JPET Fast Forward. Published on December 11, 2006 as DOI: 10.1124/jpet.106.116004
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Single Exposure to a 5-HT_{1A} Receptor Agonist (8-OH-DPAT) Produces a Prolonged Heterologous Desensitization of 5-HT_{2A} Receptors in Neuroendocrine Neurons in Vivo

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Running Title: *In vivo* 5-HT$_{1A}$ and 5-HT$_{2A}$ crosstalk

Number of text pages: 31

Number of tables: 0

Number of figures: 5

Abstract: 237 words…..250 words max.

Introduction: 540 words…..750 words max

Discussion: 1551 words…1500 words max

Number of references: 40

Non-standards abbreviations:

Hypothalamic paraventricular nucleus (PVN); adrenocorticotropic hormone (ACTH); 5-hydroxytryptamine (serotonin, 5-HT); (-)-1-(2,5 dimethoxy-4-iodophenyl)-2-amino-propane HCL ((-)DOI); (+)8-hydroxy-2-(N,N-di-n-propylamino)tetralin ((+)-8-OH-DPAT); {*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride (WAY100635), corticotrophin releasing factor (CRF).

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Abstract

We previously demonstrated colocalization of serotonin 1A (5-HT_{1A}) and serotonin 2A (5-HT_{2A}) receptors in oxytocin and corticotrophin releasing factor (CRF) neurons in the hypothalamic paraventricular nucleus (PVN). Since a functional imbalance between hypothalamic 5-HT_{1A} and 5-HT_{2A} receptors has been implicated in several neuropsychiatric disorders, this study investigated if acute *in vivo* activation of 5-HT_{1A} receptors in the PVN results in desensitization of 5-HT_{2A} receptor signaling. Functional desensitization of hypothalamic 5-HT_{2A} receptors was assessed via reduction in oxytocin and ACTH responses to the 5-$HT_{2A/2C}$ receptor agonist (-)DOI. We report here that a single systemic injection of the 5-HT_{1A} receptor agonist (+)8-OH-DPAT (200 μg/kg) significantly reduced the 5-HT_{2A} receptor-mediated oxytocin responses for at least 72 h. Direct intra-PVN injection of (+)8-OH-DPAT (0.2 nmoles) 24 h prior to a submaximal dose of (-)DOI (0.35 mg/kg) significantly inhibited the 5-HT_{2A} receptor-mediated increases in both oxytocin and ACTH (-39% and -16%, respectively). Additionally, the (+)8-OH-DPAT-induced-desensitization of the 5-HT_{2A} receptor mediated oxytocin, but not ACTH, response was inhibited in rats pretreated with either a systemic (0.1 mg/kg) or intra-PVN (10 nmoles) injection of the 5-HT_{1A} receptor antagonist WAY100635. This is the first *in vivo* demonstration of a prolonged heterologous intracellular desensitization of 5-HT_{2A} receptors after acute activation of 5-HT_{1A} receptors. These findings may provide insight into the long-term heterologous interactions between 5-HT_{1A} and 5-HT_{2A} receptor signaling that could occur in response to antidepressants, antipsychotics or drugs of abuse that target these receptor subtypes.

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Introduction

Serotonin (5-HT), a known stimulator of the hypothalamic-pituitary-adrenal axis, plays an important role in mood disorders and impulse control (Carrasco and Van de Kar, 2003). Among the seven families of serotonin receptors, changes in hypothalamic serotonin 1A (5-HT_{1A}) receptors and serotonin 2A (5-HT_{2A}) receptors have been associated with the etiology of mood disorders, the therapeutic effect of antidepressants and the effects of drugs of abuse (Sargent, et al., 2000;Schiller, et al., 2006;Carrasco and Van de Kar, 2003;Amargos-Bosch, et al., 2004;Stockmeier, et al., 1997;Levy, et al., 1994).

5-HT_{1A} and 5-HT_{2A} receptors mediate opposing or compensatory functions in a variety of cellular and behavioral events (Amargos-Bosch, et al., 2004;Araneda and Andrade, 1991;Hensler and Truett, 1998;Valdez, et al., 2002). Indeed, several *in vivo* studies 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). For example, 5-HT_{1A} receptor-mediated behaviors such as flattened posture and reciprocal forepaw treading, and hypothermia are attenuated by prior 5-HT_{2A} receptor activation (Berendsen and Broekkamp, 1990;Hensler and Truett, 1998). On the other hand, chronic or subchronic treatment with full or partial 5-HT_{1A} receptor agonists can reduce 5-HT_{2A} receptor-mediated behaviors (Eison and Yocca, 1985;Yocca, et al., 1990;Ootsuka and Blessing, 2006) and reduce 5-HT_{2A} receptor density (Eison and Yocca, 1985;Schechter, et al., 1990;Taylor and Hyslop, 1991). However, whether this effect is due to a direct interaction of 5-HT_{1A} receptors with 5-HT_{2A} receptors in cells that colocalize both receptors; or due to stimulation of somatodendritic 5-HT_{1A} autoreceptors producing a general reduction of serotonergic neurotransmission, remains unknown.

5-HT_{1A} receptors and 5-HT_{2A} receptors exhibit overlapping distributions in various brain regions (Burnet, et al., 1996;Amargos-Bosch, et al., 2004;Araneda and Andrade, 1991). Colocalization of 5-

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HT_{1A} and 5-HT_{2A} receptors has been demonstrated in frontal and prefrontal cortex (Amargos-Bosch, et al., 2004;Araneda and Andrade, 1991). Similarly, we previously reported varying degrees of co-localization of 5-HT_{1A} and 5-HT_{2A} receptors in oxytocin and corticotrophin releasing hormone (CRF) neurons in the hypothalamic paraventricular nucleus (PVN) (Zhang, et al., 2004). Furthermore, we demonstrated that activation of 5-HT_{2A} receptors in PVN produced a delayed and reversible heterologous reduction in the ACTH and oxytocin responses to a 5-HT_{1A} receptor agonist, (+)8-OH-DPAT ([(+)8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide]) (Zhang, et al., 2001;Zhang, et al., 2004). The maximal desensitization that occurred at 2-4 h appeared to recover by 24 h after the (-)DOI injection (Zhang, et al., 2001;Zhang, et al., 2004).

Heterologous desensitization occurs between different receptor systems where the responsiveness of one receptor system is regulated negatively by activation of another receptor system (Berg, et al., 1998). However, homologous or heterologous receptor desensitization typically occurs following sustained exposure to agonists. The present study demonstrates a prolonged heterologous desensitization of 5-HT_{2A} receptors by acute *in vivo* activation of 5-HT_{1A} receptors in neuroendocrine neurons in the PVN. Functional desensitization of 5-HT_{2A} receptors was assessed from reductions in the magnitude of increases in the plasma levels of oxytocin and ACTH after an injection of the 5-$HT_{2A/2C}$ receptor agonist (-)DOI (Van de Kar, et al., 2001;Zhang, et al., 2002). These findings, demonstrating crosstalk between 5-HT_{1A} and 5-HT_{2A} receptors, may provide insight into the etiology of mood disorders or the effects of drugs of abuse, and could provide novel strategies for their treatment.

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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-19:00 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 (IACUC). Every effort was made to minimize suffering and discomfort to the animals. The number of rats per group was the minimum needed for statistically meaningful evaluation of the data.

Drugs: (+)8-OH-DPAT [+8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide] was purchased from Tocris (Ellisville, MO). (-)DOI [(-)-1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane HCl] and WAY100635 ({*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride) were purchased from Sigma (St Louis, MO). (+)8-OH-DPAT, WAY100635 and (-)DOI were dissolved in 0.9% saline. All solutions were made fresh before injections and injected at a volume of 1 ml/kg.

Experimental Protocols: After arrival, the rats were housed two per cage for at least one week, followed by 4 days of handling. Rats were randomly assigned to different experimental groups (n=8 per group) to receive drug treatments. Cage-mates were assigned to the same treatment group. After receiving different drug treatments, rats were sacrificed by decapitation. The trunk 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 aliquoted and stored at -70°C for radioimmunoassays of plasma hormone concentrations.

Surgery and Cannula Implantation: Cannula implantation and intraparaventricular injection were performed according to the procedures previously described in detail (Zhang, et al., 2004). Rats were

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anesthetized with a mixture of ketamine and xylazine (100 mg/kg ketamine plus 7 mg/kg xylazine, 1.4 ml/kg, i.p.). A double-barreled guide cannula (26 gauge, 1.2 mm center-to-center distance) with its corresponding dummy cannula inserted inside (Plastic One, Roanoke, VA) was implanted into the brain above both sides of the PVN according to the following stereotaxic coordinates: 1.8 mm caudal, ± 0.6 mm lateral with respect to bregma, and 6.4 mm ventral from the skull surface. The cannulas were kept in place by dental cement attached to microscrews embedded in the skull. Postsurgery dehydration was prevented by injecting 1 ml of saline (subcutaneously) after surgery. To prevent postsurgery infection, all rats received ampicillin (50 mg/kg, s.c.) immediately after surgery, followed by 3 days of administration of sulfamethoxazole and trimethoprim suspended in the drinking water. Twelve days after cannula implantation, the rats were handled for 4 consecutive days and then randomly assigned to different experimental groups (10 rats per group). After removal of the dummy cannula, an injection cannula (33 gauge, 1.2 mm center-to-center distance, 0.5 mm projection from the tip of the double guide cannula) was inserted through the implanted guide cannula to the top of PVN.

Radioimmunoassay of plasma oxytocin and ACTH: Plasma oxytocin and ACTH were determined by radioimmuno assays previously described in detail (Li, et al., 1997). [^{125}I]-oxytocin was purchased from Perkin-Elmer (Wellesley, MA) and [^{125}I]-ACTH was purchased from Diasorn (Stillwater, MN).

Experiment #1: Time-course of the effect of (+)8-OH-DPAT on hormone responses to a subsequent (-)DOI challenge. Adult male Sprague-Dawley rats were injected with either saline (1 ml/kg, s.c.) or (+)8-OH-DPAT (200 μg/kg, s.c.) at 1, 2, 4, 24, 48 or 72 h prior to a subsequent injection of either saline (1 ml/kg, s.c.) or (-)DOI (1 mg/kg s.c.). The rats were sacrificed 30 min after the (-)DOI injection.

Experiment #2: Effects of one or two systemic injections of (+)8-OH-DPAT on 5-HT$_{2A}$ receptor-mediated hormone responses. Adult male Sprague-Dawley rats (8 per group) were randomly assigned

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to each of the following groups: (1) Saline 24h/Saline 12h, rats were injected with saline (1 ml/kg, s.c.) 24 and 12 h prior to a subsequent challenge injection of either saline (1 ml/kg, s.c.) or (-)DOI (0.35 mg/kg, s.c.); (2) (+)8-OH-DPAT 24 h/Saline 12 h, rats were injected with (+)8-OH-DPAT (200 µg/kg, s.c.) 24 h, and with saline (1 ml/kg, s.c.) 12 h, prior to a subsequent challenge injection of either saline (1 ml/kg, s.c.) or (-)DOI (0.35 mg/kg, s.c.); (3) (+)8-OH-DPAT 24 h/(+)8-OH-DPAT 12 h, rats were injected with (+)8-OH-DPAT (200 ug/kg, s.c.); 24 and 12 h prior to a subsequent challenge injection with either saline (1 ml/kg, s.c.) or (-)DOI (0.35 mg/kg, s.c.). The rats were sacrificed by decapitation 30 min after the (-)DOI injection.

Experiment #3: Effect of a direct injection of (+)8-OH-DPAT into the PVN on the 5-HT$_{2A}$ receptor-mediated neuroendocrine responses. Cannula implantation and intraparaventricular injection were performed according to the procedures described above. Saline (0.5 µl/side) or (+)DPAT (0.2 nmoles or 2 nmoles, 0.5 µl/side) were directly injected into the PVN. 24 h after the intraparaventricular injections, the rats received a challenge injection of either (-)DOI (0.35 mg/kg s.c.) or saline (1 ml/kg, s.c.). The rats were sacrificed by decapitation 30 min after the injection of (-)DOI.

Experiment #4: Effect of a systemic injection of WAY100635 on the (+)8-OH-DPAT-induced desensitization of 5-HT$_{2A}$ receptor-mediated neuroendocrine responses. Cannula implantation and intraparaventricular injection were performed according to the procedures described above. Rats were injected with either saline or WAY100635 (0.1 mg/kg, s.c.), a highly specific 5-HT$_{1A}$ receptor antagonist (Forster, et al., 1995;Chemel, et al., 2006), 30 min prior to an intraparaventricular injection of either saline (0.5 µl/side) or (+)8-OH-DPAT (0.2 nmoles, 0.5 µl/side). 24 h after the intraparaventricular injections, the rats received an injection of (-)DOI (0.35 mg/kg s.c.) or saline. The rats were sacrificed by decapitation 30 min after the challenge injection of (-)DOI.

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Experiment #5: Effect of an intraparaventricular injection of WAY100635 on the (+)8-OH-DPAT induced-desensitization of 5-HT$_{2A}$ receptor signaling. Cannula implantation and intraparaventricular injection were performed according to the procedures described above. Rats received an intraparaventricular injection of either saline (0.5 µl/side) or WAY100635 (10 nmoles, 0.5 µl/side). 30 min later the rats received an injection of either saline (1 ml/kg, s.c.) or (+)8-OH-DPAT (200 ug/kg, s.c.). 24 h after the intraparaventricular injections, the rats received a challenge injection of (-)DOI (0.35 mg/kg s.c.) or saline, and sacrificed by decapitation 30 min later.

Statistics: All data are expressed as the mean ± S.E.M., where *n* indicates the number of rats per group. Hormone data were analyzed by two or three-way analyses of variance (ANOVA). Group means were compared by a Newman-Keuls multiple-range test (Steel and Torrie, 1960). GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD, USA) was used for all statistical analyses.

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Results

Time-course of the effect of (+)8-OH-DPAT on hormone responses to a subsequent (-)DOI challenge.

Figure 1 illustrates the effects of a single injection of either saline or (+)8-OH-DPAT on the basal and (-)DOI-stimulated increases in plasma levels of oxytocin. We previously demonstrated that the sensitivity of 5-HT_{2A} receptors in hypothalamus can be measured from the magnitude of increases in the plasma levels of oxytocin and ACTH, after an injection of (-)DOI (Van de Kar, et al., 2001;Zhang, et al., 2002). Saline or the 5-HT_{1A} receptor agonist (+)8-OH-DPAT (200μg/kg, s.c.) were injected at different time points (1, 2, 4, 24, 48 and 72 h) before a subsequent saline or (-)DOI challenge(1 mg/kg, s.c.). Saline or (+)8-OH-DPAT pretreatment did not significantly alter plasma oxytocin levels to a subsequent saline injection at any of these time points. (-)DOI significantly increased plasma oxytocin levels in rats pretreated with either saline or (+)8-OH-DPAT (Fig.1), but the magnitude of hormone responses to the subsequent challenge with (-)DOI was attenuated in rats pretreated with (+)8-OH-DPAT.

The three-way ANOVA for oxytocin indicated significant main effects of (+)8-OH-DPAT pretreatment ($F_{(1,149)}$ =173.38, p<0.0001) and the (-)DOI challenge ($F_{(1,149)}$ =750.86, p<0.0001), but no significant main effect of time ($F_{(5,149)}$ =0.56974, p>0.72). There was a significant interaction between (+)8-OH-DPAT pretreatment and (-)DOI challenge ($F_{(1,149)}$ =152.97, p<0.0001), but there was no main interaction between (+)8-OH-DPAT pretreatment and time ($F_{(5,149)}$ =1.230, p>0.29), and between (-)DOI challenge and time ($F_{(5,149)}$ =0.30635, p>0.90). There was no significant interaction between (+)8-OH-DPAT pretreatment, time and (-)DOI challenge ($F_{(5,149)}$ =1.16399, p<0.3296). The Newman-Keuls test indicated that (+)8-OH-DPAT pretreatment significantly decreased plasma oxytocin responses to (-)DOI challenge at all the points examined, 1, 2, 4, 24, 48 and 72 h, with a maximal effect between 24 and 72 h (approximately 70% inhibition). However, there were no significant (p>0.01) differences in the (+)8-OH-DPAT-induced desensitization of 5-HT_{2A}-mediated oxytocin release at any of the time points

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studied. These data indicate that a single injection of (+)8-OH-DPAT produces a long-lasting inhibition of 5-HT_{2A} receptor-mediated neuroendocrine responses.

Effects of one or two systemic injections of (+)8-OH-DPAT on 5-HT_{2A} receptor-mediated hormone responses.

Oxytocin

Figure 2 illustrates basal and (-)DOI-stimulated increases in plasma levels of oxytocin (Fig.2A) and ACTH (Fig.2B) in rats pretreated 12 and 24 h prior with saline or (+)8-OH-DPAT. (+)8-OH-DPAT (200µg/kg, s.c.) pretreatment was administered once (24 h prior) or twice (24 and 12h prior) prior to an injection of a submaximal dose of (-)DOI (0.35mg/kg). Pretreatment with either one or two injections of (+)8-OH-DPAT did not significantly alter basal oxytocin levels. (-)DOI significantly increased oxytocin plasma levels in rats pretreated with saline and (+)8-OH-DPAT. The 5-HT_{2A} receptor-mediated oxytocin (Fig.2A) responses were inhibited (-66%) when (+)8-OH-DPAT was injected 24 h prior to the subsequent challenge with (-)DOI. A similar degree of inhibition (-72%) was observed in rats pretreated with (+)8-OH-DPAT 24 and 12 h prior to the subsequent challenge with (-)DOI.

The two-way ANOVA for oxytocin indicated a significant effect for (-)DOI challenge ($F_{(1,50)}$ =177.009, $p<0.0001$) and for (+)8-OH-DPAT pretreatment ($F_{(2,50)}$ =47.0975, $p<0.0001$). There was a significant interaction between (-)DOI challenge and (+)8-OH-DPAT pretreatment ($F_{(2,50)}$ =46.2009, $p<0.0001$). The Newman-Keuls test indicated that (+)8-OH-DPAT administered once or twice significantly ($p<0.01$) decreased the plasma oxytocin responses to (-)DOI. There were no significant differences in the 5-HT_{2A} receptor-mediated release of oxytocin between rats pretreated with one or two injections of (+)8-OH-DPAT.

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ACTH

Pretreatment with either one or two injections of (+)8-OH-DPAT did not significantly alter basal ACTH levels. (-)DOI significantly increased ACTH plasma levels in rats pretreated with either saline or (+)8-OH-DPAT. The 5-HT_{2A} receptor-mediated ACTH (Fig.2B) responses were inhibited (-17%) when (+)8-OH-DPAT was injected 24 h prior to the subsequent challenge with (-)DOI. This inhibition was significantly greater (-44%) in rats pretreated with (+)8-OH-DPAT at both 24 and 12 h prior to the subsequent injection of (-)DOI.

The two-way ANOVA for ACTH indicated a significant effect of (-)DOI ($F_{(1,49)}$ =258.979, $p<0.0001$) and for (+)8-OH-DPAT pretreatment ($F_{(2,49)}$ =8.22037, $p<0.0009$). There was a significant interaction between (-)DOI challenge and (+)8-OH-DPAT pretreatment ($F_{(2,49)}$ =7.98, $p<0.001$). The Newman-Keuls test indicated that (+)8-OH-DPAT administered once (24 h) significantly ($p<0.05$) decreased plasma ACTH responses to (-)DOI. The inhibition of the (-)DOI-induced hormone responses was greater ($p<0.01$) in rats pretreated with two injections (24 and 12 h) of (+)8-OH-DPAT, compared with rats that received a single administration. These data indicate a cumulative effect on the (+)8-OH-DPAT-mediated inhibition of 5-HT_{2A} receptor-associated ACTH release.

Effect of a direct injection of (+)8-OH-DPAT into the PVN on the 5-HT_{2A} receptor-mediated neuroendocrine responses.

Oxytocin

Figure 3 illustrates the effect of the intraparaventricular administration of the 5-HT_{1A} receptor agonist (+)8-OH-DPAT (0.2 and 2 nmoles) on the 5-HT_{2A} receptor mediated neuroendocrine responses. (+)8-OH-DPAT was injected 24 h prior to the subsequent systemic injection of (-)DOI. (+)8-OH-DPAT (0.2 nmoles) significantly inhibited (-39%) the (-)DOI-mediated release of oxytocin (Fig.3A). A comparable attenuation (-43%) of the (-)DOI-mediated release of oxytocin was produced by a ten-fold higher dose (2 nmoles) of (+)8-OH-DPAT (Fig.3A).

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The two-way ANOVA for oxytocin showed a significant main effect of (+)8-OH-DPAT treatment ($F_{(1,51)}$ =13.33, p<0.0001) and (-)DOI challenge ($F_{(1,51)}$ =255.326, p<0.0001). There was also a significant interaction between (+)OH-DPAT treatment and (-)DOI challenge ($F_{(1,51)}$ =13.85, p<0.0001). The Newman-Keuls test indicated that both doses of (+)8-OH-DPAT (0.2 and 2 nmoles) significantly (p<0.01) inhibited the (-)DOI-mediated oxytocin release.

ACTH

Intra-PVN injection of (+)8-OH-DPAT (0.2 nmoles) significantly inhibited (-16%) the (-)DOI-mediated release of ACTH (Fig.3B). A comparable attenuation (-24%) of the (-)DOI-mediated release of ACTH was produced by a ten-fold higher dose (2 nmoles) of (+)8-OH-DPAT (Fig.3B).

The two-way ANOVA for ACTH showed a significant main effect of (+)8-OH-DPAT treatment ($F_{(1,46)}$ =3.2875, p<0.05) and (-)DOI challenge ($F_{(1,46)}$ =384.063, p<0.0001). There was also a significant interaction between (+)8-OH-DPAT treatment and (-)DOI challenge ($F_{(1,46)}$ = 3.4223, p<0.05). The Newman-Keuls test indicated that both doses of (+)8-OH-DPAT (0.2 and 2 nmoles) significantly (p<0.05) inhibited the (-)DOI-mediated ACTH release.

Effect of a systemic injection of WAY100635 on the (+)8-OH-DPAT-induced desensitization of 5-HT_{2A} receptor-mediated neuroendocrine responses.

Oxytocin

Figure 4 illustrates the effect of a systemic administration of the 5-HT_{1A} receptor antagonist WAY100635 on the (+)8-OH-DPAT induced desensitization of the 5-HT_{2A} receptor-mediated neuroendocrine responses. (+)8-OH-DPAT (0.2 nmoles) was injected directly into the PVN 30 min after the WAY100635 pretreatment (0.1 mg/kg, s.c.). 24 h later the rats were challenged with a submaximal dose of (-)DOI (0.35mg/kg, s.c.) and sacrificed 30 min later. None of the pretreatment paradigms significantly altered basal oxytocin levels. (-)DOI significantly increased plasma oxytocin levels in rats pretreated with either saline or (+)8-OH-DPAT. The oxytocin responses were inhibited (-37%) in rats

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pretreated with (+)8-OH-DPAT 24 h prior to the subsequent challenge with (-)DOI (Fig.4A). WAY100635 did not modify the (-)DOI-induced oxytocin release in saline pretreated rats, but blocked the (+)8-OH-DPAT-induced inhibition of the 5-HT_{2A} receptor-mediated oxytocin release (Fig.4A).

The three-way ANOVA for oxytocin showed a significant main effect of (+)8-OH-DPAT treatment ($F_{(1,47)}$ =18.996, p<0.0001) and (-)DOI challenge ($F_{(1,47)}$ =531.4505, p<0.0001), but not WAY100635 pretreatment ($F_{(1,47)}$ =3.1336, p<0.08). Significant main interactions were found between WAY100635 pretreatment and (+)8-OH-DPAT treatment ($F_{(1,47)}$ =3.9483, p<0.05), between WAY100635 pretreatment and (-)DOI challenge ($F_{(1,47)}$ =3.89456, p<0.05), and between (+)8-OH-DPAT treatment and (-)DOI challenge ($F_{(1,47)}$ =17.64105, p<0.0001). There was also a main interaction between WAY100635 pretreatment, (+)8-OH-DPAT treatment and (-)DOI challenge ($F_{(1,47)}$ =3.96844, p<0.05). The Newman-Keuls test indicated that (+)8-OH-DPAT treatment significantly (p<0.01) inhibited the (-)DOI-mediated oxytocin release (Fig.4A) and that in rats pretreated with WAY100635, the magnitude of (+)8-OH-DPAT-induced inhibition of 5-HT_{2A} receptor stimulation of oxytocin (-19%) was significantly (p<0.01) different than their respective counterparts that did not receive the 5-HT_{1A} receptor antagonist (Fig.4A).

ACTH

As shown in Fig. 4B, none of the pretreatment paradigms modified the basal ACTH levels. (-)DOI significantly increased plasma ACTH levels in rats pretreated with saline or (+)8-OH-DPAT. The ACTH responses were inhibited (-19%, p<0.05) in rats pretreated with (+)8-OH-DPAT 24 h prior to the subsequent challenge with (-)DOI (Fig.4B). WAY100635 did not modify the (-)DOI-induced ACTH release in saline pretreated rats, nor did it significantly block the (+)8-OH-DPAT-induced inhibition of the 5-HT_{2A} receptor-mediated ACTH release (Fig.4B).

The three-way ANOVA for ACTH showed a significant main effect of (+)8-OH-DPAT treatment ($F_{(1,47)}$ =4.6042, p<0.03) and (-)DOI challenge ($F_{(1,47)}$ =575.0295, p<0.0001), but not WAY100635 pretreatment ($F_{(1,47)}$ =0.0782, p<0.78). Significant main interactions were found between (+)8-OH-DPAT

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treatment and (-)DOI challenge ($F_{(1,47)}$ =4.6536, p<0.03). The Newman-Keuls test indicated that (+)8-OH-DPAT treatment significantly (p<0.05) inhibited the (-)DOI-mediated ACTH release (Fig.4B). However, there were no significant differences in the (-)DOI-mediated ACTH release between rats pretreated with saline and (+)8-OH-DPAT versus WAY100635 and (+)8-OH-DPAT.

Effect of an intraparaventricular injection of WAY100635 on the (+)8-OH-DPAT induced-desensitization of 5-HT$_{2A}$ receptor signaling.

Oxytocin

Figure 5 illustrates the effect of a intraparaventricular administration of the 5-HT$_{1A}$ receptor antagonist WAY100635 on the desensitization of the 5-HT$_{2A}$ receptor mediated neuroendocrine responses produced by a systemic injection of (+)8-OH-DPAT. WAY100635 (10 nmoles) was injected directly into the PVN 30 min prior to a single injection of (+)8-OH-DPAT (200µg/kg, s.c.). 24 h later the rats were challenged with (-)DOI (0.35mg/kg, s.c.) and sacrificed 30 min later. As shown in Fig.5A, none of the pretreatment paradigms significantly altered basal oxytocin levels. (-)DOI significantly increased plasma oxytocin levels in rats pretreated with saline or (+)8-OH-DPAT. However, the oxytocin responses were inhibited (-63%) in rats pretreated with (+)8-OH-DPAT 24 h prior to the subsequent challenge with (-)DOI (Fig.5A). WAY100635 did not modify the (-)DOI-induced oxytocin response in saline pretreated rats. In contrast, WAY100635 injected directly into the PVN partially blocked the (+)8-OH-DPAT-induced inhibition of the 5-HT$_{2A}$ receptor-mediated oxytocin release.

The three-way ANOVA for oxytocin showed a significant main effect of (+)8-OH-DPAT treatment ($F_{(1,57)}$ =43.87, p<0.0001) and (-)DOI challenge ($F_{(1,57)}$ =332.656, p<0.0001), but not WAY100635 pretreatment ($F_{(1,57)}$ =2.4204, p<0.12). Significant main interactions were found between WAY100635 pretreatment and (+)8-OH-DPAT treatment ($F_{(1,57)}$ =4.0403, p<0.04), and between (+)8-OH-DPAT treatment and (-)DOI challenge ($F_{(1,57)}$ =45.05108, p<0.0001). There was also a main interaction between WAY100635 pretreatment, (+)8-OH-DPAT treatment and (-)DOI challenge ($F_{(1,57)}$ =4.0003, p<0.05). The Newman-Keuls test indicated that pretreatment with (+)8-OH-DPAT significantly (p<0.01)

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inhibited (-63%) the (-)DOI-mediated oxytocin release (Fig.5A). This effect was partially blocked in rats that were injected directly into the PVN with WAY100635.

ACTH

As shown in Fig.5B, none of the pretreatment paradigms significantly altered basal ACTH levels. (-)DOI significantly increased ACTH plasma levels in rats pretreated with saline or (+)8-OH-DPAT. The ACTH responses were inhibited (-25%) in rats pretreated with (+)8-OH-DPAT 24 h prior to the subsequent challenge with (-)DOI. Direct intra-PVN injections of WAY100635 did not modify the (-)DOI-induced ACTH release in saline pretreated rats, nor did it significantly alter the (+)8-OH-DPAT-induced inhibition of the 5-HT_{2A} receptor-mediated ACTH release.

The three-way ANOVA for ACTH showed a significant main effect of (+)8-OH-DPAT treatment ($F_{(1,60)}$ =3.9919, $p<0.05$) and (-)DOI challenge ($F_{(1,60)}$ =354.0162, $p<0.0001$), but not WAY100635 pretreatment ($F_{(1,60)}$ =0.00416, $p<0.948$). Significant main interactions were found between (+)8-OH-DPAT treatment and (-)DOI challenge ($F_{(1,60)}$ =4.7653, $p<0.03$). The Newman-Keuls test indicated that (+)8-OH-DPAT treatment significantly ($p<0.05$) inhibited (-25%) the (-)DOI-mediated oxytocin release, and this effect was reduced (-14%) by WAY100635 but this difference was not statistically significant ($p>0.05$) (Fig.5B).

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Discussion

This study provides the first *in vivo* demonstration that acute activation of 5-HT_{1A} receptors produces a prolonged functional heterologous desensitization of 5-HT_{2A} receptors in neuroendocrine neurons of the PVN. Data from a variety of pharmacological approaches that support the 5-HT_{1A} receptor-mediated heterologous desensitization of 5-HT_{2A} receptor function include: **(1)** the attenuated 5-HT_{2A} receptor-mediated release of oxytocin from 1-72 h after a single injection of (+)8-OH-DPAT (Fig.1); **(2)** the reduced 5-HT_{2A} receptor-mediated increases of both oxytocin and ACTH at 24 h after a single injection of (+)8-OH-DPAT (Fig.2); **(3)** the greater reduction in the 5-HT_{2A} receptor-mediated ACTH responses produced by two injections of (+)8-OH-DPAT (Fig.2B); **(4)** the blunted (-)DOI-mediated increases in both oxytocin and ACTH by a prior direct intraparaventricular injection of (+)8-OH-DPAT (Fig.3); **(5)** the blockade of intra-PVN (+)8-OH-DPAT-induced desensitization of the 5-HT_{2A} receptor mediated oxytocin responses by pretreatment with WAY100635 (Fig.4); and **(6)** the blockade of the (+)8-OH-DPAT-induced desensitization of the 5-HT_{2A} receptor-mediated oxytocin responses by direct intraparaventricular administration of WAY100635 (Fig.5).

We previously demonstrated that 5-HT_{1A} and 5-HT_{2A} receptors are co-expressed in oxytocin and CRF containing neurons in the PVN (Zhang, et al., 2004). Immunohistochemical double labeling of 5-HT_{1A} or 5-HT_{2A} receptors, and oxytocin revealed that a high percentage of oxytocin neurons in the PVN were immunopositive for 5-HT_{1A} and 5-HT_{2A} receptors (94% and 97%, respectively) (Zhang, et al., 2004). CRF-immunoreactive neurons in the PVN also expressed 5-HT_{1A} and 5-HT_{2A} receptor immunoreactivity (Zhang, et al., 2004), although 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 might directly interact with 5-HT_{1A} receptors through their signaling proteins to regulate hormone release. Consequently, the *in vivo* heterologous desensitization of 5-HT_{2A} receptors after 5-HT_{1A} receptor activation presumably is mediated via intracellular rather than transynaptic mechanisms, although additional studies are needed to confirm this hypothesis.

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We have previously demonstrated that activation of 5-HT_{2A} receptors in the PVN induced a heterologous desensitization of 5-HT_{1A} receptors within individual neuroendocrine cells (Zhang, et al., 2001;Zhang, et al., 2004). Activation of 5-HT_{2A} receptors produced a delayed and reversible reduction of the ACTH and oxytocin responses to a 5-HT_{1A} receptor agonist (Zhang, et al., 2001). The maximal desensitization occurred at 2-4 h, with recovery back to control responses by 24 h after the (-)DOI injection. The desensitization was dose-dependent and it shifted the oxytocin and ACTH dose-response curves of (+)8-OH-DPAT to the right (increased ED_{50}), with no change in their maximal responses (E_{max}) (Zhang, et al., 2001).

In contrast, the present studies indicated that activation of 5-HT_{1A} receptors in the PVN produced a faster and more persistent inhibition of 5-HT_{2A} receptor-mediated neuroendocrine responses. The heterologous desensitization of 5-HT_{2A} receptors was evident at least one hour after a single (+)8-OH-DPAT injection and persisted for at least 72 h, the last time-point measured in this study. It is unlikely that the reduction of (-)DOI-mediated hormone responses after a (+)8-OH-DPAT injection is due to the hormone-depleting effect of 5-HT_{1A} receptor activation as the desensitization of the 5-HT_{2A} receptor-mediated neuroendocrine responses was found even 3 days after a single (+)8-OH-DPAT injection (Fig.1). If the pretreatment with (+)8-OH-DPAT depleted the oxytocin, CRF or ACTH stores, this would have been recovered during the first few hours after the single (+)8-OH-DPAT injection. Thus, the long-lasting (+)8-OH-DPAT-induced attenuation of hormone responses to the (-)DOI challenge is likely due to a persistent desensitization of hypothalamic 5-HT_{2A} receptor signal transduction.

In the present studies, respective changes in 5-HT_{2A} receptor-mediated oxytocin and ACTH neuroendocrine responses were determined as these are mediated by different neurons and utilize different post-receptor regulatory mechanisms and signaling cascades. The oxytocin cells located in the PVN send their axons directly to the posterior lobe of the pituitary and release oxytocin into the blood stream (Carrasco and Van de Kar, 2003). Therefore, oxytocin is the most direct indicator of events occurring in the hypothalamus. On the other hand, the ACTH response represents a functional index

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that is amplified subsequent to hypothalamic activation of 5-HT receptors. ACTH is released by corticotrophs in the anterior lobe of the pituitary (Carrasco and Van de Kar, 2003). This difference in amplification may explain our observation that (+)8-OH-DPAT produced a greater attenuation of the oxytocin response to (-)DOI (Fig.2 and Fig.3), while the inhibition of the ACTH response to (-)DOI was less marked (Fig.2 and Fig.3). Also, differences in the degree of 5-HT_{1A} and 5-HT_{2A} co-localization on oxytocin versus CRF-containing cells may be responsible for differences in the magnitude of reduction in oxytocin and ACTH following single versus repeated doses of (+)8-OH-DPAT. As there is a lower degree of co-localization of 5-HT_{1A} and 5-HT_{2A} receptors in CRF-containing neurons of the PVN compared with oxytocin-containing neurons (Zhang, et al., 2004), repeated doses of (+)8-OH-DPAT may be needed to produce the greater reduction in the 5-HT_{2A} receptor-mediated ACTH responses. Alternative explanations include: (1) systemic (-)DOI injection may also activate 5-HT_{2A} receptors in other nuclei that are involved in ACTH release, such as the amygdala (Beaulieu, et al., 1986;Feldman, et al., 1998), that could mask the effects occurring in hypothalamic CRF neurons; and (2) 5-HT_{1A} receptor-associated signaling pathways in oxytocin containing cells in the PVN could be different than those found in CRF containing cells. This is supported by studies demonstrating that 5-HT_{1A} receptors are coupled to different G-proteins within different brain areas (Lin, et al., 2002;La Cour, et al., 2006).

As (+)8-OH-DPAT injected directly into the PVN produces 5-HT_{2A} receptor desensitization (Fig.3 and 4), the activation of hypothalamic 5-HT_{1A} receptors is required to produce long-term desensitization of 5-HT_{2A} receptor-mediated neuroendocrine responses. WAY 100635, which exhibits an high affinity for 5-HT_{1A} receptors (Ki = 2.2 nM) that is 10-100 times higher than its affinity for other 5-HT receptors or dopamine D_1, D_2, and D_4 receptors (Forster, et al., 1995;Chemel, et al., 2006), was used to demonstrate the specificity of the effect of 5-HT_{1A} receptors. Accordingly, the heterologous desensitization of the (-)DOI-mediated oxytocin responses was prevented when WAY100635 was injected systemically (Fig.4) or directly into the PVN (Fig.5). These observations suggest that 5-HT_{1A} receptors interact directly with 5-HT_{2A} receptors in oxytocin containing-neurons of the hypothalamic PVN. Our previous immunocytochemical observations and the high degree of colocalization of 5-HT_{1A}

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and 5-HT_{2A} receptors in oxytocin-containing cells (Zhang, et al., 2004) suggest that a direct intracellular interaction could occur between these receptor signaling systems.

On the other hand, pretreatment with (+)8-OH-DPAT has a less marked effect on 5-HT_{2A} receptor-mediated ACTH responses. Furthermore, systemic (Fig.4) or direct intra-PVN (Fig.5) administration of WAY100635 did not block the 5-HT_{1A} receptor-induced reduction of the (-)DOI-mediated ACTH responses. The ACTH responses were inhibited (19%, Fig.4B) in rats pretreated with a direct intra PVN injection of (+)8-OH-DPAT 24 h prior to the subsequent challenge with (-)DOI. However, there were no significant differences in the 5-HT_{1A} receptor-induced inhibition of the 5-HT_{2A} receptor-mediated ACTH responses between rats pre-treated with a systemic injection of either saline or WAY100635 (Fig.4B). Similarly, (-)DOI-induced ACTH responses were inhibited (-25%) in rats pretreated with (+)8-OH-DPAT (Fig.5B). However, there were no differences in the 5-HT_{1A} receptor-induced inhibition of the 5-HT_{2A} receptor-mediated ACTH responses between rats that received a direct intra PVN injection of either saline or WAY100635. Given the small magnitude of 5-HT_{2A} receptor desensitization by 5-HT_{1A} receptor activation and the partial blockade of this effect by WAY100365, the evidence of a direct crosstalk between 5-HT_{1A} and 5-HT_{2A} receptors in CRF-containing cells in the PVN is inconclusive.

The mechanisms underlying the heterologous desensitization of 5-HT_{1A} receptors by 5-HT_{2A} receptors in PVN await further investigation. Our previous studies *in vivo* demonstrated that activation of 5-HT_{1A} receptors in PVN activates MAP kinase signaling, specifically the phosphorylation of p42/44 extracellular signal-regulated kinase (ERK) (Sullivan, et al., 2005). This effect was completely abolished by WAY100635 pre-administration (Sullivan, et al., 2005). The relatively rapid onset of the 5-HT_{1A} receptor-mediated desensitization of hypothalamic 5-HT_{2A} receptors (by 1 h after activation of 5-HT_{1A} receptors) suggests that post-translational modification mechanisms may be involved. However, the long-lasting effects on 5-HT_{2A} receptor signaling after activation of 5-HT_{1A} receptors could involve activation of several transcription factors (Mazzucchelli and Brambilla, 2000;Milanini-Mongiat, et al., 2002;Sweatt, 2001). We speculate that 5-HT_{1A} receptor-induced activation of phosphorylated ERK

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(pERK) could participate in the signal transduction pathways involved in the intracellular regulation of 5-HT_{2A} receptors.

Desensitization of 5-HT_{2A} receptors has been proposed as the mechanism underlying the therapeutic efficacy of antipsychotic, anxiolytic, and antidepressant agents (Meltzer, et al., 2003;Roth and Xia, 2004). Consequently, drugs with 5-HT_{2A} receptor antagonist properties have been undergoing clinical evaluation for the treatment of several disorders such as schizophrenia, depression, anxiety, migraine, sleep and feeding disorders (Meltzer, et al., 2003;Roth and Xia, 2004;Amargos-Bosch, et al., 2004). If indeed, 5-HT_{1A} receptor activation can negatively regulate 5-HT_{2A} receptor function, then novel antidepressants and antipsychotics that stimulate 5-HT_{1A} receptors may have a greater pharmacological impact in some neurons or brain regions.

In summary, the present studies provide *in vivo* pharmacological evidence that 5-HT_{1A} receptors may directly crosstalk with 5-HT_{2A} receptors to regulate neuroendocrine function. Since a functional imbalance between hypothalamic 5-HT_{1A} and 5-HT_{2A} receptors could modify the organism's response to stressors (Berendsen, 1995;Zhang, et al., 2004); antidepressants (Carrasco and Van de Kar, 2003); and drugs of abuse (Levy, et al., 1994), elucidation of the specific mechanisms mediating interactions between hypothalamic 5-HT_{1A} and 5-HT_{2A} receptors may lead to novel targets for the development of psychotherapeutic drugs.

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Footnotes

This work was supported in part by NS034153, DA013669 and DA07741.

Dr. Louis D. Van de Kar, a good friend, colleague and internationally recognized scientist passed away on September 4, 2004. His contribution and memory will endure through all of those whose lives he enriched.

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Legends for Figures:

Figure 1. Time-course of (+)8-OH-DPAT (200µg/kg, s.c.)-induced reduction of the oxytocin response to a (-)DOI (1.0 mg/kg, s.c.) challenge. The data represent the means ± S.E.M. of 6-8 rats per group. ** significant difference from the respective saline-challenged group ($p<0.01$); ## significant difference ($p<0.01$) from saline/(-)DOI group (three-way ANOVA and Newman-Keuls multiple range test).

Figure 2. Inhibition of the (-)DOI (0.35 mg/kg, s.c.)-induced (A) oxytocin, and (B) ACTH neuroendocrine responses by one (12 h) or two (24 and 12 h) pre-administrations of the 5-HT_{1A} receptor agonist (+)8-OH-DPAT (200 ug/kg, s.c.). The data represent the means ± S.E.M. of 6-8 rats per group. ** significant difference ($p<0.01$) from the respective saline-challenged groups; ## significant difference ($p<0.01$) from saline 24h/saline 12h/(-)DOI group; # significant difference ($p<0.05$) from saline 24h/saline 12h/(-)DOI group; && significant difference ($p<0.01$) from DPAT 24h/Saline 12h/(-)DOI group (two-way ANOVA and Newman-Keuls multiple range test).

Figure 3. Inhibition of (-)DOI (0.35 mg/kg, s.c.)-induced (A) oxytocin, and (B) ACTH neuroendocrine responses by a direct injection of (+)8-OH-DPAT (0.2 nmoles or 2 nmoles, 0.5 µl/side) into the hypothalamic paraventricular nucleus. The data represent the means ± S.E.M. of 8-10 rats per group. ** significant difference ($p<0.01$) from the respective saline-challenged group; ## significant difference ($p<0.01$) from saline/(-)DOI group; # significant difference ($p<0.05$) from saline/(-)DOI group (two-way ANOVA and Newman-Keuls multiple range test).

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Figure 4. Effect of a systemic injection of WAY100635 (0.1 mg/kg, s.c.) on the (+)8-OH-DPAT (0.2 nmoles, 0.5 μl side)-induced desensitization of 5-HT_{2A} receptor-mediated (A) oxytocin and (B) ACTH, neuroendocrine responses. The data represent the means ± S.E.M. of 8-10 rats per group. ** significant difference ($p<0.01$) from the respective saline-challenged group; ## significant difference ($p<0.01$) from saline/saline/(-)DOI group; # significant difference ($p<0.05$) from saline/saline/(-)DOI group; && significant difference ($p<0.01$) from saline/DPAT/(-)DOI group (two-way ANOVA and Newman-Keuls multiple range test).

Figure 5. An intraparaventricular injection of WAY100635 (10 nmoles, 0.5 μl side) partially blocked the (+)8-OH-DPAT (200 ug/kg, s.c.) induced-desensitization of 5-HT_{2A} receptor signaling. The data represent the means ± S.E.M. of 8-10 rats per group. ** significant difference ($p<0.01$) from the respective saline-challenged group; ## significant difference ($p<0.01$) from saline/saline/(-)DOI group; # significant difference ($p<0.05$) from saline/saline/(-)DOI group; & significant difference ($p<0.05$) from saline/DPAT/(-)DOI group (two-way ANOVA and Newman-Keuls multiple range test).

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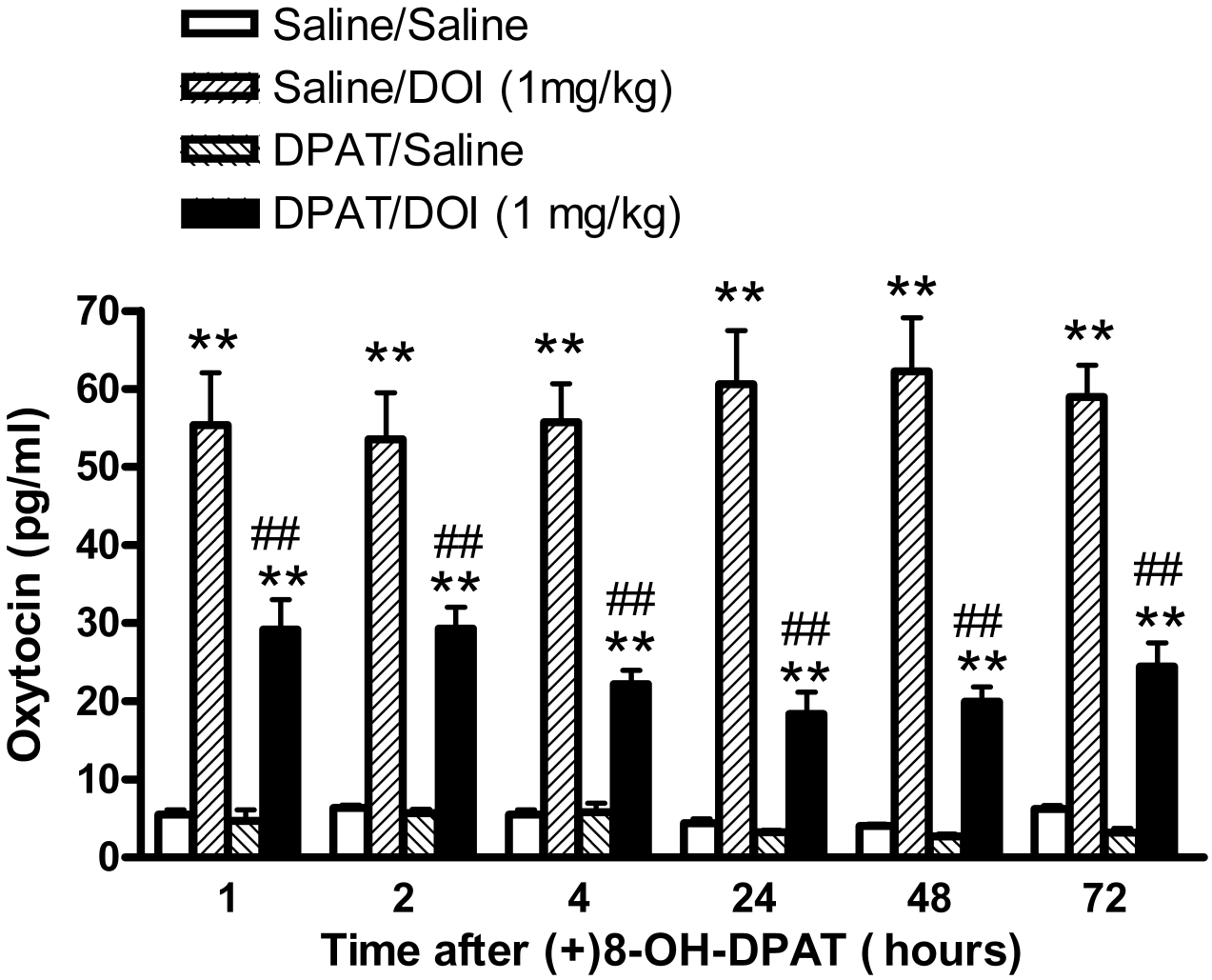

Figure 1

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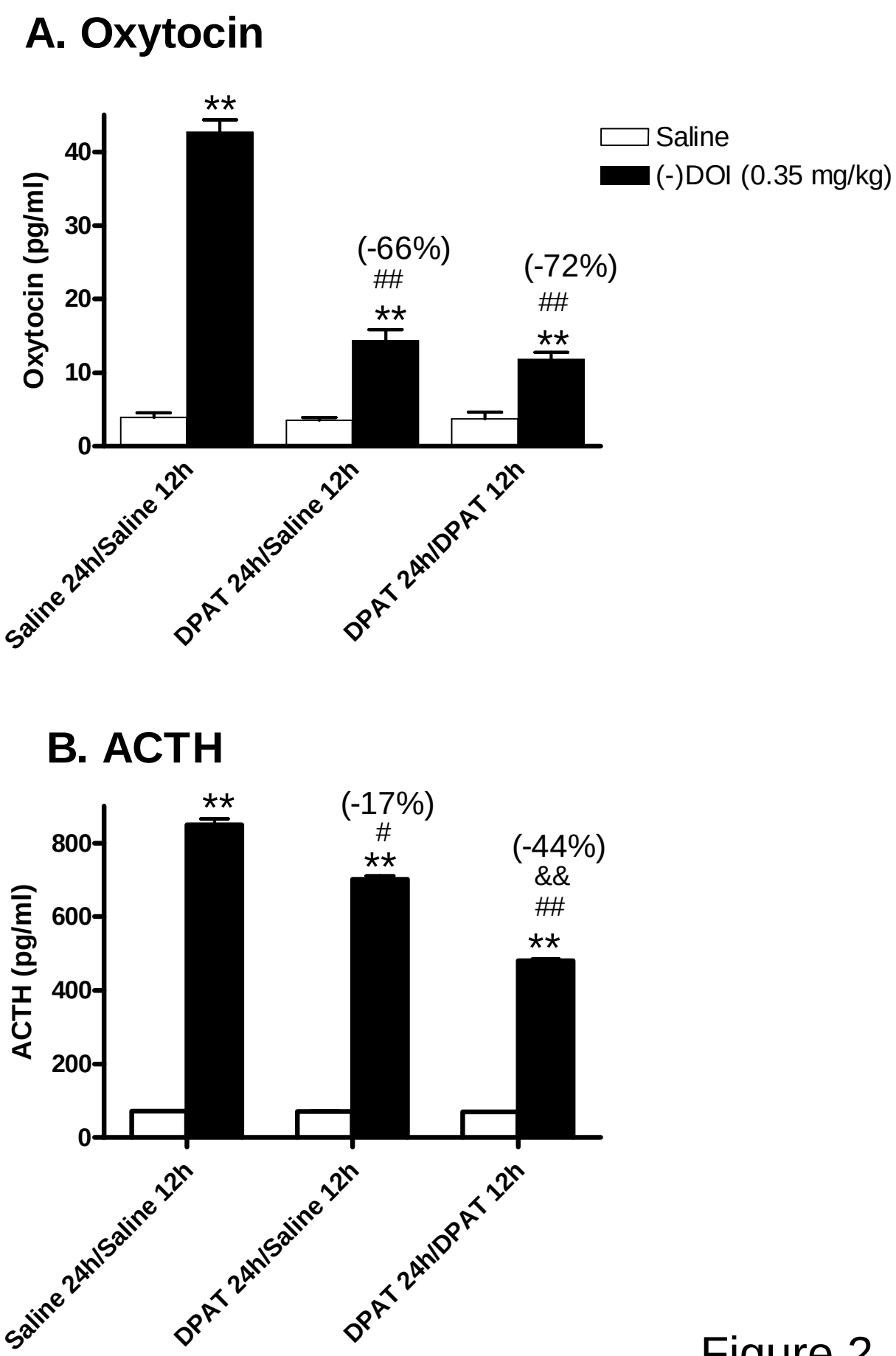

Figure 2

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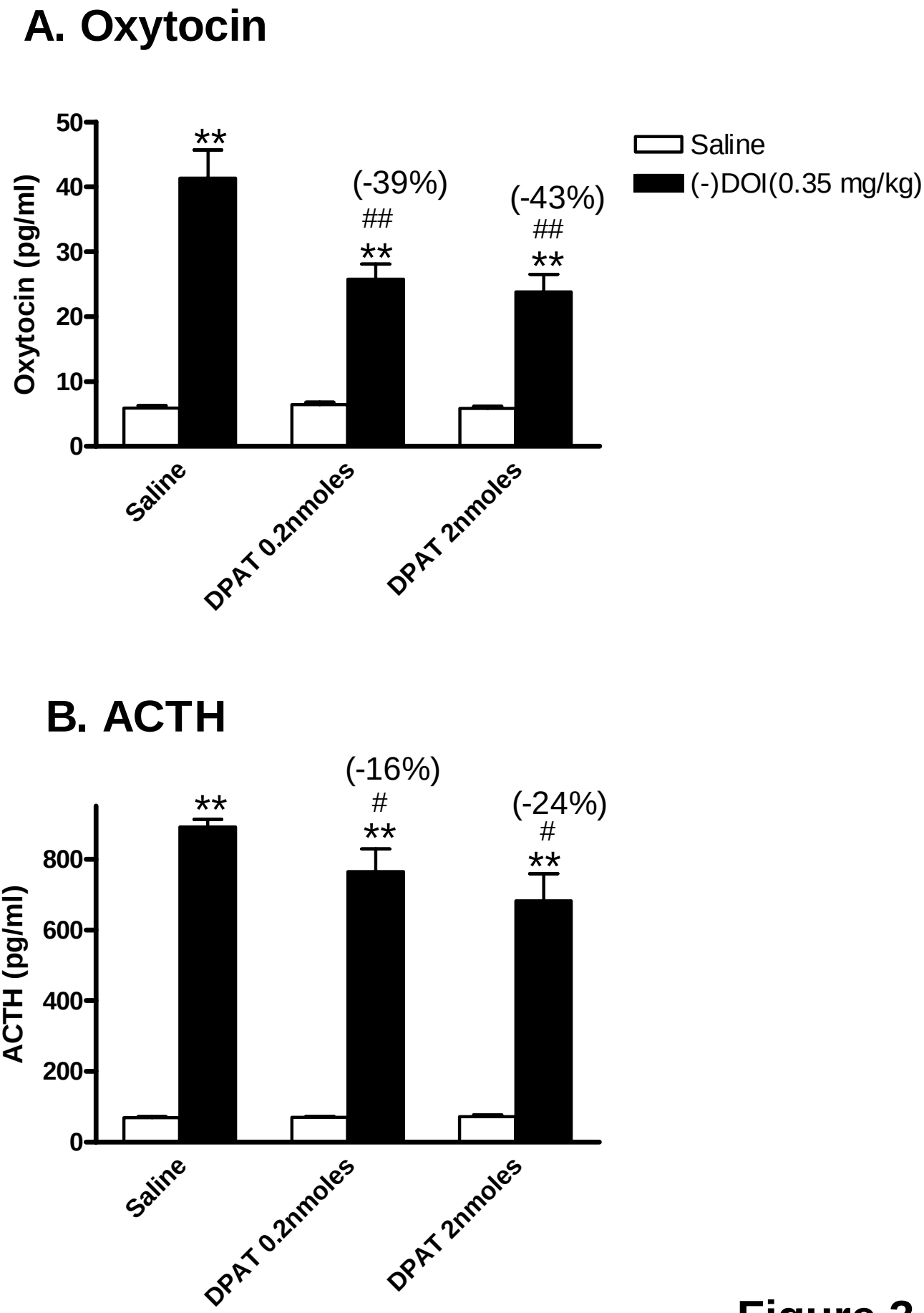

Figure 3

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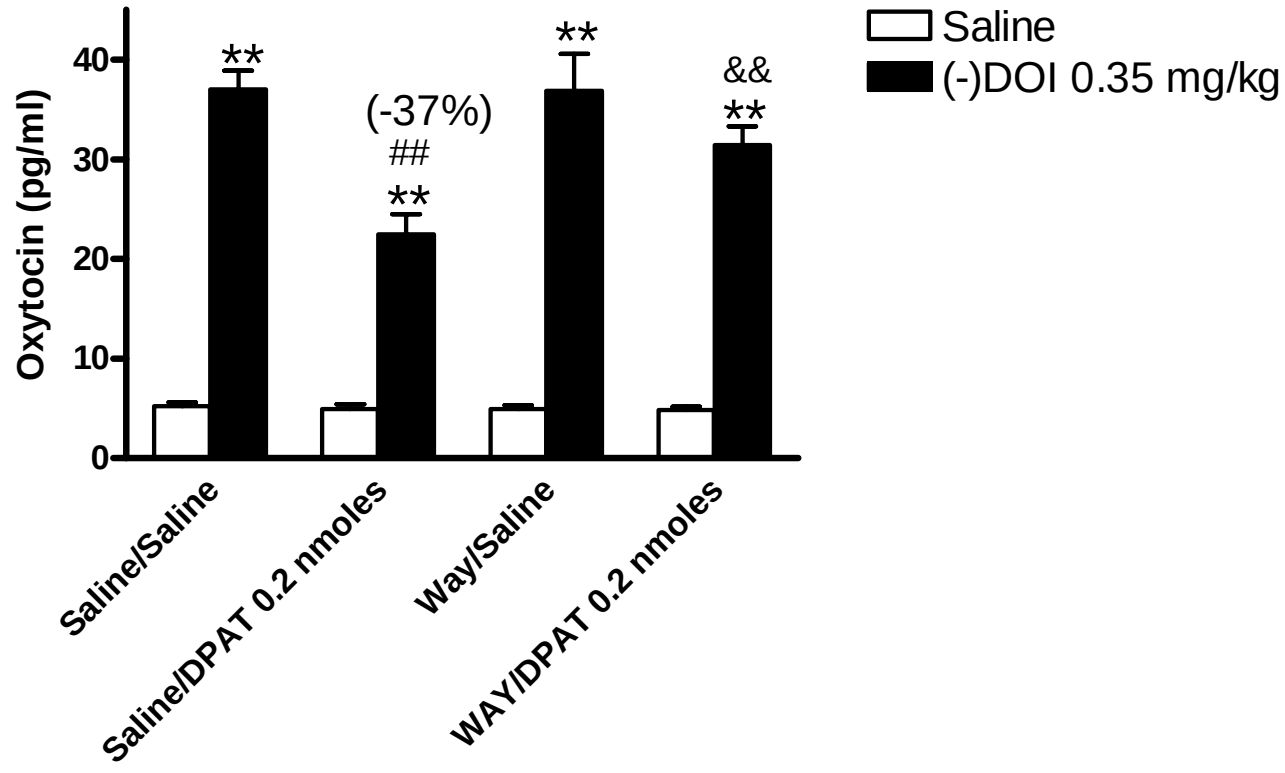
A. Oxytocin



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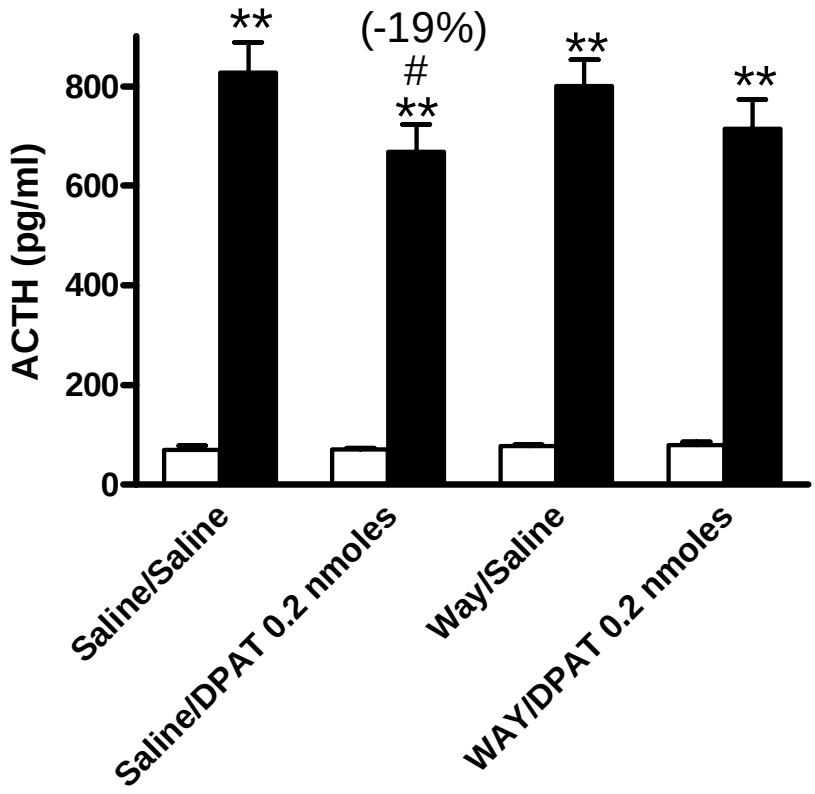

Figure 4

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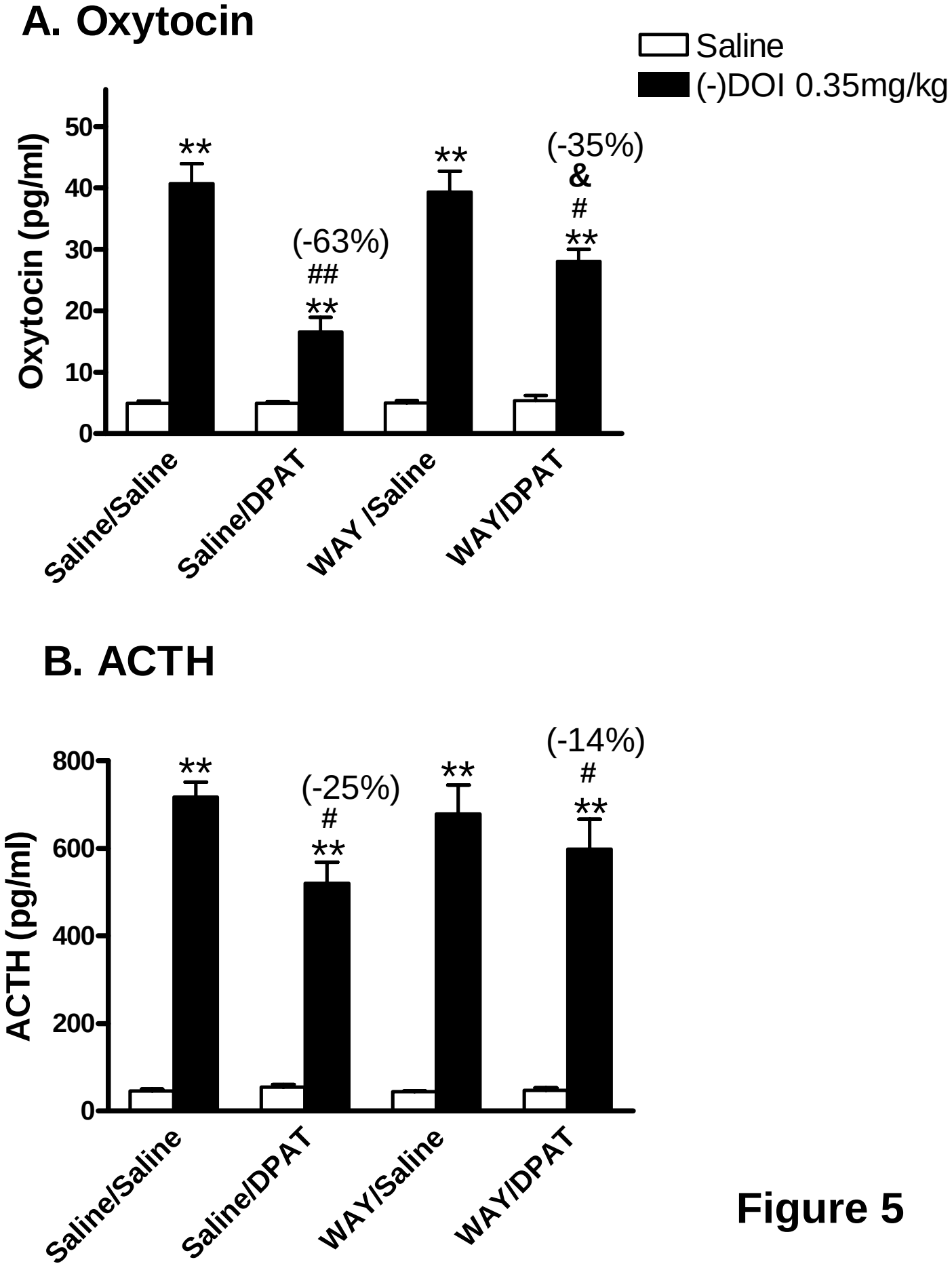

Figure 5

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