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Termination of acute stress response by the endocannabinoid system is regulated through LSD1-mediated transcriptional repression of 2-AG hydrolases ABHD6 and MAGL

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Abstract

Acute environmental stress rarely implies long lasting neurophysiological and behavioral alterations. On the contrary, chronic stress exerts a potent toxic effect at the glutamatergic synapse whose altered physiology has been recognized as a core trait of neuropsychiatric disorders. The endocannabinoid system (ECS) plays an important role in the homeostatic response to acute stress. In particular, stress induces synthesis of endocannabinoid (eCB) 2-arachidonyl glycerol (2-AG). 2-AG stimulates presynaptic cannabinoid 1 (CB1) receptor contributing to stress response termination through inhibition of glutamate release, restraining thereafter anxiety arousal. We employ mouse models of stress response coupled to gene expression analyses, unravelling that in response to acute psychosocial stress in the mouse hippocampus, ECS-mediated synaptic modulation is enhanced via transcriptional repression of two enzymes involved in 2-AG degradation: α/β-Hydrolase Domain containing 6 (ABHD6) and Monoacylglycerol Lipase (MAGL). Such a process is orchestrated by the epigenetic corepressor LSD1 who directly interacts with promoter regulatory regions of *Abhd6* and *Magl*. Remarkably, negative transcriptional control of *Abhd6* and *Magl* is lost in the hippocampus upon chronic psychosocial stress, possibly contributing to trauma-induced drift of synapse physiology toward uncontrolled glutamate transmission. We previously showed that in mice Lysine Specific Demethylase 1 (LSD1) increases its hippocampal expression in response to psychosocial stress preventing excessive consolidation of anxiety-related plasticity. With this work we unravel a nodal epigenetic modulation of eCB turn over, shedding new light on the molecular substrate of converging stress-terminating effects displayed by ECS and LSD1.

List of abbreviations used

ABHD6,	α/β-Hydrolase domain containing 6
2-AG,	2-Arachidonyl Glycerol
ASDS,	Acute Social Defeat Stress
CB1R,	Cannabinoid 1 Receptor
CoREST,	Corepressor of REST
CSDS,	Chronic Social Defeat Stress
eCB,	Endocannabinoids
ECS,	Endocannabinoid System
FAAH,	Fatty Acid Amide Hydrolase
HDAC2	Histone Deacetylase
IEGs,	Immediate Early Genes
LSD1,	Lysine Specific Demethylase 1
MAGL,	Monoacylglycerol lipase
NAPE-PLD	N-Acyl Phosphatidyl Ethanolamine-specific PhosphoLipase D
REST,	RE-1 Silencer Transcription Factor
RPSA,	Ribosomal Protein SA
SPF,	Specific Pathogen Free
TSS,	Transcription Start Site

Introduction

In the last few years many pieces of evidence concurred to consolidate a link between facing a stressful life and the prevalence of mood and anxiety disorders(Rusconi & Battaglioli 2018; Pizzagalli 2014; McEwen 2005; Krishnan & Nestler 2008). Even if the majority of the population can neutralize negative behavioral effects of traumatic experiences, all forms of psychiatric disorders in particular depression, anxiety and addiction can be precipitated or worsened by trauma in *vulnerable* individuals(Han & Nestler 2017; Sala *et al.* 2004; Bagot *et al.* 2014). We now know that neuropsychiatric disorders are often associated to defects in synaptic physiology(Popoli *et al.* 2012; Sanacora *et al.* 2012). For this reason, a broader knowledge of those pathways involved in stress response and directly subserving synaptic function is pivotal to studies aimed at widening the list of available therapeutic targets.

During stressful experiences, glutamatergic circuitries of a number of brain areas involved in stress response are engaged to immediately respond to potential environmental

danger(Treccani *et al.* 2014; Popoli *et al.* 2012; Chattarji *et al.* 2015), timely improving cognition and reactivity. In particular, stress-increased glutamatergic neuroplasticity consolidates avoidance-subserving (therefore adaptive) memory traces of the negative event. The other way around, excessive glutamate activity could generate too vivid associative memories between negative contingences and their contexts that can turn maladaptive, exacerbating approach-avoidance conflict–a core trait of anxiety and in general of many neuropsychiatric disorders (DSM-V)(APA 2013). Thus, proper modulation of glutamatergic neurotransmission upon stress is vital for adaptive behavioral response. Within this frame, a better understanding of those mechanisms instrumental to guarding against excessive glutamate activity at the synapse (Lutz *et al.* 2015) fulfills renewed interest.

The most characterized synaptic stress response mechanism is represented by the endocannabinoid system (ECS), a homeostatic set of processes controlling neurotransmitter release upon neuronal activation(Lutz *et al.* 2015; Morena *et al.* 2016). Upon acute stress 2-arachidonyl glycerol (2-AG) levels raise in different rodent brain areas including the hippocampus, mPFC and the hypothalamus(Hill *et al.* 2011; Wang *et al.* 2012; Evanson *et al.* 2010). This is not surprising as increased 2-AG concentration in brain tissues is elicited by neuronal activity, and environmental stress bolsters glutamatergic circuitry activation(Huang *et al.* 2005). 2-AG is generated from postsynaptic membrane phospholipids in response to metabotropic receptor activation and binds presynaptically to the cannabinoid 1 receptor (CB1R), leading to inhibition of glutamate release within a negative feedback mechanism. At excitatory synapses such an inhibition, known as depolarization-induced suppression of excitation (DSE) terminates stress response(Hill *et al.* 2011) and anxiety feeling(Morena *et al.* 2016; Lutz *et al.* 2015). 2-AG synthesis is dependent on a two steps biochemical process induced by glutamate-mediated activation of group I $G\alpha_q$-protein coupled receptors (mGluR1-5) followed by phospholipase C-β1 activation, relative diacylglycerol (DAG) production and subsequent DAGLα-mediated conversion to 2-AG(Savinainen *et al.* 2012). Similarly to every known signal transduction pathway, also endocannabinoid (eCB) synthesis holds termination nodes. In further detail, 2-AG degradative enzyme α/β hydrolade 6 (ABHD6) is involved in guarding 2-AG at the site of generation (the post synapse), while monoacylglycerol lipase (MAGL), representing the primary hydrolase responsible for 2-AG signaling termination in the mammalian brain, is responsible for 2-AG degradation presynaptically, in close vicinity to CB1R(Savinainen *et al.* 2012).

In the nucleus, a recently identified stress response mechanism is orchestrated by the histone demethylase Lysine Specific Demethylase 1 (LSD1). LSD1 is a transcriptional corepressor. *Vice*

versa, neuroLSD1, its neurospecific splicing isoform including micro-exon E8a and devoid of repressive activity, positively regulates transcription. Together, LSD1 and neuroLSD1 opposingly modulate Immediate Early Genes (IEGs) transcription in response to environmental stress regulating learning, memory and emotional behavior (Wang *et al.* 2015; Laurent *et al.* 2015; Rusconi *et al.* 2016; Rusconi *et al.* 2017). In particular, neuroLSD1 facilitates stress-induced IEGs transcription(Rusconi *et al.* 2016), instrumentally to memory consolidation (Wang *et al.* 2015) and with an anxiogenic effect(Rusconi *et al.* 2016). On the contrary, LSD1 exerts together with CoREST and HDAC2 a negative control toward IEGs transcription, memory formation, and synaptic plasticity(Guan *et al.* 2009; Toffolo *et al.* 2014; Wang *et al.* 2015; Rusconi *et al.* 2016). Another important implication of LSD1 and neuroLSD1 in stress response is their behavior as stress modifiers. At the nuclear level, in parallel with IEGs transcription, stress engages a slower and homeostatic post-transcriptional mechanism aimed at skipping micro-exon E8a, leading to neuroLSD1 reduction(Rusconi *et al.* 2016; Rusconi & Battaglioli 2018; Rusconi *et al.* 2017). This molecular event modifies IEGs responsivity, but theoretically also transcription of other LSD1 targets, during a peculiar window that follows stress-induced neuronal activation. This window is indeed defined by transient modification of LSD1/neuroLSD1 ratio in favor of LSD1, which in case of rodent social defeat stress gradually returns to resting levels when stress ceases. All these findings, together with the low anxiety-like phenotype of neuroLSD1^{KO} mice, suggest a functional convergence between LSD1 system and the ECS in stress response termination and suppression of anxiety (Wang *et al.* 2015; Rusconi *et al.* 2016), which is why we decided to search for a possible regulatory LSD1-ECS crosstalk.

Here we report *Magl* and *Abhd6* as novel LSD1 targets. In response to a single session of social defeat stress (ASDS) both 2-AG degrading enzymes MAGL and ABHD6 are transcriptionally repressed as a consequence of neuroLSD1 stress-induced reduction. Our results describe a new regulatory LSD1-ECS crosstalk aimed at potentiating ECS-mediated homeostasis via transient repression of negative eCB regulators, highlighting a genomic mechanism involved in stress termination via 2-AG modulation.

Material and Methods

Experimental animals. Animals were housed in a SPF animal facility. Three months old male CD1 ex-breeder (approximate weight 30 g) provided by Charles River Laboratories (Calco, Italy) (RRID:IMSR_CRL:22). Ten-week-old male C57BL/6N (RRID:IMSR_CRL:027) and neuroLSD1 knock-out (neuroLSD1^{KO}) littermates (approx. weight 22-25 g), deriving from

either our colony or from Charles River facility (*ad hoc* back up colony), were housed at controlled temperature (20–22 °C) with food and water ad libitum in a 12-h light/dark cycle (lights on at 7:00 a.m.). They were individually caged only during the testing period. Animals sacrificed (decapitation) on the same day were processed in arbitrary order and were not given anesthetics to limit molecular interference with the processes analyzed. No exclusion criteria were applied, nor animals died during the experiments; death did not represent experimental end point in any case. Animal trials did not result in excessive pain, which is why we did not use strategies to pharmacologically reduce animal suffering (see below). In this study, we used a total number of 126 mice including 24 CD1 mice and 102 C57BL/6N (wild type and neuroLSD1^{KO}). Among C57BL/6N we used 28 naïve mice and 74 manipulated mice including 17 controls and 57 stressed animals. All experimental procedures involving animals followed the Italian Council on Animal Care guidelines (Legislative Decree no. 26, March 2014) and European regulations (2010/63/UE) and were approved by Italian Ministry of Health (no. 275/2015 and 322/2018). Every effort was made to accomplish to the "3R" regulations i.e. Reduction of animal number, Refinement of experimental procedures (see below), Replacement with simpler research models, which has been not possible during this study. All current rules and regulations regarding animal usage were strictly adhered to.

Acute Social Defeat Stress (ASDS). In short, CD1 aggressor mice were used to defeat 10-weeks-old C57BL/6N wild-type mice or neuroLSD1 knock-out (neuroLSD1^{KO}) mice in a single session of SDS. The physical interaction time between the experimental mouse and a CD1 aggressor mouse was 5 minutes long, performed at 8:30 a.m. In order to reduce animal suffering, the operator took all measures to avoid physical damages to stressed mice, including tight control upon CD1 aggression also reducing when necessary physical interaction time. Thanks to this precaution it has not been necessary to administer analgesics and further medications. After this contact, the two mice were separated by a perforated Plexiglas divider to allow the psychological stress through visual and olfactory interactions with the aggressor. Control mice were housed in the opposite sides of a Plexiglas divider in cages identical to those used to perform the paradigm. Importantly, control mice were kept in a different room to avoid control conditioning. Respectively after 2 or 7 hrs (ASDS 2h, ASDS 7h) of olfactory and visual contact, molecular analyses were performed in both control and stressed mice. Animals were also analyzed 17 hrs after the discontinuation of a 7 hrs-long acute defeat stress (ASDS 7h + resting).

Chronic Social Defeat Stress (CSDS). We employed a modified protocol of CSDS, validated to induce enduring depression-like phenotypes as anxiety and social-avoidance behaviors. Briefly, aggressive CD1 ex-breeder mice were used to defeat 8/9 weeks old C57BL/6 mice.

C57BL/6 mice were exposed to a novel CD1 aggressor for 5 min/day, at 8:30 a.m. over 10 consecutive days. After the 5 min physical contact, experimental mice and CD1 aggressor were separated by a perforated Plexiglas divider in order to allow sensory interaction for 7 hours, after which mice were brought back to their home cage. Note that our CSDS protocol has been modified compared to the original method described in (Golden *et al.* 2011), and in our version, animals are allowed to recover, returning to their home cages between each 7 hour-long stress session. This measure should be included within our procedures to refine the use of animals limiting their suffering.

Total *RNA Extraction, qRT-PCR Analysis, and rqfRT-PCR.* TRIzol reagent (15596026, Invitrogen) was used for total RNA isolation from hippocampal extract(Colombo *et al.* 2009). Residual DNA was removed treating the purified RNA with RNase-free DNase set contained in the retrotranscription reaction kit Maxima^{TM} H Minus cDNA Synthesis Master Mix with dsDNase (M1682, Thermo Scientific). qRT-PCR analysis was performed as described elsewhere(Spreafico *et al.* 2018). Expression of target genes was normalized on Ribosomal protein SA (RPSA). RqfRT-PCR was used to measure exon E8a splicing inclusion in mature LSD1 endogenous transcripts, as described in(Rusconi *et al.* 2014).

Chromatin immunoprecipitation. A total of 4 mice were used. Hippocampi of 10 weeks old mice were dissected and cut in 400um slices using a chopper (McIlwain tissue chopper, Ted Pella, Redding, CA). Slices were rapidly incubated in 1% formaldehyde (F8775, Sigma Aldrich) for 8 minutes at RT for cross-linking purposes then transferred in 0.125 M glycine (AC04021000, Scharlau) for 15 minutes and homogenized in lysis buffer: 10 mM Tris- HCl pH 8 (BP153, Fisher Bioreagents), 1 mM EDTA (AC09650500, Scharlau), 0.5 mM EGTA (E3889, Sigma Aldrich) , 100 mM NaCl (131659.1211 PanReac AppliChem), 0.1 % Na-deoxy-cholate (30970, Sigma Aldrich) 0.5% N-laurylsarcosine (L9150, Sigma Aldrich) containing protease and phosphatase inhibitors (Pierce protease and Phosphatase Inhibitors Mini tablets, EDTA free A32961, Thermo Scientific). Lysates were sonicated twice with a Bandeline Electronic Sonicator for 30 seconds at 30% power to generate fragments with an average length of ~500-200 bp, as determined empirically by agarose gel electrophoresis of the fragmented chromatin sample. Immunoprecipitation was performed overnight with 40 µg of sonicated chromatin in 600 µl of lysis buffer containing Triton 1% (10789704001, Sigma Aldrich), PMSF 0.2 mM (93482, Sigma Aldrich) and 1.5 µg of rabbit monoclonal anti-LSD1 mAb, (RRID:AB_2070132). A sample with pre-immune IgG (mock) normal rabbit IgG antibody (RRID:AB_1031062) was included as a control. The samples were then incubated with Dynabeads protein G (10003D, Invitrogen) at 4°C for 2 hours. After immunoprecipitation, the mock supernatant was kept apart as input

sample. The beads were washed sequentially at 4°C (for 5 minutes each) with 1ml of low salt buffer: 0.1% SDS (2811 J.T. Backer), 2mM EDTA, 1% Triton X-100, 20mM Tris-HCl pH 8, 150 mM NaCl twice, and high salt buffer: 0.1% SDS, 2mM EDTA, 1% Triton, 20mM Tris-HCl pH 8, 500 mM NaCl and TE buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8.0. At the end the beads were washed with TE-NaCl buffer: 10 mM Tris-HCl, 1 mM EDTA, 50 mM NaCl. Elution was performed in 100µl of fresh elution buffer: 1% SDS, 50 mM Tris-HCl pH8, 1 mM EDTA. Cross-linking was reversed overnight at 65°C. After cross-link reversal, RNAsi A (R4642, Sigma Aldrich) was added to each sample to eliminate the RNA and the samples were incubated for 40 minutes at 37°C. The samples were then digested with proteinase K (EMR023100, EuroClone) for 1 hour at 56°C and DNA was obtained using a phenol:chlorophorm extraction protocol. DNA was recovered by standard methods in 40 µl of 10 mM Tris-HCl pH 8. Promoters were analyzed by quantitative real-time PCR carried out using Power SYBR Green PCR Master Mix (A25742, Applied Biosystem) with a QuantStudio 5 Real-Time PCR System (A28575, Applied Biosystem) according to manufacturer's instructions. The primers used in the PCR real-time reaction are listed in the supplementary methods section. ChIP experiment data result from at least three independent experiments, and all quantitative real-time PCR experiments were performed in duplicate. Data were then plotted as fold enrichment over mock. The experimenter was unaware of sample type, assignment code was disclosed following CT analyses.

LSD1 ChIP-seq analyses were performed within a previous publication(Wang *et al.* 2015). We loaded available ChIP-seq data, retrievable at the annotated link: Gene Expression Omnibus GSE63271(ftp://ftp.ncbi.nlm.nih.gov/geo/samples/GSM1544nnn/GSM1544937/suppl/GSM1544937%5FLsd1%5FKClminus%5Fhs60l3%5F3%2Eucsc%2EbedGraph%2Egz) on UCSC genome browser (https://genome.ucsc.edu).

Western blot analyses. Protein extraction from mouse hippocampus was performed as in(Colombo *et al.* 2009). Immunodecoration methods to comparatively quantify protein levels in the different experimental groups is described elsewhere(Colombo *et al.* 2017; Zamberletti *et al.* 2019). Hippocampal protein extracts were analyzed by a blind experimenter who received stressed and control hippocampi both from wild type and KO animals with a labeling whose code was only disclosed at the end of the analysis. Proteins were visualized by ECL detection (1705061 Bio-Rad) and bands were detected with a G:BOX Chemi XT4 camera and captured with GeneSys software (RRID:SCR_015770). Optical density of the bands was quantified using Image Pro Plus 7.0 software (RRID:SCR_016879), normalized and expressed as arbitrary units.Antibodies used: rabbit polyclonal anti-cannabinoid CB1 receptor (CB1) 1:1000

(RRID:AB_327840); rabbit polyclonal anti-N-acyl-phosphatidylethanolamine-hydrolysing phospholipase D (NAPE-PLD) 1:3000 (RRID:AB_1962525); rabbit polyclonal anti-fatty acid amide hydrolase (FAAH) 1:2000 (RRID:AB_327842); goat polyclonal anti-diacylglycerol Lipase α (DAGLα) 1:1000 (RRID:AB_1658310); rabbit polyclonal anti-monoacylglycerol lipase (MAGL) 1:1000 (RRID:AB_10079049); rabbit polyclonal anti-ABHD6 1:1000 (NBP2-57800 Novus Biologicals); rabbit polyclonal anti-β Tubulin 1:2000 (RRID:AB_10000656).

Randomization. In social defeat stress paradigm, the two main categories are control mice and stressed mice at the different time points. To assign mice to control groups we used *simple randomization* with a computer-generated sequence in order to avoid biased experimenter selection of smaller or bigger (although in range) wild type of knock out mice. We used "Research Randomizer software" https://www.randomizer.org just selecting 1 random set of numbers (controls). We randomized animals three times to select a total of 7 (two randomizations) wild type and 6 (a single randomization) knock out controls. Animals were identified with progressive numbers. No randomization was performed while comparing wild type and knock out animals in basal conditions.

Statistical analyses. We employed the number of experimental animals predicted by statistical sample size determination using the appropriate parameters (Power 0.8, α 0.05, β 0.2), within two independent study groups whose primary (continuous) endpoint is an average. We estimated two sample sizes: the first took into consideration the effect of stress on LSD1 splicing modulation (mean 1, 50%±6; 18% decrease) and related variation of LSD1 targets ABHD6 and MAGL expression: group numerosity 7 individuals. To this aim we used previous data published in(Rusconi *et al.* 2016). The second entailed the effect of LSD1-related genotype on gene expression (mean 1, 100±35; mean 2, 60): group numerosity 12 individuals. To this aim we used previously published data related to LSD1 and neuroLSD1 as transcriptional modifiers(Wang *et al.* 2015).

Data are shown as mean ± SEM. For single comparisons, we performed unpaired Student's *t* Test; for multiple comparisons, we either used one or two ways ANOVA variance analyses associated to Tukey's *post hoc* test, only in case of statistical significance of the ANOVA test. To this aim we used GraphPad PRISM 6.0 software (RRID:SCR_002798). Verification of normal data distribution was performed for all experimental datasets taking advantage of the tool http://www.statskingdom.com/320ShapiroWilk.html, which applies the Shapiro-Wilk test. Once assessed normal distribution of the data, to retrieve outliers and exclude them accordingly from statistical analyses, we took advantage of the website

https://www.graphpad.com/quickcalcs/Grubbs1.cfm, which applies the Grubbs test also known as the maximum normalized residual test.

Study pre-registration. This study was not pre-registered.

Results

1. Acute social defeat stress decreases ABHD6 and MAGL expression.

In rodents, upon different paradigms of acute environmental stress, the endocannabinoid 2-AG undergoes delayed (tens of minutes) increase in the hippocampus(Wang *et al.* 2012), mPFC(Hill *et al.* 2011) and hypothalamus(Evanson *et al.* 2010). This kinetics is compatible with multilayered controls including on demand synthesis at the synapse(Lutz *et al.* 2015), hormonal glucocorticoid-mediated regulation(Atsak *et al.* 2012) and possibly, transcriptional mechanisms. We investigated transcriptional processes involved in stress-induced 2-AG regulation in the mouse hippocampus by analyzing mRNA and protein levels of the main synthetic enzyme DAGLα, along with 2-AG pre- and post-synaptic degrading enzymes, respectively ABHD6 and MAGL hydrolases, and the CB1 receptor (CB1R). Taking advantage of a single session of social defeat stress (ASDS) we analyzed DAGLα, MAGL and ABHD6 levels in stressed C57BL/6N male mice compared to controls with qRT-PCR and western blotting. ASDS entailed 5 minutes of physical confrontation between an ex-breeder CD1 mouse and an experimental C57BL/6N intruder, followed by 2 or 7 hours of psychological stress only maintaining visual and olfactory interaction between the two mice. As previously observed(Rusconi *et al.* 2016) by relative quantity fluorescent PCR (rqf-PCR), the relative amount of LSD1 and neuroLSD1 isoforms underwent splicing modification in response to stress. In particular, neuroLSD1 isoform was significantly reduced in hippocampi of stressed mice at both time points. Data are plotted in Fig. 1A as neuroLSD1 fraction among all LSD1 isoforms, expressed as 100% (corresponding to percentage of mature transcripts including exon E8a). Note that exon E8a splicing decrease entails the relative increase of the transcriptional corepressor LSD1 (that indeed is characterized by exon E8a exclusion, not shown). We then performed qPCR analyses evaluating relative mRNA levels of endocannabinoid-related genes. 7 hours after the beginning of the stress both ABHD6 and MAGL relative mRNA levels were significantly decreased compared to controls (Fig. 1B-C). Interestingly, no transcriptional changes were detected after the 2 hour-long stress, suggesting that the process requires a longer time interval. After 7 hours of psychosocial stress we observed decreased ABHD6 also at the protein level, while at this time point, we did not observe changes in MAGL protein suggesting higher MAGL protein stability (Fig. 1B-C). Notably, along

with recovery (ASDS 7h + resting), LSD1/neuroLSD1 splicing ratio returned to resting conditions (Fig. 1A), together with ABHD6 and MAGL transcript levels. Within the same analysis we did not observe DAGLα and CB1R transcriptional modifications (Fig. 1D-E). However, we noticed that ASDS exerts other modifications of the endocannabinoid system at the protein levels that are not related to transcriptional processes. Indeed, DAGLα protein is decreased upon ASDS probably through a post-translational mechanism (Fig. S1A). Notably, this particular stress paradigm seems not to modify expression of synthetic (NAPE-PLD) and degradative (FAAH) enzymes of the other well-known eCB, anandamide (see supplementary Fig. S1B-C). These data suggest that in the hippocampus of naïve wild type mice, stress elicits a delayed transcriptional repression of 2-AG degrading enzymes, potentially important to sustain eCB responses to the negative experience. Considering that transcriptional modulation of ABHD6 and MAGL occurs within the same time window in which LSD1/neuroLSD1 splicing ratio is imbalanced in favor of the transcriptional corepressor LSD1, we hypothesized that LSD1 could play a part in this process.

2. Epigenetic corepressor LSD1 is recruited to *Abhd6* and *Magl* promoters in the hippocampus

To investigate LSD1 direct involvement in transcriptional mechanisms underlying ECS regulation, we tested its physical interaction with *Abhd6* and *Magl* gene promoters. We further analyzed publicly available data sets from LSD1 ChIP-seq experiments previously performed in primary mouse neuronal cultures, deposited at the Genome Expression Omnibus (GEO) under the subseries entry GSE63271(Wang *et al.* 2015). Interestingly, this analysis highlighted significant enrichment of LSD1 protein occupancy at the level of *Abhd6* and *Magl* genes, in close proximity to the transcription start site (TSS) (Fig. 2A). Notably, the database showed no LSD1 interaction with *Daglα* and *Cnr1* (not shown), respectively 2-AG biosynthetic enzyme and the CB1 receptor. Interestingly these two genes are not modulated by acute psychosocial stress (Fig. 1D), further indicating LSD1 selectivity for 2-AG degrading enzymes. At this point, we analyzed the mouse hippocampus in order to verify whether similar association of LSD1 to *ABHD6* and *MAGL* promoters could be conserved in vivo. We performed ChIP experiments in the hippocampus in resting conditions confirming LSD1 promoter occupancy at the level of inherent genomic regions (Fig. 2B). Such an observation is consistent with a regulatory role of LSD1 in the negative modulation of pre- and post-synaptic 2-AG degrading enzymes. The transcription factor REST/NRSF is one of the best characterized transcriptional repressors recruiting LSD1/CoREST coprepressor complex on chromatin (Shi *et al.* 2004). Notably, using

antibodies against REST/NRSF to immunoprecipitate hippocampal chromatin, we scored a significant enrichment of this transcriptional repressor at *Abhd6* promoter compared to the mock condition using the same primers employed to score LSD1 enrichment. This result suggests that at least for *Abhd6* gene, REST/NRSF might be responsible for LSD1 tethering to its site of action at the DNA level.

3. Acute stress-dependent MAGL and ABHD6 downregulation is lost in a mouse model devoid of LSD1 splicing modulation

In the hippocampus, acute psychosocial stress elicits downregulation of two key enzymes involved in 2-AG hydrolysis. We already provided a perspective on the possible LSD1-mediated repressive mechanism responsible for ABHD6 and MAGL transcriptional decrease, accounting on LSD1 ability i) to physically associate with *Abhd6* and *Magl* promoter regions (Fig. 2B) and ii) to increase its repressive potential via downregulation of its dominant negative isoform neuroLSD1 in the same window of ABHD6 and MAGL transcriptional decrease (Fig. 1A). To provide a causal relationship between transient LSD1/neuroLSD1 ratio modulation and downregulation of 2-AG degradative enzymes, we analyzed ABHD6 and MAGL expression in neuroLSD1^{KO} mice, a genetic mouse model in which we only deleted microexon E8a, thus preserving LSD1 expression. As expected, neuroLSD1^{KO} mice display a decreased hippocampal amount of both ABHD6 and MAGL mRNA in resting conditions (Fig. 3A). These data are consistent with the fact that corepressor LSD1 is the only isoform present in the brain of neuroLSD1^{KO} mice. Being absent the pro-transcriptional neuroLSD1 isoform, basal target genes transcription is decreased compared to wild type (Rusconi *et al.* 2014; Rusconi *et al.* 2016). ABHD6 protein followed mRNA profile, being accordingly reduced in neuroLSD1^{KO} mice. On the contrary, MAGL showed a tendency to increase. Such a discrepancy between mRNA and protein might suggest a compensatory post-translational mechanism restricted to MAGL, present in the genetically modified mouse.

To provide further evidence supporting direct functional role of LSD1 in modulating ABHD6 and MAGL in response to stress, we performed social defeat experiments in neuroLSD1^{KO} mice. As shown in Fig. 3B, upon a single session of social defeat stress in knock outs (where LSD1/neuroLSD1 ratio cannot be modulated), neither ABHD6 nor MAGL are modulated in the hippocampus compared to unstressed mice at both the mRNA and protein levels. Thus, ABHD6 and MAGL transcriptional downregulation upon acute social stress requires splicing modulation of the two LSD1 isoforms, aimed at reducing neuroLSD1 with concomitant increase

of LSD1. Altogether these data are consistent with a primary role of LSD1 gene function in reducing ABHD6 and MAGL mRNA levels upon stress.

4. Chronic social defeat stress alters LSD1 splicing response impairing MAGL and ABHD6 negative modulation.

We recently proposed that upon chronic stress, typical adaptive modifications observed in response to acute stress are less efficiently elicited(Rusconi & Battaglioli 2018; Gerosa *et al.* 2019). Therefore we asked whether LSD1/neuroLSD1 splicing response to acute stress would be modified following a paradigm of chronic social defeat stress. We applied a modified version of CSDS in which, after each daily stress session lasting 7 hours (see methods and scheme in Fig. 4), mice returned to their home cage. This resting phase allowed LSD1/neuroLSD1 ratio modification to recover, returning to resting values after the first stress sessions, (CTR vs ASDS + resting in Fig. 4A). With the same efficiency, also after the 9th session (CTR vs CSDS x9 + resting in Fig. 4A) LSD1/neuroLSD1 ratio recovered to resting values. However, after the 10th session of the chronic protocol, stress response in terms of LSD1/neuroLSD1 splicing modification (analyzed after the standard seven hour-long stress) is less efficient. Indeed, although the ratio LSD1/neuroLSD1 still undergoes modification in favor of LSD1 (CSDS x9 + resting vs CSDS x10 in Fig. 4A), such a change is significantly reduced compared to the one observed during the first stress session (CTR vs ASDS in Fig. 4A). As stated above, ABHD6 and MAGL transcriptional downregulation upon acute social stress requires splicing modulation of the two *LSD1* isoforms. In line, when stress reiteration hampers LSD1 splicing response, target modulation is disrupted. Indeed, and consistently with their nature as LSD1 targets, also ABHD6 and MAGL levels were invariant in response to the tenth stress session (Fig 4B-C) further supporting a transcriptional causal relationship between LSD1 splicing modulation and eCB regulation. These data are compatible with two possible interpretations: i) stress has elicited an adaptive habituation in a way that homeostatic splicing modulation is no longer required; ii) allostatic load associated to stress reiteration has desensitized LSD1-based mechanisms devoted to limiting the toxic effects of stress. Although further experiments are required to identify the functional meaning of LSD1 splicing disengagement by chronic stress, we evidenced a relevant difference in acute vs chronic LSD1/ECS-related molecular stress response. Consistently with both interpretations, upon chronic stress ABHD6 and MAGL lose their tunability, which might limit 2-AG contribution to stress termination.

Discussion

Trauma-induced transient shift of LSD1/neuroLSD1 ratio in favor of the corepressor LSD1(Rusconi *et al.* 2017; Rusconi *et al.* 2016) is operated by a neurospecific splicing mechanism leading to decrease of neuroLSD1. This process has been described as homeostatic check point of stress-induced IEGs activation and plasticity(Rusconi *et al.* 2016). We show that neuroLSD1 decrease is also instrumental to transiently reduce transcription of the two main enzymes involved in 2-AG clearance, ABHD6 and MAGL, in a window that functionally fits stress-elicited 2-AG increase(Evanson *et al.* 2010; Hill *et al.* 2011; Wang *et al.* 2012). Stress-dependent transcriptional repression of 2-AG degrading enzymes within the ranges described so far (Fig. 1B) likely allows a fine, mild and continuous modulation of the 2-AG tone.

LSD1-induced downregulation of 2-AG degrading enzymes should contribute to enhancing CB1R stimulation upon acute stress. However, excessive or prolonged 2-AG increase can lead to desensitization(Schlosburg *et al.* 2010). In this light, in conditions of chronic stress, transient nature of LSD1/neuroLSD1 splicing modulation should physiologically limit CB1R desensitization. Finally, neuroLSD1 is restricted to neurons and absent in glial cells which substantially concur to 2-AG degradation(Zibetti *et al.* 2010). For this reason, LSD1/neuroLSD1-mediated modulation of 2-AG skips glial contribution(Savinainen *et al.* 2012), another evidence in support of a highly-controlled pathway to increase 2-AG concentration. All these features provide a role for LSD1/neuroLSD1 as fine ECS tuners guaranteeing a balance in reinforcing 2-AG activity while avoiding CB1R desensitization. This finding unravels a cross-talk between two pathways known to buffer stress transduction: the ECS –a synaptic homeostatic system with a marked role in stress protection(Lutz *et al.* 2015; Morena *et al.* 2016)– and the nuclear system represented by LSD1 and neuroLSD1 with established implications in neuroplasticity-related transcriptional homeostasis (Rusconi *et al.* 2016; Rusconi *et al.* 2017; Gerosa *et al.* 2019; Rusconi & Battaglioli 2018). With this observation, LSD1 role in stress transduction is extended to the transcriptional regulation of ECS-mediated synaptic homeostasis, strengthening the implication of LSD1-orchestrated epigenetic regulation in first-line cognitive and emotional brain adaption to the environment.

In recent years two papers reviewed the role of endocannabinoid 2-arachidonyl glycerol in environmental stress response termination as instrumental to behavioral anxiety and fear discontinuance(Morena *et al.* 2016; Lutz *et al.* 2015). Notably, 2-AG not only displays a stress-terminating activity, but is also instrumental to stress resiliency, as systemic 2-AG augmentation enhances resiliency in stress-susceptible mice and *vice versa,* 2-AG depletion and/or CB1R blockade turn resilient mice into susceptible(Patel *et al.* 2016). It is worth noting that specific MAGL inhibitor JZL184 –which rapidly and efficiently increases 2-AG levels– does

exert an acute anxiolytic activity, a feature already appreciable in basal conditions but markedly enhanced upon stressful paradigms. Unfortunately, therapeutic potential of JZL184 toward stress-induced psychopathology is severely limited as it engages a rapid 2-AG-dependent CB1R desensitization upon chronic administration, quickly leading to worsened anxiety symptomatology soon after the first days of administration(Long *et al.* 2009; Schlosburg *et al.* 2010). Better knowledge of how 2-AG levels are modulated within a physiological response to stressful conditions might unravel novel possible pharmacological avenues of intervention to treat stress-induced psychiatric disorders. Within this frame, deciphering the biological rules of ABHD6 and MAGL modulation can be highly relevant to design new strategies to exogenously control 2-AG levels in stress susceptible individuals who underwent psychiatric disorders.

Acute stress does not usually lead to long term behavioral alterations. For this reason, acute stress response-related molecular processes can be considered mostly adaptive(Rusconi & Battaglioli 2018). *Vice versa*, chronically repeated stressors lead to allostatic load through disruption of the same mechanisms that successfully neutralize acute stress. This is why identifying corruption nodes is fundamental to involve them in protection from, and susceptibility to neuropsychiatric disorders possibly including drug addiction. Remarkably, upon chronic stress reiteration, stress-related tunability of LSD1/neuroLSD1 ratio decreases over time such that at the tenth social defeat stress session (Fig 4A), splicing process regulating LSD1/neuroLSD1 ratio is unable to respond to the paradigm. These data suggest that neurospecific splicing modulation of LSD1 might represent one of those adaptive molecular nodes that can be jeopardized and eventually dismissed by chronic stress. As a result, upon chronic stress, LSD1 and neuroLSD1 relative amount is only weakly modified. This is reminiscent of the loss of transient ABHD6 and MAGL transcriptional downregulation with the functional perspective of impeding the otherwise adaptive 2-AG upregulation. Such an effect could be possibly involved as risk factor in cannabinoid-related, but also unrelated addictive behaviors. Indeed, taking into consideration that stress represents one of the main precipitating factors in drug addiction(Bagot *et al.* 2014), rupture of those mechanisms, including LSD1-driven homeostasis sustaining proper ECS functionality, might favor THC seeking as a surrogate of the lacking endogenous 2-AG. All this said, considering pro-resiliency effect of 2-AG(Patel *et al.* 2016), chronic stress desensitization of LSD1/neuroLSD1 splicing modulation and related loss of transcriptional repression over 2-AG degradative enzymes, is likely linked to stress susceptibility and addictive behaviors.

Notably, we observed that the transcriptional repressor REST/NRSF, first to be described to recruiting the LSD1/CoREST/HDAC1/2 corepressor complex in non-neuronal cells(Ballas *et al.* 2001), co-occupies *Abhd6* promoter regions together with LSD1 in the hippocampus. A certain number of recent papers describes the relevance of REST/NRSF in the brain, in protecting against the detrimental effects of epileptic excitotoxicity(McClelland *et al.* 2014; Hu *et al.* 2011; Zullo *et al.* 2019). In this regard, environmental stress-induced glutamate secretion from limbic pyramidal neurons can be so pronounced to entail glutamate spill-over from the dendritic spines, leading to extra-synaptic glutamate receptors activation that is proper of excitotoxic epileptic discharges(Yang *et al.* 2005). Thus, our data suggest that homeostatic mechanisms involving REST/NRSF activity can be shared in response to both epileptic and psychiatric-relevant stimuli. Interestingly, epilepsy and neuropsychiatric disorders including anxiety and depression are often comorbid. Although we did not observe the same REST/LSD1 association at the level of *Magl* promoter in the region assayed by our ChIP primers, we cannot exclude their association at the level of other sites within *Magl* promoter. However, LSD1 is known to associate with different transcription factors(Rusconi *et al.* 2016; Sun *et al.* 2010; Adamo *et al.* 2015) and in some cases also displayed the ability to bind target DNA without the need of transcription factors (Pilotto *et al.* 2015), which justifies that although acting on functionally convergent pathways of 2-AG degradation, LSD1 can be in turn regulated by different factors.
LSD1-operated acute stress-induced decrease of 2-AG degradative enzymes at the mRNA and protein levels in the mouse hippocampus represents a physiological, innate fine-tuning mechanism to increase 2-AG activity. Altogether, our observations suggest a novel road map to regulate 2-AG without recurring to direct degrading enzymes inhibition, based on pharmacological LSD1/neuroLSD1 splicing regulation. Many papers report pharmacological exon skipping strategies based on specific administration of degradation-refractory antisense oligonucleotides (AON)(Hua *et al.* 2010; Hua *et al.* 2011). Pharmacological LSD1 splicing modulation aimed at decreasing neuroLSD1 represents therefore a promising chance to treat long-term behavioral signs of chronic stress reiteration, simply reinstating a physiological process of chronic stress coping that is susceptible to allostatic load.

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The authors declare no conflict of interest.

Bibliography

Adamo, A., Atashpaz, S., Germain, P. L. et al. (2015) 7q11.23 dosage-dependent dysregulation in human pluripotent stem cells affects transcriptional programs in disease-relevant lineages. *Nat Genet* **47,** 132-141.

APA (2013) *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition.*

Atsak, P., Hauer, D., Campolongo, P., Schelling, G., McGaugh, J. L. and Roozendaal, B. (2012) Glucocorticoids interact with the hippocampal endocannabinoid system in impairing retrieval of contextual fear memory. *Proc Natl Acad Sci U S A* **109,** 3504-3509.

Bagot, R. C., Labonté, B., Peña, C. J. and Nestler, E. J. (2014) Epigenetic signaling in psychiatric disorders: stress and depression. *Dialogues Clin Neurosci* **16,** 281-295.

Ballas, N., Battaglioli, E., Atouf, F. et al. (2001) Regulation of neuronal traits by a novel transcriptional complex. *Neuron* **31,** 353-365.

Chattarji, S., Tomar, A., Suvrathan, A., Ghosh, S. and Rahman, M. M. (2015) Neighborhood matters: divergent patterns of stress-induced plasticity across the brain. *Nat Neurosci* **18,** 1364-1375.

Colombo, G., Clerici, M., Altomare, A. et al. (2017) Thiol oxidation and di-tyrosine formation in human plasma proteins induced by inflammatory concentrations of hypochlorous acid. *J Proteomics* **152,** 22-32.

Colombo, G., Rusconi, F., Rubino, T., Cattaneo, A., Martegani, E., Parolaro, D., Bachi, A. and Zippel, R. (2009) Transcriptomic and proteomic analyses of mouse cerebellum reveals alterations in RasGRF1 expression following in vivo chronic treatment with delta 9-tetrahydrocannabinol. *J Mol Neurosci* **37,** 111-122.

Evanson, N. K., Tasker, J. G., Hill, M. N., Hillard, C. J. and Herman, J. P. (2010) Fast feedback inhibition of the HPA axis by glucocorticoids is mediated by endocannabinoid signaling. *Endocrinology* **151,** 4811-4819.

Gerosa, L., Grillo, B., Forastieri, C. et al. (2019) SRF and SRFΔ5 Splicing Isoform Recruit Corepressor LSD1/KDM1A Modifying Structural Neuroplasticity and Environmental Stress Response. *Mol Neurobiol*.

Golden, S. A., Covington, H. E., Berton, O. and Russo, S. J. (2011) A standardized protocol for repeated social defeat stress in mice. *Nat Protoc* **6,** 1183-1191.

Guan, J. S., Haggarty, S. J., Giacometti, E. et al. (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* **459,** 55-60.

Han, M. H. and Nestler, E. J. (2017) Neural Substrates of Depression and Resilience. *Neurotherapeutics*.

Hill, M. N., McLaughlin, R. J., Pan, B. et al. (2011) Recruitment of prefrontal cortical endocannabinoid signaling by glucocorticoids contributes to termination of the stress response. *J Neurosci* **31,** 10506-10515.

Hu, X. L., Cheng, X., Cai, L. et al. (2011) Conditional deletion of NRSF in forebrain neurons accelerates epileptogenesis in the kindling model. *Cereb Cortex* **21,** 2158-2165.

Hua, Y., Sahashi, K., Hung, G., Rigo, F., Passini, M. A., Bennett, C. F. and Krainer, A. R. (2010) Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev* **24,** 1634-1644.

Hua, Y., Sahashi, K., Rigo, F., Hung, G., Horev, G., Bennett, C. F. and Krainer, A. R. (2011) Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature* **478,** 123-126.

Huang, C. C., Yang, C. H. and Hsu, K. S. (2005) Do stress and long-term potentiation share the same molecular mechanisms? *Mol Neurobiol* **32,** 223-235.

Krishnan, V. and Nestler, E. J. (2008) The molecular neurobiology of depression. *Nature* **455,** 894-902.

Laurent, B., Ruitu, L., Murn, J. et al. (2015) A Specific LSD1/KDM1A Isoform Regulates Neuronal Differentiation through H3K9 Demethylation. *Mol Cell* **57,** 957-970.

Long, J. Z., Li, W., Booker, L. et al. (2009) Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat Chem Biol* **5,** 37-44.

Lutz, B., Marsicano, G., Maldonado, R. and Hillard, C. J. (2015) The endocannabinoid system in guarding against fear, anxiety and stress. *Nat Rev Neurosci* **16,** 705-718.

McClelland, S., Brennan, G. P., Dubé, C., Rajpara, S., Iyer, S., Richichi, C., Bernard, C. and Baram, T. Z. (2014) The transcription factor NRSF contributes to epileptogenesis by selective repression of a subset of target genes. *Elife* **3,** e01267.

McEwen, B. S. (2005) Glucocorticoids, depression, and mood disorders: structural remodeling in the brain. *Metabolism* **54,** 20-23.

Morena, M., Patel, S., Bains, J. S. and Hill, M. N. (2016) Neurobiological Interactions Between Stress and the Endocannabinoid System. *Neuropsychopharmacology* **41,** 80-102.

Patel, S., Shonesy, B. C., Bluett, R. J., Winder, D. G. and Colbran, R. J. (2016) The Anxiolytic Actions of 2-Arachidonoylglycerol: Converging Evidence From Two Recent Genetic Endocannabinoid Deficiency Models. *Biol Psychiatry* **79,** e78-e79.

Pilotto, S., Speranzini, V., Tortorici, M. et al. (2015) Interplay among nucleosomal DNA, histone tails, and corepressor CoREST underlies LSD1-mediated H3 demethylation. *Proc Natl Acad Sci U S A* **112,** 2752-2757.

Pizzagalli, D. A. (2014) Depression, stress, and anhedonia: toward a synthesis and integrated model. *Annu Rev Clin Psychol* **10,** 393-423.

Popoli, M., Yan, Z., McEwen, B. S. and Sanacora, G. (2012) The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. *Nat Rev Neurosci* **13,** 22-37.

Rusconi, F. and Battaglioli, E. (2018) Acute Stress-Induced Epigenetic Modulations and Their Potential Protective Role Toward Depression. *Front Mol Neurosci* **11,** 184.

Rusconi, F., Grillo, B., Ponzoni, L. et al. (2016) LSD1 modulates stress-evoked transcription of immediate early genes and emotional behavior. *Proc Natl Acad Sci U S A* **113,** 3651-3656.

Rusconi, F., Grillo, B., Toffolo, E., Mattevi, A. and Battaglioli, E. (2017) NeuroLSD1: Splicing-Generated Epigenetic Enhancer of Neuroplasticity. *Trends Neurosci* **40,** 28-38.

Rusconi, F., Paganini, L., Braida, D. et al. (2014) LSD1 Neurospecific Alternative Splicing Controls Neuronal Excitability in Mouse Models of Epilepsy. *Cereb Cortex*.

Sala, M., Perez, J., Soloff, P., Ucelli di Nemi, S., Caverzasi, E., Soares, J. C. and Brambilla, P. (2004) Stress and hippocampal abnormalities in psychiatric disorders. *Eur Neuropsychopharmacol* **14,** 393-405.

Sanacora, G., Treccani, G. and Popoli, M. (2012) Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. *Neuropharmacology* **62,** 63-77.

Savinainen, J. R., Saario, S. M. and Laitinen, J. T. (2012) The serine hydrolases MAGL, ABHD6 and ABHD12 as guardians of 2-arachidonoylglycerol signalling through cannabinoid receptors. *Acta Physiol (Oxf)* **204,** 267-276.

Schlosburg, J. E., Blankman, J. L., Long, J. Z. et al. (2010) Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat Neurosci* **13,** 1113-1119.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A. and Casero, R. A. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119,** 941-953.

Spreafico, M., Grillo, B., Rusconi, F., Battaglioli, E. and Venturin, M. (2018) Multiple Layers of. *Int J Mol Sci* **19**.

Sun, G., Alzayady, K., Stewart, R., Ye, P., Yang, S., Li, W. and Shi, Y. (2010) Histone demethylase LSD1 regulates neural stem cell proliferation. *Mol Cell Biol* **30,** 1997-2005.

Toffolo, E., Rusconi, F., Paganini, L. et al. (2014) Phosphorylation of neuronal Lysine-Specific Demethylase 1LSD1/KDM1A impairs transcriptional repression by regulating interaction with CoREST and histone deacetylases HDAC1/2. *J Neurochem* **128,** 603-616.

Treccani, G., Musazzi, L., Perego, C. et al. (2014) Stress and corticosterone increase the readily releasable pool of glutamate vesicles in synaptic terminals of prefrontal and frontal cortex. *Mol Psychiatry* **19,** 433-443.

Wang, J., Telese, F., Tan, Y. et al. (2015) LSD1n is an H4K20 demethylase regulating memory formation via transcriptional elongation control. *Nat Neurosci* **18,** 1256-1264.

Wang, M., Hill, M. N., Zhang, L., Gorzalka, B. B., Hillard, C. J. and Alger, B. E. (2012) Acute restraint stress enhances hippocampal endocannabinoid function via glucocorticoid receptor activation. *J Psychopharmacol* **26,** 56-70.

Yang, C. H., Huang, C. C. and Hsu, K. S. (2005) Behavioral stress enhances hippocampal CA1 long-term depression through the blockade of the glutamate uptake. *J Neurosci* **25,** 4288-4293.

Zamberletti, E., Gabaglio, M., Piscitelli, F. et al. (2019) Cannabidivarin completely rescues cognitive deficits and delays neurological and motor defects in male. *J Psychopharmacol* **33,** 894-907.

Zibetti, C., Adamo, A., Binda, C. et al. (2010) Alternative splicing of the histone demethylase LSD1/KDM1 contributes to the modulation of neurite morphogenesis in the mammalian nervous system. *J Neurosci* **30,** 2521-2532.

Zullo, J. M., Drake, D., Aron, L. et al. (2019) Regulation of lifespan by neural excitation and REST. *Nature* **574,** 359-364.

Figure Legends

Fig. 1 Acute Social Defeat Stress decreases neuroLSD1 isoform and transcription of ABHD6 and MAGL. On top, experimental stress scheme. Initial number of animals used for each group is indicated, this number also corresponds to the total number of animals. Effect of acute stress (2 or 7 hours long, as well as stress recovery, 7h stress plus 17h resting) on (A) LSD1/neuroLSD1 relative amount in the mouse hippocampus analysed by rqfRT-PCR(Rusconi *et al.* 2014) (n=5-7 mice per condition), and on (B-C) endocannabinoid regulators ABHD6 and MAGL, expression. Transcription was expressed as relative mRNA levels, analysed by qPCR, normalized on RPSA, and protein levels were expressed as fold increase over control conditions, band densitometry values normalized over β-tubulin (n=5-11 mice per condition). (D-E) Endocannabinoid regulators DAGLα and CB1R mRNA expression analysed as in (B-C) (n=6-8 mice per condition). Results are shown as the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001 assessed with one-way ANOVA variance test. Tukey post-hoc test. $^{\#}$p<0.05; assessed by Student's *t* test.

Fig. 2 LSD1 occupies regulatory promoter regions of *Abhd6* and *Mgll* genes in the mouse hippocampus. (A) Custom LSD1 ChIP-seq tracks loaded on UCSC Genome Browser (Mouse genome, version July 2007, NCBI37 mm9). Data were obtained from mouse primary cultured neurons(Wang *et al.* 2015). In the upper panels an overview of LSD1 association to *Abhd6* and *Mgll* genes, in green. Boxed in red a detail of transcription start sites (TSS) flanking genomic regions of interest with LSD1 peaks, magnified in the lower panels. qPCR amplicons used in our "candidate gene" Chromatin Immunoprecipitation (ChIP) analysis are shown. (B) Candidate gene ChIP on *Abhd6* and *Mgll* promoter regions, performed in the mouse hippocampus using the indicated antibodies. Enrichments over the Input condition are shown. Internal unrelated negative control is shown CTR (-) and positive controls taking as probe an already validated LSD1 target, c-fos(Rusconi *et al.* 2016) (n=3-4 mice per condition). Results are shown as the mean ± SEM. (A) $^{\#}$p < 0.05 assessed by Student's *t* test.

Fig. 3 Acute stress does not elicit ABHD6 and MAGL modulation neuroLSD1^{KO}, a mouse model devoid of neuroLSD1. (A) neuroLSD1^{KO} mice (generated by genomic deletion of *LSD1* exon E8a, indicated as KO) show in resting conditions differential levels of ABHD6 and MAGL in the hippocampus. Transcript expressed as relative mRNA levels, analysed by qPCR, normalized on RPSA. Protein band densitometry assessed as normalized values over β-tubulin and expressed as fold increase over CTR (mRNA analyses n=10-12, protein analyses n=5-6 mice

per condition). (B) Experimental scheme indicates the initial number of animals used, which also correspond to the total number of animals. Comparison of acute stress-induced modulation of *Abhd6* and *Mgll* mRNA in neuroLSD1^{KO} mice. mRNA and proteins analysed as in (A); framed are data relative to wild type mice mRNA levels already showed in Fig. 1B, re-plotted here as reference and included in the statistical comparison between wild type and neuroLSD1^{KO} littermates (mRNA analyses n=5-11 mice per condition) (protein analyses n=5-6 mice per condition). Results are shown as the mean ± SEM. $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$; assessed with Student's *t* test; **p < 0.01; p*** < 0.001 assessed with two-ways ANOVA variance test. Tukey post-hoc test.

Fig. 4 Stress-induced LSD1/neuroLSD1 ratio modulation, and relative Abhd6 and Mgll transcriptional regulation are restricted to acute stress response. On top, experimental scheme. Initial number of animals used for each group is indicated, this number also corresponds to the total number of animals. (A) Comparison between acute and chronic stress-mediated LSD1/neuroLSD1 ratio modulation in the mouse hippocampus analysed by rqf-RT-PCR(Rusconi *et al.* 2014). Framed are data relative to controls, 7 hour-long ASDS treated mice and recovered from stress (ASDS plus resting) already showed in Fig. 1A, re-plotted here as reference and included in the statistical comparison (n=5-7 mice per condition). (B-C) Comparison between acute and chronic stress-mediated modulation of ABHD6 and MAGL at the transcriptional level analysed with q-PCR normalized on RPSA (framed are data relative to controls and 7 hour-long ASDS treated mice, already showed in Fig. 1B-C, re-plotted here as reference and included in the statistical analysis) (n=6-9 mice per condition). Results are shown as the mean ± SEM. *p < 0.05; **p < 0.01; assessed with one-way ANOVA variance test. Tukey post-hoc test.

Figures

Figure 1

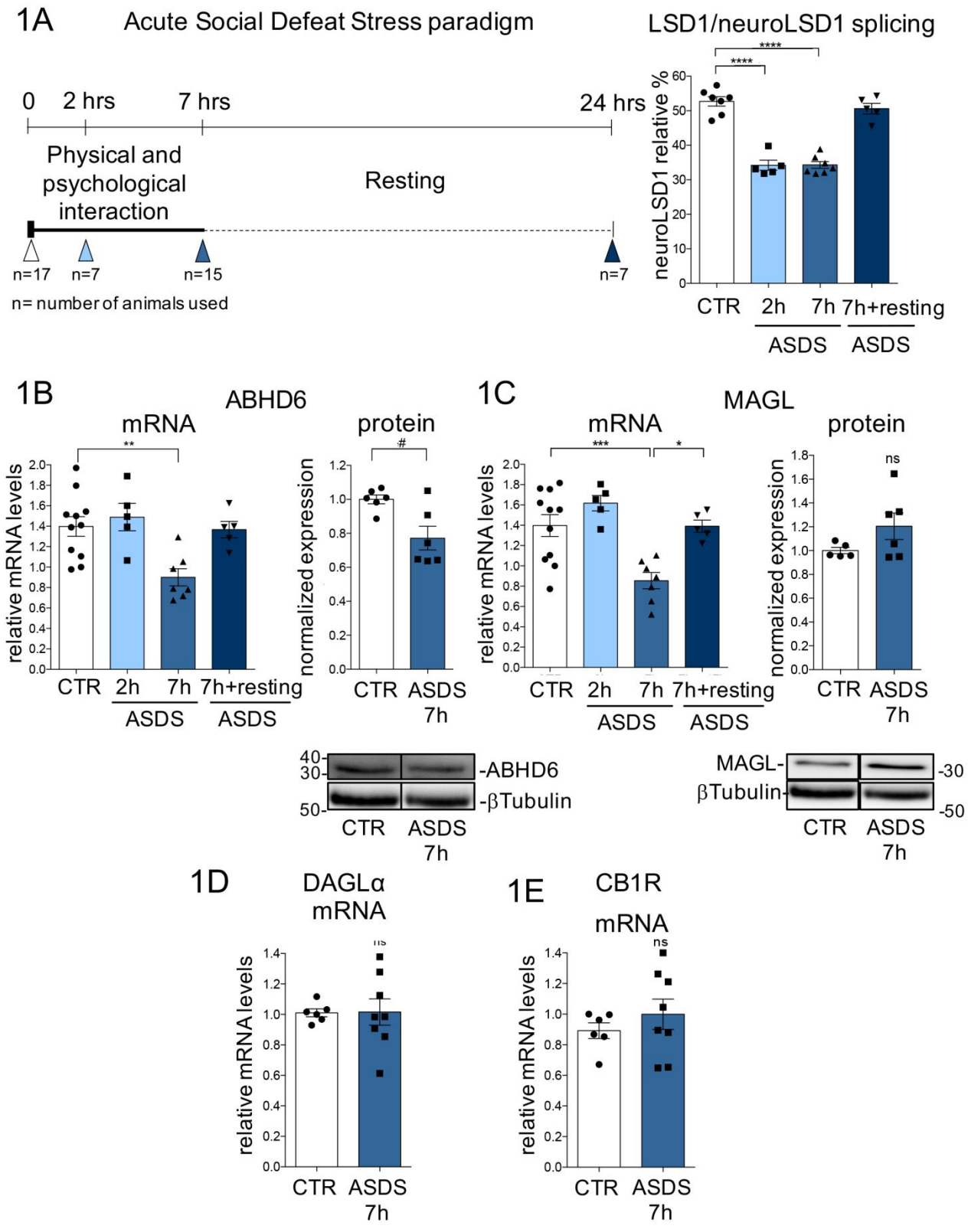
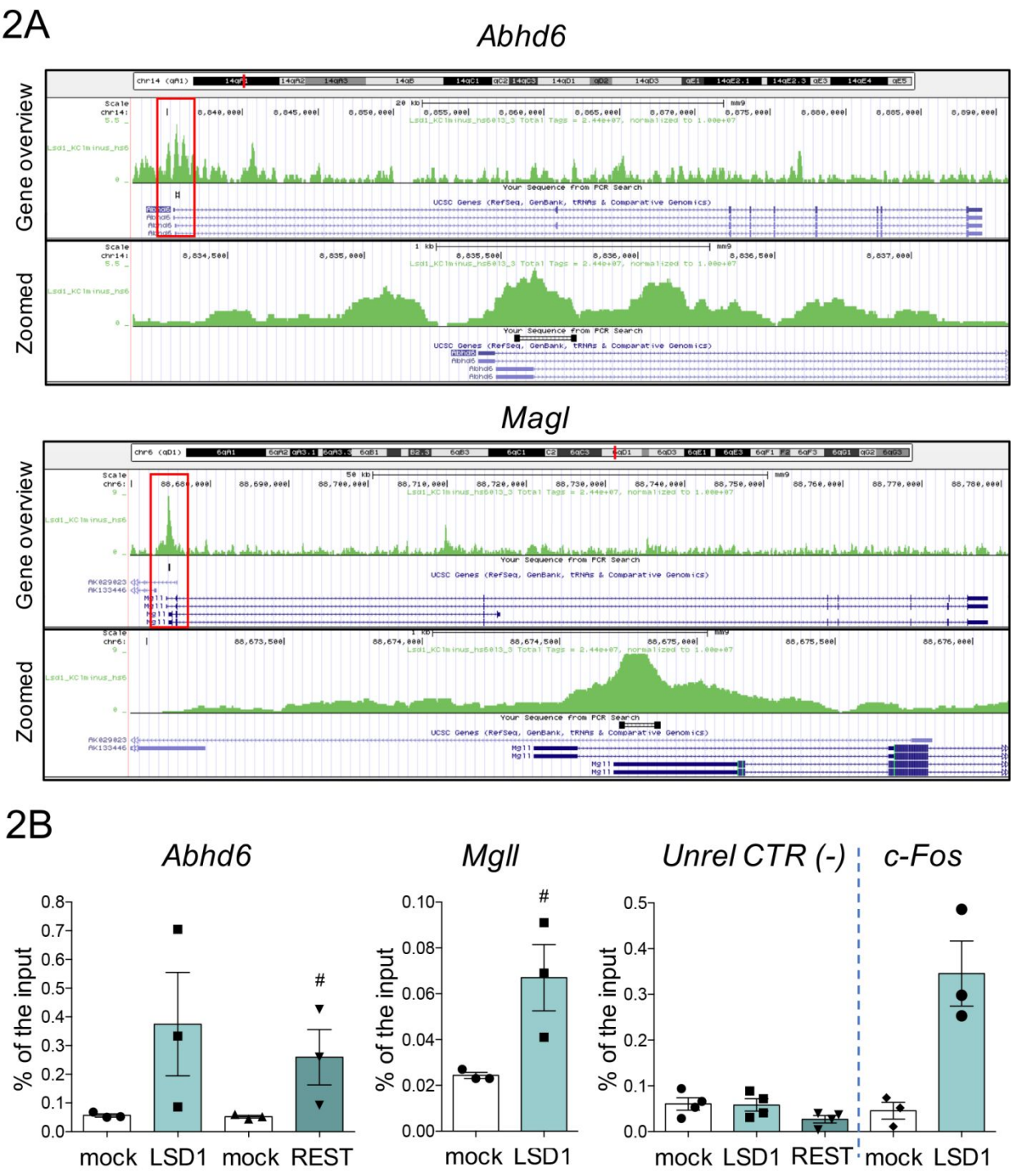


Figure 2


2A
Abhd6
Gene overview
Zoomed
Magl
Gene overview
Zoomed
2B
Abhd6
Mgll
Unrel CTR (-)
c-Fos
% of the input
mock
LSD1
mock
REST
#
% of the input
mock
LSD1
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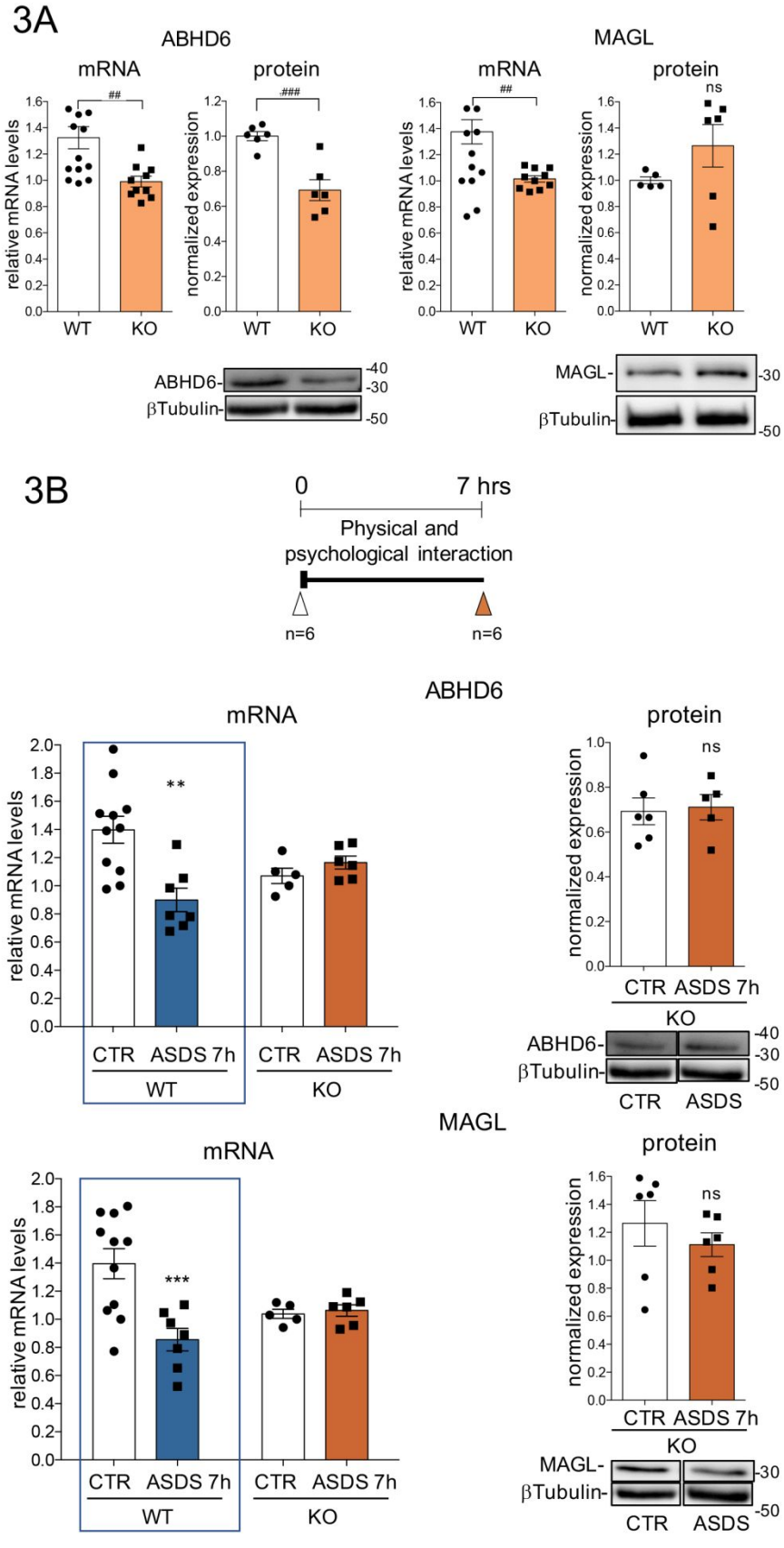
mock
LSD1
REST
mock
LSD1

Figure 3


3A
ABHD6
mRNA
protein
MAGL
mRNA
protein
relative mRNA levels
normalized expression
##
###
ns
WT
KO
ABHD6-
βTubulin-
MAGL-
-40
-30
-50
3B
0
7 hrs
Physical and psychological interaction
n=6
n=6
ABHD6
mRNA
protein
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ns
CTR ASDS 7h
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MAGL
mRNA
protein
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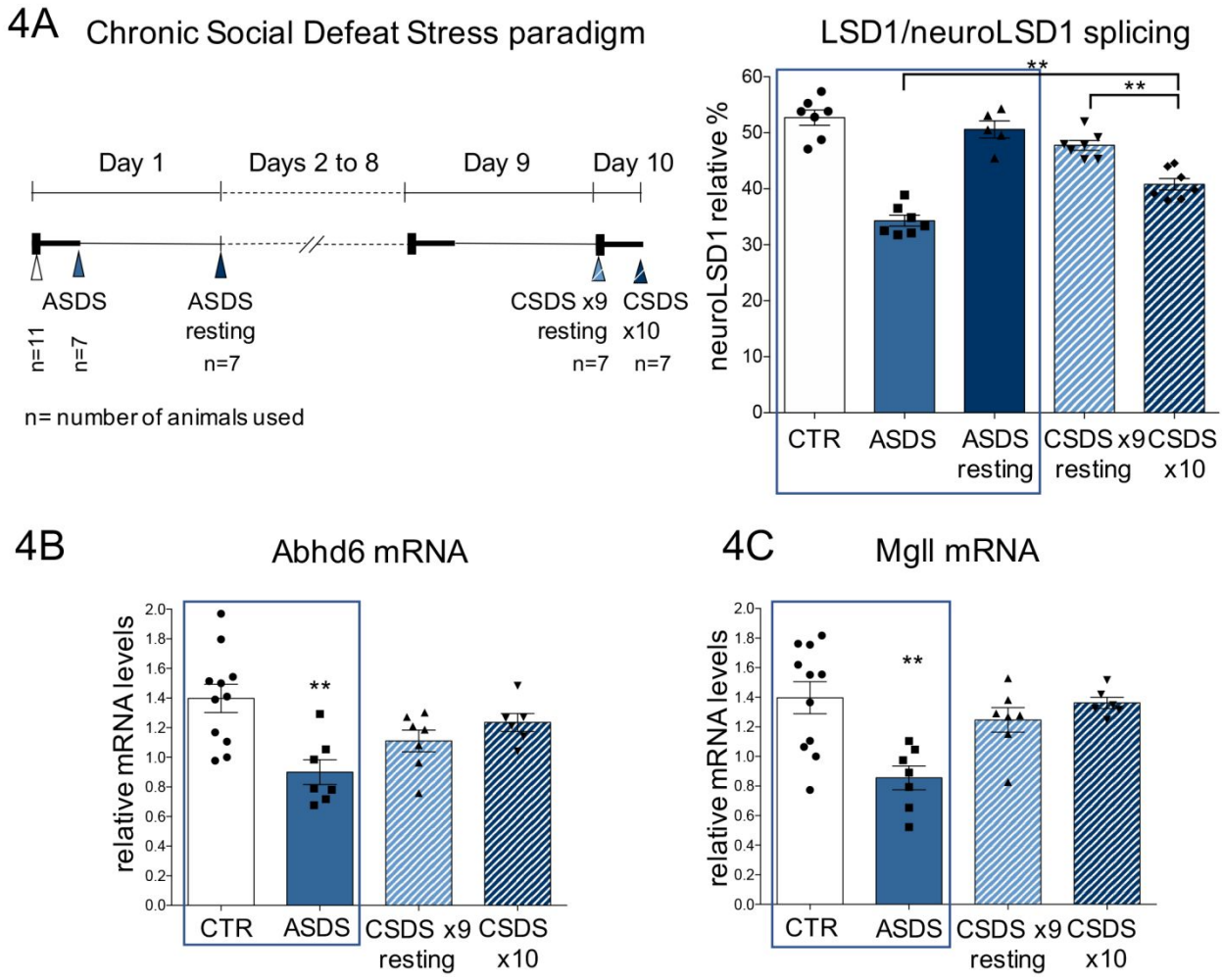
ns
MAGL-

Figure 4


4A
Chronic Social Defeat Stress paradigm
Day 1
Days 2 to 8
Day 9
Day 10
ASDS
ASDS resting
CSDS x9 resting
CSDS x10
n=11
n=7
n=7
n=7
n= number of animals used
LSD1/neuroLSD1 splicing
neuroLSD1 relative %
CTR
ASDS
ASDS resting
CSDS x9 resting
CSDS x10
4B
Abhd6 mRNA
relative mRNA levels
4C
Mgll mRNA
relative mRNA levels

Supplementary Material Longaretti et al.

Termination of acute stress response by the endocannabinoid system is regulated through LSD1-mediated transcriptional repression of 2-AG hydrolases ABHD6 and MAGL

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Supplementary Figure S1

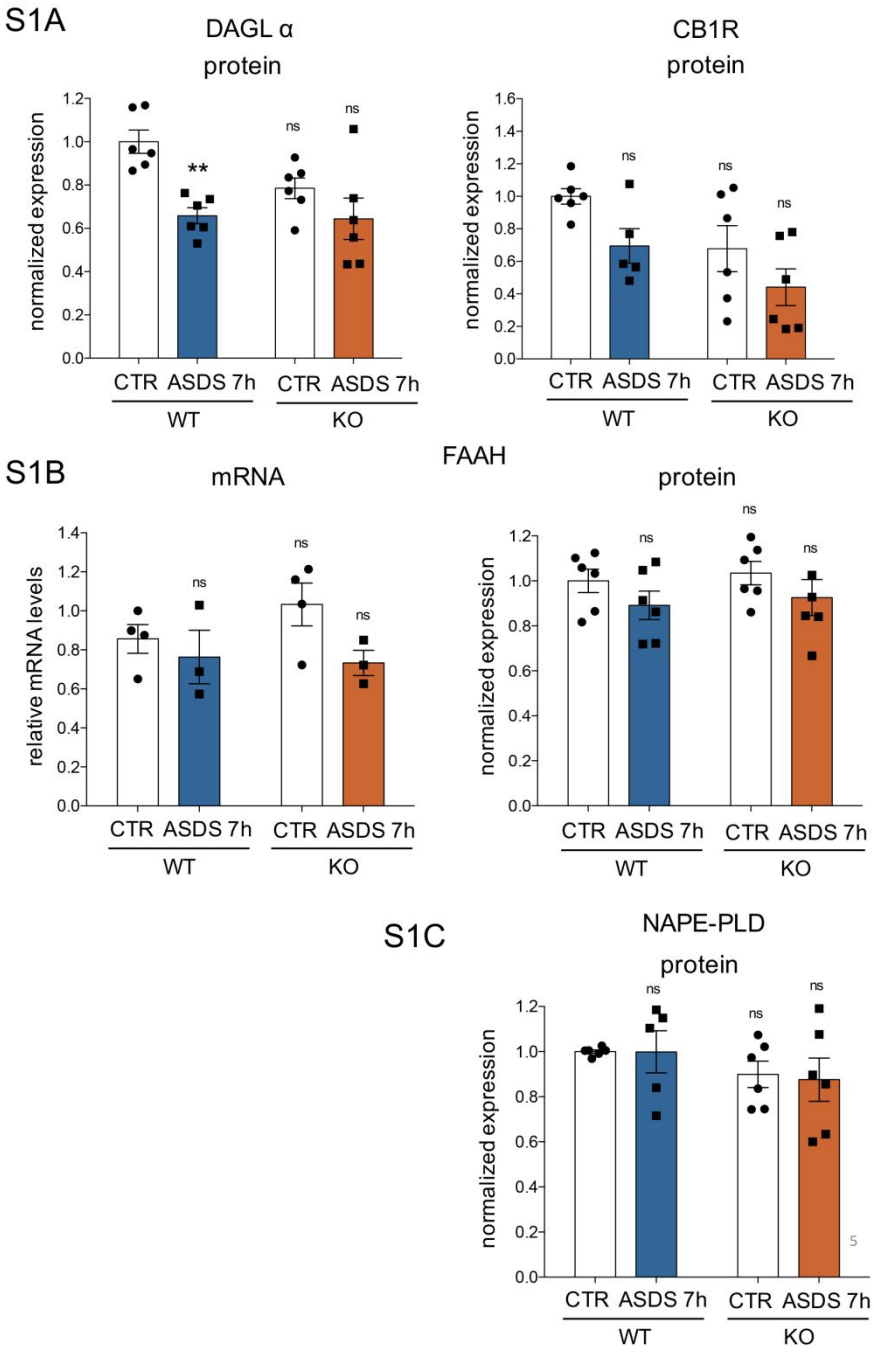

Figure S1. Effect of ASDS on expression of DAGLα, CB1R, and on biosynthetic and degradative anandamide enzymes.

(S1A) DAGLα and CB1R protein expression in wild type and neuroLSD1^{KO} mice. Protein band densitometry assessed as normalized values over β-tubulin and expressed as fold increase over CTR (n=5-6 mice per condition). (S1B) Anandamide biosynthetic and degradative enzymes are not modified by acute social defeat stress and unbound from neuroLSD1 genetic deletion.

Comparison of Fatty Acid Amide Hydrolase (FAAH) mRNA and protein levels in control and ASDS treated mice (both wild type and neuroLSD1^{KO}). Transcripts expressed as relative mRNA levels, analysed by qPCR, normalized on RPSA. Protein band densitometry assessed as normalized values over β-tubulin and expressed as fold increase over CTR (n=3-6 mice per condition). (S1C) Comparison of N-arachidonyl-PE Phospholipase D (NAPE-PLD) protein levels in control and ASDS treated mice (both wild type and neuroLSD1^{KO}). Protein analysis preformed as in (S1A-B) (n=6 mice per condition). Significance of mRNA and protein levels variation assessed with two-ways ANOVA variance test; ns, not significant. **p < 0.01; assessed with two-way ANOVA variance test. Tukey post-hoc test.

Supplementary Material and Methods

Sequences of primer oligonucleotides are indicated below and listed in the following sections.

Primers for splicing quantification

mLSD1_FW
5'-CTACCATTTCATCTTTTTCTTTTGG-3'
mLSD1_REV
[6FAM] 5′-AGTGAGCCGGAAGAGCCGTCTG-3′

Primers for mRNA quantification

mRPSA_FW
5'-ACCCAGAGGAGATTGAGAAGG-3'
mRPSA_REV
5'-TGGGGAACTGCTGAATGGGC-3'

mABHD6_FW
5'-AGTTCTGTTACTCCTTCCGGG-3'
mABHD6_REV
5'-CTTCGGAAGGAACTTGACCAC-3'

mMGLL_FW
5'-CCTGGTCAATGCAGACGGACA-3'
mMGLL_REV
5'-GCTCCATGGGACACAAAGATG-3'

mDALG_FW
5'-GCCCACATACTTTGCCATCTG-3'
mDALG_REV
5'-CTCCAGCACCTTATTGAGCC-3'

mCB1_FW
5'-CCTCTAACTTCCTTCAGGGGT-3'
mCB1_REV

Termination of acute stress response by the endocannabinoid system is regulated through LSD1-mediated transcriptional repression of 2-AG hydrolases ABHD6 and MAGL

Authors

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In this issue

Environmental stress engages physiological responses primed by synaptic activation. In the nucleus direct psychosocial stress effects induce a program of neuroplasticity-related gene expression instrumental to memory consolidation of the negative experience. On the behavioral point of view, stress response directly elicits anxiety arousal and increased vigilance. A slower homeostatic response to stress entails nuclear, synaptic and behavioral adaptations instrumental to stress response termination. We show that direct targets of LSD1 transcriptional repression at the nuclear level are 2-AG degrading enzymes ABHD6 and MAGL. In response to stress their expression is reduced helping to maintain active 2-AG concentrations via LSD1-mediated mechanism.