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Adult and developing human cerebella exhibit different profiles of opioid binding sites

Ian S. Zagon, Denise M. Gibo and Patricia J. McLaughlin

Department of Anatomy, The M.S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033 (U.S.A.)

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The binding of [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO), [^{3}H][D-Pen^{2,5}]enkephalin (DPDPE), [^{3}H]ethylketocyclazocine (EKC), and [^{3}H][Met^5]enkephalin (MET) was used to examine μ-, δ-, κ-, and ζ-receptors, respectively, in the developing (birth to postnatal day 19) and adult human cerebellum. Specific and saturable binding of all ligands was recorded in developing brains, and of [^{3}H]DAGO, [^{3}H]DPDPE, and [^{3}H]EKC in adult cerebellum; all data fit a single homogeneous binding site for each ligand. However, the ontogenic profile of opioid receptor subtypes differed. δ- and κ-receptor capacities were 7.8- and 3.6-fold, respectively, greater in infant cerebellum than in adults. The μ-receptor decreased over 7-fold in both binding affinity and capacity after day 2; by adulthood, the binding affinity was the same as in newborns but only one-half the binding capacity was recorded. The concentration of ζ-receptors was 20-fold greater in subjects 2–19 days of age than in newborns. These data demonstrate the presence, and distinct developmental profiles, of opioid receptors in human cerebellum. Although the function of μ-, δ-, and κ-receptors in human cerebellum are unclear, the growth-related ζ-receptor is present at a time of cell replication and differentiation but is not detected in mature cerebellum.

INTRODUCTION

Laboratory studies have revealed a great deal of information about opioid receptors in the adult and developing brain[6,7,14,18,22], but investigations of opioid receptors in humans have been limited[11,12,17]. In particular, there is a paucity of information on opioid receptors in developing human central nervous system (CNS). To our knowledge, only two studies in regard to opioid receptors and the ontogeny of the human CNS have been reported, and both have utilized fetal human tissue. Magnan et al.[15], using 21-week-old human fetuses, found binding to [^{3}H]etorphine in the brain, cerebellum, and spinal cord and, subsequently reported[16] μ and κ, but not δ, opioid sites in the brain of 20-week-old fetuses.

In addition to the development of opioidergic systems, the endogenous opioids also appear to play an important role in regulating embryologic events. Opioids may serve as trophic factors not only influencing the ontogeny and expression of neurotransmitter functions[8,18], but also as growth factors in cell proliferation and differentiation of the nervous system[36–39]. Recently, [Met^5]enkephalin has been identified as one of the most important opioid peptides in regulating cell replication in developing rat brain[7,39]; ligands selective for other opioid receptors (e.g., δ, κ) did not influence cell proliferation. Based on these functional studies, along with data from binding assays[33–35], the putative receptor involved with these growth controlling properties appears to be a new opioid receptor, zeta (ζ), already reported in neural tumor tissues and cells[34,35], but also identified in developing rat cerebellum[33].

In the present study, opioid receptors in the developing human nervous system were examined. Since the cerebellum has been an excellent model in laboratory investigations for understanding the role of endogenous opioids as trophic factors, this study focused exclusively on the human cerebellum. To provide a broad picture, ligands and methodology selective for μ-, δ-, κ-, and ζ-receptors were employed. In order to place the ontogeny of opioid receptors in perspective, comparison of the developmental profile of the human cerebellum with that of adults was also conducted.

MATERIALS AND METHODS

Human tissue

Human cerebellum (hemispheres only) was collected at autopsy. The tissuses from 10 infants and 15 adults were examined; see Table I for ages and causes of death. All subjects had no history of neurological or psychiatric problems, and gross and histological reports indicated no pathology of the nervous system. Tissues were generally obtained within 6–12 h of death, homogenized immediately, and stored at -70 °C.

Correspondence: I.S. Zagon, Department of Anatomy, The M.S. Hershey Medical Center, Hershey, PA 17033, U.S.A.

Ligand binding analysis

Ligand binding analysis was performed as reported earlier[34,35]. In brief, cerebellar tissues were homogenized (Polytron, setting 6, 2 × 10 s) in a solution of cold 50 mM Tris-HCl buffer with bacitracin (0.1 mg/ml), leupeptin (1 μg/ml), thiorphan (0.6 μg/l), EGTA (1 mM), and phenylmethylsulfonyl fluoride (0.6 mg/l), pH 7.4 at 4 °C; this buffer will be termed Tris/all. The homogenates were centrifuged (39,000 *g*) and pellets rehomogenized (2 × 10 s) in a solution of cold 50 mM Tris/all buffer, centrifuged at 39,000 *g*, resuspended, and incubated at room temperature (22 °C) for 20 min to dissociate endogenous opioid peptides. Aliqots of 0.95 ml were incubated for 90 min at 22 °C with 50 μl of the labeled ligand; the final protein concentration was 0.7–1.0 mg/ml. The incubation was terminated by rapid filtration through Whatman GF/B glass fiber filters under vacuum pressure with a Brandel Cell Harvester (Gaithersburg, MD). Filters were rinsed 3 times with 5-ml vols. of ice-cold 50 mM Tris-HCl, dried at 60 °C for 1 h, and counted in a 2:1 solution of Aquasol-toluene by liquid scintillation spectometry (Beckman LS-3801). Non-specific binding was determined in the presence of excess (100 nM) cold ligands. Duplicate tubes of homogenates were assayed for each concentration utilized, and each experiment was replicated 2–4 times. Protein concentrations were determined using the Bradford BioRad or Lowry techniques with gamma globulin as a standard.

Labeling of the putative μ-site was with the highly selective ligand [^{3}H][D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO). For the putative δ-binding site, [^{3}H][D-Pen^{2,5}]enkephalin (DPDPE), in the presence of unlabeled DAGO (100 nM) to suppress μ-binding was utilized. For selective labeling of the putative κ-site, the binding of [^{3}H]ethylketocyclazocine (EKC) in the presence of unlabeled DAGO (100 nM) and [D-Ala^2, D-Leu^5]enkephalin (DADLE) (100 nM) to suppress μ- and δ-binding, respectively, was employed. For the putative ζ-binding site, [^{3}H][Met^5]enkephalin (MET) was used. Non-specific binding for μ-, δ-, κ-, and ζ-receptor assays was determined in the presence of excess (100 nM) unlabeled DAGO, DPDPE, EKC, and MET, respectively.

To examine the competitive inhibition of various compounds to opioid binding sites, homogenates of human cerebellum were incubated for 90 min (22 °C) with concentrations ranging from 10^{-12} M to 10^{-3} M unlabeled drugs, and a single concentration of labeled ligand (approximately the K_d value).

Chemicals

[^{3}H]MET (spec. act. 26 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL). [^{3}H]DAGO (spec. act. 35 Ci/mmol), [^{3}H]DPDPE (spec. act. 51.5 Ci/mmol), and [^{3}H]EKC (spec. act. 15 Ci/mmol) were purchased from Dupont New England Nuclear, Boston, MA. The following compounds were obtained from the indicated sources: MET, DADLE, DPDPE, leupeptin, bacitracin, phenylmethyl-sulfonyl fluoride, Sigma (St. Louis, MO); β-funaltrexamine (β-FNA), Research Biochemicals (Wayland, MA); U50,488, Upjohn Diagnostics (Kalamazoo, MI); DAGO, thiorphan, Peninsula (Belmont, CA); ICI 174,864, Cambridge

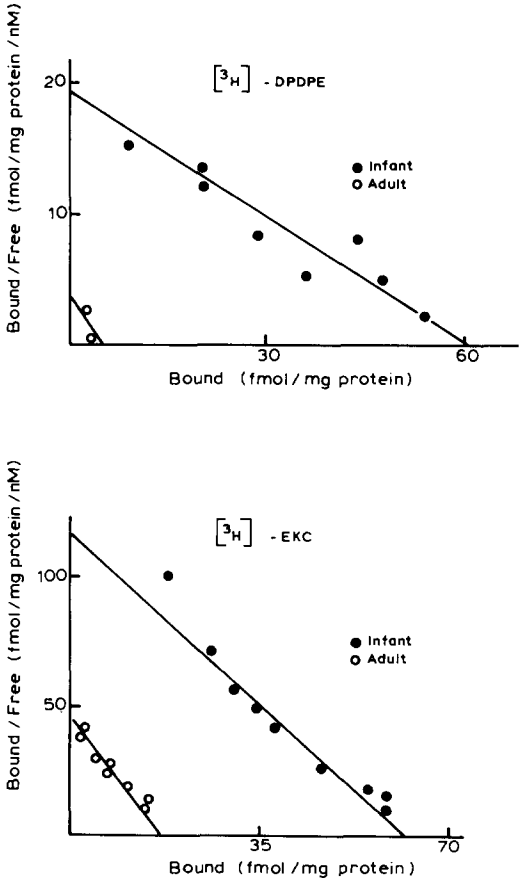

Fig. 1. Representative Scatchard plots of the specific binding of [^{3}H]DPDPE and [^{3}H]EKC to homogenates of developing and adult human cerebellum. See Table II for data as to binding affinities and binding capacities, and for statistical comparisons. Each Scatchard plot represents data from 7–10 concentrations. In order to place the Scatchard plots of infant and adult on the same scale, only a limited portion of some plots are depicted.

TABLE I

The age, sex, and cause of death in the infants of adults studied

Infant age is given as the estimation in weeks of gestation and the age at mortality after birth. Age of adults is given in years.

Gestation (weeks)	*Age*	*Sex*	*Cause of death*
28	1 h	M	Multiple fetal anomalies
30	9 h	M	Pneumonia
30	9 days	M	Respiratory failure
32	24 h	M	Cardiopulmonary arrest
33	9 days	M	Pulmonary emphysema
36	2 h	F	Pulmonary insufficiency
38	13 h	F	Cardiopulmonary arrest
38	24 h	M	Respiratory arrest
38	2 days	F	Congenital diaphragmatic hernia; persistent fetal circulation
38	19 days	F	Cardiac failure; congenital heart disease
Human adults			
	50	M	Multiple myeloma
	64	F	Myocardial infarction; ventricular arrhythmia
	65	M	Sepsis; leukemia
	66	M	Cardiorespiratory arrest
	69	M	Pneumonia
	71	M	Sepsis; pneumonia; sarcoma
	71	F	Squamous cell carcinoma
	75	M	Coronary artery disease
	75	M	Coronary artery disease
	75	M	Myocardial infarction
	78	M	Myocardial infarction
	79	M	Pneumonia
	80	M	Hypertension
	91	M	Non-pathological causes

Research Biochemicals (Valley Stream, NY); EKC, Sterling Winthrop (Rensslaer, NY); naltrexone hydrochloride, National Institute on Drug Abuse (Rockville, MD).

Analysis

Receptor binding data were analyzed with a Lundon I (Saturation Isotherm Binding Analysis) computer program (Lundon Software, Cleveland, OH); this analysis utilizes non-linear least-squares regression. Saturation curves and Scatchard plots were computed directly by this program.

Competition data were analyzed with the Lundon II Competition Data Analysis Program. The inhibition constant (K_i) was calculated from the IC_{50} values using the method of Cheng and Prusoff[4].

Values for binding affinity (K_d) and binding capacity (B_{max}) were analyzed using a two-way analysis of variance (ANOVA) with ligand and age as factors. Subsequent planned comparisons for each ligand were made using Newman–Keuls tests; 95% confidence limits were considered significant.

RESULTS

Specific and saturable binding was recorded for all of the radiolabeled ligands utilized. In all cases, computer analysis of binding showed that the data best fit a one-site model. No detectable differences in binding parameters were recorded between tissues of different post mortem intervals nor between fresh and frozen (up to several months) tissue homogenates. In general, binding in the developing cerebellum exceeded that of adult tissues (Figs. 1,2 and Table II). With the exception of [^{3}H]DAGO binding, the binding affinity (K_d) of radiolabeled DPDPE, MET, and EKC did not differ between infants and adults. With respect to [^{3}H]DAGO, a distinct difference was noted in K_d between individuals of 2–19 days and infants of less than 2 days as well as adults; the K_d of 2- to 19-day-old infants was approximately 8-fold greater than other adults or infants of less than 2 days.

The two-way ANOVA for binding capacity values was highly significant; $F = 14.58$ (df = 3,59) $P < 0.001$.

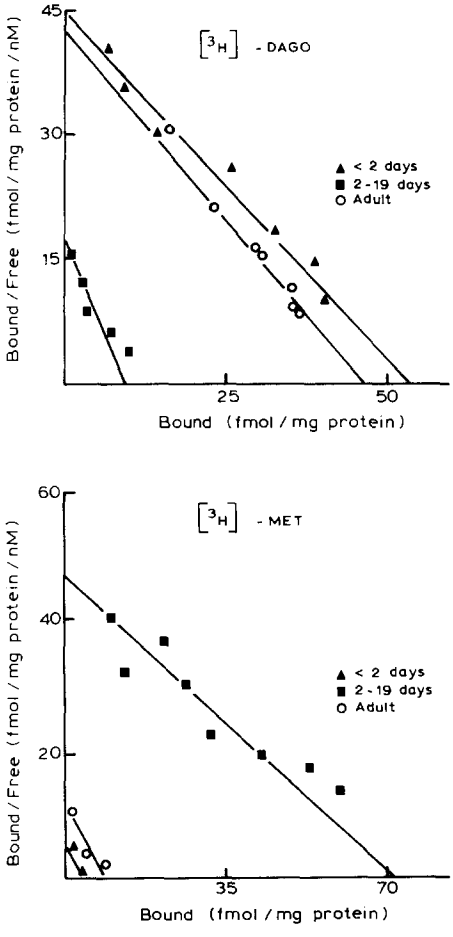

Fig. 2. Representative Scatchard plots of the specific binding of [^{3}H]DAGO and [^{3}H]MET to homogenates of developing and adult human cerebellum. See Table II for data as to binding affinities and binding capacities, and for statistical comparisons. Each Scatchard plot represents data from 7–10 concentrations. In order to place the Scatchard plots of developing and adult cerebellum on the same scale, only a limited portion of some plots are depicted.

TABLE II

Opioid binding sites in developing and adult human cerebellum

Ligand	*Age*	*K_d (nM)*	*B_{max} (fmol/mg protein)*
[^{3}H]DPDPE	Infant	5.3 ± 0.3	62.2 ± 3.4**
	Adult	5.7 ± 1.7	7.9 ± 2.3
[^{3}H]EKC	Infant	0.7 ± 0.3	62.8 ± 1.9**
	Adult	1.0 ± 0.2	17.0 ± 2.7
[^{3}H]DAGO	Birth–2 days	2.1 ± 0.2†	74.6 ± 4.1**†
	2–19 days	17.5 ± 5.2**	10.6 ± 4.3*
	Adult	2.1 ± 0.6	35.9 ± 4.7
[^{3}H]MET	Birth–2 days	1.0 ± 0.3	3.2 ± 0.4†
	2–19 days	2.4 ± 0.3	69.8 ± 12.9**
	Adult	1.7 ± 0.2	7.8 ± 2.4$^{\triangle}$

Values represent means ± S.E.M. for 3–14 saturation binding isotherms. Significantly different from adult values at $P < 0.05$ (*) or $P < 0.01$ (**). Significantly different from 2–19 day values of $P < 0.01$ (†). $^{\triangle}$ = No binding was detected with the addition of 5 nM DAGO.

TABLE III

Competition values of [Met^5]enkephalin in binding assays of [^{3}H]DAGO, [^{3}H]DPDPE, and [^{3}H]EKC using homogenates of infant and adult human cerebellum

The K_i is given as the mean ± S.E.M. as computed from 2–3 independent experiments. Concentration of each radiolabeled compound was 2 nM.

Ligand	*K_i (nM)*	
	Infant	*Adult*
[^{3}H]DAGO	16.2 ± 4.2	4.7 ± 0.4
[^{3}H]DPDPE	>10^{-6} M	282 ± 124
[^{3}H]EKC	132 ± 25	49 ± 19

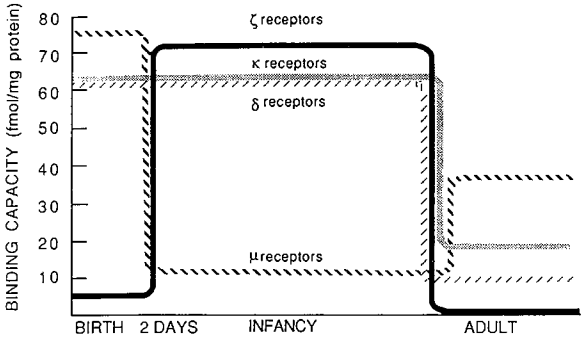

Fig. 3. A schematic interpreting the ontogeny of opioid receptors in the developing and adult human cerebellum. At birth, μ-, δ-, and κ-receptors are relatively abundant, but only a limited number of ζ-receptors are recorded. Within 2 days after birth, assays revealed that both the binding affinity and capacity of μ-receptors had decreased substantially. However, the concentration of ζ-receptors was increased markedly shortly after birth. In adults, all opioid receptors were decreased in number and the ζ-receptor was not detected; addition of DAGO to the [^{3}H]MET binding assay in order to suppress μ-receptor binding, revealed no specific and saturable binding to [^{3}H]MET, the ligand used to monitor ζ-receptors.

Detailed inspection of the data for binding capacity of [^{3}H]DAGO and [^{3}H]MET revealed distinct differences between infants of less than 2 days and those from 2–19 days of age. In the case of [^{3}H]DAGO, maximal binding capacity in the cerebellum of infants less than 2 days of age was 7-fold higher than individuals from ages 2 to 19 days (Fig. 2 and Table II); this difference was statistically significant. Adults exhibited roughly one-half the maximal binding capacity of infants less than 2 days of age, but about 3.5-fold more than infants of 2–19 days; in both cases, these differences were significant. With respect to binding of [^{3}H]MET, the greatest binding capacity occurred in infants of 2–19 days, being approximately 10-fold greater than that of cerebellum from adults, and about 20-fold greater than that of individuals from birth to 2 days.

Further examination of the ζ-receptor was performed. In infants of 2–19 days, competition studies using cold MET with [^{3}H]DAGO, [^{3}H]DPDPE, or [^{3}H]EKC revealed K_i values that showed little cross-reactivity of MET for binding sites recognized by these radiolabeled ligands (Table III). In the adult, MET was highly competitive at [^{3}H]DAGO binding sites. Additional competition studies using [^{3}H]MET and cold ligands selective for other receptor types (Table IV) in the developing and adult cerebellum showed that μ-receptor ligands such as DAGO and β-FNA were competitive in [^{3}H]MET binding assays. Ligands selective for δ-receptors, such as DPDPE and ICI-174,864, were not competitive in [^{3}H]MET assays. EKC, which recognizes a variety of receptor types, was competitive with [^{3}H]MET binding, but the selective κ-receptor ligand U50,488 was not cross-reactive with radiolabeled MET. The opioid antagonist naltrexone was very competitive in displacing [^{3}H]MET in both infants and adults.

TABLE IV

Competition values of opioid compounds for the binding of [^{3}H]MET (2 nM) in developing and adult human cerebellum

The K_i is given as the mean ± S.E.M. as computed from 2–3 independent experiments.

Ligand	K_i *(nM)*	
	Infant	*Adult*
DAGO	5.0 ± 1.0	3.7 ± 1.3
β-FNA	5.0 ± 0.3	3.2 ± 1.0
DPDPE	$>10^{-5}$ M	$>10^{-6}$ M
ICI174,864	$>10^{-2}$ M	$>10^{-4}$ M
EKC	4.5 ± 0.8	4.5 ± 0.1
U50,488	$>10^{-5}$ M	$>10^{-2}$ M
Naltrexone	2.5 ± 0.0	2.2 ± 0.2

Since MET was highly competitive at [^{3}H]DAGO sites in the adult cerebellum, the relationship of μ- and ζ-receptors in adult cerebellum were examined using blocking studies. The introduction of 5 nM DAGO in [^{3}H]MET binding assays of adult human cerebellum, in order to suppress binding to μ-receptors, completely eliminated specific and saturable binding of [^{3}H]MET. The addition of 5 nM MET in [^{3}H]DAGO assays, to suppress binding to ζ-receptors, did not alter binding capacity; a B_{max} of 50.4 fmol/mg protein was recorded for assays of [^{3}H]DAGO and a B_{max} of 48.3 fmol/mg protein for assays of [^{3}H]DAGO with the addition of 5 nM MET.

DISCUSSION

The results of this study demonstrate that opioid receptors are present in the developing and adult human cerebellum. Utilizing binding assays with ligands selective for 4 opioid receptor subtypes, our data indicate that for each receptor subtype there are more opioid receptors in developing cerebellum than in adult tissue. However, the ontogenic profile of opioid receptor subtypes differ (see schematic drawing in Fig. 3). In the case of δ- and κ-receptors, both of these subtypes were found in abundance in cerebellar tissue from infants, with 3.6- to 7.8-fold fewer receptors recorded in adulthood. The μ-receptor was found to be in greatest number at birth, with the binding affinity and capacity of this receptor decreasing over 7-fold in tissues from 2- to 19-day-old humans. In adult cerebellum, the μ-receptor had the same binding affinity as in newborns, but only one-half the binding capacity. The profile of the ζ-receptor was in distinct contrast to that of the μ-receptor, with the exception that no changes in binding affinity occurred. At

birth, the concentration of the ζ-receptor was only about 1/20th of that found in the cerebellum of 2–19 day infants. In adulthood, ζ-receptors were not detected, with low amounts of binding of [^{3}H]MET apparently due to cross-reactivity of the ligand for the μ-receptor.

Early studies[15] in the 21-week-old human fetus concerned with opioid receptors and the developing human brain and cerebellum have reported the binding of [^{3}H]etorphine, a non-selective opioid ligand which recognizes a variety of opioid receptor subtypes. Our studies using tissues from individuals obtained shortly after birth, confirm the existence of opioid receptors in the developing human cerebellum, and now demonstrate that at least 4 opioid receptor subtypes are present. Our data also show for the first time the existence of at least 3 opioid receptor subtypes in the adult human cerebellum. Earlier studies of the adult human cerebellum, often performed with ligands that were non-selective for an opioid receptor subtype and/or methodology that did not protect tissues from enzymatic degradation[11,12], revealed that the cerebellum contained low concentrations of opioid receptors in comparison to other regions. However, some autoradiographic data indicated the presence of moderate to dense opioid receptor sites in the human cerebellum[17]. Our results which have utilized binding assays with ligands and methodology selective for opioid receptor subtypes, as well as protease inhibitors, demonstrate the presence of μ-, δ-, and κ-receptors in the adult human cerebellum. Further studies defining the distribution and localization of these opioid receptors with selective ligands, as well as examination of the function of these receptors, in the adult human cerebellum are clearly warranted.

The results of this study using human cerebellar tissues must be viewed with the perspective that all materials were obtained at the time of death. Certainly consideration of post mortem changes as noted by others[21,24,31] must be kept in mind when interpreting these results with human tissues. However, our study did employ a cocktail of protease inhibitors which minimized post mortem changes; these inhibitors were reported earlier to be important in assaying the ζ-receptor[34,35]. Moreover, we uncovered no differences in data obtained from our binding assays when fresh tissues or those stored at −70 °C for extended periods of time were compared, a situation similar to that described for the σ-receptor in human brain[32]. This would suggest that, within the bounds of optimal tissue preparation, some opioid receptors in human cerebellum may be fairly stable entities.

The possible functions of opioid receptors in the adult nervous system have been discussed by others[1,2,14,22], but the importance of these receptors during ontogeny requires clarification. As in animal studies which have shown that opioid receptors appear early in life[6,7], and sometimes at levels greater than in adults[28–30], the situation in human cerebellum is quite similar. Whether these opioid receptor subtypes reflect the ontogeny of neurotransmitters, or serve other purposes during development, requires further study.

The ζ opioid receptor has been found to be related to growth of normal and abnormal cells and tissues[33–35], mediating cell proliferative events through interaction with [Met^5]enkephalin[7,39,40], an opioid peptide derived from proenkephalin A. Not only does this functional property support the identification of a unique opioid receptor, but the binding characteristics of the ζ-receptor in developing rat cerebellum[33] and in neural tumor cells from tissues[34] and culture[35], serve to distinguish the ζ-receptor from other opioid receptor subtypes. Our study now demonstrates that the ζ-receptor is present in the human cerebellum at birth, and is abundant during infancy. Since the human cerebellum develops over a lengthy period that also encompasses the postnatal period[9,23], the presence of the ζ-receptor during critical stages of cell proliferation, migration, and differentiation are consistent with earlier hypotheses suggesting that this receptor is related to growth[36–39]. It is interesting to note that the human cerebellum undergoes active growth in utero[9,23], but the number of ζ-receptors increases dramatically shortly after birth. This may indicate that birth, or the stage of development at this time, is an important stimulus for the expression of the ζ-receptor. Alternatively, our data may suggest that individuals monitored before day 2 could have been abnormal in regard to some aspects of the endogenous opioid systems, altering the number of ζ-receptors. However, no pathological problems were reported in the cerebella used in our study. Additionally, some receptor subtypes (e.g., δ) exhibited similar binding affinities and capacities prior to and after postnatal day 2. Consistent with the idea that birth is an important feature in the expression of the ζ-receptor, it is important to note that whether individuals were 28,32, or 38 weeks of gestation when born, the binding capacity of the ζ-receptor was low compared to individuals of similar gestational age examined after the 2nd postnatal day. For example, 2 infants of 38 weeks gestation age who died within the first 2 days of birth, exhibited low ζ-receptor number. In contrast, 2 infants of 38 weeks gestation age who survived for longer than 2 days had binding capacities that were over 20-fold greater. The absence of the ζ-receptor in adult cerebellum from humans is also consonant with the idea that this growth-related receptor plays an important role in developmental neurobiological events, but not in mature tissues. Of course, examination of discrete stages throughout the

ontogeny of the human cerebellum (e.g. fetus) is needed in order to formulate a more complete profile of opioid receptors during development.

Finally, the clinical implications associated with the presence of opioid receptors in the developing and adult cerebellum are of interest. Although the functional importance of μ-, δ-, and κ-receptors needs to be defined in the cerebellum, possible disorder in the ontogeny of these systems and attendant repercussions of such conditions warrant attention. Similarly, the implications of problems in the ontogeny of the ζ-receptor also may be of importance to the integrity of the developing and adult cerebellum. Recent information has linked Tourette's syndrome[13,26], Rett's syndrome[3], autism[25], epilepsy[27], self-injurious behavior[10,25], and cancer[40,41] to the endogenous opioid systems (i.e., opioids and receptors). Further study of opioid receptors during normal and abnormal development may reveal vital information with respect to the etiology and pathogenesis of diseases processes. Indeed, during the course of this study, the cerebellum of an individual with metastatic adenocarcinoma of the cerebellum was found to have high levels of the ζ-receptor[20]. These results indicate that the ζ-receptor is associated with proliferating neural cells, and appears to play a role in normal, as well as abnormal, development.

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