Pharmacology Biochemistry & Behavior, Vol 16, pp 373–375, 1982 Printed in the U S A

Characteristics of Type 1 and Type 2 Benzodiazepine Receptors in the Ovine Brain

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Received 1 October 1981

VILLIGER J W, K M TAYLOR AND P D GLUCKMAN *Characteristics of Type 1 and Type 2 benzodiazepine receptors in the ovine brain* PHARMAC BIOCHEM BEHAV 16(3) 373–375, 1982 —Analysis of the displacement of ^{3}H-diazepam binding to membranes prepared from the ovine frontal cortex by the triazolopyradiazine CL218,872 yielded a Hill coefficient significantly below unity By analogy with similar studies of this drug in rat brain this suggested the existence of Types 1 and 2 benzodiazepine receptors The degree of displacement of ^{3}H-diazepam by CL218,872 (200 nM, Type 1 800 nM, Type 2) in homogenates of brain regions differed, the rank order being cerebellum > parietal cortex > frontal cortex ≃ temporal cortex ≃ hippocampus > striatum Displacement of ^{3}H-diazepam by CL218,872 was enhanced by 10^{-5} M GABA in the striatum (at 200 nM and 800 nM CL218,872) and cerebellum (at 200 nM CL218,872) Benzodiazepine receptors in the ovine frontal cortex were least sensitive to CL218,872 (200 nM) in young fetuses (54–68 days gestation) and achieved adult levels of sensitivity by late gestation Finally, the potency of CL218,872 to displace ^{3}H-diazepam was not effected by the ^{3}H-ligand concentration (0 5 nM or 5 0 nM), suggesting that Types 1 and 2 benzodiazepine receptors are not identical to the high and low affinity ^{3}H-diazepam binding sites we have previously identified in the ovine brain

Benzodiazepine receptors Triazolopyridazine CL218,872 Ovine brain

THE mammalian central nervous system (CNS) contains binding sites for ^{3}H-benzodiazepines which have the pharmacological and biochemical characteristics of membrane bound benzodiazepine receptors [6,8] While initial studies suggested that ^{3}H-benzodiazepine bound to a single receptor, there is now growing evidence that multiple benzodiazepine receptors are present in CNS tissue [7] For example, Lippa and his associates showed that 3-methyl-6-[3-(trifluoromethyl)phenyl] - 1,2,4 - triazolo - [4,3 - b]pyridazine (CL218,872), a non-benzodiazepine with high anxiolytic potency but low hypnotic and anticonvulsant potency, displaced the binding of ^{3}H-diazepam with a shallow displacement curve which yielded a Hill coefficient of approximately 0 5 [4] Although other factors such as negatively cooperative site-site interactions or ternary receptor complex formation could explain this finding, Lippa *et al* have postulated the existence of multiple benzodiazepine receptors—those for which CL218,872 has high affinity ($K_d \simeq 100$ nM, termed Type 1) and those for which CL218,872 has low affinity ($K_d \simeq 1000$ nM, termed Type 2) [5] Types 1 and 2 benzodiazepine receptors have been characterized in the rat brain with regard to regional distribution [5,9], ontogeny [3] and sensitivity to GABA [2] We report here the existence and characteristics of Types 1 and 2 benzodiazepine receptor in the ovine brain Also, since Scatchard analysis of ^{3}H-diazepam binding to ovine frontal cortex is curvilinear suggesting high ($K_d \simeq 2$ 0 nM) and low $K_d \simeq 20$ nM) affinity binding sites (Villiger *et al*, submitted), we have examined the compatability of such a receptor subdivision with the Types 1 and 2 benzodiazepine receptor classification

METHOD

Adult ewes (pregnant and non-pregnant) and infant lambs were killed with an overdose of sodium pentobarbital injected intravenously Fetuses, whose stage of gestation was accurately known, were obtained by Caesarian section and killed by decapitation Brains were rapidly removed, dissected and the selected regions placed on dry ice before storage at −20°C They were homogenized with an Ultra-Turrax homogenizer in 50 volumes of ice-cold 50 mM Tris HCl buffer (pH 7 4 at 20°C) The homogenate was then centrifuged at 50,000 g for 10 minutes at 4°C The pellet was washed 5 times by resuspension in further 50 volume aliquots of 50 mM Tris buffer and recentrifugation The final pellet was suspended in 20 volumes of 50 mM Tris buffer before being subdivided and stored at −20°C

^{3}H-Diazepam (76 8 Ci/mmole, New England Nuclear Corp, Boston) binding was determined by incubating the ^{3}H-benzodiazepine with 200 μl of homogenate (10 mg tissue wet weight), test compound (where applicable), and 50 mM Tris HCl buffer (to give a final volume of 2 0 ml) at 0–4°C for 30 min Following the addition of 5 ml of ice-cold buffer, assays were terminated by filtration under reduced pressure through 2 5 cm Whatman GF/B glass fibre discs Filters were then washed with two further 5 ml aliquots of buffer Radioactivity present on the filters was determined by liquid scintillation spectrometry at a counting efficiency of 28–30%

Non-specific binding was defined as that occurring in the presence of 3 0 μM unlabelled diazepam Specific binding

was defined as total binding (in the absence of unlabelled drug) minus non-specific binding All data is presented as specific binding, which was 90–95% of total binding

Hill plots were calculated according to Bennett [1] and the line of best fit drawn using a computerized linear-regression analysis Deviation of Hill plot slopes from unity was calculated using a t-statistic with the formula $t_{n-2} = \frac{b-1}{b} \times \sqrt{F}$ where b = the slope of the regression line and F = the F-ration for the linear regression

All drugs used were kindly supplied by F-Hoffman-La Roche Co Ltd , Basel, Switzerland

RESULTS

Analysis of the displacement of ^{3}H-diazepam (0 5 nM) from binding sites in homogenates of frontal cortex by CL218,872 (10^{-5}–10^{-8}) yielded a Hill coefficient of 0 77±0 09, which differed significantly ($p<0$ 05) from unity (Fig 1, inset) This suggested the differential sensitivity of ^{3}H-diazepam receptors to CL218,872 in accord with the Type 1 and Type 2 benzodiazepine receptor concept [5]

To test if the high and low affinity benzodiazepine binding sites in ovine frontal cortex were differentially sensitive to CL218,872, we compared CL218,872 IC_{50} values when membranes prepared from the same brain were incubated at 0 5 nM and 5 0 nM ^{3}H-diazepam There was no significant difference between IC_{50} values which were 283±72 nM at 0 5 nM ^{3}H-diazepam and 300±58 nM at 5 0 nM ^{3}H-diazepam ($p>0$ 1, n=3) Thus the high and low affinity benzodiazepine receptors in frontal cortex do not appear to have differential sensitivity for CL218,872

Since Type 1 and 2 receptors have been localized in regions of the rat brain both biochemically [5] and by using autoradiography [9] we have investigated the sensitivity of ^{3}H-diazepam binding sites to CL218,872 in several regions of the adult sheep telencephelon and cerebellum The benzodiazepine binding sites were labelled with 0 5 nM ^{3}H-diazepam, and percent displacement by 200 nM and 800 nM CL218,872 examined These assay parameters were similar to those employed by Young *et al* [9] and, by definition, provide an index of the number of Type 1 (200 nM CL218,872) and Type 2 (800 nM CL218,872) receptors Also, in order to examine if Type 1 and Type 2 receptors are coupled to GABA receptors, we tested the effect of 10^{-5} M GABA on CL218,872 displacement of ^{3}H-diazepam As shown in Table 1, the degree of displacement of ^{3}H-diazepam by both 200 nM and 800 nM CL218,872 was in the rank order cerebellum > parietal cortex > frontal cortex ≈ occipital cortex ≈ temporal cortex ≈ hippocampus > striatum This result is consistent with those previously reported for the rat [5,9] Interestingly, 10^{-5} M GABA significantly ($p<0$ 01) increased the sensitivity of the benzodiazepine receptor to 200 nM CL218,872 in the striatum and cerebellum and to 800 nM CL218,872 in the striatum

We also examined the effect of 200 nM CL218,872 on ^{3}H-diazepam (0 5 nM) binding on membranes prepared from frontal cortices of ovine fetuses (Table 2) The displacement of ^{3}H-diazepam by CL218,872 was lowest in fetuses aged 54–68 days (21 2%) and increased significantly ($p<0$ 05) by late gestation to 45 8% A similar level of displacement was maintained to adulthood This result suggests that the number of benzodiazepine receptors able to be classified as Type 1 receptors develops primarily in late gestation

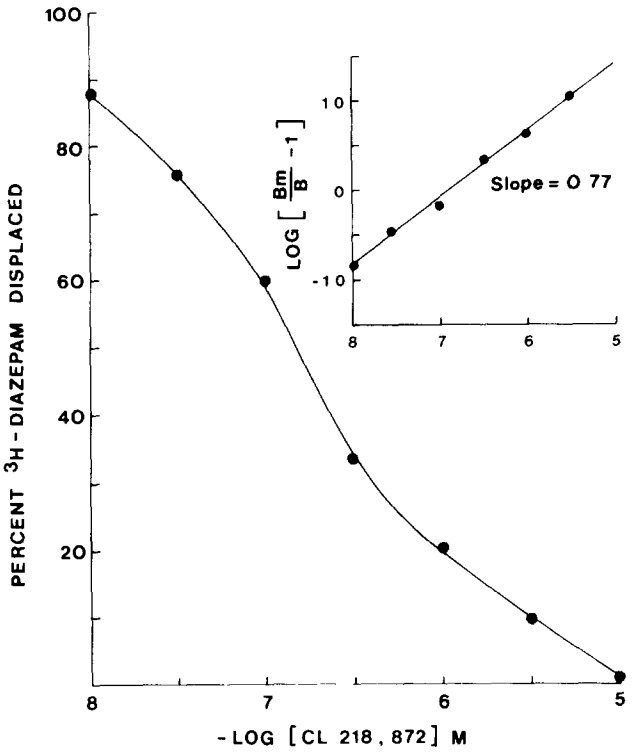

FIG 1 Displacement of 0 5 nM ^{3}H-diazepam by the triazolopyridazine CL218,872 in homogenates of the ovine frontal cortex Inset Hill plot of the displacement data

DISCUSSION

The concept of Type 1 and Type 2 benzodiazepine receptors has been introduced to explain the finding that the triazolopyridazine CL218,872 displaces ^{3}H-benzodiazepine binding to rat brain preparations with a shallow displacement curve which yields a Hill coefficient significantly less than unity [2, 3, 4, 5, 9] We have obtained similar results with CL218,872 displacement of ^{3}H-diazepam binding to homogenates of ovine frontal cortex, suggesting the presence of Types 1 and 2 benzodiazepine receptors in ovine brain Our studies indicated that the regional distribution of Type 1 and Type 2 receptors in the sheep brain is similar to that in rat brain with the highest number of Type 1 receptors in cerebellum and lowest number in striatum

Previous evidence based on the displacement by CL218,872 of ^{3}H-flunitrazepam from crude synaptosomes prepared from rat cortex suggested that Types 1 and 2 receptors are not modulated by GABA [2] We found that 10^{-5} M GABA significantly enhanced CL218,872 (200 nM) displacement of ^{3}H-diazepam (0 5 nM) from striatal and cerebellar membranes, suggesting that GABA is capable of modulating Types 1 and 2 receptors in the ovine brain under certain conditions The discrepancy between the Klepner *et al* [2] result and our finding could reside in the different tissue preparations and ^{3}H-benzodiazepines used, the different brain regions assayed, or represent a species difference between rodent and ovine brain

Our finding that the brain of the immature ovine fetus (54–68 days gestation) contains fewer Type 1 benzodiazepine

TABLE 1

THE EFFECT OF 10^{-3} M GABA OF CL218,872 DISPLACEMENT OF ^{3}H-DIAZEPAM (0 5 nM)

Region	Percent displacement of specifically bound ^{3}H-diazepam Addition: 200 nM CL218,872	200 M CL218,872 $+10^{-5}$ M GABA	800 nM CL218,872	800 nM CL218,872 $+10^{-5}$ M GABA
Frontal Cortex	40 ± 8	44 ± 8	69 ± 5	71 ± 6
Parietal Cortex	47 ± 6	50 ± 5	71 ± 4	71 ± 4
Occipital Cortex	35 ± 5	41 ± 8	58 ± 8	63 ± 3
Temporal Cortex	38 ± 6	45 ± 3	63 ± 2	61 ± 3
Hippocampus	38 ± 6	48 ± 1	59 ± 5	66 ± 3
Striatum	19 ± 1	30 ± 2*	39 ± 4	58 ± 3*
Cerebellum	62 ± 2	74 ± 2*	88 ± 3	89 ± 2

Values represent the mean ± s e m percentages of 3 adult brains

*Significantly ($p<0$ 01, two-tailed t-test) greater than CL218,872 displacement in the absence of GABA Percent displacement by CL218,872 in the absence and presence of 10^{-5} M GABA were compared respectively to control specific binding in the absence and presence of GABA

receptors than brains from older animals is consistent with studies in the rat showing later post-natal development of Type 1 receptors [3] The earlier development of the ovine benzodiazepine receptor (using parturition as a reference point) coincides with the prenatal brain growth spurt in the sheep as compared to postnatal brain growth spurt in the rat [1]

We have previously found that Scatchard analysis of ^{3}H-diazepam binding to ovine frontal cortex is curvilinear, suggesting the presence of high ($K_D \simeq 2$ 0 nM) and low ($K_D \simeq$ 20 nM) affinity binding sites (Villiger *et al* submitted) Since CL218,872 displacement of ^{3}H-diazepam binding to homogenates of frontal cortex was not significantly affected by the ^{3}H-diazepam concentration employed (0 5 nM vs 5 0 nM), we suggest that Type 1 and Type 2 benzodiazepine receptors are not identical to the high and low affinity ^{3}H-diazepam binding sites previously identified

TABLE 2

THE DEVELOPMENT OF SENSITIVITY OF THE BENZODIAZEPINE RECEPTOR TO CL218,872 IN HOMOGENATES OF THE OVINE FRONTAL CORTEX

Conceptual Age (days)	Percent displacement by 200 nM CL218,872
54–68 (Fetus)	21 2 ± 12 4(4)*
96–147 (Fetus)	45 8 ± 4 5(6)
180–205 (Lamb)	42 0 ± 18 0(3)
>730 (Adult)	40 0 ± 8 0(3)

Parturition in the sheep occurs at approximately conceptual age 147 days

*Significantly different from 96–147 day group at $p<0$ 05 (two-tailed t-test) Values given are the mean ± s e m of the number of brains given in parenthesis

ACKNOWLEDGEMENTS

We thank Sharon Wilkins for excellent technical assistance This work was supported by a grant from the N Z Neurological Foundation J W V is an M R C Postdoctoral Fellow and P D G is a Senior Fellow of the M R C

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