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.4,5 In contrast, dopamine does not influence basal arachidonic acid release6–9 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.11–13

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.14 An association between this polymorphism and schizophrenia has been suggested, but also questioned,15,16 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 se;17 this finding also gained support from a recent meta-analysis,18 and also from a recent primate study19 (see also a recent report by Lohmueller and coworkers20). 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 HindIII and XbaI 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

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.

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 Ultracompotent 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 × 10^6 cells per ml into multwell 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 fluorescent cells were washed (15 minutes, twice) with Earle’s assay (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:

\[ E = \left( \frac{(\min - \max)}{1 + (C/EC_{50})^k} \right) + \max, \]

where min = minimum response, max = maximum response, C = drug concentration, EC_{50} = drug concentration causing half-maximum response, and k = curve slope constant. Graphs were generated and parameters computed using the KaleidaGraph^TM 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 B_max (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 (57% of vehicle treated controls; IC_{50} = 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 potently 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 μM) 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. IC_{50} = 0.83 nM and maximum inhibitory effect = 56.9% of vehicle treated controls, p < 0.001. (B) Effects of various concentrations of dopamine on basal PGE2 production in transfected CHO cells. Fitted curves represent four-parameter logistic equation as described. IC_{50} = 1.1 pM and maximum inhibitory effect = 90.5% of vehicle treated controls, p < 0.001.
allele (to 75% of vehicle treated controls; IC50 = 1.1 pM), but not influenced in cells expressing the ser-9 variant (fig 1).

Similar effects of dopamine on cAMP and PGE2 were found in two other control clones expressing the corresponding receptor (not shown).

Attempts to counteract the effect of dopamine on PGE2 in cells expressing the gly-9 allele by means of the suggested D2/D3 antagonist haloperidol were not successful; in contrast, haloperidol unexpectedly reduced PGE2 concentrations per se: dopamine (30 nM): 74.5 (4.4)% of vehicle treated controls, p<0.001 vs vehicle; haloperidol (100 nM): 79.8 (4.8)% of vehicle treated controls, p<0.01 vs vehicle; haloperidol + dopamine: 64.6 (3.1)% of vehicle treated controls, p<0.001 vs vehicle; n = 14.

Prompted by these findings, we investigated the effect of various concentrations of haloperidol per se on PGE2 production in cells transfected with the gly-9 variant; these experiments confirmed that haloperidol, like dopamine, reduces PGE2 production. This effect was modest in magnitude (to 84% of controls), but observed already at low concentrations (fig 2). Furthermore, a PGE2 reducing effect was also found with three other presumed D2/D3 receptor antagonists—raclopride, eticlopride, and spiperone (data not shown). Like dopamine, haloperidol failed to reduce PGE2 levels in cells expressing the ser-9 receptor (not shown). Supporting the notion that the effects of dopamine and haloperidol on PGE2 production in cells expressing the gly-9 variant were indeed mediated by this receptor, neither dopamine nor haloperidol influenced PGE2 in non-transfected cells (not shown).

In line with previous studies, the effect of dopamine on forskolin induced cAMP formation in cells transfected with the ser-9 D3 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 Gi/Go subtype: dopamine (30 nM): 72.0 (2.5)% of vehicle treated controls, p<0.001 vs 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 PGE2 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 vs vehicle; PTX (200 ng/ml, 24 hours) + dopamine (30 nM): 77.3 (5.6)% of PTX treated controls, p<0.05 vs PTX; n = 12. In another experiment, this effect was found also to be unabated in the presence of cholera toxin (10 μg/ml; 24 hours) (not shown).

DISCUSSION
This is the first in vitro study demonstrating functional importance at the cellular level of the ser9gly polymorphism in the dopamine D3 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 D3 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 PGE2 production. The latter effect was not counteracted by pertussis toxin or by cholera toxin, suggesting that it was exerted by a mechanism involving neither Gi/G0, nor G proteins (see below). The differences between the two receptor variants with respect to effects on cAMP and PGE2, 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 PGE2 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, and also when situated in the amino terminal domain 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. Of interest in this context is a study in transfected HEK 293 cells showing that one isoform of the melatonin1D receptor mainly influences cAMP, and another isoform of the same receptor mainly influences cGMP.

The molecular mechanisms underlying the switch from the cAMP pathway to the PGE2 pathway induced by the ser9gly substitution in the D3 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 D3 receptor has been shown to undergo such N-terminal glycosylation, 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 β1 adrenergic receptors, resulting in altered agonist promoted trafficking. Moreover, corresponding serine substitutions at aminoterminal glycosylation sites have been shown to affect transduction properties in transfected secretin receptors.

Haloperidol acted as an antagonist with respect to the influence of the ser-9 D3 receptor on cAMP formation, but, intriguingly, appeared to display an agonist-like profile with respect to the influence of the gly-9 variant on PGE2 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 agonists into antagonists, and also anecdotal reports of dopamine D2/D3 receptor antagonists displaying paradoxical agonist-like effects in various experimental paradigms. Of interest in this context are recent studies by Baker and coworkers on β adrenergic receptors, 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,19 stereotypic circling,20 and catalepsia21 in rodents, and it has also been suggested that prostaglandins may be involved in psychosis.22,23 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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