Experientia 41 (1985), Birkhäuser Verlag, CH-4010 Basel/Switzerland

Serotonergic and cholinergic interaction in the regulation of pituitary-adrenal function in rats

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Summary. It has been proposed that the central serotonergic inputs which modulate pituitary-adrenal secretion are mediated by cholinergic neurons. We have tested this hypothesis in intact rats. Male Sprague-Dawley rats were injected with cholinergic and serotonergic agents which enhanced transmitter function and with receptor blocking agents. Agents were injected, singly and in combination, into both unstressed and stressed animals. Since the response to cholinergic agents might be due to changes to vasopressin release, Brattleboro (vasopressin deficient) rats were also injected with cholinergic agents. The level of plasma corticosterone at 1-h post-injection was determined.
Results indicate that the serotonin receptor blockade decreased the stimulatory, cholinergic effect of physostigmine. Cholinergic receptor blockers did not significantly reduce the corticosterone rise induced by 5-hydroxytryptophan. These results do not support the hypothesis of cholinergic mediation of serotonergic input. Nicotinic and muscarinic receptors appeared to exert opposing influences on the system. The nicotinic receptor antagonist was able to block the stimulatory effect of physostigmine. The muscarinic receptor antagonist significantly elevated plasma corticosterone levels. No differences were found in the effect of physostigmine on Brattleboro rats as compared to controls. These data are interpreted as suggesting that 1) the acetylcholine-induced stimulation of pituitary-adrenal function is mediated, in part, by serotonergic neurons; and 2) stimulation of nicotinic receptors is facilitatory whereas stimulation of muscarinic receptors is inhibitory to pituitary-adrenal function.
Key words. Serotonin; acetylcholine; corticosterone; ACTH; CRF; Brattleboro rat; stress.

1. Introduction

Numerous studies using neurotransmitters have attempted to define the mechanisms regulating the secretion of corticotropin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH)[5, 15, 17, 21]. These studies suggest that both serotonin and acetylcholine stimulate the release of CRF, while norepinephrine and GABA inhibit its secretion. In 1976, Jones and his co-workers, using hypothalamic fragments incubated in vitro, proposed a model of the neurotransmitter regulation of CRF release[16]. This model suggests that serotonin stimulates the release of CRF by a cholinergic neuronal system that synapses directly onto CRF neurosecretory cells in the hypothalamus. Yet, other in vitro studies[5] have offered evidence that such a serotonin-acetylcholine interaction may not exist. It has been shown that atropine, a muscarinic receptor blocker, does not decrease 5-hydroxytryptophan rises in plasma corticosterone levels[9, 19, 26]. No other studies on serotonergic-cholinergic interaction using pharmacologic agents have been reported.
In the present in vivo study, intact rats were used to determine whether serotonergic systems modulating pituitary-adrenal secretion exert their effect via cholinergic neurons.

2. Materials and methods

2.1 Animals and agents

Male Sprague-Dawley rats (125–150 g) were obtained from Flow Research Laboratories (Dublin, VA) and caged either individually or in groups of four, as specified in each experiment. Male homozygous Brattleboro rats and heterozygous littermates were obtained from Blue Spruce Farms (Altamont, NY). Rats were kept in a room with controlled access for 1–2 weeks prior to experimentation and given food and water ad libitum. Lights were on between 06.30 and 20.00 h. Animals weighed 200–315 g at the time of experiment.
The following cholinergic and serotonergic agents were used: physostigmine, mecamylamine, scopolamine hydrobromide, L-5-hydroxytryptophan (5-HTP), methysergide maleate, neostigmine bromide and methscopolamine bromide. The mode of action of each of these drugs and the dosages employed are summarized in the table. Mecamylamine (Merck & Co., West Point, PA) and methysergide maleate (Sandoz, East Hanover, NJ) were obtained as gifts. All other agents were purchased from Sigma Chemical Co. (St. Louis, MO). Drugs were dissolved in 0.9% NaCl (pH 6.5–7.5); Tween-80 was used

Drugs and dosages

Drug	Dosage (mg/kg)	Reference	Action
Physostigmine	0.25	7, 25, 27	Acetylcholinesterase inhibitor
Mecamylamine	1.5	20	Nicotinic receptor blocker
Scopolamine hydrobromide	1.0	25	Muscarinic receptor blocker
L-5-hydroxytryptophan	100	10, 23	Serotonin precursor
Methysergide	25	22, 26	Serotonin-receptor blocker
Neostigmine bromide	0.02*	25	Acetylcholinesterase-inhibitor (peripheral)
Methscopolamine bromide	0.5**	24	Muscarinic receptor blocker (peripheral)

* Injected simultaneously with scopolamine. ** Injected simultaneously with physostigmine.

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to suspend 5-HTP and methysergide in solution. Agents were injected i.p. in a volume of 1.6 ml/kg.
Methscopolamine bromide (a muscarinic receptor blocker which does not cross the blood-brain barrier) was administered concomitantly with physostigmine in order to reduce peripheral effects. Similarly, neostigmine bromide was injected along with scopolamine in order to control for its peripheral effects.

2.2 *Experimental procedures*

All experimental procedures were conducted between 06.30 h and 08.00 h with the exception of the stress study. In the stress study experimentation extended from 06.30 h to 10.30 h. Blood from each rat was collected and the plasma was stored at −20 °C until corticosterone content was determined by radioimmunoassay. Plasma corticosterone concentration was used as an indirect measure of CRF release.
Study on the effects of cholinergic and serotonergic agents in unstressed rats. Six groups of rats, 10 per group, were housed singly prior to experimentation. Each group was injected with one of the following agents: 1) saline, 2) physostigmine, 3) mecamylamine, 4) scopolamine, 5) 5-HTP, and 6) methysergide. Rats were decapitated 1-h post-injection; decapitation occurred within one minute after initial contact with the animal's cage.
Study on the interactions of serotonergic and cholinergic agents in unstressed rats. Five groups of rats, 10 per group, were housed singly prior to experimentation. Each group was injected with one of the following combinations of agents; 1) physostigmine plus 5-HTP, 2) physostigmine plus methysergide, 3) mecamylamine plus 5-HTP, 4) mecamylamine, scopolamine, plus 5-HTP, and 5) mecamylamine plus methysergide. Blood was collected as before for plasma corticosterone determination.
Study on the interaction of agents in stressed rats. Sixteen rats were divided into two groups, each of which was subjected to experimentation once every 4–5 days. Rats were injected with one of the following agents or agent combinations: 1) saline, 2) physostigmine, 3) mecamylamine, 4) scopolamine, 5) 5-HTP, 6) methysergide, 7) 5-HTP plus mecamylamine, and 8) 5-HTP plus scopolamine. Immediately following injection, each rat was stressed by being placed on a horizonal shaker (160 oscillations per min; 15 cm excursion) for 40 min followed by exposure to inhalant anesthesia (Metofane) for 10 min. At the end of this period, blood (100–200 µl) was obtained by clipping a toenail from a hind foot. Each rat received five different injections, with the order of agent injection being counterbalanced throughout the 1-month experimental period. This provided a sample size of 10 for each of the eight agents.
Study on the effects of agents in Brattleboro rats. Eight homozygous and eight heterozygous (control) Brattleboro rats were housed singly. Basal blood samples were obtained from six rats in each group (toe-clip). There were four treatment groups: 1) saline, 2) physostigmine, 3) physostigmine plus mecamylamine, and 4) mecamylamine. Each rat was injected (i.p.) with each agent in a counterbalanced design. Blood samples were obtained by nail-clip 1-h post-injection.

2.3 *Corticosterone determination*

Plasma corticosterone concentrations were determined by radioimmunoassay using corticosterone-21-succinate antiserum purchased from Endocrine Sciences (Tarzana, CA). The assay procedure followed was that suggested by Endocrine Sciences, with minor modifications[28]. Steroid was extracted from plasma samples with absolute ethanol (20–50 µl plasma in 1–3 ml ethanol). Standards and samples were assayed in triplicate. The linear range of the assay was between 2.5 and 250 pg/tube, with sensitivity extending to 0.1 pg/tube. The intra-assay coefficient of variation was 12.5%, and there was a variation of 8.5% between assays.

2.4 *Statistical analysis*

Dunnett's multiple comparison procedure[8] was used to compare the mean corticosterone values of treated groups with those of the control group. Duncan's Multiple Range Test was used for comparisons among experimental groups.

3. *Results*

Cholinergic neurons were stimulated in unstressed rats by injection of physostigmine, a cholinesterase inhibitor. The serotonin precursor, 5-HTP, was used to stimulate serotonergic neurons. Both physostigmine and 5-HTP significantly ($p < 0.01$) increased plasma corticosterone levels; there was no difference between the increases in corticosterone levels caused by these two agents (fig. 1a). Although scopolamine and methysergide (both receptor-blocking drugs) also caused significant increases in corticosterone levels compared to controls ($p < 0.05$), the elevated levels of plasma corticosterone caused by these receptor blockers were significantly less ($p < 0.05$) than those caused by their respective agonists.
The effectiveness of the antagonist actions of scopolamine and methysergide was evaluated by testing the ability of these drugs to counter the stimulatory effect of

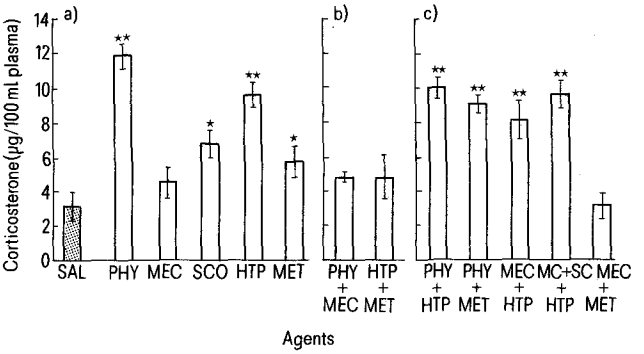

Figure 1. Effects of serotonergic and cholinergic agents on plasma corticosterone levels. Means ± SEM are shown, with levels of significance in comparison to controls at $p < 0.05$ (*) and $p < 0.01$ (**). Dosages (mg/kg) were: PHY = physostigmine 0.25; MEC = mecamylamine 1.5; SCO = scopolamine 1.0; HTP = L-5-hydroxytryptophan 100; MET = methysergide 25; and SAL = saline 0.9%. *a* Effect of agents injected singly (n = 10). *b* Agonist-antagonist interaction for serotonin (HTP+MET) and acetylcholine (PHY+MEC) drugs (n = 4). *c* Interaction of serotonergic and cholinergic agents on plasma corticosterone levels.

physostigmine and 5-HTP, respectively. When given in combination with physostigmine (fig. 1b), mecamylamine greatly reduced the corticosterone release caused by physostigmine. Similarly, when administered with 5-HTP, methysergide significantly reduced the corticosterone rise caused by 5-HTP.

The combination of physostigmine plus 5-HTP caused a significant increase ($p < 0.01$) in plasma corticosterone compared to saline controls (fig. 1c). If the effect of cholinergic stimulation is mediated by serotonergic neurons, methysergide (a serotonin antagonist) should reduce or block the stimulatory effect of physostigmine. The corticosterone levels in the physostigmine plus methysergide group were significantly lower ($p < 0.05$) than those in the physostigmine group, although higher ($p < 0.01$) than saline controls. In neither the physostigmine plus 5-HTP group or the physostigmine plus methysergide group did the corticosterone values differ significantly from those induced by 5-HTP alone.

The relative contributions of nicotinic and muscarinic receptors to the regulation of CRF were assessed in unstressed rats using receptor blockers. Mecamylamine (a nicotinic receptors blocking agent) produced a nonsignificant decrease in the effect of 5-HTP on plasma corticosterone; when scopolamine (a muscarinic receptor blocking agent) was injected along with mecamylamine and 5-HTP, this slight decrease was eliminated (fig. 1c). Mecamylamine abolished the methysergide-induced corticosterone rise.

In stressed rats, 5-HTP produced the largest increase ($p < 0.01$) in plasma corticosterone levels compared to rats injected with saline (fig. 2). Both nicotinic and muscarinic receptor blockers were evaluated for their ability to antagonize the 5-HTP induced rise in plasma corticosterone concentrations. Mecamylamine, but not scopolamine reduced the 5-HPT induced rise in plasma corticosterone.

Brattleboro rats (vasopressin deficient) were used to assess the possibility that the effects seen with cholinergic agents were due to stimulation of vasopressin release, rather than CRF release. No difference was observed in the basal level of corticosterone in homozygous Brattleboro rats (Brattleboro) as compared with their heterozygous littermates (control; fig. 3). The stress response of the Brattleboro rats to saline injections was significantly impaired (compared to their heterozygous controls; $p < 0.05$). It should be noted that since the heterozygous controls do have an intermediate level of vasopressin defect, results obtained from these animals cannot be equated with those from 'normal' rats[29].

If the rise in plasma corticosterone induced by physostigmine injection were due principally to vasopressin stimulation, one would expect that the control animals would be more responsive to physostigmine than the vasopressin deficient rats. However, Brattleboro rats did not differ from controls in their response to physostigmine injection (fig. 3). The reduction in physostigmine effectiveness seen in both control and Brattleboro rats receiving physostigmine plus mecamylamine was not statistically significant. Mecamylamine, when given alone, produced corticosterone levels in control rats that were significantly lower than those seen following saline injection ($p < 0.05$). Mecamylamine injection resulted in low corticosterone levels in both Brattleboro and control rats, similar to those seen in the Brattleboro rats following saline injection.

4. Discussion

Data from this study support the existence of serotonin neurons which mediate, at least in part, the stimulation of pituitary-adrenal function by acetylcholine in the rat central nervous system. This conclusion is based on three observations. 1) Methysergide, a serotonin receptor blocker, decreased the stimulatory effect of physostigmine. 2) Neither mecamylamine nor scopolamine (acetylcholine receptor blockers) significantly altered the corticosterone rise induced by 5-HTP. 3) If serotonin neurons mediate the effect of cholinergic stimulation, then stimulation by acetylcholine of an already maximally activated serotonin neuron would be expected to

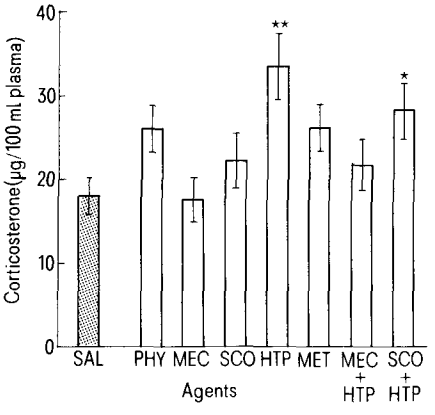

Figure 2. Effects of serotonergic and cholinergic agents on plasma corticosterone levels in stressed (horizontal shaker plus inhalant anesthesia) rats. Means ± SEM (n = 10) are shown with levels of significance in comparison to controls at $p < 0.05$ (*) and $p < 0.01$ (**). Dosages (mg/kg) were: PHY = physostigmine 0.25; HTP = L-5-hydroxytryptophan 100; MET = methysergide 25; MEC = mecamylamine 1.5; SCO = scopolamine 1.0. Combinations of drugs were given in the same respective dosages.

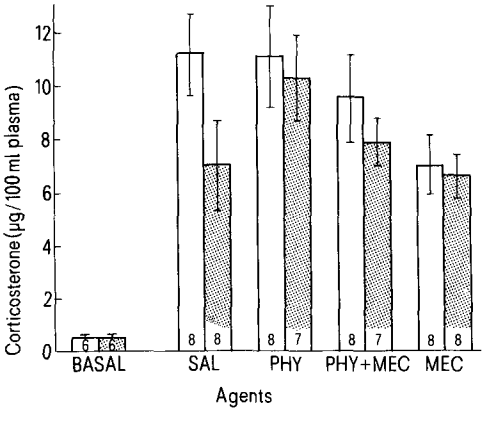

Figure 3. Effects of cholinergic agents on plasma corticosterone concentrations in Brattleboro rats. Means ± SEM are shown. Open bars give the control values; hatched bars give the Brattleboro values. The number at the bottom of each bar indicates the sample size. Dosages were: PHY = physostigmine 0.25 mg/kg, and MEC = mecamylamine 1.5 mg/kg.

produce no further release of CRF or corticosterone. The failure of 5-HTP plus physostigmine to produce a greater release of corticosterone than 5-HTP alone is in agreement with this reasoning.

The results obtained from the stress study indicate that serotonin stimulation is more effective in enhancing the release of corticosterone during stress than is cholinergic stimulation. 5-HTP injection increased plasma corticosterone levels significantly, while physostigmine produced little effect. These observations may be the result of increased cholinergic drive onto serotonergic neurons during stress. During stress, in contrast to basal conditions, the elevation in plasma corticosterone produced by physostigmine was not significant. Yet mecamylamine was able to very markedly reduce the pronounced stimulatory effect of 5-HTP. This is what would be expected if cholinergic inputs onto serotonin neurons were strongly stimulated by the stress itself.

Although the serotonin receptor blocker, methysergide, effectively reduced the stimulation of corticosterone release induced by 5-HTP, it produced an almost twofold increase in corticosterone levels when it alone was injected into unstressed rats. This observation is difficult to interpret in view of the reported inability of methysergide to increase serotonin release from the rat cortex[3]. However, the binding profile of methysergide does indicate that the compound has measurable affinity for $5HT_1$ (presynaptic) receptors[18].

Mediation by serotonin of some acetylcholine components does not imply that cholinergic inputs are unimportant. The present study, and those of others[6, 24, 26], indicate acetylcholine does play a significant role in regulating pituitary-adrenal function. Physostigmine produced a pronounced rise in plasma corticosterone in unstressed animals. This effect was significantly reduced, but not abolished by methysergide, indicating that serotonergic control of cholinergic input was not complete. Secondly, the nicotinic receptor blocking agent, mecamylamine, antagonized the stimulatory effect of 5-HTP in stressed rats. This suggests that cholinergic neurons, through their action on nicotinic receptors, can directly influence pituitary-adrenal secretion.

The data suggest that cholinergic stimulation may effect both increases and decreases in corticosterone release, with muscarinic receptor stimulation being inhibitory and nicotinic receptor stimulation being facilitatory. These conclusions are based on several observations. 1) The nicotinic receptor blocker, mecamylamine, was able to block the stimulatory effect of physostigmine in unstressed rats. 2) The muscarinic receptor blocker, scopolamine, but not mecamylamine, significantly elevated plasma corticosterone levels in unstressed animals. 3) Stressed rats given 5-HTP in combination with scopolamine had significantly higher corticosterone levels; those given 5-HTP in combination with mecamylamine did not.

Nicotinic receptor stimulation has been reported to stimulate vasopressin release, and vasopressin is generally recognized as a stimulator of ACTH release[4]. However, it is unlikely that the effects of the cholinergic drugs affected ACTH and corticosterone levels in plasma through their action on the release of vasopressin. If vasopressin mediated a significant part of the action of the cholinergic agents, one would expect that physostigmine would have less effect in a vasopressin deficient animal. This was not the case in Brattleboro rats: the effect of physostigmine was almost identical in the homozygous Brattleboro rats and their heterozygous littermates.

Although several studies have failed to show that either acetylcholine or serotonin directly releases ACTH from cultured pituitary cells[12, 14], drug action at the level of the pituitary may have contributed to our results. Muscarinic agonists have been reported to inhibit ACTH secretion[13] and methysergide has been found to be a weak stimulator of ACTH secretion[12].

Serotonin mediation of acetylcholine stimulation of CRF secretion has previously been suggested by both in vitro and in vivo studies. Buckingham and Hodges[5] using hypothalamic fragments, found that nicotinic receptor blocking agents antagonized the CRF release by acetylcholine, but not serotonin. In the same study, it was reported that a serotonin receptor blocker was able to reduce significantly the corticosterone release induced by acetylcholine. Gibbs and Vale have reported that serotonin stimulation increases both the concentration of ACTH in systemic plasma and CRF in hypophyseal portal plasma[11].

The present study suggests that serotonin mediates to a significant extent the acetylcholine stimulation of corticosterone secretion, presumably through excitation of CRF neurons. It also suggests that facilitatory and inhibitory effects may be exerted by nicotinic and muscarinic receptors, respectively.

*Acknowledgment. This work was supported by NIH Biomedical Research Support Grant 2-S07-07095-13. The authors thank Ms Karen Hoyt and Mrs Kathleen Marshall for preparation of the manuscript.

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0014-4754/85/091123-05$1.50 + 0.20/0

Short Communications

Effects of exchange transfusion with perfluorochemical emulsions on hepatic oxygen supply and blood flow in the rat[1]

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14 September 1984

Summary. Exchange-transfusion to hematocrit 20 with isotonic perfluorochemical (PFC) emulsions containing 3% hydroxyethylstarch (HES) in rats breathing 100% oxygen produced significant reductions of hepatic PO_2 and blood flow in comparison to rats hemodiluted with isotonic 3% or 6% HES solution. The results indicate that PFC and/or emulsifiers were associated with adverse effects on liver blood supply.
Key words. Perfluorochemical; hemodilution; hepatic PO_2; hepatic blood flow.

Isotonic iso-oncotic emulsions of perfluorochemicals have been studied as potential blood supplements and resuscitation fluids by virtue of their capacity to dissolve larger amounts of oxygen than solely aqueous solutions[3]. Two formulations (Green Cross Corp., Japan) have received particular attention: Fluosol-43, containing perfluorotributylamine (20% w/v), and Fluosol-DA, containing perfluorodecalin (14% w/v) and perfluorotripropylamine (6% w/v). Pluronic F-68, a nonionic detergent, is used as an emulsifier. Prior to use the emulsion is combined with hydroxyethylstarch and electrolytes. Fluosol-43 is restricted to investigations in animals due to long retention of the perfluorochemical in tissues such as liver and spleen. By contrast, the perfluorochemicals of Fluosol-DA are eliminated from the body over a period of weeks[3]. Fluosol-DA has been tested clinically in Japan with no major adverse effects reported[4].

Although clinical application of Fluosol-DA has been conducted on a restricted basis in the United States, in cases where patients refuse blood or blood products[5], there are questions about effectiveness[6] and concerns about adverse microcirculatory reactions[7,8]. Moreover, there is little information on the effects of perfluorochemical hemodiluents on tissue PO_2. The investigation reported here was undertaken to determine the relationship between changes, if any, of hepatic oxygen supply and blood flow attendant to hemodilution with perfluorochemicals in rats.

Materials and methods. Animal preparation and hemodilution procedure. Male Sprague-Dawley rats weighing 200–325 g were anesthetized by i.p. injection of pentobarbital sodium (40 mg/kg). PE-50 polyethylene cannulas were placed in the left common carotid artery and left external jugular vein. Rats breathed 100% oxygen. Following control measurements as described below, the cannulated animals were subjected to one of four treatments:

Perfluorochemical (PFC) hemodiluted. Animals in this group were exchange-transfused with either Fluosol-43 or Fluosol-DA (Green Cross Corp., Japan) prepared fresh for each rat. Hemodilution was conducted at the rate of 1.9 ml/min by simultaneous withdrawal of blood from the arterial cannula and infusion of diluent via the venous cannula until a hematocrit approximating 20 was reached. Additional volumes of diluent were added as needed to replace blood volume lost during sampling and to maintain mean arterial pressure throughout the experiment.

Hydroxyethylstarch (HES) controls. Using the same hemodilution procedure, animals were exchange-transfused to a similar hematocrit with an aqueous solution of 3% HES and electrolytes identical to that in which the PFC emulsions were mixed.