INTRODUCTION

On the basis of shortcomings of existing therapies, the broad field of depression research has formulated several key goals for the development of novel therapeutics, including faster onset of depression research has formulated several key goals for the development of novel therapeutics, including faster onset of therapeutic effects, loss of efficacy over time and serious side effects. Therefore, there is an urgent need to explore new therapeutic approaches that address these issues. Interestingly, the atypical antidepressant tianeptine already meets in part these clinical goals. However, in spite of three decades of basic and clinical investigations, the molecular target of tianeptine, as well as its mechanism of action, remains elusive. Herein, we report the characterization of tianeptine as a μ-opioid receptor (MOR) agonist. Using radioligand binding and cell-based functional assays, including bioluminescence resonance energy transfer-based assays for G-protein activation and CAMP accumulation, we identified tianeptine as an efficacious MOR agonist (Ki Human of 383 ± 183 nM and EC50 Human of 194 ± 70 nM and EC50 Mouse of 641 ± 120 nM for G-protein activation). Tianeptine was also a full δ-opioid receptor (DOR) agonist, although with much lower potency (EC50 Human of 37.4 ± 11.2 μM and EC50 Mouse of 14.5 ± 6.6 μM for G-protein activation). In contrast, tianeptine was inactive at the κ-opioid receptor (KOR, both human and rat). On the basis of these pharmacological data, we propose that activation of MOR (or dual activation of MOR and DOR) could be the initial molecular event responsible for triggering many of the known acute and chronic effects of this agent, including its antidepressant and anxiolytic actions.

MATERIALS AND METHODS

Materials

HEK-293T cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were cultured in a 5% CO2 atmosphere at 37 °C in Dulbecco’s Modified Eagle Medium (high glucose no. 11965; Life Technologies; Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (Premium Select, Atlanta Biologicals; Atlanta, GA, USA) and 100 U ml−1 penicillin and 100 μg ml−1 streptomycin (no. 15140, Life Technologies). Tianeptine sodium salt was purchased from Selleck Chemicals (Houston, TX, USA); [D-Ala2, N-Me-Phe4, Gly5-ol]-Enkephalin (DAMGO) acetic acid, nor-binaltorphimine dihydrochloride were purchased from Tocris (Houston, TX, USA); [D-Pen(2,5)]Enkephalin (DPEP) and nor-binaltorphimine dihydrochloride were purchased from Tocris Bioscience (Minneapolis, MN, USA); U-50,488 and TIP4(psi) were obtained from the National Institute on Drug Abuse Drug Supply Program; coelenterazine H was purchased from Dalton Pharma Services (Toronto,
**Receptor screening and Kᵢ determination**

Receptor screening and Kᵢ determination was generously performed by the National Institute of Mental Health’s Psychoactive Drug Screening Program, Contract no. HHSN-271-2008-00025-C (NIMH PDS). The NIMH PDS is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda, MD, USA. For experimental details, please refer to the PDS website: http://pdsp.med.unc.edu/. Tianeptine was evaluated in a primary radioligand binding screen against a panel of 51 human central nervous system receptors and transporters, including serotonin receptors (5-HT1a,b,d,e; 5-HT2a,b,c; 5-HT3,5a,6,7); nicotinic acetylcholine receptors (α2β2, α3β2, α3β4, α4β2, α4β4, α7); adrenergic receptors (α1A, 1B, 1D, 2A, 2B, 2C; 1β1-3); benzodiazepine receptor (BZP); cannabinoid receptors (CB1,2); dopamine receptor screening and Kᵢ determination was performed as described previously. 7,8,10 Briefly, transfected cells were dissociated and resuspended in phosphate-buffered saline. Approximately 200,000 cells per well were added to a black-framed, white well 96-well plate (no. 60050; Perkin Elmer; Waltham, MA, USA). Bioluminescence resonance energy transfer (BRET) experiments were performed as described previously. 7,8,10

**DNA constructs**

The mouse MOR (mMOR), the mouse DOR (mDOR) and the rat KOR (rKOR) were provided by Dr Lakshmi Devi at Mount Sinai Hospital. The human MOR (hMOR), human DOR (hDOR) and human KOR (hKOR) were obtained from the Missouri S&T Resource Center. The human G protein constructs used here have been previously described and were obtained from the Missouri S&T Resource Center unless otherwise noted. The G proteins used were: untagged Gα₁₆ (Gα₁₆); Gα₃₃ with Renilla luciferase 8 (RLuc8) inserted at position 91 (Gα₃₃-RLuc8, generously provided by C Galetis); Gβ₃ (Gβ₃); untagged Gγ₂ (Gγ₂); Gγ₂, which fused to full-length mVenus at its N terminus via the amino acid linker GSAG (mVenus-y2); YFP-Epac-RLuc (CAMYEL) was obtained from ATCC (no. MBA-277). All constructs were sequence confirmed.

**Transfection**

A total of 15 μg of cDNA was transiently transfected into HEK-293T cells (5 × 10⁶ cells per plate) in 10 cm dishes for G-protein activation (2.5 μg MOR/DOR/KOR, 0.125 μg Gα₁₆-RLuc8, 6.25 μg Gβ₃, 6.25 μg mVenus-y2) or cAMP inhibition assays (1.25 μg MOR/DOR/KOR, 10 μg CAMYEL, 1.25 μg Gα₃₃, 1.25 μg y2) using polyethyleneimine in a 1:1 ratio (diluted in Opti-MEM, Life Technologies). Cells were maintained in the HEK-293T media described above. After 24 h, the media was changed, and the experiment was performed 24 h later (48 h after transfection). Saturation curves using 3H-diprenorphine were performed to determine the expression of KOR, MOR and DOR. We estimated KOR density to be approximately five hundred thousand receptors per cell and the MOR and DOR cellular concentration to be approximately one million receptors per cell (data not shown). Bioluminescence resonance energy transfer Bioluminescence resonance energy transfer (BRET) experiments were performed as described previously. 7,8,10 Briefly, transfected cells were dissociated and resuspended in phosphate-buffered saline. Approximately 200,000 cells per well were added to a black-framed, white well 96-well plate (no. 60050; Perkin Elmer; Waltham, MA, USA). The microplate was centrifuged and the cells were resuspended in either phosphate-buffered saline (G-protein activation) or phosphate-buffered saline containing 1 μM forskolin (cAMP accumulation). After 5 min, 5 μM solution of the luciferase substrate coelenterazine H was added to each well. After 5 min, ligands were added and the BRET signal was measured at 5 min on a PHARstar FS plate reader (BMG Labtech, Cary, NC, USA). The BRET signal was calculated as the ratio of the light emitted by the energy acceptor, mVenus (450–540 nm), over the light emitted by the energy donor, RLuc8 (485 nm). After agonist stimulation, a decrease of the BRET signal was measured, which reflected a change of conformation or dissociation between the different subunits of the G protein. This drug-induced BRET signal was transformed (multiplied by –1) and normalized using the E₉₀ of the full agonists DAMGO (MOR), DPDP (DOR) or U-50,488 (KOR), which was defined as the 100% maximal response for G-protein activation or as 0% for the maximal inhibition of forskolin-stimulated cAMP accumulation in the cAMP inhibition assay. Dose–response curves were fit using a three-parameter logistic equation in GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). All experiments were performed three to six times, and data represent mean ± s.e.m. of those independent trials.

**RESULTS**

On the basis of in vivo pharmacological experiments, tianeptine has been proposed to act as an adenosine A₁ receptor (A₁R) agonist. However, we found that tianeptine had no direct activity at A₁R (Supplementary Information). Therefore, in an attempt to elucidate its primary molecular target, we screened tianeptine at a concentration of 10 μM against a broad panel of human brain receptors (>50 receptors, Psychoactive Drug Screening Program, University of North Carolina). Tianeptine bound to...
hMOR, the only hit identified in the entire panel, with a $K_i$ of $383 \pm 183$ nM (Figure 1b). We therefore further investigated its activity at this receptor (both human and mouse isoforms), as well as at the related DOR and KOR, which also couple to $G_{i/o}$ proteins. Using previously reported BRET-based assays, tianeptine showed full agonism at mMOR for G-protein activation (Figure 2a, $EC_{50}$ (mMOR, G protein) = $641 \pm 120$ nM), as well as downstream inhibition of cAMP accumulation (Figure 2b, $EC_{50}$ (mMOR, cAMP inhibition) = $1.03 \pm 0.10 \mu M$). In addition, the tianeptine-induced activation of MOR was blocked by the opioid antagonist naltrexone in a dose-dependent manner (Figure 2c). The measured potency of tianeptine at hMOR was higher than that at mMOR in both G protein and cAMP inhibition (Figure 1b, $EC_{50}$ (hMOR, G Protein) = $194 \pm 70$ nM; $EC_{50}$ (hMOR, cAMP Inhibition) = $151 \pm 45$ nM, Supplementary Information).

Next, we studied the activity of tianeptine at the DOR. We found that tianeptine binds to HDOR (Figure 1b, $K_i > 10 \mu M$) and is also a full agonist at mouse DOR, although with an order of magnitude lower potency compared with MOR for both G-protein activation (Figure 3a, $EC_{50}$ (mDOR, G Protein) = $14.5 \pm 6.6 \mu M$) and inhibition of cAMP accumulation (Figure 3b, $EC_{50}$ (mDOR, cAMP inhibition) = $9.46 \pm 1.34 \mu M$). The DOR antagonist TIPP-psi dose-dependently blocked the tianeptine-induced activation of mDOR (Figure 3c). Interestingly, we also found a difference in the potency of tianeptine between the human and mouse DOR; in this case, tianeptine was less potent at the hDOR ($EC_{50}$ (hDOR, G protein) =...
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Figure 4. Tianeptine has no apparent activity at κ-opioid receptors (KORs). (a) The rat KOR was co-expressed with Gαo-RLuc8, β1 and mVenus-γ2 to assay G-protein activation. KOR agonist U-50,488 was used as a positive control. Tianeptine has no significant agonistic activity at KOR. (b) The rat KOR was co-expressed with Gαo, β1, γ2 and the bioluminescence resonance energy transfer (BRET) sensor CAMYEL to assay inhibition of forskolin-stimulated cAMP accumulation. In this assay, tianeptine also has no significant agonistic activity at KOR. (c) The rat KOR was co-expressed with Gαo, β1, γ2 and the BRET sensor CAMYEL to measure the inhibition of cAMP inhibition by the KOR agonist U-50,488. Tianeptine does not show any antagonistic activity at KOR. Nor-binaltorphimine (nor-BNI) showed an IC50 of 28.1 ± 11.2 nM. Data represent mean ± s.e.m. of three independent experiments.

37.4 ± 11.2 μM. Thus, while the selectivity of MOR over DOR is approximately 20-fold in mouse, it is nearly 200-fold in human.

In order to complete our characterization of tianeptine’s functional activity at the opioid receptors, we assessed its activation of KOR. In both G protein activation and cAMP inhibition assays, we found tianeptine to be inactive at rat KOR (Figure 4). Tianeptine was inactive at human KOR as well (Supplementary Information). Previous reports have highlighted the antidepressant effects of KOR antagonists.13–15 In order to eliminate the possibility that tianeptine’s antidepressant effects may in fact result from activity as a KOR antagonist, we tested tianeptine’s ability to dose-dependently block the activation of KOR by control agonist U-50,488. In contrast to the known KOR antagonist, nor-binaltorphimine, tianeptine failed to inhibit the activation of KOR by U-50,488 (Figure 4c). Therefore, it is clear through both binding studies (Figure 1b) and functional assays that tianeptine has no activity at KOR.

DISCUSSION

We have described the first identification of specific molecular targets for the atypical antidepressant tianeptine, namely MOR and DOR. These are unexpected results as previous mechanistic studies have focused on tianeptine’s modulation of aminergic and glutamatergic neurotransmission.9 With regard to modulation of the glutamatergic system, previous reports have shown that tianeptine does not have appreciable affinity (K<sub>i</sub> > 10 μM) for NMDA, AMPA and kainate receptors.6 In the present work, we have demonstrated that tianeptine also has no agonist or antagonist activity at metabotropic glutamate receptors (including mGlur1a,2,4,5,6,8 receptors). It is therefore unlikely that tianeptine modulates glutamatergic neurons and synapses via a direct interaction with glutamate receptors, at least in the concentration range examined in the present study (< 10 μM). In contrast, our evidence suggests that tianeptine’s modulation of the glutamatergic system may occur indirectly, via activation of opioid receptor signaling.

In humans, a single dose of tianeptine (12.5 mg) results in ~ 1 μM maximal concentration of the drug in the plasma, whereas in rodents, standard acute and chronic dosing (10 mg·kg<sup>−1</sup>·day) leads to plasma concentrations of ~ 10 μM (in vivo brain concentrations have not been determined; an estimate in ex vivo tissue is low micromolar).16,17 Therefore, the in vivo concentration range appears sufficient for the activation of MOR (EC<sub>50</sub> ~ 0.2–1 μM, Figures 1 and 2), whereas activation of DOR (EC<sub>50</sub> ~ 12–34 μM) may only become relevant with higher dosing. Although it has been previously shown that DOR agonists have antidepressant-like effects in vivo,18 the evidence that MOR activation may have antidepressant/anxiety effects has only been suggestive.19

Taken together, these results lead us to hypothesize that activation of MOR (or possibly dual activation of MOR and DOR) is the initial molecular event responsible for tianeptine’s modulation of the glutamatergic system and for triggering many of the known acute and chronic effects of this agent, including its antidepressant/anxiety actions. MORs are widely expressed in the hippocampus and are known to modulate glutamatergic neurons and synapses through a number of mechanisms. For example, MOR activation in dentate granule cells is known to decrease protein kinase A activity, which results in a decrease of NMDA receptor phosphorylation and activity.20 This may underlie the corrective effect of tianeptine on increased NMDA receptor signaling in stressed animals.2 In addition, activation of MORs (as well as DORS) in hippocampal inhibitory interneurons decreases their activity, thereby disinhibiting CA1 glutamatergic neurons,21 consistent with reports of tianeptine’s enhancing effects on excitability and synaptic plasticity in CA1.2 We note a striking similarity between these cellular and circuit-level effects of tianeptine and those exerted by direct NMDA receptor antagonists, which also show rapid onset of antidepressant effects.1,22

Other circuit-level effects likely contribute to tianeptine’s modulation of the glutamatergic system and antidepressant action. For example, tianeptine, like known MOR and DOR agonists, increases dopamine release in the nucleus accumbens,23 and dopamine can in turn modulate glutamate release through action at presynaptic dopamine receptors on glutamatergic presynaptic terminals.

Taken together, the pharmacological results and mechanistic hypothesis presented here provide a new perspective for interpreting tianeptine’s extensive clinical and preclinical data.
The proposed hypothesis suggests specific experiments to test its validity in different experimental paradigms (for example, MOR knockout animals and MOR pharmacological inhibition). Our data are also in agreement with growing evidence regarding the importance of the opioid system in depression, anxiety and stress-related disorders.18,19,24–26

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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