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Studies conducted during 1991 examined a) the effects of tyrosine on catecholamine (dopamine; norepinephrine; epinephrine) synthesis and release; b) the effects of combining tyrosine with sympathomimetic agents on the behavioral and physiological effects of those drugs; c) the effects of melatonin on dopamine release (& vice versa); and, d) the effect of a new class of drugs, catechol-O-methyl transferase inhibitors, on dopamine release in brain, and on catecholamine-mediated cardiovascular responses.

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SUMMARY Studies conducted during 1991 examined: a) the effects of tyrosine on catecholamine (dopamine; norepinephrine; epinephrine) synthesis and release; b) the effects of combining tyrosine with sympathomimetic agents on the behavioral and physiological effects of those drugs; c) the effects of melatonin on dopamine release (and vice versa); and d) the effect of a new class of drugs, catechol-O-methyl transferase inhibitors, on dopamine release in brain and on catecholamine-mediated cardiovascular responses.

These studies are all part of a long-range program on basic science aspects of the possible use of nutrients and hormones to improve performance (including but not limited to those behavioral variables which exhibit time-dependent changes).

Ongoing and projected studies for 1992 include (besides continuation of the above): a) experiments to determine whether caffeine can potentiate effects of neurotransmitter precursors (choline, tyrosine, tryptophan) on neurotransmission; b) attempts to determine whether nicotine produces behavioral effects via serotonin release; c) preliminary studies to determine whether synthesis of the "new" neurotransmitter nitric oxide is affected by levels of its precursor amino acid arginine; and d) preliminary studies to determine whether tyrosine can potentiate actions of various analgesic agents.

STATEMENT OF WORK

1. To characterize the effects of tyrosine on epinephrine synthesis in - and release from - isolated, electrically-stimulated, superfused adrenal gland
2. To examine in rats the effects of tyrosine on behavioral and brain neurotransmitter responses to sympathomimetic amines (e.g., phenylpropanolamine; ephedrine)
3. To determine the effects of tyrosine on behavioral responses of human subjects to the above sympathomimetic amines (starting with ephedrine)
4. To continue studies on the effects of melatonin (and available tyrosine concentrations) - on potassium-evoked retinal dopamine release

5. To identify possible effects of tryptophan availability on post-synaptic responses to serotonin release (e.g., PI breakdown) in brain slices
6. To examine interactions between foods (or tryptophan) and behaviorally-active serotonergic drugs as they affect brain serotonin release (e.g., fluoxetine; d-fenfluramine)
7. To explore possible effects of melatonin on serotonin release in vivo and in vitro

STATUS OF RESEARCH

1. ISOLATED ADRENALS Studies utilizing isolated perfused rat adrenal glands were started in June, 1992. To date we have established in our laboratories a preparation in which electrical stimulation of the splanchnic nerve increases the release of the catecholamine epinephrine, as measured by reverse-phase high performance liquid chromatography with electrochemical detection. Studies currently underway are aimed at determining the ability of tyrosine to support catecholamine release during sustained periods of electrical stimulation. Results of these studies are expected to be available during February, 1992.

2. TYROSINE - SYMPATHOMIMETIC AMINES Following our demonstration of the tyrosine-specific potentiation of the presumed centrally-mediated anorectic activity (Moya Huff and Maher, 1987; Hull and Maher, 1990) of several mixed-acting sympathomimetic agents (phenylpropanolamine [PPA], ephedrine [EPH], and amphetamine [AMP]), we expanded our studies to investigate the possible influence of L-tyrosine (L-TYR) upon non-centrally mediated actions of these agents. The mixed-acting sympathomimetic agents are well known to possess thermogenic and gastroinhibitory activities that are believed to result from an action upon adrenoceptors located in non-central nervous system tissues. By measuring changes in intrascapular brown adipose tissue (IBAT) thermogenesis, gastrointestinal transit and gastric retention, in response to these agents with and without L-TYR, we aimed to further characterize the role of this amino acid in drug-induced sympathetic responses.

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were obtained at 125-150 grams and individually housed in suspended wire mesh cages with food (Purina #5001; 25% protein, 56% CHO and 5% fat) and tap water available ad libitum. Animals were acclimated to our climate-controlled animal facility for at least one week prior to experimentation.

Groups of eight rats were fasted for 24 h, and received an i.p. injection of PPA (20 mg/kg), EPH (20 mg/kg) or AMP (1.75 mg/kg) concurrently with either saline (SAL) or L-TYR (200 mg/kg) 60 min prior to sacrificing. These doses were chosen based on previous studies indicating moderate anorectic activity (Moya Huff and Maher, 1987; Hull

and Maher, 1990). Twenty min later, 1 ml of a standard "meal" containing 1% amaranth in a 1% aqueous gum arabic solution was administered intragastrically by oral intubation (Poher et al., 1989). Forty min following the "meal", animals were sacrificed by decapitation and the abdominal cavity exposed. Ligatures were placed at the esophageal and pyloric sphincters to confine the stomach contents, and the small intestine (duodenum to the ileocecal valve) was carefully removed and the omentum sufficiently separated to enable unfolding of the intestine. Special care was taken to avoid stretching. The total length of the intestine and the distance that the dye traversed were recorded to the nearest millimeter. Gastrointestinal transit is expressed as the mean percentage ($\pm$ S.E.M.) of the distance that the dye traversed in relation to the total length of the small intestine. Data were analyzed via ANOVA and Dunnett's test.

In SAL pretreated animals, PPA (20 mg/kg), EPH (20 mg/kg) and AMP (1.75 mg/kg) produced significant inhibition of gastrointestinal transit. This transit in control animals was $77.6 \pm 1.8\%$, while in PPA, EPH and AMP treated animals it was $60.6 \pm 3.1\%$ ($p < 0.01$), $67.8 \pm 3.0\%$ ($p < 0.05$) and $68.9 \pm 2.5\%$ ($p < 0.05$), respectively (Fig. 1). This constituted a 22%, 13%, and 11% inhibition of transit for the three sympathomimetics compared to control, respectively. The administration of L-TYR (200 mg/kg) alone failed to significantly alter gastrointestinal transit when compared to control animals ($77.1 \pm 3.0\%$). Additionally, the inhibitory effects on gastrointestinal transit of PPA, EPH and AMP were not significantly altered by L-TYR ($58.8 \pm 6.7\%$, $64.8 \pm 3.1\%$ and $63.6 \pm 2.5\%$, respectively, $p > 0.05$, when compared to the appropriate drug plus SAL group)(Fig. 1).

To determine gastric retention, the tied-off stomach pouch was removed and the contents emptied into a 15 ml polypropylene test tube and brought up to a final volume of 5 ml with distilled water. Stomach and contents were then vortexed and centrifuged (Sorvall-4) at 12,900 rpm for 10 min. One ml of the supernatant fluid was then analyzed for amaranth concentration using a Bausch and Lomb Spectronic 20 spectrophotometer at 365 nm. When necessary, quantitative dilutions were made with distilled water. A standard curve for amaranth (0 - 16 $\mu\text{g/ml}$) was determined just prior to testing samples. This yielded a linear relationship between the concentration of amaranth and absorbance, with a calculated linear regression coefficient of 0.99. Gastric retention is expressed as the ratio of amaranth remaining in the treated groups to that in the control group (control group equal to 1.0).

The gastric retention ratio was significantly increased by the three sympathomimetics tested. PPA, EPH and AMP produced gastric retention ratios of 3.27 ($p < 0.01$), 2.28 ($p < 0.05$) and 2.18 ($p < 0.05$), respectively (Fig. 2). L-TYR failed to alter the gastric retention ratio in SAL, PPA, EPH or AMP treated animals (1.02, 2.99, 1.82 and 2.37, respectively; $p > 0.05$ when compared to the corresponding SAL pretreated group)(Fig. 2).

To determine the thermogenic activity of these sympathomimetic agents, thirty min following an i.p. injection of either SAL or L-TYR (200 mg/kg) animals were anesthetized

with urethane (1.2 g/10 ml/kg), i.p. and the hair overlying the scapulae shaved with electric clippers. The rats were then placed on foam urethane padding to prevent heat loss from their ventral surface and a 3 cm longitudinal incision was made in the skin between the scapulae to expose the connective tissue covering the lobes of brown fat. The tissues were then carefully separated and a thermistor probe (#402, Yellow Springs Instruments Co., Inc.) was implanted such that the tip was entirely immersed within the brown adipose tissue. The thermistor probe was secured with tape and the wound was closed using surgical staples. Sixty min following the injection of L-TYR (200 mg/kg) or SAL, rats received either SAL, PPA (20 mg/kg), EPH (20 mg/kg) or AMP (1.75 mg/kg). Six min later temperatures were recorded every 4 min for 30 min using a tele-thermometer ($\pm 0.1^{\circ}\text{C}$, Yellow Springs Instruments Co., Inc.). Thermogenesis is expressed as the mean cumulative increase in $^{\circ}\text{C}$ ($\pm$ S.E.M.). Data were analyzed by ANOVA and Dunnett's test.

While SAL administration failed to significantly alter IBAT temperatures during the 30 min test period ($\pm 0.1^{\circ}\text{C} \pm 0.1$, $p > 0.05$) the administration of PPA (20 mg/kg), EPH (20 mg/kg) or AMP (1.75 mg/kg) produced a significant ($p < 0.01$) increase in IBAT temperature ($+1.6^{\circ}\text{C} \pm 0.1$ and $+1.2^{\circ}\text{C} \pm 0.2$, respectively, at 30 min) (Fig. 3). L-TYR failed to alter the IBAT temperature when administered alone ($+0.1^{\circ}\text{C} \pm 0.1$) (Fig. 3). Additionally, the coadministration of L-TYR (200 mg/kg) with each of the three mixed-acting sympathomimetics failed to potentiate the observed increase in IBAT temperature ($+1.4^{\circ}\text{C} \pm 0.2$, $+2.8^{\circ}\text{C} \pm 0.1$ and $+1.2^{\circ}\text{C} \pm 0.1$, respectively, $p > 0.05$, when compared to the corresponding SAL pretreated group) (Fig. 3).

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3. HUMAN STUDIES INVOLVING TYROSINE PLUS PHENYLPROPANOLAMINE. Our clinical studies on the effects of administering tyrosine plus sympathomimetic amines like phenylpropanolamine were delayed for more than a year by the need to obtain approval of our IND from the Food and Drug Administration (FDA).

On October 16, 1991, the FDA official responsible for this IND (Ms. Susan DeCort of the FDA Division of Neuropharmacological Drug Products), notified us that the IND had been approved so that we can plan to proceed with the study. Ms. DeCort also indicated that it would take a minimum of several months to provide written notice of this approval, but that in the interim we can proceed with the study. We are now organizing initial studies.

4. MELATONIN - DOPAMINE RELEASE. During the past year, we completed studies on the inhibition by dopamine of melatonin release from mammalian cells in tissue culture. Our experimental results clearly show that the Y79 human retinoblastoma cell line is capable of releasing melatonin (Fig. 4). This release is significantly diminished by the addition to the culture medium of 10 μ M dopamine (approximately 60%; Deng et al., 1991) (Fig. 5). Preliminary studies in our laboratory also showed that these cells could synthesize and release dopamine, a phenomenon suppressed (in retinal cells) by melatonin (Godley et al., 1989). These observations indicate that the hypothesized reciprocal relationship between dopamine and melatonin production might be fruitfully pursued using this relatively simple in vitro cell culture system.

Our specific aims for 1991 were:

1. To characterize two different cell lines with respect to the dopamine/melatonin interaction
 - A) PC12 cell line: derived from pheochromocytoma cells of the rat adrenal medulla
 - B) Y79 cell line: derived from human retinoblastoma
2. To develop an experimental protocol in which we could accurately quantify dopamine levels in the cell culture system with sensitivity and reproducibility
3. To determine whether there is parallelism in the dopamine/melatonin responses of these two cell lines
4. To explore the hypothesis that variations in tyrosine, forskolin or melatonin concentrations may influence dopamine release from these cells

Hypothesis:

1. The inhibition by melatonin of dopamine release is peculiar to a retinal cell line.
2. Dopamine release from cells depends on intracellular cyclic AMP levels.
3. The inhibitory effects of melatonin in Y79 cells may involve receptor-mediated inhibition of cyclic AMP formation, thereby depressing dopamine release.

Procedure:

To examine dopamine levels and release, cells were incubated in Krebs-Ringer-Henseleit buffer for 4 hours. Dopamine was extracted from the medium and from the cells with 0.05N HCl and measured by HPLC. The various agents tested include: tyrosine (12.5-100 μ M), forskolin (a cyclic AMP analog) (0.1-20 μ M), and melatonin (0.01-10 μ M).

Findings:

1. Addition of tyrosine (100 μ M) to the culture medium stimulated the release of dopamine by PC 12 cells from 172 ± 18.7 to 414 ± 26.8 ng/mg protein. The response to tyrosine was dose-dependent. These results confirm our earlier observations in studies on rat striatal slices and dopamine release into extracellular fluid (Milner and Wurtman, 1985; Acworth et al., 1988; During et al., 1988).
2. Addition of forskolin (10 μ M) and tyrosine (100 μ M) further enhanced dopamine release, from 131 ± 8.5 to 932 ± 36.1 ng/mg protein (Table 2).
3. In the absence of supplemental tyrosine, forskolin (20 μ M) only slightly increased dopamine release.
4. In working with Y79 cells: the amount of dopamine released was 1.5 ± 0.3 ng/mg protein/4h when cells were cultured with tyrosine (100 μ M) and forskolin (10 μ M); in the absence of tyrosine and forskolin, the release of dopamine was undetectable.

Plan for this year:

To further characterize Y79 cells, particularly as concerns the synthesis, storage and release of dopamine and to test the effects of melatonin agonists and antagonists using this cell culture system

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5. SEROTONIN, TRYPTOPHAN, AND PI BREAKDOWN

Measurement of transmembrane transducing systems ("second messengers") is an important tool in neurotransmission research, as it establishes whether and how changes in neurotransmission are, in fact, coupled to consecutive intracellular responses in the target cell. One such system, the breakdown of phosphatidylinositol (PI) to yield inositol phosphates (some of which increase intracellular calcium levels) and diacylglycerol (which activates a regulatory enzyme, protein kinase C) is known to be involved in the transmission of information by the neurotransmitter serotonin (Berridge et al., 1982). Two serotonin receptor subtypes have been shown to be exclusively linked to PI breakdown in cerebral cortex, namely the postsynaptic 5-HT₂ receptor (Conn and Sanders-Bush, 1986) and the 5-HT₃ receptor (Edwards et al., 1991), an ion channel present mainly presynaptically on nerve terminals. We are using the serotonin-PI system to examine the following working hypotheses:

1. As 5-HT increases PI breakdown in rat cortical miniprisms in vitro, tryptophan, the precursor of 5-HT, should increase PI breakdown in vitro as well. Studies from our laboratory have shown that availability of L-tryptophan modulates serotonin release from rat hypothalamic slices (Schaechter and Wurtman, 1989); our laboratory has also provided evidence that 5-HT release from rat frontal cortex, measured by microdialysis after a local perfusion with both 120 mM KCl and 100 μ M L-tryptophan is higher than after KCl alone (Gardier et al., in press). We therefore assessed both the effect of L-tryptophan and L-tryptophan + KCl on in vitro PI breakdown.

2. Chronic pretreatment of rats with drugs known to increase the amount of 5-HT present in the synaptic cleft should lead to a desensitization of PI breakdown as examined by stimulation in vitro by 5-HT. This hypothesis has evolved from our microdialysis studies on the changes in brain serotonin release induced by giving rats repeated high doses of serotonergic drugs (Gardier et al., in press). Drugs chosen for testing this hypothesis have been d-fenfluramine and dl-p-chloroamphetamine. d-Fenfluramine, a selective 5-HT releasing agent and reuptake blocker is widely used internationally in the treatment of human obesity. Chronic pretreatment with it decreases [³H]-spiperone binding sites, a measure of 5-HT₂ binding, in rat cortex (Dumbrille and Tang, 1983). dl-p-Chloroamphetamine (PCA) is a neurotoxin that, at 5 mg/kg doses, selectively destroys serotonergic cell bodies in the rat midbrain (Harvey et al., 1975) (B₉ > B₇ = B₈ according to the nomenclature of Dahlstrom and Fuxe, 1964) as well as serotonergic terminals, as shown by electron microscopy (McGeer et al., 1975; Hattori et al., 1976).

For evaluation of PI breakdown we use the lithium-inhibited [³H]-inositol monophosphate ([³H]-IP₁) accumulation assay as described by Brown et al. (1984). This assay measures the formation of [³H]-IP₁ after labelling with [³H]-myoinositol as the index of PI breakdown. Lithium is added in the final incubation in order to inhibit the turnover of IP₁, thus enhancing the signal. Sprague-Dawley rats (200-350 g) are decapitated and the brains quickly removed. The cortex is dissected and 350 x 350 μ m miniprisms are

obtained by cross cutting with a McIlwain tissue chopper. Miniprisms are then equilibrated for 60 min in oxygenated Krebs buffer, after which they are labelled for 60 min with [^3H]-myo-inositol (NEN, NET-114), washed with Krebs buffer containing 10 mM LiCl and incubated for 60 min with a lithium-containing Krebs buffer with or without a stimulatory agonist (like 5-HT itself).

In the in vitro studies with L-tryptophan, 1 mM L-tryptophan is added. In the pretreatment studies we use 100 μM 5-HT, in the presence of 10 μM tranylcypromine, for measurement of 5-HT-stimulated PI breakdown. We also stimulate with the muscarinic acetylcholine receptor agonist, carbachol (1 mM), both as a positive control and to establish whether an in vivo serotonergic pretreatment could also affect a muscarinic transmembrane signaling system in vitro.

The reactions are stopped with chloroform/methanol and the radioactively labelled phospholipids and inositolphosphates separated and then measured by liquid scintillation spectrometry. Results are expressed as a ratio of [^3H]-IP1 dpm to [^3H]-IP1 dpm + [^3H]-phospholipids dpm). Each experiment is done in triplicate; data are means $\pm$ S.E.M. Comparisons between in vitro stimulations are performed using the paired t-test; comparisons of effects of different pretreatments use the unpaired t-test.

In the pretreatment studies, we follow the pretreatment schedule used in the microdialysis studies in our laboratory (Gardier et al., in press): i.p. injections of either vehicle, 10 mg/kg/2 ml d-fenfluramine or 5 mg/kg/2 ml PCA are performed on 4 consecutive days, the decapitation taking place 48 hours after the last injection.

Effect of L-tryptophan on PI breakdown in vitro

1 mM L-tryptophan failed to alter PI breakdown in vitro: results expressed in the above mentioned ratio were 0.056 ± 0.0087 for the basal PI breakdown and 0.054 ± 0.0025 for the PI breakdown in presence of L-tryptophan ($n=8$). 30 mM KCl stimulated PI breakdown (0.1791 ± 0.0356 as compared to 0.0628 ± 0.0141) and the values of 100 μM 5-HT + 30 mM KCl (0.2443 ± 0.0250) were significantly higher ($p < 0.05$) than those for KCl alone ($n=4$). Unlike 5-HT itself, L-tryptophan did not stimulate PI breakdown in the presence of potassium ($n=7$. Basal 0.056 ± 0.0103 ; KCl 30 mM 0.153 ± 0.0296 ; KCl 30 mM + L-tryptophan 1 mM 0.178 ± 0.0425 ; as positive control: KCl 30 mM + carbachol 1 mM 0.516 ± 0.0799). Present studies involve determining whether or not this tryptophan concentration actually raised serotonin production and release. If not, other concentrations will be tested in order to conduct a fair test of hypothesis #1.

Effects of in vivo pretreatment with serotonergic drugs on PI breakdown in vitro

At first we established that the drugs that we were to use in the pretreatment study would not influence in vitro PI breakdown directly, but only indirectly, via their effects on

serotonergic transmission in vitro (basal 0.048 ± 0.0038 ; $10 \mu\text{M}$ d-fenfluramine in vitro 0.044 ± 0.023 ; $10 \mu\text{M}$ PCA in vitro 0.045 ± 0.029 ; $n=4$).

Chronic pretreatment with d-fenfluramine ($n=5$) did not change basal (in saline pretreated rats: 0.057 ± 0.0067 ; in d-fenfluramine pretreated rats: 0.060 ± 0.0111) or carbachol-stimulated PI breakdown (in saline pretreated rats: 0.213 ± 0.0313 ; in d-fenfluramine pretreated rats: 0.251 ± 0.0330), but did significantly ($p<0.05$) decrease the stimulation of PI breakdown by $100 \mu\text{M}$ 5-HT (Fig. 6).

Unlike d-fenfluramine, pretreatment with PCA significantly increased ($p<0.01$) basal PI breakdown (in saline pretreated rats: 0.057 ± 0.0069 [$n=9$]; in PCA pretreated rats: 0.118 ± 0.0224 [$n=4$]). In vitro stimulation of PI breakdown above basal values by $100 \mu\text{M}$ 5-HT was significantly impeded by PCA pretreatment (in saline pretreated rats: 0.079 ± 0.0085 [$n=9$]; in PCA pretreated rats: 0.114 ± 0.0147 [$n=4$]). Stimulation of PI breakdown by 1 mM carbachol was similarly diminished (in saline pretreated rats: 0.2121 ± 0.0224 [$n=9$]; in PCA pretreated rats: 0.2204 ± 0.0225 [$n=4$]) despite the differences in basal values.

Table 2 shows the effects of PCA pretreatment on the percentage of [^3H]-labelled products (in dpm adjusted to mg protein): myoinositol (Ins), inositol phospholipids (PL), glycerophosphoinositol (GPI), inositoltrisphosphate (IP3) and inositolbisphosphate (IP2) labelling were essentially unchanged by the pretreatment, while IP1 was increased after PCA, basically at the cost of PL.

Our failure, so far, to display an effect of L-tryptophan on in vitro PI turnover in rat cortical miniprisms could be due to the known in vitro fragility of the enzyme tryptophan hydroxylase (Cooper et al., 1982), one of the enzymes necessary to transform the precursor L-tryptophan into 5-HT. As mentioned above, this hypothesis will be tested by measuring 5-HT (by HPLC) in the incubation fluid after the 60 min drug incubation.

Our data show that chronic pretreatment with high doses of d-fenfluramine significantly decreases 5-HT-stimulated [^3H]-IP1 accumulation. (Fig. 6). This decrease is congruent with the induced decrease in [^3H]-spiperone binding sites as shown by (Dumbrille-Ross and Tanga, 1983). Our finding demonstrates that when 5-HT levels are chronically increased in the synaptic cleft by pretreatment with a purely serotonergic drug, the 5-HT-linked PI breakdown is down-regulated. Chronic pretreatment with PCA leads to an increase of basal [^3H]-IP1 accumulation, while carbachol-stimulated PI breakdown is not increased in the PCA pretreated animals, despite the differences in basal values. The desensitization of the muscarinic PI response suggests that PCA caused a continuing increase in endogenous acetylcholine within synapses and that this increase desensitized muscarinic receptors to the carbachol. It is known that serotonin tonically inhibits acetylcholine release in the cortex (Robinson, 1983); both 5-HT₂ (Quirion et al., 1985) and 5-HT₃ (Barnes et al., 1989) receptors have been suggested to mediate this effect. Evidence has been provided for the existence of 5-HT₂ receptors on cholinergic terminals in rat cortex. We currently interpret our experiments as indicating that PCA destroyed

serotoninergetic cell bodies and terminals, thus leading to a lack of 5-HT-mediated inhibition of acetylcholine release in vivo and the resulting development of receptor down-regulation with diminished sensitivity. Our data suggest for the first time that modulation of serotoninergetic activity can affect the cholinergic system on the level of a biochemical transducing system. Alonso and Soubrie (1991) had failed to find an effect of intracerebroventricular 5,7-dihydroxytryptamine lesions on carbachol-stimulated [³H]-IP1 accumulation in rat hippocampus, a result possibly due to the difference in the mechanism of action of 5-HT in these two regions (Claustre et al., 1991), namely the presence in hippocampus of 5-HT_{1A} receptors that are negatively coupled to carbachol-stimulated PI breakdown.

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Plans for 1992

1. Time course of PCA pretreatment on basal PI breakdown, 5-HT stimulated PI breakdown and carbachol stimulated PI breakdown: single injection with 5 mg/kg/2 ml PCA, i.p., decapitation after 3 h, 24 h, 4 days and 14 days. After dissection of the cortex we will simultaneously perform the PI assay with one side of the cortex and measure 5-HT concentrations by HPLC in the contralateral side of the rat cortex. Using the method described by our laboratory we have measured 200 ng/g tissue wet weight in rat cortex and 290 ng/g tissue wet weight in rat striatum.

2. Concentration response-curve for carbachol 3 days after a single injection with 5 mg/kg/2 ml PCA.

5. SEROTONINERGIC DRUGS AND NEURONAL PLASTICITY

It is well known that prolonged treatment of rats with large doses of dexfenfluramine, an anorectic drug that enhances serotonin-mediated neurotransmission by releasing this compound from nerve endings and then blocking its reuptake, can cause prolonged decreases in brain serotonin levels. (Lower therapeutic doses, which selectively suppress carbohydrate intake, do not decrease - and may actually increase - brain serotonin levels.) Using in vivo microdialysis, we previously showed that this treatment also diminished the release of serotonin that could be evoked by a single very high dose of dexfenfluramine. We observed similar, if greater, effects on serotonin release if rats received fluoxetine, a drug which, like dexfenfluramine, blocks serotonin reuptake, and so we tentatively hypothesized that the drugs produced their inhibitory effects on serotonin levels and release by markedly increasing intrasynaptic serotonin concentrations which, in time, down-regulated serotonin synthesis.

In 1991 we showed that the impairment in serotonin release caused by fluoxetine could be demonstrated by locally applying high potassium concentrations to serotonin terminals (through a microdialysis cannula). This demonstration was important inasmuch as, when acute dexfenfluramine had been used to evoke serotonin release, it could have been argued that, because of the chronic pretreatment, less of this drug was entering the nerve terminals.

We examined 5-HT and 5-HIAA release from frontal cortex evoked by high potassium chloride concentrations in rats pretreated for 3 days with high doses of the 5-HT uptake blocker fluoxetine or of dexfenfluramine, which both releases 5-HT and blocks its reuptake. The standard fluoxetine dose (30 mg/kg, i.p.) was about 4 times the drug's ED_{50} in producing a serotonin-related behavioral effect (anorexia), while the dexfenfluramine dose (7.5 mg/kg, i.p.) was about 6 times its ED_{50} . These high doses were chosen in order to elucidate the mechanism by which similar doses of fluoxetine and dexfenfluramine had been found to produce long-term changes in serotonin dynamics. Fluoxetine decreased the basal release of both compounds; dexfenfluramine decreased basal 5-HIAA efflux without affecting the release of 5-HT. Potassium-evoked 5-HT release was unchanged after dexfenfluramine pretreatment but was suppressed by fluoxetine doses as low as 7.5 mg/kg/day (Table 3). Basal release of 5-HT and 5-HIAA returned to normal after 7 days of fluoxetine pretreatment, although evoked release continued to be suppressed. These data suggest that long-term changes in brain serotonin dynamics after high doses of dexfenfluramine or fluoxetine are related to the drugs' mechanisms of action, specifically their blockade of 5-HT reuptake.

During the next year, we intend to examine our hypothesis that the reduction in serotonin release results from down-regulation by seeing whether serotonin receptor blockers protect against this phenomenon.

The general purpose of this line of research is to show the extraordinary plasticity of serotonergic neurotransmission (i.e., that a three day increase in such transmission can thereafter diminish serotonin synthesis and release for many weeks).

7. EFFECTS OF MELATONIN ON SEROTONIN RELEASE

No studies were done in 1991.

8. OTHER STUDIES: CATECHOL-O-METHYLTRANSFERASE INHIBITORS

In vivo microdialysis was used to examine the effects of two new COMT inhibitors, Ro-7592 and OR-611, on extracellular levels of dopamine, DOPAC, HVA, and 5-HIAA in rat striatum. The interactions of these COMT inhibitors with nomifensine, clorgyline and deprenyl were also studied. Ro 40-7592 (3-30 mg/kg, i.p.) decreased dose-dependently the efflux of HVA, increased that of DOPAC, and tended to increase that of dopamine. (Fig. 7). Higher doses of OR-611 (30-100 mg/kg, i.p.) also decreased the dialysate level of HVA, increased that of DOPAC, and tended to increase that of dopamine (Fig. 8). Ro 40-7592 was about tenfold as potent as OR-611. Neither of the COMT inhibitors changed dialysate levels of 5-HIAA. An OR-611 dose of 10 mg/kg, i.p. had no significant effect, in contrast to Ro 40-7592, on any of the parameters studied; this dose was thus used to differentiate between the effects of central and peripheral COMT inhibition. Both nomifensine (15 mg/kg, i.p.) and clorgyline (4 mg/kg, i.p.) alone elevated extracellular dopamine levels and lowered those of DOPAC and HVA, though there were quantitative

and temporal differences between the drugs. L-Deprenyl (1 mg/kg, i.p.) alone had no significant effect on any of the compounds measured. Ro 40-7592 (10 mg/kg, i.p.) potentiated the effect of nomifensine on dopamine efflux, and tended to increase clorgyline-induced dopamine efflux. DOPAC levels in dialysates were significantly increased by combinations of Ro 40-7592 and nomifensine or clorgyline, whereas HVA levels remained about as low as they were after Ro 40-7592 alone. Ro 40-7592 had no significant interactions with L-deprenyl. OR-611 (10 mg/kg, i.p.) did not modify the effects on dopamine metabolism of nomifensine, clorgyline, or L-deprenyl. These data show that Ro 40-7592 is a potent, centrally-active COMT inhibitor, whereas OR-611 is principally a peripherally-active inhibitor. Use of drugs which inhibit brain COMT can considerably modify dopamine metabolism. COMT inhibitors may be of clinical significance in treating patients with Parkinson's disease.

We have also investigated the cardiovascular effects of RO 40-7592 in urethane-anesthetized rats. Ro 40-7592 (30 mg/kg, i.p., a dose known to inhibit COMT by over 95%) resulted in an increase in basal systolic blood pressure from 120 ± 6 to 135 ± 6 mmHg ($p < 0.01$), and an increase in diastolic blood pressure from 75 ± 7 to 88 ± 9 mmHg ($p < 0.05$) after 60 min. Heart rate increased slightly from 365 ± 18 to 375 ± 17 bpm (NS). COMT inhibition failed to significantly alter the i.v. cumulative dose-response curves (0.3 - $10 \mu\text{g/kg}$) to epinephrine, norepinephrine (Fig. 9) and, in addition, to the α -1 adrenoceptor agonists that are not substrates for COMT (e.g., methoxamine and phenylephrine). Reflex vagally-mediated bradycardia was similarly unaltered by prior COMT inhibition. Responses to constant infusions of the above agonists were also not significantly altered. The arrhythmogenic actions of high-dose epinephrine and norepinephrine were enhanced by Ro 40-7592. Thus, only with very high doses of the catecholamines does prior inhibition of COMT result in significantly altered cardiovascular responses.

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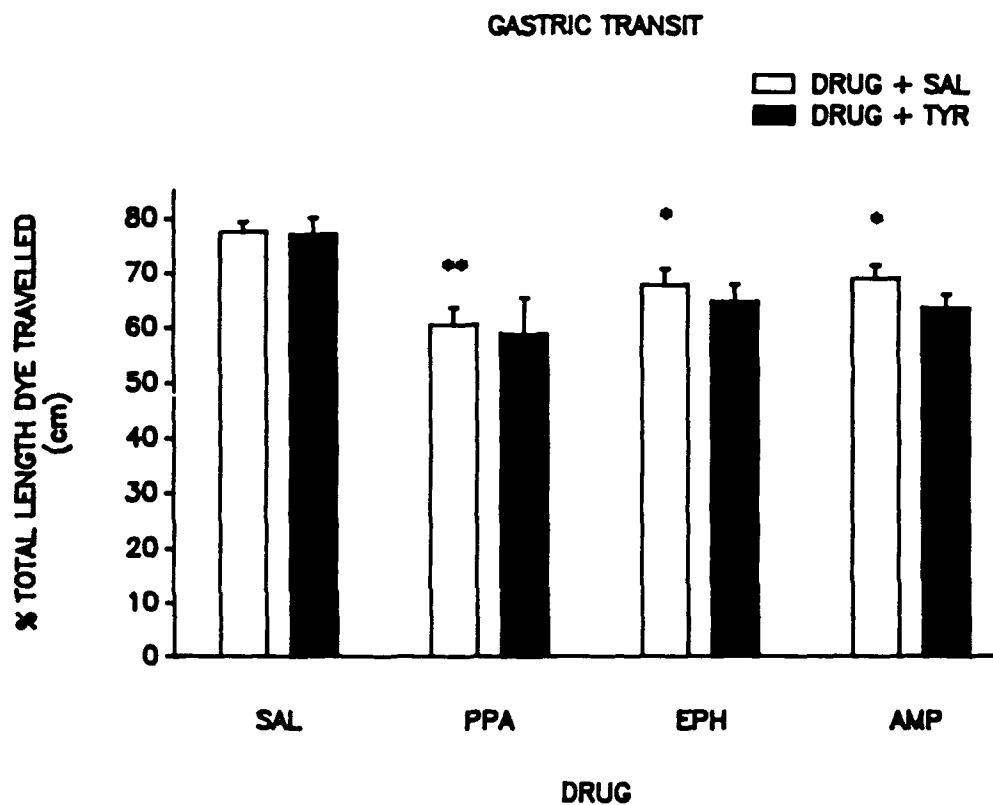


Fig. 1. Percent total length amaranth dye travelled through small intestine. Groups of eight rats were fasted for 24 h and administered either PPA (20 mg/kg), EPH (20 mg/kg) or AMP (1.75 mg/kg) with saline (SAL) or L-TYR (200 mg/kg), i.p. 60 min prior to sacrificing. Twenty min following injections, rats received 1 ml of an amaranth "meal" via oral intubation. Percent total length dye travelled is expressed as the distance amaranth dye travelled per total length of the small intestine. Values represent the mean $\pm$ S.E.M. * $p < 0.05$; and ** $p < 0.01$, significantly different from corresponding saline control as determined by ANOVA and Dunnett's test.

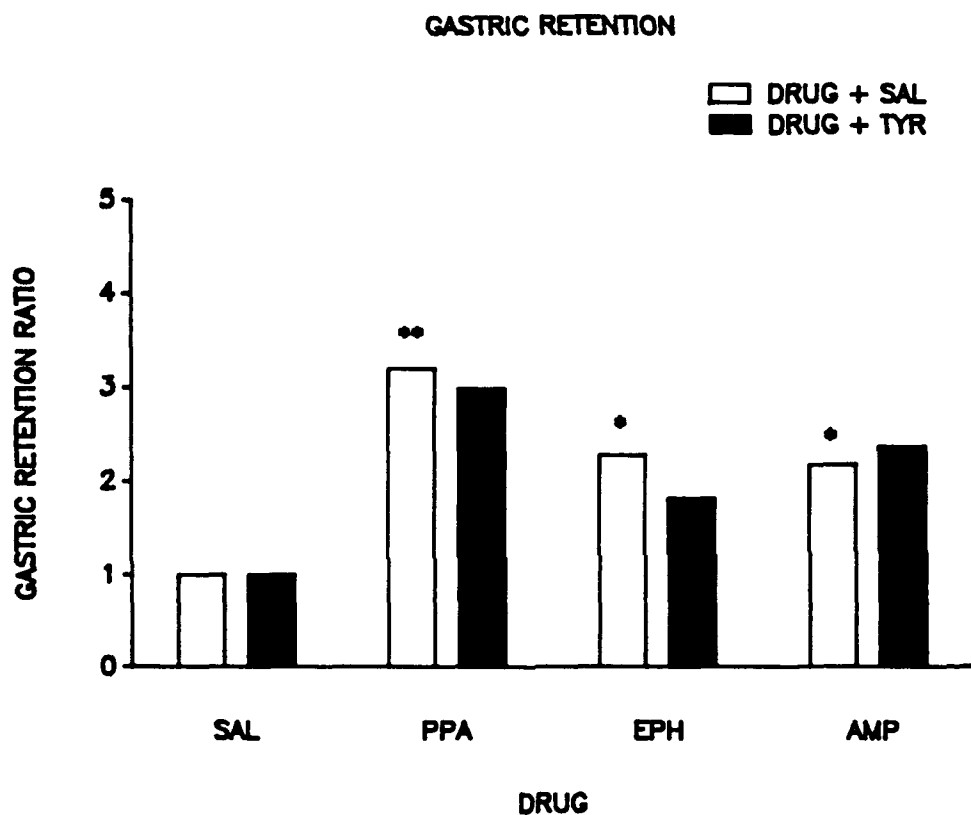


Fig. 2. Gastric retention ratio. Groups of eight rats were fasted for 24 h and administered either PPA (20 mg/kg), EPH (20 mg/kg) or AMP (1.75 mg/kg) with SAL or L-TYR (200 mg/kg) 60 min prior to sacrificing. Twenty min following injections, rats received 1 ml of an amaranth "meal" via oral intubation. Gastric retention ratio is expressed as the ratio of amaranth remaining in the treated groups to that in the control group. The control group ratio is equal to 1.0. * $p < 0.05$; and ** $p < 0.01$, significantly different from corresponding saline control as determined by ANOVA and Dunnett's test.

INTRASCAPULAR BROWN ADIPOSE TISSUE (IBAT) THERMOGENESIS

○—○ DRUG + SAL

●—● DRUG + TYR

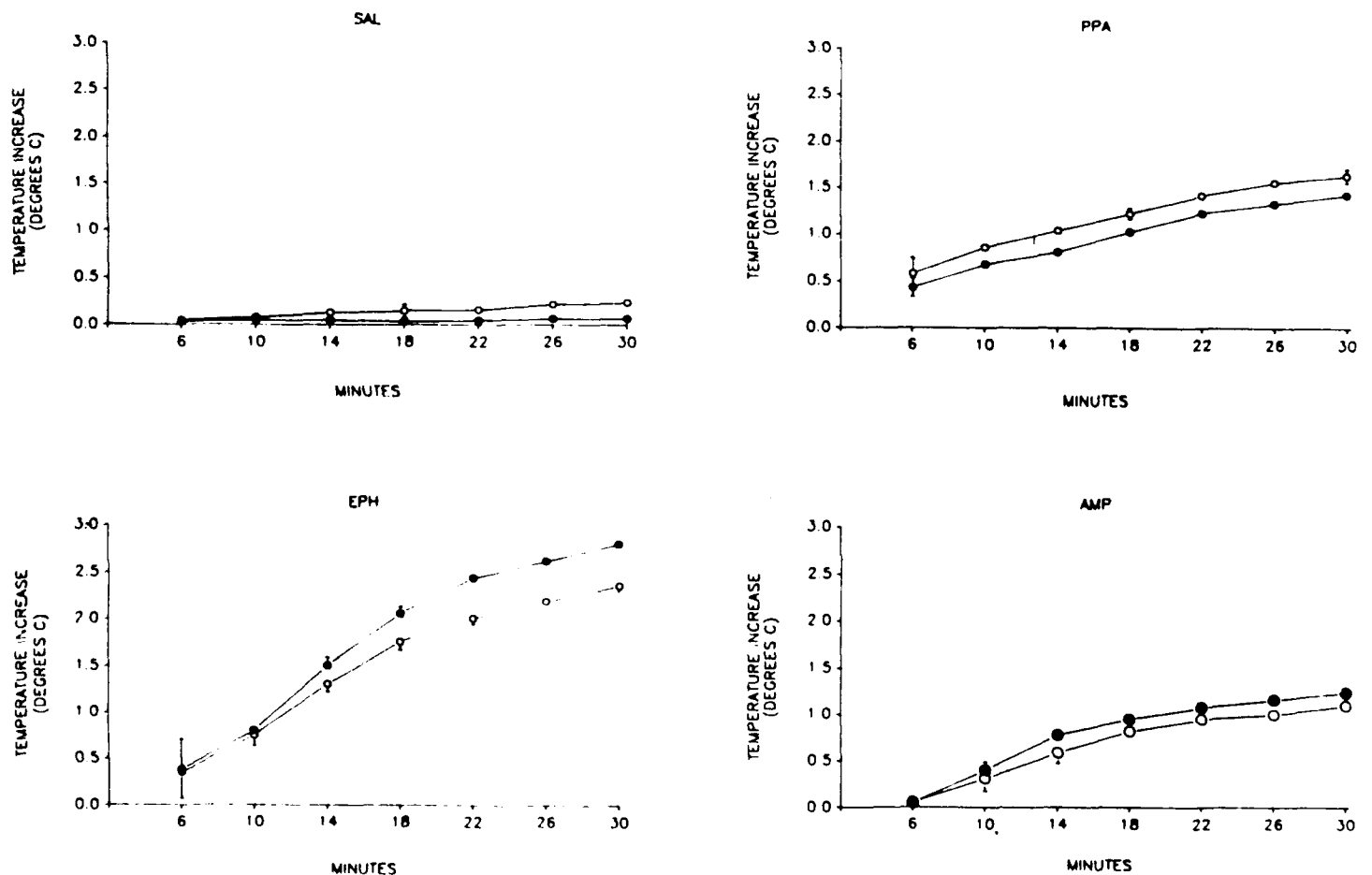


Fig. 3. Intrascapular brown adipose tissue (IBAT) thermogenesis. Groups of eight rats received an i.p. injection of either SAL or L-TYR (200 mg/kg) and 30 min later were anesthetized with urethane. Sixty min following the L-TYR or SAL injections, rats received either PPA (20 mg/kg), EPH (20 mg/kg) or AMP (1.75 mg/kg) and IBAT temperature was quantitated for 30 min with a thermistor probe. All drugs significantly ($p < 0.01$) increased IBAT thermogenesis compared to their SAL control as determined by ANOVA and Dunnett's tests. Values represent cumulative means $\pm$ S.E.M.

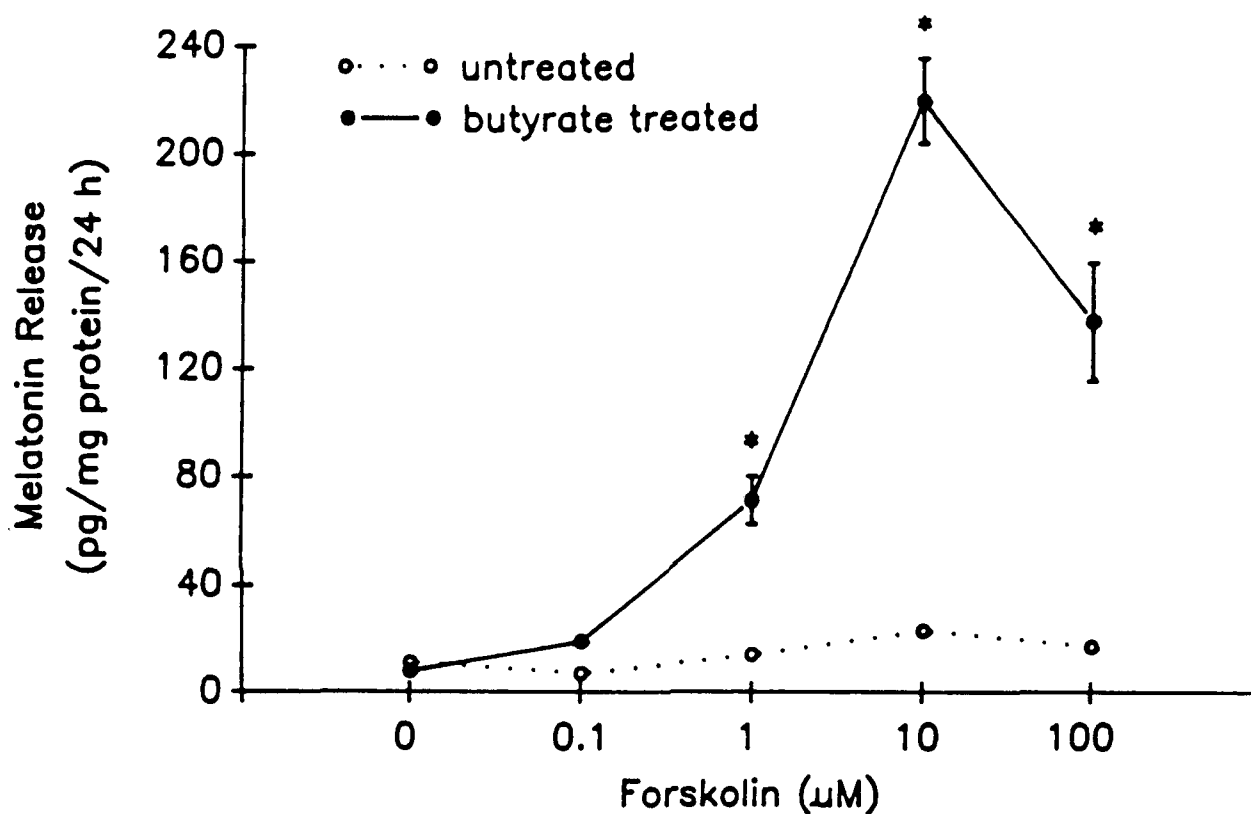


Fig. 4. Melatonin release from Y79 cells. Cells previously treated with or without sodium butyrate (3 mM) to induce differentiation were incubated in serum-free DMEM (Dulbecco's Modified Eagle Medium) in the presence of various concentrations of forskolin. After a 24 h incubation, samples of medium were collected for melatonin measurement. Data are means $\pm$ S.E.M. of 4 replicate cultures. * $p < 0.05$ vs. the group without forskolin (ANOVA, Dunnett's t-test. When error is not indicated by vertical bars, it is smaller than the size of the symbol).

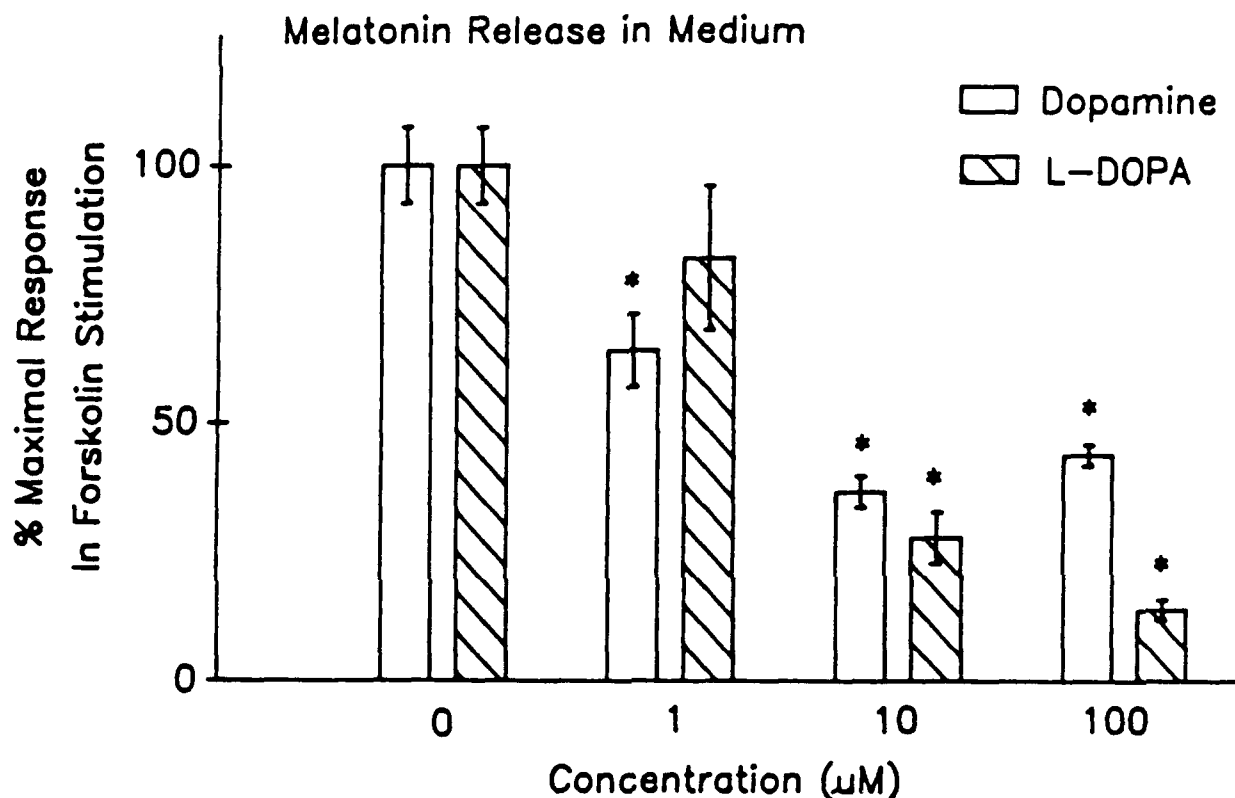


Fig. 5. Effects of dopamine or L-DOPA on forskolin-stimulated melatonin release in differentiated Y79 cells. Differentiated cells were preincubated for 10 min in serum-free DMEM containing various concentrations of dopamine or L-DOPA prior to the addition of forskolin (10 μ M). After 24 h incubation, samples of medium were collected for melatonin measurement. Data, expressed as % of the maximal forskolin-stimulated release, are means $\pm$ S.E.M. of triplicate experiments. Maximal forskolin-stimulated release was 219 $\pm$ 16 pg/mg protein/24 h. * $p < 0.05$ vs. the groups without dopamine or L-DOPA (ANOVA, Dunnett's t-test).

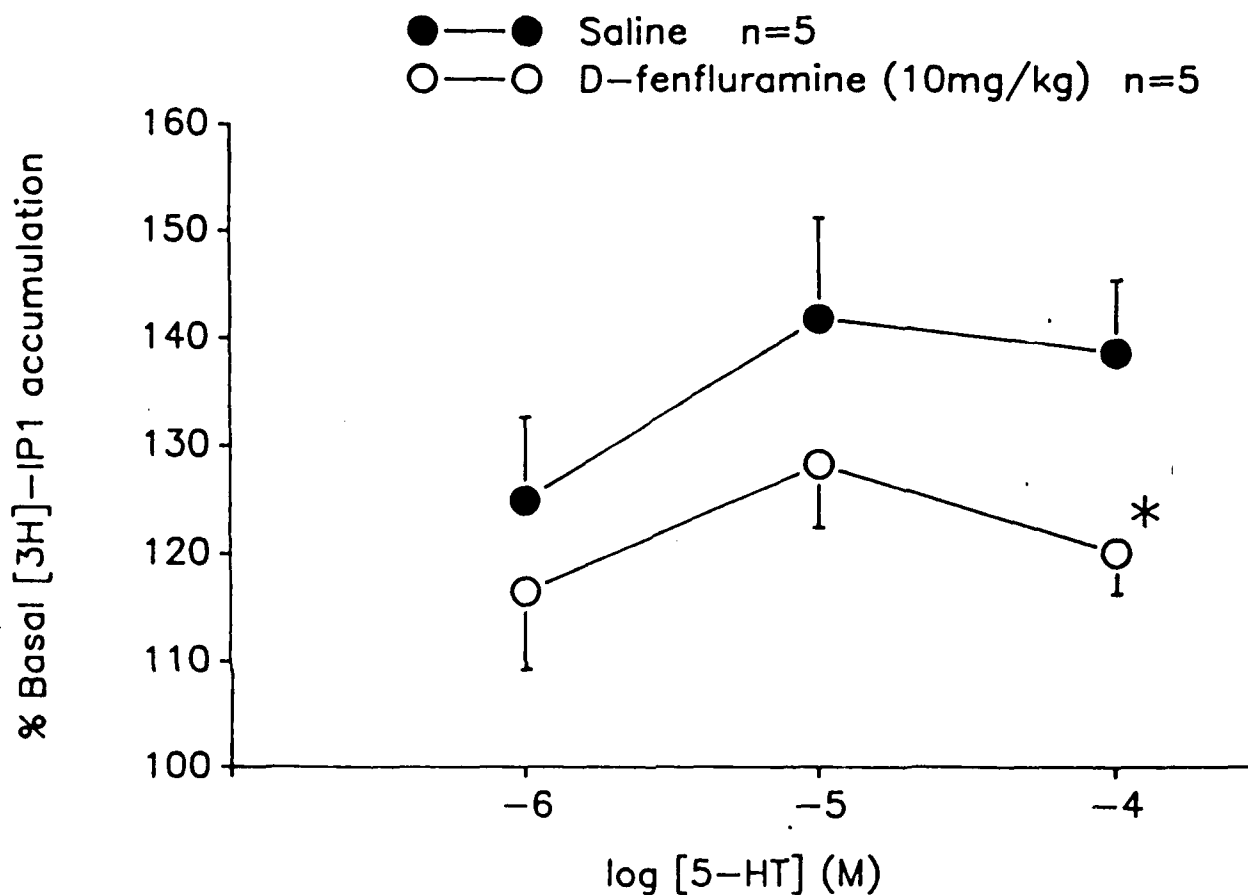


Fig. 6. Effect of chronic d-fenfluramine on 5-HT-stimulated [^3H]-IP1 accumulation was measured in cerebral cortical miniprisms from rats that had received chronic administration of either 10 mg/kg/2 ml d-fenfluramine or vehicle. Data are presented as percentage of basal [^3H]-IP1 accumulation. The [^3H]-IP1 dpm / [^3H]-IP1 dpm + [^3H]-total phospholipid dpm ratios were 0.057 ± 0.0067 for the control and 0.060 ± 0.012 for the pretreated animals. The values are the means of 5 separate experiments each done in triplicate. Each experiment included a dose-response for 1 control and 1 treated animal. Vertical bars represent S.E.M. * $p < 0.05$ (unpaired t-test).

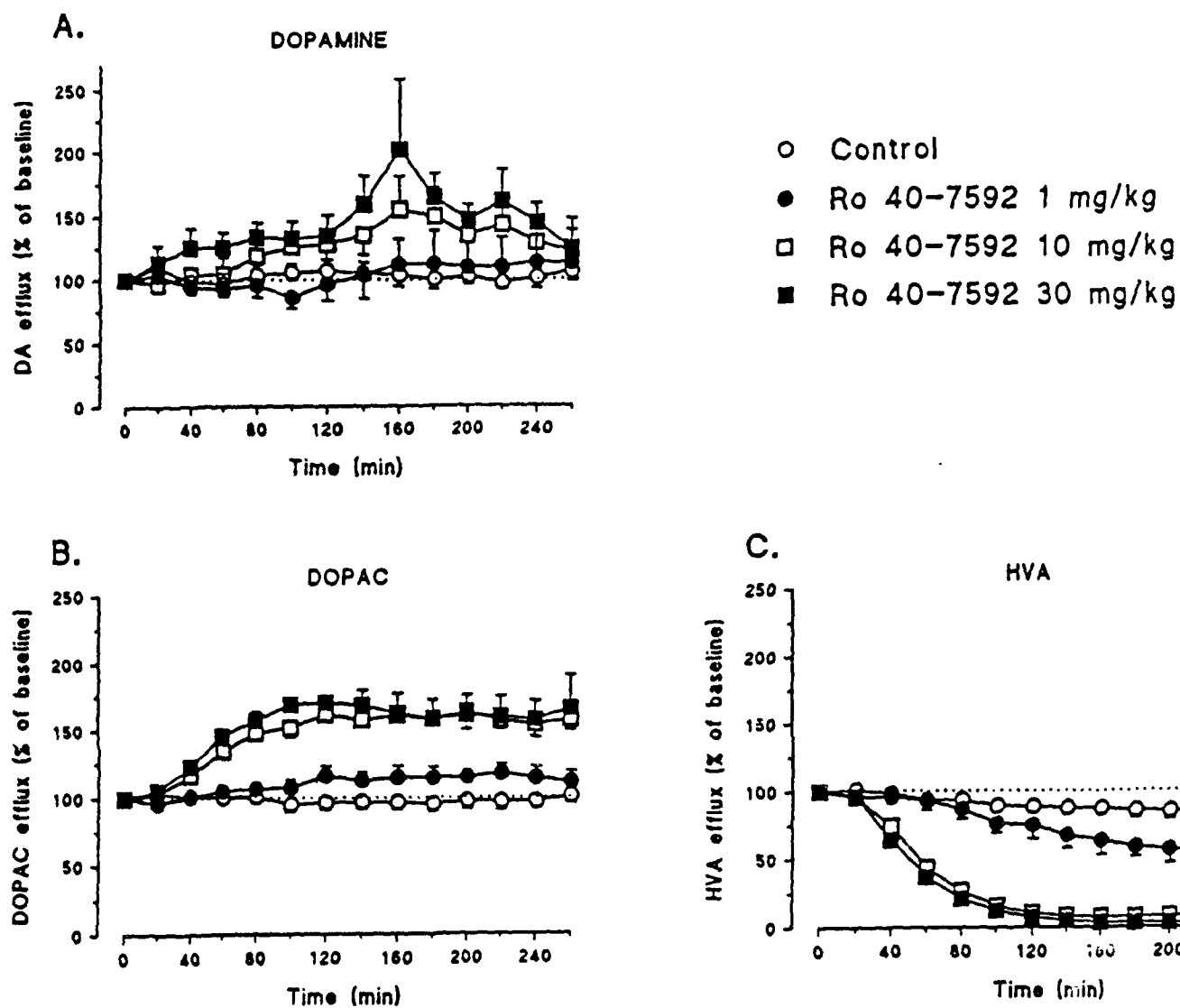


Fig. 7. Dose-response effects of Ro 40-7592 (0, 1, and 30 mg/kg) on striatal extracellular levels of dopamine (A.), DOPAC (B.), and HVA (C.). Injections were given i.p. at time = 0 min. Dialysis samples were collected for 20 min periods and assayed by HPLC-EC. Data are given as means $\pm$ S.E.M. percent variation of basal (preinjection) values; n = 6 for each group except for the 1 mg/kg group where n = 4.

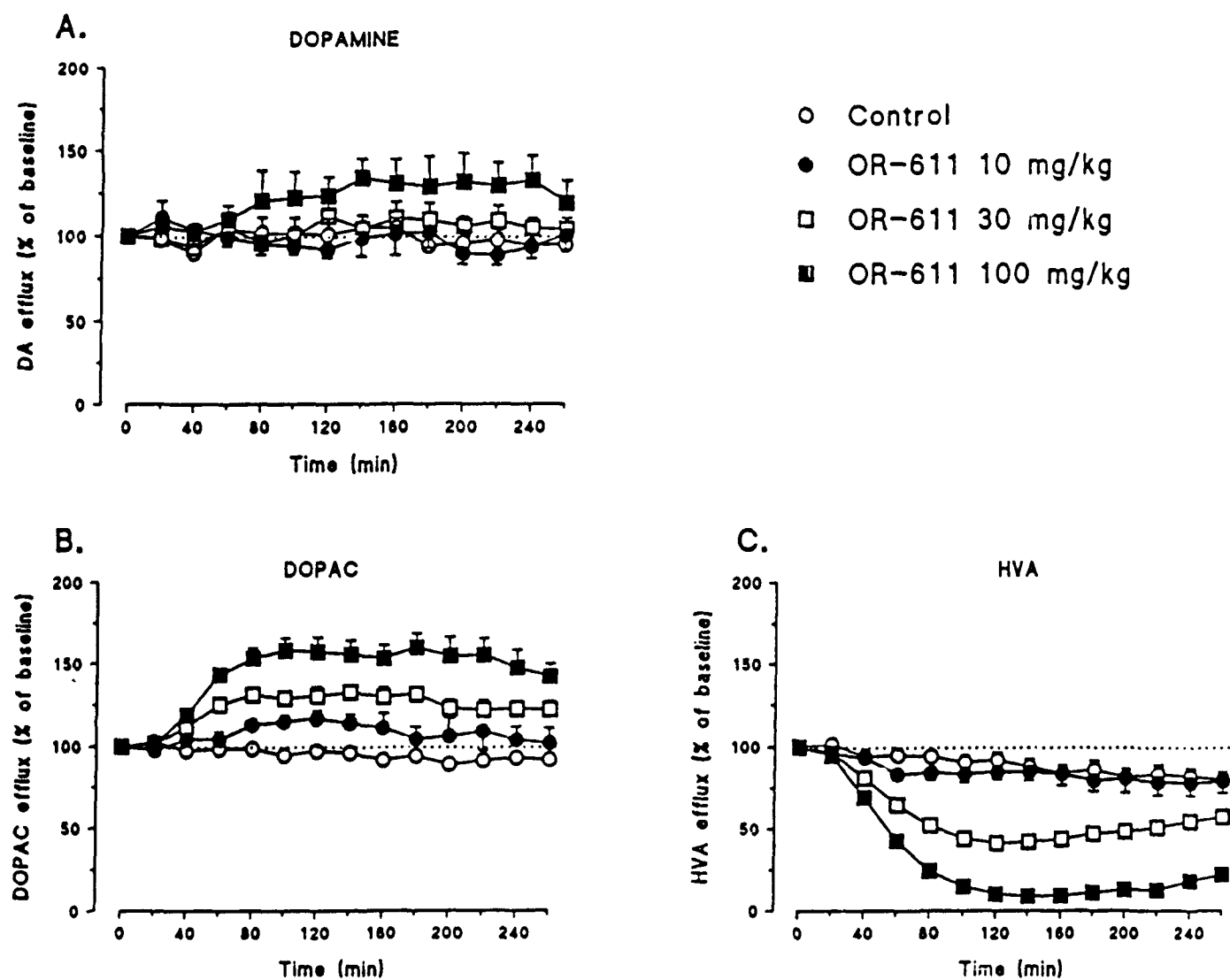


Fig. 8. Dose-response effects of OR-611 (0, 10, 30 and 100 mg/kg) on striatal extracellular level of dopamine (A.), DOPAC (B.), and HVA (C.). Injections were given i.p. at time = 0 min. Dialysis samples were collected for 20 min periods and assayed by HPLC-EC. Data are given as means $\pm$ S.E.M. percent variation of basal (preinjection) values; $n = 6$ for each group except for 100 mg/kg group where $n = 4$.

THE EFFECT OF Ro 40-7592 ON SYSTOLIC BLOOD PRESSURE

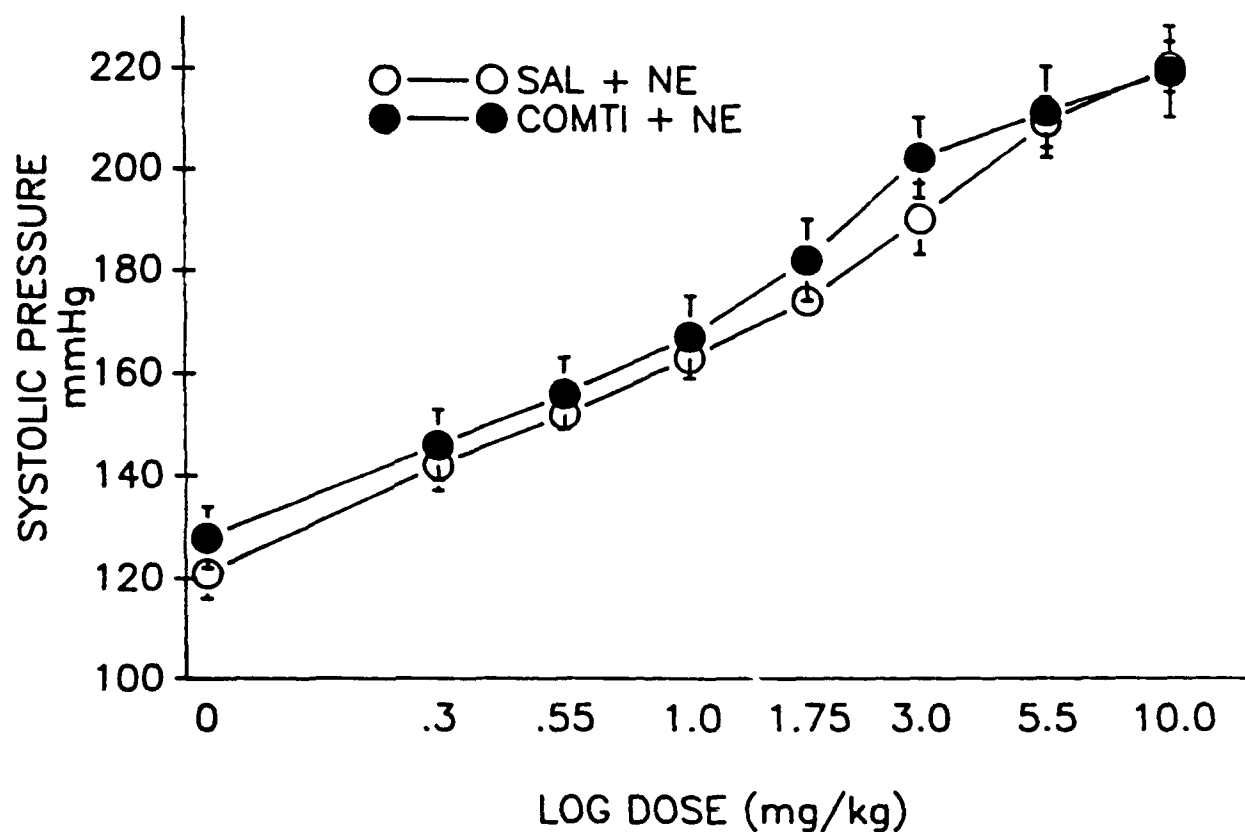


Fig. 9. Effects of Ro 40-7592 on systolic blood pressure in urethane-anesthetized rats. Cumulative dose-response curves were constructed following intravenous norepinephrine administration (0-10 mg/kg) after pretreatment with saline or 30 mg/kg, i.p. Ro 40-7592. COMT inhibitor pretreatment failed to significantly alter the norepinephrine dose-response curve.

Table 1. Effects of tyrosine on dopamine levels in PC12 and Y79 cells in medium containing forskolin (10 μ M)

Cell Type	Tyrosine (μ M)	Dopamine (ng/mg protein/4 hr)
PC12	0	131.8 $\pm$ 8.6
PC12	12.5	378.3 $\pm$ 19.0
PC12	25	627.1 $\pm$ 47.5
PC12	50	1000.5 $\pm$ 52.4
PC12	100	932.0 $\pm$ 36.0
PC12	200	877.3 $\pm$ 47.6
Y79	0	*
Y79	100	1.5 $\pm$ 0.3

*Dopamine levels were below detection limits in Y79 cells incubated in the tyrosine-free medium.

Table 2. Effect of chronic pretreatment with dl-p-chloroamphetamine (5mg/kg) on the accumulation of radioactive metabolites after [3H] myo-inositol labelling in rat cortical miniprisms

		[3H]-Ins	[3H]-PL	[3H]-GPI	[3H]-IP3	[3H]-IP2	[3H]-IP1
		(in % of total dpm/mg protein)					
Saline	basal	19.7	70.0	0.4	2.4	4.0	3.5
	5-HT (100 μ M)	15.5	72.5	0.4	2.9	3.6	5.2
	carbachol (1mM)	13.5	62.1	0.4	2.9	3.5	17.8
PCA	basal	20.5	64.2	0.9	1.7	4.4	8.2
	5-HT (100 μ M)	21.3	60.2	1.0	2.3	5.3	9.8
	carbachol (1mM)	17.7	55.9	0.9	2.4	4.4	18.6

Data are % of total dpm/mg protein calculated from means of a typical experiment done in triplicate. The experiment was repeated with similar results.

Abbreviations used: Ins, myo-inositol; PL, phospholipids; GPI, glycerophosphoinositol; IP3, inositoltrisphosphate; IP2, inositolbisphosphate; IP1, inositolmonophosphate; PCA, dl-p-chloroamphetamine

Table 3

Effects of dexfenfluramine or fluoxetine dose on potassium-evoked increase in serotonin and 5-HIAA release within frontocortical dialysates

Each value represents mean $\pm$ S.E.M. (with the number of rats in brackets) obtained from animals pretreated with vehicle (2 ml/kg, i.p.) or fluoxetine (7.5, 15, 30 or 60 mg/kg, i.p., as in text) daily for 3 days. Dialysates from rat frontal cortex were obtained before (basal) and after (evoked) perfusion with 120 mM KCl. Comparisons between effects at each trial were carried out using one-way ANOVA, followed by Fisher's t-test procedure.

Treatment	Serotonin release (fmol/20 μ l)	Serotonin release (fmol/20 μ l)	5-HIAA efflux (pmol/20 μ l)	5-HIAA efflux (pmol/20 μ l)
	Basal	Evoked	Basal	Evoked
Vehicle	7.63 $\pm$ 2.32 (5)	17.65 $\pm$ 3.98* (5)	3.82 $\pm$ 0.47 (6)	4.71 $\pm$ 0.70 (6)
D-Fen 7.5 mg/kg	4.93 $\pm$ 2.07 (6)	11.53 $\pm$ 4.15* (5)	1.08 $\pm$ 0.22** (6)	1.28 $\pm$ 0.20 (6)
Fluoxetine 7.5 mg/kg	6.10 $\pm$ 1.15 (4)	7.71 $\pm$ 1.95 (4)	1.41 $\pm$ 0.06** (4)	1.49 $\pm$ 0.09 (4)
Fluoxetine 15 mg/kg	3.33 $\pm$ 0.56* (5)	4.79 $\pm$ 0.68 (5)	1.56 $\pm$ 0.09** (5)	1.77 $\pm$ 0.19 (5)
Fluoxetine 30 mg/kg	2.95 $\pm$ 0.56* (6)	3.57 $\pm$ 0.45 (5)	1.30 $\pm$ 0.17** (6)	1.48 $\pm$ 0.12 (6)
Fluoxetine 60 mg/kg	4.65 $\pm$ 2.06 (4)	7.27 $\pm$ 2.21 (4)	1.26 $\pm$ 0.21** (4)	1.65 $\pm$ 0.33 (4)

*p<0.05 and **p<0.01 comparing basal release in saline- and drug-pretreated groups

*p<0.05 comparing basal and KCl-evoked release in each group