

LETTER TO JMG

The ser9gly SNP in the dopamine D₃ receptor causes a shift from cAMP related to PGE₂ related signal transduction mechanisms in transfected CHO cells

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The dopamine D₃ receptor is a member of the D₂ family of dopamine receptors. Several investigators have suggested that D₃ receptors are involved in the regulation of locomotion,¹ in the pathophysiology of schizophrenia,² and in drug abuse.³

Dopamine enhances basal and stimulus evoked release of arachidonic acid, as well as the production of one of its metabolites, prostaglandin E₂ (PGE₂), in CHO (Chinese hamster ovary) cells transfected with the D₂ receptor.^{4–6} In contrast, dopamine does not influence basal arachidonic acid release^{7–9} but *inhibits* stimulus evoked release in cells transfected with the rat D₃ receptor.¹⁰ On the other hand, with respect to cAMP production, the effect of dopamine is similar in CHO cells transfected with either D₂ or D₃ receptors, both receptor subtypes mediating a reduction in forskolin induced cAMP formation.^{11–12}

A single nucleotide polymorphism (SNP) in the first exon of the dopamine D₃ receptor gene, the ser9gly polymorphism, leads to a serine to glycine amino acid substitution in the N-terminal extracellular domain of the receptor protein.¹³ An association between this polymorphism and schizophrenia has been suggested, but also questioned,^{14–15} a meta-analysis suggesting the effect to be small but real in white subjects.¹⁶ Other investigators have suggested that the gly-9 allele may be associated with an increased risk of developing antipsychotic induced tardive dyskinesia rather than with schizophrenia per se¹⁷; this finding also gained support from a report containing both pooled analyses of original data and a meta-analysis,¹⁸ and also from a recent primate study¹⁹ (see also a recent report by Lohmueller and coworkers²⁰). In addition, the ser9gly polymorphism has been reported to be associated with therapeutic response to atypical antipsychotic agents,²¹ with impulsiveness and attention deficit hyperactive disorder (ADHD),²² and with obsessive traits.²³

A radioligand binding study has reported that the gly-9 variant of the receptor displays higher affinity for dopamine than the ser-9 variant,²⁴ but to our knowledge there are no functional investigations in vitro regarding possible differences between the two alleles. Usually, the possible functional consequences of a polymorphism in a gene coding for a receptor are discussed in terms of *enhanced* or *reduced* receptor responsiveness. For a receptor coupling to more than one transduction pathway, the possibility that the functional consequence of a polymorphism influencing the structure of the receptor is different for different pathways cannot, however, be excluded. In the present study, we therefore compared CHO cells transfected with either the ser-9 or the gly-9 variant of the human dopamine D₃ receptor in relation to the effect of dopamine on both forskolin induced cAMP formation and basal PGE₂ production.

Key points

- The ser9gly single nucleotide polymorphism (SNP) in the dopamine D₃ receptor gene has been associated with both schizophrenia and antipsychotic induced tardive dyskinesia, but the functional importance of this polymorphism at the cellular level is not yet known.
- In this study, the gly-9 allele induced a marked shift in intracellular transduction mechanisms: dopamine inhibited forskolin stimulated cAMP formation (to 57%) in CHO (Chinese hamster ovary) cells transfected with the ser-9 variant, but only modestly (to 91%) in cells transfected with the gly-9 allele. Conversely, dopamine inhibited PGE₂ production (to 75%) in cells expressing the gly-9 variant, without influencing PGE₂ production in cells expressing the ser-9 allele.
- In cells expressing the gly-9 allele, a paradoxical reduction in PGE₂ levels (to 84%) was also induced by the presumed D₃ antagonist haloperidol.
- This appears to be the first report suggesting that an SNP in a gene coding for a G protein coupled receptor causes a shift in the influence of the receptor from one transduction pathway to another.

METHODS

Cell culture

Both transfected and non-transfected CHO-K1 cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum (FCS), l-glutamine, and penicillin/streptomycin (Biochrome, Berlin, Germany) (= complete medium).

Cloning and stable transfection

The ser-9 ("wild type") and gly-9 variants of the human dopamine D₃ receptor were cloned into a pcDNA3 vector (Invitrogen, Carlsbad, California, USA) using polymerase chain reaction (PCR) fragments amplified from reverse transcribed human lymphocyte cDNA (obtained from blood donors homozygous for the ser-9 and gly-9 alleles, respectively). Restriction endonuclease sites for *Hind*III and *Xba*I were included in the forward and reverse primers, respectively, and the cloned DRD₃ cDNA fragment covered positions –27 to +1251 (denoting the A in the ATG start codon as

Abbreviations: CHO, Chinese hamster ovary; PGE₂, prostaglandin E₂; SNP, single nucleotide polymorphism

position +1; ORF from +1 to +1203; see GenBank accession Nos U25441 and U32499). The vector constructs were used to transform *E. coli* XL2-Blue MRF Ultracompetent cells (Stratagene, La Jolla, California, USA), and positive clones were screened and verified by PCR and DNA sequencing to assure the fidelity of the amplified DRD₃ cDNA inserts.

CHO-K1 cells were transfected with the ser-9 and gly-9 variants of the DRD₃-pcDNA3 vector constructs using a lipofectamine method (Gibco, Gaithersburg, Maryland, USA). The post-transfection selection was begun after three days, by maintaining the cells in growth medium supplemented with the G418 aminoglycoside (1 mg/ml final concentration) (Gibco). After nearly three weeks of growth in the selection medium, individual clones were isolated using sterile clone rings. The stably transfected cell clones were tested for DRD₃ expression by northern blotting, using [³²P]dCTP labelled DRD₃ and γ -actin probes.

Radioligand binding

The preparation of the membrane suspensions and the subsequent radioligand binding experiments, using [³H]-spiperone as ligand, were carried out essentially in accordance with the protocol published by Chio *et al.*²⁵

Experimental assays

For experiments, cells were seeded at a density of 1.2×10^5 cells per ml into multiwell plates in complete medium. The medium was supplemented with 5 mM sodium butyrate (Sigma, St Louis, Missouri, USA) in order to enhance receptor expression *in vitro*.^{11, 12} The next day the subconfluent cells were washed (15 minutes, twice) with Earle's balanced salt solution (EBSS) (Biochrome); thereafter the cells were incubated for 10 minutes with experimental drugs in EBSS. PGE₂ and cAMP levels were determined by the prostaglandin E₂ quantitative competitive enzyme immunoassay (EIA) (SE0100), or cAMP quantitative competitive low pH EIA (SE0355), in accordance with the manufacturer's protocol (R&D Systems, Minneapolis, Minnesota, USA).

Data analysis

Because absolute cAMP and PGE₂ levels always showed some interplate variability, they were expressed as a percentage of controls (vehicle only) from the same plate before being used for further calculations. Data are presented as mean (SEM) of normalised cAMP or PGE₂ values. Differences between groups were analysed statistically using one way analysis of variance (ANOVA) followed by Fisher's PLSD test. Percentage normalised data in the concentration-response curves were analysed according to the four parameter logistic function:

$$E = ((\min - \max) / (1 + C/EC_{50})^k) + \max,$$

where min = minimum response, max = maximum response, C = drug concentration, EC₅₀ = drug concentration causing half-maximum response, and k = curve slope constant. Graphs were generated and parameters computed using the Kaleidagraph™ program (Synergy, Reading, Pennsylvania, USA).

Drugs

All compounds were purchased from Sigma. Dopamine and IBMX were solubilised directly into EBSS. Haloperidol and forskolin were solubilised in DMSO (maximum final concentration 0.3%). Control wells were given the corresponding vehicle.

RESULTS

In radioligand binding experiments using transfected CHO cells, the ser-9 human D₃ receptor and the gly-9 variant displayed the same affinity for [³H]-spiperone, K_d for both

variants being 0.06 (0.004) nM. In contrast, the two cell lines that were compared in the functional studies differed somewhat, though not significantly, with respect to receptor density, cells expressing the ser-9 receptor displaying higher Bmax (302 (48) fmol/mg protein) than those transfected with the gly-9 variant (200 (20) fmol/mg protein) (n = 12).

In line with previous reports, dopamine concentration dependently inhibited forskolin stimulated cAMP formation in CHO cells transfected with the ser-9 human D₃ receptor (to 57% of vehicle treated controls; IC₅₀ = 0.83 nM) (fig 1). This effect was antagonised by the presumed D₂/D₃ antagonist haloperidol: dopamine (30 nM): 69.2 (3.2)% of vehicle treated controls, p < 0.001 v vehicle; haloperidol (100 nM): 92.6 (4.6)% of vehicle treated controls; haloperidol (100 nM) + dopamine (30 nM): 99.9 (4.4)% of vehicle treated controls; n = 12. In cells transfected with the gly-9 D₃ allele, the effects of dopamine on cAMP were considerably weaker, reaching a maximum of 91% of vehicle treated controls (fig 1). No effect was observed in non-transfected cells (not shown).

Conversely, basal production of PGE₂ was potentially reduced by dopamine in cells transfected with the gly-9 D₃ receptor

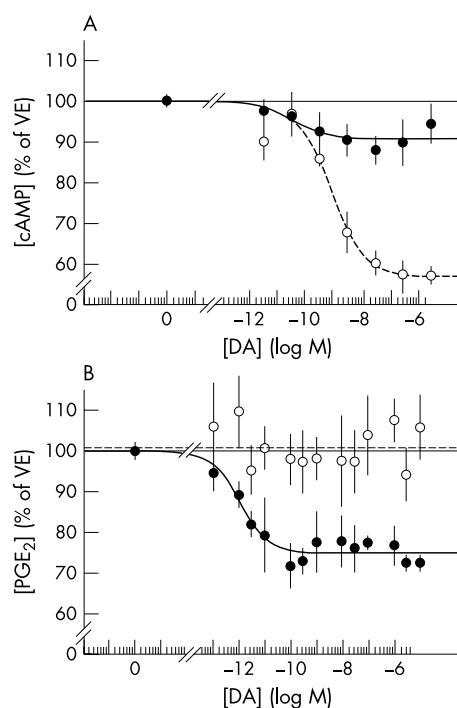


Figure 1 Effects of dopamine on cAMP formation and prostaglandin E₂ (PGE₂) production in transfected CHO cells. (A) Effects of various concentrations of dopamine [DA] on forskolin (10 μ M) stimulated cAMP formation in transfected CHO cells supplemented with IBMX (1 mM). Fitted curves represent the four-parameter logistic non-linear regression equation as described. IC₅₀ = 0.83 nM and maximum inhibitory effect = 56.9% of vehicles (VE; 100%) at \approx 100 nM for CHO ser-9 allele human D₃ receptor cells (empty dots; hatched curve). Maximum inhibitory effect = 90.5% of vehicles (VE = 100%) for CHO gly-9 variant D₃ receptor cells (filled dots; solid curve). Mean absolute cAMP concentration was 75 pmol/ml in the groups given vehicle only. (B) Effects of various concentrations of dopamine on basal PGE₂ production in transfected CHO cells. Fitted curves represent four-parameter logistic equation as described. IC₅₀ = 1.1 pM and maximum inhibitory effect = 75.0% of vehicles (VE = 100%) at \approx 3 nM for CHO gly-9 mutant human D₃ receptor cells (filled dots; solid curve). No effect was obtained in ser-9 type D₃ cells (empty dots; hatched curve). Mean absolute PGE₂ concentrations were 650 pg/ml in the groups given vehicle only. In both panels, points and error bars represent means and SEM of values in composite concentration-response curves obtained from eight separate experiments, each carried out in sextuplicate.

allele (to 75% of vehicle treated controls; $IC_{50} = 1.1 \text{ pM}$), but not influenced in cells expressing the ser-9 variant (fig 1).

Similar effects of dopamine on cAMP and PGE_2 were found in two other control clones expressing the corresponding receptor (not shown).

Attempts to counteract the effect of dopamine on PGE_2 in cells expressing the gly-9 allele by means of the suggested D_2/D_3 antagonist haloperidol were not successful; in contrast, haloperidol unexpectedly reduced PGE_2 concentrations per se: *dopamine* (30 nM): 74.5 (4.4)% of vehicle treated controls, $p < 0.001$ v vehicle; *haloperidol* (100 nM): 79.8 (4.8)% of vehicle treated controls, $p < 0.01$ v vehicle; *haloperidol* + *dopamine*: 64.6 (3.1)% of vehicle treated controls, $p < 0.001$ v vehicle; $n = 14$.

Prompted by these findings, we investigated the effect of various concentrations of haloperidol per se on PGE_2 production in cells transfected with the gly-9 variant; these experiments confirmed that haloperidol, like dopamine, reduces PGE_2 production. This effect was modest in magnitude (to 84% of controls), but observed already at low concentrations (fig 2). Furthermore, a PGE_2 reducing effect was also found with three other presumed D_2/D_3 receptor antagonists—raclopride, eticlopride, and spiperone (data not shown). Like dopamine, haloperidol failed to reduce PGE_2 levels in cells expressing the ser-9 receptor (not shown). Supporting the notion that the effects of dopamine and haloperidol on PGE_2 production in cells expressing the gly-9 variant were indeed mediated by this receptor, neither dopamine nor haloperidol influenced PGE_2 in non-transfected cells (not shown).

In line with previous studies,²⁶ the effect of dopamine on forskolin induced cAMP formation in cells transfected with the ser-9 D_3 receptor was counteracted by pretreatment with pertussis toxin (PTX), indicating that it is caused by an interaction between the receptor and a G protein of the G_i/G_o subtype: *dopamine* (30 nM): 72.0 (2.5)% of vehicle treated controls, $p < 0.001$ v vehicle; *PTX* (200 ng/ml, 24 hours) + *dopamine* (30 nM): 102.2 (3.3)% of PTX treated controls, $n = 12$. In contrast, the effect of dopamine on PGE_2 production in the gly-9 cell line was not blocked by pretreatment with PTX: *dopamine* (30 nM): 68.9 (3.8)% of vehicle treated controls, $p < 0.001$ v vehicle; *PTX* (200 ng/ml, 24 hours) + *dopamine* (30 nM): 77.3 (5.6)% of PTX treated controls, $p < 0.05$ v PTX; $n = 12$. In another experiment, this effect was found also to be unabated in the presence of cholera toxin (10 $\mu\text{g/ml}$; 24 hours) (not shown).

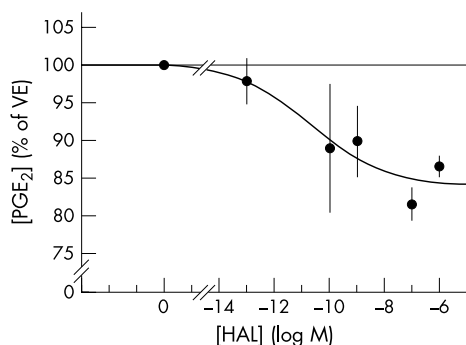


Figure 2 Effects of various concentrations of haloperidol [HAL] on basal PGE_2 production in transfected human gly-9 allele D_3 CHO cells. Points and bars represent means and SEM of values in composite concentration–response curves obtained from four separate experiments, each carried out in sextuplicate. The fitted curve represents a four-parameter logistic equation as described. $IC_{50} = 0.2 \text{ nM}$; maximum inhibitory effect = 83.9% of vehicles (VE = 100%) at $\approx 1 \text{ }\mu\text{M}$. Mean absolute PGE_2 concentration was 425 pg/ml in the groups given vehicle only.

DISCUSSION

This is the first in vitro study demonstrating functional importance at the cellular level of the ser9gly polymorphism in the dopamine D_3 receptor gene, previously reported to be associated with both schizophrenia and antipsychotic induced tardive dyskinesia. More importantly, it is also, to our knowledge, the first study ever suggesting that an SNP in the gene coding for a G protein coupled receptor may cause a shift in the influence of the receptor from one transduction pathway to another.

The main finding was that the ser-9 human D_3 receptor, but not the gly-9 variant, mediated a dopamine induced, PTX sensitive reduction in cAMP formation, whereas the gly-9 variant, but not the ser-9 allele, mediated an inhibitory effect of dopamine on PGE_2 production. The latter effect was not counteracted by pertussis toxin or by cholera toxin, suggesting that it was exerted by a mechanism involving neither G_i/G_o nor G_s proteins (see below). The differences between the two receptor variants with respect to effects on cAMP and PGE_2 , respectively, were too marked to be explained by the moderate difference between the two cell lines in relation to receptor density; moreover, the effect on PGE_2 observed only in cells expressing the gly-9 variant can hardly be explained by the fact that receptor density was lower in this cell line.

There are numerous previous studies suggesting that SNPs in genes coding for receptor proteins may enhance or reduce the effect of receptor activation on various transduction mechanisms,^{27–29} and also when situated in the aminoterminal domain³⁰ like the one examined in this study. The finding that an SNP may lead to a reduction in the effect of receptor activation on one transduction system, but to an increase in the effect on another, is novel. However, it is in line with the concept of agonist directed trafficking of receptor stimuli, according to which a compound displaying affinity for a certain receptor may act as an agonist with respect to one G protein and as an antagonist with respect to another.³¹ Of interest in this context is a study in transfected HEK 293 cells showing that one isoform of the melatonin_{1D} receptor mainly influences cAMP, and another isoform of the same receptor mainly influences cGMP.³²

The molecular mechanisms underlying the switch from the cAMP pathway to the PGE_2 pathway induced by the ser9gly substitution in the D_3 receptor remain unclear. Notably, the studied polymorphism is located in the amino terminus, which is the target for post-translational *N*-terminal glycosylation of G protein coupled receptors. The dopamine D_3 receptor has been shown to undergo such *N*-terminal glycosylation,³³ but the functional relevance of the phenomenon for this receptor is as yet unknown. However, of interest in this context is the finding that a ser-gly substitution in the *N*-terminus has been shown to eliminate glycosylation in β_1 adrenergic receptors, resulting in altered agonist promoted trafficking.³⁴ Moreover, corresponding serine substitutions at aminoterminal glycosylation sites have been shown to affect transduction properties in transfected secretin receptors.³⁵

Haloperidol acted as an antagonist with respect to the influence of the ser-9 D_3 receptor on cAMP formation, but, intriguingly, appeared to display an agonist-like profile with respect to the influence of the gly-9 variant on PGE_2 production. This finding is of interest given the suggested association between this polymorphism and antipsychotic induced dyskinesia, but it cannot yet be explained. There are, however, previous examples of single amino acid substitutions in G protein coupled receptors turning established antagonists into agonists,^{36–37} and also anecdotal reports of dopamine D_2/D_3 receptor antagonists displaying paradoxical agonist-like effects in various experimental paradigms.^{7–38} Of interest in this context are recent studies by Baker and coworkers on β adrenoceptors,^{39–40} showing that the

paradoxical agonist effects of β receptor antagonists were not G protein mediated, a finding well in line with our observation that the PGE₂ inhibiting effect of dopamine mediated by the gly-9 D₃ receptor variant was resistant to pretreatment with pertussis toxin as well as cholera toxin.

The possible clinical importance of dopamine induced arachidonic acid release and subsequent PGE₂ production remains to be elucidated. However, several studies have suggested a role for prostaglandins in dopamine related dyskinesia,⁴¹ stereotypic circling,⁴² and catalepsia⁴³ in rodents, and it has also been suggested that prostaglandins may be involved in psychosis.⁴⁴⁻⁴⁵ The possibility that the influence of the gly-9 variant of the D₃ receptor on PGE₂ production observed in this study may be of relevance both for antipsychotic induced dyskinesia and for psychosis thus deserves further attention.

Conclusions

Our results indicate that an SNP previously suggested to be associated with schizophrenia and antipsychotic induced tardive dyskinesia influences cellular transduction as well as the pharmacological profile of antipsychotic drugs. Further studies on the possibility that SNPs in genes coding for G protein coupled receptors may cause a shift from one transduction pathway to another are warranted.

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