Molecular Pharmacology Fast Forward. Published on June 9, 2009 as doi:10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #56283

Title Page:

Human 5-HT_7 receptor-induced inactivation of forskolin-stimulated adenylate cyclase by risperidone, 9-OH-risperidone and other "inactivating antagonists"

Nicole Toohey

Michael T. Klein

Jessica Knight

Carol Smith

Milt Teitler

Center for Neuropharmacology & Neuroscience, Albany Medical College, Albany, New York 12208

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title Page

Running title: 5-HT_7 inactivation of forksolin-stimulated adenylate cyclase

Corresponding author: Dr. Milt Teitler, Center for Neuropharmacology & Neuroscience, Albany Medical College, 47 New Scotland Avenue, Albany, New York 12208; 518-262-5789 (P); 518-262-5799 (F); teitlem@mail.amc.edu

Pages: 31

Abstract word count: 250

Introduction word count: 599

Discussion word count: 14922

References: 33

Tables: 2

Figures: 6

Non-standard abbreviatons: none

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract:

We have previously reported on the unusual h5-HT_7 receptor inactivating properties of risperidone, 9-OH-risperidone, bromocryptine, methiothepin, metergoline, and lisuride (Smith *et al.*, 2006;Knight *et al.*, 2009). Inactivation was defined as the inability of 10μM 5-HT to stimulate cAMP accumulation after brief exposure and thorough removal of the drugs from HEK293 cells expressing h5-HT_7 receptors. Herein we report that brief exposure of the h5-HT_7 receptor-expressing cells to inactivating drugs, followed by removal of the drugs, results in potent and efficacious irreversible inhibition of forskolin-stimulated adenylate cyclase activity. Pre-treatment, followed by removal of the inactivating drugs inhibited 10μM forskolin-stimulated adenylate cyclase activity with potencies similar to the drugs' affinities for the h5-HT_7 receptor. The actions of the inactivating drugs were pertussis toxin-insensitive, indicating the lack of G_i in their mechanism(s) of action. Methiothepin and bromocryptine maximally inhibited 10μM forskolin-stimulated adenylate cyclase while the other drugs produced partial inhibition, indicating the drugs are inducing slightly different inactive conformations of the h5-HT_7 receptor. Maximal effects of these inactivating drugs occurred within 15-30 min of exposure of the cells to the drugs. A G_s-mediated inhibition of forskolin-stimulated activity has never been reported. The inactivating antagonists appear to induce a stable conformation of the h5-HT_7 receptor, which induces an altered state of G_s, which, in turn, inhibits forskolin-mediated stimulation of adenylate cyclase. These and previous observations indicate the inactivating antagonists represent a unique class of drugs and may reveal GPCR regulatory mechanisms previously unknown. These drugs may produce innovative approaches to the development of therapeutic drugs.

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction:

The 5-HT_7 receptor is one of 14 5-HT receptors expressed in mammalian tissues (Teitler and Herrick-Davis, 1994;Gerhardt and van Heerikhuizen, 1997;Hoyer and Martin, 1997;Hoyer *et al.*, 2002;Kroeze *et al.*, 2002;Raymond *et al.*, 2001). It was discovered through homology cloning and is expressed in various areas of the human brain and in peripheral tissues, including important blood vessels in the cerebral vasculature (Bard *et al.*, 1993;Lovenberg *et al.*, 1993;Shen *et al.*, 1993;Teitler and Herrick-Davis, 1994;Hedlund and Sutcliffe, 2004). 5-HT_7 receptor antagonists are being developed for possible use in various clinical conditions including migraine (Terron, 1997), sleep (Lovenberg *et al.*, 1993), psychosis (Bard *et al.*, 1993;Lovenberg *et al.*, 1993;Shen *et al.*, 1993), and depression (Bard *et al.*, 1993;Lovenberg *et al.*, 1993;Shen *et al.*, 1993;Hedlund and Sutcliffe, 2004).

Risperidone is a highly prescribed atypical antipsychotic drug (Love and Nelson, 2000;Bhana and Spencer, 2000;Green, 2000;Schneider *et al.*, 2006). It is one of a group of drugs believed to initiate their effects through interactions with the D_2 dopamine and 5-HT_{2A} serotonin receptors (Roth *et al.*, 1994;Meltzer *et al.*, 1989). These interactions have been shown to be classical competitive antagonist interactions (Smith *et al.*, 2006;Roth *et al.*, 1994). In previous publications, using h5-HT_7 receptor-expressing HEK293 cells, we reported the rapid, potent inactivation of h5-HT_7 receptor stimulation of cAMP production by six antagonists: risperidone, 9-OH-risperidone, methiothepin, bromocryptine, metergoline and lisuride (Smith *et al.*, 2006;Knight *et al.*, 2009). The mechanism appears to involve the pseudo-irreversible interaction of the drugs with the h5-HT_7 receptor, thus occluding the orthosteric binding site and preventing stimulation by 5-HT.

However, several observations indicated a simple pseudo-irreversible blockade might not fully explain the effect of the inactivating antagonists. Risperidone and 9-OH-risperidone irreversibly inhibited only 50% of the h5-HT_7 orthosteric binding sites, while the four other inactivators irreversibly inhibited

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

all the h5-HT_7 receptor binding sites (Knight *et al.*, 2009). Also, metergoline's potency as an inactivator was significantly lower than predicted from its affinity for the h5-HT_7 receptor, while the other five inactivators' potencies matched their affinities for the h5-HT_7 receptor.

The effects of forskolin on adenylate cyclase activity have been extensively studied (Stengel *et al.*, 1982;Darfler *et al.*, 1982;Insel and Ostrom, 2003;Alousi *et al.*, 1991;Dessauer *et al.*, 1997;Tang and Gilman, 1995). While the major effect of forskolin is to directly stimulate adenylate cyclase activity, this stimulation can be regulated by GPCRs through G-proteins (Tesmer *et al.*, 2002;Taussig *et al.*, 1993;Bender *et al.*, 1984;Neer, 1986;Neer, 1978). The predominant regulation of forskolin-stimulated adenylate cyclase activity is mediated by activation of Gi/o-coupled GPCR which partially inhibit forskolin-stimulated adenylate cyclase activity (Tesmer *et al.*, 2002;Taussig *et al.*, 1993;Bender *et al.*, 1984;Neer, 1986;Neer, 1978). It has been reported that agonist-mediated modulation of forskolin-stimulated adenylate cyclase can be produced through G_s-coupled receptors(Stengel *et al.*, 1982). This effect is relatively minor and usually manifests as a potentiation of forskolin-stimulated activity. It should be noted that the GPCR-mediated regulation of forskolin-stimulated adenylate cyclase activity occurs through activity of agonists on GPCR. There appears to be no reports of acute effects of antagonists on forskolin-stimulated adenylate cyclase activity. While conducting the studies previously reported (Knight *et al.*, 2009), forskolin-stimulated adenylate cyclase activity was routinely monitored as a control for the status of cellular adenylate cyclase activity. It was anticipated that forskolin-stimulated adenylate cyclase activity would not be affected by the inactivating drugs. As described below, the inactivating antagonists produced unique effects on h5-HT_7 receptor activity, demonstrated by the persistent inhibition of forskolin-stimulated cAMP in cells exposed to this novel group of drugs. These effects provide significant information concerning the mechanism by which the inactivating antagonists produce their effects on h5-HT_7 receptor-mediated cAMP production.

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods:

cAMP Assay: Total cAMP accumulation was measured using the LANCE cAMP Detection kit (Perkin-Elmer). Cells were cultured for 18 hrs in serum-free media, with and without 100ng/ml pertussis toxin (see Results, figure 1). Cells were lifted using 1ml/dish diluted Versene (1:3 in PBS), followed by the addition of 11ml/dish HEPES buffer (20mM HEPES, 2.5mM MgSO4, 134mM NaCl, pH 7.5 at 23^{o}C). Cells were centrifuged for 3 min at 330×g, supernatant was aspirated and the cells were resuspended in HEPES buffer. Cells were pre-treated with drug, incubated 30 min at 37°C (or 15, 30, 60, and 90 min for time-course experiments), washed 3×10 min in HEPES buffer. After the third wash, cells were resuspended in stimulation buffer (prepared according to the Perkin-Elmer LANCE cAMP instruction manual). Cells were counted with a hemacytometer and added to 96-well white opaque plates. The pre-treated cells were then exposed to 10μM or 35μM forskolin for 30 min at 23°C. Detection buffer was then added (prepared according to the LANCE instruction manual, Perkin-Elmer). Control experiments demonstrated that this procedure produced no effect on the cells' responsiveness to forskolin (see Results). Control experiments also demonstrated that performing the forskolin stimulation at 37^{o}C has no effect on the properties of the inactivating antagonists (see Results). Time-resolved fluorescence resonance energy transfer was detected by the Victor3 1420 plate-reader (Perkin-Elmer).

Risperidone and metergoline pre-treatment experiments: Cells were lifted and centrifuged as above. Cells were incubated for 30 min with 10μM metergoline or 10μM risperidone at 37^{o}C. The cells were then washed 3x10min with HEPES buffer. After the third wash cells were resuspended in HEPES buffer and treated with bromocryptine or methiothepin for 30 min at 37^{o}C. Cells were then washed 3x10min with HEPES buffer. After the third wash, cells were resuspended in stimulation buffer (prepared according to the LANCE instruction manual, Perkin-Elmer). Cells were then counted using a

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

hematocytometer and added to 96-well opaque plates. The pre-treated cells were then exposed to 10μM forskolin for 30 min at room temperature. Plates were read on a Victor3 plate-reader (Perkin-Elmer).

Adenylate cyclase and Gαs immunoblots: Rabbit polyclonal A cyclase V/VI (H-130), Gαs (K-20), ERK2 and donkey anti-rabbit horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Suspended cells were treated with the specified drugs for 30 min at 37^{o}C then washed (as described above) for 3x10 min with HEPES buffer. The cells were rinsed once with 1xPBS and spun 3 min at 330 x g. The cells were lysed for 30 min on ice with RIPA cell lysis buffer with protease inhibitors. The pellets were then further disrupted by shearing the DNA with a 26-gauge needle and another 30 min incubation on ice. The cell lysates were spun for 20 min at 14000 x g at 4 degrees. The supernatants containing the total cell lysate were flash frozen and stored at -80^{o}C until needed. BCA's were done on the cell treatments in order to determine the concentration of protein in each sample. 20μg of protein/treatment was loaded on to 10% Tris-HCL polyacrylamide gradient gels (Bio-Rad Laboratories, Hercules, CA) to be separated using electrophoresis and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were then blocked for one hour with 5% non-fat dry milk before overnight incubation with the primary antibody at 4^{o}C. The membrane was washed and incubated with the secondary antibody and the bound antibodies were visualized using Pierce ECL western Blotting Substrate (Thermo Scientific, Rockford IL). The membranes were then washed, stripped, re-blotted with the ERK-2 antibody, and visualized as above.

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Results:

Figures 1Aa and 1B displays the results of screening 19 drugs for forskolin-inactivating properties. These drugs were selected based on preliminary radioligand binding studies indicating they had high to moderate affinities for the h5-HT_7 receptor. The h5-HT_7 receptor expressing HEK293 cells were first exposed to 1μM drug for 30 minutes, followed by three washouts. The cells were then exposed to 10μM forskolin for 30 minutes. Inactivation was defined as the inability, or a reduced ability, of the cells to produce cAMP in response to forskolin stimulation after the thorough washout of drug. Six of the drugs tested displayed this property: risperidone, 9-OH-risperidone, methiothepin, bromocryptine, metergoline, and lisuride. These six drugs had been previously characterized as h5-HT_7 receptor inactivators (Smith *et al.,* 2006;Knight *et al.,* 2009). The six drugs tested that had inactivating ability exhibited h5-HT_7 receptor affinities that ranged from 0.4nM-143nM. Lisuride, (0.4nM), risperidone (2nM), methiothepin (3nM), 9-OH-risperidone (10nM), metergoline (16nM), and bromocryptine (143nM) displayed inactivating properties (figure 1A). Other drugs tested that did not display inactivating properties are listed with their h5-HT_7 receptor affinities (nM) :amoxapine (69); amitriptyline (96); cyproheptadine (24); loxapine (258); mianserin (64); ritanserin (468); the selective 5-HT_7 receptor antagonist SB269970 (2); tenilapine (153); TFM (1624); trifluperazine (497); the high affinity 5-HT_2 receptor antagonist ICI169369 (393); clozapine (30); methysergide (32) and LSD (3). Of the six inactivators methiothepin and bromocryptine appeared to produce the most efficacious inhibition of 10μM forskolin-stimulation (figures 1). In order to determine if the h5-HT_7 receptor might be stimulating a G_i, thereby inhibiting forskolin-stimulated adenylate cyclase activity, we pre-treated the h5-HT_7-receptor expressing cells with pertussis toxin, which inactivates G_i (Kaslow *et al.,* 1987). Although the h5-HT_7 receptor is a well-characterized G_s-coupled receptor, it is possible that the "inactivating antagonists" are inducing a persistent state of the receptor that stimulates G_i, thereby inhibiting forskolin-

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

stimulated adenylate cyclase activity. As shown in figure 1A, pre-treatment of the h5-HT_7-receptor expressing cells with pertussis toxin, produced no main effect on the inactivating antagonists' activity ($p = 0.104$, two-way ANOVA). The lack of effect of pertussis toxin indicates G_i is not involved in the inhibition of forskolin stimulated adenylate cyclase activity. Figure 1A displays the positive control for the pertussis toxin: 5-HT_{1E}-mediated inhibition of forskolin-stimulated cAMP accumulation is blocked, indicating the pertussis toxin is active. Thus the lack of effect of pertussis toxin on the inhibition of forskolin-stimulated cAMP accumulation in the h5-HT_7 receptor-expressing cells indicates no involvement of G_i. Figure 1B displays the lack of effect of 13 antagonists, i.e. "non-inactivating antagonists", on forskolin-stimulated adenylate cyclase. Figure 1C displays the lack of effect of performing the assay at 37^o, rather than 23^o, on the inactivating antagonist drugs' activity. Figure 2 displays the lack of effect of the inactivating antagonists on forskolin-stimulated adenylate cyclase activity in HEK-293 cells not expressing h5-HT_7 receptors. Taken together the data in figures 1&2 indicate that six out of twenty drugs tested produced the inactivating effect on forskolin-stimulated adenylate cyclase activity, this effect is mediated through the h5-HT_7 receptor rather than through a non-specific mechanism, and the effect is not due to some temperature-induced alteration in the assay conditions.

In order to obtain more information on this novel ability to irreversibly inhibit forskolin-stimulated adenylate cyclase activity, concentration-response curves for the inactivating effect were produced (figure 3; table 1). The h5-HT_7 receptor expressing HEK293 cells were first exposed to increasing concentrations of drug for 30 minutes, followed by three washouts. The cells were then exposed to 10 or 35μM forskolin for 30 minutes. Risperidone, 9-OH-risperidone, bromocryptine, methiothepin, metergoline and lisuride displayed high potencies for producing the inactivation effect, with similar IC_{50} values for 10 or 35μM forskolin stimulation. The similar potencies of the inactivating drugs on 10 or 35μM forskolin-stimulated

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

activity (figure 3, table 1) indicate the mechanism-of-action is not a competitive one. An important observation from the data in figure 3 is that the inactivators appear to have different maximal levels of inhibition (table 2). Methiothepin and bromocryptine irreversibly inhibit forskolin-stimulated activity more effectivly than risperidone, 9-OH-risperidone, metergoline or lisuride. These results indicate the possibility of either one mechanism with multiple efficacies among the inactivating drugs (full and partial inactivators), or multiple mechanisms of inhibition among the inactivating drugs. The major observation was that methiothepin and bromocryptine are fully efficacious in irreversibly inhibiting forskolin-stimulated adenylate cyclase activity, while the other four inactivating drugs display less efficacy.

In order to determine if the difference in efficacies could be due to kinetic differences between the drugs in producing the inactivating effect, time-course experiments were performed (figure 4). Cells were pre-treated with each of the inactivators for 15, 30, 60, and 90 minutes and then subjected to the usual washout and assay procedures (described above). While several interesting observations were made in these experiments (see Discussion) it is clear that the lower efficacy of risperidone, 9-OH-risperidone, metergoline and lisuride, relative to methiothepin and bromocryptine, cannot be due to slower kinetics. The lesser effect observed for several of the drugs at the 90 min time point is contrary to this possibility.

Another possible rationale for the difference in efficacies could be different mechanisms involving distinct sites on the h5-HT_7 receptor mediating the effects of the inactivators. In order to obtain information on this possibility, h5-HT_7 receptor expressing cells were exposed to 10μM metergoline or risperidone (partial inactivators) for 30 minutes, washed, and then exposed to 10μM methiothepin or bromocryptine (full inactivators). Metergoline and risperidone were chosen based on previous observations indicating they display somewhat different properties as inactivators (Knight *et al.*, 2009). As shown in figure 5A, pre-treatment with metergoline resulted in a blunting of the effect of bromocrytpine but had no effect on methiothepin's inactivating activity. Risperidone pre-treatment had no

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

effect on either methiothepin or bromocryptine (figure 5B). Taken together these results indicate that methiothepin, bromocryptine, risperidone, and metergoline produce inactivation by somewhat different mechanisms (see Discussion).

Figure 6 displays the results of Western blots for adenylate cyclase (isoforms V&VI) and G_s (alpha subunit) subsequent to exposure of the h5-HT_7 receptor-expressing cells to no drug, 10μM clozapine, methiothepin or risperidone. None of the drugs produced any effect on the levels of adenylate cyclase or G_s, indicating that the loss of activity subsequent to inactivating drug treatment is not due to some dramatic effect on the cellular levels of these signal transduction components.

Discussion:

The results presented herein continue a series of unusual observations involving the h5-HT_7 receptor (Smith *et al.*, 2006;Knight *et al.*, 2009). Six drugs out of a total of 20 drugs that have been tested produce an irreversible inactivation of the h5-HT_7 receptor, as judged by inhibition of 10μM 5-HT stimulation of cAMP production. A pseudo-irreversible interaction between the "inactivating drugs" and the h5-HT_7 receptor appears to be the cause of this unusual effect (Smith *et al.*, 2006). Five of the drugs appear to produce a complete inhibition of 5-HT stimulated receptor activity, with the possible exception of metergoline (Knight *et al.*, 2009). The pseudo-irreversible block of the orthosteric binding site on the h5-HT_7 receptor appears to explain the inactivation of the receptor. However it was also noted that risperidone and 9-OH-risperidone, which potently and fully inactivate 5-HT stimulated h5-HT_7 receptor, only irreversibly block 50% of the binding sites (Knight *et al.*, 2009). This observation indicates that receptor occupancy is not sufficient to fully predict the effect of the inactivators on the h5-HT_7 receptor

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

activity. These results indicated that risperidone and 9-OH-risperidone, while producing a pseudo-irreversible complex with the h5-HT_7 receptor, might be, in addition, interacting with the h5-HT_7 receptor in a somewhat different manner than the other four inactivators.

In the present study another unusual observation is described. Forskolin-stimulated adenylate cyclase activity is due to the direct interaction of forskolin with all the isoforms of adenylate cyclase, except for the ram sperm form of the enzyme (Alousi *et al.*, 1991). The interaction of activated G_s appears to have a secondary influence on the ability of forskolin to stimulate adenylate cyclase (Tesmer *et al.*, 2002;Taussig *et al.*, 1993;Bender *et al.*, 1984;Neer, 1986;Neer, 1978). A more pronounced inhibitory influence of G_i-coupled receptors on forskolin-stimulated adenylate cyclase is a well-documented cellular mechanism (Mons and Cooper, 1995). However the involvement of G_i in the current study has been eliminated, as pertussis toxin, an irreversible inhibitor of G_i, has no effect on the inactivating properties of drugs presented (figure 1). Agonist stimulation of receptors, acting through GTP-binding proteins, have been shown to slightly increase forskolin-stimulated adenylate cyclase activity (Darfler *et al.*, 1982;Insel and Ostrom, 2003;Alousi *et al.*, 1991). Antagonist-induced inhibition of forskolin-stimulated adenylate cyclase is a novel observation. The irreversible effects of the six inactivating drugs on forskolin-stimulated adenylate cyclase activity, particularly that of methiothepin and bromocryptine, are unprecedented. Furthermore these results indicate that the inactivating drugs, particularly methiothepin and bromocryptine, must be doing more than simply irreversibly occluding the orthosteric binding site on the h5-HT_7 receptor. The effects on forskolin-stimulated adenylate cyclase suggest that the inactivators are inducing a stable, persistent, inactive state of the h5-HT_7 receptor that, in turn, is inducing an inactive state of G_s. The inactive G_s must be inducing a state of adenylate cyclase with less than maximal forskolin-stimulated potential. In other words, the effects on forskolin-stimulated activity observed reveal several things about the receptor inactivating mechanism of these drugs. First, the inactivators irreversibly

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

induce a state of the receptor that inactivates G_s, eliminating the receptor-mediated cAMP stimulation as previously reported (Smith *et al.*, 2006;Knight *et al.*, 2009). Second, the G_s/adenylate cyclase interaction may be altered, inhibiting forskolin's ability to stimulate adenylate cyclase. The similar potencies of the inactivating drugs on 10 or 35μM forskolin-stimulated activity (figure 3, table 1) indicates the mechanism-of-action is not a competitive one. The maximal degree of irreversible inhibition of forskolin-stimulation varies amongst the different inactivators and is not predicted by maximal receptor occupancy (see Knight *et al.*, 2009).

In order to determine the possibility that the lower maximal effect of metergoline was due to a slower onset of effect, time course experiments were performed (figure 4). Overall, the effect of prolonged exposure of cells to the inactivating drugs does not seem to have a major effect. However it was noted that metergoline did become less effective with prolonged exposure, especially in the 35μM forskolin experiments. This result is the opposite of what one would expect if the lower maximal effect of metergoline observed in figure 3 was due to a slower rate of onset for metergoline compared to the other inactivating drugs. The observed loss of effect of metergoline may indicate that this drug slowly dissociates, and after dissociation the receptor can become re-activated. This possibility is currently being investigated. There is a dramatic difference in the effect of 15 min lisuride exposure depending on whether 10 or 35μM forskolin is used as the stimulant (figure 4). This suggests that the effects of lisuride, at early time points may be reversed, by increasing forskolin concentrations. This possibility is also currently being investigated.

The results in figure 5A which shows metergoline pre-treatment blunts the effects of bromocryptine but does not blunt the effects of methiothepin are especially notable. These results are consistent with the complete blockade of the 5-HT_7 receptor by metergoline, preventing subsequent effects at the orthosteric site by bromocryptine. However, metergoline does not blunt the inactivating

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

effects of methiothepin. These results imply that bromocryptine and metergoline share the same mechanism, probably a pseudo-irreversible interaction with the h5-HT_7 receptor. Methiothepin appears to have properties that allow it to overcome the presence of metergoline at the h5-HT_7 receptor, i.e. methiothepin and bromocryptine induce different states of the h5-HT_7 receptor that result in the complete inactivation of the h5-HT_7 receptor.

The results in figure 5B, demonstrating that risperidone pre-treatment is ineffective in blunting the effects of either bromocryptine or methiothepin, are also notable. These results are consistent with previous results indicating that risperidone's mechanism of action is different than metergoline's (Knight *et al.*, 2009). Risperidone and 9-OH-risperidone have been shown to completely inactivate the h5-HT_7 receptor, while irreversibly blocking 50% of the receptors, while metergoline irreversibly blocks all the h5-HT_7 receptors and produces a profound inactivation of the h5-HT_7 receptor. The results in figure 4 reinforce the possibility of a different mechanism-of-action between risperidone and metergoline. Thus figure 4 indicates methiothepin, bromocryptine, risperidone, and metergoline differ in their interactions with the h5-HT_7 receptor, as judged by forskolin-stimulated adenylate cyclase activity. The details of these differences are under investigation.

The results presented herein and in the two previous papers (Smith *et al.*, 2006;Knight *et al.*, 2009) are highly unusual and unprecedented. However there has been a published paper (Krobert *et al.*, 2006) that reports similar effects with four drugs used in our studies. In this report the authors found that while mesulergine, SB269970, and clozapine produced little or no effect on the levels of ^{3}H-5-CT-labeled h5-HT_7 receptors after removal of the drugs, methiothepin reduced binding levels by 79%, with no change in affinity for the radioligand. These results correspond with the results we have reported previously: methiothepin inactivates the h5-HT_7 receptor and inhibits ^{3}H-5-HT labeling of the h5-HT_7 receptor after

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

removal: mesulergine, SB269970 and clozapine do not produce these effects (Smith *et al.*, 2006;Knight *et al.*, 2009). We have not found any other studies that studied the 5-HT_7 receptor after removal of drugs.

The findings displayed in these studies, and in the previous papers {1,426} indicate that the h5-HT_7 receptor operates in a unique manner. While many antagonists behave in a classically competitive fashion, 6 out of the 20 antagonists tested appear to induce a stable state of the receptor that a) involves pseudo-irreversible binding; b) induces a stable inactivated state of G_s, which in turn; c) induces a stable inactivated state of adenylate cyclase that includes complete or partial occlusion of the forskolin binding site. The inhibition of the forskolin-stimulated adenylate cyclase activity may not involve an occlusion of the binding site, but rather an altered, inactive conformation of adenylate cyclase that is resistant to forskolin stimulation. Studies are underway to investigate the predicted consequences of this model of h5-HT_7 function, i.e. a stable complex between the inactivating drug, h5-HT_7 receptor, G_s, and adenylate cyclase.

In summary, the results presented add another novel observation concerning the effects of inactivating drugs on the h5-HT_7 receptor, through what appears to be a pseudo-irreversible complex (Smith *et al.*, 2006;Knight *et al.*, 2009). The irreversible inhibition of forskolin-stimulated activity provides strong evidence of the production of an inactivated state of G_s by the inactivators, acting through the h5-HT_7 receptor. The different ability of the inactivators to inhibit forskolin-stimulated adenylate cyclase indicates the inactivators produce different states of the h5-HT_7 receptor. This situation is highly analogous to that of the classical case of full and partial receptor agonists adapted to this novel class of inactivating drugs. It is becoming increasingly clear that the inactivators are inducing a novel state of the h5-HT_7 receptor, revealing properties previously unobserved. As the effects of the inactivating drugs are examined in more detail, variations in the mechanism-of-action of these drugs are becoming clear. The

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

inactivating drugs appear to possess properties that produce effects on GPCRs that are distinct from competitive antagonists and thus may lead to the discovery of novel GPCR regulatory mechanisms. These novel regulatory mechanisms may be targets for the development of novel therapeutic drugs.

.

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

References:

Alousi AA, Jasper J R, Insel P A and Motulsky H J (1991) Stoichiometry of Receptor-Gs-Adenylate Cyclase Interactions. *FASEB J* **5**: 2300-2303.

Bard JA, Zgombick J, Adham N, Vaysse P, Branchek T A and Weinshank R L (1993) Cloning of a Novel Human Serotonin Receptor (5-HT7) Positively Linked to Adenylate Cyclase. *J Biol Chem* **268**: 23422-23426.

Bender JL, Wolf L G and Neer E J (1984) Interaction of Forskolin With Resolved Adenylate Cyclase Components. *Adv Cyclic Nucleotide Protein Phosphorylation Res* **17**: 101-109.

Bhana N and Spencer C M (2000) Risperidone: a Review of Its Use in the Management of the Behavioural and Psychological Symptoms of Dementia. *Drugs Aging* **16**: 451-471.

Darfler FJ, Mahan L C, Koachman A M and Insel P A (1982) Stimulation of Forskolin of Intact S49 Lymphoma Cells Involves the Nucleotide Regulatory Protein of Adenylate Cyclase. *J Biol Chem* **257**: 11901-11907.

Dessauer CW, Scully T T and Gilman A G (1997) Interactions of Forskolin and ATP With the Cytosolic Domains of Mammalian Adenylyl Cyclase. *J Biol Chem* **272**: 22272-22277.

Gerhardt CC and van Heerikhuizen H (1997) Functional Characteristics of Heterologously Expressed 5-HT Receptors. *Eur J Pharmacol* **334**: 1-23.

Green B (2000) Focus on Risperidone. *Curr Med Res Opin* **16**: 57-65.

Hedlund PB and Sutcliffe J G (2004) Functional, Molecular and Pharmacological Advances in 5-HT7 Receptor Research. *Trends Pharmacol Sci* **25**: 481-486.

Hoyer D, Hannon J P and Martin G R (2002) Molecular, Pharmacological and Functional Diversity of 5-HT Receptors. *Pharmacol Biochem Behav* **71**: 533-554.

Hoyer D and Martin G (1997) 5-HT Receptor Classification and Nomenclature: Towards a Harmonization With the Human Genome. *Neuropharmacology* **36**: 419-428.

Insel PA and Ostrom R S (2003) Forskolin As a Tool for Examining Adenylyl Cyclase Expression, Regulation, and G Protein Signaling. *Cell Mol Neurobiol* **23**: 305-314.

Kaslow HR, Lim L K, Moss J and Lesikar D D (1987) Structure-Activity Analysis of the Activation of Pertussis Toxin. *Biochemistry* **26**: 123-127.

Knight JA, Smith C, Toohey N, Klein M T and Teitler M (2009) Pharmacological Analysis of the Novel, Rapid, and Potent Inactivation of the Human 5-Hydroxytryptamine7 Receptor by Risperidone, 9-OH-Risperidone, and Other Inactivating Antagonists. *Mol Pharmacol* **75**: 374-380.

Krobert KA, Andressen K W and Levy F O (2006) Heterologous Desensitization Is Evoked by Both Agonist and Antagonist Stimulation of the Human 5-HT(7) Serotonin Receptor. *Eur J Pharmacol* **532**: 1-10.

Kroeze WK, Kristiansen K and Roth B L (2002) Molecular Biology of Serotonin Receptors Structure and Function at the Molecular Level. *Curr Top Med Chem* **2**: 507-528.

Love RC and Nelson M W (2000) Pharmacology and Clinical Experience With Risperidone. *Expert Opin Pharmacother* **1**: 1441-1453.

Lovenberg TW, Baron B M, De Lecea L, Miller J D, Prosser R A, Rea M A, Foye P E, Racke M, Slone A L, Siegel B W and . (1993) A Novel Adenylyl Cyclase-Activating Serotonin Receptor (5-HT7) Implicated in the Regulation of Mammalian Circadian Rhythms. *Neuron* **11**: 449-458.

Meltzer HY, Matsubara S and Lee J C (1989) Classification of Typical and Atypical Antipsychotic Drugs on the Basis of Dopamine D-1, D-2 and Serotonin2 PKi Values. *J Pharmacol Exp Ther* **251**: 238-246.

Mons N and Cooper D M (1995) Adenylate Cyclases: Critical Foci in Neuronal Signaling. *Trends Neurosci* **18**: 536-542.

Neer EJ (1978) Multiple Forms of Adenylate Cyclase. *Adv Cyclic Nucleotide Res* **9**: 69-83.

Neer EJ (1986) Guanine Nucleotide-Binding Proteins Involved in Transmembrane Signaling. *Symp Fundam Cancer Res* **39**: 123-136.

Raymond JR, Mukhin Y V, Gelasco A, Turner J, Collinsworth G, Gettys T W, Grewal J S and Garnovskaya M N (2001) Multiplicity of Mechanisms of Serotonin Receptor Signal Transduction. *Pharmacol Ther* **92**: 179-212.

Roth BL, Craigo S C, Choudhary M S, Uluer A, Monsma F J, Jr., Shen Y, Meltzer H Y and Sibley D R (1994) Binding of Typical and Atypical Antipsychotic Agents to 5-Hydroxytryptamine-6 and 5-Hydroxytryptamine-7 Receptors. *J Pharmacol Exp Ther* **268**: 1403-1410.

Schneider LS, Dagerman K and Insel P S (2006) Efficacy and Adverse Effects of Atypical Antipsychotics for Dementia: Meta-Analysis of Randomized, Placebo-Controlled Trials. *Am J Geriatr Psychiatry* **14**: 191-210.

Shen Y, Monsma F J, Jr., Metcalf M A, Jose P A, Hamblin M W and Sibley D R (1993) Molecular Cloning and Expression of a 5-Hydroxytryptamine7 Serotonin Receptor Subtype. *J Biol Chem* **268**: 18200-18204.

Smith C, Rahman T, Toohey N, Mazurkiewicz J, Herrick-Davis K and Teitler M (2006) Risperidone Irreversibly Binds to and Inactivates the H5-HT7 Serotonin Receptor. *Mol Pharmacol* **70**: 1264-1270.

Stengel D, Guenet L, Desmier M, Insel P and Hanoune J (1982) Forskolin Requires More Than the Catalytic Unit to Activate Adenylate Cyclase. *Mol Cell Endocrinol* **28**: 681-690.

Tang WJ and Gilman A G (1995) Construction of a Soluble Adenylyl Cyclase Activated by Gs Alpha and Forskolin. *Science* **268**: 1769-1772.

Taussig R, Iniguez-Lluhi J A and Gilman A G (1993) Inhibition of Adenylyl Cyclase by Gi Alpha. *Science* **261**: 218-221.

Teitler M and Herrick-Davis K (1994) Multiple Serotonin Receptor Subtypes: Molecular Cloning and Functional Expression. *Crit Rev Neurobiol* **8**: 175-188.

Terron JA (1997) Role of 5-Ht7 Receptors in the Long-Lasting Hypotensive Response Induced by 5-Hydroxytryptamine in the Rat. *Br J Pharmacol* **121**: 563-571.

Tesmer JJ, Sunahara R K, Fancy D A, Gilman A G and Sprang S R (2002) Crystallization of Complex Between Soluble Domains of Adenylyl Cyclase and Activated Gs Alpha. *Methods Enzymol* **345**: 198-206.

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes:

This work was supported by PHS grant no. [MH56650]

Reprint requests : Dr. Milt Teitler, Center for Neuropharmacology & Neuroscience, Albany Medical College, 47 New Scotland Avenue, Albany, New York 12208; teitlem@mail.amc.edu

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures:

Figure 1: A: Effect of drug pre-treatment on 10μM forskolin-stimulated cAMP production by HEK 293 cells stably expressing h5-HT_7 receptors, and the lack of effect of pertussis toxin on the drug effects. Cells were cultured for 18hr in serum-free media in the absence and presence of 100 ng/ml pertussis toxin. Cells were suspended in HEPES buffer and exposed to a 10μM concentration of drugs for 30 min. Cells were gently pelleted, and resuspended in HEPES buffer and incubated at 37^{o}C for 10 min. This drug washout procedure was repeated three times. Cells were resuspended and assayed for response to 10μM forskolin using the LANCE cAMP Detection kit (Perkin-Elmer; see Materials and Methods). The results are the means ± SEM of three independent experiments performed in triplicate. Risperidone, 9-OH-risperidone, bromocryptine, methiothepin, lisuride, and metergoline were significantly different from no drug treatment ($p<0.0001$, one-way ANOVA). No significant main effect for pretussis toxin treatment was observed in h5-HT_7 expressing cells ($p = 0.104$, two-way ANOVA). 5-HT_{1E} receptor-expressing cells were tested for 5-HT-mediated inhibition of 10μM forskolin-stimulated cAMP production in the absence and presence of pertussis toxin (positive control).

B: Drugs displaying no effect on forskolin-stimulated adenylate cyclase activity after thorough washout. The results are the means ± SEM of three independent experiments performed in triplicate. There was no significant effect observed with these drugs ($p=.12$, one-way ANOVA).

C: Lack of effect of temperature on drug-induced inhibition of forskolin-stimulated adenylate cyclase activity. Cells were treated as in figure 1A except that forskolin stimulation was performed at 37^{o}C rather than 23^{o}C. There was no significant main effect on inhibition of forskolin-stimulated adenylate cyclase activity ($p=0.45$, two-way ANOVA).

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2: Effect of drug pre-treatment on 10μM forskolin-stimulated cAMP production by parental HEK 293 cells. Cells were cultured for 18hr in serum-free media. Cells were suspended in HEPES buffer and exposed to a 10μM concentration of drugs for 30 min. Cells were gently pelleted, and resuspended in HEPES buffer and incubated at 37^{o}C for 10 min. This drug washout procedure was repeated three times. Cells were resuspended and assayed for response to 10μM forskolin using the LANCE cAMP Detection kit (Perkin-Elmer; see Materials and Methods). The results are the means ± SEM of three independent experiments performed in triplicate.

Figure 3: Concentration-response curves for inactivation of 10μM and 35μM forskolin-stimulated cAMP production in HEK-293 cells expressing h5-HT$_7$ receptors. Cells were suspended in HEPES buffer and exposed to buffer (control) or varying concentrations of the six drugs displaying inactivating properties (see figure 1). After the drug washout procedure (see Materials and Methods) the cells were exposed to 10μM or 35μM forskolin for 30 min. cAMP levels were determined using the LANCE cAMP Detection kit (Perkin-Elmer). The results are the means ± SEM of three independent experiments performed in triplicate. Dotted lines denote 10μM forskolin; solid lines denote 35μM forskolin.

Figure 4: Time course of maximal inhibition of forskolin-stimulated adenylate cyclase activity by h5-HT$_7$ inactivators. Cells were exposed to 10μM inactivators for varying incubation times. After the drug washout procedure (see Materials and Methods) the cells were exposed to 10μM (A) or 35μM (B) forskolin and cAMP was detected as described above. Results are the means ±SEM of three independent experiments. While there was a significant effect of time on the drugs ($p<0.001$, two-way ANOVA), this effect does not account for the difference between the maximal effects of the different drugs (see Discussion).

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5: Effect of metergoline or risperidone pre-treatment on inactivator inhibition of forskolin-stimulated adenylate cyclase. Cells were exposed to 10μM metergoline or risperidone for 30 minutes, thoroughly washed (see Materials and Methods), exposed to 10μM methiothepin or bromocryptine for 30 minutes, and thoroughly washed. After the drug washout procedure (see Materials and Methods) the cells were exposed to 10μM forskolin, and cAMP was detected as described above. Results are the means ±SEM of three independent experiments. Metergoline blunted the effects of bromocryptine ($p<0.001$) but had no effect on methiothepin's activity. Risperidone had no significant effect on methiothepin or bromocryptine (see Discussion).

Figure 6: Levels of G_s and adenylate cyclase are not altered by inactivating drug exposure: Western blots using primary antibodies specific for G_s (α subunit) (A), and adenylate cyclase (sub-types V&VI) (B) were performed. There are no detectable differences between cells exposed to no drug (lane 1), 10μM clozapine (lane 2), 10 μM methiothepin (lane 3) or 10μM risperidone (lane 4). Cells were thoroughly washed before lysing and preparation. The blots were stripped after probing for either G_s or adenylate cyclase and re-probed for total ERK-2 as a loading control. Shown are representative blots that were performed twice.

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Tables:

Table one: Potencies of the six h5-HT_7 inactivating drugs as inhibitors of forskolin-stimulated adenylate cyclase activity. Following exposure of the h5-HT_7-receptor expressing HEK293 cells to varying concentrations of drugs for 30 min, the drugs were removed by repeated washing. Activity was determined by exposing the cells to 10 or 35μM forskolin for 30 min. cAMP was detected using the LANCE cAMP detection kit (see Materials and Methods). Results are the means ± SEM of three independent experiments performed in triplicate. Also included are the K_i values determined from homogenate binding studies (Knight *et al.*, 2009). There was no effect of increasing forskolin concentration on drug potencies (p = .492, two-way ANOVA).

Drug	K_i (nM)[a]	IC50 (10μM forskolin)	IC50 (35μM forskolin)
risperidone	1.8±0.3	4.4±2.1nM	3±0.9nM
9-OH risperidone	10±1.7	4.5±0.9nM	2.2±0.3nM
bromocryptine	143±56	172±13nM	187±3.4nM
lisuride	0.4±0.2	2.9±0.7nM	1.8±0.1nM
metergoline	16±2	394±64nM	306±17nM
methiothepin	3.0±0.5	3.0±0.4nM	2.5±0.2nM

[a]membrane homogenate binding; (Knight *et al.*, 2009)

.

Molecular Pharmacology Fast Forward. Published on June 9, 2009 as DOI: 10.1124/mol.109.056283
This article has not been copyedited and formatted. The final version may differ from this version.

Table two: Maximal effects of the six inactivating drugs on 10 and 35μM forskolin-stimulated activity. h5-HT_7-receptor expressing cells were exposed to 10μM drugs for 30 min, thoroughly washed, then exposed to 10 or 35μM forskolin for 30 min (see figure three). The values listed are the percentage of forskolin-stimulated activity observed in cells not exposed to inactivators. cAMP was detected using the LANCE kit (see Materials and Methods). Results are the mean±SEM of three independent experiments.

Inactivator (10uM)	10uM forskolin (% control)	35uM forskolin (% control)
risperidone	21±3	25±2
9-OH-risperidone	25±2	30±6
methiothepin	9±4	4±3
bromocryptine	4±2	5±2
metergoline	30±3.5	31±12
lisuride	25±4	32±8

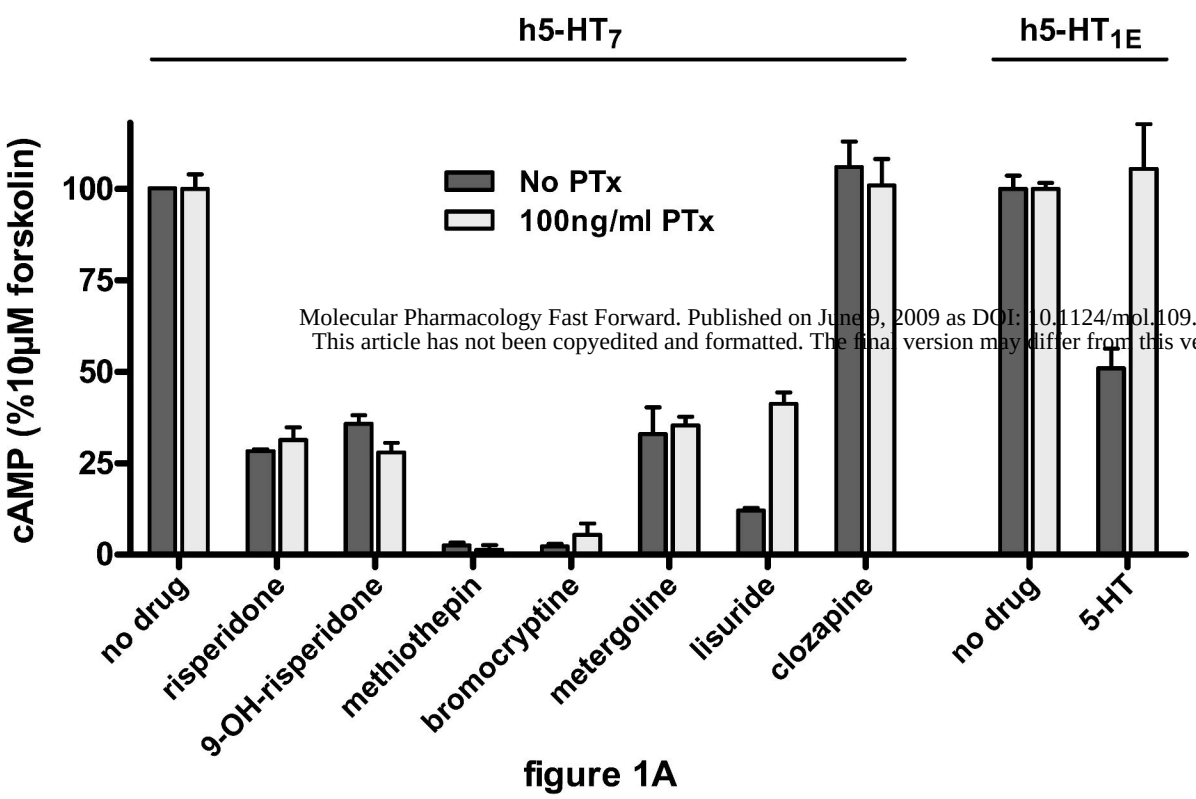


figure 1A

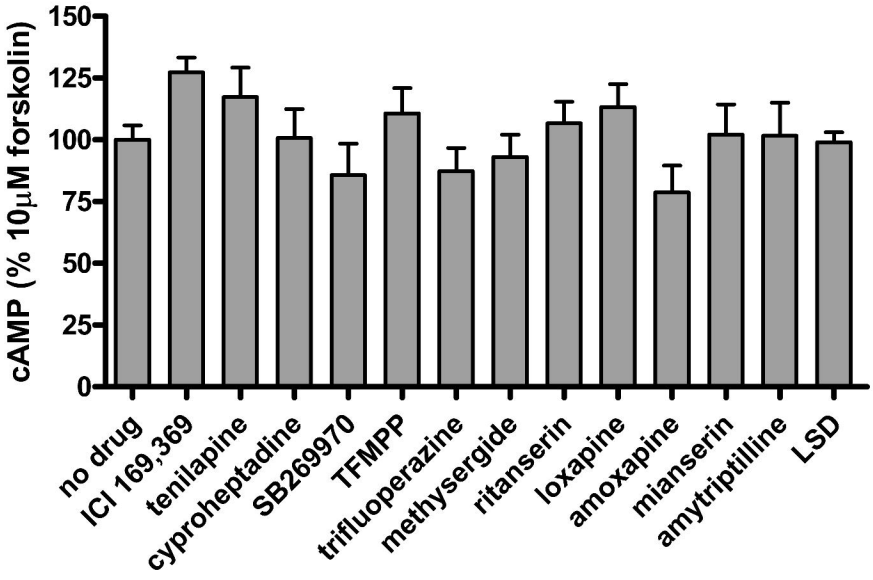


figure 1B

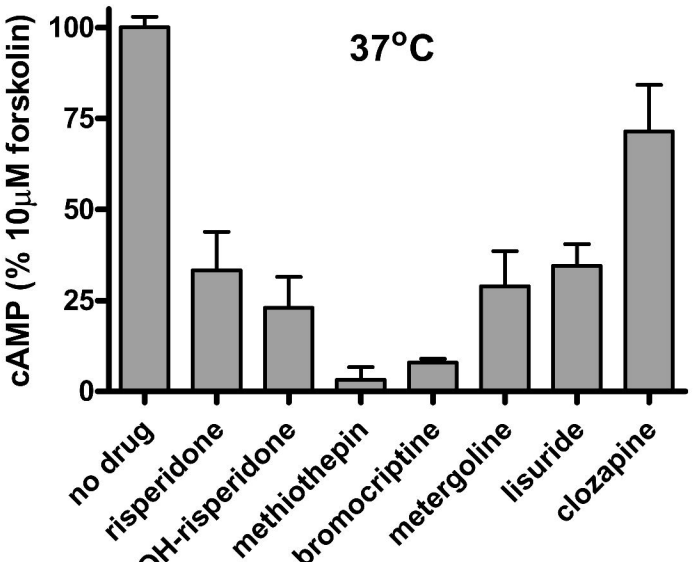


figure 1C

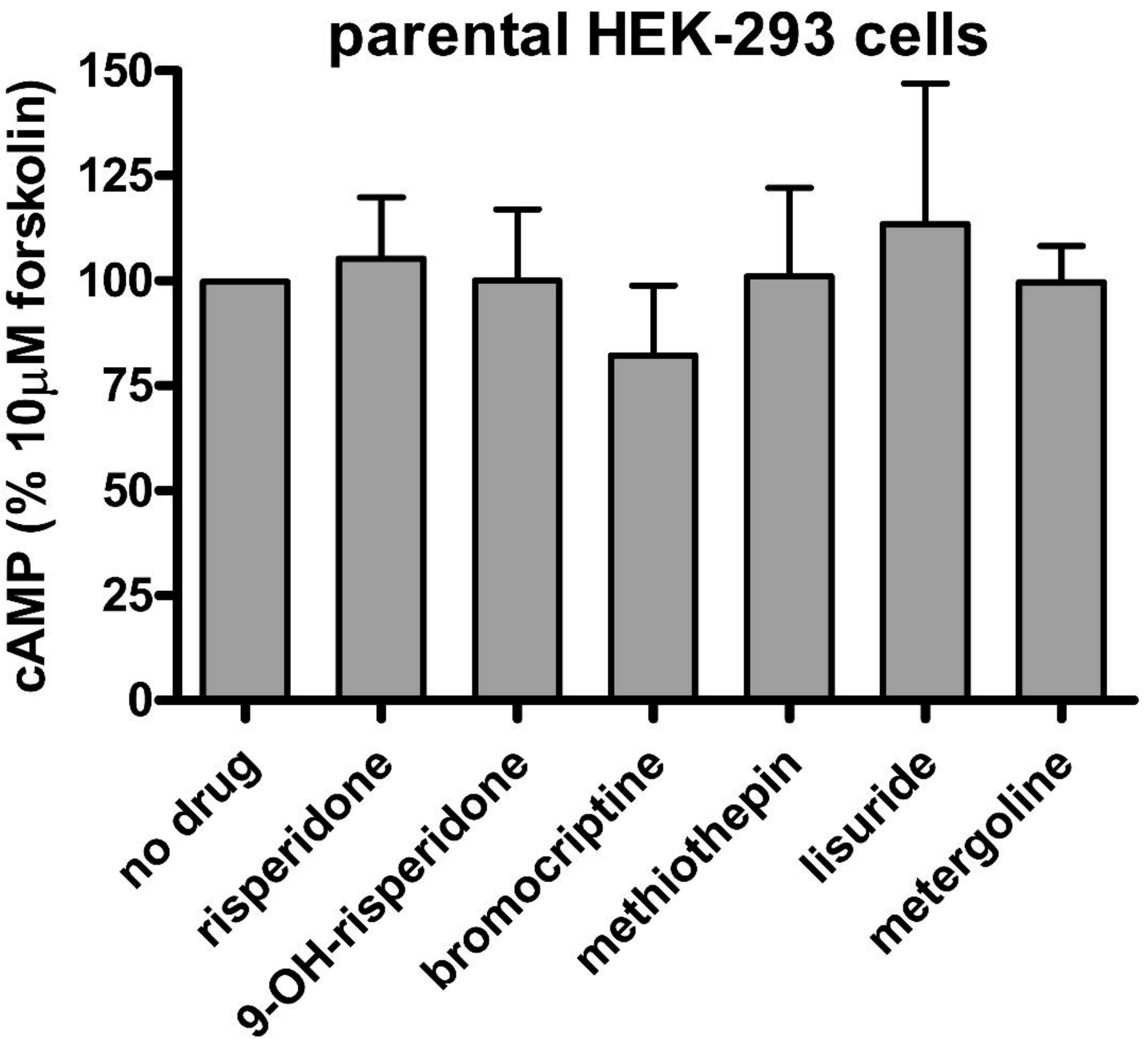
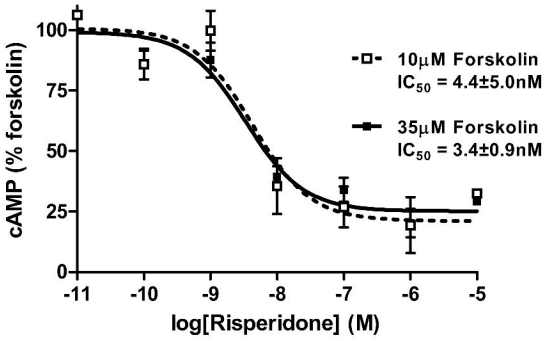
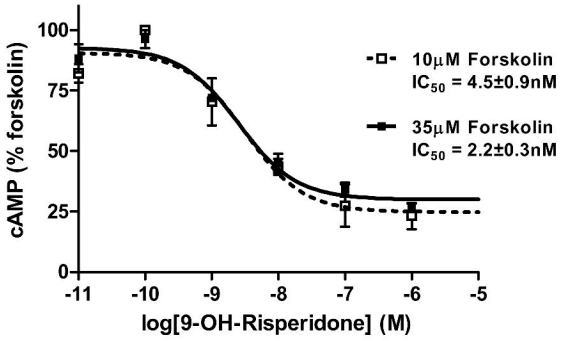
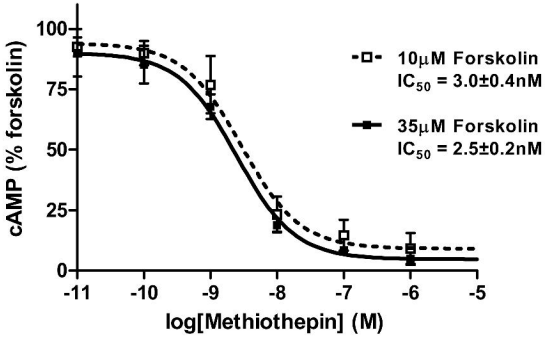
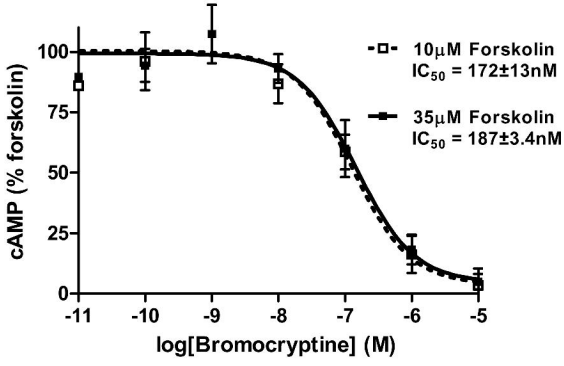

parental HEK-293 cells
cAMP (% 10μM forskolin)
150
125
100
75
50
25
0
no drug
risperidone
9-OH-risperidone
bromocriptine
methiothepin
lisuride
metergoline

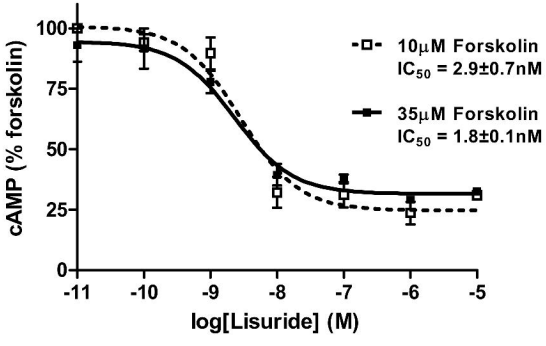
figure 2


cAMP (% forskolin)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log[Risperidone] (M)
10μM Forskolin
IC50 = 4.4±5.0nM
35μM Forskolin
IC50 = 3.4±0.9nM


cAMP (% forskolin)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log[9-OH-Risperidone] (M)
10μM Forskolin
IC50 = 4.5±0.9nM
35μM Forskolin
IC50 = 2.2±0.3nM


cAMP (% forskolin)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log[Methiothepin] (M)
10μM Forskolin
IC50 = 3.0±0.4nM
35μM Forskolin
IC50 = 2.5±0.2nM


cAMP (% forskolin)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log[Bromocryptine] (M)
10μM Forskolin
IC50 = 172±13nM
35μM Forskolin
IC50 = 187±3.4nM


cAMP (% forskolin)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log[Lisuride] (M)
10μM Forskolin
IC50 = 2.9±0.7nM
35μM Forskolin
IC50 = 1.8±0.1nM

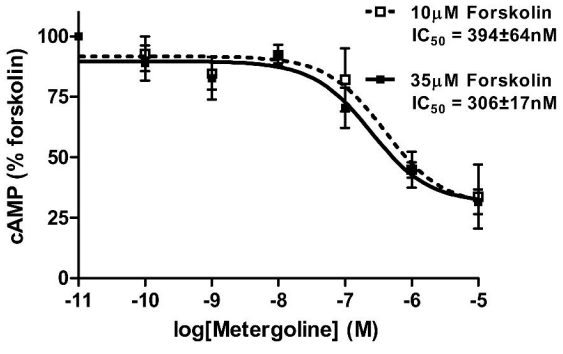

cAMP (% forskolin)
100
75
50
25
0
-11
-10
-9
-8
-7
-6
-5
log[Metergoline] (M)
10μM Forskolin
IC50 = 394±64nM
35μM Forskolin
IC50 = 306±17nM

figure 3

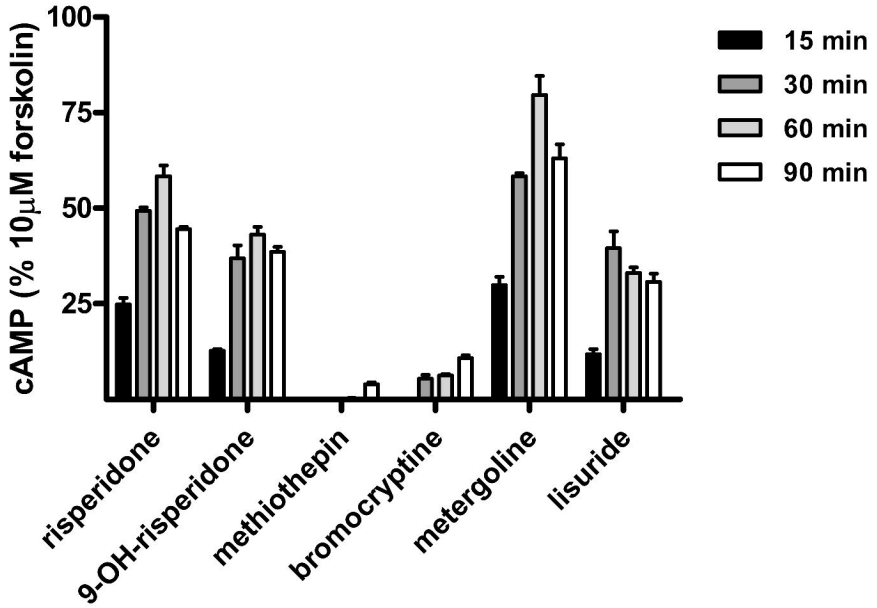


figure 4A

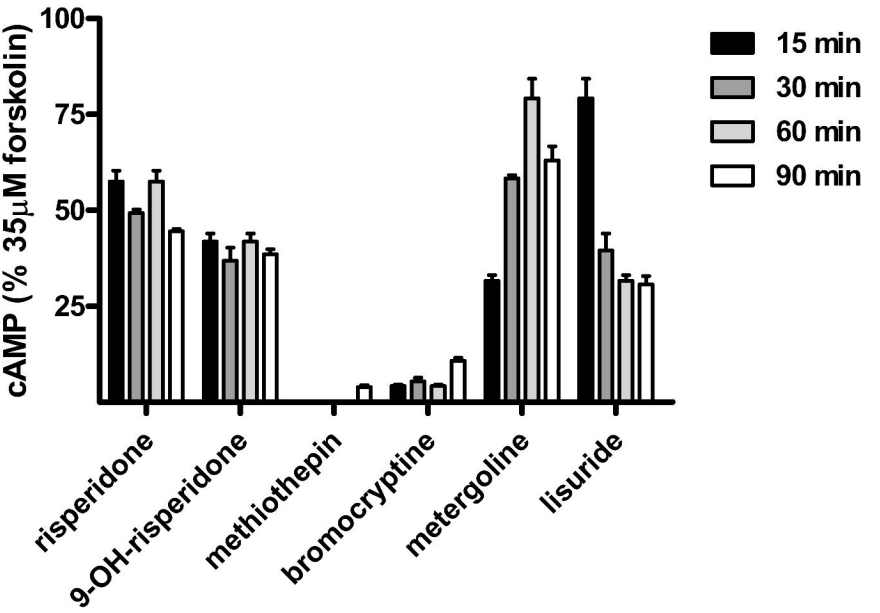


figure 4B

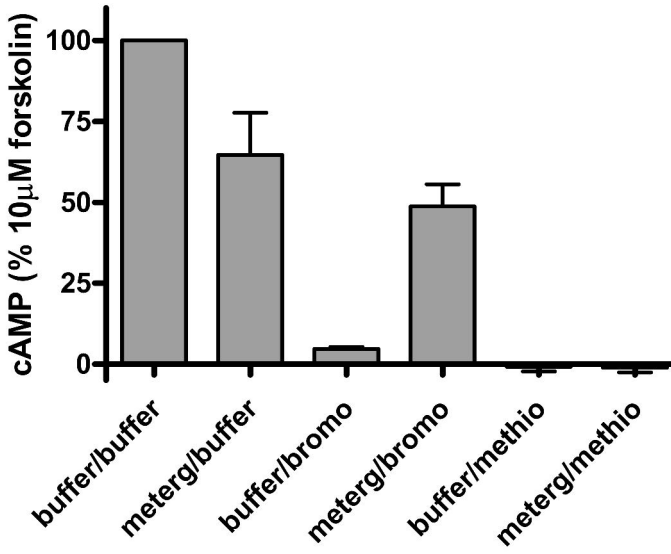


Figure 5A

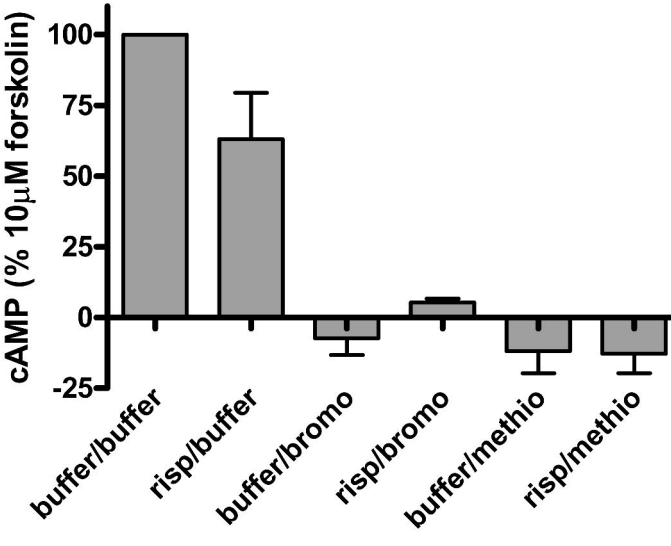
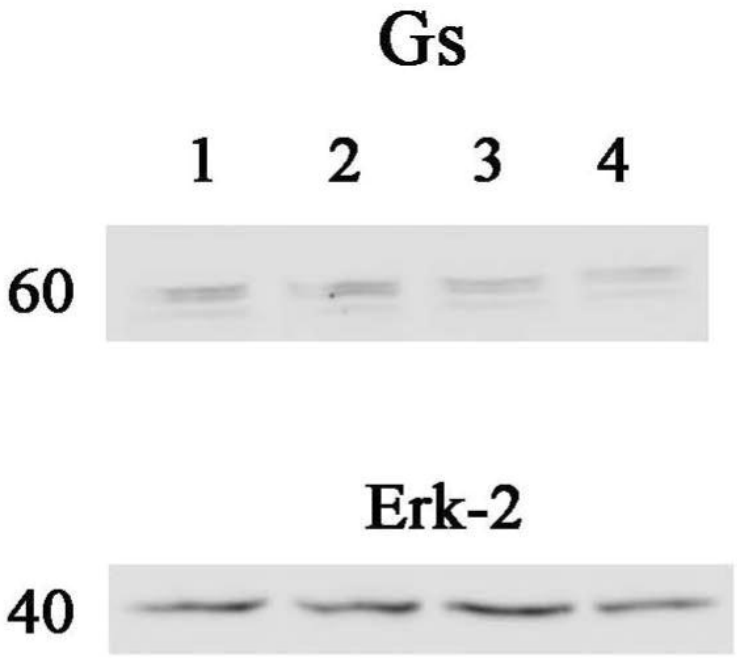


Figure 5B

A.



B.