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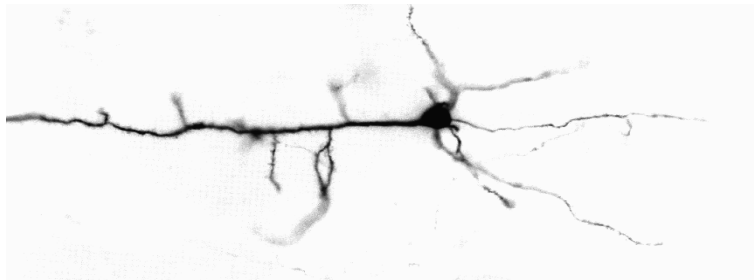
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Chapter

Steroid Receptor Coactivator-1 isoform switching in the central amygdala results in impaired contextual fear conditioning and abrogation of CRH expression regulation by glucocorticoids

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Abstract

Steroid receptor coactivator 1 (SRC-1) is a coregulator of the glucocorticoid receptor (GR) involved in the regulation of basal expression of corticotropin releasing hormone (CRH) and modulation of CRH expression by glucocorticoids in the brain. The two isoforms, SRC-1a and SRC-1e are generated by the *NCoA1* gene. SRC-1a lacks an SRC-1e specific exon. The two isoforms differ in their activities and distribution in the brain: SRC-1a is more abundant in the paraventricular nucleus of the hypothalamus and can potentiate repression at the *crh* promoter, whereas SRC-1e is more abundant in the central amygdala (CeA) and lacks repressive capacity. We hypothesized that shifting the SRC-1a:SRC-1e expression ratio in the CeA in favour of SRC-1a, using "exon skipping" would decrease the sensitivity of the CeA to glucocorticoids and therefore block the glucocorticoid-induced upregulation of CRH expression.

We injected stereotactically in the CeA of mice antisense oligonucleotides, which were designed to exclude the SRC-1e specific exon from the mRNA. Subsequently, we tested contextual- and cue-fear memory performance, anxiety responses and regulation of CRH expression by glucocorticoids in the CeA.

Our results showed in the CeA a shift of the SRC-1a:SRC-1e expression ratio in favour of SRC-1a that led to impaired consolidation of conditioned fear memory, enhanced locomotor activity in the open field test and abrogation of the glucocorticoid-induced upregulation of CRH expression in the CeA. In conclusion, our findings demonstrate that manipulation of GR downstream signaling pathways can shift responsiveness to glucocorticoids.

Introduction

The ability to orchestrate appropriate adaptive responses to stressors is indispensable for survival. The Hypothalamic-Pituitary-Adrenal (HPA) axis plays a pivotal role in the orchestration of adaptive responses. Corticotropin releasing hormone (CRH) has a key role in the regulation of the HPA axis, as its secretion from the paraventricular nucleus of the hypothalamus (PVN) along with other secretagogues to the pituitary stimulates the release of adrenocorticotropin hormone (ACTH) (1, 2). ACTH is then released into the systemic blood flow, reaches the adrenals and stimulates the production of glucocorticoids, which feedback on the brain to suppress the expression of CRH in the PVN. Another important CRH production site is the central nucleus of the amygdala (CeA), where the peptide organizes autonomic and behavioral responses to stress and is involved in fear and anxiety (3-5). A major modulator of CRH expression at both brain sites is the glucocorticoid receptor (GR). The GR is a transcription factor mediates effects of glucocorticoids on cognitive processes (e.g. memory consolidation), emotional state (e.g. fear responses) and endocrine regulation (5-7). Glucocorticoids regulate CRH expression in a distinct brain region-dependent manner: treatment with glucocorticoids results in CRH upregulation in the CeA (which may potentiate fear responses), but in downregulation in the PVN, as part of the negative feedback loop of the HPA axis (8).

The CeA is an important brain region for emotional responses such as anxiety and acquisition, consolidation and expression of conditioned fear (2, 9-11). Its function in both contextual and cue fear conditioning has been well characterized and appears to be dependent on GR and CRH expression (5). Animals conditionally lacking GR expression in the central amygdala have impairments in consolidation of conditioned fear, which can be rescued by post-training intracerebroventricular injection of CRH (5). On the other hand, increased CRH expression in the CeA may also enhance the reactivity of the HPA axis, particularly during chronic stress conditions (12, 13). High CRH expression may result in increased anxiety and depressive-like features (12, 14, 15) and may be related to psychopathology (4). The opposite direction of glucocorticoid effects on CRH in PVN and CeA illustrates the way in which these hormones act at these different sites to promote adaptation to stressors. However, these opposite effects also imply that additional factors are involved in the GR-mediated regulation of CRH expression (16).

Nuclear Receptor Coregulators are such additional proteins that are involved in steroid regulation of gene expression. Their mode of action involves binding to nuclear receptors and recruitment of other transcription factors, stabilization of the transcriptional machinery and histone acetylation either via intrinsic histone acetyltransferase activity or by recruitment of histone acetylransferases (17, 18). Most coregulators interact with multiple nuclear receptors and all nuclear receptors interact with multiple coregulators. This promiscuity of nuclear receptors and coregulators offers the aforementioned additional level of regulation of target gene expression.

SRC-1 is, arguably, the best characterized nuclear receptor coregulator and has been shown to

interact with a.o the GR, the mineralocorticoid receptor (MR), the androgen receptor and the estrogen receptor (19-22). SRC-1 knockout mice display impairments in regulation of the *crh* gene in the CeA and PVN by glucocorticoids (23). The SRC-1 gene encodes two splice variants, SRC-1a and SRC-1e, which have different expression patterns in the brain and opposite activities on the *crh* promoter (24-26). SRC-1e mRNA contains an extra exon, which has an early stop codon (Chapter 2 Figure 1). Hence, while SRC-1e mRNA is longer than SRC-1a, the SRC-1a protein is larger and presents an additional nuclear receptor binding domain (NR box). Hence, the SRC-1a protein contains four NR boxes, three of which are common between SRC-1a and SRC-1e and one specific NR box (NR box IV). SRC-1a is abundantly expressed in the PVN and can repress the *crh* promoter, whereas SRC-1e is highly expressed in the CeA and lacks repressive activity at the *crh* promoter *in vitro*. The functional significance of SRC-1 splice variants has not been clarified *in vivo*.

Antisense oligonucleotide (AON)- mediated exon skipping is a powerful and versatile technique to manipulate mRNA splicing (27). Previously (28), we have shown that a single injection of AONs targeting SRC-1 can induce a shift in the expression ratio of the two SRC-1 splice variants in favour of SRC-1a, without adverse effects and without activation of compensatory mechanisms, such as SRC-2 overexpression, or changes in total SRC-1 expression. Here, we hypothesized that this shift will lead to impaired regulation of CRH expression by glucocorticoids in the CeA, and attenuated fear behavior. Our data showed that the *crh* gene became GR resistant after changing the SRC-1 splice variant expression ratio, while we observed decreased freezing during fear conditioning testing and increased locomotor activity in the open field test.

Materials and Methods

Animals and stereotactic surgery: 11-week old (at the time of arrival) C57Bl6/j mice were purchased from Janvier (Saint-Berthin, France) and used for all experiments. All animal experiments were carried out in accordance with European Communities Council Directive 86/609/EEC and the Dutch law on animal experiments and were approved by the Leiden University animal ethical committee (protocol number: 11157). They were housed singly in individually ventilated cages upon their arrival and until the second postoperative day, thereafter moved to normal cages. Housing conditions were controlled with a 12 h light:dark cycle, with lights on at 7 am. Food and water were available *ad libitum*, except during experiments. Animals were allowed one week to acclimatize in the animal facilities and subsequently operated. The operation protocol has been extensively described elsewhere (28). Briefly, animals were anesthetized with a cocktail of Hypnorm: Dormicum: demineralized H_2O in a volume ratio of 1.3:1:3 and a dose of 5 mg/kg. Custom-made boroscillicate needles were used for the infusion connected to a Hamilton syringe. One μl of AON targeting exon 22 of SRC-1e or mismatch AON was infused bilaterally at -1.25 mm anterior-posterior, ±2.95 mm medio-lateral and -4.75 mm dorso-ventral relative to bregma, at a rate of 0.15 μl/min using an injection pump (Harvard apparatus, Holliston, MA, USA). At the end of infusions the

injection needle was left inside its injection position for 7 minutes and then retracted slowly. Afterwards, the skin incision was sutured and the animals returned to their home cage for recovery. All behavioral testing and blood sample collection took place between 9:00-13:00 h. At the end of the experiment animals were euthanized with an intraperitoneal injection of overdose euthasol (ASTfarma, Oudewater, the Netherlands), followed by decapitation and their brains were harvested, frozen in isopentane on dry ice and stored at -80ºC.

Blood samples collection: Two days after the operation and between 9-10:00 AM a blood sample was collected from each animal via a small tail incision. Tail blood samples were also collected 60 minutes after the start of the open field test, 30 and 120 minutes after the start of fear conditioning training and 60 minutes after the start of fear conditioning testing (trunk blood). Tail cut and trunk blood samples were collected in pre-cooled EDTA coated microvette CB300 tubes (Sarstedt, Etten-leur, the Netherlands) and centrifuged at 13000 rpm at 4ºC for 15 min in a table top centrifuge. Plasma was collected and stored at -20ºC.

Open field test: Three days after the operation (Figure 1a) animals were placed in a 45 cm x 45 cm with 45-cm high walls transparent glass box without a lid and were allowed to explore freely for 5 minutes. Each trial was recorded by a camera and tracked by the behavioral analysis software Ethovision XT 9 (Noldus, Wageningen, the Netherlands). Total distance walked, distance walked in a 15 x 15 cm square in the center of the platform and time spent in the center of the platform were calculated.

Fear conditioning test: Fear conditioning apparatus and protocol have been previously described elsewhere (29, 30). Briefly, the setup consisted of a 25 cm x 25 cm x 35 cm black opaque plexiglas box whose floor consisted of metal grid connected to a shock generator. A speaker connected to a noise generator was incorporated in the box. A lamp and a camera connected to a computer were placed 20 cm above the box. Each trial was digitally recorded with Observer XT (Noldus, Wageningen, the Netherlands). Five days after the operation the animals were placed in the box. Every animal was allowed to explore the box for three minutes at baseline conditions. Subsequently it received seven cue sessions (Figure 1b). The cues consisted of a bright light and a tone for 20 seconds, the last two of which were paired with a mild electric shock of 0.4 mA. Between the end of one cue session and the beginning of the next there were one minute intervals. Two minutes after the last pairing mice were returned to their home cages. To test their fear responses we returned the animals 48 hours after training to the shock box and followed the same protocol as in training, however, this time the animals did not receive any electric shocks. We calculated freezing behavior, defined as the lack of any movement apart from respiration.

Subchronic dexamethasone treatment: Starting three days after stereotactic infusion with either AONs targeting SRC-1e or mismatch AONs, mice were injected twice per day with either dexamethasone 5 mg/kg (Sigma Aldrich, Zwijndrecht, the Netherlands) or with saline for five days. At the end of the experiment, the brains were harvested, frozen in isopentane on dry ice and stored at -80 ºC. The thymi and the adrenals were also stored in PBS at 4ºC and subsequently weighed.

Laser microdissection: Brains were sectioned at a thickness of 10 μm and mounted on

polyethylene naphtalate membrane sections (Carl Zeiss, Munich, Germany). Five sections were mounted on each slide and stored at -80°C until laser microdissection. Laser microdissection was carried out on a Leica laser microdissection microscope as has been described elsewhere (31). With the assistance of appropriate software, tissue was selected, microdissected and collected in adhesive caps (Carl Zeiss).

RNA isolation, cDNA synthesis and qPCR: RNA isolation was performed as described elsewhere (32). Briefly, RNA was isolated with chloroform and precipitated with isopropanol and linear acrylamide. Subsequently, RNA pellets were cleaned with 75% ethanol, dried and resuspended with 10 µl of DEPC treated demineralized water. Quality and concentration of RNA samples were measured on an experion system (Bio-Rad, Hercules, CA, USA) using HighSens analysis kit (Bio-Rad) according to the manufacturer's instructions. For cDNA synthesis 8 µl of RNA in demineralized water treated with diethylpyrocarbonate (Sigma-Aldrich, Zwijndrecht, the Netherlands) was used in concentrations that ranged from 52 to 961 ng/µl. RNA samples were first incubated with DNaseI (Promega, Madison, WI, USA) at 37°C for 30 min in order to remove possible DNA contamination. After incubation 1 µl of DNaseI stop solution (Promega) was added to each sample followed by incubation at 65 °C for 10 min to deactivate the enzyme. RNA samples were reverse transcribed with iScript cDNA synthesis kit (Bio-Rad). Briefly, 4 µl of 5 times iScript reaction mix, 1 µl of iScript reverse transcriptase and 5 µl of Nuclease-free H2O were added to 10 µl of DNase I treated RNA. Sub-sequently samples were incubated for 5 min at 25°C followed by 30 min at 42°C and finally 5 min at 85°C in a My Thermal Cycler (Bio-Rad) machine. Quantitative polymerase chain reaction (qPCR) was performed for assessment of gene expression in the CeA of AON injected mice. A 1:1 dilution of cDNA in autoclaved demineralized water was used for qPCR. The quantification of cDNA was performed on a LightCycler 2.0 (Roche Applied Science, Basel, Switzerland) using LC FastStartDNA MasterPLUS SYBR Green I (Roche). 2.5 µl of cDNA was added to a mix of 2 µl 5 times Sybr green mix, 1 µl of both forward and reverse primers (5 µM) and 3.5 µl nuclease-free water, in LightCycler Capillaries (20 µl, Roche). All measurements were performed in duplicate. The PCR program comprised 10 min at 95°C followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec and elongation at 72°C for 10 sec, with a subsequent dissociation stage (from 65°C to 95°C, at a rate of 0.1°C/sec). The SRC-1 splice variants were quantified as an expression ratio of SRC-1a/SRC-1e; the expression of total SRC-1 was normalized against β-actin. Quantification of relative expression was calculated using the Pfaffl method (33) and normalized against the control group (mismatch AON). The forward and reverse primer sequences were: SRC-1a 5'-CCTCTACTGCAACCAGCTCTCGTC-3' and 5'-TGCTGCACCTGCTGGTTTCCAT-3', SRC-1e: 5'-TGCAACCAGCTCTCGTCCACTG-3' and 5'-GCTCCTCTAGTCTGTAGTCACCACA-3', b-actin: 5'-CAACGAGCGGTTCCGATG-3' and 5'-GCCACAGGATTCCATACCCA-3'.

Radioimmunoassay (RIA): Plasma corticosterone levels were determined with Radioimmunoassays using ^{125}I RIA kits (MP Biochemicals, Santa Ana, CA, USA) as per the manufacturer's instructions.

In situ Hybridization: Non-isotopic double label semi-quantitative in situ hybidrization was

performed using the Panomics View-RNA method (Affymetrix, Santa Clara, CA, USA). Probe sets against GR (type 6 probe) and CRH (type 1 probe) mRNA were designed by the manufacturer. 12 μm thick section cryosections were mounted on Superforst plus microscope slides (Menzel Gläser, Braunschweig, Germany). Upon thawing the sections were postfixed in 4% formaldehyde (Sigma-Aldrich, Zwijndrecht, the Netherlands). Pre-incubation steps were
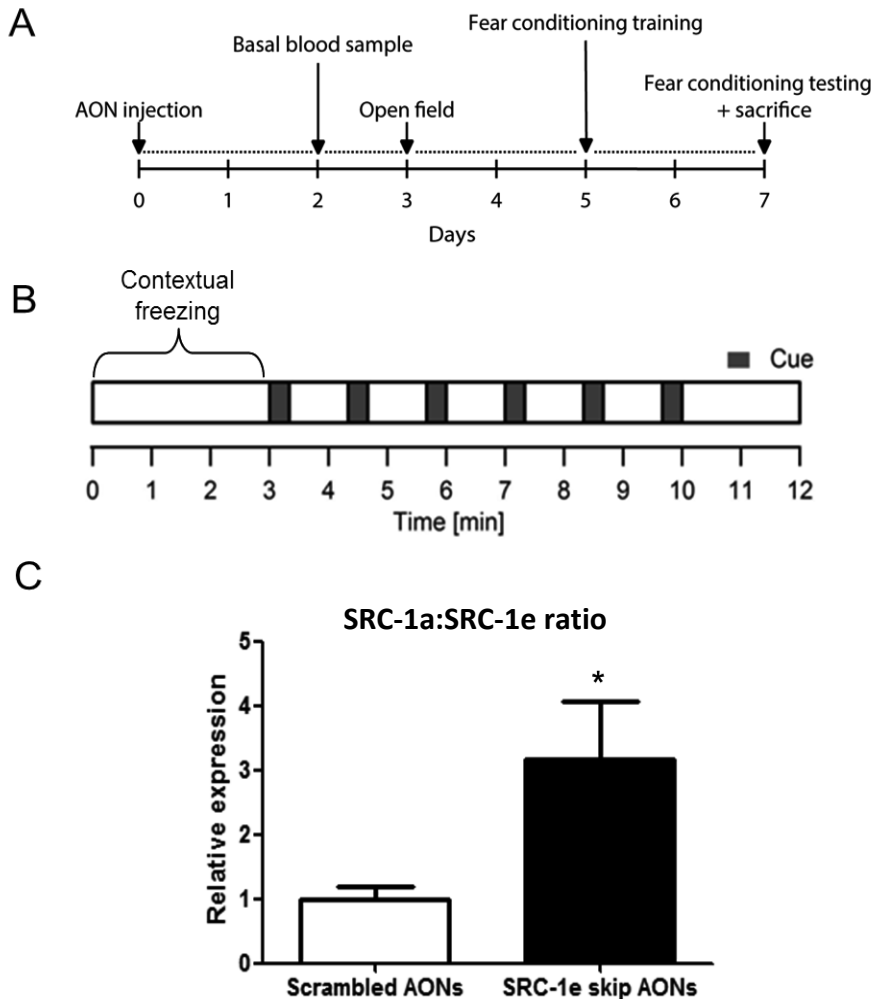

Figure 1. A. Schematic representation of the experimental design. The animals were operated 7-9 days after arrival in the animal facilities (day 0). Two days later basal blood samples were drawn. On day 3 they were introduced to an open field test. On day 5 and day 7, fear conditioning training and testing, respectively, took place. B. Fear conditioning protocol: The mice were allowed 3 minutes to explore the (*Figure 2 continued*) shock box. Afterwards, they were exposed to a strong light and sound for 20 seconds the last 2 of which coincided with a mild footshock. The interval between the end of one cue session and the beginning of the next was one minute. On training, the mice were exposed to 6 cue/shocks in total. On testing, the same protocol, but without shocks, was used. C. qPCR validation of exon skipping 7 days after an AON injection. The SRC-1a:SRC-1e ratio is significantly different between the groups (two-tailed t-test, $t_{(7)} = 2.687$, $p < 0.05$ n = 4-5 per group).

performed according to the manufacturer's instructions (https://www.panomics.com/products/rna-in-situ-analysis/viewrna-ish-tissue-assay/how-it-works). Hybridization of the probes took place for 4 hours in a Startspin thermobrite stove (Iris sample processing, Westwood, MA, USA). After hybridization slides were kept in storage buffer overnight. The next day linear amplification and visualization steps were performed following manufacturer's instructions. Slides were lightly counterstained with Mayer's hematoxylin, and DAPI (1 minute incubation at 3 µg/ml), and embedded in Innovex mounting medium (Innovex Biosciences, USA).

Slides were visualized using a Leica DRMA fluorescence microscope (Leica, Germany). For visualization of the red fluorophore, the Texas Red filter (excitation 542-582 nm, emission 604-644 nm) was used. For the blue fluorophore, the Cy5 filter (excitation 604-644 nm, emission 672-712 nm) was used. Ideally, the red fluorophore should be viewed under excitation 530 ± 20 nm, emission 590 ± 20 nm, and blue fluorophore with excitation 630 ± 20 nm and emission 775 ± 25 nm. Images were acquired through the software program ColourProc. For the images used for analysis, pictures were taken without stretching contrast. From each animal, a slice was selected and pictures were taken from the left and right CeA and the left and right PVN.

Statistical analysis: When two groups were compared, student's t-tests were performed. Differences with P values below 0.05 were considered statistically significant. For the effect of AONs and glucocorticoids on CRH expression a two-way ANOVA was performed with Glucocorticoid treatment and AON treatment as factors.

Results

Isoform expression ratio: In order to validate successful shifting of the SRC-1 splice variant ratio in the present experiment, we analyzed tissue from mice injected with AONs seven days earlier (n = 4-5 per group). qPCR analysis revealed that the SRC-1a:SRC-1e expression ratio was significantly shifted in favour of SRC-1a (Figure 1c).

Behavior: To assess basal anxiety-like behavior, we exposed animals to an open field test. Animals injected with AONs targeting SRC-1e had longer total walking distances (Figure 2a), however, no difference was found in percentage of time spent or distance walked in the center of the open field (Figure 2b).

We used a fear conditioning paradigm to assess the acquisition and consolidation of emotional memory after a shift in the SRC-1a:SRC-1e. In training, a significant trial effect and a group effect were found (Figure 2c) with animals injected with AONs targeting SRC-1e show increased freezing responses compared to control animals. However, animals injected with AONs targeting SRC-1e displayed reduced freezing upon re-exposure to the same chamber on testing day (Figure 2d). No difference was found in freezing behavior after presentation of the cue (Supplementary Figure 1). Moreover, we correlated the expression ratio of the two isoforms with the total distance walked in the open field experiment, in the subset of mice from which we had the SRC-1a:SRC-1e expression data. A strong positive correlation was

found; animals that had higher SRC-1a:SRC-1e expression ratios walked longer total distances in the open field experiment (Figure 2e).
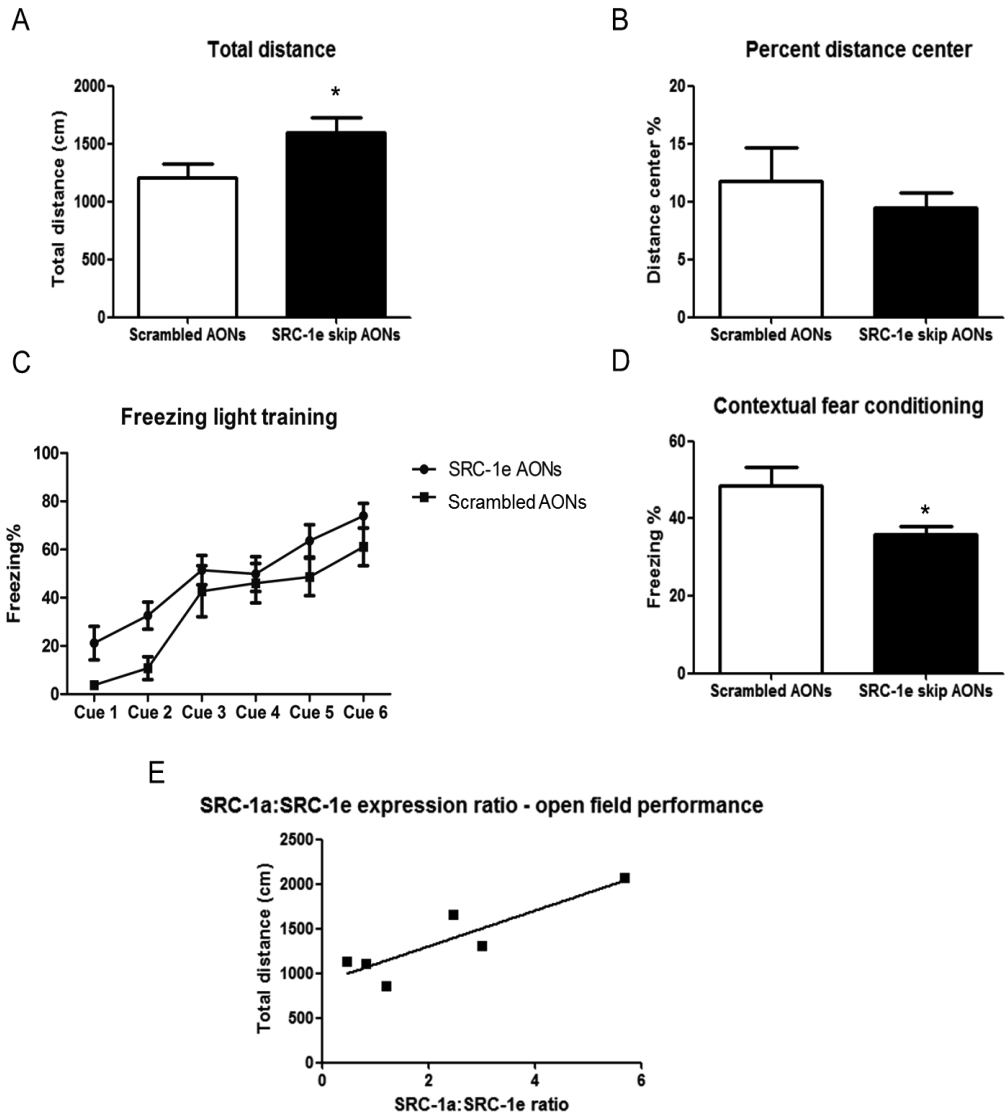

Figure 2. Behavioral profile of animals injected with SRC-1e skip AONs in the CeA. A. Total distance walked in the open field was not significantly different between the two groups (two-tailed t-test, $t_{(27)}$ = 2.3, p < 0.05, n = 13-16 per group). B. There was no difference in percent of distance walked in the center of the open field, between the groups (two-tailed t-test, $t_{(27)}$ = 0.644, p > 0.5, n = 13-16 per group). C. There was a significant (albeit small) treatment effect and a trial effect in CUE freezing during training (treatment: $F_{(1,120)}$ = 11.10, p = 0.001, trial: $F_{(5,120)}$ = 18.51, p = 0.0001, n = 9-13, per group). D. SRC-1e AONs reduced contextual fear memory consolidation measured as freezing response during reexposure to the footshock chamber (two-tailed t-test, $t_{(18)}$ = 2.313, p < 0.05, n = 10 per group). E. A significant correlation was found between SRC-1a:SRC-1e mRNA expression ratio and total distance walked in the open field (r^2 = 0.78, p<0.05, n = 6).
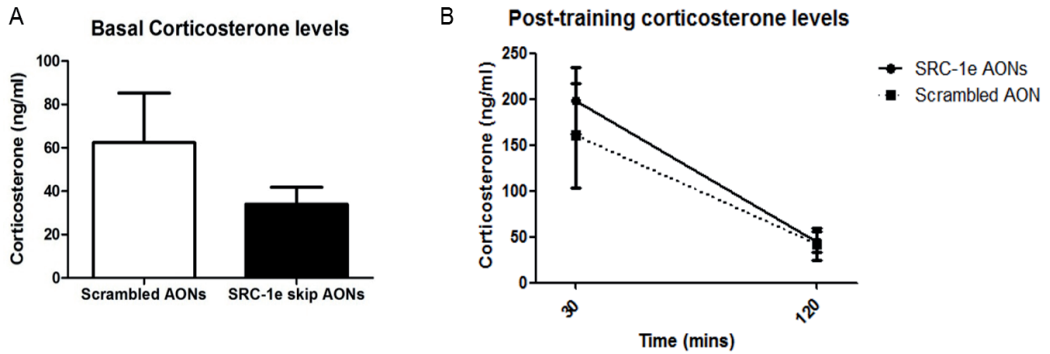
Figure 3. Lack of differential HPA axis regulation under basal conditions or in response to acute stress by SRC-1 isoform switching. A. Basal corticosterone levels do not differ between treatments (two-tailed t-test: $t_{(15)} = 1.121$, $p = 0.29$, n=8-9 per group). B. Corticosterone plasma level curves in response to acute stress. We only found a time point effect ($F_{(1,20)} = 39.85$, $p<0.001$), but no group ($F_{(1,20)} = 0.356$, $p = 0.56$) or interaction effects ($F_{(1,20)} = 0.27$, $p = 0.60$).
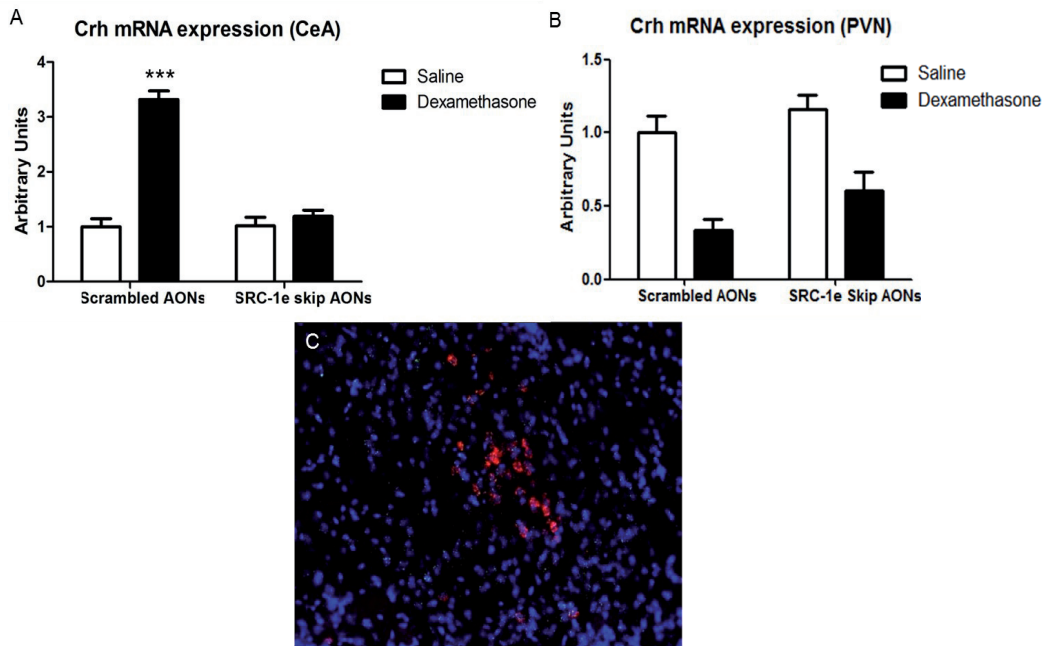

Figure 4. A. CRH mRNA in situ hybridization. In animals injected with scrambled AONs chronic dexamethasone treatment resulted in upregulation of CRH expression, which was blocked by SRC-1e skip AONs. Two-way ANOVA: AON effect, $F_{(1,17)} = 54.46$, $p<0.0001$, Glucocorticoid treatment effect, $F_{(1,17)} = 75.51$, $p<0.0001$, interaction effect, $F_{(1,17)} = 56.14$, $p<0.0001$, n = 5-7 per group. Bonferroni post hoc test: ***, $p<0.001$. B. CRH mRNA expression in the PVN. Treatment with dexamethasone significantly reduced CRH mRNA expression in the PVN ($F_{(1,12)} = 27.37$, $p<0.001$), while no AON effect was present ($F_{(1,12)} = 3.47$, $p>0.08$). C. Representative image of CRH mRNA in-situ hybridization in the central amygdala from a mouse treated with scrambled AONs and saline. Red: CRH mRNA, Blue: DAPI).

Plasma corticosterone levels: Basal corticosterone levels were not different between the groups (Figure 3a). Similarly, no differences between the two AON treatments were found after 30 or 120 minutes after fear conditioning training (Figure 3b).

CRH expression after glucocorticoid treatment: In order to test the hypothesis that the upregulation of CRH after glucocorticoid treatment is attenuated by SRC-1A, we compared the effects of 5 days of dexamethasone treatment compared to saline after injection with AONs targeting SRC-1e or scrambled AONs. Our results showed that in the scrambled AONs group there was a three-fold upregulation of CRH mRNA expression after treatment with dexamethasone, which was absent in the animals treated with AONs targeting SRC-1e (Figure 4a). In the PVN, the expected downregulation of CRH expression in response to glucocorticoids was found, independent of AON treatment (Figure 4b). Glucocorticoid treatment strongly reduced thymus weight in both groups, likewise indicating no differences in dexamethasone dosing between the groups (Figure 5a-b).
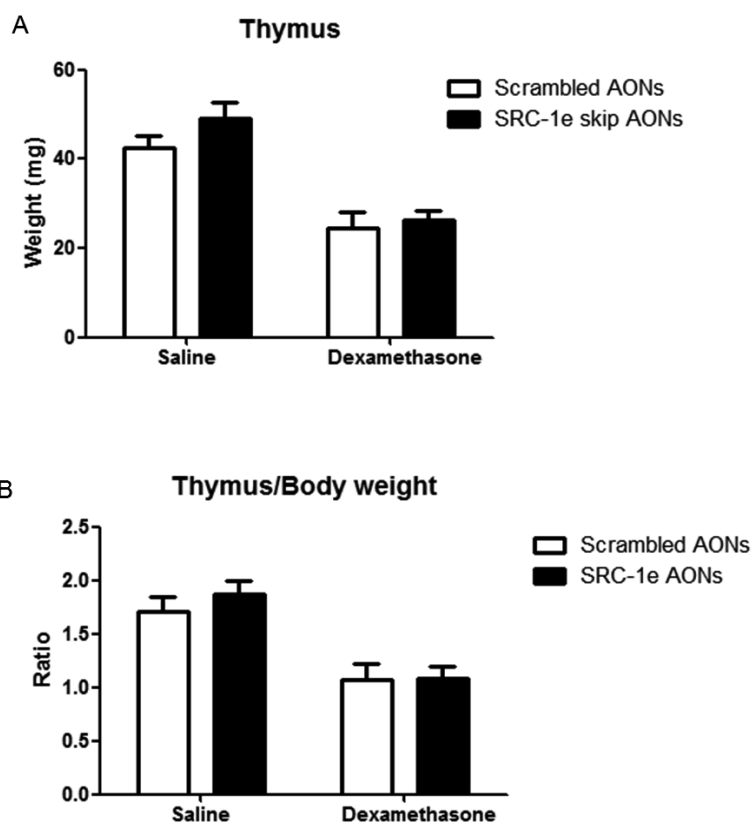

Figure 5. Effects of glucocortiocid treatment on thymus weight: A) There was a dexamethasone treatment effect on the weight of the thymi of the animals, independent of AON treatment (Glucocorticoid effect: $F_{(1,32)} = 41.01$, $p < 0.0001$, AON treatment: $F_{(1,32)} = 1.612$, $p = 0.213$, n = 8-10 per group). B) After correction for body weight, similar effects were observed (Glucocorticoid effect $F_{(1,34)} = 28.10$, $p < 0.0001$, AON treatment $F_{(1,34)} = 0.380$, $p > 0.54$) (ratios muliplied 1000X).

Discussion

In this study we manipulated the splicing of SRC-1 and we investigated its effect on stress responses and regulation of *crh* expression in the CeA by glucocorticoids. Here, we targeted exon 21 of the *Ncoa1* gene which leads to a shift towards higher expression of SRC-1a mRNA. We confirmed our previous finding (28) that seven days after a single injection of AONs targeting SRC-1e the expression ratio of two isoforms is shifted in favour of SRC-1a. Moreover, we found an effect of the expression ratio shift on contextual fear conditioning consolidation and a trend towards reduced basal anxiety as shown in an open field test. We also showed that the *crh* gene in the CeA became strongly resistant to the synthetic glucocorticoid dexamethasone. The data show that aspects of glucocorticoid effects on brain function may depend on downstream effector components in the molecular signal transduction pathway of the GR. They moreover suggest that these pathways may be targeted to overcome potentially pathogenic effects of excess glucocorticoids in stress-related disease.

Our hypothesis on the effects of changing the ratio in SRC-1 splice variants was based on a number of arguments. SRC-1a contains an additional nuclear receptor interaction domain that is possibly associated with a different affinity for the ligand-activated GR (36). In addition, the SRC-1A specific domain may lead to binding of different transcriptionally active proteins compared to the 1E isoform (34). Accordingly, SRC-1a can potentiate repression of the *crh* promoter after glucocorticoid treatment in AtT-20 cells, whereas SRC-1e lacks this repressive activity (25). Moreover, in SRC-1 KO animals, regulation of CRH expression in the CeA and the PVN by glucocorticoids is disrupted (23). Here, we observed a very strong abrogation of dexamethasone-induced CRH mRNA expression upregulation in the CeA which is in line with previous studies describing the effects of SRC-1a on the *CRH* promoter and similar to what has been observed in SRC-1 KO animals (23, 25). In contrast to SRC-1 KO animals, we did not observe a considerable effect of SRC-1e exon skipping after treatment with saline. This may have been due to the remaining expression of SRC-1e which may be adequate or even necessary for CRH expression under these conditions. The similar effects of dexamethasone on *crh* repression in the PVN, and the effects on thymus weight seem to exclude differences in steroid exposure as a cause of the observed differences.

One issue that needs to be taken into account is the stronger binding of the SRC-1a NR-IV box to the agonist bound GR compared to the central NR boxes, that has been shown in *in vitro* systems (35, 36). This may mean that the observed effect on *crh* expression and behavior may be beyond simple stoichiometry of SRC-1a and SRC-1e. Therefore, the effect of isoform switching may be higher than what would have been expected simply by the relative expression of the two isoforms. Thus, we observed a shift towards SRC-1a dependent effects of GR, such as repression of the *crh* promoter (25).

There were a number of behavioral effects of our manipulation. In the open field, the shift towards SRC-1A induced increased locomotor behavior that was proportional to the ratio between the splice variants. Moreover, after the 1e exon skip, the mice showed reduced

contextual freezing, even if both contextual and cue fear conditioning depend on amygdala function. A possible explanation for that could be the higher sensitivity of contextual fear conditioning to disruptions, and/or a ceiling effect for the cue conditioning (37). During training, SRC-1e AON injected animals showed higher reactivity to cue, while in testing the two groups had similar levels of freezing, something that may point to decreased consolidation of cue fear conditioning as well. Nevertheless, the strong effect on contextual fear conditioning suggests an important role of SRC-1 isoforms in fear memory consolidation, probably in relation to the genomic effects of glucocorticoids.

While previous studies in SRC-1 KO animals have found strong effects on their endocrine phenotype, they were accompanied by relatively mild behavioral differences (38, 39). This discrepancy has been attributed to possible developmental compensatory mechanisms such as SRC-2 upregulation in the absence of SRC-1 (23, 40). Here, we did not expect the development of strong compensatory mechanisms since we used a local manipulation on adult animals and a short term experimental setup that lasted up to seven days after AON treatment. As we have previously shown this manipulation does not change total SRC-1 expression and is not accompanied by upregulation of SRC-2 (28).

The mechanism that brings about the differences in *crh* expression and behavior may involve differential histone modification. The additional protein domain of SRC-1a contains a histone methyltransferase recruitment domain. Thus, upregulation of SRC-1a expression could well lead to higher histone methylation. Differential HAT activity may also result in decreased histone acetylation and differences in the expression of genes important for proper memory consolidation, or a direct effect of decreased *crh* expression after fear conditioning training.

We did not find a group effect on corticosterone levels at any time point which is in accordance with previous studies showing differential regulation of the HPA axis by the amygdala mainly in settings of chronic stress and sporadically after acute manipulations (5, 12, 13, 41). The lack of corticosterone plasma levels under basal conditions or after stress indicates that the local manipulation in the CeA did not block proper HPA axis function. On the other hand, the observed changes in fear memory under comparable levels of corticosterone suggest that the different relative expression ratio in the CeA may have changed its sensitivity to glucocorticoids. This is further highlighted by the abrogation of *crh* expression upregulation by dexamethasone in SRC-1e AON injected animals.

Based on our findings we suggest that a shift in the expression ratio of SRC-1a:SRC-1e may change the effects of GR on downstream targets in the context of stress and high glucocorticoid levels in the CeA by modifying its sensitivity to glucocorticoids and its selectivity regarding possible transcriptional pathways. This may have therapeutic implications in disorders characterized by high glucocorticoid levels such as psychotic depression (42), in relation to the recruitment and interaction of GR and its coregulators, either by changing the availability of the relevant coregulators [present study and (23)], or by pharmacologically targeting GR with appropriate ligands that can modulate its interactions with coregulators (36).

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Supplementary material

Contextual freezing testing

Cue freezing testing
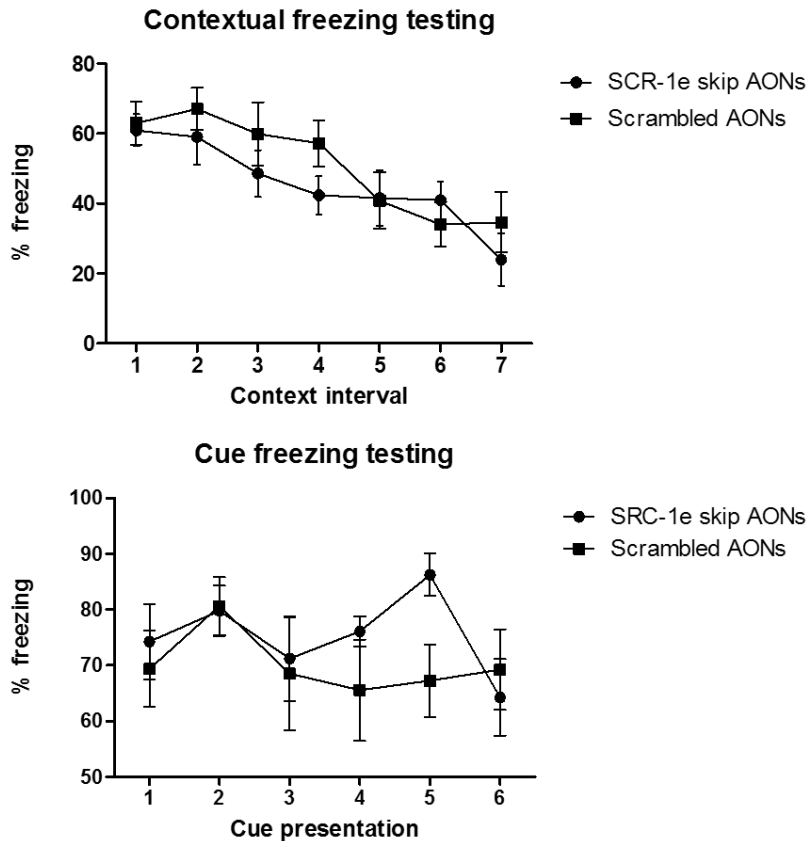
Supplementary figure 1. Percentage of time freezing in testing during the intervals between the cues (A) and during the presentation of the cues (B). A. Freezing % decreased over testing trials during reexposure to the shock box. No effect of AON treatment was found, but only a significant effect of trial ($F_{(6,133)} = 6.570$, $p < 0.001$). B. No effect was found in freezing behavior during cue presentations in the testing session.