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

Sally I. Sharp[a], Ying Hu[c], Jon F. Weymer[a], Mie Rizig[a], Andrew McQuillin[a], Stephen P. Hunt[b] and Hugh M.D. Gurling[a]

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.

Materials and methods SH-SY5Y human neuroblastoma cells were incubated with clozapine (1–20 µmol/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.

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 µmol/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 lysophosphatidic acid receptor 2 (*EDG4*) GPCR genes was upregulated in SH-SY5Y cells incubated with 5 µmol/l clozapine for 24 h. *EDG4* expression was also increased with 10–20 µmol/l clozapine treatment at 48–72 h. Clozapine significantly decreased the expression of β-arrestin, involved in GPCR desensitization, both *in vitro* and *vivo*.

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.

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-HT$_{2A}$) inverse agonism, 5-HT$_{1A}$ 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-HT$_{2A}$ (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-SY5Y 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% CO$_2$. Cells were not passaged beyond passage 21.

Cell viability assay
Cells plated at a density of 1×10^3 cells/cm^2 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-SY5Y 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 2 × 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 (C_q) values were obtained for all 84 GPCR genes and seven reference genes on the GPCR panel plate. The quadruplicate average C_q 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 C_q for each gene. The same was repeated for C_q values obtained from the second GPCR panel plate with DMSO-treated cDNA. The mean GPCR C_q 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*, *CLINT1*, disabled homolog 1 and 2 (*DAB1*, *DAB2*), Huntingtin interacting protein 1 (*HIP1*), 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 in the Roche universal

Table 1   qPCR primers and probes

Gene[a]	Ensemble ID	Sequence	5′ primer position on gene (bp)	Intron spanning	UPL probe or SYBR-Green[b]
AGTRAP_F	ENST00000376637	5′-TATGCCTGGGCCAACTTCAC	162	Y	162
AGTRAP_R		5′-GCAAGCCACCCAGAAACATG	273		
AP180_F	ENST00000521485	5′-AAGGTGACATTCCTGACCTC	1178	Y	3
AP180_R		5′-TAGATGCTGTTCAAGCGTCTC	1242		
AP2B1_F	ENST00000262325	5′-GATCTCATAGCAGATTCAAATCC	688	Y	77
AP2B1_R		5′-ATTTCAGATAATGCCGCTACG	749		
AP2A2_F	ENST00000329626	5′-GTGGTGCACCTGCTCAATG	755	Y	5
AP2A2_R		5′-CTTCTGGGTTCTTCTGTGC	842		
CLINT1_F	ENST00000296951	5′-AGCAGCAAGTCATCTGGTG	1145	Y	20
CLINT1_R		5′-ATGCAGCAGCTGAGCCAAAG	1256		
GALR2_F	ENST00000329003	5′-CTACGCCAACTCCTGCGTCAAC	923	N	54
GALR2_R		5′-CAGATCGTGCGGAAGCCTTTG	1001		
HIP1_F	ENST00000336926	5′-TGCAGGACACTCAGCTCAAAC	1820	Y	6
HIP1_F		5′-TTCGTTGGTCTTTGGCAAG	1889		
HTR1A_F	ENST00000323865	5′-AGTGAAGACGCTGGGCATCA	1029	N	SG
HTR1A_R		5′-GCAGAAGGGCAGAACAAGAG	1113		
HTR7A_F	ENST00000336152	5′-CAGCAGTAAGCACACCAACAG	1915	N	SG
HTR7A_R		5′-GGACATATGCAAAGCACCAG	1984		
ITSN2_F	ENST00000355123	5′-TGACCTTCAGCTCGTCGTC	4122	Y	53
ITSN2_R		5′-CTCCCCTTCAGTGAGAAAGC	4191		
LPAR2_F	ENST00000407877	5′-CTGGTCAAGACTGTTGTCATCA	854	Y	120
LPAR2_R		5′-CCAGGAGCAGTACCACCTG	929		
P2RY1_F	ENST00000305097	5′-AGCGGCATCTCCGTGTACA	250	N	51
P2RY1_R		5′-TGAAGTAGTAGAAGATCAGG	337		
PGK1_F	–	–	–	–	Roche PGK1 gene assay
PGK1_R		–	–	–	
PICALM_F	ENST00000341783	5′-TCTCAGGTAGCAAGTACATGG	1520	Y	5
PICALM_R		5′-CAACATTAAGGCCAGCTGAAG	1748		
RPLP0_F	ENST00000228306	5′-CGACAATGGCAGCATCTAC	816	Y	6
RPLP0_R		5′-GGTAGCCAATCTGCAGAC	931		

[a]AGTRAP, type-1 angiotensin II receptor-associated protein; AP180, clathrin coat assembly protein 180, also known as SNAP91; AP2A2, adaptor-related protein complex 2, α2 subunit; AP2B1, adaptor-related protein complex 2, β1 subunit; CLINT1, clathrin interactor 1; F, forward primer; GALR2, galanin receptor 2; HIP1, Huntingtin interacting protein 1; HTR1A, serotonin receptor 1A; HTR7, serotonin receptor 7; ITSN2, intersectin 2; LPAR2, lysophosphatidic acid receptor 2; N, no; *P2YR1,* 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.
[b]UPL, Roche Universal Library Hydrolysis Probe Number; SG, SYBR-Green.

probe library (UPL) assay design center (*https://www.roche-applied-science.com/sis/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, *DRD1*, *HTR2A*, and *SSTR3*, and two CME gene assays, *DAB1* 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 × concentrated buffer mix containing NH_4 (Bioline Ltd, London, UK), 3.2 or 6.2 mmol/l $MgCl_2$, 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 13 000*g*, 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 C_q value. The efficiency was calculated as follows: $E = 10 - (1/\text{slope})$, 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 calibrator sample of cDNA was used on all 384-well plates to control for variance between plates, along with an H_2O-negative control.

qPCR gene expression statistical analysis
### Raw data analysis
Quadruplicate C_q 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 $2^{-\Delta C_q^t}$ method (Livak and Schmittgen, 2001). The mean C_q values of each target gene were expressed relative to the mean C_q values of the two reference genes ($2^{-\Delta C_q^t}$). The mean C_q values of the calibrator were then subtracted from the relative mean C_q values on each plate. The $2^{-\Delta\Delta C_q^t}$ 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 $2^{-\Delta C_q^t}$ of the different

Fig. 1
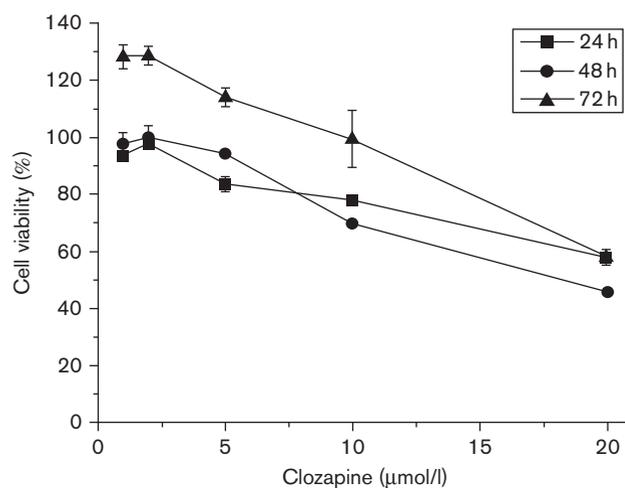
MTT SH-SY5Y neuroblastoma cell viability assay. Living cells are required for MTT cleavage in the MTT cell viability assay. SH-SY5Y cells were plated out at a density of 10^4 cells in each well of a six-well plate, incubated overnight in growth medium (DMEM, 10% fetal bovine serum), and treated with 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.

Fig. 2
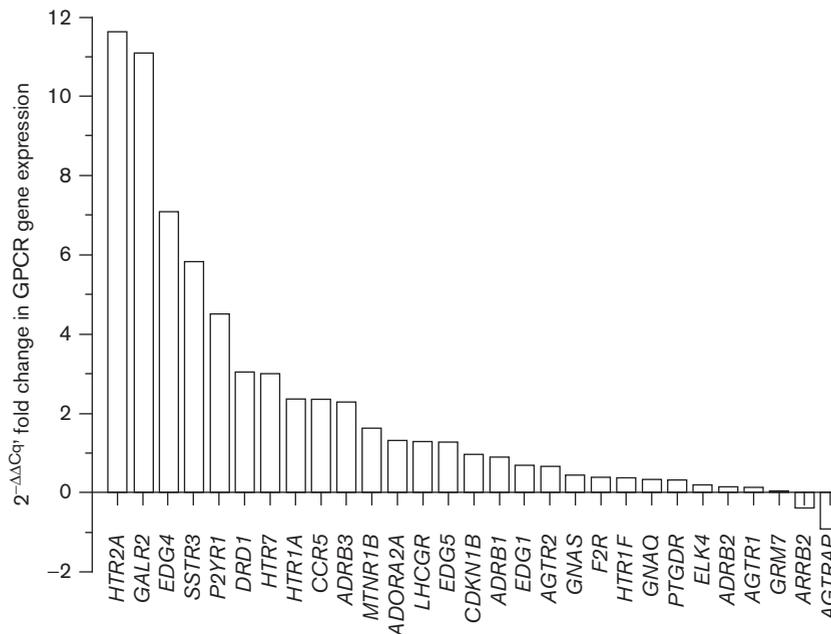
Fold change in GPCR gene expression in SH-SY5Y cells following treatment with 5 μmol/l clozapine for 24 h. ADORA2A, adenosine receptor A2a; ADRB1, β-1 adrenergic receptor; ADRB2, β-2 adrenergic receptor; ADRB3, β-3 adrenergic receptor; AGTRAP, type-1 angiotensin II receptor-associated protein; AGTR1, angiotensin II type-1B receptor; AGTR2, angiotensin II type-2 receptor; AP180, clathrin coat assembly protein 180, also known as synaptosomal-associated protein, SNAP91; AP2A2, adaptor-related protein complex 2, α2 subunit, AP2B1, adaptor-related protein complex 2, β 1 subunit; ARRB2, arrestin β-2; CCR5, chemokine (C–C motif) receptor 5; CDKN1B, cyclin-dependent kinase inhibitor 1B; CLINT1, clathrin interactor 1; DRD1, dopamine D1 receptor; EDG1, endothelial differentiation receptor 1, also known as sphingosine-1-phosphate receptor 1, S1PR1; EDG4, endothelial differentiation receptor 4, also known as lysophosphatidic acid receptor 2, LPAR2; EDG5, endothelial differentiation sphingolipid receptor 5, LPAR2; ELK4, ETS domain-containing protein, or serum response factor accessory protein 1, SAP-1; F2R, coagulation factor II (thrombin) receptor; GALR2, galanin receptor 2; GNAS, guanine nucleotide-binding protein G(s) subunit α-stimulating complex locus, also known as adenylate cyclase-stimulating G α protein; GNAQ, guanine nucleotide-binding protein G(q) subunit α; GRM7, metabotropic glutamate receptor 7; HIP1, Huntingtin interacting protein 1; HTR1A, serotonin receptor 1A; HTR1F, serotonin receptor 1F; HTR2A, Serotonin receptor 2A; HTR7, serotonin receptor 7; ITSN2, intersectin 2; LHCGR, luteinizing hormone/choriogonadotropin receptor; MTNR1B, melatonin receptor 1B; P2YR1, P2Y purinoceptor 1; PTGDR, prostaglandin D2 receptor; SSTR3, somatostatin receptor 3. Data are from nine pooled cDNA replicates. The mean fold change in GPCR gene expression, relative to six reference genes, ACTB, B2M, GAPDH, HPRT1, RPL13A, and YWHAZ, is defined as gene expression in 5 μmol/l 24-h clozapine-treated cells relative to DMSO vehicle control cells ($2^{-\Delta\Delta C_q^t}$). GPCR, G protein-coupled receptors.

clozapine drug treatment samples were compared directly with that of the DMSO vehicle sample to yield $2^{-\Delta\Delta C_q^t}$.

Removal of extreme outliers

Data for $2^{-\Delta C_q^t}$ derived from gene expression assays were initially examined by boxplot analysis to identify outliers. cDNA with $2^{-\Delta C_q^t}$ 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; IBM UK Ltd, Portsmouth, UK), but were included in the analysis. Values for cDNA more than three box lengths from the upper or the lower edge of the box, or extreme outliers, three or more SDs from the mean, were excluded from analysis. Results are given as group means with respect to time point and DMSO or clozapine treatment minus outliers along with SEM determined from the number (*n*) of replicates. Parametric statistical evaluations were performed using one-way analysis of variance (ANOVA) to compare the means between three or more treatment groups and Student's *t*-test for the comparison of two treatment groups. If

homogeneity of variance (Levene's test for equality of variances) was significantly different between subject groups, then a nonparametric analysis was carried out (Kruskal–Wallis for three or more groups; Mann–Whitney *U*-test for two groups) when appropriate (SPSS version 17.0). A cut-off significance value of *P* less than 0.05 was used.

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

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. Multivariate 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 μmol/l clozapine for 24 and 48 h, compared with vehicle. However, MTT-formazan formation decreased with increasing concentrations of clozapine (5–20 μmol/l) at 24- and 48-h incubation times, indicating decreasing SH-SY5Y cell viability. Interestingly, the rate of cell growth and survival increased by 29% with both 1 and 2 μmol/l clozapine, and by 15% with 5 μmol/l clozapine following a 72-h incubation compared with vehicle. There was no difference in cell viability between vehicle and 10 μmol/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 μmol/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-SY5Y 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, $P = 0.004$), *AP180* (Kruskal–Wallis, 48 h: $\chi^2 = 18.230$, d.f. = 5, $P = 0.003$; 72 h: $\chi^2 = 32.105$, d.f. = 5, $P = 0.0006$), *HIP1* (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, $P = 0.275$; *AP180*: one-way ANOVA, $F = 1.141$, $P = 0.360$; *HIP1*: 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 μmol/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-HT$_{2A}$ 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 (*EDG*), *EDG4* (also known as lysophosphatidic acid receptor 2, LPAR2), *EDG5*, and *EDG1* (also known as sphingosine-1-phosphate receptors *S1PR* 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

Table 2 SH-SY5Y neuroblastoma gene expression following treatment with clozapine (1–20 µmol/l) for 24, 48, and 72 h

Treatment time	Gene[a]	Clozapine treatment[b]					
		DMSO	1 µmol/l	2 µmol/l	5 µmol/l	10 µmol/l	20 µmol/l
24 h	AP2A2	0.59±0.06 (7)	0.89±0.22 (8)	0.93±0.19 (7)	**1.28±0.32 (9)**[*,c]	0.66±0.06 (7)	0.53±0.16 (5)
	CLINT1	1.88±0.32 (9)	1.36±0.17 (8)	2±0.6 (9)	1.84±0.43 (8)	1.51±0.23 (8)	0.78±0.27 (6)
	AP2B1	1.13±0.35 (4)	2.5±1.18 (5)	3.97±2.45 (3)	1.08±0.34 (4)	1.12±0.32 (4)	3.19±0.7 (4)
	AP180	2.08±0.78 (8)	1.21±0.3 (7)	1.18±0.33 (7)	1.38±0.34 (6)	1.37±0.41 (5)	2.69±0.35 (4)
	HIP1	1.75±0.63 (8)	2.4±1.22 (8)	1.92±0.81 (5)	1.33±0.58 (6)	0.69±0.21 (5)	0.84±0.15 (4)
	PICALM	1.57±0.56 (8)	1.08±0.33 (7)	2.35±1.23 (8)	1.23±0.35 (6)	1.27±0.37 (6)	1.76±0.38 (4)
	ITSN2	0.52±0.16 (8)	0.32±0.09 (7)	0.58±0.25 (8)	0.35±0.11 (7)	0.39±0.15 (7)	0.97±0.19 (6)
	AGTRAP	1.69±0.49 (9)	2.02±0.52 (7)	1.45±0.39 (9)	1.82±0.43 (7)	1.49±0.34 (8)	1.9±0.41 (7)
	P2YR1	10.4±7.4 (4)	33.55±11.12 (4)	12.02±6.48 (7)	25.42±15.71 (6)	33.4±21.26 (8)	28.23±18.17 (5)
	EDG4	1.38±0.41 (8)	1.48±0.41 (8)	1.78±0.53 (9)	1.7±0.45 (9)	1.58±0.35 (9)	1.82±0.35 (8)
	GALR2	2.01±0.94 (7)	9.73±5.68 (6)	7.85±3.77 (8)	15.45±8.12 (8)	1.28±0.53 (5)	4.53±1.96 (5)
	HTR1A	0.48±0.17 (5)	3.9±1.85 (8)	2.88±1.58 (8)	5.24±2.97 (8)	9.29±6.06 (7)	1.75±0.72 (7)
	HTR7	5.22±2.57 (6)	17.03±8.02 (7)	9.86±5.46 (7)	21.7±11.71 (6)	27.39±16.82 (6)	21.53±12.6 (6)
48 h	AP2A2	0.76±0.15 (9)	1.06±0.12 (9)	0.94±0.15 (8)	0.75±0.09 (7)	1.26±0.43 (9)	1.49±0.4 (9)
	CLINT1	0.72±0.14 (8)	0.9±0.07 (9)	0.89±0.16 (8)	0.86±0.13 (8)	0.95±0.14 (8)	1.54±0.38 (8)
	AP2B1	0.15±0.03 (8)	0.2±0.04 (9)	0.28±0.09 (9)	0.17±0.04 (8)	0.15±0.04 (7)	1.17±0.24 (9)
	AP180	0.04±0.01 (9)	0.04±0.01 (8)	0.08±0.02 (9)	0.05±0.08 (8)	0.04±0.01 (7)	1.13±0.31 (8)
	HIP1	0.1±0.02 (9)	0.11±0.02 (9)	0.15±0.04 (9)	0.08±0.02 (8)	0.07±0.02 (7)	1.39±0.37 (9)
	PICALM	0.12±0.03 (9)	0.12±0.03 (9)	0.17±0.05 (9)	0.11±0.03 (8)	0.09±0.02 (7)	0.94±0.21 (9)
	ITSN2	0.03±0.01 (8)	0.05±0.02 (9)	0.04±0.02 (8)	0.02±0.01 (7)	0.21±0.14 (8)	0.25±0.03 (7)
	AGTRAP	0.76±0.13 (9)	0.95±0.17 (9)	1.02±0.2 (9)	0.77±0.14 (9)	1.13±0.18 (9)	0.83±0.13 (9)
	P2YR1	3.89±2.24 (5)	2.32±1.32 (6)	3.42±1.17 (4)	6.71±3.68 (6)	4.1±2.09 (4)	2.13±1.02 (4)
	EDG4	0.78±0.08 (9)	0.86±0.08 (9)	0.93±0.08 (9)	0.77±0.07 (9)	1.06±0.11 (9)	**1.15±0.12 (9)**[*,i]
	GALR2	1.51±0.5 (9)	1.46±0.45 (7)	1.21±0.45 (8)	2.36±1.16 (8)	0.91±0.38 (7)	1.72±0.81 (8)
	HTR1A	0.67±0.22 (7)	0.51±0.1 (7)	1.34±0.48 (8)	4.42±2.7 (8)	0.49±0.12 (6)	2.31±1.14 (7)
	HTR7	3.04±1.78 (5)	1.56±0.48 (5)	1.19±0.37 (7)	4.87±2.86 (8)	1.54±0.94 (4)	4.04±2.57 (4)
72 h	AP2A2	137.07±18.76 (9)	131.58±16.9 (9)	105.89±17.11 (9)	118.2±23.12 (9)	118.8±18.53 (9)	148.81±26.62 (7)
	CLINT1	0.06±0.01 (9)	0.06±0.01 (9)	0.06±0.01 (9)	**0.07±0.01 (9)**[*,d]	0.06±0.01 (9)	0.07±0.01 (8)
	AP2B1	6.62±0.61 (9)	6.21±0.87 (9)	6.2±0.6 (9)	7.69±0.81 (9)	6.23±0.67 (8)	25.77±6.94 (7)
	AP180	1.74±0.06 (9)	1.77±0.16 (9)	1.78±0.15 (9)	**2.81±0.22 (9)**[*,e]	**2.28±0.09 (9)**[***,g]	32.32±12.57 (7)
	HIP1	6.63±0.83 (9)	7.64±1.24 (9)	6.74±0.89 (9)	8.6±1.02 (9)	7.9±1.16 (9)	21.18±5.46 (7)
	PICALM	12.6±1.02 (9)	11.91±1.38 (9)	11.99±0.78 (9)	**16.2±1.2 (9)**[*,f]	13.93±0.93 (9)	23.11±5.5 (8)
	ITSN2	0.26±0.03 (8)	0.32±0.07 (9)	0.26±0.05 (8)	0.32±0.05 (9)	0.41±0.11 (9)	7.76±3.73 (9)
	AGTRAP	1.14±0.1 (9)	1.18±0.08 (9)	1.08±0.07 (9)	1.24±0.1 (9)	1.27±0.09 (9)	1.26±0.14 (8)
	P2YR1	0.88±0.39 (8)	0.9±0.32 (6)	0.72±0.22 (6)	2.21±0.87 (7)	0.76±0.27 (6)	2.22±0.73 (4)
	EDG4	1.28±0.08 (9)	1.43±0.12 (9)	1.34±0.04 (9)	1.62±0.14 (9)	**1.69±0.06 (9)**[**,h]	1.48±0.19 (7)
	GALR2	1.92±0.54 (9)	2.44±0.72 (8)	1.49±0.2 (7)	3.88±1.32 (9)	1.34±0.51 (9)	2.36±0.84 (7)
	HTR1A	0.71±0.23 (6)	1.1±0.44 (7)	0.92±0.29 (8)	2.43±0.73 (8)	0.93±0.23 (8)	1.71±0.56 (7)
	HTR7	1.17±0.33 (7)	1.89±0.48 (5)	2.52±1.04 (8)	1.64±0.52 (7)	0.86±0.33 (6)	1.78±1.14 (6)

Data are mean $2^{-\Delta C_q^J}$±SEM (n). Bold indicates that the fold change in gene expression following clozapine treatment is significantly different relative to vehicle.
[a]AGTRAP, type-1 angiotensin II receptor-associated protein; AP180, adaptor protein 180; AP2A2, adaptor-related protein complex 2, α2 subunit; AP2B1, adaptor-related protein complex 2, β1 subunit; CLINT1, clathrin interactor 1; DMSO, dimethyl sulfoxide; EDG4, encoding LPAR2, lysophosphatidic acid receptor 2; GALR2, galanin receptor 2; HIP1, Huntingtin interacting protein 1; HTR1A, serotonin receptor 1A; HTR7, serotonin receptor 7; ITSN2, intersectin 2; P2RY1, P2Y purinoceptor 1; PGK1, phosphoglycerate kinase 1; PICALM, phosphatidylinositol-binding clathrin assembly lymphoid myeloid protein; RPLP0, acidic ribosomal protein P0.
[b]Gene expression relative to two reference genes: PGK1 and RPLP0.
Significant Students t-test or Mann–Whitney U-test results are shown for comparisons between treatment groups:
DMSO vs. 5 µmol/l CLZ: [c]AP2A2_24 h, $Z=-2.276$, $P=0.023$; [d]CLINT1_72 h, $t=0.634$, d.f.=16, $P=0.033$; [e]AP180_72 h, $Z=-3.228$, $P=0.01$; [f]PICALM_72 h, $t=-2.291$, d.f.=16, $P=0.036$.
DMSO vs. 10 µmol/l CLZ: [g]AP180_72 h, $t=-4.524$, d.f.=14, $P=0.0005$; [h]EDG4_72 h, $t=-3.912$, d.f.=16, $P=0.001$.
DMSO vs. 20 µmol/l CLZ: [i]EDG4_48 h, $t=-2.586$, d.f.=16, $P=0.02$.
*$P<0.05$; **$P<0.01$; ***$P<0.001$.

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: $P=0.086$), the significant difference between groups being driven by the increase in *EDG4* expression following 20 µmol/l clozapine treatment (Table 2). There was a trend toward changed *EDG4* expression after 72 h of treatment (one-way ANOVA, 24 h: $F=0.160$, $P=0.976$; 72 h: $F=2.195$, $P=0.071$). The mean comparisons of treatment groups are not significantly different for *AGTRAP*, *P2YR1*, *GALR2*, *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, $F=46.527$, d.f.=4, $N=76$, $P=6.16\times10^8$), *AP2B1* by 2.7-fold (MANOVA, $F=98.520$, d.f.=2, $N=40$, $P=4.46\times10^8$), *AP180* by 641.0-fold (Mann–Whitney U-test, $Z=-3.782$, $N=20$, $P=1.08\times$

Fig. 3
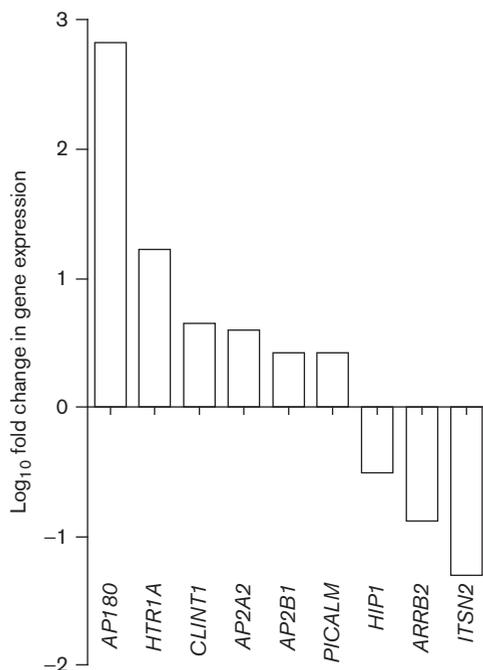
Log$_{10}$ transformation of fold change in clathrin-coated vesicles and G protein-coupled receptor gene expression in mouse brain following treatment with 12 mg/kg/day clozapine for 12 weeks: data from a microarray study. *AP180*, clathrin coat assembly protein 180, also known as synaptosomal-associated protein, SNAP91; *AP2A2*, adaptor-related protein complex 2, α2 subunit; *AP2B1*, adaptor-related protein complex 2, β 1 subunit; *ARRB2*, arrestin β-2; *CLINT1*, clathrin interactor 1; *HIP1*, Huntingtin interacting protein 1; *HTR1A*, serotonin receptor 1A; *ITSN2*, intersectin 2; *PICALM*, phosphatidylinositol-binding clathrin assembly lymphoid myeloid protein. Data are from 10 microarray replicates. The mean fold change in gene expression is defined as gene expression in 12 mg/kg/day 12-week clozapine-treated mice relative to DMSO vehicle control mice.

10^{-5}), *CLINT1* by 4.5-fold (Mann–Whitney U-test, $Z = -3.558$, $N = 18$, $P = 4.57 \times 10^{-5}$), *HTR1A* by 16.7-fold (Mann–Whitney U-test, $Z = -3.782$, $N = 20$, $P = 1.08 \times 10^{-5}$), and *PICALM* by 2.6-fold (Mann–Whitney U-test, $Z = -3.782$, $N = 20$, $P = 1.08 \times 10^{-5}$), whereas the expression of three genes was significantly decreased including *ARRB2* by 7.6-fold (MANOVA, $F = 422.140$, $d.f. = 2$, $N = 38$, $P = 6.53 \times 10^{14}$), *HIP1* by 3.2-fold (Student's t-test, $t = 12.538$, $d.f. = 18$, $N = 20$, $P = 2.48 \times 10^{10}$), and *ITSN2* by 19.9-fold (MANOVA, $F = 317.347$, $d.f. = 3$, $N = 60$, $P = 1.85 \times 10^{14}$) (Fig. 3 shows the logged mRNA fold change).

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-HT$_{2A}$ 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-HT$_{2A}$ 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/7* expression we observe following acute clozapine treatment may reflect a homeostatic upregulation of postsynaptic 5-HT$_{2A/7}$ 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-HT$_{2A}$ 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-HT$_{2A}$ 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-HT$_{2A}$ protein and mRNA (Burnet *et al.*, 1996), and hippocampal *HTR6/7* mRNA (East *et al.*, 2002). Both 5-HT$_{1A}$ 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/7* following acute clozapine exposure may result in an increased need for presynaptic inhibition by 5-HT$_{1A}$ 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-HT$_{1A}$ autoreceptors, independent of functional postsynaptic markers such as 5-HT$_{2A}$ (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 hetero-oligomers with the EDG1 receptor (Salim *et al.*, 2002) and are coexpressed with galanin receptors, which modulate serotonergic transmission (Borroto-Escuela *et al.*, 2010). Interestingly, galanin receptors are implicated in susceptibility to depression (Kuteeva *et al.*, 2010). Similarly, both *DRD1* 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

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. P2Y$_1$ receptor internalization is G protein-coupled receptor kinase (GRK) and arrestin independent but requires protein kinase C, whereas P2Y$_{12}$ and β_2 adrenergic receptors (*ADRB2*) are internalized by a separate mechanism involving GRKs and arrestin (Mundell *et al.*, 2006). Therefore, our data showing increased *P2YR1* 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 (Wolfe 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-SY5Y 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-SY5Y 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-SY5Y 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.

Acknowledgements

Conflicts of interest
There are no conflicts of interest.

References

Adem A, Mattsson ME, Nordberg A, Pahlman S (1987). Muscarinic receptors in human SH-SY5Y neuroblastoma cell line: regulation by phorbol ester and retinoic acid-induced differentiation. *Brain Res* **430**:235–242.

Agid O, Seeman P, Kapur S (2006). The 'delayed onset' of antipsychotic action – an idea whose time has come and gone. *J Psychiatry Neurosci* **31**: 93–100.

Berry SA, Shah MC, Khan N, Roth BL (1996). Rapid agonist-induced internalization of the 5-hydroxytryptamine2A receptor occurs via the endosome pathway *in vitro*. *Mol Pharmacol* **50**:306–313.

Borroto-Escuela DO, Narvaez M, Marcellino D, Parrado C, Narvaez JA, Tarakanov AO, et al. (2010). Galanin receptor-1 modulates 5-hydroxytryptamine-1A signaling via heterodimerization. *Biochem Biophys Res Commun* **393**:767–772.

Burnet PW, Eastwood SL, Harrison PJ (1996). 5-HT1A and 5-HT2A receptor mRNAs and binding site densities are differentially altered in schizophrenia. *Neuropsychopharmacology* **15**:442–455.

Burnet PW, Eastwood SL, Harrison PJ (1997). [³H]WAY-100635 for 5-HT1A receptor autoradiography in human brain: a comparison with [³H]8-OH-DPAT and demonstration of increased binding in the frontal cortex in schizophrenia. *Neurochem Int* 30:565–574.

East SZ, Burnet PW, Kerwin RW, Harrison PJ (2002). An RT-PCR study of 5-HT(6) and 5-HT(7) receptor mRNAs in the hippocampal formation and prefrontal cortex in schizophrenia. *Schizophr Res* 57:15–26.

Escamilla M, Lee BD, Ontiveros A, Raventos H, Nicolini H, Mendoza R, *et al.* (2008). The epsin 4 gene is associated with psychotic disorders in families of Latin American origin. *Schizophr Res* 106:253–257.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, *et al.* (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5:R80.

Gonzalez-Maeso J, Ang RL, Yuen T, Chan P, Weisstaub NV, Lopez-Gimenez JF, *et al.* (2008). Identification of a serotonin/glutamate receptor complex implicated in psychosis. *Nature* 452:93–97.

Gurevich VV, Gurevich EV, Cleghorn WM (2008). Arrestins as multi-functional signaling adaptors. *Handb Exp Pharmacol* 186:15–37.

Gurling H, Pimm J, McQuillin A (2007). Replication of genetic association studies between markers at the Epsin 4 gene locus and schizophrenia in two Han Chinese samples. *Schizophr Res* 89:357–359.

Heiser P, Schulte E, Hausmann C, Becker R, Remschmidt H, Krieg JC, Vedder H (2004). Effects of clozapine and its metabolites on the 5-HT2 receptor system in cortical and hippocampal cells *in vitro*. *Prog Neuropsychopharmacol Biol Psychiatry* 28:297–302.

Heusler P, Newman-Tancredi A, Loock T, Cussac D (2008). Antipsychotics differ in their ability to internalise human dopamine D2S and human serotonin 5-HT1A receptors in HEK293 cells. *Eur J Pharmacol* 581:37–46.

Iizuka Y, Sei Y, Weinberger DR, Straub RE (2007). Evidence that the BLOC-1 protein dysbindin modulates dopamine D2 receptor internalization and signaling but not D1 internalization. *J Neurosci* 27:12390–12395.

International Schizophrenia Consortium (2008). Rare chromosomal deletions and duplications increase risk of schizophrenia. *Nature* 455:237–241.

Kennedy MJ, Ehlers MD (2006). Organelles and trafficking machinery for postsynaptic plasticity. *Annu Rev Neurosci* 29:325–362.

Kurita M, Holloway T, Garcia-Bea A, Kozlenkov A, Friedman AK, Moreno JL, *et al.* (2012). HDAC2 regulates atypical antipsychotic responses through the modulation of mGlu2 promoter activity. *Nat Neurosci* 15:1245–1254.

Kuteeva E, Hokfelt T, Wardi T, Ogren SO (2010). Galanin, galanin receptor subtypes and depression-like behaviour. *EXS* 102:163–181.

Liou YJ, Lai IC, Wang YC, Bai YM, Lin CC, Lin CY, *et al.* (2006). Genetic analysis of the human ENTH (Epsin 4) gene and schizophrenia. *Schizophr Res* 84:236–243.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402–408.

Marley A, von Zastrow M (2010). Dysbindin promotes the post-endocytic sorting of G protein-coupled receptors to lysosomes. *PLoS One* 5:e9325.

McGuffin P, Farmer AE, Gottesman II, Murray RM, Reveley AM (1984). Twin concordance for operationally defined schizophrenia. Confirmation of familiality and heritability. *Arch Gen Psychiatry*. 41:pp. 541–545.

Meltzer HY, Massey BW (2011). The role of serotonin receptors in the action of atypical antipsychotic drugs. *Curr Opin Pharmacol* 11:59–67.

Mills IG, Praefcke GJ, Vallis Y, Peter BJ, Olesen LE, Gallop JL, *et al.* (2003). EpsinR: an AP1/clathrin interacting protein involved in vesicle trafficking. *J Cell Biol* 160:213–222.

Moreno JL, Holloway T, Umali A, Rayannavar V, Sealfon SC, Gonzalez-Maeso J (2012). Persistent effects of chronic clozapine on the cellular and behavioral responses to LSD in mice. *Psychopharmacology (Berl)* 225:217–226.

Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63.

Mundell SJ, Luo J, Benovic JL, Conley PB, Poole AW (2006). Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. *Traffic* 7:1420–1431.

Murray CJL, Lopez AD (1996). *The global burden of disease: a comprehensive assessment of mortality and disability from diseases, injuries, and risk factors in 1990 and projected to 2020*. Cambridge, Massachusetts: Harvard University Press, Harvard School of Public Health.

Need AC, Ge D, Weale ME, Maia J, Feng S, Heinzen EL, *et al.* (2009). A genome-wide investigation of SNPs and CNVs in schizophrenia. *PLoS Genet* 5:e1000373.

Pahlman S, Ruusala AI, Abrahamsson L, Mattsson ME, Esscher T (1984). Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. *Cell Differ* 14:135–144.

Pimm J, McQuillin A, Thirumalai S, Lawrence J, Quested D, Bass N, *et al.* (2005). The Epsin 4 gene on chromosome 5q, which encodes the clathrin-associated protein enthoprotin, is involved in the genetic susceptibility to schizophrenia. *Am J Hum Genet* 76:902–907.

Purcell SM, Wray NR, Stone JL, Visscher PM, O'Donovan MC, Sullivan PF, Sklar P (2009). Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature* 460:748–752.

Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, *et al.* (2010). Beta-arrestin- but not G protein-mediated signaling by the 'decoy' receptor CXCR7. *Proc Natl Acad Sci USA* 107:628–632.

Rizig MA, McQuillin A, Ng A, Robinson M, Harrison A, Zvelebil M, *et al.* (2012). A gene expression and systems pathway analysis of the effects of clozapine compared to haloperidol in the mouse brain implicates susceptibility genes for schizophrenia. *J Psychopharmacol* 26:1218–1230.

Robinson MS (2004). Adaptable adaptors for coated vesicles. *Trends Cell Biol* 14:167–174.

Ryder PV, Faundez V (2009). Schizophrenia: the 'BLOC' may be in the endosomes. *Sci Signal* 2:pe66.

Salim K, Fenton T, Bacha J, Urien-Rodriguez H, Bonnert T, Skynner HA, *et al.* (2002). Oligomerization of G-protein-coupled receptors shown by selective co-immunoprecipitation. *J Biol Chem* 277:15482–15485.

Salin P, Mercugliano M, Chesselet MF (1990). Differential effects of chronic treatment with haloperidol and clozapine on the level of preprosomatostatin mRNA in the striatum, nucleus accumbens, and frontal cortex of the rat. *Cell Mol Neurobiol* 10:127–144.

Sanders AR, Duan J, Levinson DF, Shi J, He D, Hou C, *et al.* (2008). No significant association of 14 candidate genes with schizophrenia in a large European ancestry sample: implications for psychiatric genetics. *Am J Psychiatry* 165:497–506.

Serretti A, De Ronchi D, Lorenzi C, Berardi D (2004). New antipsychotics and schizophrenia: a review on efficacy and side effects. *Curr Med Chem* 11:343–358.

Takakusa H, Masumoto H, Mitsuru A, Okazaki O, Sudo K (2008). Markers of electrophilic stress caused by chemically reactive metabolites in human hepatocytes. *Drug Metab Dispos* 36:816–823.

Tang RQ, Zhao XZ, Shi YY, Tang W, Gu NF, Feng GY, *et al.* (2006). Family-based association study of Epsin 4 and Schizophrenia. *Mol Psychiatry* 11:395–399.

Traub LM (2009). Clathrin couture: fashioning distinctive membrane coats at the cell surface. *PLoS Biol* 7:e1000192.

Von Zastrow M, Sorkin A (2007). Signaling on the endocytic pathway. *Curr Opin Cell Biol* 19:436–445.

Willins DL, Alsayegh L, Berry SA, Backstrom JR, Sanders-Bush E, Friedman L, *et al.* (1998). Serotonergic antagonist effects on trafficking of serotonin 5-HT2A receptors *in vitro* and *in vivo*. *Ann NY Acad Sci* 861:121–127.

Willins DL, Berry SA, Alsayegh L, Backstrom JR, Sanders-Bush E, Friedman L, Roth BL (1999). Clozapine and other 5-hydroxytryptamine-2A receptor antagonists alter the subcellular distribution of 5-hydroxytryptamine-2A receptors *in vitro* and *in vivo*. *Neuroscience* 91:599–606.

Wolfe BL, Trejo J (2007). Clathrin-dependent mechanisms of G protein-coupled receptor endocytosis. *Traffic* 8:462–470.

Yadav PN, Abbas AI, Farrell MS, Setola V, Sciaky N, Huang XP, *et al.* (2011). The presynaptic component of the serotonergic system is required for clozapine's efficacy. *Neuropsychopharmacology* 36:638–651.