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Endogenous Serotonin 5-HT_{2A} and 5-HT_{2C} Receptors Associate in the Medial Prefrontal Cortex

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Abstract

The 5-HT_{2A} receptor (5-HT_{2A}R) and 5-HT_{2C}R are localized to the same neurons within the medial prefrontal cortex (mPFC), which regulates executive function, decision-making, and reward-guided learning and memory processes. The 5-HT_{2A}R and 5-HT_{2C}R co-immunoprecipitate in the mPFC of male, Sprague-Dawley rats, while *in vitro* studies demonstrate the presence of a physical interaction between the 5-HT_{2A}R and 5-HT_{2C}R. The purpose of this study was to identify mPFC subregions in which the 5-HT_{2A}R and 5-HT_{2C}R physically interact *ex vivo* in the male Sprague-Dawley rat. We established the expression patterns of 5-HT_{2A}R and 5-HT_{2C}R in layers I-VI of the anterior cingulate cortex (ACC), prelimbic (PL), and infralimbic (IL) subregions using double-label fluorescence immunohistochemistry in male rats. We then employed the proximity ligation assay (PLA) to test the hypothesis that the 5-HT_{2A}R and 5-HT_{2C}R form a close, physical association within these mPFC subregions. Our results demonstrate subregion- and layer-specific expression of the 5-HT_{2A}R and 5-HT_{2C}R proteins using immunofluorescence and single recognition PLA, and a spatially close (within 40 nm) interaction between the 5-HT_{2A}R and 5-HT_{2C}R that occurs along a dorsal-ventral gradient in the rat mPFC.

Graphical Abstract

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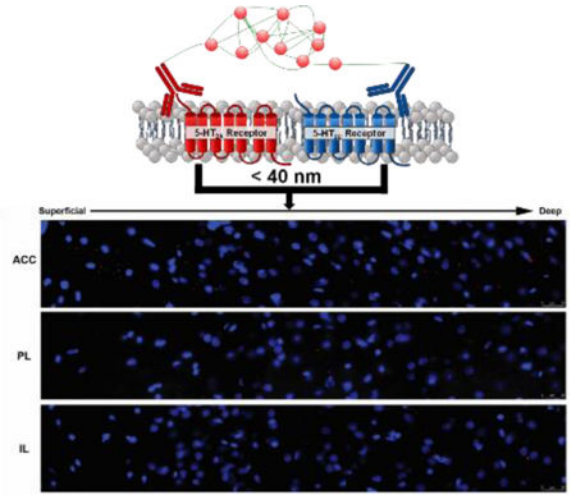
Author Contributions

A.E.P. and D.J.S. contributed equally to this work. A.E.P. and D.J.S. carried out *ex vivo* biochemical experiments, conducted statistical analyses, and drafted the manuscript. S.J.S. carried out *ex vivo* biochemical experiments and edited the manuscript. N.C.A. and K.A.C. conceptualized the projected, supervised the experimental design/interpretation/analyses, and wrote/edited the manuscript.

Conflict of Interest

The authors declare no competing financial interests.



Keywords

Serotonin; 5-HT_{2A} receptor; 5-HT_{2C} receptor; medial prefrontal cortex; proximity ligation assay; G-protein coupled receptor

INTRODUCTION

The mammalian medial prefrontal cortex (mPFC) is integrally involved in executive function, decision-making, and reward-guided learning and memory processes while perturbations of mPFC network integrity are associated with several neuropsychiatric disorders, including substance use disorders, depression, and schizophrenia (for reviews).[1–3] The mPFC is richly innervated by serotonin (5-hydroxytryptamine; 5-HT) terminals originating primarily from the dorsal raphe nucleus; this 5-HT innervation is heavily concentrated in superficial (layer I) and deep layers (layers V/VI) relative to intermediate layers (layers II/III).[4,5] Serotonin neurotransmission in mPFC is mediated by several of the 14 genetically-encoded subtypes of 5-HT receptors (5-HT_xRs), including the 5-HT_{2A}R and 5-HT_{2C}R[6,7] (for review[8]). Found in both glutamate and γ-aminobutyric (GABA) neurons in the mPFC,[9–13] the 5-HT_{2A}R and 5-HT_{2C}R are co-expressed in both neuronal cell types[13–16] and found in the same protein complex upon co-immunoprecipitation from the mPFC of male, Sprague-Dawley rats.[17]

The 5-HT_2Rs act via the "receptorsome," or the composition of membrane, cytosolic, and accessory proteins through which protein-protein interactions interface GPCR coupling to downstream intracellular signaling cascades to tailor cellular responsivity. Accumulating evidence suggests that these receptors form heteromeric protein complexes with other GPCRs which also regulate cellular signaling. The 5-HT_{2A}R is evidenced to form heteromeric complexes with the 5-HT_{1A}R,[18] dopamine D_2 receptor,[19] metabotropic glutamate $mGlu_2$ receptor,[20] and cannabinoid CB_1 receptor,[21] while the 5-HT_{2C}R reportedly forms complexes with the melatonin MT_2 receptor,[22] growth hormone secretagogue receptor 1a,[23] and the N-methyl-D-aspartate (NMDA)-gated ion channel subunit GluN2A.[24] A physical interaction between the 5-HT_{2A}R and 5-HT_{2C}R has been detected in heterologous

cellular systems via bioluminescence resonance energy transfer and luciferase complementation assays.[25, 26]

In the present study, we established the expression patterns of 5-HT_{2A}R and 5-HT_{2C}R in mPFC layers (I-VI) as well as the anterior cingulate cortex (ACC), prelimbic (PL), and infralimbic (IL) subregions of the mPFC of the male Sprague-Dawley rat using double-label fluorescence immunohistochemistry. We then employed the proximity ligation assay (PLA) to test the hypothesis that the 5-HT_{2A}R and 5-HT_{2C}R form a close, physical association within these mPFC subregions. Our results demonstrate subregion- and layer-specific expression of the 5-HT_{2A}R and 5-HT_{2C}R proteins using immunofluorescence and single recognition PLA, and a spatially close (within 40 nm) interaction between the 5-HT_{2A}R and 5-HT_{2C}R that occurs along a dorsal-ventral gradient in the rat mPFC.

RESULTS AND DISCUSSION

Localization of 5-HT_{2A}R expression in the mPFC.

Figure 1A illustrates representative, high magnification staining patterns for the anti-5-HT_{2A}R antibody RA24288 (red) and anti-5-HT_{2C}R antibody sc17797 (green) in the rat mPFC (DAPI-positive nuclei visualized in blue). The boxes in Figure 1B illustrate the mPFC subregions of interest analyzed within the ACC, PL, and IL (template from Paxinos and Watson 2005).[27] Representative images of 5-HT_{2A}R expression in the ACC, PL, and IL regions are shown in Figure 1C (left panel, red). A repeated measures one-way ANOVA demonstrated a main effect of region on 5-HT_{2A}R expression ($F_{2,4}$=14.99; $p < 0.05$). Tukey's multiple comparisons test indicated a significant difference between 5-HT_{2A}R expression in the ACC and IL ($p < 0.05$) and PL and IL ($p < 0.05$), but not between the ACC and PL (n.s.; Figure 1D). Further analyses assessed differences in 5-HT_{2A}R expression between layers within the ACC, PL, and IL (Figure 1E). A repeated measures two-way ANOVA indicated a main effect of region ($F_{2,4}$=9.416; $p < 0.05$), layer ($F_{3,6}$=54.35, $p < 0.05$), and a region x layer interaction ($F_{6,12}$=11.02; $p < 0.05$). Tukey's multiple comparisons test was used to compare 5-HT_{2A}R expression between layers within each region. In the ***ACC***, 5-HT_{2A}R expression in layer I was significantly different from layer II/III ($p < 0.05$), layer V ($p < 0.05$), and layer VI ($p <0.05$). Expression of 5-HT_{2A}R in layer II/III was significantly different from layer V ($p < 0.05$), but not layer VI (n.s.). Finally, 5-HT_{2A}R expression was significantly different between layer V and layer VI ($p < 0.05$). In the ***PL***, 5-HT_{2A}R expression in layer I was significantly different from layer II/III ($p < 0.05$), layer V ($p < 0.05$), and layer VI ($p < 0.05$). Expression of 5-HT_{2A}R in layer II/III was significantly different from layer V ($p < 0.05$), but not layer VI (n.s.). Finally, 5-HT_{2A}R expression was significantly different between layer V and layer VI ($p < 0.05$). In the ***IL***, 5-HT_{2A}R expression in layer I was not significantly different from layer II/III (n.s.), layer V (n.s.), or layer VI (n.s.). Expression of 5-HT_{2A}R in layer II/III was significantly different from layer V ($p < 0.05$), but not layer VI (n.s.). Finally, 5-HT_{2A}R expression in the IL was significantly different between layer V and layer VI ($p < 0.05$). Together, these results are the first to demonstrate that higher levels of layer-dependent 5-HT_{2A}R protein are expressed in the dorsal regions of the mPFC (ACC and PL), which is consistent with observations for RNA levels.[28]

Localization of 5-HT$_{2C}$R expression in the mPFC.

Representative images of 5-HT$_{2C}$R expression in the ACC, PL, and IL regions are shown in Figure 1C (center panel, green). A repeated measures one-way ANOVA demonstrated a main effect of region on 5-HT$_{2C}$R expression ($F_{2,4}$=23; $p < 0.05$). Tukey's multiple comparisons test indicated a significant difference between the ACC and PL ($p < 0.05$) and between the ACC and IL ($p < 0.05$), but not between the PL and IL (n.s.; Figure 1F). Further analyses assessed differences in 5-HT$_{2C}$R expression between layers within the ACC, PL, and IL (Figure 1G). A repeated measures two-way ANOVA indicated a main effect of region ($F_{2,4}$=14.01; $p < 0.05$), layer ($F_{3,6}$=37.75, $p < 0.05$), and a region x layer interaction ($F_{6,12}$=5.902; $p < 0.05$). Tukey's multiple comparisons test was used to compare 5-HT$_{2C}$R expression between layers within each region. In the ***ACC***, 5-HT$_{2C}$R expression in layer I was not significantly different from layer II/III (n.s.), but was significantly different from layer V ($p < 0.05$), and layer VI ($p < 0.05$). Expression of 5-HT$_{2C}$R in layer II/III was significantly different from layer V ($p < 0.05$) and layer VI ($p < 0.05$). Finally, 5-HT$_{2C}$R expression was not significantly different between layer V and layer VI (n.s.). In the ***PL***, 5-HT$_{2C}$R expression in layer I was not significantly different from layer II/III (n.s.) but was significantly different from layer V ($p < 0.05$), and layer VI ($p < 0.05$). Expression of 5-HT$_{2C}$R in layer II/III was significantly different from layer V ($p < 0.05$) and layer VI ($p < 0.05$). Finally, 5-HT$_{2C}$R expression was not significantly different between layer V and layer VI (n.s.). In the ***IL***, 5-HT$_{2C}$R expression in layer I was not significantly different from layer II/III (n.s.), layer V (n.s.), or layer VI (n.s.). Expression of 5-HT$_{2C}$R in layer II/III was not significantly different from layer V (n.s.) or layer VI (n.s.). Finally, 5-HT$_{2C}$R expression was not significantly different between layer V and layer VI (n.s.). Together, these data indicate a decreasing gradient of 5-HT$_{2C}$R expression moving from dorsal to ventral mPFC subregions with an increasing gradient of 5-HT$_{2C}$R expression moving from superficial to deeper layers, consistent with previously published literature.[13, 28, 29]

Single recognition PLA analyses of 5-HT$_{2A}$R and 5-HT$_{2C}$R expression in mPFC.

The PLA is an immunohistochemical methodology that can detect low levels of individual proteins as well as the subcellular localization of protein interactions (≤ 40 nm) with high specificity and sensitivity.[30–32] The PLA conducted with a single antibody (single recognition PLA) is reported to improve sensitivity through an amplification step[31] and is required to establish individual antibody concentrations prior to experiments with dual recognition PLA with both antibodies.[30] In Figure 2A, representative images of single recognition PLA detected 5-HT$_{2A}$R expression in the ACC, PL, and IL regions (red puncta). A repeated measures one-way ANOVA demonstrated a main effect of region ($F_{2,6}$=9.154; $p < 0.05$) on 5-HT$_{2A}$R PLA signal (Figure 2B). Tukey's multiple comparisons test demonstrated a significant difference in 5-HT$_{2A}$R PLA signal between ACC and IL ($p < 0.05$) and PL and IL ($p < 0.05$), but not between ACC and PL (n.s.). These results demonstrate a dorsal-ventral gradient of 5-HT$_{2A}$R expression in the mPFC, consistent with the results from immunohistochemical analyses (Figure 1D), indicating the PLA reliably assesses 5-HT$_{2A}$R expression in mPFC.

In Figure 3A, representative images of single recognition PLA of 5-HT$_{2C}$R expression in the ACC, PL, and IL regions are shown (red puncta). A repeated measures one-way ANOVA

demonstrated a main effect of region ($F_{2,6}$=6.641; $p < 0.05$) on 5-HT_{2C}R PLA signal (Figure 3B). Tukey's multiple comparisons test demonstrated a significant difference in 5-HT_{2C}R PLA signal between ACC and IL ($p < 0.05$), but not between PL and IL (n.s.) or ACC and PL (n.s.). These results demonstrate a dorsal-ventral gradient of 5-HT_{2C}R expression in the mPFC, consistent with the results from immunohistochemical analyses (Figure 1F), indicating the PLA reliably assesses 5-HT_{2C}R expression in mPFC.

Dual recognition PLA analyses of 5-HT_{2A}R and 5-HT_{2C}R expression in mPFC.

The observed immunohistochemical and single recognition PLA expression patterns of the 5-HT_{2A}R and 5-HT_{2C}R in the ACC, PL, and IL regions of rat mPFC demonstrated region- and layer-specific patterns of expression for both receptors that are consistent with previous reports.[13, 29, 33–36] The dual recognition PLA detects two proteins in complex through the employment of two proprietary, species-specific secondary antibodies conjugated to short DNA strands that complement, ligate and allow rolling circle amplification to produce fluorescent signals (see Methods).[21, 26, 31, 32, 37] Employing the dual recognition PLA, we tested the hypothesis that the 5-HT_{2A}R and 5-HT_{2C}R form a close within close spatial proximity (≤ 40 nm) within the ACC, PL, and IL. Representative images of 5-HT_{2A}R:5-HT_{2C}R complex expression in the ACC, PL, and IL regions are shown in Figure 4A (red puncta). A repeated measures one-way ANOVA demonstrated a main effect of region ($F_{2,6}$=18.95; $p < 0.05$) on 5-HT_{2A}R:5-HT_{2C}R PLA signal (Figure 4B). Tukey's multiple comparisons test demonstrated a significant difference in 5-HT_{2A}R PLA signal between ACC and IL ($p < 0.05$) and PL and IL ($p < 0.05$), but not ACC and PL (n.s.). These results suggest a dorsal-ventral gradient of 5-HT_{2A}R:5-HT_{2C}R complex expression in the mPFC. The PLA requires that two target proteins are within 40 nm of each other to produce a quantifiable signal; this resolution cannot be ensured using traditional immunohistochemical co-localization or co-immunoprecipitation analyses.[21, 26, 31, 32, 37] Thus, our report is the first to demonstrate the presence of a spatially close interaction between the two receptors in mPFC *ex vivo*.

Previous *in situ* hybridization studies indicated a dorsal-ventral gradient of 5-HT_{2A}R and 5-HT_{2C}R mRNA in the mPFC;[28] both 5-HT_{2A}R and 5-HT_{2C}R mRNA increased across layers II-III, V, and VI, but was absent in layer I.[28] While 5-HT_{2A}R *in situ* hybridization outcomes appear somewhat inconsistent with the present study, genome-wide correlation studies demonstrate that only ~40% of the variance in protein expression can be explained by changes at the mRNA transcript level.[38, 39] Further, the presence of 5-HT_{2A}R protein expression, but not mRNA levels, in layer I of the mPFC could represent thalamocortical matrix inputs reaching layer I that are 5-HT_{2A}R immunoreactive.[28] Furthermore, we corroborate previous protein biochemical studies that 5-HT_{2A}R protein expression is highest in layer V and lowest in layers II/III of the PL[13] while 5-HT_{2C}R protein expression is highest in deep layers of the PL.[13, 29] The present study extends the characterization of 5-HT_{2A}R and 5-HT_{2C}R protein expression in mPFC subregions to include the ACC and IL alongside the PL. The anatomical connectivity of mPFC is subregion-dependent; for example, while the dorsal and ventral mPFC innervate overlapping subcortical regions, dorsal mPFC neurons predominantly project to the dorsal striatum (i.e., caudate, putamen) while ventral mPFC neurons project to the ventral striatum (i.e., nucleus accumbens,

olfactory tubercle) (for review[40]). Future studies to elucidate 5-HT_{2A}R and 5-HT_{2C}R expression on ACC, PL, or IL neurons that project to the dorsal vs. ventral striatal regions are required to illuminate the role of cortical 5-HT_{2A}R and 5-HT_{2C}R in normal behaviors including executive function, decision-making, reward-guided learning and memory processes.

The pattern of expression for 5-HT_{2A}R and 5-HT_{2C}R proteins across mPFC subregions was congruent between our immunofluorescence and single recognition PLA experiments, supporting the use of single recognition PLA for relative quantification of 5-HT_{2A}R and 5-HT_{2C}R in the mPFC. It is proposed that the $G\alpha_{q/11}$-coupled 5-HT_{2A}R[41, 42] and 5-HT_{2C}R[43–46] function via their homomeric forms. However, the extent to which single recognition PLA identifies monomeric versus homomeric forms is unexplored, and strategies to differentiate the receptor stoichiometry using the single recognition PLA remain to be established.

The present study identified a spatially close (within 40 nm) interaction between the 5-HT_{2A}R and 5-HT_{2C}R that occurs along a dorsal-ventral gradient of the mPFC in the male, Sprague-Dawley rat. These results are consistent with a previous identification of co-localization of 5-HT_{2A}R and 5-HT_{2C}R protein in the deep layers of the PL[13] in addition to another study which identified co-expression of 5-HT_{2A}R and 5-HT_{2C}R mRNA within cells of the mPFC.[15] Empirically, we observed 5-HT_{2A}R:5-HT_{2C}R complex expression in both superficial and deep layers of the ACC and PL. Thus, in addition to regulating cortical-subcortical pathways via expression on neurons in the deeper layers, the 5-HT_{2A}R:5-HT_{2C}R complex may also regulate intracortical signaling via expression on neurons in the superficial layers of the PFC.[28] Mechanistically, a reciprocal 5-HT_{2A}R:5-HT_{2C}R interaction is corroborated by previous molecular and pharmacological analyses *in vitro*.[25] Together, these findings suggest differential cellular output that may result from a 5-HT_{2A}R:5-HT_{2C}R interaction relative to when the individual receptors act independently. Thus, GPCR heteromeric complexes such as the 5-HT_{2A}R:5-HT_{2C}R complex may represent new, druggable interfaces with the potential to yield novel therapeutics for future neurotherapeutics discovery programs.

METHODS

Animals.

Male, outbred Sprague-Dawley rats (n=7; Envigo, Haslett, MI, USA) weighing 175–199 g at arrival were housed two per cage under a 12-hour light-dark cycle (lights on between 0600–1800h) with controlled temperature (21–23°C) and humidity (40–50%). Animals were acclimated for one week to the colony room before transcardial perfusion. Standard food and water were available to rats *ad libitum*. All experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* (2011) and with the University of Texas Medical Branch Institutional Animal Care and Use Committee approval.

Transcardial perfusion.

Rats were anesthetized (100 mg/kg sodium pentobarbital, i.p.) and transcardially perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde for four hours at 4°C, cryoprotected in 30% sucrose for 72 hours at 4°C, and stored at −80°C until further use.

Immunofluorescence localization of 5-HT_{2A}R and 5-HT_{2C}R expression in the mPFC.

Free-floating coronal sections (30 μm) at the level of the ACC, PL, and IL (3.5–4.0 mm anterior to Bregma, Figure 1B)[27] were washed with PBS, permeabilized with 0.4% Triton-X 100 in PBS for 60 minutes at room temperature, blocked with 5% normal donkey serum, 0.4% Triton-X 100, 0.1% glycine, and 0.1% lysine in PBS for two hours at room temperature, and incubated with primary antibodies in 5% normal donkey serum and 0.4% Triton-X 100 in PBS overnight at 4°C. The primary antibodies employed were a polyclonal rabbit anti-5-HT_{2A}R (RA24288; Neuromics, Edina, MN, USA) at a concentration of 1:500 and a monoclonal mouse anti-5-HT_{2C}R (sc17797; Santa Cruz Biotechnology, Dallas, TX, USA) at a concentration of 1:50. Both antibodies have been extensively characterized in our laboratory and others.[13, 32, 47–52] The specificity of the anti-5-HT_{2A}R antibody RA24288 in immunohistochemistry is validated in cortical tissue from 5-HT_{2A}R knockout and wild-type mice,[36] and the specificity of the anti-5-HT_{2C}R antibody sc17797 in immunohistochemistry is validated in tissue from rats receiving shRNA-mediated knockdown of the 5-HT_{2C}R in the mPFC.[17, 50] The next day, sections were rinsed 6X with PBS for 10 minutes each and incubated in secondary antibodies for 60 minutes at room temperature. The secondary antibodies used were a donkey anti-rabbit with a conjugated 594 nm fluorophore (711–585-152; Jackson ImmunoResearch, West Grove, PA, USA) at a concentration of 1:500 and a donkey anti-mouse with a conjugated 488 nm fluorophore (A21202, Invitrogen, Carlsbad, CA, USA) at a concentration of 1:200. Slices were then washed 3X with 0.4% Triton-X 100 in PBS for five minutes each and then rinsed in PBS. Sections were mounted, and slides were coverslipped with Vectashield fluorescent mounting media with 4′,6-diamidino-2-phenylindole (DAPI) to stain cellular nuclei for fluorescence microscopy (Vector Laboratories, Burlingame, CA, USA).

Tilescan images encompassing all layers of the ACC, PL, or IL (three fields of view in the dorsal-ventral direction by six fields of view in the medial-lateral direction) were acquired using a 40X objective on a Leica DFC3000 wide field camera (Leica Microsystems, Wetzlar, Germany) (for a final magnification of 400X) and automatically stitched together using the Leica Application Suite. Each hemisphere of one brain section represented one technical replicate (n=4 per one biological replicate). Each technical replicate was imaged three times (i.e., tilescans of each region were acquired). Individual channel threshold values were set at the lowest value at which a signal was not visualized on the negative control (secondary antibody only) for each technical replicate. Expression of 5-HT_{2A}R and 5-HT_{2C}R in the ACC, PL, and IL spanning all layers of mPFC was determined using mean signal intensity analyzed using the Open Source software Fiji.[53] A secondary analysis was also completed to assess expression in specific layers (I, II/III, V, and VI) of the mPFC subregions. All expression analyses were carried out using region of interest boxes with predefined dimensions. The technical replicate most representative of an individual biological replicate

was used for statistical analyses and represented within each graph. The final values used in graphical representation and statistical analyses are the mean ± standard error of the mean (SEM) for all biological replicates combined (n=3). Like all antibody-based assays, absolute expression levels of a protein cannot be compared without establishment of a calibration curve.[54] Since this technique was not utilized, expression levels are represented in a relative manner and therefore can be compared within the same antibody-based protein assessment, but not between different antibody-based protein assessments (e.g., 5-HT_{2A}R expression can be compared between regions and layers of the PFC, but cannot be compared directly to 5-HT_{2C}R expression).[55]

PLA for 5-HT_{2A}R:5-HT_{2C}R complex identification:

A commercial PLA was employed to assess the 5-HT_{2A}R:5-HT_{2C}R complex in rat mPFC *ex vivo* (Duolink® PLA, Sigma-Aldrich, St. Louis, MO, USA).[32] Coronal sections (10 μm) at the level of the ACC, PL, and IL (approximately 3.5–4.0 mm anterior to Bregma, Figure 1B)[27] were thaw-mounted directly to glass slides and allowed to dry for two hours at room temperature. Sections were rehydrated in PBS for 30 minutes at room temperature, and antigen retrieval was performed by incubating sections in citric acid (pH 6.0) at 90 °C for 20 minutes then allowing the sections to cool to room temperature for 20 minutes. Sections were rinsed in PBS 3X for three minutes each and incubated in 50 mM ammonium chloride for 20 minutes at room temperature to decrease auto-fluorescence. Sections were rinsed 5X for three minutes at room temperature in PBS and blocked for two hours at room temperature in 5% normal donkey serum and 0.4% Triton-X 100 in PBS. Sections were incubated with primary antibodies in 5% normal donkey serum and 0.4% Triton-X 100 in PBS overnight at 4°C. The polyclonal rabbit anti-5-HT_{2A}R (Neuromics RA24288, Edina, MN) was employed at a concentration of 1:500. The monoclonal mouse anti-5-HT_{2C}R employed in immunohistochemical studies (above) was not compatible with the PLA reagents; thus, a previously validated[56] polyclonal goat anti-5-HT_{2C}R (Abcam ab32887, Cambridge, MA, USA) was used in the PLA at a concentration of 1:100. The specificity of the anti-5-HT_{2C}R antibody ab32887 in immunohistochemistry is validated in tissue from rats receiving shRNA-mediated knockdown of the 5-HT_{2C}R in the basolateral amygdala.[56] The next day, sections were rinsed 5X with PBS for 10 minutes each and incubated in Duolink® secondary antibodies/probes for 60 minutes at 37°C. The secondary antibodies/probes used were donkey anti-rabbit PLUS (DUO92002), donkey anti-rabbit MINUS (DUO92005), donkey anti-goat PLUS (DUO92003) and donkey anti-goat MINUS (DUO92006) and were diluted in 5% normal donkey serum and 0.4% Triton-X 100 in PBS per manufacturer instructions. Following incubation, sections were washed in PBS for five minutes followed by 2X Wash Buffer A (DUO82049) for five minutes each at room temperature. Sections were then incubated with ligation reagents for 30 minutes at 37°C, washed with 2X Wash Buffer A for two minutes at room temperature, and incubated with amplification reagents for 100 minutes at 37°C. Ligation and amplification solutions (DUO92008) were made according to manufacturer instructions. Sections were then washed in 3X Wash Buffer B (DUO82049) for 10 minutes each at room temperature and rinsed with 0.01% Wash Buffer B. Sections were allowed to dry overnight. Slides were coverslipped using Duolink® *in situ* mounting medium with DAPI (DUO82040).

Tilescan images encompassing all layers of either the ACC, PL, or IL (three fields of view in the dorsal-ventral direction by six fields of view in the medial-lateral direction) were acquired using a 40X objective on a Leica DFC3000 wide field camera (for a final magnification of 400X) and automatically stitched together using the Leica Application Suite. Each brain section represented one technical replicate (n=3 within one biological replicate). Each technical replicate was imaged six times (i.e., tilescans of each region were acquired from both hemispheres). This process was completed for single-labeled 5-HT_{2A}R, single-labeled 5-HT_{2C}R, and dual-labeled 5-HT_{2A}R:5-HT_{2C}R sections. Three non-overlapping region of interest boxes spanning all layers of the cortex (100 μm in the dorsal-ventral direction by 600 μm in the medial-lateral direction) were then applied to each tilescan image for quantification of the PLA signal. The PLA signal was quantified using automatic threshold and classification based on intensity and size of the signal (Duolink® ImageTool, Sigma-Aldrich). A corrected total signal was calculated by subtracting signal from the nuclear and immediate perinuclear regions from the total signal. Technical replicates were averaged to determine the mean expression within a biological replicate. The technical replicate most representative of an individual biological replicate was used for statistical analyses and represented within each graph. The average corrected total signal of the negative control images (secondary antibody/probe only) was subtracted from the average corrected total signal of the experimental images (primary antibody plus secondary antibody/probe) to determine the expression of 5-HT_{2A}R, 5-HT_{2C}R, or 5-HT_{2A}R:5-HT_{2C}R for each biological replicate. The final values presented are the mean ± SEM for all biological replicates combined (n=4). Final values are relative and not absolute.

Statistical Analyses:

A repeated measures one-way ANOVA followed by Tukey's multiple comparisons test was used to assess regional differences in 5-HT_{2A}R expression (immunohistochemical and PLA experiments), 5-HT_{2C}R expression (immunohistochemical and PLA experiments), and 5-HT_{2A}R:5-HT_{2C}R complex expression (PLA experiments). A repeated measures two-way ANOVA followed by Tukey's multiple comparisons test was used to analyze expression patterns of 5-HT_{2A}R and 5-HT_{2C}R using the factors of region (ACC, PL, IL) and layer (layers I, II/III, V, and VI).

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ABBREVIATIONS

5-HT	serotonin
5-HT_{2A}R	5-HT_{2A} receptor

5-HT$_{2C}$R	5-HT$_{2C}$ receptor
ACC	anterior cingulate cortex
ANOVA	analysis of variance
DAPI	4′,6-diamidino-2-phenylindole
IL	infralimbic cortex
mPFC	medial prefrontal cortex
PBS	phosphate buffered saline
PL	prelimbic cortex
PLA	proximity ligation assay
SEM	standard error of the mean
SUD	substance use disorder

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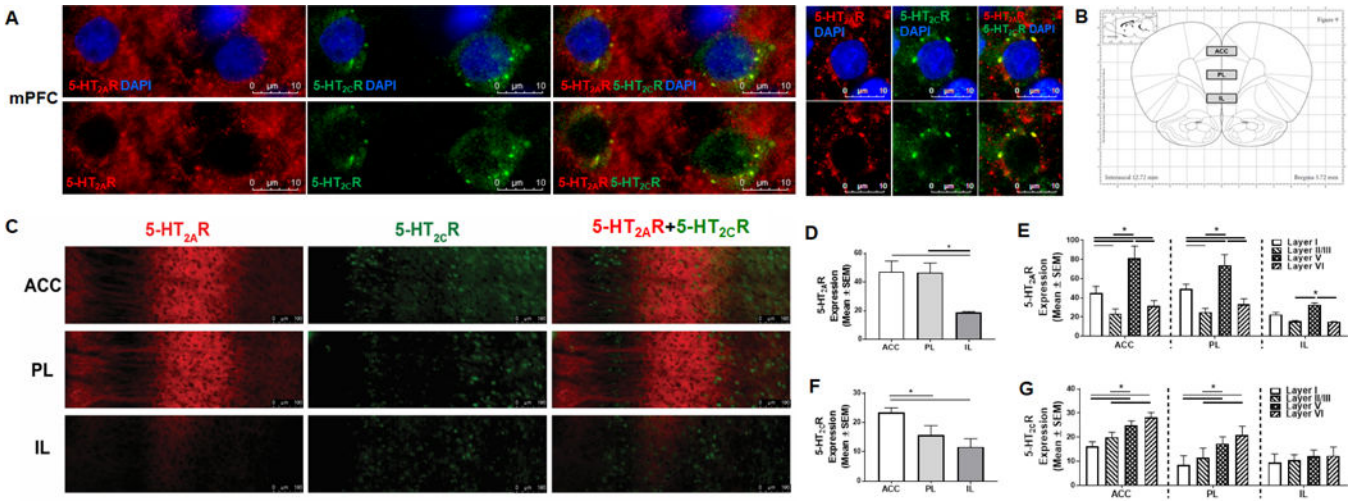

Figure 1. Immunofluorescence detection of 5-HT$_{2A}$R and 5-HT$_{2C}$R expression in rat mPFC.

Figure 1A illustrates representative staining patterns for the anti-5-HT$_{2A}$R antibody RA24288 (red) and anti-5-HT$_{2C}$R antibody sc17797 (green) in the rat mPFC (DAPI-positive nuclei visualized in blue). The boxes in **Figure 1B** illustrate the region of interest analyzed in the ACC, PL, and IL (template from Paxinos and Watson 2005)[27] Representative images of 5-HT$_{2A}$R and 5-HT$_{2C}$R expression in the ACC, PL, and IL are provided in **Figure 1C**. Significant differences in 5-HT$_{2A}$R expression between mPFC subregions (**Figure 1D**) and between layers within subregions (**Figure 1E**) were observed. Significant differences in 5-HT$_{2C}$R expression between regions (**Figure 1F**) and between layers within regions (**Figure 1G**) were also observed. For **Figures 1D–G**, 5-HT$_{2A}$R and 5-HT$_{2C}$R expression are represented as bars (mean ± SEM) for biological replicates (n=3). The lines above each bar denote statistically-significant group comparisons revealed by Tukey's multiple comparisons test (*$p < 0.05$).

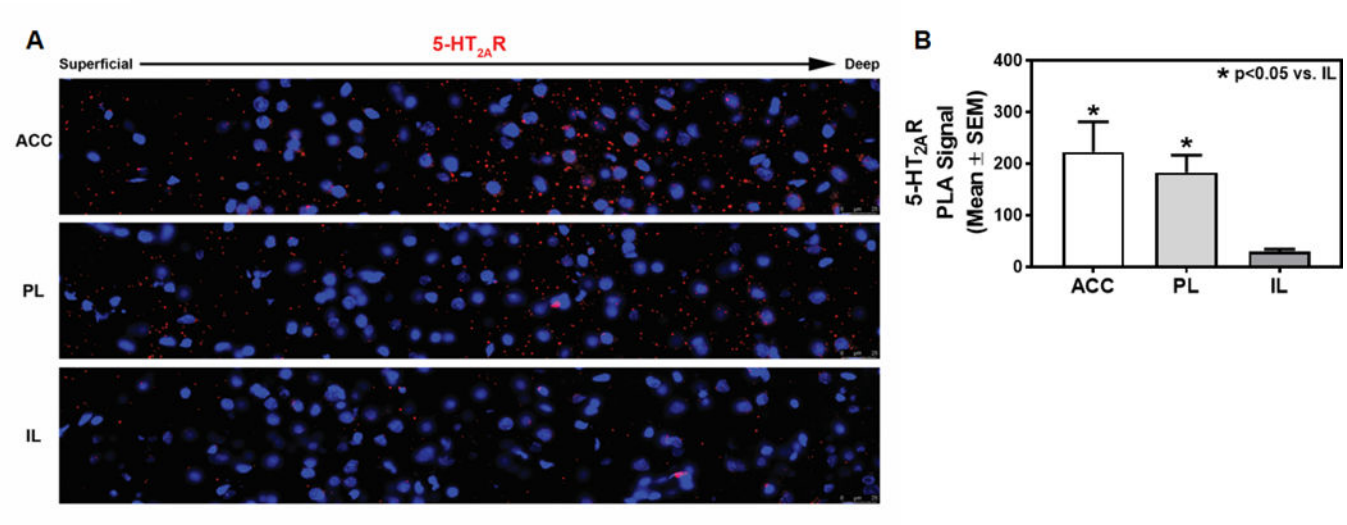

Figure 2. Proximity ligation assay (PLA) detection of 5-HT$_{2A}$R expression in mPFC.

Figure 2A illustrates representative 5-HT$_{2A}$R expression in the ACC, PL, and IL. Red puncta represent PLA signal while DAPI-staining is visualized in blue. Significant differences in PLA puncta between brain regions were observed (**Figure 2B**). The 5-HT$_{2A}$R expression is represented as bars (mean ± SEM) for biological replicates (n=4). *$p < 0.05$

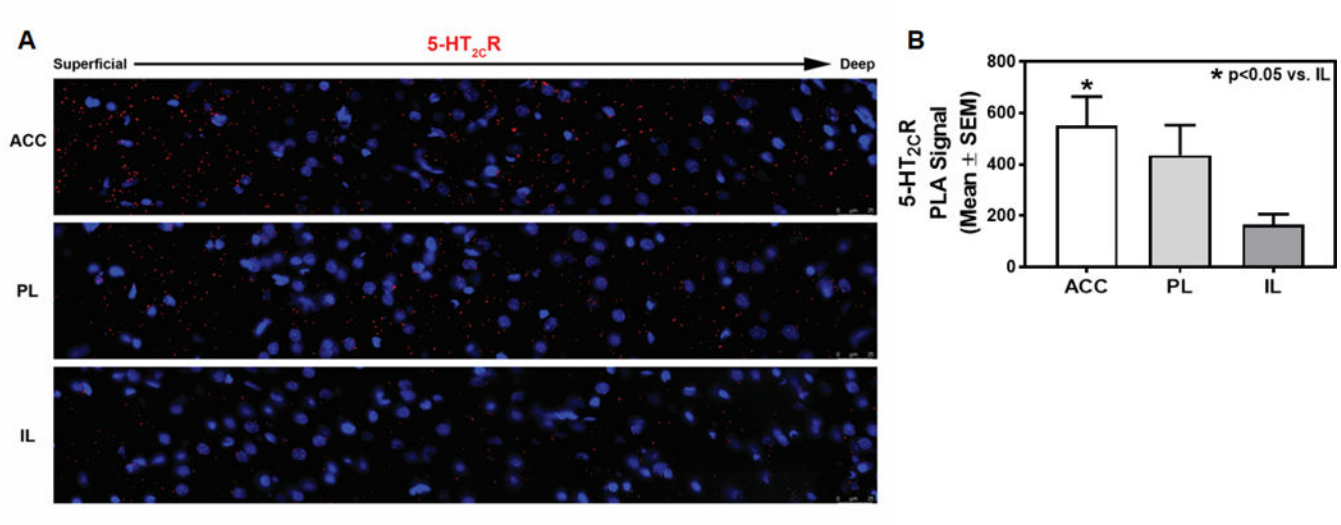

Figure 3. Proximity ligation assay (PLA) detection of 5-HT$_{2C}$R expression in mPFC.

Figure 3A illustrates representative 5-HT$_{2C}$R expression in the ACC, PL, and IL. Red puncta represent the PLA signal while DAPI-staining is visualized in blue. Significant differences in PLA puncta between brain regions were observed (**Figure 3B**). The 5-HT$_{2C}$R expression is represented as bars (mean ± SEM) for biological replicates (n=4). *$p < 0.05$

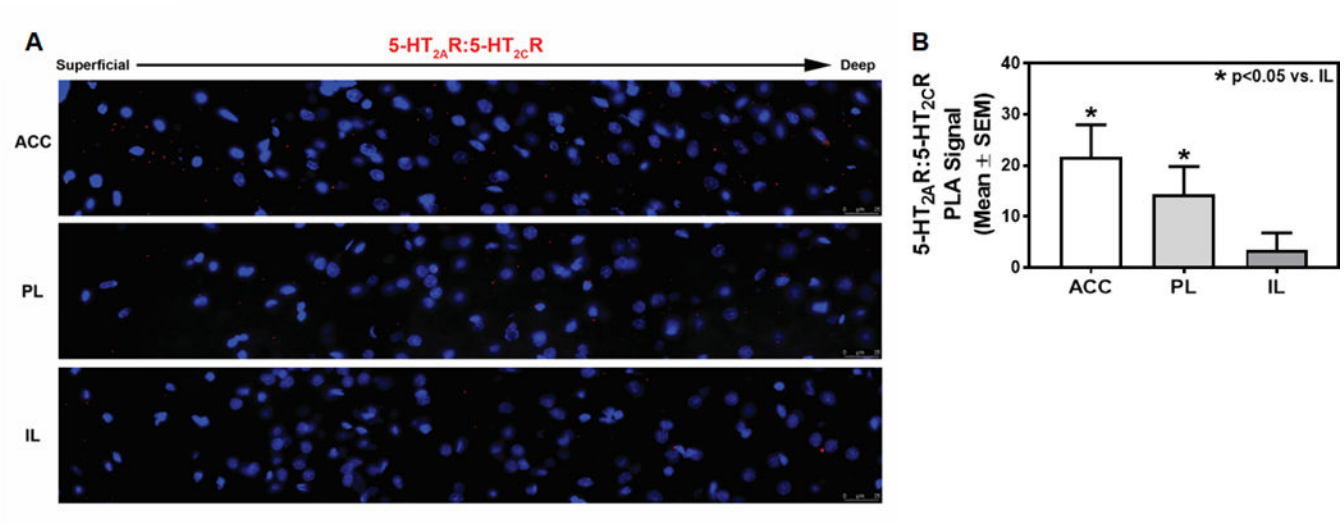

Figure 4. Proximity ligation assay (PLA) detection of 5-HT$_{2A}$R:5-HT$_{2C}$R complex expression.

Figure 4A illustrates representative 5-HT$_{2A}$R:5-HT$_{2C}$R complex expression in the ACC, PL, and IL. Red puncta represent the PLA signal while DAPI-staining is visualized in blue. Significant differences in PLA puncta between brain regions were observed (**Figure 4B**). The 5-HT$_{2A}$R:5-HT$_{2C}$R complex expression is represented as bars (mean ± SEM) for biological replicates (n=4). *$p < 0.05$