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In vitro evidence for the presence of [^{3}H]-haloperidol uptake in rat brain

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1 The neuroleptic [^{3}H]-haloperidol (HP) was taken up in synaptosomes prepared from rat brain, in a temperature-, sodium ion-, and energy-dependent process.

2 The highest concentration of uptake sites ($V_{max} = 2.37$ pmol mg^{-1} protein min^{-1}) was in the striatum with the other brain areas displaying lower (by 50–70%) values.

3 The affinity values ($K_m \approx 40$ nM) were similar in all brain areas considered.

4 The pharmacological characterization did not indicate a well-defined group of inhibitors, which suggested that HP might not use a transporter for recognized neurotransmitters.

5 The HP metabolites tested, including HPTP, were competitive inhibitors of [^{3}H]-HP uptake, an indirect indication that they may actively enter the striatal nerve endings through the same carrier.

6 Since the uptake process was partially affected by the incubation of [^{3}H]-HP in the presence of several antagonists of HP-transforming cytochrome P450 isoforms, the binding of HP at some enzyme sites inside the synaptosome cannot be excluded.

7 In conclusion, the present results suggest that HP may be actively transported in the rat brain. *British Journal of Pharmacology* (2003) **138,** 188–192. doi:10.1038/sj.bjp.0705009

Keywords: Antipsychotic; haloperidol metabolites; neuroleptic; synaptosomal uptake

Abbreviations: HP, haloperidol; HPTP, HPP^+, haloperidol pyridinium; haloperidol tetrahydropyridine; $RHPP^+$, reduced haloperidol pyridinium

Introduction

The butyrophenone neuroleptic haloperidol (HP) is an antipsychotic agent widely used for the treatment of schizophrenia and other mental disorders (Baldessarini, 1991). Depending on the disease, HP can be administered for months or years, and its therapeutic effects are often associated with the development of both acute and delayed side effects including parkinsonism and tardive dyskinesia (Gerlach & Casey, 1988). These effects appear to be consistent with the findings of morphological alterations occurring in human and animal brains after chronic treatments with HP, as well as of HP-provoked cell degeneration and death in cultured neurons and cell lines (Kim & Burkman, 1982; Bloomquist *et al*., 1994; Wright *et al*., 1998; Marchese *et al*., 2002). Some studies indicate that, after repeated administration, HP is accumulated in the rat striatum (Igarashi *et al*., 1995) and, *in vitro*, it binds to beef-eye melanin and dopamine-melanin, a putative index for its tropism towards the pigmented nerve cells in the human substantia nigra (Lyden *et al*., 1982). In spite of the evidence for the brain accumulation of HP, the mechanisms underlying this process are still unknown (Siebert *et al*., 2000). In this study it will be shown that HP is actively taken up into the rat striatum and additional brain regions.

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Methods

Freshly-dissected (Heffner *et al*., 1980) brain regions from male Sprague-Dawley rats (150–200 g) were homogenized using a Teflon-glass homogenizer (clearance approximately 0.009 inches) with 10 up and down strokes at 850 r.p.m. in 10 vol (w v^{-1}) of ice-cold 0.32 M sucrose containing 10 mM glucose and 10 mM Tris-HCl (pH 7.4). All further procedures prior to incubation were carried out at 0–4°C. The homogenate was spun at $1000 \times g$ for 10 min at 4°C and the resulting supernatant was centrifuged at $12{,}000 \times g$ for 20 min to obtain a crude synaptosomal fraction (P2). Synaptosomes were then resuspended in the assay buffer immediately before use. The standard [^{3}H]-HP (specific activity 14 Ci mmol^{-1}, N.E.N., Boston, MA, U.S.A.) uptake assays were run at 37°C for 3 min; both synaptosomal suspensions (90–150 μg protein/0.5 ml final vol) and reaction mixture were preincubated at 37°C for 3 min prior to starting the uptake reaction. The assay buffer contained (in mM): NaCl 20, KCl 5, $MgCl_2$ 1, glucose 10, $CaCl_2$ 1.2, HEPES 10, Na-methyl sulphate 100. The pH of the assay buffer was adjusted to 7.4 with NaOH (resulting in approximately 5 mM additional Na^+ in the assay buffer). In Na^+-dependence assays, choline chloride (20 mM) was used to replace the chloride coming from NaCl and the different concentrations of sodium were added by using sodium methyl sulphate. When the concentrations of K^+ were changed, choline chloride (5 mM) was used to replace the chloride coming from

KCl and potassium was added by using potassium methyl sulphate. In Cl^--dependence assays Cl^- ions were added by using choline chloride. The uptake was routinely measured after 3 min incubation, while in time-dependence experiments the uptake was terminated either immediately (0 time) or after 1, 3, 5, 7, 10, 15 and 30 min incubation. The uptake reaction was stopped by rapid filtration under vacuum through glass-fibre (Whatman GF/C) filters using a single filter holder (Millipore), thereafter the filters were washed with 4×5 ml of ice-cold 0.9% NaCl solution. The total time for the four washes was about 6 s, after which the filters were removed immediately and placed into vials containing 10 ml of scintillation liquid (Ultima Gold mv, Packard, Meridien, U.S.A.).

Tritium remaining on the filters was counted in a liquid scintillation counter (Tri-Carb 2900, Packard, Meridien, U.S.A.). The values of [^{3}H]-HP uptake obtained from synaptosomes incubated over ice (blanks) were subtracted from corresponding samples at 37°C. In the inhibition experiments drugs were added in a volume of 5 μl/0.5 ml at the start of the pre-incubation period. Inhibition curves were plotted and analysed by computer with appropriate softwares (Kell 6.0, Biosoft, Cambridge, U.K.; Prism 3.0, Graph Software, San Diego, CA, U.S.A.). Kinetic parameters (K_m and V_{max}) were estimated using a linear regression after Lineweaver-Burk transformation of the data. Statistical analysis was performed using one- or two-ways ANOVA followed by Newmann-Keuls *post hoc* test.

Results

Na^+-ions increased the [^{3}H]-HP uptake in a concentration-related manner, the half-maximal stimulating effect was observed at a Na^+ ion concentration of approximately 40 mM and the maximal effect was seen at 100 mM Na^+ (Figure 1, top panel). Conversely, chloride ions decreased the accumulation of [^{3}H]-HP with a maximal inhibition at 145 mM (Figure 1, top panel). Potassium-ions (5–20 mM) and glucose (10–50 mM) did not affect the accumulation process ($n=4$ data not shown).

Ouabain (100 μM) decreased by 42% residual radioactivity in synaptosomes (2.3 vs 4.0 pmol mg^{-1} protein min^{-1} in controls, $n=4$ data not shown). The reduction of transport in the presence of ouabain confirms the sodium-dependency of transport, as the sodium gradient is maintained by the activity of sodium-potassium ATPase. Because active transport processes are generally dependent upon physiological temperatures, we measured the amount of [^{3}H]-HP accumulation into striatal synaptosomes at different temperatures. As it can be observed in Figure 1 (bottom panel), the [^{3}H]-HP uptake was temperature-dependent and it was much higher at 37°C than at 25°C or 50°C. When [^{3}H]-HP was added to the incubation medium after a 3 min preincubation period, the time course of [^{3}H]-HP could be divided roughly into three phases (Figure 1, bottom panel). A fast increase in synaptosomal [^{3}H]-HP content was seen during the first 3 min, followed by a stable level between 3 and 5 min. Finally a decline in synaptosomal retention for [^{3}H]-HP was seen from 5 min onward. The uptake of [^{3}H]-HP (0.005–0.5 μM) under standard assay conditions in striatal synaptosomes is shown in Figure 2. Kinetic analysis

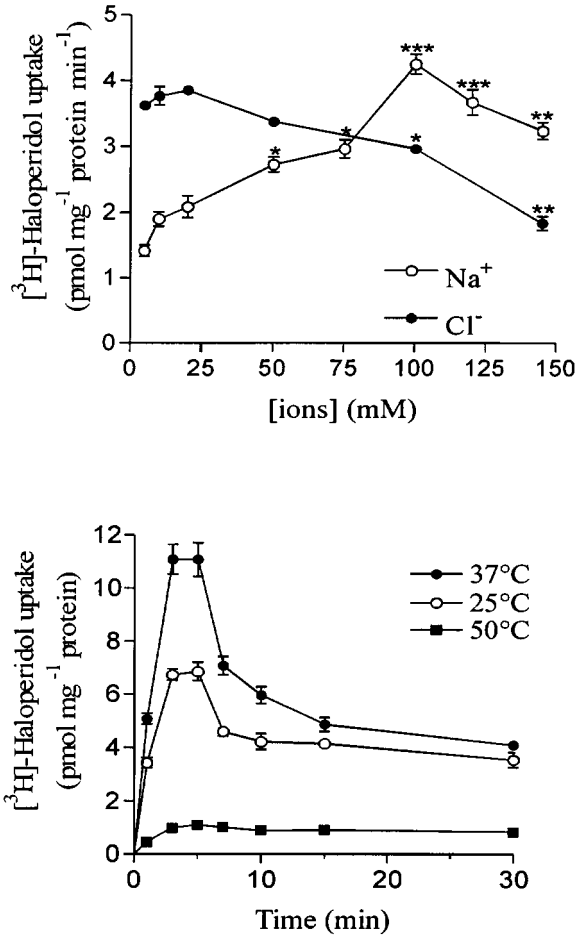

Figure 1 (*Top panel*) Effects of Na^+-ions and Cl^- ions on striatal [^{3}H]-HP (0.4 μM) uptake. In order to ascertain Na^+-dependency, choline chloride (20 mM) was used instead of NaCl and different concentrations of sodium were obtained with Na-methyl sulphate, which was also substituted for NaCl in chloride-dependency assays. Values represent mean ± s.e.mean of four independent experiments performed in triplicate. Newman–Keuls test for multiple comparisons was applied after one-way ANOVA (*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to 5 mM NaCl or 5 mM Cl^-, respectively). (*bottom panel*) Time course and temperature-dependency of striatal [^{3}H]-HP uptake. Synaptosomes were incubated at 25°C, 37°C, or 50°C in the standard assay medium with 0.4 μM [^{3}H]-HP for 0–30 min before uptake was terminated by filtration. Non carrier mediated uptake was measured at 4°C. Values are mean ± s.e.mean of six independent experiments.

of Lineweaver-Burk plots revealed a single uptake component, with a K_m value of 0.041 ± 0.002 μM and a V_{max} of 2.37 ± 0.28 pmol mg^{-1} protein min^{-1}.

When the uptake of [^{3}H]-HP into synaptosomes was compared in different brain regions, the highest concentration of uptake sites was observed in the striatum, with the V_{max} values in all other areas considered being 3.5–2.5 fold lower (Table 1).

The inhibitory potencies of several compounds at inhibiting [^{3}H]-HP accumulation are listed in Table 2. The HP metabolite, haloperidol tetrahydropyridine (HPTP), was the most potent blocker showing a K_i value of 37 nM, with haloperidolpyridinium (HPP^+) and reduced haloperidol pyridinium ($RHPP^+$) being about 30 and 60 fold less active, respectively. Several potent antagonists of dopamine uptake

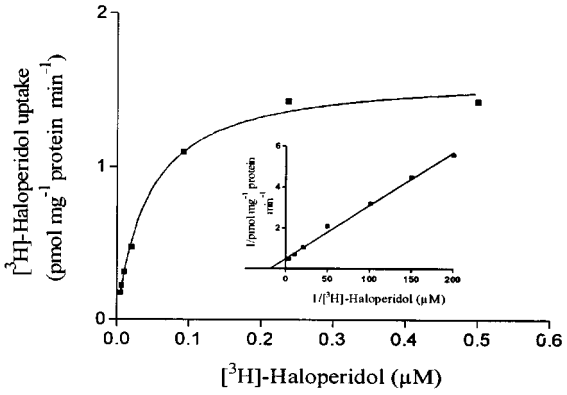

Figure 2 Concentration-dependent striatal [^{3}H]-HP uptake and (*inset*) its representative double-reciprocal plot: kinetic parameters indicated a V_{max} of 2.40 pmol mg^{-1} protein min^{-1} and a K_m of 0.042 μM. Triplicate aliquots of striatal synaptosomes were incubated at 37°C for 3 min with increasing concentrations (0.005–0.5 μM of [^{3}H]-HP.

Table 1 Regional distribution of [^{3}H]-haloperidol uptake in the rat brain

Area	K_m (μM)	V_{max} (pmol mg^{-1} prot min^{-1})	n
Striatum	0.041±0.002	2.37±0.28	8
Cerebellum	0.038±0.003	0.98±0.10**	4
Hippocampus	0.043±0.003	0.96±0.06**	6
Frontal cortex	0.045±0.002	0.85±0.02**	3
Olfactory tuberculum	0.038±0.005	1.10±0.06*	3
Nucleus accumbens	0.033±0.002	0.68±0.03**	3
Hypothalamus	0.045±0.007	0.92±0.01**	3

Aliquots of synaptosomes were incubated at 37°C for 3 min in 0.5 ml of medium with increasing concentrations of [^{3}H]-HP (0.005–0.5 μM). Values are mean±s.e.mean of *n* different experiments performed in triplicate. Data were analysed as Lineweaver-Burk plots. Newman–Keuls test for multiple comparisons were applied after one-way ANOVA (*$P<0.01$, **$P<0.001$ compared to control).

such as mazindol, GBR 12909 and cocaine were only poorly active while the most potent compounds were heterogeneous agents such as the 5-HT uptake inhibitor fluoxetine and several 5-HT- and dopamine-receptor antagonists displaying K_i values in the low (<2 μM) concentration range. The neurotoxins MPTP and MPP$^+$ were weak inhibitors of the process.

To determine the type of inhibition of HP transport by its metabolites, the initial velocity of uptake at increasing concentrations (0.005–0.5 μM) of [^{3}H]-HP in the absence and presence of HPTP (40 nM, 1 μM, 10 μM), RHPP$^+$ (10 μM) and HPP$^+$ (10 μM) was measured. HP metabolites displayed a clear competitive-type inhibition with increased Michaelis-Menten constants (K_m) and unchanged V_{max} values (Table 3 and Figure 3).

When [^{3}H]-HP was incubated with three inhibitors of HP transforming cytochrome P450 isoforms, its uptake was decreased through different times of incubation (Figure 4).

Finally, permeabilization of the loaded synaptosomes after a brief (2 min) exposure to 0.2% (2 w v^{-1}) saponin almost abolished the [^{3}H]-HP content in organelles (−59%), a finding suggesting that measured radioactivity reflected [^{3}H]-HP accumulated in synaptosomes ($n=3$ data not shown).

Table 2 Pharmacological characterization of haloperidol uptake in the rat striatum

Drug	K_i (μM)
HPTP	0.037±0.002
Ritanserin	0.8±0.1
Fluoxetine	0.9±0.1
RS 102221	1.0±0.1
HPP$^+$	1.1±0.1
cis-Flupentixol	1.1±0.1
Spiperone	1.4±0.2
Flunarizine	1.8±0.2
Lidoflazine	1.8±0.4
Ketanserin	2.2±0.2
RHPP$^+$	2.5±0.3
GBR 12909	3.3±0.7
Fluvoxamine	3.7±0.4
Mazindol	4.0±0.4
SKF 525A	9.1±0.9
MK 801	10±2.1
Ketoconazole	14±1.2
Quinidine	17±2.2
Clozapine	19±2.1
Risperidone	23±0.5
MPTP	24±2.3

The following compounds displayed K_i values >100 μM: cocaine, dopamine, tetrabenazine, L-deprenyl, L-glutamate, nifedipine, SCH-23390, L-sulpiride, nipecotic acid, nimodipine, serotonin, pargyline, xylamine, glycine, verapamil, yohimbine, diltiazem, guvacine, MPP+. Striatal synaptosomes were incubated at 37°C for 3 min with [^{3}H]-HP (0.05 μM), in the absence or presence of at least eight concentrations of competing drugs (ranging between 10^{-10} and 10^{-3} M). K_i values were calculated with Kell program, based on a K_m value of 0.05 μM for [^{3}H]-HP. Values are mean±s.e.mean of four different experiments performed in triplicate.

Table 3 Summary of effects of HPTP, RHPP$^+$ and HPP$^+$ on the kinetic constants for the transport of [^{3}H]-haloperidol in the rat striatum

Drug	K_m (μM)	V_{max} (pmol mg^{-1} prot min^{-1})	n
Control	0.040±0.002	2.59±0.02	9
HPTP (40 nM)	0.082±0.005*	3.09±0.18	3
HPTP (1 μM)	0.285±0.009*	3.15±0.08	3
HPTP (10 μM)	0.310±0.006*	2.31±0.11	3
RHPP$^+$ (10 μM)	0.092±0.009*	2.67±0.30	3
HPP$^+$ (10 μM)	0.081±0.009*	2.23±0.25	3

Aliquots of synaptosomes were incubated at 37°C for 3 min in 0.5 ml of medium with increasing concentrations of [^{3}H]-HP (0.005–0.5 μM) in the absence and presence of different concentrations of HPTP, RHPP$^+$ and HPP$^+$. Values are mean±s.e.mean of *n* different experiments performed in triplicate. Data were analysed as Lineweaver-Burk plots. Newman-Keuls test for multiple comparisons was applied after one-way ANOVA (*$P<0.001$ compared to control).

Discussion

The present experiments demonstrated that [^{3}H]-HP is taken up into brain synaptosomes with a high affinity, low capacity process obeying the rules of ion-, temperature- and energy-dependency.

The characterization of this process did not reveal a well recognizable pharmacological class of potent inhibitors

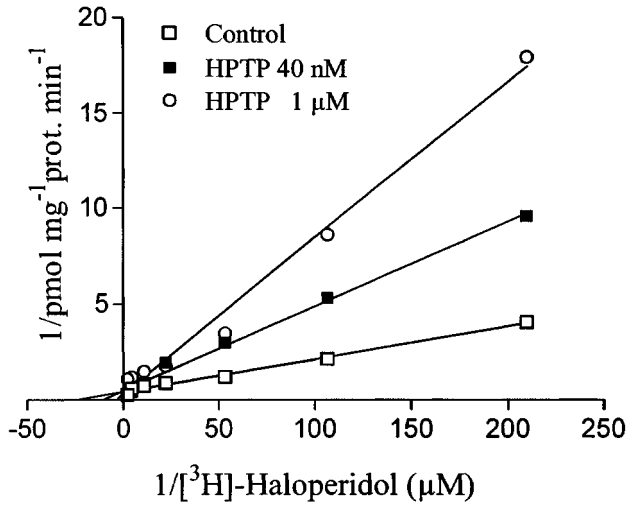

Figure 3 Representative Lineweaver-Burk plots for [^{3}H]-HP uptake in the absence and presence of 40 nM and 1 μM HPTP in rat striatal synaptosomes, demonstrating a competitive-type inhibition. Similar results were obtained in two additional experiments. The respective Kinetic parameters are summarized in Table 3.

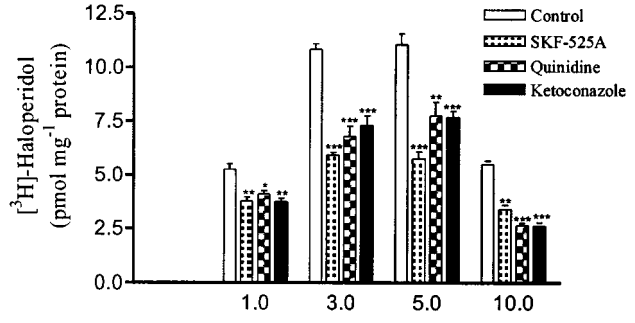

Figure 4 The effects of cytochrome P-450 inhibitors on the time course of striatal [^{3}H]-HP uptake (0.4 μM). Rat striatal synaptosomes were preincubated for 3 min in the absence (control) and presence of 50 μM Quinidine, 50 μM SKF 525-A or 50 μM Ketoconazole (all dissolved in DMSO, the controls contained an equivalent amount of the solvent) and the incubation was run for different time periods after which the samples were filtered. Values represent mean $\pm$ s.e.mean of three independent experiments. Statistical analysis was carried out using two ways ANOVA ($F_{drug}(3.8)=83.09$, $P<0.01$; $F_{time}(3,8)=507.4$, $P<0.01$; $F_{interact}(3,8)=11.47$, $P<0.01$) followed by Newman–Keuls *post-hoc* test (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs respective controls).

among the classical neurotransmitter-transporter antagonists and miscellaneous agents used, thus suggesting that the neuroleptic haloperidol might undergo a modest accumulation *via* a non-classical transporter. To this purpose it is of interest that the present K_i values for HP metabolites tested ranged widely (>60 fold), whereas the same compounds displayed rather homogeneous affinities towards dopamine (DAT), serotonin (SERT) and choline (HACU) transporters in rat neuronal cultures and in human transport-transfected COS-7 cells (Siebert *et al*., 2000). This would suggest that the putative HP carrier differs from DAT, SERT and HACU.

HP and its tetrahydropyridine dehydration product HPTP in humans and rats are converted to HPP^+ (Subramanyam *et al*., 1991), a neurotoxic agent strictly resembling the well known parkinsogenic toxins MPTP and MPP^+ (Rollema *et al*., 1994), which although being chemically similar to the HP metabolites, did poorly affect [^{3}H]-HP uptake. Conversely, the HP metabolites HPTP, HPP^+ and $RHPP^+$ did antagonize competitively the uptake process with fairly high affinities, an indirect indication that they may substitute for HP on the same neuronal carrier.

Furthermore, the incubation of synaptosomes with SKF-525A, ketoconazole and quinidine, three inhibitors of HP-transforming cytochrome P450 (CYP450) isoforms, provoked a decreasing trend in the time-course of [^{3}H]-HP uptake. It could be argued that a decrease in radioactivity due to a reduction in labelled HP or newly-formed, labelled HPP^+ can not be determined by this type of uptake experiments. However, since it has been clearly demonstrated that in brain homogenates incubated with HP the formation of HPP^+ is minimal (Igarashi *et al*., 1995), the present finding can not be univocally explained by a decrease, due to CYP450 antagonists, in the amount of radioactive HP metabolites contributing to the overall uptake measured. A possible explanation may be that once transported, [^{3}H]-HP binds at different CYP450 isoenzymes sites, a process that may concur to entrap the radioactivity inside the synaptosomes. Moreover, since chemically heterogeneous cytochrome P450 inhibitors induce a wide range of effects including the inhibition of different uptake systems (Vaugeois *et al*., 1992; Sriram *et al*., 1995; Clement *et al*., 1998), the possibility of a direct interaction with the putative [^{3}H]-HP uptake can not be excluded. HP and its metabolites have been previously hypothesized to be non-transported inhibitors or very weak substrates of the monoamine carrier (Siebert *et al*., 2000). In contrast, it is presently suggested that the radioactivity measured mostly represents the synaptosomal accumulation of HP transported *via* a monoamine neurotransmitter-unrelated carrier. It has been shown that HP and its metabolites are present in the rat striatum only at nM concentrations after a single administration which is consistent with the present low capacity characteristics of HP uptake (Igarashi *et al*., 1995). The brain levels of HP and HPP^+ markedly increase after multiple administrations of HP (Igarashi *et al*., 1995), suggesting that a long-term accumulation of carrier-transported trace amounts of HP and related metabolites cannot be excluded and might be relevant to the purposes of the well demonstrated, delayed extrapyramidal side effects of this widely used neuroleptic (Gerlach & Casey, 1988; Marchese *et al*., 2002).

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