225P THE IDENTIFICATION OF NEW LIGANDS SELECTIVE FOR δ -OPIOID RECEPTORS IN GUINEA-PIG BRAIN

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There is increasing interest in the development of selective ligands for subtypes of opioid receptors. It is recognised that there are three main types of opioid receptor, namely u, κ and δ, and there is substantial evidence for the existence of subtypes of these opioid receptors. A series of 7-benzylidene-7-dehydronaltrexone (BNTX) derivatives have been synthesisied as potential tools for studying δ opioid receptors. We now report the affinities of BU9635 (7-benzylidene-N-cyclopropylmethyl-7,8-dihydro-14β-formyl-amino-codeinone), BU9636 (14β-acetamino-6-benzylidene-N-cyclopropyl-methyl-7,8-dihydrocodeinone), BU9637 (7-benzylidene-N-cyclopropylmethyl-7,8-dihydro-14β-formyl-amino-morphinone) and BU9646 (14β-acetamino-7-benzylidene-N-cyclopropylmethyl-7,8-dihydro-morphinone) for types of opioid receptor in guinea pig brain membranes.

Guinea pig (Dunkin-Hartley, male, 350-500g) brains were homogenised (10vol 50mM Tris-HCl buffer, pH 7.4) and pelleted by centrifugation (32,000g). Membranes were washed twice by centrifugation and frozen (-70°C) until use. Aliquots of thawed membrane (300μg protein) were incubated (1hr, 22°C) with either 2nM [^{3}H]DPDPE, 1nM [^{3}H]DAMGO or 1nM [^{3}H]U69593 to label δ, μ and κ sites respectively (Smith *et al.*, 1989). Specific binding was determined with naloxone (10μM). Bound ligands were separated by filtration and determined by scintillation counting. Results were analysed by Prism (GraphPAD Software, 1994).

Displacement curves for all four compounds were best fit to a single site in each case. BU9635 demonstrated some 9 fold selectivity for δ over μ sites and 160 fold for δ over κ subtypes. BU9636 was of low affinity for opioid receptors but had some selectivity for δ over μ and κ sites. BU9637 had high affinity for δ sites being 15 fold selective for δ over μ and 41 fold selective for δ over κ sites (Table 1). BU9646 showed selectivity for δ over μ and κ binding sites (Table 1).

Table 1 Affinities of BU9635, BU9636, BU9637 and BU9646 for δ, μ and κ opioid receptors in guinea pig brain membranes.

	Ki (nM)		
	δ	μ	κ
BU9635	85.0±14.5	1095±256	12577±9057
BU9636	939.2±340.8	4638±1137	6527±2115
BU9637	2.2±0.9	32.8±4.4	90.4±20.0
BU9646	10.0±1.3	167.3±59.3	147.0±16.3

Data are mean Ki values ± s.e.mean from four experiments performed in triplicate.

These data indicate that of a series of BNTX derivatives, BU9637 displays high affinity and selectivity for δ opioid receptors relative to μ an κ subtypes, in guinea pig brain membranes. Whether BU9637 is an agonist or antagonist at δ opioid receptors is unknown at present.

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226P COMPARISON BETWEEN THE NMDA GLYCINE SITE ANTAGONISTS GV150526 AND 7-CHLOROKYNURENIC ACID IN A FUNCTIONAL PREPARATION OF NEURONS FROM EMBRYONIC RAT HIPPOCAMPUS

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In this study we have pharmacologically characterized the novel antagonist for the glycine site of the NMDA receptors, GV150526 ((E)-3[(phenylcarbamoyl)ethenyl]-4,6-dichloroindole-2-carboxylic acid sodium salt) in a functional preparation. 7-chlorokynurenic acid (CLK) was used as a standard compound.

Hippocampal neurons (HN) from embryonic SD rats of 18 days were cultured as described by Goslin and Banker (1991). Whole-cell patch-clamp recordings (Hamill et al., 1981) were performed in vitro on neurons from day 7 to 22. The extracellular solution contained (mM): NaCl 140, KCl 5, $CaCl_2$ 1, HEPES 10, glucose 10, pH adjusted to 7.4 with NaOH. Tetrodotoxin (0.1 μM) and strychnine (1 μM) were added routinely in agonist dose-response curves. Cells were voltage clamped at -60 mV in the whole-cell recording mode. Patch pipettes had a resistance of 5-8 MΩ when filled with the following intracellular solution (mM): CsCl 140, EGTA 11, $MgCl_2$ 4, Mg-ATP 2, HEPES 10. pH was adjusted to 7.3 with Trizma. Data were digitized (100 Hz), filtered (40 Hz) and stored on-line using the pCLAMP software and TL-1 DMA interface.

NMDA currents were measured by averaging the steady state of the response. NMDA and glycine elicited minimal or no membrane currents when applied alone, but activated large currents when given together. In the presence of a saturating concentration of NMDA (100 μM), glycine (0-10 μM) induced dose-related ionic currents in HN with a maximum of 1293±80 (mean±s.e., n=45) pA. The pEC50 and the slope factor of the dose-response curve of glycine were 6.65 (95% C.L. 6.61-6.69; n=15) and 1.35 (95% C.L. 1.20 - 1.50; n=15), respectively. GV150526 (0.01-10 μM) and CLK (0.01-100 μM) produced a complete inhibition of the currents induced by NMDA (100 μM) and glycine (3 μM). The estimated pIC_{50}s were 6.97 (95% C.L. 6.88-7.09; n=15) for GV150526 and 5.38 (95% C.L. 5.15-5.91; n=14) for CLK. From these values an estimation of the apparent pK_Bs (as described by Leff and Dougall, 1993) were obtained which were 8.11 (95% C.L. 7.99-8.20) and 6.49 (95% C.L. 6.26-7.02), respectively. The antagonism produced by GV150526 was completely reversed by increasing the concentration of glycine (0.03-100 μM; n=12). The antagonism was competitive since the resulting analysis (Arunlakshana & Schild, 1959) showed a slope factor of the Schild plot of 0.93±0.10 (mean±s.e.) not significantly different from one ($p > 0.05$). Constraining the slope factor to unity the estimated apparent pK_B was 8.26 (95% C.L. 7.94-8.57). On the other hand, the antagonism produced by 0.1 μM GV150526 was not reversed by increasing the concentration of NMDA (1-300 μM; n=10).

These results suggest that in functional preparations of HN glycine and NMDA induced currents only in the presence of both agonists. GV150526 was found to be a competitive antagonist of the glycine site of the NMDA receptor with a potency of about 40 fold greater than that of CLK. Moreover, there was a clear correspondence between the apparent pK_Bs obtained for GV150526 by using the methods of Leff and Dougall (1993) and Arunlakshana and Schild (1959).

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227P CAPSAICIN INCREASES THE DESENSITIZATION RATE OF ATP-EVOKED INWARD CURRENTS IN ADULT RAT DORSAL ROOT GANGLION (DRG) NEURONES *IN VITRO*

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Capsaicin (caps) opens Ca^{2+}-permeable, cation-specific ion channels in a sub-population of DRG neurones (Docherty et al., 1996). Similarly, ATP activates Ca^{2+}-permeable, cation-specific ion channels in DRG neurones by activating P_{2X} receptors (Bean, 1990). We have compared the responses to ATP of caps-sensitive and caps-insensitive DRG neurones.

DRG neurones were isolated from adult rats and maintained in culture as described (Docherty et al., 1996). Membrane currents were recorded using the whole cell voltage-clamp method as described (Docherty et al., 1996). ATP (10 μM) was applied for 10 s at a holding potential (V_h) of -60 mV and caps (0.5 μM) was applied for 1 min at V_h = +40 mV.

Of 51 neurones to which both caps and ATP were applied 28 responded to neither compound, 12 responded to both compounds, 8 responded only to ATP and 3 responded only to caps. The response to ATP was a rapidly developing inward current that was either monophasic (11/20, only one peak) or multiphasic (9/20, at least two peaks). In all cases the response desensitized in the continued presence of drug. We measured the maximum peak inward current and the amplitude after 1, 5 and 10 s (see table). The response to ATP was similar in both the caps-sensitive (n=12) and caps-insensitive (n=8) cells. We did notice, however, that the kinetics of desensitization of the ATP response in caps-sensitive cells depended on whether ATP was applied before or after caps. The decay of the ATP-induced current was significantly faster in cells that had already been exposed to caps leading to a significantly reduced amplitude (see table) at 1 s ($P<0.05$) and 5 s ($P<0.05$).

Capsaicin may activate a cellular mechanism which alters the kinetics of desensitization of P_{2X} receptors in DRG neurones.

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	i_{peak}(nA)	i_{1s}(nA)	i_{5s}(nA)	i_{10s}(nA)
(a) all ATP-sensitive cells (n=20)	1.17 ± 0.20	0.28 ± 0.08	0.12 ± 0.04	0.07± 0.03
(b) ATP applied before capsaicin (n=7)	1.11 ± 0.28	0.30 ± 0.08	0.09 ± 0.02	0.06 ± 0.02
(c) capsaicin applied before ATP (n=5)	1.52 ± 0.49	0.05 ± 0.02 *	0.02 ± 0.00 *	0.03 ± 0.02

Table 1. Data (mean ± sem) are the maximum peak inward current (i_{peak}), and the current after 1 s (i_{1s}), 5 s (i_{5s}) and 10 s (i_{10s}) evoked by ATP (10 μM). Data in groups (b) and (c) were compared by unpaired, 2-tailed Students' t test (* indicates $P<0.05$).

228P PHARMACOLOGICAL CHARACTERISATION OF THE VANILLOID RECEPTOR IN THE RAT DORSAL SPINAL CORD

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Capsaicin is known to stimulate the release of neurotransmitters from primary afferent neurones projecting into the rat dorsal spinal cord (Saria *et al.*, 1986). These neurones release calcitonin gene-related peptide (CGRP) and transmit nociceptive and thermoceptive information to the brain (Wiesendeld-Hallin *et al.*, 1984). In the present study, release of CGRP from the rat dorsal spinal cord has been used to pharmacologically characterise the vanilloid (capsaicin) receptor on these neurones.

Rat dorsal spinal cord was sliced into 300 μm x 300 μm sections on a McIlwain chopper. Slices were washed, re-suspended in 20 ml oxygenated Krebs solution containing 0.1% bovine serum albumin (BSA) and 200 μl aliquots were added to each well of a 96-well Millipore filtration plate. Each animal produced enough cord to fill one 96-well plate. Tissues were incubated for 10 min with antagonist or vehicle at 37°C, followed by a 10 min incubation with agonist before collection of the filtrate under vacuum. Filtrate was assayed for CGRP by radioimmunoassay (RIA) and compared against a standard curve. Each concentration of agonist was added in triplicate and results meaned to form a single value. A control concentration-effect curve to capsaicin was constructed in each plate, allowing data to be expressed as % of maximum response to capsaicin in each animal. Results are expressed as mean ± s.e.mean.

Capsaicin, olvanil and resiniferatoxin (RTX) evoked concentration-dependent increases in CGRP release with pEC_{50} values of 6.55 ± 0.07 (n = 14), 6.19 ± 0.15 (n = 7) and 7.90 ± 0.24 (n = 8) respectively. Olvanil and RTX were partial agonists with respect to capsaicin, with intrinsic activities of 0.63 ± 0.55 and 0.57 ± 0.08. All agonist concentration-effect curves were bell-shaped. Capsazepine had no effect at 3 μM but caused a 51 ± 7%, 49 ± 6% and 62 ± 8% reduction in the maximum response to capsaicin, olvanil and RTX at 10 μM. Similarly, ruthenium red was without significant effect at 3 μM, but caused a 67 ± 7%, 60 ± 8% and 53 ± 10% reduction in the maximum response to capsaicin, olvanil and RTX at 10 μM. Neither antagonist had any effect on basal CGRP release.

The present study characterises pharmacologically the vanilloid receptor on the unmyelinated neurones projecting into the rat dorsal spinal cord. The pharmacology of this receptor varies greatly from that previously published for peripheral terminals of this nerve (contractile responses of rat vas deferens) in which RTX was 30,000 times more potent than capsaicin and olvanil was inactive (Wardle *et al.*, 1996). While these effects may be explained in terms of pharmacokinetic differences, the possibility of multiple classes of receptor cannot be excluded.

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229P THE RELATIVE CONTRIBUTION OF α_2-ADRENOCEPTOR SUBTYPES TO THE ANTINOCICEPTIVE ACTION OF DEXMEDETOMIDINE AND CLONIDINE IN RODENT MODELS OF ACUTE AND CHRONIC PAIN

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The α_{2A}-adrenoceptor subtype has been proposed as the main α_2 receptor mediating antinociception in rodents (Millan *et al.*, 1994). However, the use of non-selective ligands in these studies meant that the involvement of additional α_2 subtypes (α_{2B} /α_{2C}) could not be excluded. The aim of the present study, therefore, was to provide a clearer delineation of the α_2 subtype(s) involved in this response.

The role of the α_{2A}-adrenoceptor (α_{2A}AR) was investigated using a mouse strain (129SvJ) expressing the point mutation, D79N, in the receptor (MacMillan *et al.*, 1996). In addition, mice were used containing a null mutation for either the α_{2B} (129SvJ/C57BL) or α_{2C} (129SvJ/FVB) gene (Link *et al.*, 1996). For acute nociception studies, the mouse (female, 20-30g) tail-immersion test was used in which the last 3.5 cm of the tail was dipped into a water bath at 52°C and the latency to flutter the tail taken as the end-point. The maximal latency allowed was 15s to avoid tissue damage. Locomotor activity was determined by the number of photobeam breaks within a 30 min observation period in computerized cages. Rectal temperature (°C) was recorded with a thermometer probe. In a rat (male, Sprague-Dawley, 350-400g) model of chronic neuropathic pain involving permanent ligation of spinal nerves L5 and L6 (SNL), the tactile allodynic response (50% gram threshold to paw withdrawal, 3-4 weeks post-surgery) was assessed using a series of calibrated von Frey filaments (Chaplan *et al.*, 1994). The role of the α_{2A} and α_{2C} subtypes in the response was investigated by suppression of receptor expression through chronic administration (15nmol per 5µl injection, i.t., b.i.d. for 3 days) of antisense oligodeoxynucleotide (ODN, 20mer) constructed against each receptor. All drugs were dissolved in water.

The α_2-agonist, dexmedetomidine (DEX, 10-300µg kg^{-1}, i.p.), 1 h post-dose, produced a dose-dependent antinociceptive response in C57BL control mice with an ED_{50} value (± s.e. mean, n=10) of 83±23 µg kg^{-1} (i.p.) and a maximum response equating to 53±8% of the maximum possible effect (15s) for the test. DEX (100µg kg^{-1}, i.p., n=8-10) also caused a significant antinociceptive effect in both the α_{2B}AR (11.1±0.7 s DEX vs. 4.7±0.4 s vehicle, VEH, P<0.01) and α_{2C}AR (10.4±1.3 s DEX vs. 3.7±0.2 s VEH, P<0.01) knock-out and WT control (13.4±1.1 s DEX vs. 6.0±0.6 s VEH, P<0.01) mice. In contrast, in the α_{2A} mutant mice, DEX (100µg kg^{-1}, i.p., n=8-10) was ineffective (5.0±0.5 s DEX vs. 4.4±0.5s VEH, P>0.1) compared with WT controls (10.8±1.5 s DEX vs. 3.8±0.2 s VEH, P<0.01). In the 129SvJ WT controls, DEX (100µg kg^{-1}, i.p., n=10) produced a 91% reduction in locomotor activity (8±2 counts DEX vs. 87±7 VEH, P<0.01), and a -5°C reduction in rectal temperature (32.2±0.2 DEX vs. 37.1±0.2°C VEH, P<0.01), while in the α_{2A} mutant mice it was completely ineffective against both parameters. In the SNL model, the α_2-agonist clonidine (CLON, 7.5-30µg, i.t.) produced a dose-dependent reduction in tactile allodynia with an ED_{50} value of 9.4±1.2 µg, i.t. Chronic dosing with the antisense ODN probe (15nmol per i.t. injection) to the α_{2A} subtype produced a significant reduction in the anti-allodynic effect of CLON (15µg, i.t.; 8.2±2.0g antisense treated vs. 12.7±1.5g untreated control, P<0.05). Pre-treatment with either a mismatch ODN (13.8±1.2 g) or washout (72 h) of the antisense probe (13.4±1.2 g), restored the anti-allodynic effect of CLON. Vehicle groups in all cases had a mean range of 1.0-1.7 g. In contrast, treatment with the antisense ODN probe to the α_{2C} subtype (15nmol, i.t.) produced severe hindlimb paralysis while a slightly lower dose (5nmol, i.t.) had no effect on CLON (15.0±0g).

In conclusion, the present study has confirmed that, in rodents, the predominant α_2-adrenoceptor involved in acute, nociception and chronic, neuropathic pain is the α_{2A} subtype.

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230P A NOVEL MODEL FOR NEUROPATHIC PAIN IN THE GUINEA-PIG: COMPARATIVE ANALGESIC ACTIVITY IN A MODEL OF INFLAMMATORY HYPERALGESIA

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The recent development of models of chronic neuropathic pain in rats has aided our understanding of some of the pathomechanisms associated with nerve injury. While many drug regimes have been proposed for the treatment of neuropathic pain, few show efficacy. Based on the studies in the rat we have developed a novel model for chronic neuropathic pain in the guinea pig. Using this model we investigate the efficacy of the standard analgesics (aspirin, morphine) and drugs showing clinical efficacy in neuropathic conditions. Drug activity is compared to that in a model of inflammatory hyperalgesia in the guinea pig (Patel et al, 1995).

Male Dunkin Hartley guinea pigs (200-220g, n=6/group) were anaesthetised with enflurane in N_2O_2:O_2 and peripheral nerve damage induced to the left sciatic nerve using partial ligation (Seltzer et al, 1990) or loose-ligation (Bennett and Xie, 1988). Inflammatory hyperalgesia was induced by intraplantar injections of 1% carrageenan. Mechanical and thermal hyperalgesia were assessed using an Ugo Basile analgesymeter and Ugo Basile Plantar Test respectively. Drugs were tested 14 days post-surgery in neuropathic animals or 24hrs following carrageenan injection in the inflammatory model. Drug effects were expressed as the ED50 values (the dose in mg/kg at which a 50% reversal of hyperalgesia is achieved) and mean ± sem percentage reversal of hyperalgesia compared by student's t-test. Drugs were given orally in a suspension of 1% tragacanth, except for morphine which was dissolved in saline and administered subcutaneously.

Although some degree of autotomy (less than 10%) was observed following partial ligation the incidence of autotomy in the Bennett model was 100% within 2 days of surgery and therefore we did not pursue this method of ligation. Partial ligation of the guinea pig sciatic nerve produced significant mechanical hyperalgesia 5 days post-operatively which reached a maximum after 12 days and was evident for at least 29 days. No significant thermal hyperalgesia was observed therefore only mechanical hyperalgesia was tested for the drug studies.

As shown below in table 1 inflammatory hyperalgesia is reduced dose-dependently by both aspirin and morphine. Aspirin was devoid of activity in the neuropathic model and morphine was only weakly active. Conversely, the anticonvulsant, carbamazepine and the antidepressant amitryptiline are inactive in the inflammatory model but are highly potent at reducing the hyperalgesia associated with neuropathic pain. At 100mg/kg side effects (impairement of motor function and sedation) were observed with carbamazepine.

The profile of drug activity in the neuropathic guinea pig model is similar to that observed clinically in patients with neuropathy. We propose that the guinea pig model for neuropathic pain may be a useful functional model for potential analgesic actions in man.

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Table 1: Comparisions of ED50 values and maximum reversal of mechanical hyperalgesia 3 hrs following drug administration in the guinea pig inflammatory and neuropathic models.

Compound	Dose range (mg/kg)	Neuropathic model		Carrageenan model	
		ED50 (mg/kg)	% maximum reversal	ED50 (mg/kg)	% maximum reversal
Morphine (s.c.)	1-10	>10	22.7 ± 8.2	1.85	79.9 ± 12.2 *
Aspirin (p.o.)	30-300	>300	7.9 ± 5.0	>300	40.1 ± 7.9 *
Carbamazepine (p.o.)	0.3-30	2.99	68.0 ± 8.6 *	>30	9.1 ± 3.4
Amitryptiline (p.o.)	0.3-100	0.24	82.2 ± 5.2 *	>100	12.8 ± 3.2

* P < 0.01 compared to the relevant control group.

231P THE EFFECTS OF SYMPATHECTOMY ON THE NERVE GROWTH FACTOR (NGF)-INDUCED NOCICEPTIVE RESPONSES IN NORMAL AND NEUROPATHIC RATS

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In adult animals, NGF has been ascribed a role in inflammatory hyperalgesia whereby increased levels of NGF are found in inflammatory exudates and the injection of NGF into naïve animals induces hyperalgesia via a mechanism mediated by the sympathetic nervous system (Andreev *et al*, 1995). Conversely, NGF can reduce the hyperalgesia associated with neuropathic pain states (Ren *et al*, 1995). A role for the sympathetic nervous system in the maintenance of neuropathic pain is also proposed (Kinnman & Levine, 1995). In this study we investigated the effect of chemical sympathectomy on the NGF-induced nocicepive changes in both normal and neuropathic rats.

Following partial ligation of the left sciatic nerve under enflurane anaesthesia (Seltzer *et al*, 1990) male Sprague-Dawley or Wistar rats (120-140g; n = 6/group) rats develop a hyperalgesia to mechanical and thermal stimuli in the ligated paw. Mechanical hyperalgesia (M) was assessed with an Ugo Basile Analgesymeter and thermal hyperalgesia (T) was measured using an Ugo Basile Plantar Test apparatus. Chemical sympathectomy (SX) was produced by the intravenous injection of 6-hydroxydopamine (2 x 50mg/kg , 24h apart). This technique produces an effective sympathectomy as assessed using the pressor response to tyramine as a functional index. In neuropathic rats, SX was performed 13 days after surgery. In all cases the studies were performed one day after SX. Animals were tested 2 weeks after surgery (bodyweight 200-250g). Normal rats were age-matched. Data are presented as mean ± sem. Statistical analysis was performed using ANOVA and Tukey's HSD test.

As shown in table 1, an intra-plantar injection of 200ng NGF into normal rats reduced mechanical and thermal pain thresholds in the injected paw; this effect was maintained for up to 24h. In neuropathic rats NGF was analgesic, causing a reversal of the hyperalgesia associated with the neural damage. SX did not affect baseline paw withdrawal in normal rats and did not prevent the NGF-induced mechanical hyperalgesia but did prevent NGF-induced thermal hyperalgesia.

In neuropathic rats SX reduced both mechanical and thermal hyperalgesia and the analgesic effect of NGF treatment was reversed, thus it became hyperalgesic, reducing baseline thresholds to pre-SX levels.

Table 1: The effect of sympathectomy on the NGF-induced nociceptive responses in normal and neuropathic rats.

	Normal rats		Neuropathic rats	
	M	T	M	T
Controls	**102.5±1.7**	**9.6±1.6**	**59.2±2.0**	**6.2±0.7**
+NGF	**65.8±1.5**	**5.1±0.6**	**75.0±1.3**	**8.5±1.1**
(v control)	(p< 0.001)	(p<0.001)	(p<0.001)	(p<0.01)
SX	**102.5±1.1**	**8.9±1.3**	**68.3±1.1**	**9.5±1.0**
(v control)	(N/S)	(N/S)	(p<0.01)	(p<0.001)
+NGF	**75.0±3.4**	**8.8±1.1**	**60.8±2.4**	**5.4±1.1**
(v SX)	(p<0.001)	(N/S)	(p<0.05)	(p<0.01)
(v NGF control)	(N/S)	(p<0.001)	(p<0.001)	(p<0.001)

Mechanical (M;g) and thermal (T;s) withdrawal thresholds for treated (left) paw. Readings shown are 4 hours after NGF injection.

These results show contrasting hyperalgesic and analgesic actions of NGF dependent on whether the animal is normal or has peripheral nerve injury. The results also reveal a complex interplay between the sensory and sympathetic nervous systems in the development and maintenance of hyperalgesia.
The data supports the hypothesis that in normal animals NGF-induced thermal hyperalgesia is SX dependant and suggests a novel mechanism for the hyperalgesic actions of NGF revealed following SX in neuropathic animals.

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232P NOCICEPTIVE THRESHOLDS IN EXPERIMENTAL DIABETES: EFFECTS OF A NK_1 ANTAGONIST

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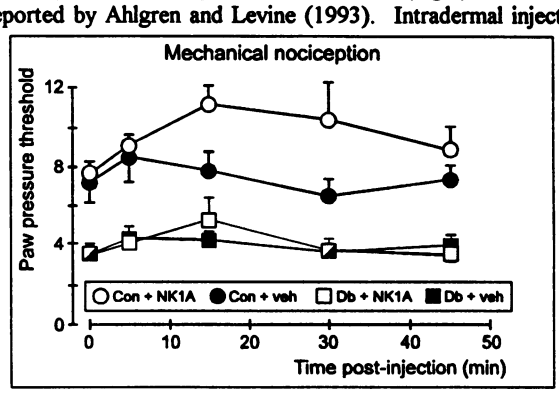
In sciatic nerves of diabetic rats, the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) are depleted, along with the neurotrophin, nerve growth factor (NGF). Reduced peptide levels are reversed by either insulin or NGF treatment. Diabetic neuropathy is characterised by a loss of sensation, correlated with depleted neuropeptides in primary afferents, but hypoaesthesia may be preceded by painful cutaneous neuropathy. We therefore studied nociceptive thresholds for different stimuli in diabetic rats with the effects of a NK_1 antagonist.

Male Wistar rats were made diabetic by the administration of streptozotocin (55 mg/kg i.p, Sigma). Diabetic rats had blood glucose >15mM and two control groups were used (age-matched and weight-matched), since diabetic rats failed to gain body weight during the study. Responses to formalin injection in the hindpaw (50 µl of a 2.5% solution), noxious heat (tail immersion in water at 52°C) and pressure applied to the hindpaw were measured. At 5 wk diabetes, heat responses were similar to both control groups, but at 6 and 10 weeks diabetes, responses were similar to age-matched controls, though lower than those of weight-matched rats. Formalin injection induces a biphasic behavioural response; diabetic rats showed significantly decreased first phase responses and a less marked second phase reduction in comparison to both control groups. Ten week diabetic rats displayed mechanical hyperalgesia against age-matched controls (Fig.1) as has been reported by Ahlgren and Levine (1993). Intradermal injection



of the NK_1 antagonist [D-Pro^2-D-Trp^{7,9}]- SP (10µg in 25µl) increased the mechanical threshold in control rats, suggesting involvement of SP in this response, whereas hyperalgesia in diabetic rats was unaltered by the NK_1 antagonist (Fig.1).

In summary, these results show that nociceptive responses in diabetic rats are altered according to the nature of the noxious stimuli. Decreased SP levels in diabetic rats explain the decreased response to formalin and the absence of effect of the NK_1 antagonist in the pressure test. It remains to be determined whether repeated systemic treatment with a NK_1 antagonist would reverse the mechanical hyperalgesia of diabetic rats.

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233P FLFQPRFamide (NPFF) INHIBITS THE FAST WAVE OF THE EVOKED FIELD POTENTIAL IN THE RAT ISOLATED SPINAL CORD PREPARATION

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In our previous study, we demonstrated that NPFF potentiated the amplitude of monosynaptic reflex (MSR) in the rat isolated spinal cord preparation (Huang *et al*, 1996). Since NPFF may play an important role in spinal nociception, we have attempted to further investigate the actions of NPFF on dorsal horn field potentials in the same preparation. The dissection procedure was as previously described (Bagust *et al*, 1985), but the cord was hemisected and a microelectrode filled with 1M NaCl was used to record dorsal horn field potentials evoked by single stimuli delivered to a lumbar dorsal root (0.5ms, 5 times threshold, 15sec intervals).

In five experiments bath application of NPFF was tested at concentrations between 0.1nM and 10μM . There was a dose dependent decrease in the amplitude of the fast component of the dorsal horn field potential, but no effect was observed on the slow wave. NPFF began to induce a significant decrease at 0.1nM, the fast wave amplitude decreased to 87.2% ± 5.0% (mean±s.e.mean, n=5) ($p<0.05$, Student's t-test) compared with control. The amplitude was further decreased at concentrations between 1nM and 10μM (1nM: 59.9%±8.7%, $p<0.01$; 10nM: 48.5%±7.3%, $p<0.001$; 0.1μM: 43.3%±3.0%, $p<0.001$; 1μM: 38.1%±7.7%, $p<0.001$; 10μM: 36.4%±8.9%, $p<0.001$). Following completion of the experiment, the effect of NPFF was rapidly reversed on washing and the amplitude returned to 85.0%±9.1% of the control value.

The decrease of the fast component of the field potential by NPFF may imply that this peptide has an inhibitory effect on the presynaptic site of the primary afferent fibre (Gouardères *et al*, 1996). Taken together with our previous results, the MSR amplitude potentiation induced by NPFF may be due to an inhibitory effect on primary afferent presynaptic inhibition. However, the mechanism of NPFF in spinal nociception requires further investigation.

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234P EFFECT OF ANALOGUES OF $CGRP_{8-37}$ ON PIGLET ISOLATED BASILAR ARTERIES AND ^{125}I-CGRP BINDING TO SKNMC CELL MEMBRANES

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In the present study we have compared the affinities of two analogues of $CGRP_{8-37}$ (see table 1) in a piglet cerebral blood vessel and in radioligand binding studies using the endogenous human CGRP receptor in SKNMC cells.

Rings of piglet (male or female, 2-5kg) basilar arteries were set up for isometric recording in Krebs-Henseleit solution at 37°C and contracted with a submaximal concentration of U46619 (U-19). A cumulative concentration-response curve (CRC) to human α-CGRP was then constructed. Tissues were washed and recontracted with U-19. Antagonist or vehicle was added when the contraction to U-19 had reached a plateau and a second CRC to CGRP was constructed. Radioligand binding studies using ^{125}I-CGRP (31.3 pM) as a ligand were performed as described by Semark *et al.*, (1992) on membranes prepared from the human SKNMC cell line.

CGRP (0.01-100nM) caused marked, concentration-dependent relaxations of U-19-precontracted pig isolated basilar arteries (mean pIC_{50} value of 8.9 ± 0.1 and mean maximum relaxation of 102 ± 5.3 % of the U-19 contraction, n=56). CRCs were repeatable if an interval of 1 h was allowed between them. CGRP-induced relaxations were not affected by L-NAME (100 μM, n=3). $CGRP_{8-37}$ (1μM) caused rightward shifts of the CGRP CRCs. In one set of experiments, two different incubation times with $CGRP_{8-37}$ were studied. With a 10 min preincubation the pA_2 estimate was 7.1 ± 0.1 (n=3) whilst with a 30 min incubation the pA_2 estimate was 6.0 ± 0.2 (n=3). Therefore, all subsequent antagonist experiments were carried out with a 10 min antagonist incubation except for L-NAME (20 min).

Both peptide analogues caused rightward shifts of the CGRP CRCs. $CGRP_{8-37}$ and $[Pro^8]CGRP_{8-37}$ had approximately 10-fold higher affinity than $[Ala^{16}]CGRP_{8-37}$ (see table 1). $CGRP_{8-37}$ and both of the analogues caused increases in tone in most tissues ($CGRP_{8-37}$ (1μM) = 24.8±3.5% U19 tone, n=12). The peptides also inhibited ^{125}I-CGRP binding to SKNMC cell membranes. $CGRP_{8-37}$ and $[Pro^8]CGRP_{8-37}$ had approximately 10-fold higher affinity than $[Ala^{16}]CGRP$ (see table 1).

Table 1. Effect of peptide analogues on CGRP responses in piglet basilar arteries and ^{125}I-CGRP binding in SKNMC cells

Peptide	Piglet basilar artery		^{125}I CGRP binding	
	pA_2 (± se mean)	n	pIC_{50} (± se mean)	n
$CGRP_{8-37}$	7.0 ± 0.1	12	8.5 ± 0.1	17
$[Pro^8]$ $CGRP_{8-37}$	7.1 ± 0.1	8	8.3 ± 0.1	4
$[Ala^{16}]$ $CGRP_{8-37}$	6.0 ± 0.1	3	7.4 ± 0.2	3

In conclusion, the piglet basilar artery may be a useful preparation for the assay of CGRP receptor antagonists. In this preparation, CGRP is a potent endothelium-independent vasodilator. The analogues of $CGRP_{8-37}$ tested in the present study showed similar rank order of affinity to the order of affinity in radioligand binding studies. However, the analogues all caused an increase in tone in this tissue, a finding which merits further investigation. In addition, the apparent loss in affinity for $CGRP_{8-37}$ at the longer incubation time, may suggest that breakdown is occurring.

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235P EFFECTS OF TEMPERATURE ON THE BINDING OF CALCITONIN GENE-RELATED PEPTIDE AND ANALOGUES TO THE GUINEA-PIG CEREBELLUM AND VAS DEFERENS

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Receptors for calcitonin gene-related peptide (CGRP) have been divided into two classes, $CGRP_1$ and $CGRP_2$. The CGRP antagonist, $CGRP_{8-37}$, has a pA_2 greater then 7 against $CGRP_1$ receptors, but is a much weaker antagonist against $CGRP_2$ receptors (Mimeault *et al.*, 1992). The guinea-pig vas deferens expresses $CGRP_2$ receptors. However, whilst in functional assays using this tissue $CGRP_{8-37}$ had a pA_2 of less than 6.0, in radioligand binding assays it had nanomolar affinity. This is similar to its affinity at $CGRP_1$ receptors as measured by radioligand binding (Mimeault *et al.*, 1992). A possible explanation of this paradox is that, whilst pA_2 determinations were carried out at 37°C, radioligand binding was done at 4°C. The affinity of $CGRP_{8-37}$ for the $CGRP_1$-like receptor found on rat, L6 skeletal myocytes is reduced by increasing temperature (Poyner *et al.*, 1992). In this study, the temperature dependency of $CGRP_{8-37}$ binding has been examined in vas deferens and cerebellum.

Experimental methods were as described previously (Mimeault *et al.*, 1992). Briefly, cerebellum or vas deferens membranes from 300 g male, Sprague-Dawley guinea-pigs were incubated with 40pM [^{125}I]-CGRP (Amersham) with concentrations of 100pM to 1 μM of either human α-CGRP or human α-$CGRP_{8-37}$ in 0.5ml of 100mM NaCl, 20mM Hepes (pH 7.5), 0.1% bovine serum albumin, 0.4mM bacitracin. Incubations were for 2h at 4°C, or 0.5h at 25°C. Preliminary experiments established that these times were sufficient to establish equilibrium. Incubations were terminated by rapid filtration using a Brandel Cell Harvester. Binding curves were fitted using EBDA/LIGAND to obtain $-\log_{10}(IC_{50})$s (pIC_{50}s) and Hill coefficients (Hn). Values were compared at 4°C and 25°C by Students t-test.

For CGRP binding to the vas deferens, pIC_{50}/Hn values (means±s.e.mean) were respectively 8.73±0.12/0.89±0.20 (n=3, 4°C) and 8.62±0.20/0.77±0.20 (n=3, 25°C); for $CGRP_{8-37}$ they were 8.78±0.11/0.77±0.17 (n=4, 4°C) and 8.50±0.13/1.31±0.21 (n=5, 25°C). In a single experiment at 37°C, the pIC_{50} for $CGRP_{8-37}$ was measured as 9.15. For CGRP binding to the cerebellum, pIC_{50}/Hn values were 9.83±0.12 /0.55±0.13 (n=3, 4°C) and 10.1±0.07/0.92±0.11 (n=3, 25°C); for $CGRP_{8-37}$ they were 9.42±0.15/0.90±0.13 (n=3, 4°C) and 8.84±0.03/0.93±0.10 (n=3, 25°C).

This data indicates that there is no significant effect of temperature on the binding of CGRP or $CGRP_{8-37}$ to the vas deferens and so it is unlikely that the low pA_2 observed for $CGRP_{8-37}$ is due to a temperature effect. Little has been reported concerning the guinea-pig cerebellar CGRP receptor (van Rossum *et al.*, 1993). Unlike the situation with the vas deferens, there is a significant decrease ($P<0.05$) in the pIC_{50} for CGRP8-37 for this receptor upon increasing temperature, but this is small (less than fourfold). In the guinea pig tissues examined here, it would seem that CGRP binding is not greatly affected by temperature.

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236P THE EFFECTS OF FLUNIXIN MEGLUMINE AND PHENYLBUTAZONE ON CYCLOOXYGENASE IN INFLAMED TISSUE AND PLATELETS

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Non-steroidal antiinflammatory drugs (NSAIDs) produce their effects partially by inhibiting cyclooxygenase (COX) (Vane 1971).There are two COX isoforms existing in mammalian cells. Cyclooxygenase 1 is a constitutive enzyme present in most cells including platelets and is involved in physiological processes whereas COX2 is an inducible enzyme, normally expressed following stimulation and involved in the inflammatory response (Kujubu *et al.*, 1991). The present study was carried out to investigate the effects of the NSAIDs of flunixin meglumine (FM) and phenylbutazone (PBZ) on carrageenan-induced COX and platelet COX in 8 sheep *in vivo*.

The study was carried out using a 3 ways Latin square cross-over design. Flunixin meglumine (1.1 mg/kg), PBZ (4.4 mg/kg) or placebo (PLB) were administered intravenously to 8 male sheep previously prepared with subcutaneous tissue-cages. An acute inflammatory response was induced by the injection of 0.3 ml of 1% carrageenan into the cages 20 minutes before the drug administration and was maintained by a second injection of 0.2 ml carrageenan 8 hours after the drug administration. Prostaglandin (PG) E_2 concentration in inflamed tissue-cage exudate was determined as an estimate of inducible COX activity and platelet COX activity was estimated by measuring serum thromboxane (TX) B_2 generation (Lees *et al.*, 1987). Exudate leukotriene (LT) B_4 was measured to estimate the effects of the drugs on 5-lipoxygenase. Prostaglandin E_2, TXB_2 and LTB_4 were quantified by radioimmunoassays and drug concentrations were measured by high performance liquid chromatography. Data were analysed by a mixed model analysis of variance. Pharmacodynamic analysis was carried out using PCNONLIN 4.0 (SCI Software).

Intra-caveal injection of carrageenan induced the generation of exudate PGE_2 from an undetectable level (<0.14 nM) at zero time to 101.15 nM at 12 h and was accompanied by increases in skin temperature over the inflamed cages and exudate LTB_4 with a maximal increase of 1.52±0.33 °C and a maximal value of 4.84±0.92 nM at 12 h after carrageenan injection, respectively.

Intravenous injection of FM abolished or significantly inhibited exudate PGE_2 and serum TXB_2 formation up to 32 h ($P<0.05$). The maximal inhibitory effects (E_{max}) for exudate PGE_2 and serum TXB_2 generation were 100 %. The drug concentration which produced 50 % of E_{max} (IC_{50}) was estimated to be 0.017 μM and <0.00014 μM for serum TXB_2 and exudate PGE_2 generation, respectively. Flunixin meglumine also significantly inhibited the increase in skin temperature over the tissue-cages for 6 h. Phenylbutazone produced only partial inhibition of exudate PGE_2 and serum TXB_2 generation. The E_{max} for serum TXB_2 was 75.33 % and the IC_{50} for TXB_2 was 35.96 μM. A maximal exudate PBZ concentration of 72.37 μM only produced 10 % inhibitory effect against exudate PGE_2 generation. It did not inhibit the skin temperature rise over inflamed cages ($P>0.05$). Neither FM nor PBZ inhibited exudate LTB_4 generation (Maximal LTB_4 values at 12 h were 4.60±0.75 and 4.26±0.82 nM, respectively), indicating that they are not 5-lipoxygenase inhibitors.

In conclusion, FM and PBZ have significantly distinct effects on carrageenan-induced COX and platelet COX. Flunixin meglumine, but not PBZ, is a potential COX inhibitor, and antiinflammatory and antipyretic drug in sheep *in vivo*. We would hypothesise that platelet COX represented COX1 and carrageenan induced COX probably represented COX2, although further studies are required to confirm this.

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237P INDIVIDUAL NEURONES ISOLATED FROM FOUR REGIONS OF RAT BRAIN EXPRESS MIXED POPULATIONS OF NMDA RECEPTOR SUBTYPES

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The NMDA receptor is a hetero-oligomeric assembly of subunits consisting of a common NR1 together with one or more distinct NR2 subunits arranged, putatively, as a pentamer. Of particular interest is the subunit stoichiometry of native receptors and whether individual neurones express multiple receptor subtypes. Ifenprodil, a non-competitive NMDA receptor antagonist has ~300-fold higher affinity for dimeric recombinant receptors comprising NR1a/NR2B compared to NR1a/NR2A subunits (Williams, 1993; Priestley *et al.*, 1995) and has been shown to discriminate subpopulations of NMDA receptors expressed by rat cultured neurones (Priestley *et al.*, 1994). However, NMDA receptor subunit expression patterns are known to be developmentally regulated and cultured neurones may not express the adult phenotype. In the present experiments we have used ifenprodil to study the subunit composition of receptors expressed by juvenile (adult phenotype) rat neurones.

All experiments were performed on whole-cell voltage-clamped (holding potential = -60mV) neurones enzymatically dissociated from 21-30 day old rat cerebral cortex, hippocampus (CA1), striatum or substantia nigra. Neurones were isolated from each brain region using an oxygenated PIPES-buffered salt solution containing papain (1mg ml^{-1}), Dnase (0.1mg ml^{-1}) and cysteine (1mM). After ~1h incubation at room temperature, tissue blocks were dissociated by trituration through Pasteur pipettes with increasingly constricted tip diameters. The final cell suspension was plated onto glass coverslips which, after cell adhesion, were transferred to a perfused chamber (details in Priestley *et al.*, 1995) for electrophysiological experiments.

Previous data obtained with recombinant NMDA receptors has shown 20μM ifenprodil to have essentially no effect at NR1a/NR2A receptors but to almost completely antagonise responses at NR1a/NR2B receptors (Priestley *et al.*, 1995). When 20μM ifenprodil was evaluated on neurones isolated from any of the above brain regions, whole-cell currents evoked by combined applications of 100μM NMDA + 10μM glycine were neither antagonised completely nor were they unaffected but were attenuated to some intermediate extent (Table 1).

Table 1. Antagonism of NMDA response by 20μM ifenprodil

Brain region	Mean current amplitude (pA)	Ifenprodil antagonism (% control current)	Number of rats / cells
CA1	513 ± 102	57 ± 2	2 / 20
Striatum	164 ± 20	38 ± 3*	3 / 20
Cortex	515 ± 151	52 ± 2	4 / 19
Substantia nigra	203 ± 43	35 ± 3*	6 / 20

* values significantly different from CA1 and cortex ($P < 0.01$, ANOVA followed by Newman-Keuls test) but not from each other.

These experiments suggest that individual cortical, CA1, striatal and nigra neurones do not express, exclusively, dimeric subunit assemblies resembling NR1/NR2A or NR1/NR2B combinations. Rather, it appears as though mixed receptor populations are expressed, one having a high affinity for ifenprodil and a second with lower affinity. Whether these receptor populations represent dimeric or trimeric subunit combinations which vary in the ratio of NR2A/NR2B content is, at present, unclear.

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238P EVIDENCE FOR A ROLE OF N-METHYL-D-ASPARTATE (NMDA) RECEPTORS IN DARK-REARING EVOKED APOPTOSIS IN THE LATERAL GENICULATE NUCLEUS (LGN) OF RABBIT

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In mammals, light-deprivation during early postnatal development disrupts the physiological organisation of the visual system. In fact, dark-rearing causes cell shrinkage and decreases the number of Y cells in the LGN, leading to loss of visual responses in cortical neurones and reduction in visual acuity (Rauschecker, 1991). Interestingly, blockade of NMDA subtype of glutamate receptors during development reduces retinogeniculate transmission (Miller *et al.*, 1989) and prevents the shrinkage of LGN neurones that results from monocular deprivation in the new-born kitten (Bear *et al.*, 1990), supporting a role for NMDA receptors in the refinement of the visual pathway. We now report data suggesting a role for abnormal NMDA receptor activation in the mechanisms of dark-induced apoptosis in the LGN of adult rabbit. Adult male albino rabbits (2.5±0.5kg) were exposed to darkness for 48 h; control animals received injections of saline (1ml/kg, i.p. twice daily) whereas test groups received (R,E)2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP040116) or MK801, two selective NMDA receptor antagonists, during dark exposure. Serial brain coronal sections were cut and processed for *in situ* detection of DNA fragmentation according to the TUNEL technique (see Gavrieli *et al.*, 1992) and morphological characteristics of adjacent brain sections were assessed under light microscopy using haematoxylin and eosin (H&E) staining. In control experiments, exposure of rabbits (n=6) to darkness for 48 h induced *in situ* DNA fragmentation in areas of the brain sections (n=6 per brain) corresponding to the LGN (Monnier & Gangloff, 1961) and involving 8.5±0.5 (mean±s.e.mean) cells counted in the LGN (x100 magnification). H&E staining of adjacent brain sections revealed nuclear chromatin marginalization and condensation. CGP040116 (15mg/kg, i.p. twice daily, n=6 rabbits) reduced the number of TUNEL positive cells to 2.2±0.4 ($p<0.05$ vs control); a lower dose of CGP040116 (1.5mg/kg, n=6 rabbits) was ineffective. Treatment with MK801 (3mg/kg, i.p. twice daily, n=6 rabbits) reduced the number of positive cells to 4.0±0.2 ($p<0.05$ vs control). In no instance was DNA fragmentation seen in sections (n=6 per brain) obtained from rabbits (n=6) exposed to 12 h light/ 12 h dark cycle. In conclusion, the present data support the hypothesis that abnormal activation of NMDA receptors is involved in the mechanisms of apoptosis induced by light-deprivation in the LGN of rabbit.

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239P EVIDENCE THAT GLUTAMATE REGULATES DOPAMINE SYNTHESIS VIA AROMATIC L-AMINO ACID DECARBOXYLASE

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The biosynthesis of dopamine involves two enzymes - tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC). TH is classically regarded as being the rate limiting enzyme, although recently it has been found that the activity of AADC can be modulated by dopamine (Zhu et al.,1992). In addition Hadjiconstantinou et al. (1995) reported that the NMDA ion-channel blocker, MK 801, increased brain AADC activity, suggesting endogenous glutamate also acts to regulate dopamine synthesis. This study compares the AADC activating action of MK 801 with that of several other NMDA or AMPA glutamate antagonists.

Male Wistar albino rats (240-260g) were injected with glutamate antagonists and sacrificed 0.5h (NBQX) or 1h later (other drugs). The brain was removed and placed into ice-cold saline whereafter the striatum (CS) and the substantia nigra (SN) were dissected out. These were then homogenised in 0.25M ice-chilled sucrose and centrifuged for 10 min. 20µl aliquots of supernatant were added to an incubation mixture comprising: 50 mM sodium phosphate buffer (pH7.2), 0.5mM L-Dopa, 0.01 mM pyridoxal-5'-phosphate, 0.1 mM EDTA, 0.17 mM ascorbic acid, 0.1 mM pargyline and 1 mM mercaptoethanol (total volume 400 µl), and incubated for 20 min at 37°C. The reaction was stopped by addition of 80µl of ice-cold 0.5M perchloric acid containing isoprenaline as internal standard. Dopamine was then quantified by HPLC with electrochemical detection and protein by the method of Lowry et al. (1951). Data were analysed by one way ANOVA and group differences were evaluated by Newman Keuls test.

Basal enzyme activity (nmol DA/mg protein/20 min) was found to be 19.7 ± 1.0 for CS and 22.0±1.9 for SN. Drug doses were selected on the basis of their efficacy in earlier behavioural experiments. MK 801 (0.01mg/kg) was ineffective whereas 0.1 and 1mg/kg increased enzyme activity ($p<0.001$) in both CS and SN (38.9±2.6 and 72.7±13.4, 30.9±0.9 and 47.0±2.5 respectively) as found earlier by Hadjiconstantinou et al. (1995). Other NMDA ion channel blockers showed the following enzyme activities in the CS and SN respectively: memantine (20mg/kg) 27.2±4.2 and 31.6±2.9 ($p<0.01$); dextromethorphan (40mg/kg) 66.5±12.7 and 135.4±34.3 (both $p<0.001$) and the clinically used anti-parkinson drug budipine (12.5mg/kg) 45.9±3.6 and 93.5±11.9 (both $p<0.001$). The NMDA glycine site antagonist (+) HA966 (5mg/kg) elevated AADC activity in the CS (28.7±2.2,$p<0.001$) but not in the SN (26.2±3.7). By contrast, the NMDA polyamine site antagonist eliprodil (10mg/kg) was ineffective in both brain areas (22.7±2.5 and 28.4±0.9) as was the glutamate binding site antagonist CGP40116 (5mg/kg) (27.5±2.7 and 27.5) and the AMPA antagonist, NBQX, (10mg/kg) (16.9±1.1 and 25.7±1.0).

These findings show that glutamate antagonism by ion-channel blockade in the NMDA receptor appears to be the site of action which most potently increases AADC activity. Furthermore, our findings show for the first time a mechanism of action of the anti-parkinson drug budipine involving AADC and highlights the important role that modulation of this enzyme may have in Parkinson patients treated with L-Dopa .

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240P EFFECTS OF NEURONAL CALCIUM CHANNEL ANTAGONISTS ON SODIUM CHANNELS *IN VITRO* AND IN GLOBAL ISCHAEMIA *IN VIVO*

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Compounds with activity at neuronal calcium and/or sodium channels may be of possible therapeutic interest for treatment of stroke. NNC 09-0026 ((-)-trans-1-butyl-4-(4-dimethylaminophenyl)-3-(4-trifluoro -methylphenoxymethyl) piperidine dihydrochloride), SB 201823-A (4-[2(3,4-dichlorophenoxy)ethyl]-1-pentyl piperidine hydrochloride) and NS 649 (2-amino-1-(2,5-dimethoxyphenyl)-5-trifluoromethyl benzimidazole) have been shown to be neuronal calcium channel antagonists with anti-ischaemic activity. CNS 1237 (N-acenaphthyl-N'-4-methoxynaphth-1-yl guanidine) has been reported to be both a Ca^{2+} and Na^{+} channel antagonist.

In the present study we have examined the effects of these compounds on human ω-conotoxin sensitive N-type voltage-dependent calcium channels (VDCC) expressed in HEK293 cells (Bleakman *et al*, 1995). Cells were voltage-clamped at Vh=-90mV and whole-cell calcium currents (ICa) evoked by depolarisation to +10mV for 50msec. IC_{50} values for steady state inhibition of ICa were as follows (in µM ± SEM): NNC 09-0026, 1.2 ± 0.3, CNS 1237, 4.2 ± 1.8, SB 201823-A, 10.6 ± 2.2 and NS 649, 45.4 ± 17.8. The activity of these compounds was also examined on Na^{+} channels in acutely isolated cerebellar Purkinje neurons *in vitro*. At a concentration of 10µM (n = 3) the inhibition produced was as follows: CNS 1237, 98± 1, SB 201823-A, 71±2, NNC 09-0026, 51 ± 7 and NS 649 5 ± 6.

The compounds were also examined in the gerbil model of global cerebral ischaemia. Male Mongolian gerbils (60-80g) were used in these experiments. Ischaemia was induced by 5 min of bilateral carotid artery occlusion under halothane anaesthesia. Sham operated animals were included as controls. Compounds were administered pre- and post-occlusion. 5 days after surgery the animals were perfused transcardially with 0.9% saline followed by 10% buffered formalin. For histological evaluation 5 µm coronal brain sections were cut, stained with haematoxylin and eosin and the viable pyramidal cells in the CA1 hippocampal region were counted (n = 8 animals per group).

The number of viable cells (mean ± SEM) per 1 mm of the CA1 was 215 ± 7 (sham operated), 10 ± 2 (ischaemic control), 44 ± 15 (NNC 09-0026 30mg/kg i.p.), 49 ± 19 (CNS 1237 30mg/kg i.p.), 11 ± 2 (SB 201823-A 10 mg/kg i.p.) and 17 ± 4 (NS 649 50 mg/kg i.p.). Therefore, NNC 09-0026 and CNS 1237 provided significant neuroprotection when administration was initiated before occlusion while SB 201823-A and NS 649 failed to protect.

These results indicate that the neuronal calcium antagonists tested in the present studies block sodium channels. Both NNC 09-0026 and CNS 1237 showed good activity on Ca^{2+} and Na^{+} channels and this may contribute to the observed neuroprotection.

Bleakman, D., Bowman, D., Bath, C.P. *et al.* (1995) Neuropharmacology 7, 753-765.

241P CLOZAPINE DRUG DISCRIMINATION IN RATS: A PHARMACOLOGICALLY SPECIFIC STIMULUS

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Clozapine is an effective discriminative stimulus in rats (Nielsen, 1988; Moore et al. 1993), allowing the pharmacological. actions of clozapine to be analysed in this *in vivo* behavioural assay. Female Wistar rats (n = 20; 220-275g) maintained at 21 degrees C were trained in daily 15 minute sessions to discriminate clozapine at 5 mg/kg (i.p., dissolved in 0.1M HCl, in distilled water at a pH of 6 administered 30 mins before each session) in a quantal two lever Fixed Ratio 30 food rewarded operant assay (Goudie et al. 1989). The clozapine discrimination was learned relatively rapidly (70 sessions at 5 sessions per week) to a high level of accuracy (the group level of accuracy of lever selection was at least 90% correct per day throughout the whole study). Generalisation tests were separated by at least two training days. As anticipated, in subsequent tests clozapine induced dose-related full generalisation between 0.625 to 5 mg/kg (the ED50, defined by log/linear least squares regression analysis, was 2.12 mg/kg ; r^2 for the regression line was 0.99 which therefore provided a very good fit to the data). 100% drug lever selection was seen at the 5 mg/kg training dose in the generalisation test, as expected. In the generalisation tests no dose of clozapine suppressed operant responding relative to the control (vehicle) baseline. Thus clozapine could be discriminated without any overt behavioural effect of the drug.

To assess the specificity of the clozapine discrimination generalisation tests were run with two drugs (4 doses/drug) known to be discriminable in rats:- amphetamine (0.25-2 mg/kg) and pentylenetetrazol (2.19-17.5 mg/kg). Neither of these drugs induced more than 10% drug lever selection at any dose. Amphetamine suppressed the group mean level of operant responding at the highest dose by 69%. Thus no generalisation to clozapine was seen even with a dose of amphetamine with very marked effects on behaviour. Further generalisation tests with 4 doses of haloperidol (0.03-0.25 mg/kg) revealed that the maximal level of drug lever selection observed was only 23% at the highest dose tested, which suppressed operant responding by as much as 89%.

These data demonstrate the pharmacological specificity of the clozapine cue, and that the standard typical neuroleptic haloperidol differs from clozapine in this assay. Other data (Taylor et al., 1996) indicate that only close structural congeners of clozapine induce marked generalisation to the clozapine discriminative stimulus. Thus the clozapine discrimination assay appears to be a reliable procedure to use in vivo to detect clozapine-like drugs.

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242P CLOZAPINE DRUG DISCRIMINATION IN RATS: EFFECTS OF ATYPICAL NEUROLEPTICS

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We have reported (Goudie et al. 1996) that clozapine is a pharmacologically specific discriminative stimulus in rats, and that the typical neuroleptic haloperidol does not generalise to it. We now report on actions of novel atypical or putative atypical neuroleptics in rats discriminating clozapine (for details of animals, and procedures see Goudie et al. 1996).

In female Wistar rats (n=20) discriminating clozapine (5 mg/kg) the following drugs were tested for generalisation:- risperidone, olanzapine and amisulpiride (Moore et al. 1993), sertindole (Sanchez et al. 1991) and JL13 (5-(4-methylpiperazin-1-yl)-8-chloro-pyrido [2,3,-b] [1,5] benzoxapine, Liegeois et al. 1994). Each compound was tested with at least 4 doses. All drugs were administered 30 mins before testing except amisulpiride (4 hrs).

Risperidone (0.05-0.4 mg/kg) produced weak partial generalisation, with a maximal level of 40% at the highest dose, which suppressed responding by 88%. Olanzapine (0.31-2.5 mg/kg) also produced weak partial generalisation, with a maximal level of 38% at the highest dose, which suppressed responding by 52%. Amisulpiride produced minimal generalisation, with a maximal level of 25% at the highest dose, which suppressed responding by 90%. Sertindole (0.625-5 mg/kg) produced 50% generalisation at the highest dose, at which responding was suppressed by 70%. JL13 (0.625-20 mg/kg) produced the most generalisation. This was dose-related, although only up to 10 mg/kg, which induced a maximal level of 70% generalisation. At 20 mg/kg generalisation dropped to 53%. The doses of JL13 tested suppressed responding in a dose-related fashion up to a maximum of 42%.

Thus different drugs induced different degrees of generalisation (JL13 > sertindole > olanzapine = risperidone > amisulpiride). Only very close structural congeners of clozapine, such as JL13 (Bruhwyler et al. 1992), induced substantial generalisation, although even JL13 did not generalise fully, and it only did so at doses which suppressed responding, whereas clozapine produced 100% drug lever selection at doses without any effect on responding (Goudie et al. 1996). In summary, clozapine discrimination may be used to assess drugs *in vivo* for clozapine-like properties. Further studies are required to define the receptors involved in clozapine discrimination. However, since JL13 produced the most marked generalisation, despite its very low affinity for D2 receptors (Bruhwyler et al. 1992; Liegeois et al, 1994), and since haloperidol did not generalise, such receptors *may* not play a critical role in the clozapine stimulus.

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243P AVERSIVE PROPERTY OF CLOZAPINE REVEALED IN A MURINE PLACE-CONDITIONING PARADIGM

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Previously the atypical antipsychotic, clozapine has been shown to possess discriminative stimulus properties in rats (Wiley & Porter, 1992). The present investigation evaluated the effects of clozapine in a place conditioning paradigm in the mouse in order to investigate whether the drug possessed a reinforcing potential.

Male adult BKW mice (32-48g) and a three chambered apparatus were used for conditioning (Ali & Kelly, in press). Baseline preferences were determined on three separate occasions by allowing each mouse access to the entire apparatus and recording the time spent in each outer chamber over the 15 min session. The average of the three sessions served as the pre-conditioning score (s±sem). Mice (n=7-8/dose group) were conditioned with clozapine (2.5, 5.0 or 10.0 mg/kg, i.p.) or vehicle (0.9% saline, 0.1ml/kg, i.p.) by confinement to one of the two outer chambers for 45 min. On alternate days they received the other treatment and were confined to the opposing chamber such that each mouse received 4 clozapine and 4 saline pairings. 24 h after the final conditioning trial, mice were tested as in the pre-conditioning phase, in a drug-free state. Effects on locomotor activity were determined in separate groups of mice (n=10) which received vehicle or clozapine (2.5-10mg/kg, i.p.) and were placed in photocell cages and counts recorded for 45 min. The procedure was repeated over 4 days and on the fifth day mice were tested in a drug free state to mimic the place conditioning protocol. Data were analysed by 2-way ANOVA followed by post-hoc t-test analysis.

Clozapine (2.5, 5.0 and 10.0 mg/kg) conditioning reduced the time spent in the clozapine conditioned chamber from 299±15s to 243±28s, from 263±15s to 147±18s and from 286±19s to 182±27s, respectively which was significant ($F(2,12)=13.1$, $p<0.05$ by post-hoc Dunnett's t-test) at 5.0 mg/kg. Saline conditioning did not affect preferences (286±11s compared to 318±33s pre- vs post-conditioning preferences). Clozapine also induced significant decreases in locomotor activity on day 1 ($F(3,27)=25.5$, $p<0.01$), day 3 ($F(3,27)=5.8$, $p<0.05$) and day 4 ($F(3,27)=2.5$, $p<0.05$) of testing when compared with the control group. However, on the drug-free test day there was no significant difference in locomotor activity between the vehicle control group and the clozapine treated groups (503±82, 697±105, 530±92 and 408±52 counts/45 min) vehicle or clozapine (2.5, 5.0 and 10.0 mg/kg) respectively.

The demonstration of a conditioned place aversion with clozapine in the mouse does not support the findings of Wiley & Porter (1992) who demonstrated that clozapine possessed dicriminative stimulus properties in a study using a two-lever drug discrimination procedure in the rat and would suggest that clozapine lacks positive reinforcing potential as demonstrated in a place conditioning model. Whilst it cannot be excluded that the decrease in locomotor activity and species variation may have influenced the outcome of the study, the difference in results probably reflects the different aspects of the drugs effects which are modelled in the two techniques.

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244P EFFECTS OF CLOZAPINE AND MDL 100,907 IN THE RAT GELLER-SEIFTER MODEL OF ANXIETY

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Animal models may be able to predict compounds with efficacy against the negative symptoms of schizophrenia e.g. social withdrawal (Ellenbroek et al., 1991). We studied the effects of the atypical antipsychotic, clozapine and the 5-HT_{2A} receptor antagonist MDL 100,907 in the rat Geller-Seifter model of anxiety. Clozapine has proven clinical efficacy against negative symptoms (Brunello et al., 1995); MDL 100,907 awaits clinical evaluation.

Male Sprague-Dawley rats (400-500g) fed a restricted diet, were trained to press a lever for a food reward, and to associate a light cue with both a high level of reward (Fixed Ratio, 5) and a contingent footshock, in 5 x 3 min periods. These were alternated with 5 x 3 min unpunished periods with rewards on average every 30 seconds (see Kennett et al., 1995). Clozapine was administered orally 60 min pretest in a suspension of 1% methylcellulose and water and MDL 100,907 was dissolved in saline and administered i.p. 30 min pretest. Data is cited as mean ± s.e.m., n = 6-7 per group. Significance was tested by 2-way ANOVA (treatments x subjects) followed by a 2-sample t-test. Comparisons were made between levels of responding on two preceding vehicle treated days and expressed as % change.

The mean (± s.e.m.) number of lever presses on the two days preceding challenge with clozapine or MDL 100,907 was 699.8 ± 26.8 during unpunished, as opposed to 27.2 ± 0.7 during punished trials. Clozapine increased punished responding at 2.5 (31.2 ± 14.0% $p<0.05$) and 5 mg/kg (138.1 ± 66.95% $p<0.01$) without altering unpunished responding. However at 10 mg/kg, the highest dose tested, the anxiolytic-like effect (measured by a selective increase in punished responding) was lost and there was a significant reduction in unpunished responding (-52.0 ± 15.8% $p<0.01$). MDL 100,907 had little effect on unpunished responding across the dose-range tested (0.5, 2, 5 and 10 mg/kg) but caused significant increases in punished responding at 2 (24.6 ± 11.2% $p<0.05$) and 10 mg/kg (97.5 ± 51.5% $p<0.05$).

Clozapine shows some anxiolytic-like activity in the Geller-Seifter model until the onset of non-specific effects at 10 mg/kg. MDL 100,907 also showed some anxiolytic-like activity but this was only substantial at high doses. The doses of clozapine used in this model were similar to, and those of MDL 100,907 much higher than, those required to produce antagonism of amphetamine-induced hyperlocomotion in rats (Moser et al., 1996). MDL 100,907 has also been tested in another model of anxiety (separation-induced vocalisation in rat pups) with similar weak effects at high doses (see Kehne et al., 1996).

To further characterise the Geller-Seifter model as a predictor of compounds with efficacy against negative symptoms, other novel neuroleptics e.g. olanzapine need to be tested in this paradigm.

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245P DISCRIMINATIVE STIMULUS PROPERTIES OF THE PUTATIVE DOPAMINE D_3 RECEPTOR AGONIST, (+)-PD 128907, IN THE RAT

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The present study has examined the discriminative stimulus properties of (+)-PD 128907, the most selective D_3 receptor agonist currently available.

Male Sprague Dawley rats (200-250g; n = 43) were trained to discriminate (+)-PD 128907 (30 µg/kg, s.c.) from saline (1ml/kg) in a two lever, operant drug discrimination paradigm with a fixed ratio, FR10, schedule of food reinforcement. Animals were tested 10 min after dosing and the number of responses made on each lever before the first food pellet was obtained and the total number of responses made during the 15 min test session recorded. Lever selection was considered correct if rats made ≤ 5 presses on the inappropriate lever before completing 10 responses on the correct lever. Experiments commenced once the criterion of 90% correct responses in the preceding 2 week period was achieved (n = 4-10 rats/dose).

All rats learned to discriminate (+)-PD 128907 (30 µg/kg) from saline with 49 ± 3.4 (mean ± s.e. mean) training sessions required to achieve the performance criterion. As expected, injection of (+)-PD 128907 (3.75 - 30 µg/kg, s.c.) dose-dependently increased drug lever selection (ED_{50} = 11 ± 2 µg/kg) with 100% of animals choosing the (+)-PD 128907 lever at the training dose of 30 µg/kg. The discriminative stimulus effects of (+)-PD 128907 were enantiomerically selective and injection of 30 µg/kg (-)-PD 128907 failed to induce drug lever responding. In generalisation tests, the non-selective dopamine $D_{2/3}$ receptor agonists apomorphine (6 - 60 µg/kg), (-)-quinpirole (15 - 60 µg/kg) and (±)-7-OH-DPAT (6 - 60 µg/kg) increased (+)-PD 128907 lever selection (ED_{50} = 15 ± 8, 16 ± 2 and 17 ± 1.4 µg/kg respectively). In contrast, the D_1 selective agonist, (±)-SKF 81297 (100 - 600 µg/kg, s.c.) failed to induce drug lever responding at the doses tested.

Pretreatment with the $D_{1/5}$ receptor antagonist, R-(+)-SCH 23390 (5 or 10 µg/kg, s.c.), or the D_4 receptor antagonist, L-745,870 (3-{[4-(4-chlorophenyl)piperazin-1-yl]methyl}-1*H*-pypyrrolo[2,3-b] pyridine; 100 or 1000 µg/kg, p.o.) 30 or 60 min prior to (+)-PD 128907 injection, failed to block drug lever selection. In contrast, pretreatment with the non-selective $D_{2/3}$ receptor antagonists, haloperidol (5 - 30 µg/kg, s.c.), raclopride (3 - 100 µg/kg, s.c.), spiperone (5 - 10 µg/kg, s.c.) or (+)-butaclamol (10 - 100 µg/kg, s.c.), 30 min prior to (+)-PD 128907 injection, dose-dependently reduced the number of rats choosing the (+)-PD 128907 lever (ED_{50} = 12 ± 3, 16 ± 0.5, 8.4 ± 0.4 and 42 ± 4 µg/kg respectively). Similar effects were also observed in rats i.p. dosed with the 10 fold selective D_2 receptor antagonist, L-741,626 (3-{[4-(4-chlorophenyl)-4-hydroxy] piperidin-1-yl} methyl-1*H*-indole; K_i at rat D_2 and D_3 receptors = 12 and 120 nM respectively; ED_{50} = 1200 ± 200 µg/kg). In contrast, the >41 and 133 fold selective dopamine D_3 receptor antagonists, L-745,829 (3-[4-(chlorophenyl)piperazin-1-yl]methyl-2(1H)-quinolone; K_i at rat D_2 and D_3 receptors = >1900 and 46 nM respectively; 0.5 - 20 mg/kg, p.o.) and GR 103691 (K_i at human dopamine D_2 and D_3 receptors = 40 and 0.3 nM respectively (Murray *et al.*, 1995); 0.3 - 3 mg/kg, s.c.) failed to block the (+)-PD 128907 discriminative stimulus.

In conclusion, low doses of the putative dopamine D_3 receptor agonist (+)-PD 128907 induced a reliable discriminative stimulus response in the rat. However, pharmacological analysis suggests that the dopamine D_2 receptor is the subtype involved.

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246P EXTRACELLULAR GLUTAMATE AND ASPARTATE ARE ABNORMALLY ELEVATED IN THE ENTOPEDUNCULAR NUCLEUS OF PARKINOSINAN RATS: REVERSAL WITH DOPAMINE $D_{2/3}$ BUT NOT D_1 AGONISTS

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Recently, Parkinson's Disease has begun to be regarded as a condition in which a number of extrapyramidal pathways, aside from the nigrostriatal system alone may be dysfunctional (Greenamyre, 1993). Specifically, evidence suggests that the striatopallidal output pathway to the entopeduncular nucleus (EPN) and substantia nigra may become hyperactive if parkinsonism is induced experimentally by dopaminergic lesioning (Mitchell *etal*, 1989). This branched pathway is thought to use glutamate as neurotransmitter (Albin *etal*, 1989) and thus has led to the notion that glutamate receptor antagonists may alleviate parkinsonian rigidity and akinesia (Starr, 1995). In the current study, we have monitored output of glutamate and aspartate (using bilateral microdialysis) from the EPN of rats rendered parkinsonian using reserpine to deplete cerebral dopamine stores. In addition, we have attempted to normalise EPN amino acid output with dopaminergic agonists and correlate this with a restoration of motor activity.

Male Wistar rats (250-350g) were implanted with concentric dialysis probes into both EPN, under halothane anaesthesia (4% for induction, 1.5% for maintenance). Following surgery, control animals received saline and all others, reserpine (4 mg/kg i.p.). Microdialysis was commenced 24h later, probes perfused with artificial CSF (Whitton *etal*, 1990) at a rate of 0.5µl/min. In all cases, 4 samples (30 min) were collected prior to the administration of drugs. In an initial set of experiments, control rats received veratridine (100 µM in perfusate bilaterally for 30 min), whilst in further sets, reserpine treated animals received either the $D_{2/3}$agonist quinpirole (4 mg/kg ip.) or the D_1 partial agonist SKF 38393 (30 mg/kg ip.). Dialysates were analysed for aspartate (ASP) and glutamate (GLU) content using HPLC with fluorimetric detection.

All figures represent combined data from left and right EPN, since 2-way ANOVA did not disclose significant differences between sides. * denotes significant differences between groups confirmed with a *post-hoc* student's t- test. Basal releases of EPN amino acids (pmols/10µl± s.e.m; n=4 rats per group) were as follows: *Controls*; ASP 9.5±3.2, GLU 15.3±2.5. *Reserpine treated;* ASP 17±2.3*, GLU 56.7±10.3*. (* $p<0.05$ against control values). Veratridine (100 µM) provoked a sustained (90 min) increase in both ASP (peak 23.7±3.0 *) and GLU (peak 41±7.0*), supporting the notion that a proportion of amino acid release in the EPN is depolarization dependant. Both quinpirole and SKF 38393 resulted in marked behavioural arousal in reserpine-treated rats, characterised by rearing, sniffing and grooming. The motor restorative effects of quinpirole were long lasting (approximately 4h) and were accompanied by a dramatic fall in EPN output of GLU (max. reduction to 18±2.0*) and ASP (max reduction to 9.2±2.5*; $p<0.05$ against reserpine treatment in both cases). In contrast, SKF 38393 failed to significantly reduce reserpine induced increases in either ASP (max. reduction to 15±3.2) or GLU (max. reduction to 32±7.0).

These findings provide the first direct demonstration that GLU and ASP levels are elevated in EPN of rats rendered parkinsonian. Furthermore, our initial studies suggest that the striatopallidal output pathway is under D_2 dopaminergic control and activation of these (presumably striatal) receptors restores pathway activity to normal.

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247P A SIMPLE METHOD FOR MEASURING ENDOGENOUS AND RADIOLABELLED DOPAMINE RELEASE FROM RAT STRIATAL SLICES

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There are many situations when rapid information is required on the effects of drugs on neurotransmitter release in brain. The most common method is measurement of radiolabelled release from superfused brain slices or synaptosomes *in vitro*. However, the method requires expensive equipment and is not readily adapted to the measurement of endogenous release.

The method described here is adapted from one used to measure synaptosomal release (Ebstein *et al.*, 1982) and allows rapid assessment of striatal dopamine (DA) (radiolabelled and endogenous) release and its response to temperature, potassium and a DA agonist and antagonist under semi-superfusion conditions using readily available inexpensive chromatographic columns (Bio-Rad Laboratories Ltd.). Rat striatal slices were placed in each column (25 µl/column) and then incubated in normal Krebs-Henseleit buffer (KHB) (5 min to 10 min) at 0 °C, room temperature, and 37 °C and eluate collected as basal release. Release was then stimulated by incubating slices in high [K⁺]KHB which gave submaximal stimulation (10 mM for [^{3}H] and 20 mM for endogenous DA release)and eluate collected as K⁺-stimulated release. The DA agonist (pergolide, 100 µM) and antagonist (eticlopride, 10 µM) were added to both normal and high [K⁺]KHB. Released DA was measured in the eluates using either liquid scintillation spectroscopy or high performance liquid chromatography with electrochemical detection.

High potassium (10 or 20 mM) increased ($p<0.01$) both radiolabelled and endogenous DA release at 37 °C but not 0 °C compared to basal release (Fig. 1). The non-selective D_1/D_2 receptor agonist, pergolide reduced the effect of K⁺-stimulation on both [^{3}H] and endogenous DA release ($p<0.01$ and $p<0.05$, respectively). Eticlopride (1 µM) increased basal release of [^{3}H] ($p<0.01$) and endogenous ($p<0.001$) DA but only increased the K⁺-stimulated release of [^{3}H]DA ($p<0.05$).

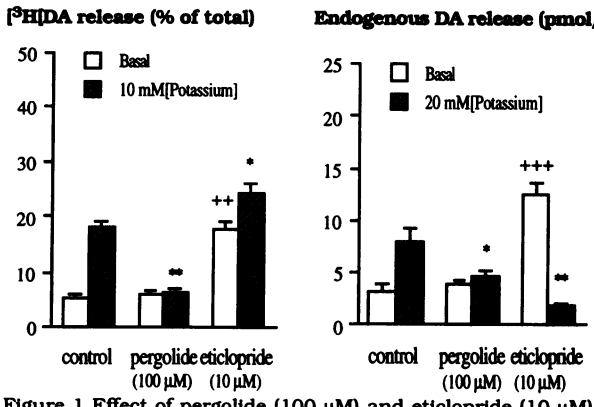

Figure 1 Effect of pergolide (100 µM) and eticlopride (10 µM) on [^{3}H] and endogenous DA release at 37 °C. ++P<0.01 and +++P<0.001 compared to basal release in control, *P<0.05, **P<0.01 compared to K⁺-stimulated release in control using Mann Whitney U test, n=6 in all groups.

The present method allows rapid comparison of endogenous and radiolabelled release using identical conditions and confirms that [^{3}H]DA release measured using this method is sensitive to temperature, depolarisation and drugs acting on the D_2 striatal autoreceptor. However, the difference in the response obtained with eticlopride between endogenous and [^{3}H]DA release might be due to depletion of endogenous DA under the experimental conditions (10 min exposure to eticlopride prior to 20 mM[K⁺]). The results support the view that radiolabelled release of DA need not necessarily mirror endogenous release (Herdon *et al.*, 1987).

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248P DIFFERENTIAL INTERACTION OF DIZOCILPINE WITH DOPAMINE D1- AND D2-INDUCED CATALEPSY

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Dopamine D_1 and D_2 receptor antagonists cause catalepsy in the rat when administered systemically, or when injected focally into the corpus striatum (CS) or nucleus accumbens (NAC; Ossowska et al., 1990). The catalepsy induced by systemic treatments is reversed by the NMDA ion channel antagonist dizocilpine (Verma and Kulkarni, 1992), indicating a functional interaction between D_1, D_2 and NMDA receptors in this condition. The site(s) from which the anticataleptic effect of dizocilpine arises are less clear, and so this study investigates whether NMDA receptors in the CS and/or NAC are involved in modulating D_1- and D_2-dependent catalepsy.

Male Wistar albino rats (200-300 g) were used. Catalepsy was induced by injecting the D_1 antagonist SCH 23390 i.p. (1 mg/kg), or bilaterally into the CS or NAC (1 µg in 0.5 µl distilled water) under halothane anaesthesia, or by injecting the D_2 antagonist raclopride i.p. (3 mg/kg) or into the CS or NAC (10 µg in 0.5 µl). Dizocilpine (0.2 mg/kg i.p.) was injected 10 min before or 45 min after neuroleptic. Alternatively the dizocilpine was injected directly into the CS or NAC (10 µg in 0.5 µl) and neuroleptic given 45 min later. Control rats received vehicle. All drug doses were found to be optimal from pilot studies. Animals were placed singly into observation cages with their front paws over a 8 cm high bar and descent latencies measured (maximum 360 sec) every 15 min for a maximum 150 min. Data from groups of 6-11 rats were analysed by ANOVA and Student's t-test.

The intensities and time-courses of the catalepsy induced by 1 mg/kg SCH 23390 and 3 mg/kg raclopride were not significantly different. Pre- and post-treatment with dizolcipine (0.2 mg/kg i.p.) reversed SCH 23390-induced catalepsy (95.7% and 83.8% inhibition at 150 min versus controls respectively, both $p<0.0001$) more effectively than raclopride-induced catalepsy (39.7%, $P>0.05$ and 53.2%, $P<0.005$ at 135 min). SCH 23390 (1 µg/0.5 µl) gave rise to profound catalepsy from the CS and NAC. This was reversed by systemic dizocilpine (0.2 mg/kg) with similar efficacy versus the CS (90.1% at 30 min, $P<0.0001$) and NAC (91.2%, $P<0.0001$%), except that the rate of reversal was faster with NAC-derived catalepsy. Similar results were obtained with systemic dizocilpine versus intraCS (88.2% at 30 min, $P<0.0001$) and intraNAC raclopride (90.1%, $P<0.0001$), but this time the CS-derived catalepsy was more rapidly affected. Pretreating the CS or NAC with dizocilpine (10µg/0.5 µl) was equivalent in preventing the catalepsy induced by systemic SCH 23390 (e.g. 87.2% and 87.9% respectively at 45 min, both $P<0.0001$), whereas with raclopride induced catalepsy dizocilpine was clearly more effective in the CS (97.8% at 45 min, $P<0.0001$) than in the NAC (0% at 45 min, $P>0.05$).

The more profound and longer-lasting reversal of D_1 than D_2 receptor-mediated catalepsy by systemic dizocilpine, may be related to the differential interaction of dizocilpine with D_1 and D_2 receptor mechanisms in the CS and NAC.

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249P THE EFFECT OF 7-OH-DPAT ON RESPONDING FOR A CONDITIONED REINFORCER IN THE RAT

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Evidence for the importance of dopamine (DA) D_1 and D_2 receptor subtypes in mediating reward comes from studies of the effects of dopaminergic manipulations on responding for a conditioned reinforcer (Beninger and Ranaldi 1992). More recently it has been suggested that the D_3 receptor is also involved in the mediation of reward (Chaperon and Thiebot, 1995). The aim of the present study was to investigate the role of D_3 receptors in the mediation of conditioned reinforcement by comparing the effects of the D_3/D_2 receptor agonist 7-OH-DPAT with a range of other DA agonists.

24 Female Lister Hooded rats maintained at 85% of their free feeding weight (250-300g) were trained to associate the delivery of a food pellet with the presentation of a conditioned stimulus (CS) over a 12 day period (Smith *et al.*, 1996). In the test phase two levers were introduced into the chamber. Responding on the non-conditioned reinforcement lever (NCR) had no programmed consequence, whilst responding on the conditioned reinforcement lever (CR) resulted in presentation of the CS, but with no accompanying pellet delivery. Drugs and vehicle were administered i.p. 30min prior to testing and test sessions were of 30min duration. Data was subjected to square root transformation before subsequent analysis by 2-way ANOVA followed by Dunnett's t-test.

Administration of SKF38393 (0.3-3mg/kg) did not significantly affect responding for a conditioned reinforcer ($F(3,20) = 0.27$ N.S.). Injection of bromocriptine significantly enhanced responding on both CR and NCR ($F(3,20) = 11.9$ $P<0.001$). Apomorphine significantly ($F(3,20) = 16.3$ $P<0.001$) enhanced responding on both CR and NCR at low doses and significantly attenuated responding selectively on CR at a higher dose. Both quinpirole ($F(3,20) = 14.7$ $P<0.001$) and 7-OH DPAT ($F(3,20) = 3.2$ $P<0.05$) significantly and selectively reduced CR responding (Table 1).

In agreement with previous studies (Beninger and Ranaldi 1992) the DA agonists apomorphine and bromocriptine had a non-specific effect to increase responding for a conditioned reinforcer. In contrast, treatment with the D_2 receptor agonist quinpirole and the D_3/D_2 receptor agonist 7-OH-DPAT attenuated responding for conditioned reward. These data are the first to show that 7-OH-DPAT reduces the ability of a secondary reinforcer to maintain responding. Further studies are needed to determine the role of the D_3 receptor in the mediation of this effect.

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Table 1 The effect of DA agonists on responding for a conditioned reinforcer

Bromocriptine			Apomorphine			Quinpirole			7-OH DPAT		
Dose (mg/kg)	CR	NCR	Dose (mg/kg)	CR	NCR	Dose (mg/kg)	CR	NCR	Dose (mg/kg)	CR	NCR
0.0	6.8±0.5	1.8±0.5	0.0	6.1±0.3	1.8±0.2	0.00	6.4±0.9	2.7±0.4	0.00	6.8±0.4	2.8±0.3
0.3	7.7±0.7	2.1±0.4	0.3	5.6±0.4	4.6±0.8	0.01	7.0±0.8	2.9±0.7	0.01	4.2±0.9	2.9±0.7
1.0	9.2±0.4††	5.1±0.8††	1.0	7.2±0.3††	6.4±0.6††	0.10	4.2±0.6	2.8±0.7	0.05	3.4±0.8*	2.9±0.6
3.0	8.0±0.4	5.9±0.7††	3.0	1.4±0.5**	2.1±0.3	1.00	1.8±0.4**	2.7±0.5	0.10	2.1±0.7**	2.7±0.5

Data are expressed as mean±s.e.m. square root of lever responses on CR or NCR (n = 6 per group). Effect of drug compared to vehicle treatment; significant attenuation: * $P<0.05$, ** $P<0.01$, significant increase: †† $P<0.01$ (Dunnett's t-test).

250P EFFECT OF RACLOPRIDE AND SULPIRIDE ON 7-OH-DPAT-INDUCED COGNITIVE DEFICIT IN THE MARMOSET

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The putative D_3/D_2 receptor agonist 7-OH-DPAT induces cognitive impairments in an object discrimination task in the marmoset (Smith *et al.*, 1996). The dopamine D_2 receptor antagonist raclopride has been shown to possess relatively high affinity for D_3 receptors at high doses (Malmberg et al.,1993) and also to antagonise the D_3 receptor mediated hypothermia response to 7-OH-DPAT (Millan et al., 1994). The present studies were designed to assess the effects of raclopride and the selective D_2 receptor antagonist(-)sulpiride on 7-OH-DPAT induced reversal learning impairments in the common marmoset (*Callithrix jacchus*).

Subjects were four adult (3 female, 1 male >18months old; 290-380g) laboratory-bred common marmosets. Cognitive performance was evaluated in a serial object discrimination task using the Wisconsin General Test Apparatus (Domeney *et al.*, 1991). Animals were trained to 90% criterion (27 correct object choices in 30 trials) in a food-rewarded serial object discrimination task. Subsequently animals were trained to 90% criterion in both the initial and reversal tasks of a same-day reversal paradigm. Animals were tested daily between 0900 and 1200 h. Drugs were administered by s.c. injection (1 ml/kg); 7-OH-DPAT (6 μg/kg) or vehicle (0.9% saline) was administered 15 min prior to behavioural testing. Potential antagonists raclopride (6-50 μg/kg) and (-)sulpiride (10 mg/kg) or vehicle were administered 40 min prior to treatment with 7-OH-DPAT or vehicle. Data were calculated as mean±s.e.m trials and mean±s.e.m errors to criterion (6 correct consecutive trials) for the initial and reversal task (30 trials per task) and analysed by a one way ANOVA with Dunnett's t-test.

Initial task performance was not impaired by 7-OH-DPAT 6 μg/kg (12±2 trials; 6±2 errors to criterion) compared to vehicle (10±2 trials, 5±1 errors). However, reversal task performance was significantly impaired by 7-OH-DPAT 6 μg/kg (26±3 trials, 16±1 errors; $p<0.001$ compared to vehicle 14±2 trials, 6±1 errors). Pretreatment with raclopride 6 μg/kg (26±2 trials, 15±1 errors; $p<0.001$) and 25 μg/kg (20±3 trials, 11±1 errors; $p<0.001$) or (-)sulpiride 10 mg/kg (23±3 trials, 11±2 errors; $p<0.01$-0.001) failed to attenuate the reversal task impairments induced by 7-OH-DPAT compared to raclopride and sulpiride control groups respectively. However, raclopride 50 μg/kg significantly attenuated the reversal task impairment induced by 7-OH-DPAT (13±3 trials, 6±1 errors; $p<0.001$ compared to 7-OH-DPAT 6 μg). Raclopride (6-50 μg/kg) and (-)sulpiride (10mg/kg) alone did not alter initial or reversal task performance.

The failure of (-)sulpiride or low doses of raclopride to antagonise the reversal learning impairments induced by 7-OH-DPAT suggests that these effects were not mediated by an agonist action at D_2 receptors. However, the ability of a high dose of raclopride to antagonise the reversal learning impairments suggests that these cognitive deficits produced by 7-OH-DPAT may be mediated by activation of the D_3 receptor subtype. Future studies in the marmoset will investigate the effects of selective D_3 receptor antagonists on 7-OH-DPAT induced cognitive deficits.

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251P DIFFERING EFFECTS OF TYPICAL AND ATYPICAL NEUROLEPTICS ON INTRACRANIAL SELF-STIMULATION MAY DEPEND ON α_2 RATHER THAN 5-HT_2 ANTAGONISM

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The binding profiles of some new antipsychotic drugs have led to various explanations for their relative freedom from extrapyramidal side-effects, including possible roles for 5-HT_2- or α_2-receptor antagonism (Meltzer *et al.*, 1989; Nutt, 1994). These atypical neuroleptics can also be distinguished from conventional neuroleptics by their failure to produce decremental within-session responding in operant behavioural studies (Sanger & Perrault 1995), and by early recovery from their depressant effect on electrical self-stimulation (Herberg et al. 1995). Some property of these drugs appears to counteract their inhibitory effect on pedal pressing, and might also contribute to the rarity of extrapyramidal effects. We investigated whether 5-HT_2 or α_2-antagonist properties of atypical neuroleptics might be responsible. Adult male PVG hooded rats (n=8 or 9) weighing 250-300 g were anaesthetised with halothane, implanted with bipolar ventral tegmental electrodes and trained to operate a pedal for stimulation available on a VI-10 s schedule (permitting reinforcement at minimum intervals averaging 10 s). Drugs were injected SC 30 min before testing, and data from test sessions were submitted to analysis of variance. Neuroleptics were tested in doses that had approximately equivalent effects after 30 min. Risperidone (0.2-0.9 mg/kg) produced a dose-dependent depression of responding [$F(1,42)=9.40$, $P<0.01$] followed by recovery to baseline levels after 200 min ($P<0.01$) at which time receptor occupancy would still be virtually undiminished (Sumiyoshi *et al.* 1994). Co-administration of the 5-$HT_{2A/2C}$ agonist, (±)-2,5-dimethoxy-4-iodoamphetamine (DOI, 0.8 mg/kg) with risperidone (0.9 mg/kg) failed to delay recovery from depression. However, recovery from risperidone was significantly weaker [$F(1,18)=9.42$, $P<0.01$] when an α_2-adrenoceptor agonist (clonidine 0.015 mg/kg) was substituted for DOI. Clonidine (0.015 mg/kg) also inhibited recovery from depression seen with another atypical neuroleptic, clozapine (6.0 mg/kg) [$F(1,24)=24.38$, $P<0.01$]. Finally, the prototypical neuroleptic, chlorpromazine (1.0 mg/kg), led to depressed responding without early recovery [$F(1,28)=1.01$, N.S.] unless accompanied by an α_2 antagonist, idazoxan (3.0 mg/kg). The combination of chlorpromazine and idazoxan was followed by recovery [$F(1,112)=20.70$, $P<0.01$] similar to that seen after atypical neuroleptics. These results support the proposed rôle of α_2-adrenoreceptor antagonism in determining the effects of atypical neuroleptics (Nutt 1994).

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252P EFFECT OF REPEATED TREATMENT WITH ANTIDEPRESSANT DRUGS ON THE BEHAVIOURAL RESPONSE TO D_1-LIKE AND D_2-LIKE DOPAMINE RECEPTOR AGONISTS IN THE RAT

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Increases in brain dopamine function may be important for the therapeutic effect of antidepressants (Willner, 1995). We recently reported that the enhancement of postsynaptic dopamine function by repeated electroconvulsive shock (ECS) requires the concomitant activation of D_1-like and D_2-like receptor subtypes (Smith & Sharp, 1995). Thus, ECS-treated rats showed an enhanced behavioural response to SKF 38393 (D_1-like agonist) and quinpirole (D_2-like agonist) administered in combination, but not when these drugs were given separately. Certain antidepressant drugs, notably tricyclic antidepressants (TCAs), may also enhance postsynaptic dopamine function but in a different way to ECS. Thus, Serra *et al.* (1990) reported that repeated TCAs potentiate the motor stimulant effects of quinpirole, but not SKF 38393. How the effects on dopamine function of other classes of antidepressant drugs compare with ECS and TCAs is not clear. Here we investigate the effects of a TCA (desipramine [DMI]), a selective serotonin reuptake inhibitor (fluoxetine [FLU]]) and a monoamine oxidase inhibitor (tranylcypromine [TCP]) on the behavioural response to selective D_1-like ((±)-SKF 38393, (±)-SKF 81297) and D_2-like ((-)-quinpirole, RU 24213) receptor agonists in the rat.

Groups of male Sprague-Dawley rats (200-250 g) were injected i.p. twice daily for 14 days with saline (1 ml/kg), FLU (2.5, 5 or 10 mg/kg), DMI (10 mg/kg) or TCP (2.5 mg/kg). Twenty four h after the final injection, rats were habituated to activity cages for 2 h and then injected s.c. with one of the following: quinpirole (0.25 mg/kg), RU 24213 (0.75 mg/kg), SKF 38393 (7.5 mg/kg), SKF 81297 (0.5 mg/kg) or SKF 38393 (7.5 mg/kg) plus quinpirole (0.25 mg/kg). The locomotor response was then monitored for a 60 min period using automated photocell activity meters. Data were analysed statistically using one way ANOVA and *post-hoc* Student's unpaired t-test.

A summary of results is presented in Table 1. In brief, repeated treatment with DMI or FLU (10 but not 5 or 2.5 mg/kg) significantly enhanced the locomotor stimulant effect of the D_2-like agonists, quinpirole and RU 24213, but not that of the D_1-like agonists, SKF 38393 and SKF 81297. In contrast, repeated treatment with TCP reduced the locomotor stimulant effects of SKF 38393 but had no significant effect on any other dopamine agonist. The locomotor response to a combined SKF 38393/ quinpirole challenge was not altered by any antidepressant treatment.

	Activity counts /60 min			
Agonist	Vehicle	Flu (10)	DMI (10)	TCP (2.5)
Quinpirole	815 ± 156	3066 ± 872 *	2149 ±506 *	1251 ± 266
RU 24213	551 ± 100	5275 ± 1603‡	2957 ± 784*	1319 ± 385
SKF 38393	739 ± 99	757 ± 102	639 ±73	234 ± 23 ‡
SKF 81297	1329 ± 369	1080 ± 503	1429 ± 313	1119 ± 112
SKF 38393+ Quinpirole	703 ± 178	1000 ± 326	954 ± 482	999 ± 302

Table 1. Effects of antidepressants (mg/kg) on activity counts (mean ± s.e.mean) induced by dopamine agonists. N=5-6 rats/group. * $P<0.05$, ‡ $P<0.01$ *vs.* corresponding vehicle group.

In summary, we find that repeated treatment of rats with DMI and FLU (the latter only in high doses) enhances the behavioural response to D_2-like but not D_1-like dopamine receptor agonists. In contrast, the behavioural response to these agonists was not altered consistently by repeated treatment with TCP. These results support the view that certain antidepressant drugs, as well as ECS, enhance dopamine function at the postsynaptic level. The effect of the drugs can be revealed using D_2-like receptor agonists alone, but ECS appears to require a combination of D_1-like and D_2-like receptor agonists (Smith & Sharp, 1995).

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253P THE ACTION OF M-CHLOROPHENYLBIGUANIDE AND ITS INTERACTIONS WITH 5-HT RECEPTOR ANTAGONISTS IN THE RAT SOCIAL INTERACTION TEST

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A 5-HT_3 receptor involvement in the behavioural responding of the rat to the social interaction test is indicated by the disinhibitory effects of the 5-HT_3 receptor antagonists (Costall *et al.*, 1990). Electrophysiological experiments have shown that m-chlorophenylbiguanide (MCPBG) is a relatively selective agonist at the 5-HT_3 receptor (Kilpatrick *et al.*, 1990). The present study investigates the ability of MCPBG to modify rat behaviour in the social interaction test and attempts to characterise the 5-HT receptor involvement.

Male Lister Hooded rats (250-300g) (University of Bradford bred) were housed in groups of 5 and kept in conditions of standard temperature (21 ± 1°C) and on a 12h light/dark cycle with lights off at 19h 00 min. Rats received a 40 min pretreatment with vehicle, ondansetron, ritanserin, methysergide or GR113808 followed by vehicle or MCPBG, with testing 40 min after the last treatment. The social interaction between two naïve rats under conditions of unfamiliarity and high illumination was measured by timing the sniffing of partner, crawling under or climbing over partner, genital investigation and following of partner measured over 10 min. (For detailed methodology see Costall *et al.*, 1990).

MCPBG caused a significant inhibition of rat social interaction by 74%: the inhibition was antagonised by the 5-HT_3 receptor antagonist ondansetron (0.1μg kg^{-1}) (which failed to significantly modify social interaction when administered alone) but not by the 5-HT_1/5-HT_2/5-HT_4 receptor antagonists methysergide, ritanserin and GR113808 (Gale *et al.*, 1994). [The changes in social interaction were not accompanied by any non-specific changes in locomotor activity (line crossings in the range 122 ± 13 to 133 ± 16)].

Table 1. The ability of MCPBG to inhibit rat social interaction and its interaction with 5-HT receptor antagonists

	Social interaction (s)
vehicle + vehicle	67.8 ± 8.9
vehicle + MCPBG (0.01 mg kg^{-1})	17.4 ± 3.5*
ondansetron (0.01 μg kg^{-1}) + vehicle)	62.0 ± 8.4
ondansetron (0.01 μg kg^{-1}) + MCPBG	15.5 ± 3.8*
ondansetron (0.1 μg kg^{-1}) + vehicle	81.1 ± 11.8
ondansetron (0.1 μg kg^{-1}) + MCPBG	63.2 ± 10.1^{+}
ritanserin (1.0 mg kg^{-1}) + vehicle	74.6 ± 9.5
ritanserin (1.0 mg kg^{-1}) + MCPBG	14.8 ± 3.3*
methysergide (1.0 mg kg^{-1}) + vehicle	60.7 ± 6.2
methysergide (1.0 mg kg^{-1}) + MCPBG	22.4 ± 3.5*
GR113808 (0.1 mg kg^{-1}) + vehicle	75.1 ± 10.3
GR113808 (0.1 mg kg^{-1}) + MCPBG	21.3 ± 4.6*

n = 6 pairs. *$P<0.01$ compared to vehicle + vehicle and $^{+}P<0.01$ compared to vehicle + MCPBG; one-way ANOVA followed by Dunnett's t-test.

It is concluded that even under conditions of unfamiliarity and high illumination MCPBG can inhibit rat social interaction. The ability of ondansetron to attenuate the anxiogenic profile of MCPBG in the rat social interaction test is supportive of a 5-HT_3 receptor involvement in the actions of MCPBG and in behavioural responding to the aversive situation.

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254P EFFECT OF PAROXETINE AND MIANSERIN ON AMPHETAMINE-INDUCED HYPERLOCOMOTION

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Many antidepressants, including mianserin and paroxetine, have been reported to enhance amphetamine-induced hyperlocomotion following chronic administration in rodents (Maj et al., 1984; Arnt et al., 1984). We have investigated whether a pharmacokinetic interaction can explain this interaction by correlating locomotor response with the level of amphetamine in rat brain following amphetamine alone and in combination with mianserin or paroxetine.

Male Sprague Dawley rats (approx 300g) were group housed in a 12 hour light cycle with free access to food and water. Testing (9-12/group) consisted of placing the animals, 30 min post-d-amphetamine (5.4-54.3 μmol/kg i.p.) or $vehicle_1$ (0.9% saline), individually into black perspex automated locomotor activity monitoring cages (57Lx17Wx25D cm) under red light. After an acclimatization period of 10 min, the number of times the animals travelled the length of the box (transits) during a further 10 min were recorded. In the interaction experiments, animals were dosed with paroxetine (27 μmol/kg p.o.), mianserin (33 μmol/kg p.o.) or $vehicle_2$ (1% methyl cellulose solution) 90 min prior to the administration of either d-amphetamine (10.8 μmol/kg i.p.) or $vehicle_1$. Animals were tested as described above. Immediately after testing, brains were removed from animals (5-6/group) that received d-amphetamine. The brain samples were homogenised in 0.4 M perchloric acid, and d-amphetamine levels were determined by HPLC. Results (expressed as mean±s.e.m.) were analysed by 1-way ANOVA followed by Dunnett's test or Newman Keuls test or by Student's t-test, as appropriate.

d-Amphetamine produced a bell-shaped dose dependent increase in locomotor acitivity with doses of 5.4, 10.8 and 21.7 μmol/kg producing significant ($p<0.01$) effects (16.9±2.8, 15.7±1.4 and 17.9±3.0 transits, respectively, vs 4.9±1.0 for saline). There was a linear correlation ($r = 0.9975$) between the dose and brain level of d-amphetamine detected. Mianserin and paroxetine had no effect on locomotion alone, but significantly ($p<0.05$) increased locomotion in animals administered d-amphetamine (10.8 μmol/kg; 1.5 mg/kg) from 12.4±1.4 to 21.0±2.0 and 29.9±4.0 transits, respectively. The brain level of d-amphetamine in control animals was consistent across experiments. Mianserin had no effect on the brain level of d-amphetamine (17.9±1.8 vs 15.6±2.5 nmol/g tissue in $vehicle_2$ group), but paroxetine produced a significant ($p<0.01$), 3-fold increase (44.1±1.1 nmol/g tissue).

The augmentation of d-amphetamine-induced hyperlocomotion by single administrations of mianserin or paroxetine is in contrast to previous reports in which chronic administration was required (Maj et al., 1984; Arnt et al., 1984). Mianserin produced a significant augmentation without an alteration in the brain level of d-amphetamine. Although a pharmacokinetic interaction was observed in the case of paroxetine, the degree of locomotor activity augmentation achieved is above that predicted by the resultant brain level of d-amphetamine.

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255P FLUOXETINE IN THE PRESENCE OF RITANSERIN ENHANCES THE POTENCY OF DIAZEPAM TO DISINHIBIT BEHAVIOUR IN THE MOUSE LIGHT DARK TEST

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The ability of 5-HT_4 receptor antagonists to attenuate and 5-HT_2 receptor antagonists to enhance the disinhibitory effects of diazepam in the mouse light/dark test indicates that the effects of diazepam may involve a raised endogenous 5-HT function (Costall & Naylor, 1994). If this hypothesis is correct then fluoxetine, by enhancing the synaptic activity of 5-HT by inhibition of 5-HT reuptake (Stanford, 1996), may facilitate the actions of diazepam. This is investigated in the present study using the mouse light/dark test.

Male albino BKW mice (Bradford strain) weighing 25-30g received an intraperitoneal injection of vehicle or fluoxetine (10 mg kg^{-1}) or ritanserin (1.0 mg kg^{-1}) alone, vehicle + diazepam, ritanserin + diazepam, fluoxetine + diazepam or ritanserin + fluoxetine + diazepam, with 40 min between treatments and with behavioural testing 40 min after the last treatment. Mice were placed into the centre of the light compartment of the test box and the latency of first movement from the light to the dark compartment and line crossings were video recorded over a 5 min period (for detailed methodology see Costall *et al.*, 1989).

In the absence of non-specific changes in line crossings, the latency values for vehicle treated control animals or those receiving ritanserin or fluoxetine alone or ritanserin + fluoxetine were in the range 10.1 ± 1.3 to 11.4 ± 1.6s. The ED_{50} of diazepam to increase the latency of first movement from the light to the dark compartment is shown in Table 1. Using diazepam in a dose range of 0.001-1.0 mg kg^{-1}, the maximum increase in latency induced in vehicle, fluoxetine, ritanserin or ritanserin + fluoxetine treated animals was indistinguishable between the 4 treatments and was in the range 25.5 ± 2.8 to 27.5 ± 2.7s.

Table 1 The effect of ritanserin/fluoxetine to modify the dose of diazepam required to increase the latency of first movement from the light to the dark compartment of the test box

Treatment	Diazepam ED_{50} mg kg^{-1}
Vehicle	0.27 ± 0.015
Fluoxetine	0.23 ± 0.016
Ritanserin	0.055 ± 0.003*
Ritanserin + Fluoxetine	0.0031 ± 0.0004*+

Animals were used in groups of 5 and the experiments repeated twice. *P<0.001 compared to vehicle control, +P<0.05, compared to 'ritanserin', one way ANOVA followed by Dunnett's t-test.

In the presence of ritanserin, fluoxetine enhanced the disinhibitory potency of diazepam 87-fold, greatly in excess of the 4-fold increase afforded by ritanserin itself. Fluoxetine alone failed to modify the disinhibitory potency of diazepam. This 'inactivity' of fluoxetine may reflect an increased 5-HT activity at both the inhibitory and disinhibitory behavioural mechanisms, producing a null response. The data also indicates that the effects of the fluoxetine interactions relate to pharmacological rather than a pharmacokinetic consequence of fluoxetine inhibiting the metabolism of diazepam in man (see Lemberger *et al.*, 1988) or rat (Costall *et al.*, unpublished data).

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256P THE EFFECT OF PAROXETINE, FLUOXETINE AND CLOMIPRAMINE ON 20 kHz ULTRASOUND DEFENSE BEHAVIOUR

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20-30kHz ultrasonic vocalisations are emitted by rats in response to aversive stimuli or predator threat (Blanchard *et al* 1993). Artificially generated 20kHz ultrasound produces defence-like behaviour in rats which is associated with activation of the brain aversive system (Aspley *et al* 1995). This behaviour can be manipulated pharmacologically (Beckett *et al* 1996) and the effect of acute treatment with selective serotonin reuptake inhibitors and a tricyclic antidepressant on this response is investigated in the present study.

Male hooded Lister rats (250-350g, n=6-8) were injected I.P with paroxetine (5 and 15mg/kg) in distilled water :5% tween 80, fluoxetine (5 and 10mg/kg) in saline and clomipramine (10 and 20mg/kg) in saline, 30 minutes before being placed in an open field arena containing a wall-mounted speaker. After 2 minutes the rats were exposed to 1 minute of 20kHz ultrasound (65, 72 and 75dB) followed by 2 minutes without sound. This was repeated for each sound intensity with an inter-procedure interval of 1 minute. Animal behaviour was analysed using a computer tracking system as distance travelled and speed (Beckett *et al* 1995).

Exposure to 20kHz ultrasound produced an intensity related defence response characterised by hyperlocomotion. Treatment with 5mg/kg paroxetine significantly decreased distance travelled whereas no significant effect was observed with 15mg/kg (Fig.1a). Both doses of fluoxetine significantly attenuated this response (Fig.1b) whereas neither dose of clomipramine had a significant effect though there was a trend to decrease the response (Fig. 1c). Administration, however, of all the drugs significantly reduced pre-stimulus (basal) locomotor activity.

Similar results were obtained for maximum and average speeds.

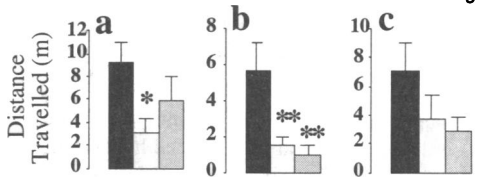

Figure 1: Effect of a) paroxetine (5 □ and 15mg/kg ■), b) fluoxetine (5 □ and 10mg/kg ■) and c) clomipramine (10 □ and 10mg/kg ■) on distance travelled during 1 minute of 20kHz ultrasound (75dB) (mean ± s.e mean) compared to control (n)* p<0.05 vs control ** p<0.01 vs control. One-way ANOVA with post-hoc Duncan's NMR.

The results further demonstrate the ability of 20kHz ultrasound to induce defence behaviour in the rat. While acute treatment with two SSRI's and a tricyclic antidepressant decreased the response, this may reflect a reduction in basal locomotor responsiveness rather than a specific effect on CNS mechanisms active in the control of aversive behaviours. These results indicate the need for careful interpretation of behaviour involving changes in locomotor activity.

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257P COMPARATIVE MODULATION OF HIPPOCAMPAL 5-HT RELEASE BY THE 5-HT$_{2C}$ RECEPTOR ANTAGONIST SB 206553 AND PAROXETINE

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The role of the 5-HT$_{2C}$ receptor in the treatment of depression is poorly understood. However, this receptor is implicated in the basic mechanism of action of several antidepressant drugs e.g. the selective serotonin re-uptake inhibitor paroxetine (Kennett, 1993). In addition; the 5-HT$_{2C}$ receptor is located in relatively high densities in brain loci associated with depression i.e. the hippocampal CA1 region. In the present study we have investigated the effects of local and systemic administration of the novel 5-HT$_{2C}$ receptor antagonist SB 206553 on 5-HT release from the hippocampus of the freely moving rat and compared the effects with those of the therapeutically effective anti-depressant paroxetine.

Male Sprague-Dawley rats (250-350g) were anaesthetised with medetomidine (Domitor: 0.4mg kg^{-1}, i.m.) and fentanyl (Sublimaze: 0.45mg kg^{-1}, i.p.) and a concentric-style microdialysis probe, manufactured in house with a 5mm x 0.5mm semi-permeable Hospal membrane for dialysis, was inserted into the hippocampus (Paxinos & Watson, 1982: AP -4.8mm, L -5.0mm, V -8.0mm, with reference to bregma). Anaesthesia was reversed with atipamezole (Antesedan: 1.0mg kg^{-1}, i.p.) and the analgesic nalbuphine (Nubain: 2.0mg kg^{-1}, i.p.). Following 24 hour recovery, microdialysis experiments were performed on unrestrained animals and the probe perfused with artificial cerebrospinal fluid (NaCl 125, KCl 3.0, $MgSO_4$ 0.75 and CaCl 1.2 mmol/l; pH 7.4) at a rate of 2µl min^{-1}. Samples were collected every 20 min for 5 h and assayed for 5-HT using high performance liquid chromatography with electrochemical detection (HPLC-ECD) (Hutson *et al.*, 1995) The perfusate levels of 5-HT are expressed as percent of the mean of absolute transmitter collected in the four pre-injection control samples. Data were analysed by repeated measures analysis of variance, the time x group interaction term was considered statistically significant when $p<0.05$.

5-HT efflux was not affected by acute systemic administration of paroxetine (10 mg kg^{-1}, i.p., n=3) or SB 206553 (10 mg kg^{-1}, p.o., n=5). However, administration of paroxetine (10µM, n=8) or SB 206553 (10µM, n=8) perfused locally via the probe for 100 min, significantly increased 5-HT release to a maximum of 394 ± 143% and 269 ± 75% of pre-infusion control levels respectively. Following a 30 min pre-treatment with the 5HT$_{1A}$ receptor antagonist WAY-100635, 1mg kg^{-1}, s.c., SB 206553 (10mg kg^{-1}, p.o.) elicited a significant increase in 5-HT efflux to 239 ± 39% of pre-infusion control levels.

A plausible explanation for the lack of effect on terminal 5-HT levels by systemically administered SB 206553 or paroxetine is that of negative feedback induced by 5-HT in the raphe via somatodendritic 5HT$_{1A}$ receptors. Subsequent blockade of 5HT$_{1A}$ receptors with WAY-100635 abolishes the negative feedback and SB 206553 enhances extracellular 5-HT, presumably via antagonism of 5-HT$_{2C}$ receptors at the level of the raphe.

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258P NITRIC OXIDE MODULATES 5-HT RELEASE IN RAT VENTRAL HIPPOCAMPUS

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Activation of N-methyl-D-aspartate (NMDA) receptors has previously been found to decrease 5-hydroxytryptamine (5-HT) release in rat hippocampus in vivo (Whitton et al., 1994). NMDA receptor stimulation leads to the generation of nitric oxide (NO) and the application of NO donors have been found to increase extracelluar 5-HT levels in the striatum of anaestheised rats. (Guevara-Guzman et al., 1994). Additionally, it has been shown that 5-HT, acting via 5-HT1A receptor, antagonizes NMDA induced increase in NO/cGMP synthesis in adult rat hippocampus suggesting, therefore an interaction between NO formation, serotonergic and glutamatergic systems (Strosznajder et al.,1996).

In the present study we have investigated whether NO has a regulatory role over 5-HT release in ventral hippocampus. In order to do this we have used in vivo microdialysis to investigate the effects of two nitric oxide synthase (NOS) inhibitors:L-nitro-arginine methyl esther (L-NAME) a competitive, non selective inhibitor and 7-nitroindazole monosodium salt (7-NI) which is claimed to be selective for the NOS isoform found in the brain on 5-HT release in the ventral hippocampus. We have also studied the effects of the NO donor S-nitroso-acetyl-penicillamine (SNAP) on 5-HT release in this structure.

Male Wistar rats (280-320g) were anaesthetised with chloral hydrate (400mg/kg) and implanted with concentric dialysis probes into the ventral hippocampus. The following day all rats were dialysed (0.5µl /min) with artificial cerebrospinal fluid (composition in mM; KCl 2.5; NaCl 125; $MgCl_2$ 1.18; $CaCl_2$ 1.26.) . After one hour equilibration period , four 30 mins samples were collected to establish basal release of 5-HT. L-NAME, 7-NI and SNAP were infused via the dialysis probe and the experiment continued for up to 7h. All dialysates were analyzed for 5-HT content using HPLC with electrochemical detection. Basal levels of 5-HT were found to be 94±12fmol/10µl (mean ±s.e mean, n=30).

Continuous infusion of 100µM L-NAME throughout the entire experiment (360 mins) did not alter extracelluar 5-HT levels, however 1mM L-NAME increased 5-HT release by up to 250±31% ($p<0.05$) and this was maintained for 240 mins after commencing L-NAME infusion, eventually returning to basal levels, in spite of continuing infusion with the drug . When 7-NI was infused at two different concentrations (10 and 100µM) for the duration of the experiment 5-HT release decreased by up 60±20% ($p<0.05$) showing no significant difference between the two concentrations. Since L-NAME increased and 7-NI decreased extracelluar levels of 5-HT we investigated the effects of the NO donor SNAP on extracelluar 5-HT. Infusion of SNAP at 1mM for 30 mins did not significantly alter dialysate 5-HT, whereas at 500µM the NO donor increased release of 5-HT by 150±10% ($p<0.05$). However, at 5mM SNAP decreased the levels of 5-HT by up to 70 ±5% ($p<0.05$) below basal levels and this was maintained for 360mins.

These data suggest that NO plays a regulatory role over 5-HT release in the ventral hippocampus of freely moving rats. However, there appear to be discrepancies in the effects observed with different NOS inhibitors, implying possible differences in the role of different NOS isoforms in the regulation of 5-HT release in this structure. The data with SNAP suggest that NO exerts a biphasic effect on 5-HT release, previously observed with other neurotransmitters, for example glutamate (Segieth et al., 1995). Therefore these data are consistent with glutamatergic control over 5-HT release in hippocampus mediated via NO formation.

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259P ANTI-DEPRESSANT TREATMENT FAILS TO MODIFY SOCIAL COMPETITION IN TRIADS OF RATS

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Social hierarchies in groups of rats based on competition for palatable substances can be disrupted by administration of both anxiolytic and anxiogenic agents suggesting that social competition could be a useful model of anxiety (Gentsch et al. 1988, Joly and Sanger 1991, 1992). However it has also been shown that hierarchies measured in terms of agonistic interactions can be disrupted by administration of clinically effective antidepressants (Mitchell and Redfern 1992). The aim of the present study was to investigate whether the behaviour expressed in the social competition paradigm could be manipulated by administration of antidepressant agents.

Baseline levels of competition were established for a 5 week period during 5 min access to sweetened milk in triads of male Lister Hooded rats weighing 256±5g at the start of the study. For each group the social hierarchy was established by observing which animal had access to the spout at 5s intervals throughout the testing period (e.g. 60 observations for one 5 min trial). All groups were tested twice a week. The effect of amitriptyline (5.0, 10.0 and 20.0 mg/kg i.p.) on the access to sweetened milk was assessed 30 min after treatment of either the subordinate or dominant rat (n=7). The effect of fluoxetine (5.0 and 10.0 mg/kg i.p., once daily for 21 days) on the access to sweetened milk by the subordinate rat was assessed 30 min after dosing on day 21 of treatment (n=5-9). In both studies chlordiazepoxide (cdp) (2.5 mg/kg i.p.) was incorporated into the experimental design as the positive control. Data were analysed using one factor ANOVA followed by Dunnett's t-test and are presented as mean ± s.e.m.

During the initial 5 week study the triads of rats developed stable hierarchies consisting of dominant, intermediate and subordinate animals which had access to the drinking spout for 41.5±1.9%, 28.5±2.5% and 21.0±1.4% of the 5 min testing period respectively (n=35). Amitriptyline did not significantly affect social competition at any of the doses tested following administration to the dominant rat in each group, 39.9±2.0%, 42.0±1.3%, 38.8±1.6% and 41.1±2.3% respectively, or the subordinate rat in each group, 24.8±1.8%, 26.2±2.2%, 26.7±1.4% and 29.1±3.9% respectively. However cdp (2.5 mg/kg i.p.) caused a significant increase in social competition following administration to the subordinate rat [$F(4,24) = 2.8$, $p<0.05$], 24.8±1.8% increased to 34.8±2.9% (n=7). Chronic treatment with fluoxetine to the subordinate rat did not affect social competition, 17.9±2.2%, 13.7±5.8% and 7.7±4.9% respectively. However administration of cdp significantly increased social competition of the subordinate animal [$F(3,24) = 9.1$, $p<0.001$], 17.9±2.2% to 35.7±3.2% (n=5-9).

These data show that stable hierarchies of rats measured in terms of access to sweetened milk are not disrupted by acute amitriptyline or chronic fluoxetine treatment. These compounds have been shown to be effective in other animal models of depression (Muscat et al. 1992, Porsolt et al. 1978) which suggests that social competition in the rat may not be a suitable model for detecting the action of antidepressant agents.

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260P EFFECT OF DESTRUCTION OF THE ASCENDING 5-HYDROXYTRYPTAMINERGIC PATHWAYS ON SWITCHING BETWEEN ALTERNATIVE RESPONSES IN AN OPERANT SCHEDULE

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The ascending 5-hydroxytryptaminergic (5HTergic) pathways are believed to play a role in the inhibitory regulation of operant behaviour controlled by positive reinforcement (see Ho *et al*, 1995). Destruction of the pathways alters performance in operant behaviour paradigms which entail temporal regulation of behaviour (Ho *et al.*, 1995). Al-Zahrani *et al.* (1996) proposed that this effect may be mediated by an increase in the rate of switching between behavioural states. We report here the effect of lesions of the 5HTergic pathways on performance in a timing schedule in which behavioural switching can be explicitly measured (Gibbon & Church, 1981).

Under halothane anaesthesia, 14 female Wistar rats (250-300 g) received injections of 5,7-dihydroxytryptamine (4 μg base in 2 μl vehicle) into the dorsal and median raphe nuclei; 15 rats received sham lesions (method: Ho *et al*, 1995). They were then trained in operant conditioning chambers to press levers for food-pellet reinforcers (45 mg). Daily training sessions consisted of 24 60-s trials in which reinforcers were available for responding either on lever A (1 pellet) or lever B (2 pellets). At a random time-point, t, following the onset of each trial, a response on either lever resulted in withdrawal of both levers and delivery of a reinforcer after a delay (d_A s following a response on lever A, 60-t s following a response on lever B). The experiment consisted of 4 phases (60-80 sessions) in which the value of d_A was 1, 2, 4 and 8 s (in random sequence). Then the rats were killed and their brains dissected for assay of 5HT, 5-hydroxyindoleacetic acid (5HIAA), noradrenaline and dopamine by high-performance liquid chromatography with electrochemical detection (method: Ho *et al.*, 1995).

In each phase, both groups showed increasing relative response rate (response rate on lever B expressed as percentage of overall response rate) as a function of time from trial onset. The time at which relative response rate attained a value of 75% (T_{75}, s) was inversely related to the value of d_A, and did not differ significantly between the two groups. The maximum rate of switching (S_{max}, switches min^{-1}) was significantly higher in the lesioned group than in the control group (Table 1). The levels of 5HT and 5HIAA in the parietal cortex, hippocampus, amygdala and nucleus accumbens of the lesioned group were less than 12% of those of the control group (t-test, $P<0.01$ in each case), but the levels of noradrenaline and dopamine did not differ significantly between the groups ($P>0.1$ in each case).

Table 1: Indices of timing and switching behaviour, mean ± s.e.mean

Index[a]	Group	Value of d_A (s) 1	2	4	8
T_{75}	Control	43.8 ±2.6	37.1 ±2.6	37.2 ±2.1	23.9 ±2.5
	Lesioned	44.4 ±1.9	39.0 ±2.4	38.6 ±1.8	26.7 ±2.0
S_{max}	Control	13.0 ±2.0	10.8 ±1.9	10.6 ±1.6	9.9 ±1.3
	Lesioned*	20.3 ±2.3	18.1 ±2.0	18.2 ±1.8	15.9 ±2.2

[a] see text for units; * difference from control: $P<0.001$ (ANOVA)

The finding that switching rate was increased following destruction of the 5HTergic pathways is consistent with the suggestion that these pathways may contribute to the inhibitory regulation of switching between behavioural states (see Al-Zahrani *et al.*, 1996).

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261P 5-HT_2-LIKE RECEPTOR-MEDIATED DEPOLARIZATION OF 5-HT-CONTAINING DORSAL RAPHE NEURONES *IN VITRO*

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Somatodendritic 5-HT_{1A} receptors mediate a hyperpolarization of 5-hydroxytryptamine (5-HT) containing neurones of the dorsal raphe nucleus (DRN). We have shown that, in the guinea pig DRN *in vitro* (Craven *et al.*, 1994), the inhibitory effects of exogenously applied 5-HT (30 μM) and the 5-HT_{1A} receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT, 30 nM) are abolished by the 5-HT_{1A} receptor antagonist WAY-100635 (30 nM) (Fletcher *et al.*, 1996). We now report that, in the presence of WAY-100635 (30 - 100 nM), relatively high concentrations of exogenously applied 5-HT (30 - 300 μM) can depolarize 5-HT neurones.

Transverse midbrain slices (350 μm) were prepared from anaesthetized male Dunkin Hartley guinea pigs (200 - 400 g, 100 - 250 mg kg^{-1} pentobarbitone), submerged in a recording chamber and perfused with an artificial cerebrospinal fluid at 30 °C (see Craven *et al.*, 1994) containing 30 - 100 nM WAY-100635. Intracellular recordings were made from presumed 5-HT containing DRN neurones (see Craven *et al.*, 1996) using 2 M KCl filled microelectrodes (40 - 120 MΩ). 5-HT and 8-OH-DPAT were applied via the perfusate for 1 - 2 min. In experiments with antagonists, 100 μM 5-HT was applied for 1 min and reapplied 40 - 50 min later following a 30 - 40 min incubation with the antagonist. All drugs were initially made up in water, prazosin being dissolved with the aid of acetic acid. Cumulated results are expressed as the median value (range, n) and samples compared by the Mann-Whitney test.

In the presence of WAY-100635, 5-HT (30 - 300 μM) gave a concentration-dependent depolarizing, often excitatory response of 5-HT neurones (n=12) which was not mimicked by 8-OH-DPAT (1 μM, n=3). The depolarization was often accompanied by an increase in the apparent input resistance of the neurone and could be recorded in the presence of the Na^+ channel blocker tetrodotoxin (1 μM, n=9). The response to a 1 min application of 100 μM 5-HT tended to run down with time, being reduced by 27% (0 - 46, n=5) after 40 - 50 min. Treatment with the α_1-adrenoceptor antagonist prazosin (500 nM), which blocked the depolarization of 5-HT neurones by phenylephrine (10 μM, n=3), did not significantly affect the response to 5-HT over this period [reduced by 16% (0 - 33, n=4)]; however, the 5-HT_2 receptor antagonists ketanserin (100 nM), mesulergine (100 nM) and lysergic acid diethylamide (LSD, 1 μM) significantly reduced the depolarizing response ($P<0.05$) [reduced by 100% (73 - 100, n=4), 67% (58 - 100, n=4) and 100% (100 - 100, n=3), respectively].

These results indicate that exogenously applied 5-HT can depolarize 5-HT containing DRN neurones by a Na^+ action potential-independent mechanism that is likely to involve the activation of a 5-HT_2-like receptor and the closure of K^+ channels. Thus, 5-HT can have opposing effects on 5-HT neurones by the activation of 5-HT_{1A} and 5-HT_2-like receptors, as seen in other neurones including those of the nucleus prepositus hypoglossi *in vitro* (Bobker, 1994).

R.M.C. is a Wellcome Prize Student.

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262P INTERACTION OF GR113808 WITH ANXIOLYTIC AND PUTATIVE ANXIOLYTIC AGENTS IN THE MOUSE LIGHT DARK TEST

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GR113808 and other 5-HT_4 receptor antagonists attenuate the disinhibitory effects of diazepam in the mouse light dark test (Costall & Naylor, 1995). The present study investigates the interaction of GR113808 with other anxiolytic and putative anxiolytic agents in the mouse test.

Male albino BKW mice (Bradford strain) weighing 25-30g received an intraperitoneal injection of anxiolytic/putative anxiolytic drug treatment (doses were selected as the minimum dose causing a maximal behavioural change) plus vehicle (veh.) or GR113808 (GR, 10μg kg^{-1}), with 40 min between treatments and with behavioural testing 40 min after the last treatment. Mice were placed into the centre of the light compartment of the test box (orientated towards the door to the dark chamber) and the latency of first movement from the light (L) to the dark (D) compartment and line crossings was video recorded over a 5 min period (for detailed methodology see Cheng *et al.*, 1994).

Chlordiazepoxide and the other agents increased the latency of first movement from the light to the dark compartment. The administration of GR113808 (1.0-100μg kg^{-1}) alone failed to modify behaviour but co-administration with the anxiolytic/putative anxiolytics antagonised the disinhbitory profiles of all agents tested. Such changes were not accompanied by non-specific changes in locomotor activity (line crossings). It is concluded that the 5-HT_4 receptor may play a role in drug induced disinhibitory behaviour in the mouse light dark test.

Table 1 Effect of chlordiazepoxide and other agents and their interaction with GR113808 in the mouse light dark test

Treatment	Latency L>D (s)
Chlordiazepoxide (2.5mg kg^{-1}) + veh.	23.2 ± 6*
Chlordiazepoxide (2.5mg kg^{-1}) + GR	10.2 ± 1.0$^+$
Buspirone (1.0mg kg^{-1}) + veh.	16.8 ± 1.3*
Buspirone (1.0mg kg^{-1}) + GR	9.0 ± 1.2$^+$
Ondansetron (0.01mg kg^{-1} + veh.	23.6 ± 1.9*
Ondansetron (0.01mg kg^{-1} + GR)	11.5 ± 1.1$^+$
R(+)Zacopride (0.001mg kg^{-1}) + veh.	22.6 ± 1.9*
R(+)Zacopride (0.001mg kg^{-1}) + GR	9.1 ± 1.4$^+$
Sulpiride (0.1mg kg^{-1}) + veh.	19.8 ± 2.6*
Sulpiride (0.1mg kg^{-1}) + GR	12.2 ± 1.2$^+$
Devazepide (0.1mg kg^{-1}) + veh.	23.7 ± 2.0*
Devazepide (0.1mg kg^{-1}) + GR	10.5 ± 1.1$^+$
Losarten (0.01mg kg^{-1}) + veh.	23.4 ± 2.0*
Losarten (0.01mg kg^{-1}) + GR	10.7 ± 0.7$^+$

The veh. (saline) + veh. (saline) and the veh. + GR113808 (10μg kg^{-1}) control responses ranged from 9.2 ± 1.0 to 12.3 ± 1.4 and 10.3 ± 1.4 to 11.8 ± 1.7 (s) respectively. Values (mean ± s.e. mean; n = 10) *$P<0.01$ compared to veh. + veh. controls; $^+P<0.01$ compared to appropriate anxiolytic/ putative anxiolytic + veh. control (one way ANOVA followed by Dunnett's t-test; assessed on veh. + veh. and anxiolytic/putative anxiolytic + veh./GR113808 data).

Cheng, C.H.K., Costall, B., Kelly, M.E. *et al.* (1994) *Eur. J. Pharmacol.* 255, 39-49.
Costall, B. & Naylor, R.J. (1996) *Br. J. Pharmacol.*, in press

263P EFFECT OF PRENATAL EXPOSURE TO MDMA ('ECSTASY') ON CEREBRAL 5-HT CONTENT OF NEONATE RATS

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The extensive recreational use of MDMA ('ecstasy') by young people means that it is being ingested by many women of child bearing age. Since there is considerable evidence that the drug can produce neurotoxic degeneration of 5-HT neurones in the brain (see Green *et al.*, 1995), this in turn gives rise to concern that the foetus may be at risk during development. There appears to have only been one study on the biochemical and behavioral effects of prenatal exposure to MDMA (St. Omer *et al.*,1991), and the doses of MDMA used were low. We have now therefore examined the effect of high doses of MDMA to the dam on the cerebral monoamine content of the pups.

Pregnant female Wistar rats were injected with MDMA (20mg kg^{-1} i.p.) twice daily at 09h 00min and 17h 00min on days 14-17 of the gestation period. Rectal temperature was measured for 6 h following the first of each daily injection. The hyperthermic response to MDMA was progressively attenuated so that the 7th dose produced almost no temperature change. Eleven days after cessation of treatment (7 days after parturition) brain 5-HT and 5-HIAA content was measured in the brains of both the dams and the neonates using h.p.l.c. with electrochemical detection (Colado & Green 1995). The concentration of indoles was decreased by over 50% in the hippocampus (Figure 1a) and cortex of the dam while the indole content of the hippocampus + cortex of the neonates was unchanged (Figure 1b).

These data confirm that exposure to MDMA produces neurotoxic damage in the brains of the adult rat but indicate that prenatal exposure does not appear to induce damage to the neonate, consistent with the observation that the drug given to neonates also fails to produce damage (Broening *et* al., 1994).

Broening, H.W., Bacon, L. & Slikker, W. (1994) J Pharmacol Exp Ther **271**, 285-293.
Colado, M.I. & Green, A.R. (1995) Eur J Pharmacol **280**, 343-346.
Green, A.R., Cross, A.J. & Goodwin, G.M. (1995) Psychopharmacology **119**, 247-260.
St.Omer, V.E. *et al.*, (1991) Neurotoxicol Teratol **13**, 13-20.

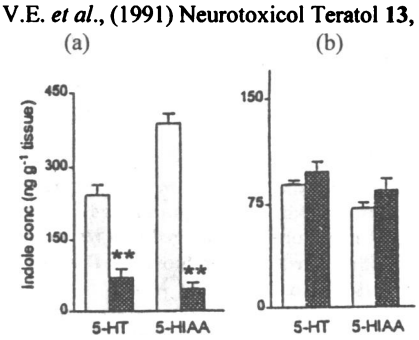

Figure 1: The 5-HT and 5-HIAA conc. (mean ± s.e. mean, n = 5-15) in (a) the hippocampus of adult females injected with saline (open bars) or MDMA (closed bars, 20mg kg^{-1}x8) and (b) hippocampus + cortex of their female pups 7 days after parturition. ** Different from saline injected $p<0.01$ (ANOVA + Newman-Keuls test).

M.I.C. thanks CICYT (SAF 1560/95), CAM (AE 00206/95) and Astra Spain for financial support.

264P EVIDENCE THAT MDMA ('ECSTASY'), BUT NOT FENFLURAMINE, INCREASES FREE RADICAL FORMATION IN RAT BRAIN

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MDAM ('ecstasy' and fenfluramine (Fen) induce degeneration of 5-HT pathways in brain of rodents (see Green *et* al., 1995). The free radical scavenger PBN, prevents degeneration induced by MDMA (Colado & Green, 1995) but not Fen (Murray *et al.*, 1997), suggesting a role for free radicals in damage produced by MDMA but not Fen. We have now examined whether increased free radical formation can be detected in rat brain in vivo following injection of these 2 compounds.

Male Dark Agouti rats (170-200g) were implanted with microdialysis probes in the hippocampus. The next day probes were perfused at 1µl min^{-1} with artificial c.s.f. containing salicylic acid (0.5mM) and samples collected for analysis of 2,3- and 2,5-Dihydroxybenzoic acid (2,3- and 2,5-DHBA. After collection of 3 x 30 min baseline samples, MDMA (15 mg kg^{1}) or Fen (15 mg kg^{-1} i.p.) was injected. The concentration of 2,3-DHBA in the dialysate rose rapidly following MDMA and was increased for over 6.5 h following injection (Fig). In contrast, the 2,3-DHBA concentration did not change following Fen (Fig). Neither MDMA nor Fen modified the 2,5-DHBA concentration (Data not shown). MDMA also produced an acute hyperthermic response (+2.5°C) following administration, while Fen did not modify rectal temperature. One week after administration both MDMA and Fen had depleted hippocampal 5-HT (measured by h.p.l.c. with electrochemical detection) by 49% and 63% respectively.

The data indicate that MDMA increases free radical formation in the brain, (see Chiueh *et al.*, 1992) a plausible explanation for the neurotoxic degeneration this drug produces. Fen did not increase free radical formation suggesting a different neurotoxic mechanism and weakening the proposal linking the clinical safety of Fen to the putative safety of ecstasy (Sanders, 1996).

Colado, M.I. & Green, A.R.(1995) Eur J Pharmacol **280**, 343-346.
Chiueh, C.C., *et al.*, (1992) Free Rad Biol Med **13**, 581-538.
Green, A.R., Cross, A.J. & Goodwin, G.M.(1995) Psychopharmacology **119**, 247-260.
Murray, J.K., *et al.*, (1997) Neuropharmacology (In Press).
Saunders, N. (1996) Br Med J **313**, 423.

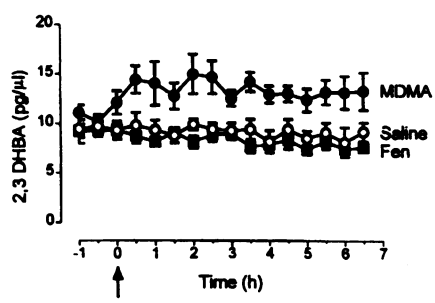

Fig. Effect of MDMA and Fen given at arrow on 2,3-DHBA concn in dialysate (mean ± s.e. mean in pg μl^{-1} n = 6-10). There was a significant effect of MDMA (ANOVA $F(1,18) = 15.78$ $p<0.05$).

M.I.C. thanks CICY(SAF 1560/95), CAM (AE 00206/95) and Astra Spain for financial support.

265P THE EFFECTS OF THE BENZODIAZEPINE INVERSE AGONIST ETHYL-BETA-CARBOLINE-3-CARBOXYLATE (BCCE) ON FOOD AND WATER INTAKE IN PIGS

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Benzodiazepine (BDZ) inverse agonists act at BDZ receptors to produce effects that are opposite to those of the classical BDZs, and are thus anxiogenic and proconvulsant (Vellucci et al., 1986). These drugs also inhibit food intake (Cooper et al., 1989), however, it remains to be determined whether the inhibition of feeding is behaviourally specific or secondary to their anxiogenic properties. In the present study, we investigated the effects of low doses of the BDZ inverse agonist βCCE on food and water intake in pigs. We argued that if the drug was behaviourally specific for food then it should inhibit food but not water intake.

Prepubertal Large White boars (b.wt. 28-35 kg) were chronically prepared under halothane anaesthesia with jugular catheters, as described previously (Ebenezer and Parrott, 1993). The pigs lived in metabolism cages and were trained to perform operant responses to obtain food and water.

Experiment 1. The pigs (n=6) were maintained on the following feeding schedule: at 10.00h a buzzer signalled that the feeders were activated for 1h; the pigs were also given an additional meal of 400g of their pelleted food at 17.00h. Water was available *ad libitum*. Twenty min prior to the start of their morning feeding period, the pigs were injected intravenously (iv) with either saline (vehicle) or βCCE (15, 30 or 60 μg kg^{-1}), within a 2 ml volume. The number of food reinforcements obtained was monitored on a computer-based data logging system. A repeated measures design was used with the pigs receiving all treatments in a random order.

Experiment 2. The pigs (n=6) were maintained on a 20h water-deprivation schedule. At 12.00h a buzzer signalled that the water delivery system was activated for the next 4h. Food was available from 10.00h until 15.00h. Twenty min prior to the onset of the drinking period, the pigs were injected iv with either saline (vehicle) or βCCE (15 or 60 μg kg^{-1}). The number of reinforcements obtained by the animals was monitored for 30 min. Analysis of the data obtained in Experiment 1 showed significant effects of drug treatment on 60 min food intake ($F_{(3,15)}$=14.95, P<0.001. *Post-hoc* tests revealed that βCCE (30 and 60 μg kg^{-1}) significantly decreased the number of food reinforcements obtained from a control mean value ± s.e.mean of 96.7 ± 10.2 to 72.8 ± 13.6 (P<0.01) and 61.3 ± 10.7 (P<0.001) respectively. The 15 μg kg^{-1} dose was without effect (number of food reinforcements = 82.3 ± 15.1). By contrast, water intake was not affected by either the 15 or 60 μg kg^{-1} doses of βCCE ($F_{(2,10)}$=1.154, ns). None of the doses used in this study produced overt abnormal behaviour during the course of the experiment.

The results of this study show that iv administration of low sub-convulsant doses of the BDZ inverse agonist βCCE decreases food intake in pigs in a dose-related manner, but has no effect on water intake. These results thus provide support for the notion that the inhibitory effect of the BDZ inverse agonists on food intake is behaviourally specific.

Cooper, S.J., Bowyer, D.M. and van der Hoek, G. (1989) Brain Res. 494, 172-174.
Ebenezer, I.S. and Parrott, R.F. (1993) NeuroReport 4, 495-498.
Vellucci, S.V., Herbert, J. and Keverne, E.B. (1986) Psychopharmacol. 90, 367-372.

266P EFFECT OF THE BENZODIAZEPINE-RECEPTOR AGONIST DIAZEPAM ON A MORPHINE DISCRIMINATIVE STIMULUS

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Addicts frequently abuse heroin in combination with a benzodiazepine (BZ)-receptor agonist. BZ/opioid interactions have been reported previously (eg Millan & Duka, 1981) but not in animal tests relevant to drug abuse; we therefore investigated the effect of diazepam in a morphine drug discrimination.

Rats (male; Lister) were trained to discriminate morphine (2 mg/kg, s.c.) from saline using a food reinforced operant technique (e.g., Stolerman, 1989). Once subjects were trained, generalisation tests were carried out to construct morphine dose-reponse curves in the presence of different doses of diazepam (i.p.). Morphine produced dose-related increases in drug lever responding ($F(4,16) = 121.2$, $p < 0.001$) whereas diazepam alone did not significantly affect this measure ($F(4,16) = 1.42$, n.s.). However, the larger doses of diazepam (3 & 6 mg/kg) tended to shift the dose-response curve for morphine to the right and 2-Way ANOVA revealed a significant interaction ($F(16,64) = 2.89$, $p < 0.01$). This appeared to be mainly due to a small enhancement of the effect of 0.5 mg/kg morphine and a reduction in the effect of 1.5 mg/kg morphine, by diazepam 3 mg/kg (Table 1). The pattern of results for rates of responding was different: morphine had no effect ($F(4,16) = 0.9$, n.s.) but there was a significant overall effect of diazepam ($F(4,16) = 6.12$, $p < 0.01$) with no interaction ($F(16,64) = 1.51$, n.s.). Alone, the largest dose of diazepam (6 mg/kg) reduced lever pressing (Table 1).

In conclusion, there was a dissociation between the effect of diazepam on a morphine-induced discriminative stimulus and rates of responding. Large doses of diazepam tended to block or disrupt stimulus control by morphine, but this effect was small. The qualitative or quantitative nature of the diazepam-induced change in opioid stimulus control remains to be determined.

Millan, M.J. & Duka, T. (1981) Mod. Prob. Pharmacopsy., 17, 123
Stolerman, I.P. (1989) Psychopharmacology, 97,131

Table 1 Effect of diazepam in combination with morphine in rats trained to discriminate morphine from saline

	Percent Drug Lever Responding (mean ± sem)					Responses (mean ± sem)				
morphine	diazepam (mg/kg)					diazepam (mg/kg)				
(mg/kg)	0	0.3	1	3	6	0	0.3	1	3	6
0	2(±2)	2(±2)	5(±2)	13(±6)	19(±10)	164(±29)	203(±53)	203(±59)	140(±69)	59(±32)*
0.5	8(±5)	19(±9)	14(±5)	13(±5)*	17(±5)	172(±50)	189(±52)	197(±49)	123(±32)	68(±29)
1	56(±6)	62(±8)	49(±7)	55(±15)	33(±16)	204(±50)	123(±30)	211(±49)	144(±42)	44(±30)
1.5	81(±5)	84(±6)	78(±6)	54(±7)**	67(±11)	182(±29)	185(±46)	185(±63)	160(±48)	77(±69)
2	82(±8)	88(±4)	84(±8)	81(±7)	68(±10)	131(±41)	129(±33)	140(±32)	102(±26)	111(±35)

2-tailed paired t test: * $p < 0.05$ ** $p < 0.01$ vs. diazepam vehicle plus corresponding morphine dose. n=5.

267P EFFECT OF ENVIRONMENTAL ENRICHMENT ON THE RESPONSE TO DIAZEPAM IN RATS

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In our laboratory, housing of rats in an 'enriched' environment from weaning has been found to reduce anxiety, as measured by the social interaction test, in female, but not male, rats (Larkin & Brett, unpublished results). The present experiment was undertaken to investigate whether environmental enrichment would alter the formation of heirarchies, and the effects of an anxiolytic drug on those heirarchies, in the rat social competition test which has been proposed as an animal model of anxiety (Joly & Sanger, 1991).

Male and female weanling rats (55-75g) were housed in single-sex groups (n=6-8) of three. Control animals were housed in grid-bottomed cages and remained unhandled for four weeks. The environmentally enriched groups were housed in somewhat larger cages and were provided with 'toys', which were changed twice weekly. After four weeks rats undergoing both treatments were offered sweetened condensed milk for 15 min after a period of water deprivation on 3-5 days, in order to familiarise them with the milk. Competition was assessed 3 times per week for 4 weeks, in order to determine the establishment of heirarchies, by observing which animal(s) had access to the drinking spout every 5s during a period of 5 min access to sweetened milk. The effect of diazepam (DZP) 2.5 mg/kg i.p. on the access of the subordinate rat to the drinking spout 30 min after treatment was tested twice, with 1-2 undrugged social competition tests in the interval. Data (score of 1 for each observed access to the spout) were converted to % drinking for each rat in the group. % drinking for each subordinate rat after administration of drug was compared with a baseline value which was the mean of values for days preceding the drug injection. Data were analysed using Wilcoxon's signed rank test.

Heirarchies appeared to be established by both male and female rats in both housing conditions. In males housed in the control environment, DZP significantly increased the % drinking of the subordinate animal (baseline (mean ± s.e.mean): 29.5 ± 1.2; DZP trial 1: 63.3 ± 5.5, $p<0.01$ compared with control; DZP trial 2: 50.8 ± 9.6, just misses significance at the 5% level), but failed to do so in males housed in the enriched environment (baseline: 24.7 ± 2.6; DZP trial 1: 18.3 ± 4.1; DZP trial 2: 24.2 ± 4.7). In the female groups there was no significant effect of DZP in either the controls or animals housed in the enriched environment (controls: baseline: 27.6 ± 1.5; DZP trial 1: 35.5 ± 5.6; DZP trial 2: 32.2 ± 3.5; enriched environment: baseline: 25.5 ± 3.3; DZP trial 1: 34.7 ± 8.9; DZP trial 2: 35.3 ± 6.3). This may indicate that this test is not sensitive to anxiolytics in female rats, or that female rats have altered sensitivity to the anxiolytic used.

These results indicate that housing male laboratory rats in a more stimulating environment mitigates the effect of an anxiolytic benzodiazepine. This provides an interesting parallel with previous work which has shown that chronic handling abolishes the anxiolytic effect of DZP in the elevated plus maze, without a clearly demonstrable effect on measures of anxiety *per se* (Brett and Pratt, 1990).

Brett, R.R. & Pratt, J.A. (1990) *Eur. J. Pharmacol.* 178, 135-138.
Joly D. & Sanger, D.J. (1991) *Behav. Pharmacol.* 2, 205-213.

268P SYSTEMIC MINERALOCORTICOID RECEPTOR BLOCKADE WITH SPIRONOLACTONE REDUCES COGNITIVE DEFICITS INDUCED BY CHOLINERGIC DISRUPTION IN AGED (24 mo) RATS

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Central cholinergic (ACh) blockade with scopolamine (SCOP) produces cognitive impairments in a variety of species (Fibiger, 1991). Our previous research demonstrated that ACh blockade increased anxiety-like behaviour and corticosterone secretion in rats (Bhatnagar et al., 1994; Smythe et al., 1996). We subsequently reported that central infusions of the mineralocorticoid receptor (MR) antagonist spironolactone (SPIRO), reduced SCOP-induced water maze impairments (Smythe et al., in press). In the present study we report on the effects of systemic MR blockade on SCOP-induced effects in a water maze task in aged (>24 month) rats. Adult male and female, Lister hooded rats (350-500 g) >24 months of age (retired breeders) served as subjects. On the first test day (acquisition phase), rats were pretreated with vehicle (VEH; saline+30% propylene glycol+ 5% ethanol, 1ml/kg IP), or SPIRO (25 mg/kg IP), 20 min before being injected with either VEH or 0.5 mg/kg SCOP IP and tested 20 min later in a water maze (n=6/group). Rats were given 4 trials of training to locate the platform, with each trial limited to a 60 sec maximum. 24 hours later the same animals were tested as before in the absence of any drug treatment (retention phase). Data (latencies taken to locate the platform, aggregates of the trials blocked together) were assessed by ANOVA (applying a Greenhouse-Geiser correction) and *post hoc* testing was performed using Bonferroni corrected t-tests (further details see Smythe et al, in press).

ANOVA revealed a significant 3-way pretreatment x test drug x test day interaction $F(1,20)=9.9$, $P<.005$. As shown in Fig. 1, VEH-VEH treated rats showed improved performance on Day 2 compared to Day 1 ($P<.05$), while SCOP-VEH treatment animals were still impaired on Day 2 ($P<.05$) compared to VEH-VEH rats on Day 2. While SPIRO produced no clear effect on maze performance over Days 1 and 2, it very effectively reduced the retention deficit produced by SCOP as shown by the significantly lower latencies in the SPIRO-SCOP rats compared to the VEH-SCOP animals on Day 2 ($P<.01$).

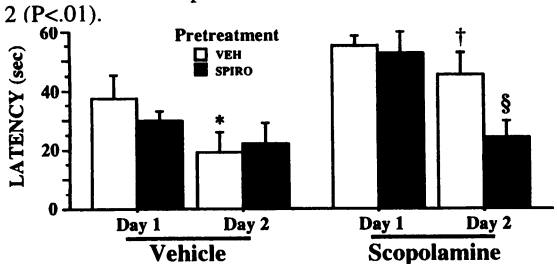

Figure 1. The effect of SCOP, SPIRO and SPIRO+SCOP on latencies to locate a submerged platform during the acquisition and retention phases in a water maze test. Data shown are means ± -SEM.
*significantly different from VEH-VEH group Day 1 ($P<.05$);
†significantly different from VEH-VEH group Day 2 ($P<.05$);
§significantly different from VEH-SCOP group Day 2 ($P<.01$)

These data corroborate our earlier results, and show that systemic MR blockade can effectively reduce cognitive impairments induced by SCOP. The fact that aged rats show this response suggests that MR blockade may be a useful treatment for age-related cognitive impairments.

Bhatnagar, S. *et al.* (1994) *Neurosci. Abs.* 20, 935.
Fibiger, H.C. (1991) *Trends Neurosci.* 14, 220-223.
Smythe, J.W.*et al.* (1996) *Pharmacol. Biochem. Behav.* 56, 57-61.
Smythe, J.W. *et al. Pharmacol.Biochem. Behav.* (in press).

269P THE EFFECTS OF MINERALOCORTICOID RECEPTOR BLOCKADE WITH SPIRONOLACTONE ON REACTIVITY TO NOVELTY IN MARMOSETS (*CALLITHRIX JACCHUS*)

J.W. Smythe, A.G. Smith & B. Costall, Neuropharmacology Research Group, Dept. of Pharmacology, Univ. of Bradford, Bradford BD7 1DP

It has been reported recently that corticosterone (CORT) enhances an animal's reactivity to novelty, an effect mediated via mineralocorticoid receptors (MR) (Oitzl *et al.*, 1992). Subsequently, our group discovered that intra-hippocampal infusions of the MR antagonist spironolactone (SPIRO) had an anxiolytic effect in rats tested in the Black-White box (Smythe *et al.*, in press), which we suggested was due to its ability to decrease the animals' reactivity to the aversive test situation. In the present study we have investigated the effects of systemic SPIRO injections on reactivity in marmosets confronted by a novel observer.

Six male and female, adult marmosets (*Callithrix jacchus*) weighing between 300-450g served as subjects. Each animal was acclimatized to handling over a 2 month period, prior to testing. On the test day each animal was injected with either vehicle (VEH; 30% propylene glycol+5% ethanol+65% saline) or SPIRO at 25 or 100 mg/kg s.c. and returned to their home cages. Twenty min following drug administration, an experimenter moved to within 50 cm of the cage and made direct eye contact with the animal for 2 min. This procedure was repeated at 60, 120 and 180 min for each animal. Behaviours during the 2 min test were recorded via a camera and VCR system; the tapes were later scored for: 1) time spent at the cage front observing the experimenter; 2) number of threat-related postures; and 3) locomotor activity (jumps from cage front to rear). Each animal was tested with all doses of SPIRO over a 4 week span in a counterbalanced design. Data were analyzed by non-parametric techniques including Freidman's ANOVA test, and pairwise comparisons were performed using the Wilcoxan matched-pairs test.

ANOVAs on the time forward data at 20 and 60 min revealed overall significant group differences (P's<.04 and .02 respectively). As shown in figure 1, at these times SPIRO significantly reduced time forward (P's<.05). There was no effect at 120 min, but a significant group effect at 180 min (P<.04). Pairwise comparisons here revealed that SPIRO at 100 mg/kg significantly increased time forward compared to the VEH group (P<.05). There were no group effects at any time point for postures and locomotor activity (jumps).

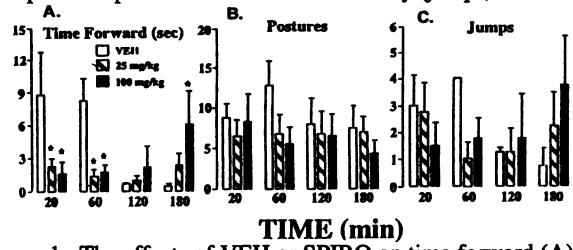

Figure 1. The effects of VEH or SPIRO on time forward (A), postures (B) and jumps (C) in marmosets exposed to a novel observer. Data shown are means ± SEM.
*significantly different from VEH at same time point (P<.05)

The decreased time forward produced by SPIRO at 20 and 60 min in the absence of obvious motor impairments and threat-related postures, suggests that the animals were not fearful or defensive, but were less responsive to the observer. While VEH-treated animals showed habituation to the test and were unresponsive by 180 min, the SPIRO animals became increasingly reactive, perhaps due to the diminishing influence of the drug. These data support previous findings that MR are important regulators of the response to novelty.

Oitzl, M.S. *et al.*, (1992) *Eur. J. Neurosci.* 6, 1072-1079
Smythe, J.W. *et al.*, *Pharmacol. Biochem. Behav.* in press

270P THE EFFECTS OF SPIRONOLACTONE ON ANXIETY-LIKE BEHAVIOUR (ALB) INDUCED BY INTRAHIPPOCAMPAL SCOPOLAMINE INFUSIONS IN THE RAT

C. Timothy, D. Murphy, B. Costall and J.W. Smythe, Neuropharmacology Research Group, Dept. of Pharmacology, Univ. of Bradford, Bradford BD7 1DP

We have demonstrated that hippocampal cholinergic blockade increases corticosterone (CORT) secretion in rats (Bhatnagar et al., 1994). We have also reported that CORT modulates anxiety-like behaviour (ALB) in the rat via mineralocorticoid receptors (MR) (Smythe et al., in press); however, the brain regions and mechanisms of its action have not been identified. Given that intrahippocampal scopolamine (SCOP) has anxiogenic-like actions in the rat (Smythe et al., 1996), we investigated whether or not SCOP-induced ALB would be blocked by the MR antagonist spironolactone (SPIRO).

Adult male, Lister Hooded rats weighing 300-450g were implanted bilaterally with hippocampal cannulae (A-P -3.3; M-L ±2.5; D-V 2.3 mm, Paxinos and Watson, 1986) under pentobarbital anaesthesia (60 mg/kg i.p.), 3 weeks prior to testing. On the test day animals were injected with either SPIRO (100 mg/kg i.p.) or vehicle (VEH, intralipid) 10 min prior to receiving intrahippocampal infusions of VEH (saline) or SCOP (3µl at 10µg/µl). Rats were placed into the white chamber of the black-white box immediately after intrahippocampal infusions. Animals were videotaped and scored for latencies to exit and re-enter the white chamber. Data were analysed by two-way ANOVA and post-hoc testing was performed using Scheffe's test.

ANOVA on latency data revealed a significant pre-treatment by post-treatment interaction $F(1,20)=6.8$, $p<.01$. As shown in Fig. 1, SCOP treated rats exited the white chamber faster than any of the other groups. ANOVA on latency to re-enter the white chamber also revealed a significant pre-by post-treatment interaction $F(1,20)=4.63$, $p<.04$. As shown in Fig. 1, SCOP treated rats displayed extreme reluctance to re-enter the white chamber, an effect that was blocked by SPIRO.

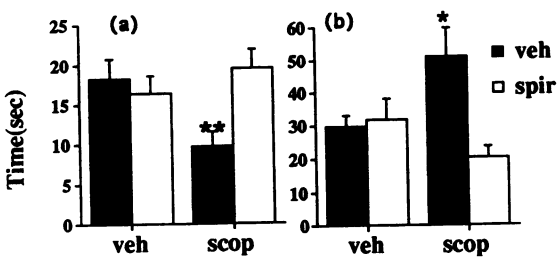

Fig. 1. The effects of SCOP, SPIR and SCOP+SPIR on (a) latency to exit the white chamber; and (b) latency to re-enter the white chamber in the black-white box.
* p=0.04 and ** p=0.01 compared to all other groups

These data replicate our earlier findings showing that intrahippocampal SCOP increases ALB in rats. Oitzl et al., (1992) have shown that MR blockade reduces reactivity to novelty in rats. Thus, in the present study, SCOP may elevate ALB by increasing reactivity to the aversive test situation, a response that is antagonized by MR blockade.

Bhatnagar, S. *et al.* (1994) *Neurosci. Abs.* 20, 935.
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271P CORTICOSTERONE MODULATES HIPPOCAMPAL THETA FREQUENCY AND POWER VIA MINERALOCORTICOID BUT NOT GLUCOCORTICOID RECEPTORS

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Hippocampal cholinergic theta is a rhythmic, sinusoidal waveform that occurs in alert, immobile rats presented with threatening stimuli (Bland, 1986). Intrahippocampal cholinergic blockade increases corticosterone (CORT) secretion elicited by acute stress (Bhatnagar *et al.*, 1994). Systemic injections of the mineralocorticoid receptor (MR) antagonist spironolactone (SPIRO) increases stimulation thresholds required to elicit theta (Murphy *et al.*, in press). Here we examined the effects of direct intrahippocampal infusions of SPIRO or the glucocorticoid receptor (GR) antagonist RU38486 on theta activity.

Adult male, Lister hooded rats weighing between 350-500 g were anaesthetized with isofluorane and implanted with jugular catheters. They were then switched to urethane (0.8 g/ml) anaesthesia and placed in a stereotaxic frame. A theta recording electrode was placed in the stratum moleculare of the hippocampus (A-P -3.3, M-L 2.5, D-V 2.7 mm). A bipolar stimulating electrode was positioned in the dorso-medial posterior hypothalamus (DMPH) to activate theta. A 30 gauge cannula was positioned 1-2 mm from the theta recording electrode for local drug infusions. Rats were administered either 150 ng of SPIRO (n=5) or RU38486 (n=3) (infusions of 3µl over 45 sec) in a vehicle made of 1% ethanol+saline, and DMPH-stimulated activities were monitored for 45 min. Changes in stimulated theta frequencies (Hz) and power (mV) of the signals were analyzed using ANOVA followed by Bonferroni corrected t-tests.

There were no interactions between stimulation intensity with drug or time, and data were collapsed across stimulation intensities. ANOVA revealed a main effect of drug treatment on theta frequency with $F(1,29)=14.9$, $P<.001$. Figure 1 shows that theta frequencies were lower in SPIRO treated rats compared to the RU38486 group, an effect that was significant at 45 min post-drug administration ($P<.01$). ANOVA also demonstrated a significant effect of drug treatment on power values $F(1,29)=4.3$, $P<.05$. SPIRO-treated rats displayed lower theta energies compared to the RU38486 animals, an effect that was significant at 30 min post-drug administration ($P<.05$).

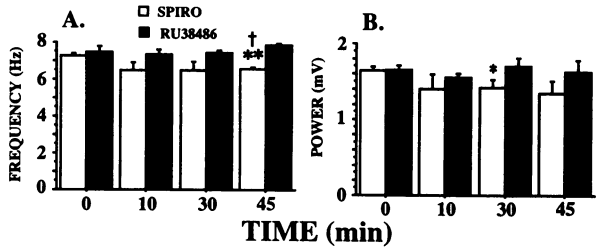

Figure 1. Hippocampal theta (A) frequency and (B) power, under stimulated conditions at various times following SPIRO or RU38486 infusions (Means ±SEM are shown). Significantly different from corresponding group at 0 min *($P<.05$), **($P<.01$), and from other drug group at same time point †($P<.01$).

In agreement with our previous data, hippocampal theta is regulated by CORT acting through MR, but not GR. These data show that CORT targets hippocampal cholinergic systems perhaps as part of a central arousing mechanism.

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272P IMMUNOHISTOCHEMICAL AND FUNCTIONAL CHARACTERISATION OF NTW8 MICROGLIAL CELLS

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Microglial cells, the phagocytic immune cells specific to the CNS, have been implicated in neurodegenerative disorders such as Alzheimer's disease. We have produced and characterised a microglial cell line, NTW8. Along with immunohistochemical analysis, function was studied by measuring the release of known microglial products, nitric oxide (NO), prostaglandin E_2 (PGE_2) and tumour necrosis factor-α (TNFα) following stimulation by lipopolysaccaride (LPS) or β-amyloid (1-42) peptide (βÅ; Meda *et al.*, 1995; Minghetti and Levi, 1995). The NTW8 cells were also compared to primary rat microglial (PRM) cells. The NTW8 microglial cell line was derived from a transgenic mouse which had incorporated a transgene encoding the immortalising oncogene, SV40 T-antigen. PRM cells were prepared using the method of Giulian & Baker (1986). The NTW8 cells displayed either a ramified or ameboid morphology. NTW8 and PRM cells stained positively with the macrophage markers anti-CD11b and ED1 but were not stained with antibodies to microtubule associated protein 2 or glial fibrillary acidic protein, specific for neurones and astrocytes, respectively.

The cells were cultured in HEPES-buffered Dulbecco's modified Eagles medium with 10% (v/v) foetal calf serum and incubated at 37°C in a humidified 5% CO_2 atmosphere. NO was detected in medium using the Griess assay and ELISA's were used to measure PGE_2 and TNFα. Experiments were performed 3 or 4 times in triplicate. Stimulation of NTW8 and PRM cells with LPS (10ng ml^{-1}) or βA (30µM) in combination with interferon-γ (INFγ; 10 or 100U ml^{-1}, respectively, for 24h) caused the release of NO, PGE_2 and TNFα (Table 1). A time-course (0-48h) of LPS (+INFγ) induced activation revealed that release of NO and PGE_2 from NTW8 cells was time-dependent with a lag of 8 and 4h, respectively. For NTW8 cells, the NO synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME; 0.3-1000µM) or the cyclooxygenase (COX) inhibitor indomethacin (0.1-300nM) caused a concentration-dependent inhibition of LPS (+INFγ) induced release of NO or PGE_2, respectively (IC_{50} values; L-NAME 107[82-141]µM, indomethacin 3 [2-4]nM; geometric mean with [95% confidence limits]).

	NTW8 cells			PRM cells		
Stimulus (+INFγ)	NO µM	PGE_2 ng ml^{-1}	TNFα ng ml^{-1}	NO µM	PGE_2 ng ml^{-1}	TNFα ng ml^{-1}
Control	0±0.2	0.1±0.02	0.2±0.2	0.2±0.2	0.4±0.2	0.04±0.02
LPS	34±1*	1.7±0.2*	2.0±0.3*	29±4*	1.9±0.9	1.3±0.2*
Control	0.7±1	0.15±0.03	0.02±0.02	0.3±0.1	0.4±0.2	0.08±0.04
βA	23±3*	0.5±0.1*	0.6±0.1*	29±8*	1.6±0.6	1.0±0.2*

Table 1: Release of NO, PGE_2 and TNFα following treatment (24h) with LPS or βA (+INFγ) from NTW8 and PRM cells. Values are the mean±s.e.mean.*$P<0.05$ compared to control; Students unpaired t-test.

These results demonstrate that NTW8 cells have the characteristics of primary microglial cells; they both express a macrophage specific antigen and release NO, PGE_2 and TNFα upon activation. These results are consistent with βA and LPS inducing COX-2 and NO synthase in NTW8 cells.

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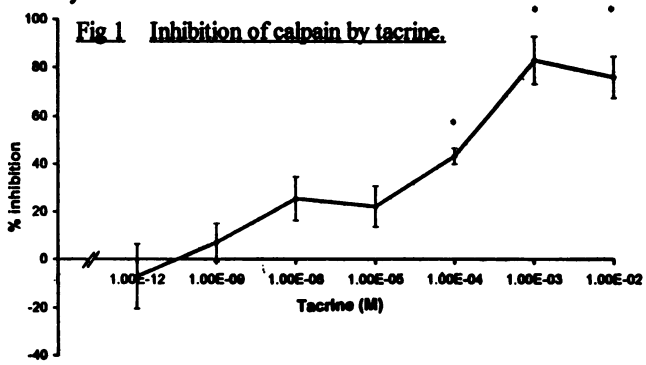
There are similarities in the structural requirements for inhibitory activity of compounds against acetylcholinesterase and proteases. This is illustrated by the existence of several series of irreversible inhibitors which possess both types of inhibitory action, for example, the biochemical reagent phenyl methyl sulphonyl fluoride (PMSF) (Moss and Fahrney, 1978; Turini *et al.*, 1969). Calpain is a widespread intracellular protease which is activated and induced to translocate to the membrane by elevated intracellular calcium levels. Since this protease is not inhibited by PMSF or benzamidine, it can also be a troublesome component of protein containing isolates. We therefore decided to establish whether reversible acetylcholinesterases could act as inhibitors of the enzyme. Here we report the activity of the most interesting compound in this study, the aminoacridine derivative tacrine.

Purified rabbit muscle calpain (Sigma, Poole, UK) was diluted in 50 mM Tris-Cl buffer (pH 7.5) containing 1 mM dithioerythreitol and 1 mM EGTA to 1 unit/ml. 180 µl substrate solution (0.2% N,N-demethylated casein in 50 mM Tris-Cl buffer (pH7.4) with 0.4 M $CaCl_2$ and 5 mM dithioerythreitol) was preincubated with 10 µl inhibitor dissolved in distilled water (or with distilled water alone) at 30 °C in a shaking water bath for two minutes. 10 µl enzyme solution was added to initiate the reaction. After 10 minutes, the reaction was terminated by addition of 200 µl of 5 % trichloroacetic acid (TCA), and the reaction solutions were then transferred to ice and centrifuged at 14 000 x g for 2 minutes at 4 °C. Proteolysed casein was measured as TCA soluble protein using the BioRad protein assay reagent micromethod, and absorbance at 595 nm compared to standard curves of casein together with appropriate drug concentrations. This was to take into account the fact that tacrine caused an increase in absorbance that was dose dependent, but did not interfere with assay sensitivity or linearity.

Fig 1 Inhibition of calpain by tacrine.



Tacrine was found to be an effective inhibitor of calpain *in vitro* (* = $p < 0.05$, Anova and post-hoc Bonferroni t-test) (n = 4). It seems that tacrine may be a useful biochemical reagent where calpain poses a contamination problem. We would also suggest that the significance of calpain inhibition in the clinical action of this and related drugs is worthy of further investigation.

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274P DIFFERENTIAL MODULATION OF THE α4-1β2 AND α7 NEURONAL NICOTINIC RECEPTOR SUBTYPES BY ETHANOL

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We have previously found that agonist responses at the α3β4 nicotinic receptor subunit combination can be both inhibited and enhanced by ethanol (EtOH), with enhancement dominating at high EtOH concentrations (>30mM) (Covernton & Connolly, 1995). However, it is widely believed that two other nicotinic receptor subtypes; those containing α4 and β2 subunits, and those containing the α7 subunit; are more abundant in the brain (M^{c}Gehee & Role, 1995, review).

Xenopus oocytes were injected with cRNA encoding either the α7 or the α4-1 and β2 subunits. Current responses to 3-10µM ACh (α4-1β2) or 10-30µM nicotine (α7) were obtained under voltage clamp conditions (V_H=-60mV). Consistent responses to a given concentration of agonist were obtained, followed by a co-application with EtOH and then a recovery response. The control responses preceding and following the co-application with EtOH were averaged. The response in the presence of EtOH was expressed as a % of the average of the controls. Pipette solutions contained: Current pipette, 0.25M CsF, 0.25M CsCl, 100mM EGTA, pH 7.2; Voltage pipette, 3M KCl. External recording solution in which drugs were applied contained: 115mM NaCl, 2.5mM KCl, 1.8mM $BaCl_2$, 10mM HEPES (pH 7.2) and 1µM atropine. Ca^{2+} was replaced by Ba^{2+} to minimize the activation of endogenous Ca^{2+}-activated Cl^- current.

The mean inward current (±S.E.M.) obtained for α7 with 10µM nicotine was 188.6±22.4nA (n=36), and for α4-1β2 with 10µM ACh was 360.9±53.1nA (n=27). Nicotinic responses in both α7 and α4-1β2 subtypes were not affected by concentrations up to 30mM EtOH. Agonist-induced current responses for the α4-1β2 subtype were increased to 112.1±2.8% ($p<0.02$, Students' t-test, n=5, range: 107.4-122.5%) of the control value by 100mM EtOH, and increased to 190.1±13.6% ($p<0.005$, n=5, range: 144.6-219.0%) by 300mM EtOH. The effects of high concentrations of EtOH on α7 current responses were more varied: with 100mM EtOH the mean response was 115.5±7.31% (n=6, range: 98.9-141.6%), and with 300mM EtOH the mean response was 107.6±6.7% (n=6, range: 88.4-130.7%). The results suggest that the different neuronal nicotinic AChR subtypes have distinct patterns of modulation by EtOH. They also suggest that, as well as the α3β4 subtype, the α4-1β2 and α7 subtypes could be mediators of the synergistic addictive processes involving both nicotine and alcohol addiction (Kozlowski *et al.*, 1993).

We wish to acknowledge the support of the M.R.C. (U.K.), the Royal Society, S.H.E.R.T., the Strathclyde Molecular Biology Laboratory and R&D Fund; and the technical contributions of Fiona Kempsill and Angela Garman.

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275P FAILURE OF α-BUNGAROTOXIN TO MODIFY NICOTINE-INDUCED CHANGES IN LOCOMOTOR ACTIVITY IN RATS

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It is well documented that chronic nicotine treatment sensitises rats to the locomotor activating effects of the drug (Clarke 1990). However, the nicotinic receptor subype involved in this response is unknown. Since it has been suggested that α7 receptors may be involved in various behavioural responses to nicotine (McGehee et al 1995), the aim of this study was to investigate the involvement of the neuronal α7 nicotinic receptor in the expression of the locomotor activating effects of chronic nicotine.

Groups of male Sprague-Dawley rats (weight 300-600g, n=8/group) were given 14 daily injections of nicotine (0.4mg kg^{-1} sc) or saline. Fifteen minutes after their daily injection, rats were placed in an enclosed arena for 15min (Vale and Balfour 1989). The arena was divided into nine equal areas and locomotor activity was determined by recording the number of times the rat moved between squares (line crossings). The effects of nicotinic receptor antagonists on the locomotor responses to nicotine were then investigated. Mecamylamine (1mg kg^{-1} sc) was administered 15 min prior to nicotine or saline. In another experiment, additional groups of rats chronically treated with nicotine or saline, were implanted with bilateral cannulae into the lateral ventricles under halothane anaesthesia. Following recovery, the selective α7 receptor antagonist α-bungarotoxin (αBgTx) was administered (0.1 nmoles/5μl bilaterally) 5 min prior to nicotine or saline. Locomotor activity data was analysed by two way ANOVA.

Mecamylamine, but not αBgTx blocked the increase in locomotor activity induced by chronic nicotine treatment (Table 1). Locomotor responses to nicotine were not influenced by the administration of icv saline pretreatment (table 1).

Table 1. The effect of mecamylamine and αBgTx on nicotine-induced locomotor activity.

Pretreatment on test day	Chronic treatment	
	Saline	Nicotine
Saline	37±10	134±3*
Mecamylamine	11±3	25±9
Saline (icv)	50±13	193±43*
αBgTx (icv)	55±17	166±31*

Values (mean±s.e.mean) indicate the number of line crossings. P<0.01 compared to appropriate chronic saline group.

These data suggest that the rat brain α7 containing receptor is not involved in the expression of the locomotor activating effects of chronic nicotine treatment. Since the increase in locomotor activity was blocked by the administration of mecamylamine, which is a less specific antagonist than α-bungarotoxin, it is likely that a nicotinic receptor which binds nicotine with high affinity, such as the putative α4β2 or α3β4 receptor, is involved in this interaction between nicotine and behaviour.

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FK is a BBSRC student supported by Merck Sharp and Dohme. We thank SHERT for laboratory facilities.

276P ANTAGONIST BINDING TO THE D_2 DOPAMINE RECEPTOR AND THE ROLE OF CONSERVED SERINE RESIDUES

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Three serine residues are conserved in the putative fifth transmembrane spanning region of the dopamine receptors. These serine residues (Ser 193, 194, 197) have been mutated individually to alanine residues (Woodward *et al.*, 1996). Whereas the binding of many antagonists e.g. haloperidol, was unaffected by these mutations there were specific effects on the binding of certain compounds. For example, the affinity of remoxipride was increased (~10 fold) by the Ala 193 mutation, the affinity of (-)-sulpiride was reduced (~5 fold) by the Ala 194 mutation and the affinity of domperidone was reduced (~13 fold) by the Ala 197 mutation. In order to investigate whether the effects of the mutations were independent and additive, we have constructed receptors with double and triple alanine mutations. These have been expressed in COS-7 cells and the effects determined in ligand binding assays.

In competition experiments versus [^{3}H]spiperone, there was no significant effect of the mutations on the binding of a wide range of antagonist drugs e.g. haloperidol so that the mutations did not affect the gross conformation of the receptor (Table 1). The multiple mutations generally had the effect predicted from the effects of the single mutations. For example, in those mutants containing the Ala 193 mutation the affinity of remoxipride was increased whereas in those mutants containing the Ala 197 mutation the binding of domperidone was reduced. For sulpiride, mutants containing the Ala 194 mutation show reduced affinity with the exception of the Ala 193/194 mutant. These results support the idea that there are specific hydrogen bonding interactions between the serine residues and certain drugs and that the effects of the mutations can be largely considered as independent with only slight effects on the local conformation of the protein (see for example the small effect of the triple mutation on the affinity of haloperidol).

Supported by the Wellcome Trust

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Table 1. The binding of antagonists to native and mutant D2 dopamine receptors. (Ki, nM, mean±S.E.M., for three or more experiments; *P<0.05 versus native, ANOVA)

	haloperidol	(-)-sulpiride	domperidone	remoxipride
native	1.99±0.27	18.2±2.5	3.3±1.5	331.8±2.7
Ala 193/194	1.07±0.07	18.3±1.0	4.1±1.1	24.8±2.1*
Ala 193/197	0.85±0.21	12.4±3.0	94.2±15.9*	19.4±2.3*
Ala 194/197	1.32±0.46	39.0±0.8*	24.2±5.0*	257.0±92.3
Ala 193/4/7	0.55±0.10*	61.7±3.7*	77.7±6.7*	21.2±4.8*

277P SODIUM SENSITIVITY OF SUBSTITUTED BENZAMIDE BINDING AT D_2 DOPAMINE RECEPTORS EXPRESSED IN CHO AND Sf21 CELLS

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The substituted benzamides are a group of sodium sensitive ligands which have recently been shown to be inverse agonists at the D_2 dopamine receptor (Hall & Strange 1996).
We have expressed the long isoform of the rat D_2 dopamine receptor in Sf21 insect cells, using the baculovirus expression system, and in CHO cells to investigate the tissue dependence of their sodium sensitivity and the relationship between sodium sensitivity and inverse agonism.
Membranes were prepared for ligand binding experiments from CHO and insect cells as previously described (Gardner *et al.*1996; Woodcock *et al.*1995 respectively).. The K_D of [^{3}H]spiperone was determined by saturation assay and the Ki of all other ligands determined by competition assay as described in Gardner *et al.*1996. Membranes (20µg) were incubated in Hepes buffer, pH 7.4, containing either 100mM NaCl or 100mM NMDG for 3hr at 25°C with [^{3}H]spiperone and drugs where appropriate. Incubation was terminated by rapid filtration and radioactivity determined. Data were analysed using the computer program LIGAND.
For the D_2 receptor expressed in insect cells we observed an increased sodium sensitivity for the binding of ligands from each of the four classes of substituted benzamides compared to CHO cells (see Table 1.) The degree of sodium sensitivity varied among the substituted benzamides in both systems as did the difference in sodium sensitivity between the two systems. The sodium sensitivity of these ligands was not, however, related to their inverse agonism. It would also appear that the conformation of the dopamine receptor expressed in insect cells is different to that adopted in CHO cells, such that the affinity of the substituted benzamides is reduced whilst the sodium sensitivity of these compounds is greatly increased.

We thank the BBSRC and Carbury Herne for financial support.

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Table 1. The binding of ligands to $D_{2(long)}$ dopamine receptors in Sf21 and CHO cells. (pKi, mean ± s.e, 3 experiments)

Compound	Sf21 pKi		Affinity change	CHO pKi		Affinity change
	NMDG	Na^+		NMDG	Na^+	
Spiperone	9.46±0.04	9.72±0.05	1.8	10.02±0.08	10.08±0.13	1.2
Clebopride	5.89±0.07	8.11±0.05	165	7.07±0.09	8.40±0.11	21.4
Metoclopramide	4.81±0.05	6.68±0.14	73.3	5.26±0.13	6.93±0.04	46.8
Nemonapride	7.83±0.15	9.57±0.09	54.8	8.89±0.19	10.04±0.19	14.1
Remoxipride	~4.2	5.66±0.22	~28.9	~5.1	5.95±0.14	~7.9

278P INVESTIGATION OF METABOTROPIC GLUTAMATE RECEPTOR SUBTYPES IN RAT BRAIN USING [^{35}S]GTPγS BINDING

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Metabotropic glutamate receptors (mGluR's) constitute a family of G-protein coupled receptors that are subdivided into three groups according to sequence homology, transduction mechanisms and pharmacological properties (Knöpfel et al., 1995). In this study we have used [^{35}S]GTPγS binding to investigate group 2 and group 3 mGluR's in rat brain membranes. In addition, this technique was used to assess the regional distribution of these subgroups in rat brain slices using *in vitro* autoradiography.

For [^{35}S]GTPγS binding in tissue homogenates, rat cortex was prepared and assayed acccording to Sim et al., 1995. Briefly, 50µg of protein was incubated with GDP (300µM), in the presence or absence of test drugs, for 30 min. [^{35}S]GTPγS (100pM) was subsequently added and incubated for a further 30 min. The reaction was terminated by rapid washing with ice-cold Tris-HCl buffer. Autoradiographic studies were performed according to Sim et al., 1996, with minor modifications.

The group 2 specific ligand, L-CCG-1 ((2S,1'S,2'S)-2-(carboxycyclopropyl) glycine), produced a dose-dependent stimulation of basal [^{35}S]GTPγS binding in tissue homogenates (see Table 1) which could be antagonised by 500µM of the mGluR antagonists, MAP4 ((S)-2-Amino-2-methyl-4-phosphobutanoic acid), MPPG ((RS)-α-Methyl-4-phosphonophenylglycine) and MCPG ((+)-α-Methyl-4-carboxyphenylglycine). The group 3 specific agonist L-AP4 (L(+)-2-Amino-4-phosphonobutyric acid) produced a small stimulation of binding which was biphasic and was shifted to the right by MAP4 (100µM).

Table 1. [^{35}S]GTPγS Binding in Rat Cortex

			app. pA_2		
	pEC_{50}	% Stim.	MPPG	MAP4	MCPG
L-CCG-1	6.1 ± 0.1	47 ± 3	4.8 ± 0.2	3.4 ± 0.1	4.1 ± 0.1
L-AP4	6.4 ± 0.4	19 ± 1	nd	4.3 ± 0.4	nd
	3.7 ± 0.4			4.9 ± 0.4	

nd = not determined

In autoradiographic studies L-CCG-1 (10µM) and L-AP4 (300µM) both stimulated [^{35}S]GTPγS binding in the cortex by 40 ± 10% and 30 ± 7% respectively.
In conclusion, [^{35}S]GTPγS binding can be used to study the activity and distribution of specific subgroups of metabotropic glutamate receptors in rat brain.

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279P CHARACTERISATION OF [^{3}H]-GLUTAMATE BINDING TO THE mGluR1α AND mGluR1β SUBTYPE OF METABOTROPIC GLUTAMATE RECEPTORS

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In this study we have developed a [^{3}H]-glutamate radioligand centrifugation binding assay to pharmacologically characterise the human 1a and 1b subtypes of metabotropic glutamate receptors (mGluR1a and mGluR1b) expressed transiently in HEK293T cells.

[^{3}H]-glutamate (10-30nM), membranes (30μg per tube) and test compounds were incubated in a final volume of 0.5ml in assay buffer (30mM Tris-HCl, 2.5mM $CaCl_2$, pH 7.4), for 30mins at 20°C. The reaction was terminated by centrifugation (17,000g, 20mins, 4°C). The supernatant was aspirated and pellets were washed with 1ml of ice-cold assay buffer and recentrifuged (17,000g, 5mins, 4°C). Final pellets were collected for liquid scintillation counting. 0.3μM quisqualate was used to define non-specific binding. Data was analysed by computer-assisted non-linear analysis (ALLFIT for competition studies). Data from homologous [^{3}H]-glutamate displacement binding was fitted to the model (Bo = ((BmaxL)/(L + Kd + A)) + NSB) to determine Bmax and Kd values (DeBlasi et al, 1989).

Specific binding of [^{3}H]-glutamate was > 85% (typically 600 dpm and 4000 dpm for mGluR1a and mGluR1b respectively). No specific binding was detected to membranes prepared from mock transfected HEK293T cells nor did the presence of glutamine in transfection culture media affect the levels of specific [^{3}H]-glutamate binding. A Bmax = 6 pmol^{-1}mg protein and 23 pmol^{-1}mg protein and a Kd = 0.35μM and 0.40μM was calculated for mGluR1a and mGluR1b respectively. In competition studies the affinities of various mGluR agonists and antagonists were determined. These are listed in table 1.

Table 1: Affinity values of compounds for the inhibition of [^{3}H]-glutamate binding to mGluR1a and mGluR1b. Results are given as mean ± S.E.M. from three separate experiments.

	mGluR1a		mGluR1b	
Compound	pKi	n_H	pKi	n_H
Glutamate	6.5 ± 0.1	1.0 ± 0.1	6.2 ± 0.1	1.2 ± 0.1
Quisqualate	-	-	8.0 ± 0.3	0.8 ± 0.1
1S,3R-ACPD	5.2 ± 0.2	1.1 ± 0.1	5.4 ± 0.2	0.9 ± 0.1
RS-MCPG	<5	2.3	-	-
S-4C-PG	6.2 ± 0.2	1.5 ± 0.4	5.7 ± 0.3	2.5 ± 0.1

These compounds show the same rank order of potency as that obtained when measuring mGluR1 stimulated phosphatidylinositol hydrolysis (Pickering D.S. *et al.*, 1993).

We have developed a centrifugation binding assay to measure specific [^{3}H]-glutamate binding to mGluR1a and mGluR1b.

Abbreviations: 1S,3R-ACPD, (1S, 3R)-1-aminocyclopentane-1,3-dicarboxylate; RS-MCPG, (RS)-α-methyl-4-carboxyphenylglycine; S-4C-PG, (S)-4-carboxy-phenylglycine.

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280P BDNF PROMOTES THE GROWTH AND SURVIVAL OF HIPPOCAMPAL GABAergic NEURONES IN PRIMARY CULTURE

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The neurotrophin brain-derived neurotrophic factor (BDNF) can promote the growth and survival of CNS neurones. The growth of foetal hippocampal GABAergic cells was promoted after 7 days in BDNF, an effect that is regulated by GABA itself (Marty *et al.*, 1996). Also, the survival of postnatal dentate gyrus cells has been shown to be increased by BDNF (Lowenstein & Arsenault, 1996). We wished to determine the effect of BDNF on the growth and survival of GABA-like immunoreactive (GABA-IR) hippocampal neurones cultured from neonatal rats.

Postnatal (P3-P4) rat hippocampal cells were grown on coverslips at low density (plating density 70 cells / mm^2) using a culture method modified from Baughman *et al.* (1991). After 24 hours in culture, BDNF (20 ng/ml) was added to the growth medium. At 1 or 7 days after treatment, the cells were fixed with 4% paraformaldehyde, treated with an antiserum to GABA (dilution 1:1000, Sera-lab, U.K.) and stained using a peroxidase based reaction with diaminobenzidine as the chromogen. The visual area of the cell somata of GABA-IR neurones in a sample area of 3.6 mm^2 was measured using a computerized image analysis system (MCID) in control and treated cultures. The experiments were carried out in a double blind manner and repeated 4 times for the two treatments. Pooled data were expressed as median (interquartile range, n) and compared using a two-tailed Mann Whitney U-test.

In cultures treated with BDNF, the GABA-IR neurones had a significantly larger cell body size. At 1 day following treatment, neurones had a median size of 74 μm^2 (49-95, n=305) compared with 56 μm^2 (38-79, n=306) for untreated cultures (P<0.001). At 7 days, treated neurones were 117 μm^2 (81-153, n=345) compared with 86 μm^2 (60-116, n=249) (P<0.001). BDNF also appeared to promote the survival of neurones since at 1 day following treatment the numbers of cells in both control and treated samples were similar (305 cf. 306) while at 7 days there were fewer cells in control compared to treated samples (249 cf. 345). Additionally it was noted that after 7 days treatment with BDNF the GABA-IR neurones showed more extensive neurite outgrowth compared with controls.

These results show that BDNF stimulates the growth of GABA-IR hippocampal neurones in culture after only 24 hours, an effect that is still apparent after 7 days. The data also show that BDNF promotes neuronal survival after 7 days. Our findings support and extend those of Marty *et al.* (1996).

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281P COLOCALIZATION OF NEUROTRANSMITTERS IN THE BASAL GANGLIA OF THE RAT

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There is increasing evidence of the colocalization of neurotransmitters in the central nervous system. In the basal ganglia, a group of subcortical nuclei involved in the control of movement, the amino acid neurotransmitter, γ-aminobutyric acid (GABA), has been shown to be colocalized with neuropeptides, but there is also evidence for the presence of GABA in nitric oxide synthase (NOS) positive neurons and glutamate in cholinergic neurons. The object of the present study was to determine directly whether axon terminals or boutons of cholinergic neurons in the basal ganglia (striatum, subthalamic nucleus (STN) and entopeduncular nucleus (EP)) also contain amino acid neurotransmitters. A second objective was to determine whether axon terminals and boutons of NOS-positive neurons in the striatum contain GABA; the data were compared to populations of known GABA-negative terminals. This was achieved using double immunocytochemistry at the electron microscopic level (Bevan & Bolam, 1995; Clarke *et. al.*, 1996).

Pentobarbitone-anaesthetised rats (n=6; Sprague Dawley) were perfuse-fixed and the basal ganglia sectioned on a vibrating microtome. The sections were then immunostained to reveal NOS, choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), or parvalbumin (PV) immunoreactivity using peroxidase methods. The immunostained sections were then re-sectioned for electron microscopy and subjected to the post-embedding immunogold method to reveal GABA or glutamate immunoreactivity. Amino acid immunoreactivity associated with populations of peroxidase-labelled and unlabelled axonal terminals or boutons was quantified by determining the density of immunogold particles overlying them. The values were corrected for background labelling and normalized (index of immunoreactivity).

In the striatum TH- and ChAT-immunopositive boutons and terminals forming asymmetrical synapses (Asym) are GABA-immunonegative (Table 1). NOS-immunopositive terminals were GABA-positive as a population (Table 1) but analysis of the immunoreactivity of individual boutons suggests that there are both GABA-positive and -negative boutons. In confirmation of previous findings, boutons containing PV, and terminals forming symmetrical synapses (Sym) are GABA-positive (Table 1). In the EP and STN ChAT-immunopositive terminals are significantly enriched in glutamate immunoreactivity ($p<0.001$, Mann-Whitney U Test) compared to terminals forming symmetrical synapses, suggesting that these terminals may also use glutamate as a neurotransmitter (Table 1).

In conclusion, these data demonstrate that ChAT-immunopositive terminals in the EP and STN, which are probably derived from the mesopontine tegmentum, are enriched in glutamate suggesting that at least some co-release glutamate and acetylcholine. Furthermore the terminals of at least some NOS neurons in the striatum contain and probably release GABA.

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Table 1. Indices of GABA and glutamate immunoreactivity in immunolabelled and non-immunolabelled boutons in the striatum, entopeduncular nucleus and subthalamic nucleus in the rat. Figures are mean ± s.e.mean of the number of boutons in brackets. N/T = not tested.

	Striatum	Entopeduncular Nucleus		Subthalamic Nucleus	
Bouton Types	GABA immunoreactivity	Glutamate immunoreactivity	GABA immunoreactivity	Glutamate immunoreactivity	GABA immunoreactivity
Asym	1 ± 0.05 (n = 470)	3.325 ± 0.12 (n = 56)	1.0 ± 0.07 (n = 59)	4.264 ± 0.19 (n = 82)	1.0 ± 0.15 (n = 28)
TH	1.74 ± 0.18 (n = 89)	N/T	N/T	N/T	N/T
ChAT	2.79 ± 0.25 (n = 87)	1.618 ± 0.08 (n = 64)	1.916 ± 0.24 (n = 46)	2.497 ± 0.12 (n = 136)	1.69 ± 0.27 (n = 21)
NOS	5.45 ± 0.44 (n = 93)	N/T	N/T	N/T	N/T
PV	12 ± 0.52 (n = 90)	N/T	N/T	N/T	N/T
Sym	12.45 ± 0.48 (n = 148)	0.999 ± 0.04 (n = 108)	7.061 ± 0.34 (n = 71)	0.976 ± 0.07 (n = 118)	13.106 ± 0.8 (n = 32)

282P CHANGES IN EXPRESSION OF mRNA ENCODING $GABA_A$ RECEPTOR SUBUNITS α2, α5 AND γ2 IN RESECTED HUMAN EPILEPTIC TEMPORAL LOBE DEMONSTRATED BY *IN SITU* HYBRIDISATION

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A functional impairment of GABAergic inhibition has been proposed as one mechanism which may underlie increased seizure susceptibility in human temporal lobe epilepsy (TLE). Changes in expression of mRNA encoding several subunits of the $GABA_A$ receptor have been demonstrated in animal models of TLE with in-situ hybridisation (Kamphuis *et al.*, 1995) and loss of the α1 subunit has been demonstrated in epileptic human hippocampus using immunohistochemical techniques (Wolf *et al.*, 1994). We have examined expression of mRNA encoding the α2, α5, and γ2 subunits of the $GABA_A$ receptor in hippocampi resected from ten patients (average age 31 years) with intractable TLE due to hippocampal sclerosis (HS) and 6 neurologically normal controls (average age 69 years) using in-situ hybridisation histochemistry.

Hippocampi samples were frozen in an embedding matrix on dry ice within minutes of resection and stored at -80°C. Control hippocampi were frozen 5.5-28 hours post-mortem. Cryostat sections (10μm) were cut at -15°C to -20°C and mounted onto charged microscope slides. Sections were fixed with 4% paraformaldehyde in ice-cold phosphate buffered saline (PBS) pH 7.2 for 5min, washed in fresh PBS, dehydrated using a series of alcohols and stored in 95% ethanol at 4°C until assayed. Oligonucleotides were labelled with [^{35}S] on the day of the hybridisation ($1.3\text{-}2.5\times10^9$dpm/μg) and diluted to a concentration of 5×10^6 dpm/ml in hybridisation buffer (Sirinathsinghji *et al., 1995*). Sections were removed from alcohol, allowed to dry, and hybridised overnight at 42°C. Sections were then washed in standard saline citrate (SSC) 2 x 30min at 55°C, rinsed in 1 x SSC and 0.1 x SSC (1min at room temp), dehydrated using a series of alcohols, air-dried and apposed to [^{3}H]-Hyperfilm (Amersham) for 17 days at room temperature. Film optical densities (converted to atmol/mm^2 [^{35}S]) were measured in six hippocampal subregions identified by cresyl violet-staining following hybridisation. Non-specific hybridisation was assessed in the presence of 100-fold excess of unlabelled oligonucleotide. Neuronal densities of hippocampal subregions were obtained, using a 3-D counting method (Williams & Rakic, 1988) on paraffin-embedded samples of epileptic and control hippocampus.

Neuronal density values obtained from six epileptic and five control hippocampi revealed significant neuronal loss in all subregions examined in the epileptic tissue (11±1% to 58±4% of control density). Increased expression of mRNA encoding all three subunits examined was observed in the epileptic subiculum compared with autopsy controls (α2 280±42% $p<0.01$, α5 199±24% $p<0.05$ and γ2 320±43% $p<0.05$ Student's *t*-test). A significant decrease in γ2 mRNA was observed in the CA2 stratum pyrimidale (8.4±4% of control, $p<0.05$). No significant change in expression of mRNA encoding the α2, α5 and γ2 subunits was detected in any of the other hippocampal subregions measured. However, if corrected for neuronal loss, a significant increase in levels of mRNA encoding the α2 subunit was apparent in epileptic hippocampi in three subregions (dentate gyrus 366±71% $p<0.05$, CA4 692±119% $p<0.01$ and CA1 677±146% $p<0.01$ of control). Subiculum was not assessed. A similar pattern was observed with the α5 and γ2 subunits. These results indicate an upregulation of expression of α2, α5 and γ2 mRNA in remaining neurons in HS, an age-related decrease in expression in autopsy controls or perhaps the selective preservation of neurons expressing these particular subunits. However, the effects of difference in age or sampling method between the two groups cannot be excluded.

Control autopsy brain tissue was provided by the Parkinson's Disease Society Brain Bank, Institute of Neurology, London and support for this research was provided by Merck Sharp & Dohme.

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283P EXPRESSION AND PHARMACOLOGY OF THE HUMAN $GABA_A$ RECEPTOR δ SUBUNIT

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We have cloned and sequenced a cDNA encoding the δ subunit of the human γ-aminobutyric acid type A ($GABA_A$) receptor. This subunit represents 10% of the $GABA_A$ receptor subunits and is located in specific brain areas including the cerebellum, cerebral cortex, striatum and hippocampus. The largest density is found in the cerebellum where approximately 27% of all $GABA_A$ receptors contain a δ subunit (Quirk *et al.*, 1995).With this in mind and due to the exclusive location of the α6 subunit in cerebellar granule cells we have expressed the human recombinant δ subunit with human α6 and β3 in *Xenopus* oocytes. Using conventional two-electrode voltage-clamp, the actions of GABA, zinc and pentobarbital (PB) were examined on this receptor combination and the results compared to α6β3 and α6β3γ2s receptors. Injection of the δ subunit alone or with an α6 failed to produce any currents to GABA or PB.

GABA concentration-response curves revealed mean (95% CL, n=5) EC_{50} values on α6β3, α6β3δ and α6β3γ2s of 0.56 (0.43-0.73), 0.58 (0.42-0.78), and 1.5 (1.05–2.13) μM respectively. These EC_{50} values were not significantly different (Student's *t*-test $p>0.05$) providing no evidence that the δ subunit was being expressed. Published data have demonstrated that $GABA_A$ receptors display different sensitivity to block by the divalent cation zinc (Draguhn *et al.*, 1990). Inhibition curves to zinc produced clear subunit dependent differences in mean IC_{50} values demonstrating that the δ subunit was being expressed along with α6 and β3 (Table 1).

The direct activation of $GABA_A$ receptors by PB produced a maximum response greater than that obtained with a saturating concentration of GABA on all three receptor subunit combinations. The maximum PB response on α6β3 receptors was 9.5 times larger than the maximum GABA response (1495 ± 539 vs 80 ± 12 nA). The addition of a third subunit into the receptor lowered this ratio to 3.2 with α6β3δ (498 ± 170 vs 80 ± 16 nA) and 1.6 with α6β3γ2s (517 ± 26 vs 372 ± 43 nA). Although PB produced a large response relative to maximum GABA on α6β3 receptors, the mean EC_{50} value was approximately 3 fold higher than on α6β3γ2s and α6β3δ (Table 1).

In summary we have cloned and sequenced a cDNA encoding the human δ subunit and have expressed this subunit with an α6 and β3. This receptor shows clear differences from α6β3γ2s and α6β3 in its sensitivity to zinc and PB, but no significant difference in its sensitivity to GABA.

Table 1. Zinc and Pentobarbital affinity.

	Zinc IC_{50} (μM)	Pentobarbital EC_{50} (μM)
α6β3	0.42 (0.34 - 0.52)*	153 (120 - 194)
α6β3δ	8.6 (7.7 - 9.8)*	62 (47 - 81)**
α6β3γ2s	143 (85 - 238)*	53 (42 - 66)**

Values are mean (95% CL, n≥3). * significantly different from each other, ** significantly different from α6β3 ($p<0.05$ *t*-test).

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284P ALANINE SCANNING MUTAGENESIS IN TRANSMEMBRANE DOMAIN SIX OF THE RAT m_1-MUSCARINIC ACETYLCHOLINE RECEPTOR (RAT m_1-RECEPTOR)

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The rat m_1 receptor is a member of the super-family of G protein-coupled receptors. The receptor consists of seven transmembrane domains (TM) and it is the TMs that are thought to be involved in ligand binding. The aim of this study is to identify the residues in TM 6 of the rat m_1 receptor that play a role in ligand binding by using a scanning mutagenesis approach.

Experimental procedures used were the same as described by Page, *et al.*, (1995) unless otherwise stated. The rat m_1 receptor was in a pCD expression vector. The residues Tyr381 to Val387 were systematically mutated to Ala using the Chameleon™ Double-Stranded, Site-Directed Mutagenesis Kit, from Stratagene. COS-7 cells were transfected with the mutant plasmids to enable membrane preparations to be produced which were used in radioligand binding assays.

Saturation assays contained 0.01 - 3 nM (–)-[^{3}H]*N*-methylscopolamine ([^{3}H]-NMS) or 0.001 - 1 nM (–)-[^{3}H]*N*-quinuclidinylbenzilate ([^{3}H]-QNB), 10 - 20 μg/ml membrane preparation, and vehicle (0.3 % (v/v) DMSO in dH_2O) or 1 μM atropine in vehicle (non-specific binding). Competition assays were carried out with 0.3 nM [^{3}H]-NMS or 20 pM [^{3}H]-QNB, membrane preparation (as above) and competing ligand, vehicle or 1 μM atropine in vehicle. Assays were all performed in triplicate in polystyrene tubes and were incubated at 30 °C for 60 min ([^{3}H]-NMS) or 180 min ([^{3}H]-QNB). Data obtained from the radioligand binding assays are summarised in Table 1.

The results show (compared to Wild-Type): 1) Most of the mutants in TM 6 do not influence the binding of [^{3}H]-NMS and [^{3}H]-QNB apart from Y381A, which only affects [^{3}H]-NMS, and N382A, which affects both ligands. 2) Y381A and L386A show reduced affinity for acetylcholine. The data suggest that Tyr381 and Leu386 play a role in ligand binding. The function of Asn382 is not clear since N382A may have its effect on ligand binding by altering receptor expression and folding. Further studies are required to investigate this. It seems that the other residues looked at, in TM 6, do not play a major role in ligand binding. [^{3}H]-NMS and [^{3}H]-QNB may bind in different orientations since Y381A discriminates between the two ligands.

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Page, K.M., Curtis, C.A.M., Jones, P.G. *et al.* (1995) *Eur. J. Pharmacol.* 289, 429-437.

Table 1. Summary of data obtained from radioligand binding assays on rat m_1 muscarinic acetylcholine receptor mutants.

	[^{3}H]-NMS binding			[^{3}H]-QNB binding			Acetylcholine affinity[¶]		
	B_{max} (fmol/mg)	pK_d	n	B_{max} (fmol/mg)	pK_d	n	pIC_{50}	n_H	n
Wild-Type	657 ± 42	9.90 ± 0.03	6	656 ± 12	10.80 ± 0.11	3	4.89 ± 0.04	0.93 ± 0.04	5
Y381A		7.08 ± 0.03 [§]	3	143 ± 19	10.63 ± 0.15	4	3.33 ± 0.18 ***	1.11 ± 0.07	3
N382A	N.M.B.			N.M.B.					
I383A	732 ± 94	9.96 ± 0.05	4	948 ± 98	10.89 ± 0.10	4	4.63 ± 0.03 **	0.93 ± 0.03	3
M384A	510 ± 56	10.03 ± 0.03	4	565 ± 81	10.83 ± 0.02	4	4.94 ± 0.05	0.88 ± 0.05	4
V385A	584 ± 14	9.80 ± 0.02	4	1000 ± 16	10.73 ± 0.03	3	4.55 ± 0.11 *	0.86 ± 0.03	5
L386A	791 ± 45	9.97 ± 0.03	3	773 ± 229	10.89 ± 0.01	3	4.30 ± 0.12 **	0.94 ± 0.05	4
V387A	725 ± 41	9.95 ± 0.04	3	744 ±161	10.95 ± 0.04	3	4.85 ± 0.06	0.92 ± 0.05	4

[¶] Hill analysis was used to calculated the pIC_{50} and n_H from data obtained from competition studies using [^{3}H]-NMS, apart from Y381A where [^{3}H]-QNB was used. *t*-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. Wild-Type pIC_{50}; [§] pK_i value obtained by fitting data from competition studies using [^{3}H]-QNB and *N*-methylscopolamine to a one-site model of ligand binding; N.M.B.: No measurable binding. Values are mean ± s.e.mean. pIC_{50} and pK_i values were corrected using the Cheng-Prusoff equation.

285P DIFFERENTIAL REGULATION OF HUMAN α_1-ADRENOCEPTOR SUBTYPES BY PHENYLEPHRINE TREATMENT

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We have recently demonstrated that the receptor agonist, phenylephrine, and the phorbol ester, phorbol-12-myristate-13-acetate (PMA), concentration-dependently down-regulate endogenously expressed α_{1B}-adrenoceptors in the canine MDCK cell line (Yang et al. 1996). We have now used the same design to compare the agonist and PMA-induced regulation of human α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors.

Rat-1 fibroblasts stably expressing the human α_1-adrenoceptor subtypes were provided by Pfizer Central Research (Sandwich, Kent, UK). Subconfluent cells were cultured in the absence and presence of the indicated drugs for the indicated times. After washout of the treatment drugs, radioligand binding experiments were performed with membrane preparations from these cells and [^{3}H]prazosin as the radioligand as described (Yang et al. 1996). Agonist-induced inositol phosphate formation was determined as described (Slivka & Insel 1987). Data are mean ± s.e. mean of n experiments. Statistical significance of differences was assessed by paired t-tests or repeated measures analysis of variances with $P < 0.05$ considered significant.

The expression density of α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors was 798 ± 9, 3311 ± 270 and 400 ± 71 fmol/mg protein (n = 15-16), respectively, in untreated cells. All further data are expressed as % of the paired control values.

Surprisingly incubation with 1-100 nM PMA for 24 h did not significantly alter the expression density of any of the three α_1-adrenoceptor subtypes.

Incubation with 10 nM - 100 µM phenylephrine for 24 h concentration-dependently down-regulated α_{1A}- and α_{1B}-adrenoceptors. Maximal down-regulation was ≈ 60% and 70% (n = 4; $P < 0.05$), respectively, and the threshold concentration for statistically significant down-regulation was ≈ 10 and 0.1 µM phenylephrine, respectively. In time course experiments with 100 µM phenylephrine maximum down-regulation was greates after 8-24 h. In contrast incubation of α_{1D}-adrenoceptor expressing rat-1 cells with phenylephrine for 24 h yielded concentration-dependent increases of receptor density by 79 ± 24% (n = 6; $P < 0.05$). In time course experiments with 100 µM phenylephrine α_{1D}-adrenoceptor upregulation was greatest after 8 h of incubation. Studies on 100 µM phenylephrine-induced inositol phosphate formation were not easy to interpret since some of the pretreatments significantly altered basal inositol phosphate accumulation for some of the subtypes. Overall, however, pretreatment effects on phenylephrine-induced inositol phosphate formation were consistent with those on receptor number for α_{1A}- and α_{1B}-adrenoceptors while no sensitization of α_{1D}-adrenoceptors was detected.

We conclude that regulation of human α_1-adrenoceptors by agonist exposure occurs in a subtype-selective manner. The comparison of the present with our previous data (Yang et al. 1996) indicates that the sensitivity to regulation by PMA may depend on the cellular environment of receptor expression.

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286P *IN VITRO* CHARACTERISATION OF RABBIT URETHRAL α_1 ADRENOCEPTORS

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Rabbit urethral smooth muscle tone is believed to be maintained by the sympathetic nervous system, mainly via postjunctional α_1-adrenoceptors (Chen & Brading, 1992). The aim of the present study was to characterise the α_1-adrenoceptor subtypes involved in the contractile response of the urethra in vitro.

Preparations of proximal female adult urethra were set up under a 1g tension in Krebs solution (37°C, bubbled with 5% CO_2 in O_2) containing idazoxan (0.5 µM) and propranolol (1.0 µM) for determination of isometric contractions.

Cummulative concentration response curves (CRC) were constructed for a range of agonists. The α_{1A} selective compounds A-61603, PNO-49B and SDZ NVI-085 (Knepper *et al.*, 1995; Muramatsu *et al.*, 1995) were potent agonists on rabbit urethra. Agonist rank order of potency (mean pEC_{50}, % max. response) was A-61603 (8.3±0.04, 100) > oxymetazoline (7.1±0.07, 100) > PNO-49B (5.7±0.07, 66) = SDZ NVI 085 (5.7±0.08, 58) > methoxamine (5.1±0.05, 70) = phenylephrine (5.1±0.07, 100). After exposure of tissues to chloroethylclonidine (CEC; 100 µM, 30 min, n=8) the maximal response to phenylephrine was reduced by 25 ± 4% with no change in pEC_{50}. This agonist profile, together with relative resistance to CEC, suggests that responses are mediated by α_{1A} adrenoceptors.

Antagonist data is shown in Table 1, together with binding affinities at cloned α_{1A} adrenoceptors as previously reported by Kenny *et al*, 1996. Prazosin, WB 4101, benoxathian and BMY 7378 antagonised phenylephrine mediated contractions in a competitive manner and Schild analysis yielded slopes not significantly different from unity. High concentrations of (+)-tamsulosin and RS-17053 were insurmountable, therefore pA_2 approximations were made at the lowest concentration tested.

In comparison to binding affinities at cloned human α_{1A} adrenoceptors the low functional affinity estimates for prazosin, WB-4101 and RS 17053 does not support a role for functional α_{1A} adrenoceptors. The low affinity of BMY-7378 does not support a role for α_{1D} adrenoceptors, and the affinity estimate for (+)-tamsulosin is not consistent with an α_{1B} subtype.

Table 1. Antagonist potencies on rabbit urethra (pA_2, slope) and binding affinities (pKi) to cloned human α_{1A} adrenoceptors.

COMPOUND	Rabbit Urethra	α_{1A} binding affinity
Prazosin	7.8 ± 0.1, 0.9	9.7
BMY-7378	5.5 ± 0.1, 0.9	6.2
Benoxathian	8.2 ± 0.1, 0.9	8.9
RS-17053	6.6 (1 µM)	8.6
(+)-Tamsulosin	8.6 (0.01 µM)	8.4
WB-4101	8.4 ± 0.1, 0.8	9.3
5-methyl-urapidil	8.5 ± 0.04, 0.7	8.5

Taken together, these data suggest that contractions of the rabbit urethra are mediated principally, but not exclusively, by the putative α_{1L} -adrenoceptor, consistent with other tissues from rabbit and human lower urinary tract (Ford *et al.*, 1996; Chalmers & Kenny, 1996).

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287P EFFECTS OF DEXMEDETOMIDINE ON LEVELS OF BIOGENIC AMINES AND METABOLITES IN MICE WITH TARGETED INACTIVATION OF THE α_{2C}-ADRENOCEPTOR GENE

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The aim of this study was to examine the effects of dexmedetomidine (d-MED), a specific α_2-adrenoceptor agonist which has high affinity for all three α_2-subtypes, on the levels of monoamine neurotransmitters and their metabolites and histamine in mice genetically manipulated so that they do not express one of the α_2-subtypes, the α_{2C} receptor i.e. so-called α_{2C}-knock out mice (α_{2C}-ko)

The characteristics of the α_{2C}-ko mice, including the method of production have been reported elsewhere (Link *et al.* 1995).The wild type mice were the same strain as the α_{2C}-ko mice (predominantly C57BL/6J with contribution of DBA/2J and 129/Sv strains); they are known to express α_{2C} receptors in several distinct brain regions. Mice were injected with d-MED (doses 5 - 120 μg/kg s.c., controls receiving saline). Each group consisted of 5 - 11 mice, average weight 31.6 ± 0.3 g s.e. mean). One hour after injection, the mice were sacrificed and the following biogenic amines and their metabolites analysed by HPLC with electrochemical detection; noradrenaline, its metabolite, methoxyhydroxyphenyl glycol (MHPG), dopamine, its metabolites dihydroxyphenylacetic acid and homovanillic acid (HVA), serotonin, its metabolite 5-hydroxyindoleacetic acid (5HIAA) and precursor tryptophan. Histamine was detected by fluorometry. Statistical analysis was 2 factor ANOVA (dose; strain) followed by Scheffe's test.

ANOVA did not reveal any dose or strain related differences for any of the biogenic amines but all the metabolites decreased significantly after d-MED (P=0.001). Only MHPG (P=0.009) and HVA (P=0.011) showed strain dependent differences (table 1).

Table 1: Concentrations (± s.e. mean) of MHPG and HVA in mouse brain 1 hour after s.c. injection of d-MED

d-MED (μg/kg)	MHPG (nmol/g) wild	MHPG (nmol/g) α2C-ko	HVA (nmol/g) wild	HVA (nmol/g) α2C-ko
0 (11)	0.26±.02	0.25±.01	1.25±.03	1.17±.04
5 (11)	0.20±.01	0.17±.01#	1.10±.03	1.10±.04
10 (11)	0.18±.01	0.16±.01#	1.09±.04*	1.07±.04
20 (11)	0.16±.01#	0.13±.01#	1.08±.04*	0.99±.03*
30 (11)	0.17±.02#	0.15±.02#	1.04±,04#	0.96±.03#
60 (5)	0.18±.01	0.15±.02#	0.90±.02#	0.90±.03*
120 (5)	0.15±.01#	0.16±.02#	1.03±.02*	0.93±.03*

Significant difference from saline (*P<0.05; #P<0.01)

The dose response curves were similar but α_{2C}-ko mice were slightly more sensitive to the MHPG lowering effect of d-MED and slightly resistant to the corresponding effect to HVA (table 1). This resistance was also seen with 5HIAA. Already at 5 μg/kg d-MED significantly (P<0.05) decreased brain 5HIAA levels by 19.0 ± 0.3 % in wild type mice but 10 μg/kg was needed to get the same extent of decrease in the α_{2C}-ko mice.

In conclusion, α_{2C}-adrenoceptors play only a minor, if any, role in mediating the effects of d-MED to inhibit the release of biogenic amines in mouse brain.

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288P α_2-ADRENOCEPTOR BINDING IN RAT BRAIN FOLLOWING CHRONIC INFUSION OF A PHOSPHOROTHIOATE ANTISENSE OLIGONUCLEOTIDE TO THE α_{2D} SUBTYPE

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At least four subtypes of α_2-adrenoceptor have been identified based on their pharmacology, amino acid sequence and chromosomal location (Mackinnon *et al.*, 1994). Highly selective agonists and antagonists are not yet available, however, it may be possible to target these subtypes using antisense technology. We have investigated whether central administration of an antisense oligonucleotide (ODN) to the RG20 gene that codes for the α_{2D} subtype (Nunes, 1995), affects the binding of the α_2-adrenoceptor antagonist [^{3}H]RX821002 (Hudson *et* al., 1992) in rat brain.

Male Wistar rats (270-300g) were anaesthetised with sodium pentobarbitone (90mg kg^{-1}) and placed in a stereotaxic frame. Osmotic pumps (Alzet, 1µl h^{-1}, 1nmol µl^{-1}) were filled with the ODN (sense or antisense) or vehicle (H_2O). ODN or vehicle were delivered to the right lateral ventricle *via* an implanted (0.92mm caudal to bregma, 1.4mm lateral and 3.5mm below the dura) steel cannulae. The pump was located subcutaneous in the midscapular region, the cannulae fixed using dental cement and the region sutured. Three days after implantation rats were anaesthetised (as above), brains removed, membranes prepared and binding assays performed according to the methods of Hudson *et al.* (1992). These data were analysed using Prism software (GraphPad, 1994).

Saturation binding studies to the brain membranes revealed no significant difference (unpaired Students *t*-test) in [^{3}H]RX821002 binding between the groups of animals with B_{MAX} values of 435.8±16.6, 429.2±23.7 and 448.0±15.1 fmol mg^{-1} protein, for vehicle, antisense and sense respectively (Fig 1, n=5). There was little change in affinity (Fig 1) between groups.

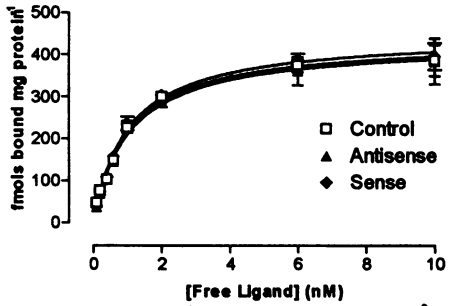

Figure 1. Saturation binding curves for specific [^{3}H]RX821002 binding to brain membranes from vehicle, antisense or sense treated rats. Values are mean ± s.e.mean.

Although [^{3}H]RX821002 labels all α_2-adrenoceptor subtypes, the predominant subtype in the cortex is the α_{2D} (Mackinnon *et al.*, 1994). These data show a 3 day central infusion of phosphorothioate antisense ODN (sequence as described by Nunes, 1995) to the RG20 gene does not reduce the density of [^{3}H]RX821002 binding in the rat whole brain membranes. At present whether the ODN enters the neurones is unknown.

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ER holds a BBSRC CASE studentship with Knoll

289P L-ALANINE SUBSTITUTION OF CONSERVED RESIDUES WITHIN THE PANCREATIC POLYPEPTIDE SEQUENCE SIGNIFICANTLY REDUCES NEUROPEPTIDE Y ACTIVITY AT A Y_2-LIKE RECEPTOR

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Neuropeptide Y (NPY) is a 36 amino acid enteric neuropeptide which exerts antisecretory effects upon rat jejunum mucosa by activating a Y_2-like receptor (Cox & Krstenansky, 1991). Replacement of single residues in the NPY sequence with L-alanine (Ala) allows evaluation of the relative importance of particular amino acids in NPY-receptor activation. Previous studies with rat jejunum and a series of Ala-NPY analogues (using single additions of peptide, 300 nM) showed a greater loss of activity when conserved residues were replaced, especially at the C-terminus (Cox *et al.*, 1994). In the present study we have chosen eight Ala-NPY analogues and performed complete concentration-response curves to establish the relative importance of conserved residues (positions 5, 8, 20, 27, 36) and nonconserved residues (positions 13, 30, 34) for full biological activity.

Preparations of rat jejunum mucosa (male Sprague-Dawley, 200-250g) were voltage-clamped at zero potential (WP Instruments) in Ussing chambers as previously described (Cox & Krstenansky, 1991). Cumulative peptide additions (1 - 300 nM) were made to the serosal compartment once a stable basal short-circuit current (s.c.c.) was achieved and concentration-response curves constructed from pooled data. Peptides analogues were synthesised and purified as described previously (Beck-Sickinger *et al.* 1994). EC_{50} values (and corresponding 95% intervals) were calculated using Graphpad Prism.

Jejunal preparations exhibited a mean basal s.c.c. value of 20.2 ± 2.2 $\mu A.cm^{-2}$ (n=62) and resistance was 56.4 ± 4.1 $\Omega.cm^2$ (n=62). The EC_{50} values obtained from concentration-response curves for each analogue are shown in Table 1 (p and h indicating porcine or human sequences, respectively).

Table 1: Summary of EC_{50} with Ala-substituted NPY analogues.

Analogue	EC_{50} (nM)	95% Confidence Intervals	n
p NPY	37.4	32.2 - 43.5	18
h Ala^5 NPY	678.3	435.8 - 1,100.0	4
p Ala^8 NPY	>1,000		5
h Ala^{13} NPY	540.0	372.8 - 782.4	5
p Ala^{20} NPY	345.7	302.7 - 394.9	4
p Ala^{27} NPY	>1,000		5
p Ala^{30} NPY	45.7	36.8 - 56.8	4
p Ala^{34} NPY	>2,000		4
p Ala^{36} NPY	~1,000		4

Replacement of Gln^{34} by Ala abolished biological activity in this Y_2-like system, while Ala^{30} was well tolerated. The native residues, $Pro^{5, 8, 13}$ and $Tyr^{20, 27}$ are critical in maintaining the correct tertiary structure of NPY thereby providing an optimal configuration for receptor activation, and substitution of amidated Tyr^{36} by Ala also significantly reduces agonist activity.

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290P STABLY TRANSFECTED NEUROPEPTIDE Y Y_1 RECEPTORS EXHIBIT A PYY-PREFERRING PHENOTYPE WHEN EXPRESSED IN A HUMAN COLONIC EPITHELIAL CELL LINE

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The pancreatic polypeptides neuropeptide Y (NPY) and peptide YY (PYY) are potent antisecretory agents in gastrointestinal preparations (Cox, 1993). In order to analyse the interactions between these peptides and a defined receptor subtype in epithelial cells, the human adenocarcinoma cell line, HT-29, has been stably transfected with the cDNA sequence encoding the NPY Y_1 receptor (Holliday & Cox, 1996). In this study we have examined the relative potencies of PYY, NPY and their Y_1 selective, [Leu^{31}, Pro^{34}] substituted analogues (Pro^{34} PYY, Pro^{34} NPY) in one of the resultant clones, called Y1-7, using both functional and binding assays.

Y1-7 cells were grown to confluence on collagen-coated filters (area 0.2 cm^2) and voltage-clamped at 0 mV as described by Cuthbert *et al.* (1987). Peak changes in short-circuit current (s.c.c.) were measured in response to basolateral application of various peptides, and are quoted as $\mu A.cm^{-2}$. Concentration-response curves used to calculate EC_{50} values were constructed from single peptide additions. Displacement binding studies were carried out using Y1-7 membranes with [^{125}I]-PYY (20 pM) as the radioligand. Incubations at 25°C were terminated after 120 min by rapid filtration over GF/B filters soaked in 0.3% polyethylenimine (for details, see Holliday & Cox, 1996). IC_{50} values from quadruplicate experiments were converted to binding affinities (K_i) by the Cheng-Prusoff equation.

Confluent Y1-7 epithelial layers exhibited a basal resistance of 32.8 ± 0.9 $\Omega.cm^2$ and basal s.c.c. of +6.9 ± 0.5 $\mu A.cm^{-2}$ (n = 343), and responded to the addition of a maximal concentration of vasoactive intestinal polypeptide (VIP, 30 nM) with a sustained increase in s.c.c. of +38.6 ± 1.6 (n = 209). Following 30 nM VIP, the maximal responses to porcine (p) PYY (100 nM, -5.6 ± 0.8; n = 11), human Pro^{34} PYY (100 nM, -5.4 ± 0.6; n = 6), pNPY (1 µM, -4.1 ± 0.7; n = 3) and $pPro^{34}$ NPY (1 µM, -4.2 ± 1.2; n = 7) were of similar size and time-profile. EC_{50} values for the inhibition of VIP-elevated s.c.c. revealed the following order of potency: PYY (8.7 nM) > Pro^{34} PYY (32.7 nM) > NPY (102.8 nM) > Pro^{34} NPY (169.7 nM). Binding affinities estimated from displacement studies were as follows: PYY (pK_i= 8.71 ± 0.14, n = 8) ≥ Pro^{34} PYY (8.67, n = 1) ≥ NPY (8.43 ± 0.22, n = 3) > Pro^{34} NPY (8.01 ± 0.21, n = 3). Both PYY and NPY (and their respective Y_1-selective analogues) therefore act as full agonists in Y1-7 epithelial cells expressing the transfected Y_1 receptor. However PYY is markedly more potent than NPY (11.8 fold) in inhibiting VIP-stimulated s.c.c. in this clone. The order of binding affinities also suggests a selectivity for PYY and its analogues that is not apparent when this receptor is transfected into HEK293 cells (Krause *et al.*, 1992).

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291P ISOPRENALINE-STIMULATED cAMP ACCUMULATION AND REPORTER GENE ACTIVATION IN CHINESE HAMSTER OVARY (CHO-K1) CELLS EXPRESSING β_2 ADRENOCEPTORS TO DIFFERENT LEVELS

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Reporter genes can be transfected into cells to provide a simple method of monitoring changes in gene expression (Berger *et al.*, 1988). In this study we have transfected a reporter gene encoding secreted placental alkaline phosphatase (SPAP, Cullen, *et al.*, 1992) into CHOK1 cells (already containing the cDNA for the human ß2adrenoceptor (ß2AR))under the control of a cyclic AMP (cAMP) response element (CRE; 6 copies) The production of SPAP should therefore be regulated by changes in the intracellular levels of cAMP. Here we compare the ability of isoprenaline to stimulate cAMP accumulation in, and SPAP secretion from, two CHOK1 cell lines expressing the ß2 AR.

CHOK1 cells expressing the human ß2 AR to different levels (Specific [^{125}I] Iodocyanopindolol binding: CHO.ß2/4; B_{MAX} 310 ± 70 pmol/mg protein, K_D 92 ± 17pM; CHO.ß2/6, B_{MAX} 50 ± 1 pmol/mg protein, K_D 64 ± 20pM; n=3-6) were secondarily transfected with the SPAP reporter vector and cells selected with hygromycin (50µg/ml). [^{3}H]-cAMP accumulation was measured in [^{3}H]-adenine prelabelled cells (McCrea & Hill, 1993) at 37°C for 15mins in the presence of the PDE inhibitor rolipram (100µM) and data expressed as [^{3}H]-cAMP production as a percentage conversion from total [^{3}H]adenine. SPAP secretion into the incubation medium was measured (10µl aliquot) after 24h incubation with isoprenaline ,by hydrolysis of the chromogenic substrate p-nitrophenol phosphate (monitored at 405nm after 1hour incubation at 37°C; Cullen *et al.*, 1992). Endogenous (non-recombinant) alkaline phosphatase activity in the medium was inactivated by heating samples to 65°C prior to assay.

	Basal	E_{Max}	-Log EC_{50} (M)
a) [^{3}H]-cAMP (% Conversion)			
CHO.ß2/4	3.8±1.4	9.9±2.7	**8.6±0.2**
CHO.ß2/6	0.6±0.2	4.3±1.3	**7.1±0.2**
b) SPAP secretion (mU/ml)			
CHO.ß2/4	0.049±0.003	0.110±0.015	**8.7±0.2**
CHO.ß2/6	0.038±0.001	0.054±0.006	**7.3±0.4**

Table 1. Concentration-response parameters (mean ± s.e.means) for isoprenaline stimulated cAMP accumulation and SPAP secretion (n=3-4).

Concentration-response data for isoprenaline-stimulated cAMP accumulation and SPAP secretion were very similar in the two cell lines (Table 1). These data indicate that measurement of SPAP secretion may be used to monitor pharmacological characteristics of receptors which stimulate cAMP accumulation in recombinant cells. In addition, the SPAP assay does not require destruction of cells so responses can be monitored at intermittent intervals over long periods of time

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292P DESENSITISATION-INDUCED CHANGES IN THE BINDING OF AGONIST AND ANTAGONIST RADIOLIGANDS TO THE TRANSFECTED HUMAN 5-HT_{2A} RECEPTOR

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Receptor desensitisation occurs via several processes, including receptor/G-protein uncoupling and down-regulation (Lohse, 1993). To investigate the processes involved in 5-HT_{2A} receptor desensitisation, we have examined changes in the binding of the partial agonist [^{125}I](±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane ([^{125}I]DOI) and the antagonist [^{3}H]ketanserin to the human 5-HT_{2A} receptor expressed in SH-SY5Y cells associated with functional desensitisation as revealed by the PI response to 5-HT.

SH-SY5Y/5-HT_{2A} cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 5% dialysed foetal calf serum. This was replaced with serum-free DMEM 16 hours prior to assay, after which time cells were exposed to 5-HT. After rinsing in phosphate buffered saline, cells were scraped, collected and membranes prepared. After washing three times by centrifugation (36,000g/4°C) membranes were resuspended in incubation buffer (50mM Tris, 5mM $MgCl_2$, 1mM EGTA, pH7.4). Membranes were incubated with [^{3}H]ketanserin (0.4-8nM) or [^{125}I]DOI (0.5-10nM) for 60 min at 37°C and the reaction terminated by rapid filtration over Whatman GF/C filters. Non-specific binding was determined by co-incubation with 10µM mianserin for [^{3}H]ketanserin and either 10µM 5-HT or 100µM guanylimidodiphosphate (GppNHp) for [^{125}I]DOI. [^{3}H]Inositol phosphate production was measured as previously described (Briddon *et al.*, 1995).

[^{3}H]Ketanserin bound with high affinity to a single population of sites on SH-SY5Y/5-HT_{2A} cell membranes (B_{max}= 474 ± 68 fmol/mg protein, pK_d= 9.08 ± 0.06, n=11, mean±s.e. mean). [^{125}I]DOI binding could be dissociated into a high affinity component sensitive to incubation with the stable GTP analogue GppNHp (B_{max}= 164 ± 29 fmol/mg protein, pK_d= 9.02 ± 0.08, n=9) and a lower affinity component which was insensitive to GppNHp (B_{max}= 215 ± 25 fmol/mg protein, pK_d= 8.31 ± 0.05, n=9). Prior exposure of cells to 10µM 5-HT for 1 hour caused a significant reduction (38%) in the number of [^{3}H]ketanserin labelled sites (B_{max}= 331 ± 8 and 204 ± 15 fmol/mg protein, control and treated respectively, n=3, p<0.01, unpaired t-test), with no change in its affinity for the receptor (pK_d= 9.11±0.11M and 8.98±0.12M). The number of higher-affinity GTP-sensitive [^{125}I]DOI sites was also significantly reduced (69%) without a change in their affinity (B_{max}= 155±24 and 48±10 fmol/mg protein, control and treated respectively, n=3, p<0.05; pK_d= 8.92±0.19M and 8.56±0.11M). In contrast, neither the capacity nor affinity of the lower-affinity GTP-insensitive [^{125}I]DOI binding was changed (B_{max}= 170±27 and 170±45 fmol/mg protein, pK_d= 8.23 ±0.09M and 8.09±0.13M, n=3 control and treated respectively). A parallel reduction in 5-HT_{2A} receptor-mediated [^{3}H]inositol phosphate production was seen after exposure of cells to 5-HT (10µM/1 hour) (E_{max} = 24.0±3.0 and 7.27±0.14 % conversion of [^{3}H]phospholipids to [^{3}H]IP_X in control and exposed cells, respectively, n=3, p<0.05).

Functional desensitisation of the transfected human 5-HT_{2A} receptor is therefore accompanied by a substantial decrease in GTP-sensitive agonist binding and a relatively smaller loss of antagonist labelled receptors. This suggests that both receptor/G-protein uncoupling and receptor down-regulation are implicated in the loss of 5-HT_{2A} receptor function associated with agonist desensitisation.

S.J.Briddon is a Wellcome Prize Student.

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293P INVESTIGATION INTO THE PARADOXICAL DOWN-REGULATION BY ANTAGONISTS OF HUMAN 5-HT$_{2A}$ RECEPTORS EXPRESSED IN SH-SY5Y HUMAN NEUROBLASTOMA CELLS

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Prolonged exposure to an agonist causes a reduction in number and/or function of most G-protein coupled receptors, whereas chronic antagonist exposure results in increased receptor number/function. However, in the case of the 5-HT$_2$ receptor family, down-regulation occurs following prolonged treatment with agonists and, paradoxically, some antagonists also (Sanders-Bush, 1990). In order to investigate the mechanisms underlying this atypical regulation we have assessed the ability of a number of antagonists to regulate human 5-HT$_{2A}$ receptors stably expressed in SH-SY5Y human neuroblastoma cells.

SH-SY5Y cells expressing the human 5-HT$_{2A}$ receptor were grown to confluency in Dulbecco's Modified Eagle's Medium (DMEM) with 5% dialysed foetal calf serum (dFCS), then transfered into either pure DMEM (control) or DMEM plus drug (treated) for 24 hours. In the case of 1 hour treatments, drug was added 23 hours later. Cells were washed and incubated in pure DMEM for 30 minutes, then harvested by scraping. Membranes were prepared by homogenisation in 5mMTris/EDTA (pH7.4) and washed three times by high speed centrifugation with a 30 minute incubation at 37°C. Following re-suspension in incubation buffer (50mM Tris, 5mM $MgCl_2$, 1mM EGTA and 1mg/ml ascorbic acid, pH 7.4), membranes were incubated with [^{3}H]ketanserin (0.2-6nM) for 60 minutes at 37°C. Reactions were terminated by rapid filtration through Whatman GF/C filters. Non-specific binding was determined using 10μM mianserin.

Cells were treated with serotonergic antagonists at concentrations ten times their Ki to ensure equivalent receptor occupancy (approximately 90%). No significant decrease in specific [^{3}H]ketanserin binding was observed following 24 hour treatment with 30nM spiperone, 3nM flupenthixol, 25nM amitriptyline or 70nM methysergide (n≥ 3 in each case). Ketanserin (30nM and 100nM) also failed to cause significant down-regulation after 24 hours (n=3). Although 24 hour treatment with cyproheptadine at 30nM caused a complete loss of specific binding (n=2), further experiments at lower concentrations (10 and 3 nM, n≥3 in each case) did not cause any significant reduction in receptor number but did cause a concentration dependent decrease in [^{3}H]ketanserin binding affinity. This suggests the initial observations were due to residual drug, despite a stringent wash-out procedure, and not a true regulatory phenomenon. Despite being reported to up-regulate receptor levels (Carmona *et al.*, 1993), SR46349B (2nM) did not alter [^{3}H]ketanserin binding in these cells. Mianserin (100nM) and clozapine (100nM) both caused a significant down-regulation of receptor number (Bmax=55±4% of control n=5, p<0.01; and Bmax=70±7% of control, n=4, p<0.05, respectively). Affinity for [^{3}H]ketanserin was not altered by clozapine, indicating this effect is not due to residual drug. No change in Bmax was observed following 1 hour treatment with 100nM clozapine. 24 hour treatment with 10nM clozapine did not cause significant down-regulation of receptor number, however 1μM clozapine caused a large decrease in [^{3}H]ketanserin affinity (K_d>10nM, n=2) preventing accurate estimation of Bmax.

These results demonstrate that the 5-HT$_{2A}$ receptor stably expressed in SH-SY5Y cells can be down-regulated following prolonged treatment with certain antagonists. In the case of clozapine, this effect is time and concentration dependent. Our results also indicate that the ability of an antagonist to cause down-regulation of the human 5-HT$_{2A}$ receptor is not simply due to occupation of the receptor.

Alex Mitchell is an MRC Research Student.

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294P DIFFERENTIAL EFFECTS OF CHRONIC CLOZAPINE AND HALOPERIDOL ON 5-HT$_{2C}$ AND 5-HT$_7$ RECEPTOR LEVELS IN THE RAT BRAIN

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The atypical antipsychotic drug clozapine (CLOZ) has high affinity at several 5-HT receptors (Roth et al., 1994). To gain insight into the mechanism of action of this drug we have investigated the effects on receptor density of two of these subtypes, 5-HT$_{2C}$ and 5-HT$_7$, following chronic administration of either CLOZ or the typical antipsychotic haloperidol (HAL), which has much lower affinity at these receptors (Roth et al., 1994).

Adult male hooded Lister rats (300-350g) were held under a 12 h light-dark cycle with food and water *ad libitum*. Rats were randomly allocated to one of three groups (each n=12) receiving either CLOZ (20 mg kg^{-1}), HAL (0.3 mg kg^{-1}) or vehicle (0.1M HCl, 0.09M lactic acid in 0.154M saline) i.p. once daily for 21 d. 24-26 h after the last injection rats were decapitated and the hypothalamus and cortex (excluding frontal cortex) removed for radioligand binding, striatum (STR) and nucleus accumbens (NA) for HPLC and trunk blood collected for plasma corticosterone radioimmunoassay. 5-HT$_7$ receptor density was measured in pooled hypothalami using [^{3}H] 5-HT (with 3μM pindolol to prevent 5-HT$_{1A}$ and 5-HT$_{1B}$ binding, modified from Sleight et al., 1995). 5-HT$_{2C}$ receptor levels were determined by [^{3}H] mesulergine binding (Fone et al., 1996). Tissue levels of DA and HVA were measured in STR and NA using HPLC-ECD.

Chronic administration of CLOZ, but not HAL, significantly reduced the B_{max} of 5-HT$_{2C}$ cortical binding to 41% of vehicle (p<0.01) without altering the K_D. However, neither CLOZ nor HAL altered the B_{max} or K_D of hypothalamic 5-HT$_7$ binding. HAL reduced the HVA/DA ratio in both the STR and NA (to 48%, p<0.001, and 67% of vehicle, p<0.01, respectively), whereas CLOZ reduced this ratio only in NA (to 79% of vehicle, p<0.05). Additionally, CLOZ, but not HAL, significantly elevated plasma corticosterone (156% above vehicle, p<0.05).

As reported previously, chronic CLOZ had region-specific effects on dopamine function, reducing dopaminergic activity in the mesolimbic system without effecting dopamine turnover in nigrostriatal terminals, consistent with the lack of extrapyramidal effects of this drug. HAL, which produces both antipsychotic and extrapyramidal side effects, reduced dopaminergic activity in both mesolimbic and nigrostriatal terminal regions. In addition to its differential effects on dopaminergic function, this study shows that chronic CLOZ also selectively regulates certain 5-HT receptors in different brain regions, down-regulating cortical 5-HT$_{2C}$ receptors but having no effect on 5-HT$_7$ binding in the hypothalamus. These findings suggest that the 5-HT$_{2C}$ receptor may mediate some of the effects of CLOZ, but would appear to exclude the 5-HT$_7$ receptor as a contributing factor in the long term effects of CLOZ. The lack of altered 5-HT$_7$ receptor density in the hypothalamus also argues against an involvement of this subtype in the central regulation of corticosterone release.

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Table 1. Effect of chronic treatment (mean ± s.e.mean). n = 12 except † n =11. * p<0.05, **<0.01, ***p<0.001. Fisher's Protected LSD following ANOVA.

	cortical 5-HT$_{2C}$ B_{max} (fmol mg protein^{-1})	hypothalamic 5-HT$_7$ B_{max} (fmol mg protein^{-1})	plasma corticosterone (ng ml^{-1})	striatal HVA/DA ratio	nucleus accumbens HVA/DA ratio
vehicle	136.0±6.5	59.7±11.0	48.5±4.1	†0.075±0.005	†0.072±0.004
haloperidol	109.6±16.0	53.7±1.2	40.9±6.6	†0.036±0.004***	†0.048±0.003**
clozapine	55.6±4.7**	51.4±5.9	75.4±10.3*	0.063±0.005	†0.057±0.005*

295P [^{3}H]GR125743 LABELS BOTH HIGH AND LOW AFFINITY STATES OF h5-HT$_{1B}$ AND h5-HT$_{1D}$ RECEPTORS

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Despite there being several potent radioligands for the labeling of 5-HT$_{1B}$ / 5-HT$_{1D}$ receptors, they have all been agonists. [^{3}H]GR125743 (Amersham International) is the first commercially available antagonist for the labeling of these sites *in vitro*. In the present study, using [^{3}H]5-HT and [^{3}H]GR125743, we have compared the receptor binding of agonists and antagonists in cell lines expressing h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors.

Recombinant h5-HT$_{1B}$ and h5-HT$_{1D}$ receptors were stably expressed in CHO cells (see Watson *et al.* 1996). Membranes (approx. 1x10^6 cells/incubation well) were resuspended in Tris buffer (50mM Tris HCl, 10mM $MgCl_2$, 6μM ascorbate, 0.6μM pargyline) and incubated with either [^{3}H]5-HT [4nM] or [^{3}H]GR125743 [1nM], at 37°C for 45 min. Incubations were terminated by filtration over GF/B filters. Non-specific binding was defined using 10μM 5-HT.

In saturation binding studies [^{3}H]GR125743 labeled almost 15 times as many sites in the h5-HT$_{1B}$ membranes as [^{3}H]5-HT (Bmax = 45.9 ± 17.6 and 3.2 ± 1.2 pmol/mg protein respectively, n = 3), but a similar number of sites in the h5-HT$_{1D}$ membranes (Bmax = 9.9 ± 0.9 and 8.7 ± 1.6 pmol/mg protein respectively, n = 3). At the h5-HT$_{1B}$ receptor, agonists competed with greater potency against [^{3}H]5-HT than [^{3}H]GR125743, whereas at the h5-HT$_{1D}$ receptor these potencies were similar (Table 1). Antagonist competition curves were similar with both radioligands in both cell lines.

Table 1. Competition for binding to 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors labelled with [^{3}H]5-HT and [^{3}H]GR125743.

	5HT1B [^{3}H]5-HT pKi	5HT1D [^{3}H]5-HT pKi	5HT1B [^{3}H]GR125743 pKi	5HT1D [^{3}H]GR125743 pKi
5-HT*	8.4±0.1	8.4±0.1	6.7±0.1	8.2±0.1
5-CT*	8.7±0.1	8.9±0.1	6.7±0.1	8.3±0.1
L-694247*	9.0±0.1	8.7±0.1	7.7±0.1	8.2±0.1
Methio.#	8.0±0.1	7.9±0.1	7.6±0.1	7.5±0.1
GR127935#	8.6±0.1	8.6±0.1	8.3±0.1	8.3±0.1
GR125743#	8.7±0.1	8.5±0.1	8.1±0.1	8.3±0.1

*=agonists, #=antagonists values are mean(n≥3) ± s.e.mean .

We attributed the differences at the h5-HT$_{1B}$ receptor to [^{3}H]5-HT binding mainly to the high affinity state of the receptor, whereas [^{3}H]GR125743 labels both high and low affinity states. This was further substantiated by the effect of adding GTPγS to the incubation. GTPγS (>1μM) reduced specific binding of [^{3}H]5-HT (38.3±4.21% of controls) with less effect on binding of [^{3}H]GR125743 (93.3±1.11 of controls). At the h5-HT$_{1D}$ receptor, GTPγS reduced specific binding by both radioligands (49.8±3.58% and 64.2±1.72% of controls for [^{3}H]5-HT and [^{3}H]GR125743 respectively).

In conclusion, [^{3}H]GR125743 labels both agonist high and low affinity states of the h5-HT$_{1B}$ receptor of which the latter predominate; whereas at the h5-HT$_{1D}$ receptor [^{3}H]GR125743 preferentially labels the agonist high affinity state.

Watson J., Burton M.J., Price G.W. *et al.* (1996) Eur. J. Pharmacol. 314, 365-372

296P FUNCTIONAL CHARACTERISATION OF AGONISTS AT SEROTONIN 5-HT$_{1A}$ RECEPTORS

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The human 5-HT$_{1A}$ serotonin receptor has been expressed in a recombinant CHO cell line (CHO-5-HT$_{1A}$) and the ligand binding properties of the receptor characterised using the binding of [^{3}H]8-OH-DPAT and [^{3}H]spiperone. Agonists showed higher affinities when competing against [^{3}H]8-OH-DPAT than [^{3}H]spiperone (Sundaram *et al.* 1993) and we now seek to understand agonism at this receptor in more detail.

Receptor-stimulated [^{35}S]-GTPγS binding is a quantitative method of characterising the functional activity of some G protein-coupled receptors, see for example (Gardner *et al.* 1996). We have therefore examined whether ligands with a higher affinity for the state defined by the agonist [^{3}H]8-OH-DPAT provided an agonist profile in a [^{35}S]-GTPγS binding assay.

Washed membranes were prepared from CHO-5-HT$_{1A}$ cells and stored as in Gardner *et al.* (1996). For the [^{35}S]-GTPγS binding assay 50μg of membrane protein was incubated in 20mM HEPES buffer pH 7.5 plus 100mM NaCl, GDP (3μM) and drug(s) in a volume of 0.9ml for 30 minutes at 30°C. 100μl of [^{35}S]-GTPγS (1nM) was then added and incubated for a further 20 minutes. Bound radiolabel was collected by rapid filtration and radioactivity determined. Dose response curves for agonists were used to determine the EC$_{50}$ and maximal response (Table 1.). Maximal responses were expressed relative to that given by 10μM serotonin, which is typically ~100% stimulation over the basal response.

The results of these experiments show that agonist stimulation of [^{35}S]-GTPγS binding can be used to determine potencies and maximal activities for agonists at the 5-HT$_{1A}$ receptor. Partial agonism can also be determined with this protocol. Compounds that showed a higher affinity in ligand binding assays versus [^{3}H]8-OH-DPAT all exhibited agonism in the present functional system.

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Table1. Stimulation of [^{35}S]-GTPγS binding by serotonergic agonists acting at serotonin 5-HT$_{1A}$ receptors. The pEC50 values are mean values ± SEM (n ≥ 3). Percentage stimulation was determined relative to the agonist response defined by 10μM serotonin.

	pEC$_{50}$		Relative Stimulation
Buspirone	7.18±0.05	(3)	57.6
5 Carboxamidotryptamine	8.89±0.03	(3)	74.2
Lisuride	8.98±0.12	(3)	94.7
8-OH-DPAT	7.97±0.09	(7)	67.9
Serotonin	7.63±0.06	(3)	100

297P VISUALISATION OF 5-HT$_1$ RECEPTORS USING [^{35}S]GTPγS BINDING IN RAT BRAIN

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[^{35}S]GTPγS binding is a method which can be used to measure G protein activation and can be applied to tissue homogenates as well as whole tissue sections. This technique was used to study the regional distribution of functional 5-HT$_1$ receptors in rat brain and to characterise these sites using selective 5-HT receptor antagonists.

Autoradiographic studies were performed according to Sim et al. (1995) with minor modifications. [^{35}S]GTPγS binding in rat cortex homogenates is described in Watson and Bruinvels (this meeting). The effect of increasing concentrations of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (DPAT) and the 5-HT$_{1A/1B/1D}$ receptor agonist SKF 99101 (3-(2-dimethylaminoethyl) 4-chloro-5-propoxyindole hemi-fumarate, Hatcher et al. 1995) on [^{35}S]GTPγS binding was studied in rat cortex homogenates. Stimulation occured in a concentration-dependent manner yielding pEC$_{50}$ and Emax values (expressed as a percentage of basal levels) of 6.9±0.2 / 121±2% (DPAT) and 6.2±0.1 / 125±1% (SKF 99101). In rat brain sections both agonists (10 and 100 μM, respectively) induced stimulation in hippocampus (HIPPO) and entorhinal cortex (ENTCX, see Table) and these effects were blocked using the selective 5-HT$_{1A}$ receptor antagonist WAY 100635 (1 μM) but not by the selective 5-HT$_{1B/1D}$ receptor antagonist SB 216641 (1 μM, see Table, N-[3-(2-dimethylamino)ethoxy-4-methoxy-phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxydiazol-3-yl)-(1,1'-biphenyl)-4-carboxamide, Price et al. 1996). SKF 99101 increased [^{35}S]GTPγS binding in substantia nigra (SN) and caudate-putamen (CPU) but this effect remained unchanged in the presence of WAY 100635 or SB 216641, although the latter compound showed a trend to block stimulation in SN. Interestingly, WAY 100635 did not stimulate [^{35}S]GTPγS binding in any brain region whereas SB 216641 (1μM) produced a small but significant increase in ENTCX (121±3%).

Table: [^{35}S]GTPγS binding (±SEM) expressed as percentage of basal stimulation in various rat brain regions

	DPAT	SKF 99101	DPAT / WAY 100635	SKF 99101/ WAY 100635	SKF 99101/ SB 216641
HIPPO	145±13*	267±41*	97±9†	122±5†*	216±48*
ENTCX	130±8*	253±46*	98±1†	127±8#*	231±48*
SN	141±21	209±30*	86±9	156±24	148±21
CPU	97±4.6	145±12*	106±11	130±2*	160±21*

*p<0.05 stimulation above basal; †p<0.05 antagonism of stimulation, #p=0.051 (ANOVA, n≥4)

These results suggest that 5-HT$_{1A}$ receptors mediate [^{35}S]GTPγS stimulation in rat hippocampus and entorhinal cortex. Surprisingly, DPAT has a lower efficacy than SKF 99101 in most brain regions. The latter compound also produced [^{35}S]GTPγS binding in substantia nigra and caudate-putamen. However, this is possibly not 5-HT$_{1B/1D}$ mediated since SB 216641 could not block this effect.

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298P CLONING, EXPRESSION AND PHARMACOLOGY OF A TRUNCATED SPLICE VARIANT OF THE HUMAN 5-HT$_7$ RECEPTOR

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The human 5-ht$_7$ receptor gene encodes a G protein coupled receptor with a predicted size of 445 amino acids (Bard et al., 1993). We have recently isolated a human 5-HT receptor from a human placental cDNA library (Clontech) using PCR with primers designed from the sequence of the human 5-ht$_7$ receptor gene (Bard et al., 1993). This novel receptor is identical to the previously published human sequence, but with a truncation in the carboxy terminus. The sequence indicates that this receptor mRNA contains a 5 base-pair insertion which introduces an 'in frame' stop codon and shortens the carboxy tail of the receptor by 13 amino acids. Interestingly, a similar splice variation has been reported for the rat 5-ht$_7$ receptors (Ruat et al. 1993; Lovenberg et al., 1993). The amino acid sequence of the C-terminal amino acids of each rat and human 5-ht$_7$ receptor variant are shown in Figure 1.

Figure 1: Comparison of rat and human 5-ht$_7$ receptor carboxy-terminal amino acid sequences.

	421	431	441
Rat-long:	ALKLAERPER	SEFVLQNSDH	CGKKGHDT*
Rat-short:	ALKLAERPER	SEFVL*	
Human-long:	ALKLAERPER	PEFVLQNADY	CRKKGHDS*
Human-short:	ALKLAERPER	PEFVL*	

When expressed in HEK 293 cells, the human short splice variant 5-ht$_7$ receptor (7.3 pmol·mg^{-1} protein) binds to 5-HT receptor agonists and stimulates intracellular cAMP accumulation with the following rank order of potency (pEC$_{50}$, n=3-4): 5-CT (8.7±0.11)>5-Methoxytryptamine(8.1±0.20)>5-HT(7.5±0.13)> Tryptamine(5.6±0.36)>8-OH-DPAT(5.3±0.28)>6-methoxytrypt-amine(5.0±0.06). The pharmacological profile of the short splice variant is similar to the published (Bard et al., 1993) long-form of the human receptor (Table 1).

The human tissue distribution of the two splice variants was studied using PCR primers specific to each variant. Both forms of the receptor were found to be expressed in the brain, heart, and regions of the intestinal tract. These data suggest that both the rat and human 5ht$_7$ receptor exist in two isoforms, a long- and truncated-splice variant, with similar operational and distributional characteristics. At present, the physiological significance of the splicing variation is unknown.

Table 1: Antagonist affinities (pKi) at human recombinant 5-ht$_7$ receptor isoforms expressed in COS-7 or HEK 293 cells.

	Long form	Short form
Methiothepin	8.43	8.87 ± 0.08
Metergoline	8.19	8.45 ± 0.06
Pirenperone	ND	8.19 ± 0.08
Methysergide	7.08	7.57 ± 0.13
Spiperone	6.95	7.65 ± 0.08
Mesulergine	7.74	7.87 ± 0.12
Ketanserin	5.87	6.44 ± 0.17
Haloperidol	ND	6.30 ± 0.11
Pindolol	<5	<5
Miancerin	ND	7.32 ± 0.15

Competition binding assays were performed with [^{3}H]5-HT at the human long 5-ht$_7$ receptor expressed in COS cells (Bard et al., 1993), and [^{3}H]5-CT at the human short 5-ht$_7$ receptor expressed in HEK 293 cells as described by To et al. (1993). Data shown are mean ± s.e. mean, n=3-4 .

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299P PHARMACOLOGICAL CHARACTERISATION OF A CLONED BOVINE P2Y$_1$-PURINOCEPTOR TRANSFECTED INTO JURKAT CELLS

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In the absence of potent and selective antagonists, pharmacological sub-classification of P2-purinoceptors has been based largely on agonist potency orders. Misleading potency orders may occur as a result of agonist hydrolysis by ecto-ATPases (Kennedy & Leff, 1995), receptor desensitisation by endogenously released ATP (Filtz *et al.*, 1994) and the presence of multiple receptors (Dainty *et al.*, 1991). The use of transfected cloned receptors may circumvent these problems allowing definitive potency orders to be obtained. This study describes the pharmacological characterisation of a cloned P2Y$_1$-purinoceptor (Henderson *et al.*, 1995).

Changes in $[Ca^{2+}]_i$ in cell suspensions(1 x 10^6 cells ml^{-1}) following loading with FURA-2 were measured by standard fluorescent techniques. Agonist concentration-effect (E/[A]) curves were constructed in log unit increments. Cuvettes of cells (2 ml) were used for single response determinations. When used, apyrase (2U ml^{-1}) was pre-incubated during Fura-2 loading (45 min) to break down endogenous ATP. The ectoATPase inhibitor ARL 67156 (300μM; Kennedy & Leff, 1995) was added 15s before the agonist. Responses were expressed as a % of that to 10μM 2-MeSATP.

Apyrase pre-treatment caused a significant increase in the maximum response to 2-MeSATP and AMP but had little effect on potency. All subsequent experiments used cells pretreated with apyrase. ARL 67156 had no effect on responses to 2-MeSATP, ATP or ATPγS. Generation of E/[A] curve data yielded the following potency order: 2-MeSADP > 2-ClATP > ADP = 2-MeSATP > ATPγS, ATP > AMP with ATP and AMP being partial agonists (Fig 1). Operational model fitting using the comparative method (Leff *et al.*, 1990) of the ATP and AMP data yielded agonist affinity (pK_A) estimates of 5.44 ± 0.09 and 4.15 ± 0.16 (mean ± s.e., n=3) respectively.

The results of this study suggest that tonic desensitisation of transfected P2Y$_1$-purinoceptors by endogenous ATP/ADP occurs in these cells but

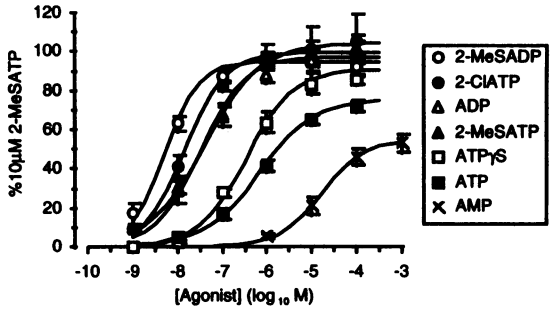

Fig 1. Agonist E/[A] curves (mean ± s.e., n=5-8)

is alleviated by pre-incubation with apyrase. Under the conditions used ecto-ATPase activity does not affect agonist activities and therefore the potency order obtained is definitive for this receptor. In addition, the low efficacies of ATP and AMP in this system, allowed us, to our knowledge, to make the first functional estimates of agonist affinities at P2Y$_1$-purinoceptors. Such estimates provide more robust receptor classification information than agonist potency orders and should prove useful in future classification studies. These data also suggest that chemical modification of ATP and/or AMP may lead to the development of selective P2Y$_1$-purinoceptor antagonists.

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300P REGULATION OF CYCLIC AMP BY ATP, ENDOTHELIN AND HISTAMINE IN PRIMARY CULTURES OF RAT BRAIN CAPILLARY ENDOTHELIAL CELLS

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The specialised endothelial cells of brain microvasculature are important in the development and treatment of a variety of brain disorders. These cells are controlled by cell surface receptors for local mediators such as histamine, endothelin and nucleotides such as ATP (Purkiss et al, 1994; Vigne et al, 1990; Nobles et al, 1995). These studies have largely concentrated on the control of phospholipase C and Ca^{2+} in these cells. Here, we have investigated the control of cyclic AMP by these mediators in primary cultures of brain endothelial cells.

Brain capillaries were prepared and highly purified endothelial cells were grown from these capillaries, by adaptations of the procedure of Rubin et al (1991). Cells were used, without passage, in 96 well plates. Cells were stimulated for 10 minutes with agonists as indicated, with forskolin present during this time where appropriate. Cyclic AMP was measured in cell extracts using a protein binding procedure (Brown et al, 1971).

In the absence of forskolin the addition of 300 μM ATP stimulated the accumulation of cyclic AMP, while histamine (300 μM) and endothelin-1 (100 nM) had no effect. The presence of 5 or 10 μM forskolin produced substantial and similar rises in cyclic AMP content. ATP (300 μM), present with forskolin, stimulated a much enhanced accumulation of cyclic AMP, while the presence of histamine (100 μM) and endothelin-1 (100 nM) reduced the forskolin-elevated level of cyclic AMP (Table 1).

Table 1

Forskolin	Basal	ATP	Histamine	Endothelin-1
0	0.51 ± 0.08	1.32 ± 0.12	0.58 ± 0.01	0.46 ± 0.01
5 μM	2.94 ± 0.54	7.93 ± 0.99	1.07 ± 0.03	0.72 ± 0.03
10 μM	1.63 ± 0.58	8.17 ± 0.41	0.82 ± 0.03	1.04 ± 0.08

Data are mean ± s.e.mean, n = 3, of cyclic AMP per well (pmols).

To ask whether the stimulation of cyclic AMP by ATP was due to release of prostaglandins, acting on adenylyl cyclase linked prostaglandin receptors, the stimulations with ATP were repeated in the presence of indomethacin (1 μM, 15 min prior to, and during, stimulation with ATP). Indomethacin produced a small (about 20 %) but significant reduction in the effects of ATP. These results show that ATP increases, while histamine and endothelin-1 decrease, the levels of cyclic AMP in forskolin stimulated brain capillary endothelial cells. The receptor types responsible for these effects are unknown.

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301P REGULATION OF INOSITOL POLYPHOSPHATE ACCUMULATION BY ENDOTHELIN-1, HISTAMINE AND NUCLEOTIDES IN PRIMARY CULTURES OF RAT BRAIN ENDOTHELIAL CELLS

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The blood brain barrier is formed by the specialised endothelium cells of the cerebral capillaries. In spite of the fact that these cells are important in a variety of pathological conditions relatively little is known about their biochemistry and physiology. Previous studies on these cells have shown that they possess cell surface receptors for a variety of important endogenous agonists e.g. histamine, endothelin and certain nucleotides (Vigne et al, 1990 & 1994). The present study addresses the consequences of agonist binding, in primary cultures of rat brain endothelial cells, at some of these receptors on the activation of phospholipase C and on intracellular calcium concentration ($[Ca^{2+}]_i$).

Adult female wistar rat brain capillaries were prepared, and highly purified endothelial cells grown from these capillaries by adaptations of the method described by Rubin (1991). Cells were used as primary cultures either grown in 96 well plates for total inositol phosphate ($[^3H]$-$InsP_x$) measurements or grown on modified 35mm petri dishes for the measurement of $[Ca^{2+}]_i$ using fura-2. Cells were labelled 24-48 hr with $[^3H]$-inositol for the $InsP_x$ assay and then stimulated for 16min with agonist. $[Ca^{2+}]_i$ was measured as previously described for these cells (Albert et al, 1996).

A previous report from this laboratory has shown that histamine (100μM, HIST), ATP (100μM), UTP (300μM), ADP (100μM) and 2-methylthio ATP (2-MeSATP; 30μM) all caused a transient increase in $[Ca^{2+}]_i$, consistent with a release of Ca^{2+} from intracellular stores, while endothelin-1 (ET-1; 100nM) was without effect on $[Ca^{2+}]_i$ (Albert et al, 1996).

Using total $[^3H]$-$InsP_x$ accumulation as a measure of phospholipase C (PLC) activation we found that histamine and 2-MeSATP, at the above concentrations, failed to cause any increase over basal levels (Table 1). In contrast ATP, UTP, ADP and ET-1 caused significant increases in total $[^3H]$-$InsP_x$ accumulation (Table 1).

Table 1-Fold stimulation of total $[^3H]$-$InsP_x$ accumulation.

ATP	ADP	UTP	ET-1	2-MeSATP	HIST
2.7±0.3	2.0±0.4	3.0±0.7	4.2±0.5	1.2±0.1	1.0±0.1
n=10, *	n=4, *	n=7, *	n=6, *	n=7, ns	n=3, ns

Data are mean ± s.e. mean, fold increases in $[^3H]$-$InsP_x$ over basal. *= $P<0.05$; ns = not significant, Student's t-test.

These results suggest that ATP, ADP and UTP release Ca^{2+} from intracellular stores as a result of PLC-activation. The results obtained with 2-MeSATP and HIST may indicate the existence of a separate signalling pathway for Ca^{2+} release from stores or may be the response to a transient increase in Ins(1,4,5) P_3 not detected by the assay used. The significance of the failure of ET-1 to increase $[Ca^{2+}]_i$ while inducing a large increase in $[^3H]$-$InsP_x$ accumulation requires investigation at the level of Ins(1,4,5) P_3. Experiments are continuing to address these issues.

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302P ATP-DEPENDENT INCREASES IN INTRACELLULAR CALCIUM CONCENTRATION IN HEK293 CELLS

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Extracellular ATP has been shown to elicit cellular responses as a consequence of raising intracellular calcium concentration ($[Ca^{2+}]_i$) in a *Xenopus* renal epithelial cell line (Mori *et al.*, 1996). We have investigated the mechanism of $[Ca^{2+}]_i$ elevation produced by purinergic receptor agonists in a human embryonic kidney cell line (HEK293), using fura-2 based imaging and whole cell voltage clamp electrophysiology.

HEK293 cells were plated onto glass coverslips 2h prior to loading with fura-2/AM (6μM) for 30 minutes in buffer solution (composition in mM: NaCl, 138; $MgCl_2$, 1; $CaCl_2$, 5; KCl, 5; HEPES, 10 and glucose, 10; adjusted to pH 7.40 with NaOH). Cells were washed in dye free buffer for 30 minutes. $[Ca^{2+}]_i$ was measured using microspectrofluorimetric imaging in a total of 145 cells (mean basal $[Ca^{2+}]_i$=118±3nM). Agonist induced responses of ≥ 40nM were used in the present analysis. In separate experiments cells were also examined using whole cell voltage clamp electrophysiology. Cells were voltage clamped at -70mV to study the effects of purinergic agonists. The intracellular solution was as follows (composition in mM: CsCl, 135; $MgCl_2$, 1; HEPES, 10; diTrisphosphocreatine, 14; MgATP, 3.6; BAPTA, 15; and 50U/ml creatine phosphokinase adjusted to pH 7.1 with CsOH). All experiments were conducted at room temperature (22°C) and results are given as the mean ± s.e.mean

Application of 3μM 2-methylthioATP (2-MeSATP), but not ATP (3μM, n=64 cells), produced increases in basal $[Ca^{2+}]_i$ in HEK293 cells ($\Delta[Ca^{2+}]_i$=70±5nM, n=24 cells). Both the $P2_Y$ receptor specific 2-MeSATP and ATP produced changes in $[Ca^{2+}]_i$ at 10μM (2-MeSATP, $\Delta[Ca^{2+}]_i$=88±3nM, n=101 cells; ATP, $\Delta[Ca^{2+}]_i$=62±4nM, n=20 cells, $P<0.05$). However, uridine trisphosphate (UTP) and the $P2_X$ receptor preferring agonist α,β-methyleneATP (α,β-MeATP) had no effect on basal $[Ca^{2+}]_i$ at concentrations up to 30μM. These results suggest a rank order of potency for the purinergic agonists of 2-MeSATP>ATP>UTP=α,β-MeATP.

2-MeSATP (10μM)-evoked increases in $[Ca^{2+}]_i$ were present but reduced by 72±6% ($P<0.05$, n=32 cells) when experiments were conducted in solutions without added calcium and containing EGTA (100μM). In addition thapsigargin (1μM) reduced 2-MeSATP (10μM)-induced increases in $[Ca^{2+}]_i$ by 77±5% ($P<0.05$, n=22 cells).

Under conditions of whole cell voltage clamp recording with pipette solutions containing BAPTA (15mM), no inward currents were observed upon application of ATP (10μM).

Based upon the observed activity of ATP and related analogues (Barnard *et al.*, 1994), the present results suggest the involvement of a $P2y_1$ receptor in mobilising calcium from intracellular stores in HEK293 cells.

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303P THE RELATIONSHIP BETWEEN RESPONSES OF A REPORTER GENE LINKED TO THE ADENOSINE A_1 RECEPTOR AND THE UNDERLYING CHANGES IN CYCLIC AMP

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Reporter genes encode the synthesis of an easily-measurable protein under the control of a genetic element which responds to changes in intracellular second messengers. We have used a cell line expressing both the human adenosine A1 receptor and a cyclic AMP response element controlling the synthesis of secreted placental alkaline phosphatase (SPAP). Although A1 receptors have been shown to inhibit both adenylate cyclase and an associated reporter gene (Castanon and Spevak, 1994), it is not known how faithfully changes in reporter secretion follow changes in cAMP levels.

Chinese hamster ovary cells were stably transfected with the gene for the human adenosine A1 receptor and a construct coding for SPAP under the control of 6 tandem cAMP response elements. Cells were grown to confluence, pre-incubated for 15min with adenosine agonists before addition of 10μM forskolin, and incubated for 5h at 37C. SPAP was measured by production of coloured product from phosphatase substrate (Sigma). In parallel experiments cAMP was measured using a commercial assay (Amersham). Potency values are shown as mean IC_{50} [95% confidence limits] from *n* experiments.

Forskolin stimulated SPAP output from the cells, and this was inhibited by adenosine agonists with an order of potency consistent with A1 receptor activation. Thus, the most potent compounds tested were the non-selective agonist, N-ethylcarboxamidoadenosine (NECA; 3.5[2.2-5.8]nM, *n*=21), and the A1-selective agonists cyclopentyladenosine (1.3nM, *n*=2) and GR79236 (Gurden et al., 1993; 1.7[0.4-7.0]nM, *n*=4); the least potent compounds tested were agonists selective for A2a and A3 receptors (respectively CGS21680 ([[2-[4-(2-carboxyethyl) phenyl]ethyl]amino]-N-ethylcarboxamido-adenosine; 350[71-1700]nM, *n*=4) and iodobenzyl-N-methyl-carboxamidoadenosine (33[11-100]nM, *n*=4). At least 3h delay after forskolin addition was necessary before measurable quantities of SPAP were produced. However, a time-limited stimulation of cAMP (induced by forskolin and terminated by addition of NECA) caused subsequent SPAP production which was approximately linear with the duration of the stimulation from 15 - 90min.

Forskolin-stimulated cAMP levels reached a maximum at 30min and remained elevated for a further 2.5h. The forskolin-induced increase was concentration-dependently inhibited by NECA at each time-point studied with similar potency values. However, the potency with which NECA inhibited cAMP (15[11-21]nM, *n*=4) was about 4-fold lower than for inhibition of SPAP, indicating amplification of the reponse between second messenger and reporter gene.

One interesting discrepancy between SPAP reporter and cyclic AMP assays was observed when, instead of using forskolin, salmon calcitonin was used to activate an endogenous Gs-coupled receptor. NECA (10^{-10} - 10^{-6}M) failed to inhibit calcitonin-induced stimulation of SPAP, whereas it inhibited calcitonin-induced elevation of cAMP.

In summary, the output from this cAMP-linked reporter gene generally reflects underlying changes in intracellular cAMP levels very closely. However, the discrepancy between experiments using forskolin and calcitonin suggests the existence of an alternative pathway to reporter gene activation which is independent of cAMP.

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304P FURTHER CHARACTERISATION OF ADENOSINE A_{2A} RECEPTOR ANTAGONIST RADIOLIGAND [^{3}H]-SCH 58261: STUDIES ON THE HUMAN CLONED A_{2A} RECEPTOR

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We have previously reported that [^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo [1,5-*c*] pyrimidine, ([^{3}H]-SCH 58261), is an antagonist radioligand which labels the A_{2A} adenosine receptor subtype both in the central nervous system (rat striatum) and periphery (human platelets) (Ongini & Fredholm, 1996). We now describe that [^{3}H]-SCH 58261 labels the human cloned A_{2A} adenosine receptor stably transfected in Chinese hamster ovary (CHO) cells. When incubated for 30 min at 25 °C, pH 7.4, 0.5 nM [^{3}H]-SCH 58261 bound to CHO membranes with specific binding of 66 (61-72)%. Association and dissociation kinetics (n=4) were monophasic with the following rate constants (geometric mean, with 95% confidence limits in parentheses): K_{obs} = 0.78 (0.39-1.53)/min and K_{-1} = 0.46 (0.25-0.87)/min from a $T_{1/2}$ = 1.5 (0.8-2.8). These values gave a kinetic dissociation constant (Kd) of 0.75 (0.64-0.88) nM. Saturation experiments (n=4) showed that [^{3}H]-SCH 58261 bound to a single class of receptors with a Kd value of 2.3 (1.7-3.2) nM and an apparent Bmax value of 526 (476-581) fmol/mg of protein. The presence of 100 μM GTP did not modify binding parameters. The ability of several adenosine receptor agonists (5'-N-ethylcarboxamido- adenosine,NECA, 2-hexynyl-NECA,HE-NECA, 2-[4-(2-carboxyethyl)-phenetylamino]-5'-N-ethylcarboxamidoadenosine, CGS 21680, R- and S-N^6-phenylisopropyladenosine,R- and S-PIA, N^6-cyclohexyladenosine ,CHA, and antagonists 5-amino-9-chloro-2-(2-furyl)-[1,2,4]-triazolo [1,5-*c*] quinazoline,CGS 15943, SCH 58261, xanthine amine congener, XAC, (*E*,18%- *Z*, 82%)7-methyl-8-(3,4-dimethoxy-styryl)-1,3-dipropylxanthine, KF 17837S, 8-cyclopentyl-1,3-dipropylxanthine, DPCPX, in inhibiting 0.5 nM [^{3}H]-SCH 58261 binding was examined in competition experiments (n=3-6).

Binding affinity of selected adenosine receptor agonists and antagonists to human cloned A_{2a} receptors

AGONIST	Ki (nM)	ANTAGONIST	Ki (nM)
HE-NECA	5.2 (4.3-6.3)	CGS 15943	0.5 (0.4-0.8)
NECA	66 (40-110)	SCH 58261	1.1 (0.8-1.6)
CGS 21680	221 (156-311)	XAC	3.6 (2.0-6.3)
R-PIA	684 (558-838)	KF 17837S	19 (11-34)
S-PIA	10416 (6821-15904)	DPCPX	284 (178-452)

The order of potency of both agonists and antagonists was consistent with an interaction occuring at A_{2A} receptors. Hill coefficients were not significantly different from unity. Thus, [^{3}H]-SCH 58261 has a high receptor affinity for human cloned A_{2A} receptors (Kd=2.3 nM) similar to that observed in rat (Kd=0.7 nM) and porcine striatum (Kd=1.3 nM), and in peripheral tissues such as human platelets (Kd=0.9 nM) and porcine coronary arteries (Kd=2.2 nM). Moreover, in all the membranes preparations examined adenosine receptor agonists and antagonists displaced [^{3}H]-SCH 58261 with comparable affinity and order of potency (Zocchi et al., 1996 ; Dionisotti et al., 1996; Belardinelli et al., 1996). The present results show that [^{3}H]-SCH 58261 is an excellent probe which will promote further progress in understanding the function of the A_{2A} adenosine receptor subtype.

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