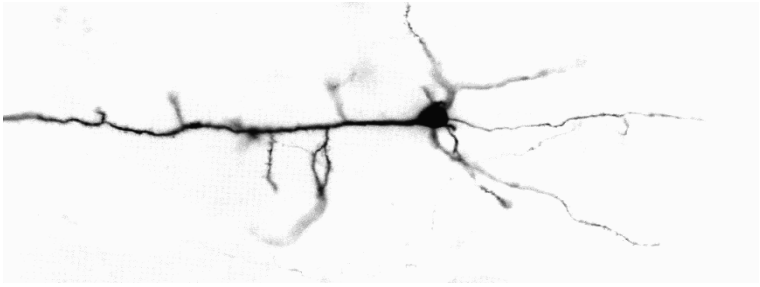
Chapter 4

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).

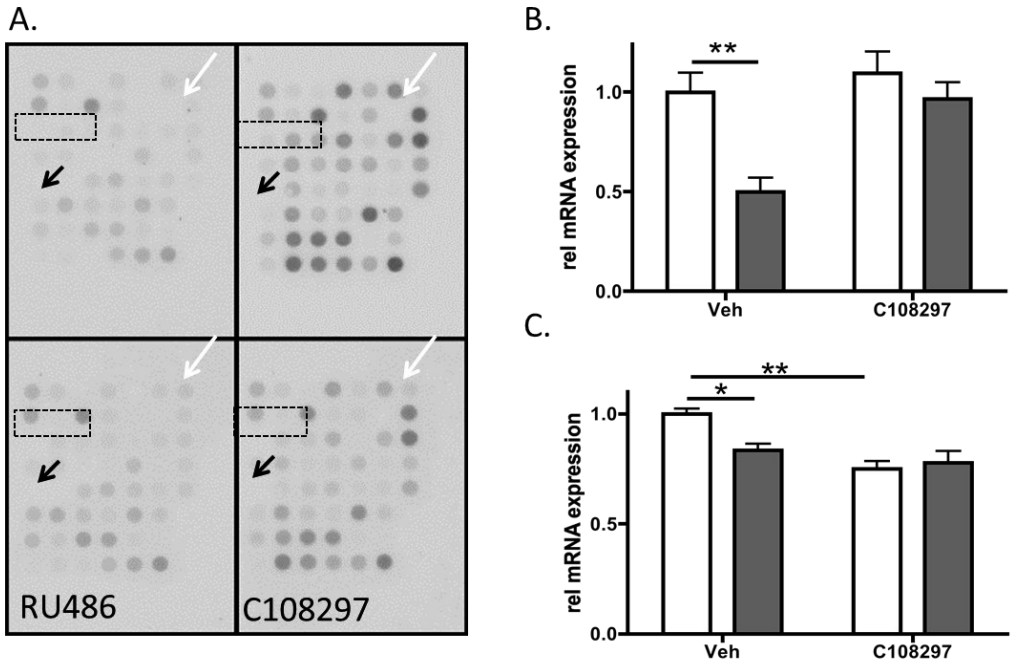

Figure 1. C108297 behaves like a selective modulator in vitro and in vivo. A. Ligand-induced interactions between the GR-LBD and coregulator motifs. DEX induced many interactions compared with DMSO. RU486 induced modest interactions with corepressor motifs (black arrow: NCoR1). C108297 showed an intermediate 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.

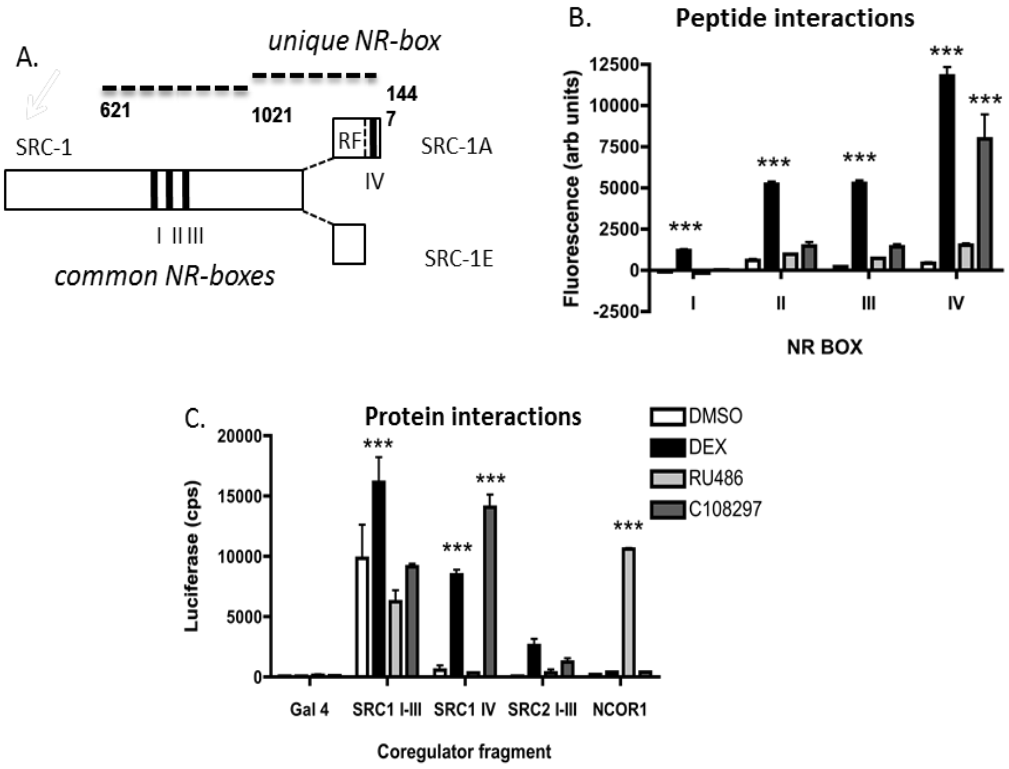

Figure 2. SCR-1 splice variant 1A is selectively recruited by GR-C108297. A. Protein structure of SRC-1 harboring three NR central boxes (roman numerals). SRC-1A harbors a repressor function (RF) and the additional NR-box IV. Protein fragments marked by dotted lines refer to C. B. MARCoNI quantification 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

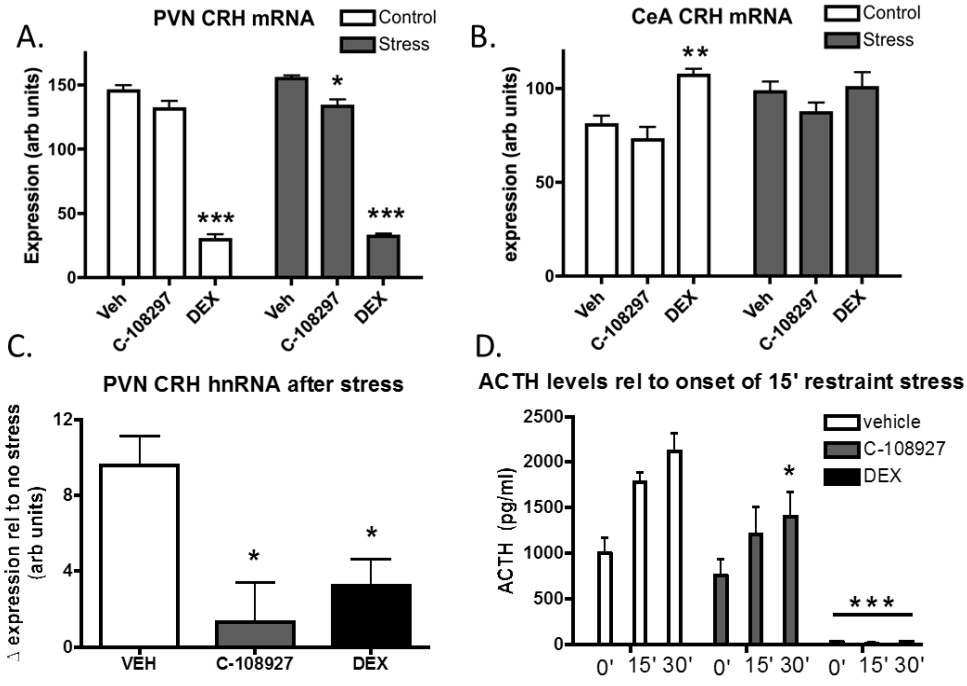

Figure 3. Selective GR modulation in the stress system. C108297-agonism in ADX rats after subchronic treatment compared with the prototypic agonist DEX. A. In the PVN, where SRC-1A is expressed at high levels, DEX led to strong down-regulation of CRH mRNA ($P < 0.001$). C108297 had a modest agonist effect 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 ($P < 0.05$).

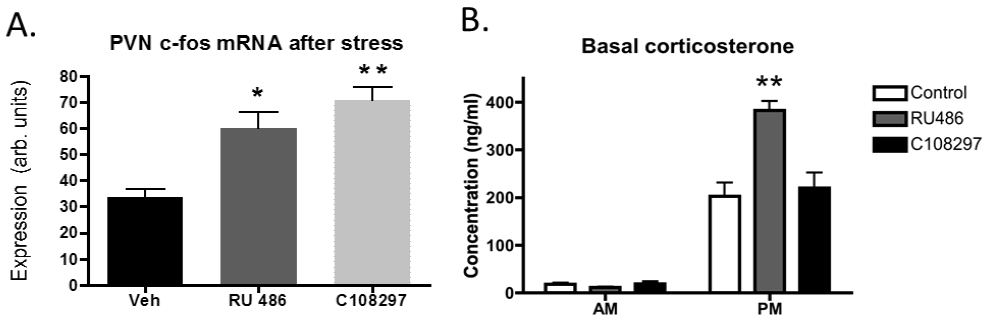

Figure 4. Selective GR modulation in the stress system: antagonism in adrenally intact rats after subchronic treatment compared with the prototypic antagonist RU486. A. The acute c-fos response to stress in the PVN was enhanced both by pretreatment with RU486 and C108297. B. RU486 treatment led to increased 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.

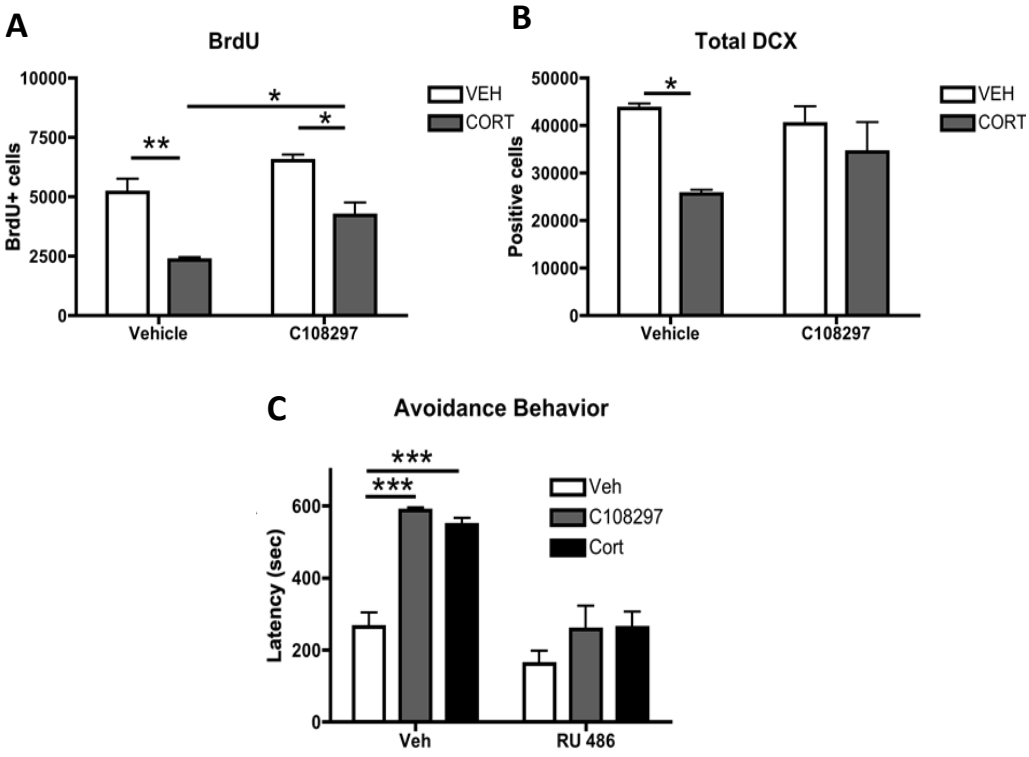

Figure 5. C108297 acts as GR antagonist in neurogenesis and as agonist in memory retention. A. Chronic corticosterone suppressed the number of BrdU positive cell, and 4 d of C108297 treatment increased this number. BrdU scores were significantly higher in animals that received C108297 in combination 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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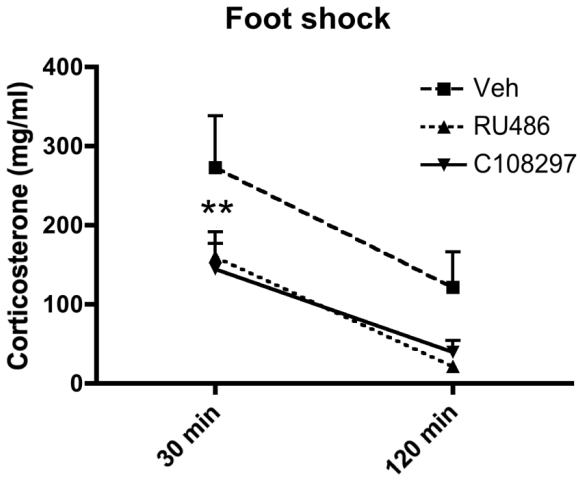
Supplementary material



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'.