Selective Glucocorticoid Receptor Modulation

TARGETING THE BRAIN UNDER STRESS

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Cover image: Oligonucleotides injection (green) in the central nucleus of the amygdala of the mouse brain.

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TARGETING THE BRAIN UNDER STRESS

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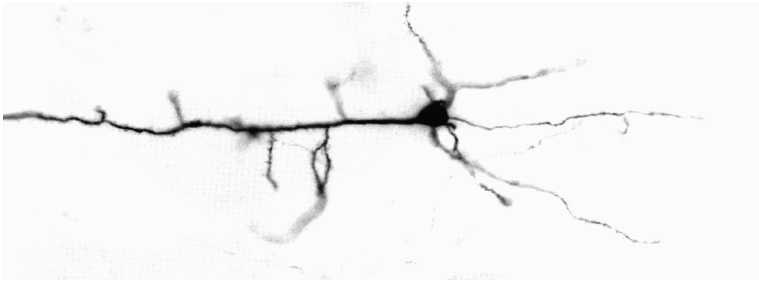
Table of contents

Chapter

1

General Introduction



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Introduction

Stress

Every stimulus that threatens (or is perceived as threatening to) the homeostasis of an organism is called a stressor (1, 2). The ability to appraise and retain or restore homeostasis via appropriate adaptive behavioral and physiological (stress) responses is crucial for survival (2). The appraisal of a stimulus as a stressor takes place in brain areas such as the amygdala, the hippocampus and the prefrontal cortex. An important component of the stress response is the secretion of glucocorticoids via the HPA axis and of catecholamines via the sympathetic nervous system which orchestrate a number of adaptations both in the brain and the periphery (2, 3). Inability to cope with stressors or prolonged exposure to them may lead to stress-related disorders such as depression, anxiety, post-traumatic stress disorder (PTSD), etc. As stress-related psychopathology results in considerable societal, financial and public health consequences, there has been increasing interest in better diagnoses and improved treatments for these disorders.

Amygdala-central amygdala

The amygdala (Figure 1) plays a central role in the orchestration of fear conditioning, anxiety and stress responses. It consists of diverse nuclei with distinct connectivity, neurochemical and morphological profiles (3). Anatomically, the amygdala is divided in the central nucleus of the amygdala (CeA), which expresses corticotropin releasing hormone (CRH) (Figure 1b-c), the basal nucleus of the amygdala and the lateral nucleus of the amygdala (3). It is believed that the basolateral nucleus (BLA), which contains primarily glutamatergic neurons (4), is the locus of associative learning of fear conditioning, while the CeA is the main output region of the amygdala, mainly involved in coordinating the expression of fear conditioning (5-7). The communication between the BLA and the CeA may be mediated by the intercalated cell masses. These are mainly GABAergic cells that are located between the BLA and the CeA and may play a gating role between the BLA and the CeA (8, 9). Recently, it has been shown that the CeA may also be involved in the learning phase of fear conditioning (10).

Importantly, the BLA sends and receives inputs from other brain regions such as the hippocampus, prefrontal cortex (PFC), hypothalamus, the ventral tegmental area and the nucleus accumbens (11, 12). Thus, the amygdala can be involved in a wide spectrum of processes and behaviors such as fear, anxiety and addiction (11, 12).

HPA axis

The main neuroendocrine regulator of stress responses is the HPA axis (Figure 2). Various stimuli and input from brain regions such as indirect input from the CeA (13) can induce the production of CRH in the paraventricular nucleus of the hypothalamus (PVN) and its secretion in the portal vessel system to activate the corticotrophs in the anterior pituitary. There, CRH

stimulates the production of adrenocorticotropic hormone (ACTH) and its release into the blood flow. Eventually, ACTH will reach the adrenal cortex where it binds to melanocortin 2 (MCR2) receptors and can stimulate the production of the glucocorticoids cortisol (human) or corticosterone (rodent). Glucocorticoids are then secreted into the blood flow and may exert a broad spectrum of effects, both peripherally and centrally that are mediated by two different receptors, the Glucocorticoid receptor (NR3C1 or GR) and the Mineralocorticoid receptor (NR3C2 or MR). In the central nervous system the receptors mediate the effects of the hormones on learning, memory and stress related behavior, as well as their inhibition of the expression of CRH in the PVN and ACTH in the anterior pituitary, as part of a negative feedback loop that prevents persistent elevation of glucocorticoid levels.

Glucocorticoids may also result in suppression of the HPA axis via their effects in the PFC and the hippocampus. Activation of GR in the PFC can result to release of endocannabinoids (CB). CB can then decrease GABA release onto prefrontal pyramidal cells which in turn increases glutamatergic input to the hypothalamus and inhibits the HPA axis (14-16). GR knockdown in the PFC may result in increased HPA axis responses to acute stress (17). Similarly, glucocorticoids in the ventral hippocampus also result in inhibition of the HPA axis stress responses (15).

Apart from activation by stressful situations, glucocorticoids are also secreted in a circadian fashion organized by inputs from the suprachiasmatic nucleus (SCN) to the PVN (18, 19). The

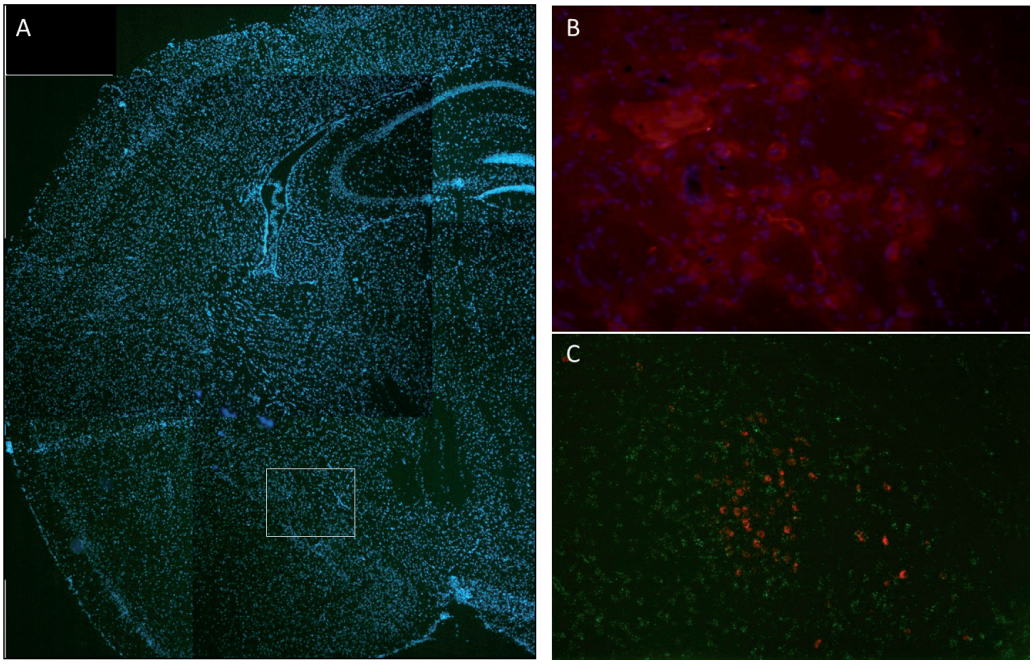

Figure 1. A. Fluorescent image of a mouse brain section stained with hoechst (blue) (10X magnification). The white box indicates the location of the central amygdala. B. The white box from picture A in magnification. CRH positive cells are immunofluorescently labeled red, while their nuclei are stained with Hoechst (blue) (63X magnification). C. In situ hybridization for CRH mRNA (red) and GR mRNA (green) in the CeA.

circadian rhythm of the glucocorticoids consists of hourly pulses of the hormone that have their largest amplitude at the start of the active period. (19). The stress-induced secretion of glucocorticoids is superimposed on these rhythms and its magnitude depends on the phase of the pulse (20-22).

CRH

CRH is a 41-amino acid peptide which was discovered in hypothalamic extracts in 1981 by W.W. Vale and was shown to stimulate the production of ACTH by cultured pituitary cells (23). CRH shows a wide expression pattern in stress-relevant areas in the brain including the PVN, the CeA, the bed nucleus of the stria terminalis, the prefrontal cortex and the hippocampus (24, 25). It plays a pivotal role in the regulation of glucocorticoid levels via its secretion from the PVN, in response to stress, while it orchestrates behavioral stress responses in the central amygdala (26). In line with these functions, its expression is tightly regulated by glucocorticoids. Interestingly, this regulation is region-specific: in the CeA the CRH expression is upregulated after treatment with glucocorticoids, whereas in the PVN it is downregulated, as part of the HPA axis' negative feedback loop (24, 27-29). CRH overexpression may result in increased anxiety behavior (30, 31), while, the *crh* promoter is epigenetically regulated in response to several stimuli including treatment with glucocorticoids, maternal deprivation and stress (27, 32-34).

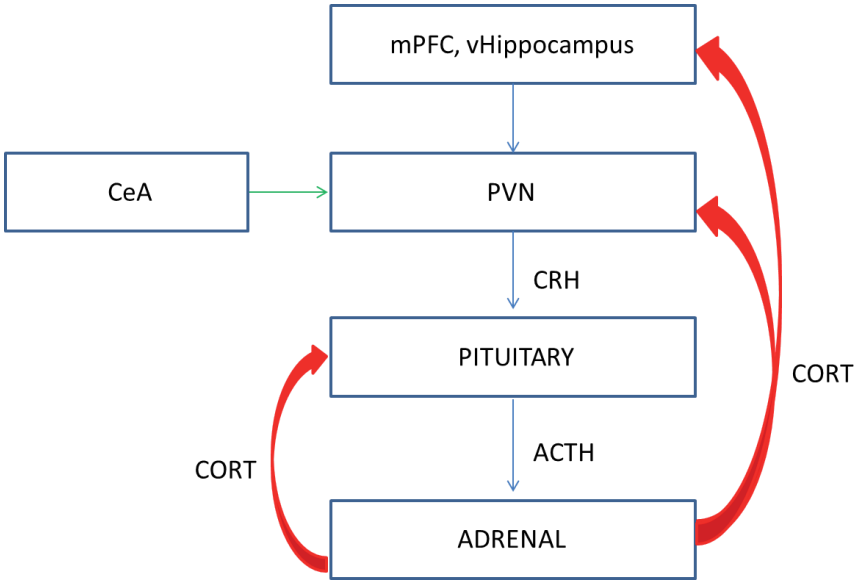

Figure 2. The Hypothalamus-Pituitary-Adrenal axis: In response to a variety of stimuli, such as indirect input from the central amygdala (CeA), corticotropin releasing hormone (CRH) is secreted from the paraventricular nucleus of the hypothalamus (PVN) into the pituitary stimulates the expression and secretion of adrenocorticotropin hormone which reaches the adrenal cortex and stimulates the production of corticosterone. Corticosterone, in turn, represses the expression of CRH and ACTH in the PVN and pituitary, respectively. Glucocorticoids in the mPFC and the ventral hippocampus also result in inhibition of the HPA axis.

GR and MR

GR and MR are nuclear receptors. All nuclear receptors consist of functional domains that can be directly coupled to their function as transcription factors. The relationship between the structure and the function of the GR (and MR) has been extensively studied (35, 36). In short, the GR protein contains domains that arise from eight exons (2-9, exon 1 of the mRNA is not translated): exon 2 codes for the N-terminal half of the protein which contains the major transcriptional activation domain τ1, exons 3 and 4 code for the central part of the protein which contains two zinc fingers involved in DNA binding and homodimerization. The C-terminal region of the protein, encoded by exons 5-9, include among others, the domains responsible for transcriptional activation (τ2) and ligand binding (Figure 3a) (35-37).

In the absence of ligand, MR and GR are bound to chaperone protein complexes in the cytoplasm. Upon ligand binding, a conformational change takes place that leads to the dimerization of the nuclear receptor and its translocation to the nucleus. There, with the assistance of coregulators, the nuclear receptor can bind to glucocorticoid response elements (GREs) on the DNA and activate or repress the expression of specific genes. The receptors are thought to mainly form homodimers, act as monomers in conjunction with other, non-receptor, transcription factors, or heterodimerize with other steroid receptors (38, 39). The activity of receptors depends also on the type and local concentration of the ligand (40, 41) and on the pattern of ligand exposure in time (42). However, additional regulation can take place at multiple levels. These may include the expression levels of the receptor (43), its posttranslational modifications (44), its interactions with molecular chaperones in the cytoplasm (45, 46), dimerization and translocation to the nucleus (47), the presence and function of kinases, such as SGK-1 (48), DNA binding and its interactions with proteins involved in transcription, either transcription factors or coregulator proteins (49).

Transcription factors that bind to regulatory DNA in conjunction with GR (and to a much lesser extent MR) are being discovered at a substantial rate by genome wide localization of receptor binding using ChIP-sequencing, and subsequent statistical analysis of DNA motifs that overlap with or surround the receptor binding sites. Some of the identified transcription factors will bring the receptors to the DNA by way of 'tethering' mechanisms, like those involved in classic transrepression in the immune system (50). There are also those transcription factors that bind in the vicinity (within hundreds of base pairs) of the steroid receptors, and are in some way involved in modulating their function. In generic cell lines, AP -1 has been shown to act as a 'pioneer' and make the DNA accessible for GR binding through chromatin modification (51). The exact nucleotide content of the GRE is associated with GR's dependence on such priming mechanisms.

It is also conceivable, or even likely, that factors that bind in the vicinity of MR and GR interact functionally in larger complexes on the DNA, analogous to what happens at composite GREs where GR binds directly adjacent to other transcription factors (52). In the rat hippocampus, it has been shown that GC-rich motifs for transcription factors MAZ1 and SP1 occur in conjunction with GR binding to the DNA, suggesting either a pioneering function, or a functional interaction with these factors (53). Recently, the first ChIP sequencing data for

GR were published for neuronally differentiated PC12 cells. Interestingly, GR binding occurred in the vicinity of AP-1 sites, as expected, but the authors also described recognition sites for a number of completely new transcription factors in the vicinity of GR binding. These data suggest that the effects of GR (and MR) are modified by other signalling pathways that we are just beginning to discover (54).

Coregulators

GR and MR make use of so called nuclear receptor coregulators, a large and rather diverse group of proteins that are involved in transcriptional modulation. The coregulator proteins do not interact with the DNA (*i.e.* they are not transcription factors), but mediate and modulate the effects of transcription factors on actual transcription. Individual coregulators may interact with either one or several members of the nuclear receptor superfamily. Some of these coregulators are also important for neuronal plasticity *per se* and they may form a substrate for the modifying effects of MR and GR on neuronal plasticity.

The recruitment of coregulators by nuclear receptors may take place in a cell-type- and promoter-specific manner (55). These interactions can regulate the stability of the transcriptional machinery, lead to recruitment of additional transcription factors and transcriptional coactivators or corepressors, and acetylate or deacetylate DNA histones either by intrinsic histone (de)acetylase activity or by recruitment of histone (de)acetylases. Histone acetyltransferases (HATs) are proteins that can catalyze the addition of an acetyl group to Lysine residues of histones. Histone acetylation may promote gene transcription via chromatin availability and binding of transcription factors (56). This model indicates that coregulators do not act in isolation but in protein complexes that may involve transcription factors, coregulator -coregulator interactions and RNA molecules (57).

Steroid receptors can recruit coregulators via their AF-1 and via their AF-2 domain. Because of their high LBD sequence similarity MR and GR share many of their AF-2 interacting coregulators (which incidentally receive more attention, based on experimental advantages in studying the ligand dependent AF-2, rather than the AF-1 which is ligand independent when studied in isolation). However, a number of MR-specific coregulators have been reported, such as Eleven-nineteen Lysine-rich Leukemia (ELL) and RNA helicase A (RHA) (58, 59).

AF-2-coregulator interactions are based on the presence of so-called NR-boxes in the coregulator protein: amino acid motifs that have an LxxLL sequence at their core. Agonist binding to the receptor causes a conformation shift that allows interactions with these NR-boxes (60). Coregulators may have several NR-boxes, which may lead to interaction with multiple nuclear receptors that have different affinities for each NR box. The total number of (AF-1 and AF-2) nuclear receptor coregulators is now over 300 (61). One may (crudely) estimate that 10 or 20 percent of these may be relevant for MR and/or GR dependent transcription, based on screenings for AF-2 interacting coregulators and the predicted higher selectivity of the AF-1 coregulators reported in literature.

Nuclear receptor-coregulator interactions depend on the amino-acid sequence of their nuclear receptor-interaction domain, as well as the presence and activation status (*i.e.* conformation) of other co-expressed steroid receptors and the overall availability of coregulators (62). Interestingly, the coregulator repertoire may allow opposite transcriptional effects of glucocorticoids on the same gene promoter in different cell types (63). Moreover, increasing coactivator availability can reverse the transcriptional repression of one steroid receptor by another (57, 64). Finally, in some cases concomitant ligand dependent degradation of nuclear receptors and coregulators by the proteasome is important for their transcriptional activity (65). This may restrict the availability of coregulators to other nuclear receptors, hence, focusing cellular function to specific pathways. Thus, coregulators form a major factor in glucocorticoid responsiveness that is, however, far from completely understood.

Several coregulators are abundantly expressed in the brain, showing wide distribution (66, 67). These include members of the best studied classes of coregulators, the p160 Steroid Receptor Coactivator (SRC) family member SRC-1 (68, 69), CBP/p300 (70, 71) and corepressors SMRT and NcoR (66). Others, such as SRC-3 (also a p160 family member), seem to be expressed mainly in the hippocampus (68, 72). These coregulators often colocalize in cells in relevant brain regions with steroid receptors, presumably able to modulate steroid sensitivity, underlining their importance for normal steroid receptor functionality (73-75).

Given the importance of coregulators in setting steroid sensitivity, a number of laboratories have studied regulation of coregulator expression in the brain. Factors that alter expression of particular coregulators in the brain include sex (76, 77) and age (78, 79), while the regulation of SRC-1, NcoR and SMRT by thyroid hormone and estrogen has been reported (66). Treatment with testosterone, restraint stress, the time of the day and photoperiod may also influence the expression of coregulators (75, 80, 81), as well as elevation of glucocorticoids

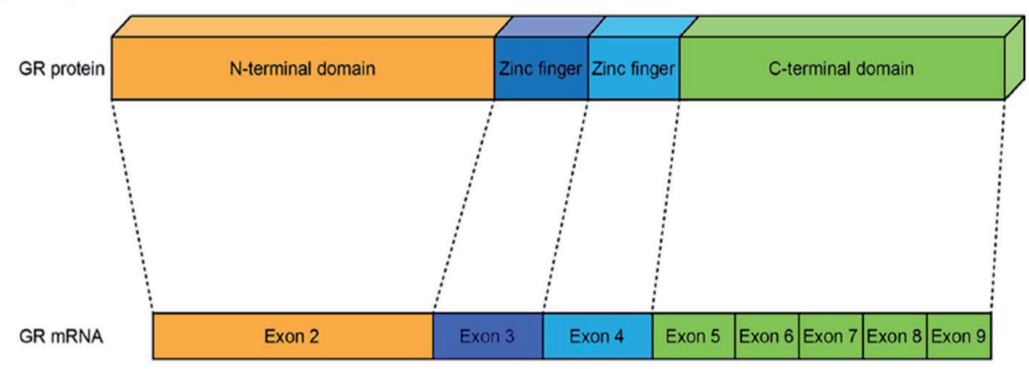

Figure 3. Relation between GR mRNA and protein. The 8 coding exons of the *GR* gene and the protein domains they code for. Exon 2 codes for the N-terminal domain of the protein which contains the major transcriptional activation domain τ1. Exons 3 and 4 code for two zinc-finger domains that are involved in DNA-binding and homodimerization. Finally exons 5–9 code for the C-terminal end of the protein which contains the domains for transcriptional activation and ligand binding.

(82). However, the majority of these studies investigated the expression of p160 family members, and these studies certainly do not keep pace with the speed at which new coregulators have been discovered. All in all, there seems to be little compelling evidence to suggest that regulation of coregulator expression in the adult brain is a major regulatory event. It rather has been argued that post-translational modifications of coregulators could have a major impact on their function (83).

SRC-1

SRC-1 was one of the first coregulators to be discovered (64). It can interact with ligand-bound steroid receptors, including GR, MR (82), estrogen (ER) and progesterone (PR) receptors. It can recruit other coregulators such as CBP/p-300 (84) and possesses HAT activity (85). It shows wide expression and distribution in the brain and is transcribed from by the *NCoA-1* gene which codes for two different splice variants (SRC-1a and SRC-1e). The SRC-1e mRNA contains an additional exon with an earlier stop codon than SRC-1a (86). Therefore, SRC-1e protein is shorter despite the longer SRC-1e mRNA. At the protein level, SRC-1a contains four Nuclear Receptor interaction domains (LLXLL motifs or NR boxes) while SRC-1e contains three. Interestingly, the C-terminal SRC-1a-specific NR box is the one that has the highest affinity for GR compared with the central ones (87). The splice variants show differential distribution in the brain (69), and in cell lines they have differential effects on transcription via MR, GR and ER (88, 89). Regarding adaptation to stress, SRC-1a and 1e have opposite activities in relation to the potentiation of GR repression of the *crh* promoter by glucocorticoids (63).

In vivo, SRC-1 is necessary for GR-dependent gene regulation in the core of the brain stress system. Knockout mice show strong GR resistance for the downregulation of both CRH mRNA in the hypothalamus, and POMC mRNA in the anterior pituitary (28, 90). Despite this rather dramatic transcriptional phenotype, the activity of the HPA-axis is almost normal in these mice, even if they tend to have slightly higher stress-induced corticosterone secretion. Interestingly, SRC-1 is also involved in CRH expression in the central nucleus of the amygdala. Not only do SRC-1 knockout mice lack the *up*regulation of CRH mRNA in response to glucocorticoids, they also show lower basal CRH expression in the central amygdala than wild type littermates, suggestive of GR-independent effects of this coregulator (28). Conversely, the majority of GR target genes are normally expressed and regulated in SRC-1 knockouts. It is still unknown to which extent SRC-1 can influence learning and memory and stress reactions as a coregulator of GR. Overexpression of SRC-2 in the lack of SRC-1 may be responsible for the lack of behavioral differences between SRC-1 KO and wild type animals (91, 92). A more general role of SRC-1 in neuronal function is suggested by a delayed development of purkinje cells in the cerebellum of SRC-1 knockout mice, but the nuclear receptor that is linked to this phenotype is unknown (72).

SRC-1 has an important role in sexual behavior and differentiation, as indicated by its expression in brain areas relevant for sexual function, coexpression and interactions with ER and PR in the brain (73, 93, 94) and the effects of their blockade in such functions. For

example, depletion of SRC-1 with oligodeoxynucleotide treatment leads to disruption of estrogen- and progesterone-induced sexual behavior in female rats (91). Similarly, inhibition of SRC-1 expression by repeated administration of locked nucleic antisense oligonucleotides targeting SRC-1 in the hypothalamus-preoptic area of male Japanese quail leads to reduction of testosterone-dependent sexual behavior (95). Moreover, antisense oligonucleotide targeting of SRC-1 in the hypothalamus could increase lordosis behavior in androgenized female and male rats (96). These results underscore involvement of SRC-1 in the signaling of multiple nuclear receptor types in the brain.

SGRMs

Particular neuromodulatory effects that are mediated by NRs such as MR and GR depend on specific interactions with downstream proteins. This offers a new level of pharmacological modulation of NR function beyond the classical agonists or antagonists as it is possible to selectively activate or block particular NR-coregulator interactions, while leaving processes that depend on other coregulators unaffected. This principle of selective hormone receptor modulators (SHRMs), may lead to the development of ligands that can exert the desired experimental or clinical effects, with a minimum of undesired side effects.

The most prominent type of selective modulation for glucocorticoid signalling has been GR ligands that have anti-inflammatory efficacy, but limited effects on metabolism or osteoporosis (97, 98). However, also in relation to the brain, it may be beneficial to distinguish between different effects of glucocorticoids. For example, blocking detrimental effects of chronically elevated glucocorticoid exposure with full antagonists will lead to disinhibition of the HPA axis and in this way will counteract efficient antagonism. It is also unlikely that blocking all effects of GR on emotional and cognitive processes will be the optimal way to counteract negative effects of stress. Lastly, induction in the brain of a pro-inflammatory state by pharmacological blockade of GR in astrocytes and/or microglia may not be desirable (99). Selective GR (or in fact: MR) modulators may therefore also be beneficial in stress-related psychopathology. They most certainly will be useful to dissect the molecular mechanisms of glucocorticoid action in experimental settings.

Originally, it has been tried to base selective GR modulation on the dissociation of effects that depend on DNA binding by the receptor, and classical transrepressive effects directly on pro-inflammatory transcription factors NF-kB and AP-1 (100). The GR ligand ‘Compound A’ is an example of this mechanism, as it induces inhibition of NF-κB-dependent pro-inflammatory transcription, but is unable to induce DNA binding of GR (101, 102). However, part of the anti -inflammatory effects mediated by GR do depend on binding by GR to classical GREs (103). Coghlan et al. (104) showed a GR ligand that retained anti-inflammatory effects while preventing the GR effects on glucose metabolism and impact on bones, and demonstrated that the specificity of the compound resulted from the specific GR-coregulator interactions. An arylpyrazole-type of GR ligand was reported to have selective agonism with respect to induction of decreased hippocampal neurogenesis without affecting skeletal muscle protein synthesis, bone or skin collagen synthesis or splenic lymphocyte counts (105). This particular

"ligand 5" was shown to have transcriptional effects on only a small number of target genes in cell lines (106). Although its mechanism of action is unknown, 'ligand 5' proves the point that GR effects relevant for modulation of brain may be quite selectively targeted with selective modulator types of drugs.

Selective receptor modulators for MR have not been studied much, as full MR antagonism has been a major clinical goal in cardiovascular disease. However, MR agonism in the brain may be of benefit in relation to particular psychiatric disorders, such as depression (107), where its expression has been shown to be decreased in several brain areas (108). The development of selective MR modulators is currently taking place and it will be exciting to see what the potential of such ligands will be (109).

Antisense oligonucleotides

Antisense oligonucleotides (AONs) (Figure 4) are small pieces of modified RNA or DNA that can hybridize to RNA. In this manner they can generate different effects depending on the AON chemistry and target site (see Figure 5). Initially, AONs were used to induce gene knockdown (110). This can be achieved through RNase H, an ubiquitous enzyme that cleaves RNA:RNA or RNA:DNA hybrids (Figure 5a). The AONs used for this application are generally modified with a phosphorothioate backbone, which increases AON stability and enhances uptake of the AON over cell membranes. Gene knockdown can also be achieved using AONs targeting the translation start site (translation block, Figure 5b). Here, AONs can be modified further to render them RNase H resistant by addition of a methyl or methoxy-ethyl group to the 2'O sugar ribose, which is the target cleavage site of the RNase H enzyme. Alternatively, nucleotides have been modified even further, e.g. using phosphorodiamidate morpholino oligomers (PMOs), peptide nucleic acids or locked nucleic acids. PMOs have been used for developmental studies in zebrafish embryos (111, 112). Multiple RNase H dependent AONs are in clinical trials including one against high-grade glioma in phase IIb (commercial name: trabedersen) (113), and one has even been registered as a drug for cytomegalovirus induced retinitis (commercial name: vitravene) (114).

However, with the availability of shRNA and siRNA, which generally gives a more robust gene knockdown (or complete knockout when cre-recombinase systems are used), the use of AONs is often not the method of first choice to achieve knockdown (in spite of advantages related to cellular uptake - see below). Meanwhile, other AON applications that use different mechanisms of action are gaining more interest. The best-known application is the manipulation of splicing. Using AONs that target splice sites or exonic/intronic inclusion signals located within exons or introns, exons can be hidden from the splicing machinery, resulting in the skipping of the target exon (Figure 5c). This can have multiple applications, e.g. switching from one isoform to another, skipping an aberrantly introduced exon to restore the normal transcript, or introducing an out-of-frame deletion to knock down expression of a gene. The latter approach may also be considered as a complementary method to AON-induced knockout through RNAse H dependent cleavage of RNA:DNA hybrids (115). Exon skipping resulting in the expression of truncated, non-functional proteins may be of particular

interest in relation with genes or gene pathways which are considered “undrugable”. Since specific ligands or antagonists cannot always target molecules of interest, AON-mediated RNA targeting can be a good alternative to achieve partial and/or reversible knockdown of such proteins.

Finally, another application of exon skipping is to reframe transcripts allowing the production of an internally deleted, partially functional protein rather than a prematurely truncated non-functional protein (Figure 5c). This has been extensively studied as a therapeutic approach for Duchenne Muscular Dystrophy (DMD). Protein restoration has been shown in patient-derived cell cultures and in animal models this led to a rescued phenotype (116-118). After encouraging results in phase I and I/II clinical trials (119-123), this approach is currently tested in phase III clinical trials. As will be detailed below, this strategy to generate deletion variants of proteins bears much promise for experimental neuroscience too. In other cases, intron splicing silencers may be targeted, resulting in exon inclusion and therefore increase of the expression of a gene or isoform. Here, the most prominent application is rescue of spinal muscular atrophy by AON mediated stimulation of the expression of a functional homologue (see below) (124-127).

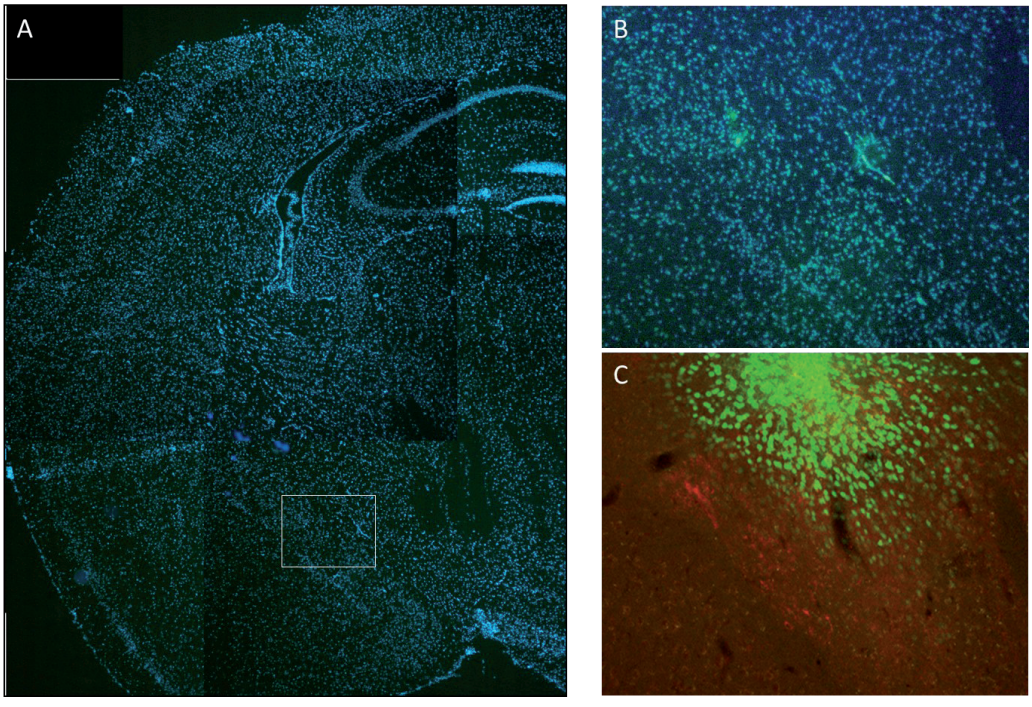

Figure 4. A. Fluorescent image of a mouse brain section stained with hoechst (blue) (10X magnification). The white box indicates the location of the central amygdala. B. The white box from picture A in magnification. AONs (green) are colocalized with hoechst in the cellular nuclei (20X magnification). C. Colocalization of AONs (green) and CRH expression (red) in the CeA (20X magnification).

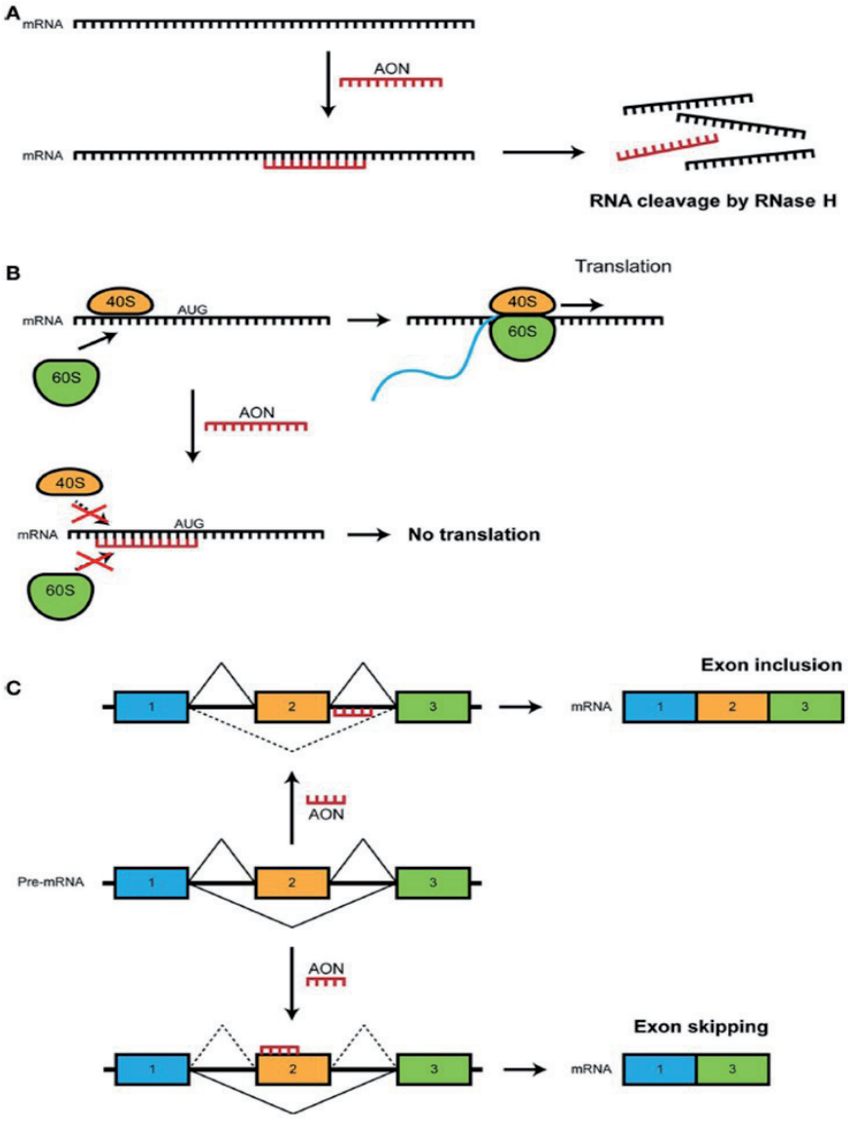

Figure 5. Schematic representation of different modes of action of antisense oligonucleotides. A. RNase H- dependent pathway. Binding of antisense oligodeoxyribonucleotides (AONs) with a phosphorothioate backbone results in a RNA:DNA hybrid, which activates Rnase H. RNaseH will cleave them RNA and prevents the translation in to a protein. B. RNase-independent translational block. 2' OH modified Rnase H-resistant oligomers targeting the translation start site prevent translation and elongation. AONs binding to the AUG initiation site or downstream prevents binding of the ribosomal units or results in steric blockage. C. Alternative splicing. 2' OH modified RNaseH-resistant or alternatively modified AONs complementary to the target pre-mRNA can result in: (1) inclusion of an exon by binding to the exonic splicing silencers (ESEs)or intronic splicing silencers (ISSs), (2) exclusion of an exon by binding to the 3 or 5 slice sites or exon-internal sequences, resulting in an in-frame transcript and translation of a shorter partly functional protein. Full lines indicate possible splicing events while dashed lines indicate non-possible events.

Specificity

A very important aspect of all splicing-modulation or gene-silencing operations is specificity to the selected target. siRNAs exert their actions in the cytoplasm via interactions with the RNA-induced silencing complex (RISC) in the cytoplasm (128). Off-target effects appear when siRNA strands interact with partially complementary regions of mRNAs other than the fully complementary target mRNAs (129-131). AONs development has faced the same issues in the past (132) and the solutions included modifications of the backbone to reduce base-pair affinity, thus reducing off-target effects (133, 134). Luckily, these modifications can be applied to siRNAs as well (132). A problem that might arise is cell death due to oversaturation of cellular RNA pathways by siRNAs (135) that are necessary for normal cellular function. However, this problem does not exist with AONs since they exert their activity in the nucleus without the need for anything equivalent to the RISC complex (136).

Cellular Delivery

In all instances of RNA or DNA interference in the brain, delivery is an issue. *In vivo* manipulation of gene expression with shRNA very often depends on the use of viral vectors (137-139), as do CRE-recombinase mediated gene excision (26) or gene overexpression models (140, 141). However, AONs after reaching the brain, are readily taken up by neurons, and are therefore independent of viral transduction of neurons (Figure 4b-c).

Delivery of viral vectors has been associated with various levels of toxicity in the brain, mainly depending on viral type used. For example, AAV vectors have been shown to induce neurotoxicity when delivered to the CNS (138, 142-144), although serotypes may differ in that aspect (145). Other viral types, such as retrovirus, show milder toxicity, but they are not suitable for investigation of long term effects and have limits in the cellular types they can infect (146). Lentivirus causes less inflammatory and immune responses, but it still shares the disadvantage that pre-existing immunity to the parental wild-type virus may cause an accentuated immune response. In contrast, for 2-O'-modified-phosphorothioate AONs only very mild toxicity has been reported, which did not interfere with their desirable effects (124, 147) after delivery in the brain via the ventricles, or in cultured neuronal cells (148). Although it has been shown that phosphorothioate AONs and siRNAs can have an immunostimulatory effect via toll-like receptors (TLRs) (149, 150), appropriate 2-O' modifications, such as 2-O'-methylation can suppress these effects (149, 151, 152). The toxic effects that have been reported in some studies after AON delivery in the brain may be due to the vehicle used (153). Results from our group showed no immune response to 2-O'-methyl-phosphorothioate AONs over saline treatment after a single local injection in the Central nucleus of the Amygdala (CeA) of the mouse brain (Chapter 2 of this thesis) (86).

Compared to viral delivery methods, AONs have a very rapid uptake and initiation of the effect (154, 155) (within minutes to hours), which allows for administration between different stages of the same experiment (155, 156). Secondly, AONs administration allows better dosage control that can give the optimal effect while reducing potential toxic effects due to

e.g. complete or too high levels of knockdown (116, 124, 136). In contrast, virally-mediated methods tend to produce an all-or-nothing effect, particularly when cre-recombinase systems are used (26, 157). Another characteristic of AON targeting is the possibility to discontinue treatment (136). Although AONs have a longer half-life than, for instance, siRNAs (136), eventually they are degraded allowing gene expression to return to basal levels. Viral vectors, however, have a virtually permanent action, although long term effects may depend on viral type (146). Obviously, in instances where long-term manipulation is the goal, a single treatment with a long term effect may be desirable (124). Finally other advantages include rapid production and lack of GMO safety related issues, since no genetically engineered viruses are involved and there is no risk of recombination or reversion to wild type virus (146, 158).

On the other hand, even when methods of virus-independent, direct delivery of siRNA are considered, for example based on conjugations (159) several other issues appear. These methods are characterized by various inherent challenges, such as high degradation rate of the siRNA, low cellular uptake and efficiency (160), and induction of interferon responses (135, 161, 162). In comparison, AONs have a lower turnover rate (136), more prolonged action (130) and, as they are single stranded rather than double stranded, better cellular uptake (Chapter 2).

In conclusion, AON treatments appear as an attractive approach not only in cases where they restore protein function (such as DMD) but in many other cases where modulation of gene expression is required. Moreover, they offer advantages over other approaches such as siRNA interference that may be very advantageous in certain contexts.

Brain Delivery of AONs

A major challenge of both AON and shRNA applications in neuroscience and in particular for possible clinical use in neurodegenerative disorders is the actual delivery to the brain. The blood brain barrier (BBB) is a physiological obstruction for molecules to enter the brain and molecules can only enter the brain interstitial fluid by transport through the brain capillary endothelial cells (163). Intravenous or intraperitoneal administration of phosphorothioate oligonucleotides in rodents showed a very low uptake in brain (164, 165). Increased brain uptake of AON after peripheral delivery can be achieved by increasing the permeability of the BBB (166) or through encapsulating the AON in liposomes conjugated to monoclonal antibodies (167, 168). Another way to solve this problem is by local injections in the desired brain region if spatial specificity is important or by injection in the cerebrospinal fluid if broad distribution in the brain is deemed more important.

Direct injection in specific brain regions is a method that has been widely used both in rodent studies and in human patients (169). Experimentally, they offer insight in local effects of widespread factors (170), and can have the advantage of contralateral controls in the same animal. Moreover, it provides the options of single injections or repeated/continuous delivery via cannulation. Importantly, it also offers the possibility of reducing the injected dose, thus,

decreasing potential toxic or immunogenic effects. In human patients intracranial delivery is used in the context of glioblastoma treatment with AONs (113).

The alternative of intraventricular (or intrathecal) delivery into the cerebrospinal fluid has also proven successful. Continuous infusion into the ventricle of rodent and nonhuman primate brains showed significant concentrations of AON throughout the brain, brain stem and spinal cord. Significant reduction of targeted mRNA indicated that the AON is readily taken up by cells (136). The advantage of ventricular infusion through a surgically implanted pump is that there is constant delivery where the dosage can be accurately regulated (171). Furthermore, the disadvantage of the AONs' restricted ability to cross the BBB also is a clear advantage, since after ventricular infusion the AONs will remain in the brain (124) thereby reducing side effects on peripheral organs like liver and kidney that readily take up AONs.

In conclusion, while AONs for use in the CNS cannot be administered systemically, they have excellent entry into cells once they passed the BBB. For several backbone chemistries, it has been shown that local injection and distribution via the CSF seem to be devoid of any major toxicity.

Knockdown

The most widely used application of AON-mediated RNA targeting in the CNS has been the downregulation of gene expression through intranuclear RNase H-mediated cleavage of DNA:RNA hybrids (110, 153) (Figure 4a). Thus, the AON in this case is targeted against an mRNA sequence of interest (153). This approach offers an alternative, with certain advantages, to knockdown induced by viral vectors and siRNAs which are mediated by the RISC complex. We present a few recent examples from which the advantages of 'classical' knockdown use of AONs is apparent.

Ma et al., (2011) used AONs to knock down BDNF expression in various brain areas and studied its involvement in conditioned taste aversion memory formation (154). They showed that BDNF synthesis in the CeA is necessary for the consolidation of long term memory formation of conditioned taste aversion. Likewise, AONs have been also used to knock down the expression of CRH in the CeA, temporally (155, 156). In a series of experiments targeting CRH mRNA it was shown that CRH plays an important role in contextual fear conditioning consolidation in the CeA (155). Furthermore, it was shown that CRH involvement in this context may be important up to 24 hours after training for successful consolidation of contextual fear (156). These studies illustrate the advantage of infusing AONs at different time points (154).

AON-mediated knockdown has been combined with other gene-silencing techniques to serve specific experimental purposes, or even to elucidate the mechanisms behind, for instance RNA interference. Hemmings-Mieszczak et al. (2003) used mixtures of siRNAs and AONs to achieve a higher degree of reduction of the expression of the pain receptor $P2X_3$, *in vitro*, and a more pronounced functional outcome. The effect was stronger when the siRNA and the AON targeted mRNA sequences distant from each other, because of steric hindrance masking their

complementary sequences (172).

AONs were recently used in an elegant way to inhibit the expression of proteins associated with the RISC complex. AON-mediated downregulation of Argonaute proteins Ago1 and Ago2, combined with modified cleavage deficient siRNAs, showed that off-target effects of siRNAs are independent from Ago2 cleavage, but they require interaction with Ago proteins and the RISC complex (130). A similar approach was used to investigate the involvement of the RISC complex in pre-rRNA processing. Targeting of Dicer, Drosha or Ago2 lead to impairments in pre-rRNA processing, suggesting a role of these proteins in the biogenesis of rRNA (173). The great advantage of AON-mediated knockdown here is that its action depends on an entirely different mechanism from siRNA allowing interference with one without affecting the other.

Thus, RNase H-mediated cleavage of DNA:RNA hybrids still is broadly used in basal and clinical research. In addition, exon skipping and inclusion offer a number of possibilities that are unique for AONs.

Aim of the thesis

Modulation of sensitivity to glucocorticoids may be of therapeutic interest for psychopathology. However, due to the pleiotropic effects of glucocorticoids, a global approach such as treatment with GR agonists or antagonists may have serious adverse effects. Here we attempted to regulate the sensitivity of discrete GR-dependent pathways to glucocorticoids, in relation to stress, using two different approaches: the first approach we used was the local modulation of splicing of SRC-1, a coregulator of the GR, in the CeA and the shift of the expression ratio towards the splice variant that represses the CRH promotor; the second approach used here, was the targeting of the GR with ligands that may act as selective modulators and have differential effects on specific GR-dependent pathways.

Outline of the thesis

In chapter 2 we investigated the cellular uptake, efficacy and adverse effects of treatment with AONs targeting the SRC-1e specific exon in the brain. In chapter 3 we studied the functional effects of a shift in the expression ratio of the two isoforms in favour of SRC-1a in the CeA. In chapter 4 we tested a novel GR ligand (C108297) with mixed agonist and antagonist properties on the regulation of crh expression and the HPA axis, regulation of gene expression in the hippocampus and fear memory consolidation. In chapter 5 we used a similar approach to test another novel GR ligand (C118335) with mainly agonist properties. In chapter 6 a synthesis of the concepts presented here is attempted.

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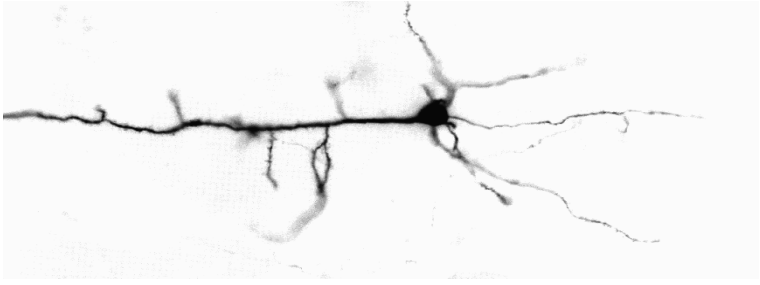
Chapter

Antisense-mediated isoform switching of Steroid Receptor Coactivator-1 in the central nucleus of the amygdala of the mouse brain

2



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Abstract

Antisense oligonucleotide (AON)-mediated exon skipping is a powerful tool to manipulate gene expression. In the present study we investigated the potential of exon skipping by local injection in a specific brain nucleus. For proof of principle of feasibility we studied uptake by different cell types, translocation to the nucleus and potential immunostimulatory effects at different time points after a local injection in the central nucleus of the amygdala (CeA) of the mouse brain of a control AON targeting human dystrophin with no targets in the murine brain. To evaluate efficacy we targeted the splicing of steroid receptor coactivator-1 (SRC-1), a protein involved in nuclear receptor function. This nuclear receptor coregulator exists in two splice variants (SRC-1a and SRC-1e) which display differential distribution and opposing activities in the brain, and whose mRNAs differ in a single SRC-1e specific exon.

We found that AONs were taken up by corticotropin releasing hormone expressing neurons and other cells in the CeA, and translocated into the cell nucleus. Immune responses after AON injection were comparable to those after sterile saline injection. A successful shift of the naturally occurring SRC-1a:SRC-1e expression ratio in favor of SRC-1a was observed, without changes in total SRC-1 expression.

We provide a proof of concept for local neuropharmacological use of exon skipping by manipulating the expression ratio of the two splice variants of SRC-1, which may be used to study nuclear receptor function in specific brain circuits. We established that exon skipping after local injection in the brain is a versatile and useful tool for the manipulation of splice variants for numerous genes that are relevant for brain function.

Introduction

Alternative splicing in the brain has gained significant attention recently and may be important for a vast number of processes [1] such as synaptic function [2] and learning and memory [1]. Examples of alternatively spliced genes include the D2 receptor gene [3], the corticotropin releasing hormone (CRH) receptor genes [4] and the cannabinoid receptor genes [5, 6]. A limitation to the study of the roles of the various splice variants in brain function is that very often specific ligands or inhibitors are lacking. Furthermore, transgenic approaches may be both costly and time consuming, and/or depend on viral delivery which may induce immune responses [7].

Single stranded DNA or RNA antisense oligonucleotides (AONs) that target RNA transcripts can be used to manipulate gene expression in different manners. DNA:RNA or RNA:RNA hybrids can be cleaved by RNase H resulting in knockdown of gene expression. A similar effect can be achieved via steric hindrance of the ribosomal complex by an AON resulting in mRNA translation arrest and blocking of protein expression [8]. A third mechanism involves the hybridization of an AON to intronic/exonic inclusion sequences of primary RNA transcripts, thus rendering specific exons inaccessible to the splicing machinery and leading to skipping of the exon [9]. In a similar fashion, AONs can hybridize to intronic/exonic exon exclusion sequences and result in inclusion of target exons [7, 10].

To date, modulation of splicing by AONs has been used as a potential treatment approach for several diseases, including Duchenne muscular dystrophy (DMD) and models of spinal muscular atrophy (SMA) [10-12]. Effective protein restoration in DMD via exon skipping has been shown in patient derived cell cultures, animal models, (reviewed in [9] and even in clinical trials [13, 14]. Similar results have been obtained in SMA via the related mechanism of exon inclusion [10, 15-17].

Despite the potential of splicing modulation, AONs have been used in an experimental setting mainly to induce knockdown of gene expression [18-20], while antisense-mediated modulation of splicing has not been used widely as a research tool in the brain. One of the obstacles preventing their more widespread application in the central nervous system (CNS) is their inability to cross the blood-brain-barrier of adult animals [21]. Nevertheless, when AONs are applied directly to the CNS via intracerebroventricular (ICV) or intrathecal administration, the results show considerable potential [10, 12, 21] and long-lasting effects [10, 21].

In this study we evaluated the efficacy and occurrence of immune-related side effects after a single local AON injection in the central amygdala of the mouse brain. As proof of principle, we targeted steroid receptor coactivator-1 (SRC-1), a gene that codes for two splice variants, SRC-1a and SRC-1e, which only differ in one exon (Figure 1; [22, 23]). SRC-1 can act as a coregulator of glucocorticoid receptor (GR) dependent transcription [24], as well as of other nuclear receptors [25]. The SRC-1 splice variants show differential activity and distribution in the brain [26]. The splice variants have been shown to exert opposite effects on the GR-mediated regulation of the *crh* gene [27].

We targeted exon 22 of the *SRC-1* gene (Figure 1) using AONs, examined their cellular uptake by different cell types, exon skipping efficacy over time and potential immunostimulatory effects. For cellular uptake and potential immunostimulatory effects we used an AON targeting human dystrophin that has no known targets in the murine genome, in order to investigate the target-independent physico-chemical properties of 2-O'-methyl modified phosphorothioate oligonucleotides. Our results showed adequate uptake by cells in the CeA and translocation into the cell nucleus, combined with detectable isoform switching until at least 7 days after a single injection and a practically complete lack of immunostimulatory effects compared with vehicle injection.

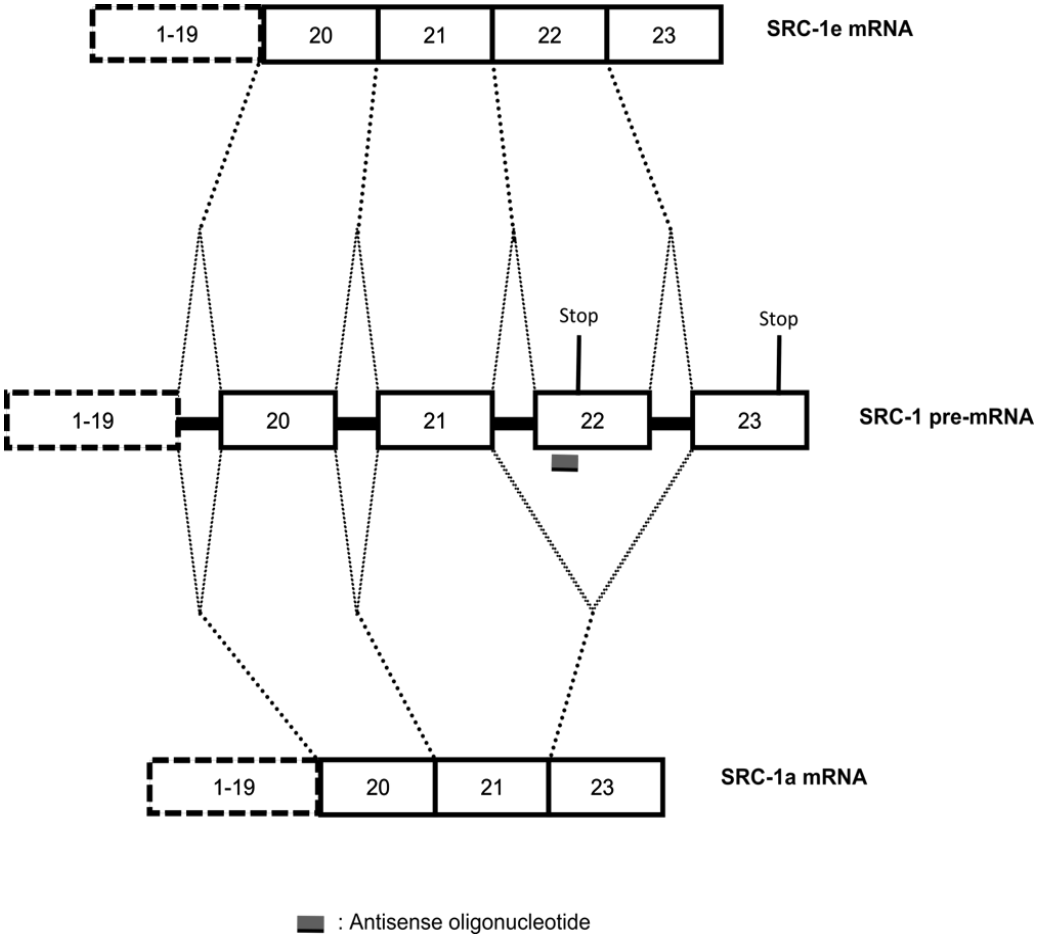

Figure 1. Schematic representation of the mRNA of the two SRC-1 splice variants. Boxes represent exons and thicker full lines represent introns. Dashed lines indicate possible splicing events. The approximate position of stop codons is also marked. If exon 22 is included, SRC-1e is expressed. Exon 22 contains an earlier stop codon, therefore SRC-1e protein is shorter than SRC-1a. AONs targeting exon 22 can render it inaccessible to the splicing machinery and therefore, shift the expression of SRC-1 towards SRC-1a. (Adapted from Kalkhoven et al., 1998).

Methods

Animals, stereotactic surgery and tissue processing: C57bl/6j male mice between the ages of 12-14 weeks (Janvier SAS, France) were used for all experiments. Animals were singly housed in individually ventilated cages at a 12 hour light cycle with lights on at 7 am. Food and water were available *ad libitum*. 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: 10128). Animals were anesthetized with a cocktail of Hypnorm-Dormicum-demineralized water in a volume ratio of 1.33:1:3. The depth of anesthesia was always confirmed by examining the paw and tail reflexes. When mice were deeply anesthetized they were mounted on a Kopf stereotact (David Kopf instruments, Tujunga, CA, USA). For every experiment, animals were bilaterally injected with 0.5 µl of the appropriate solution (sterile saline, AONs at a concentration of 400 pmol/µl in sterile saline (Eurogentec, Liège, Belgium)) in the central amygdala (coordinates relative to bregma: -1.25 mm anterior-posterior, ±2.95 mm medio-lateral and -4.75 mm dorso-ventral) [50]. For injections, customized borosilicate glass micro-capillary tips of approximately 100 µm in diameter, connected to a Hamilton needle (5 µl, 30 gauge) were used. The Hamilton syringe was connected to an injection pump (Harvard apparatus, Holliston, MA, USA) which controlled the injection rate set at 0.15 µl/ min. After surgery the animals were returned to the home cage and remained undisturbed until sacrifice, with the exception of daily weighing in order to monitor their recovery from surgery. To assess mRNA expression, animals were decapitated after an intraperitoneal injection (i.p.) of overdose Euthasol (ASTfarma, Oudewater, the Netherlands), brains were removed quickly and snap frozen on dry ice. For detection of the presence of AONs over time and putative immunostimulatory effects, mice were sacrificed with transcardial perfusion with a solution of 4% paraformaldehyde (PFA) (Sigma-Aldrich, Zwijndrecht, the Netherlands) in 0.1 M phosphate buffer saline (PBS) after an i.p. injection of overdose Euthasol, 1, 3, 5, 7 and 14 days after the injection. Each time point contained 3-7 animals. In order to assess potential immunostimulatory effects we used animals injected with 0.5 µl sterile saline (vehicle) that were sacrificed either 3 or 7 days (4 per group) after the injection as controls for the respective time points. Three additional animals were sacrificed without having been operated on. After sacrificing the animals, the brains were removed and postfixated overnight in 4% PFA at 4^{o}C. Subsequently they were cryoprotected in 15% and 30% sucrose, snap frozen on dry ice and stored at -80^{o}C.

Antisense oligonucleotides: Two different green fluorophore labeled AONs were used: one targeting human dystrophin, which has no known targets in the mouse (CGCCGCCAUUUCUCAACAG), labeled with a fluorescein amidite (FAM) fluorophore and one targeting exon 22 of SRC-1, that is specific for the SRC-1e splice variant (CUGUAGUCACCACAGAGAAG), labeled with Alexa Fluor® 488 . The AON against exon 22 of SRC-1e was administered in order to investigate whether it can induce exon skipping, whereas the AON against human dystrophin was used as control to study cellular uptake and

potential immunostimulatory effects. AONs were modified with a full-length phosphorothioate backbone which increases AON stability and cellular uptake and consisted of 2-O'-methyl RNA to render them RNase H resistant and to counterbalance potential immunostimulatory effects caused by the phosphorothioate modified backbone [34, 35, 51, 52].

Immunofluorescence: Brains were sectioned at a thickness of 25 μm on a Leica cryostat and sections stored in antifreeze solution [30% ethylene glycol (Merck, Darmstadt, Germany), 20% glycerol (Sigma-Aldrich), 0.02 M Na_2HPO_4 (Merck), 6.6 mM NaH_2PO_4 (merck)] at -20°C until use. Before use sections were washed in PBS to remove anti-freeze. Subsequently sections were incubated in 0.5% triton X-100 (Sigma-Aldrich) in PBS for 30 min to increase permeability of the cells and washed with PBS. Blocking with 2% normal donkey serum (Brunschwig Chemie, Amsterdam, the Netherlands) in PBS-B^{TSA} for 45 min was followed by an overnight incubation with primary antibody at room temperature (table 2). Afterwards, the primary antibody was washed out with PBS and incubation with the secondary antibody (table 2) followed for 2.5-3 h. The secondary antibody was washed followed by 10 min incubation with Hoechst (1:10000) (Hoechst 33258, pentahydrate, bis-benzymide, Invitrogen, Breda, the Netherlands) and another PBS washing step. Finally, the sections were mounted on glass slides, dried and coverslipped with Aqua Polymount (Polysciences Inc, Eppelheim, Germany). Slides were stored at 4°C until observation.

Microscopy: Confocal imaging was performed on a Nikon Eclipse TE 200-E microscope. Confocal images were collected as z-stacks at a magnification of 200 or 600 times with a z

Table 1. Antibodies and dilutions used for all immunofluorescent stainings. The fluorophore of all secondary antibodies was Alexa Fluor® 594

	Primary antibodies			**Secondary antibodies**		
Marker	Type	Manufacturer	Dilution	Type	Manufacturer	Dilution
CRH	Goat poly-clonal	Santa Cruz biotech-nology, Heidelberg, Germany	1:250	Donkey anti-goat	Invitrogen, Breda, the Netherlands	1:100
NeuN	Mouse mono-clonal	Chemicon, Amster-dam, the Netherlands	1:200	Donkey anti-mouse	Invitrogen, Breda, the Netherlands	1:500
GFAP	Mouse mono-clonal	Santa Cruz biotech-nology, Heidelberg, Germany	1:1000	Donkey anti-mouse	Invitrogen, Breda, the Netherlands	1:500
IBA-1	Goat mono-clonal	Santa Cruz biotech-nology, Heidelberg, Germany	1:200	Donkey anti-goat	Invitrogen, Breda, the Netherlands	1:400
CD-45	Rat poly-clonal	Serotec, Düsseldorf, Germany	1:1000	Donkey anti-rat	Invitrogen, Breda, the Netherlands	1:500

step size of 0.5 μm and an image size of 1024×1024 pixels. When two or more markers were determined in a single section, the different channels were imaged separately to avoid artifacts due to overlap of the emission wavelengths of the fluorescent labels. The same settings were used to obtain images for quantification (e.g. at different time points, between subjects or between groups for the same marker). Z-stacks were converted to .avi format and then stored as single image .tiff files using the z-projection function of Image J (NIH, Bethesda, MD) with standard deviation as projection type. Images of damaged sections or images with artifacts were excluded from further analysis. Finally, to examine cellular uptake and colocalization of different markers we merged different channels of the same image in Image J.

Image processing: Appropriate thresholds were applied to correct for background. For each marker the positive stained area was presented as a percentage of the total area of the visual field. In order to reduce measurement bias, holes or ruptures in the tissue were not taken into account for the calculation of total area. For determination of immune responses 3-4 pictures were used per brain and the mean value of those was used as the sample value.

Diffusion of the AONs: For determination of the diffusion of the AONs in brain we measured the diffusion of the green fluorescence in the medio-lateral axis and the dorso-ventral axis in Image J on 4 or 5 10 μm-thick sections per brain (n=6) which were taken 80 μm apart from each other. Images were taken on a Nikon eclipse 6800 fluorescent microscope at 100X magnification. Before measurements, appropriate background correction was applied. Lines were drawn along the medio-lateral and dorso-ventral axes and their length was measured in pixels. With help of a calibration slide we converted the values from pixels to μm. The positive area for green fluorescence was also measured and total positive volume was calculated according to Cavalieri's rule. Mean and maximum diffusion distances were calculated as well as total volume per sample.

Laser microdissection and RNA processing: Cryosections at a thickness of 10 μm were taken from snap frozen brains and mounted on polyethylene naphthalate membrane slides (Carl Zeiss, Munich, Germany). Up to 5 sections were mounted on a slide with adjacent sections being on different slides. The slides were stored at -80^{o}C until laser microdissection. Laser microdissection was carried out on a Palm laser microdissection microscope as has been described elsewhere [53, 54]. Briefly, sections were observed under fluorescent light in order to determine regions that had taken up AONs. With the assistance of appropriate software the desired regions were selected, microdissected and collected in adhesive caps (Carl Zeiss). Collected tissue was then stored in Trizol (Invitrogen) at 4^{o}C until RNA isolation, which was always carried out the same day as laser microdissection in order to preserve RNA quality. RNA isolation was performed as has been described elsewhere [55]. Briefly, RNA was isolated with chloroform and precipitated with isopropanol and linear acrylamide. RNA pellets were rinsed with ice cold ethanol 75%, air-dried and resuspended with RNase-free DEPC-treated demineralized water. Quality and concentration of RNA samples were measured on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) using the RNA 600 Pico LabChip according to the manufacturer's instructions.

cDNA synthesis: RNA samples were first treated with DNAse I (Invitrogen) to remove

potential genomic DNA contamination. Subsequently, RNA samples were reverse transcribed with iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Briefly, 4 µl of 5 times iScript reaction mix, 1 µl of iScript reverse transcriptase and 5 µl of Nuclease-free H_2O were added to 10 µl of DNase I treated RNA. Subsequently samples were incubated for 5 min at 25°C followed by 30 min at 40°C and finally 5 min at 85°C in a PTC-200 DNA engine cycler (Bio-Rad).

qPCR: 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 Master^{PLUS} 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 and GR was normalized against β-actin. Quantification of relative expression was calculated using the Pfaffl method [56] and normalized against the control group (dystrophin AON). The forward and reverse primers used for the different genes were respectively: 5'-CCTCTACTGCAACCAGCTCTCGTC-3' and 5'-TGCTGCACCTGCTGGTTTCCAT-3' for SRC-1a; 5'-TGCAACCAGCTCTCGTCCACTG-3' and 5'-GCTCCTCTAGTCTGTAGTCACCACA-3' for SRC-1e; 5'-CGACCGCAGAGCAGCAGTTA-3' and 5'-GCCGCTCAGTCAGAGAGCTG-3' for total SRC-1; 5'-CCCTCCCATCTAACCATCCT-3' and 5'-ACATAAGCGCCACCTTTCTG-3' for GR; 5'-TTTCCCACAGCAGTACGCAT-3'and 5'-TAATTTGGCCGCTGTCCCAT-3' for SRC-2 5'-CAACGAGCGGTTCCGATG-3' and 5'-GCCACAGGATTCCATACCCA-3' for β-actin.

Statistical analysis: For comparisons between two groups an independent t-test was used. For comparisons among multiple groups one-way ANOVA was used followed by Tukey's post-hoc test (for comparison of immunostimulatory effects between all groups) or Dunnett's post-hoc test (for comparison of fluorescence intensity at different time points with fluorescence intensity after one day post-injection). All data are presented as mean ± SEM.

Results

Cellular uptake: In order to investigate the cell types and intracellular destination of 2-O'-Methyl phosphorothioate AONs we performed immunofluorescent detection of CRH (which is expressed in the CeA), NeuN and Hoechst after local injection of an AON targeting human dystrophin, which has no known targets in the mouse. Our results showed that fluorescently labeled AONs were taken up by neurons in general, as well as neurons expressing CRH in the CeA, and also translocated into the cell nucleus (Figure 2A-D). Quantification showed that

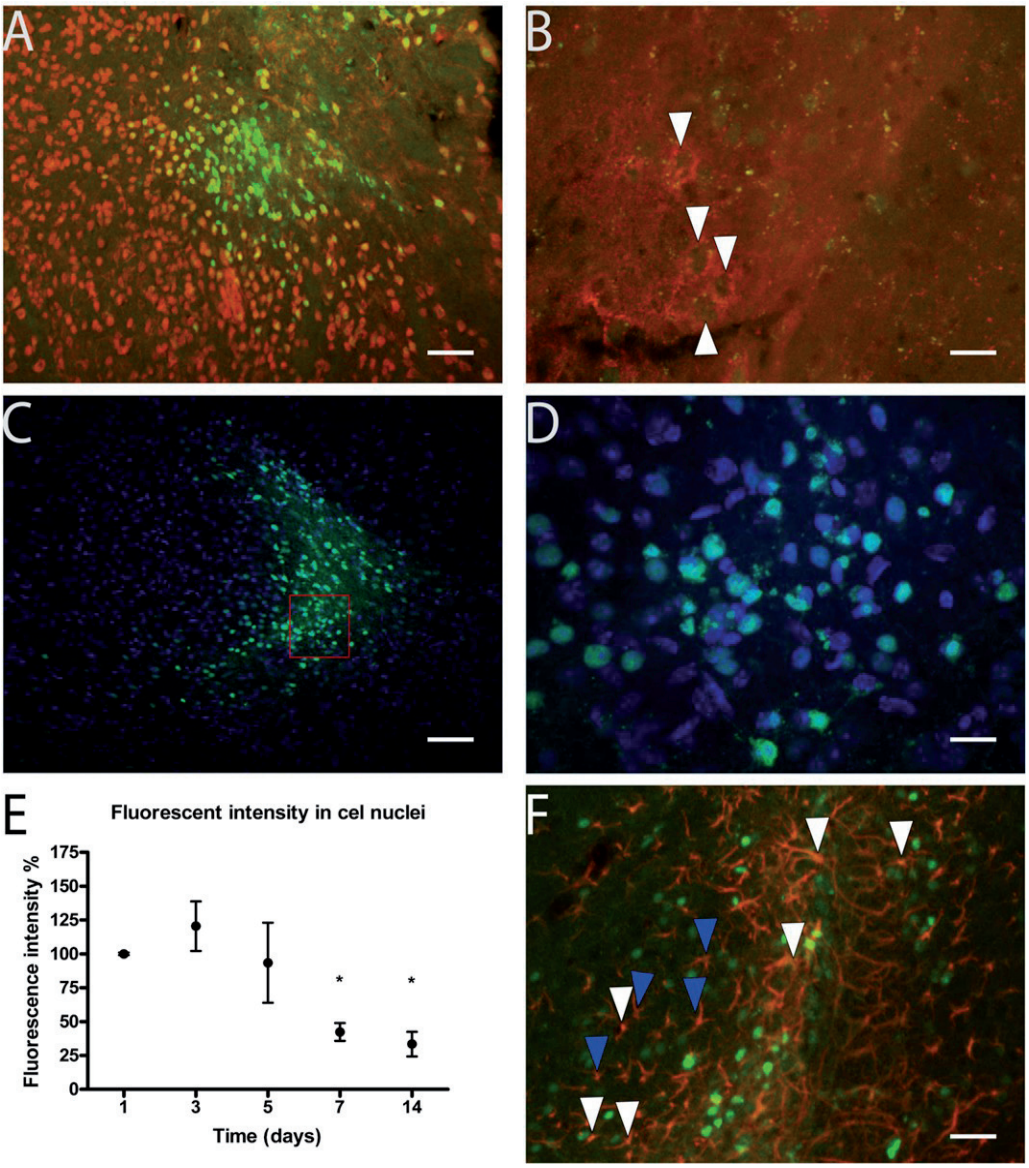

Figure 2. AON uptake by different cell types and nuclear localization in the central amygdala (CeA). A. Uptake of AONs by neurons. The green fluorescence of the labeled AONs is colocalized with NeuN (red), a marker of neurons. Scale bar 50 µm B. Uptake of AONs by cells expressing CRH. AONs (green) are located in the nuclei of those cells (white arrowheads), surrounded by CRH in the cytoplasm (red). Scale bar 15 µm. C, D. Localization of AONs (green) in the cell nucleus, colocalized with the nuclear marker Hoechst (blue). Scale bar 50 µm. The area within the red square is magnified in D (scale bar 15 µm). E. Fluorescent intensity in the cell nuclei after an injection of AONs. Fluorescence on day 1 was normalized to 100%. One-way ANOVA ($F_{(4,14)}$= 7.845, $p<0.01$) followed by Dunnett's post hoc test (all groups compared to the 1 day group) showed a significant decrease of fluorescence after 7 and 14 days (Dunnett's test $p<0.05$ in both cases). Between the 7- and 14-day groups there was no further decrease. F. Uptake of AONs (green) by GFAP (red) positive astrocytes. Several astrocytes have taken up AONs while others have not. White arrowheads indicate a few examples of astrocytes that took up AONs whereas blue arrowheads indicate a few examples of those that did not. Scale bar 30 µm.

67.5% ± 2.6 of the cells that had taken up the AONs was NeuN positive. This indicates that the AONs can indeed be taken up by neurons in the brain and translocate to the nucleus where splicing events take place.

AON detection: In order to determine the stability of AONs in the brain after local injection we measured the intensity of the green fluorescence originating from the fluorophore conjugated to AONs in the brains of animals sacrificed 1, 3, 5, 7 and 14 days after a single injection with an AON targeting human dystrophin. Fluorescence intensity did not differ significantly between 1 and 3 days but subsequently decreased over time to less than 50% in 7 days (Figure 2E). After 7 days, fluorescence intensity remained stable until the last detection time point, 14 days post-injection. In these calculations only green signal colocalized with Hoechst (cell nuclei) was taken into account, thus restricting our analysis to a functionally relevant subcellular compartment.

Diffusion of AONs: In order to investigate the specific targeting of a selected brain region we measured the diffusion of the AONs around the injection site. Our results indicated a well localized targeting of about 0.1 mm^3 (table 2).

Table 2. Diffusion of the AONs in the brain in the mediolateral, dorsoventral and anterior-posterior axes. Lengths in μm, volumes in mm^3. Data shown as mean ± SEM

Measurement	Size	SEM
Mean mediolateral diffusion	505	70
Mean dorsoventral diffusion	671	84
Mean anterior-posterior diffusion	350	34
Maximum mediolateral diffusion	903	-
Maximum dorsoventral diffusion	1015	-
Mean Volume	0.11	0.03

Immunostimulatory effects: We analyzed two different markers for microglia activation (CD -45 and IBA1) and one marker for astrocytes (GFAP). CD-45 is a marker of activated microglia, whereas IBA-1 is a constitutive marker of microglia [28, 29]. We compared AON-injected (with an AON targeting human dystrophin) to saline-injected animals 3 and 7 days after the injections. Moreover, we included an untreated group of animals to assess the effects of the injections. No differences were observed between saline and AON treated animals at either time point (Figure 3-4). AON uptake was also observed in a subset of GFAP positive astrocytes (Figure 2F). Little or no uptake by microglia was observed.

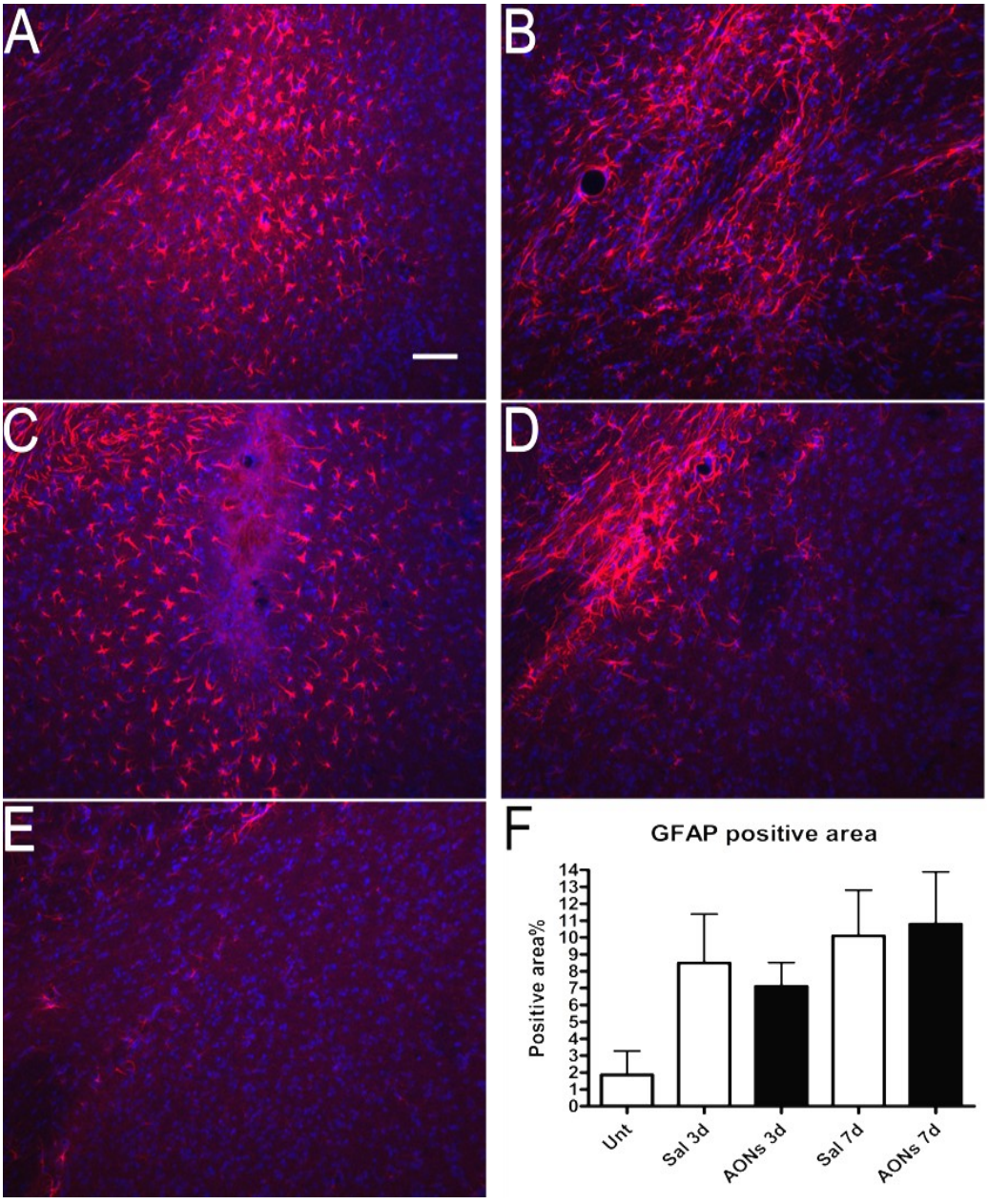

Figure 3. GFAP immunoreactivity 3 or 7 days after a single injection in the CeA. A. 3 days after a single saline injection. B. 7 days after a single saline injection. C. 3 days after a single injection of AONs. D. 7 days after a single injection of AONs. E. GFAP immunoreactivity in the CeA of an untreated mouse. F. Quantification of GFAP immunoreactive area shown as percentage of the total area of visual field. One-way ANOVA followed by Tukey's post hoc test revealed no significant differences between the respective AON and saline injected animals (one-way ANOVA $F_{(4,17)}$=1.266, p>0.32, N=3-7 animals per group). In conclusion, a single AON injection did not induce stronger astrocytosis than saline. Scale bar 50 μm. Red: GFAP, blue: Hoechst. Green signal (AONs) has been omitted for clarity.

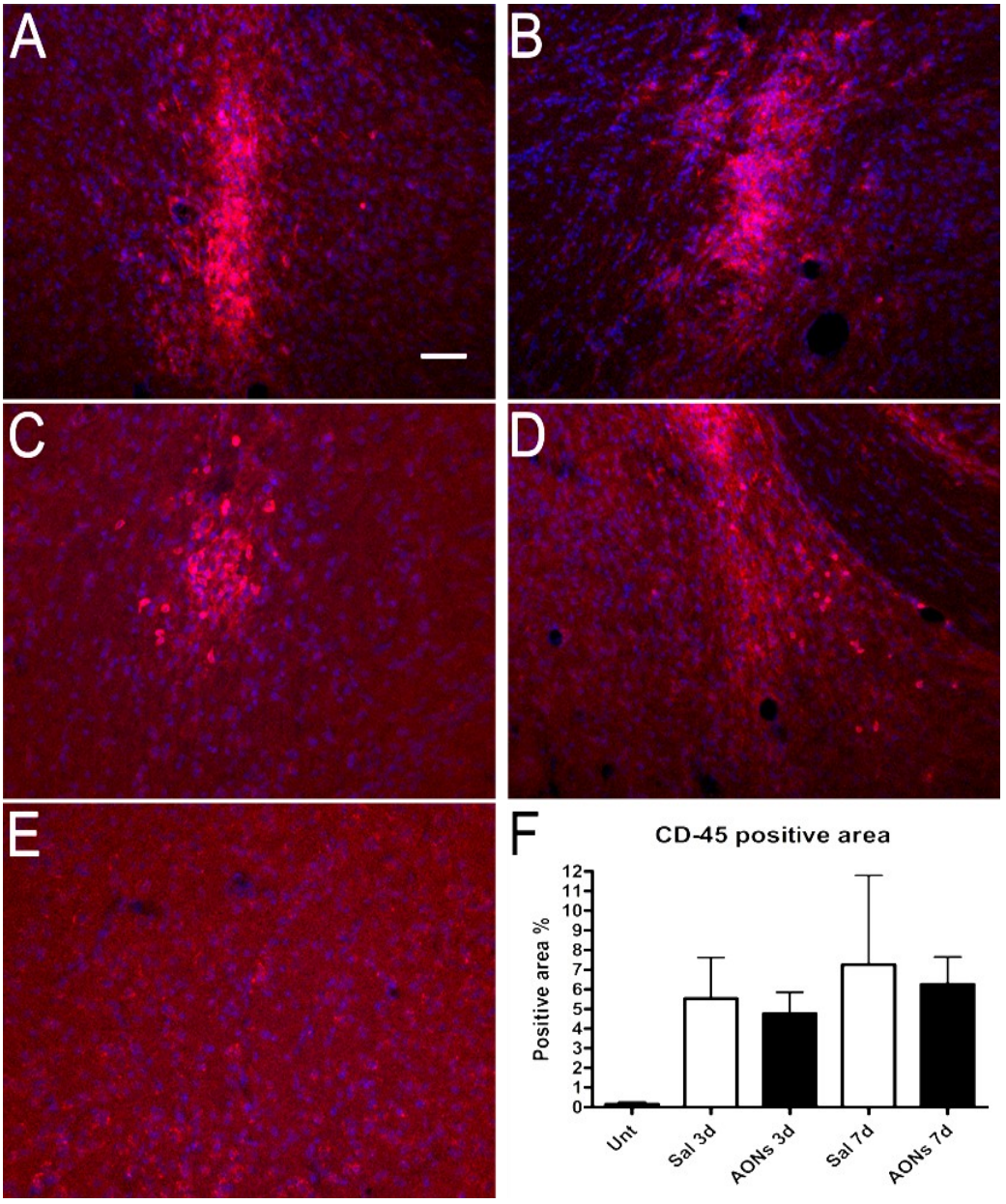

Figure 4. CD-45 immunoreactivity 3 or 7 days after a single injection in the CeA. A. 3 days after a single saline injection. B. 7 days after a single saline injection. C. 3 days after a single injection of AONs. D. 7 days after a single injection of AONs. E. CD-45 immunoreactivity in the CeA of an untreated mouse. F. Quantification of CD-45 immunoreactive area shown as percentage of the total area of visual field. One-way ANOVA followed by Tukey's post hoc test revealed no significant differences between the respective AON and saline injected animals (one-way ANOVA $F_{(4,17)}$=1.092, p>0.39, N=3-7 animals per group). Quantification of IBA-1 immunoreactive area had similar results (one-way ANOVA $F_{(4,17)}$ =1.535, p>0.23, data not shown). In conclusion, a single AON injection did not induce stronger microglia activation than saline. Scale bar 50 μm. Red: CD-45, blue: Hoechst. Green signal (AONs) has been omitted for clarity.

Isoform switching: In order to determine the efficacy of AONs treatment on exon skipping in the brain we used qPCR analysis to measure the expression ratio of the two isoforms in the CeA, 3 and 7 days after a single injection with either an AON against SRC-1e or a control-AON. Three days after the injection the SRC-1a:SRC-1e ratio showed a 2-fold shift in favor of SRC-1a in the group injected with AONs against SRC-1e, in comparison to the control-AON injected group. However, total SRC-1 expression was not different between the groups (Figure 5). Seven days after injection the expression ratio was still significantly higher in the animals injected with AONs against SRC-1e (approximately 1.5-fold higher than their control injected counterparts) without a difference in total SRC-1 expression. As an additional control for specificity, mRNA for GR (which may be one of the target nuclear receptors of SRC-1) was not significantly different between the groups either at the 3- or the 7-days time point. In view of previously reported upregulation of SRC-2 in SRC-1 knockout mice (Xu, 1998), we determined SRC-2 mRNA. We did not find a significant difference between the two groups regarding SRC-2 expression 3 days post injection. SRC-2 expression was 0.7 ± 0.2 for animals injected with AONs targeting SRC-1e and 1.0 ± 0.3 for animals injected with human dystrophin (independent t-test, $t_{(6)}$=0.8511, p>0.42).

Discussion

In this study, we investigated the efficacy in AON-mediated isoform switching, AON uptake by different cellular types and the putative immunostimulatory effects of AONs, in order to evaluate their potential use as a tool in experimental brain research.

Our results showed that it is possible to alter the expression ratio of the two SRC-1 isoforms with a single injection of AONs targeting exon 22 of the transcript of SRC-1e. Three days after the injection the isoform expression ratio showed a 2-fold increase in favor of SRC-1a, whereas 7 days after a single injection of AONs the respective difference was approximately 1.5-fold in favor of SRC-1a. In order to confirm that this was a genuine effect and was not influenced by downregulation of total SRC-1 we also investigated total SRC-1 expression in the two groups, which was shown to be comparable and not significantly different at both time points. We also investigated the expression of GR to control for possible differences as a consequence of off-target non-homologous binding of the AONs. We selected GR as an additional control, because SRC-1 is involved in GR-dependent pathways. Our results showed that GR mRNA expression is not significantly different between animals injected with either an AON targeting SRC-1e or a control AON 3 or 7 days after a single injection. This finding indicates no difference in non-homologous targeting between the specifically targeted and the control AONs and is also relevant for future experiments attempting to unravel the role of SRC-1 and its isoforms in GR dependent pathways as any effects can be attributed solely to SRC-1 isoform switching. Since SRC-2 has been shown to be upregulated in the absence of SRC-1 during development [30], we investigated its expression 3 days after injection in order to rule out an effect of SRC-1 isoform switching on SRC-2 expression. We did not find SRC-2 upregulation, which is in line with the absence of effects on total SRC-1. Although a larger-scale transcriptome and/or proteome analysis would be necessary to investigate all potential

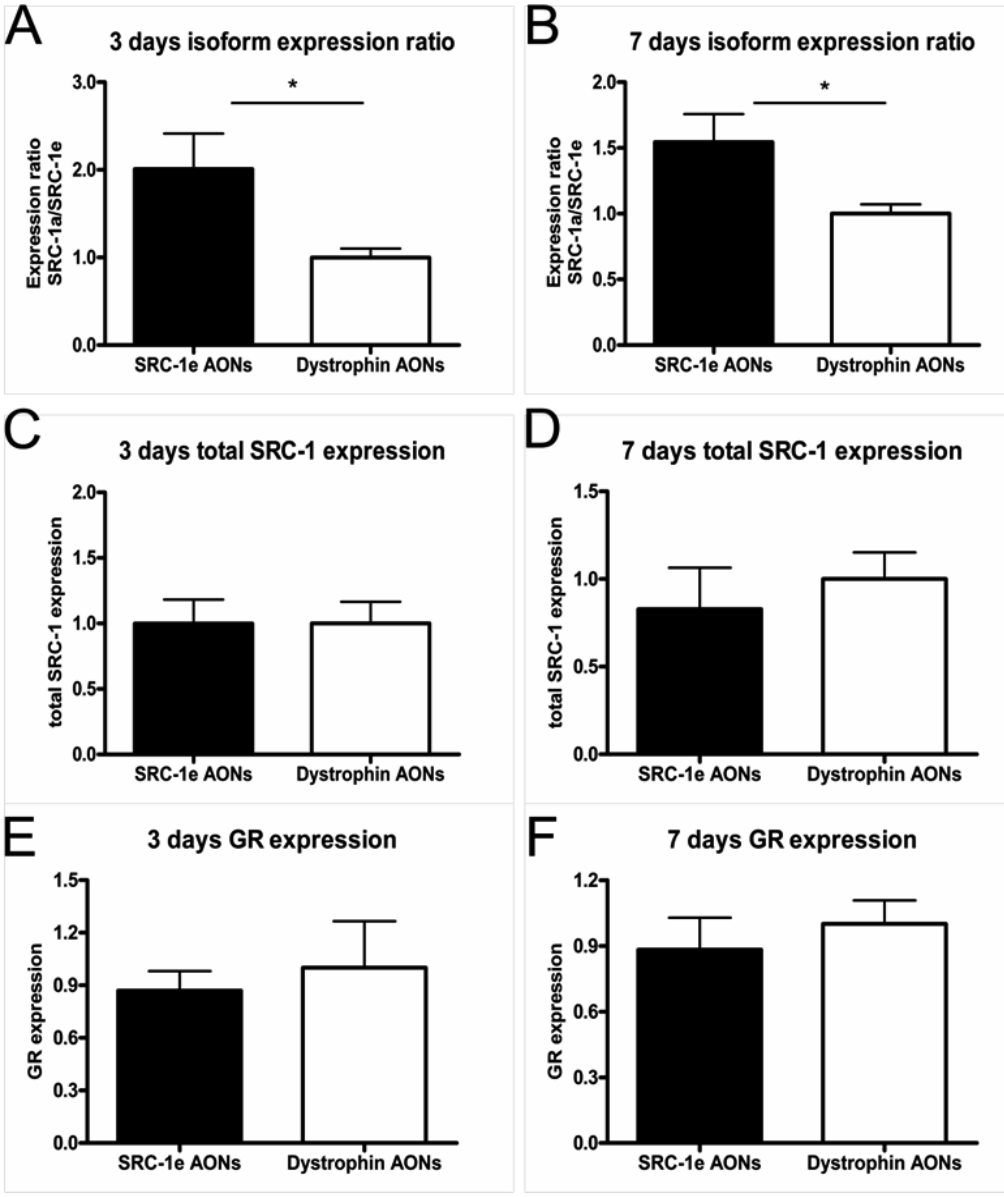

Figure 5. qPCR analysis of gene expression 3 or 7 days after a single injection. A. Relative expression of the SRC-1a/SRC-1e 3 days after a single injection of AONs. AON treatment targeting exon 22 of SRC-1e leads to a 2-fold difference of the expression ratio of the two isoforms in favour of SRC-1a (independent t-test, $t_{(6)}=2.414$, $p<0.05$, n=6-7 per group). B. Relative expression of the SRC-1a/SRC-1e 7 days after a single injection of AONs. AON treatment targeting exon 22 of SRC-1e lead to 1.5-fold difference of the expression ratio of the two isoforms in favour of SRC-1a (independent t-test $t_{(8)}=2.420$, $p<0.05$, n=5 per group). C, D. Treatment with AON targeting exon-22 of SRC-1e had no effect on total SRC-1 expression compared to control 3 (C) or 7 days (D) after a single injection (independent t-tests, $t_{(11)}=0.006$, $p>0.99$ and $t_{(7)}=1.304$, $p>0.57$ respectively, n=4-7 per group). E, F. GR expression remained unchanged between animals injected with AON targeting exon 22 of SRC-1e and controls 3 (E) or 7 days (F) after a single injection (independent t-tests, $t_{(11)}=0.479$ $p>0.64$ and $t_{(7)}=0.662$, $p>0.52$, n=4-7 per group).

off-target effects of the AONs used in this study [31], our results from total SRC-1, SRC-2 and GR mRNA expression indicate high specificity. In addition, since AONs do not obligatorily interfere with endogenous pathways unlike siRNAs they cannot saturate the cellular miRNA machinery [21], thus avoiding a source of off-target effects.

Regarding the effect size of the AONs' efficacy, it is important to note that the dissected area, particularly at longer distances from the injection site may contain cells that did not take up AONs. For the group treated with AONs against SRC-1e that would mean a dilution of the effect. Therefore, the actual efficacy of exon skipping could well be higher than observed.

The detection of isoform switching 7 days after a single injection of AONs allows animals sufficient time for post-operational recovery and performance of additional experiments, for instance, behavioral experiments. In addition, we were able to detect fluorescence of AONs up to 14 days after injection, which is probably accompanied by isoform switching to some extent, although the decrease of the expression ratio of the two isoforms between 3 and 7 days indicates that the effect size may decrease over time. If longer lasting effects are required, potential solutions may involve higher doses and repeated or continuous administration [21]. Persisting effects have been shown even 6 months after termination of continuous infusion of AONs for 7 days in the ventricles of the brain [10], and even single administration may have long-lasting effects [32].

Astrocytosis and microglia activation may confound any findings in relation to brain function. We found no differences in the immune responses caused by a single injection of AONs or a single injection of sterile saline 3 or 7 days after the injections. The time course of astrocytosis and microgliosis that we observed both in vehicle and AON treated animals was similar to what has been previously reported for saline injections [29]. It is unlikely we have reached a plateau in immune responses with saline, since it has been shown in the past that administration of lipopolysaccharide causes substantially stronger immune responses than saline [29] and particularly upregulation of CD-45. Immunostimulatory effects that have been observed in other studies may have been caused by the vehicle used [33], or immune responses elicited by simulation of Toll-Like receptors (TLRs) through the phosphorothioate backbone of the AONs [34]. However, 2-O'-modifications may act as TLR antagonists [35], which may account for the lack of immune responses in our study (in spite of the high local concentrations of AONs), as well as in others [21]. Hua et al., 2010 reported an upregulation of IBA-1 mRNA expression after continuous infusion for 9 days of 2-O'-Methyl modified AONs but not of 2-O'-Methoxyethyl AONs compared to saline. This discrepancy between the current study and the study of Hua et al. may be due to the different experimental setup. The current study used a local single injection of ~1 μg of AONs instead of a continuous ICV administration of 10 μg or more per day for 9 days that induced significant upregulation of IBA-1 in the spinal cord, or 30 μg or more that was necessary to induce significant upregulation of IBA-1 in the brain. Administration of 10 μg of AONs per day was not enough to cause significant IBA-1 upregulation in the brain. Although it is difficult to compare final local concentrations of the two approaches, our results show that we probably remain well within the "safe" range regarding the induction of immune responses. Nevertheless, this indicates that side effects of AON treatment may also depend on design, dose, frequency or

delivery of treatment and one should be aware of potential risks [7].

Before AONs can exert their effect, it is crucial that they cross the cell membrane and the nuclear membrane, since splicing takes place in the nucleus [36]. How AONs are taken up and how they are transported to the nucleus is not known. It has been shown in models of DMD that because of the lack of dystrophin protein, affected muscle cells can more easily take up AONs due to the altered properties of their muscle fiber membranes and a more open endothelium [37]. However, mechanisms of AON uptake by intact neurons in the CNS are probably different and may involve utilization of trafficking pathways for cellular uptake of AONs including absorptive endocytosis, pinocytosis and clathrin-, caveolin-, actin-, dynamin-dependent and -independent pathways [36, 38-40]. Moreover, AON cellular uptake may exploit the natural pathways of cell-to-cell nucleic acid transportation that may be also involved in micro-RNA transportation [41]. It is very likely that different physical and chemical properties of AONs depending on their chemistry, 2-O'-modifications and length may also be determining factors for the manner and efficiency of uptake [42]. The AON chemistry used in the current study has been shown to be advantageous for nuclear uptake [43].

We also showed that cells of interest in the CeA can take up AONs; NeuN and CRH positive cells represent neurons and cells expressing CRH, a hormone crucial for fear conditioning and orchestration of stress responses in the brain [44], and a putative target of SRC-1 mediated regulation [45]. NeuN positive cells account for the majority of cells taking up AONs. Moreover, we observed sporadic AON uptake by astrocytes and little or no by microglia. The low uptake by microglia cells may be due to either the properties of those cells, or the fact that they seem to arrive at the injection site probably after AONs have been already taken up by other cells. The fraction of AON-positive astrocytes was substantially lower than for NeuN-positive cells. Other studies suggested that in primates AON uptake by astrocytes may be more substantial [21]. On the other hand, GFAP staining visualizes only part of the total population of astrocytes [46] since some astrocytes do not express GFAP [47]. Hence, it is possible that GFAP negative astrocytes may have taken up AONs. To summarize, based on our findings we can conclude that generally neurons in the brain take up AONs, without, however, being able to rule out the possibility that different populations of neurons may display uptake at different rates, efficiencies or even complete lack of AON uptake. In the injected areas in the CeA, though, the vast majority of NeuN positive cells take up AONs.

It is important to mention that we did not detect the AONs directly, but rather the fluorophore with which they were labeled. Since this can be cleaved off, it would be possible that we detected fluorophores that were not bound to the AONs. However, that is not likely since uptake takes place very rapidly after injections, when little or no degradation of the AON-fluorophore complex is expected. Moreover, the considerable effect on exon skipping 3 and 7 days after an injection indicates AON activity which coincides with detection of fluorescence in the cells. For this study we made the assumption of equal stability between the two AONs.

Although the addition of a fluorophore increases hydrophobicity, hence cellular trafficking, it also increases its size. Therefore, the diffusion we observe here might be an over- or an

underestimation of what it would be without the fluorophore attached. Importantly, efficacy has been shown to be similar between labeled and unlabeled AONs [48][49]. Finally, our measurements of the diffusion of the AONs indicate that a specific brain region can be targeted with minimal leakage to adjacent areas. The diffusion observed here is likely a function of the targeted area, the volume and AON concentration and the injection rate and it may not be possible to directly extrapolate to other situations. Nevertheless, one would assume that with an optimal combination of volume and concentration smaller regions may also be targeted with reasonable specificity.

In conclusion, we have shown that it is possible to induce specific exon skipping and subsequent isoform switching of SRC-1 in the CeA without noticeable adverse effects. Our future work will address the functional consequences of SRC-1 isoform switching, as well as the many additional genes that are potential targets of such. This use of isoform switching with AONs has great potential that it must be considered not only in cases where it can restore aberrant gene expression and function, but also as an important molecular tool for manipulation of gene expression that constitutes an alternative to RNA interference or knock-out models.

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Competing interests

Annemieke M. Aartsma-Rus reports being an employee of Leiden University Medical Center and coinventor on patent applications for antisense sequences and exon skipping technology. Leiden University Medical Center has licensed the rights to part of these patents exclusively to Prosensa Therapeutics. The inventors specified on the patents (including Annemieke M. Aartsma-Rus) are jointly entitled to a share of royalties paid to Leiden University Medical Center, should the therapy eventually be brought to the market. The other authors declare absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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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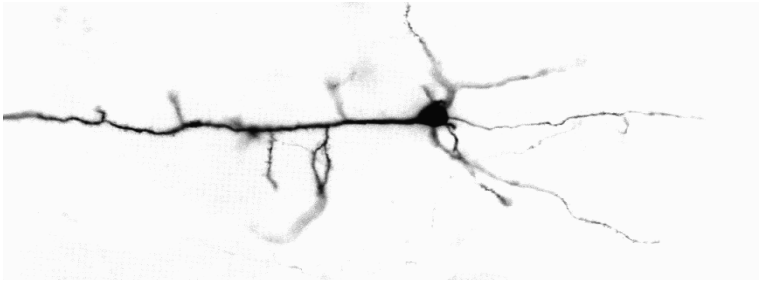

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In preparation

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 -80^{o}C.

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 4^{o}C for 15 min in a table top centrifuge. Plasma was collected and stored at -20^{o}C.

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 ^{o}C. The thymi and the adrenals were also stored in PBS at 4^{o}C 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^{o}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^{o}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 ^{o}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

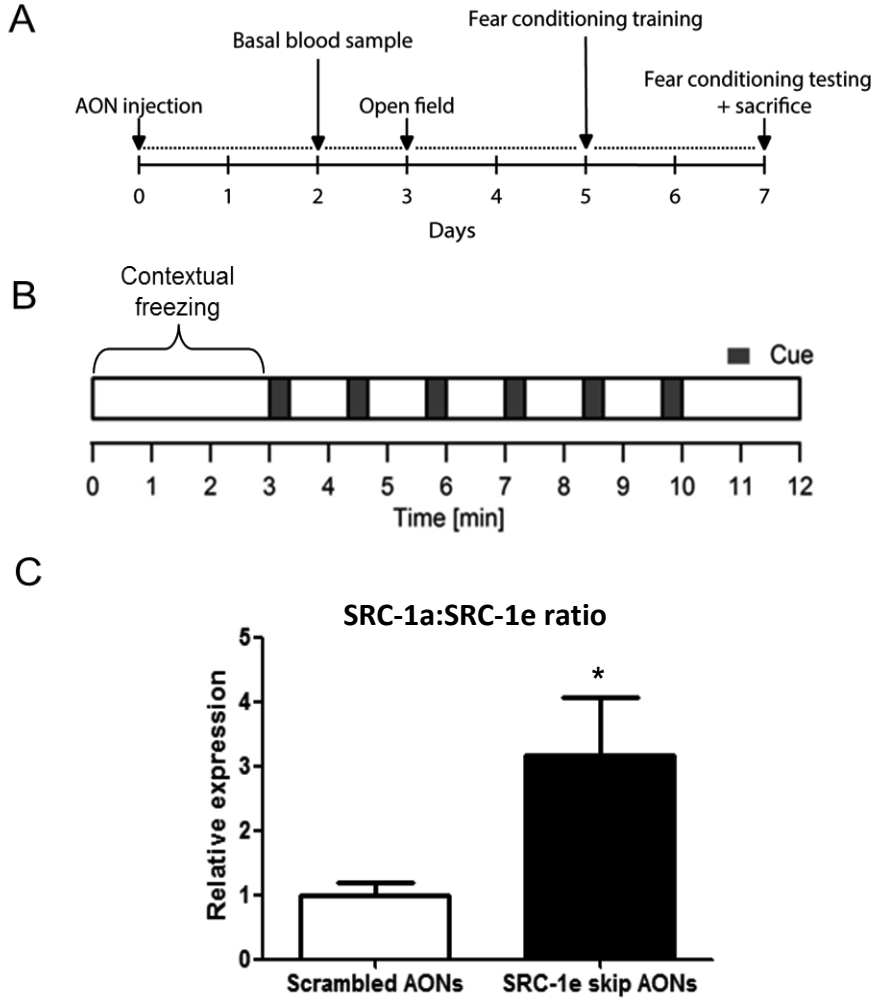

Figure 1. A. Schematic representation of the experimental design. The animals were operated 7-9 days after arrival in the animal facilities (day 0). Two days later basal blood samples were drawn. On day 3 they were introduced to an open field test. On day 5 and day 7, fear conditioning training and testing, 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).

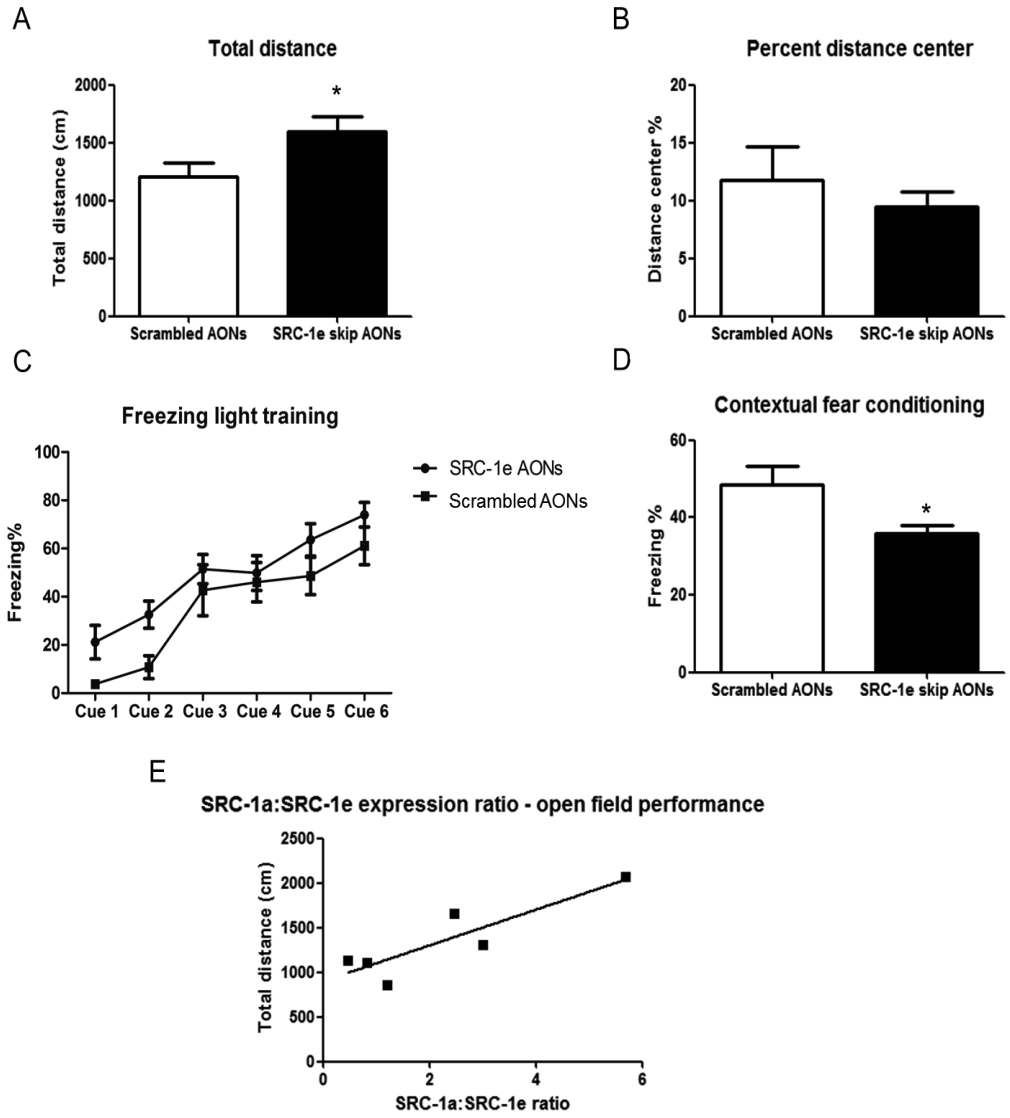

Figure 2. Behavioral profile of animals injected with SRC-1e skip AONs in the CeA. A. Total distance walked in the open field was not significantly different between the two groups (two-tailed t-test, $t_{(27)} = 2.3$, $p < 0.05$, n = 13-16 per group). B. There was no difference in percent of distance 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).

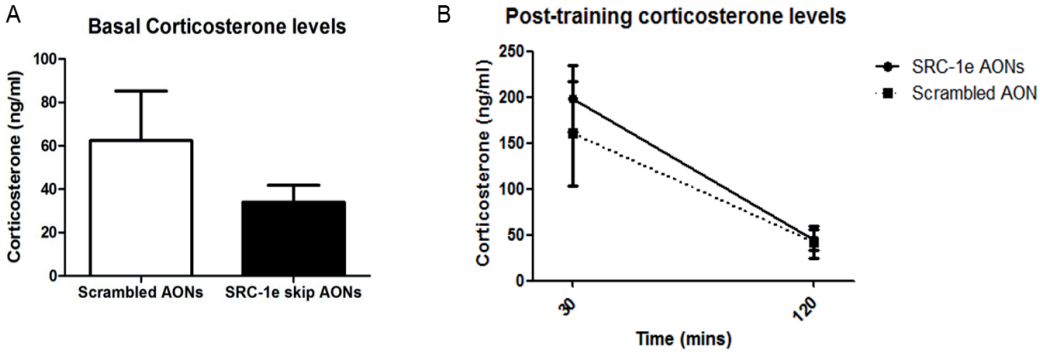

Figure 3. Lack of differential HPA axis regulation under basal conditions or in response to acute stress by SRC-1 isoform switching. A. Basal corticosterone levels do not differ between treatments (two-tailed t-test: $t_{(15)} = 1.121$, $p = 0.29$, n=8-9 per group). B. Corticosterone plasma level curves 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 ($F_{(1,20)} = 0.27$, $p = 0.60$).

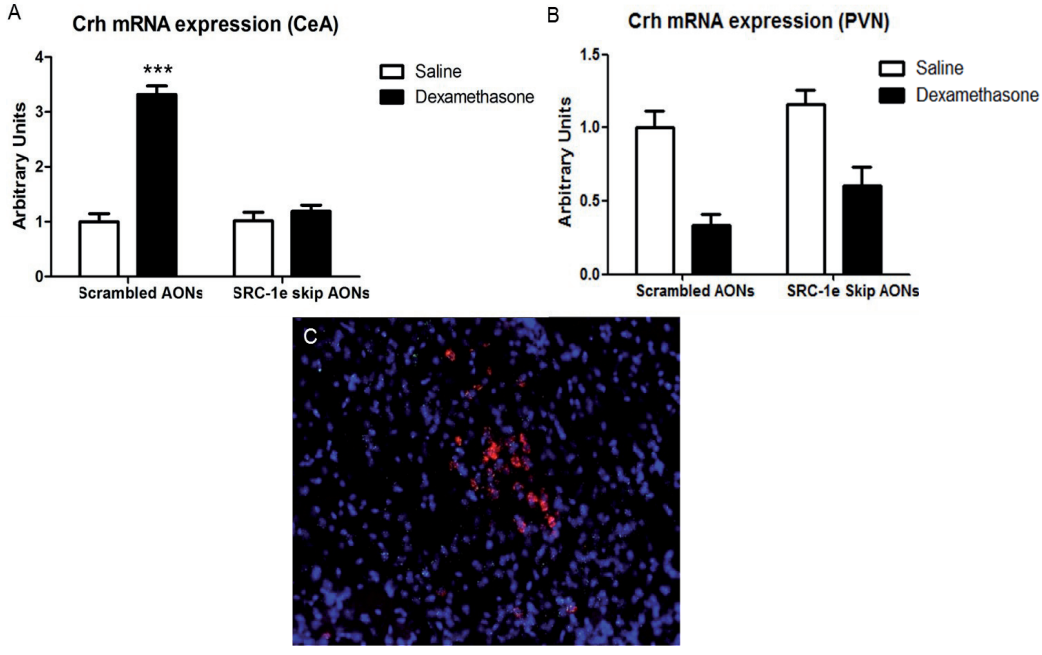

Figure 4. A. CRH mRNA in situ hybridization. In animals injected with scrambled AONs chronic dexamethasone treatment resulted in upregulation of CRH expression, which was blocked by SRC-1e skip AONs. Two-way ANOVA: AON effect, $F_{(1,17)} = 54.46$, $p<0.0001$, Glucocorticoid treatment effect, $F_{(1,17)} = 75.51$, $p<0.0001$, 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).

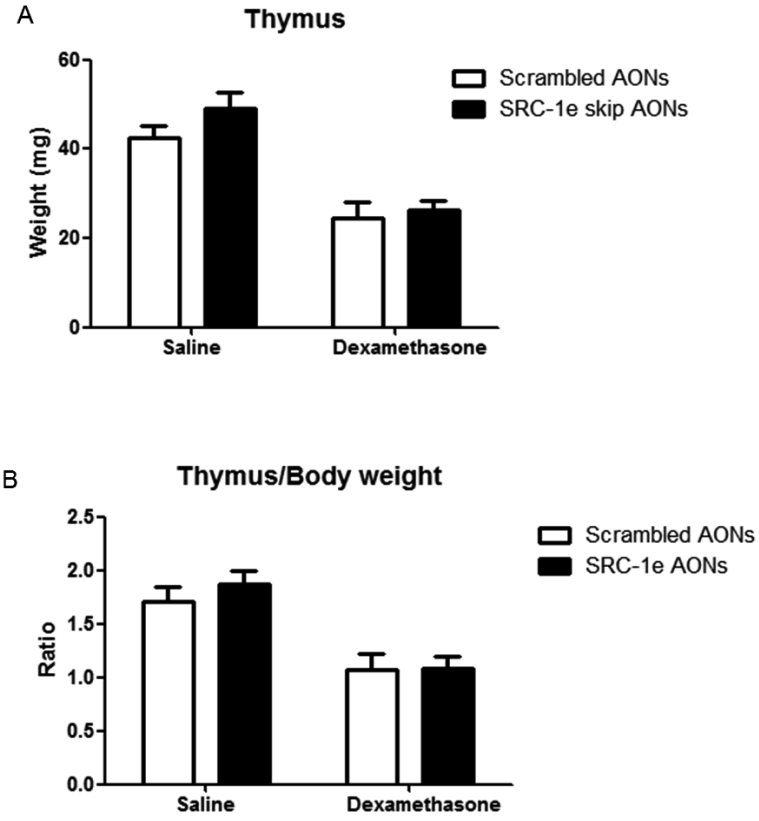

Figure 5. Effects of glucocortiocid treatment on thymus weight: A) There was a dexamethasone treatment effect on the weight of the thymi of the animals, independent of AON treatment (Glucocorticoid effect: $F_{(1,32)} = 41.01$, $p < 0.0001$, AON treatment: $F_{(1,32)} = 1.612$, $p = 0.213$, n = 8-10 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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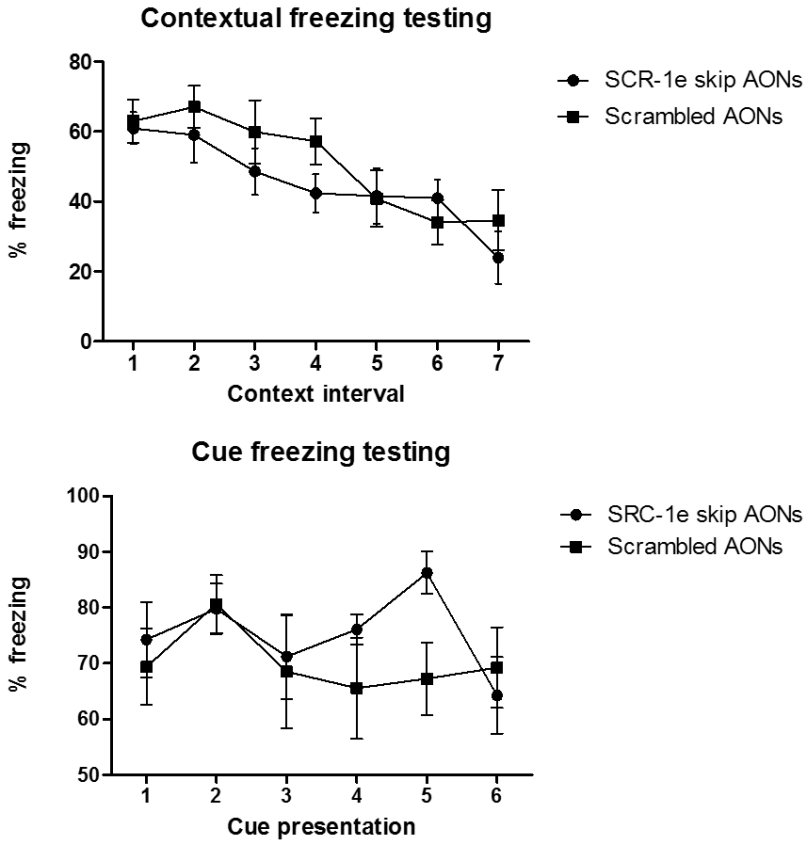
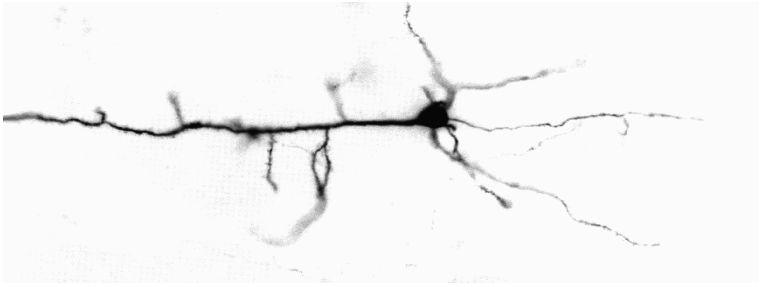
Supplementary material



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.

Chapter 4

Differential targeting of brain stress circuits with a selective glucocorticoid receptor modulator



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Abstract

Glucocorticoid receptor (GR) antagonism may be of considerable therapeutic value in stress-related psychopathology such as depression. However, blockade of all GR-dependent processes in the brain will lead to unnecessary and even counteractive effects, such as elevated endogenous cortisol levels. Selective GR modulators are ligands that can act both as agonist and as antagonist, and may be used to separate beneficial from harmful treatment effects. We have discovered that the high-affinity GR ligand C108297 is a selective modulator in the rat brain. We first demonstrate that C108297 induces a unique interaction profile between GR and its downstream effector molecules, the nuclear receptor coregulators, as compared to the full agonist dexamethasone and the antagonist RU486 (mifepristone). C108297 displays partial agonistic activity for the suppression of hypothalamic corticotropin-releasing hormone (CRH) gene expression, and potently enhances GR-dependent memory consolidation of training on an inhibitory avoidance task. In contrast, it lacks agonistic effects on the expression of CRH in the central amygdala and antagonizes GR-mediated reduction in hippocampal neurogenesis after chronic corticosterone exposure. Importantly, the compound does not lead to disinhibition of the hypothalamus-pituitary-adrenal axis. Thus, C108297 represents a novel class of ligands that has the potential to more selectively abrogate pathogenic GR-dependent processes in the brain, while retaining beneficial aspects of GR signaling.

Introduction

Adrenal glucocorticoid hormones are essential for adaptation to stressors, but prolonged or excessive exposure to glucocorticoids has been consistently implicated in the development of stress-related psychopathologies, such as depression (1). Antagonism of their most abundant receptor type, the glucocorticoid receptor (GR), can be beneficial in stress-related psychiatric disease, *e.g.* in order to abrogate psychotic and depressive features in patients with Cushing's syndrome (2) and in patients suffering from psychotic major depression (3). The GR is widely distributed in the brain (4) where it affects many different processes including learning and memory (5, 6) adult neurogenesis (7), and neuroendocrine negative feedback regulation (8). Although GR antagonism of particular processes may be of therapeutic benefit, blocking other GR-mediated effects may actually counteract the potential therapeutic efficacy. For example, GR antagonists interfere with glucocorticoid negative feedback and lead to increased cortisol levels (9, 10), which inadvertently activate mineralocorticoid receptors to which corticosteroids bind in the brain, and diminish the efficacy of antagonism at relevant sites.

The GR is a nuclear receptor (NR) that affects gene transcription through a number of transcriptional mechanisms. For several NRs 'selective receptor modulators' exist. These can act as an agonist as well as an antagonist depending on the tissue or gene targets, with the estrogen receptor ligand tamoxifen as a well-known example (11). Selective GR modulators (SGRMs) may be used to separate beneficial from unwanted glucocorticoid effects. Anti-inflammatory SGRMs with diminished side effects have been pursued, based on the distinction between GR effects that depend on direct DNA binding and those that take place via protein-protein interactions between the GR and other transcription factors (12). Selective receptor modulation may also be based on specificity of ligand-induced interactions between the GR and its major downstream effector molecules, the NR coregulators (13).

Many receptor-coregulator interactions depend on the receptor's ligand-binding domain (GR-LBD) and on specific coregulator amino acid motifs that contain an LXXLL sequence, known as 'Nuclear Receptor-boxes' (NR-boxes). These interactions are governed by the conformation that is induced by a particular ligand and may be screened for *in vitro* (14). The importance of individual coregulators for brain GR function is largely unknown, but an exception is steroid coactivator-1 (SRC-1 or NCoA1). SRC-1 is necessary for GR-mediated negative gene regulation in the hypothalamus-pituitary-adrenal (HPA) axis (15, 16), and for the induction of corticotropin releasing hormone (CRH) gene expression in the central nucleus of the amygdala (CeA)(16). Its two splice variants SRC-1A and 1E seem to exert opposite effects on CRH expression (17). Selective activation of GR interactions with SRC-1A, brought about via an SRC-1A specific NR-box, would be expected to separate GR-mediated effects on CRH expression in the hypothalamus and amygdala.

Here we show proof-of-principle for selective GR modulation in the brain with relevance for stress regulation, cognition and psychopathology. We show that a previously described selective high-affinity GR ligand induces a unique coregulator interaction profile that

distinguishes between the two splice variants of SRC-1. C108297 (or compound 47 from ref (18)) has a K_i of 0.9 nM for GR, and of >10 μM for progesterone, mineralocorticoid and androgen receptors (18). It shows GR antagonism in relation to GR-dependent CRH mRNA regulation in the amygdala and corticosterone-induced reduction in hippocampal neurogenesis.

The agonistic effects of C108297 include enhanced memory consolidation of emotionally arousing training and a suppression of hypothalamic CRH expression. The compound does not lead to net inhibition of glucocorticoid negative feedback as indicated by unaltered circulating corticosterone levels.

Materials and Methods

Peptide interaction profiling: Interactions between the GR-LBD and coregulator NR-boxes were determined using a MARCoNI assay with 55 immobilized peptides each representing a coregulator-derived NR-box (PamChip #88011, Pamgene Int, Den Bosch, The Netherlands) (14). Each array was incubated with a reaction mix of 1nM GST-tagged GR-LBD, ALEXA488-conjugated GST-antibody and buffer F (PV4689, A-11131 and PV4547; Invitrogen), and 1 μM DEX, RU486, C108297, or solvent (DMSO, 2%). Incubation was performed at 20°C in a PamStation96 (PamGene). GR binding to each peptide on the array, reflected by fluorescent signal, was quantified by tiff image analysis using BioNavigator software (PamGene).

Two-hybrid studies: To generate fusions to the DNA binding protein Gal4, partial coregulator cDNAs were cloned into the pCMV-BD vector (Stratagene): SRC-1 residues 621-1020, SRC-1A residues 1021-1441, and NCOR1 residues 1962-2440 (45) . COS-1 cells were transfected using Lipofectamine2000 (Invitrogen) with a combination of a Gal4-coregulator fusion plasmid, the pGR-VP16 transactivator plasmid and the pFR-Luc reporter gene (Stratagene). Twenty four h after transfection the medium was replaced with medium containing 0.1% DMSO, DEX, RU486, or C108297 (all 1 μM). The next day the medium was replaced with 0.1ml Hank's Balanced Salt Solution plus 0.1ml Steady light (Perkin Elmer) and luminescence was counted on a Topcount instrument (Packard).

Animal experiments: Animal experiments were carried out in accordance with the EC Council Directive of November 24 1986 (86/609/EEC), certificates and licenses granted under the Animals (Scientific Procedures) Act 1986 by the UK Home Office, or approved by the Local Committees for Animal Health, Ethics, and Research of the Dutch universities involved. Male rats were used, housed in temperature-controlled facilities on a 12 h day-night schedule with food and water available *ad libitum*. Modes of administration and duration of drug treatment differed in accordance with the standards used in the different *in vivo* paradigms.

Binding to brain GR: Group-housed Sprague Dawley rats were orally dosed with corticosterone (3 mg/kg) or C108297 (20 mg/kg and 100 mg/kg) dissolved in 10% DMSO/90% methylcellulose (0.5% w/v). After 3 h the rats were sacrificed and half-brains were snap-frozen in liquid nitrogen. For receptor binding, half-brains were homogenized in

freshly prepared buffer [0.2 M KH2PO4 (pH 7.4), 1 mM EDTA, 1 mM DTT; 4 °C], containing a protease inhibitor mixture (Sigma; P8340; 50 µL/g tissue) and phosphatase inhibitor mixtures 2 and 3 (Sigma; P5726and P0044; 1:100 dilution), using a Bead Ruptor at 4 °C for 15 min. Free GR ligands were cleared by incubation of 500 µL of homogenates (15-min incubation on ice) with dextran-coated charcoal (Sigma; C-6197) and centrifuged in a bench top Microfuge (17,000 × g; 4 °C; 10min). Receptor binding was determined by incubating 50 µL of homogenate with 2.5nM [3H]dexamethasone (Amersham; TRK645) at 4 °C for 18 h in a total volume of 100 µL of assay buffer [10 mM potassium phosphate buffer (pH 7.6), containing 5 mM DTT, 10 mM sodium molybdate, 100 µM EDTA, and 0.1% BSA). Nonspecific binding was determined by addition of 20 µM unlabeled dexamethasone. Unbound ligand was removed by addition of 15 µL of 10% dextran-coated charcoal and centrifugation at 3,080 × g for 10 min at 4 °C. The supernatant (65 µL) was transferred to a Packard Optiplate, and 125 µL of MicroScint40 was added. [3H]Dexamethasone activity was quantified as counts per minute by counting on a Perkin Elmer Topcount.

Hippocampal gene expression: The other halves of the brains were cut at 200-µm-thick coronal sections. Sections were mounted on glass slides (Gerhard Menzel). Eight tissue punches were taken from the CA1-CA2 area of the hippocampus with a Harris UniCore hollow needle (Electron Microscopy Sciences; 1.2 mm internal diameter), with one punch per section starting around 2.56 mm posterior to Bregma (46). Tissue was stored in TRIzol (Invitrogen) at –80 °C until further processing.RNA isolation, cDNA synthesis and qPCR have been described elsewhere (47). Validated hippocampal GR target genes were selected from micro-array analysis (Rat Genome 230 2.0 Arrays; Affymetrix, Santa Clara, USA) (22). Quantitative PCR was performed on a LightCycler 2.0 (Roche Applied Science) using LC FastStartDNA MasterPLUS SYBR Green I according to the manufacturer's instructions (Roche). Tubulin β2a (Tubb2a) was used to normalize expression (6). The forward and reverse primers used were, respectively, as follows: 5′-GCAAATCCGGCGCATCTCAG-3′ and 5′-TGCGGTGGTCTGGCAATTCT-3′ for Drd1a (coding for the dopamine 1A receptor), 5′-GGTCACAGCGGCAGATAAAAAGAC-3′ and 5′-TCGGCATTGCGAGTTCCAG-3′ for Bdnf and 5′-GAGGAGGGCGAGGATGAGGCTT-3′ and 5′-GACAGAGGCAAACTGAGCACCAT-3′ for Tubb2a. Tubulin β2a (Tubb2a) was used to normalize expression (48).

Subchronic treatment: agonism in relation to CRH and the HPA axis: Group-housed Wistar rats (200-220 gram, Harlan, The Netherlands) underwent adrenalectomy in the morning as described (49). One week later, animals were treated twice daily (s.c., 1 ml/kg) with vehicle (polyethylene glycol-300), C108297 (20 mg/kg) or DEX (0.5 mg/kg) (25). On day 5, three h after the morning injection, half of the animals underwent 30 min of restraint stress. A tail cut sample was collected 15 min after the onset of restraint. Animals were killed by decapitation either under basal conditions, or at 30 min after onset of the restraint. CRH and c-fos mRNA, and CRH hnRNA were quantified by in situ hybridization on whole PVN and CeA as described previously (25). Corticosterone and adrenocorticotropin (ACTH) were measured by radioimmuno assay (MP Biomedicals Inc., CA., USA).

Subchronic treatment: antagonism in relation to CRH and the HPA axis: Procedures

were as described above but in intact rats, this time using RU486 (40 mg/kg) as a reference drug. Tail cuts that were performed at 08:00 h and 20:00 h of day 4 for basal plasma corticosterone levels. To determine acute stress responses in naïve rats, we subjected rats to an acute 0.4 mA footshock in an inhibitory avoidance shock box (49), with or without a single pretreatment with the doses of RU486 and C108297 that were used in the subchronic setting.

Neurogenesis: Group-housed Wistar rats (200 grams) were habituated to the animal facility for 10 days. Corticosterone (Sigma, C-2505; 40 mg/kg) or vehicle (arachidus oil) was injected (s.c.) daily at 09:00 h for 21 days. Animals received C108297 (50 mg/kg) or vehicle (0.1% ethanol in coffee cream (Campina, Woerden, The Netherlands)) by gavage on the final 4 days of corticosterone treatment at 09:00 h and 16:00 h. Animals were sacrificed one day after the last treatment. All animals received 5-bromo-2-deoxyuridine (BrdU) (200 mg/kg, i.p) on day 1, 3 h after the first corticosterone injection. Tissue processing for immunostainings was performed as described (50). Data on vehicle treated groups were also reported elsewhere (50).

Inhibitory avoidance behavior: One-trial inhibitory avoidance training and retention was performed as described (30), using single-housed Wistar rats (300-350 g, Charles River, Germany) and a footshock intensity of 0.5 mA for 1 s. RU486 (40 mg/kg) or vehicle (polyethylene glycol) was administered (s.c.) one h before the training session. C108297 (20 mg/kg) or corticosterone (1 mg/kg) was dissolved in DMSO and administered (100 µl, s.c.) immediately after the training trial, so that treatment did not interfere with memory acquisition. Retention was tested 48 h later. A longer latency to enter the former shock compartment with all four paws (maximum latency of 600 s) was interpreted as better memory.

Statistical analysis: Data were analysed using Graphpad Prism using (as appropriate) 1- or 2-way ANOVA followed by Tukey's/Bonferoni post hoc test respectively, and Kruskal-Wallis for data that deviated from a normal distribution.

Results

C108297 displays selective modulator activity in vitro: To explore possible selective modulator activity of C108297 based on the GR-coregulator interactions, we used a MARCoNI peptide array (14) to determine interactions between (recombinant) GR-LBD and coregulator NR boxes (figure 1A). Reference drugs were the full agonist dexamethasone (DEX) and the prototypical antagonist RU486 at saturating doses. Without ligand, GR displayed only weak interactions with coregulator motifs. DEX induced significant interactions between GR-LBD and 28 motifs from coactivator proteins. RU486 induced modest interactions with motifs from two corepressor proteins, NCoR and SMRT (19). C108297 induced interactions with a subset of the motifs that were recruited after DEX treatment, suggesting selective modulator activity. C108297 did not induce interactions with NCoR and SMRT motifs. For quantitative analysis, see figure 2. The partial recruitment of coregulator motifs of C108297-bound GR suggests that the compound combines agonistic and antagonistic effects (dependent on the gene-specific coregulator use by GR).

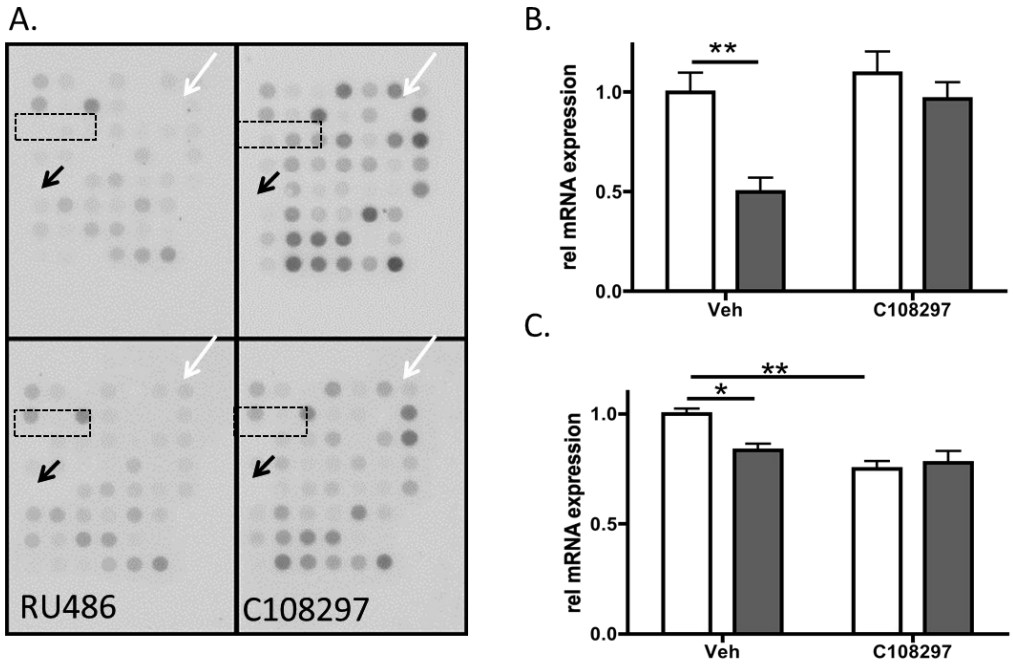

Figure 1. C108297 behaves like a selective modulator in vitro and in vivo. A. Ligand-induced interactions between the GR-LBD and coregulator motifs. DEX induced many interactions compared with DMSO. RU486 induced modest interactions with corepressor motifs (black arrow: NCoR1). C108297 showed an intermediate profile. GR-LBD interactions with the central motifs from SRC-1 were much weaker or absent (boxed), but others were retained (white arrow indicates SRC-1 motif IV). B. Hippocampal Drd1a mRNA was regulated by corticosterone after vehicle but not C108298 treatment. C. BDNF mRNA was down-regulated by both corticosterone and C108297. Asterisks indicate significant differences from the control group (*P < 0.05; **P < 0.01).

C108297 reaches the brain: We tested whether C108297 can reach the brain in order to affect GR-dependent processes. Three h after oral treatment of rats, C108297 (20 mg/kg) led to 35 ± 15% occupancy of brain GR binding determined *ex vivo* in 1 hemisphere, compared to the negative control. This level of occupancy did not differ from that observed for the positive control of 3 mg/kg corticosterone (well above the ED50 of 0.6 mg/kg (20)), which resulted in 44 ± 15% GR occupancy. This degree of occupancy is considered effective for many corticosterone effects via GR (e.g. (21)), and the dose of 20 mg/kg C108297 was used in all other *in vivo* experiments described below, with the exception of the work on neurogenesis that was initiated earlier.

C108297 displays gene-specific agonism and antagonism on GR target genes in vivo: To confirm gene-specific antagonism of C108297, we tested mRNA regulation of two previously characterized hippocampal GR target genes (22). Rats were treated with 3 mg/kg corticosterone with or without pretreatment with C1082987 (20 mg/kg), or with C108297 alone. For Drd1a mRNA (coding for the dopamine 1_A receptor) 2-way ANOVA showed main effects of corticosterone ($p < 0.01$) and C108297 ($p < 0.05$), but no interaction (but

endogenous corticosterone was present). Drd1a mRNA was significantly lower after corticosterone (3 mg/kg) treatment, but not after (pre-)treatment with C108297 (20 mg/kg) (figure 1B). For BDNF regulation, 2-way ANOVA showed main effects of corticosterone, C108297 (both $p < 0.05$) and an interaction ($p < 0.001$). C108297 by itself down-regulated BDNF mRNA levels, and did not prevent the corticosterone effect (figure 1C).

C108297 distinguishes between SRC-1 splice variants: Out of many potential coregulators of GR, SRC-1 is among the few that have been linked to regulation of specific GR target genes (15, 16). Its splice variants SRC-1A and 1E may mediate different effects in relation to stress adaptation (17). As C108297 seemed to differentiate between the SRC-1 splice variants, we focused on these for further analysis. Quantitative analysis of the MARCoNI data showed that C108297 differentiates between the three NR-boxes that are common to the two SRC-1 splice variants and NR-box IV that is unique to SRC-1A (23) (figure 2A). Two-way ANOVA indicated highly significant differences between ligands, motifs and a strong interaction between the two ($p < 0.001$ for main effects and the interaction). DEX was able to induce strong GR interactions with all four SRC-1 motifs, but C108297 induced substantial agonist-like binding only for the SRC-1A specific NR-box (figure 2B), confirming potentially selective recruitment of these splice variants by the GR-C108297 complex.

We validated the ligand-directed differential recruitment of SRC-1 splice variants using larger protein fragments in a two-hybrid system in mammalian COS-1 cells (figure 2C). Two-way ANOVA showed significant effects of drug, protein fragment and an interaction ($p < 0.001$ for all effects). Both DEX and C108297 induced a strong GR-LBD interaction with a 420 amino acid fragment containing the SRC-1A specific NR-box IV. DEX, but not C108297, induced interactions with the SRC-1 domain containing the three central NR-boxes. A fragment from the corepressor NCoR was recruited by GR-LBD only after incubation with the antagonist RU486. Thus, the ligand selective interactions of GR also occurred with large protein fragments in cell line context.

C108297 has selective partial agonist activity in the brain of adrenalectomized rats: The selective modulator type interactions of GR with SRC-1 variants led to the hypothesis that C108297 *in* vivo acts as an agonist for GR-mediated regulation of the *Crh* gene in the core of the HPA axis, but not in the CeA (17, 24). To test agonism, we used adrenalectomized rats in a 5 day treatment paradigm in which half of the animals underwent a single restraint stress on day 5, 30 min before sacrifice. This paradigm allows measurement of a number of both basal and stress-induced HPA-axis variables (25). It is well established that CRH expression in the hypothalamic paraventricular nucleus (PVN) and CeA both respond to treatment with our control agonist DEX, but in an opposite direction (26).

CRH mRNA in both brain regions responded to drug but not to acute stress (2-way ANOVA, drug effect PVN: $p < 0.001$; CeA: $p = 0.011$, stress effect not significant). In the PVN (figure 3A) CRH mRNA was strongly suppressed by DEX. C108297 also showed modest agonism that reached significance in the stressed animals. CRH mRNA in the CeA (figure 3B) was increased after DEX treatment in non-stressed animals, but unaffected by C108297. In the stressed rats the differences between the treatment groups failed to reach significance. A more

substantial agonist effect of C108297 was observed for stress-induced CRH hnRNA in the PVN. This response was equally strongly suppressed by DEX and C108297 (figure 3C, 1-way ANOVA $p < 0.001$). In the CeA, the levels of CRH hnRNA were below detection, even after prolonged exposure of the films. Thus C108297 showed (partial) agonism in the PVN, but not in CeA.

In order to assess other (ant)agonist-like effects of C108297 on HPA-axis activity, we determined basal and acute restraint stress-induced ACTH secretion after 5 days of treatment (figure 3D; 2-way ANOVA effects of time after onset of stress, drug-pretreatment ($p < 0.001$) and an interaction ($p < 0.01$)). DEX led to a complete suppression of basal and stress-induced ACTH release. Subchronic C108297 treatment did not affect basal ACTH levels in these ADX animals, but led to a modest suppression of stress-induced ACTH release, possibly indicating a weak agonistic effect.

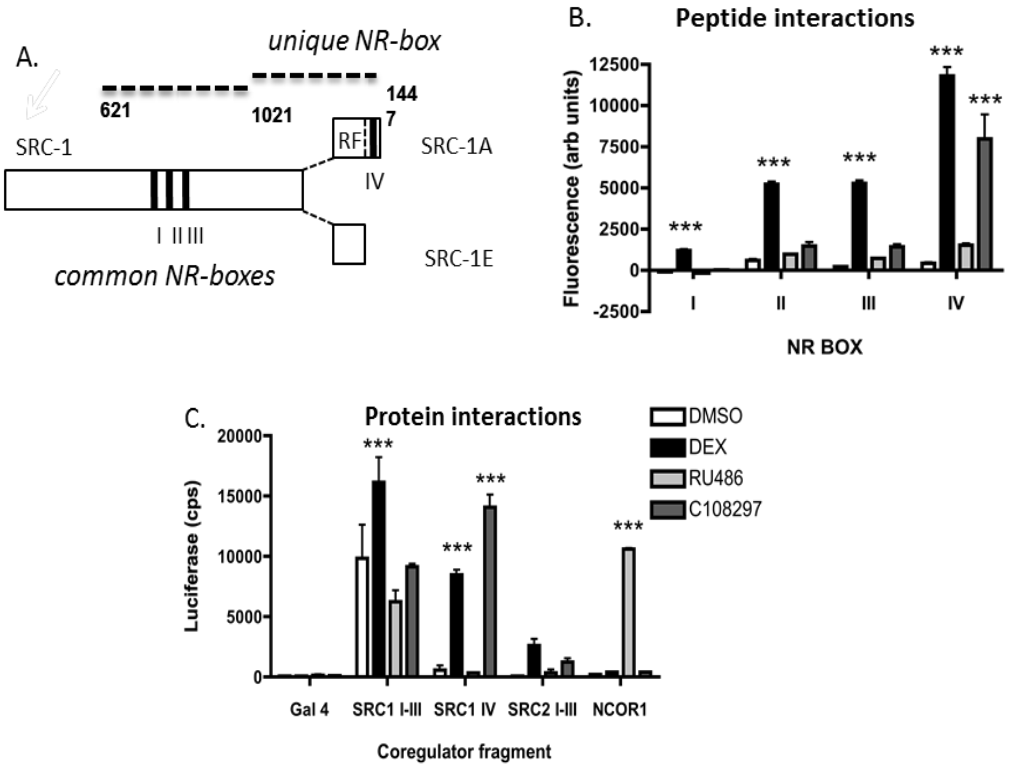

Figure 2. SCR-1 splice variant 1A is selectively recruited by GR-C108297. A. Protein structure of SRC-1 harboring three NR central boxes (roman numerals). SRC-1A harbors a repressor function (RF) and the additional NR-box IV. Protein fragments marked by dotted lines refer to C. B. MARCoNI quantification showed that unlike DEX, C108297 induced interactions only between GR and NR-box IV. C. In a two-hybrid assay only DEX induced interaction with the SRC-1 fragment common to both splice variants. The SRC-1A–specific protein fragment was also recruited by GR-C108297. A fragment of corepressor NCoR1 only interacted after incubation with RU486. Asterisks indicate significant difference from the control condition ($P < 0.001$).

C108297 has selective antagonist activity in adrenally intact rats: In order to determine neuroendocrine antagonistic effects against endogenous corticosterone we compared 5-day treatment of C108297 (20 mg/kg) with RU486 (40 mg/kg) in adrenally intact rats, followed by restraint stress on day 5 in half of the animals. The stressor strongly induced expression of both CRH hnRNA in the PVN (2-way ANOVA $p < 0.001$) and led to a modest increase in CRH mRNA (2-way ANOVA $p < 0.05$), but these parameters were not affected by drug treatment (not shown), consistent with a lack of GR involvement in the immediate curtailing of the transcriptional CRH response in acute stress situations (27). There was no effect of subchronic drug treatment or the stressor on amygdala CRH mRNA. The only central measure that responded to subchronic drug treatment in intact rats was the c-fos response to restraint-stress in the PVN (1-way ANOVA $p < 0.001$). Both RU486 and C108927 treatment led to elevated c-fos mRNA expression 30 min after the onset of stress (figure 4A).

With regard to stress-induced activation of the HPA-axis, the two compounds also led to similar changes, indicative of antagonism by C108297. At 15 min after the onset of the

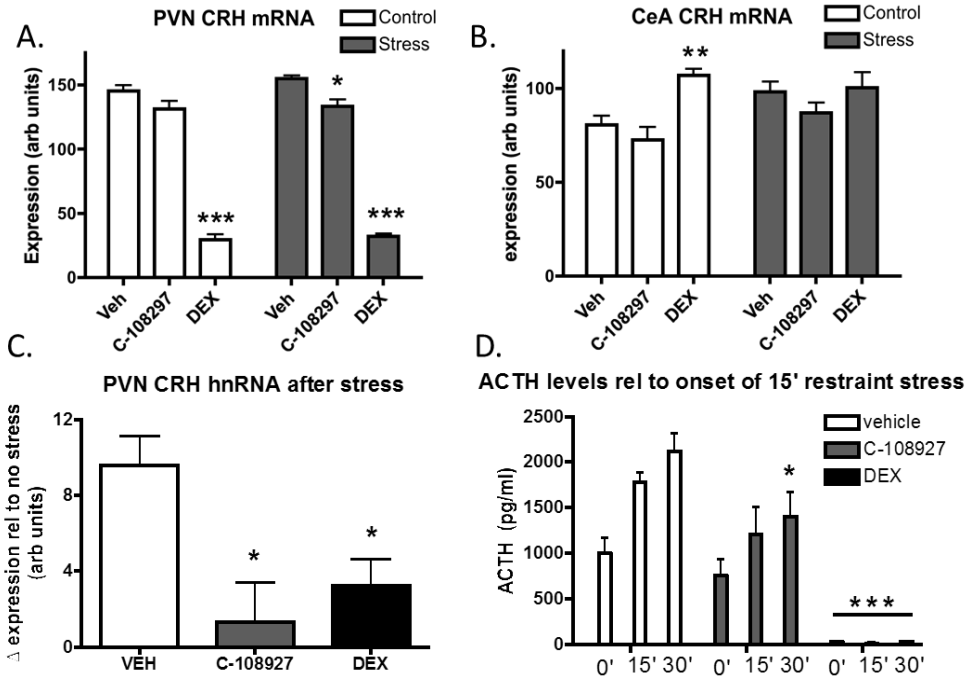

Figure 3. Selective GR modulation in the stress system. C108297-agonism in ADX rats after subchronic treatment compared with the prototypic agonist DEX. A. In the PVN, where SRC-1A is expressed at high levels, DEX led to strong down-regulation of CRH mRNA ($P < 0.001$). C108297 had a modest agonist effect that reached significance in the stressed group ($P < 0.05$). B. In the CeA, DEX up-regulated CRH mRNA in nonstressed rats ($P < 0.05$), but C108297 was without effect. C. The acute response of the Crh gene in response to restraint stress was strongly attenuated both by pretreatment with DEX and C108297. D. DEX led to a complete blockade of the HPA axis ($P < 0.001$), whereas C108297 leads to a very weak attenuation of the adrenocortical stress response ($P < 0.05$).

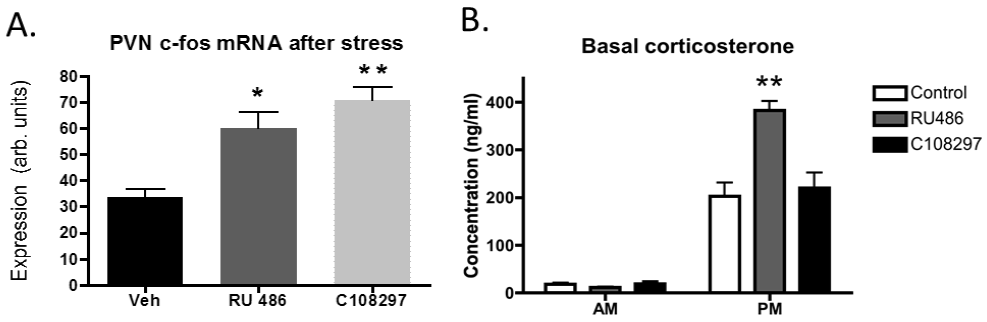

Figure 4. Selective GR modulation in the stress system: antagonism in adrenally intact rats after subchronic treatment compared with the prototypic antagonist RU486. A. The acute c-fos response to stress in the PVN was enhanced both by pretreatment with RU486 and C108297. B. RU486 treatment led to increased circadian peak levels of plasma corticosterone. C108297 does not have this effect. *P<0.05; **P<0.01.

restraint stress, corticosterone levels were about 25% lower in both the RU486 (301 ± 69 ng/ml) (28) and C108297 (273 ± 52 ng/ml) treatment groups, compared to controls (409 ± 36 ng/ml). In contrast, RU486 increased the amplitude of the basal diurnal corticosterone rhythm by increasing evening corticosterone levels without affecting AM levels, as described (9), but C108297 did not have this antagonistic effect (figure 4B, $p < 0.001$ for drug, time and interaction effects).

Agonism and antagonism on neurogenesis and behavior: In order to further evaluate the efficacy of C108297 in animal models with relevance for psychopathology, we evaluated the effect of C108297 in two paradigms: corticosterone-induced suppression of neurogenesis, and memory consolidation of inhibitory avoidance training.

C108297 was tested for reversal of GR-dependent reduction in adult neurogenesis after 3 weeks of treatment with a high dose of corticosterone (40 mg/kg/day). RU486 was earlier shown to fully normalize the reduction in neurogenesis induced by corticosterone or chronic stress (29). In a comparable design, C108297 (50 mg/kg) was administered during the last four days of corticosterone treatment. Two-way ANOVA indicated that the number of cells that stained for BrdU (a marker for newborn cell survival) was affected by chronic corticosterone treatment ($p = 0.008$) and by C108297 treatment ($p < 0.001$), but there was no significant interaction. Post-hoc analysis revealed that the difference between C108297 and vehicle groups only reached significance in animals treated chronically with corticosterone (figure 5A). The number of doublecortin (DCX) positive cells in the dentate gyrus, indicative of neuronal differentiation of newborn cells, was affected by chronic corticosterone treatment ($p = 0.002$), but not by C108297 ($p > 0.4$), although there was a trend towards an interaction ($p = 0.089$). Post-hoc analysis indicated a significantly lower number of DCX positive cells after chronic corticosterone treatment only in the group treated with the vehicle for C108297 (figure 5B). Thus, C108297 partially counteracted the effects of chronic corticosterone treatment.

To determine whether C108297 affected memory consolidation, rats were trained on an aversively motivated single-trial inhibitory avoidance task, which is known to be potentiated by GR activation (30). A corticosterone (1 mg/kg) treatment was included as a positive control. Retention test latencies, as assessed 48 h after training, indicated a significant drug treatment effect (Kruskal-Wallis test, $p < 0.001$, figure 5C). Rats treated with either corticosterone or C108297 had significantly longer retention latencies than vehicle-treated rats ($p < 0.001$). This effect could be blocked by RU486 pretreatment. These findings indicate that C108297 has substantial GR agonism in this paradigm.

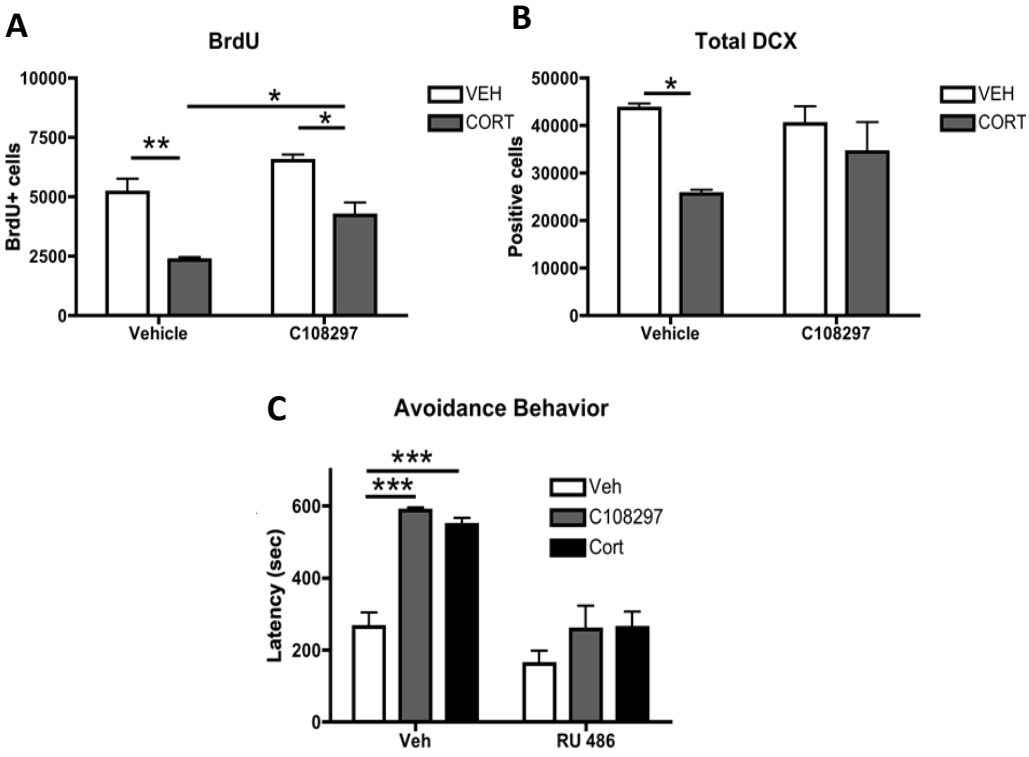

Figure 5. C108297 acts as GR antagonist in neurogenesis and as agonist in memory retention. A. Chronic corticosterone suppressed the number of BrdU positive cell, and 4 d of C108297 treatment increased this number. BrdU scores were significantly higher in animals that received C108297 in combination with chronic corticosterone, compared with corticosterone-treated animals that did not receive C108297. B. Total DCX-positive cells were significantly fewer after 3 wk of corticosterone treatment but not in animals that also received C108297. C. Acute posttraining C108297 (20 mg/kg) or corticosterone (1 mg/kg) led to long 48-h retention test latencies in the inhibitory avoidance task, and these effects were blocked by pretreatment with RU486. Significant differences: *P <0.05; **P < 0.01; ***P < 0.001.

Discussion

High levels of circulating glucocorticoids as a consequence of acute or chronic stress are known risk factors in the development of psychopathologies, either as predisposing factors or during precipitation of disease. GR antagonists have therapeutic potential (28, 31), but given the ubiquitous expression of the GR they have many undesired side-effects (32). Disinhibition of the HPA-axis is a side effect that actually counteracts the goal of any such treatment (*i.e.* blockade of GR signaling). SGRM compounds that combine antagonistic and agonistic GR properties may lead to a better-targeted interference with stress-related brain processes.

Based on the C108297-induced interactions between GR and its coregulators, we hypothesized and confirmed that this compound is a selective GR modulator, with relevance for the brain. Interestingly, clear antagonist effects on the brain were accompanied by lack of negative feedback inhibition of the HPA-axis, which in itself suggests the possibility of antagonizing a number of GR effects without affecting systemic basal glucocorticoid levels, and the associated change in activity of, for example, mineralocorticoid receptor-dependent processes (33). C108297 is expected to have selective modulator effects also in peripheral tissues that we did not examine here (34). We did not determine binding to MR and PR or specific MR/PR readouts here, but previous studies showed 0% displacement from MR and 26% from PR at 10 μM C108297, *i.e.* over a 1000-fold selectivity for GR (18). In peripheral tissues we cannot exclude some binding to PR with the 20 mg/kg dose C108297, but under non-saturating conditions for brain GR, activation of other steroid receptors is unlikely. Selective targeting to the brain may constitute a particularly efficacious way to interfere with a number of central GR-dependent processes, with very few side effects.

In the MARCoNI assay the overall strength of the GR bound to C108297 interactions with coregulator motifs is somewhat lower than for GR bound to DEX, suggesting that C108297 is a partial agonist. Some of the antagonist effects that we observed after a single dose *in vivo* may indeed reflect partial agonism relative to circulating corticosterone. However, because some of the coregulator interactions become zero while others still reach substantial levels, the molecular profile is that of a selective modulator. It is unclear at this point, whether the GR follows a two-state agonist conformation, with C108297 leading to a similar conformation to DEX, but less stable (35), or whether C108297 leads to a unique conformation of the GR-LBD. C108297 clearly differs from the well-known (but non-selective) antagonist RU486, as it lacks the capacity to induce interactions with domains from corepressors NCoR and SMRT, and the associated intrinsic (repressive) activity that may come from those interactions (19).

Reversal of glucocorticoid-induced effects was observed for expression of the *Drd1a* gene in the hippocampus. This effect may be of relevance for reversal of negative effects of glucocorticoids on cognition (36). Given chronically C108297 also antagonized the effects of corticosterone on adult neurogenesis. Here, C108297 seemed to be less potent than RU486 (31), perhaps because of a lack of interactions between GR and the classical corepressors. Notwithstanding, reversal of decreased neurogenesis may be relevant for antidepressive effects (37). In relation to regulation of brain CRH, the compound seems to have beneficial

effects in the context of stress-related psychopathology, as was predicted by its interactions with the coregulator SRC-1 splice variants (16, 17). The compound lacked efficacy for the potentially anxiogenic induction of CRH via GR (38) even in ADX rats. It showed a mild degree of agonism on basal CRH expression in the PVN, and pretreatment had a substantial suppressive (agonistic) effect on stress-induced CRH transcription (39). Moreover, there was a clear lack of antagonism by C108297 on basal regulation of the HPA axis, which is an important advantage over complete antagonists like RU486 when trying to interfere with central consequences of hypercorticism (9).

C108297 does not cause an overall dampening of brain stress responses. Like RU486, it enhanced stress-induced neuronal activity in the PVN, either indicating changed responsiveness of the parvocellular neurons, or changed activity of neuronal afferents to the PVN. The apparent agonism on BDNF expression (21) also shows that some consequences of stress may be mimicked by the compound. The GR-dependent increased consolidation of inhibitory avoidance memory also is in line with well-known stress effects, and can be either adaptive or maladaptive (6, 40).

Our data emphasize the multiple levels of GR-mediated control over the HPA-axis. For example, RU486 as well as C108297 led to an increased c-fos response to stress in the PVN, but to an attenuated stress-induced ACTH release. This dissociation has been observed by others after direct and acute manipulation of the PVN (41). The extent to which CRH and c-fos respond to stressors in 'naïve' rats is in general highly dependent on multiple factors, including the type of stressor and time after stress (42, 43).

A small part of the selective GR modulation *in vivo* may be explained by differential recruitment of SRC-1A and 1E, and the role of numerous GR-coregulator interactions in mediating the many effects of GR activation on brain will be subject to further research. SGRMs such as C108297 and their molecular interaction profiles, combined with knowledge of the regional distribution of coregulators in the brain, can in future assist in dissecting the molecular signaling pathways underlying stress-related disorders. In fact, although our analysis was necessarily not comprehensive (*e.g.* in relation to non-genomic GR signaling (44)), C108297 itself may have a beneficial profile compared to a situation of hypercortisolism.

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Conflict of interest

HH and JB are employed by Corcept Therapeutics, and made C108297 available. Corcept

financed part of the costs of the experiments. RH is employed by Pamgene Int, who made MARCoNI arrays available for this study.

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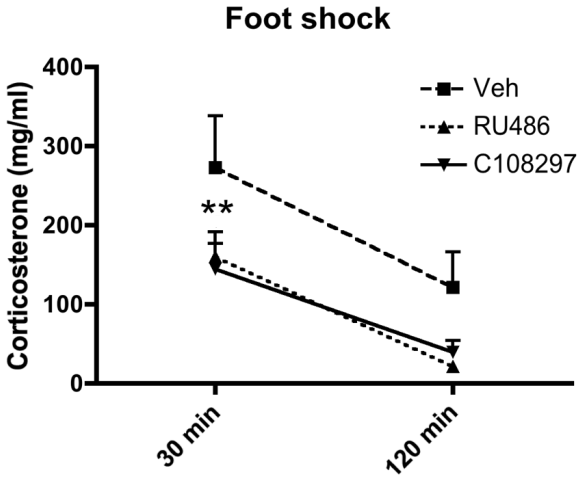
Supplementary material

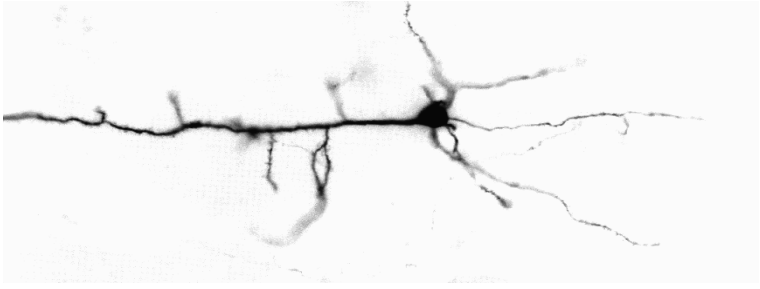


Supplementary figure 1. C108297 and RU486 45' before the stressor lead to reduced corticosterone response to a 0.4 mA footshock. Two way ANOVA show main effects of time and drug, but no interaction. Post-hoc test significant for both compounds at t = 30'.

Chapter

C118335 antagonizes glucocorticoid receptor-dependent effects on gene expression and fear memory consolidation

5



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In preparation

Abstract

Blockade of glucocorticoid effects may be relevant for various disease conditions characterized by excess of glucocorticoid levels, such as Cushing's disease and psychotic depression. However, classical antagonists such as RU486, which also binds the progesterone receptor (PR), may not be sufficiently selective for the glucocorticoid receptor (GR). In addition, RU486 may lead to disinhibition of the HPA axis, thus resulting in higher glucocorticoid levels that counteract its antagonism. Here we investigated the functional profile of a novel selective GR ligand (C118335). C118335 does not bind to the PR, but retains modest affinity for the mineralocorticoid receptor. Our results showed that C118335 induced a unique GR-coregulator interaction profile with preferential recruitment of the Steroid receptor coactivator-1a nuclear receptor box. C118335 antagonized the effects of corticosterone on SGK-1 and FKBP5 expression in the CA1-CA2 region of the hippocampus and attenuated memory consolidation in an inhibitory avoidance test. Finally, we did not find disinhibition of the HPA axis after treatment with C118335. In conclusion, we offer here a proof-of-principle for the efficacy of this compound, which shows a more selective antagonistic profile and may be of interest for the treatment of the effects of hypercortisolemia.

Introduction

Orchestration of appropriate responses to stressors is indispensable for survival. In neuroendocrine realm such responses are largely mediated by the HPA axis and glucocorticoids (1, 2). However, if glucocorticoid responses are excessive or prolonged, vulnerability to psychopathology is enhanced (e.g major depressive disorder) (1). In such cases, antagonism of the glucocorticoid receptor (GR) may be of therapeutic interest (3). GR shows a widely distributed expression pattern and is involved among others in neuroendocrine negative feedback regulation (4) and learning and memory processes (5, 6).

In order to mediate glucocorticoid effects on transcription, the GR, similarly to other nuclear receptors, needs to interact with other proteins, among which several classes of transcriptional coregulators. To date, several hundred coregulators that interact with nuclear receptors have been discovered. They differ in their expression patterns in the brain, as well as in their affinity for different ligand-bound nuclear receptors (7). The differences in expression patterns of nuclear receptors and coregulators in different brain tissues may be the basis for the gene- and tissue-specific effects of glucocorticoids that are often observed in different contexts (8, 9). This variability of nuclear receptor-coregulator interactions may also offer a new approach for neuropharmacological intervention in psychopathology.

Due to the pleiotropic effects of cortisol and corticosterone on diverse processes, full antagonism may not always be desirable, as it may block the pathogenic as well as the beneficial effects of these naturally occurring glucocorticoids. Moreover, the classical GR antagonist RU486 is not specific for the GR, but can also bind the progesterone receptor (PR), resulting in serious adverse effects (10). Finally, full GR antagonism also blocks the negative feedback loop of the HPA axis, thus resulting in even higher levels of circulating glucocorticoids, which may still exert effects via the other receptor of glucocorticoids in the brain, the mineralocorticoid receptor (MR) (11, 12). Therefore, there have been continuous attempts to develop ligands with the highest possible specificity for GR that can also target specific GR-dependent pathways (13-16).

Here, we investigated the effects on the brain of a novel GR ligand (C118335) that shows selectivity for GR over AR and PR, but with modest affinity for MR (17). We studied the effects of this compound on SGK-1, BDNF and FKBP5 expression in the CA1-CA2 region of the hippocampus and the dorsal striatum, stress-related behavior and regulation of the HPA axis. Gene selection was based on known GR-targets and on the involvement of these genes in GR signaling. We found that C118335 had antagonistic effects on glucocorticoid-induced SGK-1 and FKBP5 expression in the brain, and showed mild suppression of the HPA axis after stress. In line with the gene expression findings, it showed antagonistic effects on memory consolidation of an inhibitory avoidance response.

Methods

Peptide interaction profiling: Interactions between the GR ligand binding domain (LBD) and coregulator NR-boxes were determined on a MARCoNI assay. The method has been previously described, in detail, elsewhere (9, 18). Briefly, each array was incubated with a reaction mixture of 1 nM GST-tagged GR-LBD, ALEXA488-conjugated GST- antibody, and buffer F (PV4689, A-11131, and PV4547; Invitrogen, Bleiswijk, the Netherlands) and vehicle (2% DMSO in water), Dexamethasone (DEX; 1 μM), RU486 (1 μM), or C118335 in various concentrations. Incubation was performed at 20 °C in a PamStation96 (Pamgene International, Den Bosch, the Netherlands). GR binding to each peptide on the array, reflected by fluorescent signal, was quantified by analysis of .tiff images using BioNavigator software (Pamgene International).

Animals: 10-14 week old male Sprague-Dawley rats were used. The rats were group housed with food and water available *ad libitum* under a 12:12 dark:light regime. For gene expression studies, 5-7 rats per group were injected subcutaneously with vehicle (90% PEG, 10% DMSO), C118335 (100 mg/kg), followed 30 mins later by an injection of corticosterone (3 mg/kg) or vehicle. Three hours after the second injection animals were sacrificed by an intraperitoneal injection of overdose Euthasol (ASTfarma, Oudewater, the Netherlands) followed by decapitation. Their brains were harvested and snap frozen in isopentane on dry ice and subsequently stored at -80 ^{o}C. Trunk blood was also collected in EDTA coated tubes, centrifuged and plasma collected and stored at -20 ^{o}C until further processing. All experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and experiments were approved by the Local Committees for Animal Health, Ethics, and Research of the Dutch universities involved (DEC protocol: 12167).

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

Punching: 200 μm thick sections were taken on a Leica 3050 cryostat (Rijswijk, the Netherlands) and mounted on uncoated glass slides (Menzel-Gläser, Braunschweig, Germany). Subsequently tissue was punched out from the caudate putamen and the CA1-CA2 region of the dorsal hippocampus using appropriate Harris Uni-core punching needles (Tedpella, Redding, CA, USA).

RNA isolation, cDNA synthesis and qPCR: The samples were homogenized on a TissueLyser II (Retsch Qiagen, Haan, Germany) in 1 ml Trizol, centrifuged and 200 μl of chloroform (Merck KGaA, Darmstadt, Germany) was added to each sample. After centrifugation, the aqueous phase (top phase) was taken and 5 μl of 5 mg/ml linear acrylamide (Ambion, Austin, USA), as a carrier, and 500 μl isopropyl alcohol (Merck KGaA, Darmstadt, Germany) were added, followed by centrifugation and removal of the supernatant. Then, the RNA pellet was washed twice with 75% ethanol (Merck KGaA, Darmstadt, Germany), air-dried and dissolved in demineralized H_2O. The purity and concentration of the RNA samples

were measured on the Nanodrop 1000 (Isogen Life Science, De Meern, The Netherlands). The integrity of the samples was measured on Standardsens chips on a Bio-Rad experion system (Hercules, USA).

For cDNA synthesis, RNA samples were pretreated with DNase (Promega, Madison, USA) to remove potential genomic DNA contamination according to the manufacturer's specifications. For the incubation a MyCycler^{tm} Thermal Cycler (Bio-Rad, Hercules, USA) was used. Subsequently, cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad). Four µl 5x iScript reaction mix, 1 µl iScript reverse transcriptase (RT) and 5 µl nuclease free water were added to each DNase pretreated sample. A control sample without RT treatment was also included in which the 1 µl RT was replaced by 1 µl nuclease free water. The samples were placed in a MyCycler^{tm} Termal Cycler and incubated for 5 minutes at 25^{o}C, 30 minutes at 42^{o}C and 5 minutes at 85^{o}C (11).

Quantitative polymerase chain reaction (qPCR) was performed to measure gene expression in the different brain regions. The efficiency of the used primers was first measured for each gene in each region. To perform the qPCR the FC FastStartDNA Master^{plus} SYBR Green I (Roche Applied Science, Basel, Switzerland) kit was used. 2.5 µl per cDNA sample was added to a mix of 2 µl 5x Sybr green mix, 0.5 µl 10 µM of both the forward and reverse primers (table 1) and 4.5 µl DEPC H_2O to a total volume of 10 µl. For the reactions 20 µl LightCycler Capillaries (Roche) were used placed in a LightCycler Sample Carousel 2.0 (Roche). The carousel was centrifuged on a LC Carousel Centrifuge 2.0 (Roche), subsequently placed in a LightCycler 2.0 (Roche) to perform qPCR. All samples were measured in duplicate. The samples were incubated for 10 minutes at 95^{o}C, followed by 45 replication cycles (10 seconds denaturation at 95^{o}C, 10 seconds annealing at 60^{o}C and 10 seconds elongation at 72^{o}C) and finally a melting curve was made (65^{o}C to 95^{o}C, 0.1^{o}C/s).

Inhibitory avoidance: One-trial inhibitory avoidance training and retention was performed as has been described elsewhere (19), using single-housed male Wistar rats (10-14 weeks of age; Charles River) and a foot-shock intensity of 0.38 mA for 1 s. C118335 (20 or 80 mg/kg) or corticosterone (1 mg/kg) was dissolved in DMSO and administered (100 µl, s.c.) immediately after the training trial, to prevent interference with memory acquisition. Retention was tested 48 h later. A shorter latency to enter the former shock compartment with all four paws (maximum latency of 600 s) was interpreted as weaker memory.

Statistical analysis: To analyze the levels of Cort in the trunk blood a t-test with a significance level of $P<0.05$ between the vehicle and the C118335 treated group was used. In order to determine whether treatment with corticosterone increased the corticosterone-circulating levels a two-way ANOVA was used. For the analysis of the Ct values from the qPCR the mathematical model from Pfaffl (20) was used. Tubulin and ACTB were used as reference (housekeeping) genes. The geometric mean of these two genes was used as the reference value. The Grubbs' outlier test was conducted and outliers were excluded from the analysis. The values were analysed by one-way ANOVA followed by Tukey's post-hoc test with a significance level of $P<0.05$. In the inhibitory avoidance test the Kruskal-Wallis test was used followed by Dunn's test.

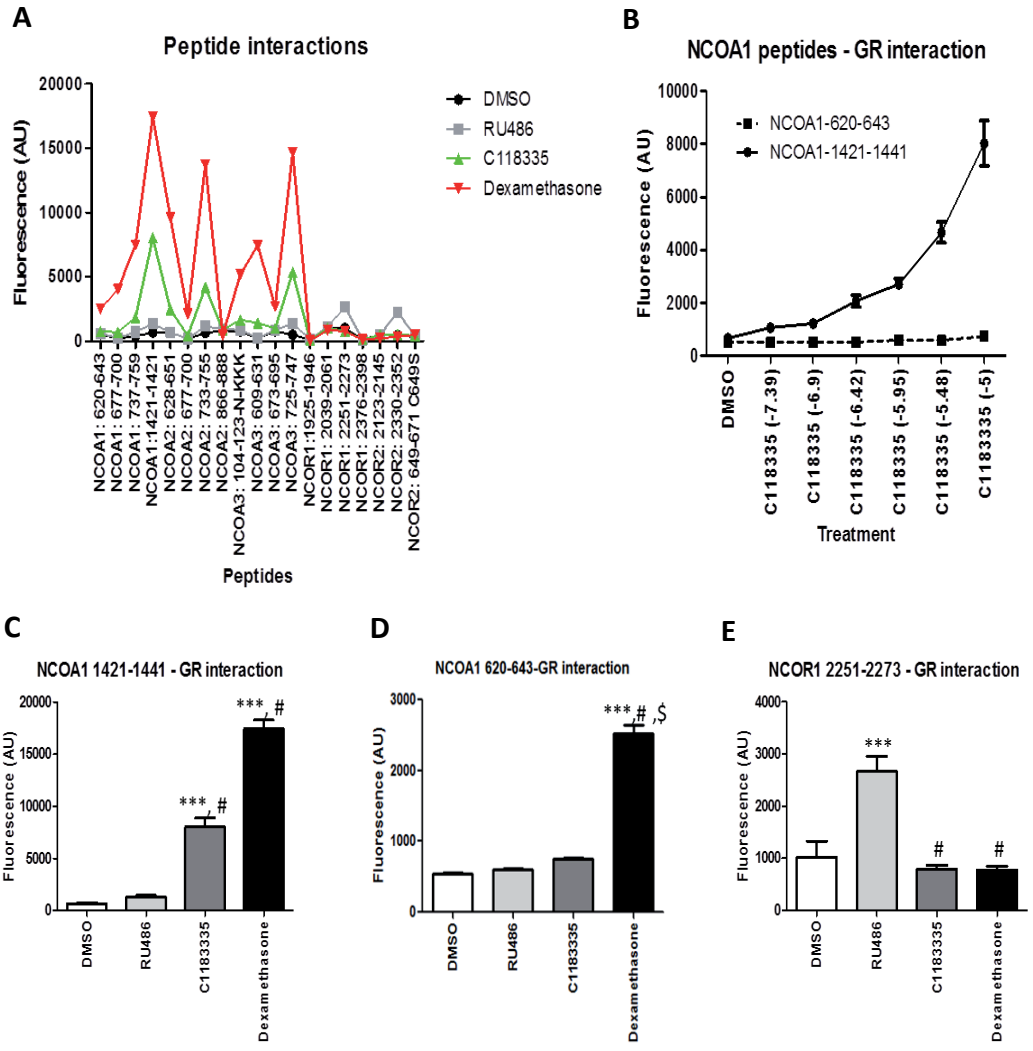

Figure 1. C118335 did not induce as many GR-LBD – coregulator peptide interactions as dexamethasone. However, it induced partial recruitment of the SRC-1a specific NR box-IV (NCOA1-1421-1441). A. Overview of the ligand-induced interactions between GR-LBD and coregulator motifs after treatment with DMSO, the classical antagonist RU486, the novel GR ligand C118335 and dexamethasone. B. C118335 induced GR-LBD – SRC-1 NR-box IV interactions in a dose-dependent manner, while it did not induce considerable GR-LBD- SRC-1 NR-box I (NCOA1-620-643) interactions at any concentration. C. C118335 induced significantly stronger interactions between GR-LBD and SRC -1 NR-box IV than DMSO, though not as strong as the dexamethasone induced interactions (one-way ANOVA, $p<0.0001$, $F_{(3,15)}= 168.6$, tukey's post hoc test: ***, $p<0.001$ compared to DMSO group; #, $p<0.001$ compared to RU486 group. D. C118335 did not induce SRC-1 NR-box I –GR-LBD interaction, unlike dexamethasone: One-way ANOVA: $p<0.0001$, $F_{(3,15)}= 227.6$, tukey's post-hoc test: ***, $p <0.001$ compared to DMSO group, #, $p<0.001$ compared to RU486 group, $, $p<0,001$ compared to C118335 group. E. C118335 did not induce interactions with the corepressor motif NCOR1-2251-2273 : One-way ANOVA : $p< 0.0001$, $F_{(3,15)} = 16.89$, tukey's post hoc test: ***, $p<0.001$ compared to DMSO group; #, $p<0.001$ compared to RU486 group.

Results

C118335 induces a unique GR-LBD – coregulator interaction profile: C118335 generally did not induce as many interactions as dexamethasone (Figure 1a). However, it selectively recruited a number of NR boxes, such as the SRC-1 NR-box IV in a dose-dependent manner (Figure 1b, c), but to a lesser extent than dexamethasone (Figure 1c). On the other hand, C118335 did not induce interactions between the GR-LBD and SRC-1 NR-box I, unlike dexamethasone (Figure 1d) and in contrast to RU486, it did not recruit corepressor motif NCOR1 2251-2273 (Figure 1e). This suggests that the compound will act as an antagonist on most processes that depend on the coregulators represented at the array, but may show substantial partial agonism for others.

Trunk blood corticosterone levels: Animals treated with C118335 had significantly lower corticosterone plasma levels than controls (Figure 2a). As expected corticosterone-treated animals had higher corticosterone plasma levels than the respective control treated groups (Figure 2b). Of relevance for the interpretation of the gene expression data, vehicle animals also had relatively high levels of plasma corticosterone.

C118335 attenuates the corticosterone-induced upregulation of FKBP5 and SGK-1 but enhances BDNF expression, in the CA1-CA2 region of the hippocampus: Treatment with corticosterone in the absence of other ligands resulted in a strong upregulation of FKBP5 expression in the CA1-CA2 region of the hippocampus. Treatment with C118335 resulted in suppression of the corticosterone-induced FKBP5 upregulation (Figure 3a). Similarly,

Table 1. Primer sequences used for qPCR analysis and the expected product sizes.

Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product length (bp)
Actb	TGAACCCTAAGGCCAACCGTG	ACACAGCCTGGATGGCTACG	90
BDNF	GGTCACAGCGGCAGATAAAAAGAC	TTCGGCATTGCGAGTTCCAG	188
FKBP5	CAGAGCAGGATGCCAAGGAA	TCCCATGGTCTGACTCTCG	95
SGK1	AGAGGCTGGGTGCCAAGGAT	CACTGGGCCCGCTCACATTT	129
Tubb2a	GAGGAGGGCGAGGATGAGGCTT	GACAGAGGCAAACTGAGCACCAT	121

C118335 resulted in decreased expression of SGK-1 regardless of glucocorticoid treatment in these adrenally intact animals (Figure 3b). C118335 increased BDNF expression in the hippocampus, but this effect was blocked by corticosterone treatment (Figure 4).

C118335 attenuates the corticosterone-induced upregulation of SGK-1 in the striatum, but it had no effect on FKBP5: C118335 had no effect on FKBP5 expression (Figure 5a). However, SGK-1 expression was upregulated in the striatum after treatment with corticosterone, an effect which was blocked by pretreatment with C118335 (Figure 5b).

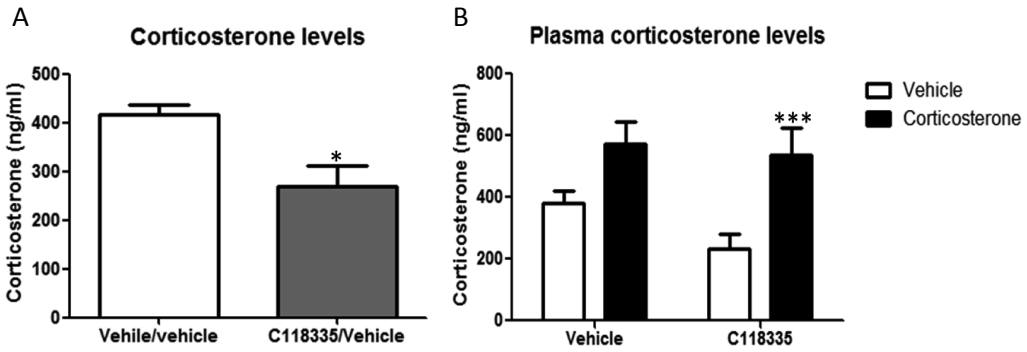

Figure 2. A. Endogenous corticosterone levels after treatment with vehicle or C118335. Rats treated with C118335 had lower plasma corticosterone levels compared to vehicle (two-tailed t-test: $t_{(10)}$=2.346, p=0.04). B. Two-way ANOVA reveled a Glucocorticoid treatment effect, where treatment with corticosterone increased circulating corticosterone plasma levels ($F_{(1,21)}$ = 14.71, p=0.001). N = 5-6 per group. Bonferroni post-hoc test: ***, p<0.001. No Compound effect was found in this analysis.

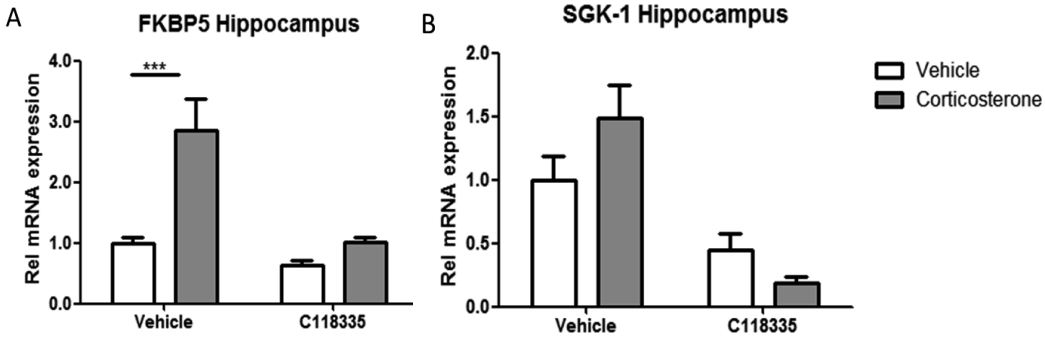

Figure 3: FKBP5 and SGK-1 expression in the hippocampus. A. C118335 can block the corticosterone-induced upregulation of FKBP5 in the hippocampus. There was a significant Glucocorticoid treatment effect ($F_{(1,20)}$ = 16.56, p<0.001), a significant Compound treatment effect ($F_{(1,20)}$ = 16.23, p<0.001) and a significant Glucocorticoid X Compound interaction ($F_{(1,20)}$ = 7.301, p< 0.05). B. C118335 downregulated SGK-1 expression in the hippocampus regardless of glucocorticoid treatment. A compound effect was observed ($F_{(1,20)}$ = 25.71, p<0.001) and a marginally non-significant interaction effect ($F_{(1,20)}$ = 4.127, p<0.06). Bonferroni post-hoc test, ***, p<0.001, n=5-6 per group.

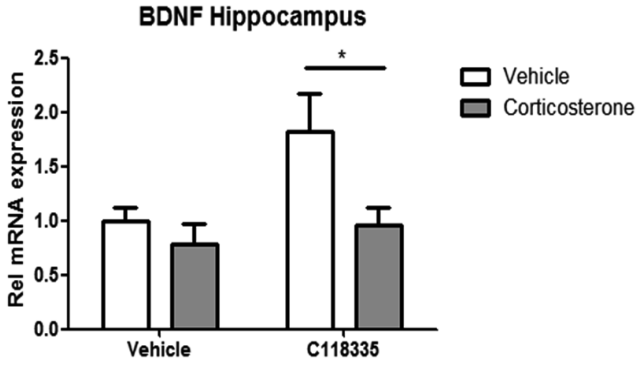

Figure 4: C118335 had effects on BDNF expression in the hippocampus. C18335 treatment resulted in upregulation of BDNF expression, but this effect was blocked by treatment with glucocorticoids. A significant Glucocorticoid treatment effect was found ($F(1,20) = 5.093$, $p<0.05$. The Compound effect was marginally non-significant ($F(1,20) = 4.286$, $p = 0.052$). Bonferroni post-hoc test: *, $p<0.05$, n=5-6 per group.

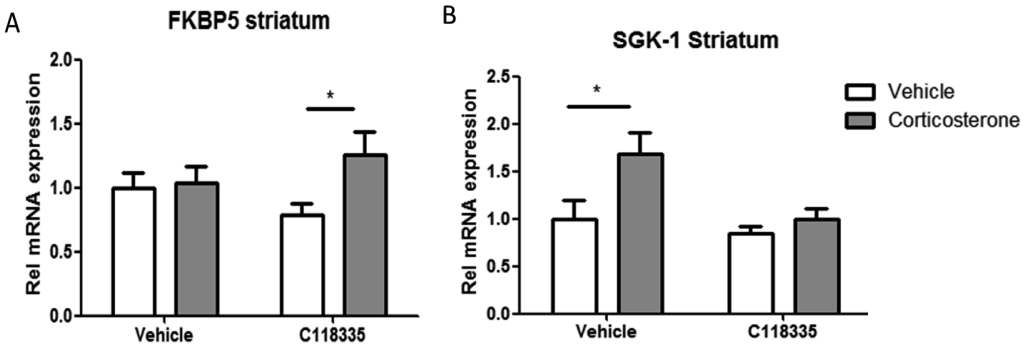

Figure 5: SGK-1 expression in the striatum. A. No Compound effect was found on FKBP5 expression in the striatum ($F_{(1,21)}=0.003$, $p>0.95$. However, a trend towards a Glucocorticoid treatment effect was found ($F_{(1,21)}=3.274$, $p=0.084$). B. C118335 treatment prevented the corticosterone-induced upregulation of SGK-1. There was a significant Glucocorticoid treatment effect ($F_{(1,20)} = 8.197$, $p<0.01$) and a significant Compound effect ($F_{(1,20)} = 8.295$, $p<0.01$.

C118335 resulted in decreased memory consolidation in an inhibitory avoidance test: To determine the effect of C118335 on stress-related behavior we used an inhibitory avoidance paradigm. In order to examine the potential dose responsiveness, we used two doses of the compound (20 and 80 mg/kg). Our results showed that immediate post-training treatment with C118335 resulted in decreased latency to enter the dark compartment in testing two days later only when the higher dose was administered. Treatment with 20 mg/kg C118335 had no effect on the latency to enter the dark compartment at re-exposure to the task (Figure 6).

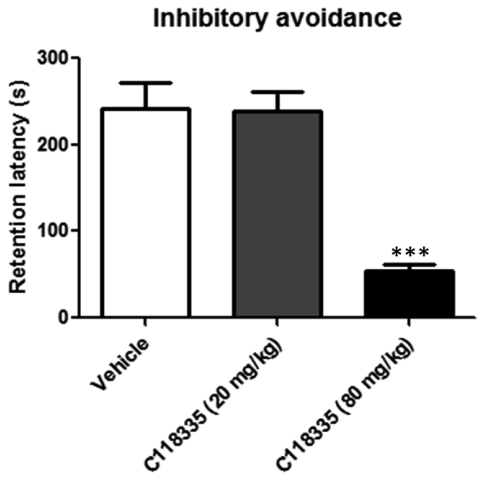

Figure 6: C118335 showed an antagonist effect in an inhibitory avoidance test only at a higher dose (80 mg/kg). The lower dose of 20 mg/kg did not have an effect on the consolidation in the inhibitory avoidance test (Kruskal-Wallis test, Kruskal-Wallis statistic =18.63, $p<0.001$, n=9-11 per group, ***, Dunn's post-hoc test, $p<0.001$).

Discussion

Blocking the undesired effects of glucocorticoids, in both the brain and the periphery, may be of relevance for a number of conditions such as Cushing's disease and psychotic depression (21-24). However, the available antagonists are, however, not specific for GR and their use may be accompanied by adverse effects that decrease their therapeutic potential. RU486, for instance, binds also the progesterone receptor and can induce abortion. Moreover, it disinhibits the HPA axis resulting in even higher cortisol levels, thus counteracting its effects (25). Therefore, it is necessary to search for more specific GR ligands in order to minimize potential side effects.

A possible level of regulation of nuclear receptor function arises from modulation of nuclear receptor-coregulator interactions (7). Recently, we characterized the selective GR modulator C108297 in a wide array of tests (9). This compound behaved both as agonist and antagonist depending on the context and brain region. Its effects were, at least to some extent, attributed to the unique profile of GR-coregulator interactions it could induce (9).

In this study we investigated the effects of the novel GR ligand C118335 on glucocorticoid dependent gene expression in vivo and on fear memory consolidation in an inhibitory avoidance test. In contrast to RU486, this compound does not bind to the progesterone or the androgen receptor, but it retains some affinity for the MR (17). Our findings suggest a strong antagonist profile of C118335, both in gene expression and inhibitory avoidance. Interestingly, however, treatment with C118335 did not disinhibit the HPA axis.

C118335 induced only a modest subset of the dexamethasone-induced GR-LBD - coregulator interactions, with about 50% efficacy for the strongest interactions. Interestingly, however, it recruited the SRC-1a specific NR box (SRC-1 NR-box IV) in a dose-dependent manner. SRC-1a potentiates repression of *crh* promoter activity *in vitro* (26), while it may also be necessary for appropriate *crh* expression regulation by glucocorticoids in the PVN, as well (8). In the current study using adrenally intact rats it is not possible to discriminate between pure antagonistic effects and partial agonism relative to endogenous corticosterone. In contrast to RU486, C118335 did not recruit any corepressor motifs. This suggests differences in the mode of action of C118335 compared to RU486 and the lack of corepressor recruitment may prevent the abrogation of all GR-mediated effects, thus it may lack some of the RU486-associated adverse effects.

Despite the high corticosterone plasma levels of the vehicle group in the present study, additional exogenous corticosterone treatment further increased the corticosterone levels and this increase was accompanied by induced changes in gene expression in the brain. On the other hand, the high corticosterone levels may have masked potential agonistic properties of C118335. C118335 treatment resulted in blockade of corticosterone-induced upregulation of FKBP5 and SGK-1. Both genes are GR-target genes, but also play an important role in mediation of the transcriptional effects of GR. SGK-1 may prolong the GR effects even in the absence of glucocorticoids, while it has been found increased in depressed patients (27). Here we found downregulation of SGK-1 expression below basal levels, which may be indicative of an effect at two levels: a direct effect on SGK-1 expression regulation by GR and an indirect effect on transcriptional activity due to decreased SGK-1 expression (27). On the other hand, FKBP5 may have inhibitory activity on GR function and it is involved in the ultrashort intracellular negative feedback loop of GR activity (28, 29). Although these two target genes exert opposite actions on GR signaling, the fact that the corticosterone-induced upregulation of both was blocked may indicate an overall dampening of the transcriptional effects of GR. Nevertheless, SGK-1 and FKBP5 may also be regulated in a brain region-specific fashion, thus making predictions of the net GR-dependent transcriptional outcome difficult.

BDNF is another gene regulated by glucocorticoids (30-32), also involved itself in GR signaling (32-35). C118335 treatment upregulated BDNF expression in the hippocampus, however, it was not enough to counteract the effect of higher corticosterone levels. The lack of efficacy of corticosterone treatment may reflect the relatively high endogenous corticosterone levels, which may have led to low BDNF expression levels (9, 36). The observed upregulation of BDNF expression in the hippocampus may be of relevance for psychopathology, as similar effects have been reported after treatment with antidepressants in rodents and humans (37-39).

The effects on glucocorticoid levels may be explained by the effects of C118335 on GR-coregulator interactions. C118335 preferentially recruited the SRC-1a-specific NR-box. SRC-1a potentiates the repression of the *crh* promoter and may be involved in the regulation of CRH expression by glucocorticoids in the PVN by the GR (8, 26). Interestingly, in studies in SRC-1 KO mice it has been shown that SRC-1 is involved in negative feedback of the HPA axis at the pituitary and the PVN (8, 40). Considering the relative abundance of the two SRC-1 splice variants, these effects are likely mediated by SRC-1a (41). It remains to be seen whether

this is relevant (and to which extent) in conditions of chronic stress or prolonged hypercortisolemia.

There was a clear antagonist effect of C118335 on fear memory consolidation in an inhibitory avoidance test. This was in line with previous findings, as immediate post-training GR antagonism has been shown to interfere with memory consolidation. The considerable effect of C118335 treatment observed here may also be related to the downregulation of SGK-1 expression below basal levels. Finally, the expected weak antagonism on MR may also be relevant to the effects of C118335 on memory consolidation.

In conclusion, we offer here a proof-of-principle for the efficacy of a novel GR antagonist which, in contrast to RU486, does not bind the progesterone receptor and induces a distinct GR-LBD – coregulator motif interaction profile. Therefore, C118335 may have an interesting novel therapeutic potential in the treatment of hypercortisolemia-induced psychopathology.

Acknowledgments

We would like to thank I.M. Mol for assistance with *in vivo* experiments.

Conflict of interest

HH and is employed by Corcept Therapeutics, and made C118335 available. Corcept financed part of the costs of the experiments. RH is employed by Pamgene Int, who made MARCoNI arrays available for this study.

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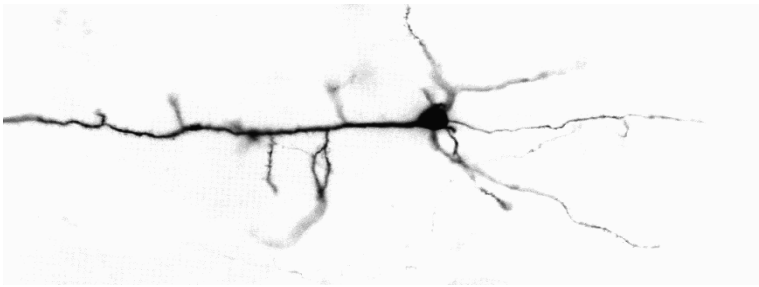
Chapter

6

General Discussion



Discussion

The appropriate orchestration and expression of responses to stressors is crucial for survival and involves the coordination of multiple systems in the brain and the periphery (1-4). The HPA axis plays a central role in the regulation of stress responses via control of glucocorticoid hormone levels. Glucocorticoids, in turn, exert a wide range of effects, including effects on memory, behavior and metabolism, that are mediated by their receptors MR and GR. Importantly, glucocorticoids can block the expression of CRH in the PVN and ACTH in the pituitary, thus controlling their expression via a negative feedback loop (5, 6).

Due to their coordinating effects, the function of GR and MR must be tightly regulated in a tissue-specific fashion rather than simply follow the changes in concentration of their ligands in a uniform way. This tissue-specific regulation may take place at multiple levels, such as the expression of the receptor, the bio-availability of free ligand in plasma, the expression of enzymes that modify the ligand, the expression of other nuclear receptors, the presence of other transcription factors and the expression and availability of coregulators (4). The latter, may create a bottleneck, as competition of nuclear receptors for coregulators may be the limiting factor when multiple signals are received at the same time.

Several coregulators have been shown to be important for brain function and particularly for learning and memory and stress responses. Notable examples, apart from SRC members of the p160 family, are the coregulators of CREB CBP/p300 and pCAF, members of the CREB regulated transcription coactivator (CRTC) family, the coregulators of steroid hormone receptors RIP-140, Ube3a and proteins involved in the SWI/SNF chromatin remodeling complex (7-20). Not surprisingly, mutations or deletions of these coregulators often result in impairments in learning and memory, decreased neuronal plasticity, inappropriate regulation of stress responses or abnormal brain morphology (21).

Here, we studied the importance of coregulator recruitment in relation to stress and effects of glucocorticoids in two different ways: First, we tried to manipulate the sensitivity of the central amygdala to glucocorticoids and interfere with proper stress responses, via induction of alternative splicing of the well-described coregulator and member of the p160 family SRC-1. Secondly, with the use of novel selective ligands of the GR we tried to interfere with GR-coregulator interactions and selectively block a subset of GR-dependent functions while leaving others intact. Subsequently, we studied the effects of these ligands on stress-induced CRH expression, fear-related memory consolidation and GR-dependent gene expression in the brain *in vivo*.

SRC-1 isoform switching in the CeA

First, we showed that AON-mediated exon skipping in the CeA is a feasible technique to modulate splicing of the *NCoA1* gene (22). We compared the immunostimulatory potential of a random 2-O'-Me ribonucleotide with a phosphorothioate backbone, which had no known targets in the murine genome or transcriptome, to saline. Our results showed no differences

between treatments in any of the markers of astrogliosis or microglia activation we used. Previous studies using similar concentrations of AONs as in our study, also reported no immunogenicity, although this might be the case for higher AON concentrations (23). In fact, the 2-O'modification used in the design of the AON may have acted as a Toll-like receptor antagonist, thus decreasing potential immunostimulatory effects (24, 25).

Secondly, we showed adequate uptake of the AONs from neurons and localization of the AONs in the cell nuclei. Both findings were important, because they indicated success in transfecting the desired cell type and cell compartment, although the underlying mechanism remains largely unknown ((26) for an overview of theories that have been proposed regarding the cellular uptake and intracellular trafficking of AONs), it is important that they are taken up and end up in the nucleus, since splicing takes place in the nucleus. Therefore, for any experimental or therapeutic effect of the AONs, this condition should be met.

Finally, we showed that a single injection of AONs targeting the SRC-1e specific exon in the CeA could result in exon skipping and a shift in expression ratio of the two SRC-1 splice variants in favour of SRC-1a, three and seven days post- injection. The expression ratio shift was not accompanied by differences in the expression levels of total SRC-1, indicating that the effects were selective for the SRC-1e specific exon, leaving total expression levels intact.

Taken together, our results showed that exon skipping may be an appropriate technique for interference with gene expression in the brain, either for experimental or therapeutic purposes. In our hands, it was characterized by specificity for SRC-1e, leaving total SRC-1 expression unaltered, as well as GR and SRC-2 expression, limited immunogenicity and high efficiency. Compared to siRNA methods or the use of viral constructs, it may offer the advantage of not causing cell death, since it does not use any intracellular machinery, thus it limits its interference with normal cellular functions (27, 28).

The fact that AONs were still detectable and active seven days after a single injection may be useful for their applications as experimental tools, as it may be possible to avoid more invasive administration to the brain such as cannulation or repeated administration. Although this may still be necessary for longer experimental designs, for our purposes a single injection was sufficient to establish the desired SRC-1a:SRC-1e expression pattern throughout the experiment (23, 29).

Considering that the majority of genes expressed in the brain undergo alternative splicing AON-mediated exon skipping has high potential (30). If one considers also the use of AON-mediated exon skipping to selectively remove exons with known or unknown functions, thus leading to the expression of truncated proteins or internal deletions, the possibilities become endless. Similarly, alternative splicing may also be relevant for therapeutic interventions, either via splice variant selection or by restoration of the reading frame of mutated pre-mRNA molecules. Obviously, there are many more considerations before moving to human use such as safety, administration and efficacy; however, for some disease models AON-mediated exon skipping has shown very promising results (23, 31-33).

Functional consequences of SRC-1 isoform switching in the CeA

As the naturally occurring expression pattern of the two SRC-1 splice variants in the CeA favors SRC-1e (34), we sought to investigate what the effects of a shift of their expression ratio in favor of SRC-1a would be on the regulation of CRH expression by glucocorticoids as well as on stress-related behavior and fear memory. The CeA is an important area for the orchestration of appropriate responses to stressors and acquisition and expression of fear conditioning. GR signaling has been shown to be indispensable for those functions, as local knockdown of GR expression in the CeA results in fear conditioning impairments which can be rescued by ICV administration of CRH (35). In addition, GR knockdown in the CeA results in abrogation of CRH expression regulation by glucocorticoids (35). Moreover, it has been shown that SRC-1 expression in the CeA is necessary for proper regulation of CRH expression by glucocorticoids and normal basal CRH expression in the CeA (36). Finally, the two SRC-1 splice variants appear to have different effects on the regulation of the *crh* promoter; SRC-1a represses the crh promoter, whereas SRC-1e lacks repressive capacity (37).

To test basal anxiety and consolidation of fear memory, we used two well-described paradigms: the open field and fear conditioning, respectively. Subsequently, we tested the effects on SRC-1 isoform switching on the regulation of CRH expression by glucocorticoids in the CeA. Our results suggested that a shift in expression ratio in favor of SRC-1a in the CeA leads to increased locomotion and impairments in a fear conditioning paradigm, as well as abrogation of CRH mRNA induction by chronic exposure to the synthetic glucocorticoid dexamethasone. These findings underline for the first time *in vivo* the importance of SRC-1 for glucocorticoid signaling, as well as the differential effects of the two SRC-1 splice variants on the *crh* promoter. Interestingly, we found a positive correlation between the SRC-1a:SRC-1e expression ratio and the total distance walked in the open field, which may indicate a direct relationship between the expression ratio of the two splice variants locomotor activity.

The most striking effect was the complete blockade of the dexamethasone-induced CRH expression upregulation in the CeA after the expression ratio shift of the two splice variants. Here, it is important to emphasize the difference between the two SRC-1 splice variants in their affinity for the GR; the SRC-1a-specific NR box has higher affinity for the GR than the three central NR boxes (38). Thus, the effects of SRC-1a in the CeA may be amplified due to its higher affinity for the GR, rather than dependent on simple stoichiometry of the two splice variants.

Another open question regards the cause of the observed behavioral differences. The fear conditioning results could be, at least to some extent, explained by the known effects of the two splice variants on CRH expression (36, 37). Kolber et al., showed that GR-dependent expression of CRH in the CeA is necessary for proper acquisition and consolidation of fear conditioning (35). However, we did not find differences in CRH expression after saline treatment (which are expected to be very close to basal levels), therefore, the differences in open field could not be easily explained in relation to CRH expression and function. Similarly, there were no differences in HPA axis reactivity at basal conditions or after stress. Importantly, basal CRH expression in the CeA may not be dependent on GR at all, as shown

by the modest effects observed after adrenalectomy (39). Considering the mode of action of coregulators, it is plausible that there are more GR-target genes differentially regulated by the two SRC-1 splice variants. To cast light to this issue further research is necessary employing broader gene expression analysis techniques such as mRNA microarrays or RNA sequencing to identify those "elusive" genes. In addition, given the interactions of coregulators with other nuclear receptors, such as the estrogen receptor (40), it would be useful to profile the interactions of the two SRC-1 splice variants with other coregulators or pathways of other transcription factors and nuclear receptors. For example, SRC-1 is known to interact with CBP/p300, a coregulator of CREB (41). CREB plays an important role in the activation of the CRH promoter, therefore, it would be essential to understand the extent of interplay between CREB- and GR-dependent transcriptional pathways and the role of the SRC-1 isoforms therein.

In conclusion, splicing modulation and shifting of the expression ratio of naturally occurring splice variants may be of relevance for brain function. Furthermore, manipulation of downstream components of GR signaling may be of relevance for psychopathology, since they offer higher specificity than, for instance GR antagonism or GR knockdown. Finally, it suggests that SRC-1 and its splice variants may be possible targets for manipulation and of therapeutic relevance for psychopathology.

Interactions of liganded GR with coregulators

There is no comprehensive overview of the coregulators that interact with MR and/or GR. Moreover, for known coregulators, we have often little knowledge about the neuromodulatory actions in which they may be involved. The expression of all putative coregulators for MR and GR is available for both mouse and human in databases such as the Allen Brain Atlas (for a number of examples see: (42)). To interpret the expression data in a meaningful way, it is important to know which of the putative coregulators can interact with the receptors. The approach we used in chapters 4 and 5 to investigate the induced interactions by different ligands between the GR and a set of coregulators was the MARCoNI assay. This assay measures one-to-one binding of a given NR to a set of coregulators. The latter are represented as helical peptides of functional NR-box motifs, or their repressor protein equivalent (CoRNR-box), selected from a broad base of literature. This set (>150) of peptides is immobilized in a micro-array format and NR binding is quantified using fluorescently labeled antibody (43). The NR-coregulator interaction profile serves as a sensor for receptor conformation and thus status of the AF-2 of the receptor (44). Functional modulation, e.g. by ligand, mutation or post -translational modification of NRs, recombinant but also in whole-cell lysates (45) can hence be studied by quantification of coregulator interactions. Since this approach involves the use of only the LBD region, we lack relevant information regarding AF-1 (which may also be ligand independent (46)), interactions with other transcription factors (and transrepression activity mediated by them) (47), as well effects on non-genomic GR signaling (48).

Assays like these will be of great assistance to identify relevant coactivators for individual members of the nuclear receptor superfamily. Combining functional interaction data with

expression data like those in the Allen Brain Atlas may bring us a long way to defining the coregulators that are involved in MR and GR signaling in particular brain regions.

Targeting GR with novel GR ligands

Besides targeting directly the expression or splicing of coregulators, it may be useful to modify the interactions between the GR and the coregulators that are present in a certain cellular context. In this regard, pharmacological modulation of the GR may be of particular interest both in the brain and the periphery. Classically, pharmacological manipulations were restricted to the use of agonists or antagonists. However, this approach has some limitations. The use of antagonists such as RU486, for instance in the treatment of the effects of hypercortisolemia, is characterized by some disadvantages which limit their therapeutic potential. One important issue is selectivity for the GR. RU486 binds also the progesterone receptor, thus acting as an abortifacient. There have been several attempts to design ligands with increased affinity for the GR compared to other receptors (49-51). The second important issue is that total GR antagonism may disinhibit the HPA axis, resulting in the elevation of glucocorticoid levels. In addition, it may not be desirable to block all GR-dependent effects, since some of them are beneficial for proper cognitive and memory functions. Hence, the use of selective GR ligands has been attempted to provide more specific modulation of the GR and block certain pathways while leaving others intact. These include attempts to develop GR ligands that retain their anti-inflammatory properties, without effects on metabolism (52-56).

In chapter 4 we profiled the novel selective GR ligand C108297. We found that it induced a unique GR-coregulator interaction profile, resembling features of both agonists and antagonists. In particular, several GR-coregulator interactions were blocked, however, the SRC-1a specific NR box was preferentially recruited. On the other hand, there was no induction of GR-corepressor interactions. We also found mixed effects on gene expression in the brain with both agonistic and antagonistic effects. Notably, there was no disinhibition of the HPA axis, and we found agonistic effects on inhibitory avoidance but antagonism in the effects of corticosterone on adult neurogenesis. C108297 showed mild suppression of post-stress CRH expression levels in PVN, but lacked any effects in the CeA.

In chapter 5 we studied the effects of a novel GR ligand (C118335) on gene expression in the brain and inhibitory avoidance behavior. This compound induced in vitro a GR-coregulator interaction profile which resembled that of an antagonist, with some notable exceptions, such as the preferential recruitment of SRC-1 NR-box IV. Moreover, it was shown to be efficient against olanzapine-induced increase of body weight in rats, suggesting an RU486 like efficacy (57). We found that C118335 antagonized corticosterone-induced gene expression in the brain, and attenuated the consolidation of an inhibitory avoidance test. Interestingly, C118335 did not disinhibit the HPA axis. Taken together, our data suggest that C118335 may be an improved GR antagonist compared to RU486. The two novel ligands that were tested showed distinct molecular interactions in the Marconi assay, which partly explained their *in vivo* efficacy. However, we are not able to predict the pharmacology of the compounds with a single assay, because the receptors can act via at least three distinct action mechanisms that

may be separately targeted. First, non-genomic signalling can take place either via membrane-associated variants of the classical receptors (58, 59), or via cytoplasmic receptors (60). Second, transcriptional signalling can occur in a manner that depends on interaction with other transcription factors. AP-1 and NF-kB are well-known examples, but which interactions bear most relevance for the brain is mostly unknown (61). Thirdly, GR and MR can bind to the DNA in their classical GRE-dependent manner, and subsequently interact with any of tens of other transcription factors and coregulator proteins that constitute the actual signal transduction of the receptors.

MR and GR always mediate hormone actions in a given cellular context – which may affect fear, memory, reward, or other aspects of cognitive and emotional processing, depending on the demands on the organism. The receptors do so via cross-talk with other signalling cascades that are activated, for instance, by glutamatergic or noradrenergic excitatory input. Much of the cross-talk may take place at the level of transcriptional coregulators that are common to the signal transduction of MR/GR and the cAMP-coupled transcription factor CREB (41). Furthermore, cross-talk may also take place at the DNA level, either by one factor pioneering the binding site of another, or by binding to the same coregulator or transcription factor (46, 62).

In order to make progress, basic knowledge of possible coregulators of MR and GR can be combined with the comprehensive expression databases that are available. The first reports on genome-wide DNA targets by ChIP-seq (61, 63) should be complemented with similar profiles of coregulators. However, the outcome of such experiments will depend on the particular context the animal is in (see (64) for an example of liver targets of GR in fed or fasted state). Of course, a better use of available transgenic (knock-in) mouse lines that allow functional dissection of GR (and MR) signalling pathways (such as the $GR^{dim/dim}$ (65) or CBP^{KIX} mice (66), or mice with altered GR:MR expression ratio (67)) may be used to a larger degree. Lastly, the selective receptor modulators that are already available, and of which the mechanism is understood, may be used to distinguish between different signalling pathways, using straightforward pharmacological approaches. The useful application of existing SGRMs, and the development of novel selective modulators for both MR and GR may not only help to understand how glucocorticoids modulate brain function, but also may be used in future for therapeutic use in stress-related psychopathology. In this regard, our data suggest that C108297 and C118335 may be good candidates.

Modulation of nuclear receptor function via targeting of coregulators

Although the work described here has focused on GR-function, the common mechanism of action of nuclear receptors allows for generalization of the model. Because of the broad expression of these receptors in many cell types and tissues, targeting with classical agonists/ antagonists has been often proven suboptimal due to side effects. However, many coregulators show a more specific and limited expression pattern such as SRC-3 in the brain where it is expressed mainly in the hippocampus, cortex and olfactory bulbs and the differential distribution of the (68, 69) splice variants of SRC-1 (34). Moreover, selective recruitment of

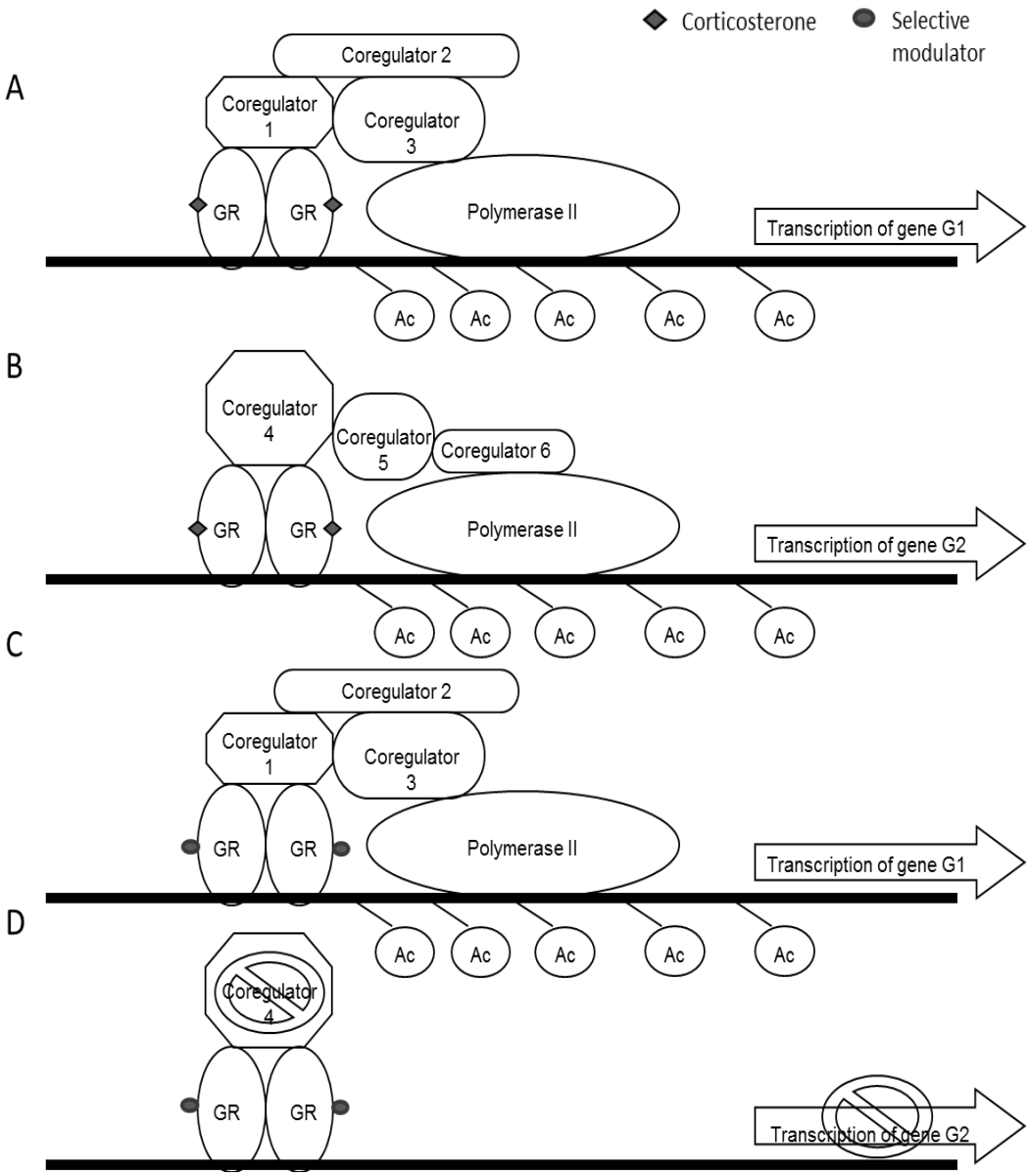


Figure 1. Proposed model of the function of selective modulators. A-B. The glucocorticoid receptor is bound to its natural ligand corticosterone, dimerized and on chromatin. It can recruit a number of different coregulators that interact directly with it (1,4), which can, in turn, recruit other coregulators (2,3,5 and 6). These GR-coregulator complexes can then stabilize the transcriptional machinery, acetylate histones and activate the transcription of genes G1 and G2. C-D. When GR binds a selective modulator it only induces/allows interaction with coregulator 1, but not 4. Therefore, only transcription of G1 takes place, while the transcription of G2 is blocked.

coregulators may change the directionality of the transcriptional effects of nuclear receptors towards the transactivation or transrepression of specific genes. Therefore, the use of ligands that result in specific recruitment of coregulators may be advantageous. An example that illustrates this principle is the use of the GR ligands C108297 and C118335 that show antagonistic effects without disinhibiting the HPA axis.

Alternatively, it is possible to modulate the expression of coregulators locally. Because of the plethora of interactions between coregulators and various nuclear receptors, global deletion of coregulators may not be ideal since it would affect different nuclear receptor-dependent pathways and may induce the development of compensatory mechanisms (36, 70). Even relatively subtle manipulations may have broader effects and this is something that needs to be taken into account for both experimental and therapeutic approaches.

Conclusions

From the research described here the following conclusions can be drawn:

-Antisense mediated exon skipping is a feasible method to study the function of genes locally in the brain.

-Shifting of the SRC-1a:SRC-1e expression ratio in favour of SRC-1a changes glucocorticoid sensitivity in the CeA, as measured by abrogation of the dexamethasone-induced upregulation of CRH expression in this cell group and the impaired fear-motivated behavior.

-C108297 is a selective modulator of the GR with mixed agonist and antagonist function that can antagonize some of the GR-dependent effects without leading to disinhibition of the HPA axis.

-C118335 is a novel GR ligand with a mainly antagonistic profile antagonizing GR-dependent effects on gene expression in the brain and impaired consolidation of fear memory.

-The approaches described here may offer new possibilities for the targeted modulation of GR -dependent effects in the brain.

Future perspectives

Despite the work described here, several questions remain unanswered. Future research should be oriented to cast light on the function of the SRC-1 splice variants in response to chronic stress and particularly whether this manipulation in the CeA would result in alterations of HPA function. In addition, since most of the *in vivo* work regarding SRC-1 function has been performed on SRC-1 KO animals which develop well-documented compensatory mechanisms, it would be worthwhile to attempt to interfere with total SRC-1 expression either via virally-mediated knockdown or with the use of AONs. This strategy would permit to investigate the effects of SRC-1 ablation on GR-signaling in the absence of compensatory mechanisms. Another relevant open question is the function of SRC-1 in response to stress in other brain region beyond the CeA.

At a different level there are outstanding questions regarding the gene targets of each splice variant/coregulator and which protein cocktail is recruited to each particular context. There has been success recently in developing ligands that recruit coregulators in a selective and specific manner (71). Therefore, knowledge of coregulator recruitment to the promoters of certain genes may assist the development of ligands that can affect the expression of genes with high specificity depending on cellular context.

Coregulators can be involved in epigenetic regulation of gene expression either via own activity or via recruitment of appropriate proteins. Thus, studying their epigenetic effects in relation to the changes that appear after exposure to stress (72, 73), early life adversity (74) or acquisition, consolidation and recollection of traumatic memories (75) may provide a new level of possibilities for regulation.

Finally, development of new selective GR or MR modulators, and better characterization of the currently available molecules is promising to open new avenues for the successful treatment of stress-related psychopathology.

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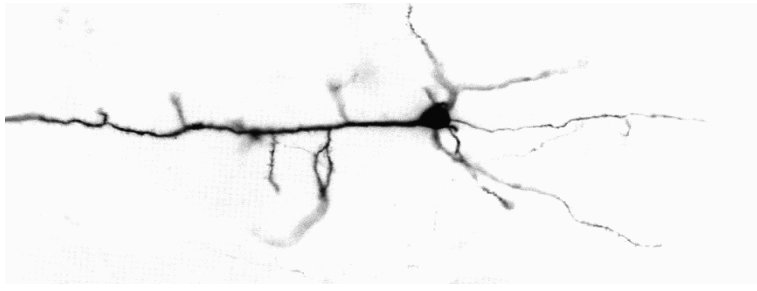
Chapter

7

Summary

Samenvatting



Summary

The hypothalamus-pituitary-adrenal (HPA) axis and its glucocorticoid end product orchestrate the stress response, which is crucial for adaptation and survival. The main effectors are glucocorticoid hormones, which act via by the mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) and involves modulation of gene expression. Here we focus on the GR. One target gene of the GR is the *Crh* gene, which is expressed in the paraventricular nucleus of the hypothalamus (PVN) and the central nucleus of the amygdala (CeA). In the PVN, GR mediates the feedback action of the glucocorticoids on stress-induced CRH synthesis and release, while in the central amygdala the glucocorticoids are involved in the regulation of expression of emotional states.

Given the pleiotropic effects of glucocorticoids, treatment with GR agonists or antagonists may be accompanied by side effects. Therefore, the ability to modulate specific GR-dependent pathways may provide an opportunity to develop more selective drugs. One possibility of increased selectivity might be to target proteins that interact with the GR.

In order to mediate the genomic effects of glucocorticoids the GR often needs to operate in synergy with other proteins (coregulators) that are involved in transcriptional modulation. Steroid receptor coactivator 1 (SRC-1) is a coregulator of the GR that is necessary for the regulation of *Crh* expression. Its two isoforms SRC1a and SRC1e are encoded by the *Ncoa1* gene. SRC-1a lacks a SRC-1e specific exon. The two isoforms differ in their activities and distribution in the brain: SRC-1a is more abundant in the PVN and can potentiate repression at the *Crh* promoter, whereas SRC-1e is more abundant in the CeA and lacks repressive capacity.

In this thesis studies are reported aimed to modulate the function of the GR by targeting GR-coregulator interactions. To achieve this goal, we used two different approaches. Firstly, we manipulated the splicing of SRC-1 with antisense oligonucleotides (AONs) administered in the CeA to change the relative expression of the two SRC-1 splice variants. Secondly, we used two novel GR ligands that allowed certain GR-coregulator interactions while preventing others, thus resulting in a mixed GR-coregulator interaction profile, which exhibited a spectrum of both agonist and antagonist activities.

In **chapter 1** the role of the HPA axis was introduced with focus on the effects of glucocorticoids on the regulation of *Crh* expression and the function of the amygdala. Moreover, we introduced the possibility to manipulate gene expression and splicing by antisense oligonucleotides for the study of the importance of GR-coregulator interactions.

In **chapter 2** we described the validation of the antisense-mediated exon skipping of SRC-1 *in vivo* in the CeA, in terms of cellular uptake, efficacy and potential immunostimulatory effects. After a single injection with either a control AON or saline in the mouse CeA, we investigated the uptake by neurons and possible immune responses induced by this treatment in the brain. The results showed that AONs were readily taken up by neurons in the brain and there were no differences between AONs and saline with regard to the elicited immune responses

following a single injection in the CeA.

Subsequently, we investigated the effects of an AON targeting the SRC-1e specific exon on the expression ratio of the two SRC-1 splice variants. Using laser microdissection, the cells that had taken up AONs were collected and the expression of SRC-1a, SRC-1e, total SRC-1 and GR was quantified with qPCR. Our results showed that the SRC-1e specific AON treatment resulted in an increased SRC-1a:SRC-1e expression ratio, three and seven days post-injection. This shift in favour of SRC-1a was not accompanied by differences in expression of total SRC-1, SRC-2 or GR in the CeA.

These results indicate that AON-mediated exon skipping is an efficient method to manipulate the splicing of the *NcoA1* gene with limited adverse effects.

In **chapter 3**, we described the effects of shifting the expression ratio of SRC-1a:SRC-1e in favour of SRC-1a, in the CeA, on behavior and regulation of *Crh* expression by glucocorticoids. We found that animals injected with the AON targeting the SRC-1 specific exon showed higher locomotion in an open field test, which was positively correlated with the SRC-1a:SRC-1e expression ratio. We also observed impaired contextual fear memory consolidation in a fear conditioning paradigm. The differences in behavior were observed despite the lack of effects on HPA axis activity either at basal conditions or after exposure to a stressor. Moreover, SRC-1 exon skipping completely blocked the dexamethasone-induced *crh* expression in the CeA. The expected downregulation of *crh* expression in the PVN in response to dexamethasone was still present suggesting that an altered SRC-1a:SRC-1e expression ratio in the CeA does not affect *Crh* expression in the PVN.

The effects on behavior, particularly on fear memory consolidation, and (foremost) the abrogation of dexamethasone-induced upregulation of *Crh* expression in the CeA suggest a differential sensitivity of the CeA to glucocorticoids as a result of the manipulation of SRC-1 splicing. In conclusion, targeted changes of the available pool of coregulators may result in preferential activation or repression of specific pathways in the brain.

In parallel, we tried to selectively modulate glucocorticoid responsive pathways with pharmacological manipulations of the GR. In **Chapter 4** the experiments were reported with a novel selective GR ligand (C108297). We tested the effects of C108297 on GR-coregulator interactions, gene expression in the hippocampus, HPA axis activity, *Crh* expression in the CeA and PVN, as well as adult neurogenesis and fear memory consolidation. We found that C108297 showed agonism and antagonism, but in different pathways. It induced a mixed GR-coregulator interaction profile, allowing some of the interactions while inhibiting others. C108297 had agonistic effects on fear memory consolidation, as well as weak agonistic effects on the regulation of the HPA axis in response to stress. On the other hand, C108297 showed antagonism on the expression of Drd1a in the hippocampus and it blocked the effects of corticosterone treatment on adult neurogenesis. Taking into account the mixed properties of C108297, we can conclude that the compound acts as selective modulator rather than an agonist or antagonist.

The pharmacological profile of C108297 suggests a potential improvement over the current

treatment regimens with GR antagonists. Its lack of affinity for other nuclear receptors may exclude some of the adverse effects associated with the current GR antagonists such as RU486. Moreover, the mixed agonist and antagonist effects indicate the differential impact of C108297 on different GR-dependent pathways, which may also result in a more specific regulation. Taken together, the current results provide a proof-of-principle for the use of selective GR ligands to limit the effects of hypercortisolemia.

In **chapter 5**, the findings with another novel GR ligand (C118335) were described. C118335 lacks affinity for the progesterone receptor, but has retained low affinity for the MR. C118335 induced a distinct GR-coregulator interaction profile. Most of the interactions were blocked. However, in contrast to the classical antagonist RU486, it did not recruit corepressors, but induced, to some extent interactions with SRC-1 NR-boxes. Interestingly, C118335 induced considerable interactions with the SRC-1a specific NR-box. At the functional level, C118335 had antagonistic effects on the regulation of gene expression by glucocorticoids in the hippocampus and the striatum, while it impaired fear memory consolidation. Despite its antagonist properties, C118335 did not lead to disinhibition of the HPA axis under mild stress.

Similarly to C108297, C118335 may also represent an improvement to current therapeutic agents in relation to hypercortisolemia, although the effects of the two compounds differ significantly, particularly regarding their effects on memory consolidation and gene expression. Nevertheless, the weak MR affinity of C118335 may also be relevant in such conditions.

Finally, in **chapter 6**, we attempt a synthesis of the concepts presented in this thesis. A model emerges where GR-coregulator interactions have a prominent role in the modulation of GR-dependent pathways. These interactions can be, in turn, modulated in two different ways: either via manipulation of the availability of coregulators or their splicing (chapters 2 and 3), or by treatment with ligands that can alter the recruitment of coregulators by the GR (chapters 4 and 5). In this process, antisense-mediated manipulation of splicing was shown to be an effective experimental tool to study gene function in the brain (chapter 2). Moreover, a step was made towards the clarification of the *in vivo* role of the two SRC-1 splice variants in relation to regulation of *crh* expression and fear memory consolidation (chapter 3).

In addition, two novel compounds were tested, both of which had distinct properties compared to GR agonists or the GR antagonist RU486. C108297 (chapter 4) acted as a selective modulator with agonist as well as antagonist properties. C118335 (chapter 5) showed antagonism in most functions tested. In conclusion, the approaches described here may offer new possibilities for the targeted modulation of GR-dependent pathways in the brain.

Samenvatting

Glucocorticoïd hormonen reguleren als eindproduct van de hypothalamus-hypofyse-bijnier (HPA) as de reactie op stress die cruciaal is voor aanpassing en overleven van het organisme. Deze hormonen oefenen doorgaans hun werking uit door binding aan mineralocorticoïd receptoren (MR) en glucocorticoïd receptoren (GR) via modulatie van genexpressie. Ons werk heeft vooral betrekking op de effecten die via de GR tot stand komen.

Omdat glucocorticoïd hormonen vele processen in het hele lichaam beïnvloeden, kan toediening van GR agonisten (activatoren) en antagonisten (blokkers) met veel bijwerkingen gepaard gaan. Modulatie van specifieke GR-afhankelijke signaaltransductie biedt een mogelijkheid om selectief werkende glucocorticoïden te ontwikkelen. Daartoe zouden eiwitten, die specifieke glucocorticoïd effecten bij GR-activatie tot stand brengen, beïnvloed moeten worden. Voor de GR effecten die via gentranscriptie verlopen is daarvoor de klasse van *coregulator* eiwitten van groot belang.

Het eiwit Steroïd Receptor Coactivator-1 (SRC-1) is zo'n coregulator. SRC-1 is van belang gebleken voor specifieke effecten die via GR verlopen in de hersenen. Het betreft regulatie van het *Crh* gen, dat codeert voor *corticotropin releasing hormone* (CRH) dat de reactie op stress orkestreert. In de nucleus paraventricularis van de hypothalamus leidt GR-activering tot onderdrukking van CRH, in het kader van negatieve terugkoppeling binnen de HPA as. In de centrale nucleus van de amygdala leidt GR-activering juist tot stimulering van CRH productie, en dit is van belang voor expressie van emoties. Zonder SRC-1 vindt noch de stimulatie noch de remming van CRH door glucocorticoïden plaats.

Het SRC-1 eiwit komt in twee varianten voor, SRC-1a en SRC-1e. Dit zijn zogenaamde *splice* varianten van hetzelfde gen, het *Ncoa1* gen (voor 'nuclear receptor coactivator 1'). Het vóórkomen van deze twee varianten verschilt tussen hersengebieden. SRC-1a is veel aanwezig in de hypothalamus, waar het de expressie van het *Crh* gen onderdrukt. SRC-1e komt relatief meer voor in de amygdala en blijkt niet in staat tot onderdrukking van het *Crh* gen.

De doelstelling van het onderzoek dat in dit proefschrift beschreven is, was om de functie van GR te moduleren door de interactie met specifieke coregulatoren te manipuleren in de hypothalamus en amygdala van proefdieren. Hiertoe zijn twee benaderingen gebruikt. Ten eerste hebben we de verhouding tussen de hoeveelheid SRC-1a en SRC-1e gemanipuleerd met *'exon skipping'*. Kleine stukjes DNA (*antisense oligonucleotiden* of AONs) zijn hiertoe toegediend in de amygdala van muizen om het 'splicing' proces te beïnvloeden en daarmee de GR-transductie sterker via de SRC-1a variant te laten verlopen. Ten tweede hebben we twee nieuwe synthetische steroïden gebruikt die selectief binden aan de GR. Waar GR bezetting door lichaamseigen hormonen ('volle agonisten') leidt tot een groot aantal GR-coregulator interacties, staan de twee nieuw gebruikte verbindingen maar een klein aantal van dergelijke interacties toe. Dat leidt tot een GR-coregulator profiel dat het midden houdt tussen dat van een agonist en een antagonist van de receptor. Daarmee bootsen deze stoffen sommige aspecten van glucocorticoïdwerking na, maar kunnen ze andere effecten blokkeren.

In **hoofdstuk 1** is de rol van de HPA-as bij aanpassing aan stress beschreven, met nadruk op de effecten die glucocorticoïd hormonen hebben op de regulatie van het *Crh* gen, en de functie van de amygdala. Bovendien wordt het principe van manipulatie van genexpressie via 'exon skipping' besproken in het kader van het onderzoek naar stress en GR functie.

In **hoofdstuk 2** is de werkzaamheid en eventuele ongewenste bijwerkingen beschreven van *exon-skipping* na AON-behandeling in het muizenbrein. Na één injectie van AONs in de centrale nucleus van de amygdala, zijn de effecten 3, 7 en 14 dagen na de injectie vastgesteld. De AONs bleken opgenomen te worden in de zenuwcellen en er werd niet meer schade of aspecifieke weefselreacties waargenomen dan wanneer er een fysiologisch zout oplossing toegediend werd. De bepaling van de werkzaamheid werd gedaan door met de *laser microdissectie* techniek kleine hoeveelheden weefsel te verzamelen, waarin door fluorescente de opname van AONs gemarkeerd was. In dat weefsel werd de expressie (het vóórkomen) van mRNA coderend voor de relevante SRC-1 varianten en voor totaal SRC-1 mRNA bepaald met de kwantitatieve PCR techniek. De resultaten lieten zien dat het zinnig is om de *exon-skip* methode te gebruiken teneinde de rol van SRC-1 varianten in het muizenbrein te onderzoeken.

In **hoofdstuk 3** zijn vervolgens de effecten beschreven van een veranderde verhouding tussen de SRC-1a en SRC-1e varianten in de amygdala (ten gunste van SRC-1a) op stress-gerelateerd gedrag *per se*, en op de gevoeligheid van de centrale amygdala voor glucocorticoïd hormonen wat betreft expressie van het *Crh* gen, en gedragseffecten van deze hormonen.

De dieren hadden na behandeling met AONs veranderde gedragsreactiviteit in een 'open veld' test, en ook minder herinnering aan een aversieve situatie in een klassiek conditioneringsexperiment. Deze gedragsveranderingen gingen evenwel niet gepaard met verschillen in hormonale stressreactie. Opmerkelijk was dat na AON behandeling de dieren in het geheel niet meer reageerden op behandeling met glucocorticoïden, wat betreft regulatie van het *Crh* gen in de amygdala. Ook gedragsveranderingen die in controle-dieren gezien werden na hormoonbehandeling traden niet op na *exon-skipping*.

Deze resultaten laten zien dat de beschikbaarheid van coregulatoren bepalend is voor de effecten van glucocorticoïden op stress-circuits in het brein. Omdat coregulatoren specifiek betrokken zijn bij een deel van de effecten die uitgeoefend worden via de GR, betekent dit dat de effecten van het glucocorticoïd hormoon cortisol *gestuurd* kunnen worden door manipulatie van de beschikbaarheid van coregulatoren.

In **hoofdstuk 4** is als alternatief voor de *exon-skipping* de farmacologische aanpak beschreven. Hiertoe is een nieuwe selectieve ligand van de GR gebruikt, de synthetische verbinding C108297. Aan de hand van de moleculaire interacties die optraden tussen de GR en verschillende coregulator eiwitten, kon voorspeld worden dat deze stof een zogenaamde *selective modulator* is, die zowel agonisme als antagonisme kan vertonen, afhankelijk van het specifieke proces. Hier is dus niet de beschikbaarheid van coregulatoren in de cel bepalend, maar de affiniteit van de door ligand gebonden receptor voor specifieke coregulatoren.

C108297 bleek inderdaad zowel als agonist als antagonist te werken op GR-afhankelijke genexpressie in de hippocampus van de rat. Er was sterk agonisme bij consolidatie van

angstherinneringen, zwak agonisme op de activiteit van de HPA-as, maar functioneel antagonisme op *Crh* expressie in de amygdala en neurogenese in de hippocampus.

Dit zijn de eerste resultaten die laten zien dat 'selectieve modulatoren' van de GR werkzaam kunnen zijn in hersengebieden die betrokken zijn bij de aanpassing aan stress. Zulke stoffen kunnen nuttig zijn om de effecten van glucocorticoïden beter te begrijpen, maar ook om – waarschijnlijk eerder dan de AONs die gebruikt werden in de eerder beschreven proeven – in klinische omstandigheden met een mate van selectiviteit GR-afhankelijke processen te beïnvloeden bij stress-gerelateerde ziekte.

In **hoofdstuk 5** is een andere nieuwe GR ligand, C118335 beschreven. Ook deze stof induceert een uniek interactie-profiel tussen de GR en haar coregulatoren. De interacties lijken op die van de meest gebruikte antagonist (mifepristone of RU486), maar suggereren een hogere mate van agonisme via interacties met enkele coregulatoren. Bovendien heeft C118335 ook affiniteit voor de MR.

Functioneel werkte C118335 met name als antagonist wat betreft regulatie van klassieke GR-afhankelijke genen in de hippocampus en het striatum. De stof had een verrassend sterk remmend effect op consolidatie van angstherinneringen. Ondanks dit antagonisme, werd geen ontremming van de HPA-as waargenomen.

C118335 is daarmee een 'selectieve modulator' met een meer antagonist-achtig werkingsprofiel, die evenwel geen duidelijk antagonisme laat zien op de HPA-as. Ook kunnen stoffen als deze experimenteel en – mogelijk – klinisch gebruikt worden om ongewenste effecten van hoge cortisolspiegels tegen te gaan, waarbij een groot aantal zowel MR- als GR afhankelijke effecten geblokkeerd zullen worden.

Ten slotte heb ik getracht in **hoofdstuk 6** tot een synthese te komen van de concepten die in eerdere hoofdstukken gepresenteerd zijn. Centraal hierbij is het idee dat GR- (en MR-) coregulator interacties selectief te activeren zijn, en dat daarmee gewenste en ongewenste effecten van glucocorticoïden te scheiden zijn, ook die effecten die via één receportype gemedieerd worden. Dit principe is op twee manieren aangetoond.

Eén specifieke manier is om coregulator-beschikbaarheid te beïnvloeden via *exon-skipping*, lokaal in de hersenen. De specificiteit betreft hier echter met name het GR-signaal dat beïnvloed wordt, omdat de coregulatoren vaak ook voor andere signaaltransductie processen van belang zijn. Een andere meer grofmazige manier is het gebruik van selectieve receptormodulatoren. Hierbij wordt van alle mogelijke mechanismen die via de receptor verlopen een deel geactiveerd, en een ander deel niet. Deze laatste benadering is misschien minder specifiek dan het manipuleren van één bepaalde coregulator, maar waarschijnlijk gemakkelijker toe te passen in het onderzoek en – wellicht – de kliniek. Beide benaderingen zijn evenwel uitermate geschikt om de rol van MR en GR bij aanpassing aan stress te onderzoeken, en bieden derhalve duidelijke aanknopingspunten om nieuwe geneesmiddelen te ontwikkelen die van betekenis kunnen zijn bij de behandeling van stress- en glucocorticoïd-gerelateerde ziekten.

Addendum

Publication List

Curriculum Vitae

Travel Grants - Awards

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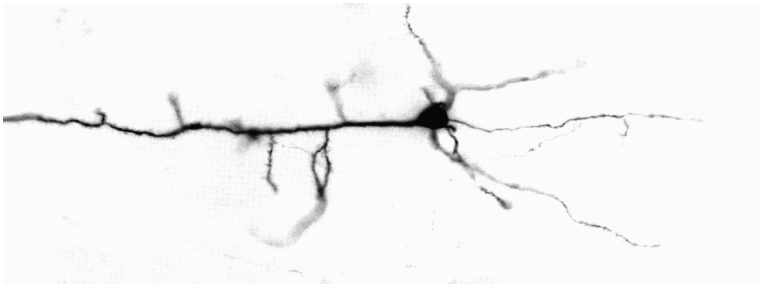

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Curriculum Vitae

Ioannis Zalachoras was born on 18th April 1984, in Thessaloniki, Greece. In 2002 he graduated from the 16th Lyceum (high school) of Thessaloniki and started his studies at the School of Biology, Faculty of Science, Aristotle University of Thessaloniki. During his Bachelor studies he followed the specialization "Molecular Biology, Genetics and Biotechnology" and completed a research internship at the Laboratory of Animal Physiology, Department of Zoology, School of Biology, Faculty of Science, Aristotle University of Thessaloniki on the project "A comparative study of the effects of five terpenes (linalool, fenchone, p-cymene, a-pinene, cineol) on the isolated sciatic nerve of the frog *Rana ridibunda"* under the supervision of Prof. Dr. George Theophilidis. In July 2007 he obtained his Bachelor's degree in Biology.

Following his graduation, he moved to Nijmegen, the Netherlands to enroll in the Master's program "Cognitive Neuroscience" at the Faculty of Social Sciences, Radboud University Nijmegen and followed the track "Neurocognition". From September 2008 till July 2009 he worked as intern at the Department of Molecular Animal Physiology, Donders Institute of Brain, Cognition and Behaviour, Nijmegen Centre for Molecular Life Sciences, Faculty of Science, Radboud University Nijmegen on the research project "Morphological and molecular dissection of the APO-SUS/UNSUS rat model for neurodevelopmental disorders" under the supervision of Prof. Dr. Gerard Martens and Dr. Michel Verheij. After the completion of this internship he obtained his Master's degree in Cognitive Neuroscience.

In September 2009, he embarked on the PhD project "Glucocorticoid receptor effector mechanisms for regulation of stress pathways in the brain" under the supervision of Dr. Onno C. Meijer and Prof. Dr. E. Ron de Kloet, initially at the Division of Medical Pharmacology of the Leiden/Amsterdam Center for Drug Research, Faculty of Science, Leiden University and after the closure of the Division of Medical Pharmacology (June 2012) at the Department of Endocrinology at the Leiden University Medical Center, until April 2014. The results of this project are reported in the present thesis.

Travel Grants - Awards

Participated in the paper that received the Top Paper award in the 2014 ENP meeting "Knockdown of the glucocorticoid receptor alters functional integration of newborn neurons in the adult hippocampus and impairs fear-motivated behavior" *Molecular Psychiatry 2013 Sep;18(9):993-1005.*

Received a FENS-Forum travel grant to attend the 9th FENS forum, Milan, Italy, in 2014.

Awarded best abstract prize Dutch Endocrine Society, NVE/ESE Basic Endocrinology course, Amsterdam, the Netherlands, 15-17/01/2014.

Received a Leiden University Fund/Nypels van der Zee travel grant to attend the 43rd Society for Neuroscience annual meeting, in 2013.

Received a travel grant from the Dutch foundation for Pharmaceutical Sciences to attend the 45th European Brain and Behaviour Society meeting, in Munich, Germany, in 2013.

Received a travel grant from the Dutch Foundation for Pharmaceutical Sciences to attend the workshop "Conceptual issues in stress research", in Erice, Italy, in 2011.