Does meptazinol bind to opiate receptors?

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Numerous studies have shown that meptazinol possesses antinociceptive activity in the mouse, rat and monkey and analgesic activity in man (for review see Stephens, Waterfall & Franklin, 1978). We have extended these studies in an attempt to determine how the antinociceptive effect is produced.

Binding studies with [^{3}H]-naloxone and [^{3}H]-meptazinol were carried out using the method of Childers, Creese, Snowman & Snyder (1979). In certain experiments, however, binding was measured in a Tris Krebs medium, pH 7.4 at 25°C. Non specific binding was determined with an excess (1000 ×) of unlabelled drug. *In vivo* binding was measured by injection of mice with [^{3}H]-meptazinol. Tissues were removed and homogenized in Tris Krebs medium ± meptazinol (10^{-4} M). Displaceable binding was the binding displaced by meptazinol *in vitro*. Antinociceptive activity was assessed using the mouse tail flick method. Studies on isolated tissues were conducted in Krebs bicarbonate medium (pH 7.4).

Meptazinol displaced [^{3}H]-naloxone only at high concentrations ($K_1 < 0.5\ \mu$M). The displacement was unaffected by sodium ions (Na^+ ratio 1.4). Scatchard analysis revealed however that there were at least two meptazinol binding sites (KD's approximately 8 and 35 nM). Although this specific binding was associated with synaptosomal material, there was also substantial non-specific binding to the myelin fraction and in the spinal cord.

In contrast to opiate induced depression of twitch in guinea-pig ileum, meptazinol (10^{-7}–10^{-5} M) potentiated the twitch. A similar phenomenon associated with an increased neurotransmitter release was observed in other tissues including atria and vas deferens.

Naloxone (2 mg/kg, i.p.) abolished the antinociceptive effect of meptazinol but did not reduce *in vivo* binding of [^{3}H]-meptazinol in mice. In experiments where *in vivo* binding of meptazinol was assessed at different dose levels (6.25–50 mg/kg, i.p.) a significant correlation ($r = 0.85$, $P < 0.001$) was observed between antinociceptive effect and binding in the spinal cord. However, as the *in vivo* binding to the spinal cord was largely non displaceable by meptazinol (10^{-4} M) added *in vitro*, this may reflect only a correlation between drug level and analgesia.

However, it remains that meptazinol displays certain features not normally associated with narcotic agonists and partial agonists.

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The binding of [^{3}H]-scopolamine to the brains of male and of female rats: the effects of ovariectomy versus the proestrous stage of the sexual cycle

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Sex hormones have profound effects on higher functions of the brain as well as on the limbic system, and act, possibly, by altering numbers and/or binding characteristics of receptors for individual neurotransmitters. It has recently been reported that 17_B oestradiol increases the number of muscarinic ([^{3}H]-QNB) binding sites in the hypothalamus, but not elsewhere, in the brains of ovariectomized female rats. (Rainbow, Degroft, Luine & McEwen; 1980.) It is not known, however, whether it has the same effect on the binding of other muscarinic antagonists. We describe here the binding of [^{3}H]-scopolamine (53.5 Ci/mmol) to membrane fractions prepared from different areas of the brains of adult rats of both sexes, females being either intact (at the proestrous stage of the sexual cycle) or ovariectomized. Assay methods were essentially those of Hulme, Birdsall, Burgen & Mehta (1978). Binding was estimated by Scatchard

analyses, control incubations containing an excess of unlabelled scopolamine. Equilibrium dissociation constants of reaction in all areas were in the order of 10 μM and did not vary with sex or with ovariectomy. The numbers of binding sites (pmoles/0.1 g protein; ± s.e.mean) in different parts of the brains of proestrous females (when oestrogen levels are high) were: cerebral cortex, 45 ± 2; amygdala 48 ± 3; hypothalamus 22 ± 3; thalamus, 30 ± 3; brain stem, 22 ± 4; cerebellum 2 ± 1.

In ovariectomized females, where oestrogen levels are minimal, values were significantly higher ($P < 0.01$) in the cerebral cortex (82 ± 4), amygdala (85 ± 2) and hypothalamus (54 ± 3). In males, significantly higher values than in the proestrous female were recorded in the cerebral cortex (95 ± 4) but in the hypothalamus, where testosterone can be converted to oestradiol *in vivo*, and in the adjoining amygdala, values intermediate between those of the proestrous and ovariectomized females (hypothalamus 33 ± 4 and amygdala 66 ± 6) were recorded.

Since the affected tissues all contain receptor proteins for oestradiol (Barley, Ginsburg, McLusky, Morris & Thomas, 1977) our results suggest that exposure to oestrogens reduces the number of scopolamine binding sites in the brain; and at first site seem to be at variance with those of Rainbow *et al.* (1980). This apparent discrepancy is probably due to a differential labelling of the sites by the different radioligands; the maximum binding capacity for [^{3}H]-QNB is approximately five times that for [^{3}H]-scopolamine in the different brain areas.

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5-Hydroxytryptamine-induced depolarization of rabbit nodose ganglia investigated by the sucrose-gap method

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Receptors for neurotransmitters and their associated ion channels may be present both on the soma membrane and the presynaptic terminal of primary afferent neurones (Deschenes, Feltz & Lamour, 1976). Investigation of the depolarizing action of 5-hydroxytryptamine (5-HT) on primary afferent cell bodies may assist understanding of any central presynaptic inhibitory action of the amine (Simonds & de Groat, 1980).

The experiments reported here show that the sucrose-gap technique may be employed to make recordings of 5-HT-induced depolarizations from somata of vagal primary afferents located in the rabbit nodose ganglion (ng) over the course of many hours. Comparison was made with responses of the same cells to other depolarizing agents and with responses of lower lumbar dorsal root (d.r.g.) and superior cervical ganglia (s.c.g.) recorded by the same technique. In the sucrose-gap apparatus employed, the sucrose compartment is separated from adjacent chambers by rubber membranes (Wallis, Lees & Kosterlitz, 1975). 5-HT, γ-aminobutyric acid (GABA) or the nicotinic agonist, 1,1-dimethyl-4-phenyl piperazinium (DMPP), were applied by bolus injection to the superfusion stream to the ganglion in quantities ranging from 0.002 to 0.2 μmol.

5-HT (0.2 μmol) evoked depolarizations of 4.5 ± 0.4 mV (mean ± s.e.mean, $n = 18$) in n.g. compared to 2.2 ± 0.2 mV in s.c.g. ($n = 20$) and 0.6 ± 0.1 mV in d.r.g. ($n = 9$). GABA (0.2 μmol) evoked depolarizations of 1.3 ± 0.1 mV in n.g. ($n = 16$) and 0.9 ± 0.1 mV in d.r.g. ($n = 9$). Thus, the responsiveness of vagal afferents to 5-HT was considerably greater than that of spinal dorsal root afferents. Responses of vagal axons to 5-HT were of much smaller amplitude than responses recorded from the ganglion.

Dose-response curves were constructed using pooled data for the effect of 5-HT on n.g. and s.c.g. The slopes of these curves were not significantly different, but the curve for s.c.g. was significantly displaced to the right (ED_{50} 0.029 and 0.098 μmol for n.g. and s.c.g., respectively).

In n.g. responses to 5-HT could be selectively antagonized by quipazine (10^{-6} M), those to GABA by picrotoxin (10^{-6} to 10^{-5} M) and those to DMPP by mecamylamine (10^{-5} M). Several analogues of 5-HT have been tested as depolarizing agents. Bufotenine was active, but less so than 5-HT; dimethyltryptamine and dimethyl-5-methoxytryptamine displayed slight activity, while tryptamine, 5-methoxytryptamine, gramine, psilocin and LSD were inactive. Neither LSD nor trazodone showed antagonist properties.

Responses evoked by 5-HT in n.g., unlike those evoked by GABA or DMPP, were greatly potentiated on removal of divalent cations from the superfusion medium. This effect was reversed on washing or by introduction of Mn^{2+} or Co^{2+} (1 mM).

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Locomotor stimulant actions of some TRH analogues

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Thyrotrophin-releasing hormone (TRH) produces several centrally-mediated behavioural effects which include stimulation of locomotor activity following injection into the rat n.accumbens, NA (Morley, 1979). Attempts have been made to enhance some of the central actions by synthesizing various analogues with modifications of the TRH tripeptide structure. The locomotor stimulant actions of several of these TRH analogues are reported here.

Female Sprague-Dawley rats (160–180 g) were fitted with bilateral cannulae for microinjection (1 µl) into both NA; locomotor activity was measured using cages fitted with a pulsed infra-red beam generator and detector unit. The following peptides were injected:– TRH (pGlu-His-ProNH_2); CG3509 (orotyl-His-ProNH_2); CG3703 (6-methyl-5-oxo-thiomorpholinyl-3-carbonyl-His-ProNH_2); RX 77368 (pGlu-His-(3,3 dimethyl)ProNH_2); PGHPA (pGlu-His-Pro-d-amphetamine). After a period of acclimatization (30 min) to the cages, locomotor activity was recorded 5–185 min after injection.

TRH (10, 20 µg) in NA stimulated locomotor activity (Table 1); the effects of TRH had ceased after 2 h. Closely similar results were obtained with PGHPA (10 µg), an analogue less-readily degraded than TRH by rat brain *in vitro* (Griffiths, McDermott, Smith & Edwardson 1981). In contrast, the locomotor stimulant activity of CG3509 and CG3703 persisted after 2 h. In the case of CG3509, the longer duration of action is probably related to the slower degradation of the parent compound because the deamidated forms of CG3509 (metabolite formed *in vitro*) was inactive after 2 h. RX 77368, the compound most resistant to degradation, was the most potent in increasing locomotor activity.

The TRH analogues tested displayed locomotor stimulant activity at least equal to, and in some cases more than, equivalent doses of TRH:– this could be due to their ability to stimulate dopamine release in NA, as is the case for TRH (Kerwin & Pycock, 1979). Their enhanced activity is probably determined by several factors including the potency of each compound as a TRH analogue and their relative resistance to degradation by cerebral enzymes. Another possibility is that metabolites formed from the analogues may modulate their actions, as has been shown for at least one TRH metabolite (Griffiths, Slater and Webster, 1981).

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Table 1 Effects of TRH and analogues in NA on locomotor activity

Compound	Dose (μg)	Activity counts (mean ± s.e.mean) 5–125 min	126–185 min	Relative degradation by rat brain in vitro*
Saline	(1 μl)	120 ± 27	10 ± 4	—
TRH	10	378 ± 61[1]	16 ± 12	1
TRH	20	413 ± 71[1]	26 ± 11	1
p-GHPA	10	386 ± 85[1]	2 ± 6	0.18
CG 3703	10	319 ± 61[1]	141 ± 26[1,2]	1.47
CG 3703 deamidated	10	209 ± 86	72 ± 46[1]	—
CG 3509	10	457 ± 81[1]	160 ± 31[1,2]	0.20
CG 3509 deamidated	10	288 ± 76[1]	5 ± 3	—
RX 77368	10	653 ± 78[1,2]	173 ± 54[1,2]	0.02

[1]Significantly greater than saline, $P < 0.05$; [2]Significantly greater than 10 μgTRH, $P < 0.05$.
$n = 6$.
*from Griffiths, McDermott, Smith & Edwardson (1981).

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Tissue glucose utilization in ethanol tolerant mice

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The effects of ethanol differ between different strains of mice. The C57BL10/ScSN(C57) strain, for example, which is diabetogenic and prefers ethanol solution to water (Goas *et al.*, 1979), is less sensitive to the depressant effects of ethanol compared to the LACG strain (Unwin & Taberner, 1980). Since ethanol is known to affect cerebral glucose metabolism (Flock *et al.*, 1970), the effects of chronic ethanol drinking on tissue glucose utilization were compared in these two strains.

Male and female mice were rendered ethanol tolerant following the chronic drinking schedule described previously (Unwin & Taberner, 1980). Blood glucose levels (BSL) and glucose uptake into cerebral cortical slices and diaphragm were determined *in vitro* (Taberner, 1973) in control and ethanol tolerant mice in both the fed state and after 24 h fasting.

The diabetogenic C57 mice showed a higher BSL than the LACGs; in both strains the males had a higher BSL than the females. The male C57 ethanol tolerant mice had significantly lower BSLs than their corresponding controls ($P < 0.01$, Student *t*-test), but the BSLs of the other groups of ethanol tolerant mice were not significantly lower compared to their controls although they were lower in LACG mice of either sex. Fasting lowered the BSL of all groups of mice compared to their corresponding controls and the BSL of fasted ethanol tolerant mice was even lower ($P < 0.05$) than corresponding fasted control mice.

Glucose uptake into either cerebral cortex or diaphragm was not significantly different between strains. However, ethanol tolerant C57 mice of both sexes showed significantly higher uptake than controls ($P < 0.05$) whereas there was no difference in the LACG mice. Glucose uptake into the diaphragm was not significantly altered by ethanol in either strain under fed or fasted conditions.

Ethanol is known to be hypoglycaemic (Metz *et al.*, 1969), due to potentiation of insulin release and possibly inhibition of glycogen synthesis. The present results indicate that ethanol is more effective in low-

ering BSL in the diabetogenic C57 mice and that this effect is still apparent during fasting. The specific increase in cerebral glucose uptake in both strains of mice may reflect the dependence of the brain on glucose for metabolism and be a protective response to lowered BSL. Further experiments are examining the significance of BSL in determining alcohol preference.

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Neurotoxicity of methytetrahydrofolate (MTHF) in rat cerebellum

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Injection of the potent neuroexcitant kainic acid into adult rat cerebellum, results in a rapid loss of inhibitory GABAergic neurones, while largely sparing the excitatory (probably glutamatergic) granule cells (Herndon & Coyle, 1977; Foster & Roberts, 1980a). Although it is still widely believed that kainate exerts its neurotoxicity through an action on glutamate receptors, both electrophysiological and neurochemical data indicate that it is unlikely to be through an action on a predominant type of receptor for this excitatory amino acid. Thus, it is feasible that kainate may interact with specific receptors for another, as yet unidentified, endogenous neuroexcitant.

Of interest, therefore, was the recent report that the pteroylmonoglutamate compound methyltetrahydrofolate (MTHF) is a potent inhibitor of [^{3}H]-kainate binding (Ruck, Kramer, Metz & Brennan, 1980). Since MTHF, like kainate possesses convulsant properties (Hommes & Obbens, 1972) we have investigated the possibility that this substance might also exhibit neurotoxic effects.

Female Wistar rats (200 g) were anaesthetized with sodium pentobarbitone (60 mg/kg i.p.), placed in a stereotaxic frame, and triple injections of MTHF (250 nmole) were made 5 mm back from Lambda at sites on, and 2.5 mm either side of the midline to a depth of 3 mm. After 14 days, animals were killed either by transcardial perfusion with Susa-Heidenhain fixative and the tissues processed for light microscopic examination of cresyl-violet-stained 10 μm sections, or by decapitation and determination of cerebellar glutamate decarboxylase (GAD) activity, and high-affinity GABA uptake.

MTHF produced approximately a 40% loss of Purkinje cells, and a further 30% of these cells appeared pyknotic; granule cells were well preserved. GAD activity and GABA uptake were reduced by approximately 20%.

Kainate and other 'excitotoxic' agents are potent stimulants of cerebellar cyclic GMP (Foster & Roberts, 1980b). MTHF was however devoid of any such activity and therefore this casts some doubt on whether its cytotoxic effects are being exerted through kainate receptors. MTHF like folate (Roberts, 1974) is an inhibitor of glutamate uptake, and it therefore seemed possible that an indirect mechanism for MTHF toxicity could at least partially be involved. In support of this idea, we have found that injections (170 nmole) of the potent glu/asp uptake inhibitor, threo-3-hydroxyaspartate is also capable of exerting neurotoxic effects in the cerebellum, although this was to a lesser extent than see with MTHF.

The demonstration of neurotoxic effects exerted by endogenous excitants such as glutamate and MTHF may have major implications for a number of neurodegenerative disorders, such as Huntingdon's disease.

G.A. Foster is an SRC Research Student.

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Analogues of thyrotrophin-releasing hormone (TRH) increase catecholamine metabolism and release in the rat nucleus accumbens *in vivo*

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The long-acting analogue of thyrotrophin releasing hormone (TRH) orotyl-histidyl-proline amide (CG 3509) stimulates locomotor behaviour when injected into the nucleus accumbens (Green, Heal, Sabbagh & Youdim, 1980) and releases endogenous dopamine (Bennett, Marsden, Metcalf, Sharp & Tulloch, 1981) *in vitro* from slices of rat accumbens. In the present study it has been shown, using electrochemical monitoring, (Conti, Strope, Adams & Marsden, 1978; Gonon, Buda, Cespuglio, Jouvet & Pujol, 1980; Brazell & Marsden, 1981) that the analogue increases catecholamine metabolism and release *in vivo*.

Graphite paste working electrodes were implanted either alone, or in combination with infusion cannulae, into regions of the nucleus accumbens of anaesthetized (chloral hydrate 450 mg/kg, i.p.) male Wistar rats (270–275 g) and electrochemical oxidation peaks were monitored using differential pulse voltammetry (Princeton Applied Research, Model 174A) as described previously (Marsden, Bennett, Brazell, Sharp & Stolz, 1981; Brazell & Marsden, 1981). All drugs were administered by infusion (1–1.5 µl over 1.5 min) either directly into the accumbens or into both lateral ventricles.

Differential pulse scans (10 mv/s, pulse rate 2/s) from −0.1 V to +0.5 V one every 8 min in the n. accumbens demonstrated always two and sometimes three oxidation peaks. The two consistent peaks were at about +0.22 V and +0.35 V, the latter corresponding to the oxidation of 5HT and 5HIAA (Brazell & Marsden, 1981). The inconsistent peak was at +0.12 V and was most obvious shortly after electrode implantation. Infusion of ascorbic acid into the accumbens produced a selective and dose related (5×10^{-5} to 5×10^{-6} M) increase in the peak at +0.12 V. The peak at +0.22 V was selectively increased by infusion of either dopamine or DOPAC (5×10^{-6} M). Furthermore the peak at +0.22 V was decreased over a period of 4 h following injection of the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine (250 mg/kg, i.p.).

Infusion of the TRH analogue CG 3509 (1 µg) into the medial region of the n.accumbens also significantly and selectively increased the peak at +0.22 V. The increase was maximal (+44%, $n=4$) ~40 min after infusion and was apparent for about 2 h. No change was observed in the peak at +0.12 V while that at +0.35 V was either unchanged or slightly decreased. The increase in the 0.22 V peak was greater (+95%, $n=3$) when a higher (5 µg) dose of CG 3509 was infused. Infusion of CG 3509 (5 µg) into the lateral ventricles also increased (+54%, $n=4$) the peak at +0.22 V in the medial accumbens but not that in either the lateral region of the accumbens or the central area of the striatum. Preliminary results with another TRH analogue, pyroglutamyl-histidyl-3,3-dimethylprolineamide (RX 7738) indicate that it has similar effects on the peak at +0.22 V. Neither of the analogues showed electroactivity *in vitro* at potentials below +0.85 V.

The high concentration of dopamine in the accumbens combined with the present results indicate that the peak at +0.22 V is due to the oxidation of dopamine and DOPAC. A contribution from the oxidation of noradrenaline however cannot be excluded. The increase in this peak by CG 3509 shows

that changes in dopamine release observed *in vitro* (Bennett *et al.*, 1981) also occur *in vivo* further indicating that certain TRH induced behaviours may involve dopamine release.

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A pH gradient may contribute to uptake into synaptosomes and synaptic vesicles

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Biogenic amines and a number of basic drugs appear to be specifically taken up by a variety of tissues, particularly synaptosomes in nervous tissue and by platelets. Uptake by both synaptosomes and platelets possesses similar kinetics, and uptake by each appears to have many features in common; it occurs against a concentration gradient, is energy dependent, saturable and competitive; similar basic molecules are taken up by each. It is now accepted that the uptake of monoamines into nervous tissue is an active process since it occurs against a concentration gradient, but the physical basis of the uptake mechanism is unclear.

The pH gradient across membranes is considered important in the transport of weak electrolytes (Jacobs, 1940). For this reason we have attempted to measure pH within synaptosomes and synaptic vesicles in order to determine whether the pH gradient was of sufficient magnitude to contribute to the observed transport phenomena.

Synaptosomes and synaptic vesicles were isolated by differential and density-gradient centrifugation (Gilbert & Wyllie, 1976). All incubations of subcellular fractions were carried out in a Tris-Krebs medium containing mM: NaCl 136, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 2.5, glucose 10, ascorbate 1, Tris 20, which was adjusted to pH 7.4 (synaptosomes) or pH 6.9 (synaptic vesicles) with HCl and gassed with pure oxygen. Water space measurements were made by resuspending the appropriate fraction (1–4 mg/ml) in Tris-Krebs containing [^{14}C]-mannitol (final concentration 1 μCi, 5 mM) and [^{3}H]-orthomethylglucose ([^{3}H]-OMG) (final concentration 1 μCi, 5 mM). The fractions were then incubated at 37°C for 1 h and filtered under vacuum through cellulose acetate filters (0.45 μm for synaptosomes, 0.025 μm for synaptic vesicles). The filters and filtrates were then counted for radioactivity after 24 h in Instagel. The intracellular water volume was calculated as the difference between the volumes of distribution of [^{3}H]-OMG and [^{14}C]-mannitol. In a separate series of experiments the pH gradient (Δ pH) across the membrane was measured from the accumulation of [^{14}C]-methylamine (0.9 μCi, 17 μM final concentration). This weak base (pKa 10.6) was assumed to distribute across the membrane (outside, O and inside, I) in response to the pH gradient so that:

$$\Delta \text{pH} = \log \left(\frac{[\text{Methylamine}]\ \text{I}}{[\text{Methylamine}]\ \text{O}} \right).$$

As described elsewhere (Wood & Wyllie, 1981) the intrasynaptosomal water volume was 6.3 ± 0.3 (6) μl/mg protein. Using this value in the calculations the intrasynaptosomal pH was found to be 6.9 ± 0.7 (7) and independent of the pH of the Tris-Krebs over the range 7.5–6.5. Similarly for synaptic vesicles the corresponding values were found to be 3.7 ± 0.6 (4) μl/mg protein and 5.8 ± 0.3 (6).

The possibility thus exists that an acidic environment within synaptosomes and vesicles contributes to the uptake of neurotransmitters and other basic substances. It is therefore feasible that substances with the appropriate ionization characteristics are passively taken up due to the presence of a pH gradient generated by energy-dependent processes.

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Effects of intrahypothalamic and intracerebroventricular injections of Δ^9-tetrahydrocannabinol on thermoregulation in restrained mice

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At ambient temperatures below thermal neutrality, Δ^9-tetrahydrocannabinol (Δ^9-THC), the main centrally active constituent of cannabis can produce marked falls in body temperature. In mice this is due mainly to an effect on heat production (Pertwee & Tavendale, 1977). The thermoregulatory system is a useful model for studying mechanisms of drug action *in vivo* and it was therefore decided to search for the sites at which Δ^9-THC acts to alter thermoregulation in mice. Physically restrained, 20 to 28 g male mice (MFI) were used at an ambient temperature of 22°C. Δ^9-THC was injected (0.5 μl) either unilaterally into the preoptic anterior hypothalamic nuclei (Nyemitei-Addo & Pertwee, 1981) or into the fourth ventricle. For intracerebroventricular injection, a cannula guide was implanted chronically to a depth of 3 mm, 1.8 mm caudal to the lambda. The tip of the injection cannula extended 1 mm beyond that of the guide. Δ^9-THC was mixed with two parts of Tween 80 by weight and then dispersed in 0.9% (w/v) NaCl solution. Rectal temperature (Tr) was used as a measure of core temperature and oxygen consumption (Vo_2) as a measure of heat production (Pertwee & Tavendale, 1977). Vo_2 was measured over consecutive 10 min periods before and after injection of drug or vehicle. Data are expressed as means ± standard errors ($n = 6$).

A dose of Δ^9-THC (5 μg) which had no effect when given intravenously significantly ($P < 0.01$) lowered Tr (1.7 ± 0.2°C) and Vo_2 (19 ± 4 ml $25g^{-1}h^{-1}$) when given intrahypothalamically. Corresponding falls after Tween (20 μg) were 0.3 ± 0.2°C ($P > 0.1$) and 15 ± 5 ml $25g^{-1}h^{-1}$ ($P < 0.05$). The responses to intrahypothalamic Δ^9-THC did not increase significantly when the dose was raised to 10 μg and were far less than those which can be elicited by intravenous injections of the drug (Pertwee & Tavendale, 1977) albeit at doses much greater than those effective intrahypothalamically. Possibly in mice, as in rats and cats (Schmeling & Hosko, 1976; 1980) Δ^9-THC may act at extrahypothalamic as well as hypothalamic sites to lower body temperature. Results consistent with this hypothesis were obtained when Δ^9-THC was injected into the fourth ventricle. This lies near midbrain, pons and medulla oblongata all of which are thought to have thermoregulatory roles. Intraventricularly, a dose of 5 μg produced maximum falls in Tr and Vo_2 of 1.3 ± 0.2°C ($P < 0.01$) and 37 ± 9 ml $25g^{-1}h^{-1}$ ($P < 0.02$) respectively. 10 μg produced falls of 2.4 ± 0.3°C ($P < 0.001$) and 62 ± 13 ml $25g^{-1}h^{-1}$ ($P < 0.01$) both significantly greater than peak falls detected after unilateral intrahypothalamic injections of the same dose (1.4 ± 0.2°C and 30 ± 5 ml $25g^{-1}h^{-1}$). Corresponding falls after Tween (20 μg) were 0.6 ± 0.2°C ($P < 0.02$) and 13 ± 4 ml $25g^{-1}h^{-1}$ ($P < 0.05$). All effects of intraventricular injection were abolished when mice were kept at a thermally neutral ambient temperature (32°C). The extent to which the responses to intraventricular injection were caused by interaction

(bilateral) with hypothalamic thermoregulatory centres after diffusion into the third ventricle is now being investigated.

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Ethanol-induced alterations in phospholipid methylation in rat brain synaptosomal membranes

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The progressive methylation of phosphatidyl ethanolamine (PE) to phosphatidylcholine (PC) has been suggested to play an important role in such synaptic events as calcium entry and receptor-response coupling including that of the β-adrenoceptor to adenylate cyclase (see Hirata & Axelrod 1980). Since ethanol is considered to act at the level of the synaptic membrane, and since alterations in both calcium entry (Friedman *et al.*, 1980) and β-adrenoceptor sensitivity (Tabakoff & Hoffman, 1980) are associated with ethanol tolerance it is of interest to consider the effects which ethanol may have on synaptosomal phospholipid methylation.

To study the effects of ethanol *in vitro* synaptosomes were prepared from rat brain by the method of Cotman (1974). Synaptosomes were then incubated in HEPES buffer (pH 7.3) for 1 h at 37°C in the presence of S-adenosyl-L-methionine (200 μM) containing 1 μCi of S-adenosyl-L-([^{3}H]-methyl) methionine (15 Ci/mmol, Radiochemical Centre, Amersham) as methyl donor. At the end of this period the synaptosomal lipids were extracted with chloroform: methanol: HCl (2:1:0.02 v/v) or, alternatively, lysed synaptosomal membranes were subjected to filtration (Millipore filters 0.45 μm) before being taken for scintillation counting for [^{3}H]. There was good agreement between the methods, synaptosomes from control animals incorporated [^{3}H] from S-adenosyl-L-([^{3}H]-methyl)-methionine at a rate of 67.1 ± 7.0 pmol mg protein^{-1} h^{-1} (mean ± s.e.mean, $n = 8$). The majority of the label in the lipid fraction was found to co-migrate with PC on thin layer chromatograms and incorporation of [^{3}H] was increased in the presence of isoprenaline (5×10^{-5}M) as described previously (see Hirata & Axelrod, 1980). It is concluded that [^{3}H]-incorporation represents mainly the methylation of other phospholipids to PC.

When ethanol was added to the incubation mixture *in vitro* synaptosomal phospholipid methylation was depressed in a concentration-dependent manner. At a pharmacologically relevant concentration (50 mM ≡ 2.3 mg/ml) ethanol produced a $25 \pm 5\%$ (mean ± s.e.mean, $n = 8$) reduction in incorporation of labelled S-adenosyl-L-methionine.

When synaptosomes were obtained from rats to which ethanol had been administered either acutely (2.5 g/kg^{-1} i.p. 30 min before sacrifice), or chronically (5–10 days by inhalation, blood ethanol concentrations 2–4 mg/ml on sacrifice) there was a tendency for [^{3}H]-incorporation to be increased above the levels found in control animals. This reached the level of significance ($P < 0.05$ in Student's test) in the animals treated chronically with ethanol ($140 \pm 10\%$ vs $100 \pm 13\%$ in controls, all values mean ± s.e.mean, $n = 8$). The addition of ethanol *in vitro* at a concentration of 50 mM to these chronically ethanol-treated rat synaptosomes depressed phospholipid methylation to a level which did not differ significantly from that of control synaptosomes in the absence of ethanol. It is possible that such an effect might represent the development of tolerance to the drug. On the other hand synaptosomes obtained from rats undergoing a

physical syndrome of withdrawal from ethanol ($n=4$) did not differ significantly in [^{3}H]-incorporation from those of control animals suggesting that phospholipid methylation is not involved in withdrawal phenomena.

Recent evidence from others (Lee *et al.*, 1981) also shows increased *in vivo* synthesis and breakdown of PC during the development of ethanol tolerance and dependence. Increased activity of arachidonic acyl: PC transferase in chronically ethanol-treated rats (Sun *et al.*, 1977) also attests to increased flux through these pathways in brain. Whether these changes contribute to the development of ethanol tolerance or dependence is a matter for continuing study.

These experiments were supported by the British Council and the Medical Research Council.

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The effects of caffeine and fasting on finger tremor

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In a trial designed to measure effects of caffeine on fat and protein utilisation during fasting, finger tremor was also measured in 7 subjects. During the fasting period, of 41–42 h duration at the time of tremor measurement, subjects consumed only clear fluids and NaCl (250 mmol) and KCl (3 mmol). The subjects fasted on two occasions and received, three times a day for two days, either caffeine (150 mg) or lactose placebo in each fast, the design being random and double blind.

Measurements were taken from an accelerometer on the middle finger (Birmingham, Williams, Wilson & Wright, 1977) of each hand with (i) the hand relaxed ('rest' tremor) (ii) the arm outstretched ('postural' tremor) (iii) the middle finger exerting an upward thrust ('work' tremor) and (iv) the forearm supported and hand extended at the wrist ('modified postural' tremor). For each measurement the root mean square (r.m.s.) of tremor amplitude was calculated. Plasma caffeine concentration 2 h after the fifth capsule (mean ± s.d.) was 8.79 ± 4.3 mg/l on caffeine and <0.9 mg/l on placebo.

The protocol was repeated in the same subjects consuming their normal diet, excluding caffeine-containing beverages. Tremor was measured 1–2 h after the sixth capsule and last meal when plasma caffeine concentrations were 9.43 ± 2.95 mg/l on caffeine and <0.9 mg/l on placebo.

Tremor amplitude tended to increase during fasting; when the subjects were taking caffeine, the increase was statistically significant ($P<0.05$) for rest and postural tremor of the dominant hand and postural and work tremor of the non-dominant hand. For non-dominant hand rest-tremor the effect of caffeine during fasting was to reduce tremor ($P<0.01$). RMS amplitudes for the dominant hand are shown in Figure 1.

Caffeine seemed to have little effect in the absence of fasting but enhanced the change in tremor, usually an increase, which accompanied fasting.

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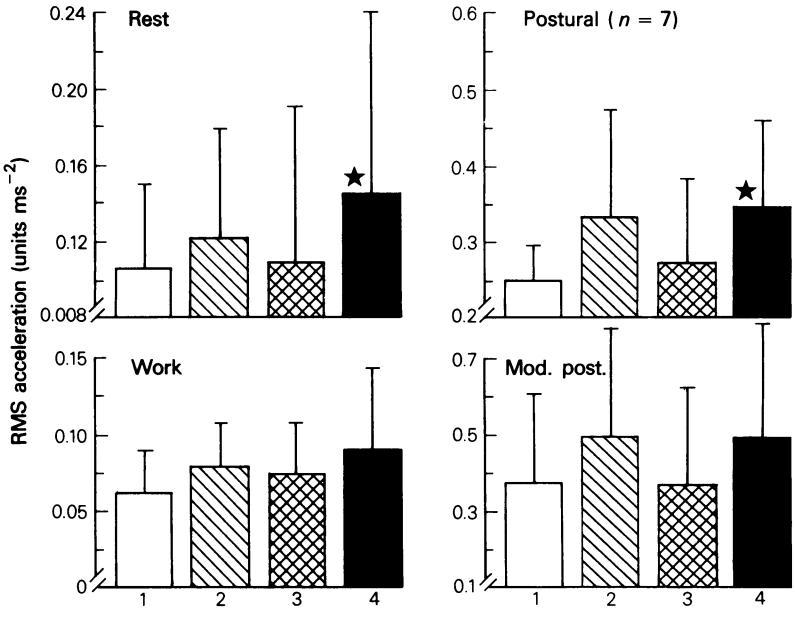

Figure 1 The means (+ 1 s.d.) of the dominant hand tremor amplitude (rms acceleration, units ms^{-2}) for the four treatments: 1. normal diet without caffeine; 2. fasting without caffeine; 3. normal diet with caffeine; 4. fasting with caffeine. * $P < 0.05$: paired t test.

Plasma protein binding and distribution in the blood of theophylline and aminophylline

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Theophylline (T, 1,3-dimethylxanthine) is only sparingly soluble in water. To facilitate its solution, it is commonly combined with ethylenediamine (EDA), the combination of T and EDA being known as aminophylline (A).

It is generally regarded that A and T are pharmacologically equivalent on a molar basis, and that A readily dissociates into its components in solution. Recently we have reported that the rate and extent of metabolism of T in man is enhanced when it is given in the form of A (Monks, Caldwell & Smith, 1981).

Possible mechanisms for this interaction might involve alterations in either the protein binding or membrane passage of T in the presence of EDA: we have thus studied these events for T, EDA and combinations thereof.

All experiments used citrated whole blood, haematocrit 48–49%. Plasma was obtained by centrifuging whole blood for 10 min at 900× g and washed erythrocytes by taking the pellet and resuspending in isotonic saline to a haematocrit of 40%. [8-^{14}C]-Theophylline and [U-^{14}C]-ethylenediamine hydrochloride were dissolved in isotonic saline. In some experiments, unlabelled T or EDA were added to the [^{14}C]-compounds (as shown in Table 1) to give compositions equivalent to aminophylline. Plasma protein binding was assayed by addition of the compound in isotonic saline to 1 ml plasma, equilibration at 37° for 30 min followed by ultrafiltration (Amicon CF 25 membranes) at 37°. Partitioning of the compounds between plasma or isotonic saline and erythrocytes was measured by addition of the compound to 5 ml of whole blood or washed erythrocytes (equivalent to 5 ml whole blood) equilibrating at 37° for 30 min and then centrifuging at 900 × g for 10 min at 37°. The supernatant was removed and counted for [^{14}C]. In some experiments, an equivalent volume of isotonic saline was then added to replace the supernatant and the procedure repeated. [^{14}C] was estimated by liquid scintillation spectrometry.

Results from the various experiments are summarized in Table 1. The plasma protein binding of T was 38% and this was independent of concentration.

Table 1 Plasma protein binding and erythrocyte uptake of theophylline, ethylenediamine and combinations thereof

	% bound to plasma protein	*Distribution in whole blood*		*Distribution in washed erythrocytes*	
		% in plasma	*haematocrit*	*% in supernatant*	*haematocrit*
[^{14}C]-T (0–50 μg/ml)	38	54	48	35	40
[^{14}C]-T (0–5 μg/ml) +EDA (0–20% w/w of T)	38	54	48	35	40
[^{14}C]-EDA (0–15 μg/ml)	n.d.	53	49	33	40
[^{14}C]-EDA (0–15 μg/ml) +T (78% w/w of EDA)	n.d.	53	49	33	40

n.d. = not detected

Addition of various amounts of EDA had no influence. EDA itself was not bound to plasma protein alone or in combination with T. The plasma/erythrocyte distribution of T in whole blood showed the extracellular concentration was slightly higher than plasma volume. This partition was not affected by EDA, which alone or with T showed the same pattern of distribution as T. Experiments with washed erythrocytes confirmed the above results. When the erythrocytes containing either drug were washed with isotonic saline, the distribution was identical, showing that there occurred no irreversible binding to the cells.

The results of this work show that the components of A behave independently and have no influence on each others plasma protein binding or passage across the erythrocyte membrane. This is consistent with the view that A does not behave as a molecular complex of T and EDA under physiological conditions, and thus other reasons must be sought for the dispositional differences between T and A in the human body.

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Further studies on the encapsulation of drugs in erythrocytes as an intravenous delivery system

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In a previous communication (Lewis & Pitt, 1980) we reported the successful encapsulation of hydrocortisone-21-phosphate and prednisolone-21-phosphate in rat erythrocytes. We also demonstrated that the preparation was not removed by the reticuloendothelial system when returned intravenously to the rats' circulation.

In this communication we have extended the use of the erythrocyte as an encapsulating vehicle to two anti-neoplastic drugs, cyclophosphamide and methotrexate. Up to 640 μg of cyclophosphamide (as determined by GLC – Pantarotto *et al.*, 1946) or 610 μg methotrexate (as determined by inhibition of dihydrofolate reductase – Werkheiser *et al.*, 1962) have been encapsulated per ml of packed red blood cells. By labelling these RBCs with fluorescein isothiocyanate (externally) prior to reinjecting back into the animal (i/v) we have been able to show that some cells encapsulated with either cyclophosphamide or methotrexate can survive for 50 days – the normal life-span of the rat erythrocyte.

Furthermore by using a fluorescent conjugate of methotrexate (F-MTX) as synthesized by Gapski *et al.*, (1975) we have demonstrated that young erythrocytes are capable of taking up more drug than the older cells. Using F-MTX a maximum encapsulation of approximately 34% was achieved (i.e. 34% fluorescent cells). Subsequent separation of these cells on a Ficoll gradient revealed that the majority of the u/v fluorescent cells were in the upper layers indicating that they were the less dense younger cells.

One factor important in determining the survival of these loaded cells *in vivo* is that the encapsulation procedure does not damage the cell membrane. We have shown that damaged cells are quickly removed from the circulation by phagocytosis. For *in vitro* tests

phagocytic leucocytes were raised in the rat by i/p saline injection. Any foreign blood cells are rapidly destroyed by these phagocytic cells and we also found this occurred with cells damaged during the encapsulation process. However, undamaged cells (encapsulating either cyclophosphamide or methotrexate) were not attacked. These results indicate that this test, using phagocytic leucocytes, could be useful in determining the viability of encapsulated cells.

A variety of compounds (e.g. steroids, antineoplastic drugs, insulin, α-1-antitrypsin and oral hypoglycemic drugs) have now been successfully encapsulated into erythrocytes and these preparations are under investigation in our laboratories for their effectiveness as delivery systems in treating various forms of disease.

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Rowachol, a mixture of six pure monoterpenes, inhibits lipogenesis in the rat when measured *in vivo*

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Rowachol (Rowa Ltd, Bantry, Eire) is a proprietary choleretic containing the following pure monoterpenes, menthol (32%), pinene (17%), menthone (6%), borneol (5%), camphene (5%) and cineol (2%) in olive oil. In man the chronic administration of Rowachol can aid cholesterol gallstone dissolution (Bell & Doran, 1979; Ellis, Middleton Bell *et al.*, 1981) and cause a 60% inhibition in hepatic S-3-hydroxy-3-methyl-glutaryl CoA reductase (HMGR) (Ellis, Bell, Clegg *et al.*, 1981). A single dose of Rowachol causes the same inhibition in hepatic HMGR when measured at its diurnal maximum in rats under reverse lighting (Clegg, Middleton, Bell *et al.*, 1980) and we wished to determine whether this effect was independent of diurnal variation in HMGR activity and whether it resulted in changes in sterol synthesis *in vivo*.

Rates of fatty acid and sterol synthesis *in vivo* were determined by measuring incorporation of injected [^{3}H]-H_2O into lipids (Lowenstein, Brunengraber & Wadke, 1975; Jeske & Dietschy, 1980). Rowachol inhibited sterol and fatty acid synthesis by $> 50\%$ in liver (Table 1) but no significant changes were found in five extrahepatic tissues. The inhibition of fatty acid synthesis may be linked to the depletion of liver glycogen in Rowachol treated animals (Table 1) since

Table 1 The effects of a single dose of Rowachol (2 ml/kg) on rates *in vivo* of hepatic sterol and fatty acid synthesis in fed rats, hepatic glycogen contents in fed rats and activities of microsomal HMGR from liver of both fed and 24 h starved rats

Treatment†	*Lipogenesis rates* in vivo *in fed rats* (μg atom of H incorporated $h^{-1} g^{-1}$) *Sterol synthesis*	*Fatty acid synthesis*	*Glycogen content in fed rats* (μmol glucose/g)	*HMGR activity in fed rats* (pmol min^{-1} mg microsomal protein^{-1})	*HMGR activity in 24 h starved rats* (pmol min^{-1} mg microsomal protein^{-1})
Controls (olive oil dosed)	5.2 ± 1.4 (6)	21.2 ± 4.4 (5)	287.6 ± 69.7 (3)	380 ± 20 (3)	98 ± 38 (4)
Rowachol	2.5 ± 0.7** (6)	9.6 ± 2.6** (5)	96.8 ± 27.4* (3)	190 ± 40** (3)	37 ± 7* (4)

† Rats (180–220 g) subjected to normal lighting (lit from 08.00–20.00) were dosed with Rowachol or olive oil (2 ml/kg) at 17.00 and killed 17–19 h later.

glycogen is a major precursor of fatty acids (Salmon, Bowen & Hems, 1974).

The terpene induced reduction in HMGR activity was not directly linked to loss of liver glycogen since Rowachol still caused inhibition in 24 h starved rats.

We conclude that a single dose of Rowachol rapidly inhibited sterol synthesis *in vivo* by specifically decreasing hepatic HMGR activity in a manner independant of diurnal control of this enzyme.

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Effect of GABA agonists, glycine and neuropeptides on the release of acetylcholine (ACh) from the rabbit retina *in vivo*

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ACh is present in all vertebrate retinas, where it is believed to be a transmitter substance (Neal, 1976a). In most species, the identity of the cholinergic neurones is unknown, but in the rabbit retina, there is strong evidence that the cholinergic neurones are restricted to a sub-population of amacrine cell (Masland & Mills, 1980). The transmitters utilized by other sub-populations of amacrine cells are believed to include GABA, glycine, dopamine and certain peptides (Neal, 1976b, Stell, *et al.*, 1981). The present experiments were undertaken to investigate the effect of these transmitters on the spontaneous and light evoked activity of the cholinergic amacrine cells.

Rabbits were anaesthetized with urethane (1.5 g/kg i.p.) and a ring (1.3 cm diameter) was sutured to the eye for support. The cornea, iris, lens and vitreous were removed and the retina was loaded with [^{3}H]ACh by exposing it to medium containing [^{3}H]Ch (10 μM). The release of [^{3}H]ACh from the dark-adapted retina during 5 min collecting periods was measured as described previously (Neal & Cunningham, 1981). The retina was stimulated with light flashes (3 Hz, 25% duty cycle, 4680 photopic lux for 5 min) and the response was monitored by recording the E.R.G.

When the retina was exposed to GABA, 3-aminopropane sulphonic acid (3APS) or muscimol there was no change in the spontaneous resting release but the light evoked release of [^{3}H]ACh was inhibited. The concentrations of the drugs which reduced the response by 50% (IC_{50}) were 900, 5 and 0.3 μM respectively. Bicuculline (5 μM) and picrotoxin (20 μM) antagonised the action of GABA and muscimol. In addition, the GABA antagonists strikingly increased both the resting release and the light evoked release of [^{3}H]ACh.

Glycine and taurine both inhibited the light evoked release of [^{3}H]ACh ($IC_{50} \simeq 1.2$ mM and 0.3 mM respectively), an effect which was blocked by strychnine (20 μM), but not by bicuculline (5 μM). In contrast to the GABA antagonists, strychnine had no effect on the spontaneous or light evoked release of [^{3}H]ACh at concentrations up to 20 μM.

The following compounds had no effect on [^{3}H]ACh release: dopamine (1 mM), 5HT (4 mM), morphine (10 μM), somatostatin, CCK, TRH, LHRH, Substance P and angiotensin at concentrations up to 1 μM.

These results suggest that cholinergic amacrine cells in the rabbit possess glycinergic and GABAergic inputs, the latter apparently being tonic in both the dark and light adapted retina.

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The effects of high pressure and of ketamine on GABA responses *in vitro*

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It is known that the high pressure neurological syndrome (HPNS), characterized by tremors and convulsions, is opposed by anaesthetics, although this action correlates poorly with general anaesthetic potency. Recently we have demonstrated (Bichard, Little & Paton, 1981) that drugs which selectively facilitate GABA transmission also decrease the HPNS, suggesting possible involvement of GABA in its aetiology.

The present experiments studied the effects of pressure and of anaesthetics on synaptic transmission with particular reference to GABA. The method of Brown & Marsh (1974), for recording post-synaptic responses from the rat superior cervical ganglion was adapted for use in the pressure chamber. Tests using artificial potential changes showed pressure did not affect the recording system. As the chamber capacity restricted the drug application to one concentration per experiment the ED_{85} and ED_{50} were chosen and the effects of pressure studied on repeated doses of each.

Helium pressure (130 atm) caused little change in GABA responses (Table 1). Results are presented as percentages for clarity but analysis of variance using the original date showed that, in total, the response amplitude was not significantly altered by pressure. Cholinergic responses, in contrast, were decreased by pressure.

Ketamine was selected as a general anaesthetic particularly effective against the HPNS (Green, Halsey & Wardley-Smith, 1977). At normal pressure ketamine considerably potentiated the responses to GABA, for example the response to GABA at ED_{50} was increased $27 \pm 2\%$ by 18 μM ketamine and $126 \pm 24\%$ by 180 μM ketamine ($n = 6$). Table 1 shows that pressure did not alter the GABA responses after potentiation by ketamine.

Whilst the dangers of extrapolation from isolated peripheral tissues to *in vivo* are recognized we suggest from these results that facilitation of GABA transmission may contribute to the anticonvulsant properties of ketamine at pressure and that it is unlikely that depression of postsynaptic responses to GABA are involved in the genesis of the HPNS.

Table 1

Concentration of GABA	*n*	*Responses before pressure*		*Responses at 130 atm helium* *Time after pressure applied (min)* 15	30	45	60	75
i) *Control conditions*								
19.4 μM	(8)	104 ± 4	99 ± 4	95 ± 8	83 ± 7	89 ± 7	97 ± 7	103 ± 10
9.7 μM	(7)	99 ± 9	98 ± 9	(107 ± 19)*	91 ± 9	87 ± 6	91 ± 10	99 ± 16
ii) *Ketamine 180 μM*								
19.4 μM	(6)	100 ± 4	98 ± 3	99 ± 5	91 ± 4	95 ± 3	95 ± 3	96 ± 5
9.7 μM	(6)	94 ± 5	96 ± 4	80 ± 7	92 ± 6	87 ± 8	95 ± 4	104 ± 6

Three control responses were obtained before application of pressure. The results are expressed as percentages of the first (omitted) control response (mean ± s.e.mean). Mean amplitude of control responses to 19.4 μM GABA was 670 μV. Separate experiments showed that ketamine, 180 μM, increased this by $103 \pm 21\%$ ($n = 6$).
*Only four results obtained.

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Anticonvulsant activity of GABA uptake inhibitors in DBA/2 mice

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Pharmacological manipulation of GABA-mediated neuronal inhibition may be a useful approach for the development of new anticonvulsant drugs (Meldrum, 1978, 1981). The recent development of active and specific inhibitors of neuronal and glial GABA uptake (see Krogsgaard-Larsen *et al.*, 1981) has provided a means of pharmacologically elevating synaptic GABA levels which may reduce susceptibility to seizures. As the known inhibitors of GABA uptake are highly polar compounds and thus unable to penetrate into the CNS after parenteral administration (Frey *et al.*, 1979) we have investigated the activity of some new, more lipid soluble, uptake inhibitors and pro-drugs following central and systemic administration.

Groups of DBA/2 mice, 21–28 days old, were injected either i.p. (0.1 ml) or i.c.v. (10 μl, under light ether anaesthesia) with drug or saline, 30–45 min before testing. Auditory stimulation (Friedland Chimes 3 in. electric bell generating 109 dB at mouse level) was applied for 60 s or until tonic extension occurred and the incidence and timing of the phases of the seizure response recorded. Statistical comparisons between groups of control and drug-treated animals were made using Fisher's exact probability test (seizure response) or Student's *t*-test (rectal temperatures).

Mice receiving the maleate salt of R.S-nipecotamide (2 μmoles i.c.v.) showed complete protection against seizures. This was preceded by reduced rectal temperature and signs of epileptiform activity in the absence of auditory provocation. The tartrate salts of (+) and (−)-nipecotamide were administered separately in a subsequent experiment (1–4 μmoles i.c.v.). Their lack of anticonvulsant activity suggested a slow or inactive hydrolysis to the free, active nipecotamide isomers.

(RS) – Nipecotic acid pivaloyloxymethyl ester reduced seizure incidence after both i.c.v. (3.2 μmoles) and i.p. (1.6–3.2 mmoles/kg) administration. After central administration, acute respiratory distress and myoclonic activity were seen, with only 50% of animals recovering normal activity before testing. Some toxicity was seen after systemic administration (ataxia, reduced locomotion) but only at the highest dose.

Mice receiving THPO (4,5,6,7-tetrahydroisoxazolo 4,5-c pyridin-3-ol) intracerebroventricularly (1–5 μmoles) were protected against all phases of the seizure response. A dose-dependent sedation occurred immediately after injection with many animals still showing reduced locomotion, ataxia and other signs of GABA toxicity (piloerection, hunched-posture) at the time of testing. After systemic administration of THPO (1–4 mmoles/kg) complete protection was again observed with slightly reduced locomotion and mild hypothermia seen only at the highest dose.

Following i.c.v. injection of either the methyl or ethyl ester of cis-4-hydroxynipecotic acid (2–4 μmoles) no anticonvulsant effects were observed although a dose-dependent reduction of locomotion and ataxia was noted after the methyl ester, accompanied by reduced rectal temperature. Following systemic administration of the methyl ester, however, (1.6–3.2 mmoles/kg) reduction in seizure incidence was observed. Marked toxicity was present at the highest dose.

These results demonstrate that GABA pro-drugs which can penetrate the blood-brain barrier after systemic administration and subsequently inhibit the high affinity uptake of GABA can reduce the incidence of audiogenically induced seizures in mice. Whether this activity can be usefully dissociated from the accompanying toxic side-effects remains to be determined.

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[^{3}H]-Nipecotic acid binding as a means of studying GABA uptake sites

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Uptake studies are often used as a tool for identifying and quantifying the presence of nerve terminals for specific neurotransmitters. However, this technique usually requires that freshly prepared synaptosomes or brain slices be used. Often it would be most informative to be able to study the kinetics and pharmacology of neurotransmitter uptake under conditions (e.g. post-mortem or frozen tissue) which are not suitable for synaptosomal or brain slice studies. For the GABA uptake systems we have tried to attack this problem by the use of specific [^{3}H]-nipecotic acid binding. Nipecotic acid (Nip) is a relatively specific inhibitor of neuronal GABA uptake without activity on post-synaptic GABA receptor binding (cf. Brehm, Krogsgaard-Larsen & Jacobson, 1979).

We have recently shown (Lloyd & Vargas, 1981) that [^{3}H]-Nip binds to membranes prepared from fresh or frozen rat brains with a single Kd (12 μM for fresh and 5 μM for frozen tissue). The pharmacology of [^{3}H]-Nip binding is very similar to [^{3}H]-Nip or [^{3}H]-GABA uptake but is totally unlike that of sodium-independent [^{3}H]-GABA binding to post-synaptic GABA receptors. We presently extend the pharmalogical observations on [^{3}H]-Nip binding and describe the regional distribution and sodium dependency in the rat brain.

Rat brains were removed, dissected into different regions and immediately frozen at −80°C. Membranes were prepared as described previously (Lloyd & Vargas, 1981) and the binding assay included NaCl (200 mM), unless otherwise indicated.

[^{3}H]-Nip binding was highly dependent on the concentration of NaCl in the medium. Without added NaCl virtually no specific binding (displaceable by 5 mM GABA) of [^{3}H]-Nip (5 μM) occurred. Specific [^{3}H]-Nip binding increased from 30 (9 pmol/mg protein) to 200 (70 pmol/mg protein) mM NaCl; 300 mM NaCl gave the maximal effect. It appeared that sodium was essential as neither KCl nor LiCl replaced the NaCl.

Scatchard analysis of saturation curves were performed for [^{3}H]-Nip binding (200 mM NaCl; 0.1–100 μM [^{3}H]-Nip) for different rat brain areas. Kd's (μM) were obtained for hypothalamus (2.4); N. accumbens (2.6); olfactory trigone (2.8); cerebral cortex (3.2); hippocampus (3.4); thalamus (3.4); olfactory bulbs (3.5); striatum (4.2); cerebellar cortex (4.4); medulla (5.9) and midbrain (7.0). The Bmax (pmol/mg protein) under these conditions were found to be, in decreasing order: cerebral cortex = 96; hippocampus = 95; olfactory trigone = 78; striatum = 72; olfactory bulbs = 63; thalamus = 62; hypothalamus = 58; N. accumbens = 53; cerebellar cortex = 44; midbrain = 40 and medulla = 26. Specific [^{3}H]-Nip binding did not occur in peripheral tissues such as heart or kidneys. This distribution is very different from that of sodium-independent [^{3}H]-GABA binding, but is similar to the distribution of GAD (Roberts, 1979) or autoradiography of [^{3}H]-GABA uptake (Bloom & Iversen, 1971).

Another possible binding site for GABA related drugs is the recently described baclofen-sensitive, bicuculline-insensitive $GABA_B$ receptor (Hill & Bowery, 1981). In the present system, baclofen in concentrations up to 5 mM did not alter [^{3}H]-Nip binding to cortical membranes.

These data support the hypothesis that [^{3}H]-Nip binds specifically to neuronal GABA uptake sites, and suggest that this ligand may be used in a quantitative manner to estimate the kinetics of GABA uptake sites in frozen or post-mortem material.

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Synaptosomal GABA release: effects of modifying the rates of GABA synthesis and degradation

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Release of neurotransmitters from synaptosomes is frequently used to determine drug effects such as direct releasing properties and modification of stimulated transmitter release. For this purpose, the neurotransmitter pool is usually prelabelled with a radioactive tracer and the radioactivity released in a superfusion system. Here we describe a novel, automated superfusion unit consisting of ten chambers in parallel and in which the upstream and downstream dead volumes were maintained at minimum values by use of a liquid-level detector.

This system has been used to study three properties of various GABA analogues: (1) direct GABA-releasing effects, (2) inhibition of GABA: 2-oxoglutarate aminotransferase (GABA-T, E.C. 2.6.1.19), and (3) irreversible effects on potassium-evoked GABA release after acute exposure of synaptosomes to the compounds.

Rat cerebral-cortical synaptosomes were obtained by a standard procedure (Gray & Whittaker, 1962), labelled with [2,3 ^{3}H] GABA and transferred to the superfusion chambers where they were perfused at 37°C at a rate of 0.5 ml/min.

Nipecotic acid, N-methylnipecotic acid, guvacine, 3-hydroxy-5-aminovaleric acid, cis-4-hydroxy-nipecotic acid, L-2,4-diaminobutyric acid and γ-vinyl-GABA (GVG, RMI 71754) released [^{3}H]-GABA, probably by an hetero-exchange mechanism, but none was as potent as GABA itself (Levi & Raiteri, 1974). Hetero-exchange should be taken into account when compounds are tested or used as GABA-uptake inhibitors.

By analysing the spontaneous release of tritium, it was possible to demonstrate inhibitory effects of several compounds (at 100 μM) on [^{3}H]-GABA catabolism in intact synaptosomes. GVG, γ-acetylenic GABA (RMI 71645), gabaculine, isogabaculine (RMI 71932) and amino-oxyacetic acid (AOAA) were potent and rapidly-acting in this respect; on the other hand (AOAA) at 10 μM and ethanolamine-o-sulphate (up to 500 μM) did not inhibit [^{3}H]-GABA catabolism.

Acute exposure (10 min) of the synaptosomes to compounds with known GABA-T inhibitory properties reduced, to varying extents, potassium-stimulated [^{3}H]-GABA release. Direct, chromatographic determination of GABA demonstrated that under these conditions [^{3}H]-GABA release did not accurately represent release of endogenous GABA. The discrepancy between GABA and [^{3}H]-GABA release probably arises from changes in the specific radioactivity of the [^{3}H]-GABA due to inhibition of GABA-T and glutamate-1-carboxy-lyase (GAD, E.C.4.1.1.15) to different extents.

The results demonstrate the usefulness of the superfusion system in detecting, in one experiment, several properties of GABA analogues and highlight potential sources of misinterpretation of stimulated-release data.

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Effect of electrical kindling on brain amino acid concentrations in rats

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Although electrically-induced kindling in laboratory animals has been widely used in studies on anticonvulsant drugs the nature of the biochemical changes underlying kindling is still largely unknown (see review by McNamara, Byrne, Dasheiff & Fitz, 1980). As disturbances in brain amino acid metabolism have been proposed as one of the underlying biochemical abnormalities in epilepsy (Bradford & Dodd, 1976) we considered it of interest to examine the effects of electrical kindling in rats on concentrations of cortical amino acids. To test if our kindling model, based on stimulation of the frontal cortex, was comparable with those used by other groups who commonly apply stimulation to discrete brain areas we also investigated the effects of chronic administration of two standard anticonvulsants, diazepam and phenytoin on kindling development.

Rats (Wistar, male, 200–250 g) were implanted with epidural screw electrodes over the frontal and parietal cortex (Goff, Miller, Smith, Smith & Wheat-

ley, 1975). Electrical stimulation (60 Hz, 1 ms pulses 1 s duration) was applied once daily via the frontal leads at increasing voltages until a threshold response characterized by the appearance of EEG after-discharge and behavioural clonus was obtained. The rats were then stimulated at threshold voltage + 30% on two occasions and then daily (Mon-Fri) at this increased voltage, at 2 h after drug (p.o.) or control fluid (Celacol 0.25% p.o.) which were given once daily seven days per week for up to 20 days. Kindling was considered to have occurred when the after-discharge duration was 10 s greater than the mean of the two responses obtained immediately prior to the commencement of drug or control fluid treatment. After-discharge duration in kindled controls progressively increased during the experiment to reach approximately 50 to 80 s above the mean of the two pretreatment responses.

Diazepam abolished kindling in a dose-related manner so that compared with controls (12/14 kindled) only 1/6 kindled at 20 mg/kg ($P = 0.007$), 2/6 at 10 mg/kg ($P = 0.04$) and 4/6 at 5 mg/kg (NS). In contrast, phenytoin at 140 mg/kg^{-1} but not at 35 and 70 mg/kg p.o. enhanced the onset of kindling: the number of shocks to the onset of kindling was 6.1 ± 0.7 ($P = 0.0007$) compared with the control value of 12.3 ± 1.6. The effects of diazepam and phenytoin in our model were similar to those reported by Racine, Livingston & Joaquin (1975), who used discrete stimulation of the amygdala in rats: this suggests that the kindling mechanisms in the two models may be similar.

Biochemical determinations were made on brains removed 24 h after the last shock. Cortical concentrations of GABA were estimated spectrofluorometrically (Jakoby & Scott, 1959) and concentrations of aspartate, glutamate, glutamine and taurine were assayed on an amino acid analyser (Spackman, Stein & Moore, 1958). Glutamine concentrations (3.37 ± 0.15 μmoles/g wet wt.) in kindled controls ($n = 9$) were significantly decreased ($P = 0.009$) compared with concentrations in shocked but non-kindled rats (4.08 ± 0.21 μmoles/g wet wt., $n = 8$). This value was not significantly different from concentrations in sham operated and in unoperated rats (4.34 ± 0.2 and 4.10 ± 0.14 μmoles/g wet wt. respectively, $n = 9$ per group). These results suggest that electrically-induced kindling may be accompanied by changes in glutamate and GABA metabolism as these amino acids are closely interrelated. In this respect it is of interest to note that GABA concentrations in kindled rats were lowered although not significantly different compared with controls (1.77 ± 0.16 and 2.17 ± 0.23 μmoles/g wet wt. respectively). There were no significant changes in the other amino acids examined. Studies on the effects of diazepam and phenytoin on brain amino acid concentrations in this model are in progress.

R.A. O'Donnell is an SRC (CASE) Student.

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The convulsant properties of methyl β-carboline-3-carboxylate in the mouse

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Ethyl β-carboline-3-carboxylate (β-CCE) has high affinity for benzodiazepine binding sites (Braestrup, Nielsen & Olsen, 1980). Recently, we have demonstrated that β-CCE has actions opposite to those of benzodiazepines and can reverse some of the pharmacological effects of diazepam in experimental animals (Oakley & Jones, 1980; Jones & Oakley, 1981). In the present experiments methyl β-carboline-3-carboxylate (β-CCM) had similar actions to β-CCE in that it had a high affinity for benzodiazepine receptors (Ki = 9.1 nM, rat cortex) and potentiated convulsions induced by leptazol, maximal electroshock, nicotine and picrotoxin in the mouse. However, unlike β-CCE, β-CCM (0.25–8 mg/kg i.v.) caused dose-related clonic/tonic seizures. At higher doses (16–32 mg/kg i.v.) fewer convulsions were seen

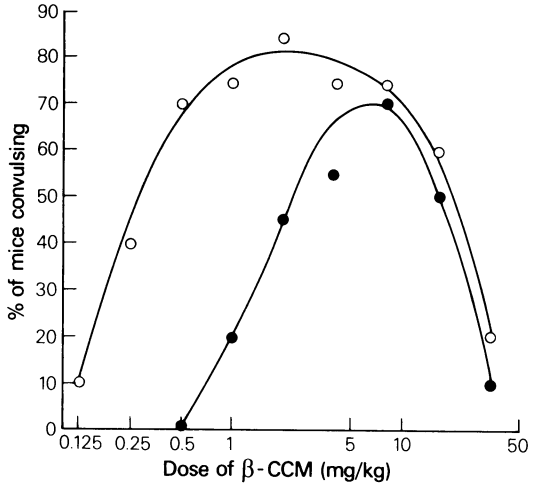

Figure 1 The effect of diazepam (8mg/kg po) pretreatment (1 h) on the convulsive effects of various doses of β-CCM (i.v.) in the mouse. β-CCM was dissolved in the smallest amount of 1 M HCl and made up to volume with saline and injected via the lateral tail vein. O–O Acacia pretreatment; ●–● Diazepam pretreatment; $n = 10–20$ at each dose level.

(Figure 1). Typically, 80–100% of a group of mice convulsed at maximally effective doses with up to 30% exhibiting tonic extensor convulsions and the remainder generalized clonic seizures.

Diazepam (Figure 1) and other benzodiazepines inhibited β-CCM induced convulsions. Other compounds with anxiolytic properties such as meprobamate ($ED_{50} = 33$ mg/kg p.o.) and phenobarbitone ($ED_{50} = 12.5$ mg/kg p.o.) also inhibited β-CCM induced convulsions whereas diphenylhydantion, 100 mg/kg p.o., and chlorpromazine, 100 mg/kg p.o., were inactive. β-CCM-induced convulsions were also antagonised by β-CCE ($ED_{50} = 1.7$ mg/kg i.v.) and propyl β-carboline-3-carboxylate ($ED_{50} = 0.7$ mg/kg i.v.).

The present results suggest that the convulsions induced by -CCM may be mediated by an action at benzodiazepine receptors. Whether antagonism of these convulsions is predictive of anxiolytic activity is the subject of further investigations.

We would like to thank Miss D.A. Powers for excellent technical assistance.

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Decreased α_2-adrenoceptor numbers in rat brain following repeated electro-convulsive shock

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There are several reports of a reduction in cortical β-adrenoceptor density after administration of repeated electro-convulsive shocks (ECS), (Pandey *et al.*, 1979; Bergstrom & Kellar, 1979), although using [^{3}H]-DHE and [^{3}H]-WB-4101 as radioligands, Bergstrom & Kellar were unable to detect alterations in α-adrenoceptor binding. However, recent studies suggest that α_2-adrenoceptors may be affected by repeated ECS since clonidine-induced hypoactivity in rats is attenuated by this treatment (Akagi, Green & Heal, 1981). Using [^{3}H]-clonidine, we have therefore investigated changes in α_2-radioligand binding in the rat after repeated ECS and found decreased binding in rat cortex, hippocampus and hypothalamus.

ECS (125 V, 1 s, 50 Hz) was administered to unanaesthetized, male, Sprague-Dawley derived rats once daily for 10 days. Animals were killed 24 h after the last ECS and the tissues stored at −18°C. [^{3}H]-clonidine binding was measured in membrane preparations from the cortices of individual rats and in membranes from brain regions pooled from 2 animals, in the case of the hippocampus and hypothalamus. [^{3}H]-clonidine binding was measured in 50 mM Tris buffer pH 7.8 over the concentration range 0.1–1.5 nM. Non-specific binding was taken as that [^{3}H]-clonidine bound in the presence of phentolamine (0.3 mM).

Our results show that [^{3}H]-clonidine binding was changed in all regions studied. In the cortex, Bmax was reduced from 18.95 ± 1.4 pmol/g in the controls to 11.7 ± 0.6 pmol/g in ECS treated animals ($n = 7$; $P < 0.001$). In the hypothalamus, Bmax was reduced from 16.39 ± 2.8 pmol/g to 6.77 ± 1.7 pmol/g after ECS, while in the hippocampus a much smaller reduction in Bmax was observed (9.60 ± 0.99 pmol/g to 7.38 ± 0.93 pmol/g). For both these brain regions $n = 4$ and $P < 0.001$. In no brain region was any change in Kd for clonidine observed.

The results show that ECS reduces the density of α_2-adrenoceptors as measured by the high affinity binding sites for clonidine. Furthermore, it seems that the changes induced by repeated ECS administration are not confined to the cortex but can also be detected in subcortical brain regions, although the magnitude of the changes seems to show regional variation.

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Temperature dependent characteristics of [^{3}H]-diazepam binding to rat cerebral cortex

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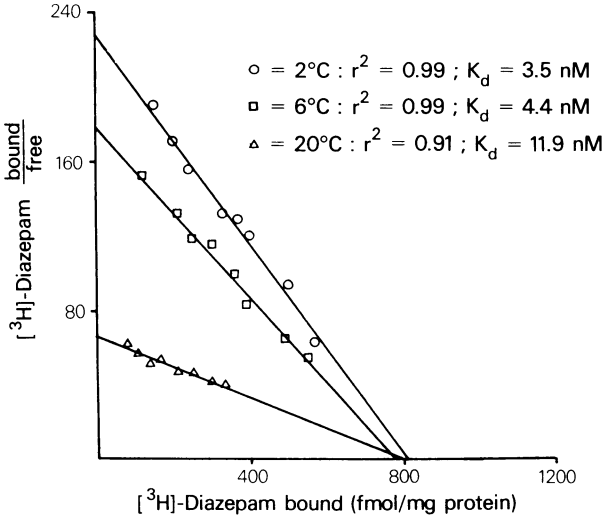

Figure 1 Scatchard analysis of [^{3}H]-diazepam binding at different temperature: Abscissa represents specific binding of [^{3}H]-diazepam to synaptosomal membrane preparation determined by incubating increasing amounts of [^{3}H]-diazepam in the absence and presence of 20 μM flurazepam and subtracting the latter from the former. Ordinate represents bound (B) [^{3}H]-diazepam as defined above divided by free (F) nM concentration of [^{3}H]-diazepam. Each point represents the mean of duplicate determinations. Four additional experiments yielded similar results.

Specific high affinity saturable mammalian C.N.S. benzodiazepine binding sites have been demonstrated (Möhler & Okada, 1977) with ligands having affinities correlating to pharmacological activity (Squires & Braestrup, 1977). Typically benzodiazepine ligand binding studies are performed at low temperatures around 0°C where binding is optimal in terms of amount of specific binding obtained (Möhler & Okada, 1977). This present experiment further characterizes the effect of temperature on benzodiazepine receptors.

A modified method of Möhler & Okada (1977) was employed using 10 mM TRIS-HCl buffer (pH 7.4) containing 1–10 nM [^{3}H]-diazepam and crude synaptosomal preparation (0.7 mg protein per assay) in a total assay volume of 0.5 ml.

Specific binding of [^{3}H]-diazepam at a free concentration of 1.5 nM varied from 203 ± 18 fmol/mg protein representing 95 ± 0.4% of total binding at 2°C to 23 ± 2 fmol/mg protein representing 77 ± 1.7% of total binding at 37°C. Scatchard analysis (Figure 1) indicates that this decrease with temperature is due to changes in affinity. Time course studies (not shown) support this conclusion. Thus, at more physiological temperatures the affinity of diazepam may be lower than estimated at 0°C and also GABA agonist induced increases in benzodiazepine affinity (Tallman *et al.*, 1978) might be more apparent at higher incubation temperatures, particularly in the case of weak or partial GABA agonists.

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An Iontophoretic study of the structure activity relationships between analogues and derivatives of ethylenediamine

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Ethylenediamine (EDA) is a powerful inhibitor of neuronal firing rates, comparable to GABA in potency, and this inhibition is bicuculline sensitive (Perkins, Bowery, Hill & Stone, 1981; Forster, Lloyd, Morgan, Perkins & Stone, 1981). The possibility that EDA is a novel GABA mimetic prompted a more detailed study of the structure-activity relationships existing between various analogues and derivatives of EDA.

Male Wistar rats were anaesthetized (urethane 1.3 g/kg, 25% w/v solution, i.p.) and placed in a stereotaxic frame and prepared for extracellular unit recording from the cerebral cortex and/or globus pallidus.

The following drugs (all 100 mM unless otherwise stated) were applied by conventional iontophoretic techniques from 7 barrelled micropipettes:– GABA, glycine hydrochloride, EDA, N-methyl-EDA (NMEDA), 1, 3, diamino propane (DAP), 1, 4, diaminobutane (DAB), 1, 5, diamino-pentane, diethylenetriamine (DELTA), 2, 3, diamino propionic acid (DAPA), 2-amino-ethanol, piperazine citrate piperidine, pyrazine, picrotoxin (saturated in 165 mM NaCl) bicuculline methobromide (10 mM), strychnine sulphate (10 mM).

Unit activity was recorded through a single glass microelectrode attached alongside, gated and counted and recorded on a polygraph with a resetting integrator as spikes per second.

GABA and EDA both inhibited all cells tested, the response characteristics being very similar. Glycine, however, was typically weak in cortex but a potent inhibitor in pallidum. Bicuculline reversed GABA and EDA responses but not glycine inhibitions in 11/19 cells tested with all three agonists. Strychnine failed to reverse EDA inhibitions at the time of glycine blockade on 7/7 cells.

Diaminopropane was less effective than EDA, but did inhibit 17/17 cells tested. Bicuculline reversed DAP and EDA or GABA in parallel whilst not affecting glycine responses.

Increasing the chain length by more than one methylene group seriously reduced the depressant properties of the compound. DAB only weakly inhibited 3/13 and diaminopentane 1/7 cells. 2-aminoethanol had no effect on 4/5 cells and excited one. Methylation or carboxylation also reduced the potency, NMEDA inhibiting 8/24, and DAPA 3/14, units tested. DELTA was largely ineffective, inhibiting 6/16 cells tested.

Piperazine depressed the firing rate on 31/33 cells, this inhibition being bicuculline sensitive (10 cells). Pyrazine and piperidine, however, were ineffective.

These results could suggest either that EDA is a novel (and potent, iontophoretically) GABA mimetic or it interacts with a novel bicuculline-sensitive receptor requiring two amine groups a critical distance apart. A third possibility is that two or more GABA molecules are normally required to activate its receptor (Takeuchi & Takeuchi, 1969; Feltz, 1971) and that EDA has the correct spacing between its nitrogens to fit onto GABA-amine acceptors.

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Selective inhibition of 5-HT uptake *in vivo*

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In an attempt to measure inhibition of amine uptake *in vivo* we have previously shown that the application of *in vitro* methodology to material prepared from drug-treated animals may be influenced by drug redistribution. A more accurate measurement was obtained by studying the uptake of radio-labelled amine following injection into the lateral ventricle (Moser & Wyllie, 1981). This work has been extended to facilitate the measurement of both NA and 5-HT uptake in the same animal.

The effects of test drugs on the uptake of NA and 5-HT *in vitro* using synaptosomes prepared from rat brain were assessed as previously described. To obtain a measurement of their action *in vivo* groups of rats were given various doses of drugs (p.o.) 1 h before the stereotaxic injection of a mixture (10 μl) containing [^{14}C]-5-HT (0.5 μCi) and [^{3}H]-L-NA (1 μCi) into the lateral ventricle. The cannula was positioned immediately following the induction of anaesthesia with pentobarbitone sodium (50 mg/kg, i.p.). An hour later the rats were decapitated, synaptosomes prepared and the radio-label counted (Wood & Wyllie, 1981).

Wy 26002 { 1-Benzoyl-3-{1-(2-napthylmethyl) piperid-4-yl} urea, HCl} a structural analogue of Wy 25093 used in earlier studies (Diggory, Dickison, Wood & Wyllie, 1980; Diggory, Dickison, Moser, Stephens & Wood, 1981) was found to be a potent and selective inhibitor of 5-HT uptake *in vitro* (Table 1). Fluoxetine and zimelidine exhibited rather less selectivity for 5-HT, whereas imipramine preferentially inhibited NA uptake. Although a similar degree of selectivity was obtained for inhibition of amine uptake by Wy 26002 *in vivo*, zimelidine exhibited considerably less selectivity for the 5-HT mechanism in the whole animal than *in vitro*.

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Table 1 Inhibition of NA and 5-HT uptake *in vitro* and *in vivo*

	IC_{50} (nM) *synaptosomal uptake*		ID_{50} (mg/kg p.o.) *intraventricular uptake*	
Drug	*5-HT*	*NA*	*5-HT*	*NA*
Wy 26002	5.6 (3.1–9.0)	449 (226–1018)	3.8 (2.6–5.1)	> 100
Fluoxetine	141 (87–213)	570 (155–1330)	3.8 (2.4–6.3)	11.9 (9.6–17.2)
Zimelidine	156 (93–247)	5300 (2950–14074)	10.2 (8.6–13.9)	16.8 (14.4–25.1)
Imipramine	245 (135–456)	18 (8–43)	9.3 (7.8–13.3)	

Values are the mean of 3–6 experiments. 95% confidence limits are shown in parentheses.

In vivo and *in vitro* comparisons of the effects of 5-HT and tryptamine in the hypothalamus

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5-HT causes a fall and tryptamine a rise in core temperature in rats after intrahypothalamic injection. Although both compounds appears to act at the same site within the preoptic region of the anterior hypothalamus (Cox, Davis, Juxon, Lee & Martin, 1981), they have different susceptibilities to blockade by indoleamine-receptor antagonists (Cox, Lee & Martin, 1981). The response to tryptamine but not to 5-HT can be abolished by pretreatment with 5,7-DHT (Cox *et al.*, 1981) suggesting that the hyperthermia caused by tryptamine may be dependent on endogenous 5-HT. It has been suggested that even at relatively low ambient temperatures there is an active heat loss mechanism in the rat (Ulman, Ford, Wilton, Borsook & Mitchell, 1980) and 5-HT may be a neurotransmitter in this pathway. Therefore a possible mode of action of tryptamine could be to inhibit this heat loss pathway by acting presynaptically to reduce 5-HT release. We decided to investigate this possibility *in vitro* by comparing the effects of 5-HT and tryptamine on K^+-evoked 5-HT release from slices of rat hypothalamus.

The method used was essentially that described previously (Ennis, Kemp & Cox, 1981) except that the slices were prepared from the hypothalamus of the rat and preloaded with tritiated 5-HT.

5-HT and tryptamine produced a dose-related inhibition of K^+-evoked tritium release over the concentration range 3×10^{-9} M to 10^{-7} M and 10^{-8} to 10^{-6} M respectively. The maximum inhibition produced by 5-HT was designated 100%. The maximum inhibition of tritium release produced by tryptamine was only 80% compared to 5-HT. The pIC_{50} values were 7.53 ± 0.11 ($n=8$) for 5-HT and 6.84 ± 0.23 ($n=8$) for tryptamine ($P<0.01$, Mann-Whitney U test). The slopes of the dose response curves were also significantly different being 62.7 ± 7.6 for 5-HT and 28.4 ± 1.9 for tryptamine.

The response to tryptamine (10^{-7} M) but not to 5-HT (5×10^{-8} M) was abolished in the presence of tetrodotoxin (10^{-7} M) indicating that tryptamine is acting through a sodium dependent mechanism, perhaps via an interneurone.

The effects of two indoleamine-receptor antagonists have been examined. Methiothepin which is reported to be an antagonist at 5-HT autoreceptors in the rat frontal cortex (Gothert, 1980) was found to be equipotent as a competitive antagonist of 5-HT and tryptamine having pA_{10} values, obtained by the method of Arunlakshana & Schild (1959) of 6.46 ± 0.09 and 6.93 ± 0.40 respectively.

Cyproheptadine, which is inactive at 5-HT autoreceptors (Cerrito & Raiteri, 1979) was a competitive antagonist of tryptamine having a pA_{10} value of 7.2 ± 0.18 but produced only a non-competitive antagonism of 5-HT at concentrations over 10^{-6} M.

The results suggest that tryptamine does not act at the 5-HT autoreceptor in the hypothalamus but may act indirectly through an interneurone to inhibit 5-HT release and could explain the *in vivo* differences.

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Further evidence for presynaptic inhibition in the olfactory cortex

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A phenomenon resembling the GABA mediated dorsal root reflex can be evoked in the lateral olfactory tract (LOT) in slices of rat olfactory cortex (Pickles & Simmonds, 1976) and it was suggested therefore that presynaptic inhibition may operate on the LOT. If this is correct, other established correlates of presynaptic inhibition should also be demonstrable. It has already been shown that GABA can depolarize the nerve fibres of the LOT (Pickles, 1979) and we now present evidence that conditioning stimulation evokes an increase in excitability of terminal regions of the LOT and that this effect is abolished by GABA antagonists.

Surface slices of rat olfactory cortex, approximately 500 μm thick, were obtained and the rostral 0.5 cm of the LOT was divided longitudinally. The medial portion of the LOT was drawn into a Ag/AgCl suction electrode for the recording of antidromic potentials. The lateral portion of the LOT was used for orthodromic conditioning stimulation via a bipolar metal electrode. Excitability of the LOT terminal regions was tested by submaximal stimulation (pulse width 200–500 μs) through a fine glass electrode containing NaCl (4 M) placed in the olfactory cortex 2–3 mm away from the LOT and 200–500 μm from the pial surface. The slices were perfused throughout with a Krebs medium containing (mM), NaCl 118, KCl 2.1, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.0, $NaHCO_3$ 25 and glucose 11, and gassed with 95% O_2/5% CO_2 at room temperature (22°–28°C).

Stimulation of the LOT terminal regions evoked a monophasic compound antidromic potential in the LOT. Antidromic potentials were evoked both before and after conditioning stimulation of the LOT and their amplitudes compared. The stimulation cycle was carefully adjusted so that the conditioning stimulation just failed, of itself, to evoke reflex antidromic activity.

Under this arrangement, the conditioned antidromic potential amplitude was increased by a maximum of 26–69% at 30–40 ms after the conditioning stimulus and then progressively returned to control values over the next 20–40 ms ($n = 6$). Perfusion with the GABA antagonists bicuculline (Sigma) 10^{-5} M ($n = 3$) or benzyl penicillin (Glaxo) 10^{-3} M ($n = 2$) abolished the conditioning, suggesting that it was entirely GABA mediated. The effects were dependant upon the terminal regions of the LOT being tested for excitability because analogous tests of excitability of the myelinated axons within the LOT showed no conditioning.

It is particularly interesting that these presynaptic actions of endogenously released GABA can occur in the absence of any demonstrable axo-axonic synapses on the LOT (Westrum, 1969).

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The inhibitory action of imipramine on monoamine transport

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Tricyclic antidepressants are thought to exert their therapeutic effects due to the chronic blockade of monoamine re-uptake. Structure activity relationships give little indication regarding either the site of tricyclic drug action or the relative selectivity for 5-hydroxytryptamine (5-HT) and noradrenaline (NA). Re-uptake blockers are not necessarily clinically effective antidepressants and some antidepressants do not inhibit re-uptake (for review see Maj, 1981).

Using purified cortical synaptosomes and a sodium correction technique to determine energy dependent uptake (Wood and Wyllie, 1981), it is found that imipramine inhibits NA and 5-HT uptake *in vitro* in a non-competitive manner (Figure 1).

Carrier-mediated monoamine transport involves at least three processes; binding to the carrier, trans-

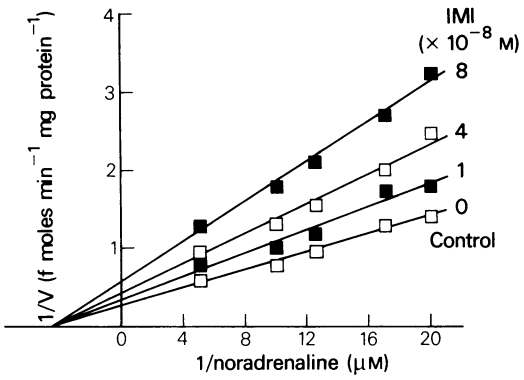

Figure 1 Inhibition of [^{3}H]-1-NA accumulation by imipramine. Purified synaptosomes prepared from rat cortex were incubated with varying [^{3}H]-1-NA and imipramine concentrations for 4 min at 37°C in a Tris Krebs medium. The reaction was terminated by rapid filtration through 0.45 μM pore size filter, and the retained radioactivity determined. Each point is the mean of three determinations.

location across the membrane, and release from and re-cycling of the carrier. By studying the kinetics of accumulation in either intact synaptosomes in the absence of sodium (substituted by lithium) or in lysed synaptosomes, it is possible to study binding to the uptake site in the absence of translocation. After sodium removal or in lysed synaptosomal preparations, the Km of the uptake process was unaltered relative to intact synaptosomes (Km for intact synaptosomes: NA, 227 ± 57 nM; 5-HT, 158 ± 48 nM), but the Vmax's were reduced by up to 75%. Using these conditions it was found that imipramine concentrations up to 10^{-6} M do not inhibit binding of NA or 5-HT to their respective carriers. Binding to this site was inhibited by unlabelled monoamines and their dihydroxylated derivatives 5,7-dihydroxytryptamine and 6-hydroxy dopamine. Ouabain (10^{-6}–10^{-3} M) inhibited translocation but not binding and only the translocation process was sensitive to alterations in sodium ion concentrations.

The inhibitory potency of imipramine against 5-HT and NA accumulation was potentiated by ATP (0.1–1.0 mM). However, the ability of unlabelled monoamine to inhibit transport was unaltered by ATP.

These results suggest that imipramine inhibits reuptake indirectly at a site removed from the monoamine carrier binding site.

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Assay of adenosine 3′,5′-monophosphate in superfusates of mouse striatal tissue

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Some dopamine receptors can be identified with and may be linked to adenylate cyclase apparently using adenosine 3′,5′-monophosphate (cAMP) as an intracellular mediator (Greengard, 1976; Iversen, 1975). Assay of cAMP as a measure of dopamine receptor activity, however, has produced conflicting reports for brain tissue both *in vitro* and *in vivo* (see Daly, 1975) which may be due in part to differences between times and techniques for sampling. The method reported here measures the continuous outflow of cAMP from brain slices and permits documentation of the time course of the cAMP response to drugs.

The striata from eight male mice (HAM/ICR Swiss) were pooled and chopped (0.2 mm × 0.2 mm) using a McIlwain tissue chopper. Slices were incubated in oxygenated (95% O_2/5% CO_2) Krebs-Henseleit solution at 37°C before transfer by pasteur pipette onto beds of Whatman GF/B glassfibre filters in four superfusion columns (5.5 mm diameter) maintained at 37°C. Krebs-Henseleit solution was circulated through the columns at a rate of 0.2 ml/min for 40 min during which time increased levels of cAMP (up to 30 pmole/ml) due to biochemical changes *post mortem* were removed and cAMP outflow became constant. The striatal slices were superfused for a further 60 min, drugs tested being dissolved in the superfusion fluid. Samples of superfusates (1 ml) were collected continuously at 5 min intervals and freeze fried. The sample content of cAMP was assayed after reconstitution in Tris EDTA buffer by a protein binding assay (Tovey, Oldham & Whelan, 1974).

Increased potassium (40 mM) and dopamine hydrochloride (0.1 mM and 1 mM) evoked rises in cAMP outflow which were inhibited by chlorpromazine hydrochloride (0.1 mM) (Figure 1). Apomorphine hydrochloride in the dose range 0.01–1 mM, contact time 10 min, had no effect on the outflow of cAMP

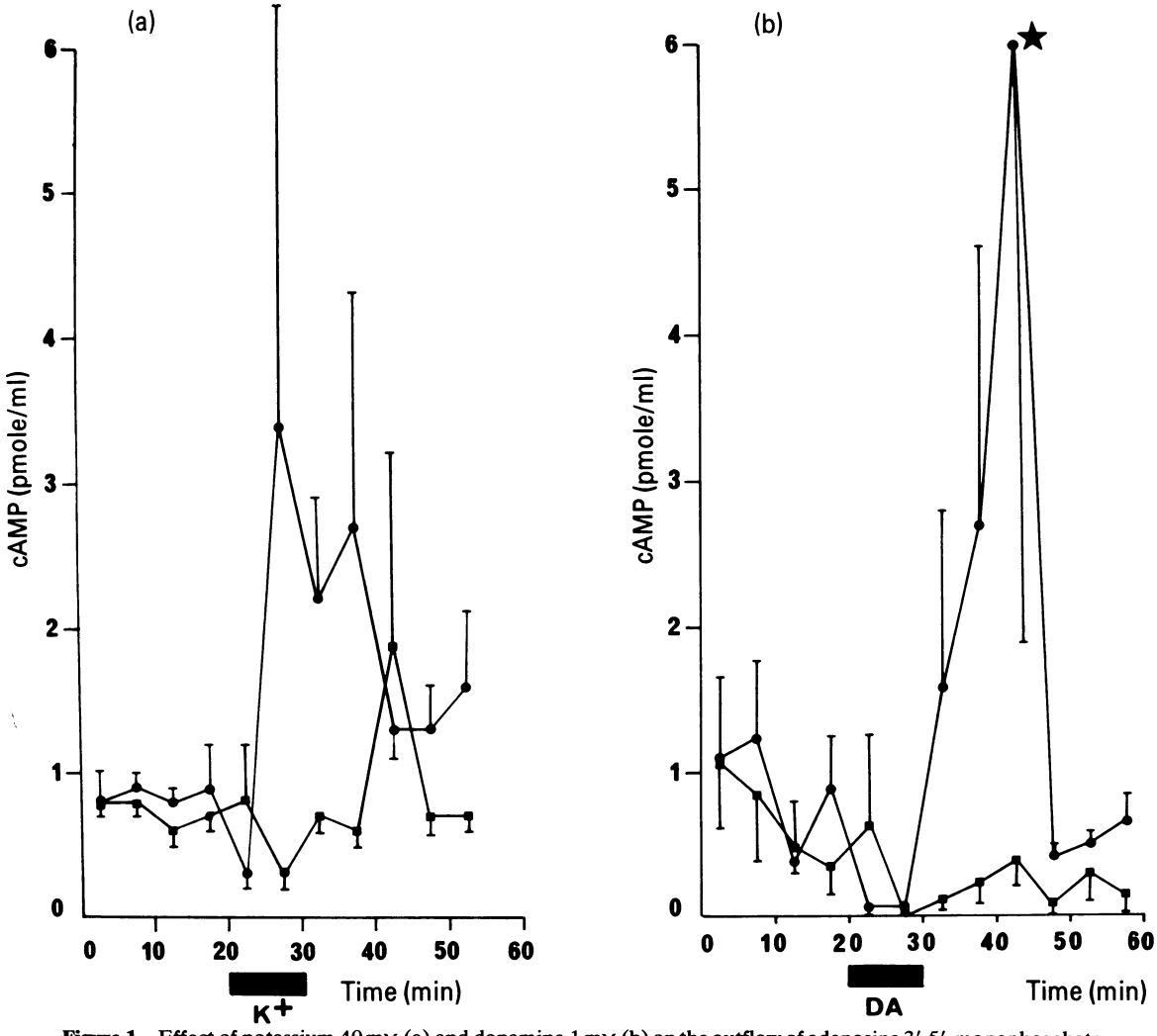

Figure 1 Effect of potassium 40 mM (a) and dopamine 1 mM (b) on the outflow of adenosine 3′,5′-monophosphate (cAMP pmole/ml) from mouse striatal slices in the absence (●) or the presence (■) of chlorpromazine (0.1 mM). Each point represents the mean ± s.e.mean ($n = 4$). Statistically significant differences are presented at the $P < 0.05$ level (*) (Mann-Whitney *U*-Test) for total outflow.

from striatal slices when compared with control levels (0.3–1.2 pmole/ml) for 40 min after introduction of the drug.

In summary this technique may be a useful method for investigating the involvement of cAMP in central dopaminergic pathways. The apparent dissimilarity between the effects of dopamine and apomorphine on the cAMP system in this model warrants further investigation.

G.G.F. is a S.R.C. Case Award student.

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Activity profiles of opiates at the μ-, δ- and κ-binding sites

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The binding of three tritiated opioids [D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin, [D-Ala^2, D-Leu^5]-enkephalin and ethylketazocine, was studied in homogenates of guinea-pig brain at 25°C (Gillan, Kosterlitz & Paterson, 1980). [D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin, a highly selective ligand for the μ-binding site (Kosterlitz & Paterson, 1981) had a K_I value of 1.86 ± 0.43 nM ($n = 4$) against the binding of [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin, compared with a K_I value of 423 ± 62 nM ($n = 4$) against the binding of [^{3}H]-[D-Ala^2, D-Leu^5]-enkephalin, a ligand for the δ-binding site. However, [D-Ala^2, D-Leu^5]-enkephalin is less selective, since the unlabelled compound exhibited a 10% cross-reactivity with the binding of [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin at the μ-binding site.

In competition studies, the high affinity binding of [^{3}H]-ethylketazocine was readily displaced by several benzomorphans but not by the μ-ligand ([D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin) or the δ-ligand ([D-Ala^2, D-Leu^5]-enkephalin). In contrast, compounds which bind to the κ-binding sites with high affinity (e.g. unlabelled ethylketazocine) showed a high degree of cross-reactivity to the μ-binding site and, to a lesser extent, to the δ-binding site (Kosterlitz, Paterson & Robson, 1981). In order to obtain a true K_I value for compounds at the κ-binding site, the binding of [^{3}H]-ethylketazocine was measured in homogenates to which unlabelled [D-Ala^2, D-Leu^5]-enkephalin (100 nM) and unlabelled [D-Ala^2, MePhe^4, Gly-ol^5]-enkephalin (100 nM) had been added. Under these conditions, the binding of [^{3}H]-ethylketazocine was selective for the κ-binding site, whereas the binding to the μ- and δ-sites was suppressed.

Using these three tritiated ligands, we measured the spectrum of activity of a large number of opiates at the μ-, δ- and κ-binding sites. Bioassays were carried out on these opiates in three different preparations (guinea-pig ileum, mouse and rat vas deferens) and their biological activities compared with the affinity values obtained in the binding assays. Morphine can be given as an example. It is an agonist with selective affinity to the μ-binding site; the replacement of N-methyl by N-allyl gives nalorphine which exhibits both agonist and antagonist activities and, in contrast to morphine, binds readily to δ- and κ-binding sites.

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Ultrastructural localization of substance P and enkephalin in the substantia gelatinosa of the spinal trigeminal nucleus

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The substantia gelatinosa of the spinal trigeminal and spinal cord is the site of termination of primary afferent sensory fibres and is thought to be involved in modulating transmission of nociceptive information. A large number of different neuropeptides and transmitters are localized in this area but for many of them their exact role in the gelatinosa is unclear (Cervero & Iggo, 80). Ultrastructural immunocytochemistry allows the detailed localization of peptides and transmitter markers and can provide an anatomical framework against which the possible function of these compounds can be evaluated. We have therefore used immunocytochemistry to examine the distribution of the peptides substance P and enkephalin in the substantia gelatinosa of the spinal trigeminal nucleus. There is evidence that

substance P is the transmitter of primary afferent nociceptive neurons and it has been proposed that enkephalin may interact with substance P fibres to inhibit presynaptically the release of the peptide from the fibre terminals (Jessell & Iversen, 77).

Male rats (250 g) were perfused through the abdominal aorta with 100 ml of fixative consisting of 4% paraformaldehyde 0.1% glutaraldehyde in 0.1 M phosphate buffer. Areas of brain were then dissected out and left in fixative for a further 2.5 h followed by 5 h in 30% sucrose phosphate buffer. Following a rapid freeze/thaw in liquid nitrogen, 40 μ sections were cut on a vibratome and then stained using the peroxidase-antiperoxidase (PAP) procedure (Sternberger, 79). Full characteristics of the antibodies against substance P and against Leu-enkephalin are described elsewhere (Cuello, Galfre & Milstein, 79; Miller, Chang, Copper & Cuatrescasas, 78) as is the staining procedure (Priestley & Cuello, 81). Following antibody staining sections were incubated in 0.06% diaminobenzidine 0.01% H_2O_2 to reveal peroxidase activity, post-fixed in osmium tetroxide, dehydrated and flat embedded on glass slides in Durcupan. Sections were examined in a light microscope and areas of interest reembedded in capsules for ultramicrotomy and subsequent electron microscopic examination.

At light microscopic level substance P immunoreactive fibres were seen to be localized in the marginal layer and superficial two thirds of the gelatinosa. Examination in the electron microscope revealed that the stained elements contained large dense core vesicles and frequently also contained small agranular spherical vesicles. Some of these stained structures made identifiable asymmetric synaptic contacts with dendrites which themselves received inputs from other unstained terminals. The unstained terminals were either similar to the substance P stained terminals or else contained flattened or pleomorphic vesicles which made symmetrical synaptic contacts (Gray 2). Enkephalin immunoreactive terminals had a similar appearance to the substance P terminals, but synaptic contacts were less frequently observed. Some animals received a Gasserian ganglion lesion and in these animals enkephalin immunoreactive terminals were seen in close apposition to dendrites which were themselves postsynaptic to degenerating terminals.

The results indicate that in the gelatinosa substance P and enkephalin are localized in synaptic terminals which make classical axo-dendritic contacts. It is possible that both peptides make contact with common dendritic elements, and that in the marginal layer substance P fibres synapse with neurons which project to the thalamus. These possibilities are now being further studied using more refined immunocytochemical procedures (Cuello, Milstein & Priestly, 1980; Priestly, Somogyi & Cuello, 1981).

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[^{3}H]-Spiperone binds to two sites in homogenates of the rat nucleus accumbens

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The nucleus accumbens septi (NAS) of the rat contains nerve terminals of ascending mesolimbic dopaminergic neurones and their associated receptors. A population of dopamine receptors appears to be localized on γ-aminobutyric acid-releasing nerve terminals in the NAS (Beart, Kuppers & Louis, 1980), and to further study dopamine receptors the characteristics of [^{3}H]-spiperone binding to membranes of the NAS have been investigated.

Membranes were prepared by homogenisation, centrifugation and washing, and used for binding studies (Quik & Iversen, 1979). Non-specific binding was defined as that not displaced by cisflupenthixol (10 μM). Kinetic parameters and IC_{50} values (concentrations causing 50% inhibition of specific binding) were determined from binding data by computerized curve fitting.

Specific [^{3}H]-spiperone binding was stereospecific, rapid, saturable, reversible and showed no cooperativity. Data from equilibrium experiments were more consistent with a two site model with the binding consisting of high and low affinity components – dissociation constants 70 pM and > 1 nM respectively. The results from dissociation experiments were also better described by a two component model.

In displacement studies [^{3}H]-spiperone binding was potently inhibited by neuroleptic drugs and ergots, but less potently by dopaminergic agonists (Table 1). Most drugs were more potent displacers of binding at 25 pM [^{3}H]-spiperone than at a ligand concentration of 0.6 nM. Drugs acting on adreno- and histamine receptors had IC_{50} values > 10 μM.

The high affinity binding sites for [^{3}H]-spiperone in the NAS may represent D-2 receptors (Kebabian & Calne, 1979). Low affinity sites are also present and their precise nature and pharmacological importance needs to be established.

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Table 1 Inhibition by drugs of the specific [^{3}H]-spiperone binding to membranes of nucleus accumbens

Compound	IC_{50} (nM)
Spiperone	0.1
Haloperidol	2
Domperidone	3
(+)-Butaclamol	6
Bromocryptine	12
Pergolide	18
Cis-flupenthixol	22
N-n-propylnorapomorphine	23
Apomorphine	47
Lergotrile	71
Sulpiride	120
Trans-flupenthixol	800
Dopamine	830
*isoADTN	1100
Cinanserin	2600
†ADTN	4000
Epinine	4900

(−)-Butaclamol and serotonin gave IC_{50} values greater than 10,000 nM. Each drug was tested at eight different concentrations in triplicate. [^{3}H]-Spiperone concentration 25 pM. *2-amino-5,6-dihydroxy-1,2,3,4-tetrahydronapthalene, †2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronapthalene.

Characterization of presynaptic dopamine receptors which modify tyrosine hydroxylase activity in rat striatal synaptosomes

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Cerrito & Raiteri (1980) proposed that the inhibitory effect of dopamine agonists on tyrosine hydroxylase (T-OH) in rat striatal synaptosomes is not mediated by receptors located externally but via an intracellular mechanism dependent upon dopamine uptake. We have investigated the sensitivity of rat striatal T-OH in synaptosomes to the presence in the medium of a number of agents with a view to further characterizing the location and nature of dopamine receptors involved in the T-OH effect.

Striata from male Charles River CD rats (130–190 g) were homogenized at 0°C in 25 ml of 0.32 M sucrose per g of wet tissue using teflon/glass pestle and tube homogenizer with 0.025 cm clearance. A synaptosome enriched pellet was obtained by centrifugation at 1000 g for 10 min at 4°C and centrifugation of resultant supernatant at 16,000 g for 20 min at 4°C. The second pellet was resuspended in 2.25 ml of incubation medium per g of original tissue to give a final composition of (mM) NaCl-125, KCl-5, $CaCl_2$-1, $MgCl_2$-1, glucose-10, ascorbate-1, Tris-HCl-50 and a synaptosomal protein concentration in the range 10–15 mg/ml. Incubations (volume 65 μl) were at pH 7.4 at 37°C. Immediately prior to use incubation medium was well oxygenated and kept at 0°C. [^{3}H]-Tyrosine (L-3,5-[^{3}H]) was purchased from the Radiochemical Centre, Amersham and purified prior to use (Boarder & Fillenz, 1978) and included in the incubations at 10 μM and 0.74 Ci/mmol. Incubation was started by shaking at 37°C and stopped after 20 min by placing in ice and adding 100 μl of 5% w/v trichloracetic acid. After mixing and centrifuging at 1000 g for 5 min supernatants were applied to double Dowex 1/Dowex 50 columns for separation of [^{3}H]-H_2O produced by tyrosine hydroxylation (Boarder & Fillenz, 1979). Results are expressed in pmol [$^3H_2$]O formed min^{-1} mg $protein^{-1}$.

Neither nomifensine nor benztropine (dopamine uptake inhibitors) at 10^{-7}–10^{-5} M significantly affected inhibition of T-OH by dopamine (3×10^{-6} M). Similar inhibitory effects of a range of dopamine agonists at 10^{-6} M were not significantly affected by benztropine (10^{-6} M). The order of potency of agonists was 6,7-ADTN > 5,6 ADTN > TL-99 >, SKF 38393 > apomorphine > dopamine > noradrenaline > M7. Weak or inactive compounds included N-propyl-3-(3-hydroxyphenyl)-piperidine, clonidine, methoxamine, piribedil.

Preincubation with phenoxybenzamine (10^{-9}–10^{-5} M) which specifically inactivates the binding of the dopamine antagonist [^{3}H]-spiperone in striatal membrane preparations (Hamblin & Creese, 1980), did not significantly alter basal T-OH or the inhibitory effect of 3×10^{-6} M dopamine. The dopamine antagonists haloperidol, sulpiride, metoclopramide and domperidone did not convincingly stimulate basal T-OH or counteract the inhibitory effects of 6,7-ADTN, dopamine, SKF 38393 or noradrenaline.

We interpret these results as support for the view that externally situated autoreceptors which are agonist preferring modify tyrosine hydroxylase activity in non-depolarized striatal synaptosomes.

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Antidepressant treatment and β-adrenoceptor function in the rat pineal

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Both repeated electroconvulsive shock (ECS) and chronic administration of a variety of antidepressant drugs reduce β-adrenoceptor binding in certain regions of rat brain (Bergstrom & Kellar, 1979; Pandey, Heinze, Brown & Davies, 1979). This reduction in ligand binding may be associated with diminished responsiveness of noradrenaline-sensitive adenylate cyclase (Vetulani & Sulser, 1975). It has also been demonstrated that repeated administration of the tricyclic antidepressant, desmethylimipramine (DMI), reduces β-adrenoceptor binding and cyclic AMP responses in rat pineal (Moyer, Greenberg, Frazer, Brunswick, Mendels & Weiss, 1979). The present experiments investigate the effect of two different antidepressant treatments on the synthesis of the pineal hormone melatonin, the production of which is dependent on β-adrenoceptor stimulation (Axelrod, 1974).

Male Sprague-Dawley derived rats, 150–175 g at the start of treatment were kept in a 12:12 light:dark schedule (lights on at 7.00 a.m.). Animals received either an ECS (125 v, 50 Hz sinusoidal for 1 s) through earclip electrodes or an injection of DMI (10 mg/kg) each given once daily for 10 days. Other treatments are shown in Table 1. 24 h after the last treatment either isoprenaline (1.5 mg/kg) or vehicle was given intravenously. 3 h later animals were killed and pineals removed. Measurement of pineal melatonin was performed using a previously described radioimmunoassay (Arendt, Paunier & Sizonenko, 1975). Experimental and control pineals were always assayed together.

Repeated ECS did not alter pineal responses to isoprenaline (Table 1). Repeated injections of DMI, however, significantly reduced the affect of isoprenaline, while a single injection did not. In addition, neither the 5-HT uptake inhibitor fluoxetine (10 mg/kg daily) nor the muscarinic antagonist, atropine (10 mg/kg daily) reduced isoprenaline responses. These results suggest that the synthesis of melatonin may be used as a functional index of β-adrenoceptor activity following treatment with antidepressant drugs.

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Table 1 Effect of various treatments on isoprenaline stimulation of pineal melatonin synthesis

Treatment	*Duration (days)*	*Vehicle*		*Isoprenaline*	
Handled	10	197 ± 28	(5)	443 ± 37	(6)
ECS	10	195 ± 27	(5)	414 ± 36	(7)
Saline	10	250 ± 11	(6)	440 ± 55	(7)
DMI	10	177 ± 10*	(6)	204 ± 21*	(6)
DMI	1	239 ± 12	(7)	366 ± 50	(6)
Saline	10	194 ± 19	(7)	466 ± 92	(7)
Fluoxetine	10	166 ± 34	(7)	444 ± 40	(7)
Atropine	10	167 ± 25	(7)	388 ± 53	(6)

Values are mean ± s.e.mean melatonin content of pineals in picogram/gland. Bracketed figure is number of pineals assayed.
*$P < 0.005$ compared to appropriate saline control.