

Serotonin Receptors Modulate GABA_A Receptor Channels through Activation of Anchored Protein Kinase C in Prefrontal Cortical Neurons

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Serotonergic neurotransmission in prefrontal cortex (PFC) has long been known to play a key role in regulating emotion and cognition under normal and pathological conditions. However, the cellular mechanisms by which this regulation occurs are unclear. In this study, we examined the impact of serotonin on GABA_A receptor channels in PFC pyramidal neurons using combined patch-clamp recording, biochemical, and molecular approaches. Application of serotonin produced a reduction of postsynaptic GABA_A receptor currents. Although multiple 5-HT receptors were coexpressed in PFC pyramidal neurons, the serotonergic modulation of GABA-evoked currents was mimicked by the 5-HT₂-class agonist (–)-2,5-dimethoxy-4-iodoamphetamine and blocked by 5-HT₂ antagonists risperidone and ketanserin, indicating the mediation by 5-HT₂ receptors. Inhibiting phospholipase C blocked the 5-HT₂ inhibition of GABA_A currents, as did dialysis with protein kinase C (PKC) inhibitory peptide. Moreover, activation of 5-HT₂ recep-

tors in PFC slices increased the *in vitro* kinase activity of PKC toward GABA_A receptor γ 2 subunits. Disrupting the interaction of PKC with its anchoring protein RACK1 (receptor for activated C kinase) eliminated the 5-HT₂ modulation of GABA_A currents, suggesting that RACK1-mediated targeting of PKC to the vicinity of GABA_A receptors is required for the serotonergic signaling. Together, our results show that activation of 5-HT₂ receptors in PFC pyramidal neurons inhibits GABA_A currents through phosphorylation of GABA_A receptors by the activation of anchored PKC. The suppression of GABAergic signaling provides a novel mechanism for serotonergic modulation of PFC neuronal activity, which may underlie the actions of many antidepressant drugs.

Key words: prefrontal cortex; serotonin receptors; GABA_A receptors; phosphorylation; anchoring proteins; RACK1; patch-clamp; single-cell mRNA profiling

Serotonin is a powerful modulator of emotional processes in the CNS. Dysfunction of serotonergic neurotransmission has long been implicated in the pathogenesis of neuropsychiatric disorders, including schizophrenia, depression, and anxiety (Breier, 1995; Dubovsky and Thomas, 1995; Abi-Dargham et al., 1997; Stockmeier, 1997). Many effective drugs for these disorders act primarily on the serotonin system (Fuxe et al., 1983; Deakin, 1988; Griebel, 1995; Meltzer, 1995; Kapur and Remington, 1996; Busatto and Kerwin, 1997; Lieberman et al., 1998). One of the main target structures of the serotonergic system is prefrontal cortex (PFC), a brain region associated with high-level, “executive” processes needed for complicated goal-directed behavior (Goldman-Rakic, 1995; Miller, 1999). PFC is composed of two major neuronal populations: glutamatergic pyramidal principal neurons and GABAergic interneurons. The axon terminals of local GABAergic neurons form numerous synapses with pyramidal projection neurons (Somogyi et al., 1983), exerting powerful inhibitory control over the excitatory output of PFC. Serotonergic projections target both types of PFC neurons in a synaptic and nonsynaptic manner (Smiley and Goldman-Rakic, 1996). Specific changes of the PFC serotonin system and PFC neuronal activity

that are found in patients with neuropsychiatric disorders (Breier, 1995; Dubovsky and Thomas, 1995; Sumiyoshi et al., 1996; Abi-Dargham et al., 1997; Stockmeier, 1997; Dean et al., 1999; Meyer et al., 1999) suggest that serotonin plays a crucial and unique role in PFC.

Molecular cloning experiments have identified at least 13 G-protein-coupled serotonin receptor subtypes, which can be grouped into several classes based on their distinct downstream signal transduction pathways. Serotonin can have both inhibitory and excitatory functions in neuronal networks through the coupling of different 5-HT receptors to distinct ion channels (for review, see Andrade, 1998). Mice lacking serotonin receptors show phenotypes ranging from epilepsy syndrome (Tecott et al., 1995) to increased impulsive aggression (Saudou et al., 1994) to elevated anxiety and antidepressant-like response (Heisler et al., 1998). Because GABA_A receptor-mediated inhibitory synaptic transmission is highly involved in epilepsy, anxiety, and depression (Macdonald and Olsen, 1994), it suggests that the phenotypes in 5-HT receptor knock-out mice may be correlated with changes in GABAergic transmission of PFC neurons. Furthermore, selective alterations in GABA_A receptors, GABA content, and GABAergic local circuit neurons have been discovered in PFC of patients with mental disorders (Benes et al., 1996; Dean et al., 1999; Ohnuma et al., 1999; Lewis, 2000). These findings led us to speculate that one potential cellular substrate of serotonin in PFC is the GABA_A receptor, and dysregulation of GABAergic transmission by serotonin in PFC is an etiological factor in neuropsychiatric diseases. To test this hypothesis, we examined the impact of serotonin on postsynaptic GABA_A receptor-mediated

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currents in dissociated PFC pyramidal neurons. Our results may provide the molecular and cellular mechanisms for serotonergic regulation of inhibitory synaptic transmission in PFC slices, as well as a framework within which the role of serotonin in normal mental functions and neuropsychiatric disorders can be better understood.

MATERIALS AND METHODS

Acute-dissociation procedure. PFC neurons from young adult (3–5 weeks postnatal) rats were acutely dissociated using procedures similar to those described previously (Yan and Surmeier, 1996). In brief, rats were anesthetized and decapitated; brains were quickly removed, iced, and then blocked for slicing. The blocked tissue was cut in 400 μ m slices with a vibratome while bathed in a low Ca²⁺ (100 μ M), HEPES-buffered salt solution [in mM: 140 Na isethionate, 2 KCl, 4 MgCl₂, 0.1 CaCl₂, 23 glucose, and 15 HEPES, pH 7.4 (300–305 mOsm/l)]. Slices were then incubated for 1–6 hr at room temperature (20–22°C) in NaHCO₃ buffered saline bubbled with 95% O₂, 5% CO₂ (in mM): 126 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 1 pyruvic acid, and 10 glucose, pH 7.4 with NaOH (300–305 mOsm/l). All reagents were purchased from Sigma (St. Louis, MO).

Slices were then removed into the low Ca²⁺ buffer, and regions of the PFC were dissected and placed in an oxygenated Cell-Stir chamber (Wheaton Inc., Millville, NJ) containing pronase (1–3 mg/ml) in HEPES-buffered HBSS (Sigma) at 35°C. After 20–30 min of enzyme digestion, tissue was rinsed three times in the low Ca²⁺, HEPES-buffered saline and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm Lux Petri dish, which was then placed on the stage of an inverted microscope.

Whole-cell recordings. Whole-cell recordings of GABA-activated currents used standard techniques (Surmeier et al., 1995; Yan and Surmeier, 1997). Electrodes were pulled from Corning 7052 glass (A-M Systems Inc., Carlsberg, WA) and fire-polished before use. The internal solution consisted of (in mM): 180 N-methyl-D-glucamine, 40 HEPES, 4 MgCl₂, 0.5 BAPTA, 12 phosphocreatine, 2 Na₂ATP, 0.2 Na₃GTP, and 0.1 leupeptin, pH 7.2–3 with H₂SO₄ (265–270 mOsm/l). The external solution consisted of (in mM): 135 NaCl, 20 CsCl, 1 MgCl₂, 10 HEPES, 0.001 TTX, 5 BaCl₂, and 10 glucose, pH 7.3 with NaOH (300–305 mOsm/l).

Recordings were obtained with an Axon Instruments 200 patch-clamp amplifier that was controlled and monitored with a IBM personal computer running pClamp (version 8.1) with a DigiData 1320 series interface (Axon Instruments, Foster City, CA). Electrode resistances were typically 2–4 M Ω in the bath. After seal rupture, series resistance (4–10 M Ω) was compensated (70–90%) and periodically monitored. The cell membrane potential was held at 0 mV. GABA (100 μ M) was applied briefly (2–3 sec) every minute. Drugs were applied with a gravity-fed “sewer pipe” system. The array of application capillaries (~150 μ m inner diameter) was positioned a few hundred micrometers from the cell under study. Solution changes were effected by altering the position of the array with a DC drive system controlled by a microprocessor-based controller (Newport, Irvine, CA).

Serotonin receptor ligands [serotonin, (–)-2,5-dimethoxy-4-iodoamphetamine (*R*(–)-DOI), 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), methysergide, risperidone, ketanserin, and cyanopindolol (Sigma)] were made freshly on the day of experiments. Second messenger reagents U73122, U74133 (Calbiochem, San Diego, CA), heparin (Sigma), phorbol-12-myristate-13-acetate (PMA), and 4 α -phorbol, PKC_{19–31} (Alexis Biochemical Co., San Diego, CA) were made up as concentrated stocks in water or DMSO and stored at –20°C. Stocks were thawed and diluted immediately before use. The amino acid (aa) sequence for the PKC anchoring inhibitory peptide RACK1 (receptor for activated C kinase)-rVI is DGGDIINALCFSPNR. The amino acid sequence for the scrambled peptide sRACK1-rVI is FDSRGIGPDINCANL. The amino acid sequence for the PKC anchoring inhibitory peptide AKAP[31–52] is KASMLCFKRRKKAALKPKKAG.

Data analyses were performed with AxoGraph (version 3.0; Axon Instruments), Kaleidagraph (version 3.0.4; Albeck Software, Reading, PA), and Statview (version 4.5; Abacus Concepts, Calabasas, CA). Box plots were used for graphic presentation of the data because of the small sample sizes (Tukey, 1977). The box plot represents the distribution as a box with the median as a central line and the hinges as the edges of the box. The inner fences run to the limits of the distribution excluding outliers. For analysis of statistical significance, paired *t* tests were per-

formed to compare the differential degrees of current inhibition between groups subjected to different treatment.

Single-neuron mRNA profiling. For the detection of serotonin receptor mRNAs in PFC pyramidal neurons, we used the single-cell reverse transcription (RT)-PCR technique similar to those described previously (Surmeier et al., 1996; Yan and Surmeier, 1996, 1997). A patch electrode was used to lift a dissociated neuron into a stream of control solution, and then the neuron was aspirated into the electrode by applying negative pressure. After aspiration, the electrode was broken, and the contents were ejected into a 0.5 ml Eppendorf tube containing 5 μ l of diethylpyrocarbonate-treated water, 0.5 μ l of RNasin (28 U/ μ l), 0.5 μ l of dithiothreitol (DTT) (0.1 M), and 1 μ l of oligo-dT primer (0.5 μ g/ μ l). The mixture was heated to 70°C for 10 min and then incubated on ice for >1 min. Single-strand cDNA was synthesized from the cellular mRNA by adding SuperScript II reverse transcriptase (1 μ l, 200 U/ μ l) and buffer [4 μ l, 5 \times first-strand buffer (in mM): 250 Tris-HCl, 375 KCl, and 15 MgCl₂], RNasin (0.5 μ l, 28 U/ μ l), DTT (1.5 μ l, 0.1 M), and mixed dNTPs (1 μ l, 10 mM each). The reaction mixture (20 μ l) was incubated at 42°C for 50 min. The reaction was terminated by heating the mixture to 70°C for 15 min and then icing. The RNA strand in the RNA-DNA hybrid was then removed by adding 1 μ l of RNase H (2 U/ μ l) and incubating for 20 min at 37°C. All reagents were obtained from Life Technologies (Grand Island, NY).

The cDNA from the RT of RNA in single PFC neurons was amplified with the PCR, which was performed with a thermal cycler (MJ Research Inc., Watertown, MA) in thin-walled plastic tubes. Reaction mixtures contained 2–2.5 mM MgCl₂, 0.5 mM each of the dNTPs, 0.8–1 μ M primers, 2.5 U *Taq* DNA polymerase (Promega, Madison, WI), 5 μ l of 10 \times buffer (Promega), and one-fourth (5 μ l) of the cDNA template made from the single cell RT reaction. The thermal cycling program for the first-round amplification was as follows: 94°C for 1 min, 52°C for 1 min, and 72°C for 1.5 min for 30 cycles. Two microliters of the first-round PCR product was used as the cDNA template for the second-round amplification: 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min for 45 cycles. Ten microliters of the second-round PCR product was separated by electrophoresis in ethidium bromide-stained 1.5% agarose gels. Negative controls for contamination from extraneous and genomic DNA were run for every batch of neurons. The tissue RT-PCR procedure (Surmeier et al., 1996) was used to detect mRNAs in PFC.

PCR primers were designed based on GenBank sequences for serotonin receptors. The primers used to amplify GAD67 (GABA synthesizing enzyme glutamic acid decarboxylase) mRNA were as follows: 5'-CAGACAAGCAGTATGACGTCTCCT and 5'-AGGAAATCGATGTCAGACTGGGTG. The size of GAD67 amplicon was 435 bp. One round (45 cycle) of PCR amplification was performed for the detection of GAD mRNA. Two rounds of nested PCR amplification were performed for the detection of 5-HT receptor mRNAs. The outer primers used to amplify 5-HT_{1A/1D} and 5-HT_{1B} receptors were as follows: 5'-AAC-TATCTCATYGGCTCC; 5'-CAGCCAGCAGAKGATRAA; 5'-TA-ACTACCTGATCGCCTC; and 5'-GAGCCAGCACACAATAAA. The outer primers used to amplify 5-HT_{2A/2C} receptors were 5'-GCCATWGCTGATATGCTG and 5'-CCASACAAACACATTGAG. The outer primers used to amplify 5-HT₄ receptors were 5'-ACAAGT GACCCCTTAC and 5'-TAGCGCTCATCATCACAG. The outer primers used to amplify 5-HT₆ and 5-HT₇ receptors were as follows: 5'-CTTCACGTCGGACTTGAT; 5'-TGTGAGGACATCGAAGAG; 5'-CGGTTCATGCCTTTTCGTTA; and 5'-ATATTCGGTACTGGCAC.

The specific inner primers for 5-HT_{1A} were 5'-TCTGTACCAGGT-GCTCAACAAG and 5'-AGAGGAAGGTGCTCTTTGGAGT. The size of 5-HT_{1A} amplicon was 638 bp. The specific inner primers for 5-HT_{1B} were 5'-ATCAGCACCATGTACACGGTCA and 5'-GACT-TGGTTCACGTACACAGGA. The size of 5-HT_{1B} amplicon was 557 bp. The specific inner primers for 5-HT_{1D} were 5'-AGATGTCT-GACTGCCTGGTGAA and 5'-TGCGTCTAAGATGCTATCAGC. The size of 5-HT_{1D} amplicon was 316 bp. The specific inner primers for 5-HT_{2A} were 5'-ATTGCCGTGTGGACCATATCTG and 5'-GCAGG-ATTCTTTTCAGATGACG. The size of 5-HT_{2A} amplicon was 460 bp. The specific inner primers for 5-HT_{2C} were 5'-GCCATCATGAAGA-TTGCCATCG and 5'-CGACGTGGTTTCTGATCTGGAT. The size of 5-HT_{2C} amplicon was 358 bp. The specific inner primers for 5-HT₄ were 5'-CCCATAATGCAAGGCTGGAACA and 5'-GGAAGGCAC-GTCTGAAAGACTT. The size of 5-HT₄ amplicon was 504 bp. The specific inner primers for 5-HT₆ were 5'-AGCCATGCTGAACG-CGCTGTAT and 5'-CAAGGCCTTCTGCTATGCTTTG. The size of 5-HT₆ amplicon was 546 bp. The specific inner primers for 5-HT₇ were

5'-CCACTTCTCTGCAACGTCTTC and 5'-GTGTTTGAGCAG-TCTCGAAAGG. The size of 5-HT₇ amplicon was 480 bp.

Protein kinase assay. Brain slices were incubated with the 5-HT₂ receptor agonist DOI or PMA for 20 min and then lysed in cold lysis buffer (0.5% Nonidet P-40, 0.1 mM EDTA, 50 mM Tris-HCl, 125 mM NaCl, 0.1 mM Na₂VO₄, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride) on ice for 30 min. Brain lysates were centrifuged and ultracentrifuged, and PKC was immunoprecipitated with rabbit polyclonal anti-PKC $\alpha\beta\gamma$ (Life Technologies) for 1 hr, followed by the addition of 50 μ l of protein A Sepharose beads and incubation for 1 hr at 4°C. The beads were pelleted by centrifugation and washed three times with lysis buffer and three times with kinase buffer (50 mM Tris-HCl pH 7.5, and 5 mM MgCl₂) and then resuspended in 30 μ l of kinase buffer. *In vitro* kinase activity was measured in the PKC immunoprecipitates using peptides derived from GABA_A receptors as substrates. The intracellular regions between the third and the fourth transmembrane domain of GABA_A receptor β 2 and γ 2 subunits contain identified PKC phosphorylation sites. The sequences for the two peptides against these regions are as follows: GABA_A- β 2, KSRLRRRASQLKITI (amino acids 402–416 in mature β 2 protein without the 24 aa signal peptide); and GABA_A- γ 2, SNRKPSKDKDKKKKNPAPT (amino acids 322–340 in mature γ 2 protein without the 38 aa signal peptide). The assay was initiated by the addition of 5 μ l of [γ -³²P]ATP (10 mCi/ml) and 1 μ l of peptide (10 mg/ml), continued for 20 min at room temperature, and stopped by boiling samples in SDS-PAGE sample buffer. In some cases, 1 μ l of the myristoylated PKC inhibitory peptide PKC_{19–31} (10 mg/ml; Promega) was added to the reaction mixture. Samples were loaded onto a 20% polyacrylamide gel and subjected to electrophoresis. The gels were vacuum dried and exposed to Biomax film (Eastman Kodak, Rochester, NY). Kinase activity was quantified by an phosphorimager.

RESULTS

Serotonin reduces GABA-activated currents in PFC pyramidal neurons

In rats, the prelimbic, infralimbic, and ventral anterior cingulate cortex represent the major subdivisions of PFC (Groenewegen, 1988). Based on their patterns of neural connectivity, these regions are thought to be functionally related to PFC in the primate (Kolb, 1984; Uylings and van Eden, 1990; Conde et al., 1995). To test the potential impact of serotonin on GABAergic signaling, we examined the effect of serotonin on GABA_A receptor-mediated currents in pyramidal neurons located in the intermediate and deep layers (III–VI) of the rat PFC. GABA was applied to these neurons that were voltage clamped using whole-cell techniques. The application of GABA (100 μ M) evoked a partially desensitizing outward current with the decay rate fitted by a single or double exponential. This current was completely blocked by the GABA_A receptor antagonist bicuculline (30 μ M, $n = 3$; data not shown), confirming mediation by the GABA_A receptor. Application of serotonin (20 μ M) caused a reduction in the amplitudes of GABA_A currents in ~90% of PFC pyramidal neurons tested ($n = 21$). Shown in Figure 1A is a plot of peak currents evoked by repeated application of GABA (100 μ M) as a function of time. GABA was applied once per minute for 2–3 sec to minimize desensitization-induced decrease of current amplitude. The serotonergic reduction of GABA_A currents had slow onset kinetics, taking 3–4 min to stabilize. The modulation was not accompanied by changes in current decay kinetics. The median reduction of peak GABA_A currents by serotonin was 25.4% ($n = 21$) (Fig. 1B). This serotonin-mediated inhibition of GABA_A currents did not result from an agonist-independent run-down of the current, because no significant decrease of the current was observed in the absence of serotonin (Fig. 1A). Application of the nonselective 5-HT receptor antagonist methysergide (10 μ M) primarily abolished the inhibition, and washing off the antagonist led to recovery of 5-HT effect on GABA_A currents (Fig. 1C,D). As shown in Figure 1C, inset, 70–90% of

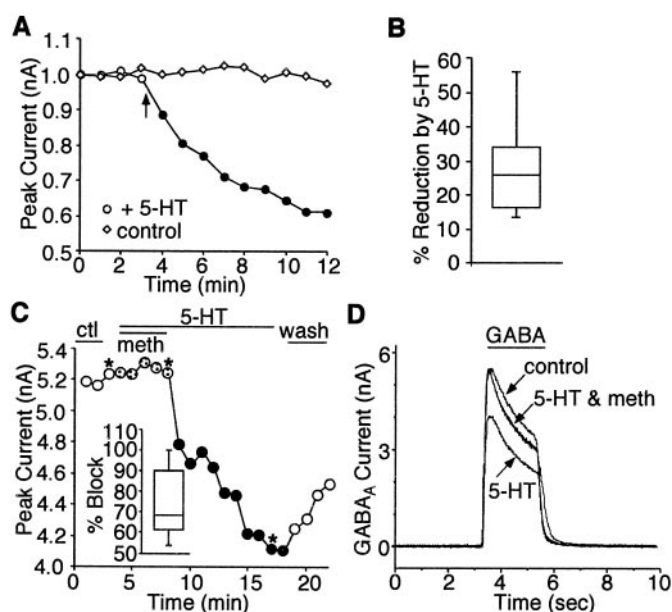


Figure 1. Application of serotonin causes a reduction of GABA_A receptor currents in PFC pyramidal neurons. *A*, Time course of peak current evoked by GABA (100 μ M) in the absence (control) or presence of serotonin (20 μ M). The starting point for continuous application of serotonin is marked by the arrow. Application of serotonin caused an inhibition of GABA_A receptor currents, whereas repeated application of GABA alone evoked a current that was stable during the whole-cell recording. *B*, Box plot summary of the percentage of reduction of peak GABA_A currents produced by serotonin in a sample of 21 PFC pyramidal neurons. *C*, Plot of peak GABA_A current as a function of time and ligand application. In the presence of the nonselective 5-HT receptor antagonist methysergide (*meth*; 10 μ M), serotonin had little effect on GABA_A currents; washing off the antagonist led to recovery of the serotonin inhibition (*wash*). The inset is a box plot summary showing the percentage of serotonin effect blocked by methysergide ($n = 8$). *D*, Representative current traces taken from the records used to construct *C* (at time points denoted by *).

serotonergic inhibition of GABA_A currents was blocked by methysergide ($n = 8$), confirming that this effect is mediated by 5-HT receptors.

PFC pyramidal neurons express multiple 5-HT receptor mRNAs

To test which 5-HT receptor may mediate the serotonergic inhibition of GABA_A currents, we first examined the expression of 5-HT receptor mRNAs in PFC neurons. The mRNAs for 5-HT₁ (5-HT_{1A}, 1B, 1D), 5-HT₂ (5-HT_{2A}, 2C), 5-HT₄, 5-HT₆, and 5-HT₇ receptors were first detected with cDNA obtained from the whole PFC tissue. As shown in Figure 2A, all of these receptor subtypes are present in this brain region. Because PFC is composed of heterogeneous neuronal populations, it is important to determine how serotonin receptors are coordinately expressed in individual PFC pyramidal neurons. Cells were harvested after recording and analyzed using the single-cell RT-PCR technique (Yan and Surmeier, 1996, 1997). Acutely isolated PFC pyramidal neurons were readily distinguished from GABAergic interneurons by their distinct morphological features: a pyramidal-shaped soma and a prominent apical dendrite. The expression of GAD mRNA was consistently negative in the harvested neurons (data not shown), confirming that they are not GABAergic interneurons. For the detection of 5-HT receptor subtypes, a two-stage PCR amplification protocol was used to minimize problems associated with

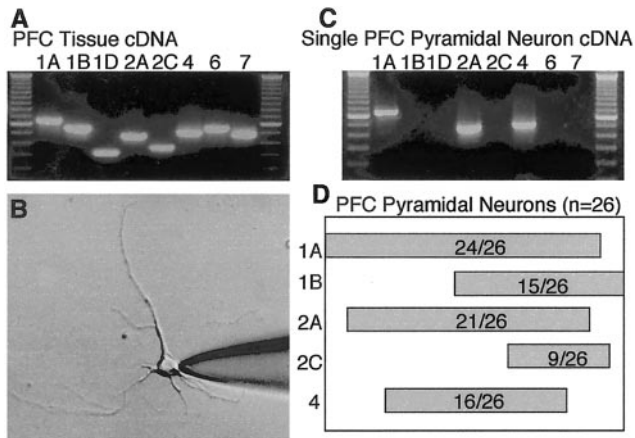


Figure 2. Multiple 5-HT receptor mRNAs are coexpressed in single PFC pyramidal neurons. *A*, Photomicrograph of an ethidium bromide-stained gel showing the expression profile of 5-HT receptor mRNAs in PFC tissue by RT-PCR. *B*, Photomicrograph of an acutely isolated PFC pyramidal neuron. After recording, the cell was harvested by the patch electrode and subject to mRNA profiling. *C*, Expression profile of 5-HT receptor mRNAs in this PFC pyramidal neuron showing the coexpression of 5-HT_{1A}, 5-HT_{2A}, and 5-HT₄ receptor mRNAs. *D*, Bar plot showing the coordinated expression of major 5-HT receptor mRNAs in a sample of 26 PFC pyramidal neurons. The extent of coexpression is indicated by the overlap of the bars.

template abundance. In the first round of amplification, each family of subunits was amplified using degenerate primer sets. In the second round, subunit-specific “nested” primers were used.

A representative picture of an acutely dissociated PFC pyramidal neuron is shown in Figure 2*B*. The mRNA profile for 5-HT receptor subtypes of this cell is shown in Figure 2*C*. The 5-HT_{1A}, 5-HT_{2A}, and 5-HT₄ receptor mRNAs were coexpressed in this neuron. In 26 individual PFC pyramidal neurons tested, nearly all of them expressed the 5-HT_{1A} receptor mRNA (24 of 26). Approximately 60% of these cells expressed the 5-HT_{1B} receptor mRNA (15 of 26). A large portion of these cells expressed the 5-HT_{2A} receptor mRNA (21 of 26), whereas the 5-HT_{2C} receptor mRNA was detected in only one-third of these cells (9 of 26). The 5-HT₄ receptor mRNA was found in ~60% of these cells (16 of 26). Other 5-HT receptor subtype mRNAs (e.g., 5-HT_{1D}, 5-HT₆, and 5-HT₇) were rarely detected (<10%). The coordinated expression of major 5-HT receptor mRNAs in the sample of 26 PFC pyramidal neurons is summarized in Figure 2*D*, from which several coexpression patterns of 5-HT receptors detectable in individual cells could be seen. For example, the simultaneous mRNA expression of all five subtypes (5-HT_{1A}, 1B, 2A, 2C, 4) was detected in 5 of 26 neurons, and coexpression of four subtypes (5-HT_{1A}, 1B, 2A, 4) was detected in 4 of 26 neurons. A subset of neurons (7 of 26) had detectable mRNA levels of three subtypes (5-HT_{1A}, 2A, 4), and other neurons (4 of 26) had only two subtypes (5-HT_{1A}, 2A). The coordinated expression of mRNAs encoding multiple 5-HT receptor subtypes suggests that 5-HT could regulate PFC functions by simultaneously activating distinct signaling cascades mediated by these receptors.

Serotonergic modulation of GABA-activated currents is mediated by 5-HT₂ receptors

Because multiple 5-HT receptors are simultaneously expressed in individual PFC pyramidal neurons, we next used subtype-specific agonists and antagonists to examine which 5-HT receptor was involved in the modulation of GABA_A currents. 5-HT_{1A} and

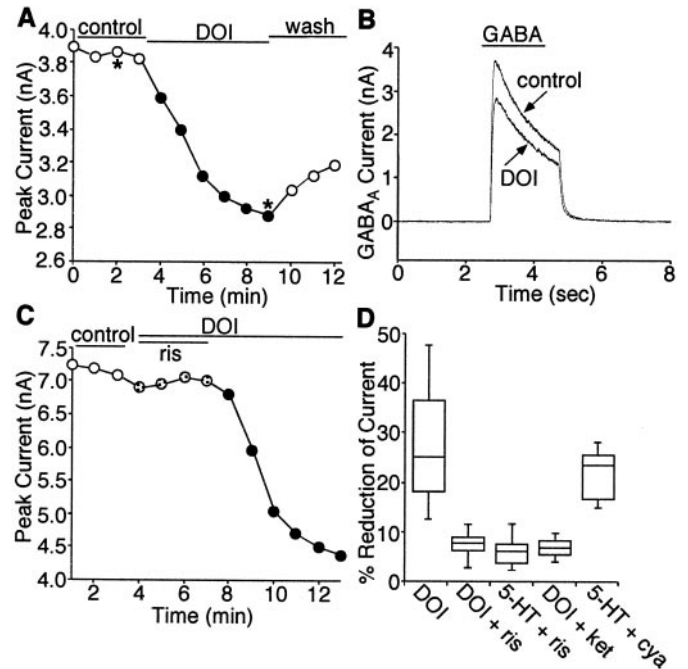


Figure 3. Serotonergic modulation of GABA_A currents is mediated by 5-HT₂ receptors. *A*, Plot of peak GABA_A current as a function of time and agonist application. The 5-HT_{2A/C} agonist DOI (10 μM) caused an inhibition of GABA_A currents. *B*, Representative current traces taken from the records used to construct *A* (at time points denoted by *). *C*, Plot of peak GABA_A current as a function of time and ligand application. The 5-HT₂ receptor antagonist risperidone (*ris*; 10 μM) blocked the effect of DOI on GABA_A currents. *D*, Box plot summary of the percentage of reduction of peak GABA_A currents produced by DOI or 5-HT in the absence and presence of different antagonists. The 5-HT₂-class antagonist risperidone (*ris*; 10 μM) and ketanserin (*ket*; 20 μM) blocked the effect of DOI and 5-HT, whereas the 5-HT₁-class antagonist cyanopindolol (*cya*; 20 μM) was ineffective.

5-HT_{2A} are the most prominent serotonin receptor subtypes expressed in PFC pyramidal neurons, so we first examined the potential role of these receptors in the modulation of GABA_A currents. In eight PFC pyramidal neurons tested, GABA_A currents were not affected by the 5-HT_{1A} receptor agonist 8-OH-DPAT (20 μM; data not shown), suggesting that the serotonergic effect on GABA_A channels was not mediated by the 5-HT_{1A} receptor. On the other hand, application of the 5-HT_{2A/C} receptor agonist DOI (10 μM) caused a reduction (27.1 ± 10.6%, mean ± SD; *n* = 24) in the amplitudes of GABA_A currents without affecting the time constants for current decay in most of the PFC pyramidal neurons tested (87.5%) (Fig. 3*A,B*), mimicking the inhibitory effect of 5-HT. Washing off DOI led to a partial or complete recovery of GABA_A currents. To verify that 5-HT_{2A/C} receptors were mediating the modulation seen with DOI or 5-HT, the ability of 5-HT₂-class antagonist risperidone (Baxter et al., 1995) to prevent the action of DOI or 5-HT was examined. As shown in Figure 3*C*, risperidone (10 μM) almost completely eliminated the effects of DOI (10 μM). Removing the antagonist restored the ability of DOI to modulate GABA_A currents. The median reduction of peak GABA_A currents by DOI was 25.1% (*n* = 24) (Fig. 3*D*), similar to the 5-HT effect (median reduction of 25.4%; *n* = 21) (Fig. 1*B*). In the presence of risperidone, the inhibition of GABA_A currents by DOI (*n* = 10) or 5-HT (*n* = 6) was both significantly blocked (median reduction of 7.8 and 5.8%, respectively) (Fig. 3*D*). Because risperidone also

blocks D₂ dopamine receptors, we further tested the DOI effect in the presence of the selective D₂ antagonist sulpiride. Application of sulpiride (10 μM) did not block the inhibitory effect of DOI on GABA_A currents (data not shown), suggesting that D₂ receptors were not involved in the action of DOI. Moreover, the DOI or 5-HT effect was greatly attenuated by another 5-HT₂-class antagonist, ketanserin (median reduction of 6.4%; *n* = 5) (Fig. 3D) but was not altered by a 5-HT₁-class antagonist, cyanopindolol (median reduction of 22.3%; *n* = 5) (Fig. 3D). These results suggest that the serotonergic effect on GABA_A channels is mediated by 5-HT_{2A/C} receptors. Our single-cell mRNA profiling experiments have shown that 5-HT_{2A} receptors are expressed in the majority of PFC pyramidal neurons (>80%), whereas 5-HT_{2C} receptors are detected in only one-third of these cells, suggesting that the effects of DOI on GABA_A currents found in most PFC pyramidal neurons can be primarily attributed to 5-HT_{2A} receptors.

5-HT₂ receptors modulate GABA_A currents via the phospholipase C β isoform-mediated pathway

We next examined the signal transduction pathways mediating the modulation of GABA_A currents by 5-HT₂ receptors. In studies using cell lines, it has been found that activation of 5-HT₂ receptors stimulates phospholipase C β isoform (PLCβ), leading to the release of inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) through the hydrolysis of membrane phosphoinositol lipids. To test whether the modulation of GABA_A currents by 5-HT₂ receptors is through the PLCβ-mediated pathway, we dialyzed the neuron with the selective PLCβ inhibitor U73122 and examined the 5-HT₂ effect on GABA_A currents under this condition. If the 5-HT₂ agonist was exerting its effect through the phospholipid cascade, then inhibiting PLCβ should block the effect of receptor activation. In agreement with this model, dialysis with the PLCβ inhibitor U73122 (4 μM) significantly reduced the ability of DOI to inhibit GABA-evoked currents, whereas the inactive analog U74133 (4 μM) had no effect. A representative experiment is shown in Figure 4. The median reduction of peak GABA_A currents by DOI was 6.2% in the presence of U73122 (*n* = 5) (Fig. 4B) and 27.5% in the presence of U74133 (*n* = 5; *p* < 0.005; paired *t* test) (Fig. 4B). These results indicate that 5-HT₂ receptors modulate GABA_A currents through the PLCβ-mediated pathway.

The 5-HT₂ modulation of GABA_A currents is dependent on PKC activation

GABA_A channels are thought to be heteropentameric structures, composed of different subunits (Macdonald and Olsen, 1994). Protein phosphorylation exerts a powerful impact on the regulation of GABA-activated currents in recombinant and native GABA_A channels (Porter et al., 1990; Kellenberger et al., 1992; Krishek et al., 1994). Because 5-HT₂ receptors couple to the PLCβ–IP₃–DAG pathway, which could lead to the activation of PKC, we tested whether the 5-HT₂-induced reduction of GABA_A currents in PFC neurons was mediated by activated PKC.

Application of the PKC activator PMA (1 μM), but not its inactive analog 4α-phorbol (1 μM), mimicked the serotonergic effect on GABA_A channels, causing an irreversible decrease of the amplitude of GABA_A currents (Fig. 5A). The median inhibition was 37.3% by PMA (*n* = 5) (Fig. 5B) and was only 5.6% by 4α-phorbol (*n* = 5; *p* < 0.002; paired *t* test) (Fig. 5B). If 5-HT₂ receptors were exerting its effect through PKC, then inhibiting the

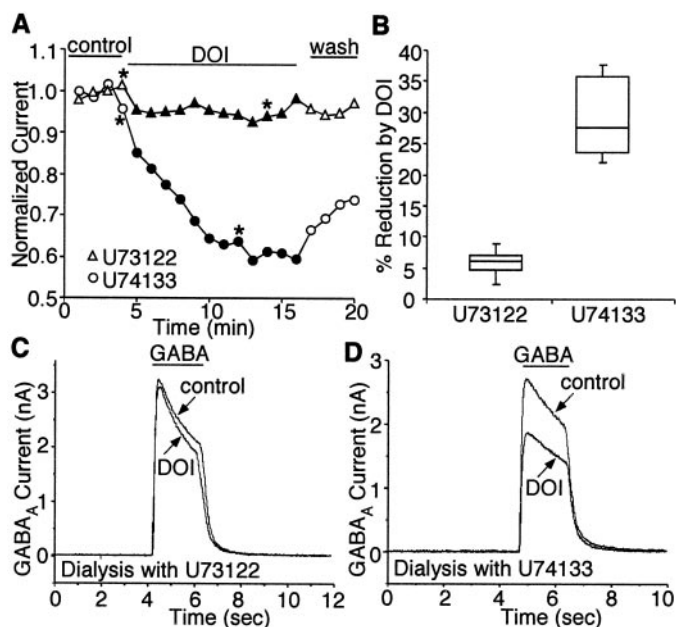


Figure 4. 5-HT₂ modulation of GABA_A currents is blocked by inhibition of PLCβ. *A*, Plot of peak GABA_A current as a function of time and agonist application with U73122 (4 μM) or U74133 (4 μM) in the recording pipette. Dialysis with the PLCβ inhibitor U73122, but not its inactive analog U74133, blocked the DOI effect on GABA_A currents. *B*, Box plot summary of the modulation of GABA_A currents by DOI in the presence of U73122 (*n* = 5) or U74133 (*n* = 5). *C*, *D*, Representative current traces taken from the records used to construct *A* (at time points denoted by *).

activation of PKC should eliminate the effect of 5-HT₂ on GABA_A currents. Because conventional PKC isoforms (PKCα, PKCβ, and PKCγ) depend on Ca²⁺ for their activation (Tanaka and Nishizuka, 1994), we used the IP₃ receptor antagonist heparin (10 U/ml) to block the elevation of intracellular free Ca²⁺ (Finch et al., 1991; Finch and Augustine, 1998) and examined 5-HT₂ modulation of GABA_A currents under this condition. As shown in Figure 5C, dialysis with heparin significantly attenuated the effect of DOI on GABA_A currents. The median reduction of peak GABA_A currents by DOI was 9.1% in the presence of heparin (*n* = 5) (Fig. 5D), whereas in control cells DOI reduced GABA_A currents by 23.5% (*n* = 6; *p* < 0.005; paired *t* test) (Fig. 5D). To further test the involvement of intracellular Ca²⁺ in 5-HT₂ modulation of GABA_A currents, we also dialyzed neurons with a high concentration (10 mM) of BAPTA, a potent and rapid Ca²⁺ chelator. As shown in Figure 5D, the median reduction of peak GABA_A currents by DOI was only 3.8% in the presence of high BAPTA (*n* = 5), which was significantly smaller than the DOI effect in control cells (*n* = 6; *p* < 0.005; paired *t* test). These results suggest that 5-HT₂ modulation of GABA_A channels requires intracellular Ca²⁺. To provide additional evidence for the involvement of PKC, we dialyzed neurons with the PKC inhibitory peptide PKC_{19–31} (20 μM). As shown in Figure 5E, PKC_{19–31} blocked the effect of DOI on GABA_A currents. The median reduction of peak GABA_A currents by DOI was 1.5% in the presence of PKC_{19–31} (*n* = 6) (Fig. 5F), which was significantly smaller than the DOI effect in control cells loaded with peptide-free internal solutions (median reduction of 30.5%; *n* = 5; *p* < 0.005; paired *t* test) (Fig. 5F). Together, these data show that 5-HT₂ receptor-mediated inhibition of GABA_A currents is dependent on PKC activation.

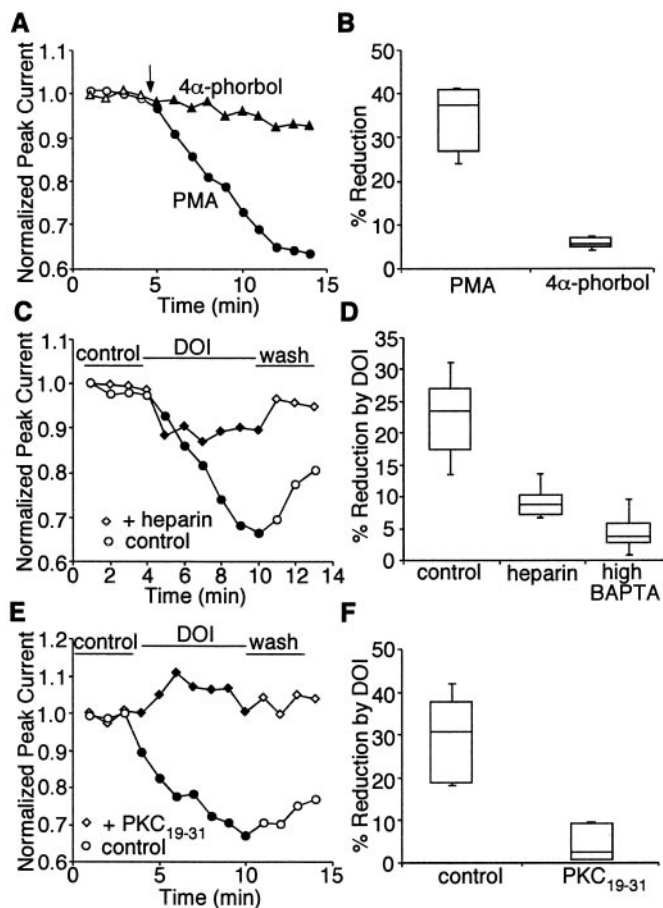


Figure 5. 5-HT₂ modulation of GABA_A currents is dependent on activation of PKC. *A*, Time course of peak GABA_A current in the presence of PMA (1 μM) or 4α-phorbol (1 μM). The starting point for continuous application of PMA or 4α-phorbol is marked by the arrow. The PKC activator PMA caused an irreversible reduction of GABA_A receptor currents, whereas the inactive analog 4α-phorbol had little effect on GABA_A currents. *B*, Box plot summary of the modulation of GABA_A currents by PMA ($n = 5$) or 4α-phorbol ($n = 5$). *C*, Plot of peak GABA_A current as a function of time and agonist application with or without heparin (10 U/ml) in the recording pipette. Blocking IP₃-mediated Ca²⁺ release with heparin significantly attenuated the effect of DOI on GABA_A currents. *D*, Box plot summary of the modulation of GABA_A currents by DOI in the absence (control; $n = 6$) and presence of heparin ($n = 5$) or the high concentration (10 mM) of BAPTA ($n = 5$). *E*, Plot of peak GABA_A current as a function of time and agonist application with or without PKC_{19–31} (20 μM) in the recording pipette. Dialysis with the PKC inhibitory peptide PKC_{19–31} blocked the effect of DOI on GABA_A currents. *F*, Box plot summary of the modulation of GABA_A currents by DOI in the absence (control; $n = 5$) and presence of PKC_{19–31} ($n = 6$).

Activation of 5-HT₂ receptors increases PKC phosphorylation of GABA_A receptor γ2 subunits

Because our electrophysiological experiments have provided strong evidence for the involvement of PKC in 5-HT₂ modulation of GABA_A currents, we hypothesize that phosphorylation of GABA_A receptors by activated PKC is the underlying molecular mechanism for 5-HT₂ inhibition of GABA_A currents. To test this hypothesis, we used biochemical methods to examine whether the activation of 5-HT₂ receptors could enhance PKC phosphorylation of GABA_A receptor subunits in PFC networks.

Multiple PKC phosphorylation sites have been identified in GABA_A receptor γ subunits and β subunits (Macdonald and Olsen, 1994). Peptides derived from the intracellular regions

between the third and the fourth transmembrane domain of GABA_A receptor β2 and γ2 subunits, which contain PKC phosphorylation sites that have been characterized previously (Moss et al., 1992), were used as substrates in *in vitro* kinase assays. Brain slices containing PFC were treated with or without the 5-HT₂ agonist DOI or the PKC activator PMA, and PKC phosphorylation of these peptides were compared in these slices. As shown in Figure 6, PKC phosphorylation of the peptide derived from GABA_A receptor γ2 subunit was significantly increased in slices treated with DOI or PMA (Fig. 6*A*). These effects were completely eliminated by including the PKC inhibitory peptide PKC_{19–31} in the *in vitro* kinase reactions (Fig. 6*B*). In contrast, PKC phosphorylation of the GABA_A receptor β2 peptide was not significantly altered by DOI or PMA treatment (Fig. 6*C*). Equal loading of PKC in the *in vitro* kinase assay was shown by Western blotting of the PKC immunoprecipitates with an antibody against PKC (Fig. 6*D–F*). Quantification of three similar experiments showed that DOI increased PKC phosphorylation of the γ2 peptide by threefold to fourfold, similar to the PMA effect, whereas DOI or PMA treatment did not significantly increase PKC phosphorylation of the β2 peptide over the high basal level (Fig. 6*G*). These results suggest that activation of 5-HT₂ receptors enhances the kinase activity of PKC, causing increased phosphorylation of GABA_A receptor γ2 subunits.

The 5-HT₂ modulation of GABA_A currents requires the anchoring of activated PKC by RACK1

In CNS neurons, ion channels and signaling enzymes are not diffusely located but are compartmentalized to particular subcellular regions by association with various anchoring proteins (Pawson and Scott, 1997). PKC, like other kinases with broad substrate selectivity, achieves the efficacy and specificity of signal transduction through anchoring protein-mediated subcellular targeting to its substrates (Mochly-Rosen, 1995). PKC has multiple binding proteins (Mochly-Rosen and Gordon, 1998). One class of proteins that binds only activated PKC isoforms in a selective manner is called RACKs (Mochly-Rosen et al., 1991). One member of this family of proteins, RACK1, binds to the GABA_A receptor intracellular domain (Brandon et al., 1999). This evidence suggests that RACK1 may be responsible for targeting PKC to GABA_A receptor channels and allowing PKC to effectively phosphorylate these receptors. We hypothesize that blocking PKC–RACK1 interaction may lead to the removal of PKC from the vicinity of GABA_A receptors, thereby attenuating PKC regulation of these channels. To test this hypothesis, we dialyzed neurons with a PKC anchoring inhibitory peptide. RACK1 contains seven WD40 motifs, an internally repeating element that is thought to be involved in protein–protein interactions. A peptide derived from the sequence within the sixth WD40 repeats of RACK1 has been found to inhibit PKC binding to RACK1 *in vitro* (Ron et al., 1994). Based on this result, we synthesized the peptide RACK1-rVI using the sequence from the sixth WD40 repeats of RACK1 and dialyzed the cell with this peptide. As shown in Figure 7, *A* and *B*, dialysis with the PKC anchoring inhibitory peptide RACK1-rVI eliminated the ability of DOI to inhibit GABA_A currents, whereas the control peptide with scrambled amino acid sequence had no effect on 5-HT₂ reduction of GABA_A currents.

To determine the specific involvement of RACK1 in 5-HT₂ modulation of GABA_A currents, we also tested the role of another PKC anchoring protein AKAP79 (Klauck et al., 1996) in this process. AKAP79 is a member of the “PKC substrate-

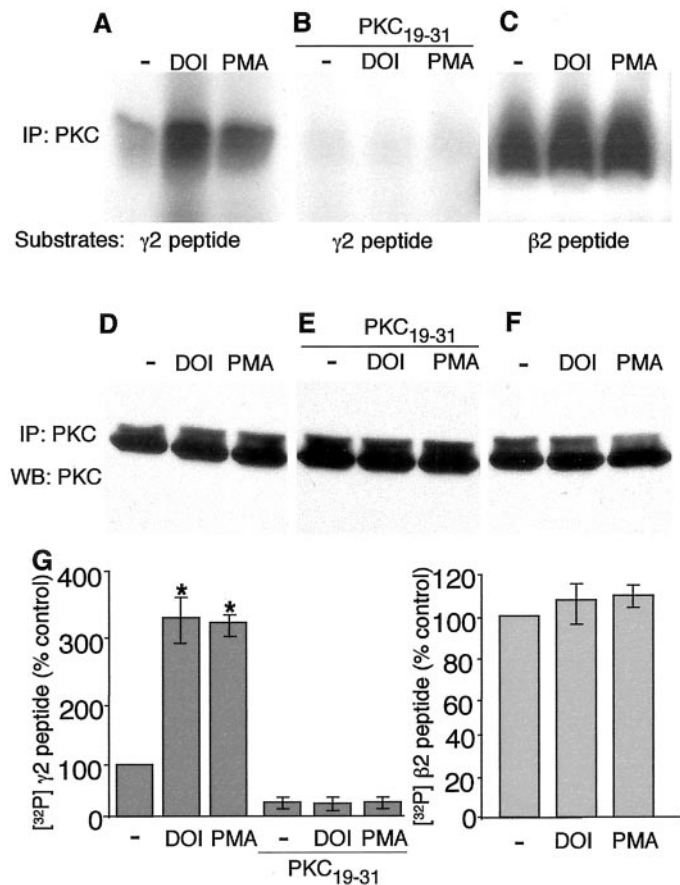


Figure 6. 5-HT₂ receptor activation increases PKC phosphorylation of GABA_A receptor subunits. *A*, *In vitro* kinase activity of PKC immunoprecipitates toward a peptide derived from GABA_A receptor γ2 subunit. Brain slices containing PFC were incubated for 15 min in the absence (–) or presence of either DOI (20 μM) or PMA (1 μM). Lysates of these slices were used for immunoprecipitation with antibodies to PKCαβγ. PKC kinase activity of the immune complex was measured using a peptide derived from GABA_A γ2 subunit as the substrate. Application of DOI or PMA enhanced PKC phosphorylation of the GABA_A γ2 peptide. *B*, Including 1 μl of the PKC inhibitory peptide PKC₁₉₋₃₁ (10 mg/ml) in the *in vitro* kinase reactions blocked PKC phosphorylation of the GABA_A γ2 peptide induced by DOI or PMA. *C*, *In vitro* kinase activity of PKC immunoprecipitates toward a peptide derived from GABA_A receptor β2 subunit. Lysates of brain slices treated with or without DOI (20 μM) or PMA (1 μM) were immunoprecipitated with PKCαβγ antibodies, and PKC activity of the immune complex was measured using a GABA_A β2 subunit peptide as the substrate. Application of DOI or PMA did not significantly alter PKC phosphorylation of the GABA_A β2 peptide over the high basal level. *D–F*, Equal loading of PKC in the *in vitro* kinase assay. Half of the brain lysates used for *in vitro* kinase assay was immunoprecipitated with PKCαβγ antibodies and Western blotted with PKCαβγ antibodies. *G*, Histogram summary of the phosphorylation of GABA_A receptor γ2 subunit-derived peptide and β2 subunit-derived peptide in PFC slices. Treatment with DOI or PMA significantly increased the phosphorylation of GABA_A receptor γ2 subunit, which was blocked by the PKC inhibitory peptide PKC₁₉₋₃₁ (left panel; *n* = 3; **p* < 0.01), but did not significantly enhance the phosphorylation of GABA_A receptor β2 subunit (right panel; *n* = 3).

binding proteins” that could be anchoring proteins for inactive PKC (Mochly-Rosen and Gordon, 1998). Biochemical studies have shown that residues 31–52 of AKAP79 are the putative PKC binding site (Klauck et al., 1996). A peptide encompassing this region of AKAP79 specifically blocked the interaction of AKAP79 with PKC in the overlay assay (Klauck et al., 1996). This peptide, named AKAP[31–52], was dialyzed to PFC pyra-

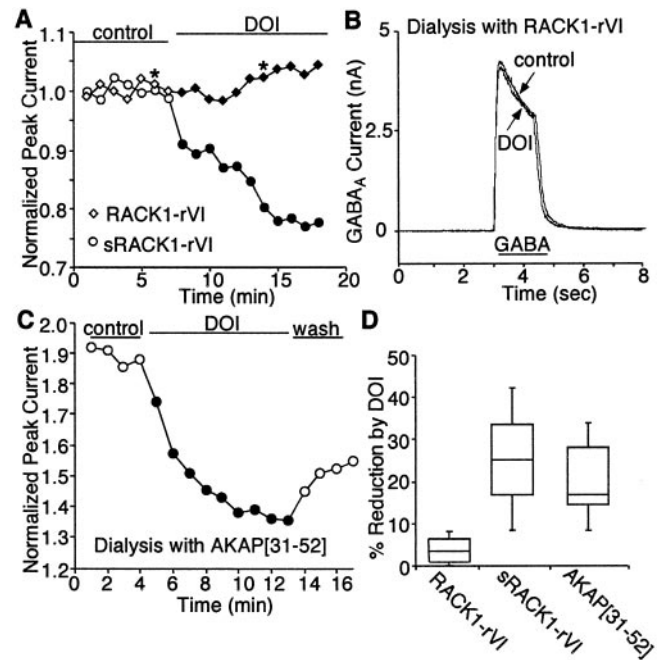


Figure 7. 5-HT₂ modulation of GABA_A receptor function requires anchoring of activated PKC to the channel by RACK1. *A*, Plot of peak GABA_A current as a function of time and agonist application with RACK1-rVI peptide (40 μM) or the scrambled peptide sRACK1-rVI (40 μM) in the recording pipette. Disruption of the interaction between PKC and RACK1 with RACK1-rVI peptide, but not the scrambled peptide sRACK1-rVI, blocked the DOI effect on GABA_A currents. *B*, Representative current traces taken from the records used to construct *A* (at time points denoted by *). *C*, Plot of peak GABA_A current as a function of time and agonist application with AKAP[31–52] peptide (10 μM) in the recording pipette. *D*, Box plot summary of the modulation of GABA_A currents by DOI in the presence of RACK1-rVI peptide (*n* = 11) or the scrambled peptide sRACK1-rVI (*n* = 5), or the AKAP[31–52] peptide (*n* = 5).

midal neurons, and DOI effects on GABA_A currents in these cells were measured. As shown in Figure 7*C*, in the presence of peptide AKAP[31–52], DOI still reduced GABA_A currents, which was similar to the DOI effect in control cells with no peptide dialyzed (Fig. 3*A*). As summarized in Figure 7*D*, the DOI effect on GABA_A currents was significantly smaller in neurons dialyzed with the peptide RACK1-rVI (median reduction of 2.9%; *n* = 11) compared with neurons dialyzed with the scrambled peptide sRACK1-rVI (median reduction of 25.5%; *n* = 5; *p* < 0.01; paired *t* test). However, the DOI effect on GABA_A currents in neurons dialyzed with the peptide AKAP[31–52] was not significantly different from that in control cells (median reduction of 17.6%; *n* = 5; *p* > 0.05; paired *t* test). These results suggest that, in PFC pyramidal neurons, serotonergic modulation of GABA_A receptors requires the anchoring of activated PKC to the vicinity of the channel by RACK1.

DISCUSSION

Because dysfunction of both serotonin and GABA neurotransmission in PFC has been implicated in mental disorders (Manji et al., 2001) and the link between them was unclear, we examined the impact of serotonin on postsynaptic GABA_A receptor functions in PFC pyramidal neurons. Our results show that application of serotonin leads to a reduction of GABA_A receptor currents, suggesting that activation of serotonin signaling can suppress the GABAergic inhibition in PFC circuits. Indeed, recordings of

pyramidal neurons in frontal cortical slices have shown that 5-HT decreases the amplitude of evoked IPSCs by 20% (Zhou and Hablitz, 1999). Although 5-HT could increase the excitability of PFC GABAergic interneurons, leading to increased presynaptic release of GABA (Zhou and Hablitz, 1999), our current study provides evidence showing that 5-HT could also decrease the postsynaptic response to GABA in pyramidal neurons through an intracellular signaling cascade. This suppression of GABAergic inhibition may provide a negative feedback mechanism for serotonergic regulation of the activity of PFC circuits.

To determine which 5-HT receptor(s) may mediate the serotonergic modulation of GABA_A receptor currents, we first need to know what 5-HT receptors are expressed in PFC neurons. With the single-cell RT-PCR technique (Lambolez et al., 1992; Monyer and Lambolez, 1995; Surmeier et al., 1996, 1997; Yan and Surmeier, 1996), mRNAs for multiple 5-HT receptors are found in individual PFC pyramidal neurons. Particularly, 5-HT_{1A} and 5-HT_{2A} receptor mRNAs are most frequently detected in these cells, and 5-HT_{1B}, 5-HT₄, and 5-HT_{2C} receptor mRNAs are also present in a subset of them. These results provide a comprehensive “blueprint” for the potential coexpression of 5-HT receptor subtypes in PFC pyramidal neurons. Moreover, our results are consistent with previous anatomical and physiological studies suggesting the possible colocalization of 5-HT₁ and 5-HT₂ receptors in PFC neurons (Goldman-Rakic et al., 1990; Araneda and Andrade, 1991). The coexpression of multiple 5-HT receptors in the same neuron provides a flexible mechanism by which serotonin may modify the function of PFC in a manner that is both selective and precise (Andrade, 1998).

With the possible coexpression of multiple 5-HT receptors in PFC pyramidal neurons, selective pharmacological tools have been used to determine the 5-HT receptor(s) involved in serotonergic modulation of GABA_A receptors. The serotonin effect on GABA_A currents can be mimicked by a 5-HT_{2A/2C} agonist and blocked by 5-HT_{2A/2C} antagonists, suggesting that 5-HT_{2A/2C} receptors play a predominant role in the serotonergic modulation of GABA_A channels. Postsynaptic 5-HT_{2A} receptors are enriched in apical dendrites proximal to the soma in PFC pyramidal neurons (Jakab and Goldman-Rakic, 1998), whereas GABA_A receptors exhibit a compartmentalized distribution on postsynaptic domains of GABAergic synapses on the soma and proximal dendrites (Nusser et al., 1996), suggesting that 5-HT_{2A} receptors may be localized in the vicinity of GABA_A receptors in PFC pyramidal neurons. This synaptic organization would enable direct serotonergic modulation of local responses to inhibitory input, thereby regulating the integration of the neuron of its myriad inputs and ultimately affecting its output via axonal projections. Intracellular recordings of PFC slices show that activation of 5-HT₂ receptors induces membrane depolarization and a burst of spikes (Araneda and Andrade, 1991; Tanaka and North, 1993). The 5-HT₂ receptor-mediated reduction of GABAergic inhibition could be one of the potential mechanisms for the excitatory actions of 5-HT₂ receptors in PFC circuits.

Several lines of evidence in the present study show that 5-HT₂ modulation of GABA_A receptor currents is through a PKC-mediated pathway. First, inhibition of the upstream signaling component PLC β , which should block the activation of PKC, eliminated 5-HT₂ receptor modulation of GABA_A currents. Second, the PKC activator PMA mimicked the 5-HT₂ effect on GABA_A currents. Third, the IP₃ receptor antagonist and high BAPTA, which should block the elevation of intracellular Ca²⁺ and the activation of conventional PKC isoforms, greatly attenu-

ated the ability of 5-HT₂ receptors to modulate GABA_A currents. Fourth, the PKC inhibitory peptide, which inhibits both autophosphorylation and substrate phosphorylation, blocked the 5-HT₂ effect on GABA_A currents. These physiological results suggest that PKC activation is required for 5-HT₂ modulation of GABA_A channels. Because membrane-translocated PKC can be converted into an effector-independent form for sustained activation (Huang, 1989) once the C-terminal sites have been phosphorylated after the activation loop phosphorylation (Newton, 1997; Dutil et al., 1998; Le Good et al., 1998), 5-HT₂ receptors can elicit physiological effects that are long lasting.

The involvement of PKC led us to speculate that PKC-induced phosphorylation of GABA_A receptor channels may be the underlying molecular mechanism for 5-HT₂ modulation of GABA_A currents. To test this, we used *in vitro* kinase assay to examine whether 5-HT₂ receptors can increase PKC phosphorylation of GABA_A receptor subunits. Treatment of PFC slices with a 5-HT₂ receptor agonist significantly increased the *in vitro* kinase activity of PKC toward the peptide derived from GABA_A receptor γ 2 subunit, but not the peptide derived from GABA_A receptor β 2 subunit. These results suggest that activation of 5-HT₂ receptors in PFC can enhance the PKC catalytic activity and potentiate PKC phosphorylation of GABA_A receptors in a subunit-specific manner. In heterologous expression systems, phosphorylation of Ser-410 in β 2, Ser-327 in γ 2, and Ser-343 in γ 2L is crucial for PKC-mediated downregulation of GABA currents (Kellenberger et al., 1992; Krishek et al., 1994), and PKC is much more effective with γ 2-containing GABA_A receptors (Krishek et al., 1994). Consistent with this, our data reveal that increased PKC phosphorylation of Ser-327 in γ 2 subunit by 5-HT₂ signaling may play a major role in mediating the serotonergic modulation of GABA_A currents in PFC neurons. Although the β 2 peptide is strongly phosphorylated in both control (–) and DOI-treated PFC slices, the lack of additional enhancement of its phosphorylation after 5-HT₂ receptor activation could be attributable to its high basal phosphorylation by endogenous constitutively active PKC.

Given the broad substrate selectivity of PKC, the control of specificity becomes a crucial issue in signal transduction. Subcellular targeting through association with anchoring proteins has emerged as an important mechanism by which signaling enzymes achieve precise substrate recognition and enhanced efficacy of signal transduction (Rosenmund et al., 1994; Gao et al., 1997; Pawson and Scott, 1997; Yan et al., 1999; Feng et al., 2000). The PKC family is composed of at least 10 isoforms, which are localized in different subcellular compartments to control different functions (Tanaka and Nishizuka, 1994). Recent studies have revealed the key role played by anchoring proteins in mediating PKC compartmentalization and functions (Mochly-Rosen, 1995; Mochly-Rosen and Gordon, 1998). RACK1, one member of the RACK family proteins, binds only activated PKC isoforms in a selective manner (Mochly-Rosen et al., 1991) and is strongly associated with neuronal GABA_A receptors in *in vitro* assays (Brandon et al., 1999). Binding to RACK1 results in augmentation of substrate phosphorylation by PKC, and blocking PKC–RACK1 interaction leads to inhibition of PKC-mediated function (Ron et al., 1994, 1995). In aged rat brain cortex, the expression of RACK1 is significantly reduced, and the RACK1-interacting PKC isoforms do not translocate from soluble to membrane during stimulation (Battaini et al., 1997), suggesting that RACK1 is particularly important in cortical functions. To test whether the targeting of activated PKC to GABA_A receptors via RACK1 may endow the enzyme to effectively phosphorylate

these receptors *in vivo*, we examined the involvement of RACK1 in serotonergic modulation of GABA_A currents in PFC neurons. Dialysis with a RACK1-derived peptide that can specifically disrupt the interaction between RACK1 and PKC (Ron et al., 1994) eliminated the serotonergic modulation of GABA_A currents, whereas a peptide that can disrupt the AKAP79–PKC complex (Klauck et al., 1996) had no effect on serotonergic modulation of GABA_A currents. These results suggest that targeting of activated PKC to GABA_A receptors via RACK1 is crucial for the regulation of GABA_A currents by the 5HT₂–PKC signaling pathway.

Together, our results show that activation of 5-HT₂ receptors decreased GABA_A receptor currents in PFC pyramidal neurons, which may provide a postsynaptic mechanism for an excitatory role of serotonin in PFC circuits. In serotonin deficit diseases (e.g., depression), the GABAergic inhibition in PFC may be over potent, leading to the decreased activity (hypoactivity). On the other hand, in serotonin excess diseases (e.g., anxiety), the GABAergic inhibition in PFC may be over suppressed, leading to the increased activity (hyperactivity). The serotonergic modulation of GABA_A receptor functions is dependent on activation of the RACK1-anchored PKC. Elucidation of the signal transduction pathway engaged in serotonergic modulation of GABA_A receptors raises the possibility that intracellular signaling components could be potential targets for novel pharmacological agents with greater therapeutic potential and fewer side effects in the treatment of neuropsychiatric disorders.

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