new spines and to stabilize them allowing their growth and maturation, thereby consolidating the encoded information under the form of a long-term memory [5].

Coupling bunches of *de novo* gene expression to stimuli-induced neuronal activation, inducing those molecular processes that concur to memory formation and potentiation exactly in the moment in which the stimulus is perceived, matches the requirements of stable dendritic spines morphological modifications allowing encoding long-term memories [6] [1]. The ability of neurons to change their shape and connective properties in response to environmental changes is called *neuroplasticity*. Neuroplasticity stands at the basis of learning, memory formation, and emotional and affective processing, the highest functions accomplished by the nervous system [1].

During my PhD program I concentrated on a milestone process underlying plasticity of neurons referred to as “activity-dependent gene transcription”. This process involves the neuronal nucleus as the final target of neurotransmitters (Glutamate), neuromodulators (examples are serotonin and the catecholamines dopamine, epinephrine and norepinephrine) and neurotrophin (BDNF and NGF) signal transduction [7]. At first, ligand binding (for example glutamate) to synaptic receptors entails important modifications of the neuronal transmembrane electric potential

mainly by Na^{+} and Ca^{++} influx through ionotropic receptors (like glutamate receptors AMPA and NMDA) giving raise to –when thresholds are overcome– action potentials and neuronal activation [8]. Thus, in response to neuronal activity, promoted by neurotransmitters binding to their specific synaptic receptors, many pathways are induced including, but not limited to Ras/MAPKs pathway and G-protein coupled receptors/cAMP pathways [9]. These pathways play both cytosolic and nuclear roles. At the cytosolic level, as a second messenger, increase Ca^{++} concentration is responsible for many biochemical modifications mainly operated via calcium-dependent kinases activation. Resulting posttranslational modifications of the synaptic proteome are mostly related to transient changes of synaptic properties underlying short-term memory formation, like early phases of synapse long-term potentiation and depression (eLTP and eLTD) [9]. These early forms of long-term neuronal plasticity are induced by ionotropic glutamate receptor trafficking and do not require gene expression. For instance, increased number of AMPA receptors at the membrane levels is sufficient to induce eLTP. On the other hand, neuronal activation also directs transduction pathways toward the nucleus that promote gene expression by inducing transcription of complex clusters of genes, in turn allowing to perpetuate early forms of synaptic plasticity to late phases (late-LTP and late-LTD) underlying long-term memory [10] [11] [12].

The first cluster of genes transcribed in response to neuronal activation is the *Immediate Early Genes* cluster (IEGs), peaking as early as 30 minutes after stimulation [6] [13] [14]. These genes are mainly represented by transcription factors that in turn activate transcription of many more downstream genes, including cytoskeletal and post-synaptic scaffolding factors that together contribute to dendritic spines reshaping inherent to memory formation [13]. IEGs are indeed fundamental to memory, since IEGs activation represents necessary condition to memory consolidation [13] [6]. Long-known transcription factors, such as *cAMP responsive element-binding protein* (CREB) and *Serum Response Factor* (SRF) represent final targets of above-mentioned transduction pathways promoted by neuronal activation and induce gene transcription in response to environmental cues [15] [16] [7]. In particular SRF directly responds to MAPK pathway activation, which is one of the most studied mechanisms of glutamate signal transduction promoting long-term typologies of neuronal plasticity [15] [16] [7]. Activity-dependent gene transcription at neuronal nuclei is extraordinarily regulated at multiple layers that go beyond transcription factor activation as final step of

neurotransmitter signal transduction. Other processes play peculiar direct and homeostatic roles toward neuromorphologic modulation including enhanced proteome variability via induction of specific programs of alternative splicing, and the different components of *epigenetic* regulation of gene expression. In this work I will present new evidences of epigenetic regulation of neuronal function mediated by a novel protein partnership between SRF and an epigenetic cofactor, *Lysine Specific Demethylase 1* (LSD1), which helps SRF accomplishing to its role in modulating activity-dependent transcription in neurons. It has been recently clarified that the set of molecular/epigenetic processes allowing environmental adaptation via inherent regulation of transcription holds a pivotal relevance in neuronal plasticity and physiology.

1. *Serum Response Factor* (SRF)

1.1 SRF: structure

The *Serum Response Factor* (SRF) is a transcription factor, part of a DNA binding proteins superfamily called MADS (MCM1, Agamous Deficiens, and SRF) superfamily box. This superfamily group of transcription factors is distinguished by the presence of a specific DNA binding and dimerization domain called MADS box, which consists of 90 amino acids and highly conserved in all eukaryotes [17].

The SRF transcription factor, by means of the MADS domain, binds DNA as a dimer at a consensus sequence named the *Serum Response Element* (SRE) which exists in the promoter of the target genes. The structure of the SRE is represented by the motif $CC(A/T)_6GG$ and for this reason is also named CArG box [15].

In humans, the gene responsible for encoding the SRF transcription factor is placed on the short arm of chromosome 6 (6p21.1) and consists of 7 exons and 6 introns. The SRF protein is composed of 508 amino acids, has a molecular weight of 67 KDa [18] and consists of an N-terminal domain, the DNA-binding domain, a dimerization domain and an interface in order to interact with other proteins; in the C-terminal region there is the transactivation domain with numerous phosphorylation sites which function as the recruitment signal for associated factors [19] [17].

The SRF transcription factor can be stimulated by numerous extracellular stimuli, like serum, growth factors, mitogens, lipopolysaccharide and, relevantly for my work,

neurotransmitters together with agents that determine an increase in intracellular calcium concentration. The SRF dimer binds the SRE sequence in the promoter of the target gene and controls transcription by means of the recruitment of molecular partners that handle the translation of a specific extracellular signal into different gene expression profiles [20] [21]. In fact, aiming to perform its activity by integrating the distinct extracellular stimuli that converge on its activation, SRF is associated with a series of cofactors. The most significant cofactors in regulating SRF activity are ternary complex proteins *Ternary Complex Factors* (TCFs) [22] and family members of myocardial transcription factors *Myocardin-Related Transcription Factors* (MRTFs) [23]. The extracellular stimulation gives rise to SRF activation by way of the signaling pathway of the Ras-Raf-MEK1/MEK2 MAP kinases that converge on the ternary complex, as well as the signaling pathway of the *calcium-calmodulin kinases dependent* (CaM) activated by the growth of intracellular calcium. The MRTFs, whose role is to bind SRF and together mediate the transcription of cytoskeletal targets, are initiated by a pathway that instead involves the activation of the Rho-GTPases (Fig. 1).

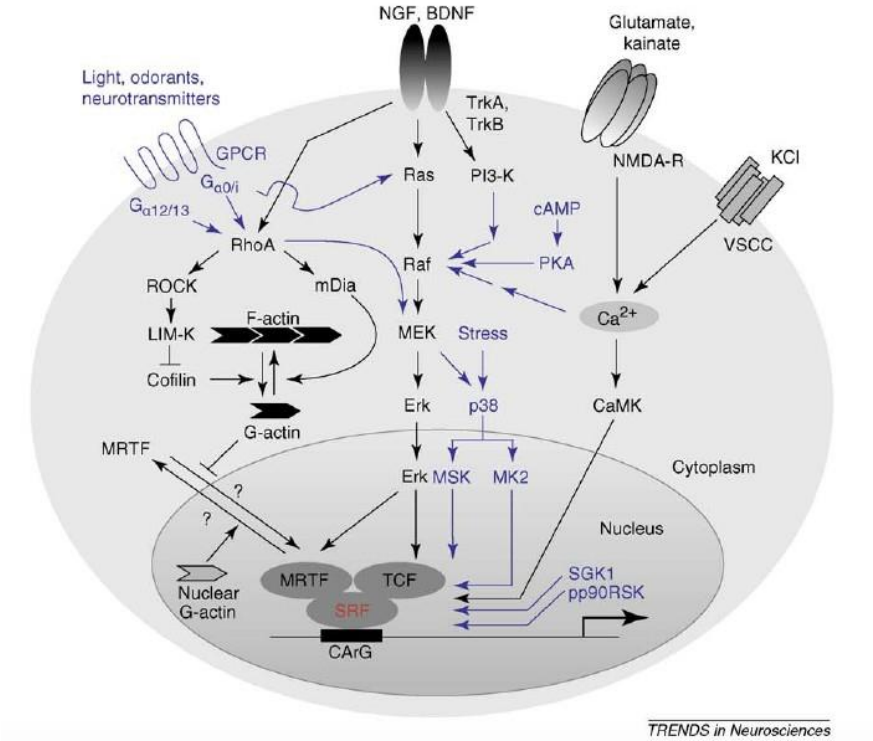

Fig. 1 Cellular pathways leading to activation of SRF in neurons. Several neuronal stimuli lead to the activation of SRF; among these there are some growth factors, such as NGF and BDNF, which activate tyrosine kinase receptors, and neuronal activity through the action of the glutamate neurotransmitter and KCl. The downstream signaling pathway involves the activation of MAP kinases, CaM kinases and RhoA-GTPases and results in the activation of SRF or MRTF and TCF cofactors (from Knöll and Nordheim 2009) [15].

1.2 SRF: function

The identification of SRF occurred in the mid-1980s, following a finding regarding the addition of serum to a culture of quiescent cells which caused rapid transcription of the *c-fos* gene, whose mRNA peaked 30 minutes as a result of the addition of serum in the medium [24]. The *c-fos* gene is a proto-oncogene, whose activation is necessary for quiescent cells to enter in the G1 phase of the cell cycle and subsequently in mitosis. Given its rapid activation in the absence of new protein synthesis, *c-fos* has been arranged as an IEG. Further investigations on the signaling pathways upstream of the rapid transcription of *c-fos*, led to the identification of a short sequence in the promoter of the 300 bp localized gene upstream of the transcription initiation site, whose name is *Serum Response Element* (SRE) since it is necessary and sufficient to make a promoter responsive to the serum [25]. Subsequent studies made it possible to identify the transcription factor that binds this regulatory element of gene expression, which is precisely called *Serum Response Factor* (SRF).

Up to now, around 160 genes have been estimated to be direct transcriptional targets of the SRF transcription factor and roughly half have been validated as a test [26].

All SRF target genes known to date comprise one or more CArG boxes within their promoter. The majority of the SRF target genes deal with cell growth and differentiation, cell migration and cytoskeletal reorganization.

Even if SRF is expressed in all tissues, its activity is specifically relevant in the development of smooth and skeletal muscle tissue, in cardiac muscle tissue, but it also has a relevant function in the development and physiology of the *nervous system*, intervening to modulating IEGs expression during the development also regulating the physiology of adult neurons. In fact, with the support of the regulation of the actin cytoskeleton and of the effectors linked to neuronal plasticity, SRF grants proper neuronal migration, synapse formation, dendritic growth and neurite differentiation, myelin formation and synaptic plasticity mechanisms, including enhancement of long-term potentiation at the base of memory and learning [15] (Fig. 2).

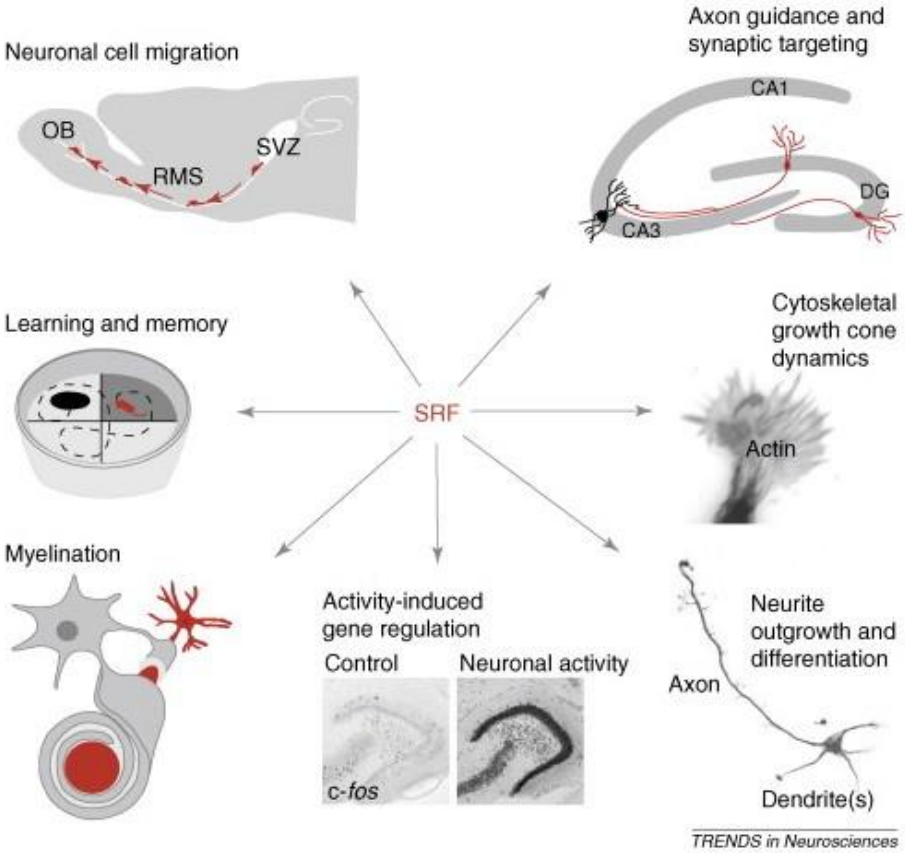

Fig. 2 Functions of SRF in the central nervous system. Within the central nervous system SRF regulates neuronal migration, axon formation, synaptic plasticity, dendritic growth, myelin formation, neurite growth and differentiation and finally the transcription of activity-dependent genes, memory training and learning (from Knöll and Nordheim 2009) [15].

As said, relevant SRF targets are the *Immediate Early Genes* (IEGs) [14], which are activated rapidly and transiently in response to extracellular stimuli and, significantly, in the brain handle the regulation of neuronal plasticity phenomena at the base of learning and consolidation of memory [13]. Genes encoding transcription factors such as c-Fos, Egr1, Egr2 and Npas4 form part of IEGs and are transcribed within 30 minutes following a stimulus. Egr1, for instance, is an SRF target that contains five CArG box sequences on its promoter [25] [27]; it is a proto-oncogene involved in different cellular procedures like regulation of cell proliferation, transformation and programmed death [27]. Other SRF target genes coding for protein effectors directly intervene in a cellular function, including Arc and Bdnf, which are generally expressed more slowly, following regulation by early-expressed transcription factors. Bdnf, for instance, is a significant neurotrophin with a role in dendritic remodeling and synaptic plasticity [28]. In this way the IEGs monitor the processes of synaptogenesis such as the growth of neurites, their maturation and the development of excitatory and inhibitory synapses [29], processes

that bring to changes in the neuron morphology and the structure of the synapses, which are the foundation of the process of learning and training of memory.

1.3 SRF: activator or repressor?

SRF is a transcription factor whose features include its high functional versatility, exerting on its target genes opposite effects of activation or repression of transcription. In neuronal cells, a genome-wide study shows that most mRNAs of SRF target genes are downregulated in SRF^{KO} neurons compared to wild type control. However, in case of lack of stimulation, a part of IEGs mRNA is upregulated in SRF^{KO} neurons compared to wild type control, suggesting that SRF can act directly or indirectly also by transcription repressor [30]. Thus, SRF transcription factor has a twofold function in regulating gene expression, which activates transcription following extracellular stimulation, and at the same time repressing transcription under basal conditions. It has been assumed that SRF can exert its functional versatility by means of cofactors recruitment of and auxiliary proteins that are responsible for mediating the effect of repression or activation on its target genes. In fact, up to now, the repression of SRF-mediated gene transcription is not yet fully understood but it is presumed that the modality through which SRF represses transcription involves the recruitment of transcriptional inhibitors or epigenetic corepressors, including histone-deacetylases HDAC4 [15] and HDAC2, the *Lysine-Specific Demethylase 1* (LSD1) and the co-repressor CoREST [31].
SRF represses the transcription of IEGs in basal conditions but has a role in activating their transcription following extracellular stimulation in an activity-dependent way.
The mechanism of transcription of IEGs relies on neuronal activity. The most significant characteristic of the transcription of IEGs is their very rapid expression as a consequence of an extracellular stimulus. At the molecular level, the rapid transcription of these genes is guaranteed by the existence of a transcription apparatus assembled on the promoter of these genes even in case of absence of neuronal activity. The promoter of IEGs under baseline conditions emerges to be bound to the RNA polymerase II complex at the level of the *Transcription Start Site* (TSS) and to activity-dependent transcription factors such as SRF. The nucleosomes placed at the IEGs promoter level are already present in basal conditions as endowed with permissive epigenetic modifications for gene transcription (like the histone transcriptional activation marker H3K4me3) [32]. As a consequence of a stimulus of neuronal activation, which causes

intense calcium entry and raise of intracellular calcium concentration, it is suggested that the recruitment of a series of proteins aim to promote gene transcription. In further detail, neuronal activity causes phosphorylation of CREB, another transcription factor similarly involved in activity-dependent gene transcription likewise SRF, which encourages the recruitment of the histone acetyltransferase CBP at the promoter level. Meanwhile, a MAP kinase (ERK1/2) phosphorylates Elk-1, a ternary complex protein, which enters the nucleus and ties SRF, making it able to assemble the transcriptional machinery at the IEGs promoter [29]. The interaction that takes place between promoters and enhancers is fundamental for the expression of IEGs. At the level of enhancers sequences, already in basal conditions, the presence of the SRE and CRE (*cAMP responsive element*) sequences allows the anchoring of SRF and CREB proteins. Subsequent neuronal activation, allows approaching of the enhancers elements to the RNA polymerase II bound in proximity of the TSS, making transcription of the IEG start. Recent theories identify the origin of the process in the formation of a double strand break upstream of the Topoisomerase IIβ-mediated promoter. The topoisomerase is situated near the transcriptional repressive factor *CCCTC-binding factor* (CTCF), which allows the formation of chromatin loops creating topological boundaries near the promoter. As a consequence of neuronal activation, CTCF is phosphorylated and loses affinity for the DNA, freeing the topoisomerase, and promoting the breaking of the DNA double strand at CTCF binding site. Double strand break of the DNA facilitates the interaction of the promoter with the enhancer, allowing enhancer to promoter bound SRF proteins to interact, a process that contributes to downstream genes transcription [33].

2. Post-transcriptional regulation, epigenetics and neuronal function

Besides the basal process of transcription, recent studies point to the importance of post-transcriptional gene regulation for proper neuronal functions, since it represents an important drive to proteome diversity. In particular, alternative splicing seems to play a major role in empowering activity-dependent regulation of neuronal morphology thus, functionality [34]. Phenotype always represents the result of uninterrupted gene-environment intercommunication and environmentally elicited neuronal signals

modulate not only activity-dependent gene transcription but also alternative splicing to obtain cellular plasticity.

The ability to couple modulation of gene expression to environmental circumstances is dependent on the possibility of neurons and glia to modify their proteome with an enhanced competence compared to other somatic cell types (Fig. 3) [35] [36] .

In addition, strong evidence indicates that a further layer of gene expression control is involved in neuroplasticity modulation, allowing the brain to change its physiology upon environmental challenges in order to write a memory trace of our experiences. This additional set of gene expression regulatory mechanisms is referred to as the epigenetic layer. During the last twenty years a considerable effort has been spent by neurobiologists to decipher the involvement of epigenetic mechanisms in establishing neuronal and behavioral plasticity [37]. Epigenetics refers to heritable changes in gene expression that are independent on DNA sequence [38]. In the brain, where most neurons are post-mitotic, epigenetics represents the molecular interface mediating modulation of gene expression in response to environment and participates to higher brain functions such as cognition, behavior and language. The epigenome has therefore a role in orchestrating transcriptional plasticity relevant to transmute environmental stimuli into morphological and functional changes [39].

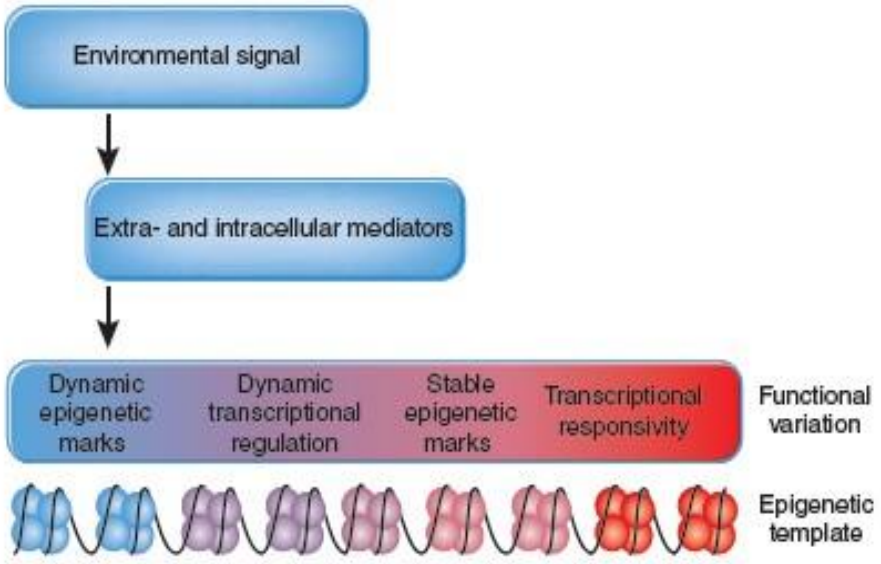

Fig. 3 The epigenetic interface. Epigenetic states laid at the junction between genome and environmental signals, helping to conduct dynamic changes in transcriptional activity through extra- and intra-cellular mediators. The epigenetic template attracts specific effectors, in a multistep process, that defines the readiness of specific genomic regions to environmentally induced intracellular signaling pathways, thus leading to more stable effects, in neural function, on the potential of transcriptional activation and variation (color-graded region) (from Vaquero et al, 2003) [39].

However, the epigenome is vulnerable and chronic or strong acute insults of different origin, for instance metabolic or stress factors can permanently alter the epigenetic asset generating the so-called epigenetic "scars" that may have pathogenic relevance. For all these reasons, epi-pharmacological manipulation of chromatin remodeling pathways could represent a novel approach in the treatment of many human disorders including neurological and neuropsychiatric disorders.

2.2 Histone modifications

The N-terminal tails of histones contain flexible and highly basic 15-30 amino acids-long sequences that are highly conserved across eukaryotic organisms and it has been proved that they act as substrates for several types of post-translational modifications, among others acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitylation, and phosphorylation (Fig. 4) [39]. Histone modifications alter chromosome function by way of at least two distinct systems. The first mechanism highlights how alterations could vary the electrostatic charge of the histone, causing morphological changes or alteration of its DNA binding affinity. The second one suggests that these modifications represent binding sites for protein recognition modules, like the bromodomains or chromodomains, which bind acetylated or methylated lysine, respectively [40].

2.3 Histone H3 *post-translational modifications* (PTMs)

At the level of N-terminal tail of histone H3 a large number of PTMs has been recognized: serine and threonine side chains are reputable phospho-acceptor sites, whereas lysine and arginine residues have various options of post-translational modification, such as acetylation and methylation (Fig. 4). In particular acetylation occurs on specific Lys residues on histones H3, leading to an open chromatin conformation. On the contrary, the methylation on different residues of Lys and Arg has distinct functions, in fact it can have both positive and negative effects on gene transcription; in the same way also different degrees of methylation can have different effects. Lysine residues can be mono-, di-, or trimethylated at the z-amine *in vivo*. Up-to-date genomic-scale investigation of histone modifications takes into consideration broad correspondence between distinct H3K4 methylation states, their genomic loci, and gene expression levels. The emerging consensus reports a

relation between uplifted levels of H3K4 trimethylation and the 5' regions of virtually all active genes, as well as a solid positive correspondence between this modification, transcription rates, active polymerase II occupancy, and histone acetylation.

Fig. 4 Schematic graph of the histone H3 N-terminal tail and its covalent *post-translational modifications* (PTMs). Different Histone H3 modifications are shown (Ac, acetylation, shown in *blue*; P, phosphorylation, shown in *orange*). Methylation (Me) is indicated on top, with *red* and *green* showing the methyl marks that are associated with repression or activation, respectively (from R.J. Sims et al. 2008) [41].

3. *Lysine Specific Demethylase 1* (LSD1/KDM1A)

3.1 Histone methylation

The discovery of *Lysine Specific Demethylase 1* (LSD1) introduced a new concept in the field of the histone methylation, facilitating the idea that histone demethylation is possible. After LSD1 finding, many studies have been carried out and mechanisms for direct histone demethylase reactions have been proposed. Relying on the catalysis reaction, histone demethylases are subdivided into two main families: Flavin-dependent histone demethylases and Jumonji domain-containing demethylases [42]. As Fig. 5 shows, the Flavin-dependent histone demethylases, causes the oxidative cleavage of the C-N methyl group bond, coupling it with a two-electron decrease of the *Flavin Adenine Dinucleotide* (FAD) cofactor. This reaction consequently produces an imine intermediate that is then non-enzymatically hydrolyzed. The methyl group is released as formaldehyde. These proteins manifest specificity for the Lysine substrate, indeed they preferentially demethylate mono- and di-methyl Lysine. In view of the fact that LSD1 is a good model for this protein family, their structure and function will be later discussed [42].

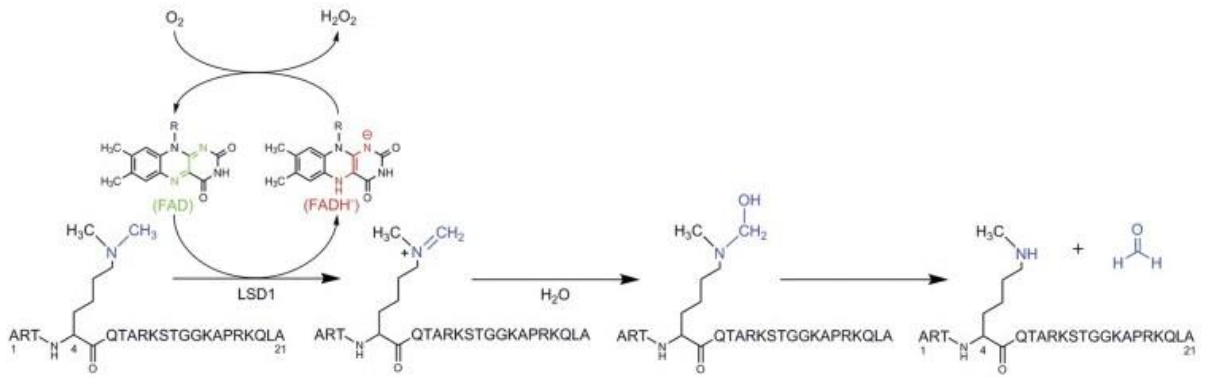

Fig. 5 Catalytic mechanisms of FAD-dependent demethylase enzymes. The FAD-dependent demethylation of Lys-4 of histone H3 proceeds through the hydrolysis of an iminium ion following a two-electron oxidation of the amine by the flavin. R, ribosyl adenine dinucleotide (from Culhane et al, 2007) [42].

3.2 LSD1 is an SRF epigenetic cofactor

During my work as a PhD student, I contributed to clarify the very relevant ability of SRF to bind to repressive epigenetic cofactors, introducing the idea that SRF versatility as a transcription factor, such as to behave as a transcriptional activator upon neural activity and as a repressor in basal conditions, at least in part relies on epigenetic cofactor recruitment [43]. Indeed, we discovered that SRF actively binds *histone deacetylases* (HDAC1/2) and *Lysine-Specific Demethylase 1* (LSD1), also named *Lysine Demethylase 1A* (KDM1A), a flavin-dependent histone demethylase that specifically eliminates methyl groups from lysine 4 of histone H3 when it is mono- or di-methylated [44] [45] [46]. Mono- and di-methylation of lysine 4 of N-terminal histone H3 tail (H3K4me1 and H3K4me2) represent histone modifications related to active gene transcription and hence, a permissive chromatin state. Therefore, LSD1 acts as an epigenetic transcriptional corepressor since it removes histone marks of transcriptional activation. LSD1 structure and functioning are extremely conserved from the yeast *Saccharomyces pombe* to humans [47] [48]. LSD1 acts in a protein co-repression complex, closely connected to the co-repressor factor for Repressor Element 1-Silencing Transcription factor (REST) also known as CoREST and with histone deacetylases HDAC1 and HDAC2, which was first characterized as a REST recruited corepressor complex [49] [50] [51] [52]. It is now known that LSD1/HDAC/CoREST co-repression complex is recruited to IEGs promoters *also* by SRF [31] performing a transcriptional repression activity on the IEGs targets, by means of deacetylation carried out by HDAC1 and 2 and demethylation catalyzed by LSD1 [53] [54]. LSD1, in humans, is a 116 KDa protein consisting of 852 amino acids and composed of the N-terminal domain called SWIRM, the amino-oxidase catalytic domain and the C-terminal tower domain [55] (Fig. 6). The N-terminal SWIRM

domain is a protein-protein interaction domain implicated in the interaction of LSD1 with other proteins of the complex and in common to many chromatin remodeling proteins. The C-terminal end of LSD1 tightly binds CoREST thanks to an intermolecular association in which the two α-helices of the tower domain are enclosed by a α-helix domain of CoREST. N-terminal histone H3 peptides bind the amino-oxidase catalytic domain of LSD1 near the flavin cofactor [56].

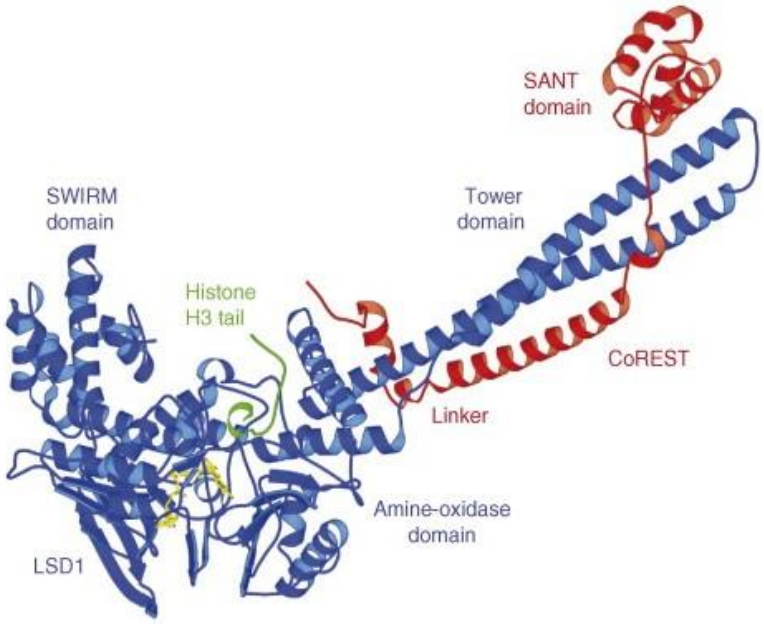

Fig. 6 Structure of LSD1-CoREST in complex with Histone H3 tail. Ribbon diagram of the structure. LSD1 is shown in *blue*, CoREST in *red*, and the Histone H3 tail in *green*. The FAD cofactor is indicated as a *yellow ball-and-stick*. The final model includes the residues 171–836 of LSD1, residues 308–440 of CoREST, and residues 1–16 of pLys4Met peptide (from Forneris et al., 2008)[56].

Early functional studies pertaining the role of LSD1 activity on the regulation of gene expression show a correlation between the demethylation of lysine 4 of histone H3 and silencing neuronal genes in non-neuronal cells. This initial functional characterization of the LSD1/HDAC/CoREST complex originates from the analysis of the molecular mechanism of REST which accurately acts between repression of neuronal genes in non-neuronal cells and in neuronal precursors [56] [57] [58] [44]. Hereafter, the involvement of LSD1 was not only noted in a wide range of cellular physiological processes such as cell maturation and differentiation, but also in pathological processes like the epithelial-mesenchymal shift mechanism of tumor cells [56] [59].

To the sake of my work, LSD1 assumes a pivotal role in the modulation of neuronal physiology, as in cooperation with SRF it negatively modulates activity-induced transcription of the IEGs cluster, impinging on memory consolidation and on emotional processing, contributing to guarantee the perfect responsiveness of IEGs transcription to

stimuli, and working to avoid transcriptional leaking in resting conditions [31] [43].

3.3 Alternative splicing of LSD1: neuroLSD1

As described earlier, a highly relevant source of proteome diversity fuels the need for enhanced cellular plasticity and homeostasis in neurons. This source is a post-transcriptional mechanism known as alternative splicing. Alternative splicing is a strategic biological mechanism responsible for creating a set of functionally different gene products from a single gene, diversifying gene functions without the need for enormous numbers of genes [60] [61]. Therefore, alternative splicing is able to increase the evolutionary complexity of mammals as well as representing a powerful mechanism in the regulation of the activity of a protein. Transcripts of neurospecific genes are highly enriched in the subsets of genes known to undergo alternative splicing events. Alternative splicing rules out a fundamental neuronal process in both cellular functions, such as synapse and axon orientation [62] [63] [34] and to more complex cognitive roles like learning and memory [64].

In our laboratory, an activity-dependent alternative splicing isoform of LSD1 was recently discovered, and related to important homeostatic neuronal functions impacting emotional processing and in particular anxiety-like behavior.

The human gene of LSD1, also recognized as AOF2 and KDM1A (GeneBank accession number NM_015013), located on the short arm of chromosome 1 (1p36.12), encodes an mRNA composed of 19 evolutionally conserved exons. In mammals, the transcript of LSD1 is subjected to alternative splicing, which leads to the formation of four different isoforms produced by single or double inclusions of two alternative exons E2a and E8a, located respectively between the second and third exons and between the eighth and ninth exons [65] [43]. Whereas the inclusion of the exon E2a takes place in all tissues, the isoform containing the exon E8a is expressed only in the nervous system and, restricted to humans, in the testis. As the inclusion of exon E8a is a process mainly involving the nervous system, LSD1 isoforms including the exon E8a (LSD1-8a and LSD1-2a/8a) have been called neuroLSD1 (Fig. 7). Since the reading frame is not altered by the inclusion of the two alternative exons, translation of the transcripts emerging from the alternative splicing of LSD1 leads to the formation of four stable proteins. In the brain, the LSD1 gene produces four different protein isoforms, which are LSD1, LSD1-2a, LSD1-8a and LSD1-2a/8a [65] [43].

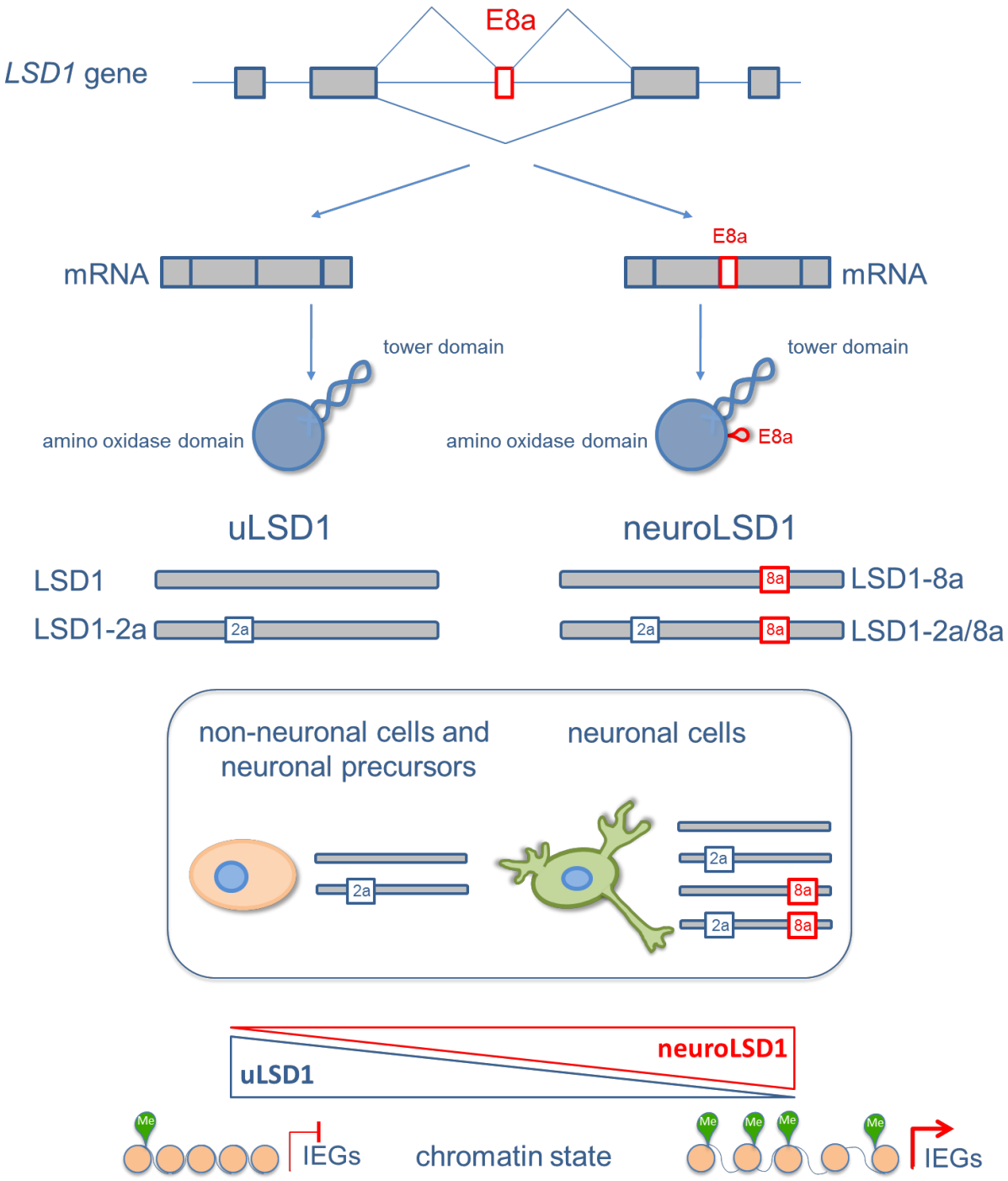

Fig. 7 Illustrative scheme describing the alternative neurospecific splicing affecting LSD1. In mammalian neurons, neuroLSD1 is generated by the inclusion of the microexon E8a by an alternative splicing mechanism. In particular, the neurospecific isoforms are characterized by a small loop encoded by the exon E8a (shown in *red*) (from Rusconi et al. 2017) [43].

The 4 amino acids encoded by the exon E8a (Asp-Thr-Val-Lys) are contained in the amino-oxidase catalytic domain and neuroLSD1 protein owns in its structure a loop that protrudes from the body of the enzyme (Fig. 8). The existence of these 4 amino acids has been proved to modify the catalytic activity of the enzyme, in fact, even if the ubiquitary enzyme LSD1 acts as a repressor of transcription by means of the demethylation of H3K4me1/2, neuroLSD1 cannot repress transcription. For this reason, neuroLSD1 has been suggested to act as a dominant negative that becoming part of the LSD1 core expression complex competes for the same LSD1 target genes and prevents its repression [65] [43].

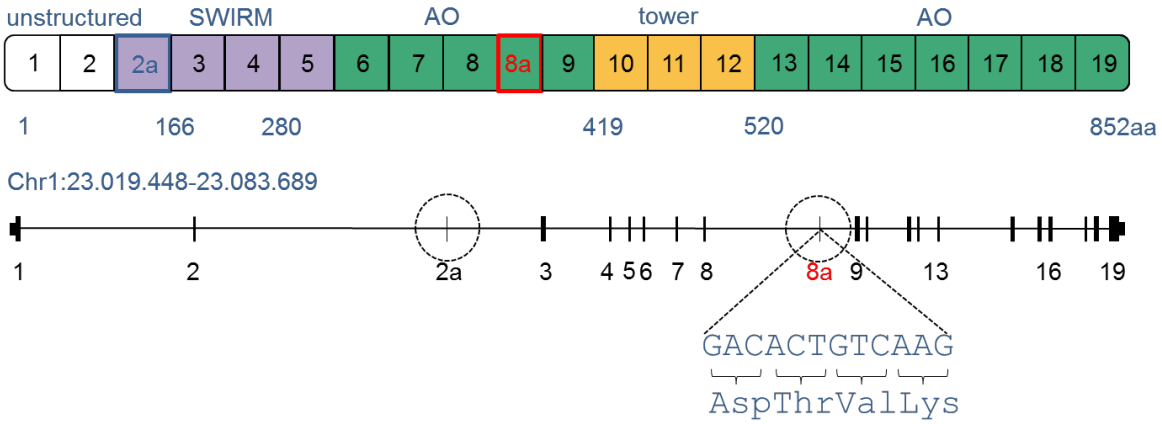

Fig. 8 Schematic representation of human LSD1 exons and protein domains. Schematic illustration of the human LSD1 protein domains together with its exons ranging from 1 to 19; in particular the location of annotated alternative exons (E2a and E8a) is shown. Functional domains are shown in different colors: N-terminal unstructured region coded by exons 1–2 in *white*, the SWIRM domain coded by exons 2– 5 in *purple*, the amino oxidase domain (AOD) coded by exons 6–9 and 13-19 in *green* and the tower domain coded by exons 10 –12 is shown in *yellow*. Position of the four amino acids is displayed (from Rusconi et al. 2017)[43].

Subsequent studies have demonstrated that the 4 amino acids loop encoded by the exon E8a can be phosphorylated at the level of threonine 369b (Thr369b) [66]. This phosphorylation provokes a conformational modification of the protein that makes neuroLSD1 unable to recruit partners of the corepressor complex. Neither CoREST, nor HDAC1 and HDAC2 can be bound by neuroLSD1 [66]. It has been demonstrated that transcriptional repressive activity of LSD1/CoREST/HDAC1/2 corepressor complex strictly relies on deacetylase and demethylase interrelationship, if LSD1 better demethylates histone H3 tails that have already been deacetylated by HDAC1/2, also deacetylase activity needs demethylation by LSD1 to efficiently proceed [67].

Interestingly, also CoREST is needed by LSD1 to accomplish to its histone demethylase function [53]. These data point to the fact that phospho-neuroLSD1 with its inability to recruit HDACs can affect repressive activity of LSD1 on its targets, acting as a dominant negative isoform depending on the relative ratio –and nuclear availability– of the two LSD1 splicing variants.

The study of LSD1 isoforms expression in rat cortex at distinct stages of development proved that relative amount of neuroLSD1 transcript compared to the total isoforms is strictly regulated during all stages of development. In the early stages of embryonic development LSD1 and neuroLSD1 isoforms are both present, with LSD1 highly dominating. In the perinatal window (E18.5-PN1) a significant growth of neuroLSD1 from 20% to 75% make it the most abundant isoform. The predominance of neuroLSD1 is maintained until the seventh day after birth (PN7); after this stage, the levels of neuroLSD1 and LSD1 stabilize around the values estimated in the adult brain, reaching grossly equal amount with small variations in the different brain areas (Fig. 9) [65].

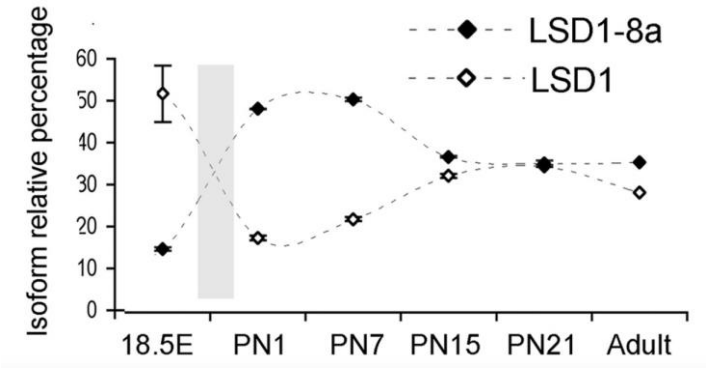

Fig. 9 The inclusion of exon E8a in the transcript of LSD1 is an event regulated during development. The graph shows the time-course of LSD1 (*blank*) and neuroLSD1 (*black*) isoforms relative percentage during development (from Zibetti et al. 2010) [65].

According to the brain area considered, mRNA levels of neuroLSD1 range from 40% to 65% of total LSD1 isoforms [65]. This kinetic of expression during development has highlighted a possible involvement of neuroLSD1 in neuronal development and maturation processes, by allowing inherent target genes transcription as a dominant negative isoform of the repressor LSD1. NeuroLSD1 is involved in numerous cellular processes, promoting neuronal differentiation and morphogenesis. The overexpression or silencing of neuroLSD1 in primary cultures of rat cortical neurons has been shown to enhance or decrease neurons morphogenesis with notable bidirectional changes in dendritic arborization and length [65]. NeuroLSD1 has been also studied for the implications its cellular roles exert on higher neuronal functions that include memory formation [68], control of emotional behavior, and in particular anxiety-like profile [31]

and neuronal excitability, evaluated in terms of neuroLSD1-mediated epileptic seizures susceptibility modulation [69]. NeuroLSD1 contribution to those neuronal morphostructural processes underlying memory formation was observed during the phenotypical characterization of a mouse model deleted for exon E8a (neuroLSD1^{KO}). This model, obtained by embryonic stem cells homologous recombination of exon E8a sequences with a neomycin cassette [69] [68], shows indeed deficits in learning and memory assessing paradigms including the *Novel Object Recognition* test [68]. An explanation has been provided by the group of Michael Rosenfeld in La Jolla who claims a role for neuroLSD1 in the beginning and elongation phase of transcription of the IEGs, for instance *c-fos*, *npas4*, *egr1* and *arc*, in response to neuronal activity [68]. Genetic ablation of neuroLSD1 indeed causes a deficit in the transcription of IEGs, involved in the processes of synaptogenesis and structural morphological changes of the synapses of neurons and therefore associated with learning and memory formation [31] [68].

Interestingly, knockout animals for neuroLSD1 also present a decreased anxiety-like phenotype, measured by inherent anxiety testing paradigms including *Elevated Plus Maze (EPM)*, *Marble Burying Test (MBT)* and *Novelty Suppressed Feeding test (NSF)*. These tests all take advantage of innate fear of rodents for void elevated spaces (EPM), for novel unidentified small objects (MBT) and for void enlightened arenas (NSF). In all these paradigms, neuroLSD1^{KO} mice show decreased tendency to anxiety and fear compared to wild type littermates. We suggested that the possible role of neuroLSD1 in the formation of a correct anxiety phenotype could depend again on mechanisms of memory formation. For what concerns anxiety, we proposed that the formation of a peculiar kind of memory, a decreased consolidation of memory related to stressful situations, is able to explain neuroLSD1^{KO} mice anxiety profile [31]. A further observation concerns the implication of neuroLSD1 in neuronal excitability, in fact neuroLSD1^{KO} mouse also display higher threshold of excitability, emphasized by a lessened susceptibility to the status epilepticus induced by Pilocarpine (PISE), a chemoconvulsant drug [69]. IEGs transcription, a process that, as said, is instrumental to enhance neurostructural plasticity, is also linked to epileptogenesis. Seizures promote gigantic waves of IEGs transcription and epileptic forms of plasticity need, to be established, a severe potentiation of glutamatergic excitation. Rescaled to pathological mechanisms, epilepsy is sustained by aberrantly increased pathways that physiologically lead to memory consolidation, which is why decreased proneness to IEGs transcription

via LSD1-mediated repression –in the context of lost demethylase activity regulation by dominant negative isoform neuroLSD1– could also reflect decreased seizure susceptibility.

3.4 Partnership between transcription factor SRF and epigenetic cofactors LSD1 and neuroLSD1

It has already been specified that initial functional characterization of LSD1 as an epigenetic corepressor arises from the analysis of the molecular system of parts of the REST transcription factor in non-neuronal cells [56] [57] [58] [44].

When we first noticed a transcriptional control of LSD1 and neuroLSD1 towards IEGs *c-fos* and *egr1* [66] we were ignoring which DNA binding factor stood at the basis of this modulation.

Since it is widely acknowledged that the expression of IEGs in the nervous system is regulated by the activity of two main transcription factors, namely SRF and CREB [16] [7], we tested their putative interaction with LSD1 by a candidate immunoprecipitation assay.

Immunoprecipitation experiment was performed *ex vivo*, on a protein extract from mouse hippocampal tissue, using anti-SRF antibodies; the immunoprecipitates were run by western blotting and the nitrocellulose membranes were immunodecorated with specific antibodies against LSD1, HDAC2 and CoREST. Such an approach showed an enrichment of the co-repressors LSD1, HDAC2 and CoREST in the immunoprecipitated fraction with anti-SRF antibodies proving a strong demonstration that not only LSD1, but the whole corepressor complex CoREST/LSD1/HDAC1/2 can be bound to SRF, and likely targeted to IEGs promoters and regulatory sequences (Fig. 10A). To complete this set of data we revealed that SRF itself can be co-immunoprecipitated with the anti-LSD1 antibody and visualized by western blot among LSD1-precipitated proteins (Fig. 10B). SRF can also interact with both LSD1 and neuroLSD1. This was assessed again by Co-IP performed in HeLa cells constitutively expressing HA-tagged LSD1 isoforms (HA-LSD1 and HA-neuroLSD1). In HA immunoprecipitates from both cell extracts SRF has been easily detected [31].

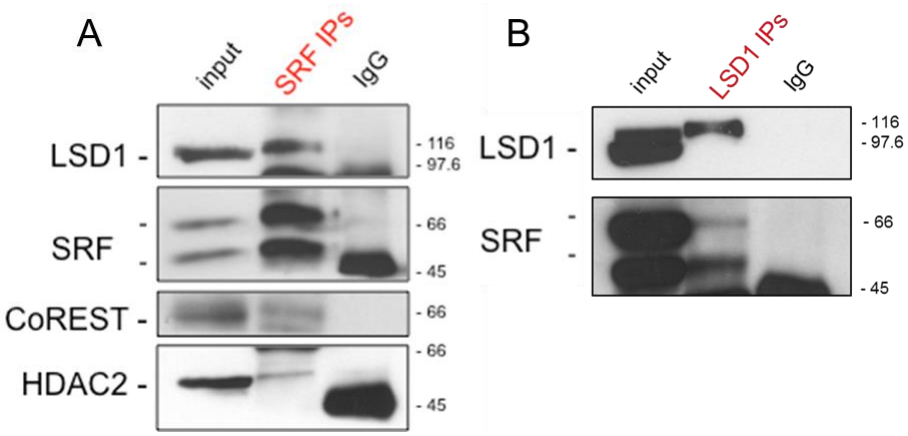

Fig. 10 Interaction between the epigenetic corepressor LSD1 and the transcription factor SRF. CoIP experiments allowed to demonstrate the interaction between LSD1 and SRF in the mouse hippocampus. Protein extract was immunoprecipitated (A) with anti-SRF antibody (SRF IPs) and (B) with anti-LSD1 antibody (LSD1 IPs); subsequently it was immunodecorated with anti-LSD1, anti-HDAC2 and anti-CoREST (A) antibody and anti-SRF antibody (B), showing that the LSD1/HDAC2/CoREST core complex is able to interact with the SRF transcription factor (from Rusconi et al., 2016) [31].

Chromatin quantitative immunoprecipitation (qChIP), a technique designed to detect enrichment of specific proteins at the level of a given sequence of DNA of interest, proved that the interaction between the LSD1 and the transcription factor SRF tethers LSD1 and its corepressor complex at the level of IEGs promoters, in the proximity of SRF consensus sequence (SRE), the so called CarG box. These experiments were fundamental to highlight the role of SRF transcription factor in determining the specificity of the LSD1/HDAC/CoREST core expression complex for IEGs promoters in the brain.

Further proof of SRF role in tethering LSD1 corepressor complex to IEGs promoters and important finding delineating a functional role for protein partnership among SRF, LSD1, neuroLSD1, CoREST, HDAC1 and 2, in neuronal molecular physiology came from the observation that in the hippocampus, at the level of neuronal IEGs promoters, positive histone marks H3K4me2 and H3K9/14ac are decreased in neuroLSD1^{KO} mice compared to wild type littermates.

This finding provided an elegant explanation of defective IEGs stress-induced transactivation, in neuroLSD1^{KO} mice, and related decrease of stress-induced anxiety, but also further demonstrated that lack of neuroLSD1, and consequent unrestrained LSD1 demethylase activity, directly affects the IEGs cluster of plasticity-related genes [31].

3.5 LSD1/neuroLSD1 and SRF-mediated regulation of IEGs transcription can be modulated by neuronal activity

We previously described that relative ratio between ubiquitous isoforms and the neurospecific isoforms of LSD1 is dynamically regulated during development with a functionally relevant neuroLSD1 increase in the perinatal window likely instrumental to promote plasticity gene transcription relevant to synapse maturation [66]. The ratio then reaches a stable balance in the adult brain in a way that in neurons, about half of mRNA molecules transcribed from *LSD1* gene include exon E8a, thus encoding neuroLSD1, and another half does not include exon E8a, encoding LSD1. At this point a critical induction proneness of IEGs transcription is set. Interestingly, such balance of IEGs transcription can be further dynamically regulated via neuronal activity-dependent control of the relative ratio LSD1/neuroLSD1. Indeed, recent studies have highlighted that neuronal activity regulates neuroLSD1 splicing *in vivo*, transiently altering in LSD1/neuroLSD1 ratio in neurons [69] [31]. Both epileptogenic stimulation of the hippocampus, as well as administration of psychosocial stress, inducing hippocampal activation, entail highly significant and fast neuroLSD1 decrease. Thus, LSD1 and neuroLSD1 regulate IEGs transcription in a highly regulated way, in the frame of feedback mechanisms in which in response to IEGs transactivation, a process restraining IEGs transcrivibility itself is engaged, which relies on increased LSD1 repressive activity through decreased levels of dominant negative isoform neuroLSD1. Hence, a neuronal stimulus of excitatory nature, rapidly inducing IEGs expression, has also the ability of influencing the typology of a subsequent IEGs response (mediated by SRF) via the modulation of splicing event regulating the ratio between LSD1 and neuroLSD1.

In our laboratory, we elegantly showed that in conditions of pharmacologically induced status epilepticus, an important neurospecific *trans*-acting splicing regulator, namely the RNA binding protein NOVA1 –directly regulating the process of alternative splicing generating neuroLSD1– rapidly decreases its expression, contributing to homeostatic neuroLSD1 downregulation [69]. It has been also shown that NOVA1 shuttles from the nucleus to the cytosol following pharmacologically induced status epilepticus, being confined outside its active compartment of action, the nucleus, and inhibited its contribution to many processes of alternative splicing regulation [69].

Another important neurospecific splicing factor, nSR100, acts as the master regulator of neuroLSD1 splicing [69] guaranteeing neuroLSD1 expression. SR family of *trans*-acting splicing regulators (to which nSR100 belongs) has been implicated in the control of alternative splicing via a very peculiar mechanism involving long non-coding RNA (lncRNA) MALAT1. Interestingly, MALAT1 takes part to the IEGs cluster of neuronal plasticity-related genes, and its expression is once again directed by the transcription factor SRF [33]. In particular it was observed that a nSR100-related protein called SRSF1 is sequestered by MALAT1 at the level of nuclear speckles, with the consequent modulation of its target events of alternative splicing [70].
During my work as a PhD student, I undertook a line of research aimed at defining a role for MALAT1 activity toward LSD1 neurospecific splicing generating neuroLSD1. My hypothesis is that psychosocial stress, via inducing transcription of lncRNA MALAT1, leads to nSR100 sequestration at the level of neuronal nuclear speckles, explaining how psychosocial stress-related hippocampal circuitry activation, via MALAT1 expression leads to neuroLSD1 downregulation.

4. Decreased memory of psychosocial stress impacts anxiety

Stress sets in motion a series of neuronal circuits in the brain that cause hippocampal neurons activation [71] [72] [73] with a consequent burst of IEGs transcription, also contributed by the transcription factor SRF [74]. Molecular analysis performed on RNA extracts of wild type mice and neuroLSD1^{HET} mice subjected to *Acute Social Defeat Stress* (ASDS) highlights a deficiency in heterozygous mice ability to transactivate IEGs after an episode of stress. Under basal conditions the transcription of IEGs in Heterozygous mice can compare to the level measured in wild type mice (Fig. 11) [31]. This means that a reduced amount of neuroLSD1 in hippocampal neurons, as a consequence of the absence of the E8a microexon for heterozygous genetic removal, results in ineffective activation of activity-dependent genes transcription following neuronal stimulation. As a consequence, stress-induced neuronal plasticity and the hence consolidation of the memory related to stressful paradigm is defective, impacting the anxiety profile of neuroLSD1^{KO} mice which is lower compared to wild type littermates.

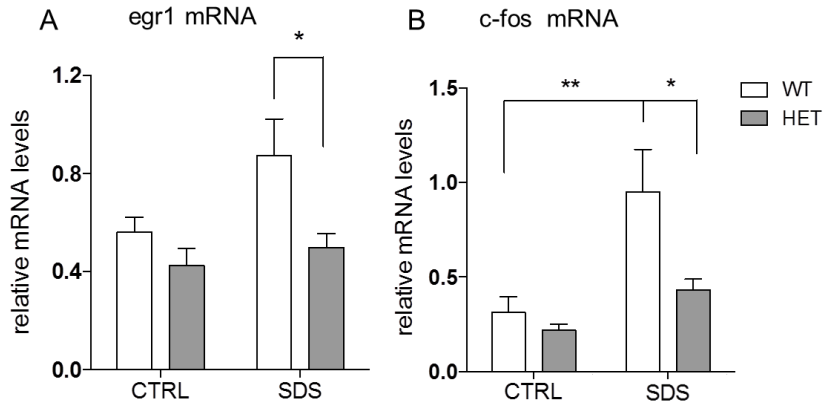

Fig. 11 The transcription of IEGs following neuronal activation is inefficient in neuroLSD1^{HET} mice. Activation of the transcription of two IEGs, *egr1* and c-*fos*, following ASDS in heterozygous mice (HET) for genetic ablation of E8a microexon is significantly reduced compared to wild type (WT) littermates (from Rusconi et al., 2016) [31].

5. A isoform of alternative splicing named SRFΔ5

As described above, functional versatility of SRF, i.e. to repress target genes transcription in resting conditions and to promote transcription upon neuronal activation, has been ascribed to the recruitment of epigenetic corepressors like LSD1 and HDAC2 [31] thanks to the presence of an annotated trans-repression domain [43] [31] [75]. Interestingly, a further possible method of transcriptional repression could also be founded on the activity of alternative splicing isoforms of the SRF transcript, which may also have tissue-specific expression. As for other members of the MADS family, the activity of SRF could be regulated according to a mechanism based on alternative splicing that produces the formation of functionally different isoforms. Four alternative splicing isoforms of SRF transcript were identified [17] [76] [77]. The *full-length isoform of SRF* (SRF-fl), which includes all the 7 exons, is 4201 bp long mRNA in humans. Alternative splicing isoforms originating from of specific exons skipping are: SRF-M or SRFΔ5 without the exon E5, which is expressed through a tissue-specific pattern, whose characterization will be highlighted in the results; SRF-S or SRFΔ4-5 without E4 and E5 exons, is present principally in the smooth and skeletal muscle; SRF-I SRFΔ3-4-5 without the exons E3, E4 and E5, is expressed at an embryonic level (Fig. 12).

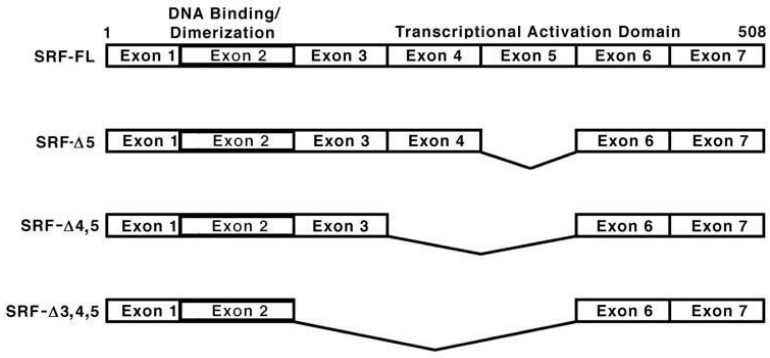

Fig. 12 SRF pre-mRNA undergoes alternative splicing by generating 4 different isoforms. Four alternative splicing isoforms of SRF transcript were identified: *full-length* (SRF-fl), without exon E5 (SRFΔ5), without exons E4 and E5 (SRFΔ4-5) and isoform without the exons E3, E4 and E5 (SRFΔ3-4-5) (from Davis et al., 2002) [77].

To the sake of my work, special attention is paid to SRFΔ5 alternative splicing isoform, defined by the absence of the exon 5. This exon measures 192 nucleotides, and is responsible for encoding a large part of SRF protein transactivation domain. SRFΔ5 protein, thus missing most of the transactivation domain, is not capable of activating gene transcription of a reporter gene sensitive to SRF [17]. However, by means of an *electrophoretic mobility shift assay* (EMSA), SRFΔ5 has been scored to be able to bind DNA like the SRE sequence on promoters of target genes. Consequently, the absence of the exon E5 does not affect the protein binding ability to the DNA sequences [17]. Subsequently, the translation of the mRNA of SRFΔ5 isoform leads to the synthesis of a 57 KDa transcription factor, missing the majority of the transactivation domain. Through a dominant negative action, this transcription factor antagonizes SRF activity as a transcriptional activator, aiming to promote the repression of target genes.

The expression of SRFΔ5 as a mechanism in charge of regulating SRF transcriptional activity has been proved to be present only in the smooth muscle of blood vessels, in the heart and muscle, where, for instance, it is able to interfere with myoblast differentiation in *in vitro* myotubes [17].

Relevantly to my work, SRFΔ5 is highly represented at the level of the nervous system. One of the aims of my project was to understand the molecular mechanism at the basis of the functional divergence between SRF and SRFΔ5 in the mammalian *central nervous system* (CNS), as well as a functional characterization of these two isoforms in terms of their relevance in modulating neuron structural plasticity.

Furthermore, through the use of a psychosocial stress paradigm performed in acute, *Acute Social Defeat Stress* (ASDS), I dealt with the analysis of the role of SRF and SRFΔ5 in response to a stress event in order to depict a functional interrelationship of stress-induced modifications of transcription factors and their epigenetic cofactors, including LSD1, neuroLSD1, as well as HDACs.

Finally, *Chronic Social Defeat Stress* allowed me to investigate a possible implication of SRF and SRFΔ5 in determining a molecular context of maladaptive neuroplasticity of the hippocampal circuit, demonstrated to be at the base of the vulnerability to psychosocial stress.

MATERIALS AND METHODS

Plasmids

Human pCGN-HA SRF plasmid was purchased precast from Addgene® (Plasmid # 11977; Addgene, Cambridge, MA, USA). Subsequently the pCGN-HA SRFΔ5 mutant was obtained from site-specific mutagenesis using the QuikChange II Site-Directed Mutagenesis Kits (Stratagene, La Jolla, CA, USA) through which we have removed the exon 5 specifically and entirely. SRF and SRFΔ5 were then excised from plasmids with HA N-terminal tag and cloned into vectors with myc C-terminal tag, in the EcoRI and BamHI sites, to obtain pCDNA3.1-SRF and pCDNA3.1-SRF Δ5. Human pBS-Splicing MG-800 was obtained as Rusconi et al.,2014 [69]. All plasmids were sequenced.

Cell cultures

For the immunoprecipitation experiments we used Neuro2a cell cultures (N2A), a mouse neuroblastoma cell line. N2a cells are grown in Dulbecco's modified Eagle's medium DMEM high glucose (EuroClone, Milan, Italy) added with 10% FBS (Fetal Bovine Serum; EuroClone, Milan, Italy), 1% of penicillin and streptomycin (EuroClone, Milan, Italy), 1% Fungizone (EuroClone, Milan, Italy), containing the antifungal Amphotericin B, and 1% GlutaMAX Supplement (Invitrogen Corporation, Carlsbad, CA, USA), as a glutamine supplement, more stable than L-glutamine. The day before transfection the cells were plated in 100mm plates (1.000.000 cells). The transfection was performed using Lipofectamine® LTX with Plus Reagent (Invitrogen Corporation, Carlsbad, CA, USA).

For the transfection experiments with antisense oligonucleotides (ASO) we used SH-SY5Y cell cultures. SH-SY5Y cells are human derived neuroblastoma cell line, originally cloned from SK-N-SH, differentiating by extending neurites to the surrounding area. Cells were grown in adhesion in 75 cm^2 flask in 12 ml of medium and kept in incubator at 37 ° C and 5% CO_2 using RPMI 1640 medium (EuroClone, Milan, Italy) added with 10% FBS, 1% GlutaMAX supplement , 1% Fungizone and 1% penicillin/streptomycin.

SH-SY5Y cells were co-transfected with pBS-Splicing MG-800 and antisense oligonucleotides (ASO) (Department of Chemical and Pharmaceutical Sciences, University of Ferrara). Two specific ASOs were used for MALAT1 and one control ASO (NC1). Each ASO was tested at 50 nM and 200 nM concentrations.

The sequences are shown in the table below, with the "m" indicating the 2'OMe

nucleotides and the asterisk the phosphorothioate bond:

ASO	Sequence 5'→3'
MALAT1 5042	mG*mG*mC*mA*mU*A*T*G*C*A*G*A*T*A*A*mU*mG*mU*mU*mC
MALAT1 5326	mC*mG*mA*mA*mA*C*A*T*T*G*G*C*A*C*A*mC*mA*mG*mC*mA
NC1	mG*mC*mG*mU*mA*T*T*A*T*A*G*C*C*G*A*mU*mU*mA*mA*mC

The day before transfection cells were plated in 6-well plates (350.000 cells/well).
The transfection was performed using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA).

Cortical and hippocampal primary neurons cultures, Immunostaining and Spine Analysis

Cortical or hippocampal neurons were prepared from embryonic day 18 (E18–E19) rat brain (Charles River Laboratories International, Wilmington, MA, USA) as previously described on Romorini et al. 2004 and Brewer et al.,1993 [78] [79], and plated on poly-D-lysine-coated slides (50 μg/ml) to 75,000/well for immunochemistry.
Primary rat hippocampal neurons (DIV4) were transfected with different plasmids (pCGN-HA SRF, pCGN-HA SRFΔ5, PCDNA3.1 SRF-myc and PCDNA3.1 SRF Δ5-myc) using calcium phosphate precipitation (transfection efficiency 1%) in parallel to empty vectors (pCGN-HA and PCDNA3.1-myc) as control condition [80]. Neuronal cells were fixed with paraformaldehyde (4%) at DIV8 for morphological analysis and at DIV18 for spine density evaluation. Cells were incubated with anti-HA (1:100 sc-805; Santa Cruz Biotechnology) or anti-myc antibodies (1:100 Abcam Ab9106, Cambridge, UK) for 3 hours at room temperature in gelatin dilution buffer (30 mM phosphate buffer, pH 7.4, containing 0.2% gelatin, 0.5% Triton X-100, and 0.8 M NaCl), and subsequently with FITC-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, ME, USA) for 1h. The pEGFP-N1 (Plasmid # 6085-1; Addgene, Cambridge, MA, USA) construct was used to mark the entire neuronal cell.
Fluorescent images were acquired using a Zeiss LSM5 510 laser-scanning confocal microscope (Oberkochen, Germany). Morphological analysis was performed using the Sholl analysis module in Fiji software [81], while spine density was evaluated with

NeuronStudio software. All measurements are given as mean standard error of the mean (SEM).

Cortical primary rat neurons (DIV13) were treated with 5μg/ml of Tubercidin (T0642; Sigma Aldrich, Saint Louis, MO, USA) and after 4 or 8 hours RNAs were extracted using the PureLink™ RNA Mini Kit (Thermo Fisher scientific, Waltham, MA, USA).

Chemical LTP

Primary rat hippocampal neurons overexpressing SRF isoforms were treated for chemical LTP at DIV18. Ten minutes before stimulation the medium was replaced by pre-warmed Stimulation solution composed by 3 μM strychnine (S0532-5G; Sigma Aldrich, Saint Louis, MO, USA) and 20 μM bicuculline (14343; Sigma Aldrich, Saint Louis, MO, USA) in Extracellular solution (140mM NaCl, 1,3 mM CaCl2, 5mM KCl, 25 mM HEPES, 33mM glucose). cLTP was induced with 200 μM glycine (G8898; Sigma Aldrich, Saint Louis, MO, USA) at 37°C. After 3 minutes the Stimulation solution was removed and replaced with pre-warmed Extracellular solution. Neurons were fixed 2 hours after and analyzed for spine density as previous described.

Protein extraction, Immunoprecipitation and Western blot

The experiments were performed as in Pilotto et al., 2016 [82]. Cell lines or brain mouse tissues were homogenized as reported in Rusconi et al., 2014 [69]. Immunoprecipitation experiments were performed as essentially described in Battaglioli et al., 2002 [57].

N2a cells were harvested in Low stringency buffer (10% glycerol, NaCl 150 mM, imidazole 10 mM, 0.5 mM EDTA, 0.5% Triton-X100, dithiothreitol 0.5 mM), added with phosphatase and protease inhibitors (Pierce™ protease and phoshatase Inhibitor tablets; Thermo Fisher Scientific, Waltham, MA, USA) prior to use. 2 mg of protein extract were immunoprecipitated with HA-conjugated Agarose beads (sc-7392; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation, the beads were washed three times with Low stringency buffer. Then immunoprecipitates were eluted with 1x sodium dodecyl sulfate, *Sample buffer,* and analyzed using Western blot.

We performed immunoprecipitation and western blot experiments with the following antibodies: anti-SRF (D71A9; Cell Signaling Technology, Danvers, MA, USA); anti-myc antibody (Abcam Ab9106, Cambridge, UK); anti-LSD1 (C69G12; Cell Signaling Technology, Danvers, MA, USA); anti-β Actin (A2228; Sigma Aldrich, Saint Louis, MO,

USA); anti-HA (sc-805; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-HA-AC (sc-7392; Santa Cruz Biotechnology, Santa Cruz, CA, USA); and normal mouse IgG-AC (sc-2343; Santa Cruz Biotechnology, Santa Cruz, CA, USA).
Western blot analyzes were conducted using the Alliance Mini HD9 acquisition system and NineAlliance 1D software (Uvitec Cambridge, UK).

Total RNA extraction and Real Time-PCR analysis

Total RNA was isolated using the Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA), and the purified RNA was retrotrascribed with Maxima Reverse Transcriptase (Thermo Fisher scientific, Waltham, MA, USA) and thanks to the presence of a strong DNAse during the reaction any residual DNA was removed.
Quantitative Real Time-PCR analysis was performed using QuantStudio 5 Real-Time PCR System (Thermo Fisher scientific, Waltham, MA, USA) using the SYBR™ Green PCR Master Mix (Thermo Fisher scientific, Waltham, MA, USA). The relative expression of the investigated genes was quantified after normalization against housekeeping genes like ribosomal protein SA (RPSA) or eukaryotic translation factor 4A, isoform 2 (EIF4A2).

Rqf RT-PCR analysis

To specifically evaluate the relative amount of each SRF isoform, we used a PCR-based method in which a FAM fluorochrome-conjugated forward primer designed on exon E4 (Sigma Aldrich, Saint Louis, MO, USA) and two non-modified reverse primers were used to amplify all the expected isoforms in a single reaction. In particular we designed one reverse primer on the junction between the exons E4 and E5, to specifically amplify SRF transcripts containing exon E5, and the other one on the junction between the exons E4 and E6, to specifically amplify SRF transcripts excluding exon E5. The two reverse primers were studied in order to generate two amplicons differing from few nucleotides, to not alter the reaction efficiency but to allow, in the same time, their discrimination using capillary electrophoresis. After having assessed in real time PCR the identical amplification efficiency of the two amplicons, we performed a unique PCR reaction using three primers together. PCR products, mixed together with an appropriate internal-lane ROX-conjugated size standard (500ROX standard; Applied Biosystems, Foster City, USA), were separated by capillary electrophoresis under denaturing conditions and the amount of each amplified product was measured as microsatellite sample, based on

levels of relative fluorescence units using the GeneMapper® software (Thermo Fisher scientific, Waltham, MA, USA).

Likewise, the Rqf RT-PCR technique was also used to measure the relative amount of LSD1 isoforms, such as in Zibetti et al., 2010 [65] and to evaluate the relative amount of exon E8a inclusion in the mature transcript resulting from minigene pBS-Splicing MG800 as Rusconi et al., 2014 [69].

Primers

Primers	Sequence 5'→3'
Cloning	pCGN-HA SRFΔ5-FW 5'- CTGTCCCGCTGGAGGAGGTC -3' pCGN-HA SRFΔ5-REV 5'- GTGGTGTCCCTCAGGTGTTC -3'
Sequencing	m SRF-FW 5'- AAGCGTCTCCCTCCCGTGACA -3' m SRF-REV 5'- CTTGCTGCCCTATCACAGCCA -3'
qualitative PCR	m SRF-FW 5'- AAGCGTCTCCCTCCCGTGACA -3' m SRF-REV 5'- CTTGCTGCCCTATCACAGCCA -3'
Rqf RT-PCR	m SRF-FW 5'- (6FAM) CTGTGCAGGCCATTCATGTG -3' m SRF-E4-5 REV 5'- GGCGGGCAACGTCACTGTCC -3' m SRF-E4-6 REV 5'- ACACCACCTGTCCCGCTGG -3' MG800 E2-FW 5'-(6FAM) CAACTTCAAGCTCCTAAGCCACTGC -3' MG800 BraII-REV 5'- CACCAGGAAGTTGGTTAAATCA -3' r LSD1-FW 5'- GAAAATGAAAGTGAGCCCGAGG -3' r LSD1-REV 5'-(6FAM) CTACCATTTCATCTTTCTCTTTTGG -3'
Real Time PCR	m SRF-FW 5'- TTCAGCTCCACCAGATGGCTG -3' m SRF-REV 5'- TTCACTCTTGGTGCTGTGGGC -3' m cFOS-FW 5'- CTATCTCCTGAAGAGGAAGAGA -3' m cFOS-REV 5'- TGATCTGTCTCCGCTTGGAGT -3' m EGR1-FW 5'- CCTTCAATCCTCAAGGGGAGC -3' m EGR1-REV 5'- AACCGAGTCGTTTGGCTGGGA -3' m NPAS4-FW 5'- GGTGGTGAGACTTCAAGCCAA -3' m NPAS4-REV 5'- TCCGTGTCACTGATAGGGTAG -3' h/m RPSA-FW 5'- CAACAACAAGGGAGCTCACTC -3' h/m RPSA-REV 5'- CTTCTCAGCAGCAGCCTGCT -3'

	r RPSA-FW 5'- ACCCAGAGGAGATTGAGAAGG -3' r RPSA-REV 5'- TGGGGAACTGCTGAATGGGC -3' EIF4A2 FW 5'- GGTCAGGGTCAAGTCGTGTT -3' EIF4A2 REV 5'- CCCCCTCTGCCAATTCTGTG -3' r/m nSR100-FW 5'- GGGGTGTAATCACTGGGTCG -3' r/m nSR100-REV 5'- GAGCTGGTTTGCGTGGAGGG -3' m NOVA1-FW 5'- TCTTCCCCAACTACCACCAAG -3' m NOVA1-REV 5'- GTTCAGGTTCTCCACTCACAG -3' m MALAT1-FW 5'- GCTGTTGGCACGACACCTTC -3' m MALAT1-REV 5'- ACTGTGAACCAAAGCCGCAC -3' h MALAT1-FW 5'- AGGGAAAGCGAGTGGTTGGT -3' h MALAT-REV 5'- GAAATCGGCCTACGTCCCCA -3' r MALAT1-FW 5'- AGGGAAAGCATGTGGTGCGG -3' r MALAT-REV 5'- GACGCGGCCTACAGCCCCA -3'
mice genotyping	mLSD1-FW 5'- ACGCGTCGACTCTTCAGTGCTTTCTCACTCCCA -3' mLSD1-REV 5'- ATAGTTTAGCGGCCGCCCTCTATTTTCTGAGCAGCC -3'

Animals

Animals were obtained from Charles River, Calco, Italy. 8-weeks-old male C57BL/6N wild type mice were housed individually for the time required, with free access to food and water, in controlled temperature (20–22 °C) with a 12-h light/dark cycle (lights on at 7:00 AM). All procedures involving animals were carried out in accordance with the European regulations (2010/63/UE) and Italian Council on Animal Care Guidelines (Legislative Decree no. 26, 2014) and were approved by the Italian Ministry of Health (n° 275/2015) and by University of Milan IRB n° 27/2014. All efforts were made to minimize the number of subjects used and their suffering. During all the procedures we have diligently followed the present rules and regulations regarding the treatment of animals.

Acute Social Defeat Stress

We used a modified protocol of the SDS test [31]. In particular, in the acute SDS, 8 weeks C57BL/6N experimental mouse is placed in direct interaction with an ex-breeder CD-1 aggressor mouse of 4-6 months of age, for 5 minutes. After direct physical interaction, a

visual and olfactory interaction phase occurs in which, for the next 2 or 7 hours, the experimental mouse remains in the same cage of the aggressor mouse separated from the latter thanks to the insertion of a protective perforated Plexiglas barrier, that allows the maintenance of a sensory interaction. At the end the mouse is sacrificed and the hippocampus is taken for molecular analysis. Another group of mice subjected to acute stress after 7 hours of sensory interaction is placed in their cage and sacrificed after 24 hours to verify a possible recovery of the molecular response to psychosocial stress. As controls, C57BL/6N mice of the same age were used, placed two per cage and separated by plexiglass. To ensure effective application of social stress, we selected CD-1 mice that showed strong aggressive behavior.

Chronic Social Defeat Stress

The chronic SDS consists in the repetition for 10 days of the psychosocial stress that alternates the physical interaction and the psychological/sensorial interaction [83] [84]. The C57BL/6N experimental mouse is placed in direct interaction with the aggressor CD-1 aggressor mouse for 5 minutes and then the two mice remain in visual and olfactory interaction for the remaining time of the 24-hour period. This cycle is repeated for 10 consecutive days in which the C57BL/6N mice are alternated daily in order to change the aggressor CD-1 mouse avoiding an accustoming and reducing variability. As controls, C57BL/6N mice housed two per cage, separated from the plexiglass and exchanged daily similarly to the experimental mice.

This treatment systematically leads to the development of a marked denial of social contact associated with reminiscent behavioral changes of a depressive phenotype in most animals, called Susceptible, and to the identification of a subgroup of animals called Resilient that do not avoid social contact. The distinction between susceptible and resilient to stress is made on the basis of an Interaction test performed 24 hours after the end of the stress. In particular, in this test, the C57BL/6N mouse subjected to stress or control is placed in the cage with a new CD-1 mouse, unable to interact with the experimental mouse because it is immobilized in a metal cage. Thanks to the use of a video camera and software (ANY-Maze®), the behavior of the mouse is automatically monitored and the Social Interaction ratio (SI ratio) is calculated. In particular the SI ratio represents the relation between the time spent in the interaction area in the presence of the CD-1 mouse and the elapsed time in the same area in the absence of the

CD-1 mouse. For the controls the SI ratio is equal to 1; if the SI ratio is less than 1, the mouse is classified as susceptible to stress (SUS), if it is higher than 1 as a resilient (RES). The hippocampus is collected 24 hours after the end of the test, therefore 48 hours after the end of the stress. As above, in order to ensure optimal application of social stress, we selected CD-1 mice that showed strong aggressive behavior.

Data Analysis and Statistical Methods

We used the minimum number of animals needed to reach statistically significant results. Statistical analysis was conducted using PRISM 6 software (GraphPad) using the Student's *t* test for single comparisons and the ANOVA one-way test for multiple comparisons. Data are shown as means ± SEM.

RESULTS

CHAPTER I

1. SRFΔ5 brain expression and developmental regulation in the mouse hippocampus

SRF acts as a multifaced transcription factor due to its ability to promote transcription following specific stimulation as well as to function as a repressor at rest conditions [15] [31]. SRF repressive activity has been outlined and functionally localized within the N-terminal domain [18]. For instance, prototypical SRF target *c-fos*, is not only transactivated in response to stimuli but also actively repressed in basal conditions [21]. We can refer to this state of repression as poised repression [21] and is at least partly mediated by SRF ability to recruit a structured HDAC-containing complex [85] [86] [31], including the LSD1/CoREST/HDAC2 corepressor complex [31].

In the brain, as we formerly observed, SRF protein is present in two different isoforms, the well-known and characterized full-length 508-amino-acids protein with an electrophoretic mobility at 67 KDa and a distinct and faster migrating band at 57 KDa at the expected molecular weight of SRFΔ5. The nature of SRF alternative splicing isoform was to be unambiguously proved [31]. As SRF gene undergoes different events of alternative splicing, the p57KDa band origin has been further investigated.

Fig. 13A shows the alternative splicing events that generate SRF and SRFΔ5. We compared the expression of the two SRF protein isoforms in mouse hippocampus within neuronal and non-neuronal cell lines. Migration of recombinant myc-tagged SRF and SRFΔ5 fusion proteins overexpressed in Neuro2a neuroblastoma cell line (N2A), recapitulates the hippocampal endogenous SRF and SRFΔ5 SDS-PAGE immunoreactivity profile (Fig. 13B). The presence of the two SRF protein bands in mouse, rat and human hippocampus has been assessed (Fig. 13B).

In order to verify the compatibility of the second SRF immunoreactive band at 57 KDa and SRFΔ5, we performed a RT-PCR on different mouse tissues using primers to amplify all possible SRF-related amplicons in the same semi-quantitative PCR reaction, including or skipping exon E5. Fig. 13C highlights a high prevalence of a 351bp band, corresponding to full-length SRF, in neuronal and non-neuronal tissues. On the other hand, the 159bp amplicon predicted to belong to SRFΔ5 was more expressed in several brain areas. Importantly, sequence analysis of the 159bp band showed the presence of

the junction between exons E4 and E6, confirming that the 159bp amplicon derives from SRFΔ5 transcript. (Fig. 13D).

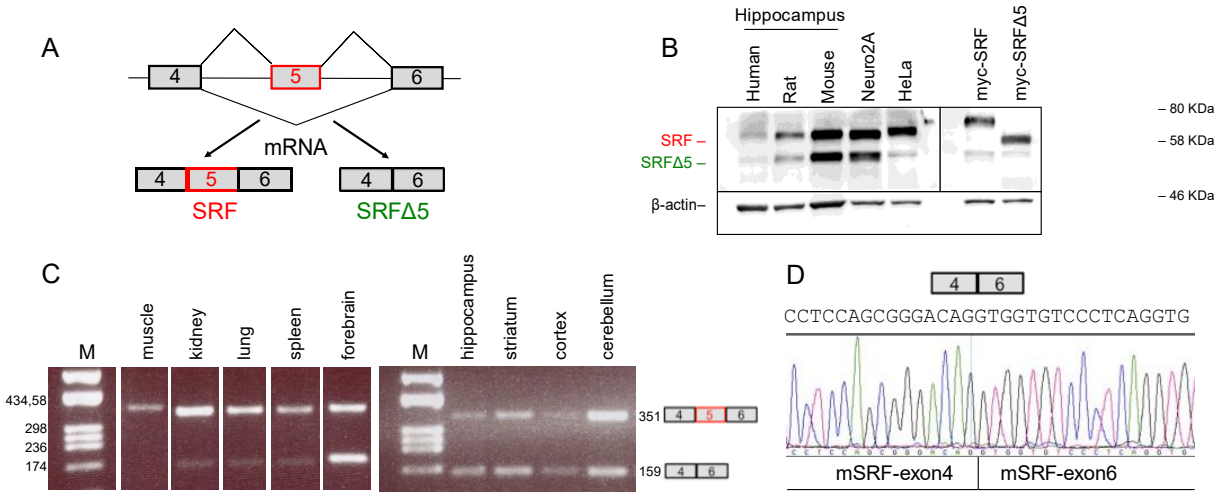

Fig. 13 SRF alternative splicing isoform, namely SRFΔ5, is preferentially expressed in brain tissue. A) Schematic drawing of splicing event on SRF pre-mRNA generating SRFΔ5. B) SRF and SRFΔ5 proteins are highly expressed in human, rat, mouse hippocampus and in N2a cell lines; SRFΔ5 is barely detectable in HeLa cell lines. Migration of the myc-tagged SRF isoforms is displayed. C) SRF and SRFΔ5 expression in different non-neuronal and neuronal tissues. D) Electropherogram from purified hippocampus 159bp SRF-related band shows skipping of exon E5. Splice junction between exon E4 and E6 is shown.

Subsequently, the relative amount of SRF and SRFΔ5 splicing ratios was analyzed by a more quantitative analysis using relative quantity fluorescent PCR (Rqf RT-PCR [65]). This analysis has been performed by employing three primers generating two amplicons of the same size (a specific one for SRF and a second one for SRFΔ5). Primers have been selected to produce amplicons with the same PCR efficiency. In particular, we used a common forward primer fluorescinated localized on exon E4, and two reverse primers, the first specific for SRF on exon E4-E5 splicing junction, and the second specific for SRFΔ5 on exon E4-E6 splicing junction. With this approach, the preferential brain expression of SRFΔ5 (Fig. 14A) was further validated.

Subsequently, using the same Rqf RT-PCR technique, we characterized in parallel during development in the mouse hippocampus, SRFΔ5 and SRF mRNA relatively to the sum of all SRF isoforms (Fig. 14B). As highlighted, SRFΔ5 relative mRNA level is stable from the perinatal window (PN 1-8) to adulthood (months 7-12). On the contrary, in the mouse hippocampus, SRFΔ5 protein level grows during development, representing the 30% of all SRF proteins during the perinatal window (PN1-8, Fig. 14C), and reaching about 50% during the adolescence. This value is then stabilized during the adult life.

A poor correspondence can be observed between protein levels and mRNAs of the two

SRF isoforms, in a way that it is difficult to hypothesize that growth in protein levels along with age depends on a transcriptional mechanism. Indeed, SRFΔ5 mRNA levels slightly increase during brain maturation (PN 9-15) from 15 to 23%.

On possible reason of the weak correspondence between mRNA and protein expression, might be related to a differential protein stability. Given the role of SRF protein in facilitating plasticity genes transcription and since enhancing SRF function promotes neuronal growth [87] [88], low levels of the dominant negative SRFΔ5 in postnatal brain might be functional to the process of neuronal maturation. In the adulthood, conversely, decrease of SRF isoform, by the densitometric analysis that compares relative intensity of the two SRF bands, could represent a possible window of the fine modulation of SRF/SRFΔ5 shared targets [17].

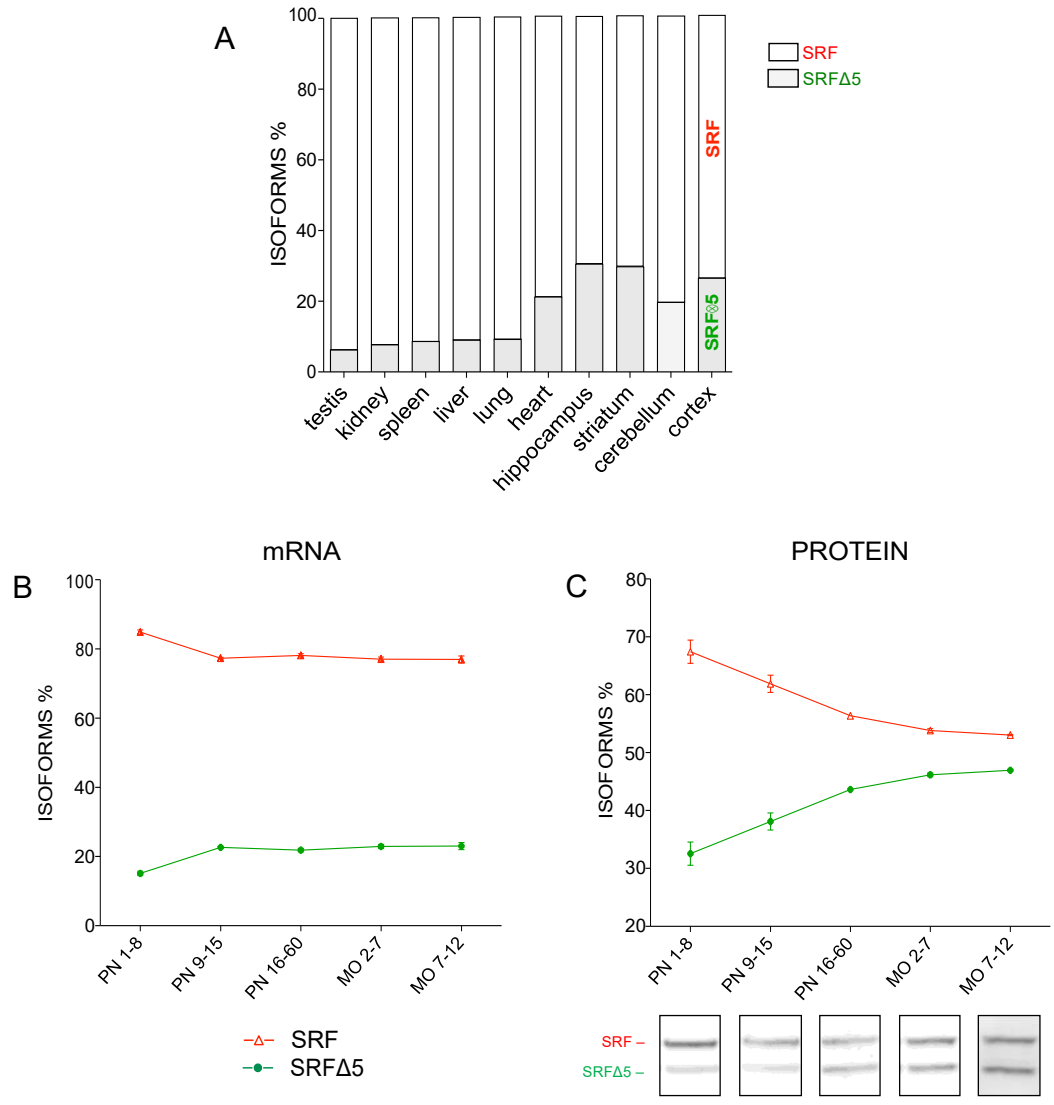

Fig. 14 SRFΔ5 is enriched in the brain and developmentally regulated in the mouse hippocampus. A) SRF and SRFΔ5 relative percentage in non-neuronal and neuronal tissues. B-C) SRF isoforms relative percentage during development: assessed by Rqf RT-PCR (B) and by Western blot (C). Data are presented as mean ± SEM.

2. SRFΔ5 is able to interact with corepressor LSD1 and to heterodimerizes with SRF.

Since SRFΔ5 lacks an important portion of the transactivation domain at the C-terminus of the protein (encoded by exon E5) but is still able to bind its DNA responsive elements Serum Responsive Elements (SRE) [17], it has been proposed that SRFΔ5 acts as an inhibitor of SRE-dependent transcriptional activity. Concerning what was just mentioned, SRFΔ5 could serve as a dominant negative SRF isoform, competing with SRF for SRE binding and preventing targets transactivation. On the contrary, SRFΔ5 preserving the whole inhibitory domains [18] at the N-terminal, should keep similarly to SRF, through the recruitment of corepressors, an active role in keeping target genes repressed.

In particular, we decided to investigate the ability of SRFΔ5 to bind the epigenetic corepressors LSD1, HDAC2 and CoREST which has been proved to play a significant role in the SRF-mediated regulation of transcriptional modulation of the target genes in the hippocampus [66] [68] [31]. To this purpose, we performed immunoprecipitation analysis overexpressing in Neuro2A cell line (N2A) a plasmid of HA-SRF or HA-SRFΔ5, showing that both isoforms can interact with corepressor complex and in particular with LSD1, HDAC2 and CoREST with similar affinity (Fig. 15A). Furthermore, given the capacity of the SRF MADS-box domain to form homodimers in a constitutive way, we analyzed the ability of SRFΔ5 to heterodimerize with SRF thus increasing the probability of SRFΔ5 to modulate SRE-containing promoters in accordance to its cellular quantity and in relation to the amount of SRF. Specifically, we performed an immunoprecipitation analysis overexpressing in N2A differently tagged SRF isoforms, showing that HA-tagged SRFΔ5 is able to bind myc-SRF and that HA-tagged SRF can interact with myc-SRFΔ5 (Fig. 15B). Fig. 15B shows a similar ability of the two proteins to interact with each other thus leading to the formation of SRF-SRF or SRFΔ5-SRFΔ5 homodimers; in addition, we observed that also the heterodimer SRF-SRFΔ5 can be formed.

Next, in order to evaluate possible differential molecular features between the two SRF isoforms, we further investigated possible differences in coactivator recruitment. To this aim, we selected among the recently proposed SRF cofactors, CTCF [33] and a well-known brain enriched SRF partner, MKL1 [89]. We performed co-immunoprecipitation experiments with HA-SRF and HA-SRFΔ5 to assess the relative affinity of endogenous

MKL1 and CTCF with the two SRF isoforms. As shown in Fig. 15C, we observed a remarkable decrease in SRFΔ5 capacity to interact with CTCF compared to SRF. Also, in the case of MKL1, the binding affinity for SRFΔ5 resulted reduced compared to SRF, although only as trend (Fig. 15D).

In conclusion SRFΔ5 represents a constitutive repressor able to bind DNA and recruit corepressor factors such as LSD1, CoREST and HDAC1/2. However, it less efficiently binds coactivators. Altogether these data suggest that by means of its relative expression, SRFΔ5 could modulate SRF function.

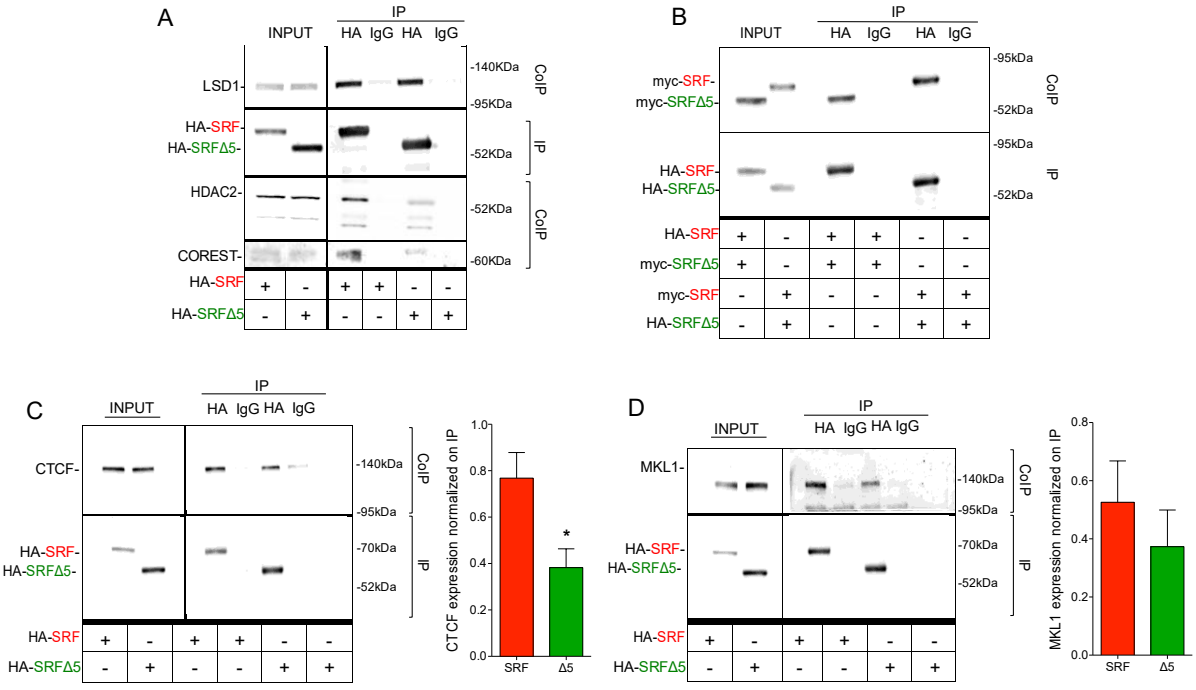

Fig.15 SRFΔ5 retains the ability to interact with the corepressor LSD1 and is able to heterodimerize with SRF. A) Western blot from N2a cells over-expressing HA-SRF and HA-SRFΔ5 and immunoprecipitated with anti HA-conjugated agarose beads, immunodecorated with anti-LSD1, anti-HDAC2 and anti-CoREST antibodies. B) Western blot analysis of protein extracts from N2a cells co-transfected with HA-SRF/myc-SRFΔ5 or HA-SRFΔ5/myc-SRF, immunoprecipitated with anti HA-conjugated agarose beads and immunodecorated with anti-HA and anti-myc antibodies. C-D) N2a cells transfected with HA-tagged SRF isoforms immunoprecipitated with anti HA-conjugated agarose beads and immunodecorated anti-CTCF (C) and anti-MKL1 (D). Data are presented as mean ± SEM and normalized on IP; *P < 0.05, Student *t* test.

3. SRFΔ5 is a negative regulator of dendritic spine density in hippocampal neurons

SRF is considered a master regulator of neuronal structural plasticity due to its ability to direct a specific transcriptional program of gene expression devoted to the regulation of actin dynamics [15] [23] [90]. We decided to evaluate in rat primary hippocampal neurons, the structural properties of SRFΔ5. To this purpose we overexpressed

recombinant myc-tagged SRF and myc-tagged SRFΔ5 vectors along with GFP to investigated neurites arborization at day *in vitro* 8 (DIV8) during *in vitro* maturation, and spine density in more mature neurons at DIV18 by means of confocal microscopy. The selection of these two times frames has to be related to different stages of neuronal *in vitro* maturation. Between DIV0-DIV12 the initial large morphology-related neurites growth and neuronal morphogenesis can be analyzed using Sholl analysis [81]. Later, between DIV12-DIV18, synaptogenesis starts and spine density can be assessed. In particular, in neurons transfected with SRFΔ5 compared to GFP, we outlined, by means of Sholl analysis, a trend to a simplified neurite arborization in terms of reduced number of dendritic branches, assessed as sum of total intersections between neuronal dendrites and crescent concentric circles centered in the neuronal soma. This trend becomes relevant by means of one-way ANOVA test at distances from the soma higher than 160 μm, hinting widely preserved number of primary dendrite and decreased arborization of apical dendrites (Fig. 16A). On the contrary, overexpression of myc-SRF entails little or no effect on neurite arborization when compared with control conditions. For what regards dendritic spine analysis performed in mature neurons at DIV18, overexpression of SRFΔ5 caused a highly significant decrease in dendritic spine density suggesting the functional frame of SRFΔ5 as a negative modifier of spines-related structural plasticity (Fig. 16B). Under the same conditions, the overexpression of myc-tagged full-length SRF gave, similarly to myc-SRFΔ5, a reduction in spine density compared to GFP overexpressing neurons. Yet, the comparison between such a spine density decrease and the one obtained overexpressing myc-SRFΔ5 proved to be very modest. Recent studies have shown that increase in spine density can be achieved in cultures of hippocampal neurons, by overexpression of a constitutively active SRF mutant characterized by a potent VP16 transactivation domain [90]. Thereby, we interpreted the inability of SRF to raise *per se* spine density as a further indication of the dual role of SRF as a repressor in resting conditions and as a transcriptional activator in response to stimuli [15] [31] (Fig. 16B). These data suggest that at least *in vitro*, role of SRFΔ5 in controlling dendritic spines should be principally related to the negative control of spine dynamics that can be attributed to its characteristics as a constitutive transcriptional repressor contending with SRF for target binding [17]. This is also further supported by the capacity of HA-SRFΔ5, likewise HA-SRF, to efficiently co-immunoprecipitate the histone demethylase LSD1 as well as histone deacetylase HDAC2 (Fig. 15A).

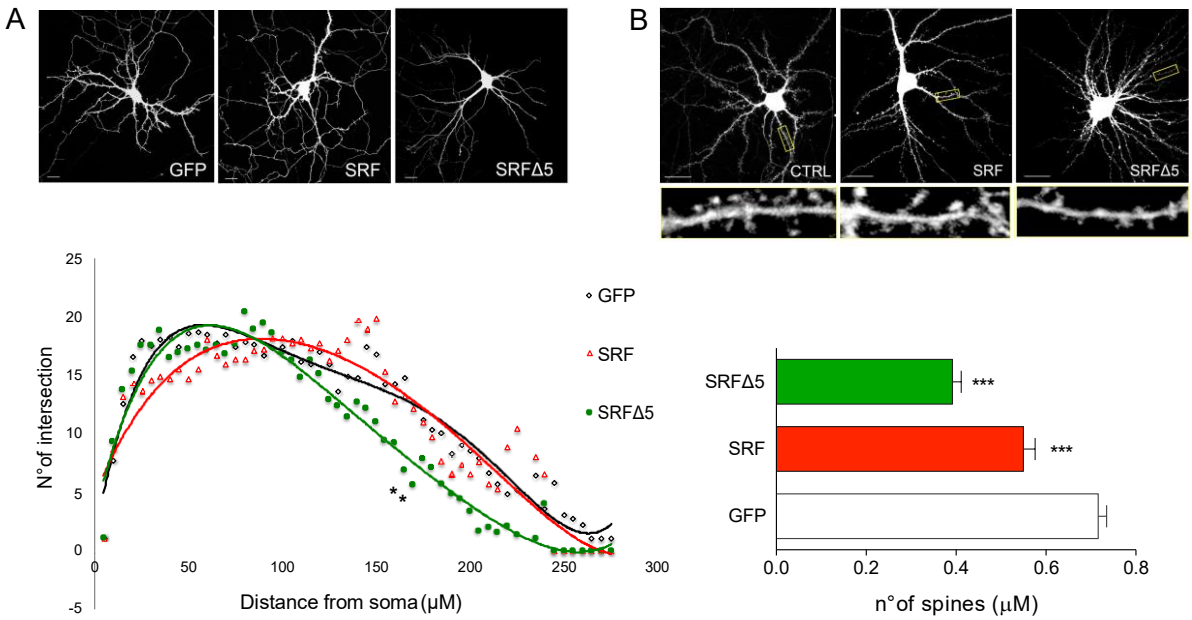

Fig. 16 SRFΔ5 is a negative regulator of dendritic spine density and neuronal morphology in hippocampal neurons. A) Morphological and Sholl-based analysis at DIV8 of GFP-positive rat hippocampal neurons transfected at DIV4 with myc-SRF and myc-SRFΔ5. B) Spine density analysis at DIV18 of GFP-positive neurons transfected at DIV4 with both SRF isoforms. Data are presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA, Bonferroni post hoc test.

4. Upon a paradigm of chemical LTP SRFΔ5 negatively modulates dendritic spines formation

SRF has an active role in repressing transcription in resting conditions; nevertheless, its best-characterized function is to promote transcription in response to stimuli thereby permitting neuroplastic changes to encode memory formation [90]. To better analyze the role of SRFΔ5 compared to that of SRF and to possibly highlight functional differences, we performed chemical LTP (cLTP). This paradigm induces *in vitro* those morphostructural changes of neurons that are inherent to dendritic spines potentiation. These modifications include increased number of spines and dimension of the spine heads together with enlarged postsynaptic densities. The same modifications are canonically observed during LTP induction at the Shaeffer collateral (CA3-CA1) in the hippocampus, which is obtained electrophysiologically and induced thanks to tetanic stimulation of hippocampal circuitry.

Morphological convergence of *in vivo* and *in vitro* LTP suggests the relevance of cLTP to study transcriptional mechanisms of memory formation in cultured neurons. Indeed, underlying this *in vitro* paradigm, a transcriptional contribution to dendritic spines stabilization is expected. Moreover, although rapid, the increase of spine density [90]

[91] [92] might involve a transcriptional role for SRF, which could be counteracted by SRFΔ5.

In particular, we transfected HA-tagged SRF, SRFΔ5 or the empty vector pCGN-HA along with GFP in hippocampal neurons at DIV4 and induced cLTP at DIV18. Mature neuronal cultures are necessary to induce cLTP, which is why we used DIV18 as the window of interest, a time point in which all spines are eventually developed and maximally functional. To induce cLTP we added to the neuronal medium 200 μM glycine, a co-agonist to the NMDA receptor that allosterically increasing the functionality of this glutamate receptor, leads to an increase of intracellular calcium which is inherent to induce morphological changes underlying potentiation. Next, after 2 hours, cells were fixed, stained for HA and dendritic spine density was scored by confocal imaging.

As Fig. 17 shows, SRFΔ5 overexpression occludes cLTP-induced growth of dendritic spine density, which is instead evident in the two samples transfected with empty vectors and SRF.

These results suggest a role for SRFΔ5 as a dominant negative SRF isoform but only in conditions of neuronal activation. cLTP in neurons overexpressing SRF, caused new spine formation, even though less efficiently in comparison with the empty vector. This outcome was unexpected seen the established role of SRF as a positive modulator of spine plasticity. Nevertheless, all literature data that highlight an active role for SRF in spine formation, were collected thanks to a chimeric fusion construct namely SRF-VP16. In particular, SRF-VP16 represents a non-physiological recombinant protein capable of promoting stimuli-independent constitutive activation of SRF targets but also leads to the loss of the SRF known transcriptional repression function. From this perspective, our data should also consider the effect of a possibly negative effect of SRF overexpression on dendritic spines morphology before the administration of cLTP, that, as Fig. 17 shows, also entails a drop of dendritic spines density, likely able to negatively affect activity dependent paradigms.

In other words, a long SRF overexpression from DIV4 to DIV18, as Fig. 16B had shown, could simplify neuronal morphology in a way that partially occludes its positive contribution at the moment of neuronal activation. Conversely, SRFΔ5 overexpression unambiguously indicates an active role as negative and constitutive regulator of neuronal plasticity, whose activity strictly relies on its cellular levels.

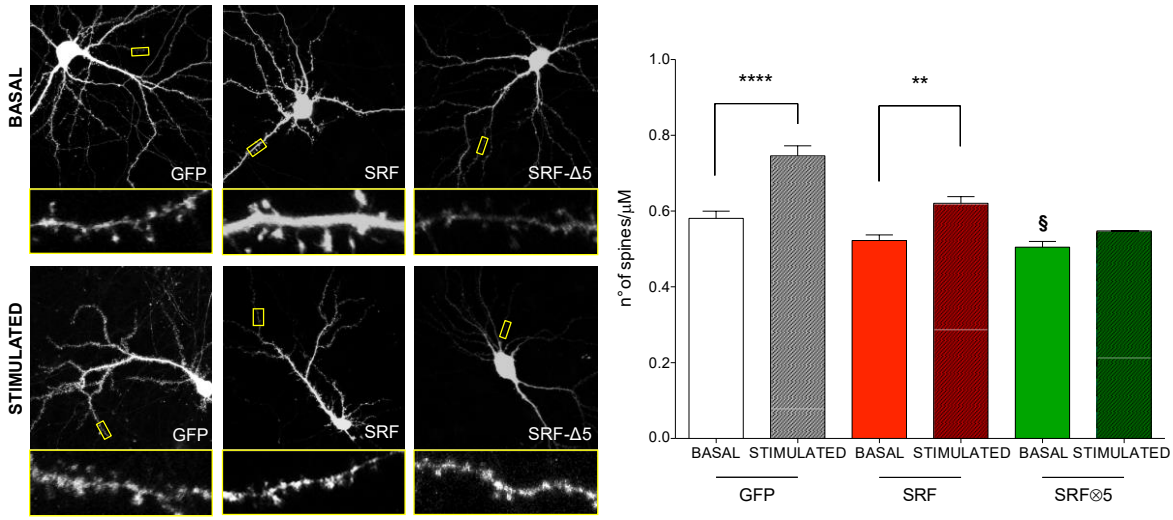

Fig.17 SRFΔ5 negatively modulates dendritic spines formation upon a paradigm of chemical LTP. Primary rat hippocampal neurons were transfected at DIV4 with HA-SRF, HA-SRFΔ5 and pCGN-HA vector. Spine density of GFP-positive neurons was evaluated with NeuronStudio software. Data are presented as mean ± SEM; § P < 0.05 vs. basal GFP condition, **P < 0.01, ****P < 0.001, one-way ANOVA, Bonferroni post hoc test.

5. SRF/SRFΔ5 protein ratio is modulated upon an acute paradigm of psychosocial stress

We recently demonstrated that long-term behavioral outcome of psychosocial stress in terms of developing a physiological anxiety-related profile depends on efficient stress-induced IEG transcription in the hippocampus [31] [93]. This activity-dependent transcription is strictly dependent on availability of correct balance of SRF corepressors and coactivators whose ratio is rapidly modulated by environmental stimuli [94] [75] [89]. Consequently, we investigated whether also the expression levels of SRF isoforms and the ratio between SRF and its dominant negative isoform SRFΔ5 could be affected upon a paradigm of psychosocial stress. For this purpose, we performed *Acute Social Defeat Stress* (ASDS), a paradigm of social stress, in *two-months* wild type mice. In particular, the experimental wild type mouse is put in interaction with a CD-1 aggressor mouse for 5 minutes, then, in order to limit physical damages for the experimental mouse, the two mice are separated by a transparent plexiglass remaining in visual and olfactory contact for few hours (2h-7h). Then we measured SRF and SRFΔ5 mRNA and protein levels during the phase of stress administration (2 and 7 hours), and after 24 of resting condition during which the animals are allowed to get better in a single cage in a different room from the CD-1 aggressor (Fig. 18 A-B-C). Our findings indicate that at the transcript level, during acute psychosocial stress, total mRNA levels of SRF isoforms remain stable after 2 and 7 hours of stress. Meanwhile, the relative percentage of SRFΔ5

transcript significantly increases after 2 hours from the beginning of the stress (Fig. 18A). Furthermore, at the protein level, both SRF isoforms tend to increase. Our results indicate that 2 hours after stress, only the increase of SRFΔ5 was statistically significant (Fig. 18B). Finally, in this time window, as shown in Fig. 18C, SRF target genes (*c-fos, egr1* and *npas4*) are transcriptionally induced by stress, which occurs also via SRF-mediated activity dependent transcription [31] [95]. Transcript data indicate that the modification of SRF and SRFΔ5 protein levels does not depend on a transcriptional process (Fig. 18A) rather, it seems that a post-transcriptional splicing-based mechanism is engaged aimed at skipping exon E5 from mature transcripts. Increased levels of the IEGs, SRF target genes, could be justified by a tendency to SRF protein stabilization. This increase is however paralleled by a concomitant increase in SRFΔ5, probably related to the strict control of this process. We interpret such a trend as an SRF/SRFΔ5-mediated homeostatic control over IEGs stress-induced transactivation, in a way that related structural modifications of hippocampal neurons could encode the memory of social stress in a buffered manner. In line, it has been shown that excessive stress-induced IEGs activity in the dentate gyrus of the hippocampus is causally linked to stress susceptibility, while a buffered expression leads to resilience [93]. As the stressful event ends, 24 hours after the end of the paradigm, SRF protein relative ratio returns to values which are similar to those observed in basal condition; in the same way, the sum of total SRF and SRFΔ5 proteins, after this time of resting condition, returns to basal level (Fig. 18B). In this time-point also *egr1, c-fos* and *npas4* IEGs targets transcription returned to resting levels (Fig. 18C). In conclusion, acute stress induces plasticity-related gene expression and meantime, it changes the level and activity of the transcription factors SRF and SRFΔ5. This expression profile of SRF and SRFΔ5 in response to an acute stress event is functionally convergent with the data related to the changes of the LSD1 and neuroLSD1 system previously published in our laboratory [31]. Increased levels of the repressor SRFΔ5 after stress, together with decreased levels of protranscription splicing isoform neuroLSD1 after the same paradigm, could concur to shut down IEGs activity-induced expression, and/or a window of IEGs unresponsiveness with a relevance for emotional processing of a stressful paradigm. This homeostatic response could have the purpose of counteracting SRF action as a transcriptional activator, contrasting possible long-term damages resulting from uncontrolled induction of pro-memorization morphological changes at the level of dendritic arbor of hippocampal neurons.

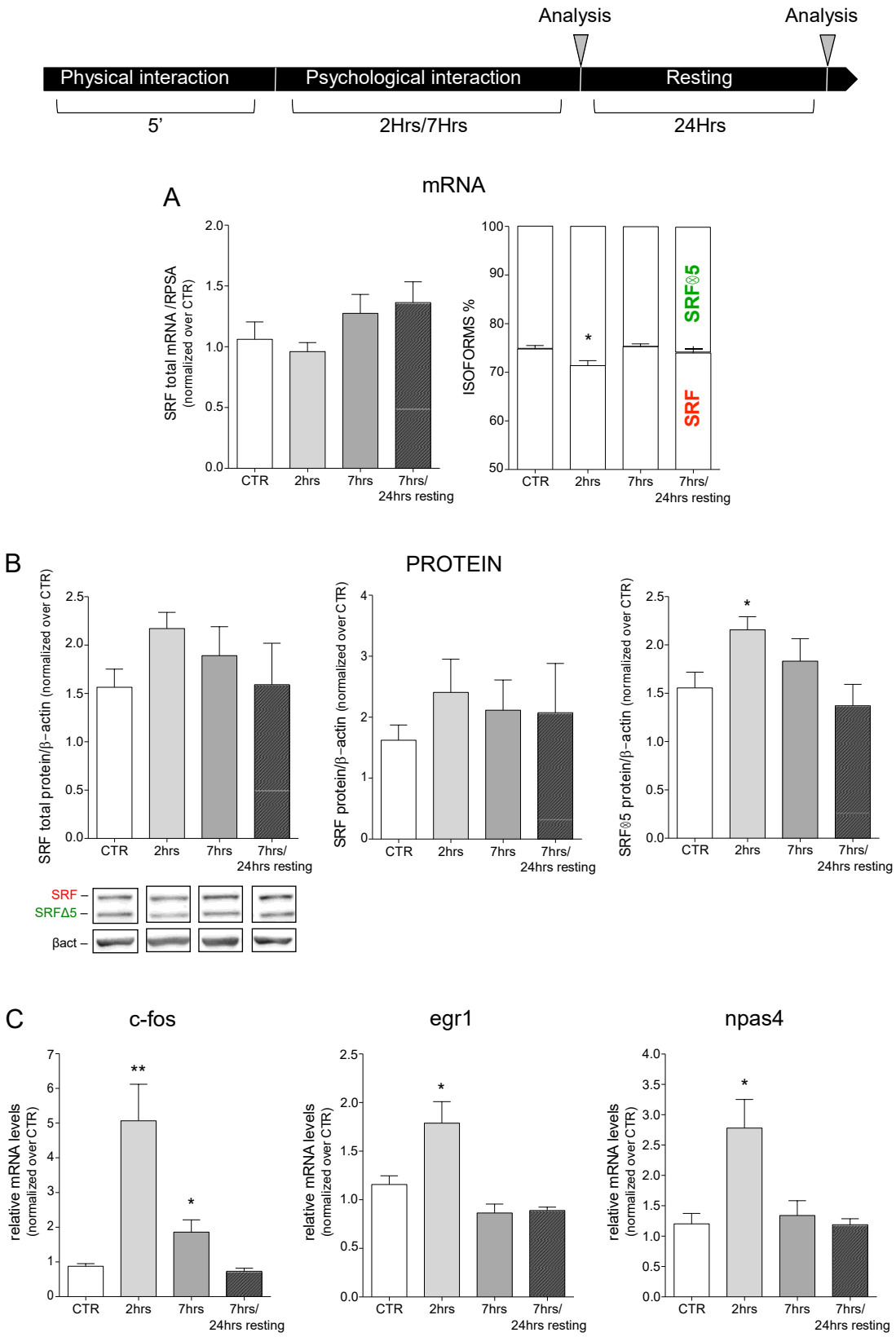

Fig.18 In mouse hippocampus *Acute Social Defeat Stress* (ASDS) transiently increases SRFΔ5 mRNA and protein. Schematic representation of *Acute Social Defeat Stress*. A) SRF transcripts analysis after 2 and 7 hours of stress paradigm and after 24 hours of resting condition: total SRF isoforms normalized on RPSA (assessed by Real Time PCR) and relative percentage of SRF isoforms (assessed with Rqf RT-PCR). B) Western blot analysis after the same social paradigm normalized over β-actin: total SRF isoforms (Sum of SRF and SRFΔ5) and protein levels analysis of SRF and SRFΔ5. C) Stress-induced transactivation of SRF validated targets *c-fos*, *egr1* and *npas4* in the mouse hippocampus after 2 and 7 hours of stress and after 24 hours of resting condition. Data are normalized over CTR condition and presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA, Bonferroni post hoc test. (n=6 to 15 mice per condition)

6. SRFΔ5 downregulation in the hippocampus is a distinctive feature of chronic stress susceptibility

In the hippocampus a paradigm of acute psychosocial stress is able to directly modify relative ratio between SRF and SRFΔ5 mRNA isoforms. Furthermore, the level of the two proteins is increased becoming statistically significant in the case of SRFΔ5. Therefore, we hypothesize that SRF could be implicated not only as transducer of stressful stimuli [96] [31] [95] but also as a stress response modifier due to its sensitivity to stressful experiences [97] (see Fig. 18A-B).

Given these results, we investigated the potential involvement of these transcription factors in hippocampal stress vulnerability pathways.

We applied *Chronic Social Defeat Stress* (CSDS), that modeling bullism and mobbing, a strong distress able to induce depressive-like behavior evaluated in terms of mice sociability [83].

This paradigm consists in the same social stress paradigm described above, but repeated for 10 days. Normally, after chronic stress mice cluster in two distinct groups: stress-susceptible (SUS) which represent the majority of animals and are characterized by the development of marked social avoidance associated with a multitude of overlapping behavioral and physiological changes reminiscent of depressive and anxiety symptoms; and stress-resilient animals (RES) corresponding to approximately one-third of the entire population that fails to develop social avoidance [83]. At the end of this experiment we investigated SRF and SRFΔ5 levels in these two different groups in the same way we did after the acute paradigm. After ten days of social stress, traits of psychopathology and particularly depressive-like behavior were assessed through Social Interaction Test, performed 24 hours after the end of stress.

In the Social Interaction Test the C57BL/6N mouse previously subjected to chronic stress is placed in the arena with a novel CD-1 mouse. CD-1 mouse is restrained in a small cage placed in a corner of the arena in order to limit any physical damages for the experimental mouse.

Thanks to the use of a video-camera and a specific software (ANY-Maze®), the behavior of the mouse is automatically monitored and the Social Interaction ratio (SIR) is calculated. SIR corresponds to the ratio between the time spent in the interaction zone in the presence of the CD-1 mouse and the elapsed time in the same area without a social

target [83]. Remarkably, susceptibility-related SIR data have been widely put in relation to depression-related behavioral abnormalities [98] [99].

A non-stressed control mouse, is normally very social and curious and tend to spend more time exploring the CD-1 mouse then far from it displaying SIR values higher then 1.2. Likewise, also resilient mice (RES) maintain an SIR higher then 1.2, retaining their interest for other mice. Vice versa, susceptible mice (SUS) are defined as those that showed an SIR lower than 0.8 being afraid or not interested in the other animal. 48 hours after the last session of the stress, and after the Social Interaction Test, mice are sacrificed and hippocampal RNA and protein are extracted and analyzed.

In particular, in RES mice, SRF and SRFΔ5 protein and mRNA levels were stable and very similar to those observed in control animals, along with the relative amount of SRF splicing isoforms (Fig. 19A-B). On the contrary, in SUS mice we identified an increase in the total SRF RNAs paralleled by a similar increase in the total SRF proteins. Curiously, we observed a small but significant increase in the relative amount of SRF protein and a concomitant decreased in SRFΔ5 relative abundance (Fig. 19B) suggesting a small change in the relative stability of the two proteins upon chronic stress.

In conclusion, these findings indicate that a prolonged stress is capable of consistently increasing cellular levels of the pro-transcriptional full-length SRF isoform only in SUS mice.

To evaluate the functional result of the modified SRF/SRFΔ5 ratio in favor of SRF, we assessed levels of IEGs in resting conditions, and observed that 48 hours after the end of the last stress, only in SUS mice, *c-fos* basal expression remained upregulated. In a similar way, *npas4* and *egr1* showed tendency toward SUS-specific increase in the same conditions. In particular in the hippocampus of those mice that upon CSDS develop depressive traits, stable overexpression of plasticity genes like egr1, c-fos and arc has recently been demonstrated and suggested as a mechanism of vulnerability [98] [99] [93].

Our finding that the transcription factor responsible for IEG modulation in response to stress, namely SRF, is positively modulated, provides a transcriptional underpinning of SUS mice restricted facilitation of IEG transcription in the hippocampus.

Thus, the increase in neuroplasticity in the hippocampus, which has been considered as one of the molecular causes for stress vulnerability, could be influenced by a particular SRF/SRFΔ5 pro-transcriptional equilibrium in resting conditions.

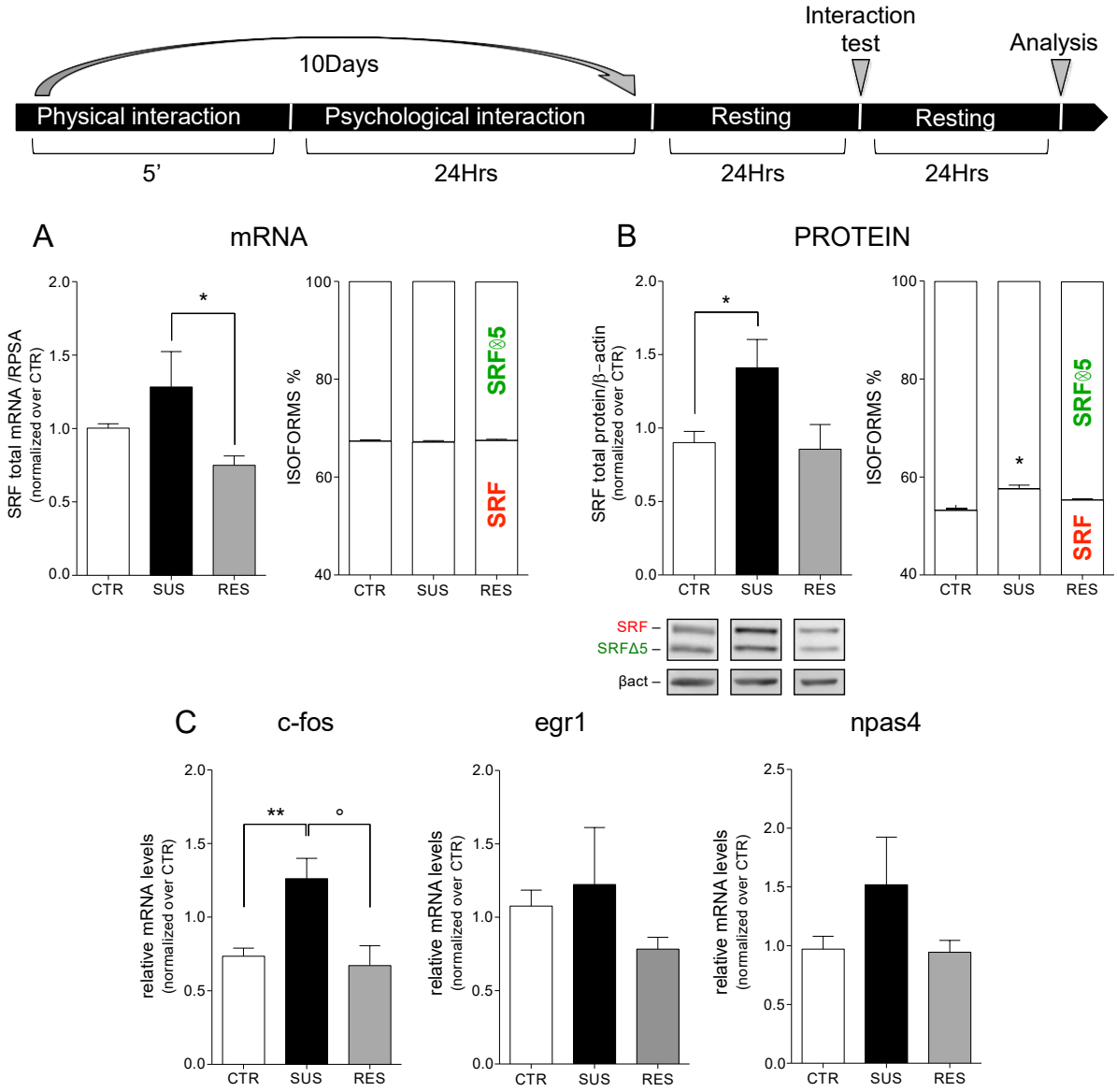

Fig. 19 SRF and SRFΔ5 mRNAs and proteins are modified by *Chronic Social Defeat Stress* (CSDS) in mouse hippocampus. Schematic representation of *Chronic Social Defeat Stress*. A) SRF transcripts analysis after 48 hours from the cease of chronic stress paradigm in susceptible, resilient and control animals: total SRF isoforms normalized over RPSA (assessed by Real Time PCR) and relative percentage of SRF isoforms (assessed with Rqf RT-PCR). B) Western blot analysis after the same social paradigm: total SRF isoforms normalized over β-actin (Sum of SRF and SRFΔ5) and relative percentage of SRF isoforms. C) Expression levels of SRF validated targets *c-fos*, *egr1* and *npas4* in the mouse hippocampus after 48 hours from the cease of stress. Data are normalized over CTR condition and presented as mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA, Bonferroni post hoc test. (ctrl mice n=24 SUS mice n=7, RES mice n=10).

CHAPTER II

1. In the mouse hippocampus, speckle-localized lncRNA MALAT1 is developmentally regulated

MALAT 1 (metastasis associated lung adenocarcinoma transcript 1, also known as NEAT2) is a large, infrequently spliced non-coding RNA, long (>6.5 kb), which is greatly conserved amongst mammals and highly expressed in the nucleus. In humans, the gene of MALAT1 is placed on the long arm of chromosome 11 (11q13.1) and it is also composed by 1 exon. First identified based upon its overexpression in different cancers, the function of MALAT1 in regular cellular physiology is still undiscovered [100].

It has been recently published that the lncRNA MALAT1 modulates splicing processes by squelching *trans*-acting splicing factors at the level of peculiar nuclear structures named speckles. One of the reported *trans*-acting RNA binding protein retained into speckles via a process involving MALAT1 is the SR protein SRSF1, also known as splicing modulator of CAMK2B, CDK7, SAT1, HMG2L1, ARHGEF1, B-MYB, and MGEA6 [70].

In the mammalian brain, and in particular in the mouse hippocampus, the neurospecific splicing process generating neuroLSD1, is finely tuned during development and the master regulator of this mechanism is nSR100, a protein belonging to the same SR family of splicing factors [69].

In order to understand whether MALAT1 could also play a role in the modulation of LSD1 splicing, we decided to evaluate MALAT1 expression in the hippocampus of wild type mice at different post-natal developmental stages.

Interestingly, as described in Fig. 20B, the expression of MALAT1 is minimal in the perinatal window and grows during the adolescence of the mouse till it reaches a plateau which is maintained during adult life. Importantly, when the levels of MALAT1 are very low, neuroLSD1 is at its maximum expression, starting to decrease exactly when MALAT1 begins to increase (Fig. 20A).

These results are consistent with the possible role of MALAT1 as exon E8a inclusion tuner in LSD1 mature transcripts and therefore on the expression of the dominant negative neuroLSD1.

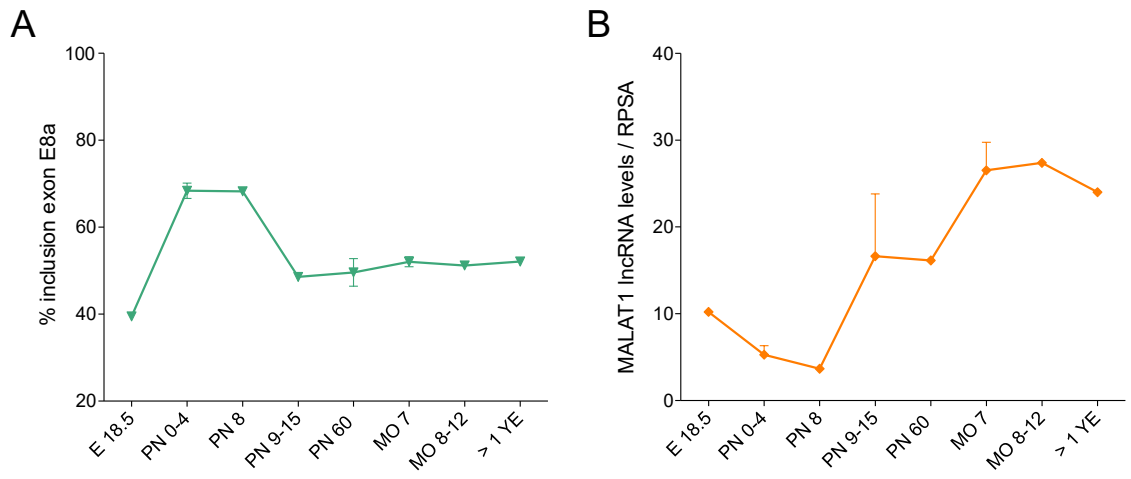

Fig. 20 Long non-coding RNA MALAT1 is developmentally regulated in mouse hippocampus. A) Percentage of exon E8a inclusion in LSD1 mature transcript assessed by Rqf RT-PCR. B) MALAT1 expression levels normalized over RPSA assessed by Real Time PCR. Data are presented as mean ± SEM.

2. Decreased levels of the lncRNA MALAT1, through the use of an antisense oligonucleotide, have an effect on the neurospecific splicing of LSD1 increasing the inclusion of exon E8a

Since the regulation of MALAT1 levels during development is compatible with a possible regulatory role of MALAT1 on the alternative splicing of LSD1, we decided to perform a minigene reporter assay to evaluate the nature of the contribution of MALAT1 on the inclusion of the alternative E8a exon in mature LSD1 transcripts. LSD1 exon E8a and its flanking introns (INT E8-E8a and INT E8a-9) have been cloned into a "minigene" namely the plasmid pBS-Splicing [101], generating the MG800 [69]. Minigene constructs are important tools for the identification and analysis of the *cis*-acting regulatory elements and *trans*-acting factors that control splicing specificity and efficiency and that regulate alternative splicing. Expression of different minigene constructs by transient transfection in different cell lines, provides a rapid assay for *loss-of-function* and *gain-or-function* analyses of *cis*-elements and *trans*-acting factors that affect splicing regulation. The Minigene Splicing Assay is highly sensitive and is based on the quantitative relative analysis of the minigene-derived transcripts including and not including exon E8a [69]. This strategy was followed because endogenous levels of neuroLSD1 in SH-SY5Y cells are too low to observe significant changes in the endogenous levels of exon E8a inclusion into LSD1 mature mRNA. In particular, the minigene reporter vector that we

employed is a hybrid construct, deriving from the low copy number pBlueScript II KS plasmid, where a minigene cassette was inserted at the level of its multiple cloning site, between the restriction sites PstI and BamHI. This cassette contains exons from fibronectin and α-globin genes under the control of the α-globin promoter and SV40 enhancer sequences, with a 3' polyadenylation site derived from the α-globin gene. In particular, the cassette is composed of the first two exons E1 and E2, deriving only from the α-globin gene, followed by two chimeric exons: one of them composed of the initial part of α-globin exon E3 and the final portion of fibronectin exon E24; the other one derives from fusion between fibronectin exon E25 and the terminal part of α-globin exon E3. These two chimeric exons were linked together by the standard fibronectin intron, which contains the restriction site NdeI (Fig. 21B). The presence of the chimeric exons allows, using specific primers annealing to the junction of each chimeric exon, the specific detection of minigene-deriving transcripts without any possible contamination from transcripts derived from endogenous fibronectin and α-globin genes. Moreover, the forward primer was designed to anneal with 3' end on the α-globin exon E2 and with the 5' end on the initial α-globin exon E3 in a way that only the mature and spliced transcripts, and not the plasmidic DNA sequences, were amplified [69].

We transfected the minigene in the context of a human neuroblastoma cell line (SH-SY5Y), and evaluated the splicing contribution of MALAT1 downregulation on exon E8a splicing inclusion. To decrease the expression levels of MALAT1 we used specific antisense oligonucleotides ASOs which, through the interaction with the lncRNA, cause an immediate degradation by endogenous RNase H, an enzyme that cleaves the RNA strand in a DNA/RNA heteroduplex. Two variants of RNase H exist in mammalian cells: RNase H1 and RNase H2. In particular it is believed that RNase H1 is responsible for ASO-directed RNA degradation. Modified antisense oligonucleotides, 2'OMe-PS ASO, were used to perform lncRNA silencing experiments. The 2'OMe-PS modifications are necessary to increase the stability and effectiveness of the ASOs. In fact, the unmodified DNA oligonucleotides are rapidly degraded by nucleases in the serum and in the intracellular environment strongly impacting their silencing efficacy. For this reason, the oligonucleotide is modified by replacing the canonic diester bonds with phosphorothioate bonds (PS), which make the ASO resistant to nucleases. However, the introduction of this type of modification strongly reduces the melting temperature of the oligonucleotide, influencing its effectiveness. To remedy this disadvantage, RNA bases

with a methyl group linked to oxygen at 2'(2'OMe-RNA) are added to the ends of the sequence. The addition of these modified bases determines the increase in the melting temperature of the oligonucleotide, thus improving its effectiveness. In this project two 2'OMe-PS ASOs specific for MALAT1 were used, as described by Lennox and Behlke et al., 2016 [102]: 5042 and 5326. For each sequence the number indicates the pairing position of the first nucleotide of each ASO. To consult the sequences of the ASOs used, refer to the "Materials and Methods" section.

To test the efficacy of each oligonucleotide and to establish the dose to be used for subsequent experiments, SH-SY5Y cells were used. All antisense oligonucleotides were tested at concentrations of 50 nM and 200 nM. The expression of MALAT1 was normalized on two housekeeping genes EIF4A2 (eukaryotic translation factor 4A, isoform 2) and RPSA (ribosomal protein SA). MALAT1 was evaluated 48 hours after transfection with each specific ASO, 5042 or 5326, by RT-qPCR. The silencing efficiency was evaluated comparing MALAT1 expression from samples transfected with a control unrelated ASO (NC1). Fig. 21A shows the results of the silencing tests. In particular, only antisense oligonucleotide 5326 was effective, reducing the level of MALAT1 of about 65% at the 200 nM dose, compared to an equivalent amount of control oligonucleotide (data on treatment with 50 nM of ASOs are not shown because there is no effect at this concentration).

Having identified an ASO able to efficiently decrease the level of endogenous MALAT1, we proceeded to verify if the inclusion rate of exon E8a in MG800 derived transcripts could be modulated by the silencing of MALAT1 in SH-SY5Y cells. The analysis was conducted on the cDNA obtained by the retro-transcription of the RNA extracted from the cells 48 hours from transfection with the 5326 antisense oligonucleotide and the pBS-Splicing MG800 plasmid, containing an 806 bp fragment comprising exon E8a and flanking introns.

The percentage of exon E8a inclusion into minigene-derived transcripts in cells transfected with the plasmid pBS-Splicing MG800 (NT) alone, the plasmid and the control oligonucleotide (NC) and the plasmid and the specific oligonucleotide (ASO 5326) were analyzed. As shown in Fig. 21B, no significant differences were observed in the percentage of exon E8a inclusion between cells transfected with the plasmid (15.46%) and cells transfected with the plasmid and the control oligonucleotide (17.11%). In contrast, treatment with the specific ASO for MALAT1 resulted in a

significant increase (24.12%) of exon E8a inclusion into minigene-derived transcripts compared to control.

All of these data are consistent and reinforce the hypothesis that an indirect control of MALAT1 on the inclusion of exon E8a in mature transcripts of LSD1 exist.

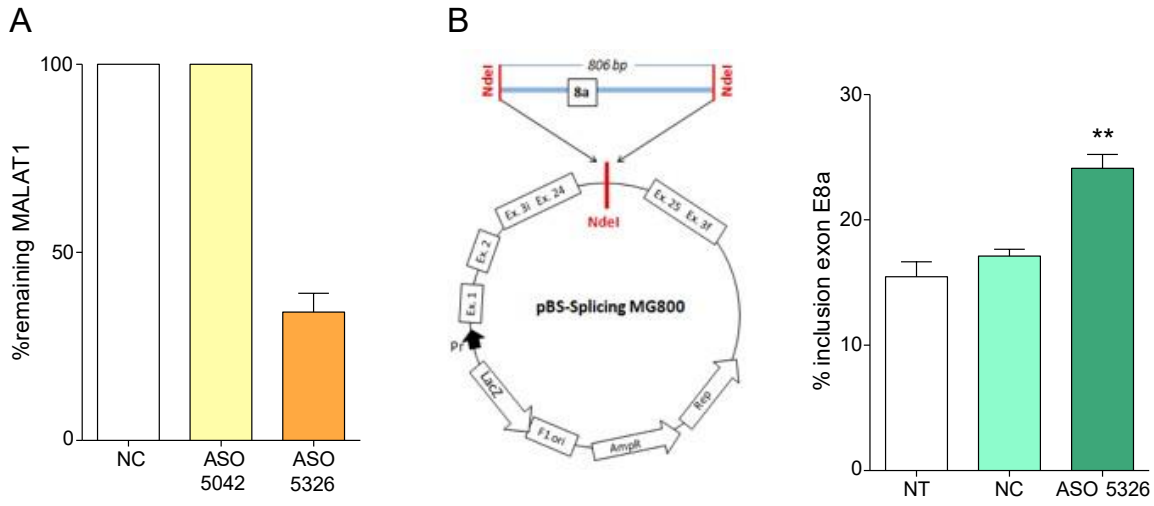

Fig. 21 Antisense oligonucleotide against MALAT1 influences LSD1 alternative splicing, leading to higher exon E8a inclusion. A) Relative levels of the lncRNA MALAT1 in SH-SY5Y 48 hours after transfection with 200nM of specific antisense oligonucleotides, ASO 5042 and ASO 5326, compared with control values obtained transfecting unrelated ASO (NC). B) Schematic representation of pBS-Splicing MG800. Percentage of the inclusion of exon E8a in minigene-derived transcript in SH-SY5Y cells transfected with pBS-Splicing MG800 and with 200 nM of ASO 5326 or an unrelated ASO (NC). Data are presented as mean ± SEM; **P < 0.01, Student *t* test.

3. Tubercidin, an inhibitor of nuclear speckle formation, negatively modulates MALAT1 increasing the levels of neurospecific splicing isoform of LSD1

So far, we have shown how MALAT1 could be involved in regulating the expression of neuroLSD1, modulating the inclusion of the microexon E8a, through the retention of nSR100 in nuclear speckles. In this section, to further prove a role for MALAT1 as neuroLSD1 modulator, we wanted to verify if Tubercidin, an antibiotic that causes structural perturbation of nuclear speckles altering expression and function of nuclear speckles components among which MALAT1, could affect alternative splicing of exon E8a. Tubercidin is an adenosine analogue, firstly identified in culture filtrates of *Streptomyces tubercidicus*, and was reported to show antibiotic and anti-tumor effects [103]. In particular, Tubercidin was shown to be incorporated *in vivo* into RNAs during transcription. Also speckle-localized lncRNAs, such as MALAT1, incorporate Tubercidin and this might affect the localization of splicing factors. One possible effect of Tubercidin

is to affect nuclear speckles interfering with the synthesis or stability of speckle-localized ncRNAs. We confirmed that Tubercidin treatment is able to reduce the basal levels of MALAT1 transcript in primary cortical neurons as it was already observed by Kurogi et al., 2014 [103] (Fig. 22B). However, the reason for the reduction of MALAT1 levels is not yet known. In particular, we treated primary cultures of rat cortical neurons (DIV13) with 5 µg/ml of Tubercidin and to proceed with the RNAs extraction at 4 and 8 hours after treatment. Notably, we observed that, already 4 hours after the treatment, there was an increase in the relative levels of neuroLSD1 expression that from a baseline level of about 56%, typical of the control sample, was increased to 60%. After 8hrs of treatment, neuroLSD1 expression reached 66% (Fig. 22A). Thus, Tubercidin is likely to induce the release of nSR100 from speckles and increase its nuclear concentration at the sites where splicing reactions take place, resulting in changes in alternative pre-mRNA splicing of LSD1. These results further support the role of lncRNA MALAT1 as fine modulator of neuroLSD1 expression in neuronal cells modulating the availability of nSR100. Curiously, by analyzing the expression levels of nSR100 by Real Time PCR, and normalizing them on the expression of the RPSA housekeeping gene, we could observe a decrease in the transcripts of nSR100 both at 4 and 8 hours after the treatment with Tubercidin (Fig. 22C). This apparently contradictory result could be explained as an attempt by the neuronal cell, to counteract the activity of splicing factors among which nSR100 massively released from speckles into the nucleoplasm.

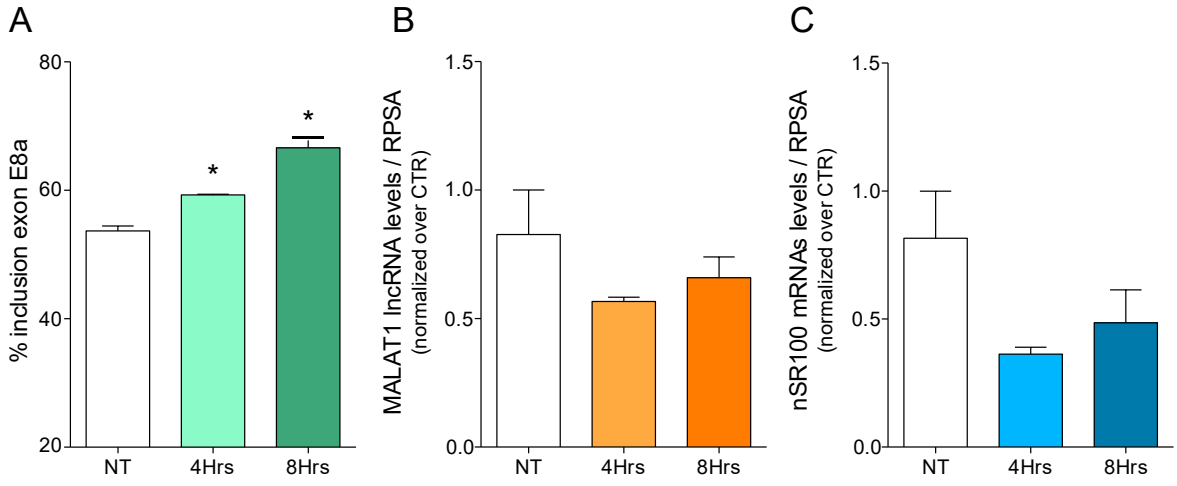

Fig. 22 Treatment with Tubercidin increases exon E8a inclusion and negatively modulates the expression of MALAT1. A-B-C) Primary rat cortical neurons (DIV 13) treated with 5 µg/ml of Tubercidin for 4-8 hours: exon E8a inclusion frequency assessed by Rqf RT-PCR (A), MALAT1 (B) and nSR100 (C) expression levels normalized over RPSA. Data are normalized over CTR condition and presented as mean ± SEM; *P < 0.05, Student *t* test.

4. lncRNA MALAT1 behaves as an IEG in response to *Acute Social Defeat Stress*

NeuroLSD1 expression is finely regulated during development by fluctuation of its *trans*-acting splicing factors [65]. However, neuronal activity and in particular the paradigm of social defeat stress, is able to rapidly and reversibly reduce exon E8a splicing. For this reason, the identification of MALAT1 as a further layer of splicing regulation, provides a possible mechanism of dynamic splicing modulation in response to neuronal activity. It has already been demonstrated that MALAT1 is a bona fide *Immediate Early Gene* which, following neuronal activation, is expressed quickly and immediately thanks to the double strand breaks mechanism [33]. We therefore applied *Acute Social Defeat Stress* on *two-months* wild type mice, in the same modalities explained in the previous chapter [31]. We could observe that already at 2 hours from the beginning of the stress, MALAT1 expression level was greatly increased as expected for an IEG (Fig. 23A). This transcriptional burst is transient since already at 7 hours, MALAT1 transcript returns to basal level. The peculiar kinetic of MALAT1 transcriptional response could be functional to the homeostatic neuroLSD1 downregulation we observed 7 hours after ASDS [31]. In other words, it is possible to hypothesize that the strong increase at 2 hours in the expression levels of MALAT1 induced by the psychosocial stress paradigm, might be directly responsible for the decrease of the levels of the dominant negative neuroLSD1 7 hours after stress. In particular, increased nuclear levels of MALAT1 would result in increased retention of the splicing factor nSR100, which plays a key role in the inclusion of exon E8a, thus reducing the expression of the neurospecific LSD1 isoform. In light of this it is possible to think of the lncRNA MALAT1 as a possible protagonist in the stress response pathway, proposing it as a possible therapeutic target in anxiety disorders. Finally, as further control, we also wanted to evaluate a possible change in the transcription levels of nSR100 and NOVA1, the two splicing factors that intervene in the alternative splicing of LSD1, two hours after the stress event. As shown in Fig. 23B two hours after the stress event the transcripts of both splicing factors are not modified; this data further reinforces our hypothesis of a central role of MALAT1 in the regulation of neurospecific LSD1 splicing in response to neuronal activity because the whole mechanism seems to be regulated only by a greater presence of the lncRNA which leads to a more retention of nSR100 in nuclear speckles and not dependent on a modification

in the transcription level of this splicing factor.

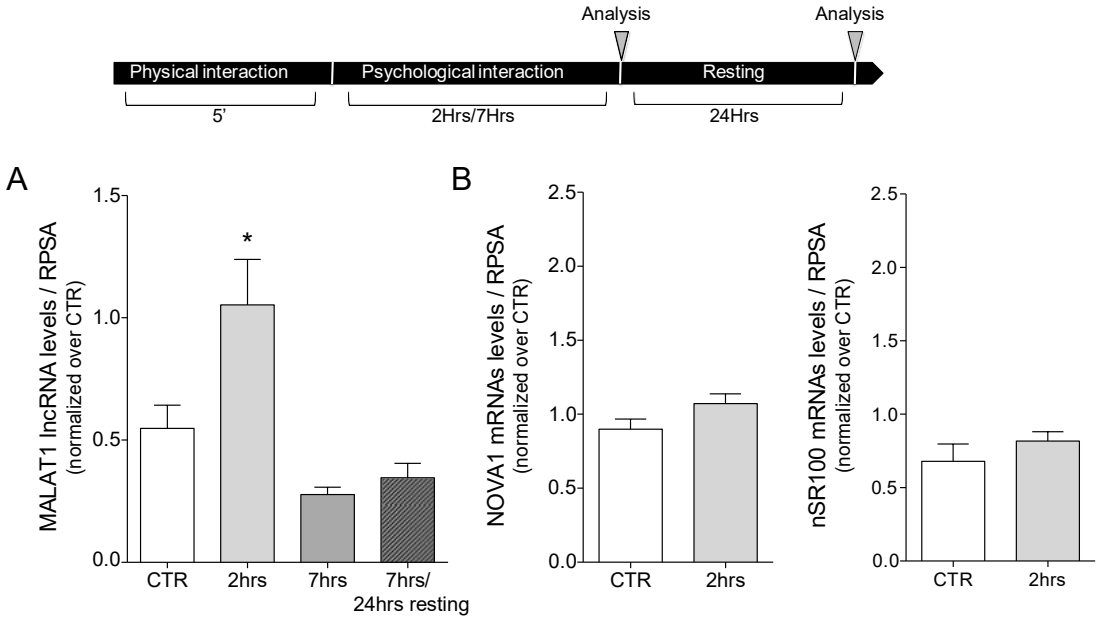

Fig. 23 lncRNA MALAT1 is transactivated by social defeat stress in mouse hippocampus. Schematic representation of *Acute Social Defeat Stress*. Transcripts analysis after 2 and 7 hours of stress paradigm and after 24 hours of resting condition. A-B-C) Expression levels of MALAT1 (A), nSR100 (B) and NOVA1 (C) normalized over RPSA. Data are normalized over CTR condition and presented as mean ± SEM; *P < 0.05, Student *t* test. (n=6 to 10 mice per condition)

5. lncRNA MALAT1 levels are stably increased following a stress paradigm performed in chronic, *Chronic Social Defeat Stress*, and represent a hallmark of resiliency

In the previous sections, we have seen how the lncRNA MALAT1 intervenes in the acute stress response pathway and how its increase causes a homeostatic decrease in neuroLSD1 expression levels leading to a more repressed chromatin state. Stress characterizes everyday life and is a critical risk factor for the onset of mood disorders and major depression [104] [105]. It is notoriously accepted that psychosocial stress specifically induces maladaptive structural neuronal responses in those susceptible individuals who undergo strong or repeated stress events [106] [107]. In particular, on the one hand the maladaptive modifications of the neuronal morphology represent a footprint of stress vulnerability; on the other hand, resilient animals avoid the onset of stress-induced depressive traits through homeostatic adaptation mechanisms [108] [109]. These pathways are instrumental to protect against the negative effects of stress through the activation of specific transcriptional programs and driving commitment of circuits linked to resilience through a dedicated modulation of neuronal structural

plasticity [99] [110]. In light of this, in this section, we wanted to analyze whether the expression levels of the lncRNA MALAT1 could be modified following a stress paradigm executed in chronic. Therefore, we performed the Chronic Social Defeat Stress, in the same way explained in the previous chapter, grouping the experimental mice, thanks to the use of the Social Interaction Test, in susceptible and resilient [83]. After 24 hours from the Social Interaction Test, and then 48 hours after the last stress event, the animals were sacrificed and the RNAs were extracted. Importantly, from Real Time PCR experiments, we could observe how only in the group of resilient animals, i.e. those mice that at the time of the Interaction test did not show depressive-like traits and displayed no changes in their sociability, showed a trend to maintain higher levels of expression of MALAT1 even 48 hours after the last stress (Fig. 24). Increased nuclear levels of lncRNA could lead to major retention of the splicing factor nSR100 in nuclear speckles and therefore reduced protein to regulate alternative splicing. In resilient individuals, all these data could be interpreted as an attempt at homeostatic adaptation that would lead to a more repressed chromatin environment with major control over the massive activation of plasticity genes that could be harmful if prolonged. Taken together these data suggest a possible role for MALAT1 in the pathway of stress response and propose it as a possible therapeutic target in neuropsychiatric disorders.

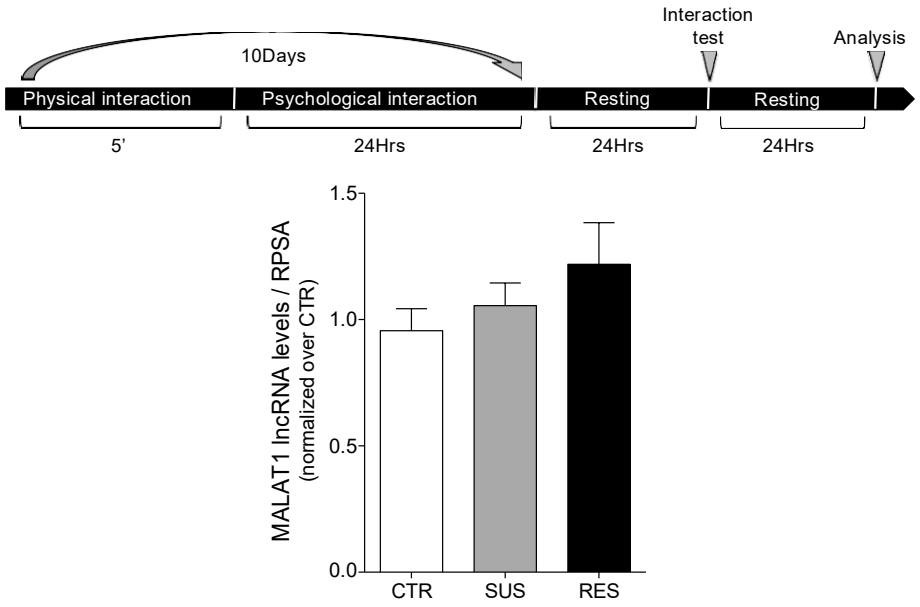

Fig. 24 lncRNA MALAT1 levels are stably increased after *Chronic Social Defeat Stress* in resilient animals. Schematic representation of *Chronic Social Defeat Stress*. MALAT1 expression levels, normalized over RPSA, after 48 hours from the cease of chronic stress paradigm in hippocampus of susceptible, resilient and control mice. Data are normalized over CTR condition and presented as mean ± SEM. (ctrl mice n=24 SUS mice n=7, RES mice n=10).

DISCUSSION

CHAPTER I

In the last twenty years, a considerable effort has been made to decipher the involvement of epigenetic mechanisms in establishing neuronal and behavioral plasticity [37]. Epigenetics deals with inheritable changes in gene expression that are independent of the DNA sequence [38]. Particularly in the brain, where most neurons are post-mitotic, epigenetics is an important molecular interface that modulates gene expression in response to the environment and participates in higher brain functions such as cognition, behavior and language. The epigenome therefore plays a role in orchestrating the relevant transcriptional plasticity to transmute environmental stimuli into morphological and functional changes. In any case, the epigenome is particularly vulnerable and acute, chronic or strong insults of different origins, for example metabolic or stress factors can permanently alter the epigenetic heritage generating the so-called epigenetic "scars" that may have pathogenetic significance. For all these reasons, the epi-pharmacological manipulation of chromatin remodeling pathways could represent a new approach in the treatment of many human disorders, including neurological and neuropsychiatric disorders.

Stress-related psychiatric disorders such as depression and anxiety are complex diseases on a pathophysiological point of view, with underlying molecular mechanisms still essentially unknown. Nevertheless, several lines of evidence show that interplay between individual genetic vulnerability and environmental risk factors, widely referred to as stress, may precipitate pathology [111] [112] [113].

Early-life stress (during the decade of childhood) mediates the risk for both anxiety and depression [114], while chronic stress in adulthood appears to precipitate depression in vulnerable individuals [115].

Many studies have shown that exposure to acute stress can change memory processes in different ways, depending on the extent, intensity and timing of the stress event [3]. In particular, it is known that stress effects are commonly mediated by activation of the *hypothalamic-pituitary-adrenal* (HPA) axis and by the release of glucocorticoids [8], which mediate fast actions in the brain that favor the release of glutamate from the cortex and the hippocampus areas [116] [117] and strengthening synaptic transmission [118]. Despite the fact that acute stress has been found to have beneficial effects on memory acquisition [3], at the same time it alters memory retrieval [4].

In this context we place LSD1, a flavin-dependent histone demethylase, and its alternative splicing isoform, called neuroLSD1. In particular, in our laboratory it has been shown that neuroLSD1 has a central role on higher neuronal functions that include memory formation [68], control of emotional behavior, and in particular anxiety-like profile [31] and neuronal excitability, evaluated in terms of neuroLSD1-mediated epileptic seizures susceptibility modulation [69].

My doctoral project was born from the discovery, during my period of master thesis, that LSD1, in addition to binding the known transcription factor REST and regulate the transcription of its target genes, was able to interact with the transcription factor SRF, *Serum Response Factor*, notoriously involved in the development and physiology of the nervous system, intervening on the modulation of IEGs expression during development. LSD1 and SRF are ubiquitous proteins, implicated in important cellular processes in many tissues. As with the epigenetic enzyme LSD1, the activity of SRF in the nervous system can also be regulated by an alternative splicing mechanism that leads to the skipping of the exon E5 and the generation of a transcription factor lacking a substantial part of transactivation domain, SRFΔ5, which acts as a dominant negative.

In the brain the alternative splicing of SRF, although not a mechanism exclusively restricted to the nervous system, together with the neurospecific splicing of LSD1, widens the possibility of combinatorial assembly by increasing the modulation of the IEGs trascription, regulated by the LSD1 corepressor complex, SRF and their respective negative dominants neuroLSD1 and SRFΔ5.

In particular, during my PhD, we focused on the characterization of the alternative splicing isoform SRFΔ5 in the mouse hippocampus and we define the role of SRF and SRFΔ5 in the acute response to a psychosocial stress and in the molecular mechanisms involved in susceptibility, at the base of the onset of depressive disorders.

The splicing mechanism leading to the generation of the SRFΔ5 isoform is regulated during development in the mouse hippocampus. In accordance with its dominant negative role, the low expression of SRFΔ5 immediately after birth and adolescence correlates with the high neuroplasticity present in this window of development of the nervous system. Then the relative percentage of SRFΔ5 transcript and protein increase with age and in the achieved equilibrium the two SRF splicing isoform cooperate to modulate the gene transcription level of their targets.

In the nervous system, SRF is well known to be an important regulator of neuronal

plasticity, by virtue of its ability to activate a transcriptional program aimed at controlling actin microfilament dynamics [15] [90].

In particular, we have shown that the over-expression of SRFΔ5 in rat hippocampal neuron cultures leads to a simplification of neurite arborization in terms of a decreased number of dendritic branches. In contrast, overexpression of SRF has no effect on dendritic arborization compared to control. Furthermore, overexpression of SRFΔ5 reduces the density of dendritic spines, consistent with its dominant negative role in transcription of SRF target genes and outlining a negative function of SRFΔ5 in the neural plasticity process. A more modest reduction in the number of spines with respect to the control is also observed following the overexpression of SRF, despite an increase in plasticity and therefore in the density of dendritic spines. The inability of SRF to increase the density of the spine by itself further supports the dual role of SRF in the nervous system, as a repressor in basal conditions and as an activator in response to stimuli [15] [31].

In particular, all literature data that highlight an active role for SRF in spine formation, were collected thanks to a chimeric fusion construct namely SRF-VP16. SRF-VP16 represents a non-physiological recombinant protein capable of promoting stimuli-independent constitutive activation of SRF targets but also leads to the loss of the SRF known transcriptional repression function. In this context, having obtained a decrease in the growth of dendritic spine density in primary hippocampal rat neurons overexpressing HA-SRFΔ5 and subjected to a chemical LTP protocol, compared to those overexpressing HA-SRF, allowed to confirm the role of SRFΔ5 as a dominant negative isoform of SRF but only in a context of neuronal activation.

The relative amount of SRF and SRFΔ5 is modified in response to acute stress and chronic stress, representing a new element that could help shape an individual's vulnerability to depressive disorder by interfering with the homeostatic mechanisms underlying resilience to a psychosocial stress. In the hippocampus, although acute stress generates a picture of maximal transcriptional activation of the plasticity genes, we observed a decrease in the relative amount of the SRF transcriptional activator and an increase in the dominant negative SRFΔ5. This could be due to the establishment of compensatory mechanisms with the aim of maintaining the neuronal plasticity of the hippocampal circuit within an adaptive interval sustained therefore by the increase of the dominant negative SRFΔ5.

The results obtained from the characterization of the SRF and SRFΔ5 system following a chronic psychosocial stress delineate a framework in which the transcriptional factor SRF, responsible for the modulation of IEGs in response to stress, is positively regulated only in susceptible individuals contributing to increased neuroplasticity in the hippocampal circuit, which is a predisposing factor to the onset of depression. Thus, in the hippocampus of resilient individuals SRFΔ5 could play a homeostatic role aimed at maintaining hippocampal neuroplasticity at an adaptive level following chronic stress. A possible origin of stress susceptibility could derive from a deficit in the homeostatic splicing mechanisms of SRF which are normally aimed at neutralizing the excessive pathological neuroplasticity of the hippocampus in response to stress through the dominant negative SRFΔ5 (Fig. 25).

Understanding the markers of susceptibility and stress resilience is a fundamental objective to shed light on the pathological mechanisms involved in the onset of depression. In fact, the discovery of the molecular processes underlying the vulnerability to psychosocial stress could in the future provide a biological rationale for the development of innovative pharmacological strategies for a new antidepressant treatment. This requires a better understanding of the central nervous system response to stress events and a better understanding of the origin of individual variability in terms of behavioral response following psychosocial stress.

In this context we propose the transcription factor SRF as a new vulnerability gene to psychosocial stress.

The regulation of SRF activity through the alternative splicing of the dominant negative SRFΔ5 is a phenomenon also present in the human brain. Therefore, in humans the presence of SRFΔ5 in the hippocampus could provide a molecular target for future pharmacological strategies for the prevention and treatment of stress-related disorders such as anxiety and depression.

The extension of these molecular analyzes to human post-mortem hippocampal tissues resulting from depressed patients compared to controls could provide the biological rationale for the use of inhibition of SRF activity in the hippocampus to counteract the molecular mechanisms underlying of susceptibility to the onset of depression.

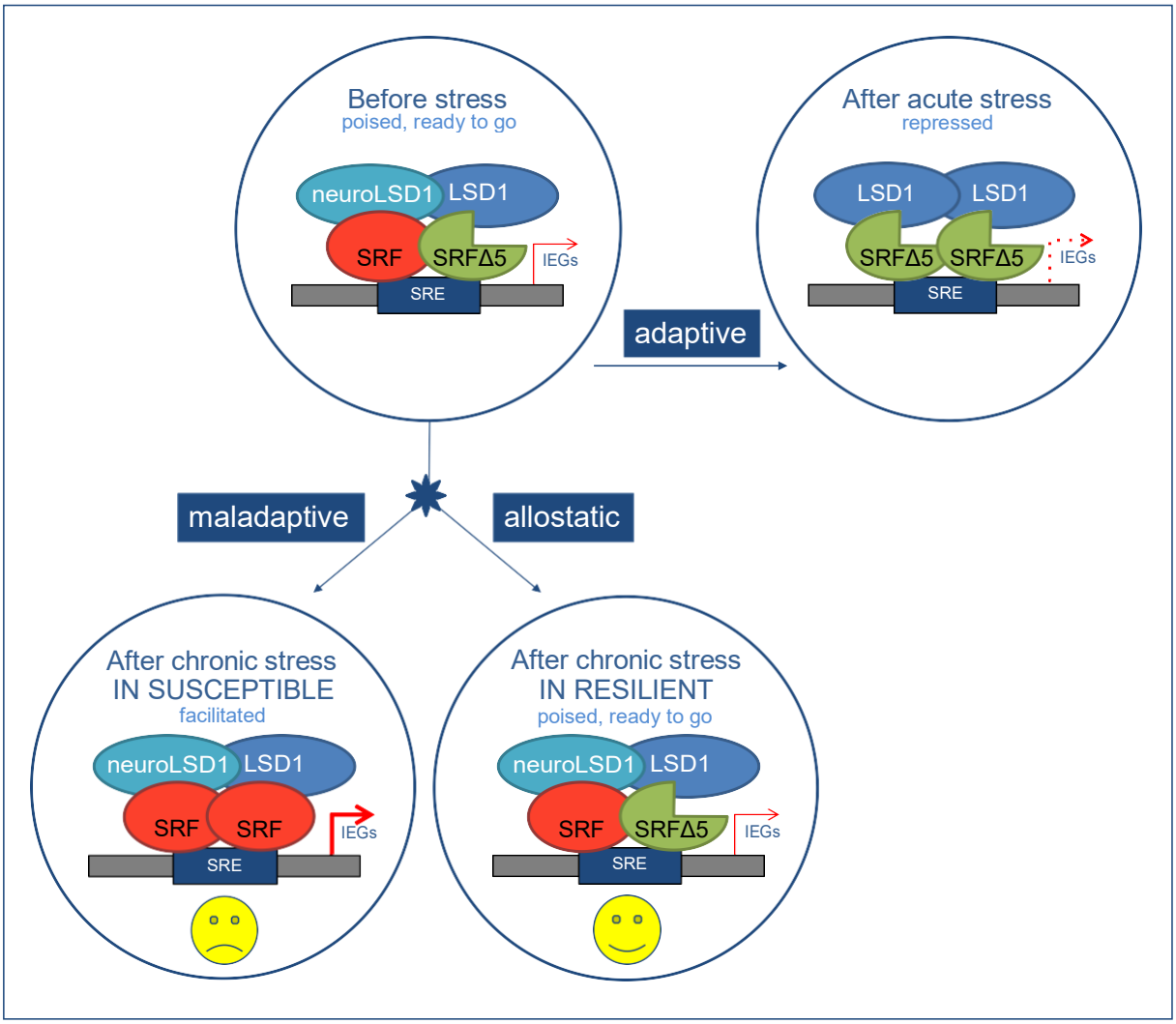

Fig. 25 Role of balance between LSD1/neuroLSD1 and between SRF/SRFΔ5 in psychosocial stress response. Schematic representation of the role of the balance between LSD1 and neuroLSD1 and between SRF and SRFΔ5 in response to acute and chronic psychosocial stress, highlighting the role of allostatic and maladaptive mechanisms in defining a framework of resilience or susceptibility to psychosocial stress.

CHAPTER II

Alternative splicing is a strategic biological mechanism that allows to create a set of functionally different gene products from a single gene, diversifying gene functions without an increase in the number of genes [60] [61]. In this way, alternative splicing helps to increase the evolutionary complexity of mammals and at the same time represents a powerful mechanism for regulating the activity of a protein.

Transcripts of neurospecific genes are known to undergo numerous alternative splicing events and thus regulate fundamental neuronal processes in both cellular functions, such as synapse formation and axon orientation [62] [63] [34] and more complex cognitive functions such as learning and memory [64].

In our laboratory neuroLSD1, an activity-dependent alternative splicing isoform of LSD1, was recently discovered and related to important homeostatic neuronal functions impacting emotional processing and in particular anxiety-like behavior [31].

It has recently been published as MALAT1, a long non-coding RNA, has a crucial role in the alternative splicing mechanism of some genes such as CAMK2B, CDK7, SAT1, HMG2L1, ARHGEF1, B-MYB, and MGEA6 through the regulation of the splicing factor SRSF1, belonging to the SR protein family. In particular, MALAT1 is mainly localized at the level of the nuclear speckles, where it seems to regulate the alternative splicing through the retention of SRSF1 in these nuclear domains and the modulation of its phosphorylation state [70].

Recently, in our laboratory, it has been discovered that alternative splicing involving LSD1 is positive regulated *in trans* by two splicing factors NOVA1 and nSR100. In particular, nSR100 is a splicing factor belonging to the SR protein family, as SRSF1, and regulates tissue-specific alternative splicing in a manner dependent on its concentration and its phosphorylation status [69].

During the last part of my PhD, we evaluated the possible role of the lncRNA MALAT1 as a negative regulator of the alternative splicing of LSD1 through interaction with the splicing factor nSR100. In particular, nSR100, as SRSF1, if retained in nuclear speckles by MALAT1 could be unable to play its role as a positive regulator of the exon E8a inclusion in the LSD1 mature transcript.

The expression of the lncRNA MALAT1 is modulated during development in the mouse hippocampus. In accordance with its role as a regulator of the action of the splicing factor nSR100, the low expression of MALAT1 immediately after birth, between PN0 and PN8, correlates with the high inclusion of exon E8a present in this development window of higher plasticity of the nervous system. After this window, the expression of MALAT1 increases with age and correlates with the decrease in the levels of the microexon E8a inclusion. MALAT1 could therefore acts as a negative regulator of the inclusion of exon E8a in the mature transcript of LSD1 limiting the expression of the neurospecific splicing isoform neuroLSD1 during development.

In a human neuroblastoma cell line (SH-SY5Y), downregulation of MALAT1 levels, through the use of antisense oligonuleotides (ASO), leads to a significant increase in the inclusion of exon E8a in mature transcripts deriving from the minigene pBS-Splicing MG800. The increased exon E8a inclusion in minigene-derived transcripts, could most

likely be due to an increase in the nuclear soluble nSR100 probably released by nuclear speckles by the MALAT1 knock down.

Likewise, in primary rat cortical neurons, the antibiotic Tubercidin leads to an increase in the frequency of exon E8a inclusion in mature endogenous transcripts of LSD1. Tubercidin acting through a double mechanism of action influences the nuclear localization of the lncRNA MALAT1. On the one hand it has a destructive action on the nuclear speckles' formation, on the other is incorporated by the speckle-localized lncRNA MALAT1 and influences the localization of the splicing factors linked to it as nSR100. Importantly, it has also been published on Kurogi et al.,2014 [103], that Tubercidin treatment causes a decrease in the expression levels of MALAT1. The nSR100 protein, free in the nucleus, could therefore become able to play its role as a positive regulator of the exon E8a inclusion. In other words, the increase of the non-restrained form of nSR100, leading to the increase of exon E8a inclusion, promotes the formation of the protrascriptional isoform neuroLSD1.

It has been published in our laboratory that *Acute Social Defeat Stress* (ASDS) causes a massive activation of the transcription of IEGs, a class of fundamental genes in the phenomena of neuroplasticity and the basis of learning and consolidation of memory, mediated by the transcription factor SRF [31]. Importantly MALAT1, following neuronal activation, is expressed rapidly and immediately thanks to the double strand breaking mechanism [33] and can be considered a bona fide *Immediate Early Genes* (IEGs). We have shown that also following a social stress paradigm, performed in acute (ASDS), the levels of MALAT1 are significantly increased after 2 hours from the beginning of the stress. This result is of particular interest considering that we have shown that many stimuli of neuronal activation, including the ASDS, generate a homeostatic response in brain tissues, including the hippocampus, leading to decrease the protrascriptional isoform neuroLSD1 and, at the same time, to increase the repressor counterpart LSD1 aimed at turning off the transcription of IEGs. This effect could be mediated by the alternative splicing mechanism in which nSR100 intervenes and might be triggered by the brief MALAT1 transcriptional wave in response to ASDS, that might temporarily sequester nSR100 within nuclear speckles being the indirect cause of neuroLSD1 homeostatic downregulation.

Although more experiments are necessary to formally demonstrate a direct interaction between MALAT1 and nSR100, this lncRNA could be considered a negative regulator of

the splicing that generates neuroLSD1.

These data provide a very interesting picture in which in response to stress in the hippocampus, several events are initiated: the first (i) and fast one is the transcription of the IEGs the main actors of the response. However, among these genes, the lncRNA MALAT1 is induced to trigger a homeostatic response to the stress inducing as second (ii) event, a reduction in neuroLSD1 and participating in the creation of an "unresponsivity window" in which SRF-driven transcription of the IEGs is buffered. In addition, also the dominant negative SRFΔ5 is upregulated by a third (iii) unknown mechanism, further contributing to decrease IEGs transcrivibility (Fig. 26).

The results obtained from the characterization of the lncRNA MALAT1 following a chronic psychosocial stress delineate a framework in which MALAT1, responsible for the negative regulation of the exon E8a inclusion in the mature transcript of LSD1, through its action on the nSR100 splicing factor, is positively regulated only in resilient individuals who manage to maintain physiological expression levels of IEGs in the hippocampus.

In other words, only the subjects resilient to chronic stress are still able to modulate the stress-related maladaptive transcription reporting the system to the basal physiological condition. In particular, this adaptive response to stress occurs thanks to the high levels of expression of MALAT1 that, only in resilient individuals, regulate in a negative way, the formation of the activity-dependent alternative splicing isoform neuroLSD1 suggesting that also this lncRNA, likewise SRF and its dominant negative isoform SRFΔ5, might all be considered possible hallmark of resilience.

In conclusion, MALAT1 plays a crucial role in the stress response pathway in which LSD1/neuroLSD1 and SRF/SRFΔ5 are involved. Furthermore, due to its high sequence conservation between rodents and human and its high expression in neuronal tissues, it represents one of the possible molecular targets underlying future pharmacological strategies for the prevention and treatment of diseases related to an excessive transcriptional activation state, such as epilepsy, or pathologies due to stress, such as anxiety and major depression.

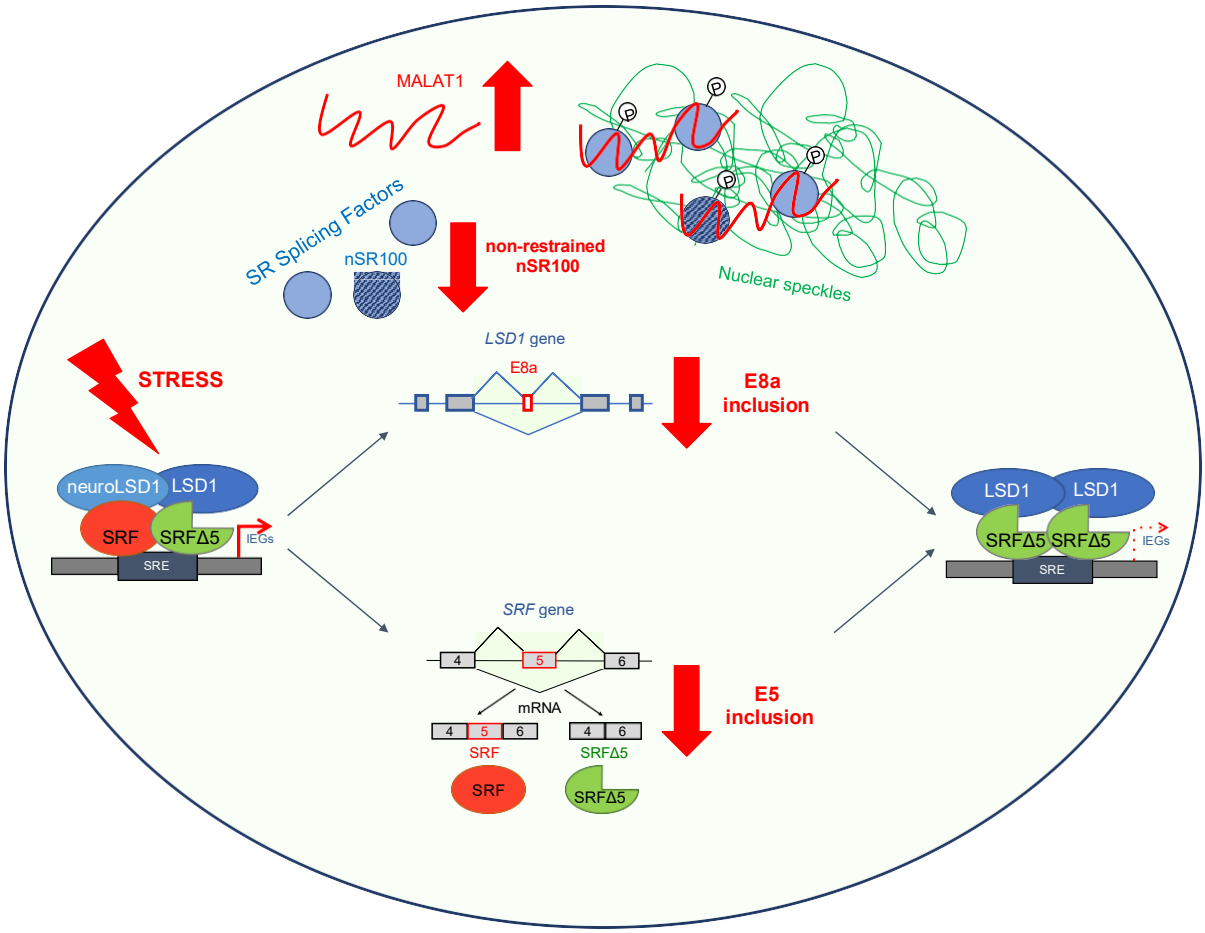

Fig. 26 In response to psychosocial stress a series of different events are triggered in the nucleus of neurons. Schematic representation of the events that characterize stress response in the nucleus of a neuron. A stress event causes a strong transactivation of IEGs, including MALAT1. Increased levels of MALAT1, through the retention of the splicing factor nSR100 in nuclear speckles, negatively regulate the exon E8a inclusion in LSD1 mature transcripts and thus promote the formation of neuroLSD1. Following stress there is also a negative regulation of the exon E5 inclusion in the SRF mature transcript which leads to the formation of SRFΔ5. All these events take part in a homeostatic response that aims to bring the system back to its basal condition.

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