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Serotonin-2C and -2A Receptor Co-expression on Cells in the Rat Medial Prefrontal Cortex

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Abstract

Neural function within the medial prefrontal cortex (mPFC) regulates normal cognition, attention and impulse control, implicating neuroregulatory abnormalities within this region in mental dysfunction related to schizophrenia, depression and drug abuse. Both serotonin -2A ($5\text{-}HT_{2A}$) and -2C ($5\text{-}HT_{2C}$) receptors are known to be important in neuropsychiatric drug action and are distributed throughout the mPFC. However, their interactive role in serotonergic cortical regulation is poorly understood. While the main signal transduction mechanism for both receptors is stimulation of phosphoinositide production, they can have opposite effects downstream. $5\text{-}HT_{2A}$ versus $5\text{-}HT_{2C}$ receptor activation oppositely regulates behavior and can oppositely affect neurochemical release within the mPFC. These distinct receptor effects could be caused by their differential cellular distribution within the cortex and/or other areas. It is known that both receptors are located on GABAergic and pyramidal cells within the mPFC, but it is not clear whether they are expressed on the same or different cells. The present work employed immunofluorescence with confocal microscopy to examine this in layers V-VI of the prelimbic

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mPFC. The majority of GABA cells in the deep prelimbic mPFC expressed 5-HT_{2C} receptor immunoreactivity. Furthermore, most cells expressing 5-HT_{2C} receptor immunoreactivity notably co-expressed 5-HT_{2A} receptors. However, 27% of 5-HT_{2C} receptor immunoreactive cells were not GABAergic, indicating that a population of prelimbic pyramidal projection cells could express the 5-HT_{2C} receptor. Indeed, some cells with 5-HT_{2C} and 5-HT_{2A} receptor co-labeling had a pyramidal shape and were expressed in the typical layered fashion of pyramidal cells. This indirectly demonstrates that 5-HT_{2C} and 5-HT_{2A} receptors may be commonly co-expressed on GABAergic cells within the deep layers of the prelimbic mPFC and perhaps co-localized on a small population of local pyramidal projection cells. Thus a complex interplay of cortical 5-HT_{2A} and 5-HT_{2C} receptor mechanisms exists, which if altered, could modulate efferent brain systems implicated in mental illness.

Keywords

5-HT; GABA; pyramidal; immunofluorescence; 5-HT_{2A} receptor; 5-HT_{2C} receptor

1. INTRODUCTION

The medial prefrontal cortex (mPFC) plays a critical executive role in working memory, attention and impulse control. Lesions of the PFC in animals (Goldman et al., 1971;Fritts et al., 1998; though see D'Esposito et al., 2006) and humans (Barbey et al., 2013;Tsuchida and Fellows, 2013) disrupt working memory. mPFC lesions also diminish the ability to attend to life-threatening or -enhancing environmental stimuli (Wilkins et al., 1987;Passetti et al., 2002;Ng et al., 2007;Lovstad et al., 2012) and to restrain behavior when needed (Perret, 1974;Muir et al., 1996;Quirk et al., 2000;Chudasama et al., 2003;although see, Eagle et al., 2008). It is thus not surprising that abnormalities in the mPFC have been associated with schizophrenia, depression and drug addiction; illnesses that are characterized with these cognitive and behavioral disturbances (Altman et al., 1996;Drevets, 2000;George et al., 2001;Brody et al., 2001;Stockmeier and Rajkowska, 2004;Mayberg et al., 2005;Lambe et al., 2007;Driesen et al., 2008;Kalivas, 2008;Covington et al., 2010;Li et al., 2011;Nocjar et al., 2012).

Serotonin, which interacts with at least 14 different receptor subtypes (Hoyer et al., 1994;Roth et al., 2000;Berger et al., 2009), is thought to play an important role in these psychological disorders (Roth and Meltzer, 1995;Kosten et al., 1998;Aghajanian and Marek, 2000;Manji et al., 2001;Nestler et al., 2002;Celada et al., 2004;Cunningham et al., 2013). The serotonin -2A and -2C receptor subtypes (5-HT_{2A}R and 5-HT_{2C}R, respectively) are widely dispersed throughout the mPFC, although density of 5-HT_{2A}Rs is higher (Leysen et al., 1982;Ashby et al., 1990;Mengod et al., 1990;Pompeiano et al., 1994;Lopez-Gimenez et al., 1997;Willins et al., 1997;Jakab and Goldman-Rakic, 1998;Clemett et al., 2000;Pandey et al., 2006;Liu et al., 2007;Yadav et al., 2011b). Both receptors are implicated in antipsychotic (Roth et al., 1992;Martin et al., 1998;Willins et al., 1999;Rauser et al., 2001;Bonaccorso et al., 2002), antidepressant (Cryan and Lucki, 2000;McMahon and Cunningham, 2001;Van Oekelen et al., 2003;Serretti et al., 2004;Millan, 2005;Opal et al., 2013) and addictive drug action (e.g. McMahon and Cunningham, 1999;Van Oekelen et al., 2003;Cunningham et al.,

2013), with those localized to the mPFC purportedly playing a vital role (e.g. Aghajanian and Marek, 1999;Tarazi et al., 2002;Filip and Cunningham, 2003;Celada et al., 2004;Ramos et al., 2005;Huang et al., 2006;Pehek et al., 2006;Carli et al., 2006;Pockros et al., 2011;Opal et al., 2013). Impaired cortical 5-HT_{2A}R and 5-HT_{2C}R function could thus contribute to a variety of neuropsychiatric diseases, but how this might occur is unclear (Meltzer and Roth, 2013).

The main signal transduction mechanism for 5-HT_{2A} and 5-HT_{2C} receptors is stimulation of phosphoinositide production (Roth et al., 1984;Conn and Sanders-Bush, 1986;Sanders-Bush et al., 1988;Araneda and Andrade, 1991;Rick et al., 1995;Garcia et al., 2007), but the direct cellular excitation induced by their activation can produce opposite effects downstream. 5-HT_{2A} versus 5-HT_{2C} receptor activation oppositely affects dopamine release within the mPFC (for review see Di Matteo et al., 2001;Alex and Pehek, 2007). The receptors also oppositely regulate behavior controlled by the mPFC (Williams et al., 2002;Winstanley et al., 2004;Mirjana et al., 2004;Ramos et al., 2005;Bubar and Cunningham, 2006;Carli et al., 2006;Pentkowski and Neisewander, 2008;Jensen et al., 2010;Pockros et al., 2011;Cunningham et al., 2013). Although indicating that a differential cellular distribution of 5-HT_{2A} and 5-HT_{2C} receptors likely exists in the brain, it is not clear whether this occurs in the mPFC.

The two major cell types in the mPFC are GABA local circuit interneurons and glutamate-containing pyramidal projection neurons (Fuster, 1997;Gabbott et al., 1997), with local GABA release playing a crucial regulatory function over mPFC pyramidal output (see Eyles et al., 2002). 5-HT_{2A}R and 5-HT_{2C}R expression has been seen on both GABA and pyramidal cells within the prelimbic mPFC (Willins et al., 1997;Jakab and Goldman-Rakic, 1998;Jakab and Goldman-Rakic, 2000;Carr et al., 2002;Santana et al., 2004;Liu et al., 2007), but whether they are individually expressed or localized on the same cell is still not clear. The present work employed immunofluorescence with confocal microscopy to examine this in the prelimbic mPFC of rats.

2. EXPERIMENTAL PROCEDURES

2.1 Animals

Six naive male Sprague-Dawley rats (Harlan, Indianapolis, USA) weighing 330–400 g were used. Rats were housed in pairs and maintained for at least 1-month after arrival in a temperature and humidity controlled rodent colony room with food and water available *ad libitum*. Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, IP) and transcardially perfused with 250ml of phosphate-buffered saline (PBS; 8g NaCl, 1.44g Na_2HPO_4, 240mg KH_2PO_4, 200mg KCl in 1L dH_2O, *p*H 7.40) and then with 500 ml of a 4% paraformaldehyde PBS solution. Brains were harvested, postfixed for 24 hours in 4% paraformaldehyde in PBS and then cryoprotected in a 30% sucrose PBS solution until they sank (~ 36–48 hrs). Brains were then rinsed in PBS, rapidly frozen on crushed dry ice and stored at −80°C until sectioning. 40 μm coronal sections containing the mPFC (bregma +3.76 to 3.20mm) were collected according to the rat brain atlas of Paxinos and Watson (2007) using a cryostat set to −23°C (Microm International, Germany). Alternating sections from the same animal were placed in two separate well-plates containing ice-cold PBS. One

well-plate with its free-floating sections was used and processed in Experiment II and the other in Experiment III (See immunofluorescent microscopy). All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local institutional animal care and use committee. All efforts were made to minimize the number of animals used and their suffering.

2.2 Antibodies

This study conducted three experiments using the primary antibodies listed in Table 1. The D-12 mouse monoclonal 5-HT_{2C}R antibody (Santa Cruz Biotechnology) has been shown to selectively detect human (Anastasio et al., 2010) and rat 5-HT_{2C}Rs (Morabito et al., 2010) in prior western blot work. Experiment I extended this by conducting both western blot and confocal immunofluorescent assessments of D-12 5-HT_{2C}R protein detection in PO1C cells that express rat 5-HT_{2C}Rs and in GF62 cells that only express rat 5-HT_{2A}Rs (Experiment Ia and Ib, respectively).

Experiment II used immunofluorescent confocal microscopy to determine whether D-12 also performed similar to other 5-HT_{2C}R antibodies by detecting 5-HT_{2C}R expression on GABAergic cells in the prelimbic mPFC (Liu et al., 2007). To localize GABA cell expression in the mPFC, the rabbit H101 anti-GAD-67 (glutamic acid decarboxylase isoform 67, Santa Cruz Biotechnology, California) and PV 25 anti-parvalbumin antibodies (Swant, Switzerland) were used as indicated in Table 1. GAD-67 is an enzyme involved in the synthesis of GABA, thus antibodies raised against the enzyme are useful in the identification of GABA-synthesizing cells in the brain. The H101 GAD-67 antibody has been shown to detect a similar number of GABA cells as other anti-GABA antibodies (Akema et al., 2005). Parvalbumin is a calcium-binding protein that is found in basket and chandelier subtype GABAergic cells (Conde et al., 1994;Gabbott et al., 1997) that directly modulate efferent signaling of cortical pyramidal neurons (Miles et al., 1996;Markram et al., 2004;Lewis et al., 2005). Specificity of the PV 25 anti-parvalbumin antibody has been validated in immunohistochemistry studies of cortical and muscle tissue from wild type versus parvalbumin knockout mice (Schwaller et al., 1999;Schwaller et al., 2004).

Experiment III used the same D-12 5-HT_{2C}R antibody to determine whether cells that express 5-HT_{2C}Rs in the prelimbic mPFC also co-express 5-HT_{2A}R s. As indicated in Table 1, the rabbit immunostar 5-HT_{2A}R antibody from Neuromics was used. We and others have validated the receptor specificity of this antibody in western blot and immunohistochemistry studies of cortical tissue from 5-HT_{2A}R knockout and wild-type mice (Magalhaes et al., 2010;Weber and Andrade, 2010;Yadav et al., 2011a). The antibody also sensitively detects changes in cortical 5-HT_{2A}R levels (Yadav et al., 2011b).

Fluorescent-conjugated secondary antibodies from Invitrogen (Eugene, OR, USA) were used in all experiments to visualize primary antibody staining: Alexa Fluor 488 goat anti-mouse (fluoresces green), Alexa Fluor 594 goat anti-rabbit (fluoresces red).

2.3 Western Blots

To determine western blot D-12 5-HT_{2C}R specificity in PO1C versus GF62 cells in Experiment Ia (see Antibodies above), PO1C and GF62 cells were pelleted by centrifugation

and then pellets were lysed in 1 mL of Hepes buffer including CHAPS and protease inhibitors to prepare lysates. Lysates were normalized for protein content. Half of the lysates for each cell type were incubated with Wheat Germ Agglutinin/lectin beads for 2 hours at 4°C. SDS sample buffer was added to the lysates and beads, which were then incubated at 67°C for 5 minutes. Beads were then spun down and 30 μL from the top of each sample was loaded onto the gel. The protein was then transferred to a nitrocellulose membrane overnight, followed by one hour incubation in blocking buffer (tris buffered saline [TBS], 0.1% Tween, 5% milk) and 2 hours incubation in the primary antibody solution (1:500 D-12 goat-anti-mouse 5-HT_{2C}R antibody, Santa Cruz, CA). The membranes were then washed several times, incubated in horseradish-peroxidase secondary antibody, and washed again several times. Finally, the membranes were incubated in western blot substrate and developed.

2.4 Immunofluorescent Microscopy

To further validate D-12 5-HT_{2C}R specificity, immunofluorescent assessments were also conducted on cultured PO1C and GF62 cells in Experiment Ib (see Antibodies above). Cells were grown on coverslips, then permeabilized with 0.3% triton X-100 (in PBS) for 15 minutes, exposed to a PBS blocking buffer that contained 5% milk, 4% normal goat serum and 0.3% triton X-100 for at least one hour, and individually incubated in a solution containing the primary antibody (D-12 anti-5-HT_{2C}R in blocking buffer, 1:100; see Table 1) for 2-hours at room temperature and then overnight at 4°C. They were then washed five times in 0.3% triton X-100, incubated at room temperature in secondary antibody (Alexa Fluor 488 goat anti-mouse, 1:200 in blocking buffer; see Antibodies) for one hour, and then washed four times in 0.3% triton X-100 and once in PBS. 5-HT_{2C}R expression was then visualized using a Zeiss LSM Confocal Microscope with digital imaging software (Carl Zeiss, Thornwood, NY).

Dual immunofluorescent microscopy of rat brain tissue was conducted in Experiment II to determine whether D-12 performs like other 5-HT_{2C}R antibodies by detecting 5-HT_{2C}R expression on GABA cells in the prelimbic mPFC (Liu et al., 2007). Experiment III determined whether 5-HT_{2C}R-IR cells within this region also expressed 5-HT_{2A}R s. Free-floating rat brain sections (see Animals above) were allowed to equilibrate to room temperature on a gentle orbital shaker for 20min. Using room temperature solutions and continued gentle shaking, sections were permeabilized in 0.3% Triton X-100 in PBS for 1hour, incubated in 0.03% Triton X-100 blocking buffer (60μl Triton X-100, 1600μl normal goat serum and 2g non-fat dry milk in 40ml PBS) for 2-hours, and then incubated in blocking buffer containing two primary antibodies (Experiment II and III:D-12 and anti-GAD-67 or anti-parvalbumin antibodies, Experiment III: D-12 and 5-HT_{2A}R antibodies; at the concentrations described in Table 1) for 2 hours and then for 72 hours at 4°C. While under continued gentle shaking, sections were allowed to return to room temperature for 20min, and then with room temperature solutions, were washed 3× in 0.03% Triton X-100 in PBS for 10min each, incubated in blocking buffer containing the secondary antibodies (Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit; 1:200 and 1:300 respectively in blocking buffer, see Antibodies) for 1hour protected from light, then washed 4× in 0.03% Triton X-100 and 1× in PBS for 10min each. Sections were then mounted onto

slides with Vectashield Fluorescent Mounting Medium (Vector Laboratories), coverslipped, sealed with clear nail polish, air dried for 20min while protected from light and then stored at 4°C until viewed. Brain tissue expression of the two fluorescence tagged antibodies within an experiment (Experiment II and III:D-12 and anti-GAD-67 or anti-parvalbumin antibodies, Experiment III: D-12 and 5-HT_{2A}R antibodies) was visualized and photographed as in our prior work (Nocjar et al., 2002; Burke et al., 2014) using a dual channel Zeiss LSM5 Confocal Microscope with digital imaging software (Carl Zeiss, Thornwood, NY). All digital photomicrographs were of single optical sections and analyzed in Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA) using the count tool. Immunofluorescent labeling by each of the two antibodies was analyzed separately under either the red or green channel, with cells identified and counted under both channels indicating co-localized immunolabeling.

3. RESULTS

3.1 D-12 5-HT_{2C} receptor immunoreactivity was selectively demonstrated in POIC versus GF62 cells

Experiments Ia and Ib were conducted to validate the receptor specificity of D-12 in POIC and GF62 cells that are known to differentially express the rat 5-HT_{2C} and 5-HT_{2A} receptors. As illustrated in Fig1A and 1B, POIC cells that express 5-HT_{2C}Rs consistently showed D-12 5-HT_{2C}R antibody expression under Western Blot and immunofluorescent microscopy assessments. However, GF62 cells that only express 5-HT_{2A}Rs showed no D12 immunoreactivity in either test, indicating that D-12 is a 5-HT_{2C}R specific antibody.

3.2 D-12 5-HT_{2C} receptor immunoreactivity was expressed in GABA cells of the rat prelimbic mPFC

Experiment II was conducted to determine whether the D12 5-HT_{2C}R antibody colabeled GABAergic cells like another 5-HT_{2C}R antibody (Liu et al., 2007). Fig 2A shows D-12 antibody expression (green fluorescence) on GABA cells (red fluorescence) in the deep layers of the prelimbic mPFC (see prelimbic area assessed in B). As seen in A, D-12 detects 5-HT_{2C}R expression within cell soma and their initial segment. Importantly, it detected 5-HT_{2C}R expression in both GAD-67 and parvalbumin GABA cells in the mPFC (see white arrows in Fig 2A, top and bottom rows respectively) as previously reported using a different 5-HT_{2C}R antibody (Liu et al., 2007). Because it acted similarly to another 5-HT_{2C}R antibody and selectively identified cells that expressed the rat 5-HT_{2C}R in the above experiment as in prior work (Morabito et al., 2010), the D-12 5-HT_{2C}R antibody was used throughout the remaining study.

3.3 5-HT_{2A} and 5-HT_{2C} receptors were expressed in a laminar overlapping fashion in the most rostral prelimbic mPFC and were co-expressed on cells in layer V

Experiment III was conducted to determine whether mPFC cells that expressed 5-HT_{2C}Rs also expressed 5-HT_{2A}Rs. Fig 3 shows a photomontage of 5-HT_{2A}R and 5-HT_{2C}R expression across the mediolateral extent of the most rostral level of the prelimbic mPFC (see AP location in the cartoon brain representation shown in B). As seen in A (middle row), a profuse laminar distribution of 5-HT_{2A}R immunoreactivity was detected in the rat mPFC

using the Immunostar 5-HT_{2A}R antibody from Neuromics (see Table1). The strongest 5-HT_{2A}R immunoreactivity was seen in layer V. Though quite profuse, the 5-HT_{2A}R immunostaining produced by the antibody is typical for this cortical region (Weber and Andrade, 2010;Yadav et al., 2011b). Under higher magnification, a punctate 5-HT_{2A}R expression could be seen on the soma and initial segment of cells in the region (see sample cell in B, center and left photo). Though impossible to identify in B due to dense 5-HT_{2A}R staining in this layer, the middle panel in A clearly shows labeled neuronal processes in more superficial layers as identified previously with this antibody in the mouse prelimbic mPFC (Yadav et al., 2011a). The bottom row of photomicrographs in A shows that 5-HT_{2C}R s in the identical mPFC tissue also had a laminar distribution; which is notably similar to that seen in an earlier report (Liu et al., 2007). However, this figure shows a population of 5-HT_{2C}R -IR cells within superficial layers II–III which was negligible in this earlier report at a more posterior location of the prelimbic mPFC (Liu et al., 2007); perhaps due to the rapid anteroposterior decrease in 5-HT_{2C}R expression demonstrated in the mPFC (Pompeiano et al., 1994). Most importantly, B shows 5-HT_{2C}R and 5-HT_{2A}R co-localization within the soma and initial segment of a sample cell in layer V of this rostral mPFC region (see black box in cartoon brain representation for approximate dorsoventral location of cell in subregion).

3.4 The majority of cells showing 5-HT_{2C} receptor immunoreactivity in layer V-VI of the prelimbic mPFC also co-expressed 5-HT_{2A} receptors

Fig 4 shows layer V 5-HT_{2C} and 5-HT_{2A} receptor expression at a more posterior level of the prelimbic mPFC in these same animals (see AP level in E). We first confirmed that 5-HT_{2C}R-IR cells (green) in layer V co-expressed the GAD-67 GABA cell marker as we found in the rostral prelimbic mPFC in experiment II (see sample cell with yellow arrow in A). Photos in B confirmed that layer V also had the same profuse laminar 5-HT_{2A}R expression (red) and cellular 5-HT_{2C}R expression (green) as we found above in the deep layers of the more rostral prelimbic mPFC (see Fig 3). Note the typical strong 5-HT_{2A}R immunoreactivity in layer V_a that rapidly diminishes laterally in layer V_b (see Fig 3; Weber and Andrade, 2010). Most notably, many 5-HT_{2C}R-IR cells in Layer V of the prelimbic mPFC were found to co-express 5-HT_{2A}Rs. The white arrow in B shows sample cells below it with 5-HT_{2C}R and 5-HT_{2A}R co-localization in layer V_a [as delineated by Weber & Andrade (2010)]. These cells can be seen more clearly in C. The yellow arrow shows the same punctate 5-HT_{2A}R expression encircling the cell's nucleus as we found above in the rostral prelimbic mPFC. The white arrowhead in B shows a representative cell with 5-HT_{2C}R and 5-HT_{2A}R co-localization in layer V_b [as delineated by Weber & Andrade (2010)]. This co-localization can be clearly seen in D. As indicated in Table2, nearly 70% of 5-HT_{2C}R-IR cells in layers V–VI of this prelimbic mPFC region coexpressed 5-HT_{2A}Rs (brain cartoon blackened box indicates area assessed according to Paxinos and Watson (2007)).

3.5 Most cells that expressed 5-HT_{2C} receptors in the deep layers of the prelimbic mPFC were GABAergic, suggesting that the majority of cells that co-express 5-HT_{2C} and 5-HT_{2A} receptors are likely GABA cells. The remaining cells with 5-HT_{2C} receptors must be

pyramidal, and we found some pyramidal-shaped cells with 5-HT$_{2C}$ and 5-HT$_{2A}$ receptor co-labeling

It is well known that layer V of the prelimbic mPFC contains a high number of pyramidal neurons with GABAergic cells dispersed throughout it. Thus we wanted to assess the approximate percentage of 5-HT$_{2C}$R-IR cells that co-expressed GAD-67 in the region versus those that did not to determine the differential expression of these receptors on GABAergic versus pyramidal neurons in the region. Table 3 shows that approximately 73% of 5-HT$_{2C}$R-IR cells in layers V-VI of the prelimbic mPFC are GABAergic (same prelimbic level assessed as in Table 2). Glutamate pyramidal projection cells are the only other cells in the region. Thus, the remaining 27% of 5-HT$_{2C}$R-IR cells in the region are likely pyramidal cells.

Fig 5 indicates that a portion of these pyramidal 5-HT$_{2C}$R-IR cells might co-express 5-HT$_{2A}$Rs. As seen in A, some cells demonstrating 5-HT$_{2C}$R and 5-HT$_{2A}$R co-expression in layer V of the prelimbic mPFC have a pyramidal shape (see arrowed sample) and tightly layered distribution that is typical of glutamate pyramidal projection cells. B shows another tightly layered 5-HT$_{2C}$R cell distribution with 5-HT$_{2A}$R co-localization (see white arrows). C however, shows a more diffuse expression of 5-HT$_{2C}$R-IR cells within the same region with GAD-67 GABAergic co-expression (see yellow arrows). Thus although the majority of cells that express 5-HT$_{2C}$ and 5-HT$_{2A}$ receptor co-localization in the mPFC are likely GABAergic, some could be pyramidal.

4. DISCUSSION

This study found that the majority of neurons expressing 5-HT$_{2C}$R-IR in layers V–VI of the prelimbic mPFC also co-expressed the 5-HT$_{2A}$R, demonstrating that a cellular subpopulation within the deep layers of the prelimbic mPFC could be directly co-regulated by 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors. These cells are likely GABAergic for the most part since 73% of 5-HT$_{2C}$R-IR cells in this region co-expressed the GABA cell marker GAD-67. Though 5-HT$_{2C}$R and 5-HT$_{2A}$R protein have each been detected previously on prelimbic GABAergic cells (Willins et al., 1997;Liu et al., 2007), this is the first demonstration that GABA cells may co-express both receptor proteins within the deep layers of the prelimbic cortex where GABAergic cells are known to provide a critical inhibitory control over efferent pyramidal projections from the mPFC (Eyles et al., 2002). Interestingly, a recent report found pyramidal shaped 5-HT$_{2C}$R-IR cells in the prelimbic mPFC (Liu et al., 2007), and we found that 27% of 5-HT$_{2C}$R -IR cells in the deep prelimbic mPFC were *not* GABAergic cells. Also, some cells with 5-HT$_{2C}$R and 5-HT$_{2A}$R co-labeling in this region had a pyramidal shape and tightly layered distribution that is typical of pyramidal cellular expression. This suggests that 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors may also be co-localized on a small population of pyramidal cells in Layer V.

It is unlikely that the evidenced cellular 5-HT$_{2C}$R and 5-HT$_{2A}$R co-immunoreactivity was due to antibody non-specificity. Both antibodies employed are specific for their respective receptor. Though there has been specificity issues raised regarding some 5-HT$_{2A}$R antibodies (Weber and Andrade, 2010), we used the Immunostar 5-HT$_{2A}$R antibody that generates immunolabeling in wild-type but not 5-HT$_{2A}$R knockout animals (Magalhaes et

al., 2010;Weber and Andrade, 2010). A gradient anteroposterior distribution of cortical 5-HT_{2A}R expression has also been identified with this antibody (Weber and Andrade, 2010) as seen in 5-HT_{2A}R binding, mRNA and gene expression work (Blue et al., 1988;Pompeiano et al., 1994;Lopez-Gimenez et al., 1997). Specificity of the D12 5-HT_{2C}R antibody employed has also been confirmed. Prior western blot work validated that D12 selectively induced immunolabeling in Chinese hamster ovary (CHO) cells that expressed the human 5-HT_{2C}R but not in parental CHO cells that lack the receptor (Anastasio et al., 2010). Immunofluorescent microscopy in the current work also detected selective D-12 immunolabeling in POIC cells that express **rat** 5-HT_{2C}Rs, but not in GF62 cells that express 5-HT_{2A}Rs. The same findings were found with western blot replicating prior work (Morabito et al., 2010). Western blot D-12 assessments also sensitively detect increases and decreases in 5-HT_{2C}R protein levels in brain tissue and mirror 5-HT_{2C}R binding, function and behavioral assessments (Morabito et al., 2010; Abbas et al., 2009). Moreover, D12 co-labeled both GAD-67 and parvalbumin -identified GABAergic cells in the deep prelimbic mPFC in the current work as previously seen with another 5-HT_{2C}R specific antibody (Liu et al., 2007;Anastasio et al., 2010), and genetic 5-HT_{2C}R knockdown reduced D-12 5-HT_{2C}R immunolabeling in mPFC tissue of rats (Anastasio et al., 2014).

We found a striking laminar distribution of both 5HT2 receptor proteins in the rat mPFC. 5-HT_{2A}R immunoreactivity was extremely profuse in the deep cellular layers of the prelimbic mPFC, particularly in layer V. In superficial layers I-III, rather sparse 5-HT_{2A}R dispersion progressed laterally to a highly localized expression on neural processes. This laminar expression is nearly identical to that reported in mouse mPFC with the same Immunostar 5-HT_{2A}R antibody (Magalhaes et al., 2010;Weber and Andrade, 2010;Yadav et al., 2011a); it is not seen if an antibody lacks 5-HT_{2A}R specificity (Weber and Andrade, 2010). Importantly, our laminar expression mirrors 5-HT_{2A}R binding (Pazos et al., 1985;Blue et al., 1988;Mengod et al., 1990;Lopez-Gimenez et al., 1997;Marek et al., 2000) and Hrt2A gene expression at the mPFC level assessed here (Weber and Andrade, 2010). A nearly identical pattern of 5-HT_{2A}R mRNA has also been reported in prior *in situ* hybridization studies (Pompeiano et al., 1994;Wright et al., 1995;Amargos-Bosch et al., 2004). Also, 5-HT_{2A}R mRNA, binding, gene expression and Immunostar protein labeling have all demonstrated that 5-HT_{2A}R expression is most profuse in the anterior mPFC (Blue et al., 1988;Pompeiano et al., 1994;Lopez-Gimenez et al., 1997;Weber and Andrade, 2010) where we found cellular 5-HT_{2A}R and 5-HT_{2C}R co-expression.

D-12 5-HT_{2C}R-IR was expressed throughout the soma and initial segment of cells. 5-HT_{2C}R protein levels were clearly low compared to 5-HT_{2A}R protein within the region, supporting prior mRNA work (Pompeiano et al., 1994). Furthermore, 5-HT_{2C}Rs showed a distinct laminar distribution as seen in prior rodent receptor mRNA and protein work at a similar anteroposterior level of the mPFC (Pompeiano et al., 1994;Liu et al., 2007). Laminar 5-HT_{2C}R binding in layer III and 5-HT_{2C}R mRNA in layer V has also been seen in primate cortex (Pazos et al., 1987;Pasqualetti et al., 1999;Lopez-Gimenez et al., 2001). We did not assess high magnification receptor co-expression in more superficial layers of the mPFC. However, low magnification identified 5-HT_{2A}R -expressing neural processes within layers II–III of the most rostral prelimbic mPFC as seen previously within the mPFC (Yadav et al.,

2011a); and dispersed among these fibers was a distinct population of 5-HT_{2C}R-IR cells. Most interesting however, was our demonstration that a laminar distribution of 5-HT_{2C}R-IR cells within the deep layers of the prelimbic mPFC co-expressed 5-HT_{2A}Rs.

In fact, 67% of 5-HT_{2C}R-IR cells in Layers V–VI of the prelimbic mPFC co-expressed 5-HT_{2A}R s. Their co-expression was seen on round and fusiform shaped cells that were widely dispersed within these layers, suggestive of GABAergic expression, and on cells with a pyramidal shape and tight linear expression in layer V where large pyramidal somata are located (Bartos et al., 2007;Shepherd, 2009;Weber and Andrade, 2010), suggestive of pyramidal cell expression. However, most 5-HT_{2C}R-IR cells in the region were found to be GABAergic. This indirectly demonstrates that 5-HT_{2C}Rs and 5-HT_{2A}Rs are likely co-expressed predominantly on GABA cells and perhaps on a small population of pyramidal cells within the deep layers of the prelimbic mPFC.

GABA interneurons, GABA long-range projection neurons and glutamate pyramidal projection cells are located within the mPFC (Fuster, 1997;Gabbott et al., 1997;Lee et al., 2014), but GABAergic cells are the major site of serotonin projection to the region (Smiley and Goldman-Rakic, 1996). 5-HT_{2A}R transcript and protein has been evidenced in mPFC GABA cells (Willins et al., 1997;Weber and Andrade, 2010). GABAergic 5-HT_{2C}R expression has also been seen, though it is region specific. In situ hybridization found few if any GABAergic cells that expressed 5-HT_{2C}R mRNA in the secondary motor cortex which is located at the most dorsal extent of the mPFC (Puig et al., 2010). However, the current study replicated earlier evidence of 5-HT_{2C}R protein expression in GAD67-identified GABA cells within the prelimbic mPFC, and we replicated this using a different 5-HT_{2C}R antibody (Liu et al., 2007; current work). We also identified subcortical GABAergic 5-HT_{2C}R protein expression with the antibody (Burke et al., 2014), directly supporting transcriptional evidence of GABAergic 5-HT_{2C}R mRNA in the region (Eberle-Wang et al., 1997). Reverse transcription-PCR has also identified 5-HT_{2C}R mRNA in GABA cells of the mPFC. In fact, Vysokanov and co-workers (1998) found GABAergic cells with 5-HT_{2C}R and 5-HT_{2A}R mRNA co-expression, directly supporting our 5-HT_2 receptor protein co-expression. Their percentage of GABAergic cells with 5-HT_{2C}R mRNA was lower than we report, but they may have assessed a more superficial layer or posterior mPFC region. 5-HT_{2C}R mRNA shows a rapid anteroposterior decrease through the mPFC (Pompeiano et al., 1994). So does 5-HT_{2C}R protein. We identified a distinct population of 5-HT_{2C}R-expressing cells within the superficial layers of the most rostral prelimbic mPFC in the current study which was notably sparse at a more posterior level of the prelimbic mPFC in another report (Liu et al., 2007). Nonetheless, 50% of the GABAergic cells that expressed 5-HT_{2C}R mRNA in the above report by Vysokanov also co-expressed 5-HT_{2A}R mRNA; strikingly similar to the 67% of 5-HT_{2C}R-IR cells that co-expressed the 5-HT_{2A}R protein within the prelimbic mPFC in our study.

Support of pyramidal 5-HT_{2C}R and 5-HT_{2A}R co-expression also exists. Each receptor has been shown to exist on the apical dendrites of cortical pyramidal cells (Willins et al., 1997;Jakab and Goldman-Rakic, 1998;Cornea-Hebert et al., 1999;Clemett et al., 2000). We assessed 5-HT_2 receptor co-expression in the deep layers of the prelimbic mPFC where large pyramidal cell bodies are located in layer V (see Weber and Andrade, 2010). Both 5-HT_{2A}R

protein (Willins et al., 1997;Weber and Andrade, 2010) and 5-HT_{2C}R protein (Liu et al., 2007) are expressed in this layer, and on pyramidal shaped cells in this layer (Willins et al., 1997;Liu et al., 2007), as we report here. Transcriptional quantification also supports pyramidal 5-HT_{2A}R expression in this layer (Vysokanov et al., 1998;Carr et al., 2002;Weber and Andrade, 2010), but is at odds regarding 5-HT_{2C}Rs. *In situ* hybridization work (Pasqualetti et al., 1999;Lopez-Gimenez et al., 2001) found 5-HT_{2C}R mRNA in this layer, but not on pyramidal cells. However, two studies that used the highly visualized single cell reverse transcription-PCR technique did identify mRNA for the 5-HT_{2C}R in mPFC layer V pyramidal cells (Vysokanov et al., 1998;Carr et al., 2002). Furthermore, they found that nearly all 5-HT_{2C}R-expressing pyramidal shaped cells co-expressed 5-HT_{2A}R mRNA (Carr et al., 2002), though only 28–53% of 5-HT_{2A}R-expressing pyramidal cells co-expressed 5-HT_{2C}R mRNA (Vysokanov et al., 1998;Carr et al., 2002). Though this evidence robustly supports our demonstration of prelimbic pyramidal-shaped cells with 5-HT_{2C}R and 5-HT_{2A}R protein co-expression, future immunohistochemical work that identifies pyramidal cells specifically with a glutamatergic cell marker could substantiate our findings.

How might 5HT-2C and -2A receptor co-expression on a cortical cell surface affect its function? Intra-mPFC administration of 5-HT_{2A}R agonists enhance local pyramidal cell excitation in a dose-dependent manner (Ashby et al., 1990;Arvanov et al., 1999;Lambe and Aghajanian, 2007), while local 5-HT_{2C}R agonism triggers GABA cell excitation and transmitter release that is thought to conversely inhibit mPFC pyramidal function (Mackowiak et al., 1999;Abi-Saab et al., 1999;Leggio et al., 2009;Zhang et al., 2010). Studies suggest that 5-HT_{2A}R pyramidal excitation is due to the receptors preferential location on pyramidal neurons (Santana et al., 2004;Celada et al., 2013). Furthermore, we and others found a preferential GABAergic 5-HT_{2C}R localization in the prelimbic mPFC (Liu et al., 2007; current work); providing a viable mechanism for the indirect 5-HT_{2C}R inhibition of cortical pyramidal function (Ashby et al., 1990;Bergqvist et al., 1999;Eyles et al., 2002). However, findings in this report do not support this clear division of receptor function. Although 70% of GABA cells expressed 5-HT_{2C}Rs in the deep layers of the prelimbic mPFC, most 5-HT_{2C}R-IR cells also co-expressed 5-HT_{2A}Rs. This indirectly suggests that prelimbic GABAergic cells largely express both receptors, and are most likely regulated by a balance in their function.

5HT-2C and -2A receptors share a high degree of homology (Roth et al., 1998) and activate many of the same second messenger signaling systems. Activation of either receptor triggers phosphoinositide and diacylglycerol production that in turn stimulates intracellular calcium release and ERK production under a similar time scale and responsivity to receptor density (Sanders-Bush et al., 1988;Araneda and Andrade, 1991;Stanford et al., 2005;Garcia et al., 2007;Seitz et al., 2012;Meltzer and Roth, 2013). Both $5HT_2$ receptors similarly activate phospholipase D and phospholipase A2 stimulation of arachidonic acid production (McGrew et al., 2002;Liu and Fanburg, 2008).

However, there are differences. 5-HT has higher affinity and potency at 5-HT_{2C} versus 5-HT_{2A} receptors (Berg et al., 2005). Agonist-directed recruitment of intracellular signaling differs between both G-protein-coupled receptors (Berg et al., 1998b). Agonist independent constitutive activation for at least some editing isoforms of 5-HT_{2C}Rs is stronger than that

of 5-HT_{2A}Rs, which would differentially affect their sensitivity to ligand stimulation and recruitment of intracellular signaling pathways (Rauser et al., 2001;Shapiro et al., 2002;Berg et al., 2005). The surrounding ligand milieu more powerfully dampens serotonergic stimulation of 5-HT_{2C}R intracellular pathways than 5-HT_{2A}R signaling systems (Seitz et al., 2012). The 5-HT_{2C}R is unique in that it undergoes RNA editing (Niswender et al., 1998;Abbas et al., 2010), which determines the receptor's trafficking, ligand response, constitutive activational status, and ability to couple to its G protein, trigger intracellular signal transduction pathways (Burns et al., 1997;Herrick-Davis et al., 1999;Berg et al., 2001;Hoyer et al., 2002;Marion et al., 2004;Berg et al., 2005;Millan et al., 2008;Werry et al., 2008;Labasque et al., 2010;Cordova-Sintjago et al., 2014) and affect behavior controlled by the mPFC (Anastasio et al., 2014). The conformation, sensitivity and trafficking of both 5-HT_2 receptors dynamically change in response to inverse agonists, antagonists or endogenous 5-HT, but the constitutional status and edited state of the 5-HT_{2C}R strongly determines how it changes, if at all (Willins et al., 1998;Porter et al., 1999;Berg et al., 1999;Gray and Roth, 2001;Van Oekelen et al., 2003;Devlin et al., 2004;Berg et al., 2005;Yadav et al., 2011b;Seitz et al., 2012;Lopez-Gimenez et al., 2013). These functional differences provide a physiological rationale for the dual expression of both 5-HT_2 receptors on a cortical GABAergic or pyramidal neuron, even though the 5-HT_{2C}R and 5-HT_{2A}R are highly homologous and both activated by 5-HT.

Perhaps their dual functional state on GABAergic cells fine-tune serotonergic control of inhibitory function in the mPFC, an important mechanism in a region where too much versus too little neurotransmitter function detrimentally affects impulsivity, attention and working memory (Arnsten et al., 1994;Goldman-Rakic, 1995;Harrison et al., 1997;Zahrt et al., 1997;Granon et al., 2000;Dalley et al., 2002;Winstanley et al., 2004;Pezze et al., 2014). Evidence supports this hypothesis. Intra-mPFC infusion with the 5-HT -2A and -2C receptor (5-$HT_{2A/2C}$ R) agonist DOI causes GABA cell stimulation that is partially blocked by 5-HT_{2C}R antagonists yet completely blocked by dual 5-$HT_{2A/2C}$R antagonism (Zhang et al., 2010). GABA released by the infusion also inhibits local pyramidal excitability (Carr et al., 2002;Wang et al., 2009).

Serotonergic heteroreceptor co-expression within the mPFC is not new (see Celada et al., 2013). 5-HT_{1A}Rs and 5-HT_{2A}Rs are co-localized on the majority of pyramidal neurons in the region (Martin-Ruiz et al., 2001;Amargos-Bosch et al., 2004;Santana et al., 2004;Puig et al., 2010) and evidence of their opposing cross-talk has been reported (Araneda and Andrade, 1991;Berg et al., 1998a;Martin-Ruiz et al., 2001;Amargos-Bosch et al., 2004;Yuen et al., 2008). However, a recent report indicated that the inhibitory effect of 5-HT_{1A}R s on NMDA-induced pyramidal excitation within the mPFC is reversed by local 5-$HT_{2A/2C}$R co-activation (Yuen et al., 2008) and cross-talk between the 5-HT_{1A}R and both 5-HT_2 receptors has been evidenced (Berg et al., 1998a;Zhong et al., 2008). The current study and others support the existence of a pyramidal subpopulation with 5-$HT_{2A/2C}$R co-expression within the mPFC (current study; Vysokanov et al., 1998;Carr et al., 2002). Though premature to suggest their involvement in this 5-HT_{1A} heteroreceptor pyramidal control, it certainly deserves further exploration.

A broader anatomical understanding of serotonergic 5-HT_2 receptor circuitry across sub-regions of the mPFC is needed. This study assessed the prelimbic mPFC where an optimal neurochemical balance is required for memory and attentional function (Arnsten et al., 1994;Bussey et al., 1997;Granon et al., 2000;Williams et al., 2002;Winstanley et al., 2003;Maddux and Holland, 2011). However, an optimal mPFC function is also required to control impulsivity (Harrison et al., 1997;Dalley et al., 2002;Winstanley et al., 2004), a behavior triggered by cortical 5-HT release (Dalley et al., 2002) and selectively regulated by the more ventral infralimbic mPFC (Chudasama et al., 2003). Infralimbic GABA and 5-HT_2 receptor function can produce an impulsive inability to control ones behavior (Passetti et al., 2003;Carli et al., 2006;Murphy et al., 2012; see also Winstanley et al., 2004). Future infralimbic 5-HT_2 receptor assessment could have particular relevance to addiction neurocircuitry. Impulsivity and cocaine-seeking are triggered in rats by an identical imbalance in cortical 5-$HT_{2A/2C}$R function (Filip and Cunningham, 2003;Pockros et al., 2011;Cunningham et al., 2013;Anastasio et al., 2014; Fink et al., 2015).

In summary, this study identified a new cortical mechanism through which serotonin might fine-tune working memory and emotional control. 5-HT_{2C}R-expressing cells in the deep layers of the prelimbic mPFC commonly co-expressed 5-HT_{2A}Rs. They also largely co-expressed GAD-67, with only 27% showing a non-GABAergic presumably pyramidal cell type. Thus this study indirectly demonstrates that 5-HT_{2C} and 5-HT_{2A} receptors may be commonly co-localized on GABA cells in the region and perhaps on a minor population of layer V pyramidal neurons. Importantly, it indicates that 5-$HT_{2A/2C}$Rs might perform a key direct interactive role in GABA's inhibitory control of pyramidal function within the prelimbic mPFC. It is not clear how $5HT_2$ receptors might interactively modulate intracellular signaling pathways within a cortical cell, but such knowledge could provide new molecular strategies in psychotherapeutic treatment for schizophrenia, depression and drug abuse.

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Abbreviations

GAD	Glutamic acid decarboxylase
mPFC	medial prefrontal cortex
5-HT	Serotonin
5-HT$_{2C}$R	Serotonin$_{2c}$ receptor
5-HT$_{2C}$R-IR	Serotonin$_{2c}$ receptor immunoreactivity
5-HT$_{2A}$R	Serotonin$_{2a}$ receptor

5-HT$_{2A/2C}$Rs	Serotonin$_{2a \text{ and } 2c}$ receptors
GABA	y-aminobutyric acid

- Most GABA cells in Layer V of the rat prelimbic mPFC expressed 5-HT_{2C} receptors
- Likewise, most 5-HT_{2C} receptor-expressing cells were GABAergic
- Most 5-HT_{2C} receptor-expressing cells also co-expressed 5-HT_{2A} receptors

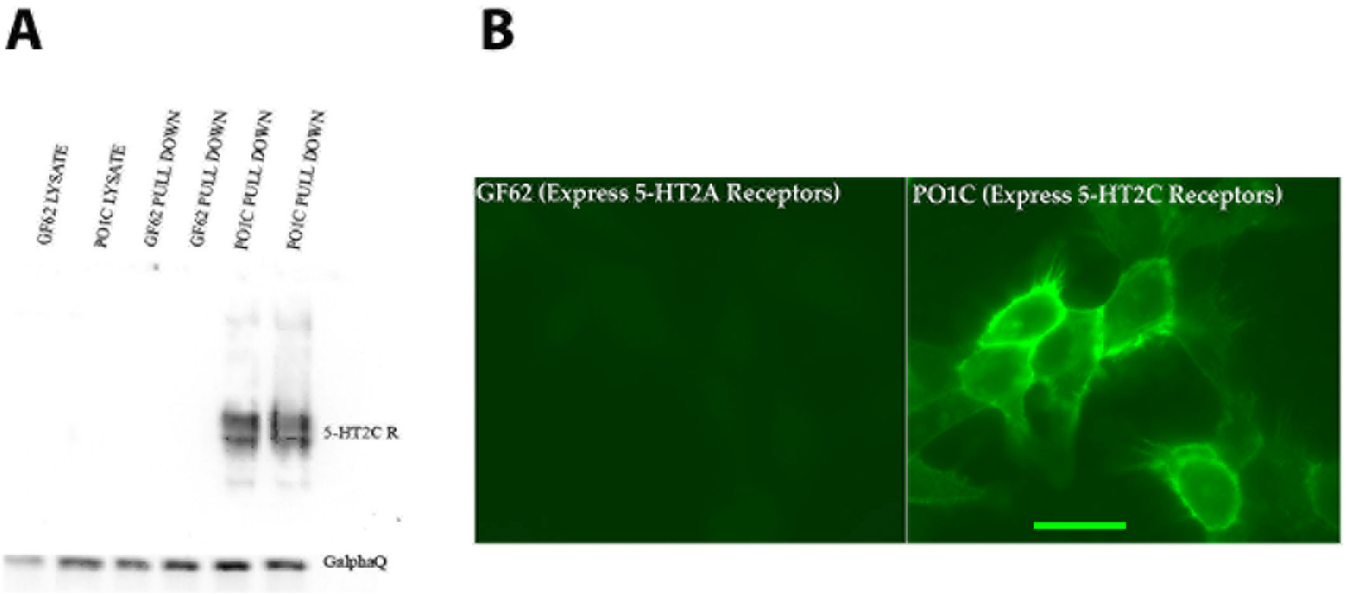

Fig1. Western blot and immunofluorescent microscopy (see Experimental Procedures) indicate that the D-12 5-HT$_{2C}$R antibody used throughout this study is specific for the 5-HT$_{2C}$R

POIC cells that express 5-HT$_{2C}$Rs consistently showed D-12 5-HT$_{2C}$R-antibody expression under both Western Blot (see A, black bars in histogram) and Immunohistochemical assessment (see B, confocal microscopy image of green cellular 5-HT$_{2C}$R immunofluorescence). However, GF62 cells that only express 5-HT$_{2A}$R s showed no D-12 antibody expression in either test (see A and B). Scale bar = 20 μm.

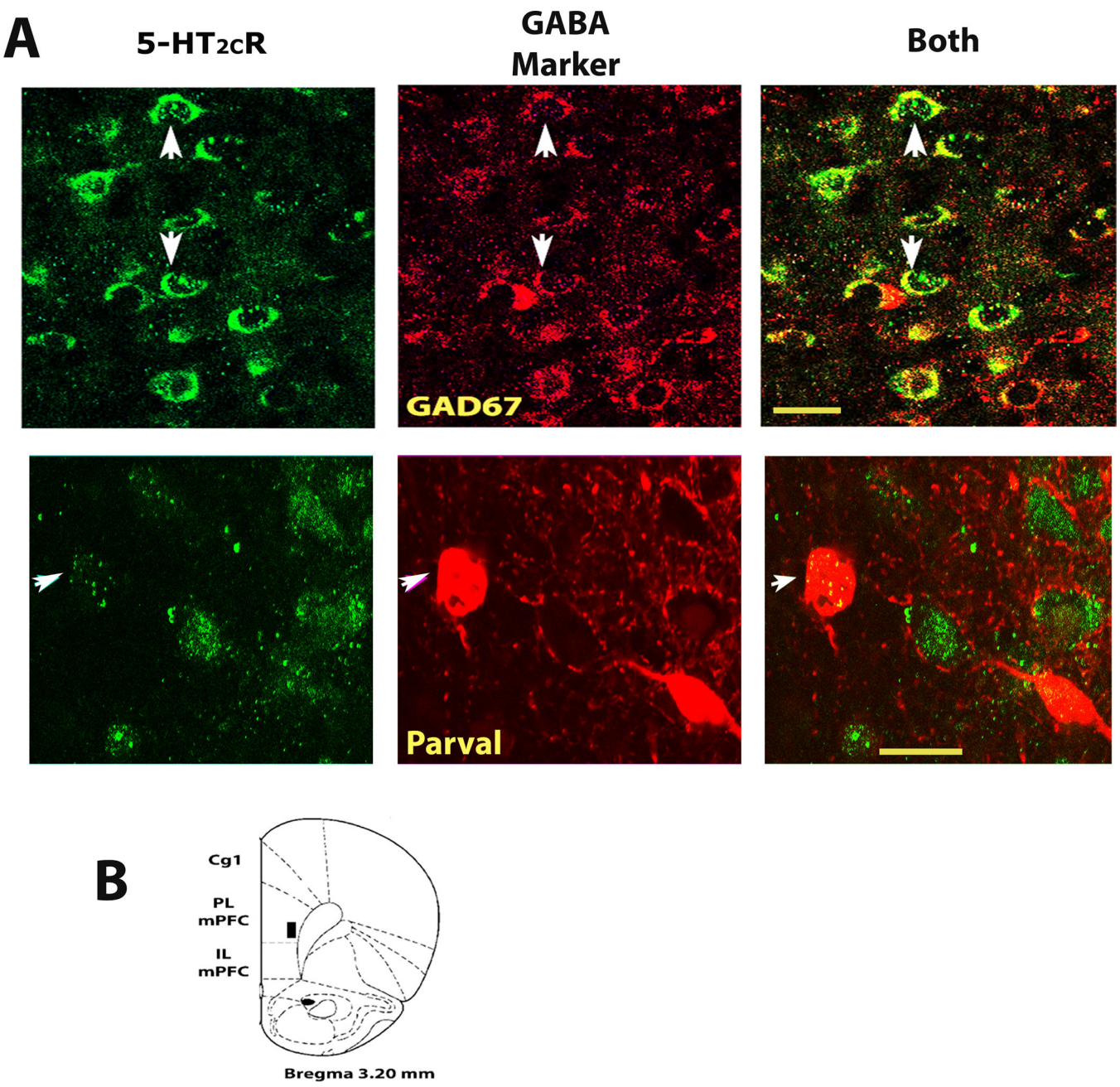

Fig2. GABA cells within the deep layers of the rat prelimbic medial prefrontal cortex (mPFC) express 5-HT$_{2C}$Rs

A, confocal photomicrographs of mPFC tissue showing D-12 5-HT$_{2C}$R antibody immunoreactivity (green florescence) and of cells expressing the GABA cell markers, GAD67 or parvalbumin (red immunostaining, top and bottom rows respectively). White arrows depict the identical cell across each row of photos. The left and middle photos show each antibody separately. Yellow staining in far right photos (under BOTH) illustrates 5-HT$_{2C}$R and GABA cell co-localization. **B**, Cartoon representation of prelimbic mPFC region assessed in this experiment (see black box) according to the rat brain atlas of Paxinos and Watson (2007). 5-HT$_{2C}$R, serotonin 2C receptor; GAD67, glutamic acid decarboxylase

isoform 67; Parval, parvalbumin; PL mPFC, prelimbic subregion of medial prefrontal cortex. All confocal photomicrographs that were used to assess dual-immunolabeling in this report, including those presented in this figure through Fig 5, were of single optical sections (see experimental procedures, section 2.4). Scale bar = 20 μm.

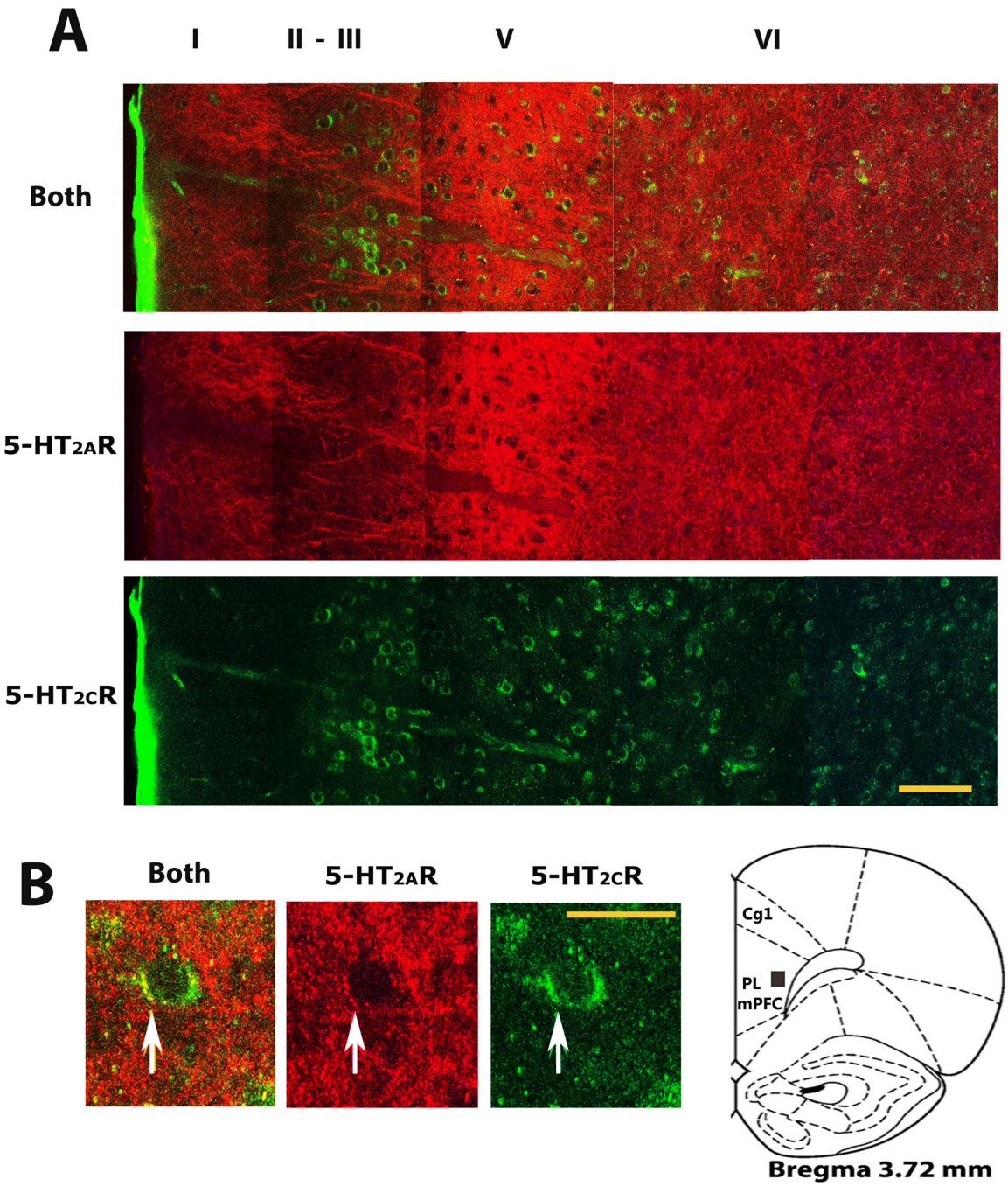

Fig3. 5-HT$_{2A}$R and 5-HT$_{2C}$R expression and cellular co-localization in the rostral prelimbic mPFC

A, All three rows show the identical confocal photomontage collected across layers I to VI of the prelimbic mPFC (cartoon in B indicates the anteroposterior brain level assessed according to Paxinos and Watson, 2007). A distinct laminar 5-HT$_{2A}$R immunoreactivity (see red, middle row), laminar 5-HT$_{2C}$R immunoreactivity (see green, bottom row) and overlapping 5-HT$_{2A}$R and 5-HT$_{2C}$R expression (see green and red staining in top row) was seen. Note the distinct subpopulation of 5-HT$_{2C}$R-IR cells amongst 5-HT$_{2A}$R immunoreactive fibers in superficial layers II–III and another within the dense 5-HT$_{2A}$R

expression in layer V. **B**, High magnification confocal image of a sample cell in layer V showing 5HT-2A and -2C receptor co-localization. The middle and end photos show the cells 5-HT_{2A}R (red) and 5-HT_{2C}R (green) immunoreactivity, while the left photo shows its 5-HT_{2A}R and 5-HT_{2C}R co-expression (yellow staining). The black box in the brain cartoon shows where the cell was sampled (Paxinos and Watson, 2007). 5-HT_{2A}R, serotonin 2A receptors; 5-HT_{2C}R-IR, serotonin 2C receptor immunoreactivity; other abbreviations and confocal microscopy, see Fig 2. Scale bar = 100μm (A) and 20μm (B).

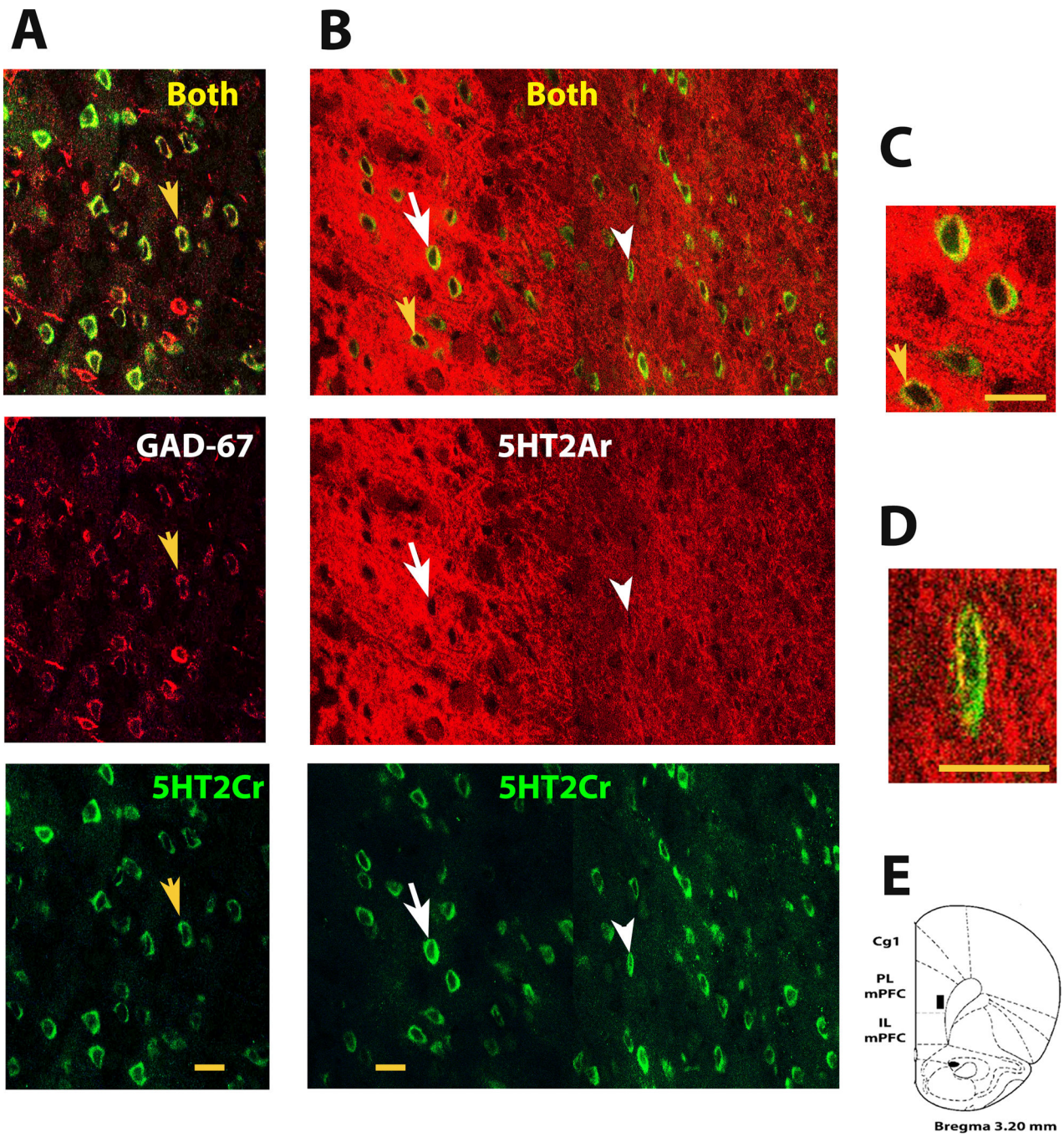

Fig4. GABA cell 5-HT$_{2C}$R expression and cellular 5-HT$_{2C}$R and 5-HT$_{2A}$R co-expression in Layer V at a more posterior level of the prelimbic mPFC

A, confocal photomicrographs of prelimbic tissue showing GAD67-identified GABA cells with 5-HT$_{2C}$R co-immunoreactivity (top photo, yellow and arrowed cells) in layer V at the anteroposterior level depicted in E. The middle and bottom photos show each antibody separately. White arrows depict the identical cell. **B,** confocal photomicrographs at the same level of the prelimbic mPFC showing 5-HT$_{2C}$R-expressing cells with 5-HT$_{2A}$R co-immunoreactivity in layer V$_a$ and layer V$_b$ (see top photo, white arrow and arrowhead,

respectively) as delineated by Weber & Andrade (2010). Cells in layer V_a that are seen between the white and yellow arrows in the top photo were magnified in C to more clearly show their 5-HT_{2C}R and 5-HT_{2A}R co-expression (yellow staining). The cell in layer V_b (see white arrowhead in B) was magnified in D to more clearly show its 5HT-2C and -2A receptor co-expression. The middle and bottom photos show each antibody separately. White arrows depict the identical cells. Abbreviations and confocal microscopy, see Fig 2 & 3. Scale bar = 20 μm.

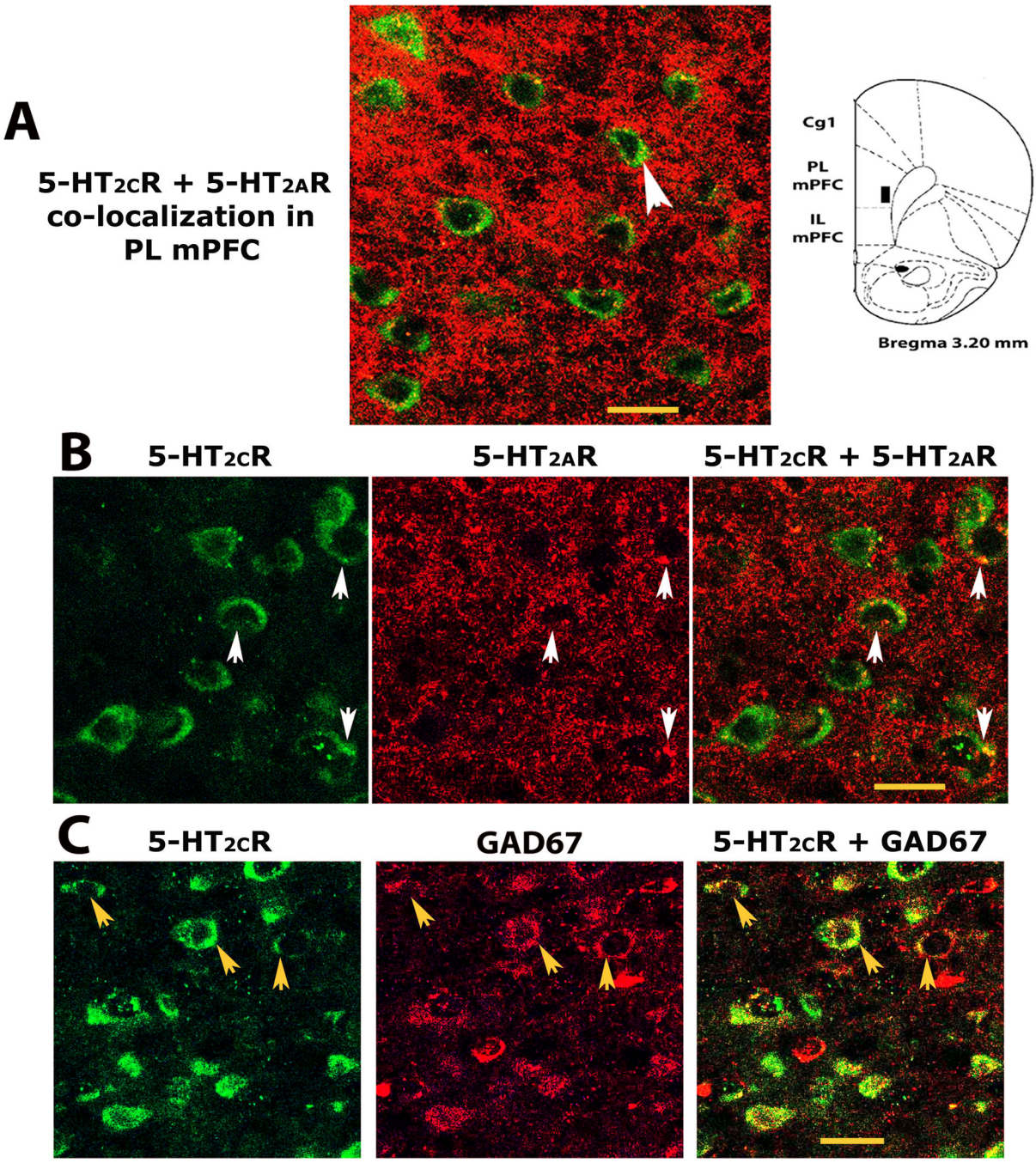

Fig5. Cells with a pyramidal shape and layered expression show 5-HT$_{2A}$R and 5-HT$_{2C}$R co-labeling in layer V of the prelimbic mPFC

A. Immunofluorescent confocal images of potential pyramidal 5-HT$_{2A}$R and 5-HT$_{2C}$R co-labeling (see sample cell above arrowhead with green 5-HT$_{2C}$R immunolabeling and yellow punctate staining around its nucleus indicative of 5-HT$_{2A}$R co-expression). **B**. A confocal image showing a population of linearly expressed pyramidal-shaped cells in layer V of the prelimbic mPFC with green 5-HT$_{2C}$R expression and punctate 5-HT$_{2A}$R co-labeling around their nucleus (see arrows in the end photo). The first and middle photos show each antibody

separately (see same arrowed cells). **C**. A comparison population of widely dispersed red GAD67-identified GABA cells in the same layer with 5-HT_{2C}R co-expression (see yellowed cells with arrows in the end photo). The first and middle photos show each antibody separately (see same arrowed cells). Abbreviations and confocal microscopy, see Fig 2 & 3. Scale bar = 20 μm.

Table 1

Primary antibodies employed within experiments.

Antibody	**5-HT_{2C}R (D-12)**	**5-HT_{2A}R**	**GAD-67 (H101)**	**Parvalbumin (PV25)**
Experiment	Exp Ia &1b, Exp II Exp III	Exp III	Exp II	Exp II
Immunogen	Human 5-HT_{2C}R C-terminus (374–458)	Rat 5-HT_{2A}R N-terminus (22–41)	Human GAD-67 N-terminus (1–101)	Rat muscle Parvalbumin calcium binding protein
Manufacturer	Santa Cruz Biotechnology, Santa Cruz,CA Sc-17797	Exp II:Neuromics, Edina, MN	Santa Cruz Biotechnology (Santa Cruz, CA)	Swant, Marly1, Switzerland
Catalog #	Sc-17797	RA24288	sc-5602	PV25
Host/clonality	Mouse monoclonal	Rabbit polyclonal	Rabbit polyclonal	Rabbit polyclonal
Dilution	1:500 (Exp 1a) 1:100 (Exp Ib) 1:50 (Exp II; Exp III)	1:100	1:50	1:2000

Table 2

Immunohistochemical localization of 5-HT_{2A}Rs on 5-HT_{2C}R-IR cells within the deep layers of the prelimbic mPFC (mean ± SEM) in experiment III

Number of 5-HT_{2C}R-IR cells	Number of cells with 5-HT_{2C}R + 5-HT_{2A}R co-labeling	% of 5-HT_{2C}R-IR cells that express 5-HT_{2A}Rs
281 ± 6.2	189 ± 9.3	67.4 ± 1.81

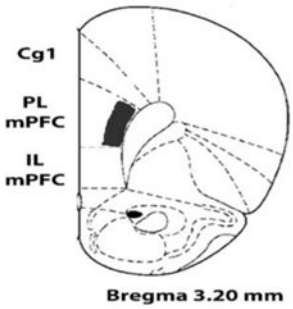

Table 3

Immunohistochemical validation of 5-HT_{2C}R-IR on GAD67 labeled GABAergic cells within the deep layers of the prelimbic mPFC (mean ± SEM) in experiment II

Number of 5-HT_{2C}R-IR cells	Number of cells with 5-HT_{2C}R + GAD67 co-labeling	% of 5-HT_{2C}R-IR cells that express GAD67	Number of GAD67 cells	% of GAD67 cells that express 5-HT_{2C}Rs
285 ± 13.0	209 ± 9.0	73.3 ± 0.18	297 ± 9.5	70.2 ± 0.78