Differential, Postnatal Ontogeny of Opiate and Benzodiazepine Receptor Subtypes in Rat Cerebral Cortex: Binding Characteristics of Tifluadom and Brotizolam

Harlan F. HILL*, Yasuo WATANABE* and Takeshi SHIBUYA*, **

*Department of Biomedical Sciences, University of Illinois College of Medicine at Rockford, Rockford, Illinois 61107-1897, U.S.A.
**Department of Pharmacology, Tokyo Medical College, Tokyo 160, Japan

Accepted May 19, 1984

Abstract—The postnatal development of $^3$H ethylketocyclazocine (EKC) binding characteristics was examined using membranes from the cerebral cortex of rats at various ages. Binding site affinity did not vary significantly between postnatal days 1 and 90. However, the apparent density of cortical binding sites increased fivefold between birth and adulthood. These results were similar to another ontogenic study of brain opiate receptor binding. Whereas EKC was equally potent as a competitor for $^3$H EKC binding in cortex from neonatal and adult rats, tifluadom was three times more potent in neonatal cortex than in adult cortex as a displacer of specific EKC binding. Brotizolam, a new thienodiazepine and a potent sedative hypnotic, also was distinctly more potent as an inhibitor of $^3$H-diazepam binding in neonatal rat brain cortex than in adult rat brain cortex. These results suggest that subtypes of benzodiazepine receptors, as well as some opiate receptor subtypes, exhibit different rates of postnatal development.

Recently, a novel benzodiazepine, tifluadom, has been described which has opiate-like neurochemical and pharmacological activity and is otherwise unlike diazepam and other antianxiety drugs (1). Namely, its analgesic effect can be prevented by naloxone, but cannot be reversed by the benzodiazepine receptor antagonist Ro 15–1788 (1). Additionally, tifluadom exhibits high affinity binding to brain opioid receptors and much lower affinity for benzodiazepine binding sites (2). This drug appears to bind selectively to $\kappa$-receptors compared to other opiate receptor subtypes (2). However, Wood et al. found that tifluadom exhibited nearly the same $K_i$ values for both $^3$H Try-D-Ala-Gly-MePhe-Glyol (DAGO) and $^3$H ethylketocyclazocine (EKC) binding sites in rat brain and concluded that, like other $\kappa$-agonists, tifluadom possesses a pharmacological profile consisting of $\kappa$-agonism as well as $\mu$-2 and $\delta$ receptor antagonism (3). Similarly, we reported previously that $\kappa$-receptor ligands inhibit electrically-evoked contractions of rabbit ileum (which has a relatively high density of $\kappa$-receptors) and of mouse vas deferens, an organ which is thought to contain few if any $\kappa$-receptor sites (4). From these results, we concluded that supposedly selective $\kappa$-agonists may act as $\mu$ or $\delta$ receptor agonists if $\kappa$-receptors are sparse in the tissue being examined.

The general ontogenic scheme for opioid receptors in rat whole brain has been described (5–8), and there is evidence that the three major sub-types of brain opioid receptors have different developmental schedules (9–11). In adult rats, cerebral cortex contains appreciable densities of $\mu$, $\delta$ and $\kappa$ receptors (12). However at birth, there are relatively few $\delta$ receptors in cortex, although the total opioid receptor density in this brain region is already about twenty percent of adult levels (10, 13). Prenatal exposures to morphine, methadone and perhaps other narcotic analgesics can interfere...
with postnatal development of central neurons and of several aspects of behavior.

Since \( \mu \) and \( \kappa \) receptors appear to proliferate mainly during the late prenatal and early postnatal period of brain development (9, 13), either one or both of these receptor sub-types could be involved directly in the developmental alterations caused by morphine and similar analgesics. However, drugs possessing selectivity for the different subpopulations of opioid receptors have not been examined for similar developmental effects.

To examine further the interaction of tifluadom with brain \( \kappa \) receptors, we decided to compare the ability of this drug to compete with \( ^3\text{H} \) EKC, the prototypic \( \kappa \) agonist, for opiate binding sites in cerebral cortex from neonatal and adult rats. In addition, since Kley et al. (14) reported that the (-)isomer of tifluadom has high selectivity for the opiate receptor, but the (+)-isomer of tifluadom binds with about equal affinity to the opiate as well as benzodiazepine receptors, the present study included parallel measurements of tifluadom binding to \( \kappa \) receptors and to benzodiazepine receptors in the cerebral cortex of neonatal and adult rats. For comparative purposes, we also examined the same cortical binding parameters of two typical benzodiazepines (diazepam and brotizolam*) at the same postnatal ages.

**Materials and Methods**

**Animals:** Sprague-Dawley rats were bred in our laboratories, and ten pregnant females were housed in separate cages after gestational day 12. At birth, litters were reduced to 8 pups each. Ten rats (one from each litter) were sacrificed for the binding assays at each study age. At each age, the numbers of male and female rats sacrificed were nearly equal. All offspring were weaned on postnatal day 22.

**Membrane preparation:** Brains of the postnatal rats were placed on an aluminum block (2–4°C) for removal of the cerebral cortex. Each whole cortex was weighed and homogenized in 10 volumes of 0.32 M sucrose (Polytron, setting 6, 5 sec \( \times 2 \)). Homogenates were centrifuged at 1,000 \( \times g \) and 0°C for 10 min. The supernatants were centrifuged at 30 min (30,000 \( \times g \), 0°C, 30 min), resuspended and centrifuged once again. The twice-washed pellets were suspended in Tris-HCl at final concentrations of 1.0 or 2.5 mg protein/ml and stored at −80°C for up to two weeks.

**Binding assay:** The total binding capacity and the equilibrium dissociation constant of \( ^3\text{H} \) EKC to cortical membranes were determined on several postnatal days with the following method, which was previously reported (15): Various concentrations of \( ^3\text{H} \) EKC between 0.5 and 20 nM were incubated with 0.1 ml of the membrane preparation (0.1–0.25 mg protein) in Tris buffer, with or without 3 nM of unlabelled EKC for 40 min at 25°C. Then, 5 ml of buffer was added immediately to the incubation mixture, and the entire contents were rapidly filtered through Whatman GF/B filters. Filters were washed twice with ice-cold 50 mM Tris buffer (5 ml) and dried. Finally, filters were transferred to scintillation vials containing 10 ml of Aquasol-2 (New England Nuclear), shaken and stored for at least 12 hr. The radioactivity was measured by liquid scintillation spectrometry (Searle, Delta 300) for 10 min per sample at a counting efficiency of 40.80–43.60%.

Specific binding of \( ^3\text{H} \) EKC was defined as total binding minus radioligand binding in the presence of 3 nM unlabelled ethylketocyclazocine. Protein concentration of the membrane preparation was determined by the method of Lowry et al. (16). Binding parameters (\( K_d \) and \( B_{max} \)) were derived from Scatchard plots of a saturation isotherm of the various concentrations of \( ^3\text{H} \) EKC for each study age.

For the competitive inhibition experiments, specific binding of 1.6 nM \( ^3\text{H} \) EKC or \( ^3\text{H} \) diazepam (1.6 nM in adult and 2.5 nM in neonatal cortex) was measured in the presence of several concentrations of each unlabelled drug. The IC50 values were obtained from probit plots of percent control specific binding of radioligand versus log

* 2-bromo-4(2-chlorophenyl)-9-methyl-6H-thieno-(3,2-f)-1,2,4-triazolo-(4,3-a)-1,4-diazepine.
Results

As seen in Fig. 1, the specific binding of all concentrations of $[\text{H}]$ EKC to cortical membranes was significantly lower than the adult value until postnatal day 28. The apparent density ($B_{\text{max}}$: femtomoles/mg protein) of $[\text{H}]$ EKC binding sites increased 5-fold between birth and adulthood (Table 1). The density of specific $[\text{H}]$ EKC binding sites in cortex increased linearly with age from postnatal day 1 to day 28, without apparent rate changes during this 4-week period. In contrast, the equilibrium dissociation constant ($K_d$) did not vary significantly during postnatal development.

The results of binding assays with cortical membranes from adult rats, using several concentrations of unlabelled EKC and of tifluadom and two other benzodiazepines as competitors for binding of 1.6 nM $[\text{H}]$ EKC or $[\text{H}]$ diazepam, are shown in Fig. 2. Both EKC and tifluadom were effective in the nanomolar range as displacers of $[\text{H}]$ EKC and were very weak as competitors for $[\text{H}]$ diazepam binding. In contrast, brotizolam and diazepam exhibited very low affinity for $[\text{H}]$ EKC binding sites and were highly effective displacers of $[\text{H}]$ diazepam binding in adult cerebral cortex.

The relative potencies of the opiates and benzodiazepines as competitors of $[\text{H}]$ EKC for opiate binding sites in the cortex of neonatal and adult rats are shown in Table 2. These results further demonstrate the similarity between the cortical binding sites for $[\text{H}]$ EKC in neonatal and adult rat brain. Notably, IC50 values for EKC were nearly identical in neonatal and adult cortex while the IC50 for tifluadom was significantly

| Age (days) | $K_d$ (nM) | $B_{\text{max}}$ (fmol/mg protein) |
|-----------|------------|-----------------------------------|
| 1         | 3.79±0.92  | 80.6±3.67*                        |
| 4         | 3.69±0.49  | 104.5±9.8*                        |
| 7         | 4.06±0.32  | 138.9±8.7*                        |
| 14        | 2.70±0.34  | 176.2±8.2*                        |
| 21        | 2.71±0.17  | 237.6±13.0*                       |
| 28        | 2.78±0.13  | 360.5±3.4                         |
| 90        | 2.81±0.25  | 361.1±9.5                         |

Cerebral cortex was dissected and specific binding of $[\text{H}]$ ethylketocyclazocine, at several concentrations, was assessed as described in the text. The data represent the means±S.E.M. of 4 separate determinations, each run in triplicate. *Significantly different from the adult (90 day) value; $P>0.01$ by 2-tailed Student's $t$. 

Table 1. Characteristics of $[\text{H}]$ ethylketocyclazocine binding to cerebral cortex membranes at various ages

Fig. 1. Saturation curves for $[\text{H}]$ ethylketocyclazocine binding in cerebral cortex from rats of different ages (postnatal (PN) 1 day–90 days). Each point represents the mean of 3–4 separate experiments; all samples in each experiment were run in triplicate. Specific binding (fmol/mg protein) is equal to the amount of tritiated ligand bound in the absence of cold ethylketocyclazocine minus the amount bound in the presence of 3 μM cold ligand.
lower in neonatal vs. adult cortex.

The results shown in Table 3 demonstrate that EKC and tifluadom have very low affinity for benzodiazepine binding sites in the cortex of both neonatal and adult rats. These results also indicate that brotizolam is a more potent competitor for 

\[^{3}\text{H}]\text{diazepam binding than is diazepam itself (see also, 17), and this difference is even more pronounced in neonatal cortex than in adult cortex.}\n
![Graph showing inhibition of 

\[^{3}\text{H}]\text{ethylketocyclazocine and }^{3}\text{H}\text{diazepam binding in cerebral cortex from adult (90-day old) rats. Specific binding of tritiated ligand was measured in the presence of various concentrations of each unlabelled drug and also in the absence of unlabelled drug in each experiment. Decreases in amount of radioligand bound at each concentration of unlabelled drug are expressed as percent inhibition. Each point represents the mean of 3–4 separate determinations, each run in triplicate.}\n
Table 2. Inhibitory potencies of unlabelled drugs on 

\[^{3}\text{H}]\text{ethylketocyclazocine binding in cortex from neonatal and adult rats}\n
| Competitor         | IC50 (nM) Neonatal | IC50 (nM) Adult |
|--------------------|--------------------|----------------|
| Ethylketocyclazocine | 0.31±0.18          | 0.30±0.02      |
| Tifluadom          | 2.48±0.69*         | 7.47±0.71      |
| Brotizolam         | 40,600±7,800       | 21,700±3,400   |
| Diazepam           | >300,000           | >300,000       |

Cerebral cortex was dissected from brains of one-day and ninety-day old rats, and suppression of 

\[^{3}\text{H}]\text{ethylketocyclazocine (1.6 nM) binding by several concentrations of each unlabelled drug was assessed as described in the text. The concentration of drug required to produce fifty percent inhibition (IC50) of tritiated ligand binding was obtained from long-probit plots. The data represent the means±S.E.M. of four separate determinations, each run in triplicate. *Significantly different from the adult value at P<0.05.}
Table 3. Inhibition by unlabelled drugs of \(^{3}\text{H}\) diazepam binding in cortex from neonatal and adult rats

| Competitor          | Neontal IC50 (nM)    | Postnatal Day 7 IC50 (nM) | Adult IC50 (nM) |
|---------------------|----------------------|---------------------------|-----------------|
| Brotizolam          | 0.49±0.17*           | 0.69±0.07                 | 1.33±0.03       |
| Diazepam            | 4.30±1.26            | 5.53±1.39                 | 5.77±0.92       |
| Tifluadom           | 34,200±7,000         | 30,800±7,000              | 19,700±2,600    |
| Ethylketocyclazoline| >300,000             | >300,000                  | >300,000        |

Cerebral cortex was dissected from rats at one, seven and ninety days of age, and suppression of \(^{3}\text{H}\) diazepam (2.5 nM) binding by unlabelled drugs was assessed as described in the text. The data represent means±S.E.M. of four separate triplicate determinations. *Significantly different from the adult value at P<0.05.

Discussion

This study afforded three major results: First is that \(^{3}\text{H}\) EKC binding site density increases in the cerebral cortex throughout postnatal development to reach adult levels by about four weeks after birth (Table 1, Fig. 1). Second is that tifluadom was three times more potent as an inhibitor of \(^{3}\text{H}\) EKC binding in neonatal than in adult cortex (Table 2).

The final result is that unlike diazepam, brotizolam differs in its potency as a \(^{3}\text{H}\) diazepam displacer in neonatal and adult cerebral cortex (Table 3).

The overall pattern of postnatal development of \(^{3}\text{H}\) EKC binding site density in cerebral cortex is similar to that found for both naloxone and naltrexone binding in rat whole brain (5, 8). Also, consistent with previous studies with the antagonist ligands, the equilibrium dissociation constant of cortical \(^{3}\text{H}\) EKC binding sites did not change significantly during postnatal maturation.

The cerebral cortex of the rat probably contains at least three main subpopulations of opiate-specific binding sites, and these subtypes of brain opiate receptors may proliferate at different rates during postnatal development (12, 13). EKC is known to bind readily to both \(\alpha\)- and \(\mu\)-opiate receptors in brain tissue; so, it is not possible to compare relative densities of these two sub-types of cortical opioid receptors during brain development on the basis of binding of this ligand alone. However, in this study, tifluadom was three times more potent in neonatal rat cortex than in adult rat cortex as an inhibitor of \(^{3}\text{H}\) EKC binding. So, assuming that tifluadom is a selective \(\kappa\) agonist as reported (2, 18), our competitive binding results suggest that neonatal cortex may have a higher ratio of \(\kappa\) to \(\mu\) opiate receptors than does adult cortex. As postnatal development progresses, the ratio of \(\kappa\) to \(\mu\) receptors in the cortex may decline due perhaps to a more rapid rate of accumulation of cortical \(\mu\) vs. \(\kappa\) receptors (13).

This explanation would be consistent with the previous report that, compared to \(\delta\) receptors, the proliferation rate for \(\mu\) receptors is increased markedly during the third and fourth postnatal weeks in rat whole brain (11). Additional study with more highly selective opioid receptor ligands will be needed to test this explanation for the age-related difference in receptor density.

Additionally, the results of this study show clearly that the novel benzodiazepine, tifluadom, is very different from diazepam and similar drugs in its receptor binding characteristics. Tifluadom has high affinity, similar to that of EKC itself, for opioid binding sites in both neonatal and adult rat cerebral cortex; diazepam and brotizolam, a new benzodiazepine very similar to diazepam pharmacologically and having very high affinity to \(^{3}\text{H}\) diazepam binding site in the rat brain cortex (17, 19), have little or no affinity for both aged cortical opioid receptors in either neocortical or adult rat brain. On the converse, tifluadom and EKC have almost no affinity for \(^{3}\text{H}\) diazepam binding sites in either neonatal or adult cortex. Similar differences in receptor binding properties between
tifluadom and other benzodiazepines in whole brain tissue have been reported by others (1, 2).

Interestingly, the IC50 value of brotizolam, for inhibition of the $^3$H diazepam binding site in neonatal rat cortex, was approximately three times lower than that in adult rat cortex. Similar to opiate receptors, differential ontogeny of benzodiazepine receptor subtypes in rat neocortices also has been demonstrated (20). According to this report, the $B_{max}$ representing the type 2 benzodiazepine receptor increased during the first postnatal week, but the type 1 receptor for benzodiazepines did not demonstrate a dramatic increase in number until the second week of postnatal life. Our present results suggest that brotizolam may have a higher affinity for type 2 benzodiazepine receptor than for type 1 receptors. This hypothesis is consistent with the results of others, comparing potencies of diazepam and brotizolam as sedative hypnotics (21, 22) and indicating that type 2 receptors are important in the production of typical benzodiazepine side effects of ataxia and sedation (23, 24).

Acknowledgements: We are grateful to Dr. H. Zeugner of Kali-Chemie AG, Hanover, for the generous gift of tifluadom and to Professor Klupp of Boehringer Ingelheim for providing us with a supply of brotizolam. Drs. Morgan and Ward of Sterling-Winthrop Research Institute generously provided ethylketocyclazocine.

References
1 Romer, D., Buscher, H.H., Hill, R.C., Maurer, R., Petcher, T.J., Zeugner, H., Benson, W., Finner, E., Milkowski, W. and Thies, P.W.: An opioid benzodiazepine. Nature 298, 759–760 (1982)
2 Romer, D., Buscher, H.H., Hill, R.C., Maurer, R., Petcher, T.J., Zeugner, H., Benson, W., Finner, E., Milkowski, W. and Thies, P.W.: Unexpected opioid activity in a known class of drug. Life Sci. 31, 1217–1220 (1982)
3 Wood, P.L., Sanschagrin, D., Richard, J.W. and Thakur, M.: Multiple opiate receptor affinities of kappa and agonist/antagonist analgesics: In vivo assessment. J. Pharmacol. Exp. Ther. 226, 545–550 (1983)
4 Oka, T., Negishi, K., Kajiwara, M., Watanabe, Y., Ishizuka, Y. and Matsumiya, T.: The choice of opiate receptor subtype by neo-endorphins. Eur. J. Pharmacol. 79, 301–305 (1982)
5 Clendenin, N.J., Petraitis, M. and Simon, E.J.: Ontological development of opiate receptors in rodent brain. Brain Res. 118, 157–160 (1976)
6 Coyle, J.T. and Pert, C.B.: Ontogenetic development of $^3$H naloxone binding in rat brain. Neuropharmacology 15, 555–560 (1976)
7 Kent, J.L., Pert, C.B. and Herkenham, M.: Ontogeny of opiate receptors in rat forebrain: Visualization by in vitro autoradiography. Dev. Brain Res. 2, 487–504 (1982)
8 Patey, G., de la Baume, S., Gros, C. and Schwartz, J.C.: Ontogenesis of enkephalinergic systems in rat brain: Postnatal changes in enkephalin levels, receptors and degrading enzyme activities. Life Sci. 27, 245–252 (1980)
9 Auguy-Valette, A., Gros, J., Gourderes, Ch., Gout, R. and Pontonnier, G.: Morphine analgesia and cerebral opiate receptors: A developmental study. Br. J. Pharmacol. 63, 303–308 (1978)
10 Tsang, D. and Ng, S.C.: Effect of antenatal exposure to opiates on the development of opiate receptors in rat brain. Brain Res. 199, 199–206 (1980)
11 Wohltmann, M., Roth, B.L. and Coscia, C.J.: Differential postnatal development of mu and delta opiate receptors. Dev. Brain Res. 3, 679–684 (1982)
12 Goodman, R.R. and Snyder, S.H.: Autoradiographic localization of kappa opiate receptors to deep layers of the cerebral cortex may explain unique sedative and analgesic properties. Life Sci. 31, 1291–1294 (1982)
13 Leslie, F.M., Tso, S. and Hurlbut, D.E.: Differential appearance of opiate receptor subtypes in neonatal rat brain. Life Sci. 31, 1393–1396 (1982)
14 Kley, H., Scheidemantel, U., Bering, B. and Müller, W.E.: Reverse stereoselectivity of opiate and benzodiazepine receptors for the opioid benzodiazepine tifluadom. Eur. J. Pharmacol. 87, 503–504 (1983)
15 Watanabe, Y., Shibuya, T., Salafsky, B. and Hill, H.F.: Prenatal and postnatal exposure to diazepam: Effects on opioid receptor binding in rat brain cortex. Eur. J. Pharmacol. 96, 141–144 (1983)
16 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951)
17 Shibuya, T., Field, R., Watanabe, Y., Sato, K. and Salafsky, B.: Structure-affinity relationships between several new benzodiazepine derivatives and $^3$H-diazepam receptor sites. Japan. J. Pharmacol. 34, 435–440 (1984)
18 Upton, N., Gonzalez, J.P. and Sewell, R.D.E.:
Characterization of a μ-agonist-like antinociceptive action of tifluadom. Neuropharmacology 22, 1241–1242 (1983)

19 Ishiko, J., Inagaki, C. and Takaori, S.: Inhibitory effects of brotizolam, a new thienodiazepine, on limbic forebrain and neostriatal dopaminergic systems in vivo and in vitro. Neuropharmacology 11, 221–226 (1983)

20 Lippa, A.S., Beer, B., Sans, M.C., Vogel, R.A. and Meyerson, L.R.: Differential ontogeny of type 1 and type 2 benzodiazepine receptors. Life Sci. 28, 2343–2347 (1981)

21 Fink, M. and Irwin, P.: Pharmacoelectroencephalographic study of brotizolam, a novel hypnotic. Clin. Pharmacol. Ther. 30, 336–342 (1981)

22 Nicholson, A.N. and Wright, C.M.: Comparative studies with thieno- and benzodiazepines: Spatial delayed alternation behavior in the monkey. Neuropharmacology 19, 491–495 (1980)

23 Klepner, C.A., Lippa, A.S., Benson, D.I., Sano, M.C. and Beer, B.: Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. Pharmacol. Biochem. Behav. 11, 457–462 (1979)

24 Lippa, A.S., Critchett, D.J., Sano, M.C., Klepner, C.A., Greenblatt, E.N., Coupel, J. and Beer, B.: Benzodiazepine receptors: Cellular and behavioral characteristics. Pharmacol. Biochem. Behav. 10, 831–843 (1979)