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Purification and subunit structure of a putative K^+-channel protein identified by its binding properties for dendrotoxin I

(affinity chromatography/mast cell degranulating peptide/β-bungarotoxin)

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ABSTRACT **The binding protein for the K^+-channel toxin dendrotoxin I was purified from a detergent extract of rat brain membranes. The purification procedure utilized chromatography on DEAE-Trisacryl, affinity chromatography on a dendrotoxin-I-Aca 22 column, and wheat germ agglutinin-Affigel 10 with a final 3800- to 4600-fold enrichment and a recovery of 8–16%. The high affinity (K_d, 40–100 pM) and specificity of the binding site are retained throughout the purification procedure. Analysis of the purified material on silver-stained NaDodSO$_4$/polyacrylamide gel revealed three bands of M_r 76,000–80,000, 38,000, and 35,000. Interestingly, the binding site for ^{125}I-labeled mast cell degranulating peptide, another putative K^+-channel ligand from bee venom, which induces long-term potentiation in hippocampus, seems to reside on the same protein complex, as both binding sites copurify through the entire purification protocol.**

Dendrotoxins are a family of neurotoxins isolated from the venom of *Dendroaspis* snakes (1). The basic polypeptides (M_r, 7000) are homologous to protease inhibitors and facilitate evoked neurotransmitter release (1, 2). Dendrotoxin I (DTX-I) is the most potent member of the dendrotoxin family known until now. Recent research has provided data strongly suggesting that blockade of K^+ channels, in particular the A-type K^+ channel, accounts for the facilitation effects of the toxins (3–6). The channel seems to be associated with the high-affinity binding site for DTX-I, which has been detected in brain membranes from rat and chicken (7–9).

Mast cell degranulating peptide (MCD) is a basic peptide of 22 amino acids isolated from bee venom (10). It induces hippocampal long-term potentiation (11) and arousal or, depending on the concentrations injected (12), convulsions and epileptogenic crisis. The toxin has also been proposed as a K^+-channel ligand (13). High-affinity binding sites for MCD have been shown to exist in brain membranes (14) and it has been observed that binding of ^{125}I-labeled DTX-I (^{125}I-DTX-I) can be inhibited by MCD and vice versa (8), suggesting a coexistence of DTX-I and MCD binding sites in the same protein complex.

Binding sites for both MCD and DTX-I are related to the binding site of a third potential K^+-channel ligand (15), β-bungarotoxin, a presynaptic toxin from the venom of the snake *Bungarus multicinctus*. The specific binding of ^{125}I-labeled β-bungarotoxin to brain membranes (16) is inhibited by MCD and DTX-I (9), and β-bungarotoxin inhibits the binding of ^{125}I-labeled MCD (^{125}I-MCD) (8) and ^{125}I-DTX-I (this paper). Thus, a K^+ channel that has binding sites for three ligands—DTX-I, MCD, and β-bungarotoxin—seems to exist in brain.

This paper reports the purification of this putative K^+ channel and its polypeptide composition.

MATERIALS AND METHODS

Materials. DTX-I and MCD were prepared and iodinated as described (8, 14). β-Bungarotoxin was from Serva (Heidelberg). DEAE-Trisacryl and activated Aca 22 were from LKB (Orsay, France). The DTX-I-Aca 22 column was prepared with 86 mg of DTX-I according to the manufacturer's recommendations. The wheat germ agglutinin (WGA)-Affigel 10 column was prepared by coupling 8 mg of WGA to 1 ml of Affigel 10 (Bio-Rad) according to the manufacturer's recommendations.

Preparation of Detergent Extract. P3 membranes from rat brain, prepared as described (14), were suspended in 20 mM Na-Hepes buffer (pH 7.4) containing 120 mM KCl, 1 mM EDTA, 10% (vol/vol) glycerol, 1% (wt/vol) Triton X-100, and 0.2% (wt/vol) soybean phospholipid (buffer I) to a protein concentration of 2–3 mg/ml. Phenylmethylsulfonyl fluoride was added to a concentration of 0.1 mM. The suspension was incubated at 4°C for 1 hr under slight stirring and then centrifuged for 1 hr at 100,000 × *g*. The supernatant of this centrifugation is referred to as detergent extract. It contained 60–70% of the ^{125}I-DTX-I binding activity and 50–60% of the protein of the P3 membranes.

Binding Assays. ^{125}I-DTX-I and ^{125}I-MCD binding to detergent extracts was determined by a modification of the filter assay described by Bruns *et al.* (17). The sample (2–50 μl) was adjusted to a volume of 190 μl with buffer I containing 2 mM $CaCl_2$ and 2 mM $MgSO_4$ instead of 1 mM EDTA. Assay mixtures for the determination of ^{125}I-MCD binding also contained 12.5 μg of compound 48/80 per ml. ^{125}I-DTX-I or ^{125}I-MCD was then added in 10 μl of the above buffer and the mixture was incubated for 20 min at 4°C. To measure nondisplaceable binding, parallel incubation mixtures contained, in addition, 100 nM unlabeled DTX-I or 1 μM unlabeled MCD, respectively. Incubation mixtures (total vol, 200 μl) were then diluted with 5–6 ml of the above buffer and filtered through GF/C filters coated with 0.5% polyethyleneimine (pH 7.5). All binding data were corrected for nonspecific binding and represent the mean of triplicate determinations.

Chromatography on DEAE-Trisacryl. DEAE-Trisacryl (39 ml) was equilibrated in a batch with buffer I and poured into a column (1.6 × 19.5 cm). After passing several column volumes of buffer I through the column, the sample (320–380 ml) was loaded at a flow rate of 16 ml/hr. The column was then washed with 3–6 column vol of buffer I and the fraction with binding activity was eluted with buffer I containing 200 mM instead of 120 mM KCl.

Chromatography on DTX-I-Aca 22. The eluate from the DEAE-Trisacryl column (16 ml) was directly loaded onto a column of DTX-I-Aca 22 (0.9 × 5 cm) equilibrated with

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Abbreviations: DTX-I, dendrotoxin I; MCD, mast cell degranulating peptide; WGA, wheat germ agglutinin; ^{125}I-DTX-I, ^{125}I-labeled DTX-I; ^{125}I-MCD, ^{125}I-labeled MCD.

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Table 1. Purification of ^{125}I-DTX-I binding sites

	Vol, ml	Protein, μg	Specific activity, pmol of ^{125}I-DTX-I bound per mg of protein	Recovery, %	Purification, -fold
Detergent extract	324	240,408	0.350	100	1
Eluate from DEAE-Trisacryl	16	20,032	3.18	76	9
Eluate from DTX-I-Aca 22	8	136	224	36	640
Eluate from WGA	2	10	1345	16	3840

buffer I containing only 0.05% (wt/vol) Triton X-100 and 0.01% (wt/vol) soybean phospholipid (buffer II). All the DTX-I binding activity was retained by the column. The column was then washed with 20 vol of buffer II, 5 vol of buffer I, and 5 vol of buffer II containing 220 mM instead of 120 mM KCl. The fraction with binding activity was eluted with buffer II containing 620 mM KCl.

Chromatography on WGA-Affigel 10. The eluate from the DTX-I-Aca 22 column was directly loaded (flow rate, 5–7 ml/hr) onto a column of WGA-Affigel 10 (0.9 × 14 cm) equilibrated with buffer II. The column was washed with 10 ml of buffer II and eluted with buffer II containing 50 mM *N*-acetylglucosamine. Usually only 60–70% of the binding activity was retained by the column, whereas 40–30% appeared in the breakthrough (see Fig. 2). Upon incubation of the unbound binding activity with fresh WGA-Affigel 10, again 60–70% was adsorbed. Therefore no evidence of receptor heterogeneity can be inferred from this experiment.

Miscellaneous Methods. Sucrose density centrifugation was performed in linear [3–20% (wt/vol)] sucrose gradients in buffer II. An aliquot (1 ml) of the eluate from the WGA-Affigel 10 column was layered on top and the gradients were centrifuged in a Spinco SW41 Ti rotor at 39,000 rpm and 4°C for 19 hr.

NaDodSO$_4$/PAGE (18) was done in 8% running gels and the gels were silver stained according to Merril *et al.* (19). Protein was determined according to Lowry *et al.* (20) or with

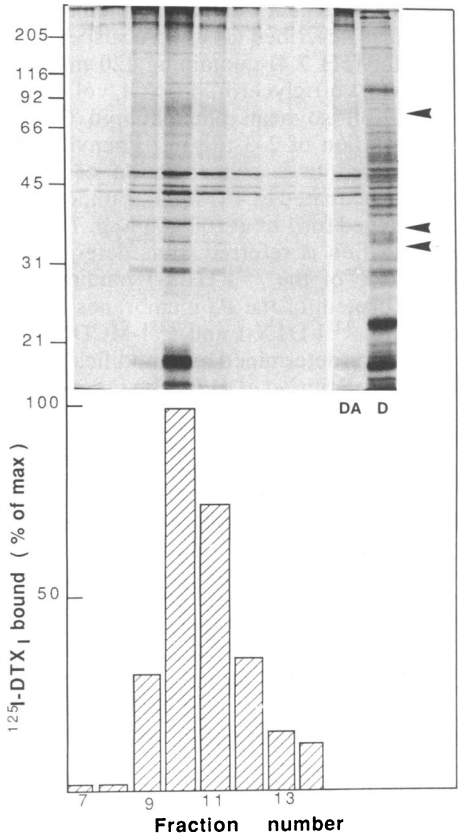

FIG. 1. Elution profile of DTX-I-Aca 22 affinity column. ^{125}I-DTX-I binding sites were solubilized from brain membranes and subjected to chromatography on DEAE-Trisacryl. The eluate from the DEAE-Trisacryl was then loaded onto the DTX-I-Aca 22 column, washed, and the fraction with DTX-I binding activity was eluted with KCl as described. (*Upper*) The protein composition of the different fractions of the eluate from the DTX-I-Aca 22 was analyzed by NaDodSO$_4$/PAGE. (*Lower*) The corresponding ^{125}I-DTX-I binding activity of the fractions. Lane D, aliquot of the starting material (detergent extract); lane DA, aliquot of the pool of the eluate from the DTX-I-Aca 22 (fractions 9–12) that was used for subsequent WGA chromatography. Arrowheads point to the position of the M_r 76,000–80,000, 38,000, and 35,000 bands. Molecular weight markers ($M_r \times 10^{-3}$) are indicated.

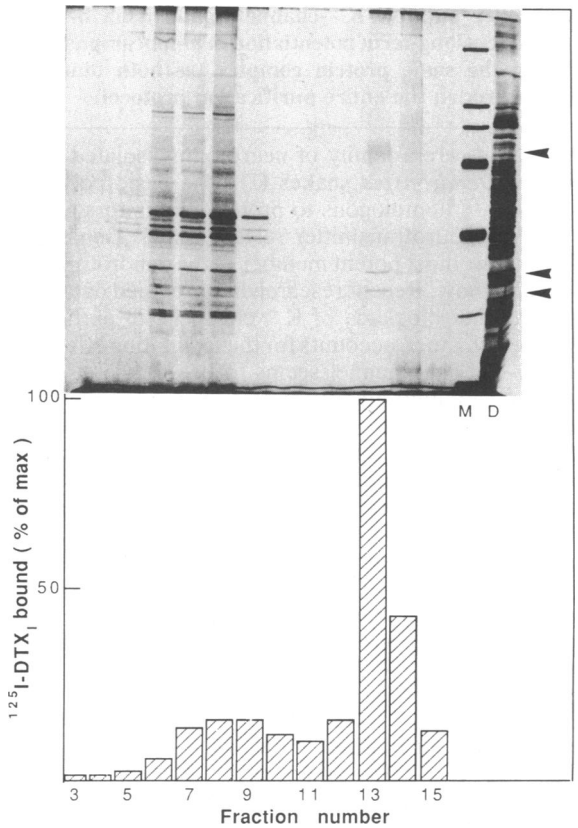

FIG. 2. Elution profile of WGA-Affigel 10 column. ^{125}I-DTX-I binding sites were purified from brain membranes by chromatography on DEAE-Trisacryl and DTX-I-Aca 22. The pool from the DTX-I-Aca 22 (Fig. 1) was loaded onto a column of WGA-Affigel 10, washed, and the fraction with ^{125}I-DTX-I binding activity was eluted with *N*-acetylglucosamine as described. (*Upper*) The protein composition of the different fractions of the WGA-Affigel 10 column was analyzed by NaDodSO$_4$/PAGE. (*Lower*) The corresponding ^{125}I-DTX-I binding activity of the fractions. Lane D, aliquot of the starting material (detergent extract). Arrowheads point to the position of the M_r 76,000–80,000, 38,000, and 35,000 bands. Molecular weight markers (lane M) are M_r 205,000, 116,000, 92,000, 66,000, 45,000, 31,000, and 21,000.

Table 2. Copurification of ^{125}I-DTX-I and ^{125}I-MCD binding sites

	^{125}I-DTX-I		^{125}I-MCD	
	Purification, -fold	Recovery, %	Purification, -fold	Recovery, %
Detergent extract	1	100	1	100
Eluate from DEAE-Trisacryl	9	41	ND	ND
Eluate from DTX-I-Aca 22	780	21	841	23
Eluate from WGA	4680	8.6	4155	7.6

Specific activity of the ^{125}I-DTX-I binding sites and the ^{125}I-MCD peptide binding sites in the detergent extract were 350 fmol/mg and 170 fmol/mg, respectively. ND, not determined.

the BCA method (Pierce), with bovine serum albumin used as a standard.

RESULTS

The binding site for ^{125}I-DTX-I was solubilized from rat brain membranes with Triton X-100. The solubilized binding sites retained their high affinity and specificity for ^{125}I-DTX-I and were stable with a half-life of 3–4 days at 4°C. Initial experiments showed that the binding component was a rare protein present in the detergent extract only to an extent of 350 fmol/mg (Table 1). Therefore, to purify adequate amounts of binding protein one had to start with large volumes of detergent extract. An ion-exchange concentrating procedure was chosen as the first step of the purification. As the binding component was found to be an acidic protein, an anion exchanger, DEAE-Trisacryl, was used that resulted in a considerable reduction of the handling volume. A 9-fold enrichment and a 40–75% recovery of the binding activity was obtained at this step (Table 1 and 2).

The incubation of glutaraldehyde-activated Aca 22 (LKB) with high amounts (86 mg) of DTX-I resulted in an affinity matrix with sufficient binding capacity for the solubilized ^{125}I-DTX-I binding site. The final substitution of the gel was moderate (3 mg of DTX-I per ml of gel). The possibility existed that this gel, because of the high positive charge of DTX-I, might have acted as an anion exchanger rather than an affinity matrix. This possibility seems unlikely as (*i*) the DTX-I-Aca 22 gave reproducibly (seven times) high enrichments (80- to 100-fold; Table 1), which would not be expected from an ion-exchange purification step, and (*ii*) the DTX-I-Aca 22 column was loaded and washed under high salt conditions—i.e., under conditions in which the ^{125}I-DTX-I binding site does not associate to anion exchangers such as DEAE-Trisacryl or DEAE-Affigel blue.

A biospecific elution of the ^{125}I-DTX-I binding component from the DTX-I-Aca 22 column with MCD (10 μM), which

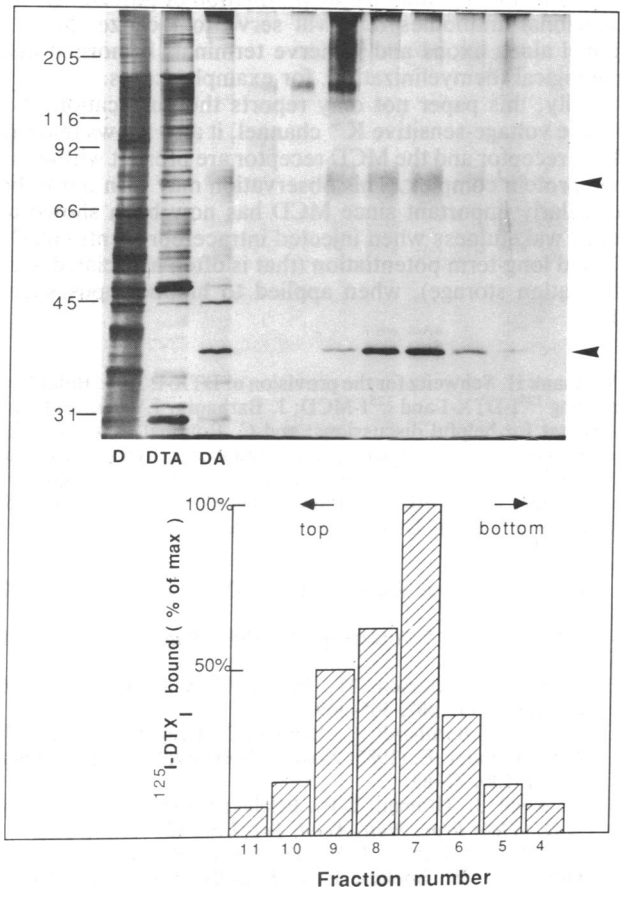

FIG. 3. Sucrose gradient centrifugation of the purified ^{125}I-DTX-I binding site. ^{125}I-DTX-I binding sites were purified from brain membranes by chromatography on DEAE-Trisacryl, DTX-I-Aca 22, and WGA-Affigel 10. The eluate from the WGA-Affigel 10 (Fig. 2) was run on a sucrose gradient [3–20% (wt/vol)] and the 13 fractions were analyzed by NaDodSO$_4$/PAGE (*Upper*) and for ^{125}I-DTX-I binding activity (*Lower*). Lanes D, DTA, and DA, aliquots of detergent extract, pool from DEAE-Trisacryl column, and pool from DTX-I-Aca 22 column, respectively. Arrowheads point to the position of the M_r 76,000–80,000 and 38,000 bands. Molecular weight markers ($M_r \times 10^{-3}$) are indicated.

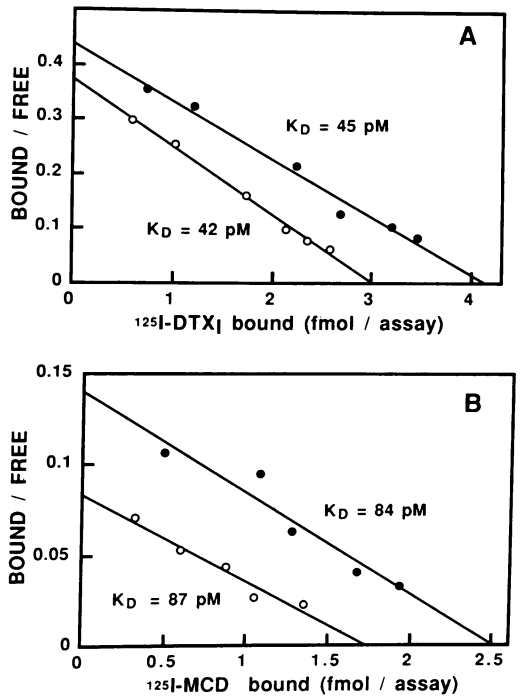

FIG. 4. Scatchard analysis of ^{125}I-DTX-I and ^{125}I-MCD binding to detergent extract and eluate from the WGA-Affigel 10 column. ^{125}I-DTX-I (^{125}I-MCD) binding sites were purified from rat brain membranes by three chromatographic steps. Binding of ^{125}I-DTX-I (*A*) or ^{125}I-MCD (*B*) to the purified sites (○) or to detergent extract (●) was determined with different concentrations of the iodinated ligands. Data are plotted according to Scatchard.

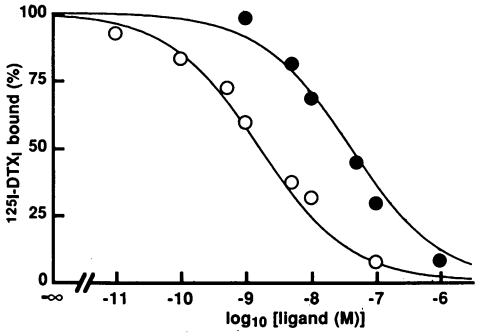

FIG. 5. Effects of MCD peptide and β-bungarotoxin on the specific binding of ^{125}I-DTX-I to its purified binding site. Binding sites for ^{125}I-DTX-I were purified, and binding of ^{125}I-DTX-I (127 pM) to these sites (2 fmol) was then determined in the absence and presence of the indicated concentrations of MCD peptide (○) or β-bungarotoxin (●).

inhibits ^{125}I-DTX-I binding, was not possible. We therefore made use of the observation that ^{125}I-DTX-I binding is inhibited by high KCl concentrations (H.R., unpublished observation) and used 0.62 M KCl for elution.

An examination of the elution profile of the DTX-I-Aca 22 column on NaDodSO$_4$/PAGE (Fig. 1) showed the presence of several contaminants in the fractions having DTX-I binding activity. The eluate from the DTX-I-Aca 22 was therefore further purified by affinity chromatography on WGA-Affigel 10 (Fig. 2). This procedure removed most of the contaminating bands. Only three bands showed a codistribution with the binding activity (Figs. 2 and 3): a broad fuzzy band of M_r 76,000–80,000, probably a glycoprotein, and two bands of M_r 38,000 and M_r 35,000. The M_r 35,000 band was present in variable amounts and tended to increase upon prolonged storage of the sample; thus, it may be a proteolytic breakdown product of one of the other bands. The molecular weights of these bands were the same when the gels were run under nonreducing conditions (data not shown). In some preparations, a band of M_r 150,000 was seen in the WGA eluate (Fig. 2). However, analysis of the WGA eluate by subsequent sucrose gradient centrifugation (Fig. 3) showed that the M_r 150,000 band, but not the others, could be separated from the binding activity.

In the purified protein, ^{125}I-DTX-I and ^{125}I-MCD have the same affinity for their binding sites as in the crude detergent extract (Fig. 4).

An interesting observation is that the ^{125}I-DTX-I and the ^{125}I-MCD binding components copurify (Table 2). Accordingly, the cross-inhibition of the binding of both ligands to their receptor sites, which was previously observed in brain membranes, was preserved (Fig. 5) in the purified material. Also the binding of ^{125}I-DTX-I [and ^{125}I-MCD (data not shown)] to the purified binding site was inhibited by β-bungarotoxin (Fig. 5).

DISCUSSION

A 3800- to 4600-fold purification of the binding protein for ^{125}I-DTX-I has been achieved starting from detergent extracts of rat brain membranes in three successive chromatographic steps. The purest fraction contains 1.4–1.6 nmol of ^{125}I-DTX-I binding sites per mg. The following considerations indicate that this purified material corresponds to a nearly homogeneous DTX-I binding protein: (*i*) Sucrose gradient centrifugation in $H_2O/^2H_2O$ and gel filtration indicated a M_r of 450,000 for the protein complex that bears the binding sites of dendrotoxin, a close relative of DTX-I (21) and β-bungarotoxin (22, 23). This protein complex is the same one that bears the binding sites for ^{125}I-DTX-I (7, 9, 24). From this molecular weight, a maximum specific activity of 2.22 nmol/mg can be calculated for the pure binding protein, which is not much higher than that found at the end of the purification (Table 1). (*ii*) No significant further purification was obtained by sucrose gradient centrifugation, a classical step in ion-channel purification that is distinct from the three steps used in Table 1. (*iii*) The protein pattern obtained with the purified preparation (M_r, 76,000–80,000, 38,000, and 35,000) is in good agreement with crosslinking data that indicated that the ^{125}I-DTX-I binding site contains a M_r 76,000 polypeptide (25).

A recently cloned A-type K^+ channel from *Drosophila* (26) seems to contain peptides of M_r 70,000 (27) and 35,000 (28). This is interesting as ion channels from *Drosophila* seem to have a similar pharmacology and structural organization as the corresponding mammalian proteins (29–31).

We conclude that the dendrotoxin-sensitive K^+ channel from rat brain is a multimeric protein consisting of polypeptide chains of M_r 76,000–80,000 and 38,000. Moreover, the broad band of M_r 76,000–80,000 could be composed of two or three closely related peptides.

The work described in this paper opens the way for further research concerning (*i*) the exact stoichiometry of the different polypeptide chains within the final putative K^+-channel complex, (*ii*) the sequencing of the polypeptide components via cDNA cloning, and (*iii*) the production of polyclonal and monoclonal antibodies that will serve to localize the K^+ channel along axons and in nerve terminals in normal and pathological (demyelinization, for example) cases.

Finally, this paper not only reports the purification of a putative voltage-sensitive K^+ channel, it also shows that the DTX-I receptor and the MCD receptor are present within the same protein complex. This observation may turn out to be particularly important since MCD has now been shown to induce wakefulness when injected intracerebroventricularly (12) and long-term potentiation (that is often associated with information storage), when applied to hippocampus slices (11).

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