The effect of clozapine on mRNA expression for genes encoding G protein-coupled receptors and the protein components of clathrin-mediated endocytosis

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\textbf{Objectives} Clathrin-mediated endocytosis (CME) is an intracellular trafficking mechanism for packaging cargo, including G protein-coupled receptors (GPCRs), into clathrin-coated vesicles (CCVs). The antipsychotic chlorpromazine inhibits CCV assembly of adaptor protein AP2 whereas clozapine increases serotonin2A receptor internalization. We hypothesized that clozapine alters the expression of CME genes modulating vesicle turnover and GPCR internalization.

\textbf{Materials and methods} SH-SY5Y human neuroblastoma cells were incubated with clozapine (1–20\,\mu{}mol/l) for 24–72\,h. GPCR and CME-related gene mRNA expression was measured using RT-PCR. We quantified changes in the same genes using expression data from a microarray study of mice brains after 12 weeks of treatment with 12\,mg/kg/day clozapine.

\textbf{Results} The expression of genes encoding adaptor and clathrin assembly proteins, AP2A2, AP2B1, AP180, CLINT1, HIP1, ITSN2, and PICALM, increased relative to the control in SH-SY5Y cells incubated with 5–10\,\mu{}mol/l clozapine for 24–72\,h. The microarray study showed significantly altered expression of the above CME-related genes, with a marked 641-fold and 17-fold increase in AP180 and the serotonin1A GPCR, respectively. The expression of three serotonergic receptor and lysophosphatic acid receptor 2 (EDG4) GPCR genes was upregulated in SH-SY5Y cells incubated with 5\,\mu{}mol/l clozapine for 24\,h. EDG4 expression was also increased with 10–20\,\mu{}mol/l clozapine treatment at 48–72\,h. Clozapine significantly decreased the expression of \(\beta\)-arrestin, involved in GPCR desensitization, both in vitro and vivo.

\textbf{Conclusion} The changes we report in CME and GPCR mRNAs implicate CCV-mediated internalization of GPCRs and the serotonergic system in clozapine’s mechanism of action, which may be useful in the design of more effective and less toxic antipsychotic therapies. "Psychiatr Genet 23:153–162 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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\textbf{Introduction}

Schizophrenia is among the top 10 causes of disability in developed countries worldwide (Murray, 1996) and has an estimated heritability of 80\% (McGuffin et al., 1984). Genetic deletions and duplications are strongly associated with schizophrenia, some of which are highly penetrant in their effects on individual susceptibility (Need et al., 2009). However, there is considerable heterogeneity, with many genes associated within specific ancestral groups (Sanders et al., 2008). Several genes that increase susceptibility to schizophrenia are regulated by or participate in endosomal transport mechanisms (Ryder and Faundez, 2009). Our own schizophrenia genetic association study shows the involvement of the clathrin-mediated endocytosis (CME)-related protein enthoprotein encoded by the clathrin interactor 1 (CLINT1) gene (Pimm et al., 2005). Genetic evidence that CLINT1 expression and structure is abnormal in schizophrenia is well replicated (Liou et al., 2006; Tang et al., 2006; Gurling et al., 2007; Escamilla et al., 2008; International Schizophrenia Consortium, 2008; Purcell et al., 2009). It is of particular interest that antipsychotic drugs are effective in the majority of patients despite the many different genetic subtypes of schizophrenia.

Antipsychotic drugs such as chlorpromazine and haloperidol are effective in the treatment of thought disorder, hallucinations, and delusions in patients with schizophrenia. The side effects of these drugs such as extrapyramidal signs and hyperprolactinemia are mediated by dopamine receptor antagonism, although these adverse effects do not characterize all antipsychotics (Serretti et al., 2004). A large number of G protein-coupled receptors (GPCRs) have an
affinity for antipsychotic drugs. The efficacy and tolerability of the atypical antipsychotic clozapine in the treatment of schizophrenia is likely to be because of strong serotonin [5-hydroxytryptamine (5-HT)] 2A receptor (5-HT2A) inverse agonism, 5-HT1A partial agonism, and weak D2/D3 antagonism (Meltzer and Massey, 2011). There is evidence that both olanzapine and clozapine, but not haloperidol, increase clathrin-mediated internalization of 5-HT2A (Willins et al., 1999). Unlike typical antipsychotics, atypical drugs may exert a beneficial effect on psychosis through a more complicated mechanism of action involving chronic molecular and structural brain changes than would be elicited by a simple drug–receptor interaction (Heusler et al., 2008).

Endosomal trafficking mechanisms regulate signal transduction and presynaptic and postsynaptic neuronal cell surface composition and function (von Zastrow and Sorkin, 2007) by controlling the release and recycling of synaptic vesicles, as well as the subcellular distribution of neurotransmitter receptors and transporters (Kennedy and Ehlers, 2006). Enthoprotin is a linker protein between the lipid bilayer of the neuronal membrane and various adaptor proteins (AP) and clathrin molecules, playing a crucial role in clathrin-coated pit formation (Mills et al., 2003). The adaptors anchor clathrin to the transmembrane, interact with the clathrin-coated vesicle (CCV) formation machinery, and select ‘cargo’ for inclusion in CCVs (Robinson, 2004). Distinct and potentially widespread mechanisms regulating GPCR surface abundance and sorting to lysosomes are dysfunctional in the brains of patients with schizophrenia (Marley and von Zastrow, 2010). Thus, clozapine may exert its positive clinical effect by regulating receptor density and trafficking. Here, we determine the effect of clozapine on CCV and GPCR gene expression in the mouse brain and human neuroblastoma cells.

**Materials and methods**

**Cell culture**

Undifferentiated human neuroblastoma SH-SYSY cells (Catalogue no. 94030304; Health Protection Agency Culture Collections, London, UK) were maintained in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, Gillingham, UK) supplemented with 10% fetal bovine serum (Invitrogen, Life Technologies Ltd, Paisley, UK) and penicillin/streptomycin (100 U/ml, 100 μg/ml final concentration; Sigma-Aldrich). Exponentially growing cells were cultured at 37°C with 95% humidified air and 5% CO2. Cells were not passaged beyond passage 21.

**Cell viability assay**

Cells plated at a density of 1 × 10⁴ cells/cm² were incubated overnight and treated for 24, 48, or 72 h with five concentrations (1–20 μmol/l) of clozapine (Sigma-Aldrich) or with 1.3% dimethyl sulfoxide (DMSO; Sigma-Aldrich) vehicle control. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) salt assay was performed to test cell viability (Mosmann, 1983). Data shown represent triplicate wells from three independent experiments.

**Drug treatment**

Clozapine dissolved in DMSO at a final concentration of 1, 2, 5, 10, or 20 μmol/l or vehicle, as described in the Cell viability section, was added to SH-SYSY cells. Cells were incubated for 24, 48, or 72 h. Drug administration was performed in triplicate and repeated in three separate cultures to yield nine replicates for each time point and concentration of clozapine or vehicle.

**RNA extraction**

RNA was extracted using the Illustra RNAspin mini RNA isolation kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and quantified using a Nanodrop ND-1000 (Labtech International Ltd, Uckfield, UK). The RNA purification kit includes an on-column DNase I digest step to avoid contamination with genomic DNA and to improve the purity of the RNA (GE Healthcare Life Sciences). All samples had a 260 : 280 nm ratio of more than 1.6. RNA was then stored at −80°C until required for reverse transcription (RT) into cDNA.

**Reverse transcription**

RNA was diluted with PCR-grade water (Sigma-Aldrich) to obtain an equal amount of substrate for RT from each RNA sample. Five hundred nanograms of Oligo(dT)15 Primer (Promega UK Ltd, Southampton, UK) was added to all RNA samples diluted to the same concentration in 11 μl and incubated at 70°C for 10 min. For RT to cDNA, master mix containing 4 μl 5 × first-strand buffer, 10 mmol/l dithiothreitol, 200 μmol/l mixed dNTPs, and 200 U SuperScript III reverse transcriptase (Invitrogen) yielding a final volume of 20 μl with RNA was prepared. The reaction was heated to 42°C for 90 min. To control for unwanted amplification of contaminating genomic DNA, no-RT samples were run that had followed the same RT procedure without the addition of the RT enzyme. Samples were diluted to yield a standardized concentration of cDNA on the basis of RNA quantification of 50 ng/μl and then frozen at −20°C until required for gene expression analysis.

**RT-qPCR panel plate to screen for GPCR gene expression**

Quantitative PCR (qPCR) to assess changes in the expression of 84 GPCR genes with the Human GPCR RealTime ready 384-well panel plate (Roche, Burgess Hill, UK) was performed using a 480 LightCycler (Roche). The copy number of each GPCR gene was measured in quadruplicate from nine pooled replicates of cDNA samples extracted from cells following treatment with 5 μmol/l clozapine for 24 h. cDNA samples were pooled to control for variation in gene expression across the nine replicates. A second Human GPCR RealTime ready 384-well panel plate was used with nine pooled cDNA samples from cells treated with DMSO for 24 h to express changes...
in GPCR gene copy number following antipsychotic drug treatment relative to vehicle. Each well contained 5 μl (5 ng) of pooled cDNA along with 5 μl of LightCycler 480 probes master mix (Roche). The amplification protocol was a one-cycle denaturing step at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s, with a measurement of hydrolysis probe fluorescence using standard FAM wavelength filter settings, and 72°C for 1 s, with a last cycle at 72°C for 10 min.

Crossing threshold quantification cycle (Cq) values were obtained for all 84 GPCR genes and seven reference genes on the GPCR panel plate. The quadruplicate average Cq of each GPCR gene measured in cDNA from cells following treatment with 5 μmol/l clozapine for 24 h was then expressed relative to each of the seven reference genes. These seven relative values for each GPCR gene were averaged to yield an overall Cq for each gene. The same was repeated for Cq values obtained from the second GPCR panel plate with DMSO-treated cDNA. The mean GPCR Cq values derived from the DMSO plate were subtracted from those of the 5 μmol/l clozapine plate to yield changes in gene copy number attributable to drug treatment. From the GPCR panel screen of 84 genes in pooled cDNA samples, nine candidate GPCR genes showing greater than two-fold change in expression or downregulated by 5 μmol/l clozapine treatment compared with DMSO were identified (Fig. 2). qPCR assays for these genes, galanin receptor 2 gene (GALR2), serotonin receptor 2A (HTR2A), endothelial differentiation receptor 4 (EDG4), somatostatin receptor 3 (SSTR3), HTR7, P2Y purinoceptor 1 (P2YR1), dopamine receptor D1 (DRD1), HTR1A, and type-1 angiotensin II receptor-associated protein (AGTRAP), were designed to confirm the effect on the expression of five doses of clozapine (1–20 μmol/l) at all three exposure times (24, 48, and 72 h) using cDNA replicates (i.e. not pooled).

In addition, qPCR assays were designed for seven CME-related genes including adaptor-related protein complex 2, α2 subunit (AP2A2), adaptor-related protein complex 2, β1 subunit (AP2B1), AP180, clathrin coat assembly protein 1 (HTR1A), intersectin 2 (ITSN2), and phosphatidylinositol-binding clathrin assembly lymphoid myeloid protein (PICALM).

### RT-qPCR GPCR, CME, and reference gene expression assays

Assays were designed using the Roche real-time ready configurator (https://www.roche-applied-science.com/sis/realtimeready/index.jsp) on the basis of those provided on the Roche GPCR panel to obtain the position of the amplicon on the gene of interest. ProbeFinder software roche universal

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*aAGTRAP, type-1 angiotensin II receptor-associated protein; AP1B0, clathrin coat assembly protein 180, also known as SNAP91; AP2B1, adaptor-related protein complex 2, α2 subunit; CLINT1, clathrin interactor 1; F, forward primer; GALR2, galanin receptor 2; HIP1, Huntington interacting protein 1; HTR1A, serotonin receptor 1A; HTR7, serotonin receptor 7; ITSN2, intersectin 2; LPAR2, lysophosphatidic acid receptor 2; N, no; P2Y1, P2Y purinoceptor 1; PGK1, phosphoglycerate kinase 1; PICALM, phosphatidylinositol-binding clathrin assembly lymphoid myeloid protein; R, reverse primer; RPLP0, acidic ribosomal protein P0; Y, yes.

bUPL, Roche Universal Library Hydrolysis Probe Number; SG, SYBR-Green.
probe library (UPL) assay design center (https://www.roche-applied-science.com/is/si/rtpcr/upl/index.jsp) was used to identify a suitable predesigned UPL hydrolysis probe (Roche) for the amplicon sequence (Table 1). Three reference genes assays were tested for stability of expression following DMSO and clozapine treatment. These comprised a predesigned assay for phosphoglycerate kinase 1 (PGK1) (Roche) and two assays designed in-house for hypoxanthine-guanine phosphoribosyltransferase (HPRT) and 60S acidic ribosomal protein P0 (RPLP0). Assays for PGK1 and RPLP0 were selected for analysis (data not shown). Where possible, intron-spanning gene-specific primers were designed (Table 1) and screened in silico for specificity using the UCSC genome browser BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start).

PCR primers were optimized in the absence of UPL hydrolysis probes for each of the nine GPCR genes. Where assays failed when the hydrolysis probe was added, the DNA intercalating fluorescent dye SYBR-Green was used instead. However, three GPCR gene assays, HTR2A, and DAB2, failed despite various optimization steps. qPCR was subsequently performed as follows: 2 μl of cDNA (50 ng) was added to a 10 μl reaction mixture containing SYBR-Green (Roche) diluted 1/10 000 where appropriate, 200 μmol/l dNTPs, 1 x concentrated buffer mix containing NH4 (Biolite Ltd, London, UK), 3.2 or 6.2 mmol/l MgCl2, 400 nmol/l of each primer, and 0.5 U of Taq DNA polymerase. Cycling conditions were one denaturing cycle of 10 min at 95 °C, followed by 45 cycles of denaturation (95 °C for 30 s), annealing (60 °C for 30 s), and extension (72 °C for 10 s), with a final 10-min extension at 72 °C. For SYBR-Green qPCR, dissociation curve analysis was carried out at 95 °C for 5 s, 65 °C for 1 min, and then 97 °C at a ramp rate of 0.11 °C/s with five fluorescence acquisitions per °C. PCR products were visualized by gel electrophoresis in 3.0% (w/v) agarose and then purified by adding an equal volume of microclean (Microzone Ltd, Haywards Heath, UK), centrifuging for 10 min at 13 000g, and resuspending in 20 μl water. The mass of the PCR product was quantified using a Nanodrop ND-1000 spectrophotometer. Using the amplicon size and a copy number calculator (http://www.uri.edu/research/gsc/resources/cndna.html), the PCR products were quantified.

Efficiencies and standard curves

Following optimization of qPCR expression assays for six GPCR, seven CME-related, and two reference genes, the efficiency of each assay was tested using five serial dilutions of purified PCR products and reference cDNA as amplification templates. The logarithm of the initial template copy number was plotted against the Cq value. The efficiency was calculated as follows: $E = 10^{(Cq - 1)/slope}$, with a value of two indicating a doubling of copy number per cycle and perfect efficiency. Efficiency values were accepted at 2 ± 5%. qPCR was performed in a 384-well plate format in quadruplicate for 1 μl cDNA, equivalent to 50 ng RNA, from each time point and treatment condition, in a 4 μl reaction mixture as described above, containing 100 nmol/l Roche UPL hydrolysis probe where appropriate using an Epmotion 5075 (Eppendorf UK Ltd, Stevenage, UK) robot and a LightCycler 480 (Roche). A caliber sample of cDNA was used on all 384-well plates to control for variance between plates, along with an H2O-negative control.

qPCR gene expression statistical analysis

Raw data analysis

Quadruplicate Cq values for each cDNA sample were averaged. Where the SEM was greater than 1%, either one of the replicate values was deleted to yield an SEM less than 1% or the entire mean value was deleted. Changes in gene transcription were evaluated using the 2−ΔΔCq method (Livak and Schmittgen, 2001). The mean Cq values of each target gene were expressed relative to the mean Cq values of the two reference genes (2−ΔΔCq). The mean Cq values of the calibrator were then subtracted from the relative mean Cq values on each plate. The 2−ΔΔCq method based on normalization with the mean of the reference genes was applied for the GPCR panel plate to determine differences in concentrations between samples. The 2−ΔΔCq of the different

![Fig. 1](https://example.com/fig1.png)

MTT SH-SYSY neuroblastoma cell viability assay. Living cells are required for MTT cleavage in the MTT cell viability assay. SH-SYSY cells were plated out at a density of 10⁵ cells in each well of a six-well plate, incubated overnight in growth medium (DMEM, 10% fetal bovine serum), and treated with clozapine (0–20 μmol/l) or DMSO. Following this, MTT (5 mg/ml stock) was added to all wells and the plates were incubated at 37 °C for 3 h. Medium was removed, the formazan MTT metabolite was resuspended in DMSO, and optical density was measured. Each point shows the mean and SD of nine replicates. DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Clozapine alters GPCR and CCV expression

Representative of the article's content:

Clozapine drug treatment samples were compared directly with that of the DMSO vehicle sample to yield $2^{-\Delta Cq}$. Removal of extreme outliers

Data for $2^{-\Delta Cq}$ derived from gene expression assays were initially examined by boxplot analysis to identify outliers. cDNA with $2^{-\Delta Cq}$ values between 1.5 and 3 box lengths (box length being the interquartile range) from the upper or the lower edge of the box were classified as an outlying value (SPSS version 17.0). All Affymetrix arrays were analyzed using Affymetrix algorithms. Microarray statistical analysis of clozapine treatment in mice

Mice were administered 12 mg/kg/day clozapine (Novartis Pharma AG, Basel, Switzerland) in drinking water at a concentration equivalent to the highest human clinical dosage in a 12-week study (10 clozapine treated and 10 untreated controls) (Rizig et al., 2012). At the end of treatment, mice brains were dissected and total RNA was extracted from the right and the left cerebral hemispheres. mRNA was prepared and hybridized to MOE430A Affymetrix arrays (Affymetrix UK Ltd, High Wycombe, UK) before being scanned using the Affymetrix GeneChip Scanner 3000 (Rizig et al., 2012). All Affymetrix arrays were analyzed using Affymetrix algorithms.

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fulfilled the recommended quality control criteria. Consistency between the intensity of replicates was investigated with box plots and histograms. Raw and normalized data are available at: http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE6512. Normalization of arrays was performed using GC-RMA (Gentleman et al., 2004) and probe values that passed detection-significance thresholds for CME-associated and GPCR genes were extracted. The log 2 output values from GC-RMA were antilogged to allow the calculation of fold changes of significant genes from the mean intensity of expression of grouped samples. Multi-variate analysis of variance (MANOVA) test on several probe sets or a Student’s t-test for a single probe was used on log_{10}-transformed data to identify significant changes in mRNA expression (SPSS version 17.0). A cut-off significance value of P less than 0.05 was used.

Results

Cell viability assay

Cell viability between 94 and 100% was unchanged after incubation with 1 or 2 μm/l clozapine for 24 and 48 h, compared with vehicle. However, MTT-formazan formation decreased with increasing concentrations of clozapine (5–20 μm/l) at 24- and 48-h incubation times, indicating decreasing SH-SYSY cell viability. Interestingly, the rate of cell growth and survival increased by 29% with both 1 and 2 μm/l clozapine, and by 15% with 5 μm/l clozapine following a 72-h incubation compared with vehicle. There was no difference in cell viability between vehicle and 10 μm/l clozapine after 72 h, suggesting that chronic exposure to lower concentrations of clozapine exerts a protective effect and actually stimulates cell proliferation. However, incubation of cells with 20 μm/l clozapine for 24, 48, and 72 h resulted in a mean cell death of 58, 46, and 42%, respectively, relative to vehicle (Fig. 1).

In-vitro CME gene expression

We show that clozapine alters mRNA expression for markers of the CME system in SH-SYSY cells (Table 2) including AP2B1 (Kruskal–Wallis, 48 h: $\chi^2 = 16.731$, d.f. = 5, $P = 0.005$; 72 h: $\chi^2 = 17.385$, d.f. = 5, $P = 0.004$), APIB2 (Kruskal–Wallis, 48 h: $\chi^2 = 18.230$, d.f. = 5, $P = 0.003$; 72 h: $\chi^2 = 32.105$, d.f. = 5, $P = 0.0006$), HIPI (Kruskal–Wallis, 48 h: $\chi^2 = 15.895$, d.f. = 5, $P = 0.007$; 72 h: $\chi^2 = 11.543$, d.f. = 5, $P = 0.042$), ITSN2 (Kruskal–Wallis, 48 h: $\chi^2 = 15.264$, $P = 0.009$; 72 h: $\chi^2 = 15.680$, $P = 0.008$), and PICALM (Kruskal–Wallis, 48 h: $\chi^2 = 15.005$, d.f. = 5, $P = 0.010$). The change in expression of PICALM comparing the five clozapine treatments with vehicle at 72 h approached, but did not reach, significance (Kruskal–Wallis, $\chi^2 = 10.965$, d.f. = 5, $P = 0.052$). There were no statistically significant changes in the expression of these CME-associated genes following the more acute 24-h treatment (AP2B1: Kruskal–Wallis, $\chi^2 = 6.333$, d.f. = 5, $P = 0.275$; APIB2: one-way ANOVA, $F = 1.141$, $P = 0.360$; HIPI: one-way ANOVA, $F = 0.585$, $P = 0.712$; ITSN2: one-way ANOVA, $F = 1.745$, $P = 0.149$; PICALM: one-way ANOVA, $F = 0.451$, $P = 0.809$). There were no significant differences between the treatment group means for AP2A2 and CLINT1 genes at any time point (one-way ANOVA, $P > 0.05$).

GPCR gene expression screening

Because of the large number of molecular targets that are implicated in the action of clozapine, we screened the expression of 84 GPCR genes in cDNA from cells incubated with 5 μm/l clozapine for 24 h. GPCR gene expression was presented relative to the average expression of six reference genes (ACTB, B2M, GAPDH, HPRT1, RPL13A, and YWHAZ) and fold change was compared from clozapine-treated cells with that of DMSO vehicle-treated cells. Expression data for one reference gene, 18S, were significantly altered by clozapine treatment and were thus excluded from analysis. Of the 84 GPCR genes screened, there were consistent data for both clozapine-treated and DMSO-treated cDNA for 29 genes only (Fig. 2). Among the GPCR genes with an increase in fold change of two or greater, clozapine treatment increased the expression of three 5-HT receptor genes (HTR1A, HTR2A, and HTR7) and DRD1. These four GPCRs are among those reported to be pharmacological targets of clozapine (Meltzer and Massey, 2011). The highest increases in gene expression following clozapine treatment were observed for the 5-HT2A gene (HTR2A), with an 11.6-fold change, and the GALR2 gene, with an 11.1-fold change relative to the control. Gene expression was downregulated for arrestin β-2 (ARRB2) and AGTRAP following clozapine treatment, with a −0.39 and −0.92 decrease in fold change relative to the controls, respectively. It is also of interest that the expression of three endothelial differentiation GPCR genes (EDG6, EDG4 (also known as lysophosphatidic acid receptor 2, LPAR2), EDG5, and EDG1 (also known as sphingosine-1-phosphate receptors SIPR 2 and 1, respectively) was upregulated between one and seven fold following clozapine treatment (Fig. 2).

GPCR gene expression

Given that the GPCR panel plate offers a rapid screening method to identify the change in gene expression from pooled cDNA, it was important to determine the changes in unpooled cDNA from cells treated with the range of concentrations over the time course. The genes with the highest fold change in expression were thus followed up with individual qPCR gene expression assays to measure the effect of drug treatment more accurately. We selected the eight genes with the highest fold increases in expression, that is, HTR2A/HTR1A (Fig. 2) and AGTRAP as this showed the highest fold decrease in expression relative to the control (Table 2). We show that EDG4 mRNA expression is altered by 48-h clozapine treatment in human neuroblastoma cells (EDG4, one-way ANOVA, $F = 2.921$, $P = 0.022$). Bonferroni adjustment of EDG4 expression from all five clozapine treatment groups
compared with the control was not significant (one-way ANOVA Bonferroni post-hoc test, 1 μmol/l: P = 1.000; 2 μmol/l: P = 1.000; 5 μmol/l: P = 1.000; 10 μmol/l: P = 0.544; 20 μmol/l: P = 0.864). The mean comparisons of treatment groups are not significantly different for AGTRAP, P2Y1, GALK2, HTR1A, and HTR7 genes at any time point (P > 0.05).

Clozapine expression profiles in the mouse brain

The full Affymetrix array list of gene mRNA expression in the mouse brain following a 12-week chronic treatment with clozapine or vehicle can be downloaded from http://www.ucl.ac.uk/~rejuhxg/ClozHalStudy. Clozapine significantly altered the expression of 1330 genes relative to control. Clozapine altered the expression of nine GPCR and CME genes in the mouse brain with increases in AP2A2 by 3.8-fold (MANOVA, F = 46.527, df = 4, P = 0.0005; EDG4, 4.48-fold, F = 2.586, df = 16, P = 0.02).
of atypical antipsychotics (Meltzer and Massey, 2011). Our study shows a marked increase in HTR2A mRNA after 24 h of clozapine treatment in vitro. Clozapine downregulates 5-HT2A cell surface expression and increases receptor internalization through CME both acutely in vitro and chronically in vivo (Berry et al., 1996; Willins et al., 1998; Willins et al., 1999). Thus, the increase in HTR2A mRNA expression we observe following acute clozapine treatment may reflect a homeostatic upregulation of postsynaptic 5-HT2A receptors. In contrast, acute clozapine exposure was shown to downregulate HTR2A expression in hippocampal cells (Heiser et al., 2004). Recently, chronic clozapine treatment was shown to reduce histone acetylation of the HTR2A promoter, corresponding with decreases in transcriptional activity, mRNA expression, and 5-HT2A density through upregulation of histone deacetylase 2 in the frontal cortex of mice and schizophrenia patients (Kurita et al., 2012; Moreno et al., 2012). In untreated schizophrenia patients, frontal cortical 5-HT2A receptors are upregulated relative to control (Gonzalez-Maeso et al., 2008) whereas in individuals with schizophrenia with a history of antipsychotic medication, there is reduced frontal cortical 5-HT2A protein and mRNA (Burnet et al., 1996), and hippocampal HTR6/7 mRNA (East et al., 2002). Both 5-HT1A mRNA levels and binding density were also increased in the prefrontal cortex of treated schizophrenia patients (Burnet et al., 1997). Our observed increase in HTR2A mRNA following acute clozapine exposure may result in an increased need for presynaptic inhibition by 5-HT1A receptors, explaining the upregulation of HTR1A in vitro and in vivo. This finding is of particular interest, given the importance of the presynaptic serotonergic system in the action of clozapine, requiring intact 5-HT releasing receptors. In contrast, acute clozapine exposure was shown to downregulate HTR2A expression in hippocampal cells (Heiser et al., 2004). Recently, chronic clozapine treatment was shown to reduce histone acetylation of the HTR2A promoter, corresponding with decreases in transcriptional activity, mRNA expression, and 5-HT2A density through upregulation of histone deacetylase 2 in the frontal cortex of mice and schizophrenia patients (Kurita et al., 2012; Moreno et al., 2012). In untreated schizophrenia patients, frontal cortical 5-HT2A receptors are upregulated relative to control (Gonzalez-Maeso et al., 2008) whereas in individuals with schizophrenia with a history of antipsychotic medication, there is reduced frontal cortical 5-HT2A protein and mRNA (Burnet et al., 1996), and hippocampal HTR6/7 mRNA (East et al., 2002). Both 5-HT1A mRNA levels and binding density were also increased in the prefrontal cortex of treated schizophrenia patients (Burnet et al., 1997). Our observed increase in HTR2A mRNA following acute clozapine exposure may result in an increased need for presynaptic inhibition by 5-HT1A receptors, explaining the upregulation of HTR1A in vitro and in vivo. This finding is of particular interest, given the importance of the presynaptic serotonergic system in the action of clozapine, requiring intact 5-HT releasing neurons with functional 5-HT1A autoreceptors, independent of functional postsynaptic markers such as 5-HT2A (Yadav et al., 2011). It is essential to further characterize the short-term and long-term clinical effects of clozapine on the serotonergic system.

In this study, the clozapine-induced increase in 5-HT receptor expression at 24 h is accompanied by increases in GALR2 and EDG4/5/1, which is sustained for EDG4 at 48 and 72 h in vitro. Serotonin receptors form heterooligomers with the EDG1 receptor (Salim et al., 2002) and are coexpressed with galanin receptors, which modulate serotonergic transmission (Borrono-Escuela et al., 2010). Interestingly, galanin receptors are implicated in susceptibility to depression (Kuteeva et al., 2010). Similarly, both DDR1 and SSTR3 (somatostatin 3 receptor) expressions were upregulated by clozapine in vitro. Chronic clozapine treatment in vivo is shown to increase preprosomatostatin mRNA in the nucleus accumbens. Dopamine regulates central somatostatinergic neurons, which play an important role in clozapine’s superior profile in the control of motor behavior (Salin et al., 1990).

In schizophrenia, changes in dopamine receptor trafficking to lysosomes because of increased receptor agonism

Discussion
Here, we show that clozapine upregulates mRNA levels of GPCRs and genes involved in CME function in vitro and in vivo. The therapeutic efficacy of most typical antipsychotics is related to dopamine D2/D3 receptor blockade, whereas serotonergic actions, notably 5-HT2A receptor antagonism, are thought to explain the differential effects of atypical antipsychotics (Meltzer and Massey, 2011). Our study shows a marked increase in HTR2A mRNA after 24 h of clozapine treatment in vitro. Clozapine downregulates 5-HT2A cell surface expression and increases receptor internalization through CME both acutely in vitro and chronically in vivo (Berry et al., 1996; Willins et al., 1998; Willins et al., 1999). Thus, the increase in HTR2A mRNA expression we observe following acute clozapine treatment may reflect a homeostatic upregulation of postsynaptic 5-HT2A receptors. In contrast, acute clozapine exposure was shown to downregulate HTR2A expression in hippocampal cells (Heiser et al., 2004). Recently, chronic clozapine treatment was shown to reduce histone acetylation of the HTR2A promoter, corresponding with decreases in transcriptional activity, mRNA expression, and 5-HT2A density through upregulation of histone deacetylase 2 in the frontal cortex of mice and schizophrenia patients (Kurita et al., 2012; Moreno et al., 2012). In untreated schizophrenia patients, frontal cortical 5-HT2A receptors are upregulated relative to control (Gonzalez-Maeso et al., 2008) whereas in individuals with schizophrenia with a history of antipsychotic medication, there is reduced frontal cortical 5-HT2A protein and mRNA (Burnet et al., 1996), and hippocampal HTR6/7 mRNA (East et al., 2002). Both 5-HT1A mRNA levels and binding density were also increased in the prefrontal cortex of treated schizophrenia patients (Burnet et al., 1997). Our observed increase in HTR2A mRNA following acute clozapine exposure may result in an increased need for presynaptic inhibition by 5-HT1A receptors, explaining the upregulation of HTR1A in vitro and in vivo. This finding is of particular interest, given the importance of the presynaptic serotonergic system in the action of clozapine, requiring intact 5-HT releasing neurons with functional 5-HT1A autoreceptors, independent of functional postsynaptic markers such as 5-HT2A (Yadav et al., 2011). It is essential to further characterize the short-term and long-term clinical effects of clozapine on the serotonergic system.

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lead to more receptors being retained on the cell surface (Iizuka et al., 2007). GPCR agonism (Traub, 2009), and less commonly antagonism (Willins et al., 1998; Willins et al., 1999; Heusler et al., 2008), results in receptor internalization from the plasma membrane to the CME pathway. Thus, post-endocytic mechanisms regulating surface expression and lysosomal degradation of GPCRs may be dysfunctional in subtypes of schizophrenia (Marley and von Zastrow, 2010). Distinct populations of CCVs sort GPCR cargo at the membrane involving differential kinase-dependent processes. P2Y1 receptor internalization is G protein-coupled receptor kinase (GRK) and arrestin independent but requires protein kinase C, whereas P2Y12 and β2 adrenergic receptors (ADRB2) are internalized by a separate mechanism involving GRKs and arrestin (Mundell et al., 2006). Therefore, our data showing increased P2Y1R and ADRB2 expression suggest that clozapine affects both kinase-dependent CCV internalization processes.

ARRB2, a functional cofactor of β-adrenergic receptor kinase that phosphorylates the agonist-occupied receptor, replaces receptor-associated G-proteins and prevents reassociation. Thus, ARRB2 plays a major role in determining homologous agonist-mediated desensitization of GPCRs and inhibits cellular responses (Gurevich et al., 2008). There is recent evidence that agonism of some GPCRs does not result in the activation of signaling pathways typical of G-proteins, but does activate MAP kinases through β-arrestins (Rajagopal et al., 2010). Interestingly, both the GPCR panel plate screen and microarray data show a downregulation of ARRB2. This decrease occurs despite a concurrent upregulation of ADRB1/2 in vitro, suggesting that clozapine treatment results in reduced inhibitory control of synaptic transmission despite increased adrenergic receptor agonism and gene expression. Arrestins regulate GPCR endocytosis through a clathrin-dependent and dynamin-dependent pathway (Wolf and Trejo, 2007). It is therefore likely that clozapine’s regulation of ARRB2 affects cell surface expression of multiple GPCRs and their trafficking through different clathrin adaptors.

We found significant increases in the gene expression of AP2A2 after 24 h along with CLINT1 and clathrin assembly proteins AP180 and PICALM after 72 h of clozapine treatment in SH-SYSY cells compared with the control. Clozapine increased AP2B1, AP180, HIP1, ITSN2, and PICALM gene expression in a dose-dependent manner at both 48 and 72 h. These increases in mRNA levels are in keeping with the maintenance of SH-SYSY cell proliferation and viability when treated with lower clozapine doses (1–10 μmol/l) for 72 h. However, high concentrations of clozapine halved cell viability, perhaps caused by the formation of electrophilic reactive metabolites (Takakusa et al., 2008). It is important to note that although the use of undifferentiated SH-SYSY cells avoids possible confounding effects on gene expression associated with the different phenotypes induced by various differentiation agents, these cells do not express typical markers of mature neurons (Pahlman et al., 1984; Adem et al., 1987). Similar to the in-vitro data, clozapine-treated mouse microarray analysis showed that AP180, AP2A2, AP2B1, CLINT1, and PICALM CME-gene mRNA levels were also significantly upregulated, whereas HIP1 and ITSN2 were downregulated.

Taking our in-vitro and in-vivo data together, clozapine increases the expression of genes encoding GPCRs and proteins that are structurally important in CME function, but it decreases the expression of ARRB2 that regulates receptor endocytosis. An increase in synaptic receptor density and/or desensitization may in turn lead to more rapid recycling of functional receptors through CCVs, or increased degradation of dysfunctional receptors through the lysosomal pathway, perhaps resulting in increased gene transcription. The early increase in GPCR gene expression is consistent with the idea that the antipsychotic response occurs in the initial days following treatment (Agid et al., 2006). Thus, CME may play a fundamental role in the mechanism of action of clozapine, which must be elucidated at the protein and cellular level to develop new treatments and preventive strategies to avoid serious side effects and improve the prognosis of schizophrenia.

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Conflicts of interest
There are no conflicts of interest.

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