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Brain and gastrointestinal cholecystokinin receptor family: Structure and functional expression

(gastrointestinal peptide receptor/neuropeptide receptor/guanine nucleotide-binding regulatory protein-coupled receptor)

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ABSTRACT **Cholecystokinin was one of the first gastrointestinal peptides discovered in the mammalian brain. In the central nervous system there is evidence for CCK_A and CCK_B receptor subtypes. The CCK_A receptors occur in a few localized areas of the central and peripheral nervous systems where they modulate feeding and dopamine-induced behavior. CCK_B receptors occur throughout the central nervous system where they modulate anxiety, analgesia, arousal, and neuroleptic activity. We have recently purified and cloned a CCK_A receptor cDNA from rat pancreas that allowed isolation of an identical cDNA from rat brain by using the polymerase chain reaction. Using low-stringency hybridization screening of cDNA libraries from rat brain and AR42-J cells, which possess large numbers of CCK_B receptors, we identified previously unreported cDNAs, the sequence of which were identical in both tissues. The cDNA sequence encodes a 452-amino acid protein that is 48% identical to the CCK_A receptor and contains seven transmembrane domains characteristic of guanine nucleotide-binding regulatory protein-coupled receptors. COS-7 cells transfected with this cDNA expressed binding sites for agonists and antagonists characteristic of a CCK_B receptor subtype. We conclude that this cDNA isolated from rat brain and AR42-J cells is a receptor of the CCK_B subtype and that the respective cDNAs for both CCK_A and CCK_B are identical in the brain and gastrointestinal system.**

The cholecystokinin (CCK) family of peptides was originally isolated from the mammalian gastrointestinal tract (1) and was one of the first gastrointestinal peptides to be discovered in the brain (2, 3), where the predominant molecular form, cholecystokinin octapeptide (CCK-8), exists in sulfated and desulfated forms (2, 4).

The receptors for CCK in both the central nervous system (CNS) and peripheral tissues can be classified into two subtypes, CCK_A and CCK_B, on the basis of their affinity (*i*) for a structurally and functionally related family of peptides with identical COOH-terminal pentapeptide sequences and differing sulfation at the sixth (gastrin) and seventh (CCK) tyrosyl residues and (*ii*) for specific antagonists. CCK_A receptors are highly selective (500- to 1000-fold higher affinity) for sulfated analogues and the antagonist L-364,718, whereas CCK_B receptors have similarly high affinity for both sulfated and nonsulfated peptide analogues (only a 3- to 10-fold higher affinity for sulfated peptide analogues) and the antagonist L-365,260 (5–8).

A CCK_A receptor subtype predominates in the gastrointestinal system but occurs also in highly localized areas of the rat CNS, where it modulates feeding and dopamine-induced behavior (9–11). We recently purified the CCK_A receptor and cloned its cDNA from rat pancreas (12). This cDNA did not hybridize to poly(A)$^+$ RNA from rat brain on Northern blot analysis, which raised the possibility that the rat brain CCK receptor with a CCK_A receptor-subtype pharmacology was different from the rat pancreatic CCK_A receptor (12).

The CCK_B receptor subtype is the predominant CCK receptor in the CNS, where it is widely distributed throughout the brain and spinal cord (10). The presence of CCK_B receptors on selective mesocorticolimbic dopaminergic neurons and other midbrain regions along with the effects of CCK analogues on behavior suggest that these receptors may modulate anxiety, neuroleptic activity, and arousal (11). CCK agonists and antagonists acting at central CCK_B receptors respectively block and enhance opiate-induced analgesia (13). CCK_B-type receptors have also been described outside the CNS in gastrointestinal smooth muscle cells (14), where they modulate gallbladder and bowel motility; and in guinea pig (15) and dog pancreas (16) and various neoplastic tissues [such as human stomach, colon (17), and lung carcinomas (18), and the rat pancreatic acinar carcinoma cell line AR42-J (19)], where they may regulate cell growth. The presence of CCK_B receptors on peripheral lymphocytes and monocytes and monocyte-derived splenic cells suggests that CCK may play a role in the long-suspected neuroendocrine modulation of the immune system (20, 21). To date, neither the CCK_B receptor protein nor its cDNA has been isolated.

Here, we report that the cDNA encoding the rat pancreatic CCK_A receptor is also present in rat brain and that both rat brain and AR42-J cells possess a distinctive cDNA† that is highly homologous to the rat CCK_A receptor cDNA and encodes a guanine nucleotide-binding regulatory protein (G protein)-coupled receptor with CCK_B receptor subtype pharmacology.

MATERIALS AND METHODS

cDNA Library Construction and Isolation of cDNA Clones. Total RNA was isolated from the rat pancreatic carcinoma cultured cell line AR42-J and from rat brain cortex by using a low-temperature guanidinium isothiocyanate/guanidine hydrochloride extraction procedure as described (22), and poly(A)$^+$ RNA was isolated by using oligo(dT)-cellulose. Separate cDNA libraries were constructed from each source of poly(A)$^+$ RNA. Oligo(dT)-primed cDNA > 2 kilobases (kb) was size-selected by agarose gel electrophoresis, electroeluted, adapted with *Eco*RI, ligated into phage λgt10 arms, and *in vitro* packaged by established methods (23). Each library (≈7.5×10^5 plaques) was screened with a ^{32}P-labeled, randomly primed probe (24) corresponding to the coding region of the CCK_A receptor cDNA isolated from rat pan-

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Abbreviations: CCK, cholecystokinin; CCK-8, CCK octapeptide; G-protein, guanine nucleotide-binding regulatory protein; CNS, central nervous system; ^{125}I-BH-CCK-8, CCK-8 conjugated to ^{125}I-labeled Bolton–Hunter reagent.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M99418).

creas (12) initially under conditions of low and later high stringency [three 20-min washes at 42°C with 2× SSC/0.1% SDS for low-stringency screening and three 20-min washes at 55°C with 0.1× SSC/0.1% SDS for high-stringency washes (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0)] (23). Several clones that hybridized at low stringency were plaque-purified from the AR42-J cell library and subcloned into pCDL-SRα at the *Xba* I site (25). A ^{32}P-labeled, randomly primed probe corresponding to the open reading frame of the AR42-J cDNA clones was used to screen another 7.5 × 10^5 plaques from the rat brain cortex library under conditions of high stringency. Several clones were purified and subcloned into the vector pCDL-SRα at the *Xba* I site.

PCR Cloning. Double-stranded cDNA was prepared from 5 μg of poly(A)$^+$ mRNA isolated from rat brain subcortex as described above. Double-stranded cDNA (≈5 ng) served as a template for PCR amplification with 0.5 μM CCK_A receptor 5′ and 3′ untranslated oligonucleotide sequences, 5′-AATGCTGCCCAGATGCTCTG-3′ and 5′-CAGTGGAC-CAGGTGGAGTTCA-3′ (26), respectively, as primers. The following cycle temperatures and times were used under standard PCR (Perkin–Elmer/Cetus) conditions: 34 cycles of denaturation at 94°C for 45 sec, annealing at 61°C for 25 sec, and extension at 72°C for 2 min with a final extension duration of 15 min.

DNA Sequencing. Both strands of two cDNA clones isolated from the AR42-J cell library were sequenced by the dideoxy chain-termination method of Sanger (27) with Sequenase 2.0 (United States Biochemical). One of the cDNA clones isolated from the rat brain cortex library and the product of PCR cloning from the rat brain subcortex cDNA were cycle-sequenced (Bethesda Research Laboratories).

DNA and Protein Sequence Analysis. Nucleotide and amino acid sequences were analyzed by the Wisconsin Genetics Computer Group software package using the GAP program (28).

Northern Blot Analysis of mRNAs. Poly(A)$^+$ RNA was isolated by using a low-temperature guanidinium isothiocyanate/guanidine hydrochloride extraction procedure as described (22) from rat pancreas, brain cortex and subcortex, striated muscle, liver, kidney, rat pancreatic acinar carcinoma cell line AR42-J, and guinea pig gallbladder. (Rats do not have gallbladders.) Four micrograms of poly(A)$^+$ RNA per lane was electrophoretically separated on a 1.4% agarose/formaldehyde gel and blotted onto Nytran (Schleicher & Schuell). The blot was hybridized separately with CCK_A and CCK_B full-length coding-region probes, which had been ^{32}P-labeled (random-primed) as described (23). The blot was washed under conditions of high stringency (three 20-min washes at 55°C with 0.1× SSC/0.1% SDS) and exposed for 24 hr in a phosphorimager (Molecular Dynamics, Sunnyvale, CA) to prepare an autoradiograph.

Expression of CCK_A and CCK_B Receptor cDNAs in Mammalian Cells. Two micrograms of pCDL-SRα containing either the CCK_A coding region (12) insert subcloned at an *Xba* I site in the sense orientation or the CCK_B insert (Fig. 1) subcloned at an *Eco*RI site in the sense orientation were transfected into nearly confluent COS-7 cells (≈1 × 10^6 cells in a 100-mm tissue culture plate) by using a DEAE/dextran method as described (26). Approximately 48 hr after transfection, cells were washed twice at 4°C with phosphate-buffered saline (PBS; pH 7.4) containing bovine serum albumin at 1 mg/ml, scraped from the plate in 4°C Dulbecco's modified Eagle's medium (DMEM) containing bovine serum albumin at 1 mg/ml, centrifuged (400 × *g*), and suspended in the same medium at 4°C (≅3 × 10^5 cells per ml). Suspended cells (500 μl) were incubated for 60 min at 37°C with 50 pM of CCK-8 radiolabeled with ^{125}I-labeled Bolton–Hunter reagent (^{125}I-BH-CCK-8; 2200 Ci/mmol; 1 Ci = 37 GBq) either with or without the indicated concentrations of unlabeled agonist or antagonist. Cells were subsequently washed three times at 4°C with 2 ml of PBS containing bovine serum albumin at 1 mg/ml by filtration on glass fiber filters (Whatman GF/C) with a suction manifold (Millipore). Filters were assayed for γ radioactivity (Packard, Auto-Gamma).

FIG. 1. Nucleotide and deduced amino acid sequences of the rat brain CCK_B receptor cDNA clone. Solid lines labeled with Roman numerals I–VII delineate the putative transmembrane domains predicted by Kyte–Doolittle criteria (29) and homology with CCK_A-subtype receptor as well as other G-protein-coupled receptor superfamily members. The solid triangles indicate four potential sites for N-linked glycosylation. Solid bars indicate the three potential sites for serine phosphorylation, and solid circles indicate cysteine residues, which are potential sites for either disulfide-bridge formation (residues 127 and 205) or palmitoylation (residue 413).

RESULTS AND DISCUSSION

Although pharmacological studies of radiolabeled ligand binding to rat brain slices indicate several small discreet areas with receptors for CCK suggestive of a CCK_A subtype (9), previous Northern blot hybridization studies using a ^{32}P-labeled, randomly primed full-length coding region probe of the CCK_A receptor from rat pancreas did not identify a poly(A)$^+$ RNA from rat brain cortex or subcortex (12). To determine whether the rat brain contained the same CCK_A receptor of the same subtype as the gastrointestinal CCK_A receptor isolated from rat pancreas, we used primers flanking the coding region of the pancreatic CCK_A receptor cDNA (12) and single-stranded cDNA from rat brain subcortex in a PCR to clone the rat brain CCK_A receptor. This resulted in a single 1.4-kb product (data not shown) that was directly cycle-sequenced (Bethesda Research Laboratories) and

found to have sequence identical to that of the rat pancreatic CCK_A receptor cDNA (12). PCR contamination from the previously cloned rat pancreatic cDNA was unlikely to account for this result because PCR reactions containing cDNA from cells not expressing CCK_A receptors or lacking DNA target sequence did not result in an amplified product.

To obtain the rat brain CCK_B receptor cDNA, we used the ^{32}P-labeled, randomly primed full-length coding region of the CCK_A receptor cDNA isolated from rat pancreas (12) to screen $\approx 7.5 \times 10^5$ plaques from two rat brain cDNA libraries constructed from cortex and subcortex under conditions of low and high stringency to isolate clones corresponding to pharmacologically described brain CCK_B receptors (5). When this approach failed to yield any hybridizing plaques, we screened a cDNA library constructed from AR42-J cells, a rat pancreatic acinar carcinoma cell line known to express predominantly (80%) CCK_B-subtype CCK receptors pharmacologically identical to brain CCK_B receptors (19). Several candidate clones were isolated only under low-stringency conditions, two of which were sequenced and found to contain identical long open reading frames highly homologous to the CCK_A receptor cDNA (data not shown).

To determine whether the nucleotide sequence of the candidate cDNA clones encoding CCK_B receptors in AR42-J cells was the same as that encoding the CCK_B receptors found in rat brain, we screened another 7.5×10^5 plaques from the rat brain cortex cDNA library, this time using the new CCK_B open reading frame sequence as a ^{32}P-labeled, randomly primed probe. Only high-stringency hybridizing clones were isolated, one of which was a 2243-base pair (bp) clone with identical cDNA sequence to those of the two clones isolated from the AR42-J cell cDNA library (Fig. 1).

A comparison of the nucleotide sequence of the CCK_B receptor cDNA to that of the CCK_A receptor cDNA (Fig. 1) reveals 54% identity (data not shown), higher than any other sequence reported to date [GenBank release 71.0; PIR-Nucleic release 36] to our knowledge. The first in-frame ATG consistent with a consensus translation initiation site (30) initiates a single long open reading frame encoding a unique 452-amino acid protein with a predicted M_r of 48,954. Similar to the CCK_A receptor (12), the sequence contains four potential N-linked glycosylation sites, three in the amino terminus and one in the third intracellular loop, which would account for the larger-than-predicted M_r of 90,000 from affinity crosslinking studies (19). There is one potential site for protein kinase C phosphorylation, on serine-82 in the first intracellular loop and two potential sites for protein kinase A phosphorylation, on serine-154 in the second intracellular loop and serine-442 in the cytoplasmic tail (31).

The predicted amino acid sequence of the CCK_B receptor is 48% identical to the CCK_A receptor (Fig. 2), which is in the expected range for receptors within the same family (33, 36), and the CCK_A receptor is the most homologous of all reported proteins (SwissProt. release 20 and PIR-Protein release 30). A hydropathy plot of the predicted amino acid sequence that uses the criteria of Kyte and Doolittle (29) and similarities to other members of the G-protein-coupled receptor superfamily identifies seven regions of hydrophobic residues corresponding to putative transmembrane domains. Several other areas of CCK_A and CCK_B amino acid sequence homology are also conserved among other G-protein-coupled receptors. The five most similar proteins were mouse gastrin-releasing peptide receptor (32), rat neuromedin B receptor (33), rat substance K receptor (34), rat substance P receptor (34), and rat neuromedin K receptor (35), which further supports the suspected (19, 37) membership in the G-protein-coupled receptor superfamily (Fig. 2). The homology between the two CCK receptor amino acid sequences diverge most notably in the length and composition of their third intracellular loops. This difference may contribute to a difference in G-protein coupling specificity, since this region has been shown to be important in G-protein coupling specificity of other receptors (38). Cysteines in the first and second extracellular domains are conserved in both receptors and may form a disulfide bridge required for stabilization of a functional tertiary structure as demonstrated for rhodopsin,

FIG. 2. Alignment of protein sequences in single-letter amino acid code of the rat CCK_B receptor (RCCKBR), rat CCK_A receptor (RCCKAR), mouse gastrin-releasing peptide receptor (MGRPR), rat substance K receptor (RSKR), rat substance P receptor (RSPR), and rat neuromedin B receptor (RNMBR). By using the PILEUP program sequence analysis package of the Genetics Computer Group (28), deduced amino acid sequence of the CCK_B receptor was aligned for maximal homology to the deduced protein sequences of the CCK_A receptor and the five sequences [mouse gastrin-releasing peptide (32), rat neuromedin B (33), rat neuromedin K (34), rat substance K (35), and rat substance P (36) receptors] found to be the most homologous upon searching the SwissProt. release 20 and PIR-Protein release 30 protein data banks. Shown here is the result of this alignment, with shaded areas denoting conserved amino acids. The number of residues in the variable C terminus not displayed are in parentheses. Solid lines labeled with Roman numerals indicate the seven putative transmembrane domains (see Fig. 1).

β-adrenergic, and muscarinic receptors (39–41). A cysteine in the C-terminal region conserved in many of the G-protein-coupled receptors may be a membrane-anchoring palmitoylation site as demonstrated for rhodopsin and the β_2-adrenergic receptors (42, 43). An aspartate commonly found in the third transmembrane domain of charged amine-binding receptors is absent, as expected, in these peptide hormone receptors (38).

Northern blot analysis revealed that the CCK_A receptor cDNA probe hybridized to a single poly(A)$^+$ RNA of ≈2.7 kb from pancreas and AR42-J cells and 4.4 kb from guinea pig gallbladder, but not to mRNA from rat brain, striated muscle, liver, or kidney (Fig. 3 *Upper*). The absence of hybridization to rat brain mRNA is not surprising for such a cellularly diverse tissue with CCK_A receptors present only in small discreet areas and is consistent with the demonstrated need for the application of PCR cloning when hybridization screening of a large number of plaques from a rat brain cDNA library failed to detect any positive clones. The possibility of another more abundant brain CCK receptor with CCK_A subtype pharmacology is unlikely in the absence of hybridization under conditions of low stringency (data not shown) but cannot be ruled out. High-stringency Northern blot hybridization to poly(A)$^+$ RNA from the same tissues with a CCK_B receptor cDNA probe (Fig. 3 *Lower*) revealed a single hybridizing transcript of ≈2.7 kb with the expected intensity and distribution in rat brain subcortex, cortex (9), and AR42-J cells (19) and absence of hybridization to mRNA from rat pancreas, striated muscle, kidney, or liver or from guinea pig gallbladder. The size of the hybridizing mRNA is close to the size of the CCK_B receptor cDNA clones isolated from AR42-J cells.

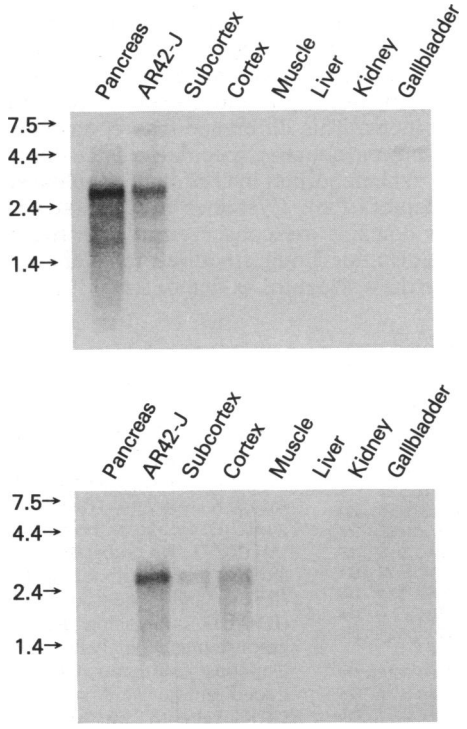

FIG. 3. Northern blot analysis of RNA from rat and guinea pig tissues; sizes are shown in kb. Poly(A)$^+$ RNA was prepared from rat and guinea pig tissues and the rat pancreatic acinar carcinoma cell line AR42-J. Four micrograms of poly(A)$^+$ RNA from each source per lane were probed under conditions of high stringency. (*Upper*) A randomly primed ^{32}P-labeled probe of the CCK_A receptor coding region hybridized with an ≈2.7-kb mRNA from the rat pancreatic acinar carcinoma cell line AR42-J and rat pancreas and with an ≈4.4-kb mRNA from guinea pig gallbladder. No hybridizing mRNA could be identified from rat cortex or subcortex, muscle, liver, and kidney, respectively. (*Lower*) A randomly primed ^{32}P-labeled probe of the CCK_B receptor coding region applied to the blot shown in *Upper* hybridized with an ≈2.7-kb mRNA from AR42-J cells and rat cortex and subcortex. No hybridizing mRNA could be identified from rat pancreas, muscle, liver, or kidney or from guinea pig gallbladder.

To confirm that the two receptors cloned from rat brain correspond to the CCK_A and CCK_B receptor subtypes, pharmacologic characterization of the recombinant receptors expressed on COS-7 cells was performed. COS-7 cells transfected with the full-length cDNA inserts of either the CCK_A (12) or CCK_B (Fig. 1) receptor subcloned in the vector pCDL-SRα, were incubated with the radiolabeled ligand ^{125}I-BH-CCK-8 alone or in the presence of increasing concentrations of unlabeled CCK receptor agonists or antagonists. In COS-7 cells transfected with the vector containing the CCK_A receptor cDNA insert, CCK-8 was >1000 times as potent as gastrin-17-I in inhibiting binding of ^{125}I-BH-CCK-8, and the CCK_A receptor-specific antagonist L-364,718 was nearly equipotent to CCK-8 and 100 times more potent than the CCK_B receptor-specific antagonist L-365,260 (Fig. 4 *Upper*). In COS-7 cells transfected with the vector containing the CCK_B receptor cDNA insert, CCK-8 was only 3 times as potent as gastrin-17-I, and the CCK_B receptor-specific antagonist L-365,260 was 30 times more potent than the CCK_A receptor-specific antagonist L-364,718 in inhibiting ^{125}I-BH-CCK-8 binding (Fig. 4 *Lower*). These results agree closely with previous pharmacological binding studies of CCK_A and

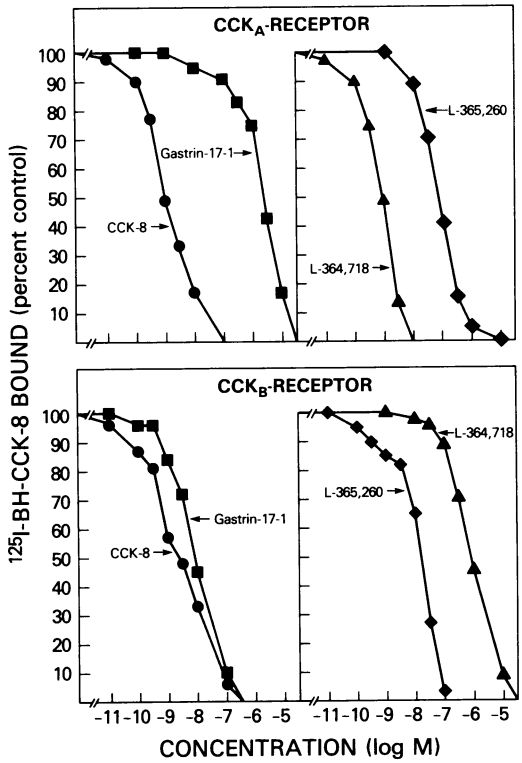

FIG. 4. Ability of CCK receptor agonists and antagonists to inhibit binding of ^{125}I-BH-CCK-8 to COS-7 cells expressing either CCK_A or CCK_B receptors. COS-7 cells were transfected with the expression vector pCDL-SRα (25) containing either the CCK_A (*Upper*) or the CCK_B (*Lower*) receptor cDNA sequence (12) (see Fig. 1). ^{125}I-BH-CCK-8 (50 pM) was incubated either alone or with increasing concentrations of agonists (CCK-8 and gastrin-17-I) (*Left*) or antagonists (L-364,718 and L-365,260) (*Right*). Data are presented as the percent saturable binding (total binding in the presence of radiolabeled hormone alone minus binding in the presence of 1 μM CCK-8). The results given are means of values from at least two experiments performed in duplicate.

CCK_B receptors from rat brain and pancreas (44) and support the classification of these receptors cloned from rat brain and pancreas as CCK_A and CCK_B receptor subtypes.

The physiologic actions of gastrointestinal CCK on the pancreas and gallbladder have been known since 1928 (45). More recently, the discovery of CCK in the brain in 1976 (2) and subsequent identification of distinct CCK receptor subtypes in 1980 (46) has rapidly led to an appreciation of CNS and peripheral nervous system CCK as a modulator of an increasing number of diverse neuropsychiatric functions encompassing satiety, anxiety, nociception, and neuroleptic-like activity (11). Whereas highly specific CCK receptor agonists and antagonists have facilitated many of these recent advances, cloning of the gastrointestinal and brain CCK_A and CCK_B receptors should enhance CCK receptor structural and functional analysis, subtype localization, and possibly the discovery of new subtypes. This should hasten the development of new more potent and selective agonists and antagonists that may be used to better understand and treat gastrointestinal and neuropsychiatric disorders.

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