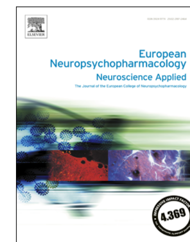
European Neuropsychopharmacology (2017) 27, 383–398

www.elsevier.com/locate/euroneuro



Clozapine as the most efficacious antipsychotic for activating ERK 1/2 kinases: Role of 5-HT_{2A} receptor agonism

Stefano Aringhieri[a,1], Shivakumar Kolachalam[a,1], Claudio Gerace[a], Marco Carli[a], Valeria Verdesca[a], Maria Giulia Brunacci[a], Chiara Rossi[b], Chiara Ippolito[b], Anna Solini[b], Giovanni U. Corsini[a], Marco Scarselli[a,*]

[a]*Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Via Roma 55, Pisa 56126, Italy*
[b]*Department of Clinical and Experimental Medicine, University of Pisa, Italy*

Received 2 August 2016; received in revised form 1 February 2017; accepted 21 February 2017

KEYWORDS
Antipsychotics;
Clozapine;
ERK1/2;
AKT;
5-HT_{2A};
Biased agonism

Abstract
Antipsychotics (APDs) are divided into first-generation antipsychotics (FGAs) and second-generation antipsychotics (SGAs) based on the concept that SGAs have reduced motor side effects. With this premise, this study examined in HeLa and other cell lines the effects of different APDs on the activation of ERK1/2 (Extracellular signal-regulated kinases) and AKT (Protein Kinase B) kinases, which may be affected in schizophrenia and bipolar disorder. Among the SGAs, Clozapine clearly resulted as the most effective drug inducing ERK1/2 phosphorylation with potency in the low micromolar range. Quetiapine and Olanzapine showed a maximal response of about 50% compared to Clozapine, while FGAs such as Haloperidol and Sulpiride did not have any relevant effect. Among FGAs, Chlorpromazine was able to partially activate ERK1/2 at 30% compared to Clozapine. Referring to AKT activation, Clozapine, Quetiapine and Olanzapine demonstrated a similar efficacy, while FGAs, besides Chlorpromazine, were incapable to obtain any particular biological response. In relation to ERK1/2 activation, we found that 5-HT_{2A} serotonin receptor antagonists Ketanserin and M100907, both partially reduced Clozapine effect. In addition, we also observed an increase of potency of Clozapine effect in HeLa transfected cells with recombinant 5-HT_{2A} receptor and in rat glioma C6 cells that express a higher amount of this receptor. This indicates that ERK1/2 stimulation induced by Clozapine could, to some extent, be

*Corresponding author. Fax: +39 050 2218717.
E-mail address: marco.scarselli@med.unipi.it (M. Scarselli).
[1]Equally contributed.

http://dx.doi.org/10.1016/j.euroneuro.2017.02.005
0924-977X/© 2017 Elsevier B.V. and ECNP. All rights reserved.

mediated by $5\text{-}HT_{2A}$ receptor, through a novel mechanism that is called "biased agonism", even though other cellular targets are involved. This evidence may be relevant to explain the superiority of Clozapine among the APDs.
© 2017 Elsevier B.V. and ECNP. All rights reserved.

1. Introduction

Antipsychotics (APDs) are widely prescribed drugs for schizophrenia and bipolar disorder, brain diseases that are characterized by psychotics features (Miyamoto et al., 2012). Generally, these drugs are divided into first-generation antipsychotics (FGAs), called typical antipsychotics, and second-generation antipsychotics (SGAs), called atypical, based on the concept that SGAs have reduced side effects such as Parkinsonism and tardive dyskinesia (Meltzer, 2013). Moreover, SGAs seem to have additional therapeutic properties such as cognitive enhancement, improvement of negative symptoms, and most importantly, prevention of progressive clinical deterioration. Recent findings suggest that SGAs might possibly slow down the loss of gray matter in schizophrenic patients at early stages as a further demonstration of their unique pharmacological profile and curative benefits (Lieberman et al., 2005; Van Haren et al., 2007).

In fact, in animal models some SGAs have shown to induce neurogenesis, synaptogenesis and increase in neurotrophins (Lieberman et al., 2008). However, to find a direct relationship between pharmacodynamics characteristics and therapeutic properties for APDs, particularly among SGAs, is complicated as these drugs have very complex receptor profiles. Many hypotheses have been formulated on this topic (Miyamoto et al., 2005). In general, the mechanism of action of typical antipsychotics is to block dopamine D_2 receptor, while for atypical other explanations have been proposed besides D_2 receptor. Indeed, for atypical antipsychotics, many works have pointed out the importance of other G protein-coupled receptors (GPCRs) such as $5\text{-}HT_{2A}$ and $5\text{-}HT_{1A}$ serotonin receptors and also muscarinic, adrenergic, glutamatergic and histamine receptors (Meltzer and Massey, 2011). Furthermore, although acute events, such as psychoses, are probably controlled by short-term effects of APDs mostly mediated by their receptor affinities, it is evident that these drugs have more complex effects, particularly in the long-term time scale, involving intracellular mechanisms that may regulate neuronal functionality and plasticity (Molteni et al., 2009; Fumagalli et al., 2009).

Among these additional intracellular mechanisms responsible for the APDs pharmacological action, kinases such as ERK1/2 (extracellular signal-regulated kinases) and AKT (protein kinase B) have received particular attention (Molteni et al., 2009; Freyberg et al., 2010). ERK1/2 pathway represents a fundamental crossroad of multiple signaling cascades involved in regulating many cellular activities induced by different receptors, either receptor tyrosine kinases (RTKs) or GPCRs (Rubinfeld and Seger, 2005). Cussac et al. (2002) showed the activation of ERK in CHO cells after treatment with Clozapine. Same results were described in the dorsal striatum of rat after the infusion of Haloperidol, while Clozapine reduced the activation of ERK1/2 (Pozzi et al., 2003). Regarding the Central Nervous System (CNS), ERK1/2 activity is relevant for synaptogenesis, neurogenesis, connectivity and neural plasticity, processes that are implicated in schizophrenia (Samuels et al., 2008, 2009). Similar to ERK1/2, AKT is one of the survival kinases with multiple biological functions in the brain and the whole body. For the CNS, AKT is important in neurodevelopment, synaptic plasticity, protein synthesis and neurotransmission (Shioda et al., 2009; Arguello and Gogos, 2008). Interestingly, AKT activation induces the phosphorylation of other kinases, such as GSK-3β, that are relevant in psychiatric disorders (Beaulieu et al., 2009). GSK-3β has recently been proposed as a contributing factor in the etiology of schizophrenia and bipolar disorder (Emamian, 2012). Lu et al. showed for the first time a role for these kinases, activated by some SGA, in neuronal survival and neurite outgrowth (Lu et al., 2004, Lu and Dwyer, 2005).

If the debate on differences between FGAs and SGAs is still an open topic, there is another ongoing discussion within this debate that tries to understand the superiority of Clozapine among the SGAs (Meltzer, 2012). Clozapine is, in fact, the most efficacious drug in treating positive and negative symptoms in schizophrenia, particularly in patients resistant to other antipsychotics, and it is considered as the "gold standard" for treating schizophrenia (Leucht et al., 2013). Unfortunately, its benefits are outweighed by relevant side effects such as the risk of severe hematological effects and metabolic syndrome (Capannolo et al., 2015). What makes Clozapine so unique compared to all other APDs is still unclear, and is probably related to its multifactorial properties on receptors and other targets. Recently, a new concept regarding GPCR function called "biased agonism" has gained attention wherein a drug can behave either as agonist or as antagonist on the same receptor depending on the specific pathway that is taken into consideration (Kenakin, 2011; Reiter et al., 2012). For a fact, Clozapine is an antagonist on $5\text{-}HT_{2A}$ serotonin receptor in relation to the G protein activation but it is an agonist on the same receptor if AKT activation is measured, as previously demonstrated by Schmid et al. (2014). With these premises, our study has compared in different cell lines, in particular in HeLa cells, the activity of FGAs and SGAs on ERK1/2 and AKT kinases. In addition, to understand their mechanism of action, we also investigated the role of $5\text{-}HT_{2A}$ serotonin receptor and other GPCRs. Our experiments have demonstrated that Clozapine has a unique profile, particularly in relation to ERK1/2 activation.

2. Experimental procedures

2.1. Reagents: chemicals and antibodies

Clozapine, Quetiapine, Olanzapine, Risperidone, Aripiprazole, Chlorpromazine, Haloperidol, Sulpiride, Serotonin, α-methyl-serotonin, PMA (phorbol 12-myristate 13-acetate), hEGF, FR180204, PD184352, GSK690693, GSK2334470, Ketanserin, M100907, Marimastat,

Tyrphostin AG 1478, Scopolamine, ICI-118/551, WAY-100/135 and MK-571, all were purchased from Sigma-Aldrich.

The primary antibodies used for Western Blotting anti-ERK-P (mouse; M9692), anti-ERK-Total (rabbit; M5670), anti-AKT-P (rabbit, P3862) and anti-AKT-Total (mouse, WH00002073M3), all were purchased from Sigma-Aldrich.

The secondary antibodies used for Western Blotting HRP conjugated A4416 (goat anti-mouse) and HRP conjugated SAB3700852 (goat anti-rabbit) were purchased from Sigma-Aldrich.

For Fluorescence Confocal-Microscopy, the primary antibody Monoclonal Anti-HA H9658 (mouse) was purchased from Sigma-Aldrich, while the secondary antibody Alexa Fluor® 488 (goat anti-mouse) was purchased from Invitrogen.

2.2. Cell cultures, transfection and DNA constructs

HeLa, C6 and SH-SY5Y cells were bought from ATCC (LGC Standards, France) and were grown in Sigma high-glucose (4500 mg/L) DMEM supplemented with 10% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 units/ml penicillin at 37 °C with 5% CO_2. The 3HA-tagged receptor constructs (in the plasmid vector pcDNA 3.1) for 5-HT_{2A} and D_2 receptors were purchased from www.cDNA.org. For the transfection with 3HA-5-HT_{2A} and 3HA-D_2 constructs, HeLa cells, which had reached around 80% confluence, were trypsinized and centrifuged in the whole medium. Cell viability was done using 0.4% Trypan Blue Solution (ThermoFisher Scientific) with Neubauer improved cell counting chamber (Sigma-Aldrich). Transfection was carried out using Neon Transfection System (Invitrogen) according to manufacturer's instructions. For each transfection, 1×10^6 viable cells were transfected with 1–2 μg of 3HA-5-HT_{2A} or 3HA-D_2 DNA constructs. After transfection, cells were plated in 12-well plates. For fluorescence microscopy, cells were plated with glass coverslips placed at the bottom of the 12-well plates. The day after, western blotting and fluorescence microscopy experiments were performed.

2.3. Western blotting, and detection of pERK(1,2)/ERK (1,2) and pAKT/AKT

Cells, at 70–80% confluence, were trypsinized, centrifuged in the whole medium. Cell viability was done using 0.4% Trypan Blue Solution (ThermoFisher Scientific) with Neubauer improved cell counting chamber (Sigma-Aldrich) and subsequently, 1.2×10^4 cells/well were plated in 12-well plates. The day after, cells were serum starved overnight before treating them with drug(s). For APDs Clozapine, Quetiapine, Olanzapine, Risperidone, Aripiprazole, Chlorpromazine, Haloperidol and Sulpiride and also ERK1/2 and AKT activators, PMA and hEGF respectively, the incubation time was 10 min. For the ATP-competitive ERK1/2 kinase inhibitor FR180204, the selective non-competitive MEK inhibitor PD184352, the ATP-competitive AKT kinase inhibitor GSK690693 and the highly specific and potent PDK1 inhibitor GSK2334470, the incubation time was 1, 1, 4 and 2 h respectively. For various antagonists Ketanserin, M100907, Scopolamine, ICI-118/551 and WAY-100/135 and broad-spectrum matrix metalloprotease (MMP) inhibitor Marimastat and ABCC multidrug resistance protein-1 (MRP1) inhibitor MK-571, the incubation time was 30 min. While for the selective inhibitor of EGFR Tyrphostin AG 1478 the incubation time was 60 min. The inhibitors and antagonists were all pre-incubated for 30 min before treating them with Clozapine for 10 min. A time-course of Clozapine-induced ERK1/2 phosphorylation at 10 μM concentration was carried out at different time intervals (1, 5, 10 and 30 min).

After incubating at 37 °C with 5% CO_2, cells were lysed in 130 μL lysis buffer containing Tris-HCl pH 8.0, NaCl, EDTA, Triton, SDS and protease inhibitor cocktail. The lysates were centrifuged to obtain proteins. Protein concentration was determined using Bradford assay (Sigma-Aldrich). The samples were denatured with Laemmli buffer by heating them at 95–100 °C for 5 min.

Proteins were separated on 8–10% polyacrylamide gel, loaded with a 25 μg of each sample, and run at room temperature for 1 h at 200 V. Proteins were transferred from gel to a PVDF membrane at 4 °C for 1h at 100 V. The membrane was blocked with 5% fat-free milk diluted in T-TBS, incubated for 1 h at room temperature.

To determine the phosphorylation activity on ERK1/2 and AKT kinases, the membrane was incubated overnight at 4 °C with mouse anti-ERK-P M9692 (1:2000) and rabbit anti-AKT-P P3862 (1:1000) antibodies, respectively. Bound primary antibodies were detected with anti-mouse secondary antibody HRP conjugated A4416 (1:20,000) for ERK-P, and anti-rabbit secondary antibody HRP conjugated SAB3700852 (1:2000) for AKT-P, both incubated at room temperature for 1 h. Membrane was washed multiple times between primary and secondary antibody incubation and post secondary antibody incubation. Finally, the membrane was incubated with Luminata Forte Western HRP Substrate (Millipore) inside a dark compartment for 3 min at room temperature before developing them on a Kodak Image Station 440 CF. The incubations were all carried out in an agitator and the antibody dilutions were all prepared in T-TBS with 5% Bovine Serum Albumin (Sigma-Aldrich).

The same membranes were stripped in an acidic solution (pH 2.0) and were re-blocked with 5% fat-free milk diluted as explained above. The above mentioned procedure was repeated for anti-ERK-Total and anti-AKT-Total with, rabbit anti-ERK-Total M5670 (1:40,000) and mouse anti-AKT-Total WH0000207M3 (1:800) primary antibodies and anti-rabbit HRP conjugated SAB3700852 (1:2000) and anti-mouse HRP conjugated A4416 (1:20,000) secondary antibodies, respectively, and the membranes were re-developed on a Kodak Image Station 440 CF machine.

ERK1/2 and AKT protein quantification was carried out using Kodak 1D image analysis software.

2.4. Analysis of gene expression

One microgram of total RNA, extracted with the miRCURY RNA Isolation kit (Exiqon, Woburn MA, USA), was reverse-transcribed using High-Capacity cDNA Reverse transcription kit (Applied Biosystems, Foster City, CA, USA) in a MJ mini-thermocycler (Biorad, Hercules, USA). Real-time PCR was performed in triplicate on an Eco-Real Time instrument (Illumina, San Diego, USA) following a standard protocol (40 cycles of denaturation at 95 °C for 15 s followed by annealing and extension at 60 °C for 1 min) with specific TaqMan Gene Expression Assays. The assays were: 5-HT_{2A} (Hs01033524_m1), GAPDH (Hs02758991_g1) for human cells, 5-HT_{2A} (Rn00568473_m1), GAPDH (Rn01775763_g1) for rat cells. The quantification of gene expression was calculated using ΔΔCT method, where CT is the threshold cycle. The amount of the target gene was normalized to GAPDH.

2.5. Immunofluorescence and receptor internalization

For immunofluorescence staining, cells were transfected and plated on glass coverslips. 24 h after transfection, cells were incubated at 4 °C for 1 h with IgG1 mouse monoclonal anti-HA H9658 primary antibody (1:1000), diluted in PBS/10%-FBS solution to label the receptor on the plasma membrane.

According to previous procedures (Scarselli and Donaldson, 2009; Scarselli et al., 2013; Deschout et al., 2014), for 3HA-5-HT_{2A} receptor internalization, cells were incubated for 30 min at 37 °C with Clozapine at 10 μM concentration or with endogenous neuromodulator Serotonin (5-HT) at 10 μM concentration. As a negative control for internalization, D_2 receptor was used with Clozapine at 10 μM concentration. After washing with PBS/10%-FBS, cells were incubated for 30 s in acidic solution (0.5% acetic acid, 0.5 M NaCl, pH 3.0 diluted in H_20) at room temperature to remove proteins present on the plasma membrane. Then, after multiple washes with PBS/10%-FBS solution, cells were fixed in 2% formaldehyde for

20 min at room temperature. Finally, cells were washed in PBS and incubated for 1 h at room temperature, away from light source, with IgG1 goat anti-mouse Alexa Fluor® 488 secondary antibody (1:500), diluted in PBS/10%-FBS solution with 0.2% Saponin (Sigma-Aldrich) to label the receptor in the internal cellular compartments. The 3HA-5-HT_{2A} receptor was also visualized in its basal conditions with and without acidic solution treatment using the same procedure.

The coverslips were carefully fixed with a drop of anti-fading agent on a microscope slide.

All images were obtained using Leica TCS SP8 confocal laserscanning inverted microscope (Leica Microsystems, Mannheim, Germany) by means of 63x oil lens with a zoom factor of 1 and at 2048 × 2048 resolution. Cells were scanned through z-axis sectioning at 250–500 nm intervals and only those planes which showed 5-HT_{2A} receptor localization were chosen as representative confocal images.

2.6. Statistical analysis

All histograms, the ratio pERK(1,2)/ERK(1,2) and pAKT/AKT (with respect to their basal activity), E_{max} ± SEM and Student's t-test calculations were done using Microsoft Excel 2013, while plots of drug concentration/response curves and their EC_{50} ± SEM values were determined using GraphPad Prism 6.1.

3. Results

3.1. APDs effect on ERK1/2 activation in HeLa cells

To study the effects of APDs (SGAs and FGAs) on ERK1/2 activation, we decided to use HeLa cells as a cellular recipient. HeLa cells are, generally, transfected with recombinant receptors, however they express a variety of functional endogenous GPCRs. We began our preliminary experiments by comparing two well-known SGAs, Clozapine and Quetiapine, with two prototypical FGAs, Haloperidol and Sulpiride, at high concentrations (Figure 1A). Then, we included in our analysis other SGAs (Olanzapine, Risperidone and Aripiprazole) and Chlorpromazine (FGA). SGAs such as Clozapine, Quetiapine and Olanzapine stimulated ERK1/2 phosphorylation in the low micromolar range, while Risperidone and Aripiprazole had a limited effect on ERK1/2 activation, up to 10 μM concentration (Figure 2). Among the FGAs, Sulpiride and Haloperidol did not induce any relevant biological response up to a concentration of 10 μM, while Chlorpromazine at submicromolar concentrations partially stimulated ERK1/2 (Figure 2). For FGAs, we decided not to exceed 10 μM because this concentration is already much higher than their putative plasma concentrations (Lostia et al., 2009; Hiemke et al., 2011).

Importantly, the efficacy (E_{max}) among the SGAs was clearly different, where Clozapine has demonstrated to be superior to all the other drugs that have been tested, including the endogenous neuromodulator 5-HT (Figure 2). Clozapine-induced ERK1/2 phosphorylation was about 4 times over the basal activity, while the strong activator PMA increased ERK1/2 activity by 6.5 times. For each treatment, the ratio pERK(1,2)/ERK(1,2) was determined with respect to the pERK(1,2)/ERK(1,2) of the basal activity. Indeed, the E_{max} of Quetiapine and Olanzapine was about half compared to Clozapine (Figure 2). To determine the E_{max} of SGAs, we examined the concentration–response curves up to a concentration of 50 or 75 μM because the plateau was already reached at these concentrations. In addition, in some cases, these compounds, at the concentration of 100 μM, reduced cell viability, and subsequently ERK1/2 phosphorylation was decreased at this high concentration (data not shown). Regarding the potency of the different SGAs for ERK1/2 phosphorylation, we found that the EC_{50} values for Clozapine, Quetiapine and Olanzapine were 26, 28 and 30 μM, respectively (Figure 2 and Table 1). For Clozapine, it is worth noting that at 5 μM concentration, it clearly began to activate ERK1/2 (Figure 2). These concentrations are close to the ones that can be reached in patients during drug treatment, where Clozapine plasma level should be around 1–2 μM (Grundmann et al., 2014). The EC_{50} value for Chlorpromazine was found to be around 1 μM, which is also close to its assumed plasma concentration (Hiemke et al., 2011), while its E_{max} was about 1/3rd compared to Clozapine (Figure 2). All the above experiments on ERK1/2 phosphorylation were carried out for an incubation time of 10 min according to previous data (Novi et al., 2004), and they are summarized in Table 1. However, for Clozapine, the time-course on ERK1/2 phosphorylation was determined and it was confirmed that the incubation time of 10 min was the one with the strongest stimulation (Figure 3). Other receptor agonists, such as Carbachol, Isoproterenol and the endogenous neuromodulator 5-HT were also tested in HeLa cells for ERK1/2 activation, where Isoproterenol was

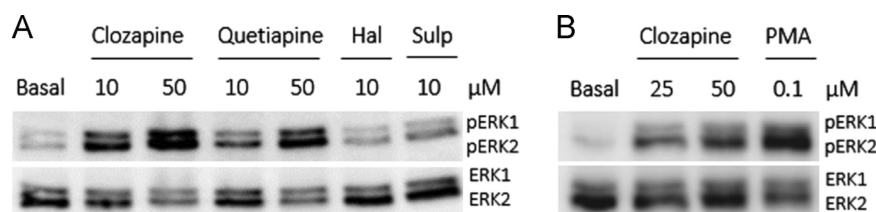


Figure 1 [A] ERK1/2 phosphorylation induced by prototypical SGAs and FGAs in HeLa cells. Representative blot comparing two prototypical SGAs (Clozapine, Quetiapine) with two prototypical FGAs (Haloperidol, Hal; Sulpiride, Sulp) at high concentrations for inducing ERK1/2 phosphorylation. Total ERK1/2 was also determined for each treatment. "Basal" represents the endogenous level of ERK1/2 phosphorylation. This experiment was repeated three times. [B] ERK1/2 phosphorylation induced by Clozapine at two different concentrations compared to the activity of PMA (phorbol 12-myristate 13-acetate). This blot is representative of experiments repeated three times.

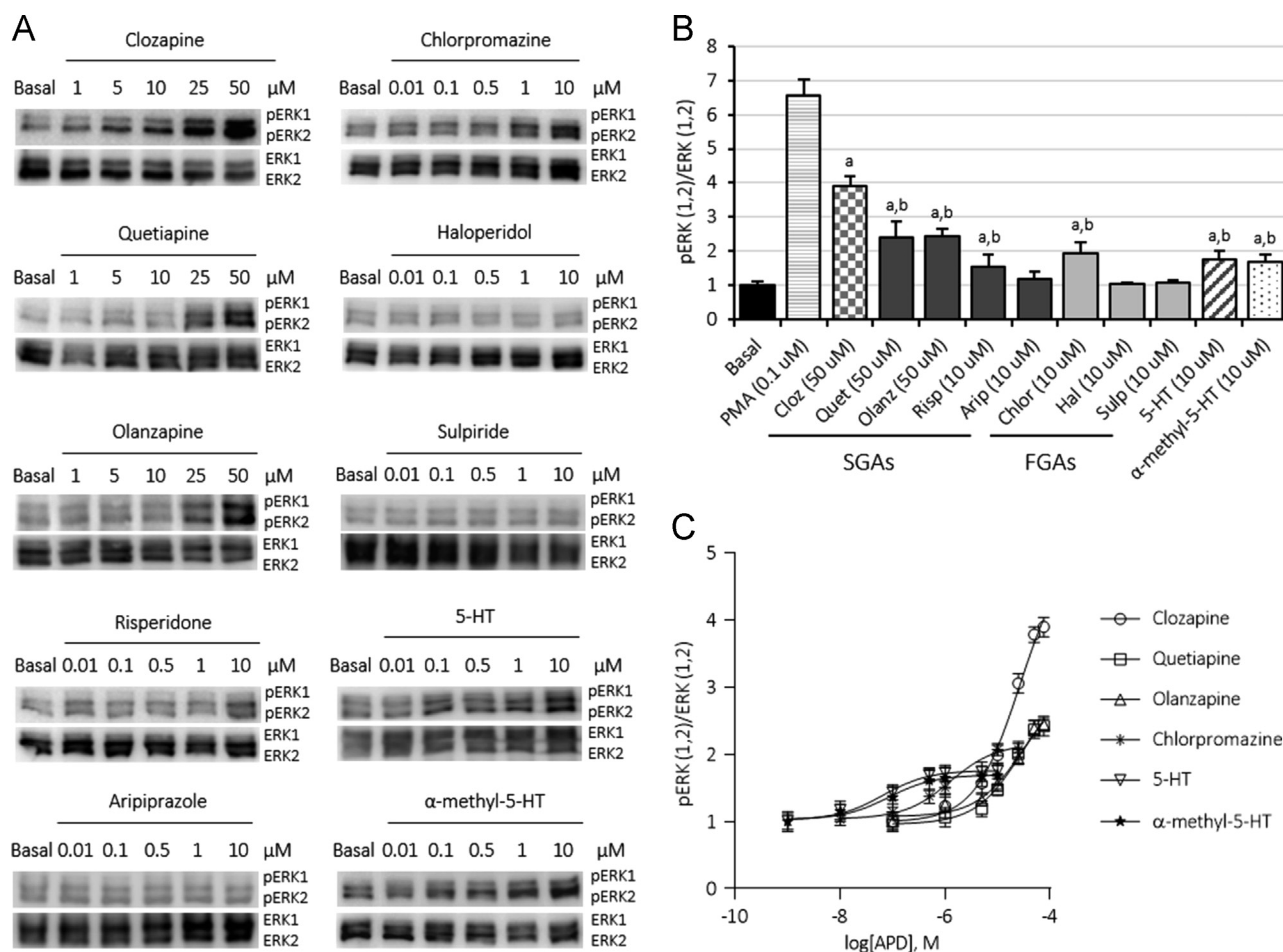


Figure 2 [A] Concentration-response course of ERK1/2 phosphorylation induced by SGAs, FGAs, 5-HT and α-methyl-5-HT in HeLa cells. Representative blots of concentration-dependent increase of ERK1/2 phosphorylation comparing efficacious SGAs (Clozapine, Quetiapine and Olanzapine) with the only active FGA (Chlorpromazine). Other compounds such as Risperidone, Aripiprazole, Haloperidol and Sulpiride showed either a limited or null response up to 10 μM concentration. The endogenous neuromodulator 5-HT and 5-HT_2 selective agonist α-methyl-5-HT were also included. Blots are representative of experiments that were repeated for at least three times. [B] Maximal effect of SGAs and FGAs for inducing ERK1/2 phosphorylation in HeLa cells. Maximal effect data (E_{max}) are expressed as fold over basal activity. For each treatment, the ratio pERK(1,2)/ERK(1,2) was determined with respect to the pERK (1,2)/ERK(1,2) of the basal activity. The maximal effect for Clozapine, Quetiapine and Olanzapine were all determined at 50 μM concentration (at plateau), while for Chlorpromazine it was calculated at 10 μM (at plateau). For all the other compounds, we found either a limited or null response up to 10 μM concentration; hence we used 10 μM to measure the maximal effect. The endogenous neuromodulator 5-HT and 5-HT_2 selective agonist α-methyl-5-HT were also tested at 10 μM concentration. The Student's t-test significance was $p<0.05$; a=compared to basal, b=compared to Clozapine at 50 μM concentration (E_{max}). [C] Concentration-response curves of ERK1/2 phosphorylation induced by SGAs and FGAs in HeLa cells. Concentration-response curves were determined for the active SGAs (Clozapine, Quetiapine and Olanzapine), the only active FGA (Chlorpromazine), 5-HT and for α-methyl-5-HT. Data are expressed as fold over basal activity. The EC_{50} and E_{max} values expressed as mean±SEM can be seen in Table 1.

found to be most effective with an E_{max} similar to Clozapine (data not shown).

3.2. Mechanism of action of Clozapine-induced ERK1/2 activation

Based on the above results, we decided to investigate the mechanism of action of ERK1/2 activation induced by APDs. Since Clozapine was clearly the most effective drug, we used it as our prototypical APD for subsequent experiments. In relation to Clozapine-induced ERK phosphorylation, we used a direct ERK kinase inhibitor (FR180204) and an upstream inhibitor (PD184352) to see if they were able to prevent Clozapine effect. Both compounds completely blocked the Clozapine effect. In addition, when used alone, they also reduced ERK1/2 phosphorylation basal activity as expected (Figure 4). Taking into consideration that HeLa cells express endogenously a different variety of GPCRs, we used different antagonists in order to evaluate their capability to block or reduce Clozapine pharmacological activity. In general, cell cultures may express some endogenous receptors at low level that still have functional activities (Friedman et al., 2002). Considering that Clozapine can be

an agonist or a partial agonist at muscarinic and serotonin (5-HT) receptors, we evaluated this possibility. In addition, we also utilized other compounds to verify their activity against Clozapine. The muscarinic receptor antagonist Scopolamine (10 μM), the β-adrenergic receptor antagonist ICI-118/551 (10 μM) and the $5\text{-}HT_{1A}$ receptor antagonist WAY-100,135 (10 μM) did not have any effect on Clozapine-induced ERK1/2 phosphorylation, thereby confirming that these receptors were not responsible for Clozapine effect (Figure 5A–C). On the contrary, Ketanserin (10 μM) and the $5\text{-}HT_{2A}$ receptor selective antagonist M100907 (10 μM) partially reduced the Clozapine effect by 38% and 42%, respectively. Ketanserin is a $5\text{-}HT_{2A}$ receptor antagonist, but it is also active on other GPCRs such as H_1 and α_1-adrenergic receptor (Sathi et al., 2008). Conversely, M100907 is a selective antagonist on $5\text{-}HT_{2A}$ receptor subtype. These data demonstrate, at least in part, the relevance of this receptor subtype in Clozapine biological activity (Figure 6). To corroborate the results regarding the relevance of $5\text{-}HT_{2A}$ receptor, we also used α-methyl-serotonin, a $5\text{-}HT_2$ selective agonist that has higher affinity for $5\text{-}HT_2$ receptors compared with $5\text{-}HT_1$ receptors (Wang et al., 2016). The ERK-induced phosphorylation by α-methyl-serotonin was similar to the endogenous neuromodulator 5-HT that conversely is a mixed $5\text{-}HT_2/5\text{-}HT_1$ agonist (Figure 2). This confirms the importance of $5\text{-}HT_2$ receptors in the activity of these two compounds. To further confirm the relevance of $5\text{-}HT_{2A}$ receptors in HeLa cells, we determined mRNA expression by using real time PCR and results indicated that HeLa cells express low but detectable amount of $5\text{-}HT_{2A}$ mRNA (Figure 7). Interestingly, in HeLa cells, the endogenous neuromodulator 5-HT, at a concentration of 10 μM, moderately increased ERK1/2 phosphorylation with an E_{max} of around 1/3rd compared to Clozapine, indirectly confirming the presence of functional 5-HT receptors in HeLa cells (Figure 2). Regarding the role of $5\text{-}HT_{2A}$ receptor in relation to Clozapine effect, we extended our examination to other cell lines by measuring ERK1/2 phosphorylation and also $5\text{-}HT_{2A}$ receptor mRNA expression at the same time. The real time PCR technique indicated that, in comparison with HeLa cells, SH-SY5Y cells did not express $5\text{-}HT_{2A}$ mRNA, while this messenger is present at a very high level in C6 cells (Figure 7). In fact, Clozapine effect on ERK1/2 activation was absent in SH-SY5Y cells up to 10 μM concentration, while it was partially increased in C6 cells in terms of potency with an EC_{50} value of 6 μM (Figure 8). Furthermore, the E_{max} value of Clozapine in C6 cells was about 25% less compared to HeLa cells. After analyzing the role of different GPCRs in Clozapine action, we also evaluated other potential targets. Considering recent data regarding Clozapine induced ERK1/2 phosphorylation through EGF receptor transactivation (Pereira et al., 2009), we used the selective EGFR inhibitor Tyrphostin AG 1478 and the MMP inhibitor Marimastat, which however did not have any effect on Clozapine activity (Figure 5D–E). Finally, we also evaluated whether Clozapine might have

Table 1 EC_{50} and E_{max} of APDs-induced ERK1/2 phosphorylation in HeLa cells are expressed as mean ± SEM. Maximal effect data (E_{max}) of SGAs and FGAs are expressed as fold over basal activity. For each treatment, the ratio pERK(1,2)/ERK(1,2) was determined with respect to the pERK(1,2)/ERK(1,2) of the basal activity. Risperidone and Aripiprazole had a limited effect on ERK1/2 activation up to 10 μM concentration, so the EC_{50} was not determined. Sulpiride and Haloperidol did not induce any relevant biological response up to a concentration of 10 μM. The E_{max} and EC_{50} of the endogenous neuromodulator 5-HT were also determined.

	EC_{50} (μM)	E_{max}
Clozapine	26.96 ± 7.90	3.90 ± 0.31
Quetiapine	28.06 ± 6.60	2.42 ± 0.47
Olanzapine	30.84 ± 7.70	2.45 ± 0.19
Risperidone	-	1.55 ± 0.35
Aripiprazole	-	1.20 ± 0.19
Chlorpromazine	1.26 ± 0.21	1.96 ± 0.31
Haloperidol	-	1.06 ± 0.04
Sulpiride	-	1.09 ± 0.08
5-HT	0.66 ± 0.18	1.75 ± 0.27
α-Methyl-5-HT	0.85 ± 0.17	1.69 ± 0.22

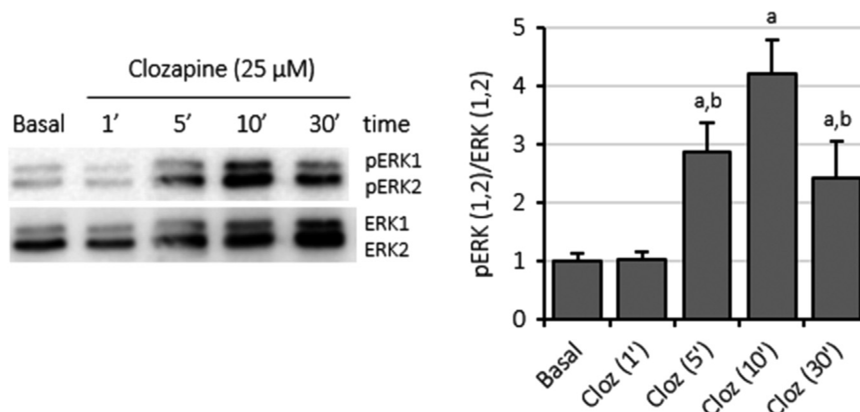


Figure 3 Time-course of Clozapine-induced ERK1/2 phosphorylation in HeLa cells. ERK1/2 phosphorylation was determined in the presence of Clozapine 25 μM at different time intervals (1, 5, 10 and 30 min). The maximal effect was found at 10 min, therefore this time interval has been used as reference. Data are expressed as fold over basal activity. This experiment was repeated three times. The Student's t-test significance was $p < 0.05$; a = compared to basal, b = compared to Clozapine at 25 μM concentration (10′).

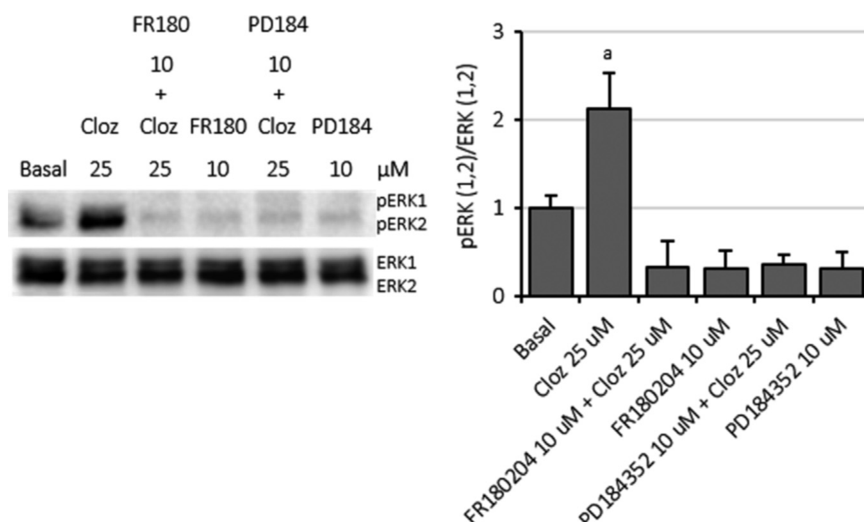


Figure 4 Effect of ERK inhibitors on Clozapine-induced ERK1/2 phosphorylation in HeLa cells. The ATP-competitive ERK1/2 kinase inhibitor FR180204 (10 μM) and the MEK inhibitor PD184352 (10 μM) blocked the Clozapine (25 μM) induced ERK1/2 phosphorylation. When used alone, both the inhibitors reduced the ERK1/2 phosphorylation basal activity. Data are expressed as fold over basal activity. This experiment was repeated three times. The Student's t-test significance was $p<0.05$; a = compared to basal.

changed the concentrations of second messengers by blocking efflux transporters of the ATP-binding cassette (ABC) family, thereby inducing ERK1/2 phosphorylation. The cAMP efflux through these transporters has been described, which may alter GPCR signaling (Cheepala et al., 2013). To evaluate this mechanism, we used the ABCC multidrug resistance protein 1(MRP1) inhibitor MK-571, and we did not find any change in terms of ERK1/2 phosphorylation in the presence of this compound (data not shown). This suggests that Clozapine is not acting through this mechanism of action.

3.3. Clozapine effect on HeLa cells transfected with recombinant 5-HT$_{2A}$ receptor: ERK1/2 activation and 5-HT$_{2A}$ receptor internalization

In order to investigate the potential role of 5-HT$_{2A}$ receptor in the mechanism of action of Clozapine, we used an alternative approach by expressing the recombinant receptor with transfection in HeLa cells. However, the strategy of over-expressing the receptor does not demonstrate the role of endogenous receptors responsible for Clozapine-induced ERK activation. In fact, over-expressed receptors could recruit signaling pathways that are not necessarily biologically relevant in vivo. In HeLa cells transfected with 5-HT$_{2A}$ receptor, we found an increase in potency by 5 times when compared to non transfected cells, with an EC$_{50}$ of 5 μM (Figure 8). Subsequently, to confirm the agonistic properties of Clozapine at the 5-HT$_{2A}$ receptors, we decided to study receptor internalization by using fluorescence microscopy in the presence of the drug. To visualize 5-HT$_{2A}$ receptor internalization, the receptor was tagged with an HA epitope and then analyzed with primary and secondary antibodies, as previously shown (Scarselli and Donaldson, 2009). After incubating for 30 min with Clozapine at 10 μM concentration and then removing receptors from the cell surface by acidic treatment, 3HA-5-HT$_{2A}$ receptors were clearly internalized and localized in endosomal compartments (Figure 9C). In contrast, 5-HT$_{2A}$ receptor was poorly localized in internal cellular structures in its basal conditions even after removing receptors from the plasma membrane with the same acidic treatment (Figure 9A). Similar to Clozapine, the presence of endogenous neuromodulator 5-HT also induced 5-HT$_{2A}$ receptor endocytosis (Figure 9D). The dopamine D$_2$ receptor was used as a negative control, whose localization on the plasma membrane was unaffected by the presence of Clozapine (data not shown).

3.4. APDs effect on AKT activation in HeLa cells

After having demonstrated that Clozapine was the most effective drug in ERK1/2 activation, we experimented APDs activity (SGAs and FGAs) on AKT, another important kinase that plays a predominant role, like ERK, in many neural processes like synaptogenesis and neurogenesis. Considering that HeLa cells were particularly sensitive to APDs action in relation to ERK1/2 activation, we decided to use the same cell line. Regarding AKT phosphorylation, again, SGAs such as Clozapine, Quetiapine and Olanzapine were active in the low micromolar range (Figure 10). In this case, the maximal effect was quite similar among these drugs with Clozapine and Quetiapine being the most efficacious (Figure 10). Clozapine-induced AKT phosphorylation was about 2.8 times over the basal activity, while hEGF, used as a positive control, increased AKT activity by 5.5 times. For each treatment, the ratio pAKT/AKT was determined with respect to the pAKT/AKT of the basal activity. The E_{max} induced by SGAs was similar to the one obtained with endogenous neuromodulator 5-HT. Indeed, the E_{max} of endogenous neuromodulator 5-HT was similar to Clozapine for AKT activation, but it was 1/3rd when compared to Clozapine for ERK1/2 (Figures 2 and 10). In relation to the other SGAs, Risperidone increased AKT phosphorylation with an E_{max} half compared to Clozapine but it was active only at high concentration with an EC$_{50}$ value of 5 μM that is far from its supposed plasma concentration (Figure 10). Conversely, Aripiprazole had a limited effect on AKT activation and

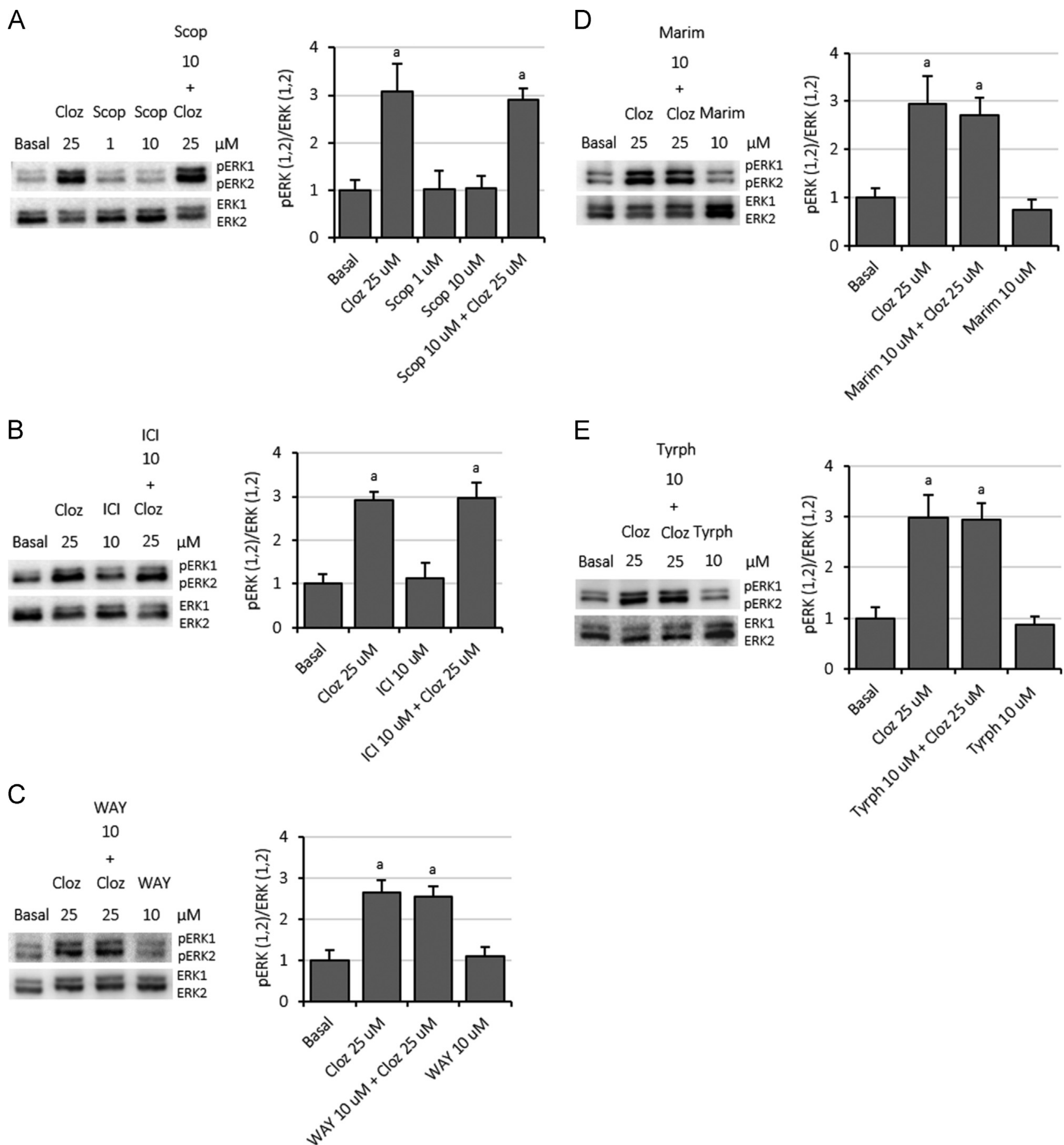


Figure 5 Effect of receptor antagonists on Clozapine-induced ERK1/2 phosphorylation in HeLa cells. [A-E] Different antagonists were evaluated for their capability to either block or reduce Clozapine-induced ERK1/2 phosphorylation. Clozapine was used at 25 μM, close to its EC_{50} value, while the antagonists were utilized at very high concentrations in relation to their receptor affinity. The muscarinic receptor antagonist Scopolamine (10 μM), the β-adrenergic receptor antagonist ICI-118/551 (10 μM) and the 5-HT_{1A} receptor antagonist WAY-100,135 (10 μM) did not have any effect on Clozapine induced ERK1/2 phosphorylation. Each of these compounds were also tested singularly as a control to verify if they were changing ERK1/2 phosphorylation. We also evaluated the effect of the broad-spectrum matrix metalloprotease (MMP) inhibitor Marimastat (10 μM) and the selective inhibitor of EGFR Tyrphostin AG 1478 (10 μM); even these did not have any effect on Clozapine activity. Data are expressed as fold over basal activity. These experiments were repeated for at least three times. The Student's *t*-test significance was $p<0.05$; a = compared to basal.

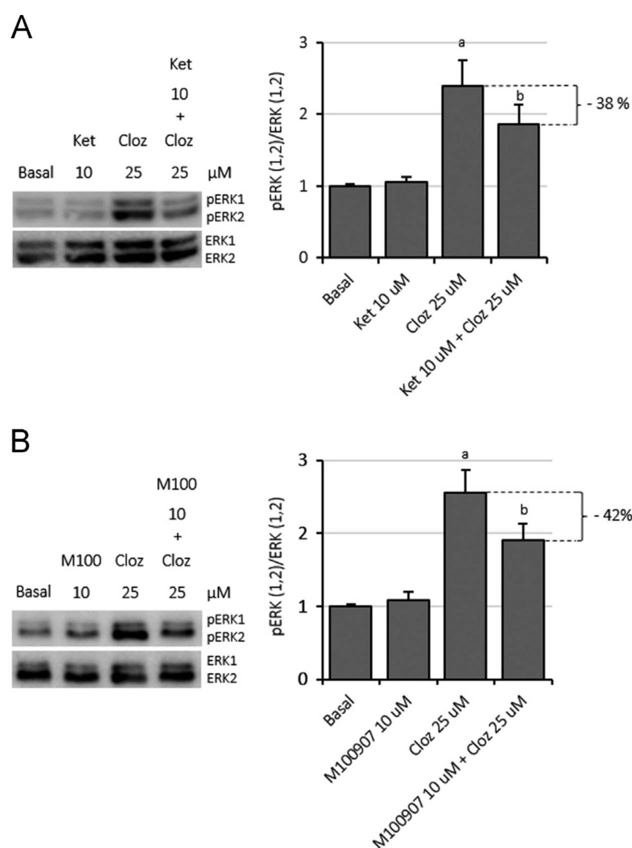


Figure 6 Effect of 5-HT_{2A} receptor antagonists Ketanserin and M100907 on Clozapine-induced ERK1/2 phosphorylation. [A] Representative blot of Ketanserin (10 μM) on Clozapine-induced ERK1/2 phosphorylation (25 μM). Ketanserin reduced the Clozapine activity by 38%. [B] Representative blot of the highly selective 5-HT_{2A} receptor antagonist M100907 (10 μM) on Clozapine-induced ERK1/2 phosphorylation (25 μM). M100907 reduced the Clozapine activity by 42%. Data are expressed as fold over basal activity. These experiments were carried out for at least three times. The Student's *t*-test significance was $p<0.05$; a=compared to basal, b=compared to Clozapine at 25 μM concentration.

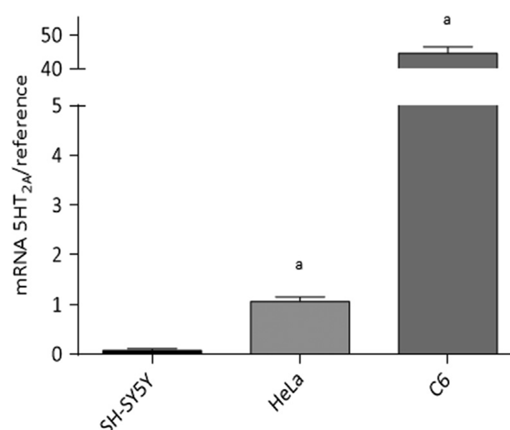


Figure 7 Constitutive mRNA expression of 5-HT_{2A} receptor measured by real time PCR in HeLa, SH-SY5Y and C6 cells. mRNA 5-HT_{2A}/mRNA reference (GAPDH) in HeLa cells was normalized to 1. Consequently, data in C6 cells were expressed with respect to HeLa cells. Data were expressed as mean ± SEM. Student's *t*-test significance was $p<0.01$; a=compared to SH-SY5Y.

exclusively at 10 μM concentration (Figure 10). The EC_{50} values determined for Clozapine, Quetiapine and Olanzapine were 9, 11 and 27 μM, respectively, indicating that the first two drugs were more potent than the last one (Table 2). Regarding the FGAs, Sulpiride and Haloperidol also had a limited effect of less than 20% compared to Clozapine, but only at a very high concentration of 10 μM (Figure 10). The FGAs clearly showed lower E_{max} compared to SGAs. However, the typical antipsychotic Chlorpromazine was able to induce AKT phosphorylation with an E_{max} 2/3rd compared to Clozapine and the EC_{50} was 0.8 μM. Chlorpromazine behaved differently among FGAs for both AKT and ERK1/2 activation. All the above experiments on AKT phosphorylation were carried out for an incubation time of 10 min and they are summarized in Table 2. Subsequently, we decided to investigate the mechanism of action of AKT activation induced by APDs and we used Clozapine as prototypical APD for these experiments as we did for ERK1/2. Regarding the mechanism of Clozapine-induced AKT phosphorylation, we used a direct AKT kinase inhibitor (GSK690693) and an upstream inhibitor (GSK2334470). Both compounds were able to block the Clozapine effect (Figure 11A), and when used alone, they reduced the basal activity as well. The presence of a small amount of AKT phosphorylation with the AKT kinase inhibitor (GSK690693) should not be correlated to AKT autophosphorylation on the T308 site, which is specifically recognized by the antibody used in our experiments, but it probably involves other kinases that may regulate AKT basal activity (Rhodes et al., 2008; Guo et al., 2016). However, we cannot completely exclude the hypothesis that AKT autophosphorylation might still occur in our HeLa cellular system. Related to GSK690693 mechanism of action, it directly inhibits AKT, but it can also target other kinases (e.g. PKA, PKC, AMPK, IKBKE) that may contribute to its mechanism. Moreover, some papers have also reported an increased basal AKT phosphorylation in the presence of this inhibitor (Rhodes et al., 2008). In addition, to inquire about Clozapine mechanism of action, we also utilized different receptor antagonists. The muscarinic receptor antagonist Scopolamine, the β-adrenergic receptor antagonist ICI-118/551 and the 5-HT_{1A} receptor antagonist WAY-100/135, none of them had any effect on Clozapine-induced AKT phosphorylation, thereby confirming that these receptors were not responsible for Clozapine activity (Figure 11B).

4. Discussion

The distinction among APDs (SGAs and FGAs) is based on the concept that SGAs have a better pharmacological profile with reduced motor side effects and additional long-term therapeutic properties (e.g. cognitive functions) that might prevent progressive clinical deterioration. However, this distinction has been re-considered by different authors (Keefe et al., 2004; Grunder et al., 2009) taking into consideration the complexity of the clinical characteristics of most of the APDs due to their broad receptor profiles and post-receptor intracellular mechanisms, which might contribute to their specific therapeutic activity. Among these post-receptor down-stream intracellular mechanisms

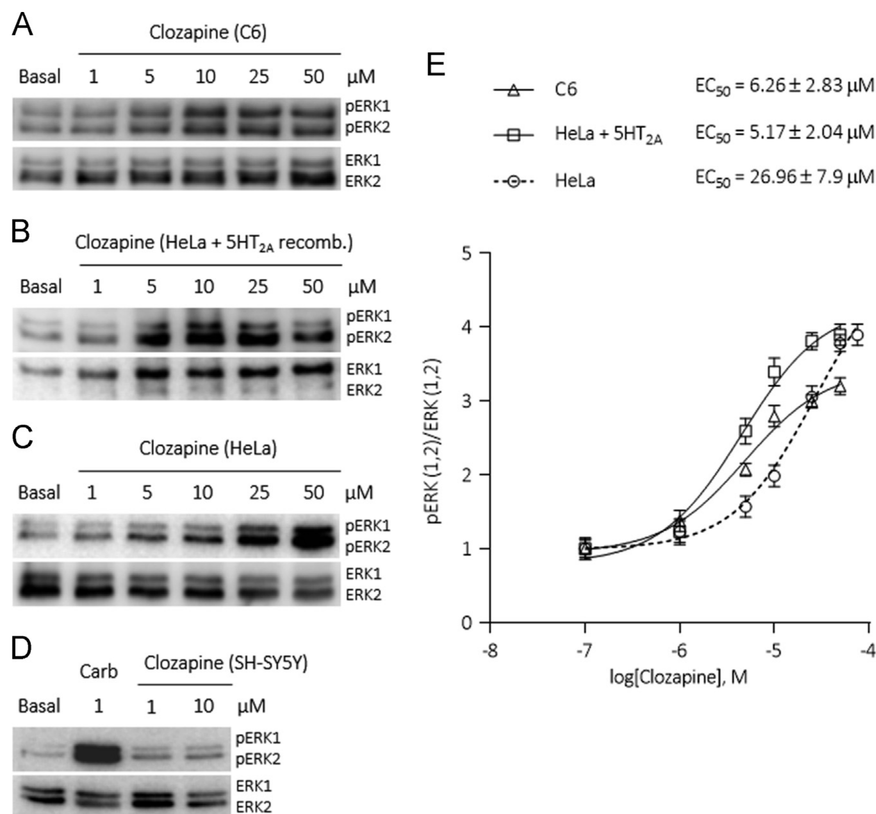


Figure 8 Concentration-response curves of Clozapine-induced ERK1/2 phosphorylation in HeLa cells transfected with recombinant 5-HT_{2A} receptor and in C6 cells. [A–C] Representative blots of concentration-dependent increase of Clozapine-induced ERK1/2 phosphorylation (1–50 μM) in HeLa cells transfected with recombinant 5-HT_{2A} receptor and in C6 cells compared with not transfected HeLa cells. [D] Clozapine activity in SH-SY5Y cells was null up to 10 μM concentration; Carbachol (10 μM) was used as positive control. [E] In the concentration-response curves, the EC_{50} values were also determined (mean ± SEM) and data are expressed as fold over basal activity. The E_{max} was found to be similar among the three curves. These experiments were carried out for at least three times.

responsible for the APDs action, kinases such as ERK1/2 and AKT have received particular attention for their relevance in many neural processes that might be implicated in schizophrenia and in other psychoses (Kyosseva, 2004; Zheng et al., 2012). For this reason, using HeLa cells as cellular model, we carried out a systematic comparison between the most frequently used APDs in relation to ERK1/2 and AKT activation. HeLa cells have a variety of endogenous receptors and can be easily transfected with recombinant receptors. Among the SGAs, Clozapine, Quetiapine and Olanzapine were active in the low micromolar range for phosphorylating ERK1/2, while Risperidone and Aripiprazole were practically unable to do the same. Furthermore, FGAs such as Haloperidol and Sulpiride were mostly inactive in relation to ERK1/2 phosphorylation. Strikingly, Clozapine was the most effective drug while other SGAs produced a response of about 50% when compared to Clozapine.

Other groups had conducted similar experiments in primary cortical neurons and in rat prefrontal cortex, where they found that Clozapine, Olanzapine and Quetiapine increased ERK1/2 phosphorylation with a different time course (Pereira et al., 2009, 2012). All these data together suggest a specific activity of SGAs, or at least some of them may contribute to explain their capability to improve cognitive functions and to delay the progression of the disease and clinical deterioration. In addition, our data in relation to the higher efficacy of Clozapine among SGAs in ERK1/2 activation may explain, in part, the therapeutic superiority of this drug on many aspects of schizophrenia and psychoses treatment. Actually, activation of ERK1/2 pathway that is involved in transcriptional regulation might contribute in improving synaptic plasticity, connectivity and neurogenesis that are impaired in schizophrenic patients. ERK1/2 activation might also be associated in enhancing the expression of neurotrophic factors, such as BDNF, GDNF and FGF2 (Park et al., 2009; Maragnoli et al., 2004). Indeed, in cellular systems and in animal models of schizophrenia, some SGAs (e.g. Clozapine and Olanzapine) have induced neurotrophic factors production (Riva et al., 1999). Furthermore, an important clinical evidence on the use of Clozapine and Olanzapine in patients has resulted in a better progression of the disease in relation to gray matter reduction (Van Haren et al., 2007).

Concerning Clozapine superiority among SGAs, different mechanisms have been proposed. Recently, Pereira et al. (2009) demonstrated in vivo that ERK1/2 phosphorylation induced by Clozapine involves EGFR transactivation, while other APDs do not. However, in our cellular systems this was not confirmed. In addition, very recently, it has also been

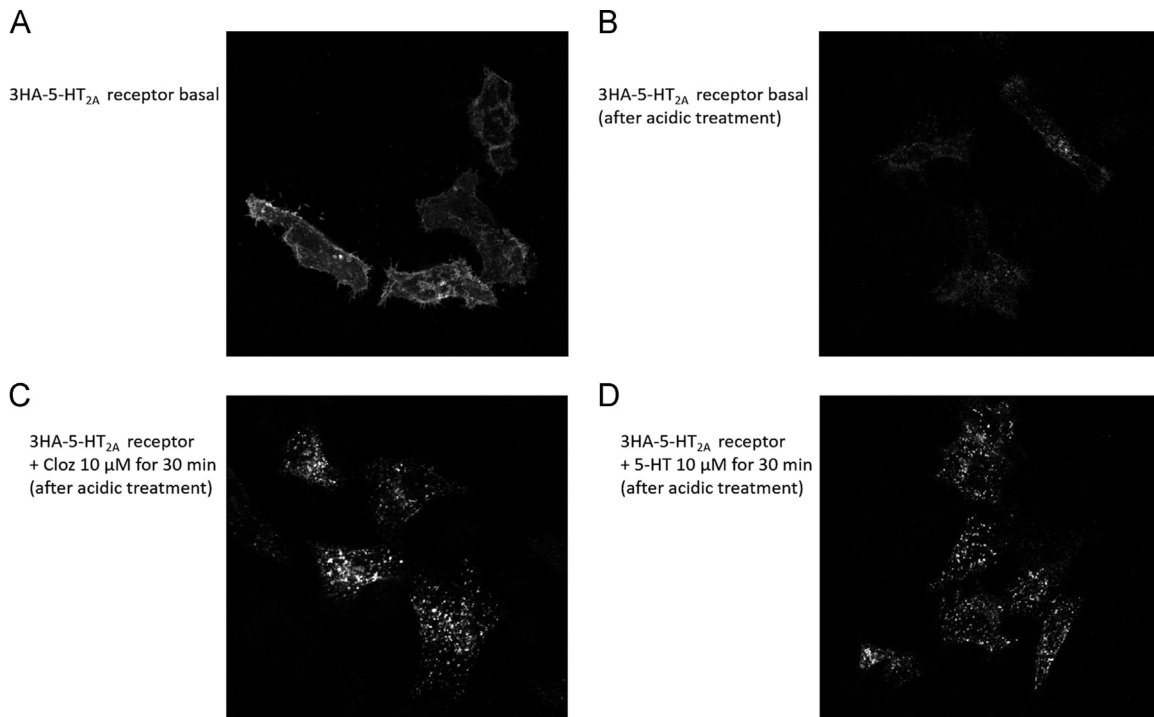


Figure 9 HA-tagged 5-HT_{2A} receptor (3HA-5-HT_{2A}) internalization induced by Clozapine in HeLa cells. [A] In basal conditions, 3HA-5-HT_{2A} receptors were mostly expressed on the cell surface. [B] In basal conditions, after removing receptors from the cell surface by acidic treatment, HA-5-HT_{2A} receptors were poorly expressed in endosomal compartments. [C] After incubating for 30 min with Clozapine at 10 μM concentration and then removing receptors from the cell surface by acidic treatment, HA-5-HT_{2A} receptors were clearly internalized and localized in endosomal compartments. [D] After incubating for 30 min with 5-HT at 10 μM concentration and then removing receptors from the cell surface by acidic treatment, HA-5-HT_{2A} receptors were clearly internalized and localized in endosomal compartments. HA-5-HT_{2A} internalization was induced by Clozapine and 5-HT to a similar extent. These images are representative of experiments, which was repeated three times.

suggested that Clozapine can behave as an agonist at 5-HT_{2A} receptors (Schmid et al., 2014). Indeed, our study indicates that ERK1/2 activation is mediated, at least in part, by 5-HT_{2A} agonism. In fact, in HeLa cells, a 5-HT_{2A} antagonist partially reduced Clozapine effect, while 5-HT_{2A} receptor transfection lead to an increase of Clozapine potency in relation to ERK1/2 activation. Besides, Clozapine potency was higher in C6 cells than in HeLa cells, where the former cell line clearly had a higher expression of 5-HT_{2A} receptors. All these data together point to the importance of 5-HT_{2A} agonism for Clozapine induced ERK1/2 activation, even though in HeLa cells other mechanisms may be involved. However, our experiments indicate that these mechanisms do not engage muscarinic, adrenergic or 5-HT_{1A} receptors. We also confirmed the agonistic properties of Clozapine using fluorescent microscopy, where 5-HT_{2A} tagged receptors internalized extensively in HeLa cells in the presence of the drug. How is it possible that Clozapine, a known 5-HT_{2A} antagonist, can be an agonist at the same receptor? These mixed properties of the drug can be explained through "biased agonism", a relatively recent concept introduced in pharmacology (Kenakin, 2011; Reiter et al., 2012). In fact, Clozapine might act as a biased agonist at 5-HT_{2A} receptor activating a specific intracellular pathway independent from G proteins but targeting effectors such as β-arrestin or others (Figure 12). A similar mechanism was recently proposed to explain how Clozapine induces AKT phosphorylation in vitro and in vivo through 5-HT_{2A} receptor (Schmid et al., 2014).

However, in relation to the mechanism of action responsible for Clozapine activity, we noticed that 5-HT_{2A} receptor antagonists were able to reduce Clozapine effect by 40% suggesting the involvement of other cellular targets, and this aspect requires further examination. Interestingly, some authors have proposed that Clozapine could promote AKT phosphorylation by acting at heterodimeric complexes such as 5-HT_{2AR}/$mGlu_{2R}$ (Fribourg et al., 2011). Whether heterodimeric complexes are involved in our experiments or not is yet to be addressed. In addition, our study seems to indicate that Clozapine is phosphorylating ERK1/2 and AKT through upstream kinases. However, we cannot exclude that in other systems Clozapine might be able to act by blocking dephosphorylation of ERK1/2 and AKT.

Importantly, Clozapine was more efficacious (E_{max}) than the endogenous neuromodulator 5-HT. This difference can be explained by the fact that 60% of Clozapine activity on ERK is not related to serotonin receptors, as 5-HT_{2A} antagonist reduced the Clozapine activity by 40%, but to other targets where 5-HT is not active. In addition, Clozapine, as a 5-HT_{2A} receptor biased agonist, may be more effective than 5-HT in activities that are independent of G proteins. In relation to AKT activation, we found that only some SGAs, such as Clozapine, Quetiapine and Olanzapine were active, while the FGAs were practically inactive on

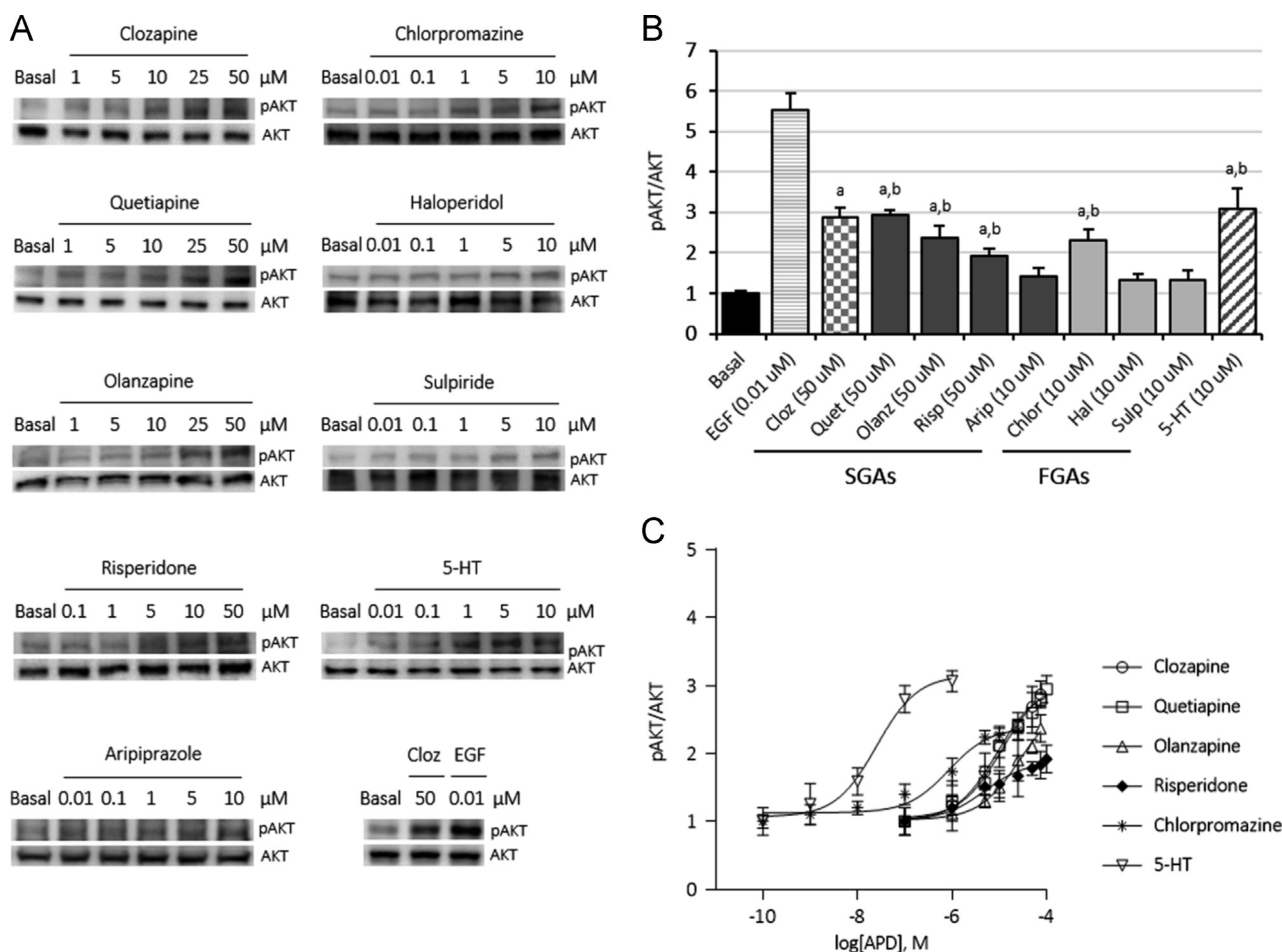


Figure 10 Concentration–response curves of AKT phosphorylation induced by prototypical SGAs and FGAs in HeLa cells. [A] Representative blots of concentration-dependent increase of AKT phosphorylation comparing efficacious SGAs (Clozapine, Quetiapine, Olanzapine and Risperidone) with the only active FGA (Chlorpromazine). Other compounds such as Aripiprazole, Haloperidol and Sulpiride showed a limited response up to 10 μM concentration (See Table 2). A representative blot of endogenous neuromodulator 5-HT is also included. The AKT phosphorylation induced by Clozapine at 50 μM concentration was compared to the activity of hEGF. Total AKT was also determined for each treatment. "Basal" represents the endogenous level of AKT phosphorylation. Blots are representative of experiments that were repeated for at least three times. [B] Maximal effect of SGAs and FGAs for inducing AKT phosphorylation. Maximal effect data (E_{max}) are expressed as fold over basal activity. For each treatment, the ratio pAKT/AKT was determined with respect to the pAKT/AKT of the basal activity. The Maximal effect for Clozapine, Quetiapine, Olanzapine and Risperidone all were determined at 50 μM concentration (at plateau), while for Chlorpromazine it was calculated at 10 μM (at plateau). For all other compounds, we found a limited response up to 10 μM concentration; hence we used 10 μM to measure the maximal effect. The endogenous neuromodulator 5-HT was also tested at 10 μM concentration. The Student's *t*-test significance was $p<0.05$; a=compared to basal, b=compared to Clozapine at 50 μM concentration (E_{max}). [C] Concentration–response curves of AKT phosphorylation induced by SGAs and FGAs in HeLa cells. Concentration–response curves were determined for the active SGAs (Clozapine, Quetiapine, Olanzapine and Risperidone), the only active FGA (Chlorpromazine) and for 5-HT. Data are expressed as fold over basal activity. The EC_{50} and E_{max} values expressed as mean±SEM can be seen in Table 2.

this pathway. Again, SGAs and FGAs seem to have different properties on particular kinases activity and this might have clinical consequences. However, in this case, Clozapine is as efficacious as other SGAs. Similar to ERK protein, AKT is also involved in many processes in the CNS, and accumulating evidence has indicated the involvement of this kinase in the pathogenesis of schizophrenia. Moreover, AKT activation induces the inhibition of its downstream effector GSK-3β. GSK-3β has been recently proposed as a contributing factor in the etiology of psychiatric disorders (Mao et al., 2009), such as schizophrenia and bipolar disorder, and it is inhibited by different drugs such as Lithium and Valproic acid (Leng et al., 2008). Apart from our data on cell cultures, in vivo, the activation of AKT signaling pathway induced by SGAs or FGAs might be mediated by different mechanisms, also like blocking D_2 receptor as previously demonstrated (Masri et al., 2008; Freyberg et al., 2010).

Among the FGAs, Chlorpromazine behaved differently compared to the other FGAs as it was able to partially induce phosphorylation of ERK1/2 and AKT. In the past, it was reported that this typical antipsychotic has a peculiar receptor profile, such as high affinity for $5\text{-}HT_{2A}$

Table 2 EC_{50} and E_{max} of APDs-induced AKT phosphorylation in HeLa cells are expressed as mean±SEM. Maximal effect data (E_{max}) of SGAs and FGAs are expressed as fold over basal activity. For each treatment, the ratio pAKT/AKT was determined with respect to the pAKT/AKT of the basal activity. Aripiprazole, Sulpiride and Haloperidol had a limited effect on AKT activation up to 10 μM concentration, so the EC_{50} was not determined. The E_{max} and EC_{50} of the endogenous neuromodulator 5-HT were also determined.

	EC_{50} (μM)	E_{max}
Clozapine	9.05±2.70	2.87±0.23
Quetiapine	11.32±3.41	2.95±0.11
Olanzapine	27.62±7.90	2.37±0.32
Risperidone	4.43±1.22	1.92±0.17
Aripiprazole	-	1.43±0.20
Chlorpromazine	0.80±0.20	2.31±0.26
Haloperidol	-	1.32±0.17
Sulpiride	-	1.34±0.23
5-HT	0.029±0.008	3.10±0.49

receptors, besides being an antagonist at D_2 receptors (Trichard et al., 1998). Indeed, a recent meta-analysis on the efficacy and tolerability of 15 APDs concluded that Chlorpromazine has a better clinical profile compared to other FGAs, particularly regarding extrapyramidal side effects and prolactin increase (Leucht et al., 2013). Both, in vitro and clinical data point out that Chlorpromazine, though a typical antipsychotic, it has a peculiar profile. This characteristic, in part, can be explained by ERK1/2 and AKT activation.

A relevant aspect regarding the translational value of this study carried out in cell cultures is the concentration of various drugs used to obtain their biological effect. For example, it is worth noticing that Clozapine clearly began to activate ERK1/2 at the concentration of 5 μM and the effect was already strong at 10 μM. These values are close enough to its supposed plasma level concentration that can be around 1-2 μM (Grundmann et al., 2014). Similar considerations can be done for Chlorpromazine, where its plasma level concentrations should be between 0.1 and 1 μM while for Quetiapine and Olanzapine this correlation is less obvious (Hiemke et al., 2011; Grundmann et al., 2014; Mauri et al.,

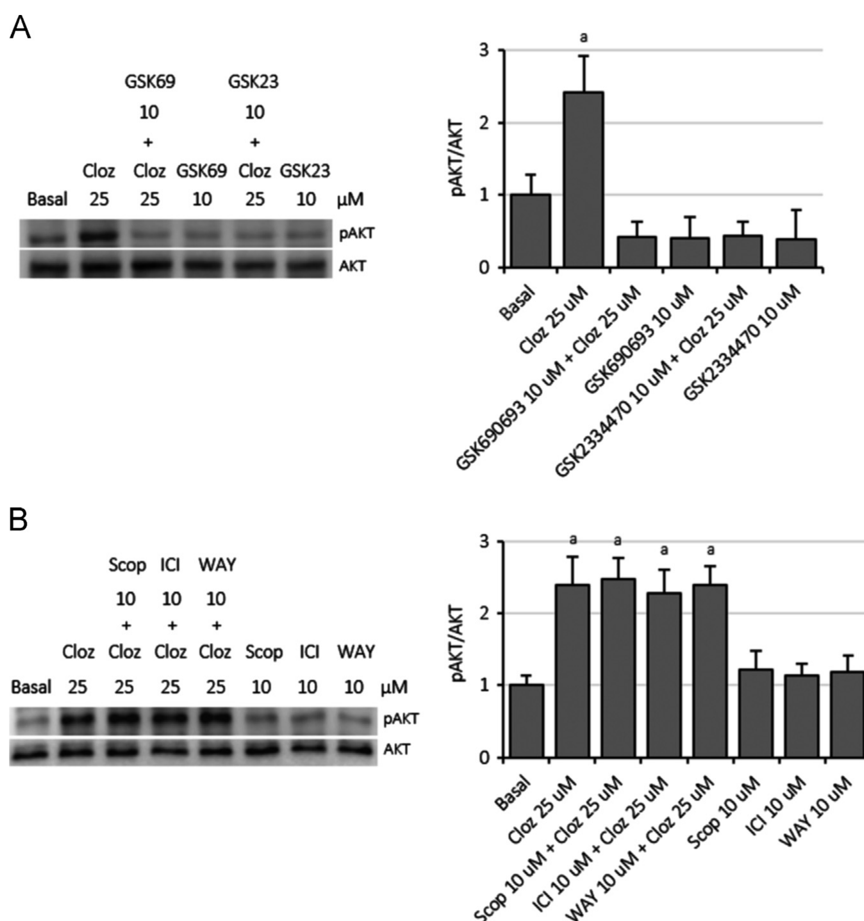


Figure 11 Effect of AKT inhibitors and receptor antagonists on Clozapine-induced AKT phosphorylation in HeLa cells. [A] The ATP-competitive AKT kinase inhibitor GSK690693 (10 μM) and the PDK1 inhibitor GSK2334470 (10 μM) blocked the Clozapine-induced AKT phosphorylation. Separately, both the inhibitors reduced the AKT phosphorylation basal activity. This experiment was repeated three times. The Student's t-test significance was $p<0.05$; a=compared to basal. [B] Different antagonists were evaluated for their capability to either block or reduce Clozapine-induced AKT phosphorylation. Clozapine was used at 25 μM, close to its EC_{50} value, while the antagonists were utilized at very high concentrations in relation to their receptor affinity. The muscarinic receptor antagonist Scopolamine (10 μM), the β-adrenergic receptor antagonist ICI-118/551 (10 μM) and the 5-HT_{1A} receptor antagonist WAY-100,135 (10 μM) did not have any effect on Clozapine induced AKT phosphorylation. Each of these compounds were also tested singularly as a control to verify if they were changing AKT phosphorylation. This experiment was repeated three times.

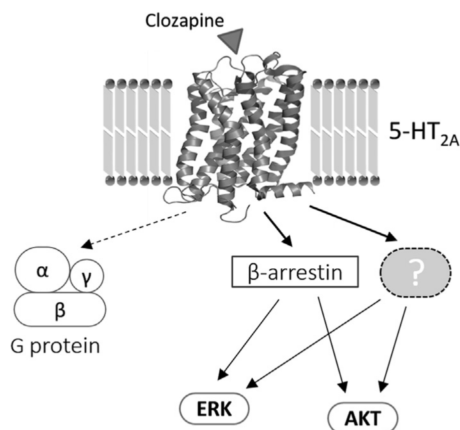


Figure 12 Clozapine biased-agonism at 5-HT_{2A} receptor. Clozapine is a biased agonist at 5-HT_{2A} receptor activating intracellular pathways independent from G proteins involving effectors such as β-arrestin or others. This mechanism is responsible for ERK1/2 and AKT activation.

2005). Importantly, the plasma level concentrations of these drugs might not correspond to their values in the CNS. However, considering their lipophilic properties and their volume of distribution, we should expect higher levels in the cerebrospinal fluid than in the blood (Hiemke et al., 2011).

In summary, our study demonstrated a relevant difference among SGAs and FGAs, particularly in relation to Clozapine superiority among the SGAs. From our data, we can propose three different categories of APDs in relation to ERK1/2 and AKT activation. In the first category, Clozapine is superior compared to all other drugs in activating ERK1/2 and it activates AKT in the low micromolar range. In the second category, Quetiapine, Olanzapine, and to a less extent, Chlorpromazine, all have a good activity regarding ERK1/2 and AKT activation in the low micromolar range. Finally, in the third category, Risperidone, Aripiprazole and other FGAs, all seem incapable to activate these kinases in a relevant manner, at least at the concentrations that could be reached during pharmacological therapy.

These differences may implicate a distinct clinical profile among APDs, particularly in the long-term treatment on preventing progressive clinical deterioration. Nevertheless, these data on cellular systems are still preliminary and, in order to improve their translational value, they would definitely benefit from in vivo validation in animal models.

In conclusion, Clozapine is the most powerful drug for activating ERK1/2 and part of this effect is due to the novel mechanism of 5-HT_{2A} receptor activation. This evidence might be relevant to explain, at least in part, the superiority of Clozapine among the APDs, as recently highlighted in a meta-analysis examining many APDs. This conclusion recommends researching for new drugs as efficacious as Clozapine with a similar complex mechanism of action, but devoid of its severe hematological side effects. As an alternative, future research should focus on reducing and/or eliminating Clozapine-induced agranulocytosis.

Role of funding source

This research was funded by Fondazione ARPA, a non-profit organization founded in 1992 (http://www.fondazionearpa.it) and by Progetti di Ricerca di Ateneo (PRA 2015).

The resources of Fondazione ARPA are aimed towards basic and scientific research, mainly for oncology, transplants and new medical and surgical techniques.

Author contributions

S. Aringhieri and S. Kolachalam carried out most of the experiments. C. Gerace, M. Carli, V. Verdesca, and M.G. Brunacci contributed to part of the experiments. C. Rossi and A. Solini, contributed to the experiments of RT-PCR, while C. Ippolito contributed to confocal microscopy. M. Scarselli conceived the general plan and wrote most of the manuscript. S. Aringhieri, S. Kolachalam and G.U. Corsini overviewed carefully the manuscript. All the authors contributed to writing the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgements

We wish to thank Prof. Giovanni Umberto Corsini for his invaluable critics, comments, suggestions and inputs which have immensely helped us during this research.

References

Arguello, P.A., Gogos, J.A., 2008. A signaling pathway AKTing up in schizophrenia. J. Clin. Invest. 118 (6), 2018–2021.

Beaulieu, J.M., Gainetdinov, R.R., Caron, M.G., 2009. Akt/GSK3 signaling in the action of psychotropic drugs. Annu. Rev. Pharmacol. Toxicol. 49, 327-347.

Capannolo, M., Fasciani, I., Romeo, S., Aloisi, G., Rossi, M., Bellio, P., Celenza, G., Cinque, B., Cifone, M.G., Scarselli, M., Maggio, R., 2015. The atypical antipsychotic clozapine selectively inhibits interleukin 8 (IL-8)-induced neutrophil chemotaxis. Eur. Neuropsychopharmacol. 25 (3), 413–424.

Cheepala, S., Hulot, J.S., Morgan, J.A., Sassi, Y., Zhang, W., Naren, A.P., Schuetz, J.D., 2013. Cyclic nucleotide compartmentalization: contributions of phosphodiesterases and ATP-binding cassette transporters. Annu. Rev. Pharmacol. Toxicol. 53, 231-253.

Cussac, D., Duqueyroix, D., Newman-Tancredi, A., Millan, M.J., 2002. Stimulation by antipsychotic agents of mitogen-activated protein kinase (MAPK) coupled to cloned, human (h)serotonin (5-HT)(1A) receptors. Psychopharmacology 162 (2), 168–177.

Deschout, H., Shivanandan, A., Annibale, P., Scarselli, M., Radenovic, A., 2014. Progress in quantitative single-molecule localization microscopy. Histochem. Cell. Biol. 142 (1), 5–17.

Emamian, E.S., 2012. AKT/GSK3 signaling pathway and schizophrenia. Front. Mol. Neurosci. 15, 5-33.

Freyberg, Z., Ferrando, S.J., Javitch, J.A., 2010. Roles of the Akt/GSK-3 and Wnt signaling pathways in schizophrenia and antipsychotic drug action. Am. J. Psychiatry 167 (4), 388–396.

Fribourg, M., Moreno, J.L., Holloway, T., Provasi, D., Baki, L., Mahajan, R., Park, G., Adney, S.K., Hatcher, C., Eltit, J.M., Ruta, J.D., Albizu, L., Li, Z., Umali, A., Shim, J., Fabiato, A., MacKerell Jr, A.D., Brezina, V., Sealfon, S.C., Filizola, M.,

González-Maeso, D.E., Logothetis, D.E., 2011. Decoding the signaling of a GPCR heteromeric complex reveals a unifying mechanism of action of antipsychotic drugs. Cell 147, 1011–1023.
Friedman, J., Babu, B., Clark, R.B., 2002. Beta(2)-adrenergic receptor lacking the cyclic AMP-dependent protein kinase consensus sites fully activates extracellular signal-regulated kinase 1/2 in human embryonic kidney 293 cells: lack of evidence for G (s)/G(i) switching. Mol. Pharmacol. 62 (5), 1094–1102.
Fumagalli, F., Frasca, A., Racagni, G., Riva, M.A., 2009. Antipsychotic drugs modulate Arc expression in the rat brain. Eur. Neuropsychopharmacol. 19 (2), 109–115.
Grunder, G., Hippius, H., Carlsson, A., 2009. The 'atypicality' of antipsychotics: a concept re-examined and re-defined. Nat. Rev. Drug. Discov. 8 (3), 197–202.
Guo, J.P., Coppola, D., Cheng, J.Q., 2016. IKBKE protein activates Akt independent of phosphatidylinositol 3-kinase/PDK1/mTORC2 and the pleckstrin homology domain to sustain malignant transformation. J. Biol. Chem. 291 (43), 22853.
Grundmann, M., Kacirova, I., Urinovska, R., 2014. Therapeutic drug monitoring of atypical antipsychotic drugs. Acta Pharm. 64 (4), 387–401.
Hiemke, C., Baumann, P., Bergemann, N., Conca, A., Dietmaier, O., Egberts, K., Fric, M., Gerlach, M., Greiner, C., Gründer, G., Haen, E., Havemann-Reinecke, U., Jaquenoud Sirot, E., Kirchherr, H., Laux, G., Lutz, U.C., Messer, T., Müller, M.J., Pfuhlmann, B., Rambeck, B., Riederer, P., Schoppek, B., Stingl, J., Uhr, M., Ulrich, S., Waschgler, R., Zernig, G., 2011. AGNP consensus guidelines for therapeutic drug monitoring in psychiatry: update 2011. Pharmacopsychiatry 44 (6), 195–235.
Keefe, R.S., Seidman, L.J., Christensen, B.K., Hamer, R.M., Sharma, T., Sitskoorn, M.M., Lewine, R.R., Yurgelun-Todd, D.A., Gur, R.C., Tohen, M., Tollefson, G.D., Sanger, T.M., Lieberman, J.A., 2004. Comparative effect of atypical and conventional antipsychotic drugs on neurocognition in first-episode psychosis: a randomized, double-blind trial of olanzapine versus low doses of haloperidol. Am. J. Psychiatry 161 (6), 985–995.
Kenakin, T.P., 2011. Biased signalling and allosteric machines: new vistas and challenges for drug discovery. Br. J. Pharmacol. 165 (6), 1659–1669.
Kyosseva, S.V., 2004. Mitogen-activated protein kinase signaling. Int. Rev. Neurobiol. 59, 201–220.
Leng, Y., Liang, M.H., Ren, M., Marinova, Z., Leeds, P., Chuang, D. M., 2008. Synergistic neuroprotective effects of lithium and valproic acid or other histone deacetylase inhibitors in neurons: roles of glycogen synthase kinase-3 inhibition. J. Neurosci. 28 (10), 2576–2588.
Leucht, S., Cipriani, A., Spineli, L., Mavridis, D., Orey, D., Richter, F., Samara, M., Barbui, C., Engel, R.R., Geddes, J.R., Kissling, W., Stapf, M.P., Lässig, B., Salanti, G., Davis, J.M., 2013. Comparative efficacy and tolerability of 15 antipsychotic drugs in schizophrenia: a multiple-treatments meta-analysis. Lancet 382, 951–962.
Lieberman, J.A., Tollefson, G.D., Charles, C., Zipursky, R., Sharma, T., Kahn, R.S., Keefe, R.S., Green, A.I., Gur, R.E., McEvoy, J., Perkins, D., Hamer, R.M., Gu, H., Tohen, M., HGDH Study Group, 2005. Antipsychotic drug effects on brain morphology in first-episode psychosis. Arch. Gen. Psychiatry 62 (4), 361–370.
Lieberman, J.A., Bymaster, F.P., Meltzer, H.Y., Deutch, A.Y., Duncan, G.E., Marx, C.E., Aprille, J.R., Dwyer, D.S., Li, X.M., Mahadik, S.P., Duman, R.S., Porter, J.H., Modica-Napolitano, J. S., Newton, S.S., Csernansky, J.G., 2008. Antipsychotic drugs: comparison in animal models of efficacy, neurotransmitter regulation, and neuroprotection. Pharmacol. Rev. 60 (3), 358–403.
Lostia, A.M., Mazzarini, L., Pacchiarotti, I., Lionetto, L., De Rossi, P., Sanna, L., Sani, G., Kotzalidis, G.D., Girardi, P., Simmaco, M., Tatarelli, R., 2009. Serum levels of risperidone and its metabolite, 9-hydroxyrisperidone: correlation between drug concentration and clinical response. Ther. Drug. Monit. 31 (4), 475–481.
Lu, X.H., Bradley, R.J., Dwyer, D.S., 2004. Olanzapine produces trophic effects in vitro and stimulates phosphorylation of Akt/PKB, ERK1/2, and the mitogen-activated protein kinase p38. Brain Res. 1011 (1), 58–68.
Lu, X.H., Dwyer, D.S., 2005. Second-generation antipsychotic drugs, olanzapine, quetiapine, and clozapine enhance neurite outgrowth in PC12 cells via PI3K/AKT, ERK, and pertussis toxin-sensitive pathways. J. Mol. Neurosci. 27 (1), 43–64.
Mao, Y., Ge, X., Frank, C.L., Madison, J.M., Koehler, A.N., Doud, M.K., Tassa, C., Berry, E.M., Soda, T., Singh, K.K., Biechele, T., Petryshen, T.L., Moon, R.T., Haggarty, S.J., Tsai, L.H., 2009. Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3beta/beta-catenin signaling. Cell 136 (6), 1017–1031.
Maragnoli, M.E., Fumagalli, F., Gennarelli, M., Racagni, G., Riva, M.A., 2004. Fluoxetine and olanzapine have synergistic effects in the modulation of fibroblast growth factor 2 expression within the rat brain. Biol. Psychiatry 55 (11), 1095–1102.
Masri, B., Salahpour, A., Didriksen, M., Ghisi, V., Beaulieu, J.M., Gainetdinov, R.R., Caron, M.G., 2008. Antagonism of dopamine D2 receptor/β-arrestin 2 interaction is a common property of clinically effective antipsychotics. Proc. Natl. Acad. Sci. 105 (36), 13656–13661.
Mauri, M.C., Steinhilber, C.P., Marino, R., Invernizzi, E., Fiorentini, A., Cerveri, G., Baldi, M.L., Barale, F., 2005. Clinical outcome and olanzapine plasma levels in acute schizophrenia. Eur. Psychiatry 20 (1), 55–60.
Meltzer, H.Y., Massey, B.W., 2011. The role of serotonin receptors in the action of atypical antipsychotic drugs. Curr. Opin. Pharmacol. 11 (1), 59–67.
Meltzer, H.Y., 2012. Clozapine: balancing safety with superior antipsychotic efficacy. Clin. Schizophr. Relat. Psychoses 6 (3), 134–144.
Meltzer, H.Y., 2013. Update on typical and atypical antipsychotic drugs. Annu. Rev. Med. 64, 393–406.
Miyamoto, S., Duncan, G.E., Marx, C.E., Lieberman, J.A., 2005. Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. Mol. Psychiatry 10 (1), 79–104.
Miyamoto, S., Miyake, N., Jarskog, L.F., Fleischhacker, W.W., Lieberman, J.A., 2012. Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. Mol. Psychiatry 17 (12), 1206–1227.
Molteni, R., Calabrese, F., Racagni, G., Fumagalli, F., Riva, M.A., 2009. Antipsychotic drug actions on gene modulation and signaling mechanisms. Pharmacol. Ther. 124 (1), 74–85.
Novi, F., Scarselli, M., Corsini, G.U., Maggio, R., 2004. The paired activation of the two components of the muscarinic M3 receptor dimer is required for induction of ERK1/2 phosphorylation. J. Biol. Chem. 279 (9), 7476–7486.
Park, S.W., Lee, J.G., Ha, E.K., Choi, S.M., Cho, H.Y., Seo, M.K., Kim, Y.H., 2009. Differential effects of aripiprazole and haloperidol on BDNF-mediated signal changes in SH-SY5Y cells. Eur. Neuropsychopharmacol. 19 (5), 356–362.
Pereira, A., Fink, G., Sundram, S., 2009. Clozapine-induced ERK1 and ERK2 signaling in prefrontal cortex is mediated by the EGF receptor. J. Mol. Neurosci. 39, 185–198.
Pereira, A., Sugiharto-Winarno, A., Zhang, B., Malcolm, P., Fink, G., Sundram, S., 2012. Clozapine induction of ERK1/2 cell signaling via the EGF receptor in mouse prefrontal cortex and striatum is distinct from other antipsychotic drugs. Int. J. Neuropsychopharmacol. 15 (8), 1149–1160.
Pozzi, L., Håkansson, K., Usiello, A., Borgkvist, A., Lindskog, M., Greengard, P., Fisone, G., 2003. Opposite regulation by typical

and atypical anti-psychotics of ERK1/2, CREB and Elk-1 phosphorylation in mouse dorsal striatum. J. Neurochem. 86 (2), 451–459.
Reiter, E., Ahn, S., Shukla, A.K., Lefkowitz, R.J., 2012. Molecular mechanism of β-arrestin-biased agonism at seven-transmembrane receptors. Annu. Rev. Pharmacol. Toxicol. 52, 179–197.
Rhodes, N., Heerding, D.A., Duckett, D.R., Eberwein, D.J., Knick, V.B., Lansing, T.J., McConnell, R.T., Gilmer, T.M., Zhang, S.Y., Robell, K., Kahana, J.A., Geske, R.S., Kleymenova, E.V., Choudhry, A.E., Lai, Z., Leber, J.D., Minthorn, E.A., Strum, S.L., Wood, E.R., Huang, P.S., Copeland, R.A., Kumar, R., 2008. Characterization of an Akt kinase inhibitor with potent pharmacodynamic and antitumor activity. Cancer Res 68 (7), 2366–2374.
Riva, M.A., Molteni, R., Tascedda, F., Massironi, A., Racagni, G., 1999. Selective modulation of fibroblast growth factor-2 expression in the rat brain by the atypical antipsychotic clozapine. Neuropharmacology 38 (7), 1075–1082.
Rubinfeld, H., Seger, R., 2005. The ERK cascade: a prototype of MAPK signaling. Mol. Biotechnol. 31 (2), 151–174.
Samuels, I.S., Karlo, J.C., Faruzzi, A.N., Pickering, K., Herrup, K., Sweatt, J.D., Saitta, S.C., Landreth, G.E., 2008. Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive Function. J. Neurosci. 28 (27), 6983–6995.
Samuels, I.S., Saitta, S.C., Landreth, G.E., 2009. MAP'ing CNS development and cognition: an ERKsome process. Neuron 61 (2), 160–167.
Sathi, Z.S., Anisuzzaman, A.S., Morishima, S., Suzuki, F., Tanaka, T., Yoshiki, H., Muramatsu, I., 2008. Different affinities of native alpha1B-adrenoceptors for ketanserin between intact tissue segments and membrane preparations. Eur. J. Pharmacol. 584 (2–3), 222–228.
Scarselli, M., Annibale, P., Gerace, C., Radenovic, A., 2013. Enlightening G-protein-coupled receptors on the plasma membrane using super-resolution photoactivated localization microscopy. Biochem. Soc. Trans. 41 (1), 191–196.
Scarselli, M., Donaldson, J.G., 2009. Constitutive internalization of G protein-coupled receptors and G proteins via clathrin-independent endocytosis. J. Biol. Chem. 284 (6), 3577–3585.
Schmid, C.L., Streicher, J.M., Meltzer, H.Y., Bohn, L.M., 2014. Clozapine acts as an agonist at serotonin 2A receptors to counter MK-801-induced behaviors through a βarrestin2-independent activation of Akt. Neuropsychopharmacology 39 (8), 1902–1913.
Shioda, N., Han, F., Fukunaga, K., 2009. Role of Akt and Erk signaling in the neurogenesis following brain ischemia. Int. Rev. Neurobiol. 85, 375–387.
Trichard, C., Paillère-Martinot, M.L., Attar-Levy, D., Recassens, C., Monnet, F., Martinot, J.L., 1998. Binding of antipsychotic drugs to cortical 5-HT2A receptors: a PT study of chlorpromazine, clozapine, andamisulpride in schizophrenic patients. Am. J. Psychiatry 155 (4), 505–508.
Van Haren, N.E., Hulshoff Pol, H.E., Schnack, H.G., Cahn, W., Mandl, R.C., Collins, D.L., Evans, A.C., Kahn, R.S., 2007. Focal gray matter changes in schizophrenia across the course of the illness: a 5-year follow-up study. Neuropsychopharmacology 32 (10), 2057–2066.
Wang, H., Hu, L., Liu, C., Su, Z., Wang, L., Pan, G., Guo, Y., He, J., 2016. 5-HT2 receptors mediate functional modulation of GABAa receptors and inhibitory synaptic transmissions in human iPS-derived neurons. Sci. Rep. 6, 20033.
Zheng, W., Wang, H., Zeng, Z., Lin, J., Little, P.J., Srivastava, L.K., Quirion, R., 2012. The possible role of the Akt signaling pathway in schizophrenia. Brain Res. 1470, 145–158.