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# PRELIMINARY COMMUNICATIONS

## ALTERATIONS OF REGIONAL γ-AMINOBUTYRIC ACID RECEPTORS IN MORPHINE TOLERANT MICE

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Several attempts have been made to ascribe a role for γ-aminobutyric acid (GABA) in analgesia and tolerance/dependence to opiates [1-5], and also in the antagonism of opiate-induced convulsions [6]. Although the binding of GABA to its receptor sites in whole brain and discrete brain regions has been studied extensively in naive animals [7-10], there is a paucity of such studies in animals rendered tolerant to morphine. The present study examines the effect of tolerance development to morphine on the distribution of GABA receptors in the whole and discrete regions of mouse brain, using [$^3$H]-muscimol, a potent GABA receptor agonist, as a ligand.

Materials and Methods

[$^3$H]-Muscimol (sp. act. 7.3 Ci/mmole) was obtained from the New England Nuclear Corp., Boston, MA. The radiochemical purity was verified using thin-layer chromatography in appropriate solvent systems. Other chemicals and reagents of an analytical grade were obtained from commercial suppliers.

Male ICR mice (TIMCO, Houston, TX), weighing 22-28 g, maintained in a room at a temperature of 24 ± 1° and under artifical 12/12 hr lighting cycles, were used; water and food were given *ad lib*. The animals were rendered tolerant to morphine by the pellet (75 mg morphine base, s.c.) implantation method of Way *et al*. [11], and decapitated 72 hr after the implantation. The control animals received placebo pellets. In acute treatment, a single acute dose of morphine sulfate (30 mg/kg, s.c.) was injected and the animals were sacrificed 60 min after injection; control animals received 0.9% w/v saline.

The whole brains were removed rapidly and the discrete regions were carefully dissected following the procedure of Glowinski and Iversen [12]. Medulla refers to medulla oblongata and pons; diencephalon refers to dien- and mesencephalon. The synaptic membrane was prepared following the method of Gray and Whittaker [13] and Terenius [14]. Briefly, pooled brain samples (4.5 g) were homogenized in 10 vol. of 0.32 M sucrose by a Potter-Elvehjem glass homogenizer and centrifuged at 1,000 *g* for 10 min. The supernatant fraction was collected and centrifuged at 20,000 *g* for 20 min to collect the pellet containing the crude mitochondrial fraction ($P_2$ fraction). The $P_2$ fraction was subjected to osmotic shock by adding 10 ml of double distilled deionized water. The suspension was centrifuged at 12,000 *g* for 20 min. The carefully decanted supernatant fraction including buffy layer, suitably divided, was layered over a discontinuous gradient consisting of 0.6 M and 1 M sucrose and

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centrifuged in a Beckman SW-27 rotor at 22,500 rpm for 60 min (Beckman LS-65 ultracentrifuge). The band between 0.6 and 1 M sucrose ($P_2B$ fraction) was collected and divided into suitable aliquots and stored at -20°. Before binding assay, the aliquots were thawed and diluted with 10 vol. of 50 mM Tris-citrate buffer (pH 7.1) and centrifuged at 25,000 g for 20 min, to obtain a pellet; the pellet was rehomogenized with a suitable volume of the Tris-citrate buffer and incubated at 37° for 30 min to dissociate endogenous inhibitors [10]. After incubation the suspension was recentrifuged at 25,000 g for 20 min to obtain a pellet which was resuspended in fresh buffer for assay. This contained 1.5 to 2 mg/ml protein.

[$^3$H]-Muscimol binding was initiated by the addition of 0.2 ml of the membrane preparation to a mixture containing the required final concentration of [$^3$H]-muscimol in a total volume of 1.0 ml. Incubations were carried out at 4° for 10 min. The binding reaction was stopped by rapidly filtering through Whatman GF/B filters. They were then washed twice with 5 ml of ice-cold 50 mM Tris-citrate buffer (pH 7.1). The conditions of binding assay adopted here were those derived from preliminary studies and found to be optimum. The filters were transferred to scintillation counting vials containing 10 ml of PCS (Phase Combining System, Amersham Co., Arlington Heights, IL). The vials were shaken for 60 min and the radioactivity was measured by a Searle Mark II 6847 liquid scintillation system at a counting efficiency of 40 percent. [$^3$H]-Muscimol was used over a concentration range of 1-100 nM; non-specific binding was determined in the presence of 1 mM unlabeled GABA. Protein content of the membrane was determined by the method of Lowry et al. [15].

Linear regression analyses were used to obtain all values of dissociation constant ($K_D$) and maximal binding capacity ($B_{max}$). When applicable data were analyzed for significance by Student's t test, a P value $<0.05$ between two means was considered significant.

## Results and Discussion

The high-affinity $Na^+$- independent, specific [$^3$H]-muscimol binding to synaptic membrane of whole and five different brain regions of naive and tolerant mice displayed saturation kinetics. Scatchard analysis of the data revealed a single population of receptors. Similar observations have been reported by other workers [10,16,17]. The Scatchard plots of [$^3$H]-muscimol binding to synaptic membranes in whole and five discrete regions of brain are presented in Fig. 1; the calculated $K_D$ and $B_{max}$ values are given in Table 1. There were marked regional differences in $K_D$ and $B_{max}$ values in naive and tolerant groups. In the naive group, the highest binding occurred in the cerebellum and the lowest in the medulla while other areas displayed intermediate values. These results are in general agreement with earlier reports [7,17,18]. The present study reveals an altered relative distribution of synaptic GABA receptors in tolerant animals; the maximum number of binding sites was increased significantly in all regions except the cortex. There were also marked alterations in the affinity of [$^3$H]-muscimol to GABA receptors in tolerant animals; in the cerebellum the affinity was increased, while in the striatum and diencephalon it was decreased. The increase in $B_{max}$ in diencephalon and medulla was three and six times higher than the corresponding controls, respectively; this is important in view of tne known influence of these regions in the modulation of pain sensation [19]. Further, the effect appears to be related to tolerance development, as an acute morphine exposure failed to alter the binding characteristics in diencephalon ($K_D$ and $B_{max}$: control 10.5 ± 6.2, 462 ± 80; morphine treated 12.8 ± 1.6, 402 ± 98).

Sodium-independent GABA receptor binding to synaptic membrane preparations has characteristics consistent with labeling post-synaptic GABA receptors [7,8]. Therefore the differential increase in the affinities and in the maximum number of receptors observed in

the present study is conceivably due to post-synaptic alterations of GABA receptors. Since there is a marked altered regional heterogeneity in the binding characteristics in tolerant animals compared to naive animals, the GABA mediated effects may involve a net result

Table 1. Effect of chronic administration of morphine on the dissociation constant ($K_D$) and maximum binding capacity ($B_{max}$) of [$^3$H]-muscimol binding to synaptic membrane in whole and discrete regions of mouse brain.*

| | Placebo | | Tolerant | |
|---|---|---|---|---|
| | $K_D$ | $B_{max}$ | $K_D$ | $B_{max}$ |
| Striatum | 5.5 ± 0.64 | 354 ± 27 | 9.5 ± 0.90+ | 766 ± 60$^{\neq}$ |
| Medulla oblongata | 7.3 ± 0.56 | 204 ± 25 | 7.0 ± 0.58 | 1197 ± 103$^{\S}$ |
| Diencephalon | 9.6 ± 0.85 | 347 ± 40 | 17.2 ± 0.72$^{\neq}$ | 950 ± 58$^{\S}$ |
| Cerebellum | 10.4 ± 1.20 | 861 ± 98 | 4.4 ± 1.10$^{+}$ | 1348 ± 87$^{+}$ |
| Cortex | 24.5 ± 2.60 | 625 ± 30 | 25.3 ± 1.80 | 732 ± 45 |
| Whole brain | 13.6 ± 1.63 | 508 ± 34 | 14.5 ± 2.1 | 733 ± 60$^{+}$ |

*Each values is the mean ± S.E. of three independent determinations, each in triplicate. $K_D$ = nM; $B_{max}$ = pmoles/g.
$^{+}P < 0.05$, $^{\neq}P < 0.01$ and $^{\S}P < 0.001$ compared to the respective placebo group.

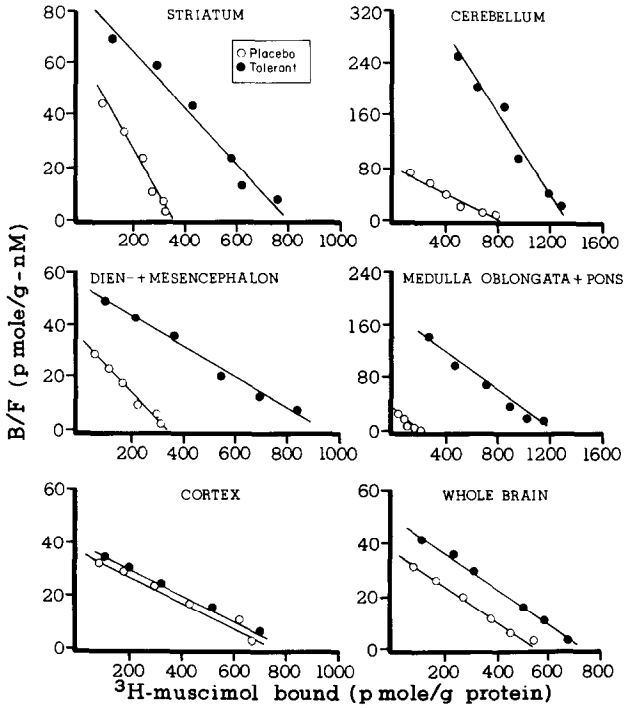


Fig. 1. Scatchard plots of $Na^+$-independent, specific [$^3$H]-muscimol binding to synaptic membranes obtained from whole and discrete brain regions of mouse. The $K_D$ and $B_{max}$ values are given in Table 1. For experimental details see text.

of the differential inputs from different regions. This may explain the divergent results obtained in in vivo studies involving the effects of GABAergic compounds (intracerebroventricular or parenteral) on morphine analgesia and tolerance/dependence [2,4,20]. The marked affinity changes observed in discrete regions were not evident in the whole brain, but only an increase in receptor density was apparent. Therefore, whole brain results may not reflect true changes occurring in different regions and due caution should be exercised in interpreting/generalizing the results of whole brain studies. In summary, the study reveals a marked regional alteration of synaptic GABA receptors' distribution and affinity in morphine tolerance and lends further support to the view that the GABA system may be involved in morphine tolerance/dependence.

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REFERENCES

1. I. K. Ho and H. H. Loh, Fedn Proc. 32, 758 (1974).
2. I. K. Ho, H. H Loh and E. L. Way, Life Sci. 18, 1111 (1976).
3. T. DeBoer, H. J. Metselaar and J. Bruinvels, Life Sci. 20, 933 (1977).
4. P. Mantegazza, R. Tammiso, L. Vicentini, F. Zambotti and N. Zonta, Pharmac. Res. Commun. 12, 239 (1980).
5. W. R. Buckett, Psychopharmacology 66, 233 (1979).
6. R. Dingledine, L. L. Iversen and E. Breuker, Eur. J. Pharmac. 47, 19 (1978).
7. S. R. Zukin, A. E. Young and S. H. Snyder, Proc. natnl. Acad. Sci. U.S.A. 71, 4802 (1974).
8. S. J. Enna and S. H. Snyder, Brain Res. 100, 81 (1975).
9. M. K. Ticku and T. Burch, Biochem. Pharmac. 29, 1217 (1980).
10. A. E. Kingsbury, G. P. Wilkin, A. J. Patel and R. Balars, J. Neurochem. 35, 739 (1980).
11. E. L. Way, H. H. Loh and F. H. Shen, J. Pharmac. exp. Ther. 167, 1 (1969).
12. J. Glowinski and L. L. Iversen, J. Neurochem. 13, 655 (1966).
13. E. G. Gray and V. P. Whittaker, J. Anat. 96, 79 (1962).
14. L. Terenius, Acta pharmac. tox. 32, 317 (1973).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, J. biol. Chem. 193, 265 (1951).
16. J. Hyttel, Psychopharmacology 65, 211 (1979).
17. F. V. DeFeudis, Neuroscience 5, 675 (1980).
18. J. S. Horng and D. T. Wong, J. Neurochem. 32, 1379 (1979).
19. B. Bishop, Phys. Ther. 60, 13 (1980).
20. G. Biggio, D. Della, V. Frigeni and A. Guidotti, Neuropharmacology 16, 149 (1977).