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Pituitary Localization of ^{3}H-Spiroperidol by an Uptake/Storage Mechanism?

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The lack of a pituitary imaging agent combined with the considerable clinical value for such an agent prompted an examination of ^{3}H-spiroperidol (^{3}HSp). Spiroperidol was selected for initial evaluation based on its high affinity for D_2 receptors which are known to be present in the pituitary. A time course study of ^{3}HSp concentration in rat pituitary and other tissues was conducted. Pituitary activity levels were found to be constant from 5 min to 4 h and were about 8 times levels in corpus striatum at 1 h. Blocking studies with (+)-butaclamol and with unlabelled spiroperidol suggested the existence of both a D_2 receptor mediated binding localization and a second uptake which is postulated to be an internalization process. Further studies involving ultracentrifugation of pituitary homogenates resulted in evidence for association of ^{3}HSp with dense subcellular particles. ^{3}HSp thus appears to be internalized by pituitary cells.

Introduction

Previous workers have evaluated radiolabelled spiroperidol derivatives as brain imaging agents. Those studies have relied on the high affinity of spiroperidol, a potent D_2 antagonist, for the dopamine receptor. In particular, [^{11}C]spiroperidol, $^{(1-3)}$3-N-[^{11}C]methyl-spiroperidol,[4,5] [^{18}F]spiroperidol[6,7] (position emitters), and ^{77}Br-*p*-bromospiroperidol[8,9] (γ emitter) showed sufficient uptake in the corpus striatum, known to contain D_2 receptors, to permit imaging in both animals and man. Although the pituitary is known to contain D_2 receptors similar to those in corpus striatum,[10–12] few of these studies[3] have addressed the possibility of pituitary imaging.

Indeed, reports on radionuclidic imaging of the pituitary are scarce. [^{99m}Tc]pertechnetate was employed as an imaging agent to detect pituitary adenomas.[13,14] Reported data clearly demonstrated the increased vascularity and activity of pituitary tumors, but pertechnetate does not functionally characterize pituitary adenomas.

Various pituitary tumor pathologies are known, and prolactin secreting adenomas (prolactinomas) are common, being found in 20–25% of all women with amenorrhea.[15,16] Pituitary tumors are usually detected by physical symptoms, abnormal hormone levels and CT scans of the sella turcica. However, CT scans do not functionally characterize pituitary adenomas; and thus diagnosis may be imprecise and often rests on association of abnormal hormone levels with a possible CT scan abnormality.

Thus the availability of imaging agents which would permit specific differentiation between pituitary pathologies and normal tissue would be of considerable clinical value. The presence of dopamine receptors capable of binding ^{3}H-neuroleptics[11] raises the possibility of imaging the pituitary with a radiolabelled D_2 receptor antagonist.

^{3}H-Spiroperidol was selected for initial evaluation based on several factors:

(1) The existence of data establishing its binding to dopamine receptors in normal pituitary tissue of rat,[10] sheep,[17] steer[17] and human.[18]

(2) Its high affinity for the D_2 receptor and its commercial availability as a tritiated compound of high specific activity.

(3) Spiroperidol is a potent antagonist of inhibition of prolactin release by dopamine or dopamine agonists.[19]

(4) Spiroperidol can be radiobrominated and the radiolabelled analog, ^{77}Br-*p*-bromospiroperidol (^{77}BrSp), exhibits similar binding to dopamine receptors and is comparable to spiroperidol in ability to stimulate prolactin release.[20] Tissue distribution studies with ^{77}BrSp in normal rats have been promis-

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ing in terms of pituitary concentration,[21] but the data are insufficient for evaluation of this compound in terms of pituitary imaging.

Thus we have studied (1) the time course of ^{3}H-spiroperidol (^{3}HSp) in male and female Fischer F344 rats, (2) the degree of D_2 receptor-mediated localization, and (3) evidence for a possible alternative localization mechanism.

Materials and Methods

The following compounds or reagents were obtained from commercial sources: ^{3}H-spiroperidol (25–30 Ci/mmol) (New England Nuclear), (+)-butaclamol hydrochloride (Research Biochemicals, Inc.), and reserpine (Sigma Chemical Company). Scintillation fluid used in counting, Ox-triti-scint, was obtained commercially (Romac).

Fischer F344 rats, male and female, were purchased (Charles River). Animals were exposed to alternating 12 h periods of light and dark and received rat chow and water *ad libitum* during the study.

Tissue distribution studies

Normals. Tissue distribution studies were performed on normal, 10 week old female rats at 5, 30 and 60 min and at 2 and 4 h post injection, and on normal, 10 week old male rats at 5 min and 4 h post injection. For these studies, approximately 25 μCi (approximately 0.3–0.4 μg) of ^{3}HSp in 0.25–0.35 mL of formulation (40% ethanol in sodium acetate buffer, pH 4.5) were administered intravenously in the femoral vein to rats anesthetized with sodium pentobarbitol. Animals, five at each time interval, were killed by decapitation and tissues removed rapidly. Representative 1–20 mg tissue samples (in duplicate) of cerebral cortex, cerebellum and corpus striatum were obtained only at $t = 1$ h. For all other time intervals, the anterior and posterior pituitary were removed, separated and processed individually. Duplicate samples of uterus and blood were also obtained. Each sample was weighed on an analytical balance interfaced to computer printout and oxidized in a Packard Model 306 Oxidizer. Samples were counted in a liquid scintillation counter with corrections made for counting efficiency and background. The values of resulting tissue concentrations were calculated as % dose/organ and/or as % kg dose/g in which body weights are normalized to 1 kg and are reported as mean ± SEM.

(+)-Butaclamol treated. Six normal female rats were treated with (+)-butaclamol (2 mg/kg) 45 min prior to ^{3}HSp injection (25 μCi). Tissue distribution studies were performed as described above at $t = 1$ h post ^{3}HSp injection.

Spiroperidol treated. Six normal female rats were coinjected with 25 μCi of ^{3}HSp and unlabelled spiroperidol (2 mg/kg). Tissue distribution studies were performed at $t = 1$ h post injection.

Reserpine treated. Normal female rats were injected with reserpine ($n = 6$) (1 mg/kg) or with an equal volume of reserpine vehicle[22] ($n = 6$) 3 h prior to injection of ^{3}HSp (25 μCi). Animals were sacrified at $t = 1$ h post ^{3}HSp injection and tissue distribution studies were conducted.

Analysis of pituitary homogenates. Twenty-four female rats were injected with ^{3}HSp (25 μCi) and sacrificed 1 h later. The anterior pituitaries were rapidly removed and divided into groups of six. The experimental protocol for tissue preparation and sucrose density gradient centrifugation was as described.[23] The final gradient was fractionated into 200 μL fractions, each fraction diluted with 12 mL aqueous scintillation fluid and counted.

In a related study, groups of eight bisected anterior pituitaries were incubated with 0.50 nM ^{3}HSp in medium 199 at 37°C for 2 h. The pituitaries were washed thoroughly and then homogenized. The remaining steps were followed as above.

Control studies consisted of preparing and centrifuging a sucrose density gradient in which only homogenizing medium containing 0.25 nM ^{3}HSp was layered on the gradient. A second control consisted of homogenizing pituitary in the presence of 0.5 nM ^{3}HSp at 0–4°C and then proceeding as described. In all controls, the final gradients were fractionated and counted. Each control was repeated a minimum of three times.

For purposes of comparison, homogenates of corpus striatal tissue were similarly analyzed. The brains from 24 rats injected with ^{3}HSp as above were rapidly removed after sacrifice and frozen. Striatal tissue from each group of 6 brains was combined and kept frozen until analysis. The tissue was washed and treated exactly as described for pituitary tissue.

Results

In order to assess the specific pituitary imaging potential of spiroperidol, a time–activity curve in the tissues of normal rats was necessary.

Time course in normal rats

The concentration of ^{3}HSp in selected tissues in normal, 10 week old female rats for time intervals from 5 min to 4 h is presented in Table 1. Over this time frame, constant levels of radioactivity were observed in the anterior and posterior pituitary which were approximately 10 times the levels observed at $t = 1$ h in cerebral cortex, cerebellum and corpus striatum. Male rats showed similar values in blood but levels in the anterior pituitaries were lower (Table 1). The values in posterior pituitary were similar for males and females at 5 min but levels had fallen by 4 h in males.

The observation that both anterior and posterior pituitary concentrations were similar was not unexpected. Earlier studies have shown that in male Sprague–Dawley rats the maximal number of binding sites for ^{3}HSp is slightly greater in the posterior

Table 1. Time course of ^{3}H-spiroperidol activity levels in normal male and female Fischer rats[a,b]

Tissue		Time				
		5 min	30 min	1 h[c]	2 h	4 h
Anterior pituitary	Female	0.238 ± 0.082	0.229 ± 0.003	0.355 ± 0.021	0.308 ± 0.027	0.299 ± 0.061
	Male	0.159 ± 0.015		0.317 ± 0.008		0.167 ± 0.010
Posterior pituitary	Female	0.306 ± 0.110	0.395 ± 0.102	0.244 ± 0.056	0.229 ± 0.039	0.308 ± 0.089
	Male	0.443 ± 0.040		0.182 ± 0.012		0.120 ± 0.017
Cerebral cortex	Female			0.034 ± 0.002		
Cerebellum	Female			0.012 ± 0.001		
Corpus striatum	Female			0.048 ± 0.003		
Blood	Female	0.032 ± 0.001	0.022 ± 0.025	0.022 ± 0.001	0.018 ± 0.001	0.022 ± 0.013
	Male	0.037 ± 0.003				0.015 ± 0.001
Uterus	Female	0.266 ± 0.032	0.121 ± 0.016	0.122 ± 0.005	0.085 ± 0.004	0.087 ± 0.011
Testes	Male			0.045 ± 0.001		

[a] Data in % kg dose/g, mean ± SEM.
[b] 10 week old Fischer F344 rats, $n = 5$.
[c] $n = 6$.

pituitary (5.9 pmol/g tissue) than in the anterior pituitary (3.8 pmol/g tissue).[10]

The approximately constant concentration of ^{3}HSp in normal pituitary tissue was expected based on time course studies of ^{3}HSp in striatal tissue as both tissues contain D_2 receptor sites. Striatal concentrations of ^{77}Br-*p*-bromospiroperidol in Sprague–Dawley rats[20] and in cats[24] have been shown to be approximately constant over a time period from 2 to 4 h.

Receptor blocking studies

^{3}HSp values at $t = 1$ h post injection in rats pretreated with (+)-butaclamol are compiled in Table 2. (+)-Butaclamol pretreatment did not significantly alter uptake of ^{3}HSp in the uterus or blood. No change in uptake in posterior pituitary was observed which is suggestive of negligible amounts of specific D_2 receptor binding in this tissue. Creese *et al.*,[28] using bovine pituitary, reported negligible specific binding in the posterior pituitary. A reduction of radioactivity was observed for the anterior pituitary which was expected based on the *in vitro* studies mentioned above. In a second blocking study, unlabelled (cold) spiroperidol was coinjected with ^{3}HSp. The results, compiled in Table 2, indicate that the unlabelled spiroperidol had little or no effect on ^{3}HSp pituitary concentration.

Cellular incorporation studies

Table 3 contains the results of ^{3}HSp uptake in rats

Table 2. ^{3}H-spiroperidol activity in female rats blocked with (+)-butaclamol or coinjected with unlabelled spiroperidol[a]

Tissue	Control	Butaclamol[b]	Spiroperidol[c]
Anterior pituitary	0.355 ± 0.021	0.240 ± 0.025	0.345 ± 0.029
Posterior pituitary	0.244 ± 0.056	0.231 ± 0.030	0.262 ± 0.046
Cerebral cortex	0.034 ± 0.002	0.014 ± 0.002	0.026 ± 0.002
Cerebellum	0.012 ± 0.001	0.010 ± 0.001	0.019 ± 0.001
Corpus striatum	0.048 ± 0.003	0.012 ± 0.002	0.027 ± 0.007
Uterus	0.122 ± 0.005	0.091 ± 0.008	0.137 ± 0.011
Blood	0.022 ± 0.001	0.017 ± 0.002	0.022 ± 0.003

[a] $n = 6$, female Fischer F344 rats; data in % kg dose/g, mean ± SEM; $t = 1$ h, 25 μCi ^{3}HSp injected.
[b] Rats injected with (+)-butaclamol (2 mg/kg) 45 min prior to ^{3}HSp injection (25 μCi). Sacrifice at $t = 1$ h post ^{3}HSp injection.
[c] Rats coinjected with unlabelled spiroperidol (2 mg/kg) and ^{3}HSp (25 μCi); ~0.3 mg total spiroperidol injected. Sacrifice at $t = 1$ h.

Table 3. The effects of reserpine and reserpine vehicle on ^{3}HSp uptake[a]

Tissue	Normals[b]	Reserpine[c]	Reserpine vehicle[d]
Anterior pituitary	0.355 ± 0.021	0.286 ± 0.033	0.355 ± 0.021
Posterior pituitary	0.244 ± 0.056	0.219 ± 0.023	0.316 ± 0.036
Cerebral cortex	0.034 ± 0.002	0.066 ± 0.008	0.076 ± 0.005
Cerebellum	0.012 ± 0.001	0.025 ± 0.006	0.018 ± 0.000
Corpus striatum	0.048 ± 0.003	0.043 ± 0.006	0.041 ± 0.002
Uterus	0.122 ± 0.005	0.117 ± 0.006	0.145 ± 0.008
Blood	0.022 ± 0.001	0.017 ± 0.001	0.015 ± 0.002

[a] $n = 6$, female Fischer F344 rats; data in % kg dose/g, mean ± SEM. ^{3}HSp (25 μCi) injected, sacrificed at $t = 1$ h.
[b] Data as in Table 1 at $t = 1$ h.
[c] Reserpine (1 mg/kg) injected 3 h prior to ^{3}HSp.
[d] Equal volume of reserpine vehicle injected 3 h prior to ^{3}HSp.

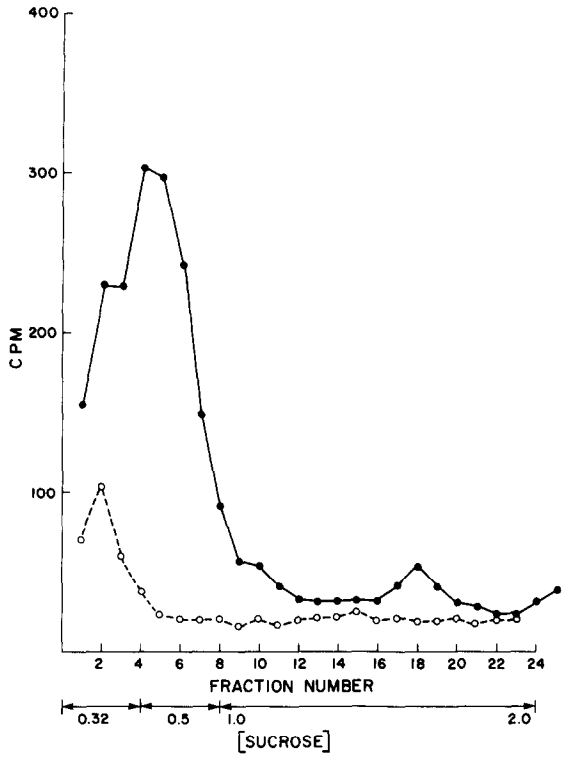

Fig. 1. CPM vs sucrose gradient fraction number or sucrose concentration in molarity for *in vivo* pituitary analysis (●——●) and for a control (○-----○).

with reserpine or vehicle pretreatment. Data from normals is included for comparison. Reserpine treatment results in an approximate 20% reduction in ^{3}HSp localization in anterior pituitary. Concentration data in other tissues were apparently unaffected by reserpine treatment.

A second set of experiments was performed in order to obtain evidence for ^{3}HSp association with dense subcellular granules to confirm that ^{3}HSp was taken into pituitary cells. In one case bisected anterior pituitaries were incubated in the presence of ^{3}HSp; in a second case anterior pituitaries were removed from rats previously injected with ^{3}HSp. All anterior pituitaries were washed and homogenized as described. Centrifugation to remove whole cells and cell debris was followed by ultracentrifugation of supernatant through a sucrose density gradient as described.[23,29] The final gradients were fractionated and counted. Figure 1 shows plots of CPM vs fraction number for the *in vivo* study and a control. The results of the incubation (*in vitro*) study were similar and are not shown. Unbound ^{3}HSp remains at the top of the gradient in both the supernatant layer and buffer layer. Two peaks of radioactivity were observed in the *in vivo* study; the first, and largest, peak contains unbound ^{3}HSp and ^{3}HSp presumably bound to light membrane fragments which remain at the top of the gradient. This was also observed for dopamine.[23] The second peak of radioactivity occurs in the same region of the gradient where dopamine associated with dense organelles was observed.[23] This second peak is strong evidence for ^{3}HSp incorporation into pituitary cells and for ^{3}HSp association with dense subcellular particles. Tissue from the corpus striatum was treated similarly. Radioactivity was found only in the top portion of the gradient as expected for membrane-bound ^{3}HSp (specific and nonspecific binding).

Discussion

Evaluation of ^{3}HSp as a model for other radiolabelled analogs or derivatives of spiroperidol was begun assuming that only two types of localization would occur in the pituitary: specific, D_2 receptor mediated, binding and nonspecific binding. That spiroperidol binds to pituitary D_2 receptors has been amply demonstrated in *in vitro* studies.[10] Binding to these receptors *in vivo* has not been clearly demonstrated but would be expected based on analogy with studies on the corpus striatum. Because evidence for receptor labelling typically includes reduction of binding in the presence of either (+)-butaclamol or unlabelled spiroperidol, the effects of these agents on ^{3}HSp pituitary concentration *in vivo* were evaluated. Treatment with (+)-butaclamol reduced ^{3}H-activity concentration in the anterior pituitary by 36% rather than 70% reduction to cerebellum levels. Previous work has shown differences between the dopamine receptors in the anterior pituitary and the striatum[30] or the caudate nucleus[1] and spiroperidol is a more potent competitor of ^{3}HSp binding in the rat striatum than in anterior pituitary.[30] These facts do not seem sufficient to explain the observed effect of (+)-butaclamol. As the time course of butaclamol effect on the pituitary is not documented, a second blocking study was conducted. The data from this study parallel data reported for ^{77}BrSp in the pituitary when unlabelled spiroperidol was coinjected.[21] It was suggested that the relative inability of unlabelled spiroperidol to displace ^{77}BrSp was due to a relatively small portion of total binding which was specific.[21] However, if this were true, then the blocking study with (+)-butaclamol should have yielded similar results, i.e. (+)-butaclamol should have had little or no effect on ^{3}HSp concentration.

Thus, although data from the (+)-butaclamol blocking study supports the presence of some specific D_2 receptor binding of ^{3}HSp, the fact that radioactivity levels are not reduced to cerebellum (nonspecific binding) levels is suggestive of an additional mode of retention. Further evidence for an additional retention mechanism consists of the negligible effect of unlabelled spiroperidol on ^{3}HSp pituitary concentration.

Cellular uptake and localization of ^{3}HSp in dense subcellular particles, possibly prolactin secreting storage vesicles, are possible as the alternative means of retention. There is some evidence in the literature to support an internalization mechanism. The endo-

genous inhibitor of prolactin secretion, dopamine, has been shown to be incorporated into prolactin-secreting storage granules.[23,29,31] An immunofluorescent study using haloperidol, a D_2 antagonist similar in structure to spiroperidol, presented evidence suggestive of haloperidol incorporation via endocytosis.[32] An uptake study using cultured pituitary tumor cells (F_4C_1 strain) showed that spiroperidol uptake could be reduced by 50% in the presence of reserpine, which was interpreted as evidence for a biogenic amine vesicular uptake.[33] The effect of reserpine on *in vivo* ^{3}HSp uptake was tested. Radioactivity levels were reduced by 20% which provides some evidence for the existence of a biogenic amine vesicular uptake mechanism for spiroperidol. Further support is seen in the finding of radioactivity associated with dense subcellular particles obtained from homogenates of the pituitary but not from homogenates of striatal tissue. This finding was confirmed for pituitary by both *in vivo* and *in vitro* experiments. If no metabolism of ^{3}HSp occurs, then the observed location of ^{3}H activity is strong evidence for the cellular uptake of ^{3}HSp and for the association of ^{3}HSp with dense subcellular particles. This process of internalization of ^{3}HSp suggests that alternative approaches to the design of radiopharmaceuticals specific for the pituitary may be useful. The release of prolactin elicited by spiroperidol and previously attributed to D_2 receptor interaction may need to be re-examined in light of this evidence.

Clearly, there is significant ^{3}HSp localization in the anterior pituitary. Analysis of a worst case scenario suggests that radiolabelled spiroperidol has excellent potential for imaging normal pituitary. In the worst case, uptake in normal (control) pituitary was 1.5% dose/g for 0.15 kg male rats. Adjusting this specific uptake for body mass gives 0.0032% dose/g for a 70 kg human (1.5% × 0.15/70). The average human pituitary weighs 0.7 g which would then concentrate 2.25×10^{-5} of the injected dose which would amount to 0.225 μCi for a 10 mCi dose. Sensitivity for various brain imaging instruments ranges from 240 to 1400 counts/min/μCi in air or 50–300 counts/min/μCi in 10 cm of water. One might, therefore, detect from 12 to 70 cpm from the hypothetical pituitary under discussion. Since specific uptake in cerebellum is only 7% of this worst case example and since the pituitary is isolated at the base of the brain, it should be well visualized above any cerebellar background. Problems with radioactivity background from blood should be minimal as the anterior pituitary-to-blood ratio is 14.3.

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