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Brief Article

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Predicting the Antinociceptive Efficacy of σ_1 Receptor Ligands by a Novel Receptor Fluorescence Resonance Energy Transfer (FRET) Based Biosensor

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Supporting Information

ABSTRACT: We have developed a novel methodology for monitoring the σ_1 receptor activation switch in living cells. Our assay uncovered the intrinsic nature of σ_1 receptor ligands by recording the ligand-mediated conformational changes of this chaperone protein. The change triggered by each ligand correlated well with its ability to attenuate formalin induced nociception in an animal model of pain. This tool may assist in predicting the antinociceptive efficacy of σ_1 receptor ligands.

INTRODUCTION

The σ_1 receptor (σ_1R) is an intriguing and unique molecule that regulates the function of a number of proteins based on its chaperone-like activity.[1,2] While it was initially described as a subtype of the opioid receptor family, the σ_1R does not present reasonable homology with any known mammalian protein. Interestingly, the σ_1R has been long thought to play a key role in neuropharmacology, mainly in learning and memory processes, depression and anxiety, schizophrenia, analgesia, and various effects of certain drugs of abuse.[1,3] On the other hand, σ_1R is expressed in nearly all mammalian cells and widely distributed in the central nervous system (CNS).[4] However, despite its ubiquity, no natural ligand for σ_1R has been clearly identified; thus, for years scientists have puzzled over the precise function of this receptor protein. Nevertheless, the σ_1R activity has been shown to be modulated by progesterone, sphingosine, and endogenous trace amines and their *N*-methyl and *N*,*N*-dimethyl derivatives.[5] Indeed, the hallucinogen *N*,*N*-dimethyltryptamine (DMT),[6] enzymatically produced in lung and brain,[5] has been proposed as a possible endogenous agonist for the σ_1R.[7]

The σ_1R has a complex pharmacology, since it is able to bind a plethora of structurally diverse drugs that possess different therapeutic and pharmacological profiles (e.g., neuroleptics, antidepressants, drugs of abuse, antitusives, and drugs for the treatment of neurodegenerative disorders, such as Parkinson's and Alzheimer's disease).[7] In addition, the coupling of σ_1R to G-proteins remains controversial, and the intrinsic activity of these drugs (e.g., agonist vs antagonist) is based on the regulation of some ion channels (e.g., small conductance calcium-activated potassium channels and voltage-gated calcium channels), G-protein-coupled receptors (e.g., dopamine D1 and μ-opioid receptors), neurotransmission (e.g., glutamatergic), and second messenger systems (e.g., PLC/PKC/InsP_3), for which the σ_1R plays a role as a regulatory chaperone protein.[2] Thus, as a consequence of both its complex pharmacology and the lack of clear functional assays to identify and unequivocally classify σ_1R ligands, the potential use of these drugs as therapeutic agents is currently unrealistic. Accordingly, we developed a σ_1R biosensor based on the cyan and yellow fluorescent proteins (CFP and YFP, respectively), which allowed the detection of ligand-mediated intramolecular conformational rearrangements of the receptor by assessing real-time fluorescence resonance energy transfer (FRET) changes in living cells. Thus, by means of this real-time FRET-based assay, we aimed to compare the intrinsic activity of putative σ_1R ligands and then to predict their in vivo functionality. Accordingly, we analyzed the antinociceptive properties of the σ_1R ligands tested in our in-cell FRET-based assay by means of the formalin test, a widely used tonic model of continuous pain. Overall, by using our intramolecular FRET-based approach (i.e., a σ_1R biosensor), we aimed to establish a close relationship between the intrinsic activity of σ_1R ligands and analgesia.

RESULTS

A major impediment to identify the intrinsic efficacy of σ_1R drugs is the lack of unambiguous biological assays to monitor receptor activity. Hence, we aimed to develop a straightforward method to predict the intrinsic nature of putative σ_1R ligands. Accordingly, we engineered a σ_1R biosensor containing the CFP and YFP to monitor ligand-mediated receptor conformational rearrangements by assessing real-time intramolecular FRET changes in living cells. Therefore, we genetically fused the CFP and the YFP to the N- and C-terminal domains of the receptor, respectively ($\sigma_1R^{CFP/YFP}$) (Figure S1A, Supporting Information) and stably expressed in HEK-293T cells (Figure S1B, Supporting Information). The recovery of the CFP emission after photobleaching of YFP confirmed that there was

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constitutive intramolecular FRET (Figure S1B, Supporting Information), as previously described.[9] The intramolecular FRET efficiency for $\sigma_1R^{CFP/YFP}$ was calculated using the donor recovery after photobleaching of the acceptor and was shown to be 15.7 ± 1.5% (n = 4) (Figure S1C, Supporting Information). Interestingly, our σ_1R biosensor retained its essential ligand binding properties (Figure S1D, Supporting Information), as described for other σ_1R.[10]

Once the putative σ_1R biosensor (i.e., $\sigma_1R^{CFP/YFP}$) was shown to exhibit normal receptorial behavior, we investigated the effects of σ_1R ligands on its constitutive FRET signal, which was measured as the bleed-through emission intensity ratio F^*_{535}/F^*_{480}. Thus, after rapid superfusion of $\sigma_1R^{CFP/YFP}$ expressing cells (Figure S1B, Supporting Information) with a canonical σ_1R agonist, 2-morpholin-4-ylethyl 1-phenylcyclohexane-1-carboxylate **1** (PRE-084),[8] the ratio F^*_{535}/F^*_{480} rapidly decreased (Figure 1A). Indeed, the symmetrical increase in CFP fluorescence and decrease in YFP fluorescence indicated that the change was due to a decrease in FRET. Interestingly, after a short delay (~700 ms), the FRET decrease followed a monoexponential time-course with a time constant (τ) of 2.93 ± 0.72 s (n = 5). Similarly, we applied a previously described σ_1R antagonist, haloperidol,[11] to cells expressing $\sigma_1R^{CFP/YFP}$ and recorded the FRET signal over time (Figure 2A). Haloperidol induced a fast increase (τ = 4.97 ± 0.90 s; n = 5) in the FRET signal, thus suggesting that σ_1R underwent a conformational change that differed from that seen in response to **1** (Figure 1A). On the other hand, the FRET signals obtained from cells expressing $\sigma_1R^{CFP/YFP}$ and treated with two other distinct σ_1R agonists (1,3-di-*o*-tolylguanidine and (+)-pentazocine) and two σ_1R antagonists (4-methoxy-3-(2-phenylethoxy)-*N*,*N*-dipropylbenzeneethanamine **2** (NE-100) and the 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine **3** (BD-1063))[8] (Figure S2, Supporting Information) confirmed that the observed decrease and increase in the FRET signals represented the general response of the σ_1R biosensor to agonists and antagonists, respectively. Next, we further examined the dose–response of ligand-mediated changes in the intramolecular FRET signal. Interestingly, both **1** and haloperidol induced opposite dose-dependent conformational changes in the σ_1R biosensor, with comparable half maximal effective concentrations (313 ± 88 and 230 ± 79 μM, respectively) (Figure 1B). Of course, these values were high compared to those determined in radioligand binding experiments,[1,10] but it has been previously shown by real-time FRET that no receptor reserve exists. Furthermore, the measurements were taken under continuous superfusion of the ligand, which creates nonequilibrium conditions.[12] Overall, we generated a ligand-sensitive FRET σ_1R biosensor that showed differential conformational rearrangements when challenged with either receptor agonists or antagonists.

Our real-time FRET-based assay allowed us to compare the ligand-mediated receptor intramolecular conformational rearrangements of putative σ_1R ligands. Hence, this novel tool would permit, in theory, predicting their in vivo functionality. To test this possibility, we performed behavioral animal studies, which have largely contributed to the pharmacological classification of σ_1R ligands.[13] Interestingly, it has been described that the σ_1R is located in CNS regions involved in pain control (i.e., the superficial layers of the spinal cord dorsal horn, periaqueductal gray matter, locus coeruleus, and rostroventral medulla).[4] Thus, a role of the σ_1R in nociception has been proposed, both in modulating opioid analgesia and in the absence of opioid drugs.[13,14]

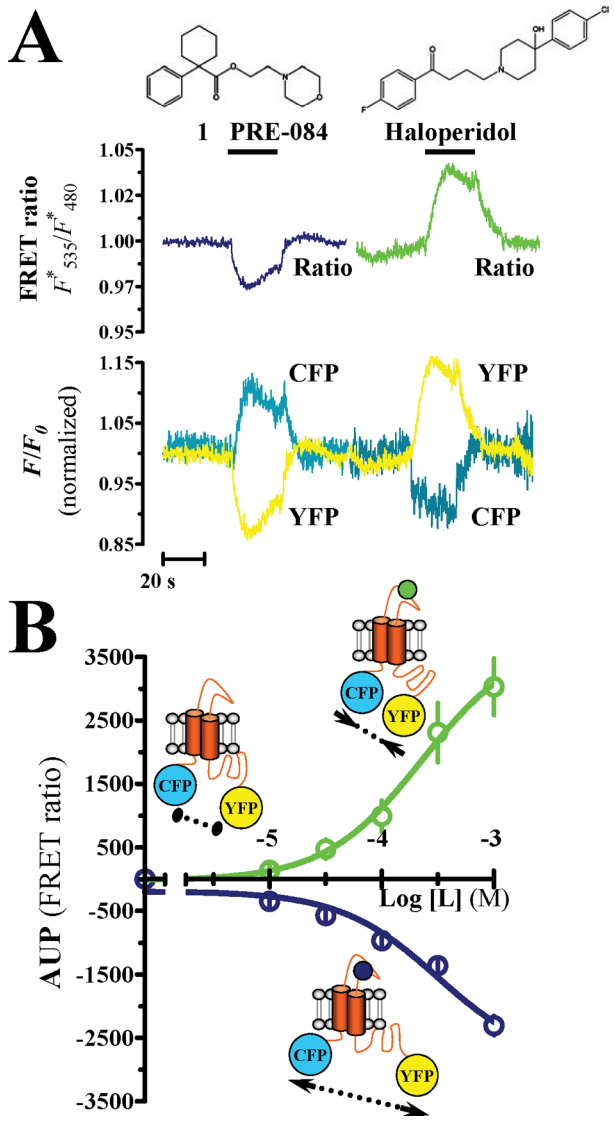

Figure 1. Ligand-mediated conformational changes of the σ_1R. (A) The fluorescence emission intensities of YFP (F_{535}) and CFP (F_{480}) were recorded simultaneously from a single human embryonic kidney 293T (HEK-293T) cell expressing $\sigma_1R^{CFP/YFP}$. The FRET was calculated as the ratio of the normalized emission intensities (F^*_{535}/F^*_{480}). The changes induced by rapid superfusion of **1** (100 μM) and haloperidol (100 μM) are shown. The traces are representative of at least 10 separate experiments. (B) Dose–response relationship for the ligand-mediated FRET response (from data similar to those shown in panel A). Single HEK-293T cells expressing $\sigma_1R^{CFP/YFP}$ were superfused with increasing concentrations of **1** (dark blue) or haloperidol (light green), and the ligand-mediated FRET was expressed as the integrated area under the peak (AUP) of the normalized FRET ratio F^*_{535}/F^*_{480} related to the HBSS treatment. The data indicate the mean ± SEM (haloperidol, n = 6; **1**, n = 8).

The formalin test is a widely used tonic model of continuous pain resulting from formalin-induced tissue injury. It is a useful model, particularly for the screening of novel compounds because it encompasses the inflammatory, neurogenic, and central mechanisms of nociception. In addition, it has previously been used for testing σ_1R ligands.[13] Therefore, as a proof of principle, we analyzed the antinociceptive properties of the σ_1R ligands tested in our in-cell FRET-based assay (Figure S2, Supporting Information). Our results showed that

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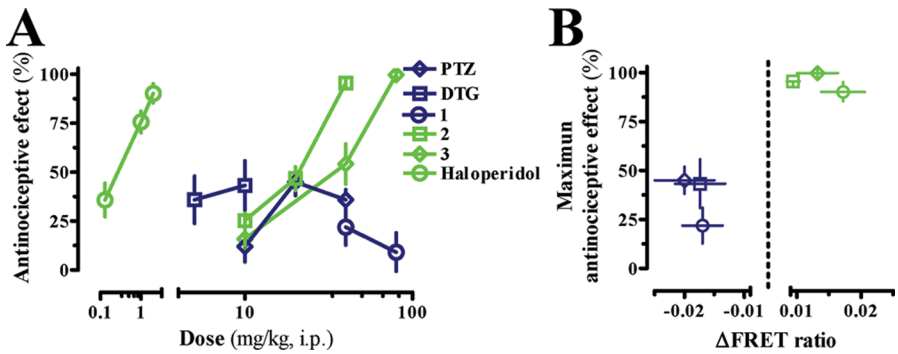

Figure 2. Antinociceptive effect of σ_1R ligands. (A) Dose−response relationship for the antinociceptive effect of several σ_1R ligands in the formalin test. Haloperidol, **1**, **2**, **3**, 1,3-di-*o*-tolylguanidine (DTG), or pentazocine was administered ip 15 min before the ipl injection of formalin, and the time spent licking or biting the injected paw was recorded. The antinociceptive effect in the phase II formalin test was calculated as the percentage of the maximum possible effect (mean ± SEM, n = 8−10). Note that the σ_1R antagonists (i.e., haloperidol, **1**, and **2**) inhibited the behavioral response to formalin in a dose-dependent manner and with a similar maximal efficacy (~100%). (B) Plot of the antinociceptive effect of the σ_1R ligands versus the changes that they induced in the normalized FRET ratio F^*_{535}/F^*_{480}. The ΔFRET ratio ($A - 1$) induced by each single σ_1R ligand at 100 μM (n = 5) is represented vs the maximum antinociceptive effect achieved (n = 8−12) for the same ligand.

formalin-induced nociception was dose-dependently abolished by the intraperitoneal administration of all of the putative σ_1R antagonists tested (Figure 2A). However, under the same experimental conditions, the proposed putative σ_1R agonists tested had either a discrete (<50%) or nonsignificant antinociceptive effect (Figure 2A). Indeed, when we correlated the antinociceptive effect and the FRET change promoted by each σ_1R ligand, a significant difference between the putative σ1R agonists and antagonists was observed. Thus, while all of the antagonists tested showed maximal antinociceptive efficacy and increased FRET signals, the agonists presented limited antinociceptive activity and reduced FRET signals (Figure 2B).

DISCUSSION

Currently, the most promising therapeutic use of σ_1R focuses on drug addiction, learning and memory, and pain.[1,13] Indeed, several ongoing clinical trials are exploring the use of σ_1R antagonists in pain treatment (EudraCT numbers 2012-000398-21, 2012-000399-41, 2012-000400-14). However, it is important to mention that candidate drugs are selected based on their efficacy in preclinical animal models, thus making the screening of large drug libraries expensive, time-consuming, and often inadequate to provide clear pharmacologic profiles. One of the main difficulties regarding the characterization of the intrinsic nature of σ_1R ligands consists of the lack of information about the mechanistic aspects revolving σ_1R activation. In such way, the effort made to provide detailed structural information of such protein is noteworthy. Thus, initial studies suggested a single transmembrane domain for the σ_1R, but nowadays the most accepted structural model proposes two putative transmembrane domains with the N- and C-terminal tails (residues 11−29 and 80−100, respectively) facing the cytoplasmatic side of the membrane.[15,16] Interestingly, it is believed that the ligand binding region that likely interacts with endogenous neurosteroids is formed by juxtaposition of its second transmembrane domain and a predicted third membrane associated region located at the C-terminal domain (residues ~176−204).[15,16] Importantly, this last domain contains two cholesterol recognition motifs and overlaps with the σ_1R chaperone domain (residues 112−223) recently characterized.[17] Nevertheless, despite all these data, the methodology for the characterization of the intrinsic nature of putative σ_1R ligands nonbased in pain animal models is still too laborious (i.e., co-immunoprecipitation assays and bradykinin-triggered calcium responses).[18,19] In view of that, it seems likely that a possible approach to definitively unravel the intrinsic drug activity of the plethora of promiscuous compounds acting on this intriguing receptor would consist of understanding the conformational changes occurring on the σ_1R when a ligand binds to and activates the receptor. To this end, we developed a dynamic σ_1R biosensor able to achieve ligand-specific conformational rearrangements with high temporal resolution. Indeed, this kind of assay, able to monitor intramolecular conformational changes in the receptor upon activation, seems to appear the most appropriate method for the σ_1R because of its chaperone activity. Interestingly, a similar approach was used to determine the kinetics and magnitude of G-protein-coupled receptor (GPCR) activation in living cells. Furthermore, GPCR biosensors also used FRET to measure intramolecular conformational changes, and in this case agonists caused a decrease in FRET, whereas inverse agonists produced an increase in the FRET signal.[20] In addition, within the GPCR agonists tested, different magnitudes and kinetics were observed; thus, FRET studies showed fast conformational changes for full agonists (rate constant $\tau \approx 50$ ms), smaller and slower changes for partial agonists ($\tau < 1$ s), and even slower changes ($\tau \approx 1$ s) in the opposite direction for inverse agonists. Interestingly, all the σ_1R compounds tested showed slow kinetics ($\tau > 1$ s) when compared to GPCRs and also putative σ_1R agonists and antagonists promoted opposite conformational rearrangements. Noteworthy, these suprasecond kinetics and differential ligand-mediated σ_1R conformational changes might be related to the receptor's ability to form ternary complexes with well-known partners because of its chaperone-like activity.

Overall, our current study describes a method able to predict the intrinsic analgesic nature of σ_1R ligands. To this end, we engineered a σ_1R biosensor capable of detecting ligand-mediated receptor conformational rearrangements, and by using this biosensor, we were able to catalogue σ_1R ligands into two categories according to the intramolecular FRET trend induced. Interestingly, these conformational-based categories matched well with the physiological classification of σ_1R ligands (i.e., agonist vs antagonist). Therefore, we were able to establish a good correlation between the intramolecular FRET trend and the antinociceptive properties observed for each single ligand.

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Consequently, our assay enables the unbiased in vitro evaluation of the intrinsic analgesic activity of σ_1R ligands, thus constituting a powerful pharmacological tool that will definitively play a key role in the σ_1R-based drug discovery in general and in the pain field in particular.

EXPERIMENTAL SECTION

Microscopic FRET Measurements. Single-cell real-time intramolecular FRET measurements were performed as previously described.[8] In this FRET approach, the donor (CFP) and acceptor (YFP) were located within the same molecule (σ_1R) (Figure S1A, Supporting Information). Briefly, HEK-293T cells permanently expressing the $\sigma_1R^{CFP/YFP}$ were seeded on poly-D-lysine-coated coverslips and allowed to grow overnight. The coverslips were then mounted in an Attofluor holder (Life Technologies) and maintained in Hank's balanced salt solution (HBSS) containing (in mM) the following: 137 NaCl, 0.34 Na_2HPO_4, 5 KCl, 0.44 KH_2PO_4, 0.5 $MgCl_2$, 0.4 Mg_2SO_4, 1.26 $CaCl_2$, 10 HEPES, 2 D-glucose, and 1 ascorbic acid (adjusted to pH 7.4 with NaOH) at room temperature. The holder was placed on a Zeiss inverted microscope (Axio Observer D1) equipped with a 63× oil immersion objective and a dual emission photometry system (Till Photonics). The sample was then illuminated with light from a polychrome V monochromator (Till Photonics). The excitation light was set at 436 ± 10 nm (beam splitter dichroic long-pass [DCLP] of 460 nm), and the excitation time was 10 ms at 10 Hz to minimize photobleaching. The emission light intensities were recorded at 535 ± 15 nm (F_{535}) and 480 ± 20 nm (F_{480}) (beam splitter DCLP of 505 nm), and FRET was monitored as the emission ratio of YFP to CFP (F_{535}/F_{480}). The FRET ratio was corrected by the corresponding spillover of CFP emission into the 535 nm channel (the bleed-through of YFP into the 480 nm channel was negligible) and by the cross-talk due to the direct excitation of YFP by light at 436 nm to give the corrected FRET ratio F^*_{535}/F^*_{480}. Eventually, the FRET efficiency between the donor (CFP) and acceptor (YFP) fluorophores was determined by the donor recovery after acceptor photobleaching according to the equation:

$$\text{FRET efficiency} = 1 - (\text{CFPpre}/\text{CFPpost})$$

where CFPpre and CFPpost are the CFP emissions (F480) before and after photobleaching YFP by 5−10 min of illumination at 500 nm.

The ligand-mediated changes in the constitutive $\sigma_1R^{CFP/YFP}$ FRET were recorded from cells with comparable CFP and YFP fluorescence levels after superfusion using a pressure-driven, computer-controlled solenoid valve perfusion system (Octaflow from ALA Scientific Instruments, solution exchange of 10−20 ms). The fluorescence signals were detected by avalanche photodiodes, digitized using an analog/digital converter (Digidata 1440, Molecular Devices), and recorded using a pCLAMP (Molecular Devices). GraphPad Prism 4 (GraphPad Software) software was used for the data analysis. The change in the FRET ratio was fitted to the equation

$$r(t) = A(1 - e^{-t/\tau})$$

where τ is the time constant (s) and A is the magnitude of the signal. For each measurement, changes in the fluorescence emission produced by photobleaching were subtracted.

Formalin-Induced Nociceptive Behavior. The formalin test was performed as previously described.[15] In brief, a diluted formalin solution (20 μL of 2.5% formalin/0.92% formaldehyde) was intraplantarly (ipl) injected into the mid-plantar surface of the right hind paw of the mouse. The formalin-induced nociceptive behavior was quantified as the time spent licking or biting the injected paw during the 45 min (divided into 9 periods of 5 min each) after the injection of formalin. The initial acute phase (0−5 min, phase I) was followed by a relatively short quiescent period, which was then followed by a prolonged response (15−45 min, phase II). Mice (n = 6−10) were administered intraperitoneally (ip) with vehicle or haloperidol (Sigma-Aldrich), **1** (Tocris), **2** (Tocris), **3** (Tocris), 1,3-di-*o*-tolylguanidine (Tocris), or (+)-pentazocine (Sigma-Aldrich) 15 min before the ipl injection of formalin (Sigma-Aldrich).

The antinociception induced by the treatments in the formalin test was calculated with equation

$$\text{antinociceptive effect (\%)} = [(\text{LTV} - \text{LTD})/\text{LTV}] \times 100$$

where LTV and LTD represent the licking/biting time in the vehicle- and drug-treated animals, respectively.

ASSOCIATED CONTENT

Supporting Information

Additional experimental procedures and supplementary Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

F.C. and J.B. conceived the project. F.C., V.F.-D., and D.Z. designed the experiments. M.G.-S., E.P.-S., P.P., and V.F.-D. performed and analyzed the experiments. F.C., V.F.-D., J.M.V., and M.G.-S. wrote the paper.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

σ_1R, σ_1 receptor; RET, fluorescence resonance energy transfer; CNS, central nervous system; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; DMT, *N*,*N*-dimethyltryptamine; HEK-293T, human embryonic kidney 293T

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