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Original Research

Chronic treatment with (−)-stepholidine alters density and turnover of D_1 and D_2 receptors in striatum[1]

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KEY WORDS (−)-stepholidine; corpus striatum; SCH-23390; radioligand assay; dopamine D_1 receptors; dopamine D_2 receptors

AIM: To study the effects of chronic administration of SPD on the density and turnover of striatal D_1 and D_2 dopamine (DA) receptors. **METHODS**: Receptor density was monitored by radio-receptor binding assay. The receptor recovery and turnover were studied after irreversible inactivation by *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydro-quinoline (EEDQ). **RESULTS**: Chronic SPD treatment (sc, 20 $mg \cdot kg^{-1} \cdot d^{-1} \times 21$ d) upregulated both striatal D_1 and D_2 receptor density. As compared to vehicle-treated rats, SPD increased D_1 and D_2 receptors by 41.5 % and 43.7 %, respectively. SPD also altered the turnover of both D_1 and D_2 receptors. The degradation rate constant ($k = 0.0082 \cdot h^{-1}$) and the synthesis rate ($r = 2.65$ $pmol \cdot h^{-1}/g$ protein) of D_2 receptors in SPD-treated rats were significantly increased *vs* vehicle-treated rats ($k = 0.0049 \cdot h^{-1}$; $r = 1.10$ $pmol \cdot h^{-1}/g$ protein). The degradation rate constant ($k = 0.0059 \cdot h^{-1}$) and the synthesis rate ($r = 3.1$ $pmol \cdot h^{-1}/g$ protein) of D_1 receptors was also increased in SPD-treated rats *vs* vehicle-treated rats ($k = 0.0048 \cdot h^{-1}$; $r = 1.8$ $pmol \cdot h^{-1}/g$ protein), but the alteration of degradation rate constant missed significance ($P > 0.05$). As a result, receptor recovery following EEDQ was accelerated The half time for D_1 and D_2 receptors recovery in SPD group were 117.5 h and 84.5 h, respectively, shorter than 144.4 h and 141.4 h in vehicle-treated rats. **CONCLUSION**: Chronic SPD treatment upregulated D_1 and D_2 receptors, and accelerated DA receptor turnover and recovery mainly by increasing receptor synthesis.

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Alteration of dopamine (DA) receptor density is involved in the pathogenesis of several disorders, such as Parkinson's disease, Huntington's disease, schizophrenia, and tardive dyskinesia[1,2]. A better understanding of how DA receptors are regulated by drugs may help to suggest novel treatments for these disorders.

Chronic treatment with DA agonist or antagonist alters striatal DA receptor density[3-5]. Changes in receptor density could result from the alterations of receptor synthesis and/or receptor degradation, and those can be analyzed with a relatively simple model by inactivating the receptors[7]. *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) has been reported to inactivate irreversibly DA receptors[8]. It was thus used in this experiment.

(−)-Stepholidine (SPD), isolated from the Chinese herb *Stephania intermedia* Lo, possesses high affinity for both D_1 and D_2 receptors with preference to D_1 receptors[9]. It has been established as a D_2 antagonist based on the following observations: 1) SPD antagonizes apomorphine (APO)-induced stereotypy in rats[10]; 2) SPD reverses dose-dependently APO-induced firing inhibition of DA neurons in substantia nigra pars compacta[11]; 3) SPD blocks the autoreceptor-mediated feedback regulation of DA synthesis in striatum[12].

However, the pharmacological effect of SPD on D_1 receptors remains unclear[13,14]. The present work aims to explore how chronic SPD treatment affects the density and turnover of striatal D_1 and D_2 receptors and further characterize SPD.

MATERIALS AND METHODS

Medication Sprague-Dawley rats (♂, clean, Shanghai

Experimental Animal Center, certificate № 005 conferred by Animal Management Committee, Chinese Academy of Sciences), weighing 208 ± s 25 g, were housed 6 - 8 per cage on a 12-h light-dark cycle with food and water *ad lib*. SPD (mp 161 - 162 ℃, $[\alpha]_D$ - 440° in pyridine), supplied by Shanghai Institute of Materia Medica, was dissolved in 10 % phosphoric acid, and adjusted with NaOH 0.1 $mol \cdot L^{-1}$ to pH 4 - 5. Rats were injected SPD (20 $mg \cdot kg^{-1}$, sc) or its vehicle (distilled water, pH 4 - 5) once daily for 21 d. EEDQ (Research Biochemicals Inc), freshly dissolved in ethanol/propylene glycol 1 : 1 (vol/vol), was injected ip 6 $mg \cdot kg^{-1}$ · 3 d after withdrawal from chronic SPD treatment. Rats were decapitated at 6, 24, 72, 120, and 196 h after ip EEDQ.

Radioreceptor binding assay Striata were homogenized in ice-cold Tris-HCl buffer (pH 7.4) 50 $mmol \cdot L^{-1}$, using a Teflon homogenater. After centrifugation at 50 000 · *g* at 4 ℃ for 20 min, the pellet was washed and spinned once more. Protein contents were determined colorimetrically[6].

In the binding experiments, $[^3H]$SCH-23390 (3.0 $PBq \cdot mol^{-1}$, Amersham), and $[^3H]$spiperone (3.7 $PBq \cdot mol^{-1}$, Amersham) were used as radioligands to label D_1 and D_2 receptors, respectively. In determining the time course of D_1 or D_2 receptor recovery, binding assay was performed using a single-concentration of the radioligand. For $[^3H]$SCH-23390 binding, the pellet was resuspended in Tris-buffered incubation solution (pH 7.4) containing $MgSO_4$ 5 $mmol \cdot L^{-1}$, edetic acid 0.5 $mmol \cdot L^{-1}$, and 0.02 % ascorbic acid. For $[^3H]$spiperone binding, the pellet was resuspended in Tris-buffered incubation solution (pH 7.4) containing NaCl 120, $CaCl_2$ 2, KCl 5, $MgCl_2$ 1 $mmol \cdot L^{-1}$, and 0.01 % ascorbic acid. Membrane suspension 200 μL (*ca* 200 μg) was added to assay tubes in triplicate to bring the total volume to 0.5 mL. SCH-23390 1 $\mu mol \cdot L^{-1}$ or haloperidol 10 $\mu mol \cdot L^{-1}$ was used as a D_1 or D_2 receptor blocker to determine nonspecific binding. For D_2 receptor assay ketanserin (40 $nmol \cdot L^{-1}$) was included to prevent the binding of $[^3H]$spiperone to serotonin receptors. After the addition of radioligand 8 $nmol \cdot L^{-1}$ (final concentration), the assay tubes were incubated at 37 ℃ for 30 min. The reaction was terminated by dilution with 5 mL ice-cold buffer and filtration through filters. Following additional rinse with 2 × 5 mL buffer the dried filters were placed in scintillation vials and the radioactivity was counted. In separate experiments, saturation analysis were done to estimate the B_{max} and K_d values under the same conditions as described above except that 8 concentrations of the radioligand (0.01 - 12 $nmol \cdot L^{-1}$ for $[^3H]$SCH-23390 binding, 0.01 - 10 $nmol \cdot L^{-1}$ for $[^3H]$spiperone binding) were used.

Data analysis Saturation isotherms were analyzed by computer software GPIP (Graph Inplot) with nonlinear fitting to estimate the B_{max} and K_d. When a single concentration of radioligand was used to label receptors, the B'_{max} value was estimated using the following equation:

$$B'_{max} = B(L + K_d)/L \quad (1)$$

where L = concentration of free radioligand, B = density of receptors specifically labeled at concentration *L*, and K_d = equilibrium dissociation constant determined by analysis of saturation isotherms.

Calculation of recovery kinetics after receptor inactivation was carried out[7]. Briefly, it was assumed that the receptor production rate (*r*) is constant during the repopulation period, and the degradation of receptors after blockade is proportional to the density of receptors at any time, giving the equation:

$$R_t = r/k(1 - e^{-kt}) \quad (2)$$

where R_t = receptor concentration at time *t* and *k* = rate constant for receptor degradation. When *t* tends to infinity R_t approaches r/k, which is equal to R_{ss}, the concentration of receptor at steady state. The logarithmic transformation $[\ln(R_{ss}/R_{ss}-R_t) = kt]$ of the experimental recovery curves yields a linear plot. The slope of the plot is equal to the rate constant of degradation (*k*). The receptor production rate $(r) = R_{ss} \times k$. The half time ($T_{1/2}$) of receptor recovery = $0.693/k$.

The mean B_{max} values throughout the observation time in SPD-treated rats were compared with those in vehicle-treated rats using a two-way ANOVA followed by an unpaired *t*-test.

RESULTS

Upregulation of D_1 and D_2 receptor density by chronic SPD treatment Saturation analyses revealed that chronic treatment with SPD increased the B_{max} of striatal D_1 and D_2 receptors by 41.5 % and 43.7 %, respectively, without any significant effect on the K_d values of D_1 and D_2 receptors (Tab 1). According to the single concentration binding method, where B'_{max} were calculated by the equation (1), the upregulation rates of D_1 and D_2 receptors were 41.0 % and 42.1 %, respectively, which were similar to those values estimated by saturation analysis.

SPD accelerated the recovery of D_1 and D_2 receptors inactivated by EEDQ EEDQ (6 $mg \cdot kg^{-1}$, ip) produced a profound decrease (about 80 % in D_1 receptors and 60 % in D_2 receptors) of receptor density (B_{max}) at 6 h, which was followed by a gradual recovery. SPD accelerated the receptor recovery following irreversible inactivation by EEDQ. By 192 h after EEDQ injection the

Tab 1. Effects of chronic treatment with SPD on D_1 and D_2 receptors. B_{max} and K_d were obtained from saturation analysis, B'_{max} from single concentration binding. The unit of B_{max} or B'_{max} was pmol/g protein, the unit of K_d was nmol·L^{-1}. $\bar{x} \pm s$. [a]$P > 0.05$, [b]$P < 0.05$ vs vehicle; [d]$P > 0.05$ vs B_{max}.

		SPD	Vehicle
D_1	B_{max}	522 ± 98[b]	369 ± 55
	K_d	1.14 ± 0.18[a]	1.09 ± 0.23
	B'_{max}	512 ± 86[d]	363 ± 50[d]
D_2	B_{max}	323 ± 84[b]	225 ± 52
	K_d	2.25 ± 0.32[a]	2.17 ± 0.29
	B'_{max}	328 ± 67[d]	231 ± 54[d]

B_{max} of D_1 receptors in SPD group returned to 77.0 % of control value, more than the recovery rate in vehicle group (68.3 %). Similarly the B_{max} of D_2 receptors in SPD group returned to 87.2 % relative to 75.3 % in vehicle group by 192 h. Besides, the recovery rates of D_2 receptors were also higher in SPD group than those in vehicle group at other assay points, indicating an accelerated receptor recovery (Tab 2).

Acceleration of the turnover of striatal D_1 and D_2 receptors by chronic SPD treatment The semilogarithmic transformation of the B_{max} against the time (h) after EEDQ injection produced straight lines, representing D_1 or D_2 receptor recovery, respectively (Fig 1).

From these plots, the kinetic parameters of receptor recovery were calculated. The rate constant of degradation and the synthesis rates of D_1 and D_2 receptors in SPD-treated rats were higher than those in vehicle-treated rats. However, the alteration of degradation rate of D_1 receptors had no significance ($P > 0.05$). Accordingly, the half-time ($T_{1/2}$) for receptor recovery in SPD group was 117.5 h for D_1 receptors and 84.5 h for D_2 receptors, both shorter than vehicle group (144.4 h and 141.4 h, respectively) (Tab 3).

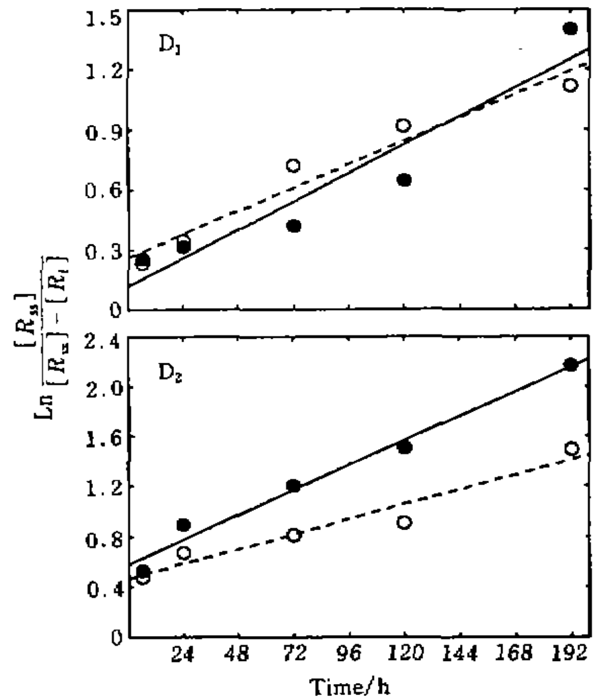

Fig 1. Semilogarithmic plots of the time course of striatal D_1 (upper panel) and D_2 (lower panel) receptor recovery in chronical treatment with vehicle (○) or SPD (●) in rats. R_{ss} = steady state receptor concentration. R_t = receptor concentration after EEDQ blockade.

DISCUSSION

In the present study, chronic SPD treatment

Tab 2. Recovery of D_1 and D_2 receptors in striatum after EEDQ treatment in chronically SPD- or vehicle-treated rats. B_{max} of 4 – 6 rats.

	Vehicle				SPD			
	B_{max}, pmol/g protein		%, control		B_{max}, pmol/g protein		%, control	
	D_1	D_2	D_1	D_2	D_1	D_2	D_1	D_2
Control	363 ± 50	231 ± 54	100	100	512 ± 86	328 ± 67	100	100
6 h	76 ± 12	85 ± 7.5	20.9	36.8	117 ± 20	132 ± 25	22.9	40.2
24 h	108 ± 21	110 ± 16	29.8	47.6	142 ± 2.2	190 ± 41	27.7	58.0
72 h	190 ± 17	125 ± 13	52.3	54.1	179 ± 12	226 ± 41	35.0	68.9
120 h	222 ± 16	134 ± 13	61.2	58.0	249 ± 16	251 ± 9	48.6	76.5
192 h	248 ± 17	174 ± 20	68.3	75.3	394 ± 53	286 ± 34	77.0	87.2

Tab 3. Recovery kinetic parameters of D_1 and D_2 receptor after EEDQ in vehicle- or SPD-treated rats. $n=5$, $\bar{x} \pm s$. [a]$P>0.05$, [c]$P<0.01$ *vs* vehicle.

Parameters		10^3 · Rate of degradation, · h^{-1}	Rate of synthesis, $pmol \cdot h^{-1}$/g protein	$T_{1/2}$, h
D_1	SPD	5.9±1.1[a]	3.08±0.45[c]	117.5
	Vehicle	4.8±0.6	1.77±0.32	144.4
D_2	SPD	8.2±0.6[c]	2.65±0.19[c]	84.5
	Vehicle	4.9±0.7	1.10±0.12	141.4

up-regulated the density of striatal D_2 receptors, which was consistent with previous observations that SPD exerted antagonistic action[10-12]. On the other hand D_1 receptor density was up-regulated by SPD, indicating a D_1 antagonistic action, which corresponded with the electrophysiological observation in reserpinized rats[14] but contradicted with the D_1 agonistic action in rotational behavior of 6-OHDA-lesioned rats[13]. This contradiction might be explained with the intrinsic activity of SPD to D_1 and D_2 receptors and the supersensitivity of DA receptors in the 6-OHDA-lesioned rats (Dong, *et al*. Biochem Pharmacol 1996; to be published). More over, SPD displayed a maximal response less than a full agonist such as DA or APO, it had biphasic action curves when coadministrated with DA or APO, all supporting SPD as a D_1 partial agonist.

Chronic SPD demonstrated a D_1 antagonistic action. This result has important implications because in normal animals the effect of an agent (eg, SPD) on D_1 receptors was often masked by the interaction between D_1 and D_2 receptors[15], it was therefore difficult to characterize a D_1 agent. Currently, we obtained the evidence demonstrating the interaction was attenuated in 6-OHDA-lesioned rats but not in chronically SPD-treated rats (unpublished data), which may explain why SPD displayed an agonistic action in 6-OHDA-lesioned rats while it acted as a D_1 antagonist in chronically SPD-treated rats.

Kinetic analyses revealed that chronic SPD treatment increased D_1 and D_2 receptor turnover and recovery, mainly by increasing the receptor synthesis rate. However, it remains unclear at present whether these increases result from the increased receptor synthesis or increased insertion of recycling receptors into membrane. To answer this question requires the experiments using protein synthesis inhibitor.

In summary, the present study indicates that SPD is a D_1 and D_2 receptor antagonist, when chronically administrated, it increases the density and turnover of both D_1 and D_2 receptors mainly by increasing the receptor synthesis.

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慢性给予左旋千金藤立定对纹状体多巴胺 D_1 和 D_2 受体密度和更新率的影响[1]

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关键词 左旋千金藤立定；纹状体；SCH-23390；放射配位体测定；多巴胺 D_1 受体；多巴胺 D_2 受体

A **目的**：观察慢性给予左旋千金藤立定(SPD)对纹状体多巴胺(DA)受体密度和更新率的影响，推论SPD的药理性质. **方法**：应用EEDQ失活DA受体，用放射受体结合分析法测定受体的密度，计算有关的动力学参数. **结果**：慢性给予SPD 21 d使纹状体 D_1 与 D_2 受体密度分别增加41 5 %和43.7 %. 并且慢性给予SPD改变DA受体的更新率，使 D_1 受体的生成速度由对照组的1 77 pmol · h^{-1}/g protein 增加至3.08 pmol · h^{-1}/g protein，D_2 受体的生成速度和降解速率分别由对照组的1 10 pmol·h^{-1}/g protein和0 0049 h^{-1}增加至2 65 pmol·h^{-1}/g protein和0 0082 h^{-1}. D_1 和 D_2 受体更新一半所需的时间分别由144.4 h和141 4 h缩短至117 5 h和84.5 h. **结论**：SPD增加DA受体的密度并加快受体更新，呈现 D_1 与 D_2 混合型阻滞剂的作用特性.

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Hypoxia effects on hypothalamic corticotropin-releasing hormone and anterior pituitary cAMP[1]

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KEY WORDS Sprague-Dawley rats; pikas; anoxia; cyclic AMP; corticosterone; anterior pituitary gland; corticotropin-releasing hormone; argipressin; norepinephrine

AIM: To study the effects of acute and chronic hypoxia on hypothalamus-anterior pituitary-adrenocortex axis. **METHODS**: Rats and pikas were exposed to different altitude and periods. Animals were injected with CRH, Arg and NE in the third ventricle of the brain of rats. **RESULTS**: Anterior pituitary cAMP and plasma corticosterone levels of rats obviously increased during 1 h of hypoxia. cAMP was increased from 2.23 ± 0.13 of control group to 7.7 ± 0 7 of 5 km and 13.4 ± 1.9 nmol/g wet tissue of 8 km, respectively. icv CRH, Arg and NE all activated HPA axis. The effects of CRH were most potent. CRH 2 μL 0.75 nmol icv increased anterior pituitary of cAMP from 3.5 ± 0 4 of control to 22.4 ± 2.2 nmol/kg wet tissue. Stimulating altitude of 5000 m resulted in a 16.9 % decrease in corticosterone level ($P < 0.05$), 8000 m resulted in a 47.5 % decrease ($P < 0.01$) after hypoxia for 25 d. Hypoxia did not activate HPA axis in pikas. **CONCLUTION**: 1) Hypoxia stress activates the secretion of corticotrophin (ACTH) via cAMP; 2) Adrenocotical function of rats decays during chronic hypoxia; 3) Arg and NE regulate the secretion of plasma corticosterone and synthesis of pituitary cAMP at the hypothalamus level; 4) Hypoxia tolerance of the pika was high.

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Corticotropin-releasing hormone (CRH), argipressin (Arg) and norepinephrine (NE) regulate the secretion of corticotrophin in anterior pituitary