



Universiteit
Leiden
The Netherlands

Targeting the brain under stress : selective glucocorticoid receptor modulation

Zalachoras, I.

Citation

Zalachoras, I. (2014, September 17). *Targeting the brain under stress : selective glucocorticoid receptor modulation*. Retrieved from <https://hdl.handle.net/1887/28734>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/28734>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/28734> holds various files of this Leiden University dissertation

Author: Zalachoras, Ioannis

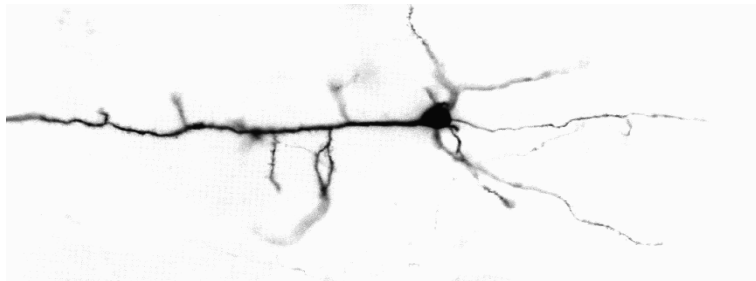
Title: Targeting the brain under stress : selective glucocorticoid receptor modulation

Issue Date: 2014-09-17

Chapter

General Introduction

1



Parts of this chapter have been previously published in:

- I. Zalachoras, M.M Evers, W.M. van Roon-Mom, A.M. Aartsma-Rus, O.C. Meijer
“Antisense-mediated RNA targeting: a versatile expedient genetic manipulation in the brain”
Frontiers in Molecular Neuroscience, 2011, Vol 4, doi: 10.3389/fnmol.2011.00010

- I. Zalachoras, R. Houtman, O.C. Meijer
“Understanding stress-effects in the brain via transcriptional signal transduction pathways”
Neuroscience, 2013, 242:97-109. doi: 10.1016/j.neuroscience.2013.03.038

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 adrenocorticotrophic 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

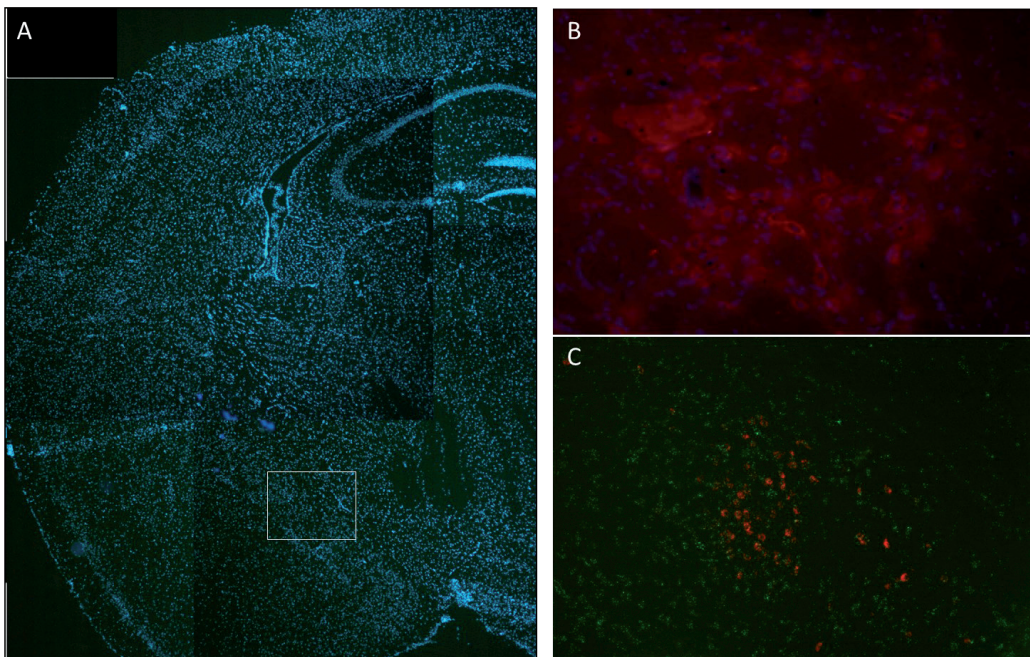


Figure 1. A. Fluorescent image of a mouse brain section stained with hoechst (blue) (10X magnification). The white box indicates the location of the central amygdala. B. The white box from picture A in magnification. CRH positive cells are immunofluorescently labeled red, while their nuclei are stained 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).

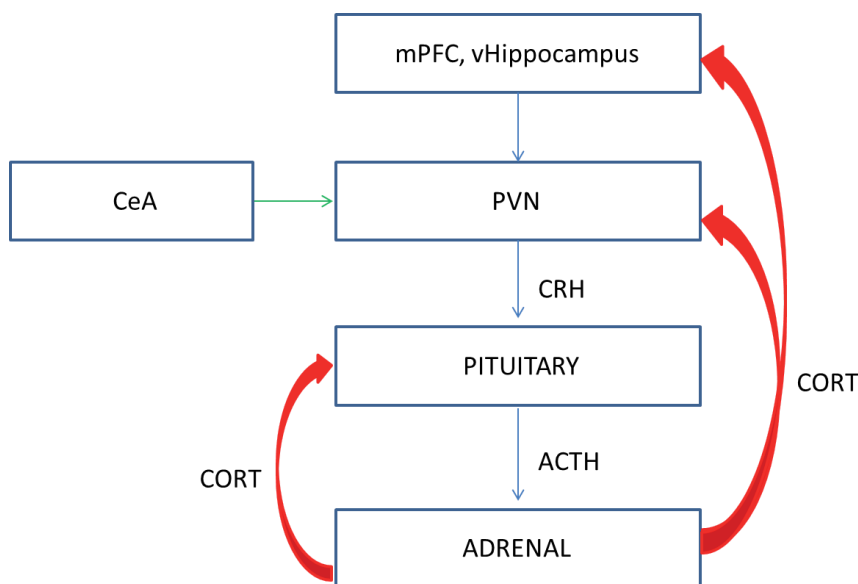


Figure 2. The Hypothalamus-Pituitary-Adrenal axis: In response to a variety of stimuli, such as indirect input from the central amygdala (CeA), corticotropin releasing hormone (CRH) is secreted from the paraventricular nucleus of the hypothalamus (PVN) into the pituitary stimulates the expression and 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 $\tau 1$, 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 ($\tau 2$) 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

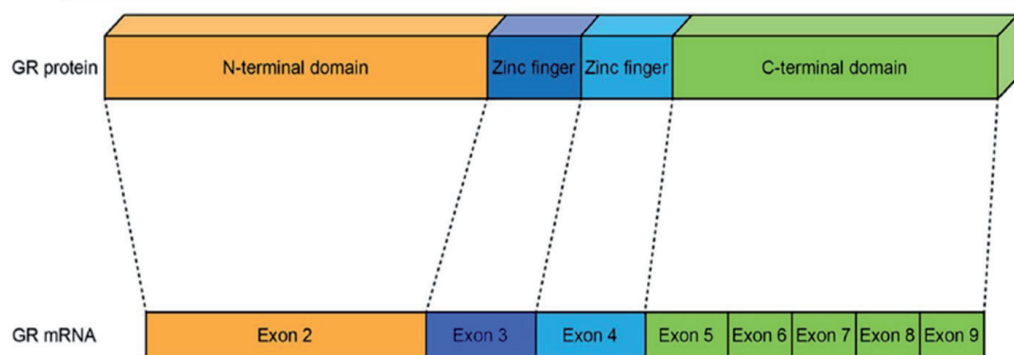


Figure 3. Relation between GR mRNA and protein. The 8 coding exons of the *GR* gene and the protein domains they code for. Exon 2 codes for the N-terminal domain of the protein which contains the major transcriptional activation domain $\tau 1$. Exons 3 and 4 code for two zinc-finger domains that are 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 *upregulation* 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- κ B and AP-1 (100). The GR ligand ‘Compound A’ is an example of this mechanism, as it induces inhibition of NF- κ B-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 methoxyethyl 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).

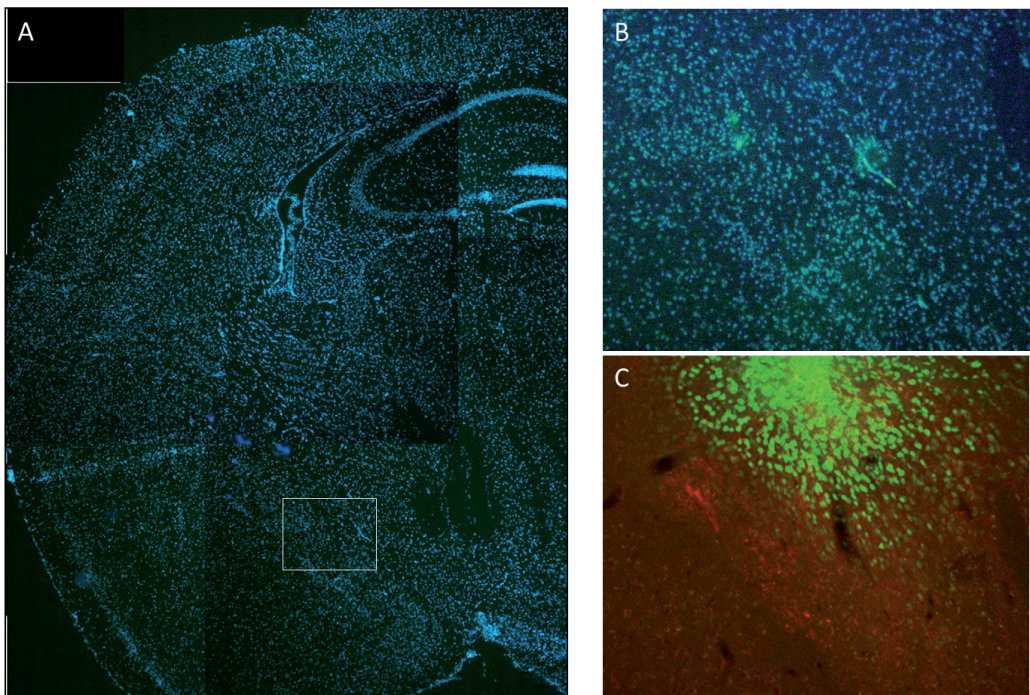


Figure 4. A. Fluorescent image of a mouse brain section stained with hoechst (blue) (10X magnification). The white box indicates the location of the central amygdala. B. The white box from picture A in magnification. AONs (green) are colocalized with hoechst in the cellular nuclei (20X magnification).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

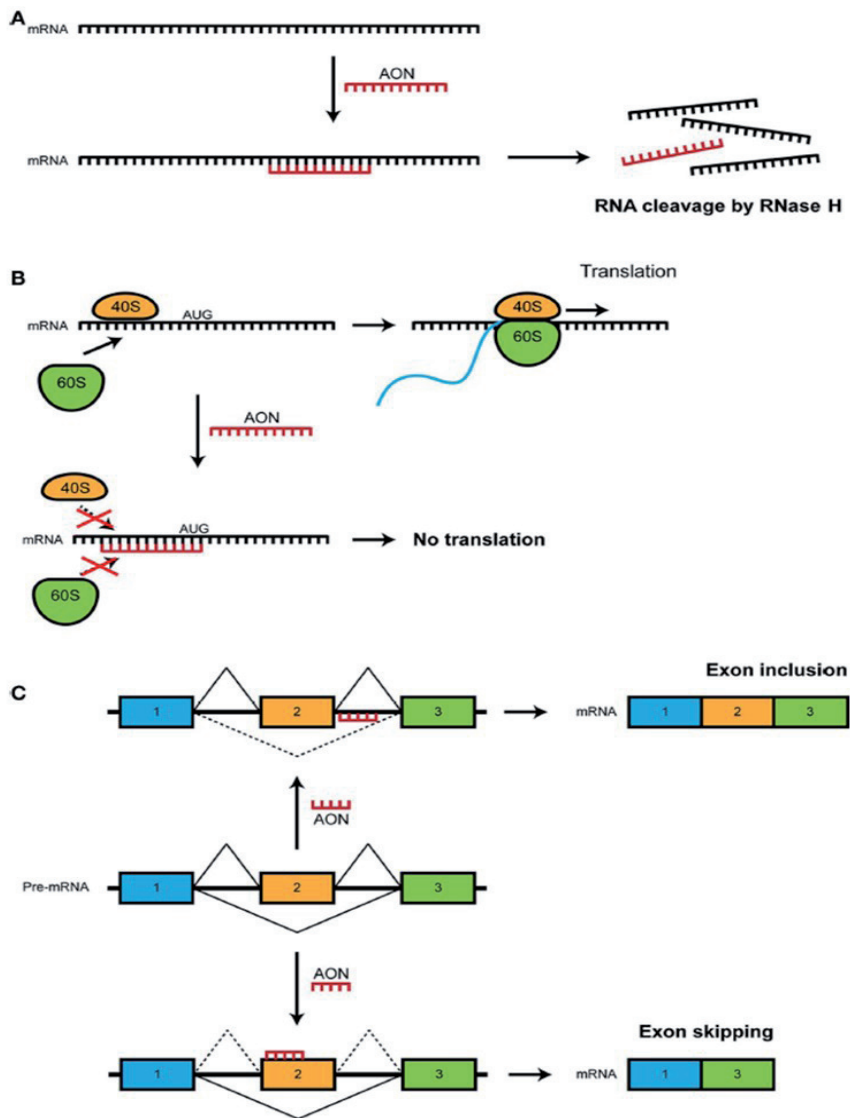


Figure 5. Schematic representation of different modes of action of antisense oligonucleotides. A. RNase H- dependent pathway. Binding of antisense oligodeoxyribonucleotides (AONs) with a phosphorothioate backbone results in a RNA:DNA hybrid, which activates RNase H. RNaseH will cleave them RNA and prevents 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' splice 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 P2X₃, *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.

References

1. Herman J (2013) Neural Control of Chronic Stress Adaptation. *Frontiers in behavioral neuroscience* 7.
2. de Kloet ER, Joels M, & Holsboer F (2005) Stress and the brain: from adaptation to disease. *Nat Rev Neurosci* 6(6):463-475.
3. Rodrigues SM, LeDoux JE, & Sapolsky RM (2009) The Influence of Stress Hormones on Fear Circuitry. *Annual Review of Neuroscience* 32(1):289-313.
4. Pape H-C & Pare D (2010) Plastic Synaptic Networks of the Amygdala for the Acquisition, Expression, and Extinction of Conditioned Fear. *Physiological Reviews* 90(2):419-463.
5. Medina JF, Christopher Repa J, Mauk MD, & LeDoux JE (2002) Parallels between cerebellum - and Amygdala-Dependant conditioning. *Nat Rev Neurosci* 3(2):122-131.
6. Maren S & Quirk GJ (2004) Neuronal signalling of fear memory. *Nat Rev Neurosci* 5(11):844-852.
7. Johansen JP, *et al.* (2010) Optical activation of lateral amygdala pyramidal cells instructs associative fear learning. *Proceedings of the National Academy of Sciences* 107(28):12692-12697.
8. Paré D, Quirk GJ, & Ledoux JE (2004) New Vistas on Amygdala Networks in Conditioned Fear. *Journal of Neurophysiology* 92(1):1-9.
9. Busti D, *et al.* (2011) Different Fear States Engage Distinct Networks within the Intercalated Cell Clusters of the Amygdala. *The Journal of Neuroscience* 31(13):5131-5144.
10. Ciocchi S, *et al.* (2010) Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature* 468(7321):277-282.
11. Gill KM & Grace AA (2013) Differential effects of acute and repeated stress on hippocampus and amygdala inputs to the nucleus accumbens shell. *The International Journal of Neuropsychopharmacology* 16(09):2013-2025.
12. Noori HR, Spanagel R, & Hansson AC (2012) Neurocircuitry for modeling drug effects. *Addiction Biology* 17(5):827-864.
13. Jankord R & Herman JP (2008) Limbic Regulation of Hypothalamo-Pituitary-Adrenocortical Function during Acute and Chronic Stress. *Annals of the New York Academy of Sciences* 1148 (1):64-73.
14. Butts KA & Phillips AG (2013) Glucocorticoid receptors in the prefrontal cortex regulate dopamine efflux to stress via descending glutamatergic feedback to the ventral tegmental area. *The International Journal of Neuropsychopharmacology* 16(08):1799-1807.
15. Myers B, McKlveen JM, & Herman JP (2013) Glucocorticoid actions on synapses, circuits, and behavior: Implications for the energetics of stress. *Front Neuroendocrinol.*
16. Hill MN, *et al.* (2011) Recruitment of Prefrontal Cortical Endocannabinoid Signaling by Glucocorticoids Contributes to Termination of the Stress Response. *The Journal of Neuroscience* 31(29):10506-10515.
17. McKlveen JM, *et al.* (2013) Role of Prefrontal Cortex Glucocorticoid Receptors in Stress and Emotion. *Biol Psychiatry.*
18. Reppert SM & Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* 418 (6901):935-941.
19. Lightman SL & Conway-Campbell BL (2010) The crucial role of pulsatile activity of the HPA axis for continuous dynamic equilibration. *Nat Rev Neurosci* 11(10):710-718.
20. Windle RJ, Wood SA, Shanks N, Lightman SL, & Ingram CD (1998) Ultradian Rhythm of Basal Corticosterone Release in the Female Rat: Dynamic Interaction with the Response to Acute Stress. *Endocrinology* 139(2):443-450.

21. Sarabdjitsingh RA, *et al.* (2010) Stress Responsiveness Varies over the Ultradian Glucocorticoid Cycle in a Brain-Region-Specific Manner. *Endocrinology* 151(11):5369-5379.
22. Sarabdjitsingh RA, *et al.* (2010) Disrupted Corticosterone Pulsatile Patterns Attenuate Responsiveness to Glucocorticoid Signaling in Rat Brain. *Endocrinology* 151(3):1177-1186.
23. Vale W, Spiess J, Rivier C, & Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* 213(4514):1394-1397.
24. Kovács KJ (2013) CRH: The link between hormonal-, metabolic- and behavioral responses to stress. *Journal of Chemical Neuroanatomy* (0).
25. Aguilera G & Liu Y (2012) The molecular physiology of CRH neurons. *Frontiers in Neuroendocrinology* 33(1):67-84.
26. Kolber BJ, *et al.* (2008) Central amygdala glucocorticoid receptor action promotes fear-associated CRH activation and conditioning. *Proc Natl Acad Sci U S A* 105(33):12004-12009.
27. Sharma D, Bhawe S, Gregg E, & Uht R (2013) Dexamethasone Induces a Putative Repressor Complex and Chromatin Modifications in the CRH Promoter. *Molecular Endocrinology*.
28. Lachize S, *et al.* (2009) Steroid receptor coactivator-1 is necessary for regulation of corticotropin-releasing hormone by chronic stress and glucocorticoids. *Proc Natl Acad Sci U S A* 106(19):8038-8042.
29. Makino S, Gold PW, & Schulkin J (1994) Effects of corticosterone on CRH mRNA and content in the bed nucleus of the stria terminalis; comparison with the effects in the central nucleus of the amygdala and the paraventricular nucleus of the hypothalamus. *Brain Res* 657(1-2):141-149.
30. Dedic N, *et al.* (2012) Assessing Behavioural Effects of Chronic HPA Axis Activation Using Conditional CRH-Overexpressing Mice. *Cellular and Molecular Neurobiology* 32(5):815-828.
31. Van Gaalen MM, Stenzel-Poore MP, Holsboer F, & Steckler T (2002) Effects of transgenic overproduction of CRH on anxiety-like behaviour. *European Journal of Neuroscience* 15(12):2007-2015.
32. Chen J, *et al.* (2012) Maternal Deprivation in Rats is Associated with Corticotrophin-Releasing Hormone (CRH) Promoter Hypomethylation and Enhances CRH Transcriptional Responses to Stress in Adulthood. *Journal of Neuroendocrinology* 24(7):1055-1064.
33. Miller L, *et al.* (2011) Histone deacetylase 1 (HDAC1) participates in the down-regulation of corticotropin releasing hormone gene (*crh*) expression. *Physiology & Behavior* 104(2):312-320.
34. Elliott E, Ezra-Nevo G, Regev L, Neufeld-Cohen A, & Chen A (2010) Resilience to social stress coincides with functional DNA methylation of the *Crf* gene in adult mice. *Nat Neurosci* 13(11):1351-1353.
35. Mittelstadt PR & Ashwell JD (2003) Disruption of glucocorticoid receptor exon 2 yields a ligand-responsive C-terminal fragment that regulates gene expression. *Mol Endocrinol* 17(8):1534-1542.
36. Giguere V, Hollenberg SM, Rosenfeld MG, & Evans RM (1986) Functional domains of the human glucocorticoid receptor. *Cell* 46(5):645-652.
37. Danielian PS, White R, Lees JA, & Parker MG (1992) Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J* 11(3):1025-1033.
38. Pearce D (1994) A mechanistic basis for distinct mineralocorticoid and glucocorticoid receptor transcriptional specificities. *Steroids* 59(2):153-159.
39. Chen S-y, Wang J, Yu G-q, Liu W, & Pearce D (1997) Androgen and Glucocorticoid Receptor Heterodimer Formation: A POSSIBLE MECHANISM FOR MUTUAL INHIBITION OF

- TRANSCRIPTIONAL ACTIVITY. *Journal of Biological Chemistry* 272(22):14087-14092.
40. Awasthi S & Simons Jr SS (2012) Separate regions of glucocorticoid receptor, coactivator TIF2, and comodulator STAMP modify different parameters of glucocorticoid-mediated gene induction. *Molecular and Cellular Endocrinology* 355(1):121-134.
 41. Yang J & Fuller PJ (2012) Interactions of the mineralocorticoid receptor – Within and without. *Molecular and Cellular Endocrinology* 350(2):196-205.
 42. Walker JJ, *et al.* (2012) The Origin of Glucocorticoid Hormone Oscillations. *PLoS Biol* 10(6):e1001341.
 43. Noguchi T, *et al.* (2010) Regulation of Glucocorticoid Receptor Transcription and Nuclear Translocation during Single and Repeated Immobilization Stress. *Endocrinology* 151(9):4344-4355.
 44. Nicolaides NC, Galata Z, Kino T, Chrousos GP, & Charmandari E (2010) The human glucocorticoid receptor: Molecular basis of biologic function. *Steroids* 75(1):1-12.
 45. Touma C, *et al.* (2011) FK506 Binding Protein 5 Shapes Stress Responsiveness: Modulation of Neuroendocrine Reactivity and Coping Behavior. *Biol Psychiat* 70(10):928-936.
 46. Hartmann J, *et al.* (2012) The involvement of FK506-binding protein 51 (FKBP5) in the behavioral and neuroendocrine effects of chronic social defeat stress. *Neuropharmacology* 62(1):332-339.
 47. Fitzsimons CP, *et al.* (2008) The Microtubule-Associated Protein Doublecortin-Like Regulates the Transport of the Glucocorticoid Receptor in Neuronal Progenitor Cells. *Molecular Endocrinology* 22(2):248-262.
 48. Anacker C, *et al.* (2013) Role for the kinase SGK1 in stress, depression, and glucocorticoid effects on hippocampal neurogenesis. *Proc Natl Acad Sci US A* 110(21):8708-8713.
 49. de Kloet ER, Fitzsimons CP, Datson NA, Meijer OC, & Vreugdenhil E (2009) Glucocorticoid signaling and stress-related limbic susceptibility pathway: about receptors, transcription machinery and microRNA. *Brain Res* 1293:129-141.
 50. De Bosscher K, Van Craenenbroeck K, Meijer OC, & Haegeman G (2008) Selective transrepression versus transactivation mechanisms by glucocorticoid receptor modulators in stress and immune systems. *European Journal of Pharmacology* 583(2–3):290-302.
 51. Biddie Simon C, *et al.* (2011) Transcription Factor AP1 Potentiates Chromatin Accessibility and Glucocorticoid Receptor Binding. *Molecular Cell* 43(1):145-155.
 52. Webster JC & Cidlowski JA (1999) Mechanisms of Glucocorticoid-receptor-mediated Repression of Gene Expression. *Trends in Endocrinology & Metabolism* 10(10):396-402.
 53. Datson NA, *et al.* (2011) Specific Regulatory Motifs Predict Glucocorticoid Responsiveness of Hippocampal Gene Expression. *Endocrinology* 152(10):3749-3757.
 54. Polman JA, *et al.* (2012) A genome-wide signature of glucocorticoid receptor binding in neuronal PC12 cells. *BMC Neuroscience* 13(1):118.
 55. Trousson A, *et al.* (2007) Recruitment of the p160 coactivators by the glucocorticoid receptor: Dependence on the promoter context and cell type but not hypoxic conditions. *The Journal of Steroid Biochemistry and Molecular Biology* 104(3–5):305-311.
 56. Oliveira AMM, *et al.* (2011) Subregion-specific p300 conditional knock-out mice exhibit long-term memory impairments. *Learning & Memory* 18(3):161-169.
 57. Tetel MJ, Auger AP, & Charlier TD (2009) Who's in charge? Nuclear receptor coactivator and corepressor function in brain and behavior. *Front Neuroendocrinol* 30(3):328-342.
 58. Pascual-Le Tallec L & Lombès M (2005) The Mineralocorticoid Receptor: A Journey Exploring Its Diversity and Specificity of Action. *Molecular Endocrinology* 19(9):2211-2221.
 59. Yang J & Young MJ (2009) The mineralocorticoid receptor and its coregulators. *Journal of Molecular Endocrinology* 43(2):53-64.

60. Huang P, Chandra V, & Rastinejad F (2010) Structural Overview of the Nuclear Receptor Superfamily: Insights into Physiology and Therapeutics. *Annual Review of Physiology* 72 (1):247-272.
61. Lonard DM & O'Malley BW (2012) Nuclear receptor coregulators: modulators of pathology and therapeutic targets. *Nat Rev Endocrinol* 8(10):598-604.
62. Rosenfeld MG, Lunyak VV, & Glass CK (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes & Development* 20(11):1405-1428.
63. van der Laan S, Lachize SB, Vreugdenhil E, de Kloet ER, & Meijer OC (2008) Nuclear receptor coregulators differentially modulate induction and glucocorticoid receptor-mediated repression of the corticotropin-releasing hormone gene. *Endocrinology* 149(2):725-732.
64. Oñate SA, Tsai SY, Tsai M-J, & O'Malley BW (1995) Sequence and Characterization of a Coactivator for the Steroid Hormone Receptor Superfamily. *Science* 270(5240):1354-1357.
65. Amazit L, *et al.* (2011) Ligand-Dependent Degradation of SRC-1 Is Pivotal for Progesterone Receptor Transcriptional Activity. *Molecular Endocrinology* 25(3):394-408.
66. Misiti S, Schomburg L, M. Yen P, & Chin WW (1998) Expression and Hormonal Regulation of Coactivator and Corepressor Genes. *Endocrinology* 139(5):2493-2500.
67. Maj M, *et al.* (2012) Novel insights into the distribution and functional aspects of the calcium binding protein Secretagoin from studies on rat brain and primary neuronal cell culture. *Frontiers in Molecular Neuroscience* 5.
68. Meijer OC, van der Laan S, Lachize S, Steenbergen PJ, & de Kloet ER (2006) Steroid receptor coregulator diversity: What can it mean for the stressed brain? *Neuroscience* 138(3):891-899.
69. Meijer OC, Steenbergen PJ, & de Kloet ER (2000) Differential Expression and Regional Distribution of Steroid Receptor Coactivators SRC-1 and SRC-2 in Brain and Pituitary. *Endocrinology* 141(6):2192-2199.
70. Alboni S, *et al.* (2011) Stress induces altered CRE/CREB pathway activity and BDNF expression in the hippocampus of glucocorticoid receptor-impaired mice. *Neuropharmacology* 60(7-8):1337-1346.
71. Malvaez M, Mhillaj E, Matheos DP, Palmery M, & Wood MA (2011) CBP in the Nucleus Accumbens Regulates Cocaine-Induced Histone Acetylation and Is Critical for Cocaine-Associated Behaviors. *The Journal of Neuroscience* 31(47):16941-16948.
72. Nishihara E, *et al.* (2003) SRC-1 Null Mice Exhibit Moderate Motor Dysfunction and Delayed Development of Cerebellar Purkinje Cells. *The Journal of Neuroscience* 23(1):213-222.
73. Tognoni CM, Chadwick JJG, Ackeifi CA, & Tetel MJ (2011) Nuclear Receptor Coactivators Are Coexpressed with Steroid Receptors and Regulated by Estradiol in Mouse Brain. *Neuroendocrinology* 94(1):49-57.
74. Tetel MJ, Siegal NK, & Murphy SD (2007) Cells in Behaviourally Relevant Brain Regions Coexpress Nuclear Receptor Coactivators and Ovarian Steroid Receptors. *Journal of Neuroendocrinology* 19(4):262-271.
75. Charlier TD, Ball GF, & Balthazart J (2006) Plasticity in the expression of the steroid receptor coactivator 1 in the Japanese quail brain: Effect of sex, testosterone, stress and time of the day. *Neuroscience* 140(4):1381-1394.
76. Bian C, *et al.* (2012) Sex differences and synchronous development of steroid receptor coactivator-1 and synaptic proteins in the hippocampus of postnatal female and male C57BL/6 mice. *Steroids* 77(1-2):149-156.
77. Duncan KA, Jimenez P, & Carruth LL (2011) Distribution and sexually dimorphic expression of steroid receptor coactivator-1 (SRC-1) in the zebra finch brain. *General and Comparative Endocrinology* 170(2):408-414.

78. Zhang D, *et al.* (2011) Expression of Steroid Receptor Coactivator-1 Was Regulated by Postnatal Development but Not Ovariectomy in the Hippocampus of Rats. *Developmental Neuroscience* 33(1):57-63.
79. Zhang D, *et al.* (2011) Alterations of steroid receptor coactivator-1 (SRC-1) immunoreactivities in specific brain regions of young and middle-aged female Sprague-Dawley rats. *Brain Research* 1382(0):88-97.
80. Bousios S, Karandrea D, Kittas C, & Kitraki E (2001) Effects of gender and stress on the regulation of steroid receptor coactivator-1 expression in the rat brain and pituitary. *The Journal of Steroid Biochemistry and Molecular Biology* 78(5):401-407.
81. Tetel MJ, Ungar TC, Hassan B, & Bittman EL (2004) Photoperiodic regulation of androgen receptor and steroid receptor coactivator-1 in Siberian hamster brain. *Mol Brain Res* 131(1-2):79-87.
82. Meijer OC, *et al.* (2005) Steroid Receptor Coactivator-1 Splice Variants Differentially Affect Corticosteroid Receptor Signaling. *Endocrinology* 146(3):1438-1448.
83. Stanisic V, Lonard DM, & O'Malley BW (2010) Modulation of steroid hormone receptor activity. *Prog Brain Res* 181:153-176.
84. Kamei Y, *et al.* (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85(3):403-414.
85. Spencer TE, *et al.* (1997) Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* 389(6647):194-198.
86. Zalachoras I, *et al.* (2013) Antisense-mediated isoform switching of steroid receptor coactivator-1 in the central nucleus of the amygdala of the mouse brain. *BMC Neurosci* 14:5.
87. Ding XF, *et al.* (1998) Nuclear Receptor-Binding Sites of Coactivators Glucocorticoid Receptor Interacting Protein 1 (GRIP1) and Steroid Receptor Coactivator 1 (SRC-1): Multiple Motifs with Different Binding Specificities. *Molecular Endocrinology* 12(2):302-313.
88. Meijer OC, *et al.* (2005) Steroid receptor coactivator-1 splice variants differentially affect corticosteroid receptor signaling. *Endocrinology* 146(3):1438-1448.
89. Kalkhoven E, Valentine JE, Heery DM, & Parker MG (1998) Isoforms of steroid receptor coactivator 1 differ in their ability to potentiate transcription by the oestrogen receptor. *EMBO J* 17(1):232-243.
90. Winnay JN, Xu J, O'Malley BW, & Hammer GD (2006) Steroid Receptor Coactivator-1-Deficient Mice Exhibit Altered Hypothalamic-Pituitary-Adrenal Axis Function. *Endocrinology* 147(3):1322-1332.
91. Apostolakis EM, Ramamurphy M, Zhou D, Oñate S, & O'Malley BW (2002) Acute Disruption of Select Steroid Receptor Coactivators Prevents Reproductive Behavior in Rats and Unmasks Genetic Adaptation in Knockout Mice. *Molecular Endocrinology* 16(7):1511-1523.
92. Xu J, *et al.* (1998) Partial Hormone Resistance in Mice with Disruption of the Steroid Receptor Coactivator-1 (SRC-1) Gene. *Science* 279(5358):1922-1925.
93. Yore MA, *et al.* (2010) Steroid receptor coactivator-2 expression in brain and physical associations with steroid receptors. *Neuroscience* 169(3):1017-1028.
94. Charlier TD, Lakaye B, Ball GF, & Balthazart J (2002) Steroid receptor coactivator SRC-1 exhibits high expression in steroid-sensitive brain areas regulating reproductive behaviors in the quail brain. *Neuroendocrinology* 76:297-315.
95. Charlier TD, Harada N, Ball GF, & Balthazart J (2006) Targeting steroid receptor coactivator-1 expression with locked nucleic acids antisense reveals different thresholds for the hormonal regulation of male sexual behavior in relation to aromatase activity and protein expression. *Behavioural Brain Research* 172(2):333-343.

96. Auger AP, Tetel MJ, & McCarthy MM (2000) Steroid receptor coactivator-1 (SRC-1) mediates the development of sex-specific brain morphology and behavior. *Proceedings of the National Academy of Sciences* 97(13):7551-7555.
97. Rauch A, *et al.* (2011) An anti-inflammatory selective glucocorticoid receptor modulator preserves osteoblast differentiation. *The FASEB Journal* 25(4):1323-1332.
98. van Lierop M-JC, *et al.* (2012) Org 214007-0: A Novel Non-Steroidal Selective Glucocorticoid Receptor Modulator with Full Anti-Inflammatory Properties and Improved Therapeutic Index. *PLoS One* 7(11):e48385.
99. Crossin KL, Tai M-H, Krushel LA, Mauro VP, & Edelman GM (1997) Glucocorticoid receptor pathways are involved in the inhibition of astrocyte proliferation. *Proceedings of the National Academy of Sciences* 94(6):2687-2692.
100. De Bosscher K, Vanden Berghe W, & Haegeman G (2003) The Interplay between the Glucocorticoid Receptor and Nuclear Factor- κ B or Activator Protein-1: Molecular Mechanisms for Gene Repression. *Endocrine Reviews* 24(4):488-522.
101. De Bosscher K, *et al.* (2005) A fully dissociated compound of plant origin for inflammatory gene repression. *P Natl Acad Sci USA* 102(44):15827-15832.
102. Reber LL, *et al.* (2012) A Dissociated Glucocorticoid Receptor Modulator Reduces Airway Hyperresponsiveness and Inflammation in a Mouse Model of Asthma. *The Journal of Immunology* 188(7):3478-3487.
103. Beaulieu E & Morand EF (2011) Role of GILZ in immune regulation, glucocorticoid actions and rheumatoid arthritis. *Nat Rev Rheumatol* 7(6):340-348.
104. Coghlan MJ, *et al.* (2003) A Novel Antiinflammatory Maintains Glucocorticoid Efficacy with Reduced Side Effects. *Molecular Endocrinology* 17(5):860-869.
105. Roohk DJ, *et al.* (2010) Differential In Vivo Effects on Target Pathways of a Novel Arylpyrazole Glucocorticoid Receptor Modulator Compared with Prednisolone. *Journal of Pharmacology and Experimental Therapeutics* 333(1):281-289.
106. Wang J-C, *et al.* (2006) Novel arylpyrazole compounds selectively modulate glucocorticoid receptor regulatory activity. *Genes & Development* 20(6):689-699.
107. Klok MD, *et al.* (2011) A common and functional mineralocorticoid receptor haplotype enhances optimism and protects against depression in females. *Transl Psychiatry* 1:e62.
108. Qi X-R, *et al.* (2012) Aberrant stress hormone receptor balance in the human prefrontal cortex and hypothalamic paraventricular nucleus of depressed patients. *Psychoneuroendocrinology* (0).
109. Yang J, *et al.* (2011) Identification of Ligand-Selective Peptide Antagonists of the Mineralocorticoid Receptor Using Phage Display. *Molecular Endocrinology* 25(1):32-43.
110. Dias N & Stein CA (2002) Antisense oligonucleotides: Basic concepts and mechanisms. *Mol Cancer Ther* 1(5):347-355.
111. Nasevicius A & Ekker SC (2000) Effective targeted gene 'knockdown' in zebrafish. *Nat Genet* 26(2):216-220.
112. Nasevicius A, Larson J, & Ekker SC (2000) Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. *Yeast* 17(4):294-301.
113. Bogdahn U, *et al.* (2011) Targeted therapy for high-grade glioma with the TGF-beta2 inhibitor trabedersen: results of a randomized and controlled phase IIb study. *Neuro Oncol* 13(1):132-142.
114. Marwick C (1998) First "Antisense" Drug Will Treat CMV Retinitis. *JAMA: The Journal of the American Medical Association* 280(10):871.
115. Aartsma-Rus A, *et al.* (2009) Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms. *Mol Ther* 17(3):548-553.

116. Heemskerk H, *et al.* (2010) Preclinical PK and PD studies on 2'-O-methyl-phosphorothioate RNA antisense oligonucleotides in the mdx mouse model. *Mol Ther* 18(6):1210-1217.
117. Heemskerk HA, *et al.* (2009) In vivo comparison of 2'-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. *J Gene Med* 11(3):257-266.
118. Aartsma-Rus A, *et al.* (2006) Therapeutic modulation of DMD splicing by blocking exonic splicing enhancer sites with antisense oligonucleotides. *Ann N Y Acad Sci* 1082:74-76.
119. Goemans NM, *et al.* (2011) Systemic Administration of PRO051 in Duchenne's Muscular Dystrophy. *N Engl J Med*.
120. Kinali M, *et al.* (2009) Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *The Lancet Neurology* 8(10):918-928.
121. van Deutekom JC, *et al.* (2007) Local Dystrophin Restoration with Antisense Oligonucleotide PRO051. *New England Journal of Medicine* 357(26):2677-2686.
122. Alter J, *et al.* (2006) Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat Med* 12(2):175-177.
123. Lu QL, *et al.* (2003) Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nat Med* 9(8):1009-1014.
124. Hua Y, *et al.* (2010) Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev* 24(15):1634-1644.
125. Nlend Nlend R, Meyer K, & Schumperli D (2010) Repair of pre-mRNA splicing: prospects for a therapy for spinal muscular atrophy. *RNA Biol* 7(4):430-440.
126. Burghes AH & McGovern VL (2010) Antisense oligonucleotides and spinal muscular atrophy: skipping along. *Genes Dev* 24(15):1574-1579.
127. Williams JH, *et al.* (2009) Oligonucleotide-mediated survival of motor neuron protein expression in CNS improves phenotype in a mouse model of spinal muscular atrophy. *J Neurosci* 29(24):7633-7638.
128. Krebs MD & Alsberg E (2011) Localized, Targeted, and Sustained siRNA Delivery. *Chemistry – A European Journal* 17(11):3054-3062.
129. Petri S, *et al.* (2011) Increased siRNA duplex stability correlates with reduced off-target and elevated on-target effects. *RNA* 17(4):737-749.
130. Vickers TA, *et al.* (2009) Off-target and a portion of target-specific siRNA mediated mRNA degradation is Ago2 'Slicer' independent and can be mediated by Ago1. *Nucleic Acids Res* 37(20):6927-6941.
131. Ma Y, Creanga A, Lum L, & Beachy PA (2006) Prevalence of off-target effects in Drosophila RNA interference screens. *Nature* 443(7109):359-363.
132. Gaglione M & Messere A (2010) Recent progress in chemically modified siRNAs. *Mini Rev Med Chem* 10(7):578-595.
133. Guterstam P, *et al.* (2008) Splice-switching efficiency and specificity for oligonucleotides with locked nucleic acid monomers. *Biochem J* 412(2):307-313.
134. Yoo BH, Bochkareva E, Bochkarev A, Mou TC, & Gray DM (2004) 2'-O-methyl-modified phosphorothioate antisense oligonucleotides have reduced non-specific effects in vitro. *Nucleic Acids Res* 32(6):2008-2016.
135. Grimm D, *et al.* (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441(7092):537-541.
136. Smith RA, *et al.* (2006) Antisense oligonucleotide therapy for neurodegenerative disease. *J Clin Invest* 116(8):2290-2296.
137. Kubo K, *et al.* (2010) Migration defects by DISC1 knockdown in C57BL/6, 129X1/SvJ, and

- ICR strains via in utero gene transfer and virus-mediated RNAi. *Biochem Biophys Res Commun* 400(4):631-637.
138. Ehler EM, Eggers R, Niclou SP, & Verhaagen J (2010) Cellular toxicity following application of adeno-associated viral vector-mediated RNA interference in the nervous system. *BMC Neurosci* 11:20.
 139. Di Benedetto B, Wefers B, Wurst W, & Kuhn R (2009) Local knockdown of ERK2 in the adult mouse brain via adeno-associated virus-mediated RNA interference. *Mol Biotechnol* 41(3):263-269.
 140. Woldbye DP, *et al.* (2010) Adeno-associated viral vector-induced overexpression of neuropeptide Y Y2 receptors in the hippocampus suppresses seizures. *Brain* 133(9):2778-2788.
 141. Ulusoy A, Decressac M, Kirik D, & Bjorklund A (2010) Viral vector-mediated overexpression of alpha-synuclein as a progressive model of Parkinson's disease. *Prog Brain Res* 184:89-111.
 142. Jayandharan GR, *et al.* (2011) Activation of the NF- κ B pathway by adeno-associated virus (AAV) vectors and its implications in immune response and gene therapy. *Proceedings of the National Academy of Sciences* 108(9):3743-3748.
 143. Driesse MJ, *et al.* (2000) Intra-CSF administered recombinant adenovirus causes an immune response-mediated toxicity. *Gene Ther* 7(16):1401-1409.
 144. Oshiro EM, *et al.* (1995) Toxicity studies and distribution dynamics of retroviral vectors following intrathecal administration of retroviral vector-producer cells. *Cancer Gene Ther* 2(2):87-95.
 145. Sanchez CE, *et al.* (2011) Recombinant adeno-associated virus type 2 pseudotypes: comparing safety, specificity, and transduction efficiency in the primate striatum. *Journal of Neurosurgery* 114(3):672-680.
 146. Kaplitt MG, Darackiev B, & During MJ (1998) Prospects for gene therapy in pediatric neurosurgery. *Pediatr Neurosurg* 28(1):3-14.
 147. Liebsch G, Landgraf R, Engelmann M, Lorsch P, & Holsboer F (1999) Differential behavioural effects of chronic infusion of CRH1 and CRH2 receptor antisense oligonucleotides into the rat brain. *J Psychiat Res* 33(2):153-163.
 148. Muller YL, Reitstetter R, & Yool AJ (2000) Antisense knockdown of calcium-dependent K⁺ channels in developing cerebellar Purkinje neurons. *Dev Brain Res* 120(2):135-140.
 149. Sioud M, Furset G, & Cekaite L (2007) Suppression of immunostimulatory siRNA-driven innate immune activation by 2'-modified RNAs. *Biochem Biophys Res Commun* 361(1):122-126.
 150. Okun E, Lathia JD, & Mattson MP (2009) Adhesion- and migration-related side effects of phosphothioated CpG oligodeoxynucleotides. *Cell Adh Migr* 3(3):272-274.
 151. Robbins M, *et al.* (2007) 2'-O-methyl-modified RNAs act as TLR7 antagonists. *Mol Ther* 15(9):1663-1669.
 152. Hamm S, *et al.* (2010) Alternating 2'-O-ribose methylation is a universal approach for generating non-stimulatory siRNA by acting as TLR7 antagonist. *Immunobiology* 215(7):559 - 569.
 153. Chiasson BJ, Armstrong JN, Hooper ML, Murphy PR, & Robertson HA (1994) The application of antisense oligonucleotide technology to the brain: some pitfalls. *Cell Mol Neurobiol* 14(5):507-521.
 154. Ma L, *et al.* (2011) Region-Specific Involvement of BDNF Secretion and Synthesis in Conditioned Taste Aversion Memory Formation. *J. Neurosci.* 31(6):2079-2090.
 155. Pitts MW, Todorovic C, Blank T, & Takahashi LK (2009) The Central Nucleus of the Amygdala and Corticotropin-Releasing Factor: Insights into Contextual Fear Memory. *Journal of Neuroscience* 29(22):7379-7388.
 156. Pitts MW & Takahashi LK (2010) The central amygdala nucleus via corticotropin-releasing

- factor is necessary for time-limited consolidation processing but not storage of contextual fear memory. *Neurobiol Learn Mem*.
157. Pfeifer A, Brandon EP, Kootstra N, Gage FH, & Verma IM (2001) Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting in vivo. *Proc Natl Acad Sci U S A* 98(20):11450-11455.
 158. Naldini L, *et al.* (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 272(5259):263-267.
 159. Iorns E, Lord CJ, Turner N, & Ashworth A (2007) Utilizing RNA interference to enhance cancer drug discovery. *Nat Rev Drug Discov* 6(7):556-568.
 160. Shim MS & Kwon YJ (2010) Efficient and targeted delivery of siRNA in vivo. *FEBS J* 277(23):4814-4827.
 161. Pan Q, *et al.* (2011) Disturbance of the microRNA pathway by commonly used lentiviral shRNA libraries limits the application for screening host factors involved in hepatitis C virus infection. *FEBS Lett* 585(7):1025-1030.
 162. Sledz CA, Holko M, de Veer MJ, Silverman RH, & Williams BRG (2003) Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 5(9):834-839.
 163. Pardridge WM (2002) Drug and gene targeting to the brain with molecular trojan horses. *Nat Rev Drug Discov* 1(2):131-139.
 164. Cossum PA, *et al.* (1993) Disposition of the 14C-labeled phosphorothioate oligonucleotide ISIS 2105 after intravenous administration to rats. *Journal of Pharmacology and Experimental Therapeutics* 267(3):1181-1190.
 165. Agrawal S, Temsamani J, & Tang JY (1991) Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. *Proceedings of the National Academy of Sciences* 88(17):7595-7599.
 166. Riley MGI, *et al.* (1998) Intra-arterial administration of carboplatin and the blood brain barrier permeabilizing agent, RMP-7: A toxicologic evaluation in swine. *Journal of Neuro-Oncology* 36(2):167-178.
 167. Brignole C, *et al.* (2003) Targeted delivery system for antisense oligonucleotides: a novel experimental strategy for neuroblastoma treatment. *Cancer Lett* 197(1):231-235.
 168. Zhang Y, Jeong Lee H, Boado RJ, & Pardridge WM (2002) Receptor-mediated delivery of an antisense gene to human brain cancer cells. *The Journal of Gene Medicine* 4(2):183-194.
 169. Olbricht WL, Neeves KB, & Foley CP (2010) Microfluidic probes in the treatment of brain-related diseases. *Drug News Perspect* 23(8):491-497.
 170. Ambroggi F, *et al.* (2009) Stress and addiction: glucocorticoid receptor in dopaminergic neurons facilitates cocaine seeking. *Nat Neurosci* 12(3):247-249.
 171. Dash AK & Cudworthli GC (1998) Therapeutic applications of implantable drug delivery systems. *Journal of Pharmacological and Toxicological Methods* 40(1):1-12.
 172. Hemmings-Mieszczak M, Dorn G, Natt FJ, Hall J, & Wishart WL (2003) Independent combinatorial effect of antisense oligonucleotides and RNAi-mediated specific inhibition of the recombinant Rat P2X3 receptor. *Nucleic Acids Research* 31(8):2117-2126.
 173. Liang X-h & Crooke ST (Depletion of key protein components of the RISC pathway impairs pre-ribosomal RNA processing. *Nucleic Acids Research*.

