Localisation of [^{3}H]-imipramine binding sites on serotonin nerve terminals

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Specific high-affinity binding sites for [^{3}H]-imipramine have been demonstrated in the brain (Raisman, Briley & Langer, 1979; Rehavi, Paul, Skolnick & Goodwin, 1980) and blood platelets (Briley, Raisman & Langer, 1979; Paul, Rehavi, Skolnick & Goodwin, 1980) of various species including man. These sites possess many of the characteristics to be expected for the specific binding site of action of a drug and are significantly reduced in the platelets of untreated severely depressed patients (Briley, Langer, Raisman, Sechter & Zarifian, 1980). Inhibition studies of the [^{3}H]-imipramine binding by a wide range of compounds suggest that the site is unrelated to any known neurotransmitter receptor (Raisman *et al.*, 1979) but that it is possibly associated with the neuronal uptake mechanism for serotonin (Langer, Moret, Raisman, Dubocovich and Briley, 1980). This would imply that [^{3}H]-imipramine binding sites are located on serotonin nerve terminals. We report here that destruction of serotonin nerve terminals in the rat hypothalamus, by electrolytic lesion of the dorsal raphe nucleus results in a major loss of [^{3}H]-imipramine binding sites.

Male Sprague-Dawley rats (250 g at the time of lesion) were lesioned by electrocoagulation of the dorsal raphe nucleus using a glass-insulated monopolar platinum electrode (0.5 mm diam). The stereotaxic coordinates were: medial, 0 mm; anterior/posterior 1.0 mm behind the interauricular line; vertical 4.5 mm from the skull. An electric current (20 mA; 100 KHz) was applied from a Grass LM4 'Lesion Maker' until the resistance increased indicating an effective electrocoagulation. Sham lesions were carried out in an identical manner but no current was passed. After 30 days both groups of animals were killed and membranes were prepared from the hypothalamus, of lesioned and sham-lesioned rats by homogenization and centrifugation as described by Raisman *et al.* (1979). The [^{3}H]-imipramine binding was measured as described previously (Raisman *et al.*, 1979).

30 days after the operation the level of endogenous serotonin in the hypothalamus was significantly decreased in the lesioned animals (control, 760 ± 48 ng/g; lesioned, 466 ± 71 ng/g, $n = 5$, $P < 0.01$). The levels of nordrenaline and dopamine were unchanged. The maximal binding of [^{3}H]-imipramine in the hypothalamus, of the same group of animals was significantly decreased compared with the sham-lesioned animals (control, Bmax 370 ± < 35 fmoles/mg protein; lesioned, 184 ± 18 fmoles/mg protein, $n = 9$, $P < 0.002$). There was no change in the affinity constant for [^{3}H]-imipramine (control, Kd 5.6 ± 0.8 nM; lesioned 5.5 ± 1.2 nM, $n = 9$). Qualitatively similar results have also been obtained in the striatum and cortex, and at 15 days after the lesion.

These data would suggest that a large proportion of the [^{3}H]-imipramine binding sites in the hypothalamus, and in the striatum and cortex, are located on serotonin nerve terminals emanating from the dorsal raphe. This localization of [^{3}H]-imipramine binding sites on serotonin nerve terminals is consistent with other recent experimental data. In a study of [^{3}H]-imipramine binding in 23 microdissected regions of the rat brain the distribution of [^{3}H]-imipramine binding sites closely paralleled the endogenous levels of serotonin (Palkovits, Raisman, Briley & Langer, 1980). Thus data from several different experimental approaches are all consistent with the hypothesis that the [^{3}H]-imipramine binding site is probably located on serotonin nerve terminals.

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Effects of depleting brain and spinal cord 5HT on footshock induced analgesia

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The analgesic effect of footshock is decreased by agents increasing the availability or release of 5-hydroxytryptamine (5HT) (Curzon *et al.*, 1980). Conversely it is increased by giving *p*-chlorophenylalanine so that 5HT is depleted throughout the brain and spinal cord by 80% or more (Tricklebank, Hutson & Curzon, 1981). We now find that considerable depletion of brain but not spinal cord 5HT by *p*-chloroamphetamine (PCA) does not increase shock provoked analgesia and have therefore examined the role of spinal cord 5HT in this phenomenon.

Responses to noxious heat (56°C) following inescapable footshock (1 mA or 2 mA for 30 s) were measured in male Sprague-Dawley rats (200–250 g) using a hot plate method (Curzon *et al.*, 1980). Latency to lick the paws was measured immediately before (L1) and immediately after (L2) shock.

Eleven days after giving either PCA (10 mg/kg, i.p.) or saline the percentage analgesia scores (L2 × 100/L1) of non-shocked animals were (mean ± s.d.) 102 ± 23 (n = 10) and 101 ± 31 (n = 10) respectively. After footshock (2 mA) the scores of PCA and saline treated rats increased significantly ($P < 0.001$), and almost identically (269 ± 60, n = 8; 264 ± 102, n = 10 respectively). Determinations of 5HT by an HPLC procedure (Curzon, Kantamaneni & Tricklebank, 1981) showed that PCA decreased 5HT by about 45% in brain regions but only by 15% and 26% in the lumbar and cervical + thoracic spinal cord respectively. The lack of an associated increase of shock provoked analgesia suggested that spinal cord 5HT might be involved in the analgesia.

This was investigated by injecting the 5HT neurotoxin 5,7 dihydroxytryptamine (5,7-DHT) intraspinally between L6 and S1 vertebrae. Rats were given desmethylimipramine (25 mg/kg, i.p.) to protect noradrenergic neurones and 1–2 h later injected with 25 μl of 5,7-DHT (1 mg/ml free base + 1 mg/ml ascorbic acid in 0.9% NaCl). control animals were identically treated except that they were injected intraspinally with vehicle. Analgesia scores without footshock determined 9 days later were 102 ± 19 (n = 11) for 5,7-DHT treated rats and 93 ± 38 (n = 5) for controls. In footshocked rats (1 mA) the analgesia score was 262 ± 59 (n = 9) after 5,7-DHT treatment which was significantly greater ($P < 0.05$) than the control value of 187 ± 58 (n = 5). Lumbar cord 5HT fell by 82% in the 5,7-DHT group. Depletion was less marked in the cervical + thoracic cord (47%) while 5HT concentrations in brain regions were not significantly altered.

Results in general confirm previous evidence that shock induced analgesia in inversely related to 5HT availability and indicate the involvement of spinal 5HT neurones in this response.

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Identification by differential pulse voltammetry of a 5-hydroxyindoleamine oxidation peak in the striatum and frontal cortex of the anaesthetized rat

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Recent studies suggest that *in vivo* electrochemical techniques can monitor changes in release and metabolism of 5-hydroxytryptamine (5-HT) (Conti, Strope, Adams & Marsden, 1978; Marsden, Conti, Strope, Curzon & Adams, 1979). Differential pulse voltammetry has been used to identify chemical components of specific oxidation peaks *in vivo* (Gonon, Buda, Cespuglio, Jouvet & Pujol, 1980; Cespuglio, Faradji, Ponchon, Buda, Riou, Gonon, Jouvet & Pujol, 1981). In the present study a 5-hydroxy-indoleamine oxidation peak in the striatum and frontal cortex of the anaesthetized rat has been identified by differential pulse voltammetry.

Graphite paste working electrodes either alone or in combination with infusion cannulae (Marsden, Bennett, Brazell, Sharp & Stolz, 1981) were stereotoxically implanted into the striatum or frontal cortex of anaesthetized (chloral hydrate, 450 mg/kg i.p.), male wistar rats (270–275 g).

Micro Ag/Ag Cl reference and auxillary electrodes were implanted on the dura surface (Conti, Strope, Adams & Marsden, 1978). Differential pulse voltammetry (Princeton Applied Research, Model 174A) was performed in the anaesthetized rat for up to 8 h after electrode placement using a scan rate of 10 mv/s pulse rate 2/s at a frequency of 1 every 8 minutes.

Differential pulse voltammetric recordings over the potential range -0.1 V to $+0.5$ V exhibited up to three distinct oxidation peaks in the striatum and frontal cortex. These peaks occurred at approximately $+0.12$ V, $+0.22$ V and $+0.35$ V. The peaks at the latter two potentials were strikingly consistant and stable between rats in the same brain region. The peak at $+0.22$ V was consistantly bigger in the striatum than the frontal cortex.

5-Hydroxytryptamine (5×10^{-6}–1×10^{-7} M) and its principal metabolite, 5-hydroxyindoleacetic acid, (5-HIAA) (1×10^{-4}– 1×10^{-5} M) oxidized *in vitro* at about $+0.35$ V, using the same electrodes as implanted *in vivo*, and gave a linear current response over the concentration ranges quoted.

Infusion of 1–1.5 μl of 5-HT (7.5×10^{-6} M) over 1.5 min produced a selective increase in the peak at $+0.35$ V of between 5–7 nA (mean increase 28% ± 1.5%, $n = 4$) while 5-HT (7.5×10^{-5} M) increased the peak at 0.35 V by about 40 nA. Similarly the infusion of 1–1.5 μl of 5-HIAA (2.5×10^{-5} M) over 1.5 min also increased the peak at $+0.35$ V by 7–10 nA (29% ± 2.3%, $n = 3$), while a higher concentration (5×10^{-4} M) increased the peak by about 30 nA. Phosphate buffered saline 0.1 M, pH 7.4 at 37°C was used as the vehicle and its infusion (1.5 μl) either had no effect or slightly reduced all the oxidation peaks. Administration of *p*-chlorophenylalanine (2×150 mg/kg i.p.), to deplete 5-HT and 5-HIAA, completely abolished the peak at $+0.35$ V, while apparently potentiating the peak at $+0.22$ V. Infusion of 5-HT (7.5×10^{-6} M) or 5-HIAA (2.5×10^{-5} M) into the 5-hydroxyindole depleted animals produced a peak of 15–20 nA ($n = 3$) or 18–24 nA ($n = 3$) respectively at about $+0.35$ V. 5-Hydroxytryptophan (100 mg/kg i.v.) administered to p-chlorophenylalanine treated rats also restored a peak at about $+0.35$ V which first appeared approximately 20 min after injection. Intraventricular 5, 7-dihydroxytryptamine (50 μg in 5 μl) also decreased the peak at $+0.35$ V.

The results indicate that differential pulse voltammetry can detect 5-HT and 5-HIAA and selectively monitor fluctuations of 5-hydroxyindoles *in vivo*.

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Presynaptic autoreceptors controlling acetylcholine release in synaptosomes from rat hippocampus

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The question whether the presence of autoreceptors modulating neurotransmitter release is a general feature of all types of presynaptic nerve endings is still open. In the case of the major biogenic amines, for example, a receptor-mediated negative feedback control of the release evoked by depolarization can easily be shown in various tissue preparations containing noradrenaline or serotonin nerve terminals, including synaptosomes (Langer, 1977; Starke, 1977; Cerrito & Raiteri, 1979). However, a reduction of dopamine release by extracellular dopamine can only be detected in rabbit striatal slices (Hertting, Reimann, Zumstein, Jackish & Starke, 1979) but not in synaptosomes, either from rabbit or rat striatum (Raiteri, Cervoni, del Carmine & Levi, 1978 and unpublished results) thus suggesting that, in brain slices, the modulation of release of a given neurotransmitter may result from interactions among various neuronal systems.

On the basis of experiments performed in hippocampal slices, Szerb (1979) interpreted the inhibition of acetylcholine (ACh) release by extracellular ACh as due to a negative feedback modulation mediated through presynaptic autoreceptors.

We have examined the ability of exogenous ACh to reduce ACh release from hippocampal synaptosomes in superfusion conditions, in which any indirect effect should be minimized. P_2 fractions were prepared from the hippocampus of adult male Sprague-Dawley rats. The crude synaptosomes were prelabelled with 0.1 μM [^{3}H]-choline for 4 min and aliquots of the suspension were superfused in several parallel superfusion chambers (Raiteri, Angelini & Levi, 19/4).

Depolarization with KCl (15 mM) evoked a release of [^{3}H]-ACh which was almost abolished in the absence of Ca^{2+}. Addition to the superfusion medium of ACh (10^{-4}–5×10^{-6} M) in presence of neostigmine (5×10^{-4} M), caused a dose-depended inhibition (50% to 20%) of the K^+-induced release. The inhibitory activity of exogenous ACh was counteracted by atropine (10^{-6}M). As expected in superfused synaptosomes, atropine per se did not cause the increase of [^{3}H]-ACh release generally found in slices. The potentiating effect of atropine in slices could be due to antagonism of the autoinhibition produced by the released ACh on its own further release.

In conclusion, the data of the present investigation represent a 'direct' confirmation of the existence of ACh autoreceptors on central presynaptic cholinergic nerve endings.

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The involvement of GABA in the high pressure neurological syndrome (HPNS)

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Increase in ambient pressure produces hyperexcitability *in vivo*, characterized by tremors, convulsions and death. In man these symptoms are a potential hazard in deep sea diving. The onset pressures for these behavioural effects are raised by many general anaesthetic agents but the underlying mechanisms are still obscure. Anticonvulsants, such as phenytoin, ethosuximide or carbamezepine do not protect against high pressure (Halsey & Wardley-Smith, 1980), although phenobarbitone is effective, more so than pentobarbitone (Beaver, Brauer & Lahser, 1977).

The role of γ-aminobutyric acid (GABA) in controlling CNS excitability led us to investigate the effects on the HPNS of drugs which interfere selectively with GABA transmission.

Male CDI mice were injected (i.p.) with the drugs (using coded solutions) before being placed, individually, in a pressure chamber. The pressure was raised (3 atm/min) using helium gas (Lever, Miller, Paton & Smith, 1971) and the pressures noted for the onset of fine tremor, coarse tremor, convulsions and death. The rectal temperatures were maintained at 37°C ± 1.5°C and the oxygen partial pressure at 1 atm. For comparison the same doses of the drugs were tested on the convulsion threshold to intravenous bicuculline (Nutt, Cowen & Green, 1980). Flurazapam, sodium valproate, aminooxyacetic acid (AOAA) and L-2,4-diaminobutyric acid (DABA) signifieantly increased the pressure required to cause convulsions and the other behavioral signs of pressure (Table 1). These drugs, acting by different mechanisms, are all thought to facilitate GABA-ergic transmission.

The effective doses of these compounds produced no obvious behavioural effects (except the higher dose of sodium valproate). It is unlikely that their effects were due to general anaesthetic action.

A positive correlation was found between effects on pressure and bicuculline convulsion thresholds ($r_s = 0.81$, $P < 0.05$) although changes in body temperature (fully controlled at pressure) may have affected the latter.

We conclude from these results that in contrast to other anti-convulsants, agents which selectively facilitate GABA-ergic transmission oppose the effects of high pressure.

A.R.B. is an M.R.C. scholar.

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Table 1

					Convulsion thresholds			
Drug	*Dose (mg/kg)*	*Pre-treatment time*	*HPNS (atm)*	*n*	*Relative increase (%)*	*Bicuculline (mg/kg)*	*n*	*Relative increase (%)*
Saline			79 ± 2	6	—	0.64 ± 0.04	8	—
Sodium valproate	400		125 ± 1*	6	58	1.39 ± 0.16*	6	117
Sodium valproate	800		133 ± 4*	6	68	1.89 ± 0.14*	6	195
Flurazepam	10		113 ± 3*	6	43	1.54 ± 0.04*	7	141
Flurazepam	20		111 ± 2*	6	41	2.09 ± 0.14*	7	227
Saline		1 h	82 ± 1	6	—	0.84 ± 0.04	5	—
AOAA	25	1 h	94 ± 2*	6	15	1.37 ± 0.04*	5	63
AOAA	35	1 h	100 ± 2*	6	22	1.50 ± 0.06*	6	79
DABA	600	1 h	95 ± 3*	6	16	1.20 ± 0.05*	5	43
Muscimol	1.0	1 h	85 ± 2	6	0	1.17 ± 0.04*	5	39

Mean values ± s.e. mean, * $P < 0.05$, Mann-Whitney 'U' test.

[^{3}H]-GABA and [^{3}H]-baclofen are ligands for the same bicuculline-insensitive site on mammalian CNS synaptic membranes

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A bicuculline-insensitive GABA receptor ($GABA_B$ site) has recently been demonstrated in the mammalian CNS (Bowery, Hill, Hudson, Doble, Middlemiss, Shaw & Turnbull, 1980a; Bowery, Doble, Hill, Hudson & Turnbull, 1980b). Many GABA analogues (e.g. isoguvacine and 3-aminopropanesulphonic acid) are inactive at this site although they are potent mimetics at bicuculline-sensitive receptors ($GABA_A$ sites). However, baclofen (β-chlorophenyl GABA) which is inactive at $GABA_A$ sites (e.g. Galli, Zilletti, Scotton, Adembri & Giotti, 1979; Horng & Wong, 1979; Olsen, Greenlee, Van Ness & Ticku, 1978; Wang, Salvaterra & Roberts, 1979) is an agonist at the $GABA_B$ receptor. By using radiolabelled binding techniques we have been able to detect high affinity, saturable binding of [^{3}H]-baclofen and [^{3}H]-GABA to this novel $GABA_B$ site but only in the presence of divalent cations.

Rat synaptic membranes were prepared according to Zukin, Young & Snyder (1974) and were either used immediately or stored at −20°C. For the assay membrane pellets were washed four times in 50 mM tris-HCl pH 7.4 buffer containing 2.5 mM $CaCl_2$ for [^{3}H]-GABA binding or Krebs-Henseleit (which contains 2.5 mM $CaCl_2$) for [^{3}H]-baclofen binding. [^{3}H]-GABA (57 Ci/mmole; 10 nM) or [^{3}H]-baclofen (8.8 Ci/mmole; 20 nM) with or without excess non-radioactive drug was added and the membranes incubated in the same medium at 20°C for 10 min before centrifugation. Specific binding was that portion displaced by non-radioactive baclofen (100 μM).

The presence of $CaCl_2$ in the Tris medium increased total [^{3}H]-GABA binding when compared with the amount bound in Ca^{++}-free solution and also revealed a displaceable component of [^{3}H]-baclofen binding. In both cases the increase in bound tritium could be completely suppressed by (−)-baclofen (100 μM) or GABA (100 μM) but not by isoguvacine or bicuculline methobromide (up to 100 μM). Since isoguvacine (40 μM) saturates $GABA_A$ site binding its presence throughout all incubation media only allowed [^{3}H]-GABA binding to $GABA_B$ sites. Under these conditions this binding represented 45 ± 1.3% of total binding (21539 ± 994 dpm/mg protein, n = 29 experiments) and saturation analysis indicated a single binding site (K_D = 77 nM, B_{max} 1.22 pmoles/mg protein; Hill coefficient = 1.06, n = 6 experiments).

Analogues of baclofen and GABA inhibited Ca^{++}-dependent [^{3}H]-baclofen and [^{3}H]-GABA binding in accordance with their biological activity (Bowery, Doble, Hill, Hudson, Shaw, Turnbull & Warrington,

Table 1 Inhibition of specific [^{3}H]-GABA (in presence of isoguvacine 40 μM) and [^{3}H]-baclofen binding by related compounds in rat brain crude synaptic membranes

	IC_{50} values (μM ± s.e. mean)	
	[^{3}H]-GABA	*[^{3}H]-baclofen*
GABA	0.08 ± 0.01	0.041 ± 0.008
(−) baclofen	0.13 ± 0.02	0.042 ± 0.009
(±) baclofen	0.13 ± 0.05	0.184 ± 0.028
β-o-chlorophenyl GABA	0.92 ± 0.08	0.74 ± 0.34
β-hydroxy GABA	1.10 ± 0.10	1.60 ± 0.15
β-p-fluorophenyl GABA	1.70 ± 0.17	1.60 ± 0.001
β-chloro GABA	4.6 ± 1.1	11.4 ± 1.69
muscimol	5.4 ± 0.71	12.3 ± 2.02
β-m-chlorophenyl GABA	19.1 ± 0.93	17.7 ± 0.3
β-phenyl GABA	9.6 ± 0.37	>100
3-aminopropane sulphonic acid	10.0 ± 0.10	10.9 ± 1.05
(+) baclofen	74.0 ± 5.7	33.3 ± 3.0
β-napthyl GABA	>100	>100
γ-hydroxy butyric acid	>100	>100
bicuculline methobromide	>100	>100
picrotoxin	>100	>100

IC_{50} values determined by probit analysis of data from 3 or more experiments for each analogue (concentration range 10 nM–100 μM; triplicate determinations at each concentration within any experiment).

1981) and with similar potencies in both binding systems (Table 1) (r = 0.903).

We conclude that [^{3}H]-GABA and [^{3}H]-baclofen bind with high affinity to the same bicuculline-insensitive receptor in the mammalian central nervous system.

DRH is an SRC student.
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The differential *in vitro* and *in vivo* potencies of ethyl-β-carboline-3-carboxylate, a potent inhibitor of benzodiazepine receptor binding

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Recently, Nielsen & Braestrup (1980) reported that ethyl-β-carboline-3-carboxylate (βCCE), a substance isolated from human urine, had high affinity for benzodiazepine receptors. In initial studies (Oakley & Jones, 1980), however, we demonstrated that it had actions opposite to those of the benzodiazepines in some *in vivo* tests. Thus, in mice it potentiated the convulsant actions of leptazol and maximal electroshock and antagonized the incoordination and inhibition of footshock-induced fighting induced by diazepam.

Table 1 The effect of βCCE injected intraperitoneally or intravenously on the maximal electroshock convulsion threshold in mice

Intraperitoneal		*Intravenous*		
Treatment	*Median convulsant shock level and 95% confidence limits (mA)*	*Treatment*	*Median convulsant shock level and 95% confidence limits (mA)*	
Saline	9.1 (8.0–10.4)	Saline	11.5 (10.1–13.0)	†10.3 (8.9–11.8)
βCCE (25 mg/kg)	9.0 (7.9–10.2)	βCCE 0.5 mg/kg	9.9 (8.6–11.3)	
βCCE (50 mg/kg)	8.4 (7.5–9.4)	βCCE 1 mg/kg	8.9 (7.8–10.1)*	
βCCE (100 mg/kg)	6.4 (5.4–7.5)*	βCCE 5 mg/kg		†6.6 (5.9–7.5)**
		βCCE 10 mg/kg		†4.7 (4.1–5.5)**

Median convulsant shock levels were determined using an 'up and down' method (Dixon & Mood, 1948) with subsequent probit analysis.
βCCE was dissolved in saline acidified with the minimum amount of 1 M HCl and injected 30 min (i.p.) or 10 min (i.v.) before testing. Control mice received acidified saline.
*$P < 0.01$, **$P < 0.001$ compared to controls.
n = 15, except †, where n = 10.

The doses required to demonstrate these effects were inordinately high (25–150 mg/kg i.p.) compared to the *in vitro* potency of βCCE at inhibiting [^{3}H]-flunitrazepam binding (k_i 0.4 nM in rat cortex). This discrepancy was not due to differing sensitivities of benzodiazepine receptors in rat and mouse brain (K_i 1.0 nM in mouse cortex). A more likely explanation was that βCCE was being rapidly metabolized and indeed, much lower doses were required to reduce the maximal electroshock convulsion threshold in mice when βCCE was injected intravenously (Table 1). This effect was maximal 10 min after injection of 10 mg/kg and had disappeared by 40 minutes. In support of this finding, βCCE was rapidly hydrolysed *in vitro* when incubated with rat or mouse blood at 37°C (T½ approximately 15 minutes).

In cynomolgus monkeys, βCCE (0.2 mg/kg i.v.) produced a transient reversal of the behavioural depressant effects of diazepam (8 mg/kg p.o.).

Our results suggest that the discrepancy between the *in vitro* and *in vivo* potencies of βCCE can be explained by its rapid metabolism. Thus, the affinity of βCCE for benzodiazepine receptors and its proconvulsant properties are likely to be directly related.

We would like to thank Miss D. Powers and Miss E.K. Pedder for technical assistance, Dr R. Storer for synthesizing βCCE and Dr R. Eastmond for performing the metabolic studies.

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Electrochemical detection of dopamine agonists

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A carbon fibre microelectrode which has been incorporated into a multibarrel ionophoresis array can be used to record unit activity in the CNS (Armstrong-James & Millar, 1979). When suitably calibrated, such an electrode can be used as the working electrode for high speed voltametry which allows the time course of dopamine (DA) concentration changes to be monitored (Millar, Armstrong-James & Kruk, 1981). By suitable switching, it is possible alternately to record unit activity or the electrochemical current due to DA. By reference to a suitable calibration curve, the concentration of DA present at the tip of the carbon fibre microelectrode following ionophoresis with a given strength of current can be measured. In experiments designed to characterize DA receptors, it is desirable to study the response of a biological system to a series of DA agonists in the presence or absence of antagonists. We report here the sensitivity of our system for detecting and quantifying ionophoretically ejected drugs which are reputed to act as DA agonists.

Experiments were made as previously described (Armstrong-James, Millar & Kruk, 1980). The substances studied were DA, epinine, apomorphine, ADTN and bromocryptine. With the exception of bromocryptine, all substances listed above could be detected *in vitro* at concentrations less than 1×10^{-7} M, and calibration curves in the range 1×10^{-7} M to 1×10^{-3} M could be constructed. In addition, curves relating ionophoretic eject current and concentration of the electroactive agonists *in vitro* and *in vivo* could be obtained. The results indicate that the high speed voltametric technique may be of value for studying the neurobiology of DA in the CNS.

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Multiplicity of dopamine-mediated behaviours during six months phenothiazine neuroleptic treatment in young but not aged rats

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The treatment of animals with neuroleptic drugs for substantial periods of their adult life represents a new strategy for the evaluation of putative mechanisms that may mediate their therapeutic efficacy and/or side effects in schizophrenic patients (Clow *et al.*, 1979; Waddington & Gamble, 1980a). In this report we describe behavioural measures during 6 months administration of the phenothiazine neuroleptic fluphenazine HCl (FPZ) to young and aged rats. We find indices of dopamine (DA) receptor blockade to be common to both study groups but that aged, unlike young animals, do not show development of DA receptor 'supersensitivity' when assessed while continuing to receive the drug.

Male Sprague-Dawley rats were given FPZ via drinking water for 1 week or 6 months (1.0–1.5 mg kg^{-1} day^{-1}). Treatment was commenced in separate groups of young (400 g, 3 months) or aged (700 g, 1 year) animals. Stereotyped behaviour was assessed both before and after challenge with apomorphine (APOM, 0.15 or 1.0 mg/kg s.c.), by rating scale (Waddington & Gamble, 1980a). All measures were made while animals continued to receive the drug at 14.00–16.00 hours.

After 1 week of FPZ administration to young or aged rats, stereotypy responses to APOM (1.0 mg/kg) were significantly attenuated ($P < 0.01$) in both colonies.

After 6 months of treatment with FPZ, responsivity to APOM (0.15 mg/kg) was completely abolished ($P < 0.01$) in both young and aged colonies. Stereotypy responses to APOM (1.0 mg/kg), however, were complexely and differentially influenced. In animals which were young when FPZ treatment commenced, sniffing responses to APOM (1.0 mg/kg) were attenuated ($P < 0.01$) but greatly enhanced locomotion was seen in 100% of animals (0% of controls, $P < 0.01$). In animals which were aged when FPZ treatment commenced, a trend towards attenuated sniffing in response to APOM (1.0 mg/kg) was seen while greatly enhanced locomotion was seen in only 33% of animals (20% in controls, NS). There was a significantly lower incidence of enhanced locomotion in aged compared with young animals given FPZ for 6 months ($P < 0.05$).

As noted previously (Clow *et al.*, 1979; Waddington & Gamble, 1980a, b), while some DA mediated behaviours can be enduringly antagonized during prolonged neuroleptic treatment others can be enhanced. Such apparent manifestation of DA receptor-supersensitivity during ongoing phenothiazine treatment does not seem to appear when neuroleptic treatment is commenced in senescence. The influence of ageing processes on dopaminergic function (Waddington & Gamble, 1980c) may extend to the functional heterogeneity and differential adaptive capacity of distinct dopaminergic substrates (Waddington *et al.*, 1979; Waddington & Gamble, 1980b) during prolonged phenothiazine treatment.

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Dopamine autoreceptors inhibiting [^{3}H]-dopamine release in the caudate nucleus of the cat: evidence for a role of endogenously released dopamine

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The question whether the dopamine (DA) autoreceptor (Farnebo & Hamberger, 1971) is physiologically active remains largely unexplored. We report that 1) the cat caudate possesses a DA autoreceptor, inhibiting electrically-evoked [^{3}H]-DA release. 2) the frequency dependence of the actions of the DA agonist pergolide and the DA antagonist S-sulpiride from 1 to 3 Hz is predicted by postulating an interaction between endogenously released DA and the DA autoreceptor.

Cat caudate slices incubated with [^{3}H]-DA were superfused continuously as previously described (Starke, Reimann, Zumstein & Hertting, 1978). Two 2 min stimulation periods (S_1, S_2) at the same frequency were applied in each experiment, with drug added before the second stimulation. The ratio of the overflow elicited by each stimulation, S_2/S_1, reflects the drug's effect on [^{3}H]-DA release. Control ratio values are given in figure legend. At 1 Hz pergolide decreased the S_2/S_1 ratio (IC_{50} = 2 nM) with maximal effect at 10 nM (S_2/S_1 = 0.18 ± 0.04, n = 6). S-sulpiride alone increased the S_2/S_1 ratio (see Fig. 1A) at 100 nM.

At 3 Hz, where approximately twice as much [^{3}H]-DA is released per unit time than at 1 Hz, there was a shift in the effects of agonist and antagonist in opposite directions. Sulpiride (100 nM) was twice as effective as at 1 Hz in enhancing [^{3}H]-DA overflow (Figure 1A), while pergolide 3 nM was half as effective in inhibiting [^{3}H]-DA overflow (Figure 1B).

It is postulated that, at 3 Hz, released DA occupies a higher proportion of autoreceptors than at 1 Hz. Thus, the antagonist produces a larger dis-inhibition at 3 Hz than at 1 Hz. With respect to the agonist the law of mass action implies a lower added occupancy of inhibitory autoreceptor sites by pergolide at 3 Hz than at 1 Hz. These frequencies of stimulation are in the range at which the DA neurons themselves fire (Guyenet & Aghajanian, 1978). Because of its high affinity and the apparent interaction with endogenously released dopamine we advance the hypothesis that the DA autoreceptor is physiologically functional.

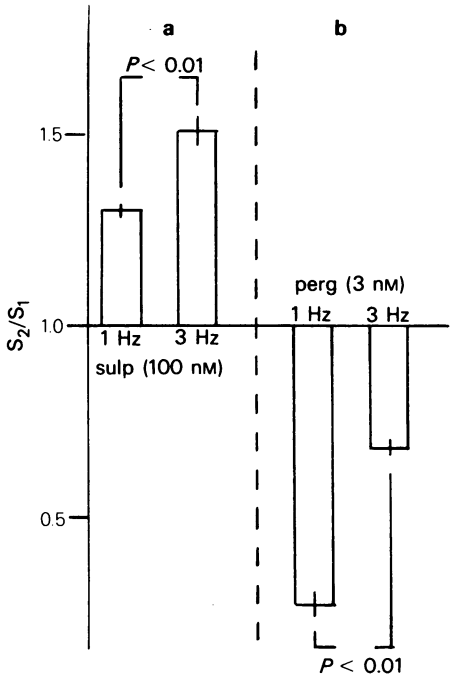

Figure 1 Frequency dependence of the effects of the dopamine agonist pergolide (PERG) and the dopamine antagonist S-sulpiride (SULP) on electrically evoked overflow of [^{3}H]-DA. Drugs were added to superfusion medium 16 min before the second stimulation was applied to slices of cat caudate. Plotted on the ordinate, the ratio S_2/S_1 assesses the action of the drug on [^{3}H]-DA overflow. In the controls S_2/S_1 at 1 Hz was 1.08 ± 0.02 (n = 17) and at 3 Hz was 1.04 ± 0.04 (n = 9). Results are the mean ± s.e. mean of 3–7 values.

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Analogues of thyrotrophin releasing hormone (TRH) stimulate *in vitro* release of endogenous dopamine from rat brain regions

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Thyrotrophin-releasing hormone (TRH) is distributed throughout mammalian brain with high levels in the hypothalamus and other forebrain regions (Brownstein, Palkovits, Saavedra, Bassiri & Utiger, 1974). It has pronounced behavioural effects independent of its endocrine function and some of these have been associated with induced release of dopamine from the nucleus accumbens (Heal & Green, 1979; Kerwin & Pycock, 1979). In other studies the septum was reported more sensitive to central administration of TRH (Kalivas & Horita, 1980). Thyrotrophin-releasing hormone analogues are available which mimic the endocrine and CNS activities of TRH but have a much longer duration of action (Fridericks, Schwertner, Herrling, Gunzler & Flohé, 1979; Dettmar, Fortune, Lynn, Metcalf & Morgan, 1980). The present study investigated (a) the regional distribution and release of TRH in rat hypothalamus, striatum, septum and nucleus accumbens, and (b) the effects of potent TRH analogues; orotyl-histidyl-prolyl amide (CG 3509) and pyroglutamyl-histidyl-3'3-dimethyl prolyl amide (RX77368) on the release of endogenous dopamine from these regions *in vitro*. The techniques used have been described previously (Bennett, Marsden, Sharp & Stolz, 1980).

Tissue levels of TRH were determined by radioimmunoassay following extraction with 90% methanol. Dissected brain regions were sliced and incubated for 25 mins in gassed (95% O_2, 5% CO_2) Krebs bicarbonate buffer, pH 7.4, containing glucose (10 mM), pargyline (5×10^{-5} M) and bacitracin (2×10^{-5} M). Test substances were added after 5 minutes. Bilateral halves were incubated separately, the contralateral half being used as a control. The supernatants were measured for TRH by radioimmunoassay or dopamine using high performance liquid chromatography (250×5 mm nucleosil column) with electrochemical detection.

Endogenous levels of TRH were highest in the hypothalamus (102.0 ± 11.8 ng/g, $n = 6$) and septum (102.8 ± 16.8 ng/g, $n = 5$), where they were about seven times higher than those in the nucleus accumbens (13.7 ± 3.5 ng/g, $n = 5$). Lowest levels were found in the striatum (6.5 ± 0.8 ng/g, $n = 6$). Basal release of TRH followed a similar pattern with highest in the hypothalamus (11.45 ± 1.78 pg/mg tissue, $n = 8$) then septum (6.19 ± 1.24 pg/mg tissue, $n = 5$), nucleus accumbens (1.88 ± 0.13 pg/mg tissue, $n = 4$) and striatum (0.63 ± 0.04 pg/mg tissue, $n = 8$). All regions showed stimulated release by potassium (56 mM) with nucleus accumbens (175%) and septum (125%) showing the largest stimulation.

Basal release of dopamine was highest in the striatum (3.47 ± 0.83 pmoles/mg tissue, $n = 6$) followed by the nucleus accumbens (1.75 ± 0.30 pmoles/mg tissue, $n = 8$), septum (0.49 ± 0.16 pmoles/mg tissue, $n = 8$) and hypothalamus (0.17 ± 0.03 pmoles/mg tissue, $n = 8$). The TRH analogue CG3509 (10^{-4} M) significantly increased release of endogenous dopamine in the hypothalamus (65%, $n = 8$, $P < 0.05$; Wilcoxon signed-rank paired test) nucleus accumbens (61%, $n = 11$, $P < 0.02$) and septum (57%, $n = 8$, $P < 0.05$) while RX77368 (10^{-4} M) increased release in the septum only (23%, $n = 7$, $P < 0.05$). Neither analogue significantly changed release of dopamine from the striatum.

The results suggest that dopaminergic systems, in the nucleus accumbens, septum and hypothalamus, may be involved in the central actions of CG3509 and RX77368. These studies indicate the need to distinguish carefully between closely associated regions such as the nucleus accumbens and septum.

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Effect of cations on [^{3}H]-sulpiride binding

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Sulpiride is a substituted benzamide which has potent dopamine-receptor blocking activity. [^{3}H]-sulpiride binds to striatal synaptic membrane preparations in a specific saturable fashion (Woodruff & Freedman, 1980; Theodorou, Crockett, Jenner & Marsden, 1979). Recently, it has been shown that sodium ions are essential for this binding (Theodorou, Hall, Jenner & Marsden, 1980), and that sodium can selectively increase the affinity of substituted benzamides in displacing [^{3}H]-spiperone binding (Stefanini, Marchisio, Deroto, Vernaleone, Collu & Spano, 1980).

We have studied the binding of [^{3}H]-(±)-sulpiride (15 nM) using partially purified rat striatal membranes. S-(−)-sulpiride (1 μM) was used to define specific binding. Appropriate filter blanks were included in each assay. In 50 mM Tris-Krebs' buffer, pH 7.4, containing nialamide (10 μM) and ascorbate (0.1%) specific binding was 208 ± 25 (mean ± s.e. mean) fmol/mg protein (n = 11). Omission of ions from the buffer significantly reduced the binding to 27.5 ± 5.2 fmol/mg protein (n = 11). Reintroduction of sodium restored the binding to control levels in a dose dependent fashion (IC_{50} 4.8 mM). Kinetic analysis indicated that this effect was due to changes in receptor number, rather than affinity. Thus, the K_D (8.9 ± 1.8 nM) in 50 mM tris-HCl containing 120 mM Na^+ was similar to that in buffer containing 10 mM Na^+ (10.6 ± 2.3 nM), but the Bmax was reduced from 330 ± 29 to 233 ± 23 fmol/mg protein. The effect was cation specific. Thus, in 50 mM tris-HCl containing 120 mM NaCl a binding value of 240 ± 4 (n = 3) fmol/mg protein was obtained. The corresponding values for binding in tris-HCl containing 60 mM Na_2SO_4, 60 mM Na_2HPO_4 and 120 mM NaBr were 260 ± 6, 235 ± 4 and 226 ± 6 fmol/mg protein respectively (n = 3 in each case).

Lithium ions could partially replace sodium (IC_{50} 9 mM) but the maximum binding was significantly reduced to 55% of that observed with sodium. The effects of lithium and sodium on submaximal concentrations were not additive. Maximal response obtained in the presence of 120 mM sodium was unchanged by the addition of 100 mM lithium. Other cations such as potassium, magnesium, calcium and manganese had no effect upon [^{3}H]-sulpiride binding at concentrations up to 50 mM.

The results show that sodium ions are necessary for [^{3}H]-sulpiride binding to striatal membranes. The effect does not appear to be due to a change in receptor affinity. Lithium appears to be the only other cation able to mimic sodium. Lithium and sodium may be useful tools in understanding the interactions of substituted benzamines with dopamine receptors.

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Repeated sulpiride administration to rats, like repeated haloperidol, induces cerebral dopamine receptor supersensitivity

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Sulpiride ((±)-N-[1′-ethyl-2′-pyrrolidinylmethyl]-2-methoxysulphamoyl benzamide) is a cerebral dopamine receptor antagonist (Jenner, Elliott, Clow, Reavill & Marsden, 1978). However, acute administration of sulpiride does not block apomorphine-induced stereotyped behaviour or *in vivo* inhibit dopamine stimulation of adenylate cyclase in striatal preparations. Following repeated administration of typical neuroleptic agents enhanced apomorphine-induced stereotyped behaviour and increased dopamine receptor numbers are apparent (see Muller & Seeman, 1978). We have therefore compared the ability of repeated administration of sulpiride to cause behavioural and biochemical supersensitivity of cerebral dopamine receptors with that of the butyophenone neuroleptic haloperidol.

Male Wistar rats (150 ± 10 g at the start of the experiment) received either sulpiride (100 mg/kg i.p. twice daily), haloperidol (5 mg/kg i.p.) or 0.9% saline for 21 days after which time the animals were allowed a 3–4 day drug-washout period.

Stereotyped behaviour was assessed 15 min following administration of apomorphine hydrochloride (0.125–2.0 mg/kg s.c.). Repeated administration of haloperidol or sulpiride caused an enhancement of the apomorphine stereotyped response over the dosage range used when compared to saline-treated animals (Table 1).

Dopamine (1–1000 uM) stimulation of adenylate cyclase activity was determined *in vitro* in striatal homogenates. Pre-treatment with either haloperidol or sulpiride caused no change in dopamine stimulated cyclic AMP formation compared to saline-treated animals (Table 1).

Specific [^{3}H]-spiperone (0.125–4.0 nM; 26 Ci/mmol; as defined using 5×10^{-6} M (+)-butaclamol), [^{3}H]-sulpiride (5–40 nM; 26.2 Ci/mmole; as defined using 5×10^{-6} M (−)-sulpiride) and [^{3}H]-N,n-propylnorapomorphine (0.0625–4.0 nM; 58.5 Ci/mmole; as defined using 1×10^{-6} M (+)-butaclamol) binding was determined in striatal preparations. In each case the number of binding sites (Bmax) was enhanced by repeated sulpiride or haloperidol administration compared to saline treated animals (Table 1). The dissociation constant (K_D) for these ligands was unaltered by prior drug treatment.

The data shows that sulpiride, like haloperidol (Tarsy & Baldessarini, 1974; Burt, Creese & Snyder, 1977), is capable of inducing behavioural and biochemical supersensitivity of cerebral dopamine receptors.

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Table 1 The effect of repeated administration of haloperidol or sulpiride on cerebral dopamine receptor function

Treatment	*Stereotypy score*	*Cyclic AMP formed (% increase over basal)*	[^3H]-*spiperone*		[^3H]-*sulpiride*		[^3H]-*N,n-propylnorapomorphine*	
			Bmax (pmoles/ g tissue)	K_D (nM)	*Bmax (pmoles/ g tissue)*	K_D (nM)	*Bmax (pmoles/ g tissue)*	K_D (nM)
Saline	2.1 ± 0.2	59.6 ± 6.5	24.0 ± 1.8	0.22 ± 0.03	33.9 ± 2.6	19.6 ± 2.6	6.8 ± 0.5	1.1 ± 0.1
Haloperidol	2.9 ± 0.2*	53.1 ± 2.4	32.3 ± 2.3*	0.23 ± 0.02	51.1 ± 2.2*	22.6 ± 3.7	12.7 ± 1.8*	1.2 ± 0.2
Salpiride	3.0 ± 0.2*	44.9 ± 4.2	29.5 ± 2.4*	0.27 ± 0.04	47.3 ± 2.7*	22.4 ± 3.3	9.7 ± 1.2*	1.3 ± 0.2

* $P < 0.05$ compared to saline treated animals.
Stereotypy was assessed 15 min following administration of apomorphine (0.5 mg/kg s.c.).
Cyclic AMP formation was stimulated by incorporation of dopamine (50 uM).

A comparative study of [^{3}H]-domperidone and [^{3}H]-spiperone binding in the rat striatum

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A number of neuroleptics have been used as radioligands for dopamine receptors but all have suffered from either problems of low affinity, high non-specific binding or relative lack of specificity. Domperidone has recently been introduced as a new ligand for dopamine receptors (Baudry, Martres & Schwartz, 1979) and the present study was conducted to compare the binding characteristics of [^{3}H]-domperidone ([^{3}H]-DOM) and [^{3}H]-spiperone ([^{3}H]-SPI) in membranes prepared from rat striatum, a brain region in which [^{3}H]-SPI appears to bind predominantly, but not exclusively, to dopamine receptors (Howlett & Nahorski, 1980).

Washed striatal homogenates were prepared from male Wistar rats by homogenization and centrifugation, and aliquots (120–150 ug protein) were incubated at room temperature for 45 min with Tris-HCl (50 mM, pH 7.6), radioligand (made up in 0.01% bovine serum albumin) and competing drugs in a volume of 1 ml (displacement experiments) or 2 ml (saturation experiments). Membranes were filtered through glass-fibre filters (Whatman GF-C, pretreated with 0.01% bovine serum albumin) and rapidly washed with 20 ml Tris buffer. Specific binding was defined as that displaced by 10^{-6} M (+)-butaclamol. Experiments were conducted in duplicate and [^{3}H]-SPI and [^{3}H]-DOM were examined in parallel within the same experiment.

Scatchard analysis of saturation experiments in the concentration range 0.02 nM–0.8 nM indicated that each ligand bound to an apparently homogeneous population of sites with similar K_D([^{3}H]-SPI = 0.06 ± 0.01 nM; [^{3}H]-DOM, = 0.08 ± 0.01 nM) but that the B_{max} (fmol/mg protein) for [^{3}H]-SPI (308 ± 29) was greater than that for [^{3}H]-DOM (205 ± 26). The ligands (0.2–0.3 nM) had similar specific binding ratios (70–80% of total binding) and showed similar profiles in their displacement by various cold drugs, but all drugs displayed greater potency against [^{3}H]-DOM than against [^{3}H]-SPI (Table 1). Dopamine had the greatest potency ratio, followed by DA agonists and selective DA antagonists, and the smallest potency ratios were found with 5-HT related antagonists and 5-HT itself. Sulpiride produced an obviously biphasic displacement curve against [^{3}H]-SPI, and iterative curve-fitting revealed that it displaced from a population comprising 70% of [^{3}H]-SPI sites with an IC_{50} of 0.31 uM, and from a population comprising almost 100% of DOM sites with an IC_{50} of 0.15 uM.

These data suggest that [^{3}H]-DOM labels predominantly a population of sites which make up about 70% of butaclamol-specific [^{3}H]-SPI sites, and the remaining 30% of [^{3}H]-SPI sites could be 5-HT related. [^{3}H]-DOM has a high affinity and high

Table 1 Displacement by various drugs of [^{3}H]-DOM and [^{3}H]-SPI from rat striatal membranes

Drug	*[^{3}H]-DOM IC_{50} (nM)*	*[^{3}H]-SPI IC_{50} (nM)*	*Potency ratio IC_{50} SPI/IC_{50} DOM*
Dopamine	120 ± 30	740 ± 170	7.9 ± 2.7
ADTN	24 ± 6	160 ± 50	6.5 ± 1.2
Bromocryptine	11 ± 2	55 ± 14	5.1 ± 1.0
Sulpiride	230 ± 50	1300 ± 500	5.2 ± 0.9
Clebopride	38 ± 7	170 ± 40	4.6 ± 0.5
Domperidone	1.5 ± 0.4	5.7 ± 0.7	4.3 ± 0.9
Haloperidol	0.8 ± 0.1	2.8 ± 0.2	3.6 ± 0.2
α-Flupenthixol	1.6 ± 0.2	4.9 ± 0.02	3.2 ± 0.3
Chlorpromazine	1.2 ± 0.2	3.4 ± 0.5	3.2 ± 0.7
Spiperone	0.8 ± 0.1	2.4 ± 0.4	2.9 ± 0.3
d-Butaclamol	4.1 ± 1.1	8.3 ± 2.1	2.5 ± 0.7
l-Butaclamol	1900 ± 400	5600 ± 300	3.4 ± 0.8
Noradrenaline	1300 ± 300	5800 ± 1800	4.6 ± 1.0
Phentolamine	1100 ± 200	2400 ± 500	2.4 ± 0.8
Pipamperone	50 ± 19	71 ± 21	1.5 ± 0.3
Mianserine	2000 ± 600	3100 ± 700	1.6 ± 0.2
5-Hydroxytryptamine	6900 ± 1000	7900 ± 2000	1.2 ± 0.3

IC_{50} values were calculated by Hill analysis. [^{3}H]-ligand concentration was 0.2–0.3 nM. A potency ratio was obtained for each experiment. Values are the means and standard errors of at least three experiments.

specific binding ratio like [^{3}H]-SPI, but appears to demonstrate a greater specificity for dopaminergic sites and as such is the more appropriate ligand for dopamine receptors.

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The effects of striatal kainic acid lesions on [^{3}H]-spiperone and [^{3}H]-cis flupenthixol binding in rat striatum

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[^{3}H]-cis flupenthixol ([^{3}H]-FPT) has previously been shown to bind to striatal membrane preparations with a pharmacological profile similar to that of dopamine-stimulated adenylate cyclase (D1 receptors) (Hyttel, 1978; Cross & Owen, 1980), and distinct from [^{3}H]-spiperone ([^{3}H]-SPIP) binding (D2 receptors). It has also been demonstrated that kainic acid (KA) lesions of rat striatum produce marked losses of dopamine-stimulated adenylate cyclase acitivity, with significantly less depletions of [^{3}H]-SPIP binding sites (Schwarcz, Creese, Coyle & Snyder, 1978). To further define the distinction between [^{3}H]-FPT binding sites and [^{3}H]-SPIP binding sites, we have studied the effects of striatal KA lesions on the binding of both ligands to dopamine receptors in rat striatum.

Unilateral striatal KA lesions, and ligand binding assays were performed as previously described (Waddington & Cross, 1978; Cross & Owen, 1980). Comparisons were made between lesioned and control striata using the paired Students '*t*' test (2 tailed).

After striatal KA lesion (n = 23), significant reductions were observed in both [^{3}H]-SPIP binding (-37%, $P < 0.01$) and [^{3}H]-FPT binding (-55%, $P < 0.01$). The decrease in [^{3}H]-FPT binding was significantly greater than the decrease in [^{3}H]-SPIP binding ($P < 0.01$). Saturation analysis revealed that for both ligands the reduction in binding was characterized by a decreased number of binding sites, with no change in affinity. A range of lesions enabled comparisons to be made between reductions in binding parameters and the degree of striatal cell loss. Reductions in both [^{3}H]-FPT and [^{3}H]-SPIP binding were significantly correlated with reductions in striatal glutamic acid decarboxylase (GAD) activity. After complete loss of striatal GAD activity, 30–40% of [^{3}H]-SPIP binding sites remained, whereas all [^{3}H]-FPT binding sites were destroyed.

These results demonstrate a differential localization of [^{3}H]-FPT and [^{3}H]-SPIP binding sites within the rat striatum. The results are consistent with [^{3}H]-FPT binding predominantly to dopamine D1 receptors, and [^{3}H]-SPIP binding mainly to D2 receptors.

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Characteristics of [^{3}H]-Spiperone binding to human lymphocytes

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Le Fur, Phan & Uzan (1980a) recently described the high affinity binding of [^{3}H]-spiperone to the lymphocytes of several mammalian species including man. Subsequently Le Fur, Meininger, Phan, Gerard, Baulac & Uzan (1980b) reported decreased binding of [^{3}H]-spiperone to lymphocytes from patients with Parkinsonism. In view of the potential importance of a possible peripheral index of dopamine receptors in the CNS we have studied the characteristics of [^{3}H]-spiperone binding to human lymphocytes.

Preparation of lymphocytes and the binding studies were carried out as described by Le Fur *et al.* (1980a). With increasing concentrations of [^{3}H]-spiperone (1–16 nM), binding curves were biphasic, similar to those reported by Le Fur *et al.* (1980a). However, Scatchard analysis of the binding data produced no evidence of saturable high affinity binding sites. In addition, the binding did not exhibit marked stereospecifity with respect to the isomers of either butaclamol or flupenthixol. Also there was no correlation between the potencies of a number of neuroleptics in displacing [^{3}H]-spiperone binding from human striatal preparations and lymphocytes (Table 1).

Table 1 Inhibition of [^{3}H]-spiperone binding to human lymphocytes and human striatum by drugs

Compound	*Lymphocyte Ki (nM)*	*Striatum Ki (nM)*
Chlorpromazine	10.6	35
Domperidone	210	1.7
Clozapine	210	—
(+) Butaclamol	456	1.9
Pimozide	420	—
Spiperone	530	0.25
cis-flupenthixol	652	3.1
(−) Butaclamol	833	⁄ 10,000
trans-flupenthixol	1110	—
Dopamine	2108	200
Serotonin	8393	—

It is concluded that the binding of [^{3}H]-spiperone to human lymphocytes is not to dopamine receptors and its use as a peripheral index of central DA receptors is unlikely to yield meaningful results.

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α-Adrenoceptor binding and autoregulation of noradrenaline release in the brain of a convulsive mutant mouse

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The Quaking mouse (neurological mutant of the C57Bl/6J strain) is characterized by tonic-clonic seizures triggered by tactile stimuli.

These convulsions are antagonized by the α_2-adrenoceptor antagonist yohimbine. This protection is reversed by the α_2-adrenoceptor agonist clonidine and by the α_1-adrenoceptor antagonist prazosin (Chermat, Lachapelle, Baumann & Simon, 1979). Therefore it was considered of interest to examine if these *in vivo* observations could be explained by changes in the binding characteristics of α-adrenoceptor sites or in the presynaptic autoregulation of noradrenaline release.

Binding of [^{3}H]-prazosin and [^{3}H]-clonidine to brain homogenates was measured as previously described (Pimoule, Briley & Langer, 1980).

Electrically-evoked release of [^{3}H]-noradrenaline from prelabelled occipital cortex slices was measured as described by Dubocovich, Galzin & Langer (1980). Two periods (S_1, S_2) of electrical stimulation (3 Hz, 2 msec, 2 min, 24 mA) were applied 44 min apart and drugs were added 20 min before S_2.

[^{3}H]-Prazosin binding revealed an increase in the number of binding sites in the mutants (Bmax

130.27 ± 9.36 fmoles/mg protein, as compared to 85.20 ± 9.48 fmoles/mg protein, for the controls, $P < 0.01$, $n = 6$) with no alteration of the Kd.

Binding of [^{3}H]-clonidine showed that both the Kd and Bmax values were increased in the brain of the Quaking mice (Kd: 5.15 ± 0.58 nM and Bmax: 112.80 ± 5.32 fmoles/mg protein, $n = 6$ for controls; Kd: 8.62 ± 1.08 nM and Bmax: 250.74 ± 7.33 fmoles/mg protein, $n =$ for the Quaking, $P < 0.001$).

In studies of [^{3}H]-noradrenaline release the percentage of total tissue radioactivity released during S_1 was 3.37 ± 0.16% for the controls and 2.98 ± 0.17% for the mutants ($n = 16$). S_2/S_1 ratios were 1.03 ± 0.03 and 0.98 ± 0.03 for the controls and mutants, respectively. The release of [^{3}H]-noradrenaline was entirely calcium dependent.

Yohimbine increased and clonidine decreased the release of noradrenaline in a concentration-dependent manner. In both cases, the curves obtained for mutant and control were identical. The maximal effects were obtained at 1 μM yohimbine (S_2/S_1: controls 3.49 ± 0.09; Quaking: 3.62 ± 0.08, $n = 3$) and at 100 nM clonidine (S_2/S_1: controls 0.17 ± 0.08; Quaking 0.14 ± 0.06, $n = 3$).

We conclude that *in vitro* binding studies support the *in vivo* pharmacological results of Chermat *et al.* (1979) since α-adrenoceptors appear to be modified in the brain of the Quaking mice. Electrically-evoked overflow of labelled noradrenaline shows that the presynaptic autoregulation of the release of this neurotransmitter through α_2-adrenoceptors, as reported for other species, exists also in the mouse. However, this autoregulation does not appear to be directly related to the seizures exhibited by the Quaking mice. Thus, the changes observed in [^{3}H]-Clonidine binding might be linked to post-synaptic rather than presynaptic release-modulating α_2-adrenoceptors (Pimoule *et al.*, 1980; U'Prichard, Bechtel, Rouot & Snyder, 1979). The increase in the numbers of α_1- and α_2-adrenoceptors may play a role in the behavioural abnormalities of the Quaking mice.

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Effects of anisomycin and electrical stimulation on substance P concentrations in the mouse nervous system

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Substance P (SP) has been proposed as a neurotransmitter in several tracts within the CNS and in primary afferent neurones. To assess the activity in these tracts under various conditions we wish to study changes in SP turnover and have attempted to do this by measuring the rate of decline in tissue SP content following the administration of anisomycin, a protein synthesis inhibitor which inhibits SP synthesis *in vitro* at 10^{-6} M. Anisomycin was chosen because it inhibits cerebral protein synthesis *in vivo* at doses which are relatively non-toxic (Flood, Bennett, Rosenzweig & Orme, 1973).

Mice were given anisomycin (100 μg/g s.c. every 2 h). This regime inhibited protein synthesis, as measured by incorporation of [^{14}C]-leucine into TCA insoluble material, by greater than 96% in dorsal root ganglia (L 3–5), lumbar spinal cord, medulla, midbrain, hypothalamus, striatum and cortex. 8 h after commencing anisomycin administration mice were killed by decapitation and SP-like immunoreactivity (SPLI) was measured by radioimmunoassay in the seven structures listed above and in the cornea, which contains peripheral terminals of SP neurones. In none of these tissues did anisomycin cause a significant change in levels of SPLI relative to saline-treated control animals, suggesting that turnover of SP must be very slow compared with that of most conventional neurotransmitters.

To determine whether this turnover could be accelerated by nervous activity pairs of mice were anaesthetized with urethane and given 2-hourly injections of anisomycin over an 8 h period. One mouse of each pair was stimulated electrically (1 Hz, 1 ms, 30 V) through fish-hook electrodes implanted in the pad and thigh of each hind-limb, throughout a 7 h period, commencing 1 h after the first dose of anisomycin had been given. Stimulation produced no significant change in the SPLI content of dorsal root ganglia L3–5 (control 730 ± 51 (mean ± s.e. mean, n = 10); stimulated 694 ± 36, n = 12, pg total) or in ventral spinal cord (control 2.05 ± 0.21, n = 10; stimulated 1.95 ± 0.23, n = 11 pg/μg insoluble protein) but a significant ($P < 0.05$) depletion in SPLI of dorsal lumbar spinal cord (control 13.4 ± 0.9, n = 10; stimulated 10.1 ± 1.2, n = 12, pg/μg protein) in which area the majority of SP-containing primary afferent terminals are found (Takahashi & Otsuka, 1975).

It is probable that neuropeptide synthesis is confined to the neuronal cell body so that changes in demand cannot be met by short-term control of transmitter synthesis in nerve terminals, as occurs with conventional neurotransmitters. The results presented here suggest that SP neurones may buffer changes in transmitter demand by maintaining a relatively large pool of peptide in their terminals which turns over slowly but that can be partially depleted by intense nervous activity.

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Behavioural effects evoked by microinjection of excitatory amino acid antagonists into the midbrain of the rat

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The compounds 2-amino-5-phosphonovaleric acid (2-APV) and γ-D-glutamylglycine (γ-DGG) have been reported to be specific antagonists at receptors for excitatory amino acids. In particular both of these compounds potently antagonize the excitation produced by N-methyl-D-aspartic acid (NMDA), a proposed excitatory amino acid agonist, in the vertebrate spinal cord (Francis, Jones & Watkins, 1980; Davies, Francis, Jones & Watkins, 1980). However, to date there have been no studies on the possible behavioural effects of these antagonists when administered at supraspinal sites. In view of the neurochemical evidence suggesting the existence of excitatory amino acid pathways to the midbrain (Taniyama, Nitsch, Wagner & Hassler, 1980) and the fact that in a previous report we have shown that the excitatory amino acid agonists kainic acid and NMDA, when injected into the substantia nigra and ventral tegmental area of the rat, produce specific behavioural responses (Dawbarn & Pycock, 1980), we have now focally applied 2-APV and γ-DGG into the midbrain of the rat and observed the behavioural effects.

Microinjections of antagonists or vehicle (0.05 M phosphate buffer, pH 7.4) were carried out under halothane anaesthesia using co-ordinates taken from the atlas of König & Klippel (1963): substantia nigra, pars compacta (SNc), A 2.4, L ± 2.0, V −2.0; substantia nigra, pars reticulata (SNr), A 1.6, L ± 2.0, V −2.4; ventral tegmental area (VTA), A 2.2, L ± 0.25, V −3.0. Both 2-APV and γ-DGG were injected either unilaterally or bilaterally at two different doses into these areas in a volume of 0.1 μl. The animals were then placed in an open field and after 5 min any behavioural changes were recorded and compared with rats receiving vehicle only.

2-APV (0.1 and 1 μg) and γ-DGG (1 and 10 μg) produced qualitatively similar results although 2-APV appeared to be the more potent drug. Bilateral injection of each dose of the antagonists into either the SNc or VTA evoked enhanced locomotor activity in the open field ($P < 0.05$–0.001). Unilateral injection into the SNc produced tight dose-related contralateral turning behaviour (1 μg 2-APV and γ-DGG induced 11 ± 2 and 6 ± 1 turns/min respectively, 20 min after injection). These motor responses were antagonized by pretreating the animals with fluphenazine (1 mg/kg, i.p., 30 min).

Bilateral injection of the antagonists into the SNr in

contrast produced a significant decrease ($P < 0.01$) in locomotor activity compared to animals injected with vehicle alone. Animals remained sedated in one corner of the field. Paradoxically unilateral injection of either 2-APV or γ-DGG into the SNr again produced dose-related contralateral turning (1 μg 2-APV and γ-DGG induced 9 ± 2 and 5 ± 1 turns/min respectively, 15 min after injection). This circling behaviour was also blocked by pretreatment with fluphenazine. Unilateral injections of both 2-APV (1 μg) and γ-DGG (10 μg) into either SNc or SNr were associated with a significant rise in the ipsilateral concentrations of the striatal dopamine metabolites homovanillic acid and dihydroxyphenylacetic acid ($P < 0.05$).

In conclusion the putative excitatory amino acid antagonists 2-APV and γDGG, when injected into the midbrain of the rat, produce specific behavioural actions which may in part be mediated through the ascending dopaminergic pathways. This data may add weight to the postulated existence of excitatory amino acid fibres in the midbrain of the rat, which may play a role in the control of movement.

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Evidence for excitatory amino acid sensitive adenylate cyclase in rat brain membrane preparations

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The transmitter action of excitatory amino acids in the central nervous system is well-established (Watkins, 1978). It has previously been shown that glutamate and aspartate increase levels of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) in brain slice preparations (Shimizu, 1975). However, these responses may be mediated by several indirect means including potassium release (Evans, 1980), adenosine release (Shimizu, 1975), or phosphodiesterase inhibitor (Shimizu, 1977).

The development of more stable and specific agonists for the glutamate and aspartate preferring receptors, namely N-methyl D-aspartic acid (NMDA) and kainic acid (KA) respectively, has added new dimensions to the possibility of studying the biochemical basis of excitatory amino acid receptor activation. We have tested the hypothesis that excitatory amino acid adenylate cyclase is associated with brain cell membranes.

Experiments were performed to examine the effect of NMDA and KA on membrane preparations enriched in adenylate cyclase activity by separation and enrichment through differential centrifugation. Rat whole brain and striatal homogenates were centrifuged at 1600 *g* for 10 min and 37,000 *g* for 30 min after prior removal of 600 *g* × 5 min and 1600 *g* × 10 min fractions respectively. In every experiment transmitter sensitivity of adenylate cyclase was assessed with dopamine (50 μM) and the primary catalytic activity with sodium fluoride (20 mM). In membrane preparations incubated with adenosine triphosphate (2 mM) and 3-Isobutyl 1-methyl xanthine (0.5 mM, IBMX), NMDA showed a time-dependent activation of adenylate cyclase which became maximal at 15 to 20 minutes. KA showed an initial time-dependent activation of adenylate cyclase, but with larger incubations often a reduction was observed in the activation of the enzyme. NMDA stimulation of adenylate cyclase was dose-dependent in the concentration range, 50 μM to 1 mM. KA activation of adenylate cyclase was dose-dependent over the 50 μM to 200 μM range, but often a reduction was observed with 1 mM and 2 mM KA.

Guanyl nucleotides are known to influence many cAMP-mediated responses. We investigated the effects of varying concentrations of guanosine triphosphate (GTP) on the NMDA induced activation of adenylate cyclase in the striatum. Basal and dopamine stimulated activity was increased by GTP (McSwigan, 1980). In contrast, a reduction in the NMDA-stimulated activity was observed with increasing concentrations of GTP.

These results suggest that there are NMDA and KA receptors in rat brain membrane preparations linked to adenylate cyclase and influenced by GTP.

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Histamine H_1-receptors and cAMP accumulation in guinea-pig cerebral cortical slices

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There is now strong evidence for the presence of histamine H_1-receptors in the brain of the guinea-pig and other species (Schwartz, 1979). Studies with [^{3}H]-mepyramine indicate that the binding characteristics of central H_1-receptors are closely similar to those of peripheral H_1-receptors (Hill, Emson & Young, 1978; Chang, Tran & Snyder, 1979). However, at present there is very little evidence for any functional response in brain mediated by H_1-receptors with properties quantitatively equivalent to those observed in binding studies. In this communication we show that the potentiation by histamine of the adenosine-stimulated accumulation of cyclic AMP in guinea-pig cerebral cortex (Daly, 1977) is a particularly suitable response for this type of analysis and that the affinities of antagonists are closely similar to those determined from binding studies.

The histamine potentiation of the adenosine-stimulated cyclase is observed only in slice preparations and it seems likely that the action of histamine is indirect, possibly being mediated by Ca^{2+} (Schwartz, 1979). In contrast, H_2-stimulation of adenylate cyclase appears to be direct and is observed in tissue homogenates in non-physiological media (Hegstrand, Kanof & Greengard, 1976). The presence of both H_1-and H_2-components can complicate interpretation of results (Palacios, Garbarg, Barbin & Schwartz, 1978), but in guinea-pig cerebral cortex the H_2-component is reported to be small (Daly, 1977).

Slices from guinea-pig cerebral cortex were prepared with a McIlwain chopper and preincubated at 37°C for 20 min with 4 μM adenine. Incubations with histamine or H_1-selective agonists were for 10 min at 37°C and were terminated by boiling. CyclicAMP was measured using the protein binding assay.

In these preparations the response to 1 mM dimaprit, an H_2-agonist, was negligible and was not significantly altered in the presence of 0.1 mM adenosine. In contrast, histamine and 2-thiazolylethylamine, an H_1-selective agonist, in the presence of 0.1 mM adenosine produced a 3–15 fold stimulation of the accumulation of cAMP, which was not significantly diminished by cimetidine (0.1 mM). The antagonism of the histamine stimulated adenosine response by four H_1-antihistamines was competitive and stereospecific (potency ratio (+)-:(−)-chlorpheniramine approx. 200). The affinity constants derived for the antagonists from the parallel shift of the dose-response curves were in very good agreement with the values obtained from the inhibition of the binding of [^{3}H]-mepyramine.

These observations indicate that at least a proportion of the sites labelled by [^{3}H]-mepyramine in guinea-pig cerebral cortex are linked to functional histamine H_1-receptors.

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A comparison of slow and fast depressant responses of rat cerebral cortical neurones to ionophoretically applied histamine

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Carette (1978) observed two types of inhibitory response to histamine applied ionophoretically to guinea-pig preoptic septal neurones, a response with rapid onset and recovery and a response with an appreciably slower time course. We have also observed two types of response on rat cerebral cortical neurones although only the fast has been described previously (Haas & Wolf, 1977). These responses have therefore been investigated.

Fast responses to histamine were recorded using conventional 7-barrelled glass microelectrodes (tips 7–10 μm) from glutamate-stimulated cells in the cerebral cortex (2–8 mm behind bregma, 2–4 mm lateral of midline) of rats anaesthetized with urethane plus pentobarbitone (0.4 g and 40 mg/kg respectively, ip). Slow responses to histamine were recorded using a single barrel attached to the side of a conventional 7-barrelled electrode so that its tip protruded 10–20 μm, from spontaneously firing cells in the cerebral cortex (0–4 mm in front of bregma, 2–4 mm lateral of midline) of rats anaesthetized with 1% halothane in oxygen. The recording barrel and that used for current balancing contained 2 M NaCl. Drug solutions were placed in the other barrels, including histamine dihydrochloride (B.D.H.), N^{τ}-methylhistamine dihydrochloride (SK&F), N^{π}-methylhistamine dihydrochloride (SK&F), 2-pyridylethylamine dihydrochloride (SK&F), 2-methylhistamine dihydrochloride (SK&F), 4-methylhistamine dihydrochloride (SK&F) and impromidine trihydrochloride (SK&F) all at 0.2 M pH 4.5, metiamide (SK&F, 0.1 M pH 4.5), and monosodium L-glutamate (B.D.H. 0.2 M pH 6.5).

The compounds N^{τ}-methylhistamine and N^{π}-methylhistamine, although inactive on peripheral histamine H_1 and H_2 receptors, produced fast depressant responses on cells responding in this way to histamine (41/41, 15/15 cells), but were never observed to produce slow responses (0/20, 0/4 cells). Similarly 2-methylhistamine, 2-pyridylethylamine, 4-methylhistamine and impromidine all produced fast depressant responses (4/4, 6/13, 4/4, 4/4 cells respectively), whereas only the selective H_2-receptor agonists 4-methylhistamine and impromidine were observed to produce slow responses (0/6, 0/8, 5/5, 7/7 respectively). The slow response to histamine could be antagonized readily, completely and reversibly by ionophoresis of the H_2-receptor histamine antagonist, metiamide (11/11 cells), whereas antagonism was much less readily observed on the fast depressant response (4/11 cells), partly perhaps due to the depressant activity of metiamide itself. Metiamide was never observed to produce a slow depression.

In conclusion, in contrast to the lack of pharmacological specificity of the fast depressant response to histamine reported here and previously (e.g. Roberts, 1980), the specificity of the slow depressant response to histamine is consistent with its being mediated by histamine H_2-receptors.

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A central cholinergic link in the neural control of the release of vasopressin

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The release of vasopressin without oxytocin in response to haemorrhage, hypotension and carotid occlusion is controlled by neuro-endocrine reflexes involving synapses in the brain stem and hypothalamus (see Bisset & Jones, 1976). In the water-loaded rat under ethanol anaesthesia, the hypotensive action of amyl nitrite induces an antidiuretic response (ADR) which is accompanied by increased excretion of vasopressin in the urine and can be blocked by a specific antibody to vasopressin (Bisset, Black, Hilton, Jones, Kanjanapothi & Montgomery, 1974; Kanjanapothi, 1975). We have found that the ADR to amyl nitrate is inhibited by barbiturates and this indicates, by analogy to the milk-ejection response to suckling, that a neuro-endocrine reflex is involved.

In the experiment of Figure 1, inhalation of amyl nitrate at A1 produced a brief fall of blood pressure followed by a prolonged ADR. A dose of hexamethonium (0.5 mg), sufficient to block the ADR to nicotine (50 μg i.v.), was then injected into a lateral cerebral ventricle (i.vent.). 65 min later a second inhalation at A2, sufficient to produce a much larger fall of blood pressure, caused only a transient decrease in urine flow concomitant with the hypotension; the prolonged ADR seen at A1 was abolished. As Figure 1 shows, muscarine (20 ng i.vent.) (Bisset & Chowdrey, 1980) also elicited an ADR which could be blocked by atropine (20 μg i.v.) Neither atropine (2 mg) nor hexamethonium (1 mg i.v.) reduced the ADR to amyl nitrite but this was blocked by injection of hexamethonium (0.5 mg) into the cisterna magna from which in the rat there is no access to the cerebral ventricles (Feldberg, 1976). This indicates that hexamethonium acts at a central site reached from the subarachnoid space. The results which were obtained repeatedly provide evidence for a cholinergic link involving nicotinic but not muscarinic receptors in the reflex pathway for release of vasopressin by hypotension.

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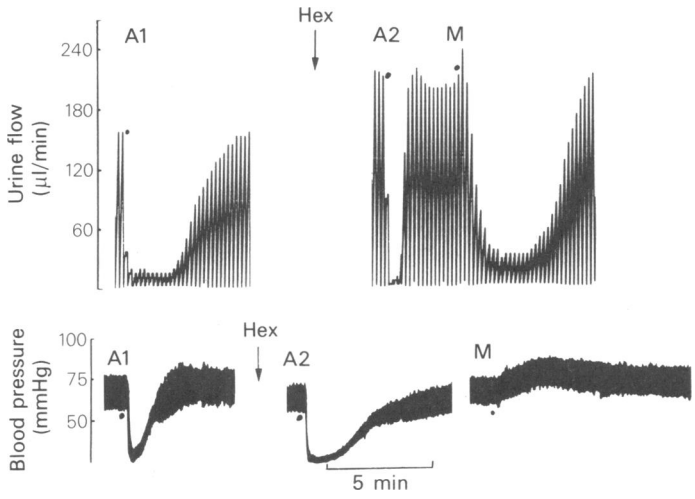

Figure 1 Urine flow (upper panel) and arterial blood pressure (lower panel) in a water-loaded rat under ethanol anaesthesia. Urine flow was measured with a drop recorder which reset to zero every minute. Drug administration indicated by a dot; Amyl-nitrite inhalation (A1, A2); Intraventricular injections of muscarine (20 ng, M) and of hexamethonium (0.5 mg, Hex, 65 min before A2) were given.

Inhibition of uterine responses to oestrogen in the ovariectomized rat

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It has previously been established that 3 h after an intravenous injection of oestradiol-17β (0.5 μg/kg) a maximal increase in blood flow and a significant increase in wet and dry weights are observed in uteri of mature ovariectomized rats (Majid & Senior, 1980). Uterine blood flow was measured using the microsphere technique. In the present experiments a similar increase in blood flow and uterine weight was observed 3 h after an intravenous injection of oestriol (1 μg/kg) but the increase in uterine wet and dry weights was not maintained. The uterine wet and dry weights 9 h after oestradiol-17β injection (165 $\pm$ 6 and 28 $\pm$ 1 mg respectively) were significantly higher ($P < 0.005$) than those 9 h after oestriol injection (116 $\pm$ 4 and 22 $\pm$ 1 mg). The uterine response to oestriol is consistent with the suggested short term occupancy of the nuclear receptors in target cells by this oestrogen (Anderson, Peck & Clark, 1975).

Pretreatment with anti-oestrogen, tamoxifen (1 mg/kg) subcutaneously 24 h before treating with oestradiol-17β inhibited the uterine blood flow response and reduced the uterine weight increases seen with oestradiol alone. Tamoxifen treatment itself produced a significant increase ($P < 0.005$) in wet and dry uterine weights but had no significant effect on blood flow. Neither actinomycin-D (0.5 mg/kg) intraperitoneally nor the prostaglandin synthetase inhibitor AH 7170 (2-m-(γ-chlorobenzoyl)phenylpropionic acid) (1 mg/kg, Glaxo Research Ltd) orally produced any significant effect on the maximal uterine blood flow 3 h after oestradiol-17β injection but both treatments inhibited the uterine water imbibition. Actinomycin-D also suppressed the uterine dry weight increase to oestradiol.

Treatment with either actinomycin-D or AH 7170 combined with tamoxifen before injection of oestradiol-17β inhibited both the uterine blood flow response and the uterine wet and dry weight increases. The combination of tamoxifen with a prostaglandin synthetase inhibitor suggests a useful and non-toxic treatment for the suppression of oestrogenic effects on the uterus.

The results also suggest that modification of occupancy time of oestrogen receptors in the nucleus and inhibition of RNA or prostaglandin synthesis may be used to suppress the responses to oestrogens in target organs.

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Inhibition of the irreversible binding of ethinyloestradiol to rat liver microsomes *in vitro*

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Ethinyloestradiol (EE_2) may induce the formation of anti-EE_2 antibodies in women taking oral contraceptives (Beaumont *et al.*, 1979). Thus formation of chemically reactive metabolites may be relevant to the toxicology of oral contraceptives, and we have therefore investigated factors which may influence the relationship between the metabolism of EE_2 and the irreversible binding of EE_2 metabolites to rat liver microsomes *in vitro*.

Liver microsomes were prepared from male Wistar rats (200–300 g) and incubated with [^{3}H]-EE_2 as described previously (Park & Whittaker, 1978). Irreversible binding of [^{3}H]-EE_2 metabolite(s) to microsomes increased linearly up to 30 min and the effect of inhibitors was determined at 20 minutes. The irreversible binding of reactive [^{3}H]-intermediates to microsomal components was measured after exhaustive solvent extraction of the microsomes. In parallel experiments, a lower concentration of EE_2 (0.01 mM) was used to allow sufficient turnover of substrate for analysis of the metabolite profile by reverse-phase h.p.l.c. using gradient elution with methanol-ammonium phosphate buffer (pH3) after extraction with ether and ethanol.

The inhibition of the irreversible binding of EE_2 metabolites to microsomes by various agents is shown in Table 1. It has been demonstrated that mixed-function oxidases are involved in the generation of reactive metabolites from EE_2 (Bolt *et al.*, 1973). Accordingly, we have found that the irreversible binding of EE_2 to microsomes is inhibited by SKF 525A, piperonyl butoxide and, to a lesser extent, by cimetidine. α-Naphthoflavone is a more potent inhibitor than metyrapone, suggesting that P-448 mixed-function oxidases are more important than P-450 mixed-function oxidases for the formation of chemically reactive metabolites.

Table 1 Inhibition of the irreversible binding of ethinyloestradiol metabolite(s) to rat liver microsomes *in vitro*.

Inhibitor (0.5 mM)	*% Inhibition*
SKF 525A	90 ± 4*
Piperonyl butoxide	75 ± 3*
Cimetidine	36 ± 7*
α-Naphthoflavone	82 ± 4*
β-Naphthoflavone	86 ± 4*
Metyrapone	45 ± 4*
β-Naphthoflavone + Metyrapone	83 ± 10*
Oestradiol	−10 ± 12
Cortisol	−9 ± 17
Norethisterone	29 ± 4*
Norgestrel	33 ± 7*
Glutathione	85 ± 3*
Cysteine	70 ± 5*
N-Acetylcysteine	62 ± 6*
Cysteamine	86 ± 6*
Dithiothreitol	75 ± 5*
2-Mercaptoethanol	78 ± 3*
Ascorbic acid	83 ± 12*

Each value is the mean (n = 5) ± s.d.
*$P < 0.001$ using Student's t-test.

It has been postulated that the chemically reactive metabolite is a quinone or semiquinone derived from 2-hydroxyethinyloestradiol (Kappus *et al.*, 1973), although others have suggested that oxygenation of the ethinyl group may be involved (Helton *et al.*, 1977). Consistent with the former hypothesis, ascorbic acid inhibited the irreversible binding of ^3H-EE_2 metabolites and produced an accumulation of 2-hydroxyethinyloestradiol. Furthermore, SKF 525A, metyrapone and α-naphthoflavone inhibited the 2-hydroxylation of EE_2. In incubations containing thiols there was a significant increase in polar metabolites, consistent with the formation of 1(4)-thioether adducts. There was no evidence for either direct inhibition of the mixed-function oxidases or reduction of the intermediate quinone by either glutathione of N-acetylcysteine.

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3,5,5-trimethylcyclohexanol (TMC): effects on cholesterogenesis, bile flow and biliary lipid secretion in the rat

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The peripheral vasodilator cyclandelate (Cyclospasmol—Brocades Ltd) is synthesized by esterifying mandelic acid with TMC. After absorption cyclandelate is rapidly hydrolyzed back to TMC and mandelic acid with 65% of the former compound being excreted in the urine in the form of various conjugates (Brocades Ltd: data on file). TMC is a 9-carbon homologue of naturally occurring 10-carbon cyclic monoterpenes such as menthol, menthone, pinene, borneol, cineole and camphene. Rowachol (Rowa Ltd, Bantry, Co Cork) is a proprietary choleretic containing all six of these plant terpenes. Rowachol, like chenodeoxycholic acid, depresses the rate-limiting enzyme for cholesterogenesis (HMGCoA reductase) (Clegg, Middleton, Bell *et al.*, 1980),

favourably alters biliary lipid composition (Doran, Keighley & Bell, 1979) and dissolves cholesterol gallstones when given alone (Doran & Bell, 1979) or in combination with bile acids (Ellis, Middleton, White *et al.*, 1981). Our group had previously studied the effects of Rowachol and all its individual constituents in the rat (Bell, Clegg, Cohn *et al.*, 1980). When therefore Rowa Ltd found that TMC had hitherto undiscovered choleretic properties they asked us to screen the compound for possible cholelitholytic activity.

When TMC was administered to night/day adapted male Wistar rats (250–300 g weight) in a dose of 3 mmol/kg body weight 41 and again 17 h prior to sacrifice, hepatic HMGCoAR levels fell by a mean of 41% ($P < 0.01$). Chronic administration of the compound for 44–51 days (10 mM TMC in the drinking water) resulted in a 58% inhibition of hepatic HMGCoAR ($P < 0.01$). Acute administration of TMC (3 mmol/kg by stomach tube 17 h prior to study) produced a significant choleresis (20.8 ± 4.1 treated cf. 10.9 ± 2.3 for the controls; $P < 0.001$)* of similar magnitude to that produced by menthol (17.1 ± 1.3; $P < 0.001$ cf. controls)*, and also tended to lower biliary cholesterol secretion relative to that of bile salt. Chronic low dose administration of TMC (1 mM in the drinking water for 15 days) again produced significant choleresis (16.5 ± 1.9 treated cf. 13.3 ± 1.2 controls; $P < 0.01$)*, while a similar dose of menthol (12.6 ± 3.0 N.S. cf. controls)* did not. Low dose TMC reduced the lithogenic index of rat bile by significantly altering the slope of the regression line relating bile salt output to cholesterol secretion ($P < 0.02$).

Studies are currently in progress to see if cyclandelate will, as predicted, have similar effects to its major metabolite TMC—if so, this commonly prescribed vasodilator may well prove to have useful cholelitholytic properties in man.

*Bile flow expressed as the calculated 24 h bile flow in ml/100 g body weight.

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3,5,5-trimethylcyclohexanol (TMC) II: studies in the cholesterol-fed rabbit

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The cholesterol-fed rabbit is a long established experimental model in which atheroma-like aortic lipid deposition occurs quickly in association with very high serum cholesterol levels. We have shown that the bile of these animals becomes markedly more lithogenic while their biliary bile acid pattern is also profundly altered (Ellis, Taylor, Clegg *et al.*, 1980). The approximately tenfold rise in serum cholesterol levels produced by feeding a 2% cholesterol diet to rabbits is associated with a 75% reduction in hepatic HMGCoA reductase actively and a fivefold rise in that of acyl-Coenzyme A cholesterol acyltransferase (ACAT) suggesting reduced endogenous cholesterol synthesis and increased export of cholesterol esters to plasma (Ellis, Taylor, Clegg *et al.*, 1980).

Rowachol (Rowa Ltd, Bantry)—a mixture of plant monoterpenes has been shown to reduce atheroma formation in the cholesterol-fed rabbit (Benko, Macher, Szarvas *et al.*, 1961). Rowachol raises serum HDL-cholesterol levels in man (Bell, Bradshaw, Burgess *et al.*, 1980) reduces biliary lithogenicity and dissolves cholesterol gallstones (Doran, Keighley & Bell, 1979). We decided to repeat the study of Benko and colleagues using not only Rowachol but also TMC—a compound which we had previously shown, like Rowachol, to affect cholesterogenesis and biliary

lipid composition in the rat (Bell, Clegg, Ellis, Middleton & White, 1981).

New Zealand white rabbits (2.5–3 kg) were fed a 2% cholesterol diet alone or with added Rowachol, or TMC (0.2% w/w), for nine weeks prior to sacrifice. The grossly elevated serum cholesterol levels found in all three groups of animals were not significantly different. Serum HDL-cholesterol levels were somewhat, but not significantly higher in the two treated groups (0.67 ± 0.2 [mean ± s.d.] Rowachol, 0.62 ± 0.2 TMC, compared with 0.47 ± 0.2 mmol/l for the control rabbits). This latter effect may in part explain why both drugs tended to reduce deposition of cholesterol in the rabbit aortae, but not significantly (19.2 ± 7.2 mg cholesterol per gram wet weight for Rowachol ($P = 0.012$), 30.0 ± 16.8 for TMC compared with 41.9 ± 11.9 for the control rabbits). The biliary lithogenic index of the control animals (0.85 ± 0.04) was significantly reduced by TMC (0.63 ± 0.04 $P < 0.005$) but not Rowachol (0.72 ± 0.05). Rowachol did not significantly affect individual bile salt composition, but TMC altered the percentages of cholic acid (reduced, $P < 0.02$) and allodeoxycholic acid (increased, $P < 0.05$). Both drugs tended to further depress HMGCoAR.

We conclude that TMC, like Rowachol, has interesting effects on experimental atheroma formation, biliary lithogenicity and cholesterol metabolism which merit further study. Similar studies are now in progress using the mandelic ester of TMC, cyclandelate.

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Metabolism of aminoglutethimide in humans: formation of *N*-formylaminoglutethimide and nitroglutethimide

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Aminoglutethimide [Elipten, 3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione] is in current use for the treatment of disseminated breast cancer and acts by inhibiting the conversions cholesterol → pregnenolone and androstenedione → oestrone (Santen, Veldhuis, Samojlik, Lipton, Harvey & Wells, 1979). Little has been reported on the metabolism of the drug. There is a marked decrease in the plasma half-life of aminoglutethimide in patients during chronic therapy implying that the drug induces its own hepatic metabolism (Santen, Veldhuis, Samojlik, Lipton, Harvey & Wells, 1979). *N*-Acetylaminoglutethimide is the only reported (Douglas & Nicholls, 1972) metabolite (4–25% of the administered dose and appearing in the urine). Our studies with non-radiolabelled aminoglutethimide have revealed an array of urinary metabolites two of which are unusual types, namely, *N*-formylaminoglutethimide and nitroglutethimide.

Ten healthy volunteers (age 22–39 years) each took aminoglutethimide (250 mg) orally. The 24 h urine was collected on an individual basis and the material extracted therefrom with dichloromethane was subjected to HPLC [Spherisorb 5 μC_6 column, 23.5° acetonitrile-water (22:78) containing 0.05% of perchloric acid, 1.5 ml/min, UV detection (254 nm)]. In addition to aminoglutethimide (retention time, T 8.1 min, 0–36.5% of the administered dose) and the *N*-acetyl derivative (T 11.0 min, 2.7–12%), *N*-formylaminoglutethimide (T 9.2 min, 0.34–0.66%) and nitroglutethimide (T 28.4 min, 0.01–0.14%) were

detected. These metabolites were characterized by chemical ionization mass spectrometry (methane reagent gas) and quantified by HPLC by reference to authentic compounds. In separate experiments small amounts of several other metabolites were detected, three of which were probably hydroxylated derivatives.

Since nitroglutethimide is an intermediate in the synthesis of aminoglutethimide from glutethimide (Hoffman & Urech, 1958) the purity of the administered drug was investigated. The products extracted with dichloromethane from a standard aminoglutethimide tablet and from the urine of volunteers given tablets from the same batch were *N*-methylated (methyl iodide-silver oxide) and subjected to GC-MS. Selected ion recording of the $[M\text{-}C_2H_4]^+$ ions for *N*-methylated glutethimide (*m/z* 203) and nitroglutethimide (*m/z* 248) indicated glutethimide to be present in both the tablet and the urine not detected by HPLC (*T* 26.0 min) owing to its low UV absorption but that nitroglutethimide was present in the urine only and is thus a genuine metabolite.

N-Formyl and nitro derivatives are unusual metabolites of primary aromatic amines. *N*-Formylation, which is mediated by kynurenine formamidase (Santti & Hopsu-Navu, 1968) and was first observed by Boyland & Manson (1966) who reported 2-formamido-1-naphthyl hydrogen sulphate as a urinary metabolite of 2-naphthylamine in several animal species (see also Gothoskar, Benjamin, Roller & Weisburger, 1979), apparently has not been observed hitherto as a metabolism pathway of a primary aromatic amine in man. One of the amino functions in dapsone [*bis*(4-aminophenyl)sulphone] was converted into a nitro group by rat liver microsomes (Tyler, Buhs & VanderHeuvel, 1973) but it is possible that non-enzymatic oxidation of an initially formed hydroxylamino derivative could have occurred. For this reason the mode of formation of nitroglutethimide must be regarded as uncertain. The contribution of *N*-formylaminoglutethimide and nitroglutethimide to the biological activity and toxicity of aminoglutethimide remains to be determined.

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The influence of varying hepatic arterial flow contribution to the rat perfused liver on systemic availability of lignocaine

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Blood flow through the liver, drug binding and hepatic metabolism are important determinants of hepatic drug extraction. The extent to which intrahepatic shunting influences drug extraction remains uncertain. Mathematical models have been evolved to predict hepatic drug clearance when flow, binding and metabolism alter (Pang & Rowland, 1977a). Clearance of lignocaine by the rat liver *in situ* perfused solely through the portal vein can be described by the 'well stirred' model when flow varies (Pang & Rowland, 1977b). Simultaneous perfusion through both hepatic artery and portal vein represents a more physiological system and we have investigated the influence of varying hepatic artery flow contribution on the clearance of lignocaine.

A perfused liver *in situ* method was developed using male Wistar rats (380–420 gm) as liver donors and Krebs-bicarbonate plus 20% washed human red blood cells as the perfusion medium. The preparation allowed separate or simultaneous perfusion of the

hepatic artery and portal vein at different flow rates. Availability of lignocaine to the systematic circulation (F) was calculated as the rate of appearance of drug in the hepatic effluent at steady state after a single pass through the liver expressed as a fraction of the rate of presentation of drug to the liver. At a constant flow 10 ml/min the value for F when the liver was perfused solely through the portal vein was 0.005 ± 0.0004 (mean ± s.e. mean) (n = 15); when the liver was perfused solely through the hepatic artery F was 0.0919 ± 0.002 (n = 9) ($P < 0.01$). When hepatic artery and portal vein were perfused simultaneously but lignocaine was administered only through the portal vein, F decreased with increasing portal vein flow fraction; when lignocaine was administered only through the hepatic artery, F increased with increasing hepatic arterial flow contribution. When lignocaine was administered simultaneously and in equal concentration to both portal vein and hepatic artery (n = 5), F could be predicted from the proportion of flow received by the liver through each route. In all cases, linear correlations were obtained when log F values were plotted against decreasing flow fractions for the portal vein (r = 0.991) and increasing flow fractions for the hepatic artery (r = 0.987).

When 15μ [^{85}Sr]-labelled microspheres were injected into the portal vein or into the hepatic artery separately the fractions recovered in the hepatic effluent were 0.059 ± 0.001% (n = 20) and 0.065 ± 0.001% (n = 6) ($P < 0.01$) respectively, suggesting minimal anatomical shunting. Differences in F for lignocaine between the hepatic arterial and portal venous routes could be due to functional shunts which increase as the flow fraction of the hepatic artery is increased. Such shunts may arise through a redistribution of flow within sinusoids as postulated by Nakai *et al.* (1979).

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Studies on a new diuretic, fenquizone

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Fenquizone (MG 13054) is a new sulphonamide diuretic *viz* 2-phenyl-6-sulphamido-7-chloro-1,2,3,4-tetrahydroquinazoline-4-one. It bears the closest structual similarity to quinethazone, which is itself chemically and pharmacologically similar to the thiazides. The diuretic pattern of fenquizone was compared with that of existing agents together with its potential to produce hyperglycaemia and hyperuricaemia. The ability of fenquizone to modify glucose transport across the isolated everted intestinal sac of the rat was also compared using the method of Barry, Matthews & Smyth (1961).

Male mice (30–40 g) were dosed orally with drug suitably suspended in 25 ml/kg 0.9% sodium chloride/0.25% carboxymethylcellulose solution, placed on a collection apparatus and urinary volume, sodium and potassium measured over the next 3 hours. Dose-response curves to fenquizone, hydrochlorthiazide and frusemide were obtained. To study plasma glucose and urate levels and urinary urate excretion, groups of mice received 10 mg/kg fenquizone daily for 5 days after which glucose and urate levels were determined colorimetrically (Asatoor & King, 1954; Caraway, 1964). Intestinal transport of glucose was studied in isolated sacs prepared from female rats (250–300 g) anaesthetized with pentobarbitone according to the method of Wilson & Wiseman (1954). Animals were dosed 1 h previously with either fenquizone (10 mg/kg), quinethazone (10 mg/kg) or hydrochlorothiazide (25 mg/kg).

With fenquizone diuresis commenced at a dose of 0.5 mg/kg when 126% of the volume dosed was excreted as urine in 3 hours. A peak of 141% volume dosed was elicited at 10 mg/kg. Control animals excreted 70% of volume dosed. Sodium excretion was 2.55 m.eq./kg control compared to a range of 4.45–6.23 m.eq./kg at doses of 0.5–100 mg/kg fenquizone. At the same dose range, potassium excretion varied between 0.92 and 1.35 m.eq./kg. These

responses were similar to those obtained using hydrochlorothiazide and were generally smaller and flatter than those obtained with the 'high ceiling' diuretic, frusemide. Blood glucose and urate levels and urinary excretion of urate were not significantly altered. On the everted sac of the rat fenquizone (10 mg/kg), hydrochlorothiazide (25 mg/kg) and quinethazone (10 mg/kg) all increased the transfer of glucose inside sac 3 (the entire jejumun and ileum being divided into 5 segments). On sac 1, fenquizone did not increase glucose transport into the sac unlike hydrochlorothiazide.

Fenquizone diuresis resembles hydrochlorothiazide rather than frusemide. It has a similar efficacy to hydrochlorothiazide but a somewhat greater potency coupled with moderate kaluresis. It did not produce hyperglycaemia and hyperuricaemia in the mouse tests. Fenquizone produced a similar effect to other diuretics on intestinal glucose transport.

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Characterization of the excitatory opiate receptors in the rat large intestine

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Opioids cause contractions of rat isolated large intestine. The response is inhibited by naloxone but is unaffected by tetrodotoxin or procaine (Nijkamp & Van Ree, 1980; Gillan & Pollock, 1980), indicating the presence of excitatory opiate receptors on intestinal smooth muscle. Several types of opiate receptor have been identified in other tissues but the nature of those mediating this response has not yet been established.

Four cm segments of ascending, mid and descending colon and the entire rectum (2 cm) were taken from male hooded rats (200–300 g) killed by cervical dislocation. Tissues were suspended under 1 g tension and were superfused (2 ml/min at 30°C), in cascade with Kreb's, saturated with oxygen and 5% CO_2. Responses were recorded isotonically. The potency (K_D) of each agonist was determined from its dose response curve after exposure of the tissue to each concentration for 20 s. Antagonist affinity was determined by pA_2 determination (Arunlakshana & Schild, 1959).

Colon preparations responded sensitively to leu-enkephalin (10^{-9}–10^{-6} M) by contracting, the maximum response evoked being 30–50% of that induced by acetylcholine. The rectum showed marked spontaneous activity and lacked a convincing opioid response although contracting to acetylcholine. Two criteria for receptor involvement in the descending colon response, stereospecificity and naloxone sensitivity were investigated. Excitatory dose-related responses to leu-enkephalin and levorphanol (10^{-6}–10^{-4} M) were obtained. Leu-enkephalin (10^{-6} M) and levorphanol (10^{-4} M) responses were markedly reduced by naloxone (10^{-6} M) but although dextrorphan did induce a contraction at 10^{-3} M this was unaffected by naloxone (10^{-6} M).

Evaluation of the receptor in the descending colon using ligands with differing profiles of specificity for the sub types of opiate receptor yielded the following dissociation constants: leu-enkephalin (1.05×10^{-8} M), BW 180 C (1.15×10^{-8} M), normorphine (8.52×10^{-8} M), Rx 3030 (7.8×10^{-7} M), ketocyclazocine (1.32×10^{-5} M) and SKF 10047 (1.11×10^{-4} M).

These data, suggesting the existence of a δ receptor, were supported by the finding that the pA_2 for naloxone antagonism of the δ ligands, BW 180 C and leu-enkephalin, were 7.4 and 7.3 respectively (Lord *et al.*, 1977). It would appear that μ receptors are also present since Rx 3030, predominantly a μ ligand, had agonist properties and was antagonized by naloxone (pA_2 8.6).

Ketocyclazocine and SKF 10047, κ and σ receptor ligands respectively (Martin, 1976) and Mr 2266 (10^{-1} M), a κ antagonist, had minimal activity.

This indicates that the descending colon, but not the rectum, of rat possesses stereospecific, naloxone sensitive excitatory opiate receptors. δ receptors appear to predominate but μ receptors can be

demonstrated. κ and σ receptor involvement is not indicated.

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BW 180 C Tyr.D-Ala.Gly.Phe.D-Leu (Wellcome).

MR 2266 (−)-2-(3-Furylmethyl)-2′-hydroxy-5,9α-diethyl-6,7-benzomorphan (Boehringer Ingelheim).

Rx 3030 Tyr.D-Ala.Gly.Me.Phe.NH.Ch_2.N(O).Me_2 (Reckitt & Colman).

SKF 10047-N-allyl norphenazocine (Smith, Kilne & French).

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Extraction of an endogenous opioid from human plasma

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Endogenous opioid substances which differ chromatographically from β-endorphin and the enkephalins have been detected in human CSF. Their concentration appears to be more variable in patients in pain (Almay *et al.*, 1978; Miller *et al.*, 1979). Sarne and his coworkers (1979), have isolated similar substances from human plasma but levels of these opioids in pain have not been studied.

Venous blood was obtained by venepuncture, centrifuged and the separated plasma frozen and stored at −20°C until extraction. Plasma samples were adjusted to pH 5.3 with 1 M HCl and boiled for 45 minutes. After centrifugation for 30 min at 10,000 rpm the supernatant was lyophilized. The lyophilisate was extracted with methanol (4 ml) and the methanol removed by rotary evaporation (80°C). The residue was resuspended in 0.5 ml Tris HCl (pH 7.4 at 25°C) and opioid activity was evaluated in triplicate using 100 μl per radioreceptor assay against met-enkenphalin (ME) as standard (Lewis & Olley, 1980). Opioid activity was expressed as pmol ME equivalent/ml.

The levels of this substance in normal subjects (6) and patients attending a pain clinic (17) was determined using 20 ml blood samples. Opioid activity was detected in 5 control (3.0–7.1 pmol ME/ml plasma) and 8 pain patients (5.7–28 pmol ME/ml plasma) ie, 1 control and 9 patients had <0.5 pmol ME equivalent/ml plasma. These results show a wide variation in the amount of opioids in the plasma of pain patients from below detection to apparently elevated levels when compared with a group of normal volunteers.

The identity and origin of the opioid remains to be elucidated but levels are considerably higher than could be attributed to β-endorphin and enkephalins as these peptides are present in very low concentration in human plasma (Akil *et al.*, 1979; Clement-Jones *et al.*, 1980).

If these substances are found to relate to those isolated from CSF and to vary in pain states then the plasma may provide a valuable but more accessible fluid than CSF for study of the role of these opioids in pain.

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The action of mecamylamine at the postsynaptic channels of cat skeletal muscle: noise analysis

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The ganglion-blocking drug mecamylamine acts also at the post-synaptic membrane of skeletal muscle. Mecamylamine changes the mechanism of action of depolarizing drugs: these may become ineffective as neuromuscular blocking drugs or may even produce a neuromuscular block bearing some similarities to that produced by competition with acetylcholine (ACh) (Bennet, Tyler & Zaimis, 1957).

In the present experiments, the mode of action of mecamylamine on cat tenuissimus muscle end-plate channels was studied, using microelectrodes to record membrane potential (at 37–38°C) and voltage clamped currents (at 21–25°C). Miniature end-plate potentials (m.e.p.p.s) and currents (m.e.p.c.s), ACh-induced voltage noise and voltage clamped current noise were analysed (Katz & Miledi, 1972; Wray, 1980). Paired *t* tests were used throughout.

Mecamylamine (10–49 μM) caused a dose dependent reduction in amplitude of m.e.p.p.s, and a slight shortening in decay time constant. For instance, mecamylamine (25 μM) caused a reduction of 59 ± 6% in amplitude and of 17 ± 4% in decay time constant ($P < 0.005$, $n = 5$ end-plates, mean ± s.e. mean).

The depolarization produced by ACh (1–2 μM) in the presence of physostigmine was reduced by mecamylamine (25 μM) from 17.5 ± 2.9 mV to 6.2 ± 1.6 mV ($P < 0.025$, $n = 4$). The depolarization produced by a single channel, *a*, was reduced by 28 ± 5% in the presence of mecamylaine (25 μM, $P < 0.005$, $n = 4$), while the noise time constant was not significantly changed. The maximum frequency of channel opening fell after mecamylamine (25 μM) from $33 \pm 13 \times 10^7$/s to $13 \pm 6 \times 10^7$/s ($P < 0.05$, $n = 4$). Furthermore, the normally slow desensitization rate, assessed from the noise (Wray, 1981), was not significantly affected by mecamylamine.

In contrast, tubocurarine (0.4 μM) produced 46% reduction in m.e.p.p. amplitude, but did not significantly reduce the above time constants or *a*.

Both the amplitude and decay time constant of m.e.p.c.s were reduced by mecamylamine (10–20 μM), with greater effect at hyperpolarized voltages. For instance, at 70 mV clamp potential, m.e.p.c. amplitude was reduced by 21% and m.e.p.c. decay time constant by 18% while at 100 mV clamp potential, m.e.p.c. amplitude was reduced by 42% and m.e.p.c. decay time constant by 36% (10 μM mecamylamine, $n = 3$).

Current noise analysis (at 60–70 mV clamp potential) showed that mecamylamine (10–20 μM) reduced channel open time by 16 ± 5% from a control value of 1.0 ± 0.2 ms ($P < 0.025$, $n = 4$), while channel conductance was also reduced, though not significantly, from a control value of 17.1 ± 6.1 pS to 11.5 ± 2.6 pS.

These results show that mecamylamine reduces open time and frequency of opening of end-plate channels, and that these changes cause a reduction in the maintained depolarization produced by a depolarizing drug such as ACh. Thus depolarization itself can no longer lead to neuromuscular block in the presence of mecamylamine.

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Effects of ATX-II on action potentials in slow- and fast-twitch mammalian skeletal muscle fibres

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The toxin ATX-II, isolated from extracts of the sea anemone *Anemonia sulcata* delays sodium inactivation in neuronal membranes leading to a prolongation of the action potential (Bergman, Dubois, Rojas & Rathmayer, 1976). It has recently been shown that ATX-II causes a sodium dependant depolarization of rat soleus (SOL) muscle fibres, as well as a prolongation of the action potential (Alsen, Harris & Tesseraux, unpublished observation). Preliminary results suggested that fibres of the rat fast-twitch muscle extensor digitorum longus (EDL), are less sensitive to the actions of ATX-II than are slow-twitch SOL fibres. We have now examined fast- and slow-twitch muscles from both rat and mouse and differences in sensitivity to ATX-II have been observed.

EDL and SOL muscles, isolated from adult rats and mice, were maintained in a physiological bathing fluid containing dantrolene sodium 10^{-5} M. Action potentials were generated and recorded using a double microelectrode technique, the muscle fibre membrane first being locally hyperpolarized to between -90 and -95 mV. ATX-II (10^{-1} M) caused a depolarization of rat SOL muscle fibre membranes by 20 mV and increased the duration of the action potential from 2.5 ± 0.1 to 6.5 ± 0.8 ms. Rat EDL muscle fibres were unaffected by this concentration of toxin. In the mouse muscles ATX-II (10^{-1} M) caused a small depolarization (< 10 mV) of SOL fibres but EDL fibres were not depolarized. The toxin had no effect on the duration of the action potential in either muscle.

It was observed that the repetitive stimulation of the muscle fibres (50 pulses at 10 Hz) in the presence of ATX-II (10^{-7} M) caused an irreversible prolongation of the action potential (from 1.7 ± 0.1 to 6.9 ± 0.8 ms) in about 50% of SOL muscle fibres but had no effect on EDL fibres. The prolongation of the action potential was not observed following lower rates of stimulation.

The results demonstrate that the difference in sensitivity to ATX-II of EDL and SOL muscles is not confined to rat muscle but is also observed in mouse muscle fibres. As ATX-II probably interacts with the sodium channel 'gate' the observations suggest that the 'gates' of fast-twitch fibres are either chemically distinct from those of slow-twitch fibres or are less accessible to the toxin. It would also appear, from the results of repetitive stimulation, that the interaction of ATX-II with the sodium channel 'gate' is either facilitated by depolarization, or requires that the 'gate' be in its open configuration. The possibility that ATX-II can activate a different population of 'gates' which may be responsible for the prolongation of the action potential is not ignored.

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Reconstruction of neuromuscular junctions in rat skeletal muscles following assault by the myotoxic neurotoxin, notexin

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Notexin, a basic toxin isolated from the crude venom of the Australian tiger snake, *Notechis scutatus scutatus* is a presynaptically active neurotoxin, and a potent myotoxin (Harris, Karlsson & Thesleff, 1973; Harris, Johnson & Karlsson, 1975). When injected into anaesthetized rats, the toxin causes an inflammatory necrotizing myopathy to the underlying muscle, with associated damage to the peripheral motor nerve. The muscle regenerates, forming immature myotubes at day 3 and fully differentiated muscle fibres by day 14.

The morphological and physiological characteristics of the restitution of neuromuscular transmission has now been studied in rat isolated soleus muscles recovering from assault by a single injection of notexin (2.0 μg in 0.2 ml, s.c. into the antero-lateral

aspect of one hind limb). Miniature end-plate potentials (mepps) were first identified 3 days after the injection of toxin, and by 5 days all junctional regions exhibited mepps. Mepp frequency remained lower than normal for 21 days. Muscle fibre action potentials could be elicited in response to nerve stimulation by 5 days, and were present in all fibres by 7 days. The quantal contents of the end-plate potentials were lower than normal for 14 days. At no time could evidence of multiple innervation be found.

Histochemical techniques applied to frozen sections (10 μm thick) of the regenerating muscle were used to demonstrate the presence of end-plate cholinesterase in normal, necrotic and regenerating muscle fibres, reflecting the preservation of the basal lamina. Since the neural sheath remains undamaged by notexin (Slack, unpublished observation) the very rapid restitution of a functioning neuromuscular junction is probably due to the preservation of the appropriate 'skeletons' (that is neural sheath and basal lamina) within which axons and muscle fibres respectively regenerate, and the preservation of the end-plate cholinesterase.

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