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ORIGINAL RESEARCH

Identification and synthesis of low-molecular weight cholecystokinin B receptor (CCKBR) agonists as mediators of long-term synaptic potentiation

Yanmei Zhang[1] · Yican Wang[1] · Yiping Guo[1] · Jinxi Liao[1] · Zhengchao Tu[1] · Yongzhi Lu[1] · Ke Ding[1] · Micky D. Tortorella[1] · Jufang He[2]

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Abstract

Recently, He et al. reported that CCKB receptors located in the neocortex of the brain when bound to their bound natural ligand, CCK peptides, enhance memory, bringing up the possibility that agonists targeting the CCKB receptor may act as therapeutic agents in diseases in which memory loss is marked as observed in dementia and Alzheimer's. In this report, we describe the synthesis of novel low-molecular weight benzoamine CCKB receptor agonists. The compounds made in this series were determined to be mostly partial agonists, although some antagonists were identified, as well, capable of triggering calcium release in a cell line that overexpresses the CCKB receptor. Compound 35 demonstrated an EC_{50} of 0.15 μM in the cell-based assay, but more importantly, several of the compounds, including 35, demonstrated a physiological effect, inducing long-term potentiation in rat brains comparable to the CCK-8 peptide albeit at much higher concentrations. Based on these findings, benzoamines may be the basis for a new series of CCKB receptor agonists in drug-discovery efforts that seek to develop therapeutics to prevent memory loss.

Keywords Cholecystokinin B receptor · Agonist · Synaptic potentiation · Memory

Introduction

The entorhinal cortex in the medial temporal lobe that forms strong reciprocal connections with the neocortex serving is the gateway from the hippocampus to the neocortex (Brown and Aggleton 2001; Canto et al. 2008; Swanson and Kohler 1986). Many dementia patients exhibit early neuro-atrophy (neurodegeneration) in the entorhinal cortex (Apostolova and Thompson 2008). Neurons in the entorhinal cortex show heavy cholecystokinin (CCK) labeling (Greenwood et al. 1981; Innis et al. 1979; Kohler and Chan-Palay 1982) and project to neocortical areas and the hippocampus (Canto et al. 2008; Swanson and Kohler 1986; Li et al. 2014). CCK is the most abundant of all neuropeptides (Rehfeld 1978), and mice lacking the CCK gene exhibit poor performance in a passive avoidance task and impaired spatial memory (Lo et al. 2008). We have previously found that the local infusion of CCK into the auditory cortex of anesthetized mice enabled the auditory neurons to start responding to lights after their pairing with an auditory stimulus (Li et al. 2014). Activation of the entorhinal cortex potentiates neuronal responses in the auditory cortex, and this effect is suppressed by the infusion of a CCKB antagonist (Li et al. 2014).

Memory is stored in neural networks via changes in synaptic strength (Kandel 2001; Martin et al. 2000). Long-term potentiation (LTP) is considered to be the basis of memory traces in different brains (Bliss and Lomo 1973; Bliss and Collingridge 1993; Buonomano and Merzenich 1998; Mulkey and Malenka 1992; Nabavi et al. 2014). In a separate physiological experiment, we found that the administration of a CCKB antagonist blocked high-frequency (HF) stimulation-induced LTP in the auditory cortex, whereas the local infusion of CCK alone induced LTP.

✉ Yanmei Zhang
zhang_yanmei@gibh.ac.cn

✉ Jufang He
jufanghe@cityu.edu.hk

[1] Drug Discovery Pipeline, Guangzhou Institutes of Biomedicine and Health, Science City, Guangzhou 510530, China

[2] Division of Life Science, Hong Kong University of Science and Technology, Clearwater Bay, N.T., Hong Kong SAR, China

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$CCK^{-/-}$ mice exhibited no neocortical LTP and deficits in memory formation. HF stimulation of CCK-containing entorhinal–neocortical projection neurons before the pairing of an auditory stimulus and electrical stimulation of the auditory cortex potentiated neuronal responses to the auditory stimulus. Furthermore, when electrical stimulation was preconditioned to a foot shock, mice showed a freezing response to the auditory stimulus after the pairing of auditory and electrical stimuli, indicating that mice had formed an association between auditory and electrical stimuli. These findings demonstrate that CCK released from the entorhinal cortex enables neocortical LTP and contributes to memory formation.

All the CCKB antagonists and agonists reported to date tend to be large-molecular weight compounds (Kalindjian and Iain 2007; Tikhonova et al. 2007; Wang et al. 2011; Blommaert et al. 1997). Therefore, the goal of this study was to develop low-molecular weight CCKB receptor agonists capable of mediating long-term synaptic potentiation in vivo. To this end, we adopted the following strategy: first, we screened an existing compound library (>30,000) in Guangzhou Institute of Biomedicine and Health, followed by studying the analytical and computational chemistry, after which we confirmed the lead molecules in a cell line overexpressing the CCKB receptor, and finally, tested the most potent agonists in a rat model for measuring long-term synaptic potentiation.

Materials and methods

Materials and chemical analysis

All materials were obtained from commercial sources. Solvents were of chromatography grade and were used without further purification. Thin-layer chromatography analysis was performed using Merck silica gel 60 F-254 thin-layer plates. Liquid Chromatography Mass Spectrometers (LC-MS) analyses were performed on Agilent 1200 HPLC/MCD electrospray mass spectrometer in positive/negative ion mode. The scan range was 100–1000 d. High-resolution mass spectra were obtained in ABSciex TOF5600 + MS using electrospray ionization. Preparative reverse-phase high-performance liquid chromatography (HPLC) was performed on a SHIMADZU LC-20AP equipped with a C18 column using a methanol/water gradient. The purity of tested compounds was ≥ 95% as determined by HPLC analysis conducted on the Agilent 1260 system using a reverse-phase C18 column with a diode array detector unless stated otherwise. Chiral GC column: CHIRALPAK OD-H, AS-H, OJ-H, 4.6 mm × 250 mm, 5 µm. Specific rotation values were recorded on AUTOPOL® IV-T (λ = 589 nm, 50 mm cell, 25 °C). 1H and 13C NMR spectra were recorded on Bruker 500 or Bruker 400 (at 500 and 125 MHz or 400 and 100 MHz, respectively) and are reported to CDCl3 (δ 1H: 7.26 and 13C: 77.16) and 19F NMR (at 471 MHz). Coupling constants (J) are given in Hz.

CCKBR cell-imaging assay

CHO/CCKb receptor stable cell line was developed by lipofectin transfection. Briefly, a 1.36 kb insert containing the full-length CDS sequence (NM_013165.2) of the rat CCKB receptor was cloned into *Hind*III/*Eco*RI sites of the mammalian expression vector pcDNA3.1(+) with neomycin as the selection marker. G418 (1 mg/ml) was added to the culture medium from the first passage after transfection and resistant colonies were obtained by limiting dilution method and were propagated in the presence of G418 (0.5 mg/ml). CCKBR, coupled to Gq protein, can induce intracellular calcium mobilization after the binding of CCK-8 ligand. All the cell lines were validated using this fluorescence-based functional assay with CCK-8s (Tocric, UK). Before choosing to develop the high-throughput screening (HTS) cell-based assay for screening CCKBR agonist compounds, the cell line from Clone2 was proven to be effective and reliable with two other commercially available CCKb receptor agonists (BBL454 and 8b; Wuxi Apptech, China), and we further subcultured 10 passages.

For fluorescence-based imaging assay (calcium flux assay), the cells were seeded in a 96-well plate at a density of 40,000 cells per well one day prior to the experiment. Dye-loading solution (Fluo-8; AAT Bioquest, US) was loaded for 30 min in a 37 °C incubator, followed by 15 min in room temperature. After washing with Hanks' Buffer with 20mM Hepes (HHBS), the compound candidate was added to the cells, and the fluorescence signal change was captured with a fluorescent microscope.

96/384-well plate screening assay

Based on fluorescence-based imaging assay, the 96/384-well plate screening assay was performed. Briefly, fluorescence dye was added to the cells using a bulk dispensing system (freedom evo 150; Tecan). After 30min of incubation, the baseline was recorded prior to candidate compound addition to the plate using the pipettor onboard the microplate reader (FlexStation 3 Multi-Mode Microplate Reader; Molecular Devices). During the first 90 s of activation, data were acquired every 10 s. EC_{50} value was calculated based on the concentration–response curve.

Homology model of CCKBR with lead agonists

Homology model of CCKBR was generated using Discovery Studio. Molecular docking was carried out by the

program Autodock Vina using the homology model as the receptor. Three-dimensional models of compounds 31, 34, and 35 were generated using the software ChemBio3D Ultra from the 2D structures drawn using ChemBioDraw. Input files of both proteins and ligands were prepared by AutoDock Tools. The parameter values of exhaustiveness and num_modes were set at 100 and 100, respectively.

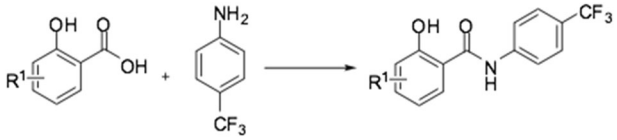
Long-term synaptic potentiation in rat brains

Anesthesia was induced using urethane sodium (2 g/kg, intraperitoneally) and maintained throughout the surgery and neuronal recordings with periodic supplements. Atropine sulfate (0.05 mg/kg, subcutaneously) was administered 15 min before the induction of anesthesia to inhibit tracheal secretions. A local anesthetic (xylocaine, 2%) was applied liberally to the incision site. Animals were prepared for surgery as previously described. Briefly, animals were mounted on a stereotaxic device and a midline incision was made in the scalp. A craniotomy was performed at the temporal lobe (–2 to –4 mm posterior and –1.5 to –3 mm ventral to bregma for mice) to access the auditory cortex and the dura matter was removed. A recording electrode array (0.5–1.0 MΩ; FHC Inc.) was stepped into layer 2/3 of the auditory cortex of rats, and a stimulation electrode with a low impedance (<100 kΩ; FHC Inc.) was positioned into layer 4 of the auditory cortex. Electrode placement was confirmed by the neuronal responses to auditory stimuli. For drug application, a glass pipette was attached to the recording electrodes. We first recorded the baseline field excitatory postsynaptic potential (fEPSPs) in response to testing electrical stimulation for 15 min, and then 0.5 µl of drug was injected at a speed of 0.1 µl/min, followed by another 60min of fEPSPs recording. Before baseline recording, an input–output curve of fEPSPs was measured. The maximum current was defined as that which elicited a saturated fEPSP. We adopted 50% of the maximum current as the testing current to examine the changes in fEPSP slope. Mean fEPSP slopes before and after the pairings were calculated by linear regression and analyzed using two-way ANOVA.

Results

Chemistry

A series of benzoamides as a new class of CCK receptor agonists were identified through the screening of a structurally diverse compound library. Compound 1 is a representative example of this group of molecules first identified as a CCK agonist. Based on the pharmacophore derived from compound 1, novel benzoamide derivatives were designed and synthesized.



Scheme 1 Synthesis of derivatives of 2-hydroxy-*N*-(4-(trifluoromethyl)phenyl)benzamide

Table 1 List of derivatives made in series 1

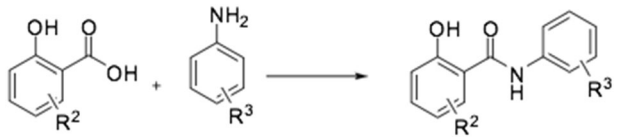
Compound (EC_{50}, µM)	R^1	Agonist	Antagonist
1	5-Cl	>10 µM	
2	2-Cl, 5-Cl		5.02 µM
3	3-Br, 5-Br		9.54 µM
4	3-Cl, 5-Cl, 6-Cl		>10 µM
5	4-F		>10 µM
6	5-F		
7	4-CF_3		>10 µM
8	5-NH_2		
9	3-NO_2, 5-NO_2		
10	5-Br		
11	5-I		
12	5-NO_2		8.95 µM
13	5-COMe	>10 µM	
14	5-COOMe	>10 µM	

EC_{50} values for CCKBR agonism or antagonism

Benzoamide derivatives were normally prepared by direct condensation using substituted benzoic acids with benzoamine in the presence of EDCI and CH_2Cl_2 at room temperature (Scheme 1). Both the benzoamines and the substituted benzoic acids were made using commercial chemicals as the starting points of modification. The derivatives in the series were designed to be 5-substituted benzamides with one 4-substituted product (Table 1). The EC_{50} value results showed no increase for CCKBR agonism.

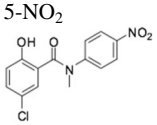
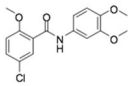
Next, more analogs were designed and synthesized by the introduction of diversified substituted groups on both benzyl amines and benzoic acid groups as seen in Scheme 2. The derivatives in series 2 were still designed to carry 5-substituted groups on benzoic acid and to introduce different groups on benzoamines. To our delight, compound 21 was discovered with the highest agonism in this series (Table 2).

Finally, after we compared the structures of compound 1 and compound 21, a series of phenylbenzamide derivatives from the modified benzyl amines with 5-chloro-2-hydroxybenzoic acid were designed and made to keep 5-chloro substituted on benzoic acids as shown in series 3 (Scheme 3). In this series, compound 35 was observed to be the most potent in CCKBR agonism with highest affinity and bioactivity (Table 3).



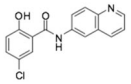
Scheme 2 Synthesis of derivatives of multiple-substituted benzamide

Table 2 List of derivatives made in series 2

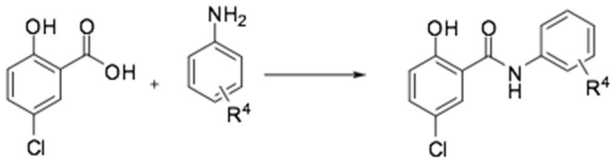
Compound (EC_{50}, μM)	R^2	R^3	Agonist	Antagonist
15	5-Br	3-CF_3, 4-CH_3		
16	5-Br	4-CMe_3		
17	5-Br	3-OMe, 4-OMe	4.43 μM	
18	5-Br	3-$OCHMe_2$		
19	5-Br	4-NO_2		>10 μM
20	5-Br	2-Cl, 4-NO_2		10.00 μM
21	5-Br	3-F, 5-F	0.21 μM	
22	5-I	2-Cl, 4-NO_2	>10 μM	>10 μM
23	5-I	4-NO_2		
24	5-NO_2	4-NO_2		10.00μM
25	5-NO_2	2-CF_3, 4-NO_2		>10μM
26	5-NO_2	3-F, 5-F		
27	5-NO_2	4-Cl		
28	5-NO_2	3-OMe, 5-OMe		
29				
30				

EC_{50} values for CCKBR agonism or antagonism

Table 3 List of derivatives made in series 3

Compound (EC_{50}, μM)	R^4	Agonist	Antagonist
31	4-Br	0.65 μM	
32	4-Cl	1.39 μM	
33	3-Cl, 4-F	10.00 μM	
34	2-Cl	0.64 μM	
35	4-NO_2	0.49 μM	
36	4-CMe_3		
37	4-I		
38	3-$OCHMe_2$		
39	2-Cl, 4-Cl		
40	3-COOMe, 5-COOMe		
41	3-COOH, 5-COOH		
42	2-Cl, 4-NO_2		6.82 μM
43	3-OMe, 4-OMe	>10 μM	
44	H		>10 μM
45	2-NO_2		>10 μM
46	4-NH_2		
47	3-F, 5-F		
48	3-NO_2		
49		3.86 μM	

EC_{50} values for CCKBR agonism or antagonism



Scheme 3 Synthesis of derivatives of 5-chloro-2-hydroxy-*N*-phenylbenzamide

CCKBR cell-imaging assay

CCKBR, coupled to Gq protein, can induce intracellular calcium mobilization after the binding of ligand CCK-8. Based on this, fluorescence-based imaging assay (calcium flux assay) was established, by which a transient rise of intracellular calcium signals upon CCKBR agonist binding can be captured by a fluorescent microscope (Fig. 1a). Candidate compounds from HTS 96-well plate screening assay were further validated in this imaging assay. One example of compound 35, which showed partial in vivo CCKBR agonist activity, is illustrated in Fig. 1b. When compared with the natural CCKB receptor agonist, cck-8 sulfate (cck-8s), compound 35 has nearly a 3-order magnitude of lower affinity/bioactivity to CCKBR (Fig. 1).

CCCKBR 96-well plate screening assay

Calcium flux assay is a well-established method suitable for high-throughput screening, on using commercially available specialized machines, like the FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices) used here. Its reliability in this study was first confirmed by measuring the EC_{50} value of CCK-8s ($EC_{50} = 1.54$ nM, Fig. 2 and Table 4), which is consistent with the previous reports. Then, we screened an existing compound library (>30,000) in Guangzhou Institute of Biomedicine and Health, and compound 1 is a representative example for this HTS. Totally, three series of compounds are described here, and their EC_{50} values are listed in Tables 1–3.

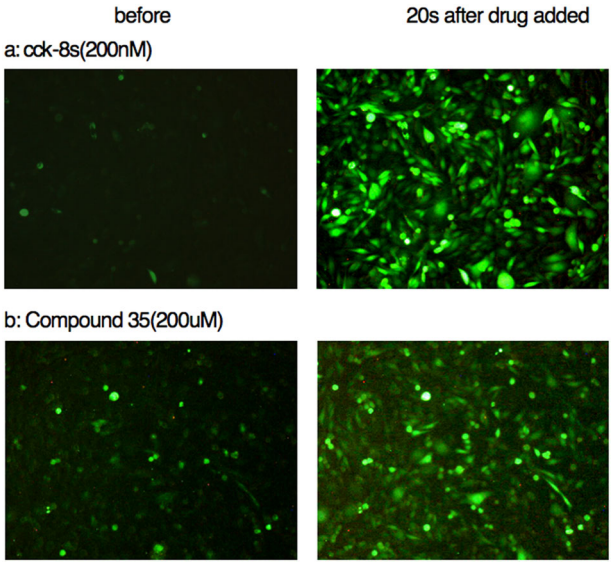


Fig. 1 Cell-based calcium influx assay measuring molecules binding to the CCKB receptors

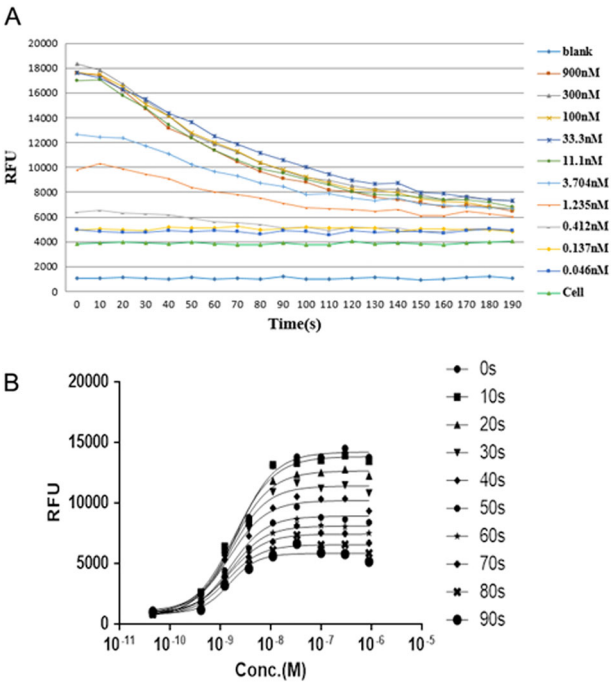


Fig. 2 EC_{50} value of CCK-8s

Homology model of CCKBR with lead agonists

To understand the potential interaction between CCKBR and ligand compounds, we carried out docking analysis for compounds 31, 34, and 35 using the homology model of CCKBR as the receptor. The binding-affinity energy (BAE) was -7.5, -8.1, and -8.6 kcal/mol for 31, 34, and 35, respectively. The predicted binding sites of these three compounds were located at the out-membrane entrance of CCKBR (Fig. 3 #A). Compound 31 docked to one side of Arg278 and compounds 34 and 35 docked to the other side. Compound 34 formed 3 H-bonds with the residues Arg278, Arg282, and Ser286 (Fig. 3 #C). Also, compound 35 formed 2 H-bonds with the residues Arg282 and Ser286 (Fig. 3 #B). However, no specific interaction was observed between compound 31 and CCKBR.

Long-term potentiation assay in rat brains

In earlier studies, we have found that no LTP can be induced with HF stimulation in the CCK-/- mouse brain, but CCK infusion into the CCK-/- mouse brain can induce a higher LTP in the neocortex when compared to normal mice, indicating overly expressed CCKB receptors in the neocortex. We used LTP induction in the CCK-/- mouse brain as the in vivo functional assay to examine the physiological effectiveness of the potential candidates popped out from the cell-based assay. Three top candidates (compound name XXX) were tested here, and two of them, including compound 35, showed physiological functions (Fig. 4).

Discussion

Targeting the CCKB receptor represents a new approach for the development of therapeutics aimed at enhancing memory. In the past, the CCKB receptor was a hot target for obesity as the receptor has been shown to bind to gastrin and CCK in the gastrointestinal tract inducing one's appetite. However, the focus at that time was on the advancement of antagonists and not so much on agonists. Also, most of the effort was centered on targeting the gastrointestinal tract; thus, central nervous system (CNS) permeability was not a priority during the development of these new compounds. Thus, many of the reported CCKB receptor agonists are not ideal for CNS applications. In addition, most of the agonists targeting the CCKB receptor tend to be high-molecular weight molecules, including 1,4-benzodiazepines, 1,5-benzodiazepines, and pyrimidine carboxamides. The purpose of our study was to identify new potent partial agonists for the CCKB receptor with a low molecular weight and good CNS.

As hoped, compounds targeting the CCKB receptor were identified as partial agonists and antagonists belonging to three chemical series, including derivatives of 2-hydroxy-*N*-(4-(trifluoromethyl)phenyl)benzamide, phenylbenzamides, and 5-chloro-2-hydroxy-*N*-phenylbenzamides. Most importantly, several of the agonists, such as compound 35, demonstrated long-term potentiation in rat brains, which is a biomarker of memory enhancement, bringing up the possibility that these drugs may be used as therapeutics for

Table 4 EC_{50} value of CCK-8s at different time points

	0 s	10 s	20 s	30 s	40 s	50 s	60 s	70 s	80 s	90 s
EC_{50} (nM)	2.21	1.90	1.73	1.62	1.54	1.65	1.64	1.53	1.37	1.53
R^2	0.9927	0.987	0.9937	0.99	0.9852	0.9806	0.9825	0.9722	0.9657	0.9597

For simplifying the comparison among compounds, EC_{50} value at 40 s was used

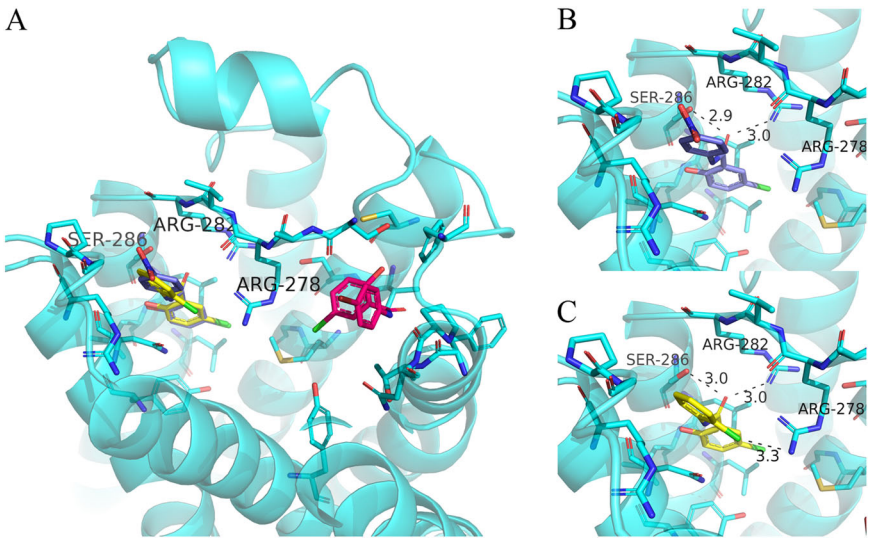


Fig. 3 The predicted interaction between ligand compounds and CCKBR homology model. **a** Docking pose of compounds 31, 34, and 35. **b**, **c** Potential interaction between CCKBR homology model and compounds 34 and 35, respectively. CCKBR homology model is shown as a cartoon and the residues with a distance less than 4 Å to the ligand compound are shown as sticks. Compounds 31, 34, and 35 are shown as pink, yellow, and dark-blue sticks, respectively (color figure online)

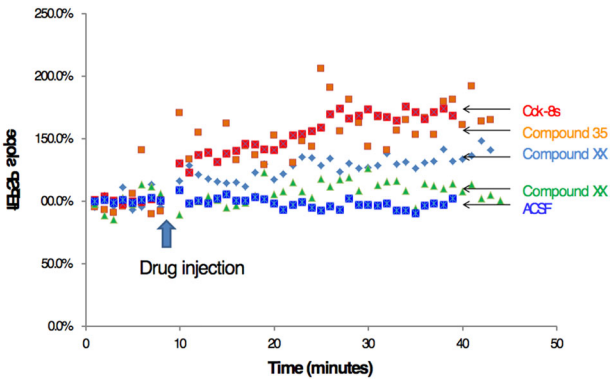


Fig. 4 Time-dependent measurement of long-term potentiation in rat brains in response to partial agonists of CCKB receptors

treating various forms of dementia. Future studies will focus on testing these compounds in animal models of memory. For example, it was recently reported that mice lacking the CCK peptide (the natural ligand of the CCKB receptor) are memory deficient in various behavioral tests, making them a great model for testing our newly discovered partial agonists.

Conclusion

In conclusion, we designed and synthesized a series of novel low-molecular weight benzoamine CCKB receptor agonists. Compound 35 demonstrated a physiological effect, inducing long-term potentiation in rat brains comparable to the CCK-8 peptide, albeit at much higher concentrations. Therefore, this novel type of benzoamines may be the basis for a new series of CCKB receptor agonists in drug discovery efforts that seek to develop therapeutics to prevent memory loss. However, more studies are required to be conducted in the animal model of memory.

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