Endocrine, *vol. 18, no. 2, 167–172, July 2002* 0969–711X/02/18:167–172/$11.50

Characterization of Oxytocin Receptors and Serotonin Transporters in Mast Cells

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Oxytocin (OT) inhibits the uptake of serotonin (5HT) into uterine mast cells. This may modulate 5HT bioavailability in the myometrium. Because 5HT is an important endogenous uterotonic compound, it has been postulated that this effect of OT may contribute to its potency as a labor inducer. This also predicts the presence of oxytocin receptors (OTRs) and transducing signals that will interact with 5HT transporters (SERT) in mast cells. In this study, OTR and SERT were characterized in murine peritoneal mast cells by radioligand-binding studies. Saturation assays for OTR showed no changes in K_d along the estrous cycle (6.95 ± 2.76 n*M* in estrus and 4.07 ± 1.73 n*M* in diestrus) but an increase in B_{max} in estrus (0.71 ± 0.08 pmol/10^6 cells and 0.37 ± 0.05 pmol/10^6 cells in estrus and diestrus, respectively). B_{max} and K_d for SERT were not affected along the estrous cycle.

The signaling between the OTR and the SERT was analyzed by measuring the extent of inhibition of OT and PMA (activator of protein kinase C on 5HT uptake and the capability of Ro318220 (specific inhibitor of PKC) to increase 5HT uptake and block the effect of the above compounds in mast cells. The results showed that in murine peritoneal mast cells in vitro (1) ovarian hormones modulate OTR but not SERT expression, (2) the magnitude of OT action on 5HT uptake depends on the number of OTRs expressed in mast cells, and (3) the signaling between OTR and the SERT is mediated through the activation of protein kinase C. It is concluded that the ovarian hormones have a modulatory action on 5HT uptake which involves OT-mediated mechanism.

Key Words: Oxytocin receptors; serotonin transporters; serotonin uptake; mast cells; estrogen; progesterone.

Introduction

Activation of oxytocin receptor (OTR) by oxytocin (OT) has been shown to play critical roles in reproductive processes, such as labor, lactation, and maternal behavior *(1,2)*. OTRs belong to the superfamily of G-protein-coupled receptors. The pathways sensitive to OT actions are those involved in the activation of protein kinase C (PKC) and mitogen-activated protein kinases (MAPKs). OTR/PKC-mediated events promote the contraction of smooth muscle cells in the myometrium, whereas OTR/$p42^{MAPK}$ activation may lead to prostaglandin E_2 (PGE_2) synthesis *(3–5)*.

Gene expression of the OTR in the uterus has been shown to be stimulated by estrogens *(6,7)*. Nevertheless, the changes observed in the uterus through the estrous cycle and pregnancy cannot only be explained by changes in the sensitivity to OT. It would appear that additional paracrine factors should also be involved *(8)*. The action of OT on uterine mast cells, through the inhibition of the uptake of the biogenic amine serotonin (5-hydroxytryptamine, 5HT) could become a critical component *(9)*.

5HT is a potent endogenous uterotonic compound. Synthetic analogs of 5HT (e.g., Ergometrine) are used in the clinical setting to induce postpartum uterine contractions in order to reduce hemorrhaging *(10)*. 5HT is also believed to be important for cervix ripening, which, in turn, determines the time of parturition, by the stimulation of myometrial smooth muscle cells to synthesize collagenase at the end of gestation *(11)*. 5HT bioavailability is determined in large part by the action of the plasma membrane 5HT transporter (SERT). The SERT-mediated 5HT accumulation is sodium and chloride dependent and is implicated in the regulation of mood and anxiety. It is the site of the initial action of widely used antidepressant and antianxiety drugs. In fact, SERTs are effectively blocked by tricyclic antidepressants such as paroxetine and imipramine *(12,13)*, nonselective stimulants such as amphetamines and cocaine *(14)*, and, most selectively, by selective-reuptake inhibitors such as fluoxetine and 6-nitroquipazine *(15)*. Rodent and human SERTs are predicted to be encoded by subunits of 630 amino acids with >90% cross-species identity. Hydropathy analysis suggest that SERTs exhibit a topology of 12 transmembrane domains. Canonical sites for protein kinases on presumed cytoplasmatic domains raise the possibility that SERTs,

Received April 8, 2002; Revised June 5, 2002; Accepted June 5, 2002.

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like other membrane transporter and ion pumps, may be regulated by phosphorylation *(16–18)*.

In addition to neurons, various cell types in the periphery, including platelets *(19)*, mast cells *(17)*, and placental *(20)* and lung endothelial cells *(21)*, uptake 5HT by a similar mechanism. Previous studies have shown that mast cells are responsible for the majority of 5HT uptake in the mouse uterus *(9)*. The ability of mouse uterine horns to uptake 5HT is significantly reduced by OT. This effect is increased in estrogen-primed uterine horns, which suggests that 5HT bioavailability may be increased by the combined effects of estrogens and OT. Moreover, regarding uterine contractility, a potentiation between OT and 5HT has been observed *(9)*. The result is the hypothesis on the establishment of an indirect mechanism by which OT may increase uterine contractility by inhibiting 5HT uptake, which contributes to its potency as a physiological labor inducer at the end of pregnancy.

Previous work from our laboratory using purified murine peritoneal mast cells showed that they are sensitive to estrogens, take up 5HT, and bind OT mechanisms similar to that for uterine mast cells *(9)*. Therefore, mouse peritoneal mast cells provide a model to study the interaction among the OTRs, SERTs, and sexual hormone receptors. The objective of this work was to characterize OTRs and SERTs and analyze the pathways involved in the action of OT on 5HT uptake in mast cells.

Results

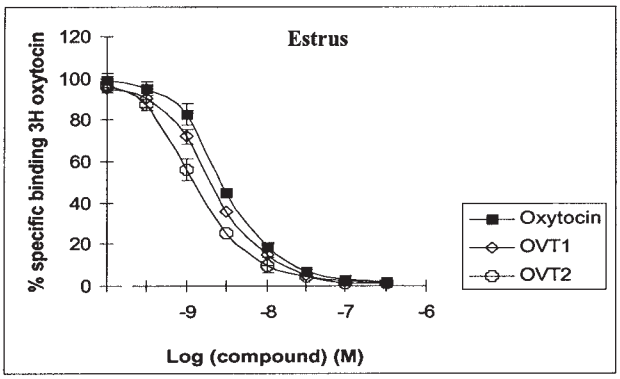
Specific Binding of ^{3}H-OT to Mouse Peritoneal Mast Cells

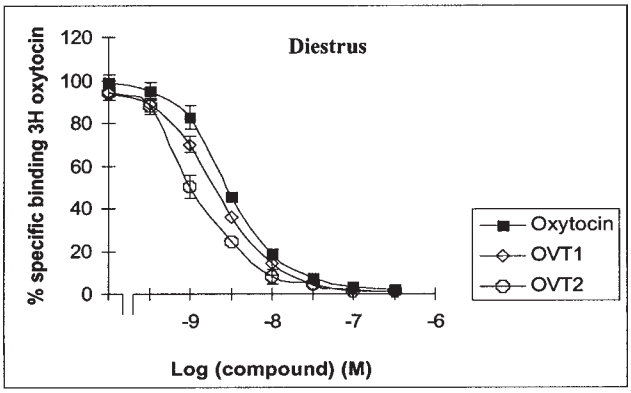
Preliminary experiments were performed in order to obtain the optimum binding conditions of ^{3}H-OT to membranes of mouse peritoneal mast cells. It was shown that maximal specific binding was obtained at 30ºC and at a pH of 7.4. The specific binding equilibrated after 30 min and remained constant for at least 4 h. Under these experimental conditions, the specific binding of ^{3}H-OT was linear, with protein concentrations ranging from 20 to 250 µg/mL.

As shown in Fig. 1, OT and the antagonists OVT_1 and OVT_2 competed with ^{3}H-OT for the binding sites according to an inhibitory pattern that suggests that ^{3}H-OT binds to a single site. Three independent experiments yielded the IC_{50} values shown in the inset of Fig. 1; they were similar in both estrous stages. Binding parameters obtained from saturation experiments are shown in Fig. 2. As shown in the inset of Fig. 2, the estimated K_d's were similar in membranes from peritoneal mast cells of mouse, both in estrus and diestrus. Nevertheless, the B_{max} value was significantly higher under estrogen than under progesterone predominance.

Effect of OT on 5HT Uptake in Peritoneal Mast Cells

^{3}H-5HT uptake was saturable in mouse peritoneal mast cells at concentrations over 250 n*M*. The double reciprocal plot of the data obtained by measuring the uptake of ^{3}H-5HT into mast cells was linear ($r > 0.99$). The estimated K_{Mapp} values were similar at estrus and diestrus (K_{Mapp} 65 ± 2 n*M* and 68 ± 3 n*M*, respectively, $n = 6$). Nevertheless, significant differences were observed in the values of the maximal uptake velocity (V_{max}) (estrus: 63.9 ± 8.2 fmol/10^6 cell/min; diestrus: 78.2 ± 4.0 fmol/10^6 cell/min; $n = 6$). OT (0.03 n*M*) inhibited V_{max} but did not modify K_{Mapp}. The effect on B_{max} was higher under estrogen predominance (72% in mast cells obtained from mice in estrus but only 23% in

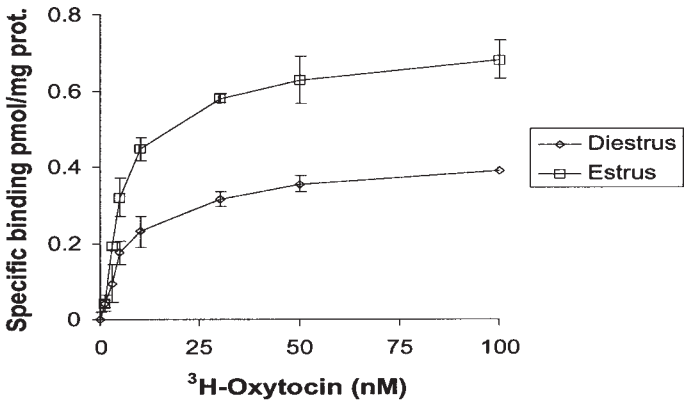




IC_{50} (nM) ±

	Estrus	Diestrus
Oxytocin	8.8 ± 0.9	9.3 ± 0.8
OVT_1	24.8±7.8	23.2±6.5
OVT_2	57.8±9.3	53.2±8.2

Fig. 1. Inhibition of ^{3}H-oxytocin binding to membranes of mouse peritoneal mast cells by oxytocin and the antagonists OVT_1 and OVT_2. Membranes were incubated for 1 h at 30ºC with 10 n*M* ^{3}H-oxytocin in the presence of different concentrations of the above compounds, as described in the Materials and Methods section. One hundred percent binding for 10 n*M* ^{3}H-oxytocin corresponded to 0.35 ± 0.02 fmol/mg protein and 0.22 ± 0.02 fmol/mg protein in estrus and diestrus, respectively. Results are the means of three independent experiments, each one performed in triplicate. Bars represent the standard deviation. The inset shows the IC_{50} values estimated both in estrus and diestrus.



Binding parameters for ^{3}H oxytocin		
	Estrus	Diestrus
Kd (nM)	6.95± 2.76	4.07 ± 1.73
Bmax (pmol/mg prot.)	0.71 ± 0.08*	0.37 ± 0.05

Fig. 2. Saturation curves for analyzing the specific binding of ^{3}H-oxytocin to mouse peritoneal mast cells membranes. Total binding was obtained by incubating the membranes at 30ºC for 1 h in the presence of increasing concentrations of ^{3}H-oxytocin, ranging from 0.10 to 100 n*M*. Specific binding was evaluated as the difference between total binding and nonspecific binding of ^{3}H-oxytocin, which was determined in the presence of 10 µ*M* oxytocin. Bars represent the standard deviation of three independent experiments. Inset: K_d and B_{max} values for the specific binding of ^{3}H-oxytocin in mouse peritoneal mast cell membranes in estrus and diestrus. *$p < 0.05$.

Table 1
V_{max} Estimated Values for SERT Transport Activity in Mouse Peritoneal Mast Cells

V_{max} (fmol/10^6 cell/min)	Control	Oxytocin (0.03 n*M*)	Inhibition	Oxytocin (0.03 n*M*) + OVT_1 (4 n*M*)
Estrus	63.9 ± 8.2	19.7 ± 5.3*	72%	61.9 ± 5.6
Diestrus	78.2 ± 4.9	60.2 ± 4.8*	23%	81.6 ± 5.5

Note: The uptake of 5HT was carried out by incubating ^{3}H-5HT with mice peritoneal mast as explained in the Materials and Methods section. Values are means ± SD for the number (six) of independent experiments set in parentheses, with a minimum of five animals per experiment. All values are statistically significant ($p < 0.05$) when compared at each stage.

*$p < 0.01$ when compared to values from their respective controls.

mast cells from mice in diestrus). This effect was blocked by using OVT_1 at a concentration of 4 n*M* (Table 1). Vasopresin at a concentration up to 1 µ*M* did not inhibit 5HT uptake in peritoneal mast cells.

Regulation of 5HT Uptake by PKC

As shown in Table 2, treatment of mouse peritoneal mast cells with 1 µ*M* PMA decreased ^{3}H-5HT uptake in estrus and diestrus (near 70%). The magnitude of the inhibition was similar to that found with 0.03 n*M* OT during estrus. An inhibitor of PKC, the compound Ro318220, at a concentration of 1 µ*M*, not only blocked the effect of OT but also evoked a significant increase of ^{3}H-5HT uptake. The stimulation evoked by Ro318220 was not reversed by OT or PMA. Staurosporine did not have appreciable effects on 5HT uptake, nor was the vehicle for PMA (ethanol) or the vehicle for staurosporine (dimethyl sulfoxide) at equivalent dilutions.

Characterization of SERT in Peritoneal Mast Cell Membranes

Membranes prepared from peritoneal mast cells displayed saturable and specific binding sites for ^{3}H-paroxetine. Results in Table 3 show that binding of ^{3}H-paroxetine does not depend on sexual hormone predominance because K_d and B_{max} values were not statistically different in estrus and diestrus. IC_{50} for imipramine values were also similar: 87.8 ± 9.8 n*M* in estrus and 93.2 ± 8.5 n*M* in diestrus.

Table 2
Regulation of ^{3}H-5HT Uptake by PMA, Staurosporine, and Ro318220; Coupling of Oxytocin Receptors to SERT

	Estrus (fmol/10^6 cell/min)	Diestrus (fmol/10^6 cell/min)
Control	63.9 ± 8.2 (6)	78.2 ± 4.9 (6)
Oxytocin, 0.03 n*M*	14.7 ± 5.3* (6)	61.0 ± 4.8* (6)
PMA, 1 μ*M*	13.4 ± 3.8* (3)	20.3 ± 9.2* (3)
Oxytocin, 0.03 n*M* + PMA, 1 μ*M*	16.0 ± 4.2* (3)	18.8 ± 3.2* (3)
Ro318220, 1 μ*M*	123.2 ± 12.6* (3)	153.6 ± 8.4* (3)
Oxytocin, 0.03 n*M* + Ro318220, 1 μ*M*	126.5 ± 9.8* (3)	149.4 ± 11.3* (3)
Staurosporine, 1 μ*M*	66.3 ± 7.4 (3)	75.7 ± 6.1 (3)

Note: Peritoneal mast cells were preincubated for 10 min with the respective compounds before the uptake assays. Parallel assays were performed in a Na-free buffer to define specific uptake. Number of independent experiments is set in parentheses.

*$p < 0.01$ when compared with their respective controls.

Table 3
SERT in Peritoneal Mast Cell Membranes; Binding of ^{3}H-Paroxetine

	Estrus	Diestrus
K_d (n*M*)	0.32 ± 0.08	0.28 ± 0.05
B_{max} (pmol/mg protein)	8.7 ± 1.2	11.4 ± 5.3
IC_{50} (n*M*)	87.8 ± 9.8	93.2 ± 8.5

Note: Membranes prepared from peritoneal mast cells displayed saturable binding of ^{3}H-paroxetine. Membranes were incubated at 30ºC for 1 h in the presence of increasing concentrations of ^{3}H-paroxetine, ranging from 1 n*M* to 1 m*M*. Nonspecific binding of ^{3}H-paroxetine was determined in the presence of 100 μ*M* imipramine. Values are means ± SD of three independent experiments performed in triplicate.

Discussion

The results from these studies show that the binding sites for OT found in mouse peritoneal mast cells have comparable pharmacological attributes as OTRs characterized previously in other tissues. Equilibrium binding of ^{3}H-OT to mast cell membranes shows a representative curve for a single binding isotherm. Scatchard plot analysis exhibit a K_d for OT binding that is similar to that reported for OTR in the uterus of guinea pigs and humans *(22)* and a B_{max} value that is highly dependent on the action of estrogens, as had been found previously *(8,23,24)*. Competition experiments showed that the specific ^{3}H-OT binding was displaced with similar affinities by cold OT and two oxytocin antagonists, the peptides OVT_1 and OVT_2 *(25,26)*. Murine peritoneal mast cells also exhibited a saturable 5HT transport. K_{Mapp} values exhibited a reduction in comparison to that observed in RBL 2H3 cells *(17)* and uterine horns *(9)* but were comparable to that observed in plasma membrane vesicles from mouse cerebral cortex *(27)* and human platelets *(28)*. Binding of ^{3}H-paroxetine to SERT in murine peritoneal mast cells showed higher K_d values than that observed in human platelets *(29)*, but similar to that found in RBL 2H3 cells *(17)*.

In this model of murine peritoneal mast cells, OT inhibited 5HT uptake. This action of OT is similar to the one observed in mouse uterine horns *(9)*. Because uterine mast cells are responsible for the majority of 5HT uptake in mouse uterine horns, the present results suggest an additional role for OTR in the uterus by providing an increase of 5HT bioavailability, a strong uterotonic endogenous amine *(9. 10)*. In addition, the interaction between OTRs and SERTs seems to be dependent on PKC, a key enzyme that regulates 5HT uptake by inhibiting cell surface expression of the transporter *(18)*.

Protein kinase C plays a complex role in signal transduction pathways. It has been shown that activation of different PKC isoenzymes in the same cell can lead to opposite effects *(30)*. However, the common structure and high homology among PKC isoenzymes make their pharmacological characterization difficult. In order to determine if PKC participates in the regulation of 5HT uptake in peritoneal mast cells, PMA, a phorbol ester that mimics the effects of DAG in a nonspecific manner, and the PKC inhibitor Ro318220 were used. The nonspecific action of PMA on PKC mimicked the inhibitory effect of OT on 5HT uptake into mast cells under estrogen predominance. On the other hand, the specific PKC inhibitor Ro318220 reversed the action of OT and increased 5HT uptake. In addition, staurosporine, a compound that inhibits serine/threonine kinases nonspecifically, had no effect. It is probable that the inhibition of specific kinases could inhibit 5HT uptake, whereas the inhibition of others can enhance this activity. Therefore, it is possible to expect that the effect of staurosporine is a consequence of a combination of these results. The action of Ro318220 to increase 5HT uptake suggests the presence of a PKC with constitutive activity that may regulate 5HT bioavailability in the mast cell. On the other hand, the lack of effect of staurosporine could indicate the presence of another kinases that may modulate SERT activity, as suggested previously *(16)*.

The findings previously described suggest that SERT activity may not be directly affected by sexual hormones, but an indirect effect could occur through OT. This is based on the fact that PMA inhibited 5HT uptake in the same magnitude in estrus and diestrus, whereas OT inhibited the uptake of 5HT in both stages of the estrous cycle, but the effect was significantly greater in mast cells obtained from mice under estrogen predominance. In addition, the maximum effect of OT is at estrus and coincides with the time of highest

expression of OTR in mast cells during the cycle. This process could have a physiological significance at term, when maximal expression of OT and OTR are observed in the uterus *(8)*.

To our knowledge, this is the first demonstration of an interaction between OTR and SERT through PKC. It is may be important to speculate that if this mechanism is conserved among different populations of mast cells (or other cells that express the SERT), it is likely to have profound effects on different functions of the body. For example, it remains to be seen whether a similar interaction may occur in the nervous system, especially in the neurons of the limbic forebrain areas, where OT and 5HT have been shown to be involved in emotional and affective behavior *(31)*.

Materials and Methods

Animals

Female albino mice (30–40 g) maintained under a dark/light cycle (12 h/12 h) in a controlled-temperature room (24–25ºC) with free access to drinking water and laboratory food were used. They were cycling regularly and the stage of the estrous cycle was determined through a cytochemical analysis of the vaginal smear and confirmed by physical and anatomical aspects of uterine horns once dissected. For the group in diestrus, animals were used on diestrus d 2. All of the procedures used with the animals were approved by Ethics Committee of the University of Concepción.

Peritoneal Mast Cells

Mice were sacrificed by decapitation early in the morning. Peritoneal mast cells were collected by washing the peritoneal cavity from female mice with 2 mL of a solution containing 153 m*M* NaCl, 5.6 m*M* KCl, 1.2 m*M* $MgCl_2$, 6.54 m*M* $NaHCO_3$, 2.8 m*M* glucose, and 1.2 m*M* ascorbic acid, as described in refs. *9* and *32*. Cells were purified using an albumin density gradient (31% w/v) as described in ref. *33*. After an incubation for 18 h at 37ºC with the above solution, cells were washed three times to eliminate albumin and restore their uptake capacity. Purity was evaluated by microscopic observation after staining the mast cells with acidic toluidine blue 0.1%, in a Newbauer hemocytometer. Crude peritoneal cell suspensions contained 2.7% mast cells; after albumin density gradient purification, they ranged over 95%. The viability of the mast cells was 90%, as determined by their ability to exclude trypan blue 0.4%.

Characterization of OT-Binding Sites in Peritoneal Mast Cells

Mast cells were homogenized in 50 m*M* Tris-HCl buffer using an polytron Janke–Kunkel/Ika-werk at maximum velocity for 5 s. The homogenates were pelleted at 850*g* for 30 min at 4ºC, the supernatant was centrifuged at 31,000*g* for 30 min at 4ºC, and the membrane pellets were resuspended with the ultraturrax in suitable volumes of 50 m*M* Tris-HCl, pH 7.4, resulting in a final protein concentration of 300 µg/mL. The binding assays were carried out for 1 h at 30ºC in a total final volume of 250 µL of 50 m*M* Tris-HCl, pH 7.4, with 2.8 m*M* glucose, 10 m*M* $MnCl_2$, 1.2 m*M* ascorbic acid, 0.1 m*M* *p*-methyl-sulfoxide (PMSF), ^{3}H-OT, and 10 µg of protein membranes. Competition assays were carried out in triplicate with 10 n*M* ^{3}H-OT, with or without the addition of OT and the OT antagonists desGly-NH_2d$(CH_2)_5$[Tyr(Me)^2, Thr^4] OVT (OVT_1) and d(CH2)5[Tyr(Me)^2, Thr^4, Tyr-NH2^9] OVT (OVT_2) at concentrations ranging from 0.1 n*M* to 0.3 µ*M*. Saturation curves were determined using 0.1–100 n*M* ^{3}H-OT. Nonspecific binding was determined using 10 µ*M* of OT.

The binding assay was terminated by adding 5 mL of ice-cold buffer to the tubes and rapidly filtering under reduced pressure through glass filters. The filters containing the membrane-bound ^{3}H-OT were then washed twice with 5 mL of Tris-HCl cold buffer. The whole procedure of filtering lasted no longer than 8 s. The filters were then transferred to scintillation vials containing 4 mL of a toluene counting solution: PPO (2 g/L), POPOP (0.7 g/L), and Triton X-100 (30% v/v). The scintillation vials were analyzed for tritium in a Beckman LS-100 scintillation counter with an efficiency of 54%.

5HT Uptake Measurements

Purified peritoneal mast cells were preincubated for 5 min in a solution containing 153 m*M* NaCl, 5.6 m*M* KCl, 1.2 m*M* $MgCl_2$, 6.54 m*M* $NaHCO_3$, 2.8 m*M* glucose, 1.2 m*M* ascorbic acid, plus the respective drugs whose effects were supposed to be analyzed: oxytocin, phorbol 12-myristate 13-acetate (PMA, an activator of PKC), and Ro318220 and staurosporine (selective and nonselective inhibitors of PKC, respectively). Total uptake assays were initiated by the addition of ^{3}H-5HT and incubation was performed for 30 min at 37ºC. Samples were diluted with 4 mL of ice-cold incubation solution and rapidly filtered under reduced pressure through glass fiber filters. The filters containing the mast cells were washed three times with 5 mL of incubation solution at 37ºC. The whole procedure of filtering lasted no longer than 10 s. The filters were then transferred to scintillation vials and analyzed for tritium, as described earlier. Corresponding incubations were conducted in a Na^+-free medium to correct for nonspecific uptake. Nonlinear curve-fit data for uptake used the generalized Michaelis–Menten model $V = V_{max}[S]^n/[S]^n + [K]^n$, to obtain $K_{M\text{app}}$ and V_{max}.

Analysis of SERT in Peritoneal Mast Cell Membranes

Total cell membranes were prepared from mouse peritoneal mast cells as described earlier. SERT density was assessed with ^{3}H-paroxetine (27.6 Ci/mmol; NEN, USA) in a binding buffer containing 50 m*M* Tris-HCl, pH 7.4, with 2.8 m*M* glucose, 10 m*M* $MnCl_2$, 1.2 m*M* ascorbic acid, and 0.1 m*M* PMSF. Initial studies demonstrated linearity of specific binding up to 30 µg of membrane protein/mL; therefore, in subsequent assays, 10 µg/mL was used. Assays, performed

in triplicate, with various concentrations of ^{3}H-paroxetine were initiated with the addition of membranes and terminated after 40 min incubation at 30ºC by rapid filtration over 0.5% (v/v) polyethylenimine-presoaked glass filters and processed as described earlier. Nonspecific binding, defined as the binding in the presence of 100 μ*M* imipramine, was subtracted from total binding to define specific binding. The concentration of imipramine to inhibit binding by 50% (IC_{50}) was estimated from curves in which 100 n*M* ^{3}H-paroxetine was displaced by increasing concentrations of imipramine *(34)*.

Statistical Analysis

Statistical significance between groups was determined by the Student's *t*-test; $p \leq 0.05$ was considered significant. Saturation isotherms for ^{3}H-OT and ^{3}H-paroxetine were subjected to Scatchard analysis. Binding parameters, dissociation constants (K_d), and maximum number of receptors (B_{max}) were calculated by using GraphPad InPlot software.

Compounds and Solutions

^{3}H-5HT (specific activity = 27.6 Ci/mmol), ^{3}H-OT (specific activity = 35 Ci/mmol), and ^{3}H-paroxetine (specific activity = 27.6 Ci/mmol) were purchased from New England Nuclear Corp. (Boston, MA). OT (5 UI/mL) is a commercial OT distributed by Novartis. Ro318220 was from LC Laboratories (San Diego, CA). OT antagonists were provided by Dr. Maurice Manning, Toledo, OH, USA. The other compounds were purchased from Sigma Chemical Co. (St. Louis, MO). Na^+-free medium contained the same compounds as normal solution, but Na^+ was replaced by Li^+.

Acknowledgments

This work was supported by research grants from FONDECYT (197-0842, 298-0059, and 200.032.010-10) from the University of Concepción.

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