Biological Profile of L-745,870, a Selective Antagonist with High Affinity for the Dopamine D4 Receptor

SMITA PATEL, STEPHEN FREEDMAN, KERRY L. CHAPMAN, FRANCES EMMS, ALAN E. FLETCHER, MICK KNOWLES, ROSEMARIE MARWOOD, GEORGE MCALLISTER, JAN MYERS, SHIL PATEL, NEIL CURTIS, JAN KULAGOWSKI, PAUL D. LEESON, MARK RIDGILL, MIKE GRAHAM, STEVE MATHESON, DENISE RATHBONE, ALAN P. WATT, LINDA J. BRISTOW, NADIA M. J. RUPNIAK, ELIZABETH BASKIN, JOSEPH J. LYNCH, and C. IAN RAGAN

Department of Biochemistry & Molecular Biology, Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road Harlow, Essex, U.K.

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ABSTRACT

L-745,870, (3-[[4-(4-chlorophenyl)piperazin-1-yl)methyl]-1H-pyrrolo[2,3-b]pyridine, was identified as a selective dopamine D4 receptor antagonist with excellent oral bioavailability and brain penetration. L-745,870 displaced specific binding of 0.2 nM [3H] spiperone to cloned human dopamine D4 receptors with a binding affinity (K) of 0.43 nM which was 5- and 20-fold higher than that of the standard antipsychotics haloperidol and clozapine, respectively. L-745,870 exhibited high selectivity for the dopamine D4 receptor (>2000 fold) compared to other dopamine receptor subtypes and had moderate affinity for 5HT2, sigma and alpha adrenergic receptors (IC50 < 300 nM). In vitro, L-745,870 (0.1-1 μM) exhibited D4 receptor antagonist activity, reversing dopamine (1 μM) mediated 1) inhibition of adenylate cyclase in hD4HEK and hD4CHO cells; 2) stimulation of [35S] GTPγS binding and 3) stimulation of extracellular acidification rate, but did not exhibit any significant intrinsic activity in these assays. Although standard antipsychotics increase dopamine metabolism or plasma prolactin levels in rodents, L-745,870 (≤30 mg/kg p.o.) had no effect in these assays. The lack of a suitable in vivo assay for D4 receptor activation prompted the use of in vivo surrogate marker assays which confirmed that doses of 5-60 μg/kg L-745,870 would be sufficient to occupy 50% D4 receptors in the brain. These results show that dopamine D4 receptor antagonism in the brain does not result in the same neurochemical consequences (increased dopamine metabolism or hyperprolactinemia) observed with typical neuroleptics.

The identification of the dopamine D4 receptor subtype in 1990 (Van Tol et al., 1990) raised considerable interest in psychiatry due to its homology and pharmacological similarities to the dopamine D2 receptor. Interest was further generated because the D4 receptor showed high affinity for the atypical neuroleptic clozapine. In the clinic, clozapine is efficacious against positive symptoms of schizophrenia and has limited efficacy for the negative symptoms of schizophrenia. Clozapine has particular efficacy in refractory patients, produces fewer and milder EPS compared to classical antipsychotics and does not stimulate prolactin secretion (Meltzer et al., 1989; Fitton and Heel, 1990; Baldessarini and Frankenburg, 1991). The clinical use of clozapine has been limited by the 1 to 2% incidence of agranulocytosis. The pharmacological mechanism of action of clozapine’s unique clinical profile has not been unequivocally explained due to its affinity for many neurotransmitter receptors (Fitton and Heel, 1990; Baldessarini and Frankenburg, 1991). All antipsychotics that are currently used to treat the symptoms of schizophrenia have a common ability to antagonize D2 receptor activity albeit with varying affinities (Creese et al., 1978; Lahti et al., 1993; Seeman and Van Tol, 1994). Clozapine binds to the D4 receptor with the highest affinity compared to other members of the dopamine receptor family that has led to speculations that the atypicality of clozapine may be related to antagonism of dopamine D4 receptors. In contrast, the EPS and hyperprolactinemia associated with neu-

ABBREVIATIONS: EPS, extrapyramidal side effects; CHO, Chinese hamster ovary cells; HEK, human embryonic kidney cells; EDTA, ethylenediaminetetraacetic acid; PEI, polyethylenimine; [3H]DTG, 1,3-Di(2-[5-3H]toly)guanidine; ERP, effective refractory period; DMEM, Dulbecco’s modified Eagle’s medium; TCA, trichloroacetic acid; PBS, phosphate buffered saline; [35S]GTPγS, guanosine 5’[γ-35S] thiotriphosphate; FCS, fetal calf serum; PEG, polyethylene glycol; HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; SHT, 5-hydroxy tryptamine; CNS, central nervous system; MED, minimal effective dose; HPLC, high-performance liquid chromatography.
roleptics is thought to be a reflection of D2 receptor antagonism in the striatum and putitory, respectively. It was evident that highly selective D4 receptor antagonists were required to evaluate whether blockade of dopamine D4 receptors alone could exert antipsychotic activity. We report the in vitro and in vivo biological profile of (3-[4-(4-chlorophenyl) piperazin-1-yl] methyl)-1H-pyrrole [2,3-b] pyridine (L-745,870): a selective, high affinity ligand for the dopamine D4 receptor (Kulagowski et al., 1996). Preliminary reports of these data have been previously communicated to the British Pharmacological Society (Patel et al., 1996).

Methods

Clonal Cell Lines

Human dopamine D2short receptors stably expressed in CHO cells were obtained from Dr. M. Graziano (Merck Sharp & Dohme, Rahway, NJ). Human D4.2 gene/cDNA hybrid construct was obtained from Dr. O. Civelli (Volum Institute, Portland, OR) and stably transfected into HEK cells. Full length human D4.2 cDNA was obtained from Dr. D. Grandy (Volum Institute and human D3 cDNA was obtained from Dr. P. Sokoloff (INSERM, Paris, France), subcloned into pcDNA3 mammalian expression vector (Invitrogen) and stably transfected into CHO and HEK cells respectively by standard techniques (Cullen, 1987). The rat D2short receptor (expressed in CHO cells) and the rat D3 receptor (expressed in rat fibroblasts) were obtained from Dr. P. Sokoloff (INSERM). Rat D4 receptors stably expressed in mouse fibroblasts were obtained from Dr. R. Todd (Washington University, St. Louis, MO).

Radioligand Binding Assays

[3H]Spiperone binding assay. Cells stably expressing the human D2, D3 and D4 receptors were lysed by homogenization (polytron, 2 × 5 sec) in 10 mM Tris HCl buffer (pH 7.4) containing 5 mM MgSO4 and spun at 50,000 × g for 15 min. The resulting pellet was resuspended in assay buffer (50 mM Tris HCl, pH 7.4 containing 5 mM EDTA, 1.5 mM CaCl2, 5 mM MgCl2, 120 mM NaCl, 0.1% acetic acid) at 20 mg wet weight/ml (human D4 HEK cells), 10 mg wet weight/ml (human D4 CHO cells), 40 mg wet weight/ml (human D2 CHO and D3 HEK cells) or 8,10 and 70 mg wet weight/ml (rat D2, D3 and D4 cells, respectively). Incubations were performed in the presence of 0.005 to 2 nM [3H] spiperone (65-140 Ci/mmol, Amersham, U.K.) or 0.2 nM [3H] spiperone for drug displacement studies, 50 μl of displacing drugs (at a final concentration range of 0.001-10 μM) and either 50 μl buffer (total binding) or 10 μM aropomorphine (nonspecific binding) in a final assay volume of 500 μl. The reaction was initiated by the addition of 75 μl membranes and allowed to proceed for 2 hr at room temperature before being terminated by rapid filtration over GF/B filters (presoaked in 0.3% PEI) with 2 × 5 ml ice cold 50 mM Tris HCl pH 7.4. Binding parameters were determined by nonlinear, least squares regression analysis using SRS 1 (BBN Research Systems, Cambridge, MA) and an in house computerized iterative procedure developed by Dr. A. Richardson.

[3H] SCH 23390 binding assay. Rat striatal membranes were used to determine specific binding of the dopamine D1/D5 receptor radioligand [3H] SCH 23390 (70-87 Ci/mmol, NEN, Boston, MA) as described by Billard et al. (1994). Cloned human D1 and D5 receptors were obtained from SEMAT (St. Albans, Hertfordshire, UK) and the radioligand binding protocol was similar to that described above except the buffer used was 50 mM Tris HCl containing 5 mM HCl, 5 mM MgCl2, 5 mM EGTA and 1.5 mM CaCl2 pH 7.4. Results were analyzed as described above.

[3H] DTG binding to guinea pig cerebellar membranes. Male Dunkin-Hartley guinea pigs (350-400 g) were euthanized by decapitation, the brains removed, cerebellum dissected and homogenized in 10 volumes of ice-cold 0.32 M sucrose and spun at 900 × g for 10 min at 4°C. The resulting supernatant was centrifuged at 22,000 × g for 20 min at 4°C. The pellet was resuspended in 10 volumes of assay buffer (50 mM Tris HCl, pH 7.4) followed by centrifugation at 22,000 × g for 20 min at 4°C. The final pellet was resuspended in 10 volumes of assay buffer and stored in 10-ml aliquots at -70°C. For the radioligand binding assay, the frozen membrane homogenate was thawed and homogenized in assay buffer (77 ml assay buffer/10 ml homogenate). 750 μl of the membrane homogenate were incubated with 5 nM [3H] DTG (1.3-Di3-[5-3H(tolyl)guanidine, 30-60 Ci/mmol, NEN) and either buffer, 10 μM haloperidol (non specific binding) or test compound in a total assay volume of 1 ml. Incubation was allowed to proceed for 90 min at 23°C and terminated by rapid filtration over GF/B filters followed by 1% PEI, followed by 3 × 5 ml washes of ice-cold assay buffer. Radioactivity was determined using liquid scintillation spectrometry.

Ion channel activities. Binding to the voltage sensitive sodium channel was evaluated by displacement of [3H] batrachotoxinin (30-60 Ci/mmol, NEN) binding to rat cerebral cortex (Catterall et al., 1981). Activity at the voltage sensitive calcium channel (diltiazem alike allosteric site) was evaluated by displacement of [3H] diltiazem (60-87 Ci/mmol, NEN) binding to rabbit skeletal muscle (Reynolds et al., 1986). Activity at voltage sensitive potassium channels (particularly IKr channels) was estimated by measurement of the ERP in the ferret papillary muscle (Baskin et al., 1991).

In Vitro Functional Studies

Adenylate cyclase studies. Human D4 receptors expressed in HEK or CHO cells were grown in confluent monolayers in 24-well plates in growing medium (DMEM containing 10% FCS). The cells were washed with 1 × 1 ml serum free DMEM containing 1 mM IBMX and incubated with test compounds at 37°C for 30 min in a final volume of 1 ml. Assays were terminated by washing the plates with 3 × 1 ml ice-cold PBS followed by addition of 200 μl of ice-cold 5% TCA for 15 min. cAMP was extracted with the addition of 3 ml water-saturated diethylther followed by evaporation to dryness of 200 μl of the aqueous layer using a “speed vac” for 1 to 2 hr. cAMP content was determined using a radioimmunounassay kit TRK 432 (Amersham International UK).

[35S] GTPγS binding assay. [35S] GTPγS (>1000 Ci/mmol, Amersham, U.K.) binding was measured essentially as described previously (Lazareno et al., 1993). hD4CHO cells were homogenized in lysis buffer (20 mM Hapes, 10 mM EDTA, pH 7.4). After centrifugation (50,000 × g, 15 min at 4°C) the pellet was resuspended in 20 mM Hapes containing 0.1 mM EDTA (pH 7.4) at 40 mg wet weight/ml buffer. Membranes were incubated with 10 μM GDP and either dopamine (1 mM-100 μM), L-745,870 (1 mM-100 μM) or buffer (basal [35S] GTPγS binding) in a final assay volume of 1 ml, for 20 min at 30°C. 100 pM [35S] GTPγS was added to all tubes and the reaction was allowed to proceed for an additional 30 min at 30°C. The reaction was terminated by rapid filtration over GF/B filters washed twice with 5 ml deionized water. Radioactivity was determined by liquid scintillation counting and the results expressed as percent stimulation over basal activity or activity relative to dopamine (10 μM) stimulated [35S] GTPγS binding (maximal stimulation defined as 100%).

Extracellular acidification measured with a microphysiometer. CHO cells expressing human D4 receptors were seeded into 12-mm disposable polycarbonate inserts (Molecular Devices Corp, Menlo Park, CA) at 3.0 × 10⁶ cells/cup in medium containing FCS. The cells were incubated for 6 hr at 37°C in 5% CO2, before changing to medium without FCS. After another 18 hr, cups were loaded into the sensor chambers of the microphysiometer and the chambers perfused with the running medium (bicarbonate-free DMEM containing 2 mM glutamine and 44 mM NaCl) at a flow rate of 50 μl/min (Neve et al., 1992; Raleigh-Susman et al., 1992). Each pump cycle lasted 60 sec. The pump was on for the first 38 sec and the acidification rate determined between 43 and 58 sec, using the Cyotosys programme (Molecular Devices Corp.). Test compounds were diluted
in running medium. Cells were exposed (4 min) to dopamine (3-3000 nM), L-745,870 (30-3000 nM) or dopamine (3-3000 nM) in the presence of 100 nM L-745,870 at 20-min intervals. Peak acidification rate to agonist in the absence and presence of antagonist was determined and concentration-response curves fitted using GraFit (Leatherbarrow, 1992).

**Pharmacokinetic Studies**

Male Sprague-Dawley rats (approximate body weight 250g) received L-745,870 (3 mg/kg) either i.v. (n = 33) or p.o. (n = 33). The vehicles used were acidified PEG400 (0.6 mM HCl) and acidified propylene glycol for p.o. and i.v. administration respectively. L-745,870 was prepared in both formulations at a concentration of 2 mg/ml. Each rat was anesthetized with isoflurane before taking a blood sample (typically > 4 ml) using cardiac puncture at one of 11 predetermined time points (n = 3 rats per time point) up to 7 hr after dosing, after which each animal was killed by decapitation or cervical dislocation. Animals were also killed at selected time points and the brains were removed for further analysis. Plasma and brain samples were frozen at -20°C and retained for further analysis. Extracts of plasma and brain tissue homogenates were analyzed by HPLC with UV detection as follows. After basification with NaOH, plasma samples were liquid/liquid extracted with ethyl acetate. After separation and evaporation of the organic phase these were redissolved in mobile phase and injected onto an appropriate HPLC system. This consisted of a KR100 5C8 column (150 × 4.6 mm i.d.) with a mobile phase of 50% MeCN in 25 mM KH2PO4 and 5 mM pentane-sulfonic acid at pH 7.0. The flow was 1 ml/min and detection was UV at 286 nm. The concentration of L-745,870 in each sample was determined using a standard curve constructed by spiking plasma from nondrug treated animals with known concentrations of L-745,870. Brain samples were treated in an analogous fashion after homogenization in distilled water at 2 ml/g. An almost identical study was also conducted, in which the hydrochloride salt of L-745,870 was dissolved in water for both the i.v. and oral formulation.

For pharmacokinetic studies in rhesus monkeys, L-745,870 was dissolved in N-methyl-2-pyrrolidinone (10 mg/ml) for i.v. administration and in 1 mM HCl (1 mg/ml) for oral administration. L-745,870 was administered in the i.v. formulation to four rhesus monkeys (6.0 kg body weight) at a dose of 1 mg/kg. Blood samples were collected as before and retained for analysis. After 4 wk, the monkeys were anesthetized with isoflurane before taking a blood sample (typically > 4 ml) using cardiac puncture at one of 11 predetermined time points (n = 3 rats per time point) up to 7 hr after dosing, after which each animal was killed by decapitation or cervical dislocation. Animals were also killed at selected time points and the brains were removed for further analysis. Plasma and brain samples were frozen at -20°C and retained for further analysis. Extracts of plasma and brain tissue homogenates were analyzed by HPLC with UV detection as follows. After basification with NaOH, plasma samples were liquid/liquid extracted with ethyl acetate. After separation and evaporation of the organic phase these were redissolved in mobile phase and injected onto an appropriate HPLC system. This consisted of a KR100 5C8 column (150 × 4.6 mm i.d.) with a mobile phase of 50% MeCN in 25 mM KH2PO4 and 5 mM pentane-sulfonic acid at pH 7.0. The flow was 1 ml/min and detection was UV at 286 nm. The concentration of L-745,870 in each sample was determined using a standard curve constructed by spiking plasma from nondrug treated animals with known concentrations of L-745,870. Brain samples were treated in an analogous fashion after homogenization in distilled water at 2 ml/g. An almost identical study was also conducted, in which the hydrochloride salt of L-745,870 was dissolved in water for both the i.v. and oral formulations.

**Functional Studies in Vivo**

**Measurement of prolactin secretion.** Male Sprague Dawley rats (n = 4/group, 150-200 g) received L-745,870 (0.01-10 mg/kg, p.o. in acidified PEG300), haloperidol (3 mg/kg s.c. in saline) or vehicle. Thirty min later, the animals were euthanized by decapitation and blood collected from the superior vena cava into heparinized tubes. Plasma was obtained by centrifugation of the blood sample at 13,000 rpm for 15 min and stored at -70°C. Prolactin content was determined using a commercially available radioimmunoassay from Amersham International (RPA 553).

**Monoamine metabolism studies.** Male BKTO mice (n = 5/group, 20-28 g) received L-745,870 (1-30 mg/kg p.o. in acidified PEG300), haloperidol (0.1-3 mg/kg s.c. in saline) or clozapine (1-50 mg/kg i.p. in acidified PEG300). After 30 min, animals were euthanized by decapitation, the striatum and nucleus accumbens dissected from the brains and stored at -70°C until assayed for dopamine, HVA, DOPAC, 5HT and 5-hydroxyindoleacetic acid by HPLC with electrochemical detection (Bristow et al., 1993).

**In vivo binding of [3H] SKF 10047 to mouse brain.** Male BKTO mice (n = 6/dose, 25-30g) received either saline, acidified PEG300, L-745,870 (0.1-10 mg/kg p.o. in acidified PEG300), haloperidol (0.01-3 mg/kg s.c.) or clozapine (1-30 mg/kg s.c.) 40 min before i.v. administration with 5 μCi [3H] SKF 10047 (40-70 Ci/mmol, NEN) via the tail vein. After 20 min, the mice were decapitated and the brains homogenized (0.4 g wet weight/10 ml ice cold Tris HCl pH 7.4). A 500-μl aliquot of the homogenate was counted to determine the overall entry of [3H] SKF 10047 into the brain and a further 500-μl sample of the homogenate was filtered over GF/B filters, followed by two 5-ml washes with ice cold Tris HCl to determine the bound fraction. Radioactivity retained on the filter was determined using liquid scintillation counting. Nonspecific binding was measured using haloperidol (3 mg/kg s.c.).

**Mescaline-induced head twitches in mice.** Experiments were carried out on male BKTO mice (25-35 g) housed in groups of 5 and maintained on a 12 hr light:dark cycle (lights on at 07.00 hr with food and water freely available. All procedures were carried out in accordance with the U.K. Home Office Animals (Scientific Procedures) Act 1986. Mice were orally dosed with either L-745,870 (3-30 mg/kg) or vehicle (10 ml/kg) 30 min before injection of mescaline (25 mg/kg s.c.). Ten min later, animals were placed in individual perspex observation boxes (230 × 280 × 210 mm) and the number of head twitches recorded for the next 10 min. Data were analyzed by analysis of variance followed by Dunnett’s t test, comparing all groups to vehicle-treated mice.

**Behavioral studies in squirrel monkeys.** The subjects were 16 “naive” adult male squirrel monkeys (Saimiri sciureus; 700-1300 g). The vehicle used for L-745,870 was acidified 0.5% methocel (pH 3.5) for oral administration. Drugs were initially administered to two animals at escalating doses of 1, 10 and 30 mg/kg p.o., dosing every 2 hr, to establish the behaviorally active dose range. L-745,870 was subsequently administered to four separate groups of four animals at 1, 10 or 30 mg/kg and their behavior compared to that observed in vehicle-treated monkeys continuously throughout a 3-hr observation period. Behavior was recorded by an observer who was blind to treatment for 2.5 min every 5 min as follows: 1) duration of locomotor activity (timed in sec); 2) abnormal motor signs (bradykinesia, ataxia, dystonia and tremor) were rated on a scale of 0 to 4 depending on frequency and/or intensity: 0 = absent; 1 = occasional/mild; 2 = intermittent/moderate; 3 = frequent/marked; 4 = continuous/severe; 3) sedation was scored as 0 = absent; 1 = head down, hunched posture, easily aroused; 2 = head down, eyes closed, head raised in response to noise; 3 = eyes open in response to noise; 4 = unable to arouse with noise. Behavioral scores were summed in 30-min time bins prior to one- or two-way analysis of variance followed by trend analysis or Dunnett’s multiple comparison t tests.

**Results**

**Receptor binding profile.** The receptor binding affinities of L-745,870, haloperidol and clozapine, for the human dopamine D2-like receptor subtypes (hD2, hD3 and hD4) are summarized in table 1. L-745,870 bound to dopamine D4 receptors with subnanomolar affinity (Kᵢ, 0.43 nM) and was 2000- and 5000-fold selective for the D4 over D2 and D3 receptors, respectively. In contrast, haloperidol was relatively nonspecific, exhibiting similar binding affinities for all D2-like receptors. Clozapine bound to D4 receptors with nanomolar affinity (Kᵢ, 10 nM) and was 7- and 20-fold selective for D4 receptors compared to D2 and D3 receptors, respectively (table 1). L-745,870 exhibited negligible affinity for human D1 and D5 receptors (IC₅₀ ≥ 10 μM).

The receptor binding affinity of L-745,870 was also determined for the rat D2-D4 dopamine receptors (table 1) and these were found to be comparable to that obtained for the
human dopamine receptor subtypes. L-745,870 bound with high affinity, albeit 3-fold weaker, to the rat D4 receptor (Kᵢ 1.5 nM) compared to the human D4 receptor (Kᵢ 0.45 nM) but still retained >1000-fold D4 receptor selectivity over other dopamine receptor subtypes (table 1). Haloperidol and clozapine also exhibited similar binding affinities for both rat and human dopamine D2, D3 and D4 receptors (table 1). L-745,870 did not displace specific binding of 0.5 nM [³H]SCH 23390 to rat striatal membranes, exhibiting little affinity for rat D1/D5 receptors (IC₅₀ ≥ 10 μM).

L-745,870 was also evaluated for interactions with other human and mammalian receptors. In most of these assays (e.g., beta adrenoreceptors, adenosine, muscarinic, neurotransin, neuropeptide receptors) L-745,870 exhibited little binding affinity (IC₅₀ > 10,000 nM). L-745,870 did however exhibit weak affinity for other receptor sites and these data are presented in order of decreasing receptor binding affinities in table 2. In comparison to its affinity for the dopamine D4 receptor, L-745,870 maintained at least 300-fold selectivity compared to these other neurotransmitter receptors. L-745,870 did bind to sigma binding sites and 5HT2A receptors with moderate affinity (Kᵢ 0.13 and 0.2 μM, respectively) and this has proved useful for estimation of in vivo D4 receptor occupancy with L-745,870 (see “In vivo occupancy studies”). L-745,870 exhibited little or no affinity for potassium, calcium or sodium channels (IC₅₀ ≥ 2.5, 9.3, >10 μM, respectively).

**Adenylate cyclase studies in vitro.** We have previously shown that the dopamine D4 receptor expressed in HEK cells is functionally coupled to the inhibition of adenylate cyclase (McAllister et al., 1995). Forskolin (10 μM) produced a 10- to 20-fold increase over basal levels of cAMP. In a typical experiment this resulted in an increase from 1.8 ± 0.01 to 16.5 ± 0.1 pmol cAMP per well (fig. 1A). This forskolin elevation of cAMP levels was significantly inhibited by 0.3 and 1.0 μM dopamine (P < .001 vs. forskolin alone, paired t test, BMDP). Activation of D4 receptors with dopamine consistently produced a maximum of 20 to 40% inhibition of the forskolin response and an EC₅₀ of 80 nM (Patel et al., 1996a). This inhibition was attenuated by pretreatment with either 300 nM haloperidol or 300 nM clozapine (data not shown). L-745,870 (.001-1.0 μM) did not exhibit any agonist activity in hD4 HEK cells (fig. 1B) but completely reversed the dopamine-1 μM mediated inhibition of adenylate cyclase (MED, 100 nM) as did haloperidol (1 μM, fig. 1C). The functional efficacy of L-745,870 was also evaluated in the hD4 CHO cell line. In radioligand binding assays, [³H]spiperone (0.005-2 nM) bound specifically and in a concentration dependent manner to hD4 CHO cell membranes. Saturation studies revealed saturable binding that on Scatchard analysis resulted in an increase from 1.8 ± 0.01 to 16.5 ± 0.1 pmol cAMP. In a typical experiment this resulted in an increase from 1.8 ± 0.01 to 16.5 ± 0.1 pmol cAMP. In a typical experiment this resulted in an increase from 1.8 ± 0.01 to 16.5 ± 0.1 pmol cAMP. In a typical experiment this resulted in an increase from 1.8 ± 0.01 to 16.5 ± 0.1 pmol cAMP. In a typical experiment this resulted in an increase from 1.8 ± 0.01 to 16.5 ± 0.1 pmol cAMP. In a typical experiment this resulted in an increase from 1.8 ± 0.01 to 16.5 ± 0.1 pmol cAMP.
levels. Dopamine (0.01-3 μM) dose dependently inhibited forskolin elevated cAMP levels with a 100% inhibition of the forskolin response at doses ≥ 1 μM (EC₅₀ 90 ± 10 nM, n = 3; fig. 2A). Although the potency of dopamine was similar in both the hD4 HEK and hD4 CHO cells the maximal inhibition of adenylate cyclase activity was greater in hD4 CHO cells. One explanation for this difference may be that hD4 receptors are more efficiently coupled to inhibition of adenylate cyclase when expressed in CHO cells.

L-745,870 (0.0001-1 μM) had no intrinsic activity (fig. 2B) but at 0.1 and 1 μM L-745,870 completely antagonized the dopamine- (1 μM) mediated inhibition of adenylate cyclase in hD4 CHO cells (fig. 2C). To confirm functional selectivity of L-745,870 to reverse agonist-mediated inhibition of forskolin-stimulated cAMP levels was evaluated in the hD2 CHO and hD3 HEK cell lines. L-745,870 (0.001-10 μM) had no significant effect on either D2 or D3 receptor mediated inhibition of adenylate cyclase (data not shown).

[^35S] GTPγS binding studies. Activation of hD4 receptors stably expressed in CHO cells with dopamine (0.001-100 μM) produced a dose-dependent stimulation of [^35S] GTPγS binding with a maximal response at 10 μM (70-90% stimulation of [^35S] GTPγS binding over basal activity) with an EC₅₀ of 33 ± 2 nM (n = 4). L-745,870 (0.001-100 μM) had no intrinsic activity alone but inhibited the response to dopamine (0.001-100 μM). L-745,870 (10-100 nM) produced rightward shifts of the dopamine dose response curve together with an apparent decrease in the maximal response to dopamine (fig. 3).

Microphysiometry studies. CHO cells expressing human D4 receptors responded to dopamine by accelerating the rate of acidification of the surrounding medium from a basal level of 100 to 300 μvolts/sec (data not shown). Data from normalized segments generated by the Cytosoft program (Molecular Devices) were then used to generate percent stimulation values. Dopamine (3-3000 nM) stimulated the rate of acidification (maximal increase in the rate of acidification 38 ± 9%, n = 3) in a concentration-dependent manner (EC₅₀ = 290 ± 37 nM). L-745,870 (30-1000 nM) had no effect alone (data not shown) but at a concentration of 100 nM, L-745,870 produced a rightward shift in the dose response curve to dopamine (300-3000 nM) with an apparent Kᵦ of 25.8 ± 9.3 nM (n = 3).

Pharmacokinetic profile of L-745,870. Table 3 summarizes the pharmacokinetic profile of L-745,870 in the rat and rhesus monkey. In the rat, within 5 min of i.v. administration, L-745,870 (3 mg/kg, i.v.) was detected in the brain at very high concentrations (11.75 ± 0.27 μg/g). L-745,870 showed excellent brain penetration with brain concentrations at least 10 times higher than corresponding plasma concentrations. The elimination half-life was calculated from the 1 to 7-hr time points of the i.v. plasma profile (plasma t½ 2.1 hr). After oral administration L-745,870 (formulated in acidified PEG 400) was rapidly absorbed, achieving maximal plasma concentration of 350 ng/ml after 15 min with excellent oral bioavailability of 66%. A similar study was later conducted with a simple aqueous solution of the hydrochloride salt of L-745,870 for both the oral and i.v. formulations and the oral bioavailability of this formulation was 60%. In the rhesus monkey, L-745,870 exhibited a plasma t½ of 2.8 hr and moderate oral bioavailability (20%).
Functional studies in vivo. To determine brain penetration of L-745,870 after oral administration, L-745,870 was administered to rats over a dose range of 30 mg/kg to 30 mg/kg p.o. (fig. 4). The amount of L-745,870 present in plasma and brain samples at various time points (15 min to 6 hr) was determined by HPLC analysis. Increasing doses of L-745,870 were reflected in corresponding increases in measured plasma and brain levels of L-745,870. Area under curve calculated from 0 to 2 hr) are presented as a more accurate estimation of total amount of drug present. The data demonstrated that plasma and brain levels of L-745,870 rose proportionally to the dose over the entire dose range used in the study and confirmed that L-745,870 has excellent brain penetration after oral administration.

Prolactin secretion in rats. Antipsychotics have been shown to increase plasma prolactin levels (Meltzer et al., 1978, 1989). The nonselective dopamine receptor antagonist haloperidol, at 3 mg/kg s.c., consistently produced a 4- to 5-fold increase in rat plasma prolactin levels. L-745,870, over a wide dose range (0.01-10 mg/kg, p.o.) had no significant effect on plasma prolactin levels compared to vehicle treated rats. Results from a typical experiment are shown in figure 5.

Effect of L-745,870 on dopamine metabolism. Antagonism of central dopamine D2 receptors by neuroleptics leads to an increase of dopamine metabolism (Karoum and Egan, 1997). L-745,870, a Selective D4 Antagonist 641

TABLE 3
Pharmacokinetic profile of L-745,870

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rat</th>
<th>Rhesus Monkey</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>3 mg/kg (i.v. and p.o.)</td>
<td>1 mg/kg (i.v. and p.o.)</td>
</tr>
<tr>
<td>Oral bioavailability</td>
<td>66%</td>
<td>20%</td>
</tr>
<tr>
<td>Plasma T1/2</td>
<td>2.1 hr</td>
<td>2.8 hr</td>
</tr>
<tr>
<td>Plasma Cmax</td>
<td>360 ng/ml</td>
<td>35 ng/ml</td>
</tr>
<tr>
<td>Brain Cmax</td>
<td>5.1 µg/ml</td>
<td>nd*</td>
</tr>
<tr>
<td>Brain/plasma ratio</td>
<td>&gt;10</td>
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</table>

*nd, Not determined.
Dopamine metabolism was defined in our study as the ratio of the dopamine metabolites to dopamine (DOPAC+HVA/DA) in striatum and nucleus accumbens. After a 30-min pretreatment, L-745,870 (1-30 mg/kg p.o.) did not exhibit any significant effect on dopamine metabolism in either brain region (fig. 6A). In contrast, haloperidol dose dependently increased dopamine metabolism (2.9- and 1.7-fold) in the striatum and nucleus accumbens respectively (MED mg/kg, accumbens/striatum: 0.03/0.1; fig. 6B). Clozapine also stimulated dopamine metabolism in both brain regions to a similar extent to haloperidol albeit with a 100- to 300-fold lower potency (MED mg/kg, accumbens/striatum: 30/50; fig. 6C).

**Estimation of dopamine D4 receptor occupancy in vivo.** The lack of activity in the previous two assays could be explained either by the unique receptor selectivity of L-745,870 or that the compound present in the CNS (see above) was not available for biological activity. To date, there are no known neurochemical or behavioral CNS effects due to activation of dopamine D4 receptors. Therefore, to confirm that L-745,870 was available for biological activity, a number of surrogate marker experiments were performed.

The weak receptor affinity of L-745,870 for sigma sites ($K_i$ 130 nM; sigma/rat D4 ratio 87), 5HT2 receptors ($K_i$ 200 nM; 5HT2/rat D4 ratio 133) and for dopamine D2 receptors ($K_i$ 960 nM; hD2/hD4 ratio 2200) was used to provide surrogate markers to estimate D4 receptor occupancy in the brain by L-745,870. The assays used were in vivo binding of [3H] SKF 10047 (sigma radioligand) in mice, mescaline-induced head twitches in mice (5HT2 behavioral assay) and the appearance of parkinsonian extrapyramidal symptoms in squirrel monkeys.

Oral administration of L-745,870 (0.1-10 mg/kg p.o.) dose dependently inhibited in vivo binding of [3H] SKF 10047 to mouse whole brain (ED$_{50}$ 3.0 mg/kg). Haloperidol and clozapine also displaced [3H] SKF 10047 binding to mouse brain with ED$_{50}$ values of 0.03 and 8 mg/kg s.c. respectively (fig. 7). The dose of L-745,870 required to occupy 50% of dopamine D2 and D4 receptors was estimated by extrapolating the ED$_{50}$ dose in the in vivo sigma binding assay using the ratio of the binding affinity of L-745,870 for sigma and D2 and D4 receptors. Thus, given that L-745,870 is 12-fold weaker at D2 (rat D2 $K_i$ 1600 nM) and has 87-fold higher affinity for the D4 receptor (rat D4 $K_i$ 1.5 nM) than the sigma site ($K_i$ 130 nM), then extrapolating the ED$_{50}$ obtained from the in vivo sigma binding study (ED$_{50}$ 3 mg/kg), the dose of L-745,870 required to occupy 50% of D2 and D4 receptors was estimated to be 37 mg/kg and 35 µg/kg, respectively. Similar calculations were performed for haloperidol and clozapine and the results are shown in table 4.

The percent displacement of [3H] SKF 10047 measured from the in vivo sigma binding assay was used to calculate the effective concentration of L-745,870 in the brain. A detailed dose response curve for inhibition of specific sigma receptor binding by L-745,870 in vitro was used to convert the percent inhibition observed in the in vivo binding assay to actual amount of L-745,870 present. At doses of 0.3 to 3 mg/kg, L-745,870 was present at an estimated concentration of 4.2 to 52 ng/ml, which would be sufficient to produce in excess of 90% occupancy of dopamine D4 receptors. In contrast, at the highest dose of 3 mg/kg, only 25% of dopamine D2 receptors would be occupied. The concentration of L-745,870 present in the brain 1 hr after a dose of 3 mg/kg p.o. was 2370 ng/ml as measured directly by HPLC compared to the estimated concentration of 52 ng/ml after administration of L-745,870 (3 mg/kg p.o.) in the in vivo sigma binding assay. This 40- to 50-fold discrepancy between the two measurements of brain levels of L-745,870 after a 3 mg/kg p.o. dose suggests that only a small proportion of the total amount of drug in the brain (as measured by HPLC) is freely available for biological activity (as measured by displacement of in vivo sigma binding).

The weak affinity of L-745,870 at 5HT2 receptors ($K_i$ 200 nM) was utilized to evaluate its ability to antagonize mescaline-induced head twitches in mice. Oral administration of
L-745,870 dose dependently and significantly attenuated mescaline-induced head twitches in the mouse (ED$_{50}$ 7.1 ± 1.3 mg/kg, fig. 8). Given that L-745,870 is 133 times more potent at the rat D4 receptor than at the 5HT2 receptor, these studies predict that a dose of 53 µg/kg p.o. should occupy 50% of dopamine receptors in the brain. This correlates well with the 50% D4 receptor occupancy dose estimated using in vivo sigma binding (35 µg/kg p.o.).

We reasoned that high doses of L-745,870 (10-30 mg/kg) might be expected to cause extrapyramidal symptoms in “naive” monkeys because the drug levels in the CNS at these doses may be sufficient to antagonize D2 receptors. The behavioral effects of L-745,870 (1, 10 and 30 mg/kg p.o; n = 4 per dose group) was determined in “naive” squirrel monkeys. At a dose of 1 mg/kg p.o., L-745,870 had no significant effect on locomotor activity compared to vehicle-treated animals (fig. 9). In contrast, in animals treated with 10 or 30 mg/kg p.o. L-745,870, motor activity was greatly reduced after approximately 90 min (P < .05 compared with vehicle treatment, trend analysis). This decrease in activity coincided with the induction of sedation at 10 and 30 mg/kg p.o. that first reached significance 120 min after drug treatment (fig. 9). Drug-induced parkinsonism (hunched and apparently rigid posture, slow shuffling gait, reduced voluntary move-
ment but no dystonia) was apparent in monkeys treated with a high dose of L-745,870 (30 mg/kg p.o.; latency of onset 60 min) but was generally mild or absent at 10 mg/kg or lower (fig. 9). Animals receiving the highest dose of L-745,870 (30 mg/kg p.o.) remained visibly subdued 24 hr later. By comparison with previous studies (N. Rupniak, unpublished data) in this species, L-745,870 resembled haloperidol (3 mg/kg p.o.) in that it decreased locomotion and induced sedation and parkinsonism; unlike haloperidol catalepsy was not observed in this dose range. The most prominent behavioral effects observed with clozapine (12 mg/kg p.o.) in this species, ataxia and tremor, were not consistently induced by L-745,870.

Therefore given the MED for L-745,870 in this “D2 surrogate assay” was found to be 10 to 30 mg/kg p.o. and the ratio between the hD2 and hD4 dopamine receptor binding affinities was 2200, extrapolating the data we can estimate that 5 to 15 μg/kg of L-745,870 would be required to occupy 50% D4 receptors in the brain. This was consistent with the estimated D4 receptor occupancy data obtained from the in vivo sigma binding assay and mescaline-induced head twitches in mice.

**Discussion**

Although antipsychotics currently used in the clinic are generally effective against the positive symptoms of schizophrenia there is still a need to design novel antipsychotics that would be efficacious against the positive and negative symptoms, lack the adverse side effects of current therapy (extrapyramidal motor symptoms, hyperprolactinaemia, agranulocytosis, seizures) and be effective in treatment-resistant patients. Approaches so far in this area have been primarily directed at mimicking the atypical antipsychotic clozapine. The superior clinical profile of clozapine (greater efficacy than typical antipsychotics against positive and possibly negative symptoms of schizophrenia, effective in some refractory patients and causing fewer EPS) has been difficult to pinpoint to a single pharmacological action because clozapine binds with high affinity to many neurotransmitter receptors (Fitton and Heel, 1990; Baldessarini and Franken, 1991). The identification of the dopamine D4 receptor subtype (Van Tol et al., 1990) led to speculations that clozapine may be exerting its antipsychotic activity via dopamine D4 receptor antagonism and also because at therapeutic doses, in schizophrenic patients, clozapine occupies a greater percentage of D4 than D2 receptors (Seeman, 1992). To test this hypothesis, we have identified a highly potent and selective dopamine D4 receptor antagonist.

The 7-azaindole L-745,870 was identified after optimization of an indole lead that was discovered through directed screening of the Merck sample collection (Kulagowski et al., 1996). This compound exhibited subnanomolar affinity for dopamine D4 receptors and had a 5- to 20-fold higher binding affinity compared to the standard antipsychotics haloperidol and clozapine, respectively. L-745,870 exhibited high selectivity for the dopamine D4 receptor (>2000-fold) compared to other dopamine receptor subtypes that was markedly

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**Table 4**

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED(_{50}) mg/kg</th>
<th>50% D2 Receptor Occupancy</th>
<th>50% D4 Receptor Occupancy</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-745,870</td>
<td>3.0</td>
<td>37 mg/kg</td>
<td>35 μg/kg</td>
</tr>
<tr>
<td>Clozapine</td>
<td>8.0</td>
<td>310 μg/kg</td>
<td>47 μg/kg</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>0.03</td>
<td>3.6 μg/kg</td>
<td>32 μg/kg</td>
</tr>
</tbody>
</table>

Mice received drug treatment 40 min before [\(^{3}H\)]SKF10047 5 μCi/mouse i.v. and in vivo binding was determined as described in “Methods.” The estimated 50% D2 and D4 receptor occupancy doses were calculated from a comparison of the receptor binding affinities of the drug for the sigma binding site and either the rat D2 or rat D4 dopamine receptor.
more selective than either haloperidol or clozapine. Because most of the preclinical evaluation of L-745,870 was performed in rodents we established that there was little species difference in terms of dopamine D4 receptor affinity of L-745,870 for the human and rat D4 receptor. L-745,870 had no or weak affinity for a number of human and mammalian neurotransmitter receptors tested (>10 μM) although it had moderate affinity for 5HT2, sigma and alpha adrenergic receptors (IC50 < 300 nM). It has been postulated that ion channel blocking properties of some antipsychotics may contribute to their adverse side-effect profile (Cunningham Owens, 1996). L-745,870 was devoid of activity at calcium, potassium and sodium ion channels. Indeed, L-745,870 had no effect on blood pressure or heart rate of anesthetized rats (R. Hargreaves and K. Woodford, unpublished data), confirming the high selectivity of L-745,870 and suggesting that L-745,870 would be unlikely to produce any cardiovascular liabilities in vivo. Functional efficacy of L-745,870 was investigated in vitro using adenylate cyclase, [35S]GTPγS binding and measurement of extracellular acidification rates in hD4 HEK and/or hD4 CHO cells. The results from these studies showed that L-745,870 behaved as an antagonist at D4 receptors: reversing dopamine-mediated inhibition of adenylate cyclase in both cell lines; antagonizing dopamine-mediated stimulation of [35S]GTPγS binding and dopamine-mediated increase in extracellular acidification rate in hD4 CHO cells. L-745,870 did not exhibit any significant intrinsic activity in any of these assays. The compound had good pharmacokinetic properties (20-60% oral bioavailability and plasma t1/2 2.1-2.8 hr) in both rat and monkey, and excellent brain penetration with high brain to plasma ratios in rat.

Classical antipsychotics (e.g., haloperidol) and atypical antipsychotics (e.g., clozapine) produce a range of biochemical and behavioral effects associated with dopamine D2-like receptor antagonism in the brain. Both classical and atypical antipsychotics increase dopamine turnover in forebrain regions and antagonize amphetamine-induced hyperactivity, whereas classical antipsychotics increase plasma prolactin levels, induce catalepsy and block apomorphine induced stereotyped behaviors (Meltzer et al., 1978; Baldessarini and Taray, 1980; Karoum and Egan, 1992). The role of D2-like receptor subtypes (D2,D3,D4) in mediating these behaviors has not been conclusively demonstrated. L-745,870 did not alter dopamine metabolism in either the nucleus accumbens or striatum at doses up to 30 mg/kg p.o. in mice, whereas haloperidol stimulated dopamine metabolism in both brain regions at doses comparable to those required for reversal of amphetamine induced hyperactivity (Bristow et al., 1996). The lack of effect on dopamine metabolism with L-745,870, at doses that would produce 100% occupancy of dopamine D4 receptors, suggests that the effects on dopamine metabolism with antipsychotics is likely to be a result of dopamine D2/D3 receptor blockade. The pituitary contains a high density of dopamine D2 receptors and compounds that possess D2 receptor antagonist activity produce hyperprolactinemia. In our study, L-745,870 did not stimulate prolactin secretion in rats at doses up to 10 mg/kg p.o. The lack of effect with L-745,870 at 10 mg/kg p.o., suggests it is not achieving sufficient D2 receptor occupancy to increase plasma prolactin levels via D2 receptor blockade, which is in agreement with the estimation of D2 receptor occupancy from our surrogate marker studies (ED50 for D2 occupancy 37 mg/kg p.o.). Furthermore the data suggest that D4 receptor antagonism does not contribute to the hyperprolactinemia commonly observed with typical antipsychotics.

The lack of a measurable neurochemical or behavioral response to D4 receptor activation in vivo, led us to look at alternative means of estimating in vivo D4 receptor occupancy in the brain with L-745,870. To do this, we utilized the modest affinity of L-745,870 for sigma binding sites, 5HT2 and dopamine D2 receptors and examined the ability of L-
745,870 to disrupt either biochemical or behavioral effects observed after activation of these surrogate markers i.e. inhibition of in vivo [3H]SKF10047 binding in mice, inhibition of mesocannical-induced head twitches in mice and induction of mild parkinsonian-like symptoms in squirrel monkeys. HPLC analysis confirmed that brain and plasma levels of L-745,870 were proportional to dose over a large concentration range (0.03-30 mg/kg p.o.). At doses of 0.1-0.3 mg/kg p.o. the brain levels of L-745,870 freely available for biological activity are sufficient to occupy >90% D4 receptors, doses at which L-745,870 has no effect on mesocannical head twitches in rats or induction of EPS in monkeys. Thus any effect of L-745,870 (>0.3 mg/kg p.o.) in these surrogate marker assays are unlikely to be due to dopamine D4 receptor blockade. The ED50 values obtained for L-745,870 in these surrogate marker studies were extrapolated to estimate the dose of L-745,870 required to occupy 50% D4 receptors, using the relative ratios between the binding affinity of L-745,870 at the D4 receptor and the respective surrogate marker. A close correlation was observed in the estimations of in vivo D4 receptor occupancy with L-745,870 using the various surrogate markers (5-60 μg/kg p.o.). The behavioral and biochemical studies were performed in mice and the extrapolated estimations of D4 receptor occupancy doses were calculated using the rat D4, rat 5HT2 and guinea pig sigma receptor affinities of L-745,870. However, when L-745,870 was evaluated for activity against mesocannical-induced head twitches in rats, the ED50 was not dissimilar to that observed in mice, illustrating that the pharmacokinetic profile and receptor binding affinities of L-745,870 probably does not differ between rats and mice (Bristow et al., in preparation). These results show that the doses of L-745,870 used in the dopamine metabolism or prolactin secretion studies in rodents were sufficient to occupy >90% D4 receptors in the brain. To validate these estimations, haloperidol and clozapine were also evaluated in these surrogate assays and the estimated doses of these compounds to occupy 50% D2 rather than D4 receptors correlated better with the doses required to stimulate dopamine metabolism (MED 0.03 and 30 mg/kg s.c., respectively) and reverse amphetamine induced hyperactivity in mice (Bristow et al., 1996).

In summary, L-745,870 has been identified as a high affinity and selective dopamine D4 receptor antagonist, with a reasonable plasma half-life, good oral bioavailability and excellent brain penetration. In vivo, L-745,870 did not alter either dopamine metabolism or basal plasma prolactin levels at doses up to 10 mg/kg p.o. in rodents. Evaluation of L-745,870 in surrogate marker assays demonstrated that this compound was freely available for biological activity in the brain and that at doses of 5 to 60 μg/kg p.o. L-745,870 would occupy 50% D4 receptors in the brain. One can speculate from these preclinical studies that L-745,870 would be unlikely to produce hyperprolactinemia in man at D4 selective doses. L-745,870 (1 mg/kg p.o.) was recently reported to be inactive against amphetamine-induced hyperactivity in mice and apomorphine-induced stereotyped behaviors in the rat although haloperidol was active in both studies (ED50 0.14 and 0.37 mg/kg p.o., respectively, Bristow et al., 1996). These data suggest that in man L-745,870 may be an improvement over typical antipsychotics as it would have a reduced liability for EPS because it failed to alter apomorphine-induced stereotypy in rats. However, if amphetamine hyperactivity in rodents is predictive of antipsychotic efficacy in the clinic then L-745,870 would not be efficacious in schizophrenic patients.

L-745,870 has been evaluated in phase II clinical trials of patients with acute schizophrenia (Kramer et al., 1997). This study was performed as a multicenter 4-wk study in which hospitalized patients received either L-745,870 (15 mg/day) or placebo. Results from 38 patients (n = 26 for drug treated and n = 12 for placebo) showed that L-745,870 was generally well tolerated; however, L-745,870 was devoid of clinical efficacy in schizophrenic patients. Thus the blockade of dopamine D4 receptors does not appear to confer antipsychotic efficacy in schizophrenic patients and is probably not the prime target through which clozapine exerts its antipsychotic activity. Unraveling the mechanism by which clozapine exerts its unique clinical profile in schizophrenia (improved reactions with low EPS) remains a pharmacological challenge.

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Send reprint requests to: Dr. Smita Patel, Department of Biochemistry & Molecular Biology, Merck Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Eastwick Road Harlow, Essex, U.K.