Desensitization of 5-HT$_{1A}$ Receptors by 5-HT$_{2A}$ Receptors in Neuroendocrine Neurons in Vivo

Yahong Zhang, Thackery S. Gray, Deborah N. D’Souza, Gonzalez A. Carrasco, Katerina J. Damjanoska, Bertalan Dudas, Francisca Garcia, Gina M. Zainelli, Nicole R. Sullivan Hanley, George Battaglia, Nancy A. Muma, and Louis D. Van de Kar

Center for Serotonin Disorders Research and Department of Pharmacology (Y.Z., T.S.G., D.N.D., G.A.C., K.J.D., B.D., F.G., G.M.Z., N.R.S.H., G.B., N.A.M., L.D.V.d.K.) and Department of Cell Biology, Neurology, and Anatomy (T.S.G.), Loyola University Chicago, Stritch School of Medicine, Maywood, Illinois

Received January 8, 2004; accepted April 2, 2004

ABSTRACT

An imbalance between serotonin-2A (5-HT$_{2A}$) and 5-HT$_{1A}$ receptors may underlie several mood disorders. The present studies determined whether 5-HT$_{2A}$ receptors interact with 5-HT$_{1A}$ receptors in the rat hypothalamic paraventricular nucleus (PVN). The sensitivity of the hypothalamic 5-HT$_{1A}$ receptors was measured as oxytocin and adrenocorticotropic hormone (ACTH) responses to the 5-HT$_{1A}$ receptor agonist (+)-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide [(+)-8-OH-DPAT] (40 µg/kg s.c.). The 5-HT$_{2A/2C}$ receptor agonist agonist (−)-DOI [(−)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino propane HCl] (1 mg/kg s.c.) injected 2 h prior to (+)-8-OH-DPAT significantly reduced the oxytocin and ACTH responses to (+)-8-OH-DPAT, producing a heterologous desensitization of the 5-HT$_{1A}$ receptors. Microinjection of the 5-HT$_{2A}$ receptor antagonist MDL100,907 [(+)–α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol; 0, 10, or 20 nmol, 15 min prior to (−)-DOI] into the PVN dose-dependently prevented the desensitization of 5-HT$_{1A}$ receptors induced by the 5-HT$_{2A}$ receptor agonist (−)-DOI. Double-label immunocytochemistry revealed a high degree of colocalization of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in the oxytocin and corticotropin-releasing factor neurons of the PVN. Thus, activation of 5-HT$_{2A}$ receptors in the PVN may directly induce a heterologous desensitization of 5-HT$_{1A}$ receptors within individual neuroendocrine cells. These findings may provide insight into the long-term adaptation of 5-HT$_{1A}$ receptor signaling after changes in function of 5-HT$_{2A}$ receptors; for example, during pharmacotherapy of mood disorders.

The neurotransmitter serotonin (5-HT) plays an important role in mood and impulse control (Caspi et al., 2003). Like other phylogenetically old neurotransmitters, serotonin has many receptors, divided into seven families (5-HT$_1$–5-HT$_7$). 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors have been particularly implicated in the regulation of mood. A functional imbalance may exist between 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in brains of patients with mood disorders (Borsini, 1994; Berendse, 1995). Long-term treatment with selective serotonin reuptake inhibitors produces a desensitization of hypothalamic 5-HT$_{1A}$ receptors both in humans and in rats (Li et al., 1994; Berlin et al., 1998; Lerer et al., 1999; Raap et al., 1999) and alters the function of 5-HT$_{2A}$ receptors (Cadogan et al., 1993; Tilakaratne et al., 1995; Li et al., 1997c; Damjanoska et al., 2003). Recent studies suggest that activation of 5-HT$_{2A}$ receptors induces a desensitization of 5-HT$_{1A}$ receptors (Zhang et al., 2001; Valdez et al., 2002). It is possible that selective serotonin reuptake inhibitors exert their therapeutic activity at least in part by restoring the balance of sensitivity between 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors. For example, combining a 5-HT$_{2A}$ receptor antagonist, such as the atypical antipsychotic drug olanzapine with fluoxetine improves the therapeutic efficacy in treatment-resistant depression (Shelton et al., 2001; Thase, 2002). Additionally, the mechanism of the antipsychotic effects of atypical antipsychotic drugs may in-

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; PVN, paraventricular nucleus; ACTH, adrenocorticotropic hormone; (+)-8-OH-DPAT, (+)-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide; CRF, corticotropin-releasing factor; MDL100,907, (+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol; (−)DOI, (−)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino propane HCl; PBS, phosphate-buffered saline; DAB, 3,3’-diaminobenzidine tetrahydrochloride; $E_{\text{max}}$, maximal response; WAY100635, N-[2-[4-(2-methoxyphenyl)]-1-piperazinyl]ethyl]-N-[2-pyridinyl]; NAN190, 1-[2-methoxyphenyl]-4-[4-(2-phthalimido)butyl]piperazine.
volve an intricate interaction between 5-HT2A and 5-HT1A receptors (Millan, 2000; Ichikawa et al., 2001). Thus, studying the interaction between 5-HT2A and 5-HT1A receptor systems could provide insight into the mechanisms underlying several neuropsychiatric disorders.

Studies in cell culture and in vivo suggest that a two-way interaction exists between 5-HT1A and 5-HT2A receptors (Darmani et al., 1990; Pranzatelli and Pluchino, 1991; Eisen et al., 1993; Eisen and Mullins, 1995; Maswood et al., 1996; Hensler and Truett, 1998; Krebs-Thomson and Geyer, 1998; Valdez et al., 2002). One study indicates that 5-HT1A and 5-HT2A receptors are expressed by neurons in the frontal cortex (Martin-Ruíz et al., 2001). 5-HT1A and 5-HT2A receptors and their mRNA are found in the hypothalamic paraventricular nucleus (PVN) (Appel et al., 1990; Wright et al., 1995; Li et al., 1997a; Gundlah et al., 1999). What is not known is whether 5-HT1A and 5-HT2A receptors are coexpressed by the same neurons. Additionally, it is not clear that oxytocin and CRF neurons express 5-HT1A and/or 5-HT2A receptors. The present studies addressed these questions.

The sensitivity of 5-HT1A receptors in the hypothalamus can be measured from the magnitude of increases in the plasma levels of oxytocin and ACTH, after an injection of the 5-HT1A receptor agonist (+)-8-Hydroxy-2-(di-n-propylamino) tetralin hydrobromide (8-OH-DPAT) (Gilbert et al., 1988; Bagdy and Kalogeras, 1993; Meller and Bohmker, 1994; Vicentí et al., 1998). Activation of 5-HT2A receptors by a peripheral injection of the 5-HT2A/C receptor agonist DOI produces a functional heterologous desensitization of the 5-HT1A receptors in the hypothalamus (Zhang et al., 2001). A peripheral injection of DOI directly activates 5-HT2A receptors in the paraventricular nucleus as the effect can be blocked by an intraparaventricular injection of the 5-HT2A antagonist (+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol (MDL100,907) (Van de Kar et al., 2001; Zhang et al., 2002). Therefore, the DOI-induced desensitization of hypothalamic 5-HT1A receptor systems could result from a direct interaction between 5-HT2A receptor signaling and 5-HT1A receptor systems in the PVN.

The present studies were intended to determine whether 5-HT2A receptors and 5-HT1A receptors are coexpressed by oxytocin and CRF neurons in the PVN and to determine whether the activation of 5-HT2A receptors in the PVN will result in desensitization of 5-HT1A receptor signaling.

### Materials and Methods

#### Animals

Male Sprague-Dawley rats (225–275 g) were purchased from Harlan (Indianapolis, IN). The rats were housed two per cage in a temperature-, humidity-, and light-controlled room (12-h light/dark cycle, lights on 7:00 AM–7:00 PM). Food and water were available ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by Loyola University Institutional Animal Care and Use Committee.

#### Drugs

(+)-8-OH-DPAT and (-)-1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane HCl ([−]-DOI) were purchased from Sigma/RBI (Natick, MA). MDL100,907 was donated by Hoechst Marion Roussel Research Institute (Cincinnati, OH).

(+)-8-OH-DPAT and (-)-DOI were dissolved in 0.9% saline. MDL100,907 was prepared by sonication in a minimal volume of 0.01 N HCl containing 10% 2-hydroxypyrrol-β-cyclodextrin (Sigma/RBI) and diluted with saline to the final concentrations of 10 and 20 mM, respectively. All solutions were made fresh before administration.

### Immunohistochemical and Immunofluorescence Labeling

#### Tissue Preparation

Sprague-Dawley rats (225–275 g) were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and perfused intracardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 0.1 M phosphate-buffered 4% formaldehyde (pH 7.4). The brains were removed and postfixed for 2 h at 4°C, then stored overnight at 4°C in 0.01 M PBS containing 30% sucrose. Serial coronal sections of the hypothalamus (30 μm) were cut with a freezing microtome and transferred into 0.01 M PBS containing 0.2% sodium azide and stored at 4°C.

**Double Labeling of 5-HT1A Receptors, 5-HT2A Receptors, Oxytocin, or CRF.** Sections from three rats were exposed to microwave radiation (400 W for 15 s) to retrieve 5-HT2A receptor antigens (Fritschy et al., 1998). The sections were permeated with 0.2% Triton X-100 (Fisher Scientific Co., Pittsburgh, PA) for 40 min and placed in 3% hydrogen peroxide for 10 min, followed by 5% blocking serum (the same species as the secondary antibody) for 1 h at room temperature. The sections were sequentially labeled for 5-HT1A receptors and 5-HT2A receptors using 3,3′-diaminobenzidine tetrahydrochloride (DAB) and nickel-DAB as the chromogens, respectively. Sections were incubated overnight at room temperature with a polyclonal guinea pig anti-5-HT1A IgG (1:2000 dilution; Chemicon International, Temecula, CA), followed by 1 h of incubation with a biotinylated goat anti-guinea pig immunoglobulin (1:1000 dilution; Vector Laboratories, Burlingame, CA) at room temperature. The sections were then incubated with avidin-biotinylated peroxidase complex (1:200 dilution; Vector Laboratories) for 40 min. The sections were then incubated in 0.05 M Tris-HCl containing 0.02% (w/v) DAB in the presence of ammonium nickel sulfate (0.25%) and 0.002% (v/v) hydrogen peroxide. The sections, mounted on uncoated glass slides, were coveredslipped using Depex mounting media (BDH Lab Supplies, Poole, UK). They immediately were photographed with a digital camera mounted on a microscope. The cover slips and sections were removed from the slides, and the sections were washed in PBS and treated with 10% hydrogen peroxide (10 min) to quench the residual peroxidase. The 5-HT2A receptor labeling was performed using a monoclonal mouse anti-5-HT2A IgG (1:200 dilution; BD Biosciences Pharmingen, San Diego, CA) and a biotinylated horse anti-mouse IgG (1:1500 dilution; Vector Laboratories). The peroxidase reaction was performed using DAB as the chromogen. Finally, the sections were rinsed, mounted on gelatin-coated slides, and overslipped for light microscopy and digital photography.

The photographic images of the first labeling (5-HT1A receptor) and the images after the second labeling (5-HT1A and 5-HT2A receptor) on the same section were compared using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Two sections (~1.53 and ~1.78 from bregma) were sampled per rat (three rats in total) for quantification analysis. The number of neurons immunopositive for 5-HT1A or 5-HT2A receptors and the number of double-labeled neurons for both 5-HT1A and 5-HT2A receptors were counted in the magnocellular and parvicellular regions, respectively, on one side of the paraventricular nucleus. The percentages of double-labeled neurons with respect to 5-HT1A or 5-HT2A receptor immunopositive neurons were calculated in each rat for magnocellular and parvicular regions, respectively. The percentages were then averaged for three rats.

The same procedure as described above was used to double stain CRF- or oxytocin-expressing cells for 5-HT1A or 5-HT2A receptors. 5-HT1A or 5-HT2A receptors were always stained first in the sequence, followed by oxytocin or CRF. Both the oxytocin antibody (1:8000 dilution; provided by S.J. Watson, University of Michigan) and CRF antibody (1:5000 dilution, John Olschowska, University of Rochester, NY) were rabbit polyclonal antibodies. The percentage of
5-HT$_{1A}$ or 5-HT$_{2A}$ receptor immunoreactive oxytocin-expressing neurons was quantified as described above. We were unable to quantify the percentages of CRF neurons in the PVN that were immunoreactive for 5-HT$_{1A}$ or 5-HT$_{2A}$, receptors due to the treatment with colchicine and the low sensitivity of detection of CRF neurons in our samples from rats that were not treated with colchicine.

**Triple Labeling of 5-HT$_{2A}$ and 5-HT$_{1A}$ Receptors and Oxytocin.** After pretreatment of the sections as described above, the sections were incubated overnight at room temperature with a monoclonal mouse anti-5-HT$_{2A}$ IgG (1:50 dilution with PBS buffer; BD Biosciences PharMingen), followed by 1 h of incubation with fluorescein isothiocyanate-conjugated affinity pure donkey anti-mouse IgG (1:200 dilution with PBS buffer; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Subsequently, the sections were stained with a polyclonal guinea pig anti-5-HT$_{1A}$ IgG (1:200 dilution with PBS buffer; Chemicon International), followed by Rhodamine-Red-X-conjugated affinity pure donkey anti-guinea pig IgG (1:200 dilution with PBS; Jackson ImmunoResearch Laboratories Inc.). Background autoimmune fluorescence was reduced by treating the sections with 1% Sudan black for 5 min. The sections were mounted in methanol to remove the Sudan Black and washed thoroughly in PBS buffer. The oxytocin neurons were immunohistochemically labeled using DAB as the chromogen. The sections were then coverslipped with immunofluorescence mounting medium (H-11002, Vector Laboratories) and examined under an immunofluorescence microscope and photographed. The sections were then rinsed in methanol to remove the Sudan Black and washed thoroughly in PBS buffer to the molecular weight of 5-HT$_{1A}$ receptors (46 kDa) in homogenates of the rat frontal cortex. After preadsorption of the antibody with its blocking peptide, the immunoreactive bands were barely detectable (Fig. 1C).

**Experimental Protocol**

Cannula implantation and intraparaventricular injection were performed according to the procedures described in detail previously (Zhang et al., 2002). Ten days after cannula implantation, the rats were handled for 4 consecutive days and then randomly assigned to different experimental groups [eight rats for saline injected groups and 13–15 rats for (-)-DOI-injected groups]. Rats received an intraparaventricular injection (0.5 µl/side) of vehicle or different doses of MDL100,907 (10 and 20 nmol, 0.5 µl/side). Fifteen minutes after the intraparaventricular injections, the rats received an injection of (-)-DOI (1 mg/kg s.c.) or saline. Two hours after the injection of (-)-DOI, the rats received an injection of saline or (+)-8-OH-DPAT (40 µg/kg s.c.) and were sacrificed by decapitation 15 min after the injection of (+)-8-OH-DPAT. The blood was collected in centrifuge tubes containing a 0.5-mL solution of 0.3 M EDTA, pH 7.4. After centrifugation, the plasma was stored at -70°C for radioimmunoassays of plasma hormones. The brains were frozen on dry ice and saved for a histological verification of the position of the cannula. Only animals with the tip of the cannula positioned on the dorsal border of the hypothalamic paraventricular nucleus and with intact neurons in this nucleus were used for data analysis.

Radioimmunoassays

Plasma oxytocin and ACTH concentrations were determined by radioimmunoassays as described in detail in previous publications (Li et al., 1993, 1997b).

**Statistical Analyses**

The data are presented as the group means and S.E.M.s and analyzed by one-way analysis of variance. Post hoc tests were conducted using Newman-Keuls multiple-range test. A computer program (GBSTAT, Silver Spring, MD) was used for the statistical analyses.

**Results**

**Specificity of the 5-HT$_{1A}$ Receptor Antibody.** A high density of 5-HT$_{1A}$ receptor-like immunoreactivity was observed in the dorsal raphe nucleus and the hypothalamic paraventricular nucleus (Fig. 1, A and D). Preadsorption of the 5-HT$_{1A}$ receptor antibody with its blocking peptide greatly reduced the immunoreactivity (Fig. 1, B and E). No 5-HT$_{1A}$ receptor immunoreactivity was observed when the 5-HT$_{1A}$ receptor antibody was omitted from the immunohistochemical procedure (Fig. 1F). Furthermore, immunoblot analysis detected two immunopositive bands corresponding to the molecular weight of 5-HT$_{1A}$ receptors (~46 kDa) in homogenates of the rat frontal cortex. After preadsorption of the antibody with its blocking peptide, the immunoreactive bands were barely detectable (Fig. 1C).

**Coexpression of 5-HT$_{1A}$ and 5-HT$_{2A}$ Receptors by Neurons in the Hypothalamic Paraventricular Nucleus (PVN).**

![Experimental Protocol](Fig. 1. Preadsorption of the 5-HT$_{1A}$ receptor antibody by its blocking peptide. A and E, 5-HT$_{1A}$ receptor-like immunoreactivity in the dorsal raphe before (A) and after (E) blocking peptide preadsorption. B and F, 5-HT$_{1A}$ receptor-like immunoreactivity in the PVN before (B) and after (F) blocking peptide preadsorption. C, no 5-HT$_{1A}$ receptor immunoreactivity was observed in the absence of the primary antibody. PVN, hypothalamic paraventricular nucleus; *, location of the third ventricle.)
Nucleus. Double-label immunohistochemistry revealed that 5-HT1A and 5-HT2A receptors are coexpressed by neurons in the PVN (Fig. 2). Nearly all magnocellular neurons were immunoreactive for 5-HT1A and 5-HT2A receptors in the PVN. A moderate number of 5-HT1A and 5-HT2A receptor immunoreactive neurons were located in the parvocellular part of the PVN. Most of the cells that expressed one of the 5-HT receptors expressed both 5-HT receptor subtypes.

We also observed coexpression of 5-HT1A and 5-HT2A receptors in the supraoptic nucleus as well as in accessory magnocellular neurons scattered between the paraventricular and supraoptic nuclei of the hypothalamus. As shown in Table 1, the percentages of 5-HT2A receptor immunoreactive neurons that were immunopositive for 5-HT1A receptors were 97% in the magnocellular region and 95% in the parvocellular region. In a reversed order, the percentages of 5-HT1A receptor immunopositive neurons that were immunoreactive for 5-HT2A receptors were 96% in the magnocellular region and 97% in the parvocellular region.

Oxytocin and CRF Neurons in the PVN Expressing Both 5-HT1A and 5-HT2A Receptors. Immunohistochemical double labeling of 5-HT1A and 5-HT2A receptors and oxytocin revealed that oxytocin neurons in the PVN were 5-HT1A and 5-HT2A receptor immunopositive (Fig. 3). Of the oxytocin neurons, 94% were immunopositive for 5-HT1A receptors, and 97% of the oxytocin neurons were immunoreactive for 5-HT2A receptors (Table 2). Immunofluorescence double labeling confirmed that 5-HT1A and 5-HT2A receptors were coexpressed by neurons in the PVN (Fig. 4, A and B). A subpopulation of the neurons coexpressing 5-HT1A and 5-HT2A receptors were oxytocin-containing neurons (Fig. 3). CRF-immunoreactive neurons in the hypothalamic PVN also expressed 5-HT1A and 5-HT2A receptor immunoreactivity (Fig. 5, A–D). We were not able to quantify the degree of coexistence of CRF neurons with 5-HT1A and 5-HT2A receptors due to the lower sensitivity of detection of CRF neurons by CRF antibodies. However, 5-HT1A and 5-HT2A receptor immunoreactivity did not appear to nearly approach the high level of coexpression observed in oxytocin neurons.

Microinjection of MDL100,907 into the PVN Prevents (−)DOI-Induced Inhibition of Oxytocin and ACTH Responses to (+)-8-OH-DPAT. Microinjection of the 5-HT2A receptor antagonist MDL100,907 into the hypothalamic PVN or subcutaneous injection of (−)DOI did not alter the basal plasma levels of oxytocin or ACTH. The injection of (+)-8-OH-DPAT significantly elevated the plasma levels of oxytocin by 845% and ACTH by 235% (Fig. 6). (−)DOI (injected 2.25 h prior to blood sampling) significantly inhibited the (+)-8-OH-DPAT-induced increase in the plasma levels of oxytocin (88%) and ACTH (79%). DOI injected alone 2.25 h prior to blood sampling did not alter the plasma levels of oxytocin or ACTH. The intraPVN injection of MDL100,907 dose-dependently reversed the inhibitory effect of (−)DOI on the oxytocin and ACTH responses to (+)-8-OH-DPAT (p < 0.01, Newman-Keuls test). MDL100,907 reversed the inhibitory effect of (−)DOI on oxytocin response to (+)-8-OH-DPAT by 47% at the dose of 10 nmol and by 90% at the dose of 20 nmol (Fig. 6A). Similarly, the inhibitory effect of (−)DOI on ACTH response to (+)-8-OH-DPAT was dose-dependently reversed by MDL100,907 at the doses of 10 (97%) and 20 (100%) nmol, respectively (Fig. 6B).

Discussion

The present studies are the first to provide in vivo evidence that 5-HT2A receptor-mediated desensitization of 5-HT1A receptor functioning occurs in the hypothalamic PVN. The data also indicate that 5-HT1A and 5-HT2A receptors are coexpressed by oxytocin and CRF neurons in the hypothalamic PVN. Thus, it is highly likely that activation of 5-HT2A receptors produces a functional desensitization of hypothalamic 5-HT1A receptor signaling in individual oxytocin and CRF neurons in the PVN.

Studies in cell culture did not indicate an interaction between 5-HT2A receptors and 5-HT1A receptors (Saitoh et al., 1995). Our previous studies in rats (Zhang et al., 2001) indicate that a single administration of the 5-HT2A receptor agonist DOI induces a functional desensitization of 5-HT1A receptors that regulate the secretion of ACTH and oxytocin. Studies from other laboratories (Hensler and Truett, 1998; Valdez et al., 2002) indicate that repeated daily injections of DOI also produce a desensitization of 5-HT1A receptor-induced hypothermia. Furthermore, repeated daily injections of DOI reduce the coupling between 5-HT1A receptors and G proteins in the anterior cingulate cortex (Valdez et al., 2002). However, these phenomena could represent an interneuronal interaction rather than a heterologous desensitization between two receptor systems expressed by the same neurons. The current studies examined the hypothesis that an interneuronal interaction between 5-HT2A and 5-HT1A receptors is possible.

8-OH-DPAT is a selective 5-HT1A receptor agonist with a high affinity for 5-HT1A receptors and 10- to 100-fold lower affinities for other serotonin receptors (Hoyer et al., 1994). The affinity of 8-OH-DPAT for 5-HT1A receptors (pKᵢ = 7.73) (Sleight et al., 1995) is about 10-fold lower than its affinity for 5-HT1A receptors (pKᵢ = 8.4) (Olivier et al., 1999). We have previously tested the specificity of the effect of 8-OH-DPAT by examining its ability to release hormones in the presence of antagonists. An increase in plasma levels of oxytocin and ACTH induced by 8-OH-DPAT is blocked by the 5-HT1A receptor antagonists WAY100635, NAN190, and pin dolol (Bagdy and Kalogeras, 1993; Critchley et al., 1994; Meller and Bohmaker, 1994; Vicent et al., 1998). Thus, an alteration in plasma levels of oxytocin and ACTH after an injection with (+)-8-OH-DPAT is not mediated by activation

Fig. 2. Colocalization of 5-HT1A and 5-HT2A receptor-like immunoreactivity in the same neurons in the hypothalamic PVN. The arrowheads point to 5-HT1A receptor immunopositive neurons; arrows point to neurons that are immunopositive for both 5-HT1A and 5-HT2A receptors.
neuroendocrine neurons in the PVN by activating 5-HT2A MDL100,907 (Van de Kar et al., 2001). Thus, DOI stimulates able to date, with similar affinities for 5-HT 2A and 5-HT2C receptors. Arrows point to oxytocin neurons that are 5-HT1A or 5-HT 2A receptors. Scale bar = 200 μm in A, B, C, and D. *, location of the third ventricle.

of 5-HT7 receptors and can be used to measure changes in the sensitivity of hypothalamic 5-HT1A receptors.

DOI is the most selective 5-HT2A/2C receptor agonist available to date, with similar affinities for 5-HT2A and 5-HT2C receptors (Hoyer, 1988; Van Wijngaarden et al., 1990). DOI-induced increase in Fos expression in neurons of the parventricular nucleus and elevation of plasma levels of oxytocin and ACTH are blocked by the 5-HT2A receptor antagonist MDL100,907 (Van de Kar et al., 2001). Thus, DOI stimulates neuroendocrine neurons in the PVN by activating 5-HT2A receptors.

Activation of 5-HT2A receptors with DOI increases the plasma levels of oxytocin and ACTH with a peak response at 15 and 30 min, respectively, after DOI injection and a return to basal levels by 1 to 2 h postinjection (Bagdy, 1996; Djamjanoska et al., 2003). At 2 h after an injection of DOI, both hormones would have returned to basal level. Our previous experiment indicates that the 5-HT2A receptor-mediated desensitization of 5-HT1A receptors is maximal at 2 h postinjection of DOI (Zhang et al., 2001). For this reason, the injection of 8-OH-DPAT was performed 2 h after the injection of DOI, at a time when the levels of ACTH and oxytocin would have returned to basal levels after the previous injection of DOI.

In our previous study (Zhang et al., 2001), the DOI-mediated heterologous desensitization of the hormone response to 8-OH-DPAT was pharmacologically characterized as a right shift in the hormone responses to 8-OH-DPAT with no change in the maximal response (E_{max}). Depletion of hormone stores by prior injection of DOI would more likely result in reduced E_{max} because insufficient hormone stores would be available for secretion. The fact that the reduction of hormone responses to 8-OH-DPAT was characterized as a right shift in the dose-response curve (increased ED50) rather than a reduced E_{max} suggests that this reduced hormone response to injection of 8-OH-DPAT is not due to hormone-depleting effects of the prior injection of DOI (Zhang et al., 2001).

MDL100,907 is a selective 5-HT2A receptor antagonist (pK_i = 9.07) with a lower affinity for other serotonin receptors (Johnson et al., 1996; Kehne et al., 1996). Low doses of MDL100,907 (0.75–18.7 nmol) microinjected into the hypothalamic PVN dose-dependently inhibit hormone responses to peripherally injected (−)DOI (Zhang et al., 2002). Accordingly, we injected doses of 10 and 20 nmol of MDL100,907 into the hypothalamic PVN to produce partial and complete blockade, respectively, of 5-HT2A receptors in the PVN. In the present study, the intracerebroventricular injection of similar doses of MDL100,907 prevented the DOI-induced desensitization of 5-HT2A receptor signaling. These observations suggest either that 5-HT2A receptors interact directly with 5-HT1A receptors in neurons of the hypothalamic PVN or that hypothalamic neurons expressing 5-HT2A receptors interact with other hypothalamic neurons that express 5-HT1A receptors. The immunocytochemical observations suggest that a direct intracellular interaction occurs between these receptor signaling systems.

Western blot analyses of 5-HT1A receptors have revealed protein bands of different molecular mass: 40, 67, and 70 kDa (Anthony and Azmitia, 1997; Zhou et al., 1999). We found two immunopositive bands corresponding to the molecular mass of 5-HT1A receptors (~46 kDa) (Raymond et al., 1999). 5-HT1A receptors have consensus sequences for post-translational modifications such as glycosylation and phosphorylation (Albert et al., 1990; Anthony and Azmitia, 1997; Zhou et al., 1999; Wu et al., 2002). 5-HT1A receptors are differently modified in different tissues; thus, antibodies for different epitopes may recognize various forms of 5-HT1A receptors that are modified by post-translational mechanisms.

The specificity of 5-HT1A receptor-like immunoreactivity observed in the hypothalamic PVN was verified by preadsorption of the 5-HT1A receptor antibody with its blocking peptide (Fig. 1). Moreover, this antibody detected a similar pattern of 5-HT1A receptor-like immunoreactivity in other brain regions as previously reported, such as dorsal raphe nucleus and hippocampus (Kia et al., 1996). Thus, the immu-

### Table 1

<table>
<thead>
<tr>
<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1A + 5-HT2A</td>
<td>98 (362/369)</td>
<td>92 (160/169)</td>
<td>94 (160/171)</td>
</tr>
<tr>
<td>Magno</td>
<td>95 (160/169)</td>
<td>99 (336/341)</td>
<td>97 (336/348)</td>
</tr>
<tr>
<td>Parvi</td>
<td>97 (362/375)</td>
<td>94 (360/382)</td>
<td>99 (360/377)</td>
</tr>
<tr>
<td>5-HT1A + 5-HT2A</td>
<td>97 (362/375)</td>
<td>97 (540/554)</td>
<td>97 (540/554)</td>
</tr>
<tr>
<td>Magno</td>
<td>99 (105/114)</td>
<td>97 (336/341)</td>
<td>96 (336/348)</td>
</tr>
<tr>
<td>Parvi</td>
<td>99 (105/118)</td>
<td>97 (336/348)</td>
<td>97 (336/348)</td>
</tr>
</tbody>
</table>

Magno, magnocellular regions; Parvi, parvicellular regions.

Fig. 3. Double immunohistochemical labeling of oxytocin and 5-HT1A or 5-HT2A receptors in the PVN. Arrows point to oxytocin neurons that are immunopositive for 5-HT1A or 5-HT2A receptors; arrowheads point to other hypothalamic neurons that express 5-HT1A receptors. Scale bar = 200 μm in A, B, C, and D.
nopositive neurons observed in the hypothalamic paraventricular nucleus most likely represent the labeling of 5-HT1A receptors. The specificity of the 5-HT2A receptor antibody was discussed previously (Zhang et al., 2002). The CRF and oxytocin antibodies have been used by us before (Javed et al., 1999; Van de Kar et al., 2001), and their immunocytochemical staining is consistent with the neuroanatomical location of oxytocin and CRF neurons in the PVN (Piekut and Joseph, 1986; Alves et al., 1998).

The hypothesis of a heterologous desensitization that presupposes a coexpression of both receptors in the same cells is supported by a high degree of coexpression of 5-HT1A and 5-HT2A receptors by neurons in the hypothalamic PVN (>95%). Neurons in the frontal cortex have also been reported to coexpress 5-HT1A and 5-HT2A receptors (Martin-Ruiz et al., 2001). We observed that 5-HT1A and 5-HT2A receptors were coexpressed throughout all magnocellular regions of the PVN. A subpopulation of the magnocellular neurons coexpressing 5-HT1A and 5-HT2A receptors are oxytocin neurons. This suggests that other magnocellular neuroendocrine cells such as vasopressin neurons also coexpress 5-HT1A and 5-HT2A receptors. CRF neurons were separately examined, using colchicine pretreatment to increase the levels of CRF in neurons. Double-labeling immunohistochemistry revealed that 5-HT1A and 5-HT2A receptors are also coexpressed by CRF neurons. However, due to the small size of CRF neurons and morphological changes in the neurons caused by colchicine pretreatment, we were unable to quantify the percentages of CRF neurons that coexpress 5-HT1A and 5-HT2A receptors. Combined, these data suggest that 5-HT2A receptors may interact with 5-HT1A receptors through their signaling proteins to regulate hormone release.

Activation of both 5-HT2A and 5-HT1A receptors in the PVN increases the plasma levels of oxytocin and ACTH (Bagdy, 1996; Zhang et al., 2002). The observation that CRF and oxytocin neurons express both 5-HT1A and 5-HT2A receptors suggests that the serotonergic stimulation of oxytocin and ACTH release occurs by direct activation of these receptors, rather than by stimulation of interneurons. Hence, the increase in plasma levels of ACTH and oxytocin after injection of a 5-HT1A receptor agonist is a clinically useful peripheral marker of the functional status of hypothalamic 5-HT1A receptor signaling.

### TABLE 2

The percentage of oxytocin neurons that are immunopositive for 5-HT1A or 5-HT2A receptors in the hypothalamic PVN

<table>
<thead>
<tr>
<th>Rat</th>
<th>Rat 1</th>
<th>Rat 2</th>
<th>Rat 3</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>(5-HT1A + Oxy)/Oxy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Section A</td>
<td>96 (94/98)</td>
<td>90 (36/40)</td>
<td>96 (71/74)</td>
<td></td>
</tr>
<tr>
<td>Section B</td>
<td>91 (74/81)</td>
<td>96 (51/53)</td>
<td>95 (63/87)</td>
<td></td>
</tr>
<tr>
<td>Section C</td>
<td>95 (155/164)</td>
<td>89 (68/76)</td>
<td>95 (151/159)</td>
<td></td>
</tr>
<tr>
<td>Section D</td>
<td>95 (117/123)</td>
<td>94 (97/103)</td>
<td>98 (142/145)</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>94</td>
<td>92</td>
<td>96</td>
<td>94</td>
</tr>
<tr>
<td>(5-HT2A + Oxy)/Oxy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Section A</td>
<td>97 (84/87)</td>
<td>96 (54/56)</td>
<td>98 (110/112)</td>
<td></td>
</tr>
<tr>
<td>Section B</td>
<td>97 (93/96)</td>
<td>94 (34/36)</td>
<td>97 (97/100)</td>
<td></td>
</tr>
<tr>
<td>Section C</td>
<td>95 (116/122)</td>
<td>95 (76/80)</td>
<td>98 (219/223)</td>
<td></td>
</tr>
<tr>
<td>Section D</td>
<td>99 (100/101)</td>
<td>96 (138/144)</td>
<td>99 (189/190)</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>97</td>
<td>95</td>
<td>98</td>
<td>97</td>
</tr>
</tbody>
</table>

**Fig. 4.** Double immunofluorescence labeling of 5-HT2A receptors (A) and 5-HT1A receptors (B) followed by immunohistochemical labeling of oxytocin (C) in the paraventricular hypothalamic nucleus. Arrowheads point to a neuron immunopositive for 5-HT1A and 5-HT2A receptor antibodies and for oxytocin antibody. * neuron immunopositive for 5-HT1A and 5-HT2A receptor antibodies but immunonegative for oxytocin antibody.

**Fig. 5.** Double immunohistochemical labeling of CRF and 5-HT1A or 5-HT2A receptors in the paraventricular hypothalamic nucleus. Arrows point to CRF neurons that are immunopositive for 5-HT1A or 5-HT2A receptors. Arrowheads point to neurons that are immunonegative for CRF but immunopositive for 5-HT1A or 5-HT2A receptors; scale bar = 200 μm in A, B, C, and D.
The mechanisms underlying the heterologous desensitization of 5-HT{sub}1A receptors by 5-HT{sub}2A receptors in the hypothalamic paraventricular nucleus await further investigation. The rapid onset of the 5-HT{sub}2A receptor-mediated desensitization of hypothalamic 5-HT{sub}1A receptors (maximal effect at 2 h postinjection of DOI) (Zhang et al., 2001) suggests that 5-HT{sub}2A receptors may directly cross talk to 5-HT{sub}1A receptors in the hypothalamic paraventricular nucleus. Our studies provide a combination of pharmacological and neuronalatomi cal evidence that 5-HT{sub}2A receptors may directly cross talk to 5-HT{sub}1A receptors that regulate neuroendocrine function in vivo. Considering the prominent role of 5-HT{sub}1A and 5-HT{sub}2A receptors in the regulation of mood, the observations of their high degree of colocalization and potential intracellular interaction could lead to the development of novel treatments for mood disorders.

Acknowledgments

The authors are grateful to Dr. Lanny C. Keil from the National Aeronautics and Space Administration Ames Research Center ( Moffat Field, CA) for the oxytocin antiserum and to the Hoechst Marion Roussel Research Institute (Cincinnati, OH) for the sample of ML100,907.

References


Cagadan AK, Marsden CA, Tulloch I, and Kendall DA (1993) Evidence that chronic administration of paroxetine or fluoxetine enhances 5-HT{sub}2 receptor function in the brain of the guinea pig. Neuropharmacology 32:249–256.


Critchley DJ, Childe KJ, Middlefell VC, and Dourish CT (1994) Inhibition of 8-OH-DPAT-induced elevation of plasma corticosterone by the 5-HT{sub}1A receptor antagonist WAY100635. Eur J Pharmacol 264:95–97.


