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Title Page

THE BULKY N(6) SUBSTITUENT OF CABERGOLINE IS RESPONSIBLE FOR AGONISM OF THIS DRUG AT SEROTONIN 5-HT_{2A} AND 5-HT_{2B} RECEPTORS AND THUS A DETERMINANT OF VALVULAR HEART DISEASE

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Running Title Page

Running title: Cabergoline and 5-HT_2 receptors

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ABBREVIATIONS: VHD, valvular heart disease; $hD_{2L}R$, human dopamine D_{2LONG} receptor; $hD_{2S}R$, human dopamine D_{2SHORT} receptor; 5-HT_{2A}R, 5-HT_{2A} receptor; 5-HT_{2B}R, 5-HT_{2B} receptor; 5-HT_{1B}R, 5-HT_{1B} receptor; [^{35}S]GTPγS, guanosine 5'-*O*-(3-[^{35}S]thio)triphosphate; SB204741, *N*-(1-methyl-1*H*-5-indolyl)-*N*`-(3-methyl-5-isothiazolyl)urea; ERK, extracellular signal-regulated kinases; GR127935, *N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-

yl)-1,1'-biphenyl-4-carboxamide; MDL100907, (*R*)-(+)-4-(1-hydroxy-1-(2,3-dimethoxyphenyl)methy1)-*N*-2-(4-fluorophenylethyl)piperidine; PD, Parkinson's disease; TGF-β1, transforming growth factor β1; CHO, Chinese hamster ovary; KHS, Krebs-Henseleit solution; PVIC, porcine valvular interstitial cell; 5-HT, 5-hydroxytryptamine (serotonin); U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostagnadin $F_{2\alpha}$; GR55562, 3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyridinyl)phenyl]benzamide; ECM, extracellular matrix.

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ABSTRACT

Fibrotic valvular heart disease (VHD) has been observed in patients with Parkinson's disease treated with dopamine receptor agonists such as pergolide and cabergoline. 5-hydroxytryptamine$_{2B}$ receptor (5-HT$_{2B}$R) agonism is the most likely cause but other 5-HTRs may also play a role in VHD. We aimed at characterizing the molecular fragment of cabergoline being responsible for agonism at 5-HT$_{2B}$ and 5-HT$_{2A}$Rs. Cabergoline with an allyl substituent at N(6) behaved as a potent 5-HT$_{2B}$R full agonist in relaxation of porcine pulmonary arteries and as a weaker 5-HT$_{2A}$R partial agonist in contraction of coronary arteries. The same was true for cabergoline derivatives with cyclopropylmethyl, propyl or ethyl at N(6). However, agonism was converted into antagonism, when the N(6) substituent was methyl. 6-Methylcabergoline retained agonism compared to cabergoline at human hD$_{2L}$ and hD$_{2S}$Rs as determined by [^{35}S]GTPγS binding. In porcine aortic valve cusps, 5-HT-induced contractions were inhibited by ketanserin (5-HT$_{2A/2C}$R antagonist) but not by SB204741 (5-HT$_{2B}$R antagonist). In porcine valvular interstitial cells, cabergoline-induced activation of ERK1/2, an initiator of cellular proliferation and activity, was blocked by MDL100907 (5-HT$_{2A}$R antagonist) and GR127935 (5-HT$_{1B}$R antagonist), whereas the stimulatory effect on ^{3}H-proline and ^{3}H-glucosamine incorporations (indices of extracellular matrix collagen and glycosaminoglycan) was blocked by MDL100907. We conclude that the bulky N(6) substituent of cabergoline is responsible for 5-HT$_{2A}$ and 5-HT$_{2B}$R agonism. The increased ERK1/2 phosphorylation and production of extracellular matrix by cabergoline are mediated by 5-HT$_{2A}$Rs. However, the moderate potency of cabergoline at native 5-HT$_{2A}$Rs suggests that these are not the preferential target in VHD in vivo.

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Introduction

Cabergoline, a dopamine D_2 receptor agonist, is used in the treatment of Parkinson's disease (PD), hyperprolactinemia, and restless legs syndrome. Several studies have shown that PD patients receiving a high daily dose of cabergoline (3 to 4 mg) had an increased risk of fibrotic valvular heart disease (VHD) when compared to PD patients who did not receive the drug (Bhattacharyya et al., 2009 for review). The risk of VHD by small cabergoline doses (0.5 to 2 mg per week) to treat hyperprolactinemia and restless legs syndrome is unclear (Kars et al., 2008; Colao et al., 2008; Tan et al., 2010; Oertel et al., 2007). Pathobiological characteristics of VHD involve fibromyoblast proliferation, thickening of the leaflets and the chords, increase in tissue rigidity, valvular stenosis and valvular insufficiency (Schoen, 2005).

The incidence of VHD as an adverse effect of drugs has been reported in patients taking appetite suppressants (e.g., fenfluramine, dexfenfluramine) or ergolines the latter to which cabergoline belongs (Roth, 2007). However, VHD seems not to be a class effect of the ergolines. Only one case report has documented VHD in the treatment of PD with bromocriptine (Serratrice et al., 2002) and some cases of VHD have been described with the antimigraine drugs ergotamine and methysergide (Bhattacharyya et al., 2009). Serious damage to the heart valves has predominantly been associated with the use of the ergot derivative dopamine agonists, pergolide and cabergoline (Antonini and Poewe, 2007; Bhattacharyya et al., 2009). In contrast, VHD has never been observed in the therapy of PD with lisuride (Hofmann et al., 2006). Obviously, the occurrence of VHD is restricted to certain representatives of the ergolines. This raises the question of whether a specific molecular fragment of the ergoline molecule may be responsible for the severe adverse effect VHD.

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It has recently been demonstrated that VHD caused by the above-mentioned drugs is predominantly associated with an activation of 5-hydroxytryptamine$_{2B}$ receptors (5-HT$_{2B}$Rs) in cardiac valves (Roth, 2007; Huang et al., 2009; Bhattacharyya et al., 2009). However, a participation of other 5-HTRs, e.g., 5-HT$_{2A}$ and 5-HT$_{1B}$, in the development of drug-induced VHD cannot be completely ruled out. Several lines of evidence suggest that 1) human heart valves also have high mRNA levels of 5-HT$_{2A}$ and 5-HT$_{1B}$Rs (Fitzgerald et al., 2000; Roy et al., 2000); 2) 5-HT-induced incompetence of porcine aortic valves was blocked by ketanserin (5-HT$_{2A/2C}$R antagonist; Chester et al., 2001); 3) 5-HT-induced up-regulation of TGF-β1, which plays a role in the pathological response to VHD, was inhibited by MDL100907 (selective 5-HT$_{2A}$R antagonist; Xu et al., 2002); and 4) cabergoline behaved as a full agonist at recombinant human 5-HT$_{2A}$ and 5-HT$_{1B}$Rs, respectively (Newman-Tancredi et al., 2002b).

Based on the observation that some ergolines induce VHD and other ones do not, we have recently demonstrated that the N(6) propyl substituent of pergolide is crucial for 5-HT$_{2A}$ and 5-HT$_{2B}$R agonism and that agonism is converted into antagonism when N(6) propyl is replaced by methyl (Görnemann et al., 2008). Consequently, it was of interest to know whether the pharmacological properties of different cabergoline derivatives (cyclopropylmethyl, propyl, ethyl, methyl, hydrogen instead of allyl as N(6) substituent; Fig. 1) follow the same pattern as those of the corresponding pergolide series (Görnemann et al., 2008). 5-HT$_{2A}$R-mediated responses were studied in endothelium-denuded porcine coronary arteries by measurement of vasoconstriction and 5-HT$_{2B}$R-mediated responses in porcine pulmonary arteries by measurement of endothelium-dependent relaxation (Görnemann et al., 2008; Glusa and Pertz, 2000). Since aortic cusp tissue exhibit contractile responses to 5-HT (Chester et al., 2000, 2001), we additionally studied the effects of 5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{1B}$R antagonists in this tissue. The

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extracellular signal-regulated kinases (ERK1/2) are signaling molecules downstream from 5-HT_{2A}, 5-HT_{2B} and 5-HT_{1B}Rs (Knauer et al., 2009; Maddahi and Edvinsson, 2008) and phosphorylation of ERK1/2 initiates processes of cell proliferation, cell activity and differentiation. Interestingly, in valve tissue, ERK1/2 has been shown to transform normally quiescient VICs into the more active myofibroblast phenotype (Xu et al., 2002; Jian et al., 2002). Therefore, we aimed at assessing whether porcine VICs would show increased ERK1/2 phosphorylation when exposed to cabergoline. A further aim of this study was to investigate the direct effect of cabergoline on collagen and glycosaminoglycan biosynthesis in porcine VICs. To demonstrate whether structural modification of cabergoline would affect the efficacy at dopamine D_2Rs, which are the major therapeutic target of this drug, we studied the effects of cabergoline and 6-methylcabergoline at recombinant hD_{2L} and hD_{2S}Rs, stably expressed in Chinese hamster ovary (CHO) cells, by measuring G protein activation using a [^{35}S]GTPγS binding assay.

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Methods

Tissue preparation. Lungs and hearts from pigs were obtained from the Lehr- und Versuchsanstalt für Tierzucht und Tierhaltung (Teltow Ruhlsdorf, Germany) and placed in ice-cold oxygenated Krebs-Henseleit solution (KHS) of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$ (1.6 mM for coronary arteries and aortic valves), 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.4). Small branches of pulmonary arteries were dissected from the lungs and coronary arteries (left anterior descending and left circumflex) from the hearts. Each of the three aortic valve cusps was cut away from the aortic root. The tissues were cleaned of fat and adhering tissue. The vessels were cut into rings (pulmonary arteries: 2-3 mm long and 2 mm wide, coronary arteries: 3-4 mm long and 2-3 mm wide). The intimal surface of coronary artery rings was gently rolled with a pair of tweezers to destroy the endothelium. Vascular rings were horizontally suspended between two L-shaped stainless steel hooks (300 µm diameter). Strips from the belly area of aortic valve cusps were tied at each end by a cotton thread for measurement of circumferential contraction (Kershaw et al., 2004). The tissues were mounted in water-jacketed 20-mL organ chambers and constantly exposed to oxygenated KHS (95:5% O_2/CO_2, pH 7.4, 37°C). Preparations were connected to an isometric force transducer (FMI TIM-1020) attached to a TSE 4711 transducer coupler and a Siemens C 1016 compensograph for the continuous recording of changes in tension.

Porcine pulmonary arteries (functional 5-HT_{2B} receptor assay). Resting tension was adjusted to 20 mN at the beginning of the experiment. During an initial stabilization period of 60 min, the bathing medium was replaced once after 30 min. The tissue rings were then stimulated at intervals of 45 min once with KCl (30 mM) and three times

with U46619 (0.01 μM) until the contractile response had become constant. After each of the stimulations, the rings were rinsed with KHS for 5 min to wash out KCl or U46619. Fifteen min after the KCl and the first U46619 stimulation had returned to baseline, resting tension was readjusted to 20 mN. The presence of endothelium was verified by the ability of bradykinin (0.01 μM) to cause relaxation following the second contraction with U46619. The relaxant response to 5-HT (or ergot alkaloid derivative) was studied after the third U46619-induced contraction had stabilized. In agonist experiments with ergot alkaloid derivatives, a non-cumulative concentration-response curve to the agonist (0.3–1000 nM) was established by adding only one concentration of agonist to each tissue. This method was employed, since it is known that many tissues respond only to the first concentration of ergots with the consequence that the cumulative concentration-response technique cannot be applied (Müller-Schweinitzer, 1990). The relaxant effect of agonists developed within 2 to 5 min. Agonist experiments were performed in the absence or presence of SB204741 (3 μM) added 30 min before the construction of the concentration-response curve. In experiments where the antagonist or partial agonist properties of ergot alkaloid derivatives were studied, a cumulative concentration-response curve to 5-HT was constructed on each tissue 60 min after the addition of the ergot alkaloid derivative. When the maximal relaxant response to 5-HT or the test agonist had been attained, relaxation was accomplished by addition of bradykinin (0.01 μM). Relaxant effects were expressed as a percentage of the relaxation induced by the agonist plus bradykinin. All experiments were performed in the continuous presence of ketanserin (0.1 μM) to block 5-HT_{2A}Rs.

Porcine coronary arteries (functional 5-HT_{2A} receptor assay). Resting tension was adjusted to 20 mN at the beginning of the experiment. The tissues were stabilized for 60 min with replacement of the bathing medium after 30 min. During the following

equilibration period (115 min) the vascular rings were stimulated twice with KCl (50 mM) for 30 min. The rings were rinsed with KHS for 5 min to wash out KCl. Resting tension was readjusted to 20 mN 15 min after the first contraction with KCl had returned to baseline. The absence of endothelium was verified by the failure of bradykinin (0.1µM) to cause relaxation after the second contraction with KCl. Cumulative concentration-response curves to 5-HT or ergot alkaloid derivative were constructed in the absence or presence of ketanserin (0.01 µM). Each concentration of agonist was administered after the preceding one had produced its maximal effect (usually after 5 to 10 min). pA_2 for ketanserin against 5-HT was 8.88 ± 0.03 (slope of the Schild plot 1.05 ± 0.05; Görnemann et al., 2008). Antagonists were added to the bathing medium 60 min before the construction of a concentration-response curve. Contractile effects were expressed as a percentage of the second KCl-induced contraction. All experiments were performed in the continuous presence of prazosin (0.1 µM), cocaine (6 µM) and indomethacin (5 µM) to block α_1-adrenoceptors and to inhibit neuronal uptake of 5-HT and vascular eicosanoid production by cyclooxygenase, respectively.

Porcine aortic valve cusps. Resting tension was adjusted to 5 mN at the beginning of the experiment. The tissues were stabilized for 60 min with replacement of the bathing medium after 30 min. The strips were then contracted with 5-HT (3 µM). During a period of 45 min the tissues were repeatedly washed with KHS. A cumulative concentration-response curve to 5-HT was constructed after further 30 min in the absence or presence of antagonist. Contractile effects were expressed as a percentage of the 5-HT-induced prestimulation.

Culture of porcine valvular interstitial cells (PVICs). Porcine hearts were transported in sterile KHS containing 100 U/mL penicillin and 0.1 mg/mL

streptomycin. Aortic and mitral valve cusps were excised under sterile conditions and scraped on both sides with a scalpel blade to remove the endothelial cells. Pieces of 2 to 5 mm were immersed in a solution of collagenase-II (2 mg/mL; Sigma-Aldrich) in Dulbecco's MEM/HAM's F-12 (1:1) (DMEM/F12) for 30 min at 37°C under agitation. Fetal calf serum was added to stop collagenase activity. The tissue pieces and cells were centrifuged, washed twice with phosphate-buffered saline (PBS) and transferred to cell culture flasks. Cells were grown in DMEM/F12 supplemented with 10% dialyzed fetal calf serum (HyClone, Logan, UT) and antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). At first confluence, the cells were cryopreserved in liquid nitrogen and used for experiments at passages 1 to 3.

Western Blot analysis of extracellular signal-regulated kinases (ERK1/2). PVICs were grown to eighty till ninety percent confluence and made quiescent by incubation in DMEM/F12 containing 0.2 % FCS for 48 hrs. 5-HT or ergolines were added for 5 min. Antagonists (1 µM MDL100907, SB204741 or GR 127935) were added at least 30 min before the treatment with agonist. After rinsing twice with PBS cells were scraped on ice and lysed with radioimmunoprecipitation assay buffer (RIPA-buffer) containing 150 mM NaCl, 50 mM Tris, 1% Triton-X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, supplemented with protease inhibitors (aprotinin 2 µg/mL, leupeptin 10 µg/mL, pepstatin A 1 µg/mL, PMSF 1 mM, EDTA 5 mM, sodium orthovanadate 1 mM, sodium fluoride 10 mM). The cell lysates were centrifuged, boiled at 95°C for 5 min and stored at –20°C prior further processing. Total protein concentrations were determined with the Bradford method. 20 µg protein from each sample were loaded for SDS-PAGE under reducing conditions and subsequently semi-dry blotted on PVDV membranes (Westran S; Whatman, Maidstone, Kent, UK). After blocking with 5% nonfat dry milk membranes were incubated with specific primary antibody reactive only

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against the phosphorylated form of ERK1/2 (1:1000; Cell Signaling Technology, Denvers, MA) and peroxidase conjugated secondary antibody (Rockland, Gilbertsville, PA). Specific bands were visualized by chemiluminiscence with LumiGLO Reagent (Cell Signaling Technology) on Kodak Biomax films. Membranes were stripped and re-probed with control antibody against both phosphorylated and non-phosphorylated ERK1/2 (1:750). The studies were performed in triplicate.

Measurement of ^{3}H-proline and ^{3}H-glucosamine incorporations. Collagen and glycosaminoglycan synthesis activity were assessed by measuring the incorporation of ^{3}H-proline and ^{3}H-glucosamine as follows: 5 x 10^{4} cells were seeded in 24-well plates and grown in DMEM/F12 medium supplemented with 10% dialyzed FCS and penicillin/streptomycin. After reaching confluence state, the medium was replaced with a low serum concentration of 0.5%. After further 24 hrs the medium was changed again and 1 µM 5-HT, cabergoline or 6-methylcabergoline was added in the absence or presence of 1 µM MDL100907, SB204741, or GR127935. Each of these drugs was added 30 min before the addition of the agonists. Cells were then incubated for 48 hrs in the presence of 0.5 µCi/mL ^{3}H-proline or 3 µCi/mL ^{3}H-glucosamine (PerkinElmer, Rodgau-Jügesheim, Germany). Cells were washed twice with PBS before precipitation with ice-cold 10% trichloroacetic acid for 1 hr at 4°C. The precipitates were solubilized in 0.3 N NaOH/0.1% sodium dodecyl sulfate solution at 37°C under gentle agitation, mixed with scintillation cocktail and measured in a β-counter. Experiments were performed in triplicate or quadruplicate. Results are presented as fold-changes compared with untreated control cells.

[^{35}S]GTPγS binding (functional dopamine D_2 receptor assay). Agonist potencies of cabergoline and 6-methylcabergoline at the human dopamine receptor subtypes D_{2L} and D_{2S} were investigated in a [^{35}S]GTPγS assay as described previously (Schlotter et

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al., 2005) utilizing membranes of CHO cells stably expressing the human D_{2L} and D_{2S} receptor (Hayes et al., 1992). Homogenates of membranes were prepared according to literature (Hübner et al., 2000) with receptor densities of B_{max} = 0.78 pmol/µg and 3.37 pmol/µg for D_{2L} and D_{2S}, respectively, diluted in HEPES buffer (20 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, 40 µg/mL saponin; pH 7.4) and incubated at 37°C with 1 µM of GDP (HEPES buffer) and the test compound (in HEPES buffer supplemented with 0.1 mM dithiothreitol) applying 8 different concentrations (0.01-10,000 nM) as pentaplicates at a final volume of 200 µL in 96-well microplates. After 30 min, 0.1 nM of [^{35}S]GTPγS (specific activity 1250 Ci/mmol, PerkinElmer) were added and the incubation continued for further 30 min. The experiment was terminated by rapid filtration through GF/B filters using an automated cell harvester, the filters were washed 5 times with ice-cold washing buffer (140 mM NaCl, 10 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4; pH 7.4), dried at 60°C for 3 hrs and the trapped radioactivity was counted in a microplate scintillation counter. Data analysis was done by normalisation of the [^{35}S]GTPγS binding data from each individual experiment related to the full effect of the reference agonist quinpirole and further pooling in a mean curve. Nonlinear regression analysis of this curve provided the EC_{50} value as a measure of agonist potency. The top value of the curve represents the maximal response compared to quinpirole (100%).

Drugs. The following drugs were obtained as gifts: cabergoline from Schering AG (Berlin, Germany), GR127935 (GlaxoSmithKline, Stevenage, UK) and U46619 from Upjohn (Kalamazoo, MI). The following drugs were purchased: 6-nor-6-cyclopropyl-methylcabergoline (6-cyclopropylmethylcabergoline), 6-nor-6-propylcabergoline (6-propylcabergoline), 6-nor-6-ethylcabergoline (6-ethylcabergoline), 6-nor-6-methyl-cabergoline (6-methylcabergoline), 6-norcabergoline from Alfarma s.r.o. (Cernosice,

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Czech Republic). Bradykinin triacetate, indomethacin, prazosin hydrochloride and quinpirole hydrochloride were from Sigma-Aldrich (Taufkirchen, Germany). Cocaine hydrochloride was from Merck (Darmstadt, Germany). 5-HT was from Acros Organics (Geel, Belgium) and ketanserin tartrate from Janssen (Beerse, Belgium). SB204741 was from Tocris (Bristol, UK) and MDL100907 from ABX (Radeberg, Germany).

Drugs were dissolved in distilled water, dimethylsulfoxide (SB204741; ergot alkaloid derivatives in the [^{35}S]GTPγS assay), 50% (v/v) ethanol (indomethacin and prazosin) or ethanol (U46619) to a 1-30 mM stock solution. In 5-HT receptor assays ergot alkaloid derivatives were made soluble in a mixture of 50% (v/v) ethanol and an equimolar amount of 1N-HCl. Stock solutions were stored at –18°C and freshly diluted in distilled water, ethanol (SB204741), PBS or in HEPES buffer before the beginning of the experiment. Final concentrations of ethanol and dimethylsulfoxide present in the organ bath did not exceed 0.1 and 0.01%, respectively.

Data presentation and analysis. Data are presented as mean values ± standard error of the mean (S.E.M) for *n* animals or *n* individual experiments. Concentration-response curves were fitted to the Hill equation by an iterative least square method (GraphPad Prism 4.0, GraphPad Soft-ware, San Diego, CA) to provide estimates of the maximal response E_{max} (percentage of the maximal response to a reference compound) and the half-maximal effective concentration pEC_{50} (the negative logarithm of the agonist concentration producing 50% of the maximal response). Affinities of partial agonists ($-\log K_P = pK_P$ values) were estimated in agonist experiments according to the method of Kenakin (1993) or in antagonist experiments according to the method of Marano and Kaumann (1976). Using the method of Kenakin (1993) we estimated the equilibrium dissociation constant K_P for the partial agonist/receptor complex by comparing equiactive molar concentrations of the full agonist A (5-HT) and the partial agonist P

(ergot derivative) according to the equation: $c(A) = m \cdot c(A)/c(P) + b$ with $m = K_P/(\varepsilon_P/\varepsilon_A - 1)$, where $c(A)$ is the molar concentration of A, $c(P)$ the molar concentration of P, m the slope and b the ordinate intercept of the regression line of $c(A)$ vs. $c(A)/c(P)$. ε_A and ε_P represent the intrinsic efficacies of A and P, respectively. If $\varepsilon_P << \varepsilon_A$, $pK_P = -\log K_P$ can be calculated from: $-\log K_P = \log m$. Using the method of Marano and Kaumann (1976) we estimated the equilibrium dissociation constant K_P for the partial agonist/receptor complex by comparing equiactive molar concentrations of the full agonist A (5-HT) in the absence and presence of the partial agonist P (ergot derivative) according to the equation: $c(A) = m \cdot c(A)^* + b$ with $m = 1/[1 + (1 - \varepsilon_P/\varepsilon_A) \cdot c(P)/K_P]$, where $c(A)$ is the molar concentration of A in the absence of P, $c(A)^*$ the molar concentration of A in the presence of P, m the slope of a weighted regression line of $c(A)$ vs. $c(A)^*$, b the ordinate intercept, and $c(P)$ the molar concentration of P. If $\varepsilon_P << \varepsilon_A$, $pK_P = -\log K_P$ can be calculated from: $\log [(1/m) - 1] = \log c(P) - \log K_P$. Antagonist affinities of silent antagonists were expressed as an apparent pA_2 value. pA_2 was calculated from a single concentration of antagonist using the following equation: $pA_2 = -\log c(B) + \log (r - 1)$ where $c(B)$ is the molar concentration of the antagonist and r the ratio of agonist EC_{50} determined in the presence and absence of the antagonist. Student's *t*-test (unpaired, two-tailed) was used to assess differences between two mean values with $P < 0.05$ being considered as significant. In the case of inhomogeneous variances the Welch test of significance was used.

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Results

Effects of cabergoline and its derivatives at endothelial 5-HT$_{2B}$ receptors in porcine pulmonary arteries. Cabergoline and its derivatives with a cyclopropylmethyl, propyl or ethyl instead of an allyl group at N(6) caused relaxant responses in porcine pulmonary arteries. The compounds behaved as full agonists in this tissue (Fig. 2). Agonist potencies of the compounds were similar to the agonist potency of 5-HT. Relaxations to the ergolines were inhibited by 3 µM SB204741 (selective 5-HT$_{2B}$R antagonist; Fig. 2). The estimated pA_2 values for SB204741 were in the same range as the pA_2 for SB204741 against 5-HT and argued for an involvement of the 5-HT$_{2B}$R in the relaxant response to the drugs (Table 1). In contrast to the compounds with bulkier N(6) substituents, 6-methylcabergoline (1.5 nM), failed to show relaxation but antagonized the relaxant response to 5-HT (apparent pA_2 10.06 ± 0.12; Fig. 3). High concentrations (1 µM) of 6-methylcabergoline induced a slight relaxation of 7 ± 3% that was the same in the presence of SB204741 (7 ± 4%, n = 4). 6-Norcabergoline (0.2 µM) induced a relaxation of 22 ± 6%. Accordingly, this drug behaved as a partial agonist and blocked 5-HT-induced relaxation (–log K_P = pK_P = 7.28 ± 0.12, n = 4). Agonist and antagonist effects of the drugs are summarized in Table 1.

Effects of cabergoline and its derivatives at smooth muscle 5-HT$_{2A}$ receptors in porcine coronary arteries. Cabergoline and its derivatives with a cyclopropylmethyl, propyl or ethyl instead of an allyl group at N(6) produced concentration-dependent contractile effects in porcine coronary arteries. The rank order of agonist potency was 5-HT ≈ 6-propylcabergoline > cabergoline ≈ 6-ethylcabergoline ≈ 6-cyclopropylmethyl-cabergoline. The drugs were partial agonists relative to 5-HT (Fig. 4). The pEC$_{50}$ values for the partial agonists were in good agreement with the –log K_P = pK_P values calculated

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from equiactive concentrations of the respective partial agonist and 5-HT (Table 2). 0.1 μM ketanserin (selective 5-$HT_{2A/2C}$R antagonist) inhibited the contractile response to the agonists. The estimated pA_2 values for ketanserin against the compounds were in the same range as the pA_2 value of the antagonist against 5-HT and argued for an involvement of 5-HT_{2A}Rs in the contractile response to the compounds (Table 2). In contrast to the compounds with bulkier N(6) substituents, 6-methylcabergoline did not induce a contraction up to a concentration of 1 μM. 6-Methylcabergoline (0.1 μM) behaved as surmountable antagonists of the 5-HT response in porcine coronary arteries (apparent pA_2 7.85 ± 0.12; Fig. 5A). The drug with a hydrogen at N(6) of the ergoline molecule, 6-norcabergoline (3 μM), acted as an insurmountable antagonists in this tissue (Fig. 5B). Agonist and antagonist effects of these drugs are summarized in Table 2.

Effects of 5-HT in isolated porcine aortic valve cusps. 5-HT induced a concentration-dependent contraction in isolated aortic cusp tissue (pEC_{50} 7.24 ± 0.07; *n* = 9). The contractile 5-HT response was inhibited by 10 nM ketanserin (apparent pA_2 8.66 ± 0.11, *n* = 4). Contractile 5-HT responses remained unaffected in the presence of 10 nM GR127935 but were blocked by 100 nM GR127935 (apparent pA_2 7.56 ± 0.13, *n* = 4). 3 μM SB204741 (*n* = 4) failed to inhibit 5-HT-induced responses (Fig. 6).

Effects of 5-HT, cabergoline and 6-methylcabergoline on PVIC ERK1/2 phosphorylation. 5-HT increased pERK1/2 in a concentration-dependent manner in PVIC cultures as shown by Western blotting (Fig. 7A). The same was true for cabergoline (Fig. 7C). The stimulatory effect of 1 μM 5-HT or 1 μM cabergoline on pERK1/2 was inhibited by 1 μM GR127935 but not by 1 μM SB204741 (Fig. 7B, D). Pretreatment with 1 μM MDL100907 strongly inhibited the effect of 1 μM cabergoline (Fig. 7D) but that of 1 μM 5-HT to a lower extent (Fig. 7B). 1 μM GR127935,

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SB204741 or MDL100907 alone had no stimulatory effect on pERK1/2 (Fig. 7E). 1 μM 6-methylcabergoline stimulated pERK1/2 to a lower extent compared to 1 μM 5-HT or 1 μM cabergoline (Fig. 7F).

Effects of 5-HT, cabergoline and 6-methylcabergoline on collagen and glycosaminoglycan synthesis in PVICs. 5-HT, cabergoline and to a minor extent 6-methylcabergoline showed an increase in collagen synthesis in PVICs as monitored by ^{3}H-proline incorporation (Fig. 8A). The stimulatory effect of cabergoline on ^{3}H-proline incorporation was inhibited by 1 μM MDL100907 but not by 1 μM SB204741 or 1 μM GR127935 (Fig. 8B). Similarly, glycosaminoglycan biosynthesis increased in the presence of 5-HT and cabergoline as monitored by ^{3}H-glucosamine incorporation (Fig. 8C). The effect of 6-methylcabergoline was less pronounced (Fig. 8C). Increased ^{3}H-glucosamine incorporation by cabergoline was inhibited by 1 μM MDL100907 but not by 1 μM SB204741 or 1 μM GR127935 (Fig. 8D).

Effects of cabergoline and 6-methylcabergoline at hD_{2L} and hD_{2S} receptors, respectively. Cabergoline and 6-methylcabergoline displayed higher agonist potency than the selective dopamine D_2R agonist quinpirole at hD_{2L} and hD_{2S}Rs, stably expressed in CHO cells (Fig. 9). Substitution of the allyl group at N(6) (cabergoline) against a methyl group (6-methylcabergoline) did not affect agonist potency neither at hD_{2L} nor at hD_{2S}Rs. Efficacy of 6-methylcabergoline was lower than that of cabergoline (Table 3).

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Discussion

Numerous studies have reported that cabergoline and pergolide have a similar risk of inducing restrictive VHD (Bhattacharyya et al., 2009 for review). From a structural viewpoint, cabergoline and pergolide do not possess a N(6) methyl substituent which is characteristic of other therapeutically used ergot alkaloid derivatives such as bromocriptine, ergotamine or methysergide. Cabergoline bears a N(6) allyl group and pergolide a similarly sized N(6) propyl group. The most striking result of the present study was that cabergoline and its derivatives which differed in their substitution pattern at N(6) (see Fig. 1) showed exactly the same pharmacological properties at 5-HT_{2A} and 5-HT_{2B}Rs as the corresponding pergolide derivatives we had previously studied (Görnemann et al., 2008). Cabergoline, 6-cylclopropylmethylcabergoline, 6-propyl-cabergoline, and 6-ethylabergoline acted as partial agonists at 5-HT_{2A}Rs and as full agonists at 5-HT_{2B}Rs, whereas 6-methylcabergoline was a silent antagonist at both receptors. Accordingly, pergolide, 6-cyclopropylmethylpergolide and 6-ethylpergolide were partial agonists at 5-HT_{2A}Rs and full agonists at 5-HT_{2B}Rs, and 6-methylpergolide was an antagonist at these receptors (Görnemann et al., 2008). Thus the replacement of an allyl or a propyl against a methyl group at N(6) of the ergoline skeleton can convert agonism into silent antagonism both at 5-HT_{2A} and 5-HT_{2B}Rs. The low efficacy partial 5-HT_{2B}R agonist properties of 6-norcabergoline (6-deallylcabergoline), a metabolite of cabergoline, appears not to be of clinical relevance; 6-norcabergoline was detected only in trace amounts in rat urine (Battaglia et al., 1993).

Cabergoline has previously been described to act as a potent, full agonist at recombinant human 5-HT_{2B}Rs expressed in CHO cells (Newman-Tancredi et al., 2002b). This is consistent with our results. However, cabergoline that was also a full

agonist at recombinant 5-HT_{2A}Rs (Newman-Tancredi et al., 2002b), behaved as a partial agonist at 5-HT_{2A}Rs of porcine coronary artery. Moreover, cabergoline showed about a 60-fold higher agonist potency at recombinant 5-HT_{2A}Rs (Newman-Tancredi et al., 2002b) than at 5-HT_{2A}Rs of porcine coronary artery. Agonist potency depends on receptor number/density and receptor-effector coupling efficiency; hence, a given drug acting on the same receptor can show large variations in efficacy and potency when different models and experimental conditions are used (Hoyer and Boddeke, 1993). Thus, efficacy and potency of cabergoline at 5-HT_{2A}Rs may be more pronounced in any other tissue including valve tissue. However, the much lower agonist potency of cabergoline at 5-HT_{2A}Rs (pEC_{50} 6.32) compared to the high potency at 5-HT_{2B}Rs (pEC_{50} 8.15) observed in the present study suggests that the 5-HT_{2A}R cannot be the preferential site of action in vivo when the daily dose of cabergoline in the treatment of PD is 3 to 4 mg. Further studies are required to show whether selective 5-HT_{2A} or 5-HT_{2B}R antagonists can prevent cabergoline-induced VHD in vivo.

5-HT may impair the function of the heart valves by its effect on the contractile elements of the valve tissue, which would alter the correct opening and closing of the valve, thereby initiating valvular insufficiency (Chester et al., 2000). The response of cusp tissue to 5-HT may be mediated by contraction of smooth muscle α-actin (α-SMA)-positive cells of the aortic valve cusps (Chester and Taylor, 2007). Indeed, 5-HT contracted porcine aortic valve leaflets, and these contractions were abolished by 1 μM ketanserin (Chester et al., 2000). In our study, 5-HT induced circumferential contractions of aortic valve cusp strips and 10 nM ketanserin caused a rightward shift of the 5-HT curve with no depression of the maximal response. The apparent pA_2 of 8.7 argues for an involvement of 5-HT_{2A}Rs in the contractile response to 5-HT in aortic valves. The failure of SB204741 to inhibit 5-HT-induced valvular contractions argues

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against a role for 5-HT_{2B}Rs in the contractile response to 5-HT. GR12793 inhibited the 5-HT contraction only at a high concentration (100 nM). It should be emphasized that GR127935 blocks 5-$HT_{1B/1D}$R-mediated responses in the (sub)nanomolar range (see PDSP K_i Database http://pdsp.med.unc.edu/indexR.html). The pA_2 of 7.6 for GR127935 rules out any contribution of this subtype in the contractile 5-HT response in aortic valve cusps and is in good agreement with the pK_i of 7.4 determined in cells expressing the human recombinant 5-HT_{2A}R (Huang et al., 2005). The observation that 5-HT elicited circumferential contractions of aortic valve cusps mediated by 5-HT_{2A}Rs is of special interest. Collagen fibers are circumferentially aligned in the fibrosa layer of the leaflets (Xu and Grande-Allen, 2010) and we could demonstrate that 5-HT or cabergoline increased collagen biosynthesis via stimulation of 5-HT_{2A}Rs (see below).

Our finding on the agonist effect of cabergoline at D_{2S} and D_{2L}Rs is in line with that previously reported (Newman-Tancredi et al., 2002a). The present study shows that agonist potency of 6-methylcabergoline at hD_{2S} and hD_{2L} Rs is retained compared to that of cabergoline. 6-Methylcabergoline behaved as a high-efficacy partial hD_{2S} and hD_{2L}R agonist.

5-HT and anorectics such as fenfluramine have a direct mitogenic effect on human VICs as shown by stimulation of ^{3}H-thymidine deoxyribose incorporation into newly synthesized DNA. This effect is mediated via 5-HT_{2B}Rs (Setola et al., 2003). Accordingly, 5-HT_{2B}Rs have been suggested to contribute to valvular proliferation in VHD. ERK1/2 plays an important role in the regulation of cell proliferation and in the production of extracellular matrix (ECM) components (Xu et al., 2002). 5-HT and norfenfluramine induced an increase in ERK1/2 phosphorylation in human VICs (Setola et al, 2003). However, Setola et al. (2003) did not block ERK1/2 activation with antagonists. In sheep aortic VICs pERK1/2 activation by 5-HT was minimally inhibited

by MDL100907 (5-HT_{2A}R antagonist; Xu et al., 2002), whereas in canine mitral VICs the 5-HT response was strongly inhibited by ketanserin (5-$HT_{2A/2C}$R antagonist) and GR55562 (5-HT_{1B}R antagonist) but not by SB204741 (5-HT_{2B}R antagonist; Connolly et al., 2009). Our results using porcine VICs that represent a mixture of aortic and mitral VICs are consistent with these studies (Xu et al., 2002; Connolly et al., 2009); we detected a minimal inhibition with MDL100907 and a strong inhibition of 5-HT-induced pERK1/2 activation with GR127935. Interestingly, cabergoline mimicked 5-HT as an activator of ERK1/2 inasmuch as the increase of pERK1/2 induced by the ergot was inhibited by GR127935 but not by SB204741. The inhibition of the cabergoline response with MDL100907, however, was more pronounced compared to that of 5-HT. pERK/1/2 activation by 6-methylcabergoline, a potent 5-HT_{2A} and 5-HT_{2B}R antagonist in the present study, was detectable but lower than that of 5-HT or cabergoline.

In the present study, we also used porcine VICs to examine the potential role of 5-HT receptor subtypes in 5-HT or cabergoline-induced incorporation of proline and glucosamine as indices of collagen and glycosaminoglycan production. Collagen is the main ECM component of the fibrosa layer of the heart valve leaflet, whereas the ECM of the spongiosa layer is rich in glycosaminoglycans (Xu and Grande-Allen, 2010). We observed that collagen and glycosaminoglycan biosynthesis were increased by 5-HT, cabergoline and to a lower extent by 6-methylcabergoline. Only MDL100907 inhibited the effect of 5-HT or cabergoline, whereas selective blockade of 5-HT_{2B} or 5-HT_{1B}Rs had no effect. The inability of 5-HT_{2B}R antagonists to block 5-HT-induced collagen and glycosaminoglycan production is consistent with observations in cultured porcine mitral valves (Barzilla et al., 2010). Admittedly, our study has several limitations. Cell cultures normally do not mirror the in vivo situation (Mekontso-Dessap et al., 2006). Moreover,

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different signaling pathways may also be involved in drug-induced VHD when different species are used. A further point is that the cabergoline-induced increase in the production of ECM components was not completely blocked by MDL100907. Thus other mechanisms may also be involved in cabergoline-induced VHD.

In summary, the present study shows that agonism of cabergoline both at 5-HT_{2B} and 5-HT_{2A}Rs can be converted into antagonism when the N(6) allyl substituent is replaced by a methyl substituent. Substitution of the N(6) allyl group by a methyl group retains agonist potency at D_2Rs which are the major therapeutic target of cabergoline. Evidence has been provided that agonist activity at 5-HT_{2B}Rs is a likely molecular mechanism of drug-induced VHD (Roth, 2007). We hypothesize that both 5-HT_{2B} and 5-HT_{2A}Rs are involved in VHD. Activation of 5-HT_{2B}Rs is mitogenic (Setola et al., 2003); the influence on cell activity (ERK1/2 phosphorylation, production of ECM components) is mediated by 5-HT_{2A}Rs. Both mechanisms may contribute to the severe side effects of cabergoline. Since the effect of 6-methylcabergoline on 5-HT receptor-mediated cellular activity was lower than that of the parent compound, we hypothesize that the N(6) allyl substituent of cabergoline is the molecular fragment that is especially responsible for agonism at 5-$HT_{2A/2B}$Rs and thus a determinant of VHD.

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Authorship Contributions

Participated in research design: Pertz and Gmeiner.

Conducted experiments: Kekewska, Pertz and Hübner.

Performed data analysis: Pertz.

Wrote or contributed to the writing of the manuscript: Pertz, Kekewska, and Hübner.

Other: Pertz acquired funding for the research.

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Legends for Figures

Fig. 1. Chemical structure of cabergoline and its derivatives.

Fig. 2. Relaxant responses to 5-HT and cabergoline (**A**) and its derivatives, 6-cyclo-propylmethylcabergoline (6-Cpm-Cab; **B**), 6-propylcabergoline (6-propylcab; **C**), 6-ethylcabergoline (6-ethylcab; **D**), in porcine pulmonary arteries in the absence and presence of SB204741 (5-HT_{2B}R antagonist). Points are mean values ± S.E.M for *n* animals indicated in Table 1.

Fig. 3. Relaxant response to 5-HT in porcine pulmonary arteries in the absence and presence of 6-methylcabergoline (**A**) and 6-nor-cabergoline (6-norcab; **B**). Points are mean values ± S.E.M from *n* animals indicated in Table 1.

Fig. 4. Contractile responses to 5-HT and cabergoline (**A**) and its derivatives, 6-cyclo-propylmethylcabergoline (6-Cpm-Cab; **B**), 6-propylcabergoline (6-propylcab; **C**), 6-ethylcabergoline (6-ethylcab; **D**), in porcine coronary artery in the absence and presence of ketanserin (5-$HT_{2A/2C}$R antagonist). Points are mean values ± S.E.M from *n* animals indicated in Table 2.

Fig. 5. Contractile response to 5-HT in porcine coronary artery in the absence and presence of 6-methylcabergoline (**A**) and 6-nor-cabergoline (6-norcab; **B**). Points are mean values ± S.E.M from *n* animals indicated in Table 2.

Fig. 6. Circumferential contractions of aortic valve cusp strips in response to 5-HT in the absence or presence of ketanserin (5-$HT_{2A/2C}$R antagonist; **A**), SB204741 (5-HT_{2B}R antagonist; **B**), and GR127935 (5-$HT_{1B/1D}$R antagonist; **C**). Points are mean values ± S.E.M from 4 to 9 animals.

Fig. 7. Western blots of porcine aortic and mitral valvular interstitial cells demonstrating the effect of 5-HT (**A, B**), cabergoline (**C, D**), and 6-methylcabergoline (**F**) on ERK1/2 phosphorylation. The effects of MDL100907 (5-HT_{2A}R antagonist), SB204741 (5-HT_{2B}R antagonist) and GR127935 (5-$HT_{1B/1D}$R antagonist) on 5-HT and cabergoline stimulation of pERK1/2 are shown in **B** and **D**, respectively. MDL100907, SB204741 and GR127935 alone had not effect on ERK1/2 phosphorylation (**E**). The blots are representative of 4 different experiments, with comparable results.

Fig. 8. Porcine aortic and mitral valvular interstitial cell culture showing the increase in collagen biosynthesis by tritiated proline incorporation (**A, B**) and glycosaminoglycan biosynthesis by tritiated glucosamine incorporation (**C,D**) induced by 5-HT, cabergoline and 6-methylcabergoline in the absence and presence of the MDL100907 (5-HT_{2A}R antagonist), SB204741 (5-HT_{2B}R antagonist) and GR127935 (5-$HT_{1B/1D}$R antagonist). Values are mean ± S.E.M. of 3 to 6 independent experiments. *$P < 0.05$.

Fig. 9. Effects of cabergoline and 6-methylcabergoline at dopamine D_{2L} (**A**) and D_{2S}Rs (**B**), stably expressed in CHO cells. [^{35}S]GTPγS binding is expressed as a percentage relative to the effect of the full agonist quinpirole. Points are mean values ± S.E.M. from 6 individual experiments.

TABLE 1

Pharmacological profile of cabergoline and its derivatives at 5-HT_{2B}Rs in porcine pulmonary arteries

Data are means ± S.E.M. from *n* animals given in parentheses. E_{max} is expressed as a percentage of the maximal relaxation induced by the agonist and bradykinin (0.01 µM) finally added at the end of each experiment.

Compounds	pEC_{50}	E_{max} (%)	pK_P	pA_2
Agonist				
5-HT	8.52 ± 0.05 (26)[a]	73 ± 2[a]		6.59 ± 0.07[b,c]
Cabergoline	8.15 ± 0.07 (5)	58 ± 6[d]		6.45 ± 0.06 (4)[c]
6-Cyclopropylmethylcabergoline	8.10 ± 0.14 (6)	75 ± 6[e]		6.63 ± 0.25 (4)[c]
6-Propylcabergoline	7.87 ± 0.03 (5)	86 ± 3[f]		6.43 ± 0.09 (4)[c]
6-Ethylcabergoline	8.08 ± 0.09 (4)	68 ± 1[g]		6.34 ± 0.20 (4)[c]
6-Norcabergoline		22 ± 6[h]	7.28 ± 0.12 (4)[i]	
Antagonist				
6-Methylcabergoline		0		10.06 ± 0.12 (4)[j]

[a]Pooled data obtained from all experiments

[b]Data from Glusa and Pertz (2000)

[c]Apparent pA_2 for SB204741 at 3 µM

[d]Not significantly different from respective 5-HT control curve (64 ± 5)

[e]Not significantly different from respective 5-HT control curve (74 ± 5)

[f]Significantly different from respective 5-HT control curve (75 ± 4)

[g]Not significantly different from respective 5-HT control curve (72 ± 2)

[h]Significantly different from respective 5-HT control curve (76 ± 8)

[i]Calculated from the antagonism of the 5-HT response by the partial agonist at a concentration of 0.2 µM (Marano and Kaumann, 1976)

[j]Apparent pA_2 tested at 1.5 nM

TABLE 2

Pharmacological profile of cabergoline and its derivatives at 5-HT_{2A}Rs in porcine coronary artery

Data are means ± S.E.M from *n* animals given in parentheses. E_{max} is expressed as a percentage of the maximal contraction to KCl (50 mM).

Compound	pEC_{50}	E_{max} (%)	pK_P[a]	pA_2
Agonist				
5-HT	6.70 ± 0.04 (10)[b]	54 ± 6[b]		8.88 ± 0.03[c,d]
Cabergoline	6.32 ± 0.08 (5)	34 ± 5[e]	6.27 ± 0.20 (5)	8.88 ± 0.11 (4)[d]
6-Cyclopropylmethylcabergoline	6.01 ± 0.15 (4)	18 ± 2[e]	6.43 ± 0.22 (4)	8.61 ± 0.14 (4)[d]
6-Propylcabergoline	6.60 ± 0.11 (4)	32 ± 6[e]	6.58 ± 0.09 (4)	8.57 ± 0.12 (4)[d]
6-Ethylcabergoline	6.22 ± 0.12 (4)	30 ± 3[e]	6.21 ± 0.03 (4)	8.75 ± 0.16 (4)[d]
Antagonist				
6-Norcabergoline		0		5.85 ± 0.07 (4)[f]
6-Methylcabergoline		0		7.85 ± 0.12 (6)[g]

[a]pK_P, calculated from equiactive concentrations of the partial agonist and 5-HT (Kenakin, 1993)

[b]Pooled data obtained from all experiments

[c]Data from Görnemann et al. (2008)

[d]Apparent pA_2 for ketanserin at 0.01 µM

[e]Significantly different from respective 5-HT control curve

[f]Apparent pA_2 for cabergoline derivative tested at 3 µM

[g]Apparent pA_2 for cabergoline derivative tested at 0.1 µM

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TABLE 3

Functional properties of cabergoline and 6-methylcabergoline at D_{2L} and D_{2S}Rs determined in a [^{35}S]GTPγS assay

Data are means ± S.E.M. from 6 to 8 individual experiments. E_{max} expressed as percentage of the maximum response to the full agonist quinpirole

Compound	D_{2L}		D_{2S}	
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)
Cabergoline	8.51 ± 0.11	88 ± 6	8.82 ± 0.06	94 ± 3
6-Methylcabergoline	8.33 ± 0.07	75 ± 4	8.68 ± 0.08	71 ± 4

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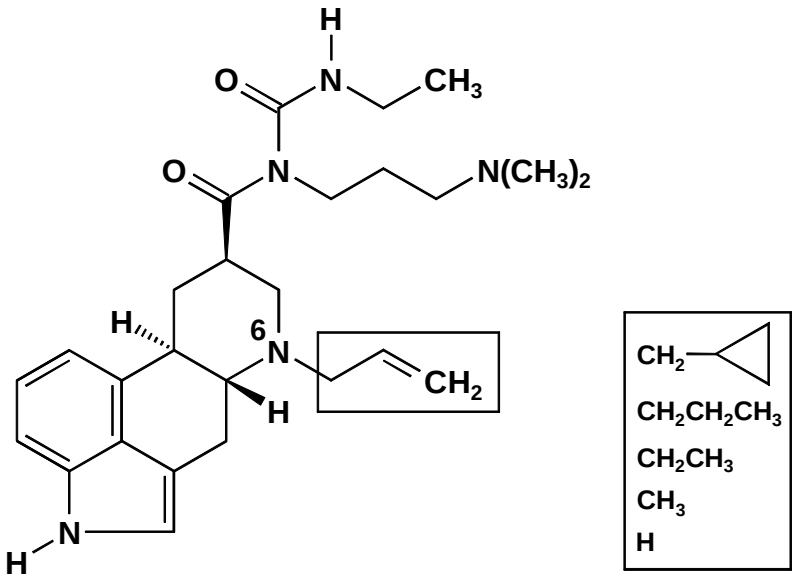

Cabergoline

Fig. 1

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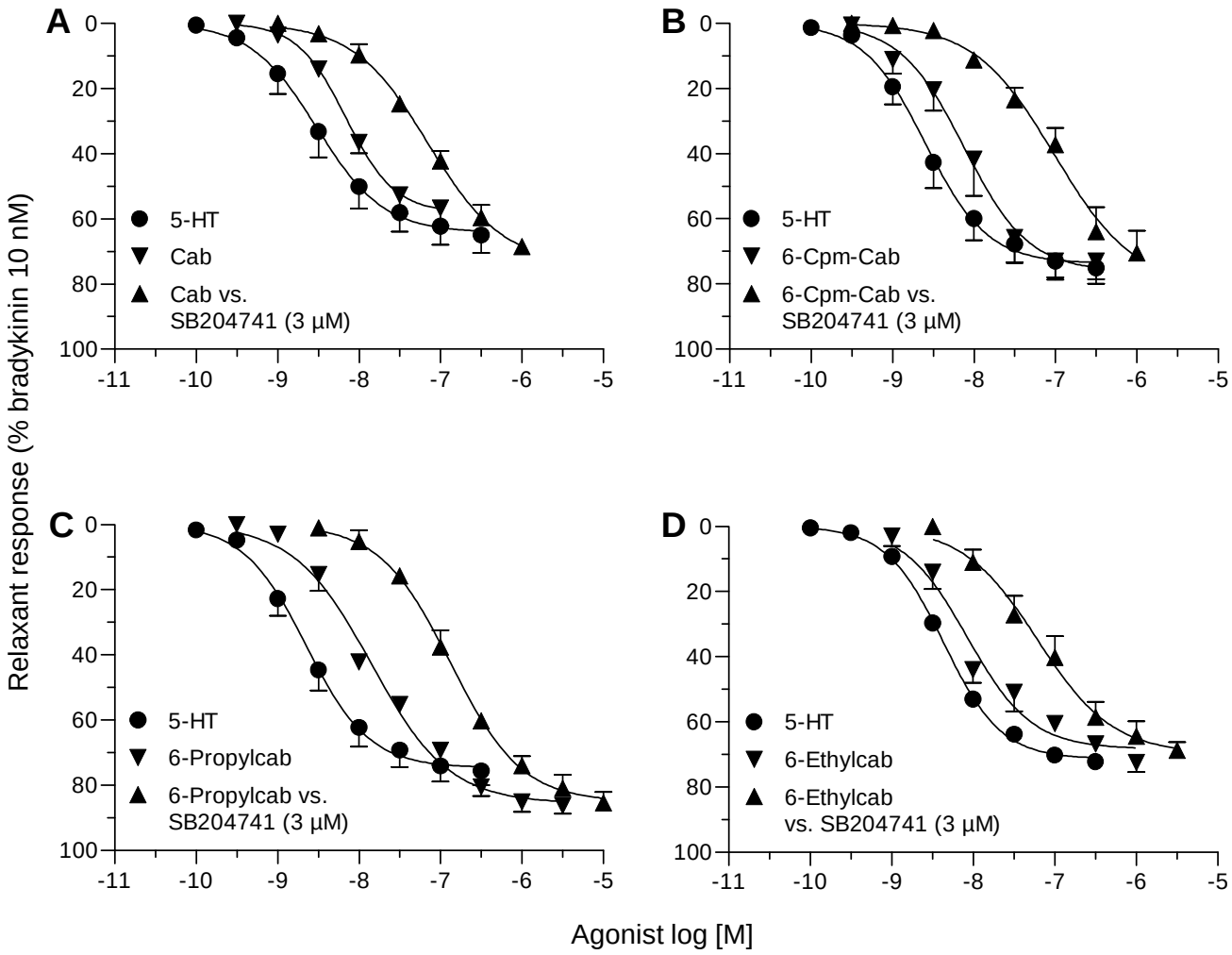

Fig. 2

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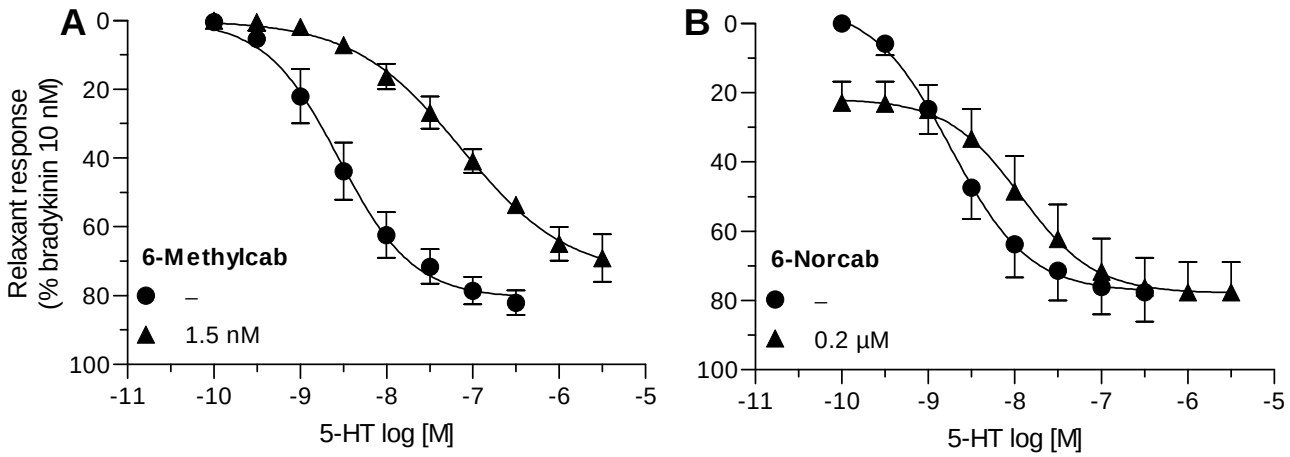

Fig. 3

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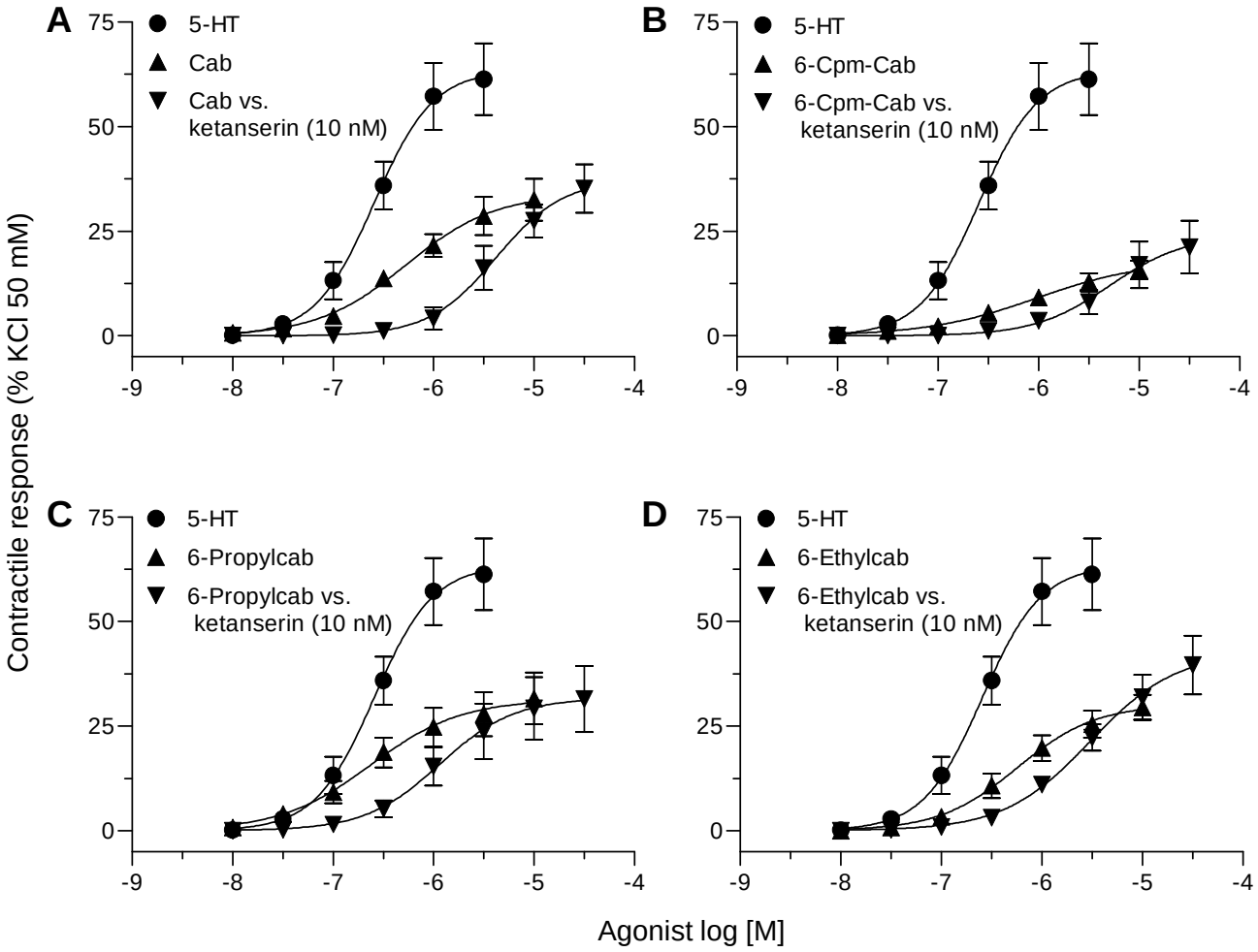

Fig. 4

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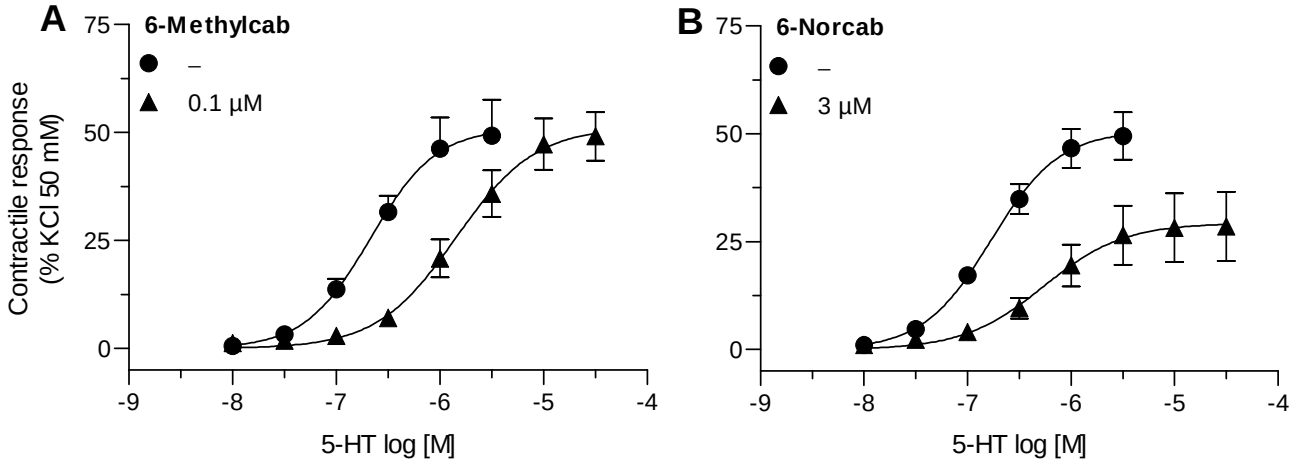

Fig. 5

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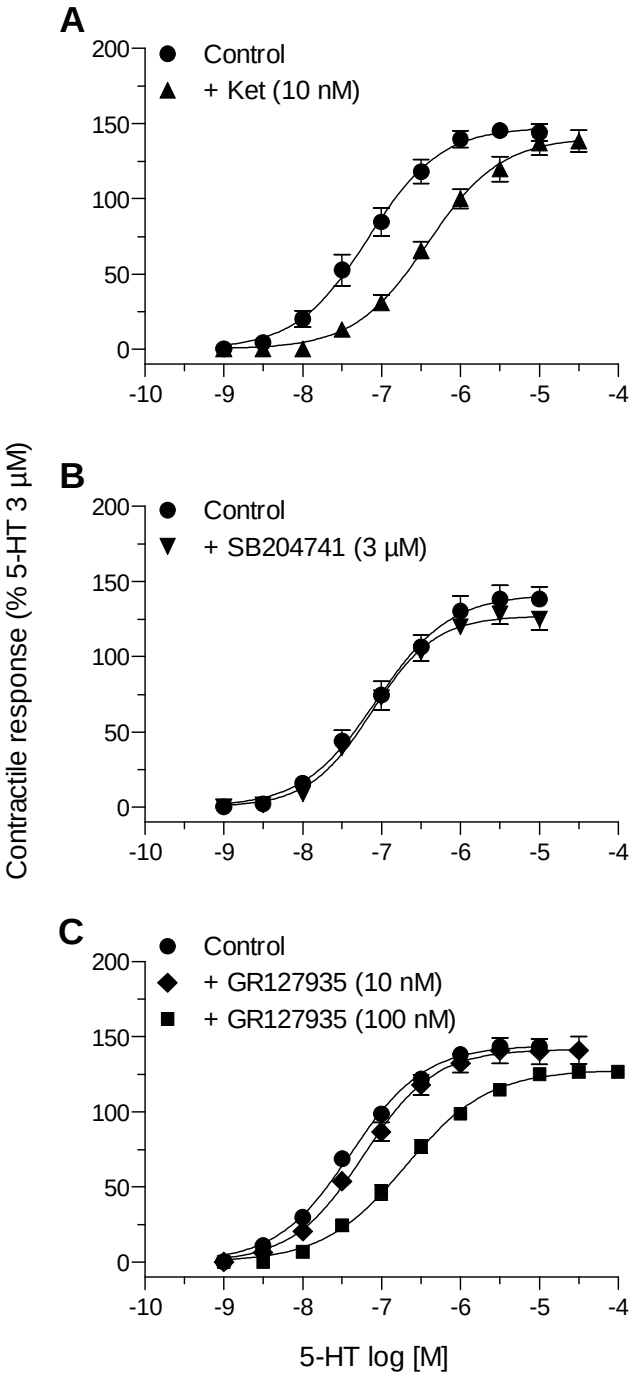

Fig. 6

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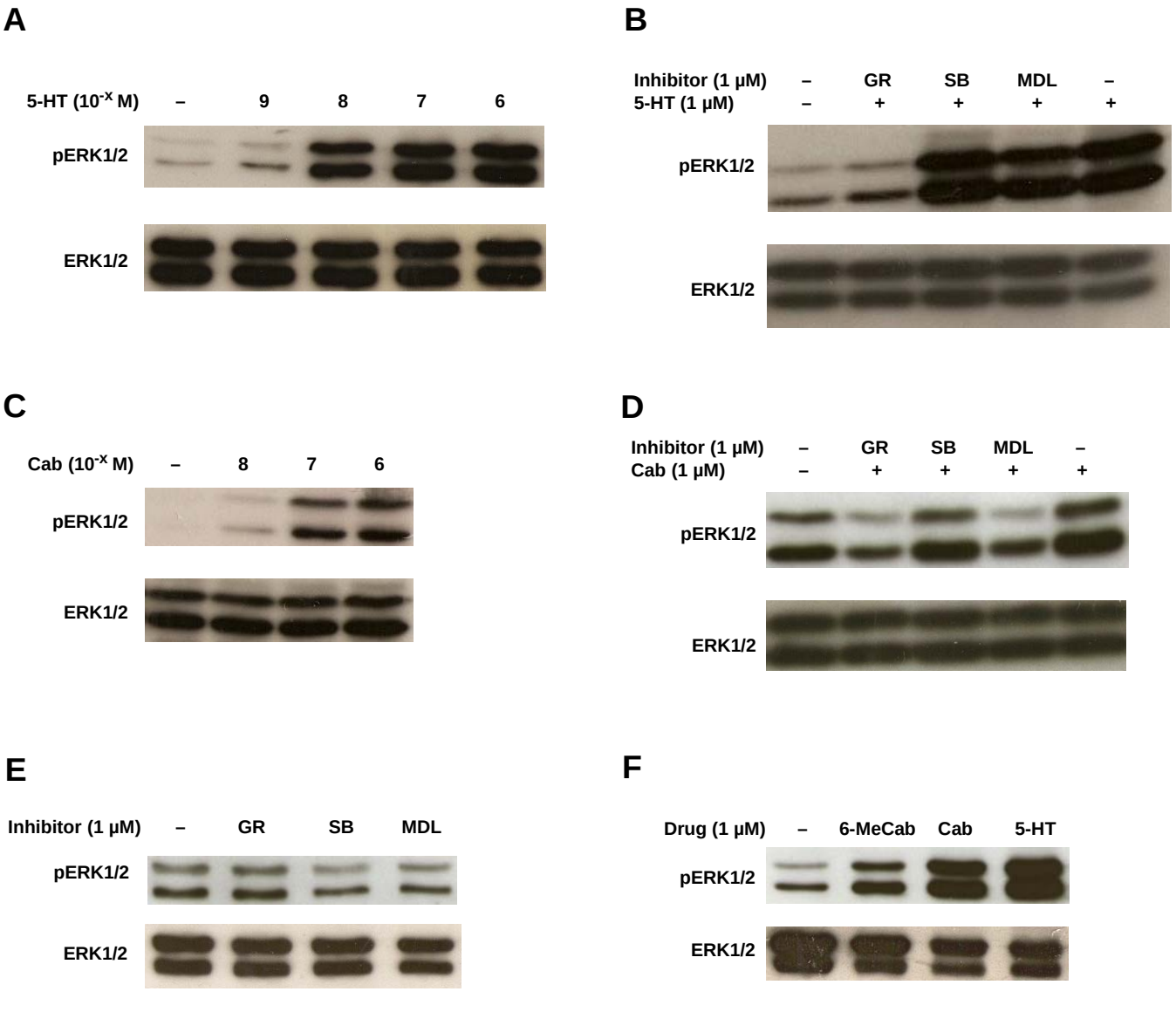

Fig. 7

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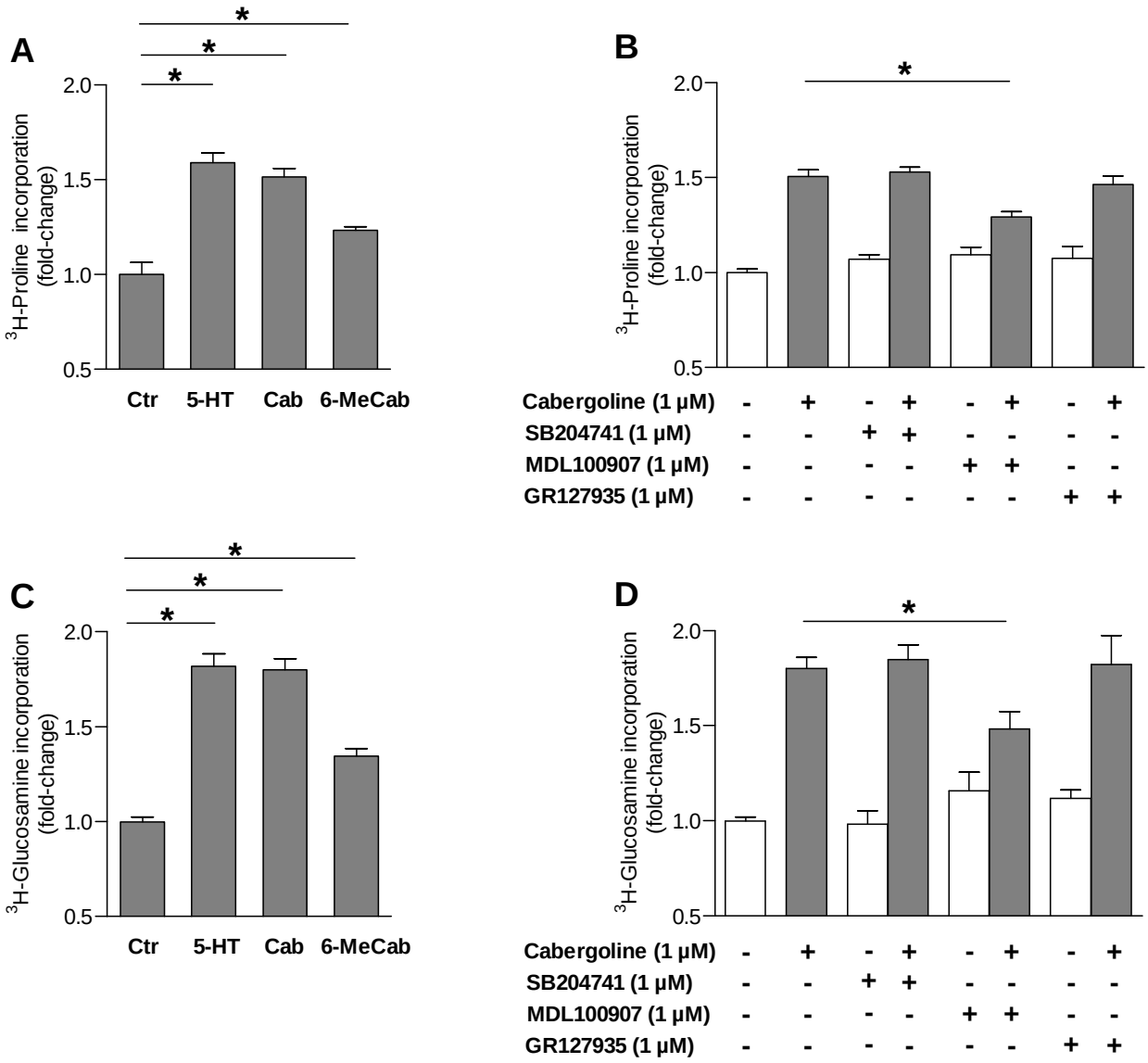

Fig. 8

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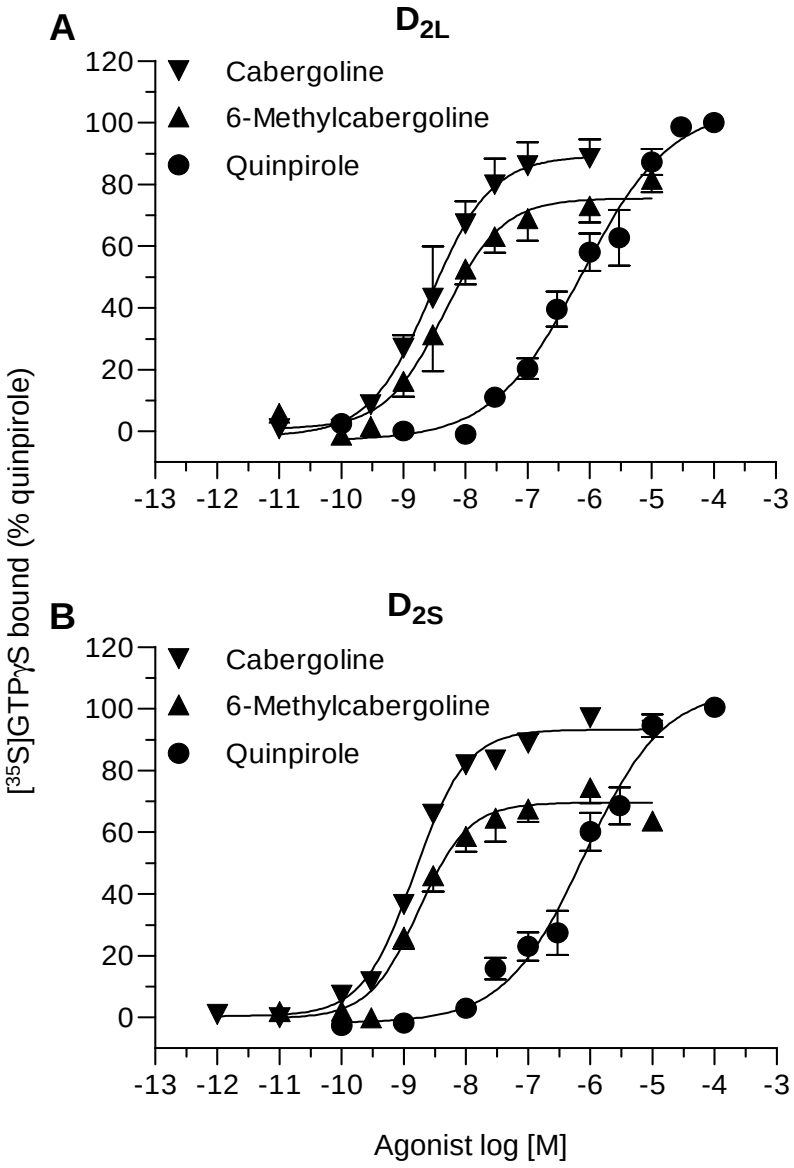

Fig. 9