# SRF and SRFΔ5 splicing isoform recruit corepressor LSD1/KDM1A modifying structural neuroplasticity and environmental stress response.

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**Abstract**

Ten to twenty percent of western countries population suffers from Major Depression Disorder (MDD). Stressful life events represent the main environmental risk factor contributing to the onset of MDD and other stress-related neuropsychiatric disorders. In this regard, investigating brain physiology of stress response underlying the remarkable individual variability in terms of behavioral outcome, may uncover stress-vulnerability pathways as a source of candidate targets for conceptually new antidepressant treatments. Serum Response Factor (SRF) has been addressed as a stress transducer via promoting inherent experience-induced *Immediate Early Genes* (*IEGs*) expression in neurons. However, in resting conditions, SRF also represents a transcriptional repressor able to assemble the core LSD1/CoREST/HDAC2 corepressor complex, including demethylase and deacetylase activities. We here show that dominant negative SRF splicing isoform lacking most part of the transactivation domain, namely SRFΔ5, owes its transcriptional repressive behavior to the ability of assembling LSD1/CoREST/HDAC2 corepressor complex meanwhile loosing its affinity for transcription-permissive cofactor ELK1. SRFΔ5 is highly expressed in the brain and developmentally regulated. In the light of its activity as negative modulator of dendritic spine density, SRFΔ5 increase along with brain maturation suggests a role in synaptic pruning. Upon acute psychosocial stress SRFΔ5 isoform transiently increases its levels. Remarkably, when stress is chronically repeated a different picture occurs where SRF protein becomes stably upregulated in vulnerable mice but not in resilient animals. These data suggest a role for SRFΔ5 that is restricted to acute stress response, while positive modulation of SRF during chronic stress matches the criteria for stress vulnerability hallmark.

**Introduction**

Everyday life stress is a critical risk factor toward the onset of depression and mood disorders[1,2]. It is commonly accepted that psychosocial stress specifically elicits maladaptive structural neuronal responses in those susceptible individuals that undergo stress-induced depressive traits[3-8]. In particular, stress remodels dendritic arbor as well as spine shape and density in different brain areas including, but not limited to hippocampus, amygdala and prefrontal cortex thereby modifying neuron excitation thresholds[7]. Notably, these different areas undergo opposite structural changes in response to stress synergistically contributing to a thorough reshaping of affective cortico-limbic brain circuitry[6]. In general, maladaptive modifications of neuronal morphology represent a footprint of stress-vulnerability; on the other side, resilient animals allostatically avoid the onset of stress-induced depressive traits via preserving *homeostatic adaptation mechanisms*[9-12]. These pathways are instrumental to protect against the negative effects of stress via activation of a specific transcriptional program –involving proper control of the Immediate Early Genes (*IEGs*)– and driving engagement of resiliency-related circuitry through dedicated modulation of neuronal structural plasticity[8,10,11,13-15]. A master integrator of the transcriptional program orchestrating *IEGs* expression in neurons in response to experience is the transcription factor Serum Response Factor (SRF)[16]. SRF has been also implicated in stress response as a mediator of neuronal adaptation underlying resilience to chronic Social Defeat Stress in the Nucleus Accumbens (NAc)[13]. Moreover, through activity-dependent control of several *IEGs*, featuring a Serum Responsive Element (SRE) at the level of genomic regulatory regions, SRF remodels neurons cytoarchitecture and morphology impinging on synaptic activity of stress-responsive circuitry, thereby modulating behavioral outcome[13,14]. SRF represents a versatile transcription factor because of its ability to promote transcription upon stimuli and to function as a repressor in resting conditions[17,18]. Consistently, we show that

SRF binds epigenetic corepressor Lysine Specific Demethylase 1 (LSD1/KDM1A) together with CoREST and HDAC2, thus recruiting at the IEGs promoters a core epigenetic corepressor complex that has already been shown to exert negative regulation of memory and synaptic plasticity in mice[19,20].

Interestingly, in the hippocampus a paradigm of psychosocial stress increases the availability of corepressor LSD1 through a transcriptional and splicing-based mechanism also involving downregulation of dominant negative neurospecific LSD1 isoform neuroLSD1[18]. It has been proposed that reduced IEGs expression in the hippocampus upon psychosocial stress concurs to resiliency via *homeostatic mechanisms*[18,20]*.* Acute stress-induced epigenetic modifications in the hippocampus to concur to restrain *IEG* transcription in response to further stressful experiences, a process that has been associated to maintaining stress-related plasticity and consequent memorization of the negative experience at an adaptive level[12,15,18,20]. Taking into account that most often, acute stress does not lead to long term psychobiologic and behavioral outcomes, learning molecular rules of acute stress response –likely desensitized via chronic stress-related allostatic overload– could represent a promising strategy to decipher molecular underpinnings of vulnerability[12]. In search for novel neurospecific modulators of *IEG* transcription in response to stress, we focused on transcription factor SRF. SRF includes three well-characterized functional domains, the MADS-box DNA binding domain, which is also involved in homodimerization, a transactivation domain located at the C-terminus of the protein that alone can activate transcription of a reporter gene[21] and a N-terminal portion where two distinct repressive domains were mapped and described as instrumental to restrain target transcription[21,22]. SRF has been indeed described to bind target DNA promoters in both basal conditions, behaving as a repressor when the IEGs are largely silenced, and upon serum-induced activity

(in proliferating cells) or neuronal activity in neurons, behaving as transcriptional activator [23-25].

It has been described that splicing mechanism leading to exon E5 skipping can regulate SRF function in the heart. The alternatively spliced isoform SRFΔ5 is a transcription factor lacking a substantial part of the C-terminal transactivation domain but retaining the ability to bind DNA. This splicing isoform antagonizes SRF function as transcriptional activator, acting as a potent repressor of SRF-dependent promoters[26]. In this work we outlined for the first time SRFΔ5 expression in mouse and human brain. In the hippocampus SRFΔ5 makes up a substantial amount of SRF protein isoforms, suggesting that alternative splicing may regulate SRF function in this brain area. Interestingly, both SRF and SRFΔ5 bind the transcriptional corepressor complex LSD1/CoREST/HDAC2 via their shared N-terminal repressor domain, but relevantly, SRFΔ5 cannot bind ELK1, one of the most characterized positive SRF cofactor[27-29]. SRF mainly regulates morphostructural-relevant transcription and consistently SRFΔ5 overexpression significantly reduces spine density in hippocampal neurons. We propose that positive and negative modulation of activity-evoked transcription via SRF and LSD1 together with their brain enriched splicing isoforms, potentially contributes to shaping psychosocial stress vulnerability.

**Materials and methods**

*Protein Extraction, Western Blot and Immunoprecipitation.* Experiments were performed essentially as in[30,31]. Cultured cell lines or tissues were homogenized as described[32]. We performed immunoprecipitation experiments and western blot experiments with the following antibodies: anti-SRF (D71A9; Cell Signaling Technology); anti-myc (Abcam Ab9106); anti-LSD1 (C69G12; Cell Signaling Technology); anti-ELK1 (9182; Cell Signaling Technology); anti-Synaptophysin (D35E4, Cell Signaling Technology); anti-HDAC2 (ab7029,

Abcam), anti-CoREST (Merck Millipore); anti-β Actin (A2228; Sigma-Aldrich); anti-HA (sc-805; Santa Cruz Biotechnology); anti-HA-AC (sc-7392; Santa Cruz Biotechnology); normal mouse IgG-AC (sc-2343; Santa Cruz Biotechnology); goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) and donkey anti-rabbit IgG horseradish (ECL).

Western blot analyses were carried out using Alliance Mini HD9 acquisition system and NineAlliance 1D software (Uvitec Cambridge, UK).

*GST Pull-Down.* Briefly, BL21 cells were CaCl$_2$-transformed with pGEX-GST, pGEX-GST-hSRF, pGEX-GST-hSRFΔ5, pGEX-GST-hSRF1-171-N-term, pGEX-GST-hSRF339-508-C-term and encoded GST-tagged proteins were expressed, batch purified by conjugation to Glutathione Sepharose 4B beads (GE Healthcare), and incubated over night with 2 months old male mice forebrain extracts obtained with a low stringency buffer. GST pull-down experiments were performed as in[33]

*Plasmids.* For coimmunoprepitation experiments: pCGN-HA-hSRFΔ5 was obtained from pCGN-HA-hSRF (Addgene, 11977) by site-specific mutagenesis using the Q5 Site-Directed Mutagenesis Kits (New England BioLabs, Massachusetts, USA); myc-hSRF and myc-hSRFΔ5 were obtained by subcloning hSRF and hSRFΔ5 into pcDNA3.1-myc/His (-). For GST Pull-down: pGEX-hSRF1-171 was obtained from pCGN-HA-hSRF by site-specific mutagenesis introducing a stop codon after amino acid 171 and then subcloning it into pGex plasmid; pGEX-hSRF339-508-C-term was generated by PCR using pCGN-HA-hSRF as template and cloned in XbaI site in pCGN-HA vector, introducing a NLS in frame with the coding sequence, and then subcloning it into pGEX plasmid.

For primary neurons transfection: myc-fused SRF and SRFΔ5 (pCDNA3.1-myc/His-hSRF and pCDNA3.1-myc/His-hSRFΔ5) were generated by PCR using pCGN-vectors as templates and cloned into expression vector pCDNA3.1-myc/His in the EcoRI and BamHI sites; pEGFP-N1

was obtained from Addgene. pEGFP-N1 construct, supplied by Addgene, was transfected to mark the entire neuronal cell. All plasmids were sequenced.

*Total RNA Extraction, qRT-PCR Analysis, and rqfRT-PCR*. TRIzol reagent (Sigma-Aldrich) was used for total RNA isolation from hippocampal extract. Any residual DNA was removed treating the purified RNA with RNase-free DNase set (Qiagen). qRT-PCR analysis was performed as described elsewhere[34]. Expression of SRF isoforms was normalized on Ribosomal protein SA (RPSA). RqfRT-PCR was used to measure exon E5 splicing inclusion in mature SRF endogenous transcripts, as described for LSD1[35] Thanks to the annotated primers (Supplemental material) we obtained two PCR products that have been scored for displaying identical amplification efficiency with qRT-PCR. A common forward primer has been designed on exon E4, and two reverse primers, the former (specific for SRF) was designed on exon E4-E5 splicing junction, and the latter (specific for SRFΔ5) was designed on exon E4-E6 splicing junction.

*Primary hippocampal neuronal cultures.* Primary hippocampal neurons were prepared from embryonic days 18-19 rat brains[36] and plated on coverslips coated with poly-L-lysine (50μg/ml) at 75.000/well for immunochemistry. Cultured neurons were cotransfected using the calcium phosphate method as described in[37]. Immature neurons were transfected at DIV4 and fixed at DIV8-18.

*Proteasome inhibition.* DIV12 primary rat hippocampal neurons were treated with MG-132 for proteasome inhibition. 20 μM MG-132 (Sigma-Aldrich) was added to the medium. Untreated samples were incubated with the drug solvent (DMSO). Protein extraction was performed after 2,5 hours of treatment.

*Morphological and spine density analyses.* Neuronal cells were fixed with 4% paraformaldehyde/4% sucrose at DIV8 for morphological analysis and at DIV18 for spine density evaluation. Images were acquired by confocal microscopy. Morphological analysis was

performed using the Sholl analysis module in Fiji software, while spine density was evaluated with NeuronStudio software.

*Chemical LTP (cLTP).* Primary rat hippocampal neurons were treated for chemical LTP at DIV18. Ten minutes before stimulation the medium was replaced by pre-warmed Stimulation solution (3 µM strychnine, 20 µM bicuculline) in Extracellular solution (140mM NaCl, 1,3 mM $CaCl_2$, 5mM KCl, 25 mM HEPES, 33mM glucose). cLTP was induced with 200 µM glycine 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.

*Experimental Animals.* 10-weeks-old male C57BL/6N wild-type mice were used and they were housed individually throughout the testing period with free access to food and water. Mice lived in controlled temperature (20–22 °C) with a 12-h light/dark cycle (lights on at 7:00 AM). All experimental procedures entailing the use of rodents followed the guidelines established by the Italian Council on Animal Care (Legislative Decree no. 26, March 2014) and European regulations (2010/63/UE) and were approved by Italian Ministry of Health. Every experiment was made to minimize the number of mice used and their suffering. We strictly followed these current rules and regulations regarding animal treatment during all the procedures.

*Acute Social Defeat Stress (ASDS).* We used a modified protocol of the SDS test[18]. In short, CD1 aggressor mice were used to defeat 10-weeks-old C57BL/6N wild-type mice in a single session of SDS. The experimental mouse was exposed to a CD1 aggressor mouse for 5 minutes. After the physical contact the two mice were separated by a perforated Plexiglas divider and the stress continued in its psychological form through visual and olfactory interactions with the aggressor. In ASDS test the control mice were housed (two per cage) in the opposite sides of Plexiglas divider in cages identical to those of experimental mice. Respectively after 2 or 7 hours of physical contact, the molecular analyses were performed in

both of control and stressed mice. Animals were also analyzed 24 hours after the cease of a 7 hours-long acute psychosocial stress.

*Chronic Social Defeat Stress*. We employed a standardized protocol of CSDS, able to induce long-lasting depression-like phenotype with anhedonia, anxiety and social-avoidance behaviors[5,38]. Briefly, aggressive CD1 retired breeder mice were used to defeat 8/9 weeks old C57BL/6 mice. C57BL/6 mice were put in direct contact for 6,5 min/day over 10 consecutive days to a novel CD1 aggressor. After the 6,5 min physical contact, experimental mice and CD1 aggressor were separated by a perforated Plexiglas divider in order to allow sensory interaction, in the context of limiting potential physical damages to the smaller intruder mice. Non-defeated control C57BL/6 mice were housed (two per cage) in the opposite side of divided cages identical to those used for the defeat. 24 hours after the cease of CSDS, defeated and control mice were subjected to the social interaction test to measure social avoidance. In the first part of the test each mouse was placed in a square arena (40 x 40 cm) with an empty small cage for 2,5 min. In the second part of the test each mouse was placed back in the arena for 2,5 min but with an unfamiliar CD1 mouse inside the small cage. The social interaction ratio (SIR, i.e. time spent in the interaction zone with the novel CD1 mouse present/ time spent in interaction zone without the CD1 mouse) was calculated. Control mice usually have a SIR >1. Defeated mice with a SIR<0,8 were considered as susceptible (SUS), while defeated mice that showed a SIR>1,2 were considered as resilient (RES). 48 hours after the cease of CSDS, mice were sacrificed and hippocampal areas collected for RNA and protein analysis. We started from a total number of 48 mice. 24 mice were stressed and 24 were used as controls. Of the 24 controls we selected 7 mice with SIR>1,2. Of the 24 stressed mice, 17 were further analyzed for SIR. 10 were RES and 7 were SUS[5]. We excluded 3 RES with 1<SIR<1,2 and 1 SUS with 0,8<SIR<1.

*Data Analysis and Statistical Methods.* We used the minimum number of animals predicted by statistical sample size determination analyses using the following parameters: POWER 0.8; α 0.05; β0.2. Data are shown as means ± SEM. The statistical analyses were performed using unpaired Student's *t* test for single comparisons and one-way or two-way ANOVA for multiple comparisons with PRISM 6.0 software (GraphPad).

**Results**

**1. SRFΔ5 is enriched in the brain and developmentally regulated in the mouse hippocampus**

SRF repressive activity has been mapped and functionally characterized within the N-terminal domain[21]. For instance, prototypical SRF target *c-fos*, is not only transactivated in response to stimuli but also actively repressed in basal conditions with the contribution of SRF[21,22,39]. This state of repression can be referred to as poised repression[22] and is at least in part mediated by SRF ability to recruit a structured HDAC-containing complex[18,40,41], among which the LSD1/CoREST/HDAC2 corepressor complex[18].

We previously observed that, also in the brain, SRF protein is present in two distinct isoforms, the canonical full-length protein of 508 amino acids with an electrophoretic mobility at 67 KDa and a specific and faster migrating band at 57 KDa, the expected molecular weight of SRFΔ5[18]. Note that it has recently been shown that in the mouse brain, knocking down SRF entails loss of both bands[42]. SRF gene undergoes different events of alternative splicing; therefore we further investigated the origin of p57 band as a predicted alternative splicing isoform. In Fig. 1A the alternative splicing events generating SRF and SRFΔ5 are schematized. We compared the expression of the two SRF protein isoforms in mouse, rat and human hippocampus as well as in neuronal and non-neuronal cell lines (Fig. 1B). Migration of recombinant myc-tagged SRF and SRFΔ5 fusion proteins overexpressed in Neuro2a

neuroblastoma cell line, recapitulates the hippocampal endogenous SRF and SRFΔ5 SDS-PAGE immunoreactivity profile (Fig. 1B). In order to unambiguously define that SRF p57 corresponded to SRFΔ5, we performed a RT-PCR on different mouse tissues using primers able to generate two families of SRF-related amplicons –including and skipping exon E5– in the same semi-quantitative PCR assay. These primers anneal on SRF exon E4 and E6 thereby generating amplicons of different molecular weight. As shown in Fig. 1C the upper band, corresponding to full-length SRF was highly prevalent in non-neuronal tissues. On the contrary, the lower amplicon predicted to belong to SRFΔ5 was expressed in the forebrain as well as in different brain areas, similarly to SRF. Notably, sequence analysis of the lower band, confirmed the presence of expected junction between exons E4 and E6, ultimately demonstrating that this band originates from SRFΔ5 (Fig. 1D). A more quantitative analysis based on relative quantity fluorescent PCR (rqfRT-PCR[35]) of SRF and SRFΔ5 splicing ratios has been performed by using a specific set of three primers generating two amplicons comparable in length (a first which is specific for SRF and a second for SRFΔ5). With this approach we further confirmed the preferential brain expression of SRFΔ5 compared to other tissues (Fig. 1E).

Next, using the same rqfRT-PCR technique we characterized SRFΔ5 expression during development in the mouse hippocampus, in parallel with SRF (Fig. 1F). As shown, SRFΔ5 relative mRNA level remains stable from PN 9-15 to adulthood (months 7-12). Conversely, SRFΔ5 protein level increases during development in the mouse hippocampus, representing 30% of all SRF proteins at the perinatal window (PN1-8 Fig. 1G), and increasing to almost 50% during adulthood (MO7-12 Fig. 1G). This weak correspondence between RNA and protein levels along with aging suggests that post-translational differences between the two SRF isoforms may occur, possibly related to differential half-life between the two proteins. In this regard we analyzed susceptibility to proteasomal degradation of endogenous SRF and

SRFΔ5 using mature primary hippocampal neurons. As shown in Fig. 1H only SRF showed significant increase upon 2,5 hours proteasome inhibition with MG-132. On the contrary, SRFΔ5 does not change its levels in these experimental conditions, escaping proteasome degradation. This result provides a possible reason why in basal conditions, in the adult hippocampus, to a relative level of 20% for SRFΔ5 encoding mRNA and 80% SRF, the protein levels of the two isoforms are almost equally expressed.

## 2. SRFΔ5 cannot bind ELK1 in the brain but retains the ability to recruit the corepressor complex LSD1/CoREST/HDAC2.

SRFΔ5 has been described as an inhibitor of SRF-dependent transcriptional activity, because it lacks a significant portion of the transactivation domain at the C-terminal (largely encoded by exon E5) but maintains the ability to bind these responsive elements at the DNA level[26]. We then probed SRF association with ELK1, long-known positive SRF coregulator responsive to MAPK pathway[29]. To this aim, we performed GST pull-down experiments using purified recombinant GST-tagged SRF and SRFΔ5 expressed in *E. coli* incubated with mouse forebrain protein extracts. As expected, GST-SRF can recruit ELK1[43]; on the contrary, using recombinant GST-SRFΔ5 as a bait, ELK1 is barely detectable by western blot analysis (Fig. 2C). We then decided to evaluate the ability of SRFΔ5 to bind the epigenetic corepressors LSD1 and CoREST, which have been demonstrated to be important to regulate SRF target genes transcriptional modulation in the hippocampus[18,34,44]. LSD1, as well as HDAC2 and CoREST are all recruited by SRF and SRFΔ5 (Fig. 2D). To better characterize the SRF domain involved in the interaction with LSD1, we generated the GST-tagged SRF N-terminus (N-SRF, amino acids 1-171), containing annotated repressive domains[21] as well as GST-tagged SRF-C-terminus (C-SRF, amino acid 339-508)(Fig. 2A). As displayed in Figure 2D, N-SRF is sufficient to pull-down the whole LSD1 corepressor complex. Synaptic protein synaptophysin

(Syp) was used as negative control. Thanks to these experiments a picture emerges in which SRFΔ5 looses the ability to bind positive coregulator ELK1 while retaining binding specificity for corepressors, at least in part justifying why SRFΔ5 has been previously defined as a SRF dominant negative splicing isoform[26].

Given the capability of MADS-box domains of SRF to constitutively form homodimers, we next scored the ability of SRFΔ5 to heterodimerize with SRF thus increasing the probability of SRFΔ5 to modulate SRE-containing promoters in accordance to its cellular amount, relative to SRF. In particular we took advantage of differently tagged SRF isoforms overexpression in Neuro2a, showing by coimmunoprecipitation experiments that HA-tagged SRF can interact with myc-SRFΔ5 and that HA-tagged SRFΔ5 binds to myc-SRF (Fig. 2E).

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

SRF is known to represent a master regulator of neuronal structural plasticity by virtue of its ability to guide a transcriptional program of gene expression aimed at regulating actin dynamics[17,45,46]. To assess neuroplastic implications of SRFΔ5 in primary hippocampal neurons we overexpressed recombinant myc-tagged SRF and SRFΔ5 vectors along with GFP, and analyzed neurites arborization at day in vitro 8 (DIV8) during in vitro maturation, and spine density in more mature neurons at DIV18 using confocal microscopy. These two time frames have to be related to different phases of neuronal in vitro maturation characterized by initial massive morphology-related neurites growth (DIV0-DIV12), followed by the process of spinogenesis, that represents an optimal window to assess spine density (DIV12-DIV18). Using Sholl analysis we outlined, in neurons transfected with SRFΔ5 compared to GFP cotransfected with pCDNA3.1 empty vector, a tendency to simplified neurite arborization in terms of diminished number of dendritic branches. This tendency becomes significant by means of two-way ANOVA test at distances from the soma higher than 160 μm, suggesting

largely preserved number of primary dendrite and decreased arborization of apical dendrites (Fig. 3A). Conversely, overexpression of myc-SRF ensued little or no effect when compared with control conditions.

For what concerns dendritic spine analysis, performed in mature neurons at DIV18, overexpression of SRFΔ5 caused a strong decrease in dendritic spine density with respect to neurons overexpressing GFP along with pCDNA3.1 empty vector. It is true that in the same conditions, also SRF overexpression led to reduction of spine density. However, the effect of SRFΔ5 is significantly stronger compared to the one of SRF (Fig 3B). These results indicate that in resting conditions both SRF and SRFΔ5 act as negative modifiers of spines-related structural plasticity. This result only apparently contrasts previous data scoring SRF as a positive regulator of spine density[46]. Indeed, to our knowledge, we are the first to overexpress full length SRF in neurons to evaluate spine density in basal conditions. Every other work describing SRF as a positive spine regulator either used overexpression of a constitutively active SRF (gain-of-function generated by fusing SRF with potent VP16 transactivation domain)[46,47], or performed the experiments upon conditions of neuronal activation[48].

Anyway, best-characterized SRF function is to promote transcription in response to stimuli allowing neuroplastic changes concurring to memory formation and consolidation[17,24,25]. For this reason we further analyzed the role of SRFΔ5 compared to SRF upon neuronal activity. It is widely recognized that long-term potentiation (LTP) of synaptic efficacy entails growth as well as increased number of dendritic spines in stimulated neurons, processes in which SRF has been proposed to play an active role[46,49-53]. Structural substrates of LTP in terms of morphological processes aimed at inherently changing the shape and number of dendritic spines are well recapitulated in vitro by the chemical version of LTP (cLTP)[54-56], that is pharmacologically induced via administration of the NMDAR co-agonist Glycine to

primary neuron cultures (see methods)[56]. In hippocampal neurons, we transfected myc-SRF, myc-SRFΔ5 or the empty vector pCDNA3.1 together with GFP at DIV8 and preformed cLTP at DIV18. Dendritic spine density was scored after 2 hours of cLTP with confocal imaging. As shown in Fig. 3C, SRFΔ5 overexpression hampers cLTP-induced increase of dendritic spine density, which remains evident instead in both empty vector and myc-SRF transfected neurons. These data, together with those presented in figure 3B, show that while SRF upon a paradigm of neuronal activation can change its activity from repressing to promoting new spine formation, SRFΔ5 preserves its negative structural function regardless the presence or absence of a specific stimulus.

## 4. Acute psychosocial stress modifies SRF/SRFΔ5 mRNA and protein ratios

Given that SRFΔ5 displays a clear behavior as synaptic restrainer (Fig. 3), we asked whether the expression levels of SRF isoforms and the ratio between SRF and its dominant negative isoform SRFΔ5 could be affected in response to psychosocial stress. Indeed, the search for molecular mechanisms linking environmental stress to modification of structural plasticity in corticolimbic brain regions important to emotion and cognition-relevant information processing has not yet fully clarified the nature of involved pathways[6]. To this aim we performed a paradigm of psychosocial stress, the acute social defeat stress (ASDS) in wild type mice[18] and measured SRF and SRFΔ5 mRNA and protein levels during the phases of stress administration 2, 7 and 24 hours after the end of the paradigm (Fig. 4A-B). Interestingly, we show that after 2 and 7 hours of psychosocial stress total SRF mRNA does not vary, whereas after 2 hours, the relative ratio between SRF and SRFΔ5 transcripts decreases. This splicing modulation favors the expression of SRFΔ5 protein, whose levels significantly increased compared to controls two hours after the beginning of the stress paradigm (Fig. 4B, right panel). Notably, SRFΔ5 stress-induced increase at both mRNA and

protein level is transient, as 24 hours after the end of the stress it returns to basal levels. On the contrary, SRF protein does not change its levels, as the small tendency to increase does not reach statistical significance (Fig. 4B).

In order to gain insight into acute and transient SRFΔ5 increase two hours after the beginning of the stress paradigm, we measured activity-induced expression of three IEGs, *egr1, c-fos* and *npas4,* which represent SRF elective targets. As expected, these targets peak after 2 hours and notwithstanding the stress continuation, at 7 hours their mRNA returns to basal levels. In this frame, it is interesting to note that the kinetic of SRFΔ5 increase coincides with the decreasing levels of its targets (Fig. 4C). It has been recently proposed that LTD-like decrease of synaptic transmission in response to psychosocial stress at the level of the ventral hippocampus represents a resiliency-like physiological traits that correlates with decreased expression of the IEGs *egr1* and *c-fos,* which is restricted to resilient mice[8,10]. In this light, our results point to a protective role of SRFΔ5 increase upon acute psychosocial stress.


## 5. SRF upregulation in the hippocampus is a hallmark of chronic stress susceptibility

SRF has already been suggested to be involved not only as transducer of stressful stimuli[13,14,18], but also as a stress response modifier thanks to the possibility to adjust its level and activity in response chronic stressful experiences in human and rodents[13]. Our data, obtained upon acute psychosocial stress, showing a transient splicing modulation increasing SRFΔ5 protein level, suggest a further layer of SRF stress-induced regulation. As acute stress-induced modifications might be predictive stress-coping mechanisms potentially corrupted by chronic stress[12,57], we decided to investigate a possible involvement of these transcription factors in hippocampal stress vulnerability pathways by applying the chronic version of social defeat stress (CSDS). After ten days of CSDS, traits of psychopathology and in particular depressive-like behavior were assessed by means of decreased social interaction

ratio (SIR) [5]. Social interaction test was performed 24 hours after the cessation of CSDS, considering as SUS those mice that showed a SIR<0.8 and RES those with a SIR>1.2. Susceptibility-related SIR values have been widely associated to depression-related behavioral abnormalities[10,58]. 48 hours after the end of the CSDS, mice were sacrificed and RNA and protein analysis in the hippocampus was carried out. In RES mice SRF and SRFΔ5 mRNA and protein levels were unmodified and very similar to those observed in control animals, as well as the relative amount of SRF splicing isoforms (Fig. 5A-B). In RES mice, phenotypical absence of depressive-like social avoidance (SIR>1.2), correlates with unmodified basal levels of IEGs in the hippocampus (Fig. 5C). Interestingly, in SUS animals we confirmed the molecular signature of increased basal levels of *c-fos, egr1* and *npas4*[10] which has been recently proposed as possible read-out of pathogenically increased excitability of this brain structure[8].

In SUS mice, total SRF mRNA was increased indicating a sustained SRF transcription reflecting significant upregulation of SRF protein and only a tendency towards increased SRFΔ5 protein (Fig.5A-B). Note that SRF splicing was unchanged. These data suggest that only in SUS mice SRF expression undergoes modification in response to chronic stress and that this modulation has a transcriptional nature. In this frame an increase of SRF transcription in a context of unchanged SRF/SRFΔ5 protein ratio is consistent with increasing overall SRF transcriptional activity towards its targets, again compatible with concomitant upregulation of the IEGs. Thus, SRF function is modified by both acute and chronic stress but with different functional relevance, likely homeostatic in response to acute stress and potentially pathogenic in response to chronic.

**Discussion**

SRF actually represents one of the most studied regulators of activity-induced transcription impacting neurostructural plasticity[17,45,59,60]. It also appears as a mediator of the impact that environmental stimuli exert on brain circuitry [13,18]. SRF function was previously shown to be regulated by alternative splicing. In particular, SRFΔ5 skipping exon 5, acts as dominant negative isoform by inhibiting SRE-dependent promoter activity in non-neuronal tissues[26]. We present evidences that SRFΔ5 is also highly expressed in the mammalian hippocampus. In the brain, while SRFΔ5 cannot bind SRF coregulator ELK1, it retains the ability to behave as a transcriptional repressor, a relevant function displayed by SRF in basal conditions. Indeed, both SRF and SRFΔ5 bind the LSD1/CoREST/HDAC2 corepressor complex thanks to their N-terminal segment, already identified as a functional SRF repressive domain[21]. Note that this domain includes at least two phosphorylation sites, described as important to modify DNA binding properties (Ser103 in human)[61], along with transactivation activity (Ser228, in human)[43]. It would be very interesting to study whether phosphorylation at these sites modifies SRF interaction with LSD1. From the functional point of view, SRF and SRFΔ5 overexpression in primary neurons has a clear impact on dendritic spines plasticity. In particular, in basal conditions their common ability to recruit a corepressor complex correlates with a definite behavior as dendritic spines restrainers. *Vice versa*, upon neuronal activity SRF and SRFΔ5 functions diverge. SRF, consistently with its role as plasticity genes transcriptional activator, endorse cLTP-induced increase of spine density while SRFΔ5 opposes proplastic impact of this in vitro paradigm, inhibiting new spines formation. These evidences sheds a renewed light on the important role played by SRF as a negative regulator of transcription in resting conditions also via LSD1 partnership. This role was reportedly proposed[17,18,21,40] and we here suggest should be considered as important as the classical view of SRF as an activity-dependent transcription inducer in

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neurons. In this regard it will be very interesting to understand the potential impact of repression-deficient SRF mutants. Indeed, as many brain diseases including epilepsy, autism spectrum disorders (ASD) and depression, share increased excitability of selected brain areas[8,10,62,63], a role for SRF/SRFΔ5 could be envisaged in the pathogenesis or as a potential therapeutic target.

Environmental stress is well known to select two typologies of individuals, those that appear to be susceptible in the long run, developing emotional and affective dysfunctions, and those who neutralize the negative effects of stress in the frame of a resilient phenotype. On a cellular and circuitry point of view these two categories differ in terms of neurostructural plasticity modifications –spine density and dendrite branching– in different brain areas, including the hippocampus, that participate to stress response[6,64]. In particular, a novel remarkable finding about neurobiology of stress-vulnerability reveals that augmented excitability of the hippocampus, modifying input/output ratio at the neuronal level can be related to depressive traits induced by chronic stress in susceptible individuals[8,10]. Such enhanced hippocampal excitability was functionally associated to increased IEGs expression in the hippocampus of susceptible mice, a finding that we also confirmed in this work, further supporting the idea that susceptible individual-restricted IEGs overexpression represents a functional biomarker of stress vulnerability[8,10]. However, an open question still remains about what molecular players could orchestrate the pathway modulations underlying the divergence that occur at the level of a susceptible or resilient hippocampi.

In this context we observed that SRFΔ5, a factor predicted to restraining susceptibility-like hippocampal modifications (both in transcriptional and structural plasticity terms) plays a role in acute stress response, transiently increasing its level upon a paradigm of psychosocial stress. In this regard, acute SRFΔ5 upregulation might play a protective role. A functionally convergent modification of SRF corepressor LSD1 occurs during the same window of acute

psychosocial stress response, when LSD1 increases through a splicing-based mechanism aimed at reducing neuroLSD1, its dominant negative isoform unable to repress transcription (also described as a transcriptional activator[44]) and relevant to shape anxiety behavior[18]. Hence, SRF target genes transcription can be attenuated by combinatorial assembly not only of homo- or heterodimers of SRF and SRFΔ5 (depending on their relative neuronal amount) but also of coregulators LSD1/neuroLSD1[18]. In other words, we can think about a fine tuning mechanism based on corepressor complex assembly, regulated by alternative splicing of the different components which, in response to environmental stress, can exert a concerted homeostatic action aimed at restraining IEGs transcription[12]. Interestingly when stress is chronically reiterated, 48 hours after stress cessation, in resilient mice SRF and SRFΔ5 are not modified. *Vice versa*, in those mice that develop social avoidance, a high face validity depressive symptom, and thereby defined susceptible to social defeat stress, SRF protein expression is sustained, remaining elevated after stress cessation. Accordingly, its transcriptional IEGs targets are as well upregulated. In this time frame, although a tendency to SRFΔ5 increase can be observed, it seems not be sufficient to counteract IEGs sustained overexpression.

Global health improvement in western countries does not encompass psychiatric disorders, whose penetrance in the general population has grown over the last decades overpassing thresholds of a burden for healthcare national systems and society[65,66]. Molecular psychiatry research has concentrated huge efforts to shed new light on molecular mechanisms of stress-resiliency and susceptibility at the basis of depression[67]. We suggest that our discovery of a hippocampus enriched repressive splicing isoform of SRF, functionally associated with the histone H3K4 demethylase LSD1, represents an important step forward to better understanding the set of protective processes aimed at buffering stimuli-induced

plasticity gene transcription, likely limiting memorization of negative experiences via epigenetically uncoupling their perception from inherent memory trace consolidation[12].

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**Figure Legends**

Fig. 1 **Alternative splicing-generated SRFΔ5 is preferentially expressed in brain tissues and modulated during development.** A) Schematic representation of splicing event generating SRFΔ5. B) Endogenous SRF and SRFΔ5 proteins in human, rat and mouse hippocampus along with Neuro2a and HeLa cell lines. Recombinant myc-tagged SRF isoforms. C) SRF and SRFΔ5 mRNA expression in different non-neuronal and neuronal tissues. D) Electropherogram from purified 159bp SRF-related band shows sequence of SRFΔ5 E4 and E6 splice junction skipping of exon E5. E) Histogram showing SRF and SRFΔ5 relative percentages in non-neuronal and neuronal tissues. F-G) Developmental modulation of SRF and SRFΔ5 mRNA and protein ratio in mouse hippocampus. Data are presented as mean ± SEM; *,#p< 0.05, **p< 0.01, ***,###p< 0.001, one-way ANOVA, Bonferroni post hoc test, * referred to PN 1-8 group, # referred to PN 9-15; (n=3 to 11 mice per condition). H) Differential susceptibility of endogenous SRF and SRFΔ5 proteins to proteasomal degradation in DIV 12 primary rat hippocampal neurons. Data are presented as mean ± SEM; *p< 0.05, Student's *t* test (n=5 per condition).

Fig. 2 **SRFΔ5 is unable to interact with ELK1 co-activator but retains the ability to bind LSD1, along with CoREST and HDAC2, and forms homo- and hetero-dimers with SRF.** A)

Schematic representation of SRF exon structure, protein domain organization and GST-tagged constructs used for interactions analysis, with relative nucleotide and amino acid extension. RD I/II Repression Domain I/II; NLS Nuclear Localization Signal; DBD, DNA-binding Domain; TAD Transactivation Domain. B) Coomassie staining of bacterially expressed GST-tagged proteins. C) GST pull-down analysis of GST-tagged SRF and GST-tagged SRFΔ5 on mouse forebrain protein extract; western blot images showing ELK1; Syp was used as negative control. D) GST pull-down assay showing GST-SRF, GST-SRFΔ5, GST-N-SRF and GST-C-SRF interactions with LSD1, CoREST and HDAC2. E) Neuro2a cells overexpressing HA-tagged and myc-tagged SRF and SRFΔ5 proteins show heterodimers formation between SRF and SRFΔ5 isoforms.

Fig. 3 **SRFΔ5 negatively modulates neuronal morphology and dendritic spine density increasing combinatorial complexity of SRF repressive function.** A) Sholl-based morphological analysis of GFP-positive hippocampal neurons overexpressing myc-tagged SRF and SRFΔ5; neurons were transfected at DIV4 and analyzed at DIV8. Data are presented as mean ± SEM; *p< 0.05, **p< 0.01 two-way ANOVA, Tukey post hoc test referred to control GFP group B) Spine density analysis of GFP-positive hippocampal neurons overexpressing the same constructs as in (A), neurons were transfected at DIV4 and analyzed at DIV18. Data are presented as mean ± SEM; ##p< 0.01 ***p< 0.001, one-way ANOVA, Tukey post hoc test, *referred to GFP condition, #referred to SRF condition. C) Chemical LTP (cLTP) experiments were performed in primary hippocampal neurons transfected at DIV8 with GFP, and myc-tagged SRF and SRFΔ5, and analyzed at DIV18. For control conditions GFP was used along with SRF and SRFΔ5 vectors. Analyses were performed by confocal microscopy. Data are presented as mean ± SEM; **p < 0.01, ****p< 0.0001, two-way ANOVA, Tukey post hoc test. §p< 0.05, §§p< 0.01, two-way ANOVA, Tukey post hoc test referred to basal GFP group. D)

Nuclear localization of myc-SRF and myc-SRFΔ5 proteins in DIV18 primary rat hippocampal neurons.

Fig. 4 **Acute Social Defeat Stress (ASDS) transiently modifies relative abundance of SRF and SRFΔ5 proteins in the hippocampus.** A) Total SRF isoforms mRNA (assessed by qRT-PCR) and relative percentage of SRF/SRFΔ5 mRNA (assessed with rqfRT-PCR) after 2, 7 and 7 hrs stress + 24 hrs resting. B) SRF and SRFΔ5 protein expression profiles (evaluated by western blot) in control mice and after 2, 7 and 7 hrs stress + 24 hrs resting. C) Stress-induced transactivation of SRF validated targets c-fos, egr1 and npas4 in the mouse hippocampus after 2 and 7 hrs ASDS and after 24 hrs from the cease of stress. Data are presented as mean ± SEM; *p< 0.05, **p< 0.01, one-way ANOVA, Bonferroni post hoc test. (n=6 to 15 mice per condition).

Fig. 5 **Chronic Social Defeat Stress (CSDS) stably modifies SRF and SRFΔ5 mRNAs and proteins in the hippocampus.** A) Total SRF isoforms mRNA (assessed by qRT-PCR) and relative percentage of SRF/SRFΔ5 mRNA (assessed with rqfRT-PCR) in control mice and mice that underwent CSDS ending up in being stress-susceptible (SUS) of resilient (RES) sorted by means of social interaction ratio (SIR) evaluation. B) SRF and SRFΔ5 protein expression profiles (evaluated by western blot) in control mice, SUS and RES. All the gene expression analyses were carried out after 48 hrs from the cease of stress paradigm. C) Hippocampus stable overexpression of SRF validated targets c-fos, egr1 and npas4 48 hrs after the cease of stress. For SIR see table. Data are presented as mean ± SEM; *p< 0.05, **p< 0.01, one-way ANOVA, Bonferroni post hoc test, °p< 0.05 Student's *t* test. (ctrl mice n=7 SUS mice n=6, RES mice n=7).
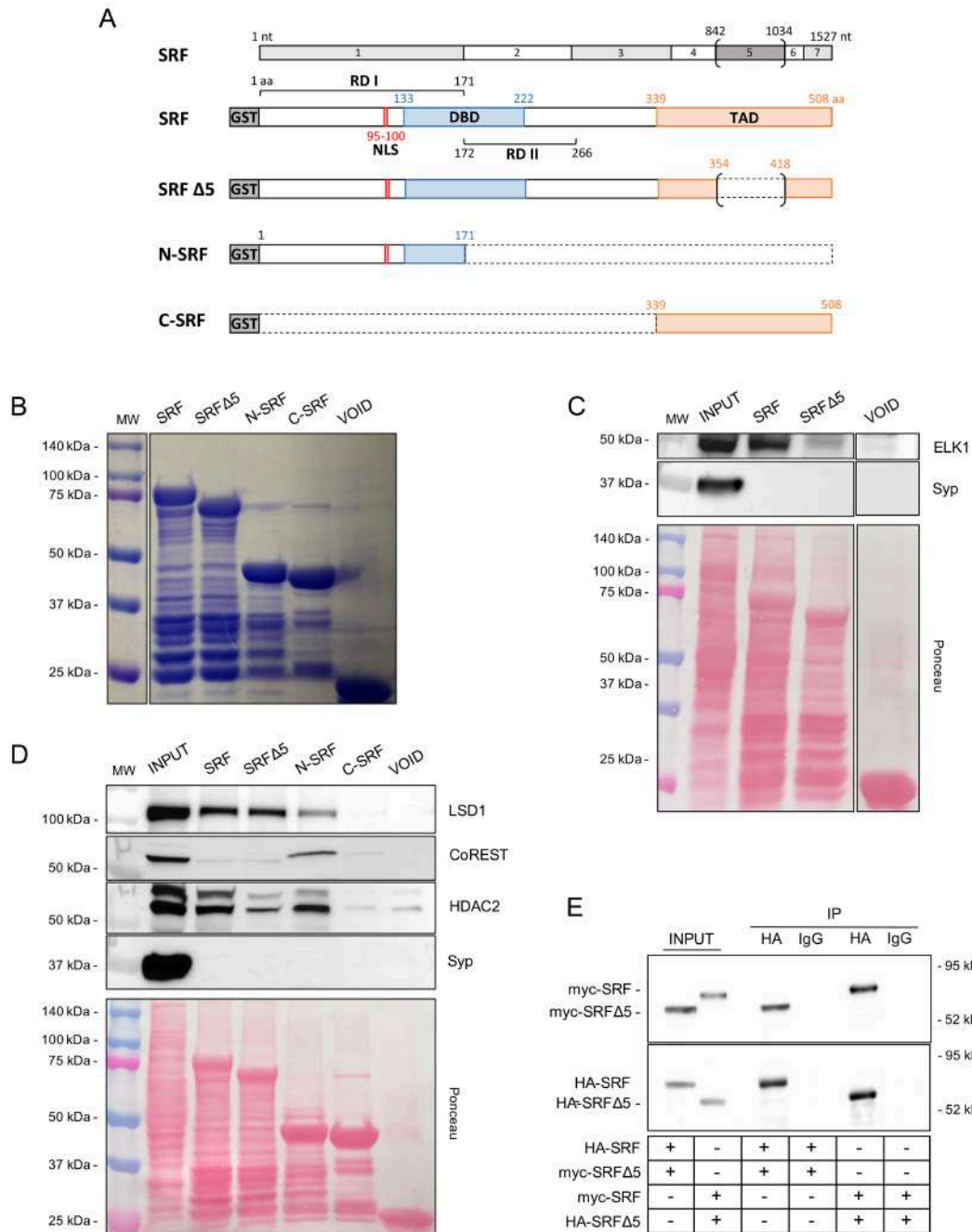
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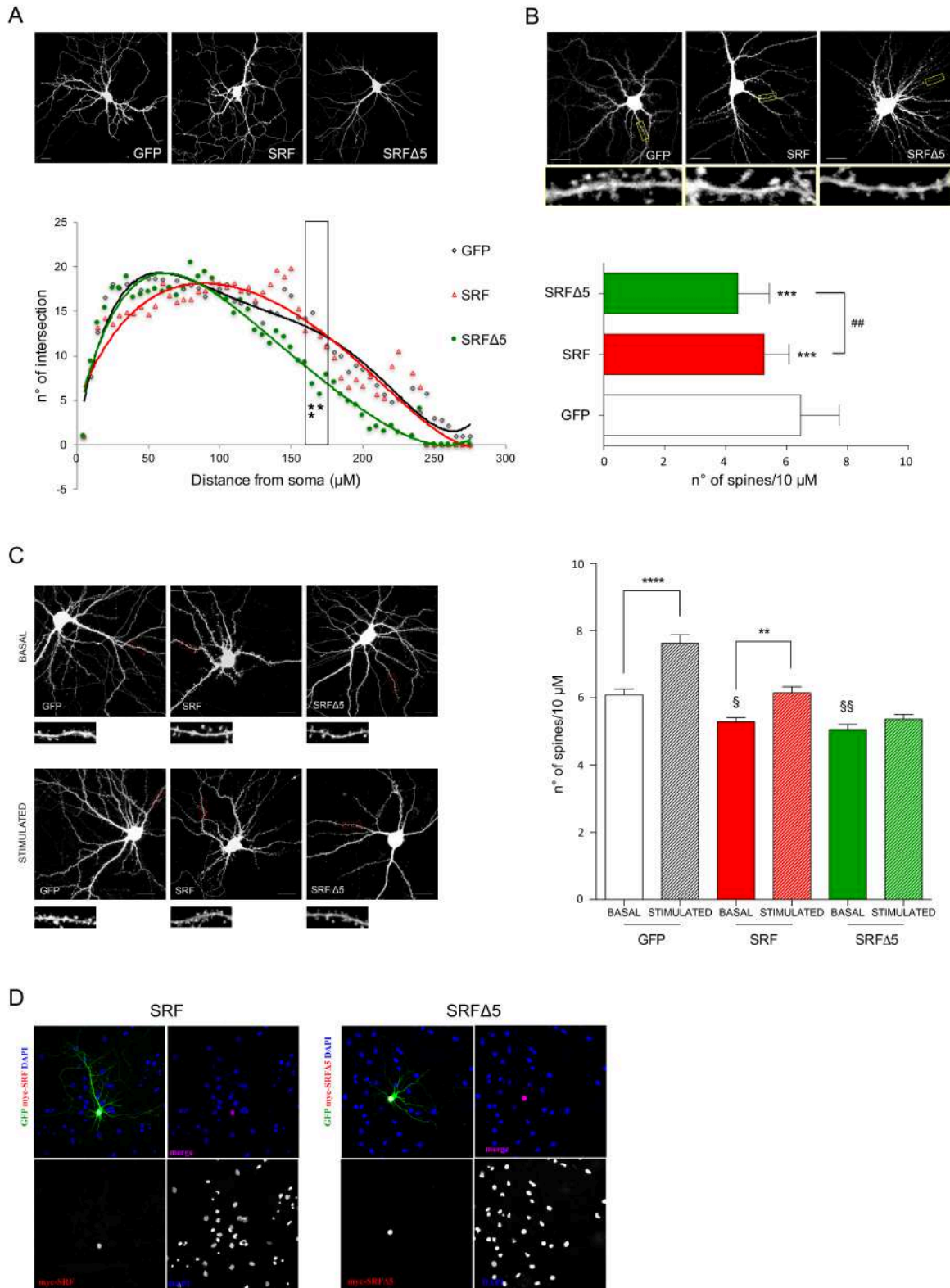
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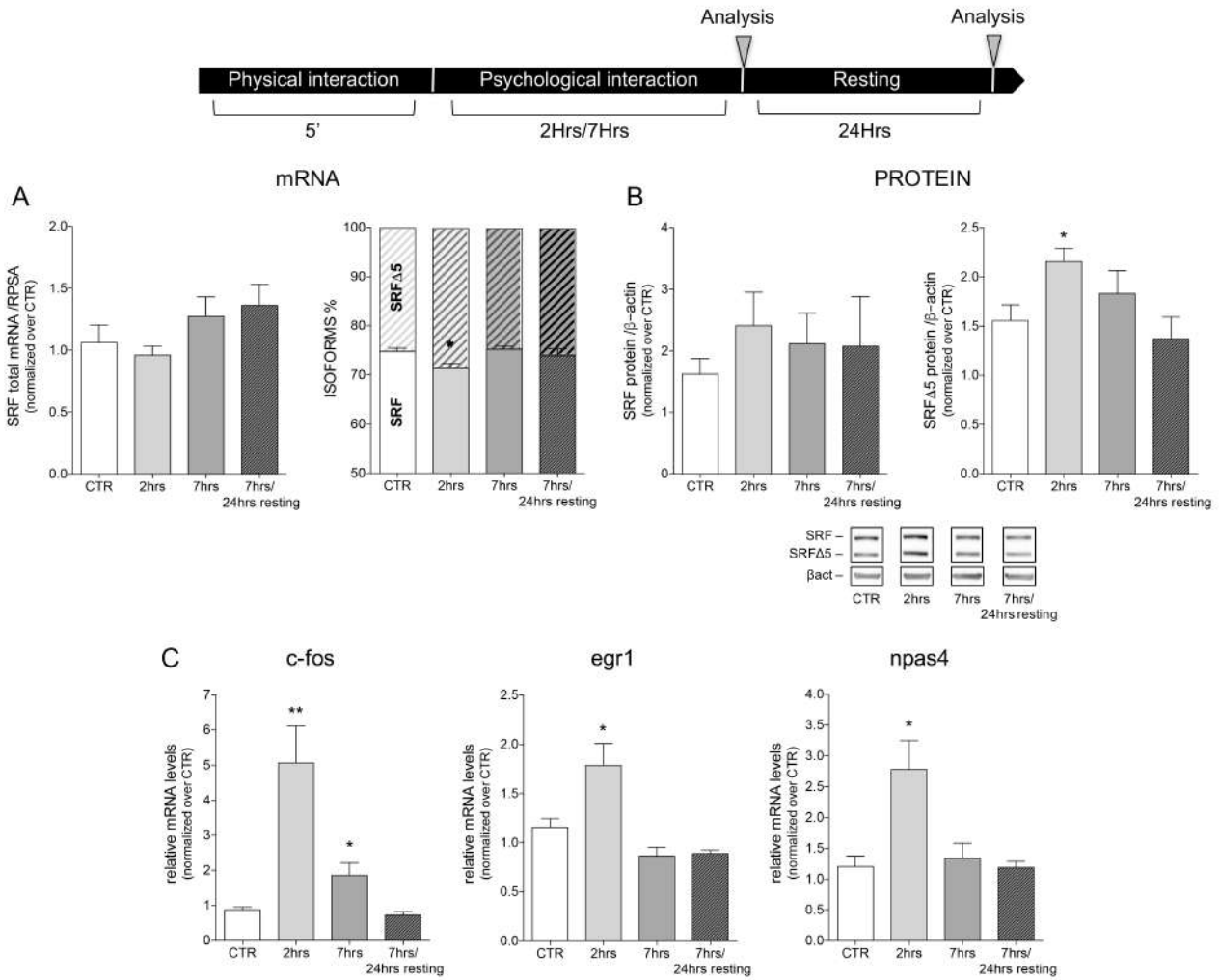
Fig. 3

A



B

C

D

Fig. 3 **SRFΔ5 negatively modulates neuronal morphology and dendritic spine density increasing combinatorial complexity of SRF repressive function.** A) Sholl-based morphological analysis of GFP-positive hippocampal neurons overexpressing myc-tagged SRF and SRFΔ5; neurons were transfected at DIV4 and analyzed at DIV8. Data are presented as
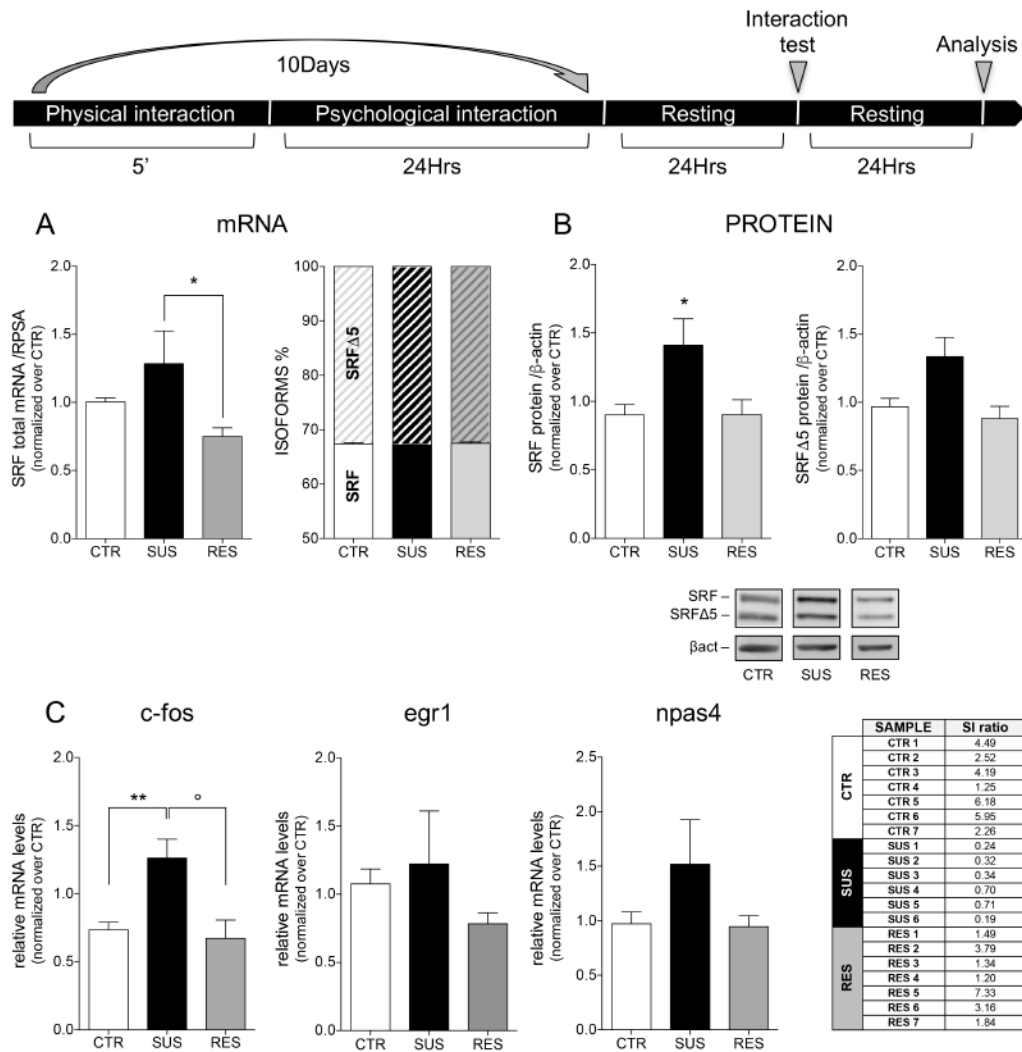
mean ± SEM; *p< 0.05, **p< 0.01 two-way ANOVA, Tukey post hoc test referred to control GFP group B) Spine density analysis of GFP-positive hippocampal neurons overexpressing the same constructs as in (A), neurons were transfected at DIV4 and analyzed at DIV18. Data are presented as mean ± SEM; ##p< 0.01 ***p< 0.001, one-way ANOVA, Tukey post hoc test, *referred to GFP condition, #referred to SRF condition. C) Chemical LTP (cLTP) experiments were performed in primary hippocampal neurons transfected at DIV8 with GFP, and myc-tagged SRF and SRFΔ5, and analyzed at DIV18. For control conditions GFP was used along with SRF and SRFΔ5 vectors. Analyses were performed by confocal microscopy. Data are presented as mean ± SEM; **p < 0.01, ****p< 0.0001, two-way ANOVA, Tukey post hoc test. §p< 0.05, §§p< 0.01, two-way ANOVA, Tukey post hoc test referred to basal GFP group. D) Nuclear localization of myc-SRF and myc-SRFΔ5 proteins in DIV18 primary rat hippocampal neurons.

Fig.4



Fig. 4 **Acute Social Defeat Stress (ASDS) transiently modifies relative abundance of SRF and SRFΔ5 proteins in the hippocampus.** A) Total SRF isoforms mRNA (assessed by qRT-PCR) and relative percentage of SRF/SRFΔ5 mRNA (assessed with rqfRT-PCR) after 2, 7 and 7 hrs stress + 24 hrs resting. B) SRF and SRFΔ5 protein expression profiles (evaluated by western blot) in control mice and after 2, 7 and 7 hrs stress + 24 hrs resting. C) Stress-induced transactivation of SRF validated targets c-fos, egr1 and npas4 in the mouse hippocampus after 2 and 7 hrs ASDS and after 24 hrs from the cease of stress. Data are presented as mean ± SEM; *p< 0.05, **p< 0.01, one-way ANOVA, Bonferroni post hoc test.  (n=6 to 15 mice per condition).

Fig. 5 **Chronic Social Defeat Stress (CSDS) stably modifies SRF and SRFΔ5 mRNAs and proteins in the hippocampus.** A) Total SRF isoforms mRNA (assessed by qRT-PCR) and relative percentage of SRF/SRFΔ5 mRNA (assessed with rqfRT-PCR) in control mice and mice that underwent CSDS ending up in being stress-susceptible (SUS) of resilient (RES) sorted by means of social interaction ratio (SIR) evaluation. B) SRF and SRFΔ5 protein expression profiles (evaluated by western blot) in control mice, SUS and RES. All the gene expression analyses were carried out after 48 hrs from the cease of stress paradigm. C) Hippocampus stable overexpression of SRF validated targets c-fos, egr1 and npas4 48 hrs after the cease of stress. For SIR see table. Data are presented as mean ± SEM; *p< 0.05, **p< 0.01, one-way ANOVA, Bonferroni post hoc test, °p< 0.05 Student's *t* test. (ctrl mice n=7 SUS mice n=6, RES mice n=7).