Production and characterization of polyclonal and monoclonal antibodies to the zeta (ζ) opioid receptor

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The opioid growth factor, [Met5]-enkephalin, is an inhibitory agent of cell proliferation and maturation that interacts with the zeta (ζ) opioid receptor to modulate growth. In order to learn more about this receptor, polyclonal and monoclonal antibodies were raised against binding subunits identified on two-dimensional gels by ligand blotting. Using Western blotting, the polyclonal antibodies and some of the monoclonal antibodies recognized all 4 binding polypeptides (32, 30, 17, and 16 kDa) in developing rat cerebellum; no reaction was recorded in adult cerebellum. In addition, other monoclonals were able to distinguish only certain subunits (e.g. 17 kDa). The monoclonal antibodies and their Fab' fragments, as well as the polyclonal antibodies, blocked the binding of [3H][Met5]-enkephalin to preparations of developing cerebellum. Both the polyclonal and monoclonal antibodies immunoprecipitated ζ opioid binding polypeptides from 6-day-old cerebellar homogenates solubilized by the zwitterionic detergent, CHAPS. Immunocytochemistry performed with polyclonal antibodies showed immunoreactivity associated with proliferating and differentiating cerebellar cells, but no specific staining was detected in the adult cerebellum. These results have identified and characterized antibodies to the ζ opioid receptor, and the antibodies were used to localize this receptor; these antibodies will be valuable to further cellular and molecular studies.

INTRODUCTION

Studies of regulatory events associated with cell proliferation, migration, and differentiation are important in order to understand the nature of normal and dysfunctional developmental processes, including oncogenesis, wound healing, and tissue regeneration. The control of developmental and neoplastic events by an endogenous opioid system (i.e. opioid peptide and receptor) was first postulated in 1983 following experiments showing that opioid antagonists profoundly influenced growth (see reviews in refs. 30, 32). An endogenous opioid growth factor (OGF), [Met5]-enkephalin, has been identified in both eukaryotes and prokaryotes that serves as an active inhibitory influence on cell replication, maturation, and survival. Immunocytochemical and immunoelectron microscopy studies have demonstrated that [Met5]-enkephalin is associated with proliferating and differentiating neurons and glia in the developing rat cerebellum; with the exception of Golgi II neurons, these cell types are not associated with [Met5]-enkephalin as a neurotransmitter. In situ hybridization studies indicate that mRNA for preproenkephalin, the prohormone for [Met5]-enkephalin, is related to replicating and maturing neural cells and suggests an autocrine and paracrine production for this growth peptide.20

The receptor related to OGF has been identified and characterized in the developing brain26 and neural tumor cells27, and termed the zeta (ζ) opioid receptor. This nuclear-associated receptor is an integral membrane protein, present during development but not adulthood, and consists of binding polypeptides of 32, 30, 17, and 16 kDa. The present study was designed to produce and characterize polyclonal and monoclonal antibodies to the binding polypeptides of the ζ opioid receptor. Moreover, utilizing these antibodies, the location of the ζ receptor in the developing rat cerebellum was determined by immunocytochemistry.

MATERIALS AND METHODS

Antigen preparation

The cerebella of 6-day-old Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were homogenized in 50 mM Tris-HCl buffer with bacitracin (0.1 mg/ml), leupeptin (1 μg/ml), thiorphan (6 nM), EGTA (1 mM), and phenylmethylsulfonyl fluoride
(PMSF) (3.5 mM) ( = Tris/all buffer), pH 7.4, submitted to subcellular fractionation, and a nuclear fraction (P1) obtained according to earlier procedures. Proteins were subjected to two-dimensional non-equilibrium pH gradient electrophoresis (NEPHGE) and the gels stained with Coomassie brilliant blue R dye. Zeta receptor binding polypeptides that were identified previously by ligand blotting were excised from NEPHGE SDS-PAGE gels, de-stained, and electroeluted (Bio-Rad II Electro-Eluter). In some cases, proteins electrotransferred to nitrocellulose were stained with Ponceau S for identification, and small pieces of nitrocellulose containing the proteins were excised and dissolved in DMSO.

**Polyclonal antibody production**

The 32- and 17-kDa binding polypeptides were emulsified in 0.5 ml of complete Freund’s adjuvant and each subunit injected intramuscularly into multiple sites of different male New Zealand white rabbits (Hazelton Laboratories, Denver, PA). Booster injections (30–40 μg) in 0.5 ml of incomplete Freund’s adjuvant were given 4 and 8 weeks after the initial injection. Antibody titer was determined by an immunodot assay with P1 homogenates of 6-day-old cerebellum using small volumes of blood obtained from the ear vein.

When antibody titer was sufficient and specific (i.e., recognition of z receptor polypeptides in cerebellum from 6-day-old rats but not adults), as determined by immunodot and immunoblot assays, rabbits were anesthetized and exsanguinated. Serum was collected and purified using ammonium sulfate precipitation and DEAE Affi-blue gel filtration. Antiserum was aliquoted into small volumes and stored at −70°C.

**Immunodot and immunoblot assays**

For immunodot assays, 10 μg of protein from the P1 fraction of 6-day and adult cerebellum were spotted onto nitrocellulose paper, dried, and washed with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl (TBS), and blocked for 1 h in 5% non-fat dried milk in Tris/all buffer. Blots were incubated overnight with polyclonal antiserum diluted 1:1,000, rinsed with TBS, blocked for 30 min with 5% non-fat dried milk, and incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit antisem. Filters were visualized with 4-chloro-1-naphthol.

For immunoblot assays, P1 fractions of 6-day-old rat cerebellum were separated by 12% SDS-PAGE under reducing conditions in a discontinuous buffer system as described by Laemmli, electrotransferred to nitrocellulose, and processed in a fashion similar to that for immunodot procedures. Western blotting of two-dimensional gels (NEPHGE) was also performed. In some cases, the secondary antibody was [125I]protein A. Filters were incubated for 2 h with [125I]protein A (50,000 cpm/ml), washed, air-dried, and exposed to Kodak XAR5 film with intensifying screens for 24–48 h at −70°C. Lipid blotted of the P1 fraction from 6-day-old rat cerebellum using [125I]enkephalin was conducted as described previously.

**Monoclonal antibody production**

Monoclonal antibodies were generated against the 32 and 17 kDa polypeptides of the z receptor isolated from two-dimensional NEPHGE gels. The procedure for producing hybrids was adapted from Harlow and Lane. In brief, antigens (30–40 μg) emulsified in complete Freund’s adjuvant were injected subcutaneously into adult male BALB/c mice; booster injections with 20–30 μg antigen and incomplete Freund’s adjuvant were administered until titer was sufficient. The final injection of antigen was performed intravenously and 3 days later immunized spleen cells (6 × 10⁶) were fused with late-phase myeloma cells (P3.x63.Ag8.653) (2 × 10⁵) in the presence of 41% polyethylene glycol (MW, 1,500) for 60 s at 37°C. Hybrids were grown in Iscove’s medium supplemented with HAT in 96-well plates with a feeder layer of spleen cells. Hybrids positive for heavy- and light-chain immunoglobulins were screened by slotblotting (Schleicher and Schuell Mannifold II Slot blot apparatus) P1 nuclear fractions of 6-day-old rat cerebellum; adult cerebellar tissue and P3.x63.Ag8.653 culture media served as negative controls. Procedures for slotblotting were similar to those described for the dot blot assays. Selective hybrids were cloned by limiting dilution and screened for specificity by Western blotting.

**Monoclonal antibody purification**

Monoclonal antibodies were purified from media using immobi-lized protein A. Culture media, adjusted to pH 8, was passed over (up to 3 times) a column of protein A attached to cyanogen bromide-activated Sepharose. The column was washed with 15 column vol's of phosphate buffer. Antibody was eluted by lowering the pH to 4.5. Antibodies were immediately neutralized with NaOH, lyophilized, and quantitated. Purity of fragments was measured by SDS-PAGE under reducing conditions.

Isotyping was carried out using a monoclonal isotyping kit (Amersham). Initial screening procedures preferentially selected for IgG immunoglobulins.

**Receptor binding assays**

Antisera were tested for their ability to inhibit [Met]enkephalin binding to preparations of 6-day-old rat cerebellum. Methodology for receptor binding assays was similar to that published earlier. Briefly, P1 homogenates were incubated with 1.5 nM [3H][Met]enkephalin and various concentrations of monoclonal or polyclonal antibody for 30 min at 22°C. Binding was terminated by rapid filtration; filters were washed, dried, and counted by liquid scintillation spectrometry. Non-specific binding was measured in the presence of excess unlabelled [Met]enkephalin. Data were analyzed using Landon II software.

In addition, the binding efficacy of each of 17 monoclonal antibodies to the z opioid receptor was assessed by binding assays. In brief, 15 μg of each monoclonal antibody (selected on the basis that this concentration of monoclonal antibody 117-3X blocked binding by approximately 50%) was added to 1.5 nM [3H][Met]enkephalin and binding assays performed.

To determine the specificity and selectivity of the monoclonal antibody in receptor binding assays, Fab', fragments were prepared by pepsin digestion following the protocol provided by the manufacturer (Sigma). The mixture was centrifuged and separated on a protein A—Sepharose column. The flow-through contained the Fab' fragments; the purity of these fragments was determined by SDS-PAGE. P1 fractions of 6-day-old rat cerebellar protein were incubated with equal concentrations of antibody, Fab', or fragments of the antibody, or γ-globulin.

**Immunoprecipitation**

P1 homogenates of 6-day-old rat cerebellum were isolated, solubi-lized in 10 mM CHAPS in Tris/all buffer with 10 mM MgCl₂, 0.4 mM DDT, and 1.6 M NaCl at 4°C for 1 h, and centrifuged for 60 min at 160,000 × g. The supernatant (1 ml) was added to 30–50 μg of ammonium sulfate-purified polyclonal antibody or 15 μg protein A-purified monoclonal antibody and incubated on ice for 60 min. 500 μl of a 10% solution of staph A (Pansorbin, Calbiochem) was added and the incubation continued on ice for 20 min; solution was vor-texed periodically. The antigen—antibody—staph A complex was sedi-mented (20 min, 2,000 × g), boiled for 5 min in sample buffer (SDS mixture of 160 mM dithiothreitol, 5% SDS, 5 mM EDTA, 50% sucrose, and 0.1% Bromphenol blue), centrifuged at 13,000 × g for 2 min and the supernatant collected. The immunoprecipitated receptor subunits were separated by SDS-PAGE using the Laemmli buffer system and 12% acrylamide gels.

**Immunocytochemistry**

Six-day-old and adult rats were anesthetized and sacrificed by decapitation. Tissues were rinsed in 0.1 M Sorenson’s phosphate buffer (pH 7.4), frozen in isopentane cooled by liquid nitrogen, and embedded in OCT medium. Cryostat sections (15 μm) were collected on gelatin-coated slides and stored at −20°C with Drierite until use. Sections were placed in ice-cold 95% ethanol (2 min) and acetone (2 min) and rinsed with SPB. Tissues were bleached for 15 min with 3% normal goat serum (NGS) in 50 mM SPB, pH 7.4.
Specimens were incubated with polyclonal antibody AO-440 (1:75) to the \( \zeta \) receptor for 40 h. The antibody was diluted in SPB with 1% NGS and 0.1% Triton X-100. Following incubation, sections were rinsed in SPB with 1% NGS and 0.1% Triton X-100, incubated for 1 h with rhodamine-conjugated goat anti-rabbit IgG (1:100). Sections were washed in SPB and distilled water and mounted with 9:1 glycerol/TBS. Immunoreactivity was visualized using an Olympus microscope with a rhodamine interference filter. Controls for specificity included sections processed with antibody and an excess of antigen (− pre-absorbed) or pre-immune serum.

RESULTS

Characterization of the polyclonal antibodies

Immunoblots of nuclear fractions (P1) from developing rat cerebellum which reacted with polyclonal antisera are shown in Fig. 1. Antisera generated against the 32 kDa (AO-721), 17 kDa (AO-440), or 16 kDa (TO-418) polypeptides each revealed 4 bands of immunoreactivity in Western blotting experiments (Fig. 1B–D). The molecular weights of these four bands were 32, 30, 17, and 16 kDa, and were identical in molecular weight to the 4 bands detected by ligand blotting (Fig. 1F). No immunoreactivity was recorded with Western blots of adult rat cerebellum (Fig. 1E). Subcellular fractionation studies and immunoblotting with polyclonal antisera to the \( \zeta \) receptor binding polypeptides demonstrated positive immunoreactivity in the nuclear fraction (P1) (Fig. 2). Occasionally light immunoreactivity of one or more binding subunits could be detected in the microsomal fraction (P3), and little or no binding was recorded in the membrane fraction (P2) or soluble fraction (S3). Immunoblotting of two-dimensional NEPHGE gels with polyclonal antibody AO-440 revealed the staining of 32, 30, 17, and 16 kDa polypeptides; each subunit had migrated to the same pH as found earlier in ligand-blotting studies\(^{28}\) (data not shown).

Characterization of monoclonal antibodies

From four fusions, an average success rate of 23% of 760 wells/mouse was found for the production of IgG hybrids. Of these, 4–5% of the successful clones were positive for \( \zeta \) receptor binding subunits as determined by immunodot assays; positive staining of the 6-day-old but not adult cerebellar protein was detected. The cultures of positive clones were expanded and screened by immunoblots. Positive subclones were further evaluated after cloning by limiting dilution. The subclones of 17 hybrids from the 4 mice were expanded for mass preparation.

The characteristics of all 17 monoclonal antibodies are presented in Table I. In addition to the polypeptide used to generate the antibody, the isotype, reactivity to \( \zeta \) polypeptides, and ability of each antibody to block OGF binding to the \( \zeta \) opioid receptor are presented. As noted in Table I, some antibodies revealed 4 bands of molecular weight 32, 30, 17, and 16 kDa (Fig. 3B–D), whereas other antibodies stained only the 32/30, 32/30 and 17, or 17 kDa polypeptides (Fig. 3D–F).

Opioid receptor binding inhibition

The ability of monoclonal antibodies to inhibit the binding of \( ^{3}H\)Met\(^{3}\)-enkephalin in P1 preparations of 6-day-old rat cerebellum was monitored. Initial experiments using monoclonal antibody 117-3X showed that 50% of the specific binding was blocked by approximately 15 \( \mu \)g of this monoclonal antibody (Fig. 4). Using this antibody concentration, the panel of 17 monoclonal antibodies was assessed for their ability to inhibit \( ^{3}H\)Met\(^{3}\)-enkephalin binding (Table I). Addition of \( \gamma \)-globulin did not influence the binding of radiolabeled Met\(^{3}\)-enkephalin to the \( \zeta \) receptor.

To elucidate further whether the blocking of receptor binding was related to non-specific binding by the Fc portion of the IgG, IgG fractions of some antibodies were digested to generate F(ab')\(_2\) fragments. Specifi-
Fig. 2. Western blotting of 6-day-old rat cerebellum fractionated through a sucrose density gradient, prepared for SDS-PAGE, and stained with Coomassie brilliant blue (A–D), or electroblotted to nitrocellulose and incubated with polyclonal antibody AO-440 generated against a 17 kDa 𝜹 receptor polypeptide. Lanes: A,E, nuclear fraction (P1); B,F, membrane fraction (P2); microsomal fraction (P3); D,H, soluble fraction (S3) supernatant. Note that immunoreactivity is demonstrated only in the P1 nuclear fraction. Molecular weight markers (×10³ Da) are indicated in the left column.

cally, IgG secreted from monoclonal antibody 117-3X was subjected to pepsin digestion in order to obtain F(ab′)₂ fragments. The F(ab′)₂ fragments were able to block binding of radiolabeled [Met³]-enkephalin to the 𝜹 receptor. Table II shows that 15 μg of F(ab′)₂ fragment or purified monoclonal antibody 117-3X inhibited the binding of 1.5 nM [³H][Met³]-enkephalin to 0.2 mg of developing rat cerebellar nuclear protein by 53% and 58%, respectively. Under identical conditions, 15 μg of γ-globulin had no effect on binding.

<table>
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<tr>
<th>Antibody</th>
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<td>G2a</td>
<td>x x x x</td>
<td>43</td>
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* % of control binding.
The ability of polyclonal antibodies to inhibit the binding of \(^{1}H[H\text{Met}^2]\text{-enkephalin}\) in P1 preparations was ascertained. At the concentrations tested, polyclonal antibody AO-440 was found to block 64% of total specific binding at 27.6 \(\mu\text{l}\), and 100% binding with 69.0 \(\mu\text{l}\) (data not shown).

**Immunoprecipitation**

Both polyclonal and monoclonal antibodies were able to immunoprecipitate \(\zeta\) opioid binding polypeptides (Fig. 5). These results suggest that both types of antibodies, each generated against the 17 kDa subunit, immunoprecipitated all four binding subunits related to the \(\zeta\) receptor.

**Cellular distribution of the \(\zeta\) receptor**

Examination of sections of cerebellum from 6-day-old animals stained with a polyclonal antibody (AO-440) to the 17 kDa subunit of the \(\zeta\) receptor revealed a progressive diminution in fluorescence from the pial surface inward. The external germinal layer was brightly fluorescent (Fig. 6A), the molecular layer somewhat reactive, and the internal granule and medullary layers only slightly immunoreactive. Higher magnification (Fig. 6C) showed the perinuclear region of the external germinal cells to be stained intensely, but the nucleus to be negative. In the external germinal layer there often appeared to be a gradient of fluorescence. Cells near the pial surface usually seemed to stain more intensely than those adjacent to the molecular layer. The nature of the immunoreactivity in the molecular layer with respect to an association with cellular elements was difficult to decipher, although Purkinje cells appeared to be immunofluorescent. The internal granule layer exhibited a mosaic pattern of staining, with

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**Fig. 3.** Panel of Western blots using monoclonal antibodies (mAb) prepared from either the 32 kDa (lanes B,G = mAb 1F; E = mAb 232-13) or 17 kDa (lane C = mAb 1G; D = mAb 117-1X; F = mAb 2G) subunits. P1 proteins from 6-day-old rat cerebellum (lanes A–F) or adult cerebellum (lane G) were subjected to electrophoresis, electrophorated onto nitrocellulose, and incubated with culture media of corresponding antibodies. Lane A stained with Coomassie blue.

**Fig. 4.** Inhibition of \(^{1}H[H\text{Met}^2]\text{-enkephalin}\) binding by different concentrations of monoclonal antibody 117-3X. P1 fractions of 6-day-old rat cerebellum at a protein concentration of 420 \(\mu\text{g/ml}\) were incubated with varying concentrations of purified monoclonal antibody 117-3X. Binding was carried out as described in Materials and Methods.

**Fig. 5.** Characterization of immunoprecipitated proteins by both a polyclonal antibody (AO-440) (B) and monoclonal antibody (117-3X) (C). P1 nuclear protein from 6-day-old rat cerebellum (A) was solubilized and immunoprecipitated as described in Materials and Methods. Approximately 35 \(\mu\text{g}\) of protein obtained from each antibody coupling was subjected to electrophoresis on 12\% SDS gels; gels were stained with Coomassie brilliant blue. Molecular weight markers are shown in the left lane (\(\times 10^3\) Da).
TABLE II

Competition of monoclonal antibody (mAb) 117-3X for κ opioid binding sites in P1 nuclear rat cerebellar preparations

Values represent means ± S.E.M. from at least 2 assays.

<table>
<thead>
<tr>
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<th>% of control binding</th>
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<tr>
<td>Control</td>
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<tr>
<td>mAb 117-3X</td>
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<tr>
<td>F(ab')2 of mAb 117-3X</td>
<td>47.2 ± 5.1</td>
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<tr>
<td>γ-Globulin</td>
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immunoreactivity related to a small proportion of cells. Neurons in the deep cerebellar nuclei, as well as glia in the medullary layer, reacted with the antibody (data not shown). No specific staining was detected in the pre-absorbed controls (Fig. 6D), pre-immune controls, or in the cerebellar cortex from adult rats (Fig. 6B).

DISCUSSION

Unlike other opioid receptors, the function (i.e. growth), temporal sequence (i.e. transient appearance during ontogeny), ligand specificity (i.e. [Met²]-enkephalin), and subcellular location (i.e. nucleus) of the κ receptor have provided a unique set of characteristics that distinguish it from other opioid receptors. This study capitalized on previous findings which utilized a ligand-blotting method and proteins electrotrans-

Fig. 6. Photomicrographs showing the location of κ immunoreactivity in the cerebellum of 6-day-old (A,C,D) and adult (B) rat cerebellum using 1:75 dilution of AO-440 polyclonal antisera generated against the 17 kDa subunit. ×330. A: note the bright staining of the external germinal layer (EGL) and the low-to-moderate immunofluorescence of the molecular layer (MOL) and internal granule layer (IGL) in the 6-day-old rat cerebellum. The pia (arrows) is extremely fluorescent. ×330. B: cerebellum of the adult rat stained with antiserum to the 17 kDa subunit showing no specific staining. ×330. C: external germinal cells of the external germinal layer with intensely stained cortical cytoplasm (arrows). ×1,110. D: control section of developing rat cerebellum stained with the 17 kDa antiserum absorbed with an excess of pure antigen. ×330. The times of exposure and printing in B and D are similar to those in A.
ferred to nitrocellulose to identify and characterize 4 polypeptides (i.e. 32, 30, 17, and 16 kDa). These polypeptides bound radiolabeled [Met\textsuperscript{5}]-enkephalin in a fashion consistent with the criteria of an opioid receptor binding site\textsuperscript{28}, including displacement by opioid antagonists and stereospecificity. Binding occurred at concentrations of peptide relevant to the \( K_d \), was blocked by unlabelled ligand and opioid antagonists, and subcellular fractionation studies showed that binding was localized to the nuclear fraction. No binding was recorded in the adult rat cerebellum. Two-dimensional protein analysis showed that the subunits were of basic pH. In the present study, \( \zeta \) polypeptides were eluted from two-dimensional gels, and both polyclonal and monoclonal antibodies produced. Evidence from a number of experiments suggests that these antibodies were specific to \( \zeta \) binding subunits. First, preliminary screening techniques using an immunodot assay showed that these antibodies reacted with homogenates of 6-day-old rat cerebellum. Moreover, no reaction was noted when adult cerebellum was examined. Second, Western blotting studies indicated that these antibodies identified one or more of the \( \zeta \) opioid receptor binding subunits. Third, receptor binding assays showed that the antibodies can block the binding of \([\text{H}]\text{Met}^5\text{-enkephalin} to homogenates of 6-day old rat cerebellum which are rich in \( \zeta \) receptors. This blockade was displaced by unlabelled [Met\textsuperscript{5}]-enkephalin, but specific and saturable binding was noted when \( \gamma \)-globulin was included in the assays. Fourth, the F(\text{ab}')\text{2} fragment retained the ability to block ligand binding, suggesting that the ability of antibodies to interfere with [Met\textsuperscript{5}]-enkephalin binding was not due to such properties as steric hinderance but rather to a specific antibody–antigen recognition site. Fifth, antibodies immunoprecipitated only those polypeptides that corresponded in molecular weight to \( \zeta \) receptor binding subunits. Sixth, immunocytochemical experiments showed that the antibodies stained developing and maturing neural cells in the developing brain, but did not react with sections from the adult cerebellum. Thus, these findings are the first to report the production and characterization of antibodies to the \( \zeta \) opioid receptor. The use of these antibodies in quantitative and qualitative studies, immunocytochemical and immunoelectron microscopy investigations, and in cloning, will be important for understanding the role and mechanisms of the \( \zeta \) receptor.

Earlier studies\textsuperscript{29} exploring the nature of the \( \zeta \) receptor using two-dimensional tryptic peptide mapping analysis of each of the four binding subunits revealed that the 32- and 30 kDa polypeptides were nearly identical (a spot homology of \( >95\% \)). Only a partial homology (\( \sim 60\% \) spot overlap) was detected between the 17- and 16 kDa binding subunits, and each of these polypeptides had a spot homology of \( \sim 40\% \) with the 32/30 subunits. It is interesting that all of the monoclonal antibodies recognized the 4 subunits, confirming that there is some homology between the binding polypeptides. Evaluation of the monoclonal antibodies indicated that over half the number reacted with all four subunits, perhaps indicating the possibility that there are dominant epitopes shared by the subunits. However, monoclonal antibodies were generated that were more restricted in their recognition. Thus, antibodies to 32/30, 17/16, or 17 were produced, supporting earlier peptide mapping studies showing some structural differences between the 32/30, 17, and 16 kDa polypeptides. These subunit-specific monoclonal antibodies should be particularly useful in future characterization of the binding subunits.

A number of previous studies have developed and characterized antibodies to opioid receptors. These investigations can be divided into three major avenues, and each depends on the source of antigen. The first approach utilizes preparations of opioid receptors to prepare polyclonal or monoclonal antibodies.\textsuperscript{1-5,18-20,24,25} The second approach utilizes the strategy of creating anti-idiotypic antibodies as tools for receptor isolation and characterization, and includes reports by Coscia et al.\textsuperscript{9}, Cupo et al.\textsuperscript{10}, Glaseal and Myers\textsuperscript{11}, Glaseal and Pelosi\textsuperscript{12}, Gramsch et al.\textsuperscript{13}, Hassan et al.\textsuperscript{15}, Myers and Glaseal\textsuperscript{16}, Ng and Isom\textsuperscript{17,22}, and Ornatowska and Glaseal\textsuperscript{19}. Finally, Carr et al.\textsuperscript{2-8} have produced anti-opioid receptor antibodies using the molecular recognition theory in which peptides specified by the strand complementary to the strand coding for a ligand (e.g. [Met\textsuperscript{5}]-enkephalin, \( \gamma \)-endorphin) are used as immunogens. The present study utilizes a totally different approach from earlier investigations, and employed well-characterized opioid binding polypeptides identified by ligand blotting\textsuperscript{28} to produce polyclonal and monoclonal antibodies. Whether the ligand-blotting technique can be applied to opioid receptors other than \( \zeta \), and the subsequent use of these binding subunits to create antibodies, merits further consideration.

The immunocytochemical results presented in this report are the first to demonstrate the topographical distribution of the \( \zeta \) receptor. The data show that the \( \zeta \) receptor is associated with proliferating (i.e. external germinal layer, EGL) and differentiating (e.g. Purkinje) cells, but not adult cerebellar cells. Cells destined to form neurons and glia revealed immunoreactivity, indicating that all types of neural cells are related to the \( \zeta \) receptor. It is interesting that receptor expression may be retained after some cells are post-mitotic. For ex-
ample, EGL cells exhibited immunoreactivity when they reached the internal granule layer, and the Purkinje neurons, which are generated prenatally, displayed immunoreactivity at least 1 week after birth. The location of the ε receptor corresponded with the distribution of the opioid growth factor, [Met5]-enkephalin, with peptide found in replicating and maturing cells but not in the adult33 (with the exception of Golgi II cells). Since the opioid growth factor and ε receptor are known to influence cell proliferation and differentiation31, these data are consonant with the concept that this endogenous opioid system plays an important role in regulating brain development.

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REFERENCES

6 Carr, D.J.J., Bost, K.L. and Blalock, J.E., An antibody to a peptide specified by an RNA that is complementary to γ-endorphin mRNA recognizes an opiate receptor, J. Neuroimmunol., 12 (1986) 329–337.