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Serotonin Receptor 2A blocker (Risperidone) has no effect on Human Polyomavirus JC Infection of Primary Human Fetal Glial cells

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Abstract

A recent report demonstrated that JCV employs serotonin receptor 2A (5HT_{2A}R) to infect the glial cells. To assess the ability of a potent 5HT_{2A}R blocker, risperidone, to inhibit JCV infection, we treated primary human fetal glial (PHFG) cells *in-vitro* with risperidone for 24 hr and inoculated with JCV(Mad1). There was no significant difference in JCV genome copies or mRNA transcripts and protein expression in treatment-naive and risperidone-treated PHFG cells. This data indicate that risperidone does not inhibit JCV(Mad1) attachment, internalization and replication in PHFG cells, and 5HT_{2A}R blockers may not be effective in treating progressive multifocal leukoencephalopathy (PML).

Keywords

JCV; human polyomavirus; PML; serotonin receptor; risperidone; mirtazapine

Progressive multifocal leukoencephalopathy (PML), a fatal, subacute demyelinating disease of the central nervous system (CNS), is caused by human polyomavirus JC (JCV) (Padgett *et al*, 1971). Although, some AIDS-associated PML patients had survival benefit when treated with highly active antiretroviral therapy (HAART) (Antinori *et al*, 2003; Lima *et al*, 2007), in the post-HAART era incidence of PML has not significantly changed (Antinori *et al*, 2001), and PML currently is the second most frequently diagnosed neurological disorder among AIDS patients (Antinori *et al*, 2001). Few reports have demonstrated beneficial effect of cytosine arabinoside (Aksamit, 2001; De Luca *et al*, 1999; Elphick *et al*, 2004) and cidofovir (De Luca *et al*, 2001) in treating PML patients, however studies have concluded that cytosine arabinoside (Enting and Portegies, 2000) and cidofovir (Marra *et al*, 2002; Wyen *et al*, 2004) therapy have no significant therapeutic benefit. Although, clinical outcome among PML patients treated with interferon is controversial (Colosimo *et al*, 1992; Geschwind *et al*, 2001; Huang *et al*, 1998; Nath *et al*, 2006), IFN is effective in *in-vitro* inhibition of JCV replication and intrathecal

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infusion of IFN may be beneficial as an adjunct therapy for PML (Co *et al*, 2007). It is important to note that there is currently no proven therapy or vaccine available for treatment and prevention of the fatal disease, PML.

A recent study demonstrated that the chimera polyomavirus JC (Mad-1/SVEΔ) consisting of JCV-SV40 promoter enhancer sequences in the backbone of JCV coding region sequences employs the 5HT_{2A}R to infect SVG-A cells, a subclone of transformed human fetal glial cells by an origin-defective SV40 mutant (Elphick *et al*, 2004). Altschuler and Kast (2005), have suggested that newer safer antipsychotic drugs such as risperidone, ziprasidone and olanzapine with significantly more potent 5HT_{2A}R receptor antagonist activity, may be useful in treating or preventing PML. Since, half of the healthy adult population shed large amounts of JCV in the urine (Agostini *et al*, 2001; Agostini *et al*, 1997; Agostini *et al*, 1996; Shah *et al*, 1997), this provides an unique opportunity to test safe and easy-to-tolerate drugs such as, risperidone and mirtazapine for anti-JCV activity, using urinary tract clearance of JCV as an additional surrogate marker for potential in treating PML (Focosi *et al*, 2007b). Sure enough, these drugs were tested and some recent case reports have suggested survival benefit among HIV naive PML patients treated with risperidone (Focosi *et al*, 2007) or mitrazepam (Owczarczyk *et al*, 2007; Verma *et al*, 2007; Vulliemoz *et al*, 2006) and demonstrated clearance of JCV from blood (Focosi *et al*, 2007). It is possible that 5HT_{2A}R blockers may be effective in controlling JCV replication *in vivo*. However, it was unclear whether the beneficial effect observed in these patients was the result of modification of immunosuppressive treatment leading to immune reconstitution or was contributed by the 5HT_{2A}R blockers risperidone or mirtazapine. We recently demonstrated that human brain microvascular endothelial cells can be infected with JCV, independent of 5HT_{2A}R (Chapagain *et al*, 2007). Although, PHFG cells *in-vitro* express 5HT_{2A}R mRNA transcripts and protein (Chapagain *et al*, 2007), it remains yet unclear, whether oligodendrocytes, the primary target cells *in-vivo* express 5HT_{2A}R (Santagata and Kinney, 2005) and if 5HT_{2A}R blockers inhibit JCV replication in oligodendrocytes and are useful in preventing or treating PML.

To assess the effect of risperidone on JCV infection and replication in PHFG cells, we first tested the toxicity of risperidone to the PHFG cells. PHFG cells were isolated and cultured from therapeutically aborted 10- to 14-weeks-old fetal brains, as described previously, after obtaining approval from the institutional review board of the Kapi'olani Medical Center for Women and Children, Honolulu, Hawai'i (Chapagain *et al*, 2006; Padgett *et al*, 1977). Approximately 5,000 PHFG cells were grown per well in a 96-well plate either with media alone or in the continuous presence of risperidone (RISPERDAL[®] oral solution, Cat#: NDC 50458-305-03, Janssen Pharmaceutica N.V., Beerse, Belgium). Risperidone a monoaminergic antagonist for the serotonin Type 2, dopamine Type 2, α-1 and α-2 adrenergic, and H1 histaminergic receptors, is widely used to treat psychotic disorders and has high affinity for 5HT_{2A}R (Morisset *et al*, 1999). The medium, with or without risperidone was changed every fifth day and cell proliferation and viability were determined by the CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) on days 0, 5, 10, 15 after-incubation with risperidone. The absorbance in each well was measured according to the manufacturer's protocol at 490 nm using a multiplate reader (Victor³, Perkin Elmer, MA), and was expressed as percentage of untreated cells. Risperidone in concentration up to 200 ng/mL was non-toxic to PHFG cells *in-vitro* and PHFG cells untreated or treated with risperidone for up to 15 days did not show any change in the cell viability based on the cell proliferation assay (Fig. 1).

We next examined the effect of risperidone in PHFG cells infected with JCV (Mad1). PHFG cells grown to 70–80% confluency in each 35-mm culture plate, were incubated for 24 hr either with medium alone or with medium containing 12.5, 50 or 200 ng/mL of risperidone and inoculated with JCV containing $1.96 (\pm 0.53) \times 10^9$ and $1.36 (\pm 0.42) \times 10^9$ TAG and VP-1

genome copies, respectively, for 2 hr in the absence or continuous presence of risperidone. Cells were harvested on days 0, 5, 10 and 15 after-inoculation, and DNA (Qiagen QIAprep® Spin Miniprep Kit; Cat #27104) and RNA (Qiagen RNeasy® Plus kit; Cat #74134), extracted from 35-mm plates, were eluted in 100 μ L and 50 μ L of elution buffer, respectively. cDNA was synthesized from 0.5 μ g of total RNA using Bio-Rad's iScript® cDNA synthesis kit (Bio-Rad Inc., Hercules, CA) in a 20 μ L reaction mixture. Two μ L of template DNA or cDNA were amplified and quantitated in the Bio-Rad's iCycler iQ™ Multicolor Real-Time PCR Detection System using Bio-Rad 2X iQ™ Taqman® supermix, 12.5 pmol each of forward and reverse primers and 5 pmole of probes specific for JCV TAG (forward: 5'-AGA GTG TTG GGA TCC TGT GTT TT-3', reverse: 5'-GAG AAG TGG GAT GAA GAC CTG TTT-3' and probe 5'-/FAM/TCA TCA CTG GCA AAC ATT TCT TCA/BHQ-1/-3') or VP-1 (forward: 5'-ACT GTC CAT ATT TGT CAA CGT ATC-3', reverse: 5'-AAG GTC CAG CTA AGG AAA AGG-3' and probe 5'-/FAM/TCT GGG TCC CCT GGA AGC TCC TCT/BHQ-1/-3') in a final reaction volume of 20 μ L. Thermal cycling was initiated with the first denaturation step of 4 min at 95°C, followed by 40 cycles of 95°C (30 sec for TAG or 10 sec for VP-1) and 60°C (45 sec for TAG) or 58°C (10 sec for VP-1). Copies of JCV TAG or VP-1 genomes or mRNA transcripts in experimental samples were calculated from the standard curve and expressed as copies of viral genome per 35-mm plate or mRNA transcripts per microgram of total RNA. Based on the TAG and VP-1 genome copies, approximately 11.2% (SD \pm 2.6%) and 9.3% (SD \pm 0.98%), respectively, of JCV was attached or internalized into PHFG cells at 2 hr after-inoculation with JCV. However, there was no significant ($p > 0.05$) difference in the JCV TAG (Fig. 2A) or VP-1 (Fig. 2B) genome copies recovered at 2 hr after-inoculation from PHFG cells untreated or treated with risperidone at concentrations of 12.5, 50 or 200 ng/mL suggesting that there was no effect of risperidone on JCV attachment or internalization into PHFG cells. Similar numbers of JCV TAG and VP-1 genome copies were recovered from drug naive and risperidone treated PHFG cells harvested at different time points after-inoculation (Fig 2A and 2B) suggesting that risperidone has no effect in JCV genome replication. There was no significant difference ($p > 0.05$) in the JCV early (TAG) or late (VP-1) gene mRNA transcripts (Fig 2C and 2D) copies recovered from naive or risperidone-treated PHFG cells. Moreover, immunoprecipitation/immunoblotting of JCV T antigen protein (Chapagain *et al*, 2007), demonstrated similar amount of JCV T-antigen protein in naive or risperidone-treated PHFG cells harvested on days 10 and 15 after inoculation (Fig. 3) suggesting that risperidone does not have any significant effect on JCV early protein expression in PHFG cells.

Since, risperidone did not have significant effect on JCV replication, it was important to demonstrate that the risperidone we employed was functional. We therefore conducted receptor binding assays on membrane preparation of HEK-293 cells transfected with 5-HT_{2A}R pDNA. HEK-293 cells were transfected with the open reading frame (ORF) of human 5-hydroxytryptamine receptor 2A (GB accession no. NM_000621, University of Missouri cDNA resource center, Rolla, MO) using Lipofectamine™ 2000 transfection reagent (Invitrogen, cat# 11668-019), according to the manufacturer's instructions. Cells were either untreated or treated with 12.5, 50, 200 or 1000 ng/mL of risperidone for 24 hr, harvested at 72 hr after transfection, RNA was extracted, and 0.5 μ g of RNA was employed for cDNA synthesis. 5-HT_{2A}R gene was amplified using the 5-HT_{2A}R primer pair [forward: GGC ACA CGG GCC AAA TTA GC and reverse: TTG CTC ATT GCT GAT GGA CTG C] in a GeneAmp Thermal Cycler 9700 (Perkin- Elmer, Wellesley, MA) with 5 μ L of cDNA in a 50- μ L reaction mixture containing 1.0 U AmpliTaq Gold (Applied Biosystems, Foster City, CA), PCR buffer with 1.5 mM MgCl₂, 0.2 mM dNTPs and 0.4 μ M of each primer. An initial pre-PCR denaturation step (4 min at 95°C) was followed by 35 three-step PCR cycles of denaturation (95°C for 10 sec), annealing (58°C for 20 sec) and elongation (72°C for 30s) followed by a final elongation step (72°C for 10 min). Simultaneously, PCR was conducted using primers for house keeping gene GAPDH [forward: AGT TAG CCG CAT CTT CTT TTG C and reverse: CAA TAC GAC CAA ATC CGT TGA CT] as an internal control (Chapagain *et al*, 2007). The amplicons were

electrophoresed on a 2% agarose gel, and the ethidium bromide fluorescence was visualized after scanning with a Bio-Rad Molecular Phosphorimager. Our data suggest that transfected HEK-293 cells expressed 5-HT_{2A}R transcripts, and risperidone treatment at all concentrations does not inhibit 5-HT_{2A}R transcripts levels (fig. 4A). 5-HT_{2A}R transfection was further confirmed by immunostaining. At 72 hr after transfection, cells were fixed with 4% paraformaldehyde for 10 min, lightly permeabilized on ice (0.2% Triton X-100 in PBS) for 20 min, and incubated with blocking buffer (5% fetal bovine serum) for 1 hr. Cells were then incubated overnight with rabbit polyclonal anti 5-HT_{2A}R antibody (Cat# sc-50396, Santa Cruz Biotech, Inc) at 1:150 dilution, washed and incubated with FITC-conjugated goat anti-rabbit IgG secondary antibody (Cat# N1034, Amersham Biosciences, 1:150) for 1 hr. The cells were washed and mounted in vectashield mounting medium with DAPI (VECTOR Laboratories; Burlingame, California). The slides were examined by the Carl Zeiss Inverted microscope and images were captured with Zeiss AxioCam MRm camera and processed with the Axio Vision Rel. 4.5 software. Our data suggest that transfected HEK-293 cells express 5-HT_{2A}R on the cell surface (fig 4B), and risperidone treatment has no significant effect on 5-HT_{2A}R immunoreactivity (data not shown). Further, ketanserin binding assay was conducted to analyze the effect of risperidone treatment on the binding of [Ethylene-³H]-labelled ketanserin hydrochloride, (Cat# R41 468, 25 μ Ci, 925 kBq) (PerkinElmer Life and Analytical Sciences, Inc, Waltham, MA) as described elsewhere (Decker et al, 2004). Briefly, HEK-293 cells were seeded on 100-mm plates and transfected with 12 μ g 5-HT_{2A}R pDNA and 24 μ L of LipofectamineTM 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. At 72 hr after transfection, cells were washed with 1X PBS, scraped and centrifuged at 1,000 rpm for 10 min. The pellet was homogenized in 50 mM Tris-HCl (pH = 7.7) with a polytron homogenizer, centrifuged at 20,000 rpm, resuspended with 25 mM Tris-HCl (pH = 7.7), and aliquoted and stored at -80°C after measuring protein concentration by the Bradford assay. On the day of assay, the protein preparation was thawed and adjusted to 5 mg protein/80 mL with 25 mM Tris-HCl (pH 7.7) and 0.8 mL of cell homogenate (0.05 mg/protein/well) was added to wells containing 100 μ L of the risperidone at the final concentration of 0.125, 1.25, 12.5, 50 or 200 ng/mL or buffer, and 100 μ L of [³H] ketanserin (2.0 nM final concentration). The mixture was incubated at 37°C for 30 min, reaction was stopped by rapidly filtering through the glass fiber filter paper, washed four times with ice-cold 50 mM Tris-HCl (pH = 7.7) using FilterMateTM Cell Harvester (Packard Instrument Company, Meriden, CT), dried for 4 hr, and the radioactivity was measured using a Tri-Carb 2900TR Scintillation Counter (Perkin-Elmer). Nonspecific binding was determined by incubating with 10.0 μ M ritanserin. Risperidone at concentrations of 0.125, 1.25, 12.5, 50 or 200 ng/mL reduced [³H] ketanserin binding to 67.58 (\pm 4.28), 13.48 (\pm 2.35), 0.21 (\pm 1.1), 0.48 (\pm 3.28) and 0.16 (\pm 0.26) percent of the binding of untreated control respectively (fig. 4D). These data indicate that risperidone significantly reduces ketanserin binding in a dose dependent manner (fig 4D), and confirms that the risperidone we employed is functional.

Our data suggest that risperidone, a potent 5HT_{2A}R blocker (Altschuler and Kast, 2005; Morisset *et al*, 1999), does not inhibit JCV entry and replication in PHFG cells *in-vitro*. This result is consistent with our previous study demonstrating 5HT_{2A}R independent JCV infection of HBMVE cells (Chapagain *et al*, 2007), however, it contradicts with the previous study that suggested JCV uses 5HT_{2A}R to infect glial cells (Elphick *et al*, 2004). This inconsistency may be attributed to the use of different JCV strains or cells. While Elphick and colleagues employed chimeric JCV that has SV40 promoter, in this study, we used the JCV(Mad1), which is predominantly found among brains of PML patients (Dubois *et al*, 2001). It is possible that chimeric JCV (Mad-1/SVE Δ) behaves differently than JCV(Mad1) found in the PML brains. Moreover, we used PHFG cells, that most closely resemble the JCV target cells population *in-vivo* whereas Elphick et al used SVG-A cells, a subclone of SV-40 transformed human fetal glial cells (Elphick *et al*, 2004). It is also possible that JCV may use different receptors to infect different target cells. Although, PHFG cells *in-vitro* express 5HT_{2A}R mRNA transcripts and

protein (Chapagain *et al*, 2007), 5HT_{2A}R immunoreactivity was not detected in oligodendrocytes of rat spinal cord (Maxishima *et al*, 2001), and there is as yet no clear report demonstrating that human oligodendrocytes express 5HT_{2A}R (Santagata and Kinney, 2005). Interestingly, a recent publication also suggests that there was only a modest effect if any of risperidone on JCV infection of SVGA cells and more importantly, there was no significant difference in JC viral load between 5-HT_{2A}R blockers-treated and -untreated SVGA cells, which concur with our findings (O'Hara and Atwood, 2008). It is possible that human oligodendrocytes may not express 5HT_{2A}R, JCV may employ receptors other than 5HT_{2A}R to infect oligodendrocytes, and 5HT_{2A}R blockers may not be effective in treating PML. Further studies are essential to demonstrate expression of 5HT_{2A}R in oligodendrocytes, the primary target cells of JCV in the human brain, and to document the effectiveness of 5HT_{2A}R blockers in reducing JCV viral load in clinical trials before widespread use of 5HT_{2A}R blockers for treatment or prevention of PML.

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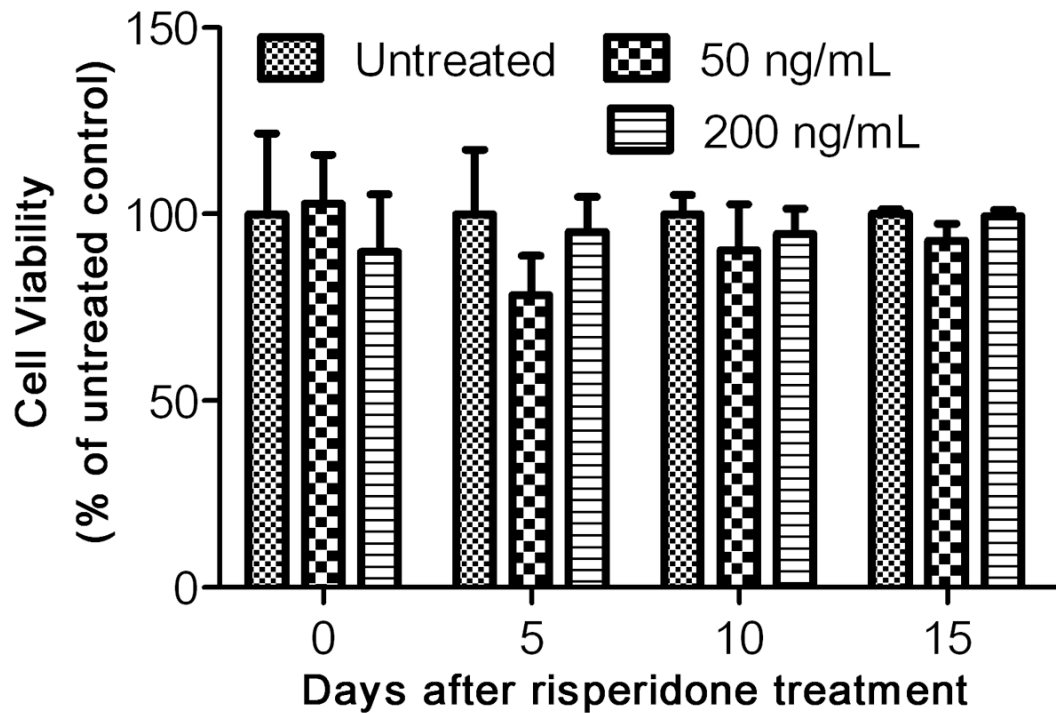


Fig. 1. Risperidone is non-toxic to PHFG cells

The cell proliferation and viability of PHFG cells grown in a 96-well plate either with media alone (untreated) or in the continuous presence of risperidone 50 or 200 ng/mL was determined by the CellTiter 96[®] AQueous One Solution Cell Proliferation Assay kit on days 0, 5, 10, 15 after-incubation with risperidone. The absorbance was measured at 490 nm and expressed as percentage of untreated-treated cells. Data are representative of mean of six independent samples in each group at each time point and error bars represent standard deviations.

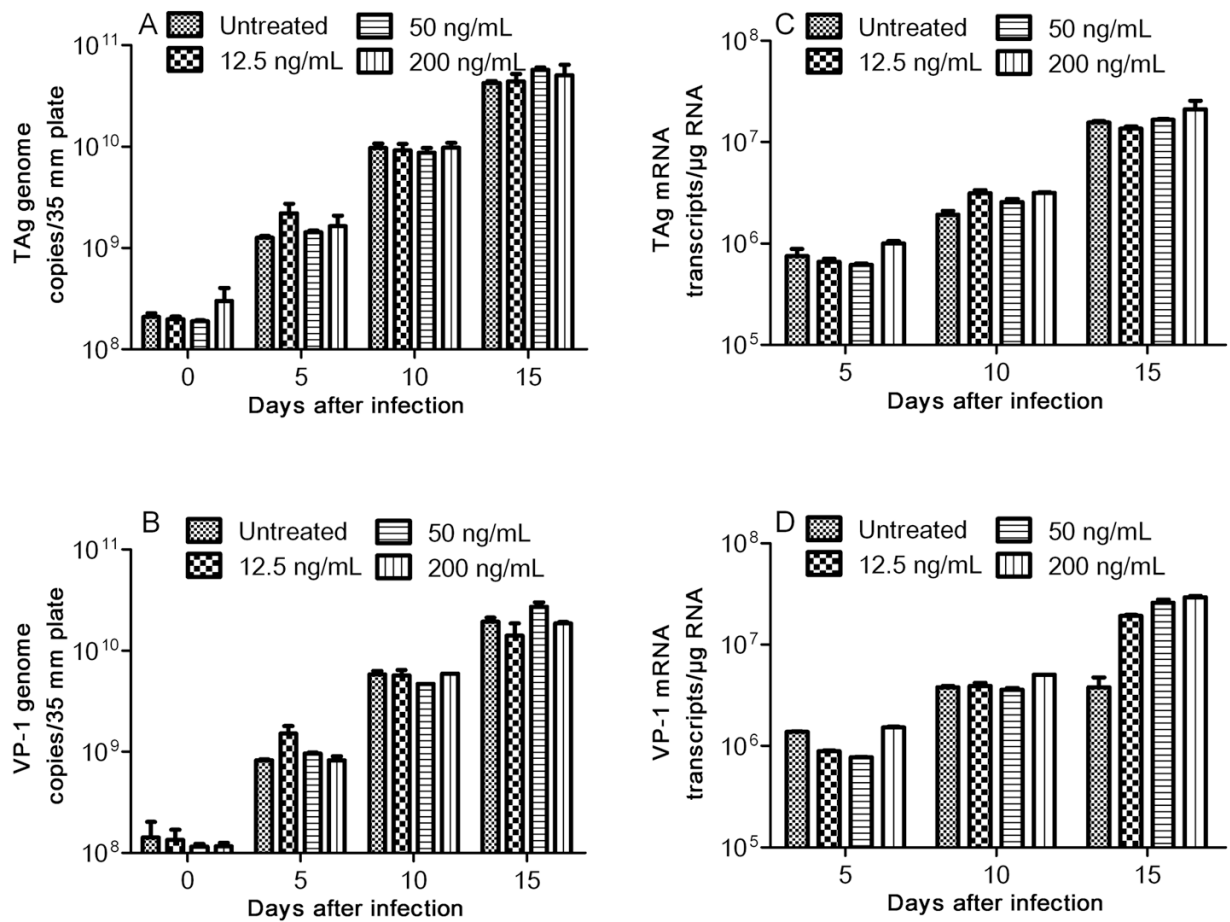


Fig. 2. Risperidone has no effect in JCV infection of PHFG cells

PHFG cells were treated with risperidone at a concentration of 12.5, 50, or 200 ng/mL for 24 hr and inoculated with JCV for 2 hr and harvested at the indicated time points for DNA or RNA extraction. JCV TAG (Fig. 2A) and VP-1 (Fig. 2B) genome or TAG (Fig. 2C) and VP-1 (Fig. 2D) transcripts were amplified and quantitated by real-time PCR or real-time RT-PCR. Data are representative of duplicate samples assayed in three independent experiments. Error bars represent standard deviations.

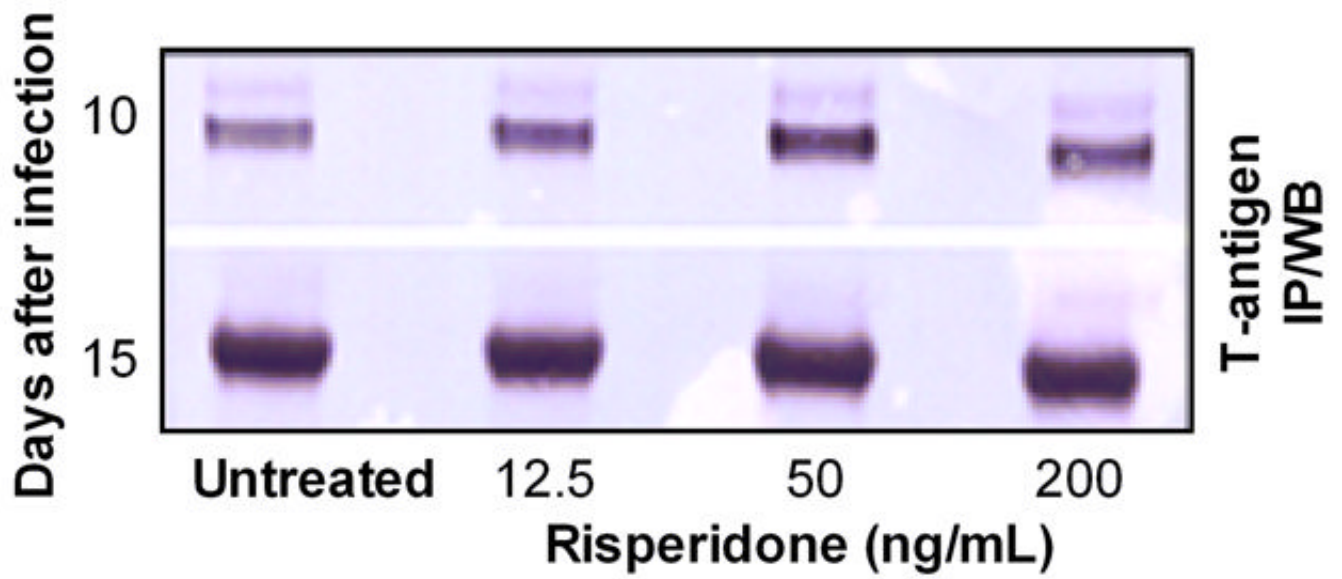


Fig. 3. Risperidone does not inhibit JCV T antigen protein expression in PHFG cells
JCV-infected and naive PHFG cells were harvested at the indicated time points and JCV T antigen protein was detected in JCV-infected HBMVE cells by immunoprecipitation and Western blotting.

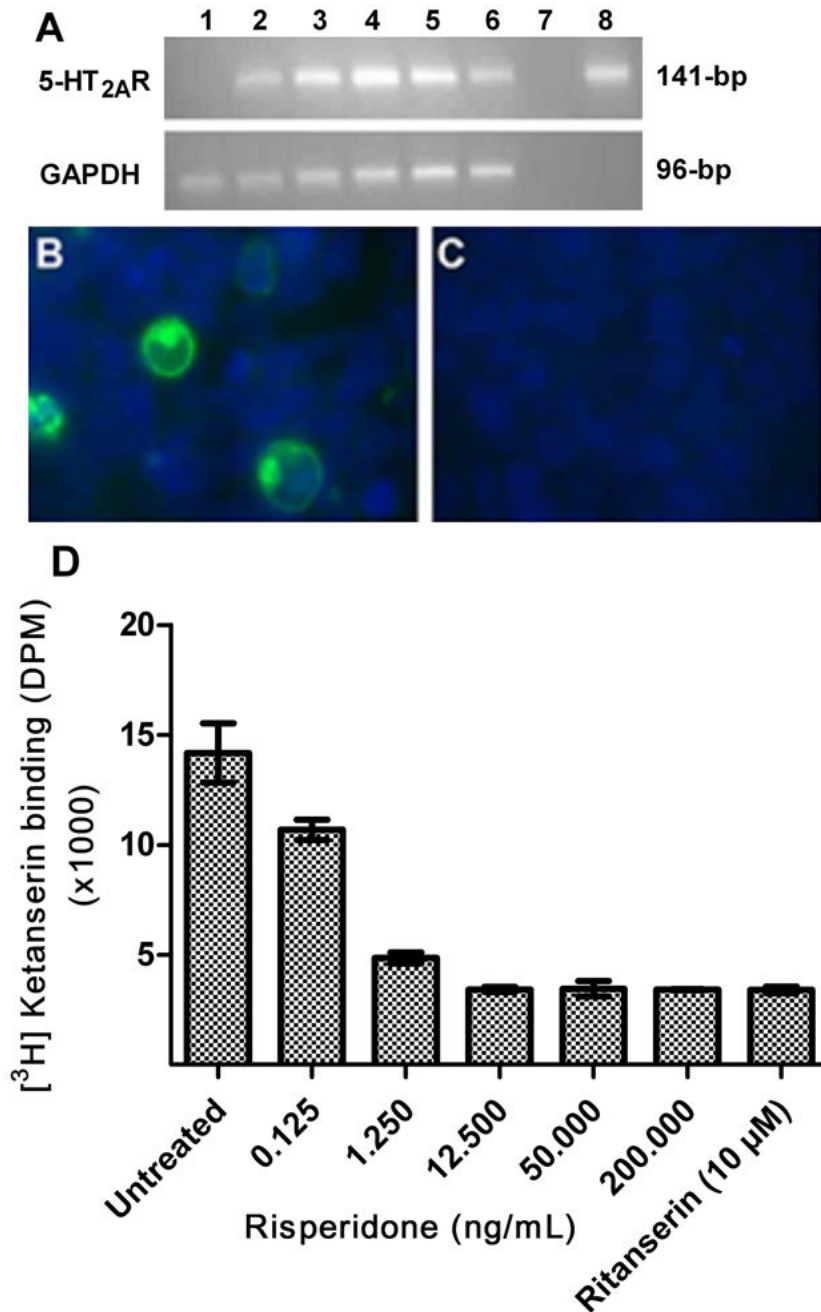


Fig. 4. Transfection of HEK-293 cells with 5-HT_{2A}R pDNA construct

(A) HEK-293 cells at 72 hr after transfection expressed 5-HT_{2A}R transcripts but risperidone treatment had no negative effect on mRNA expression. HEK-293 cells untransfected (lane 1); transfected and untreated (lane 2); transfected and treated with 12.5 (lane 3); 50 (lane 4), 200 (lane 5); and 1,000 (lane 6) ng/mL of risperidone; transfected and untreated HEK-293 cells without reverse transcriptase (negative control) (lane 7); and 5-HT_{2A}R pDNA (positive control) (lane 8). (B) Transfected HEK-293 cells demonstrated 5-HT_{2A}R immunoreactivity (green), and nuclei were stained with bisbenzidine (blue). (C) Control empty vector transfected cells. (D) Ketanserin binding is inhibited by risperidone in a dose dependent manner. [Ethylene-³H]-labeled ketanserin hydrochloride with or without risperidone was co-incubated

with HEK293 membrane protein preparation and the radioactivity was measured. Data represents the mean DPM with standard deviation.