



# Identification of the dopamine autoreceptor in the guinea-pig retina as D<sub>2</sub> receptor using novel subtype-selective antagonists

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**1** Dopamine release in the retina is subject to modulation *via* autoreceptors, which belong to the D<sub>2</sub> receptor family (encompassing the D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> receptors). The aim of the present study was to determine the receptor *subtype* (D<sub>2</sub> vs D<sub>3</sub>) involved in the inhibition of dopamine release in guinea-pig retinal discs, using established (haloperidol, (*S*)-nafadotride) and novel dopamine receptor antagonists (ST-148, ST-198).

**2** hD<sub>2L</sub> and hD<sub>3</sub> receptors were expressed in CHO cells and the p*K<sub>i</sub>* values determined in binding studies with [<sup>125</sup>I]-iodosulpride were: haloperidol 9.22 vs 8.54; ST-148 7.85 vs 6.60; (*S*)-nafadotride 8.52 vs 9.51; ST-198 6.14 vs 7.92.

**3** The electrically evoked tritium overflow from retinal discs preincubated with [<sup>3</sup>H]-noradrenaline (which represents quasi-physiological dopamine release) was inhibited by the dopamine receptor agonists B-HT 920 (talipexole) and quinpirole (maximally by 82 and 71%; pEC<sub>50</sub> 5.80 and 5.83). The concentration-response curves of these agonists were shifted to the right by haloperidol (apparent pA<sub>2</sub> 8.69 and 8.23) and ST-148 (7.52 and 7.66). (*S*)-Nafadotride 0.01 μM and ST-198 0.32 μM did not affect the concentration-response curve of B-HT 920.

**4** The dopamine autoreceptor in the guinea-pig retina can be classified as a D<sub>2</sub> receptor. ST-148 and ST-198 show an improved selectivity for D<sub>2</sub> and D<sub>3</sub> receptors when compared to haloperidol and (*S*)-nafadotride, respectively.

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**Keywords:** Guinea-pig retina; Dopamine D<sub>2</sub>/D<sub>3</sub> receptor; Haloperidol; (*S*)-Nafadotride; ST-148; ST-198; quinpirole; B-HT 920; dopamine release; autoreceptor

**Abbreviations:** B-HT 920, 6-allyl-5,6,7,8-tetrahydro-4H-thiazolo[4,5-d]azepin-2-amine; CHO, Chinese hamster ovary; PSS, physiological salt solution; S, electrical stimulation; ST-148, *N*-(4-[4-(2-methoxyphenyl)-piperazin-1-yl]-butyl)-5-(dimethylamino)-naphthalene-1-sulphonamide; ST-198, *N*-(4-[1,2,3,4-tetrahydroisoquinolin-2-yl]-butyl)-3-phenylacrylamide; t, collection period in which basal tritium efflux was determined

## Introduction

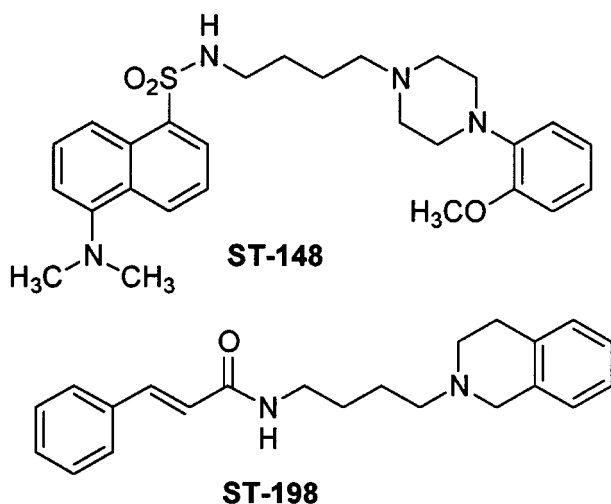
Dopamine is located in the amacrine and interplexiform cells of the inner nuclear layer of the retina (Smeets & Gonzalez, 2000). In the guinea-pig retina dopamine has recently been shown to be localized to amacrine cells type 1 and 2 (Oh *et al.*, 1999). Some inherent functions of the retina involve the release of dopamine, e.g., dark-light adaptation (Djamgoz & Wagner, 1992) or motion detection (Mora-Ferrer & Gangluff, 2000).

Retinal dopamine release is subject to modulation *via* inhibitory autoreceptors, which have been identified in the retina of teleosts (Rashid *et al.*, 1993), rabbits (Dubocovich & Weiner, 1985) and guinea-pigs (Weber *et al.*, 2001), and belong to the dopamine D<sub>2</sub>-subfamily (which encompasses the D<sub>2</sub>, D<sub>3</sub> and D<sub>4</sub> receptors; for review, see Strange, 2001). For some dopamine autoreceptors located outside the retina, e.g., in the brain, the dopamine receptor subtype within the dopamine D<sub>2</sub>-subfamily has been determined. The autoreceptor in the human neocortex and in the rat striatum can be subclassified as D<sub>2</sub> (Fedele *et al.*, 1999) whereas the

autoreceptor on the tuberoinfundibular neurones of the rat is D<sub>3</sub> (Lin *et al.*, 2000). It may be of particular interest to determine the exact receptor subtype also for the dopamine autoreceptor in the retina since dopamine in the retina is highly relevant with respect to experimental models of myopia (e.g., deprivation myopia in chickens is aggravated by the D<sub>2</sub>/D<sub>3</sub> receptor antagonist sulpiride; Schaeffel *et al.*, 1995).

In a study on guinea-pig retinal discs carried out for this purpose, we used haloperidol and (*S*)-nafadotride, which have a preference for the D<sub>2</sub>- or D<sub>3</sub>-receptor subtypes, respectively, and two novel substances ST-148 and ST-198, with a higher degree of selectivity for the D<sub>2</sub>- and D<sub>3</sub>-receptor subtypes, respectively (for chemical structures, see Figure 1). The agonists used in the present study, quinpirole and B-HT 920, possess a preference for the D<sub>3</sub> over the D<sub>2</sub> receptor (Levant, 1997; Wood *et al.*, 2000). All experiments were performed on retinal discs preincubated with [<sup>3</sup>H]-noradrenaline, which in areas devoid of noradrenergic neurones like the retina of the guinea-pig is taken up (and released from) dopaminergic cells and offers advantages over the use of [<sup>3</sup>H]-dopamine itself (Schlicker *et al.*, 1996).

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**Figure 1** Chemical structures of the novel dopamine D<sub>2</sub> (ST-148) and D<sub>3</sub> (ST-198) receptor antagonists.

## Methods

### Superfusion experiments

Male Dunkin-Hartley guinea-pigs were decapitated and the eyes were removed from the skull. The retina was carefully detached from other layers of the eye using a spatula and discs (diameter 3 mm) were punched out. For the experiments, a physiological salt solution (PSS) of the following composition was used (mM): NaCl 118, KCl 4.8, CaCl<sub>2</sub> 1.3, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, ascorbic acid 0.06, disodium EDTA 0.03, glucose 10; the solution was aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4).

Retinal discs preincubated with [<sup>3</sup>H]-noradrenaline 25 nM (specific activity 51.8–57.3 Ci mmol<sup>-1</sup>) (60 min; 37°C) were superfused with PSS (37°C) for 110 min. Tritium overflow was evoked by two 2-min periods of stimulation (3 Hz, 200 mA, 2 ms) after 40 and 90 min (S<sub>1</sub>, S<sub>2</sub>). In all experiments the PSS contained nomifensine 10 μM throughout superfusion. The antagonists under study were present in the PSS throughout superfusion, whereas the agonists were added to the PSS from 62 min of superfusion onward.

### Binding studies

Membranes of CHO cell lines stably transfected with human dopamine D<sub>2L</sub> or D<sub>3</sub> receptor DNA were taken for binding assays using [<sup>125</sup>I]-iodosulpride according to Sautel *et al.* (1995). Nonspecific binding was determined in the presence of emonapride 1 μM. K<sub>i</sub> values were derived from IC<sub>50</sub> values according to the Cheng-Prusoff equation (Cheng & Prusoff, 1973), taking into account the K<sub>d</sub> of [<sup>125</sup>I]-iodosulpride for the respective receptors. Data were obtained from at least three separate experiments.

### Calculations and statistics

Tritium overflow was calculated as the fraction of the tritium content of the slices at the beginning of the respective collection period (fractional rate of tritium efflux). Basal

tritium efflux was quantified by calculating the ratio of the fractional rate in the 5-min period immediately before S<sub>2</sub> (i.e. from 85–90 min; t<sub>2</sub>) over that in the collection period from 55–60 min (t<sub>1</sub>, i.e. in the 5-min sample collected just before the addition of the agonist to the superfusion medium). Stimulation-evoked tritium overflow was calculated by subtraction of the basal from the total tritium efflux during stimulation and the subsequent 13 min and was expressed as per cent of tritium present in the slice at the onset of stimulation (basal tritium efflux was assumed to decline linearly from the 5-min collection period before that to 15–20 min after onset of stimulation). To quantify the effects of agonists on the stimulated tritium overflow, the ratio of the overflow evoked by S<sub>2</sub> over that evoked by S<sub>1</sub> was determined. To determine the effects of antagonists on the evoked overflow, the S<sub>1</sub> values obtained in the presence and absence of the respective antagonist were compared. To quantify agonist potencies, pEC<sub>50</sub> values (negative logarithms of the concentration causing the half-maximal effect) were determined. Apparent pA<sub>2</sub> values for antagonists were calculated according to formula 4 of Furchgott (1972).

Results are given as means ± s.e. mean of *n* experiments. For comparison of mean values, Student's *t*-test was used; the Bonferroni correction was used, when two or more values were compared to the same control.

### Drugs

(–)-[Ring-2,5,6-<sup>3</sup>H]noradrenaline (NEN, Zaventem, Belgium); [<sup>125</sup>I]-iodosulpride (Amersham Int., Buckinghamshire, U.K.); tetrodotoxin (Roth, Karlsruhe, Germany); haloperidol, quinpirole hydrochloride (RBI/Sigma, Munich, Germany); B-HT 920 (talipexole; 6-allyl-5,6,7,8-tetrahydro-4H-thiazolo[4,5-d]azepin-2-amine dihydrochloride; Thomae, Biberach an der Riss, Germany); nomifensine (Hoechst, Frankfurt); emonapride (Yamanouchi, Tokyo, Japan); ST-148 (*N*-(4-[4-(2-methoxyphenyl)-piperazin-1-yl]-butyl)-5-(dimethylamino)-naphthalene-1-sulphonamide) maleate and ST-198 (*N*-(4-[1,2,3,4-tetrahydroisoquinolin-2-yl]-butyl)-3-phenylacrylamide) maleate were synthesized by H. Stark; (*S*)-nafadotride (synthesized at the Unité de Neurobiologie et Pharmacologie Moléculaire, Centre Paul Broca). Stock solutions of the drugs were prepared with water, citrate buffer (0.1 mM, pH 4.8; tetrodotoxin), lactic acid (2 M; emonapride), HCl 0.01 M (haloperidol) or DMSO (ST-148, ST-198) and diluted to the concentration required. The solvents did not affect basal and evoked tritium overflow by themselves.

## Results

### Superfusion experiments

Basal tritium efflux was expressed as t<sub>1</sub> or t<sub>2</sub>/t<sub>1</sub>. The t<sub>1</sub> value was not affected by any of the antagonists under study (Table 1). The t<sub>2</sub>/t<sub>1</sub> value was 0.58 ± 0.08 in 43 control experiments in which no agonist or antagonist was present; similar t<sub>2</sub>/t<sub>1</sub> values were obtained in experiments in which no agonist but one of the four antagonists was present (results not shown). B-HT 920 0.1–320 μM, quinpirole 0.1–100 μM, tetrodotoxin 1 μM and omission of Ca<sup>2+</sup> ions did not alter t<sub>2</sub>/t<sub>1</sub> values (results not shown).

The electrically evoked tritium overflow was expressed as  $S_1$  or  $S_2/S_1$ . To quantify the effects of antagonists (present in the medium during  $S_1$  and  $S_2$ ),  $S_1$  values were used. To quantify the effects of agonists (present in the medium during  $S_2$ ),  $S_2/S_1$  values were considered.  $S_1$  values are shown in Table 1.  $S_2/S_1$  values in agonist-free controls are given in the legends to Figures 2 and 3.

The electrically evoked tritium overflow ( $S_2/S_1$ ) was inhibited by  $96 \pm 1\%$  and  $99 \pm 1\%$  by tetrodotoxin  $1 \mu\text{M}$  or omission of  $\text{Ca}^{2+}$  ions, respectively ( $n = 5-8$ ). The dopamine receptor agonists B-HT 920 and quinpirole inhibited the  $S_2/S_1$  value in a concentration-dependent manner (Figures 2 and 3). The effect of either agonist became significant from  $1 \mu\text{M}$  onward and the maximum effect was obtained at  $100 \mu\text{M}$ ; the extent of inhibition obtained for B-HT 920  $100 \mu\text{M}$  ( $82 \pm 1\%$ ;  $n = 14$ ) was significantly ( $P < 0.005$ ) higher than that obtained for quinpirole  $100 \mu\text{M}$  ( $71 \pm 2\%$ ;  $n = 14$ ). The negative logarithm of the concentration causing the half-maximum inhibitory effect ( $\text{pEC}_{50}$ ) was 5.80 and 5.83 for B-HT 920 and quinpirole, respectively.

The concentration-response curve of B-HT 920 was shifted to the right by haloperidol  $0.01 \mu\text{M}$  but not affected by the same concentration of (*S*)-nafadotride (Figure 2A). The novel antagonist ST-148  $0.32 \mu\text{M}$  caused a dextral shift of the concentration-response curve of B-HT 920 whereas the same concentration of ST-198 failed to do so (Figure 2B). Haloperidol  $0.01 \mu\text{M}$  and ST-148  $0.32 \mu\text{M}$  also shifted to the right the concentration-response curve of the other dopamine agonist, quinpirole (Figure 3). The apparent  $\text{pA}_2$  values obtained for the dopamine receptor antagonists are listed in Table 2. Compound ST-148  $0.32 \mu\text{M}$  by itself facilitated the evoked overflow ( $S_1$ ) by 15%, whereas the other antagonists had no significant effect (Table 1).

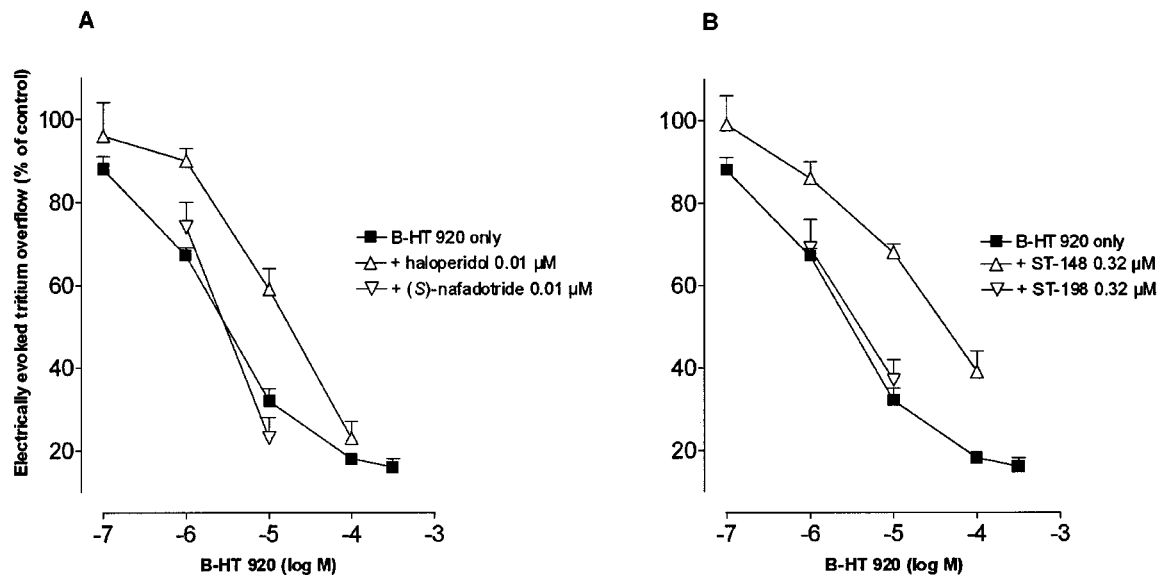
### Binding studies

Binding of [<sup>125</sup>I]-iodosulpride to hD<sub>2L</sub> and hD<sub>3</sub> receptors expressed in CHO cells has been thoroughly characterized in the studies by Sokoloff *et al.* (1992) and Sautel *et al.* (1995) and the  $K_i$  values for haloperidol and (*S*)-nafadotride (Table

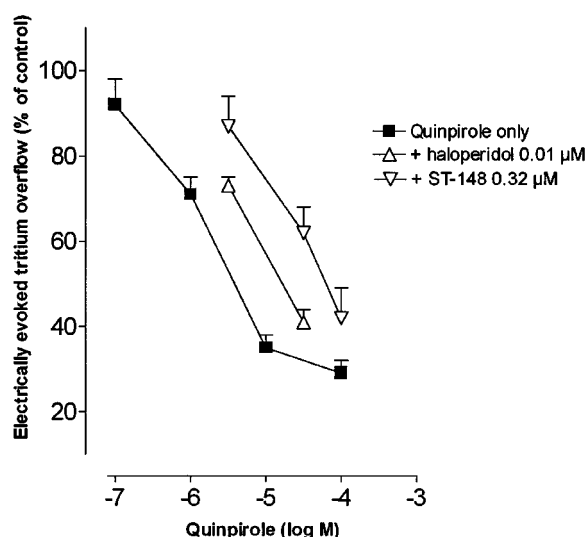
**Table 1** Influence of the antagonists on basal and electrically evoked tritium outflow in guinea-pig retinal discs preincubated with [<sup>3</sup>H]-noradrenaline

	Basal tritium efflux during $t_1$ (fractional rate; $\text{min}^{-1}$ )	Tritium overflow evoked by $S_1$ (% of tissue tritium)
Control	$0.0090 \pm 0.0012$	$17.75 \pm 0.60$
Haloperidol $0.01 \mu\text{M}$	$0.0094 \pm 0.0012$	$19.75 \pm 1.33$
( <i>S</i> )-Nafadotride $0.01 \mu\text{M}$	$0.0076 \pm 0.0011$	$18.18 \pm 1.47$
ST-148 $0.32 \mu\text{M}$	$0.0095 \pm 0.0015$	$20.48 \pm 0.96^*$
ST-198 $0.32 \mu\text{M}$	$0.0096 \pm 0.0022$	$19.80 \pm 2.64$

Tritium overflow was evoked after 40 min ( $S_1$ ). Basal tritium efflux was determined in the 5-min sample from 55–60 min ( $t_1$ ). Means  $\pm$  s.e. mean of 5–29 experiments. \* $P < 0.05$ .



**Figure 2** Effect of B-HT 920 on the electrically evoked tritium overflow from [<sup>3</sup>H]-noradrenaline-preincubated retinal discs and interaction with dopamine receptor antagonists. (A) shows the two classical dopamine receptor antagonists, in (B) the novel antagonists are depicted. The antagonists were present throughout the superfusion (110 min), B-HT 920 from 62 min onward. Tritium overflow was stimulated after 40 and 90 min ( $S_1$ ,  $S_2$ ) and the ratio of the overflow evoked by  $S_2$  over that evoked by  $S_1$  was determined; results are given as per cent of the  $S_2/S_1$  values in B-HT 920-free controls. The  $S_2/S_1$  values in the five B-HT 920-free control series were:  $0.86 \pm 0.02$  (no antagonist);  $0.70 \pm 0.02$  (haloperidol);  $0.93 \pm 0.07$  ((*S*)-nafadotride);  $0.77 \pm 0.03$  (ST-148);  $0.78 \pm 0.03$  (ST-198). Means  $\pm$  s.e. mean of 5–31 (B-HT 920 alone) and 4–7 independent superfusion experiments (in the presence of antagonists).



**Figure 3** Effect of quinpirole on the electrically evoked tritium overflow from [<sup>3</sup>H]-noradrenaline-preincubated retinal discs and interaction with dopamine D<sub>2</sub> receptor-selective antagonists. The antagonists were present throughout the superfusion (110 min), quinpirole from 62 min onward. Tritium overflow was stimulated after 40 and 90 min (S<sub>1</sub>, S<sub>2</sub>) and the ratio of the overflow evoked by S<sub>2</sub> over that evoked by S<sub>1</sub> was determined; results are given as per cent of the S<sub>2</sub>/S<sub>1</sub> values in quinpirole-free controls. The S<sub>2</sub>/S<sub>1</sub> values in the three quinpirole-free control series were: 0.86 ± 0.02 (no antagonist); 0.73 ± 0.02 (haloperidol); 0.75 ± 0.07 (ST-148). Means ± s.e.mean of 7–16 (quinpirole alone) and 4–8 independent superfusion experiments (in the presence of antagonists).

**Table 2** Apparent pA<sub>2</sub> values of the antagonists under study at the dopamine autoreceptor in the guinea-pig retina and their pK<sub>i</sub> values at recombinant hD<sub>2L</sub>- and hD<sub>3</sub>-receptors

	pA <sub>2</sub> in guinea-pig retina	pK <sub>i</sub> hD <sub>2L</sub>	pK <sub>i</sub> hD <sub>3</sub>
Haloperidol	8.46 <sup>a</sup> (8.69; 8.23)	9.22 <sup>c</sup>	8.54 <sup>c</sup>
ST-148	7.59 <sup>a</sup> (7.52; 7.66)	7.85	6.60
(S)-Nafadotride	<8 <sup>b</sup>	8.52 <sup>d</sup>	9.51 <sup>d</sup>
ST-198	<6.5 <sup>b</sup>	6.14	7.92

<sup>a</sup>Mean value of the pA<sub>2</sub> values given in parentheses, which were obtained against B-HT 920 (Figure 2) and quinpirole (Figure 3). <sup>b</sup>From Figure 2. <sup>c</sup>From Sokoloff *et al.* (1992). <sup>d</sup>From Sautel *et al.* (1995).

2) were taken from these studies. Table 2 also shows the K<sub>i</sub> values for the novel dopamine receptor antagonists ST-148 and ST-198. Compared to haloperidol and (S)-nafadotride, ST-148 exhibits an improved selectivity for hD<sub>2L</sub> receptors and ST-198 has a higher preference for hD<sub>3</sub> receptors, respectively.

## Discussion

The aim of our study was to characterize the release-regulating dopamine autoreceptor in the guinea-pig retina. The guinea-pig retinal discs were preincubated with [<sup>3</sup>H]-noradrenaline, which is accumulated in dopaminergic cells in

this avascular retina (Chase, 1982; Schlicker *et al.*, 1996) (and not in postganglionic sympathetic neurones innervating the retinal vasculature, like in porcine retina; Schlicker *et al.*, 1990). The electrically evoked tritium overflow, which is Ca<sup>2+</sup> dependent and tetrodotoxin-sensitive, therefore represents quasi-physiological dopamine release (Schlicker *et al.*, 1996). [<sup>3</sup>H]-Noradrenaline was employed instead of [<sup>3</sup>H]-dopamine itself because of the lower variability of the results (Schlicker *et al.*, 1996). In all of the experiments, nomifensine 10 μM was used to block the dopamine transporter. The amount of dopamine release (expressed as stimulation-evoked tritium overflow divided by the tissue tritium content × 100) was almost 20% and much higher than in our previous studies on guinea-pig retinal discs in which, however, a blocker of the dopamine transporter was omitted and/or a lower stimulation frequency and/or current strength were used (Schlicker *et al.*, 1996; Schlicker & Kathmann, 1998).

The antagonistic effects of haloperidol, (S)-nafadotride, ST-148, and ST-198 were studied against quinpirole and B-HT 920. The latter is also a potent α<sub>2</sub>-adrenoceptor agonist but the possibility that this property contributes to its inhibitory effect on dopamine release could be excluded (effect of B-HT 920 not antagonized by the α-adrenoceptor antagonist phentolamine; unpublished results). Surprisingly, the maximum inhibitory effect of quinpirole was less marked than that of B-HT 920 (whereas the pEC<sub>50</sub> values of both drugs were identical), suggesting that quinpirole acts as a partial agonist. This finding is reminiscent of the results obtained in the study by Wood *et al.* (2000) in which both agonists were examined at recombinant hD<sub>2</sub> and hD<sub>3</sub> receptors in microphysiometry studies.

To determine the dopamine receptor subtype involved in the inhibitory effect of B-HT 920 or quinpirole the apparent pA<sub>2</sub> values of the four antagonists were compared to their pK<sub>i</sub> values at hD<sub>2L</sub> and hD<sub>3</sub> receptors (binding studies with [<sup>125</sup>I]-iodosulpride on CHO cells) (Table 2). The apparent pA<sub>2</sub> values underestimate the true antagonist affinity since the antagonist, under the experimental conditions of the present study, is competing not only with the exogenously added agonist (B-HT 920 or quinpirole) but also with endogenously released dopamine. This is e.g. shown by the fact that dopamine release was facilitated, probably by interruption of the tonical activation of the dopamine autoreceptor, by ST-148 (and by haloperidol 0.1 μM, i.e. a 10 fold higher concentration than that used in this study; unpublished results). Taking into account this phenomenon it may be appropriate to add 0.5 log units to the apparent pA<sub>2</sub> value to get a more authentic estimate of the true affinity of the antagonists.

A look at Table 2 shows that the apparent pA<sub>2</sub> (+0.5 log units) values of the antagonists with preference for D<sub>2</sub> receptors (haloperidol, ST-148) and their pK<sub>i</sub> values at hD<sub>2</sub> receptors agree well, suggesting the involvement of D<sub>2</sub> receptors. In harmony with this view, the effect of B-HT 920 was not antagonized by the antagonists with preference for D<sub>3</sub> receptors at concentrations exceeding their K<sub>i</sub> values at hD<sub>3</sub> receptors by a factor of about 30. For comparison of potencies and affinities one may use in addition the ratios of antagonists with differing selectivity profile (Trendelenburg *et al.*, 1995). This approach also offers the advantage that the underestimation of the true antagonist dissociation constant is cancelled out. In Table 3 the four possible ratios between

**Table 3** Comparison of the ratios of the  $K_B$  values for the dopamine receptor antagonists obtained in release studies with the ratios of their  $K_i$  values obtained in binding sites.

	Autoreceptor	hD <sub>2L</sub>	hD <sub>3</sub>
(S)-Nafadotride/Haloperidol	> 2.9	5.01	0.11
ST-198/Haloperidol	> 91.2	1202	4.17
(S)-Nafadotride/ST-148	> 0.39	0.21	0.001
ST-198/ST-148	> 12.3	51.3	0.05

the antagonists with D<sub>2</sub>- and D<sub>3</sub>-receptor preference have been listed. Again the values suggest that the dopamine autoreceptor in the guinea-pig retina is a D<sub>2</sub> receptor.

One point of concern is that the functional dopamine autoreceptor has been examined in retinal discs from an experimental animal rather than from humans and that the gpD<sub>2</sub> receptor (which, to the best of our knowledge, has not yet been cloned) may differ in its pharmacological properties from the hD<sub>2</sub> receptor. In a recent study, Dubocovich *et al.* (1997) used a similar approach like in the present study, i.e. they compared the potencies of a series of compounds at the

melatonin heteroreceptor causing inhibition of dopamine release in retinal discs from an experimental animal, the rabbit, with their affinities for recombinant human melatonin receptors. Since enough native human retinal tissue is hardly available one might try in the future to perform release experiments in post mortem human retinal tissue or in cultured human retinal cells.

In conclusion, (i) the release-regulating autoreceptor for dopamine in guinea-pig retinal discs belongs to the D<sub>2</sub>-subtype of the D<sub>2</sub>-subfamily of dopamine receptors; (ii) the newly synthesized antagonists ST-148 and ST-198 strongly differentiate between the hD<sub>2</sub>- and hD<sub>3</sub>-subtype of dopamine receptors, and (iii) quinpirole acts as a partial agonist at the guinea-pig dopamine D<sub>2</sub> receptor in the retina.

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