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# Desensitization of 5-$HT_{1A}$ Receptors by 5-$HT_{2A}$ Receptors in Neuroendocrine Neurons *in vivo*[1]

Yahong Zhang, Thackery S. Gray*, Deborah. N. D' Souza, Gonzalo 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, *Department of Cell Biology, Neurology & Anatomy, Loyola University Chicago, Stritch School of Medicine, 2160 South First Avenue, Maywood, Illinois 60153

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**Correspondence**: Louis D. Van de Kar, Ph.D.

Department of Pharmacology Phone: 708-216-3263

Loyola University of Chicago, Stritch School of Medicine Fax: 708-216-6596

2160 South First Avenue e-mail: lvandek@lumc.edu

Maywood, Illinois 60153 URL:http://www.luhs.org/SerotoninResearch

Key words: serotonin, hypothalamus, signaling, hormones, ACTH, oxytocin

**List of Non-Standard Abbreviations:**

5-HT, 5-hydroxytryptamine; (-)DOI, ((-)-1-(2,5-dimethoxy-4-iodophenyl)2-aminopropane HCl);

ACTH, adrenocorticotropic hormone;

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**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 ACTH responses to the 5-$HT_{1A}$ receptor agonist (+)8-OH-DPAT (40 µg/kg, sc). The 5-$HT_{2A/2C}$ receptor agonist (-) DOI (1 mg/kg, sc), injected 2 hrs 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 (0, 10 or 20 nmoles, 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 co-localization of 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors in the oxytocin and corticotropin releasing factor (CRF) 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.

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**Introduction**

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 7 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(Berendsen, 1995;Borsini, 1994). Long-term treatment with selective serotonin reuptake inhibitors (SSRIs) 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(Valdez et al., 2002;Zhang et al., 2001). It is possible that SSRIs exerts 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 involve an intricate interaction between 5-$HT_{2A}$ and 5-$HT_{1A}$ receptors(Ichikawa et al., 2001;Millan, 2000). Thus, studying the interaction between 5-$HT_{2A}$ and 5-$HT_{1A}$ 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-$HT_{1A}$ and 5-$HT_{2A}$ receptors (Valdez et al., 2002;Hensler and Truett, 1998;Eison and Mullins, 1995;Darmani et al., 1990;Krebs-Thomson and Geyer, 1998;Maswood et al., 1996;Eison et al., 1993;Pranzatelli and Pluchino, 1991). One study indicates that 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors are

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expressed by neurons in the frontal cortex (Martin-Ruiz et al., 2001). 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors and their mRNA are found in the hypothalamic paraventricular nucleus (PVN)(Appel et al., 1990;Li et al., 1997a;Wright et al., 1995;Gundlah et al., 1999). What is not known is whether 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors are co-expressed by the same neurons. Additionally, it is not clear that oxytocin and CRF neurons express 5-$HT_{1A}$ and/or 5-$HT_{2A}$ receptors. The present studies addressed these questions.

The sensitivity of 5-$HT_{1A}$ 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-$HT_{1A}$ receptor agonist 8-OH-DPAT(Gilbert et al., 1988;Bagdy and Kalogeras, 1993;Meller and Bohmaker, 1994;Vicentic et al., 1998). Activation of 5-$HT_{2A}$ receptors by a peripheral injection of the 5-$HT_{2A/2C}$ receptor agonist DOI produces a functional heterologous desensitization of the 5-$HT_{1A}$ receptors in the hypothalamus(Zhang et al., 2001). A peripheral injection of DOI directly activates 5-$HT_{2A}$ receptors in the paraventricular nucleus as the effect can be blocked by an intra-paraventricular injection of the 5-$HT_{2A}$ antagonist MDL100,907(Van de Kar et al., 2001;Zhang et al., 2002). Therefore, the DOI-induced desensitization of hypothalamic 5-$HT_{1A}$ receptor systems could result from a direct interaction between 5-$HT_{2A}$ receptor signaling and 5-$HT_{1A}$ receptor systems in the PVN.

The present studies were intended to determine whether 5-$HT_{2A}$ receptors and 5-$HT_{1A}$ receptors are co-expressed by oxytocin and CRF neurons in the PVN and to determine whether the activation of 5-$HT_{2A}$ receptors in the PVN will result in desensitization of 5-$HT_{1A}$ receptor signaling.

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## Materials and Methods

Animals

Male Sprague-Dawley rats (225-275 g) were purchased from Harlan Sprague-Dawley Inc. (Indianapolis, IN). The rats were housed two per cage in a temperature-, humidity- and light-controlled room (12 hr 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-Hydroxy-2-(di-n-propylamino) tetralin hydrobromide [(+) 8-OH-DPAT] and (-)-1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane HCl [(-) DOI] were purchased from Research Biochemical Inc. (Natick, MA). (+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol (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 0.01N HCl containing 10 % of 2-hydroxypropyl-β-cyclodextrin (Sigma-RBI, Saint Louis, MO) 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, ip) and perfused intra-cardially 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 post-fixed for 2 hours 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-$HT_{1A}$ receptors,5-$HT_{2A}$ receptors, oxytocin or CRF*

Sections from 3 rats were exposed to microwave radiation (400 W, for 15 seconds) to retrieve 5-$HT_{2A}$ receptor antigens(Fritschy et al., 1998). The sections were permeated with 0.2 % Triton X100 (Fisher Scientific Hanover Park, IL) for 40 minutes and placed in 3% hydrogen peroxide for 10 minutes, followed by 5 % blocking serum (the same species as the secondary antibody) for 1 hour at room temperature. The sections were sequentially labeled for 5-$HT_{1A}$ receptors and 5-$HT_{2A}$ receptors using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and Nickel-DAB (Ni-DAB) as the chromogens, respectively. Sections were incubated overnight at room temperature with a polyclonal guinea pig anti-5-$HT_{1A}$ IgG (1:2000 dilution; Chemicon, Temecula, CA), followed by 1 hour incubation with a biotinylated goat anti-guinea pig immunoglobulin (dilution 1:1000, Vector Laboratories, Burlingame, CA) at room temperature. The sections were then incubated with avidin-biotinylated peroxidase complex (ABC; dilution, 1:200; Vector Laboratories, Burlingame, CA) for 40 minutes. 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 cover-slipped using Depex mounting media (BDH Lab Supplies). 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-$HT_{2A}$ receptor labeling was performed using a monoclonal mouse anti-5-$HT_{2A}$ IgG (1:200 dilution, Pharmingen, San Diego,

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CA) and a biotinylated horse anti-mouse IgG (1:500 dilution, Vector Laboratories, Burlingame, CA). The peroxidase reaction was performed using DAB as the chromogen. Finally, the sections were rinsed, mounted on gelatin-coated slides and cover-slipped for light microscopy and digital photography.

The photographic images of the first labeling (5-$HT_{1A}$ receptor) and the images after the second labeling (5-$HT_{1A}$ & 5-$HT_{2A}$ receptor) on the same section were compared using Adobe Photoshop software. Two sections (-1.53 and – 1.78 from bregma) were sampled per rat (3 rats in total) for quantification analysis. The number of neurons immunopositive for 5-$HT_{1A}$ or 5-$HT_{2A}$ receptors, and the number of double-labeled neurons for both 5-$HT_{1A}$ and 5-$HT_{2A}$ 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-$HT_{1A}$ or 5-$HT_{2A}$ receptor immunopositive neurons were calculated in each rat for magnocellular and parvicellular 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-$HT_{1A}$ or 5-$HT_{2A}$ receptors. 5-$HT_{1A}$ or 5-$HT_{2A}$ receptor were always stained first in the sequence followed by oxytocin or CRF. Both the oxytocin antibody (dilution 1:8000; provided by S. J. Watson, University of Michigan) and CRF antibody (dilutions 15:000, John Olschowska, University of Rochester) 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 pre-treatment of the sections as described above, the sections were incubated overnight at room temperature with a monoclonal mouse anti-5-HT$_{2A}$ IgG (dilution 1:50 with PBS buffer; Pharmingen, San Diego, CA), followed by 1 hr incubation with fluorescein isothiocyanate (FITC)-conjugated affinity pure donkey anti-mouse IgG (dilution 1:200 with PBS buffer, Jackson Immunoresearch, West Grove, PA). Subsequently, the sections were stained with a polyclonal guinea pig anti-5-HT$_{1A}$ IgG (dilution 1:200 with PBS buffer, Chemicon, Temecula, CA), followed by Rhodamine-Red-X-conjugated affinity pure donkey anti-Guinea Pig IgG (dilution 1:200 with PBS, Jackson Immunoresearch, West Grove, PA). Background autoimmune fluorescence was reduced by treating the sections with 1% Sudan black for 5 minutes. The sections were mounted and cover-slipped with immunofluorescence mounting medium (H-1000, Vector Laboratories, Burlingame, CA), 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. The oxytocin neurons were immunohistochemically labeled using DAB as the chromogen. The sections were then coverslipped for light microscopy and photography.

*Control Experiments: Specificity of the 5-HT$_{1A}$ receptor antibody*

The polyclonal guinea pig anti-5-HT$_{1A}$ immunoglobulin was pre-absorbed with its blocking peptide (Alpha Diagnostic Intl. Inc., San Antonio, TX). The control sections were treated exactly the same as the experimental sections except that the diluent of the primary antibody or the pre-absorbed antibody was applied instead of the primary antibody. The pre-absorbed 5-HT$_{1A}$ immunoglobulin was also verified using Western blot analysis.

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Experimental Protocol

Cannula implantation and intra-paraventricular injection were performed according to the procedures described in detail in previous publication (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 (8 rats for saline injected groups and 13-15 rats for (-) DOI injected groups). Rats received an intra-paraventricular injection (0.5 µl/side) of vehicle or different doses of MDL100,907 (10 and 20 nmoles, 0.5 µl/side). 15 minutes after the intra-paraventricular injections, the rats received an injection of (-) DOI (1 mg/kg, sc) or saline. Two hours after the injection of (-)DOI, the rats received an injection of saline or (+) 8-OH-DPAT (40 µg/kg, sc) and 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;Li et al., 1997b).

Statistical analyses:

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

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## 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 (Figure 1A, 1D). Pre- adsorption of the 5-$HT_{1A}$ receptor antibody with its blocking peptide greatly reduced the immunoreactivity (Figure 1B, 1E). No 5-$HT_{1A}$ receptor- immunoreactivity was observed when the 5-$HT_{1A}$ receptor antibody was omitted from the immunohistochemical procedure (Figure 1F). Furthermore, immunoblot analysis detected two immunopositive bands corresponding to the molecular weight of 5-$HT_{1A}$ receptors (~ 46 kD) in homogenates of the rat frontal cortex. After pre- adsorption of the antibody with its blocking peptide, the immunoreactive bands were barely detectable (Figure 1C).

### *Co-expression of 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors by neurons in the hypothalamic paraventricular nucleus*

Double-label immunohistochemistry revealed that 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors are co-expressed by neurons in the PVN (Figure 2). Nearly all magnocellular neurons were immunoreactive for 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors in the PVN. A moderate number of 5-$HT_{1A}$ and 5-$HT_{2A}$ receptor immunoreactive neurons were located in the parvocellular part of the PVN. Most the cells that expressed one of the 5-HT receptors expressed both 5-HT receptor subtypes.

We also observed co-expression of 5-$HT_{1A}$ and5-$HT_{2A}$ 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-$HT_{2A}$ receptor immunopositive neurons that were immunopositive for 5-$HT_{1A}$ receptors were 97 % in the magnocellular region and 95 % in the parvicellular region. In a reversed order, the percentages of 5-$HT_{1A}$ receptor immunopositive neurons that were immunopositive for 5-$HT_{2A}$ receptors were 96 % in the magnocellular region and 97 % in the parvicellular region.

***Oxytocin and CRF neurons in the PVN expression both 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors***

Immunohistochemical double labeling of 5-$HT_{1A}$ or 5-$HT_{2A}$ receptors, and oxytocin revealed that oxytocin neurons in the PVN were 5-$HT_{1A}$ and 5-$HT_{2A}$ receptor-immunopositive (Figure 3, Panel I). 94 % of the oxytocin neurons were immunopositive for 5-$HT_{1A}$ receptors, and 97 % of the oxytocin neurons were immunopositive for 5-$HT_{2A}$ receptors (Table 2). Immunofluorescence double labeling confirmed that 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors were co-expressed by neurons in the PVN (Figure 4A and B). A subpopulation of the neurons co-expressing 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors were oxytocin-containing neurons (Figure 3C). CRF-immunoreactive neurons in the hypothalamic PVN also expressed 5-$HT_{1A}$ and 5-$HT_{2A}$ receptor immunoreactivity (Fig. 4A-D). We were not able to quantify the degree of co-existence of CRF neurons with 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors due to the lower sensitivity of detection of CRF neurons by CRF antibodies. However, 5-$HT_{1A}$ and 5-$HT_{2A}$ receptor immunoreactivity did not appear to nearly approach the high level of co-expression 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-$HT_{2A}$ 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 % (Figure 5). (-) DOI (injected at 2.25 hr 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 hr prior to blood sampling did not alter the

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plasma levels of oxytocin or ACTH. The intra-PVN 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 nmoles and by 90 % at the dose of 20 nmoles (Figure 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 nmoles (97 %) and 20 nmoles (100 %), respectively (Figure 6B).

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## Discussion

The present studies are the first to provide *in vivo* evidence that 5-$HT_{2A}$ receptor-mediated desensitization of 5-$HT_{1A}$ receptor functioning occurs in the hypothalamic PVN. The data also indicate that 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors are co-expressed by oxytocin and CRF neurons in the hypothalamic PVN. Thus, it is highly likely that activation of 5-$HT_{2A}$ receptors produces a functional desensitization of hypothalamic 5-$HT_{1A}$ receptor signaling in individual oxytocin and CRF neurons in the PVN.

Studies in cell culture did not indicate an interaction between 5-$HT_{2A}$ receptors and 5-$HT_{1A}$ receptors(Saitoh et al., 1995). Our previous studies in rats(Zhang et al., 2001) indicate that a single administration of the 5-$HT_{2A}$ receptor agonist DOI induces a functional desensitization of 5-$HT_{1A}$ receptors that regulate the secretion of ACTH and oxytocin. Studies from other laboratories(Valdez et al., 2002;Hensler et al., 1998) indicate that repeated daily injections of DOI also produce a desensitization of 5-$HT_{1A}$ receptor-induced hypothermia. Furthermore, repeated daily injections of DOI reduce the coupling between 5-$HT_{1A}$ receptors and G proteins in the anterior cingulate cortex(Valdez et al., 2002). However, These phenomena could represent an inter-neuronal interaction rather than a heterologous desensitization between two receptor systems expressed by the same neurons. The current studies examined the hypothesis that an intra-neuronal interaction between 5-$HT_{2A}$ and 5-$HT_{1A}$ receptors is possible.

8-OH-DPAT is a selective 5-$HT_{1A}$ receptor agonist with a high affinity for 5-$HT_{1A}$ receptors and 10 to 100 fold lower affinities for other serotonin receptors (Hoyer et al., 1994). The affinity of 8-OH-DPAT for 5-$HT_7$ receptors (pKi=7.73) (Sleight et al., 1995) is about 10 fold lower than its affinity for 5-$HT_{1A}$ 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-$HT_{1A}$ receptor antagonists WAY100635, NAN190 and pindolol

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(Bagdy et al., 1993;Critchley et al., 1994;Meller et al., 1994;Vicentic 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 of 5-$HT_7$ receptors and can be used to measure changes in the sensitivity of hypothalamic 5-$HT_{1A}$ receptors.

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

Activation of 5-$HT_{2A}$ 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 1-2 hr post-injection (Bagdy, 1996;Damjanoska et al., 2003). At 2 hrs after an injection of DOI, both hormones would have returned to basal level. Our previous experiment indicates that the 5-$HT_{2A}$ receptor-mediated desensitization of 5-$HT_{1A}$ receptors is maximal at 2 hours post-injection of DOI (Zhang et al., 2001). For this reason, the injection of 8-OH-DPAT was performed 2 hours 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 not change in the maximal response (Emax). Depletion of hormone stores by prior injection of DOI would more likely result in reduced Emax as 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 Emax suggests that this reduced hormone response to

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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-$HT_{2A}$ receptor antagonist (pKi = 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 to 18.7 nmoles) microinjected into the hypothalamic PVN dose-dependently inhibit hormone responses to peripherally injected (-) DOI(Zhang et al., 2002). Accordingly, we injected doses of 10 nmoles and 20 nmoles of MDL100,907 into the hypothalamic PVN to produce partial and complete blockade, respectively, of 5-$HT_{2A}$ receptors in the PVN. In the present study, the intra-PVN injection of similar doses of MDL100,907 prevented the DOI-induced desensitization of 5-$HT_{1A}$ receptor signaling. These observations suggest that either that 5-$HT_{2A}$ receptors interact directly with 5-$HT_{1A}$ receptors in neurons of the hypothalamic PVN or that hypothalamic neurons expressing 5-$HT_{2A}$ receptors interact with other hypothalamic neurons that express 5-$HT_{1A}$ receptors. The immunocytochemical observations suggest that a direct intracellular interaction occurs between these receptor signaling systems.

Western blot analyses of 5-$HT_{1A}$ receptors have revealed protein bands of different molecular mass: 40 kD, 67 kD and 70 kD (Zhou et al., 1999;Anthony and Azmitia, 1997). We found two immunopositive bands corresponding to the molecular mass of 5-$HT_{1A}$ receptors (~ 46 kDa) (Raymond et al., 1999). 5-$HT_{1A}$ receptors have consensus sequences for post-translational modifications such as glycosylation and phosphorylation (Albert et al., 1990;Anthony et al., 1997;Wu et al., 2002;Zhou et al., 1999). 5-$HT_{1A}$ receptors are differently modified in different tissues, and thus antibodies for different epitopes may recognize various forms of 5-$HT_{1A}$ receptors that are modified by post-translational mechanisms.

The specificity of 5-$HT_{1A}$ receptor-like immunoreactivity observed in the hypothalamic PVN was verified by pre- adsorption of the 5-$HT_{1A}$ receptor antibody with its blocking peptide (Figure 1). Moreover, this antibody detected a similar pattern of 5-$HT_{1A}$ receptor-like

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immunoreactivity in other brain regions as previously reported, such as dorsal raphe nucleus and hippocampus(Kia et al., 1996). Thus, the immunopositive neurons observed in the hypothalamic paraventricular nucleus most likely represent the labeling of 5-$HT_{1A}$ receptors. The specificity of the 5-$HT_{2A}$ receptor antibody was discussed in a previous paper(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(Alves et al., 1998;Piekut and Joseph, 1986).

The hypothesis of a heterologous desensitization, that presupposes a co-expression of both receptors in the same cells, is supported by a high degree of co-expression of 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors by neurons in the hypothalamic PVN (> 95 %). Neurons in the frontal cortex have also been reported to co-express 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors (Martin-Ruiz et al., 2001). We observed that 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors were co-expressed throughout all magnocellular regions of the PVN. A subpopulation of the magnocellular neurons co-expressing 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors are oxytocin neurons. This suggests that other magnocellular neuroendocrine cells such as vasopressin neurons also co-express 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors. CRF neurons were separately examined, using colchicine pre-treatment to increase the levels of CRF in neurons. Double labeling immunohistochemistry revealed that 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors are also co-expressed by CRF neurons. However, due to the small size of CRF neurons and morphological changes in the neurons caused by colchicine pre-treatment, we were unable to quantify the percentages of CRF neurons that co-express 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors. Combined, these data suggest that 5-$HT_{2A}$ receptors may interact with 5-$HT_{1A}$ receptors through their signaling proteins to regulate hormone release.

Activation of both 5-$HT_{2A}$ and 5-$HT_{1A}$ receptors in the PVN increases the plasma levels of oxytocin and ACTH(Zhang et al., 2002;Bagdy, 1996). The observation that CRF and oxytocin neurons express both 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors suggests that the serotonergic stimulation of

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oxytocin and ACTH release occurs by direct activation of these receptors, rather than by stimulation of inter-neurons. Hence, the increase in plasma levels of ACTH and oxytocin, after injection of a 5-$HT_{1A}$ receptor agonist is a clinically useful peripheral marker of the functional status of hypothalamic 5-$HT_{1A}$ receptor signaling.

The mechanisms underlying the heterologous desensitization of 5-$HT_{1A}$ receptors by 5-$HT_{2A}$ receptors in the hypothalamic paraventricular nucleus await further investigation. The rapid onset of the 5-$HT_{2A}$ receptor-mediated desensitization of hypothalamic 5-$HT_{1A}$ receptors (maximal effect at 2 hrs post injection of DOI)(Zhang et al., 2001) suggests that post-translational modification mechanisms may be involved. Cell culture studies indicate that 5-$HT_{1A}$ receptors are subject to regulation by phosphorylation, palmitoylation and myristoylation(Raymond, 1991;Raymond and Olsen, 1994;Fields and Casey, 1995;Glick et al., 1998;Morales et al., 1998;Tu et al., 1997;Hallak et al., 1994). On the other hand, 5-$HT_{2A}$ receptors may reduce 5-$HT_{1A}$ receptor function via a cyclooxygenase-dependent metabolite of arachidonic acid(Evans et al., 2001).

In summary, 5-$HT_{1A}$ receptors and 5-$HT_{2A}$ receptors are co-localized in the same neurons, particularly in oxytocin and CRF neurons and likely other hormone-releasing neurons in the hypothalamic paraventricular nucleus. 5-$HT_{2A}$ receptors interact with 5-$HT_{1A}$ receptors, likely in the same neurons of the paraventricular nucleus, producing a heterologous desensitization of 5-$HT_{1A}$ receptors. Our studies provide a combination of pharmacological and neuroanatomical evidence that 5-$HT_{2A}$ receptors may directly cross talk to 5-$HT_{1A}$ receptors that regulate neuroendocrine function *in vivo.* Considering the prominent role of 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors in the regulation of mood, the observations of their high degree of co-localization and potential intracellular interaction could lead to the development of novel treatments for mood disorders.

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## footnotes

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**Reprint Request**: Louis D. Van de Kar, Ph.D.

Department of Pharmacology

Loyola University of Chicago, Stritch School of Medicine

2160 South First Avenue

Maywood, Illinois 60153

Phone: 708-216-3263

Fax: 708-216-6596

e-mail: lvandek@lumc.edu

## TABLES

**Table 1**: The percent of neurons that are immunopositive for $5\text{-HT}_{1A}$ and $5\text{-HT}_{2A}$ receptors in the hypothalamic paraventricular nucleus (PVN)

| | | rat 1 % | rat 2 % | rat 3 % | Average |
|---|---|---|---|---|---|
| $5\text{-HT}_{1A}R+5\text{-HT}_{2A}R$ $/5\text{-HT}_{2A}R$ | Magno | 98 (362/369) | 95 (360/377) | 97 (540/548) | **97 %** |
| | Parvi | 95 (160/169) | 92 (105/114) | 99 (336/341) | **95 %** |
| $5\text{-HT}_{1A}R+5\text{-HT}_{2A}R$ $/5\text{-HT}_{1A}R$ | Magno | 97 (362/375) | 94 (360/382) | 97 (540/554) | **96 %** |
| | Parvi | 94 (160/171) | 99 (105/118) | 97 (336/348) | **97 %** |

Magno: magnocellular regions; Parvi: parvicellular regions

**Table 2**: The percent of oxytocin neurons that are immunopositive for $5\text{-HT}_{1A}$ or $5\text{-HT}_{2A}$ receptors in the hypothalamic paraventricular nucleus (PVN)

| Rat # | rat 1 % | rat 2 % | rat 3 % | |
|---|---|---|---|---|
| $(5\text{-HT}_{1A}R+\text{Oxy})/\text{Oxy}$ | | | | |
| Sections A | 96 (94/98) | 90 (36/40) | 96 (71/74) | |
| B | 91 (74/81) | 96 (51/53) | 95 (83/87) | |
| C | 95 (155/164) | 89 (68/76) | 95 (151/159) | |
| D | 95 (117/123) | 94 (97/103) | 98 (142/145) | Average |
| Average | **94** | **92** | **96** | **94 %** |
| $(5\text{-HT}_{2A}R+\text{Oxy})/\text{Oxy}$ | | | | |
| Sections A | 97 (84/87) | 96 (54/56) | 98 (110/112) | |
| B | 97 (93/96) | 94 (34/36) | 97 (97/100) | |
| C | 95 (116/122) | 95 (76/80) | 98 (219/223) | |
| D | 99 (100/101) | 96 (138/144) | 99 (189/190) | Average |
| Average | **97** | **95** | **98** | **97 %** |

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## FIGURE LEGENDS

**Figure 1**. Pre- adsorption of the 5-$HT_{1A}$ receptor antibody by its blocking peptide. A-B, 5-$HT_{1A}$ receptor-like immunoreactivity in the dorsal raphe before (A) and after (B) blocking peptide pre-adsorption; C, The immunoreactive bands of 5-$HT_{1A}$ receptors (~ 46 kD) detected in the homogenates of rat frontal cortex (10 and 20 μg protein) was pre-absorbed by its blocking peptide; D-E, 5-$HT_{1A}$ receptor-like immunoreactivity in the PVN before (D) and after (E) blocking peptide pre- adsorption; F, No 5-$HT_{1A}$ receptor immunoreactivity was observed in the absence of the primary antibody; PVN = the hypothalamic paraventricular nucleus; * indicates location of the 3rd ventricle.

**Figure 2**. Co-localization of 5-$HT_{1A}$ and 5-$HT_{2A}$ receptor-like immunoreactivity in the same neurons in the hypothalamic paraventricular nucleus (PVN). The arrow heads point to 5-$HT_{1A}$ receptor immunopositive neurons; arrows point to neurons that are immunopositive for both 5-$HT_{1A}$ and 5-$HT_{2A}$ receptors.

**Figure 3**.  Double immunohistochemical labeling of oxytocin and 5-$HT_{1A}$ or 5-$HT_{2A}$ receptors in the PVN.  Arrows point to oxytocin neurons that are immunopositive for 5-$HT_{1A}$ or 5-$HT_{2A}$ receptors; arrow heads point to neurons that are immuno-negative for oxytocin but immunopositive for 5-$HT_{1A}$ or 5-$HT_{2A}$ receptors; scale bar = 200 mm in A, B, D and E; * marks the location of the 3rd ventricle.

**Figure 4**. Double immunofluorescence labeling of 5-$HT_{2A}$ receptors (A) and 5-$HT_{1A}$ receptors (B) followed by immunohistochemical labeling of oxytocin (C) in the paraventricular hypothalamic nucleus. "b" identifies a blood vessel; arrow heads point to a neuron

immunopositive for 5-$TH_{1A}$ & 5-$HT_{2A}$ receptor antibodies and for oxytocin antibody; * marks a neuron immunopositive for 5-$HT_{1A}$ & 5-$HT_{2A}$ receptor antibodies, but immuno-negative for oxytocin antibody.

**Figure 5**. Double immunohistochemical labeling of CRF and 5-$HT_{1A}$ or 5-$HT_{2A}$ receptors in the paraventricular hypothalamic nucleus. Arrows point to CRF neurons that are immunopositive for 5-$HT_{1A}$ or 5-$HT_{2A}$ receptors. Arrow heads point to neurons that are immuno-negative for CRF but immunopositive for 5-$HT_{1A}$ or 5-$HT_{2A}$ receptors; scale bar = 200 µm in A, B, D and E.

**Figure 6**. Intra-paraventricular nucleus injection of MDL100,907 dose-dependently reverses the inhibitory effect of (-) DOI (1 mg/kg, sc) on oxytocin (A) and ACTH (B) responses to (+) 8-OH-DPAT (40 mg/kg, sc). The data represent the mean ± SEM of 7 - 14 rats per group. * ($p < 0.05$) and ** ($p < 0.01$) significant effect of (+) 8-OH-DPAT compared with saline-challenged group; # ($p < 0.05$) and ## ($p < 0.01$) significant effect of (-) DOI compared with saline pretreated and (+) 8-OH-DPAT challenged group; a ($p < 0.05$); aa ($p < 0.01$) significant effect of MDL100,907 compared with vehicle pretreated, (-) DOI/(+) 8-OH-DPAT group. (One-way ANOVA and Newman-Keuls' multiple range test).

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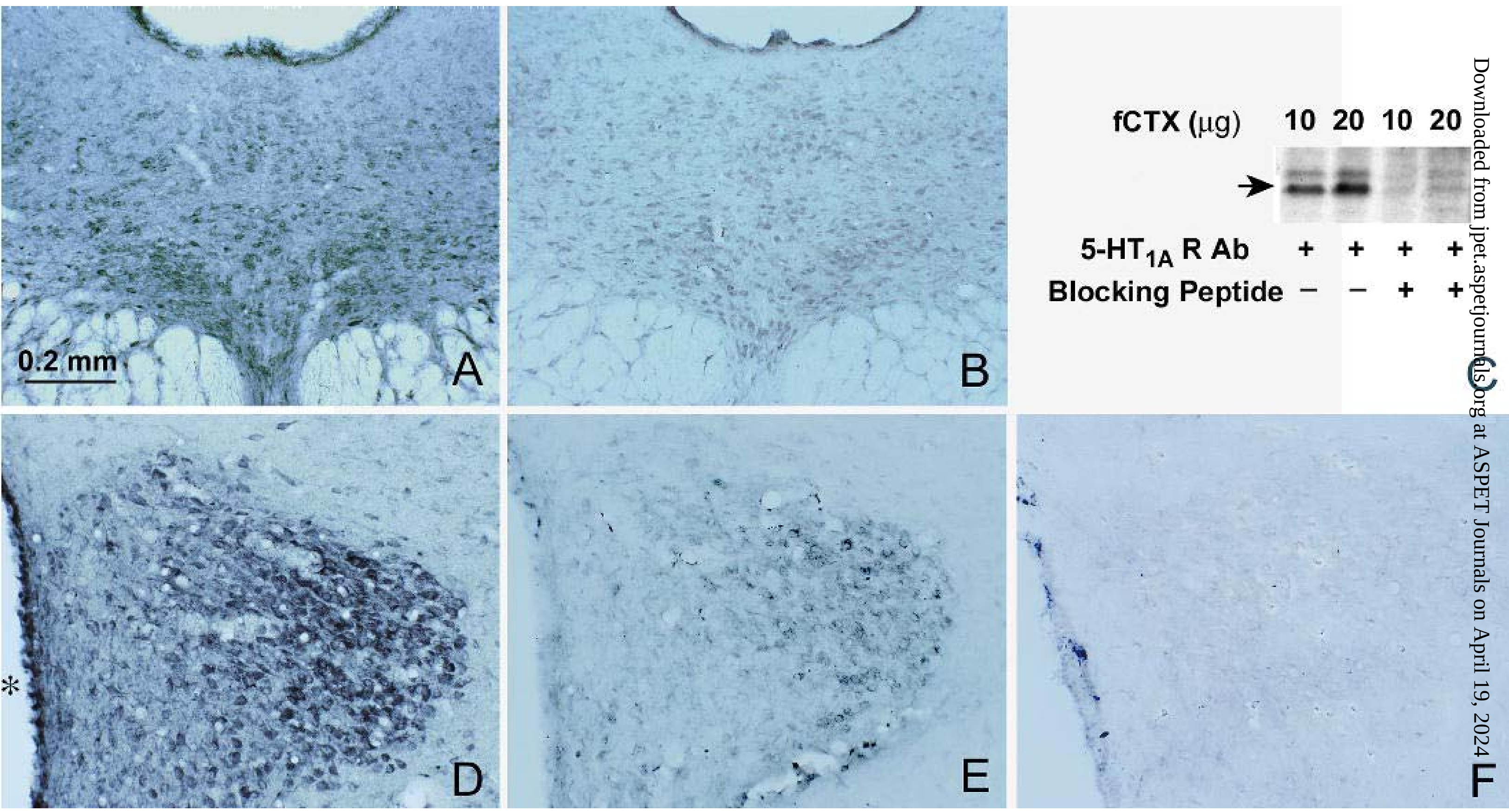

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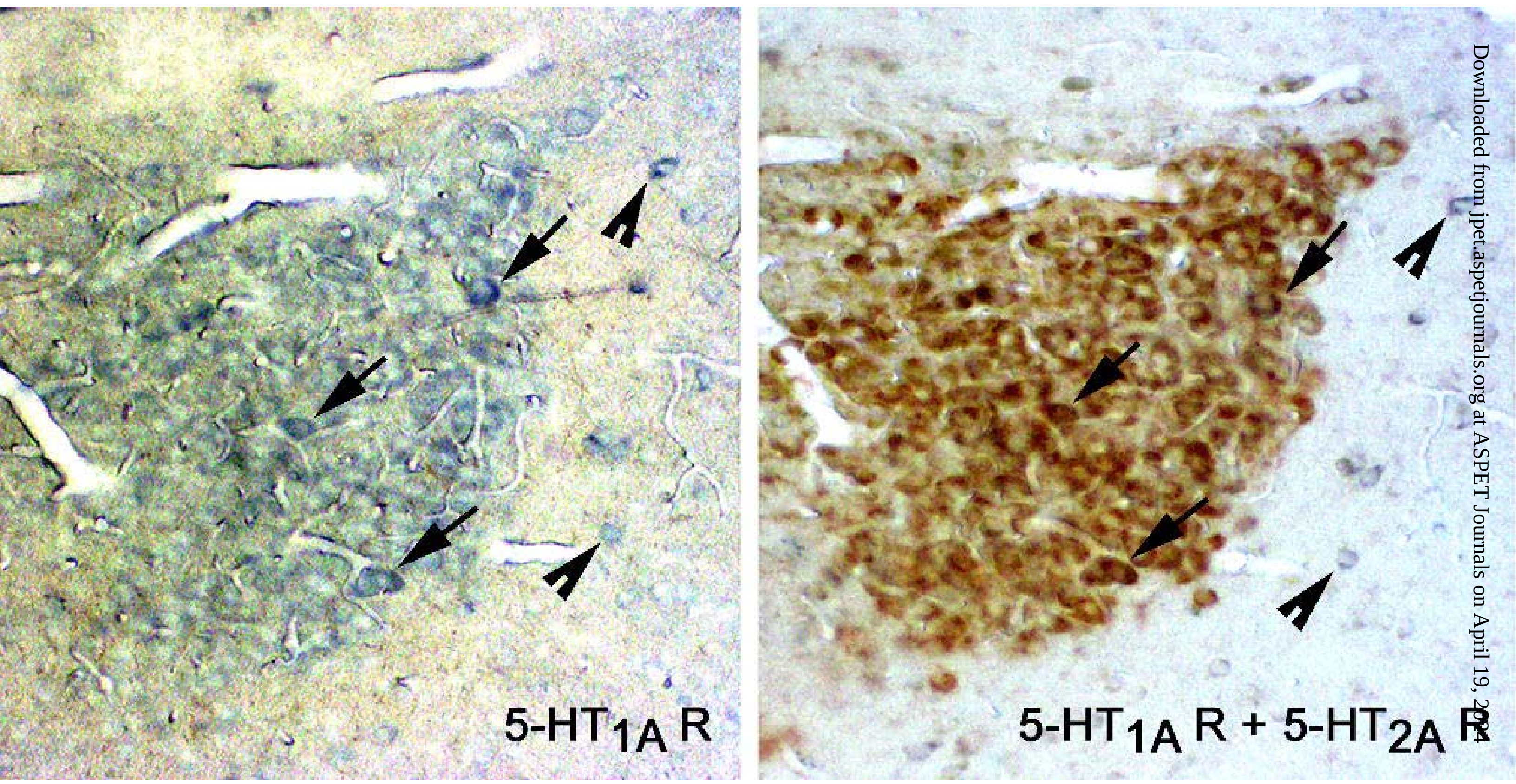

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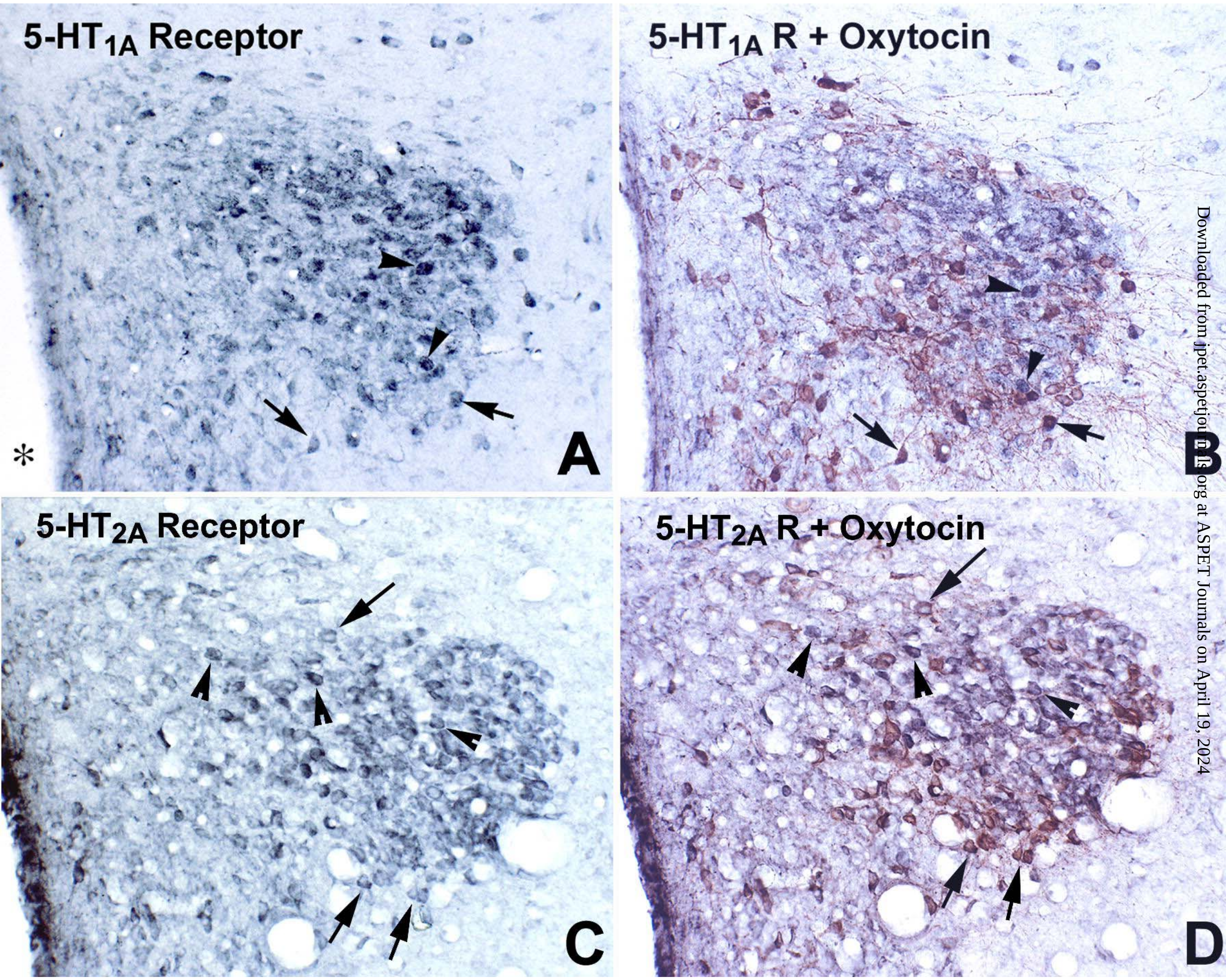

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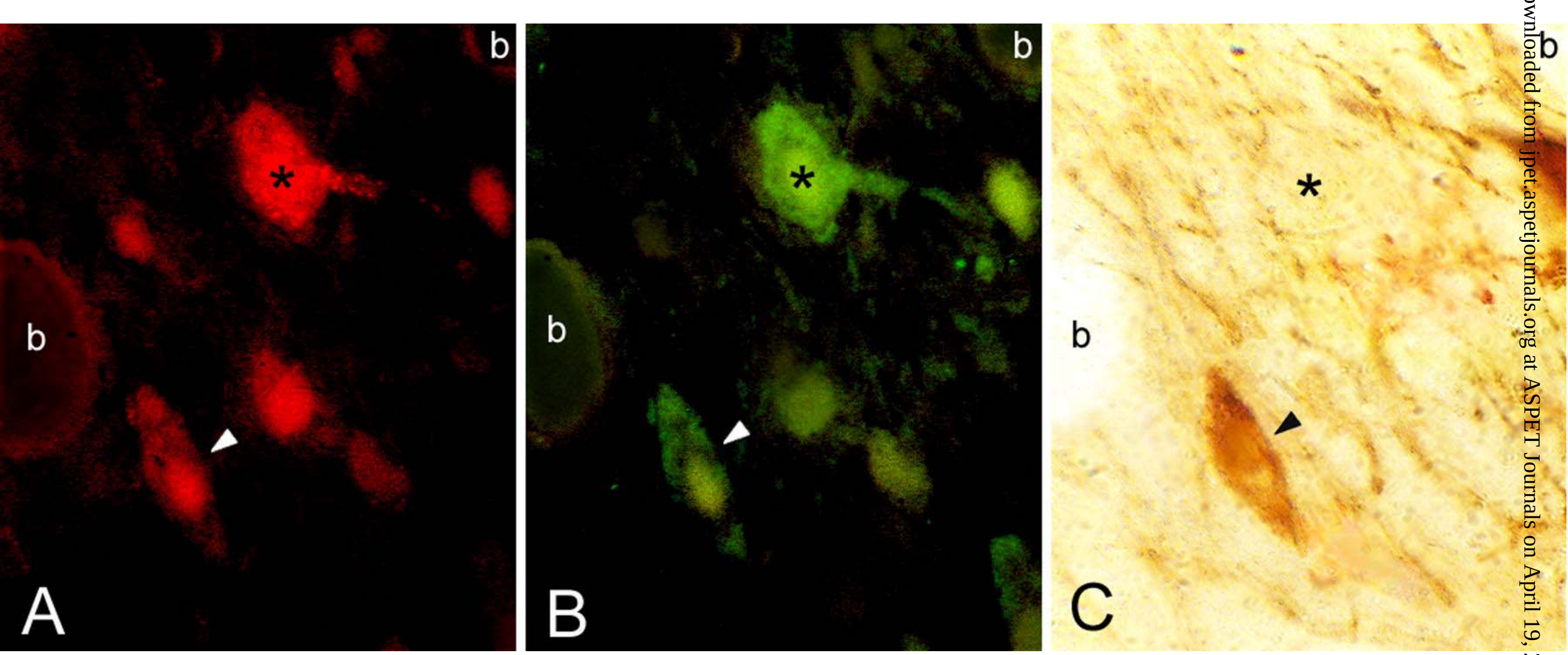

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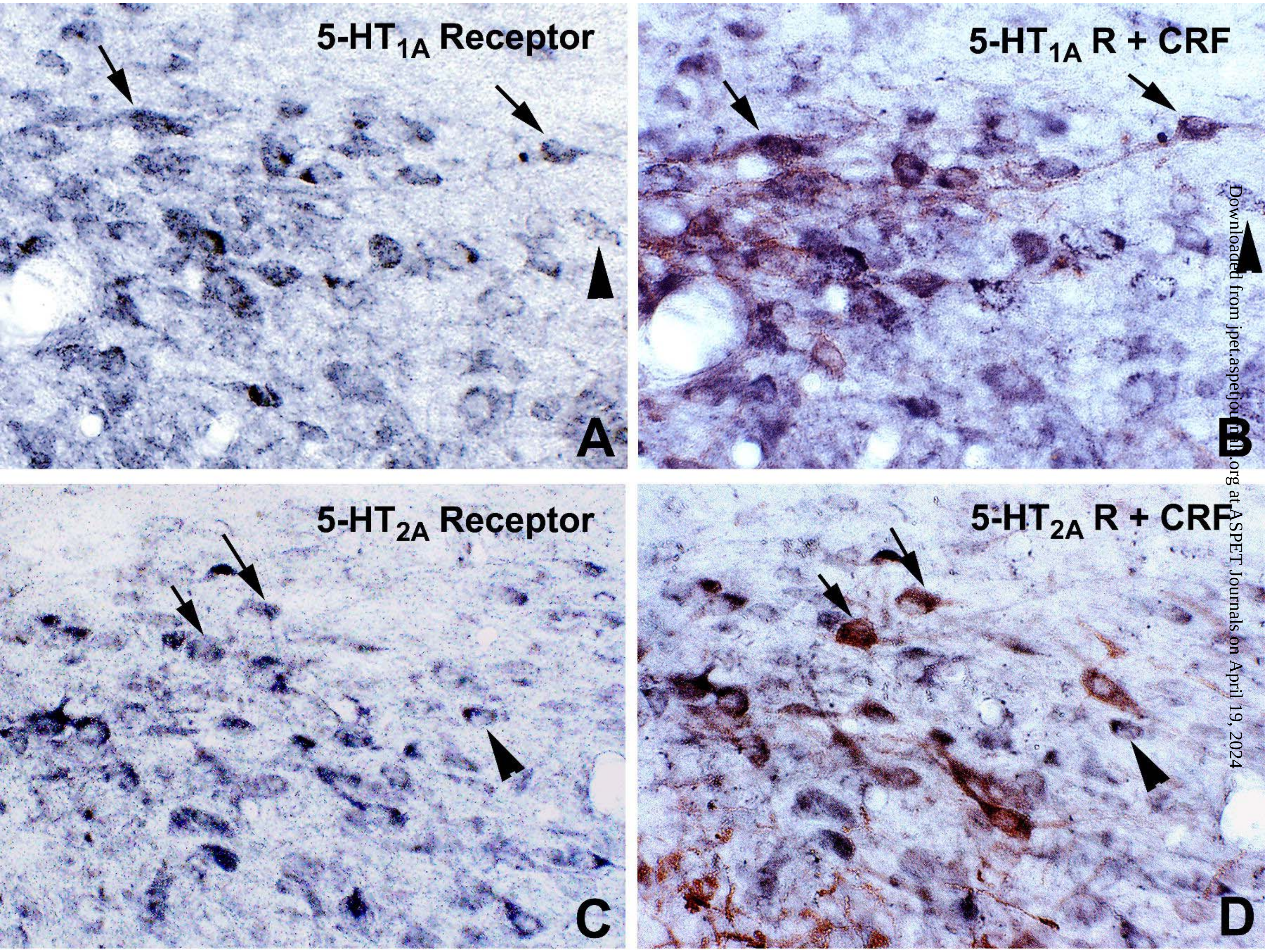

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