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Selective siRNA-mediated suppression of 5-HT$_{1A}$ autoreceptors evokes strong antidepressant-like effects

Analía Bortolozzi[1,2,3a], Anna Castañé[1,3b], Jana Semakova[5b], Noemí Santana[1,3], Gabriel Alvarado[6], Roser Cortés[1,4], Albert Ferrés-Coy[1,2], Gerónimo Fernández[6], María C Carmona[6], Miklos Toth[7], José C Perales[5], Andrés Montefeltro[6], Francesc Artigas[1,3a]

[1]Department of Neurochemistry and Neuropharmacology, IIBB - CSIC - IDIBAPS, Barcelona, Spain.
[2]Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain.
[3]Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Spain. [4]Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), Spain.
[5]Department of Physiological Sciences II, School of Medicine, Bellvitge, Barcelona University, Spain.
[6]n-Life Therapeutics, S.L., La Coruña, Spain.
[7]Department of Pharmacology, Weill Medical College, Cornell University, NY, USA.

Running title: Antidepressant effects and 5-HT$_{1A}$ receptor siRNA

[a]Correspondence should be addressed to Prof. Francesc Artigas or Dr. Analía Bortolozzi, IIBB-CSIC-IDIBAPS, 08036 Barcelona, Spain. E-mail: fapnqi@iibb.csic.es (F.A.); abbnqi@iibb.csic.es (A.B.).
[b]Anna Castañé and Jana Semakova have contributed equally to this study.

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Abstract

Depression is a major health problem worldwide. Most prescribed antidepressants, the selective serotonin reuptake inhibitors (SSRI) show limited efficacy and delayed onset of action, partly due to the activation of somatodendritic 5-HT_{1A}-autoreceptors by the excess extracellular serotonin (5-HT) produced by SSRI in the raphe nuclei. Likewise, 5-HT_{1A} receptor (5-HT_{1A}R) gene polymorphisms leading to high 5-HT_{1A}-autoreceptor expression increase depression susceptibility and decrease treatment response. Here we report on a new treatment strategy based on the administration of small interfering RNA (siRNA) to acutely suppress 5-HT_{1A}-autoreceptor-mediated negative feedback mechanisms. We developed a conjugated siRNA (C-1A-siRNA) by covalently binding siRNA targeting 5-HT_{1A} receptor mRNA with the SSRI sertraline in order to concentrate it in serotonin axons, rich in serotonin transporter (SERT) sites. The intracerebroventricular (i.c.v.) infusion of C-1A-siRNA to mice resulted in its selective accumulation in serotonin neurons. This evoked marked antidepressant-like effects in the forced swim and tail suspension tests but did not affect anxiety-like behaviors in the elevated plus-maze. In parallel, C-1A-siRNA administration markedly decreased 5-HT_{1A}-autoreceptor expression and suppressed 8-OH-DPAT-induced hypothermia (a presynaptic 5-HT_{1A}R effect in mice) without affecting postsynaptic 5-HT_{1A}R expression in hippocampus and prefrontal cortex. Moreover, i.c.v. C-1A-siRNA infusion augmented the increase in extracellular serotonin evoked by fluoxetine in prefrontal cortex to the level seen in 5-HT_{1A}R knockout mice. Interestingly, intranasal C-1A-siRNA administration produced the same effects, thus opening the way to the therapeutic use of C-1A-siRNA. Hence, C-1A-siRNA represents a new approach to treat mood disorders, as monotherapy or in combination with SSRI.

Keywords: 5-HT_{1A} receptors; antidepressant drug design; anxiety; major depression; serotonin neurons; raphe nuclei; small interfering RNA.

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Introduction

Major depression is a severe and heterogeneous psychiatric disease with high, increasing prevalence and socio-economic impact.[1-3] The serotonergic system is implicated in the etiology and treatment of mood disorders.[2] Most prescribed antidepressants, the selective serotonin (5-HT) reuptake inhibitors (SSRI) and the dual 5-HT and norepinephrine reuptake inhibitors block physiological reuptake mechanisms in serotonergic axons and thereby increase extracellular 5-HT concentration to activate postsynaptic 5-HT receptors (5-HT_{1A}R) required for clinical effects. However, this process is severely compromised by the simultaneous activation of somatodendritic 5-HT_{1A}-autoreceptors in the midbrain raphe nuclei. 5-HT_{1A}-autoreceptor activation reduces serotonergic activity and forebrain 5-HT release, an effect contrary to that required for therapeutic response.[4-6]

Hence, the limited clinical efficacy of 5-HT-enhancing drugs and their delayed action are partly due to this negative feedback mechanism. Upon chronic treatment, 5-HT_{1A}-autoreceptors desensitize, leading to the recovery of serotonergic activity and 5-HT release.[5,6] Individuals with elevated density or activity of 5-HT_{1A}-autoreceptors are more susceptible to mood disorders and respond poorly to antidepressants.[7-9]

5-HT_{1A}R antagonists might thus be useful to improve antidepressant therapy by preventing the 5-HT_{1A}-autoreceptor-mediated negative feedback. However, the activation of postsynaptic 5-HT_{1A}R is a necessary step for antidepressant effects[10] which limits the usefulness of this strategy. Thus, unlike the non-selective 5-HT_{1A}R/ß-adrenoceptor antagonist pindolol[11,12] (with a preferential action at 5-HT_{1A}-autoreceptors),[13,14] the selective 5-HT_{1A}R antagonist DU-125530 does not enhance clinical fluoxetine effects (Scorza et al., in preparation).

Here we report on a new antidepressant strategy, based on the use of small interfering RNA (siRNA) targeted to serotonin neurons, to selectively reduce the expression and function of presynaptic (but not postsynaptic) 5-HT_{1A}R. We have developed a conjugated 5-HT_{1A}R siRNA (C-1A-siRNA) directed to serotonin neurons, by covalently binding 5-HT_{1A}R siRNA molecules to the SSRI sertraline. Our working hypotheses were: *a*) the presence of sertraline would allow the selective accumulation of C-1A-siRNA in serotonin neurons, and *b*) the selective 5-HT_{1A}-autoreceptor silencing would have antidepressant effects due to the increased signaling and preservation of postsynaptic 5-HT_{1A}R activity.

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Material and Methods

Animals

Male C57BL/6J mice (Charles River) and male homozygous 5-HT_{1A}R knockout (1A-KO) mice on the same background[15] were used. 10-15-week old mice were housed under controlled conditions (22±1ºC; 12h light/dark cycle) with food and water available *ad libitum*. Animal procedures were conducted in accordance with standard ethical guidelines (European Union regulations L35/118/12/1986) and approved by the local ethical committee.

siRNAs

Four unmodified siRNAs (nt: 633-651, 852-870, 1889-1907 and 2167-2185, GenBank accession #NM_008308) targeting the 5-HT_{1A}R (1A-siRNA1 to 1A-siRNA4) were chosen for *in vivo* studies and administered as a mixture, containing an equal amount of each siRNA duplex. In addition, an unrelated siRNA duplex with no homology to mouse genome was used as negative control (nonsense siRNA – ns-siRNA). All siRNAs, consisted of two complementary 21-nucleotide RNA strand with 3´dTdT overhangs and were prepared according to the manufacturer's protocol (Ambion, Inc.). Conjugated siRNA against 5-HT_{1A}R (C-1A-siRNA) and conjugated nonsense siRNA (C-ns-siRNA) were synthesized by nLife Therapeutics, S.L. as described (International patent application PCT/EP2011/056270). Each specific siRNA sequence against 5-HT_{1A}R (1A-siRNA1 to 1A-siRNA4) and ns-siRNA was designed to carry a substitution in each 5'-end strand **(Figure 1a)**. Conjugated single strand oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC). Fractions containing full-length oligonucleotides were

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pooled, desalted, lyophilized and characterized by MALDI-TOF. Complementary strands were annealed in an isotonic RNA annealing buffer (100mM potassium acetate, 30mM HEPES-KOH pH=7.4, 2mM magnesium acetate), pre-incubated by 1min at 90ºC, centrifuged for 15s and incubated 1h at 37ºC. Annealed products were HPLC-purified and siRNA-containing fractions were lyophilized. Purity was >92%. Similarly to unmodified siRNAs, conjugated siRNAs were used *in vivo* as a mixture consisting of an equal amount of each C-1A-siRNA.

For experiments involving localization of C-1A-siRNA after intracerebroventricular (i.c.v.) infusion, each C-1A-siRNA molecule was additionally bound to biotin in the antisense strand. This was performed by a biotinylation reaction of the conjugate's OH-free end in its terminal C18 spacer.

We used a second strategy to generate a construct with a stronger stability against ribonucleases by the standard phosporamidite chemistry[16,17] as described (International patent application PCT/EP2011/056270). A sertraline-conjugated ns-oligonucleotide (single strand 18-mer oligonucleotide nonsense sequence, with 2'-O-methyl ribonucleotides at positions 1 to 3 and 16 to 18, and a DNA gap of 12 nucleobases) was synthesized with a Cy3 label at 3' attached by a phosphodiester linkage. This was coupled to sertraline to examine the penetration into serotonin neurons after i.c.v. infusion.

A siRNA targeting β-galactosidase (β-Gal-128) bearing a motif eliciting interferon production was also used.[18] Stock solutions of all siRNAs were prepared by re-suspension of the lyophilized product in RNAse-free water and stored at -20ºC until use. siRNA sequences are shown in **Supplementary Table S1.**

Treatments

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Intracerebral siRNA infusion. Mice were anesthetized (pentobarbital, 40mgkg^{-1}, i.p.) and silica capillary microcannulae (110µm-OD, 40µm-ID; Polymicro Technologies) were stereotaxically implanted into dorsal raphe nucleus (DR; coordinates in mm: anteroposterior-AP, -4.5; mediolateral-ML, -1.0; dorsoventral-DV, -4.4; with a lateral angle of 20º) or in dorsal third ventricle (D3V; AP, -2.0; ML, 0; DV, -2.1).[19] Some animals were also implanted with a microdialysis probe in medial prefrontal cortex (mPFC) (see below). siRNA microinfusion was performed with a perfusion pump at 0.5µlmin^{-1} 20–24h after surgery in awake mice. siRNAs targeting 5-HT$_{1A}$R, β-galactosidase or nonsense were prepared in artificial cerebrospinal fluid (aCSF: 125mM NaCl, 2.5mM KCl, 1.26mM $CaCl_2$ and 1.18mM $MgCl_2$ with 5% glucose) and infused at doses of 10 or 30µg of siRNA per mouse. Intra-DR siRNA infusion was repeated 24h later (1-µl aliquots; two administrations in total), while i.c.v. infusion was performed only once into the D3V (2.5-ul aliquots). Controls and 1A-KO mice received aCSF.

Intranasal siRNA administration. Pentobarbital-anesthetized mice were positioned lying on their backs. Phosphate buffered saline (PBS) or conjugated siRNAs (C-ns-siRNA and C-1A-siRNA prepared in PBS) were slowly dropped alternatively into each nostril with a micropipette tip in 5-µl aliquots. Total administered doses were 30 or 100µg of conjugated siRNAs.

RNA isolation and quantitative RT-PCR analysis

24-48h after the last administration of vehicle or siRNA, mice were killed and brains were removed. Midbrain sections containing the raphe nuclei (1-mm thick) were dissected out using a Mouse Brain Matrix (Ted Pella, Inc.), quickly frozen on dry ice and stored at -80ºC. Total RNA was isolated with Trizol solution (Invitrogen). cDNA

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synthesis was performed using Retro-tools two steep kits (Biotools BandM labs SA). Quantitative RT-PCR (7900HT Fast Real-Time PCR System, Applied Biosystems) was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) in 20µl of reaction mix. Cycle threshold (Ct) values were calculated using AbiPrism SDS 2.1 software (Applied Biosystems). Data were normalized to the amount of CyclophilinA cDNA. Results were expressed as the unitary ratio versus vehicle controls. Primers sequences are given in **Supplementary Table S1**.

Tissue preparation for in situ hybridization and receptor autoradiography

Mice were killed by pentobarbital overdose and the brains rapidly removed, frozen on dry ice and stored at -20°C. Tissue sections, 14-µm thick, were cut using a microtome-cryostat (HM500 OM, Microm), thaw-mounted onto APTS (3-aminopropyltriethoxysilane, Sigma-Aldrich) coated slides and kept at –20°C until use.

In situ hybridization

For 5-HT_{1A}R mRNA, three oligonucleotides were used simultaneously, complementary to bases: 1-48, 763-810 and 1219-1266 (GenBank accession #NM_012585). These probes were synthesized with a 380 Applied Biosystem DNA synthesizer. Labeling of the probes and *in situ* hybridization procedures were carried out as described previously.[20-22] Hybridized sections were exposed to Biomax MR film (Kodak) for 2-3 weeks with intensifying screens.

Receptor autoradiography

The autoradiographic binding assays for 5-HT_{1A} and 5-HT_{1B} receptors and serotonin transporter (SERT)[20,23,24] were performed using the following radioligands: *a)* [^{3}H]-8-

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OH-DPAT (233Cimmol^{-1}), *b)* [^{125}I]-cyanopindolol (2200Cimmol^{-1}) and *c)* [^{3}H]-citalopram (70Cimmol^{-1}), respectively (Amersham-GE Healthcare and Perkin-Elmer). 8-OH-DPAT, isoproterenol, pargyline and 5-HT were from Sigma-Aldrich and fluoxetine was from Tocris. Experimental conditions are summarized in **Supplementary Table S2**. Tissues were exposed to Biomax MR film (Kodak) together with ^{3}H-Microscales standards (Amersham-GE Healthcare). All experimental and control brains within a group were processed in duplicate and exposed to films as a batch.

Films were analysed by microdensitometry using a computer assisted image analyser (AIS, Imaging Research Inc.). 5-HT$_{1A}$R mRNA and 5-HT$_{1B}$ binding sites in selected brain regions were measured in the respective autoradiograms to obtain relative optical densities (ROD). For 5-HT$_{1A}$R and SERT binding, the system was calibrated with ^{3}H-Microscales standards to obtain fmol·mg^{-1} protein equivalents from ROD data. AIS system was also used to acquire pseudocolor images. Black and white photographs were taken from autoradiograms using a Wild 420 macroscope (Leica) equipped with a Nikon DXM1200 F digital camera and ACT-1 Nikon software. Images were processed with Photoshop (Adobe Systems, Mountain View) by using identical values for contrast and brightness.

Immunohistochemistry

Animals were killed by a pentobarbital overdose and perfused with 4% paraformaldehyde in sodium-phosphate buffer (pH=7.4). Brains were dissected, post-fixed 24h at 4°C in the same solution, and then placed in 30% sucrose in PBS for 3 days at 4°C. After cryopreservation, brains were embedded with gelatin, frozen and sectioned in a cryostat (30um coronal). Free floating sections were washed and

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permeabilized with PBS-Triton 0.3%, blocked with BlockAid (Invitrogen) plus 0.3% Triton for 2h at 23°C and then incubated with NeutrAvidin (Invitrogen) 0.0125mgml^{-1} in blocking solution (1h, 23°C). Sections were than washed and incubated overnight (4°C) with biotinylated rabbit anti-donkey antibody (Abcam) diluted 1:200, washed and incubated (2h, 23°C) with Cy3 conjugated donkey anti-rabbit antibody (Rockland) diluted 1:200 in blocking solution. This was followed by additional blocking with 10% donkey serum (Sigma-Aldrich) in PBS-Triton 0.3%, 1h, 23°C and overnight incubation with anti-tryptophanhydroxylase antibody (1:100; Chemicon-Millipore) diluted in 3% of donkey serum in PBS-Triton 0.3%. Excess of primary antibody was washed and sections were incubated 2h, 23°C with AlexaFluor 647 conjugated donkey anti-sheep antibody (Invitrogen) diluted 1:200 and YOYO-1 (Invitrogen) diluted 1:5000 in 3% of donkey serum in PBS-Triton 0.3%. Finally, sections were mounted with Mowiol (Calbiochem). Samples were analyzed using a Leica spectral confocal microscope; data were sequentially collected from each channel, merged by Leica Confocal Software and 3D projections (maximal, average and transparent) were made from z-series. Pictures were generated using Photoshop (Adobe Systems, Mountain View).

Intracerebral microdialysis

Extracellular 5-HT concentration was measured by in vivo microdialysis as previously described.[20] Briefly, one concentric dialysis probe (Cuprophan; 1mm-long) was implanted in mPFC (AP, 2.2; ML, -0.2; DV, -3.4)[19] of pentobarbital-anaesthetized mice. Experiments were performed 48-72h after surgery. To assess 8-OH-DPAT effects on extracellular 5-HT, 1µM citalopram (SSRI; Lundbeck A/S) was added to aCSF.[25] The aCSF was pumped at 2.0µlmin^{-1} (WPI model sp220i) and 30-min

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samples were collected. 5-HT concentrations were analyzed by HPLC-amperometric detection (Hewlett-Packard 1049; +0.6V) with detection limits of 1.5 fmolsample^{-1}. Baseline 5-HT levels were calculated as the average of the four pre-drug samples. Correct probe placement was verified using cresyl-violet staining.

Behavioral and physiological assessments

Mice were tested at 24-72h after treatments. Elevated plus-maze (EPM) and tail suspension tests (TST) were always conducted in this order, with one day between tests. All tests were performed between 10:00 AM-3:00 PM. On test days, animals were transported to the dimly illuminated behavioral laboratory and were left undisturbed for at least 1h before testing.

8-OH-DPAT-induced hypothermia. Body temperature was measured intrarectally using a lubricated probe inserted ~2cm and a digital thermometer (AZ9882, Panlab). Mice were singly housed in clean cages for 20min before measurements and then two baseline temperature measurements were taken. Ten minutes later, animals received 8-OH-DPAT 1mgkg^{-1} i.p. and body temperature was recorded every 15min for a total of 120min. Data are presented as a change from the final baseline measurement.

Elevated plus-maze. The elevated plus-maze was performed using a cross maze with 16x5cm arms illuminated from the top (100lux). Mice were placed in the central area, facing one of the open arms and the time and number of entries into open and closed arms in 5min were recorded (video camera system, Smart, Panlab). Results were expressed as the percentage of time and number of entries into the open arms.

Tail suspension test. Mice were suspended 30cm above the bench by adhesive tape placed approximately 1cm from the tip of the tail. Mice were monitored and recorded

using a video camera system (Smart, Panlab) and the time spent immobile was recorded for 6min.

Forced swim test (FST). Mice were individually placed into a clear cylinder (15cm diameter, 30cm height) containing 20cm of water maintained at 24-25ºC, essentially as described by Porsolt et al.[26] In this test, immobility of the mouse was scored in 2-mim bins for a total of 6min using a video camera system (Smart, Panlab).

Statistical analysis

Data are expressed as means ± s.e.m. Data were analyzed with Student's *t*-test, one- or two-way ANOVA, as appropriate, followed by post-hoc test (Newman-Keuls). The level of significance was set at $P < 0.05$ (two-tailed)

Results

Selective silencing of 5-HT_{1A}-autoreceptor expression

The potential of siRNA-based gene silencing in vivo has been limited by the lack of safe delivery to specific target cells.[27] Previously,[28] we established that local infusion of four siRNA sequences directed towards 5-HT_{1A}R (1A-siRNA) into the mice DR reduced 5-HT_{1A}-autoreceptor expression and function without affecting postsynaptic 5-HT_{1A}R, evoking a robust and rapid antidepressant-like effect. We therefore used the same 1A-siRNA sequences to construct C-1A-siRNA. Sense and antisense strands were chemically conjugated in each 5'-end strand whereas the sense strand was covalently bound to the SSRI-sertraline (**Figure 1a**). This modification was intended to direct C-1A-siRNA molecules to 5-HT axons, extremely rich in axonal varicosities (>10^6/mm^3)[29], expressing SERT, for which sertraline shows very high affinity (Ki=0.29nM, http://kidb.case.edu/pdsp.php).

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Forty eight h after stereotaxic infusion in the D3V (30µg/mouse, as in receptor expression and functional experiments; see below), the C-1A-siRNA construct was detected in midbrain serotonergic neuropil, rich in dendrites and initial axonal segments (**Figure 1b, Supplementary Figure S1**). In contrast, C-1A-siRNA was undetectable in dorsal hippocampal neurons, located much closer to the infusion site (**Supplementary Figure S2**). Additional experiments using a Cy3-coupled, sertraline-conjugated -18-mer oligonucleotide (see Methods) showed that 24h after its infusion into the D3V (30µg), the Cy3 labeled oligonucleotide was found in the cytoplasm of TPH-positive midbrain serotonin neurons (**Supplementary Figure S3).**

Next, we evaluated whether the i.c.v. infusion of C-1A-siRNA could selectively silence the 5-HT_{1A}R gene in 5-HT neurons, as observed after local infusion of unmodified 1A-siRNA.[28] Mice were stereotaxically injected into D3V with: *a)* vehicle, *b)* unmodified nonsense siRNA (ns-siRNA), *c)* unmodified 1A-siRNA, *d)* conjugated nonsense siRNA (C-ns-siRNA), or *e)* C-1A-siRNA (30µg of each siRNA). Histological examination at 1-3 days post-administration revealed that 5-HT_{1A}R mRNA and binding densities were significantly decreased in the dorsal (40% versus control) and median raphe (30%) nuclei of C-1A-siRNA-treated mice (**Figure 2a, 2c, Supplementary Figure S4**).

A similar decrease was produced by intra-DR infusion of the same unmodified 1A-siRNA sequences (**Supplementary Figure S5**). Likewise, local 1A-siRNA and C-1A-siRNA infusion reduced comparably 5-HT_{1A}R binding density in the DR (**Supplementary Figure S6**), indicating that sertraline conjugation did not alter 1A-siRNA capacity to knockdown 5-HT_{1A}R.

In contrast, C-1A-siRNA infusion into D3V did not affect postsynaptic 5-HT_{1A}R density in forebrain (**Figure 2b, 2d, Supplementary Figure S7a**) nor that of other

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genes expressed by 5-HT neurons (SERT and 5-HT_{1B}R) of the same mice (**Figure 3a-c**). Likewise, C-1A-siRNA sequences did not up-regulate inflammatory cytokines (TNFα and IFNγ) or apoptotic genes (BAX*)*, while the immunostimulatory activity of ß-galactosidase-siRNA sequence (used as positive control) dramatically increased TNFα and IFNγ expression (**Figure 3d-f**), indicating that 5-HT_{1A}-autoreceptor silencing was specifically mediated by an RNAi mechanism.

Effects of C-1A-siRNA on serotonergic function

The physiological consequences of 5-HT_{1A}-autoreceptor silencing were examined using the hypothermia response induced by the selective 5-HT_{1A}R agonist, 8-OH-DPAT, an effect mediated exclusively by presynaptic 5-HT_{1A}R in mice.[30] C-1A-siRNA-treated mice did not show hypothermia, nor did constitutive 5-HT_{1A}R knockout mice (1A-KO), while control groups displayed the expected hypothermic response (**Figure 2e, Supplementary Figure S8a)**. We next investigated the consequences of 5-HT_{1A}-autoreceptor knockdown on serotonergic function. Baseline extracellular 5-HT concentration in mPFC did not differ among experimental groups **(Supplementary Table S3).** However, 8-OH-DPAT administration (0.5mgkg^{-1}, i.p.) reduced extracellular 5-HT concentration in mPFC of vehicle (56% of baseline) and C-ns-siRNA-treated mice (60%), but not in C-1A-siRNA-treated (88%) and 1A-KO mice (99%) (**Figure 2f, Supplementary Figure S8b**).

To assess the involvement of SERT as a gate for the accumulation of C-1A-siRNA in 5-HT neurons, mice were pretreated with sertraline (20 mgkg^{-1}, i.p.; 3h before the i.c.v. C-1A-siRNA infusion). These mice displayed the fall in 5-HT release and body temperature as did controls, whereas those treated with C-1A-siRNA alone did not respond to 8-OH-DPAT in both experimental approaches (**Figure 2e-f,**

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Supplementary Figure S8). This indicates that C-1A-siRNA requires functional SERT sites to silence 5-HT_{1A}-autoreceptor expression.

C-1A-siRNA evokes antidepressant-like responses

To explore the behavioral effects of 5-HT_{1A}-autoreceptor knockdown, we examined the antidepressant- and anxiety-like responses using the forced swim test (FST), tail suspension test (TST), and elevated plus-maze (EPM) paradigm. Mice infused with C-1A-siRNA (30µg) into the D3V displayed a marked reduction of immobility time in FST compared to controls (**Figure 4a**). Moreover, C-1A-siRNA-treated mice showed a significantly lower immobility than controls in the TST, as did 1A-KO mice (**Figure 4b**). A quantitative difference between both groups was noted, likely due to the partial knockdown of 5-HT_{1A}-autoreceptors in C-1A-siRNA-treated mice and total absence in 1A-KO mice. Fluoxetine administration (10mgkg^{-1}, i.p.), used as a positive control, also reduced immobility in the FST and TST (**Figure 4a-b**). In contrast, control and C-1A-siRNA-treated mice behaved comparably in the EPM, with equal number of entries and time spent in the open arms. 1A-KO mice showed a clear anxiogenic profile, as previously described[15,31] (**Figure 4c**). At the neurochemical level, fluoxetine (20mgkg^{-1}, i.p.) increased extracellular 5-HT concentration in mPFC significantly more in C-1A-siRNA (272% of baseline) and 1A-KO mice (230%) than in controls (vehicle: 138%; C-ns-siRNA: 150%), indicating that C-1A-siRNA-induced reduction in 5-HT_{1A}-autoreceptors augments SSRI effects (**Figure 4d**).

Intranasal C-1A-siRNA administration reduces 5-HT_{1A}-autoreceptor expression and induces antidepressant effects

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The above findings support the potential of C-1A-siRNA as a new antidepressant strategy, either as monotherapy or in combination with 5-HT-enhancing drugs. However, its therapeutic use is limited by the lack of a suitable route to brain. We therefore examined the potential of the non-invasive intranasal route, as described before.[32-34] Intranasal C-1A-siRNA administration (30µg/mouse, as in i.c.v. studies) reduced 5-HT_{1A}R mRNA and binding density (27%) in the DR, but not in postsynaptic sites in the same mice (**Figure 5a-d, Supplementary Figure S7b**). Unlike in controls (vehicle and C-ns-siRNA), 8-OH-DPAT administration did not reduce body temperature and 5-HT release in mice treated intranasally with C-1A-siRNA (**Figure 5e-f**).

Given the smaller effect size of 30µg intranasal C-1A-siRNA on 5-HT_{1A}-autoreceptor expression -compared with i.c.v. infusion-, we conducted behavioral experiments using 30 and 100µg of intranasal C-1A-siRNA. Both doses significantly reduced immobility in the TST, with a greater effect of the 100µg dose (**Figure 5g**). Similarly, intranasal C-1A-siRNA (100µg) evoked a reduction of immobility time in the FST which was significantly lower than that seen in controls (**Figure 5h**). Neither dose affected anxiety-like behavior as assessed with EPM test (data not shown).

Discussion

The present findings show that C-1A-siRNA can be used to efficiently and selectively silence 5-HT_{1A}-autoreceptor expression and function *in vivo*. This represents a new therapeutic approach in neuropsychopharmacology, based on the use of RNAi. Hence, i.c.v. and intranasal C-1A-siRNA infusion *i*) reduced 5-HT_{1A}-autoreceptor expression, without affecting that of postsynaptic 5-HT_{1A}R, *ii)* suppressed the hypothermia and fall in 5-HT release induced by 8-OH-DPAT; *iii*) evoked a marked

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antidepressant-like response in the FST and TST, and *iv)* augmented the SSRI-induced elevation of extracellular 5-HT.

siRNAs are effective, safe and well tolerated in mice and non-human primates. While they are used therapeutically in other medical areas, their applicability to brain diseases has been hampered by the difficulty to target selected neuronal populations and by the lack of adequate delivery methods.[27] The present results represent a major advance in this field given the selective targeting of 5-HT_{1A}R expressed in 5-HT neurons after i.c.v. and intranasal administration. Although further investigations are required to examine the exact mechanism(s) used by C-1A-siRNA to accumulate into 5-HT neurons, available evidence suggests that C-1A-siRNA can be internalized via SERT, similarly to SSRI[35] to reach the somatodendritic region of serotonergic neurons via retrograde axonal transport. This view is supported by *i*) the selective presence of sertraline-conjugated C-1A-siNA and the C-ns-oligonucleotide in midbrain serotonergic neuropil and cell bodies, respectively, *ii*) its selective action on 5-HT_{1A}-autoreceptors (far from the infusion site), *iii*) the lack of effect on hippocampal 5-HT_{1A}R -much closer to the D3V-, and *iv*) the prevention of C-1A-siRNA effects by prior SERT blockade. The present procedure offers unprecedented efficacy and cellular selectivity, compared with previous siRNA conjugation methods used *in vivo*.[27,36-38] Hence, the 40% knockdown of 5-HT_{1A}-autoreceptors produced by single-point administration of 30µg C-1A-siRNA is comparable to previous results using prolonged infusion of unmodified siRNA to silence monoamine transporters (400 $\mu g day^{-1}$ for 1-2 weeks; 2.8-5.6mg in total, i.e., 90-180-fold the dose used herein).[39,40]

Our results agree with previous observations on the detrimental role of 5-HT_{1A}-autorerceptors in depression. Human neuroimaging studies have associated high 5-HT_{1A}-autoreceptor levels with a poor amygdale reactivity.[41] Likewise, genetic studies

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link 5-HT_{1A}R mutations leading to high levels of 5-HT_{1A}-autoreceptors with higher incidence of mood disorders and poorer response to antidepressants.[7] In rodents, disruption of the 5-HT_{1A}-autoreceptor-mediated negative feedback with 5-HT_{1A}R antagonists enhances SSRI effects.[5,6] Clinically, the non-selective 5-HT_{1A}R antagonist pindolol augments and accelerates SSRI effects, likely by a preferential interaction with 5-HT_{1A}-autoreceptors.[11,12,42] The present data support that C-1A-siRNA could be used to acutely suppress 5-HT_{1A}-autoreceptor function, without the need to wait for antidepressant-induced desensitization, thus optimizing antidepressant action.

Our results also agree with those found recently using conditional knockout mice for presynaptic 5-HT_{1A}R.[43] Both studies indicate that the selective –yet partial- reduction of 5-HT_{1A}-autoreceptor expression with unchanged postsynaptic 5-HT_{1A}R expression evokes antidepressant-like behavior and augments SSRI effects. Interestingly, the RNAi strategy can be applied in adult mice, without the unwanted neurodevelopmental effects associated to 5-HT_{1A}R suppression.[44] Although further improvements are required, the intranasal route offers a first potential way for C-1A-siRNA delivery to brain. This route allowed the same effects than the i.c.v. infusion, yet with a slightly smaller effect size, likely due to the dilution of C-1A-siRNA during brain entry. Once crossed the semi-permeable nasal blood-brain barrier, C-1A-siRNA molecules may reach the dense plexus of serotonergic axons in outer layers of the olfactory bulb, anatomically connected with olfactory regions.[45]

In summary, our results show for the first time the feasibility of using RNAi strategies for the treatment of mood disorders. The excellent cellular selectivity and efficacy obtained with acute C-1A-siRNA treatment indicate that this may be a new approach to treat mood disorders and open the way to develop RNAi-based

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therapeutic classes to silence genes, or variant alleles refractory to classical pharmacological treatments.

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Figure Legends

Figure 1. (a) Chemical structure of conjugated 5-HT_{1A}R-siRNA (C-1A-siRNA). C-1A-siRNA construct comprising specific siRNA sequences directed against 5-HT_{1A}R with chemical conjugation in each 5'-end strand. The sense strand was aminomodified by performing a 5'-C6 amino modification and condensation with a succinimide active ester of sertraline (sertraline-NH-CH2C(=O)NH(CH2)5COO-succinimide) making up the peptide linker of the conjugate (sertraline-NH-CH2C(=O)NH(CH2)5COO-sense strand-3'OH). A phosphoramidite derivative of 1',2'-dideoxyribose molecule (dSpacer CE Phosphoramidite: 5'-O-Dimethoxytrityl-1',2'-Dideoxyribose-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite) and two phosphoramidite C18 spacer arms (Spacer Phosphoramidite 18: 18-O-Dimethoxytritylhexaethyleneglycol,1-[(2-cyanoethyl)-(N,N-diisopropyl)] phosphoramidite) were covalently coupled to the complementary antisense strand by phosphodiester linkages. **(b)** Selective accumulation of C-1A-siRNA in raphe serotonin neurons. Mice received a single intracerebroventricular infusion of vehicle or biotin-labeled C-1A-siRNA (30µg) into the dorsal third ventricle and were killed 48h post-administration (*n*=3 mice/group). Laser confocal images of TPH-immunoreactive serotonin neurons (blue) showing the immune-localized biotin-labeled C-1A-siRNA (red). Right-hand panels are high-magnification photomicrographs of the frames in the left. Scale bars: white=60µm, yellow=25µm.

Figure 2. Selective 5-HT_{1A}-autoreceptor silencing by intracerebroventricular infusion of C-1A-siRNA. **(a)** Representative coronal brain sections showing the C-1A-siRNA-induced reduction of 5-HT_{1A}R expression in raphe nuclei assessed by in situ

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hybridization and [^{3}H]-8-OH-DPAT binding. **(b)** Representative coronal brain sections showing [^{3}H]-8-OH-DPAT binding to 5-HT$_{1A}$R in medial prefrontal cortex (mPFC) and hippocampus (HPC). Scale bars: white=2mm, black=500µm. **(c)** Effect of C-1A-siRNA (30µg) on 5-HT$_{1A}$R mRNA and binding in dorsal raphe nucleus (DR) (*n*=3-5). One-way ANOVA revealed a group effect on 5-HT$_{1A}$R mRNA ($F_{4,15}$=7.15, *P*<0.01) and 5-HT$_{1A}$R binding ($F_{4,13}$=5.04, *P*<0.05). **P*<0.05, ***P*<0.01 versus the rest of experimental groups. **(d)** Postsynaptic 5-HT$_{1A}$R binding in mPFC and HPC was not affected by any treatment (*n*=3-4). **(e)** The 5-HT$_{1A}$R agonist, 8-OH-DPAT (1mgkg^{-1}, i.p.) reduced body temperature in vehicle and C-ns-siRNA, but not in C-1A-siRNA and 1A-KO mice. Pretreatment (3h) with sertraline (20mgkg^{-1}, i.p.) blocked C-1A-siRNA (30µg) effect on 5-HT$_{1A}$-autoreceptor silencing (*n*=5-8). One-way ANOVA, $F_{5,31}$=25.35, *P*<0.001. **(f)** 8-OH-DPAT (0.5mgkg^{-1}, i.p.) decreased extracellular serotonin in controls, but not in C-1A-siRNA and 1A-KO groups. Pretreatment (3h) with sertraline (20mgkg^{-1}, i.p.) prevented the effect of C-1A-siRNA (*n*=5-8, $F_{6,38}$=15.74, *P*<0.001). ****P*<0.001 versus control groups, $^{\#\#\#}$*P*<0.001 versus C-1A-siRNA. Values are mean ± s.e.m.

Figure 3. (a-c) Unchanged serotonin transporter (SERT) and 5-HT$_{1B}$R expression after 5-HT$_{1A}$-autoreceptors silencing. Mice received: *a*) vehicle, *b*) C-ns-siRNA, or *c*) C-1A-siRNA (30µg) into D3V and were killed 24h after administration (*n*=5-7). **(a)** The levels of the mRNAs encoding 5-HT$_{1A}$, SERT and 5-HT$_{1B}$ (serotonin pathway) were evaluated in midbrain raphe nuclei by RT-qPCR. One-way ANOVA showed a group effect on 5-HT$_{1A}$R mRNA levels ($F_{2,13}$=6.06, *P*<0.05). **P*<0.05 *versus* vehicle and C-ns-siRNA. **(b)** Quantitative autoradiography of [^{3}H]-citalopram binding showed no differences on SERT density in the different brain areas for each treatment. **(c)** Bar

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graph showing no differences in 5-HT_{1B}R density evaluated by [^{125}I]-cyanopindolol binding in the different brain areas. mPFC-medial prefrontal cortex; HPC-hippocampus; DR-dorsal raphe nucleus; GP-globus pallidus; SN-substantia nigra. **(d-f)** Unmodified 1A-siRNA and C-1A-siRNA sequences did not induce inflammatory and apoptotic responses. **(d)** Mice received intra-DR: *a*) vehicle, *b*) unmodified ns-siRNA or *c*) unmodified 1A-siRNA (10µg by 2 consecutive days, 20µg in total) and were killed 24h after administration. mRNA levels of TNFα and IFNγ (pro-inflammatory cytokines) and BAX (apoptosis pathway) were evaluated in midbrain raphe nuclei by RT-qPCR (*n*=4-6). **(e)** Immunostimulatory β-Gal-siRNA was used as a positive control. Mice received intra-DR: *a*) vehicle, or *b*) β-Gal-siRNA (10µg by 2 consecutive days, 20µg in total) and were killed 24h after administration. TNFα and IFNγ mRNA levels were significantly up-regulated in midbrain after β-Gal-siRNA infusion (*n*=3-6, Student's *t*-test, ***P<0.001). **(f)** Bar graphs showing no differences in TNFα, IFNγ and BAX mRNA levels of mice infused with: *a*) vehicle, *b*) C-ns-siRNA or *c*) C-1A-siRNA (30µg) into D3V and killed 24h after administration (*n*=5-7). Values are mean ± s.e.m.

Figure 4. C-1A-siRNA evokes antidepressant-like responses and potentiates the neurochemical effect of fluoxetine. **(a)** Mice infused with 30µg C-1A-siRNA in the dorsal third ventricle (D3V) displayed a decreased immobility in the forced swim test (FST) (*n*=14-15). Two-way ANOVA showed a significant effect of group ($F_{1,27}$=12.98, P<0.001), time ($F_{2,54}$=150.05, P<0.0001) and interaction ($F_{2,54}$=3.18, P<0.05). A significantly greater reduction of the immobility in C-1A-siRNA-treated mice vs. controls was observed for the total period (0-6min) (P<0.01; Student's *t*-test). A comparable antidepressant response was observed after acute SSRI fluoxetine

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administration to control C57BL/6J mice (10mgkg^{-1}, i.p. 30min before FST) (n=12-13). Effect of group ($F_{1,23}$=5.18, P<0.05) and time ($F_{2,46}$=66.94, P<0.0001). Student's t-test was used to assess the action of fluoxetine for the total observation period (0-6min). *P<0.05, **P<0.01, ***P<0.001 versus respective controls. **(b)** C-1A-siRNA-treated mice also showed a reduced immobility in the tail suspension test (TST), as did constitutive 1A-KO mice (n=12-18). One-way ANOVA showed a significant effect of group ($F_{2,47}$=18.64, P<0.001). Acute fluoxetine administration (10mgkg^{-1}, i.p. 1h before TST) produced a similar antidepressant response (n=7). **P<0.01, ***P<0.001 versus vehicle mice, $^{\#\#}P$<0.01 versus C-1A-siRNA mice. **(c)** Unlike 1A-KO mice, vehicle and C-1A-siRNA mice behaved similarly in the elevated plus-maze (n=12-18). One-way ANOVA indicated a group effect on entries ($F_{2,43}$=24.18, P<0.001) and time in open arms ($F_{2,42}$=4.56, P<0.05). *P<0.05, **P<0.01, ***P<0.001 versus vehicle and C-1A-siRNA groups. **(d)** Acute fluoxetine (20 mgkg^{-1}, i.p.) increased extracellular serotonin in mPFC of C-1A-siRNA and 1A-KO mice significantly more than in vehicle and C-ns-siRNA groups (n=4-6). Effect of group ($F_{3,17}$=4.78, P<0.05), time ($F_{14,238}$=19.40, P<0.001) and interaction ($F_{42,238}$=2.57, P<0.001). *P<0.05 versus control groups. Values are mean ± s.e.m.

Figure 5. Intranasal C-1A-siRNA silences 5-HT$_{1A}$-autoreceptors and evokes antidepressant-like responses. **(a)** Representative coronal brain sections showing the C-1A-siRNA-induced reduction of 5-HT$_{1A}$R expression in raphe nuclei by in situ hybridization and [^{3}H]-8-OH-DPAT binding. **(b)** Representative coronal brain sections showing [^{3}H]-8-OH-DPAT binding to 5-HT$_{1A}$R in medial prefrontal cortex (mPFC) and hippocampus (HPC). Scale bars: white=2mm, black=500µm. **(c)** Bar graphs showing the effects of intranasal C-1A-siRNA (30µg) administration on 5-HT$_{1A}$R mRNA and

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binding in dorsal raphe nucleus (DR) (*n*=3-6). One-way ANOVA showed a significant effect of group on 5-HT_{1A}R mRNA ($F_{2,9}$=12.41, P<0.01) and 5-HT_{1A}R binding ($F_{2,8}$=37.98, P<0.001). *P<0.05, **P<0.01 versus control groups. **(d)** Unchanged densities of postsynaptic 5-HT_{1A}R binding in all experimental groups (*n*=3-6). **(e)** Systemic 8-OH-DPAT administration (1 $mgkg^{-1}$, i.p.) did not evoke hypothermia in C-1A-siRNA-treated (30µg) mice (*n*=5-7). Two-way ANOVA showed a significant effect of group ($F_{2,13}$=34.21, P<0.001), time ($F_{4,52}$=59.30, P<0.001) and interaction ($F_{8,52}$=10.89, P<0.001). ***P<0.001 versus control groups. **(f)** Reduced extracellular serotonin in mPFC of controls, but not in C-1A-siRNA mice after 8-OH-DPAT administration (0.5$mgkg^{-1}$, i.p.) (*n*=4-6). Significant effect of group ($F_{2,12}$=8.22, P<0.01), time ($F_{14,168}$=7.56, P<0.001) and interaction ($F_{28,168}$=2.28, P<0.001). **P<0.01 versus controls. **(g)** Single intranasal C-1A-siRNA administration (30 or 100µg) evoked a dose-dependent decreased immobility in the tail suspension test (*n*=10-15). One-way ANOVA showed a significant effect of group ($F_{2,34}$=8.70, P<0.001). *P<0.05, ***P<0.001 versus vehicle. **(h)** Mice treated intranasally with C-1A-siRNA (100µg) displayed a reduced immobility time in the forced swim test compared with vehicle-treated controls (*n*=13-16). Two-way ANOVA showed a significant effect of group ($F_{1,27}$=9.27, P<0.01) and time ($F_{2,54}$=102.95, P<0.0001). Student's *t*-test was used to assess the action of C-1A-siRNA for the total observation period (0-6min). **P<0.01 versus vehicle. Values are mean ± s.e.m.

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a

Sense strand

Antisense strand

b

TPH-ir

Vehicle

C-1A-siRNA

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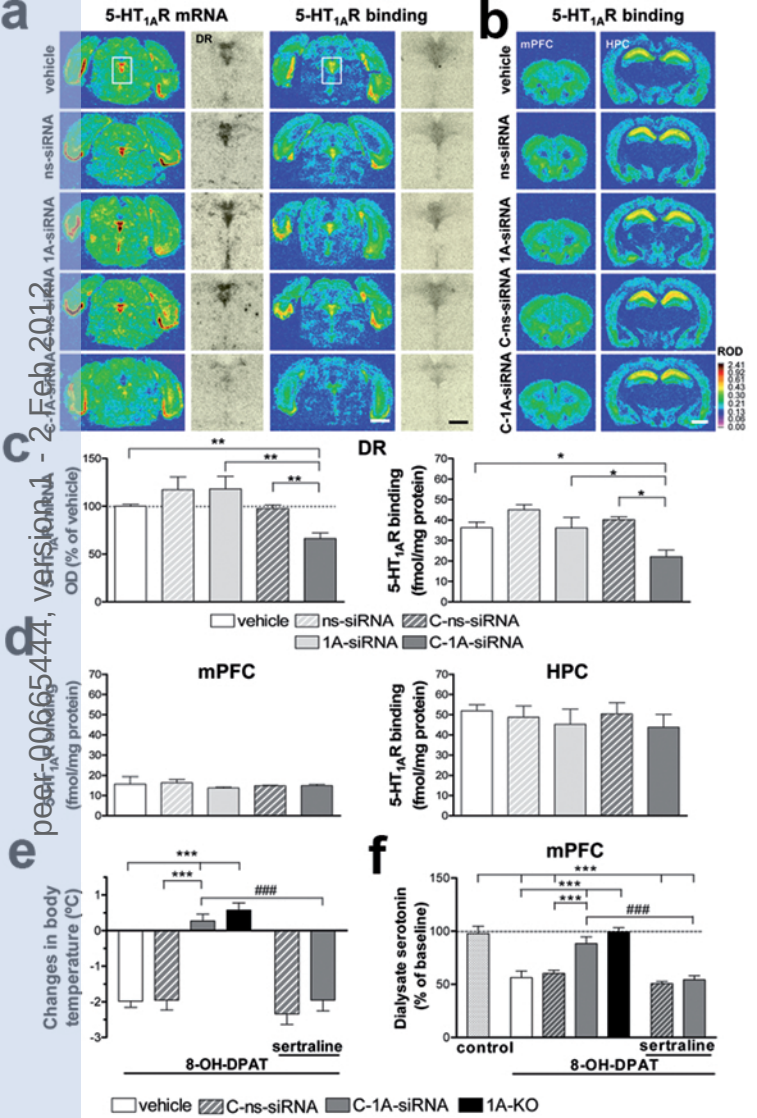

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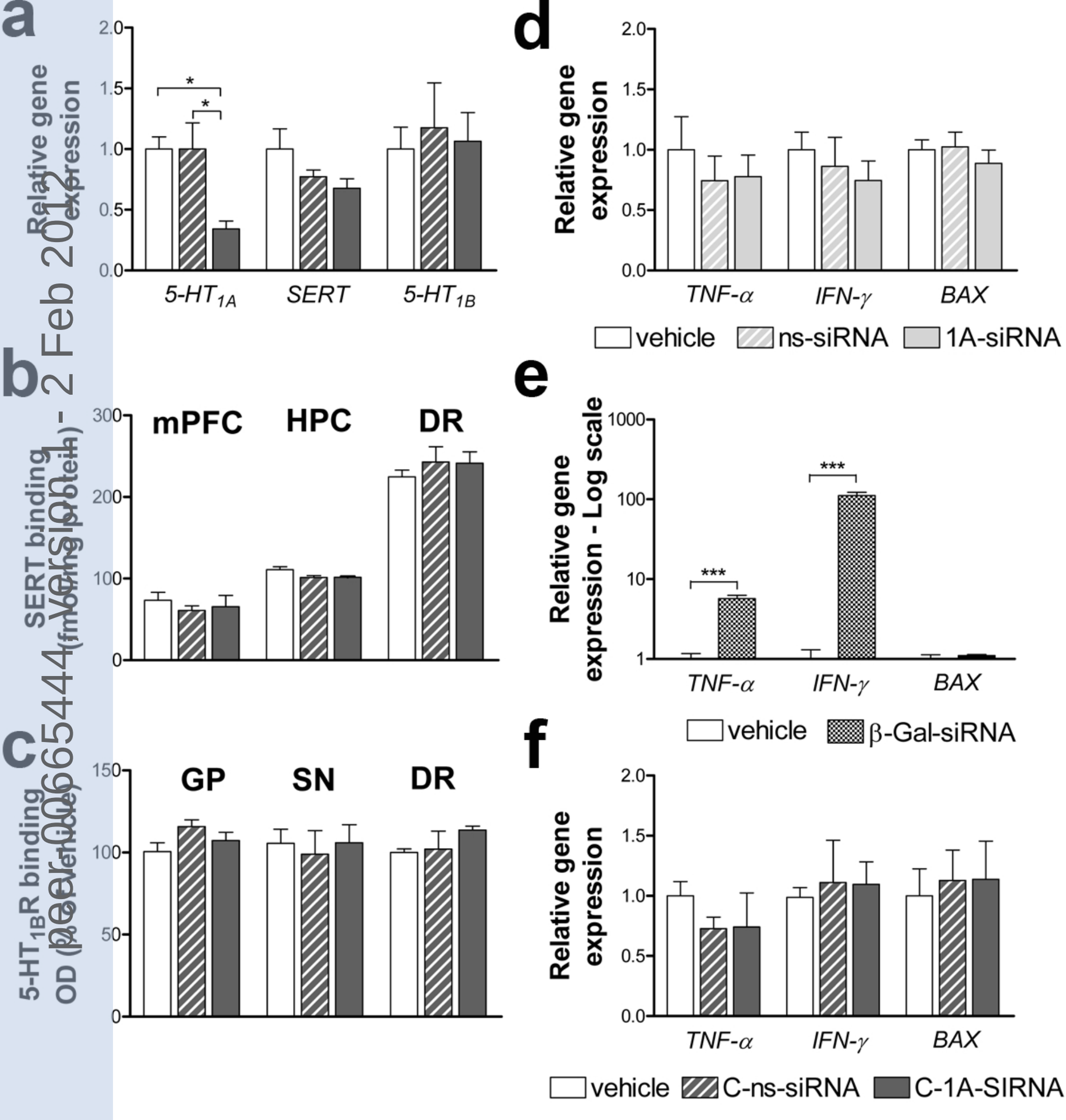

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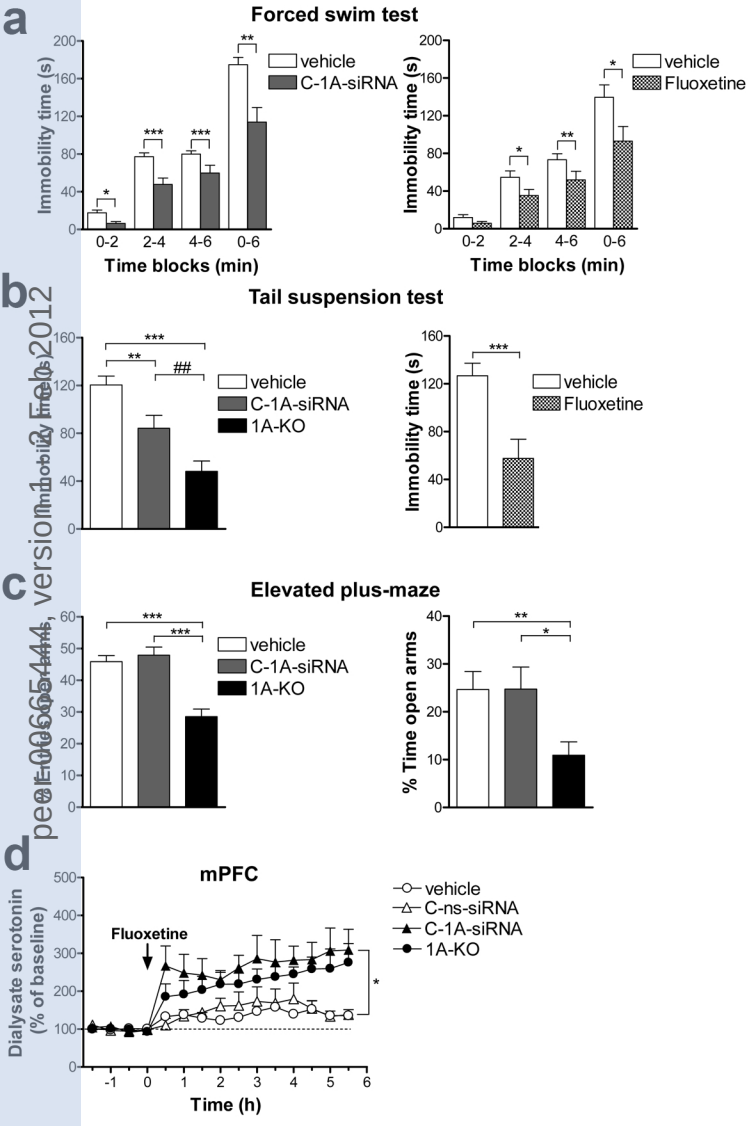

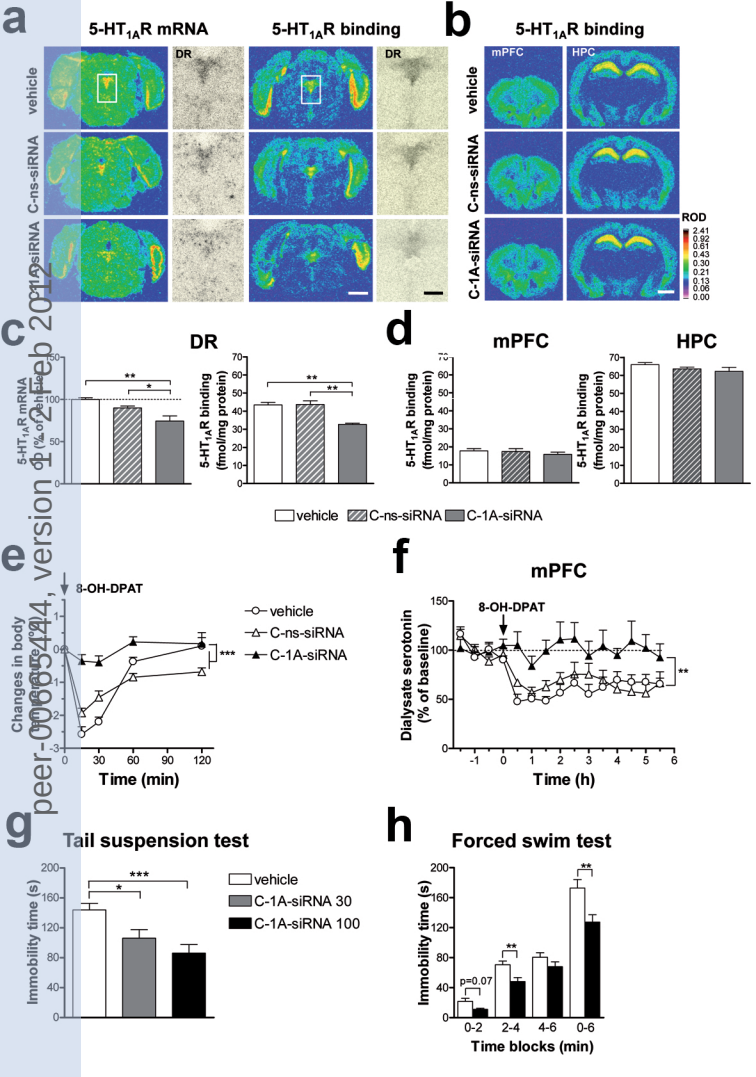

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