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Opiate Receptors in the Rat Brain

Specific Labeling of Multiple Membrane Components with [^{3}H]Etorphine?

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SUMMARY

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A crude membrane fraction (CMF) from rat brain is labeled with [^{3}H]etorphine (2×10^{-9} M) in the absence (total binding, −L) and in the presence (nonspecific binding, +L) of 2×10^{-6} M levorphanol. The ^{3}H-labeled CMF (±L) is solubilized with 1% (w/v) cholic acid (sodium salt) and centrifuged. The high-speed (100,000 × *g*) supernatant is analyzed by molecular exclusion chromatography on Sephadex G-25, Sephadex G-100, and Sepharose 6B. A comparison of the radioactivity profiles reveals, in addition to the previously identified 500,000 mol wt species [Puget *et al.*, *F. E. B. S. Lett.* **122**:199–202 (1980)] lower molecular weight, tritium-labeled entities distinct from monomolecular [^{3}H]etorphine. These small complexes are readily separated from free radioligand upon dialysis in the cold. They appear to be opiate-specific, since they are not observed in ^{3}H-labeled CMF (+L) soluble extracts.

INTRODUCTION

Opiate alkaloids and opioid peptides initiate their multiple central effects by interacting reversibly with at least two classes of stereospecific binding sites in the CNS[1] (1–5). These sites referred to as μ (morphine) and δ (enkephalin) are believed to mediate intrinsically distinct pharmacological responses, including analgesic and behavioral ones, respectively (6–9).

However, the same ligand, depending on whether its concentration is low or high, binds to one or, indiscriminately, both sites, sometimes in different ways. For example, *N*-allylnormetazocine might act as a μ agonist and a δ antagonist (10). Briefly stated, according to Chang and Cautrecasas (10), the pharmacological response elicited by opioid drugs may be qualitatively as well as quantitatively defined in terms of their concentration-dependent relative occupancy of only two categories of receptor sites in the CNS.

Morphine and enkephalin sites are carried by membrane-bound receptors about which very little is known. In particular, it remains to be determined whether opiate alkaloids and opioid peptides "bind differently to the same receptor or bind to different receptors with overlapping specificity." An answer to this question would be of considerable value in relation to how these drugs initiate their pharmacological effects in the CNS.

In a previous article (11) we have presented clear evidence for a [^{3}H]etorphine-binding component with an apparent molecular weight of 500,000 (~67 A) in rat brain membranes. However, there seemed to exist in our preparations much smaller species with similar binding characteristics. In the present article, experimental data are given which support this notion.

MATERIALS AND METHODS

1. *Preparation of the CMF from rat brain.* Adult Wistar rats were killed by decapitation. The brains (without the cerebellum) were rapidly removed and homogenized at 4° in a total volume (v_o) of 12 ml/g of tissue (wet weight) of 0.32 M sucrose in Tris-HCl, 1 mM, pH 7.4. Homogenization was completed in a Potter-Elvehjem tissue grinder by 10 strokes of a Teflon pestle, motor-driven at 800–1,000 rpm. The suspension was incubated for 30 min at 35° and centrifuged (0–2°) in a Beckman rotor (Type 30) for 30 min at 30,000 rpm. The pellet was resuspended (Polytron) in a large excess of ice-cold 50 μM Tris-HCl, pH 7.4 (hereafter referred to as "buffer") and centrifuged as before. The (washed) pellet was homogenized in one-half v_o of buffer to yield the CMF (12–14 mg of protein per milliliter).

2. *Labeling of the CMF with [^{3}H]etorphine.* Labeling of the CMF with [^{3}H]etorphine was carried out routinely

[1] The abbreviations used are: CNS, central nervous system; CMF, crude membrane fraction; HMWC, high molecular weight complexes; LMWC, low molecular weight complexes.

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by incubating for 20 min at 35° the following: 4.0 ml of CMF, 1.0 ml of buffer (with and without 20 μM levorphanol), and 5.0 ml of 4 nM [^{3}H]etorphine (41.4 Ci/mmole; the Radiochemical Centre, Amersham, England) in buffer.

The mixture was then chilled to 0°, centrifuged, and washed in a large volume of ice-cold buffer as described above. The final pellet was dispersed (Polytron) in 5.0 ml with buffer to give the ^{3}H-labeled CMF (10–11 mg of protein per milliliter).

3. *Solubilization of the ^{3}H-labeled CMF with sodium cholate.* To 2.4 ml of ^{3}H-labeled CMF were added 0.3 ml of ice-cold buffer and 0.3 ml of 10% (w/v) cholic acid (sodium salt; Sigma Chemical Company, St. Louis, Mo.). After 15 min of gentle agitation at 0°, the membrane suspension was centrifuged (in the cold) in a Beckman rotor (Type 30) for 30 min at 30,000 rpm. The supernatant (2 mg of protein per milliliter) constituted the radioactive detergent extract.

4. *Dissociation of the [^{3}H]etorphine complexe(s) in solution.* Bound and free [^{3}H]etorphine in the cholate extracts were separated by rapid filtration on short prepacked Sephadex G-25 columns (PD-10; Pharmacia Fine Chemicals, Piscataway, N. J.) equilibrated at cold room temperature with 1% (w/v) cholate in buffer.

The freshly prepared detergent solutions were made 1×10^{-6} M with unlabeled etorphine. At various time intervals, 0.3-ml aliquots were applied to the column. Fifty fractions of 20 drops were collected directly into scintillation vials and counted as described below.

5. *Molecular exclusion chromatography.* A special device was used such that six runs could be performed simultaneously under identical conditions. It consisted of six glass (Silyl-8-coated) columns (1.6 × 45 cm) held parallel on a vertical rack and connected, via SMA flow-rated pump tubes (Technicon, nominal and actual flow rate, 0.15 ml/min) to a fraction collector (Apelab) equipped with a custom-made circular plateau holding six concentric rows of sixty tubes each. Elution was carried out by a seven-channel peristaltic pump (Technicon).

The columns, in duplicate (±L) were made of about 80 ml of packed Sephadex G-25 (20–80 μm), Sephadex G-100 (40–120 μm), and Sepharose 6B (45–145 μm), all from Pharmacia Fine Chemicals. They were fully equilibrated at cold room temperature with sodium cholate (1%, w/v in buffer), then loaded with 0.5-ml aliquots of detergent extract (±L). Fractions of 11 min (1.65 ml) were collected. A portion (0.4 ml) of each fraction was mixed with 4.0 ml of home-made scintillation liquid (toluene, 0.7 liter; Triton N-101, = 0.3 liter; 2,4″-*tert*-butylphenyl)-5-(4″-biphenyl-1,3,4-oxidazole, 7 g) and counted in an Intertechnique Model SL 30 counter. Recoveries were in the range 87–98% of input.

RESULTS

1. *Labeling of the CMF with [^{3}H]etorphine and solubilization of the [^{3}H]etorphine-labeled CMF with cholic acid (sodium salt).* The CMF from rat brain was labeled with 2 nM [^{3}H]etorphine in the absence and in the presence of 2 μM levorphanol to yield the ^{3}H-labeled CMF(−L) and ^{3}H-labeled CMF(+L), respectively. Under our experimental conditions (see Step 2 under Materials and Methods), it was estimated that up to 75% of the bound radioligand was associated with specific (levorphanol-sensitive) binding sites.

After incubation of the ^{3}H-labeled CMF fractions with 1% (w/v) cholic acid (sodium salt), 60–70% of the specifically bound radioactivity was recovered in the high-speed supernatant (soluble fraction). Therefore, a second extraction was not attempted. There did not seem to be differential solubilization of specifically and nonspecifically bound radioactivity.

Prolonged dialysis (16 hr at 4°) of the soluble extracts resulted in a substantial loss (up to 50%) of radioactivity.

2. *Analysis of the cholate-solubilized radioactivity and kinetics of dissociation of bound [^{3}H]etorphine.* The cholate extracts from ^{3}H-labeled CMF(−L) and ^{3}H-labeled CMF(+L) were rapidly filtered on short (PD-10) columns of Sephadex G-25. These columns had been calibrated with blue dextran 2000 (V_o) and [^{3}H]etorphine (V_t). In the ^{3}H-labeled CMF(−L) soluble fraction, two peaks of radioactivity were observed (Fig. 1, *ND*): a major one (in V_o) corresponded to bound [^{3}H]etorphine and represented 65% of the input; the other (in V_t) corresponded to free [^{3}H]etorphine and accounted for 35% of the input. It is interesting to mention here that, when Triton X-100 was used instead of sodium cholate, more than 80% of the applied radioactivity was recovered as free [^{3}H]etorphine (not shown).

In the ^{3}H-labeled CMF(+L) extract, only trace amounts of radioactivity were found in V_o. The bulk of the counts was eluted in V_t (free radioligand). Actually, it was quite unexpected that more free [^{3}H]etorphine was present in the (−L) than in the (+L) extracts. Some rapid dissociation of initially bound radioligand must therefore have occurred at the time of solubilization.

Dialysis of the extracts resulted in nearly complete removal of free [^{3}H]etorphine (Fig. 1, *D*). However some bound [^{3}H]etorphine was also lost, either as free radioligand (dissociation) or as low molecular weight labeled complexes (see below).

Rapid filtration on PD-10 columns was also used to estimate the rate of dissociation of bound [^{3}H]etorphine in detergent solution at cold room temperature (4°). The extracts were first made 1 μM with unlabeled etorphine, and aliquots were filtered at various time intervals. Under these conditions, a time-dependent decrease in the amount of bound (V_o) and a simultaneous increase (not shown) in the amount of free (V_t) [^{3}H]etorphine were observed. A dissociation kinetics is represented in Fig. 2. It was found that only 25–30% of the initially bound radioactivity was released as free radioligand within 24 hr at 4°.

3. *Molecular exclusion chromatography of the cholate-solubilized radioactivity on various gel types.* The cholate-extracted radioactivity from the ^{3}H-labeled CMF(−L) and (+L) was analyzed by molecular exclusion chromatography on Sephadex G-25, Sephadex G-100, and Sepharose 6B. It must be emphasized here that the three runs, each in duplicate (±L) were performed simultaneously with identical aliquots of the same extract(s).

In nondialyzed extracts (Fig. 3, *ND*), 50% of the radio-

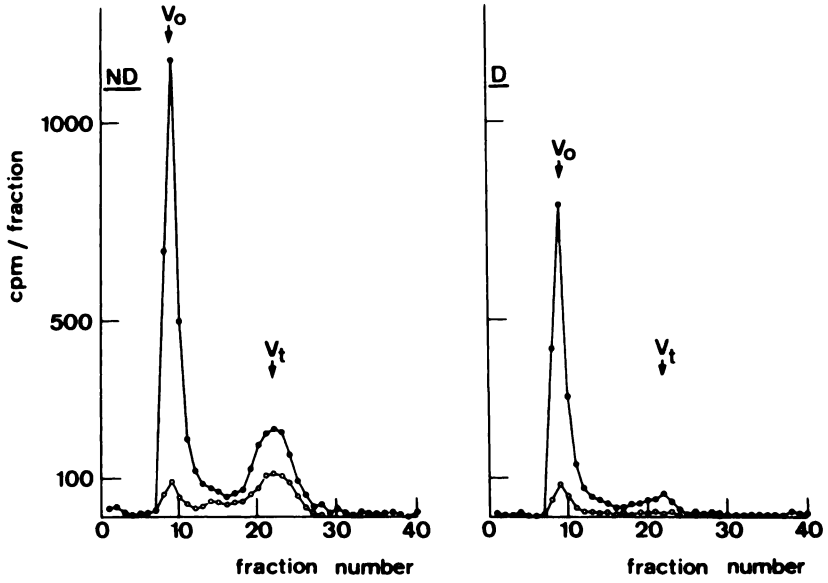

FIG. 1. *Chromatography on Sephadex G-25 of the cholate-soluble fraction from [^{3}H]etorphine labeled brain membranes*

ND. The detergent extracts were prepared from crude membranes that had been allowed to react with [^{3}H]etorphine in the absence (−L, ●) and in the presence (+L, ○) of levorphanol. (Aliquots 0.2 ml of radioactive solution were applied to short, prepacked (PD-10, Pharmacia) columns calibrated with blue dextran 2000 (V_o) and with [^{3}H]etorphine (V_t). Elution was carried out as described under Materials and Methods.

D. Same experiment as above except that the cholate-soluble fractions (+L) were dialyzed (16 hr, 4°) prior to gel filtration. Note that free [^{3}H] etorphine was nearly quantitatively removed but that some bound [^{3}H]etorphine was also lost.

activity was excluded from Sephadex G-25 and 33% was completely retained (free [^{3}H]etorphine). Some radioactivity was included (plateau above baseline) which likely consisted of dissociated, monomolecular etorphine.

In Sepharose 6B, the profile was apparently reversed, since only 22% of the radioactivity was recovered as the 500,000 mol wt species (HMWC). The remaining 78% was eluted as a broad peak in a range of much lower molecular weights. This second (major) peak was clearly asymetrical, suggesting heterogeneity.

This enormous discrepancy may have come from increased dissociation in agarose. However, filtration on Sephadex G-100, the chemical composition (dextran) of which is identical with that of Sephadex G-25, yielded a radioactivity elution profile more like that observed in Sepharose 6B: only 31% of the applied counts were excluded, the other 69% being eluted as a very broad, asymetrical peak.

To evaluate the contribution of monomolecular [^{3}H] etorphine to the second peak in each column, the extracts were first dialyzed (16 hr at 4°). As reported earlier (Results, Step 2), this step removes almost completely free [^{3}H]etorphine and some of its bound form(s). This was confirmed by filtration on Sephadex G-25 (Fig. 3, *D*). However, a second peak (LMWC) in the low molecular weight range and accounting for 47% and 58% of the input radioactivity was still observed in Sephadex G-100 and Sepharose 6B profiles, respectively.

The 500,000 mol wt species (HMWC) and the LMWC were absent in dialyzed soluble extracts from ^{3}H-labeled CMF(+L). Therefore, it was concluded that these two components (or sets of components) were opiate-specific.

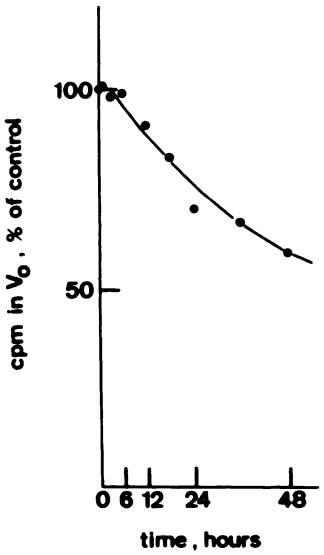

FIG. 2. *Kinetics of dissociation of bound [^{3}H]etorphine in cholate-soluble extracts*

Bound and free radioligand fractions were separated by rapid filtration on Sephadex G-25 (prepacked PD-10 columns, Pharmacia). The values indicated, expressed as percentage of control, were calculated from radioactivity elution profiles identical with the one depicted in Fig. 1.

DISCUSSION

In a recent article (11) we had characterized in rat brain membranes a component with an apparent molecular weight of 500,000 that binds opiates-specifically. This component appears to be somewhat larger than the one (380,000 mol wt) described by Simon *et al.* (12) and by Zukin and Kream (13). In the same article we suggested that, under our experimental conditions, [^{3}H]etorphine had selectively labeled other membrane constituents of

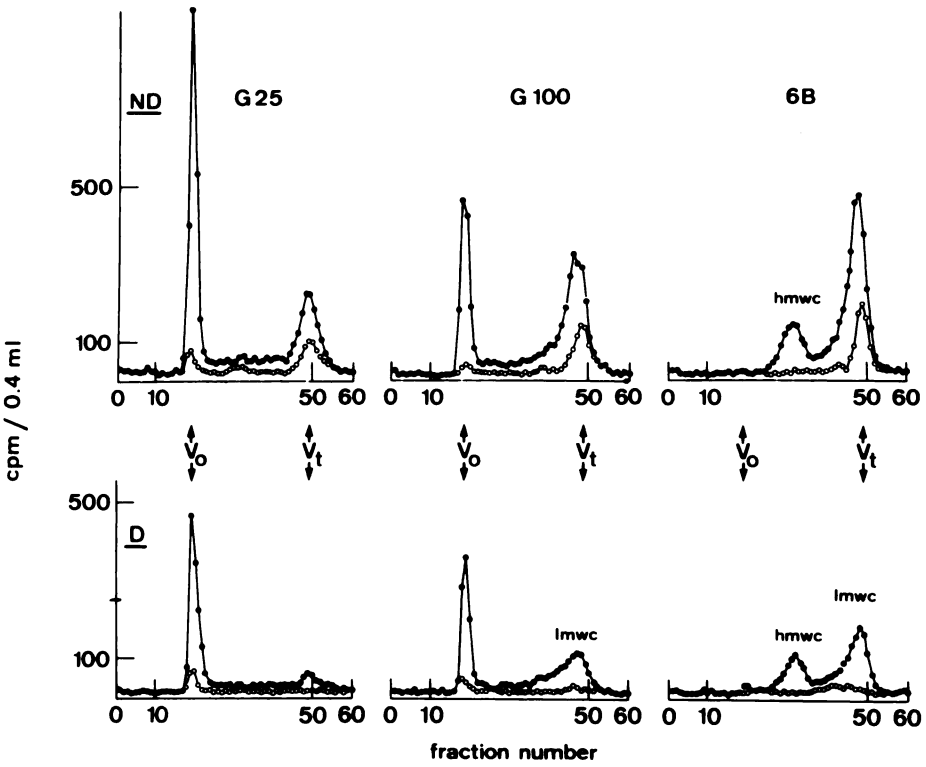

FIG. 3. *Molecular exclusion chromatography on Sephadex G-25, Sephadex G-100, and Sepharose 6B of the cholate-soluble fraction from [^{3}H]etorphine labeled brain membranes*

Rat brain membranes were incubated with [^{3}H]etorphine in the absence (−L, ●) and in the presence (+L, ○) of levorphanol. Radioactive cholate extracts (0.5 ml) (−L and +L) were applied to each column type and the six chromatographic analyses were run simultaneously as described under Materials and Methods. The columns were calibrated with blue dextran 2000 (V_o) and with [^{3}H]etorphine (V_t). Experiments such as the one illustrated here lasted a total of 24 hr (at 4°) and yielded highly reproducible results, qualitatively as well as quantitatively. *ND*, nondialyzed cholate extracts; *D*, dialyzed (16 hr, 4°) cholate extracts. The dialysis step led to clear distinction between LMWC and monomolecular [^{3}H]etorphine.

much lower molecular weight. We now present clear evidence for these small opiate-binding entities in the rat brain.

First, we used a CMF so as not to select any particular class of receptor molecules. Indeed, several classes of binding sites may be carried by different receptors with different subcellular distributions.

Second, the membranes were labeled with [^{3}H]etorphine, which does not discriminate between μ and δ sites (1, 2, 14). In addition, the affinity of [^{3}H]etorphine for the two sites is so high (10^{+10} M^{-1}) that it could be used at low concentration (low nonspecific binding). Another important correlate of the high affinity of etorphine was its not too rapid dissociation away from receptor sites, especially at low temperature (30% within 24 hr at 4°).

Third, the solubilizing agent, cholic acid (sodium salt) was chosen since it offers several advantages over other detergents. In particular, it ensures, unlike Triton X-100, substantial solubilization of the radioactivity in bound form(s). In addition, because of its very high critical micellar concentration, sodium cholate does not build up into aggregates—a possible source of artifacts, especially when dealing with drugs as hydrophobic as etorphine.

Finally, since our conclusions were to stem from comparisons between elution profiles of filtration experiments on various gel types, it was imperative that chromatographic studies be run simultaneously with the same preparation. This was readily achieved by means of a simple, custom-made device (Materials and Methods, Step 5). The only variable was the chemical nature of the molecular sieve, dextran or agarose).

In the course of the present study, we have taken great care in evaluating dissociation, and the dialysis experiment has clearly distinguished LMWC and monomolecular [^{3}H]etorphine. However, it may be argued that the LMWC(s) represent small labeled fragments generated by endogenous proteolysis. This possibility may easily be ruled out, on the following grounds: (a) binding of opiates to rat brain membranes is considerably diminished after treatment with trypsin (15), chymotrypsin (16), or pronase (17); (b) fragments of this size, labeled or not, would have been eliminated prior to solubilization: preparing the ^{3}H-labeled CMF involves three centrifugations (Materials and Methods, Steps 2 and 3); and (c) after solubilization, free [^{3}H]etorphine is present in trace amounts, which precludes any substantial labeling of still "active" fragments. In fact, (a) Labeling the CMF and solubilizing it under low proteolysis conditions (presence of 0.3 mM phenylmethylsulfonyl fluoride + 2.5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) yielded preparations which elution profile were identical with the ones in Fig. 3 (not shown). (b) When [^{3}H]-

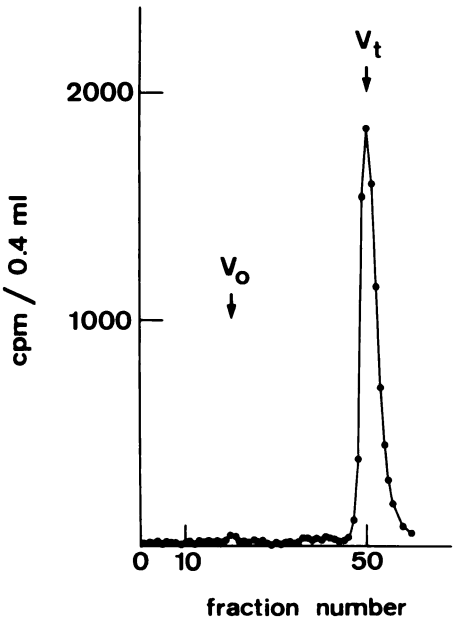

FIG. 4. *Chromatography on Sephadex G-25 of the [^{3}H]etorphine-labeled cholate-soluble fraction from brain membranes*

The unlabeled CMF was solubilized as described under Materials and Methods and mixed with [^{3}H]etorphine at a final concentration identical with the (total) one in the soluble ^{3}H-labeled CMF (−L). Under these conditions, no radioligand was recovered in bound form (V_o). For comparison, see the corresponding *ND* profile in Fig. 3.

etorphine was added after solubilization of the unlabeled CMF, no radioligand was recovered in bound form upon filtration through Sephadex G-25 (Fig. 4).

Multiple molecular forms of stereospecific opiate binding in brain membranes have already been reported by Smith and Loh (18). However, these investigators have labeled opiate receptors with tritiated methionine-enkephalinamide, a ligand which, in addition to being metabolically unstable, binds to brain membranes with much lower affinity than does [^{3}H]etorphine. Therefore, dissociation became a major pitfall in evaluating their data. Diagnostic enough in this respect is the fact that Smith and Loh (18) were unable to demonstrate the 500,000 mol wt species (our HMWC) in Sepharose 6B profiles. Instead, they described "a broad peak of radioactivity of 100,000–500,000 molecular weight" which they attributed to heterogeneity.

Our experimental conditions, which we have amply justified here, appear to be better controlled and considerably less susceptible to artifacts. Therefore we concluded, with reasonable confidence, that rat brain membranes contain, in addition to the 500,000 mol wt species (HMWC), lower molecular weight entities (LMWC) which are also opiate-specific, i.e., that bind [^{3}H]etorphine in the absence but not in the presence of unlabeled levorphanol.

The actual significance of these two components (or sets of components) remains to be elucidated. They may represent either distinct opiate receptors or different aggregation states or binding subunits of the same receptor. Along these lines it should be remembered that (a) in the rat brain, regional variations of the ratio μ to δ binding sites have been reported in biochemical binding (4, 19, 20) and autoradiographic (19, 21, 22) studies. These observations are consistent with the notion that μ and δ sites are carried by different receptors. (b) In neuroblastoma cells, enkephalin receptor sites form clusters (aggregates) in the presence of opiate agonists and antagonists (23). (c) A protein-lipid model of the opiate receptor has recently been proposed (24) according to which "the receptor consists of a complex containing two topologically distinct binding sites, one with a high affinity for enkephalins and the other for alkaloids: the enkephalin binding site is located on a protein while the alkaloid site is located on a different molecule, perhaps lipid."

Our observations are not inconsistent with any of these three possibilities. Nevertheless, it would be surprising that membrane components which differ so much in size represent distinct receptors with overlapping specificity. It is more likely that we are dealing here with different aspects (aggregates or subunits) of the same (or very similar) receptor(s).

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