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Relevance between striatal expression of Fos, proenkephalin mRNA, prodynorphin mRNA and rotation induced by *l*-stepholidine in 6-hydroxydopamine-lesioned rats[1]

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KEY WORDS stepholidine; corpus striatum; 6-hydroxydopamine; *in situ* hybridization; gene expression; enkephalins; dynorphins; *fos* genes

ABSTRACT

AIM: To study that *l*-stepholidine (SPD) regulates the expression of proenkephalin (PENK) and prodynorphin (PDYN) mRNA and Fos in the striatum after rotational test in the 6-hydroxydopamine (6-OHDA)-lesioned rats. **METHODS**: PENK and PDYN mRNA levels were examined with *in situ* hybridization, and Fos expression was detected with immunocytochemistry. The data were semi-quantified with image analyzer. **RESULTS**: (1) Following repeated SPD treatment, the rotation was kept on high activity in the 6-OHDA-lesioned rats. (2) SPD significantly elicited Fos expression in both sides of striatum, particularly in the denervated one. Repeated administration of SPD, Fos expression declined on both sides, particularly in the intact one. (3) In the denervated striatum of 6-OHDA-lesioned rats, the PENK mRNA level was extremely increased *vs* that in the intact striatum. This high level of PENK mRNA was significantly reduced by 7-d treatments of SPD. SPD also reduced the level of PENK mRNA in the intact striatum. However, the level of PDYN mRNA did not show significant change in both sides of striatum after denervation or SPD treatment. **CONCLUSION**: In the 6-OHDA-lesioned rats, the rotation induced by SPD was kept on a high activity, which was in pace with the inducement of Fos expression and the reduction of expression of PENK mRNA in the denervated striatum. But then the lesion and SPD treatment had no remarkable effect on the expression of PDYN mRNA.

INTRODUCTION

Striatum is a main structure of the basal ganglia and plays a critical role in the extrapyramidal motor response mediated via the striatal efferent neurons, ie direct circuit neurons projecting to the endopendicular nucleus and the substantia nigra pars reticula (SNR), and indirect circuit neurons to the external globus pallidus[1,2]. Such striatal direct and indirect circuit neurons are gama-amino-butyric acid (GABA)-producing, co-expressing enkephalin (ENK) and co-expressing dynorphin (DYN) respectively, which are correspondingly regulated by the D_2 and D_1 dopamine (DA) receptors[3]. DA receptor drugs can also regulate the expression of ENK and DYN at the striatal efferent neurons with different effects in the normal and denervated striatum[4,5]. Thus, the relationship between the expression of both neuropeptide is often attractive to be investigated for different functions of D_2 and D_1 receptors[6-8].

ENK and DYN are derived from their precursor peptides, ie pro-ENK (PENK) and pro-DYN (PDYN). The proprotein convertase enzymes convert PENK into six copies of Met-ENK and one copy of Leu-ENK, whereas PDYN into several DYN analogues and Leu-ENK[9,10]. Therefore, PENK and PDYN levels are more precisely correlated to the activation of D_2 and D_1 receptors in the striatum than the contents of ENK and DYN.

l-Stepholidine (SPD), a natural product extracted from Chinese herb *Stephania*, possesses dual actions, ie D_1 agonistic and D_2 antagonistic[11,12]. Its D_1 agonistic action induces contralateral rotational behavior in the unilaterally lesioned rats by 6-hydroxydopamine (6-

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OHDA)[12]. After intermittent treatment for 21 d, SPD could lowed the increased level of ENK or PENK mRNA in the lesioned rats, and elevated the level of DYN or PDYN mRNA[13]. Only could these effects explain both neuropeptides rebalanced by SPD in the 6-OHDA-lesioned status. The correlation between acute treatment of SPD and rotational function remains unclear.

The present study attempts to elucidate the relationship between SPD-induced rotational behavior and the expression of PENK mRNA, PDYN mRNA, and Fos in the denervated and intact striatum of 6-OHDA-lesioned rats.

MATERIALS AND METHODS

Chemicals (-)-SPD (Shanghai Institute of Materia Medica, Chinese Academy of Sciences), mp 161 – 162 ℃, $[\alpha]_D$-440 ℃ in pyridine, was dissolved in a small amount of H_2SO_4 0.1 mol/L, then diluted with distilled water and adjusted with NaOH 0.1 mol/L to pH 5.0 – 5.5. 6-Hydroxydopamine · Br was from Sigma. Apomorphine (APO) was from the First Pharmaceutical Co of Shenyang, and desipramine-HCl was from RBI. Antisense oligodeoxynucleotide probes to PENK and PDYN mRNA were synthesized in the Institute of Plant Physiology, Chinese Academy of Sciences. Digoxigenin labeling and detecting kits were obtained from Bochringer. Stock 20 × SSC solution (sodium chloride, sodium citrate buffer) were diluted into 4 × SSC, 2 × SSC, 1 × SSC and 0.1 × SSC in the hybridization of PENK and PDYN mRNA. NBT (nitro-blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) stock solution were in the Digoxigenin detecting kits. All of the other agents were AR.

Preparation of 6-OHDA-lesioned rats All of the experiments were carried out on Sprague-Dawley rats (230 – 250 g, grade Ⅱ) purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences (Certificate № 005). Rats for 6-OHDA-lesion were preinjected desipramine-HCl (25 mg/kg, ip, RBI) to prevent the uptake of 6-OHDA into the noradrenergic neurons. Thirty minutes later, 6-OHDA were microinjected into the left medial forebrain bundle (Coordinate: AP – 4.0 mm, ML 1.5 mm, DV 7.6 mm below the surface of brain)[14] with the 4 μL saline solution (containing 20 μg 6-OHDA · Br and 1 μg ascorbic acid) at the rate of 0.5 μL/min by an infusion pump. The needle was withdrawn after an additional 10 min.

One month after the 6-OHDA-lesion, rats were injected with apomorphine (0.2 mg/kg, ip) and the contralateral rotations were counted more than 150 cycles per 30 min. The rats were kept in housing for the bulk experimental uses.

Rotation challenged by SPD Two months later, thirty lesioned rats were divided into 5 groups (4 – 10 within each group). The group was delivered with vehicle for 7 consecutive days. Group 2 to 4 were given SPD (20 mg/kg, ip) once daily for 1, 3, and 7 consecutive days, respectively, and group 5 was given SPD (40 mg/kg, ip) for 7 consecutive days. The contralateral turn was counted within 30 min after last injection of SPD. The random rotation in the vehicle treatment was used as control.

Tissue preparation After rotational experiment, 20 out of 30 lesioned rats (4 each group) were used for the following tissue preparation. After the last experiment (corresponding 2 h after last injection), the rats were anesthesized with chloral hydrate (400 mg/kg, ip) and then perfused through the ascending aorta with saline followed by 4 % ice-cold paraformaldehyde in phosphate buffer 0.1 mol/L (pH 7.4). Brains were quickly removed and post-fixed for 8 h in above fixative at 4 ℃ before being transfered to 30 % sucrose in phosphate buffer 0.1 mol/L. Until the brains sunk in the buffer for 2 – 3 d, serial coronal sections (30 μm) were carried out with a freezing microtome and were collected for the successive experiments for Fos immunocytochemistry and for hybridization of PENK mRNA and PDYN mRNA.

Fos immunocytochemistry Coronal sections at the coordinate Bregma 1.20 mm[14] were for Fos immunocytochemistry. The sections were first washed with 0.01 mol/L phosphate buffer saline (PBS, pH 7.4) and immersed in 1 % H_2O_2 for 30 min, followed by the incubation with the block solution containing 10 % normal sheep serum, 0.3 % Triton X-100 in PBS at 37 ℃ for 1 h. Then, sections were transferred to 1∶1000 anti-Fos (Santa Cruz, USA) containing 0.3 % Triton-X 100 in PBS at 37 ℃ for 1 h and subsequently 4 ℃ for 48 h. After being rinsed with PBS, sections were incubated in 1∶200 biotinylated secondary IgG (Vector Laboratories, CA, USA) containing 2 % normal serum at room temperature for 2 h and 1∶200 avidin-biotin complex (Vector Laboratories, CA, USA) at room temperature for 2 h separately. At the intervals sections were washed fully. Then the sections were incubated in 0.05 % diaminobenzidine (DAB) and 0.003 % H_2O_2. Finally, they were mounted on gelanin coated slides, dehydrated, cleared and coversliped.

***In situ* hybridization for PENK and PDYN**

mRNA Rostral sections covering the striatum at the coordinate Bregma 1.20 mm were also treated with the in situ hybrydization procedure with some modification[15]. Sections were first rinsed shakily with PBS 0.01 mol/L, followed by the treatment of 2 mg/L proteinase K (30 min, 37 ℃). After the incubation in 0.25 % acetate anhydride and 0.1 mol/L triethanolamine for 10 min, the sections were immersed in prehybridization solution (50 % formamide, 4 × SSC) and then hybridized in the solution containing digoxigenin-labelled antisense oligodeoxynucleotide probe corresponding to the cDNA sequence encoding the rat PENK amino acid 112 – 121 or the rat PDYN mRNA nucleotides 539 – 568 (300 g/L) at 37 ℃ overnight. Then, sections were rinsed twice in 2 × SSC at room temperature for 15 min each, 1 × SSC at 37 ℃ for 30 min, and 0.1 × SSC at 42 ℃ for 30 min. After the incubation of sections in alkaline phosphatase labelled polyclonal anti-digoxigenin Fab fragment (1∶1000, Bochringer) at 4 ℃ overnight (18 h), the sections were rinsed in Buffer Ⅰ twice for 10 min each. Then, following a short rinse in Buffer Ⅲ, tissue-bound alkaline phosphatase activity was visualized by incubating the sections with NBT/BCIP in Buffer Ⅱ. The enzymatic reaction was stopped by rinsing the sections in PBS. Finally, the sections were mounted, dehydrated, and coverslipped with neutral balsam.

Image analysis Sections were employed to image analysis. The optical density for PENK and PDYN mRNA expression and positive Fos-immunoreactive nuclei within three subregions of the striata, ie dorsolateral, dorsomedial and ventrolateral parts on both sides, was determined with Q750 IMAGE ANALSER. Statistically, *t*-test was performed for the analysis of the data.

RESULTS

Rotation Thirty rats were used for rotational test. In the vehicle control group, the rats only showed occasionally contralateral turns (2.8 ± 1.1 turns per 30 min). After the first treatment of SPD (20 mg/kg), the contralateral turns of each group increased profoundly (250 – 300 turns per 30 min) and had significant difference against that of vehicle group ($P < 0.01$). But, there were no differences among the three tested groups (20 mg/kg, $P > 0.05$). At the third day, the SPD-induced rotations were increased from 236 ± 39 to 422 ± 28 turns per 30 min in second group. At d 7 treatment of SPD, the rotation was kept in the peak tendency in third group (Tab 1). Another group of lesioned-rats was given SPD with 40 mg/kg for 7 d, their rotation behavior was observed on d 1, d 3, and d 7. There was no significant difference between 20 and 40 mg/kg (Fig 1).

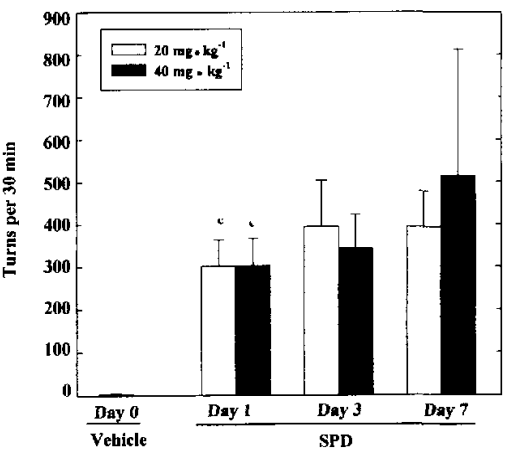

Fig 1. Rotation induced by SPD (20 mg/kg or 40 mg/kg, respectively) for 7 consecutive days. $^cP < 0.01$ *vs* vehicle.

PENK mRNA After rotational experiment, 20 out of 30 lesioned-rats were used for *in situ* hybridization for PENK and PDYN mRNA. The PENK mRNA

Tab 1. 6-OHDA-lesioned rats tested in the rotational behavior. $\bar{x} \pm s$. $^cP < 0.01$ *vs* vehicle; $^eP < 0.05$ *vs* 1st group.

Group	rat (*n*)	SPD (mg/kg, ip)	Day	Rotations d 1	d 3	d 7
1	4	vehicle	7	2.8 ± 1.1	2.1 ± 0.9	2.3 ± 0.5
2	4	20	1	249 ± 25^c	/	/
3	4	20	3	236 ± 39^c	422 ± 28^c	/
4	10	20	7	302 ± 31^c	391 ± 39	392 ± 23
5	8	40	7	303 ± 29^c	357 ± 32	511 ± 272

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expressing neurons distributed in all of the subregions of the striatum were observed with similar cell size and shape (Fig 2).

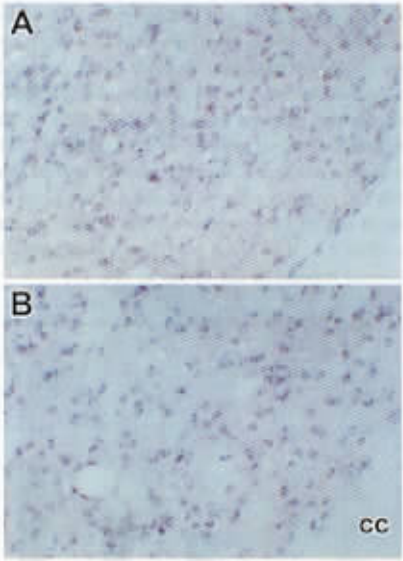

Fig 2. PENK mRNA expressing neurons in the denervated striatum of 6-OHDA-lesioned rats treated with vehicle (A) or SPD for 7 days (B).

In the denervated striatum of 6-OHDA-lesioned rats, the PENK mRNA level was increased significantly compared with that in the intact striatum ($P<0.05$). Treatment with SPD 20 mg/kg (ip) for 3 d, the elevated PENK mRNA in the denervated striatum only had a decreasing tendency ($P>0.05$). And for 7 d, SPD lowered the elevated PENK mRNA significantly in the lateral part of the denervated striatum, but not in the dorsomedial subregion against the vehicle group or with SPD for 1 d (Fig 3A). When the dose was increased to 40 mg/kg for 7 d, SPD further decreased the PENK mRNA level significantly in the three subregions of the denervated striatum ($P<0.05$), especially in the dorsolateral part of the denervated, and this level was near to that of the intact striatum of vehicle group (or called control value). Therefore, SPD reversed the elevated level of PPEK mRNA induced by 6-OHDA-lesion.

Under the same experimental conditions, treatment

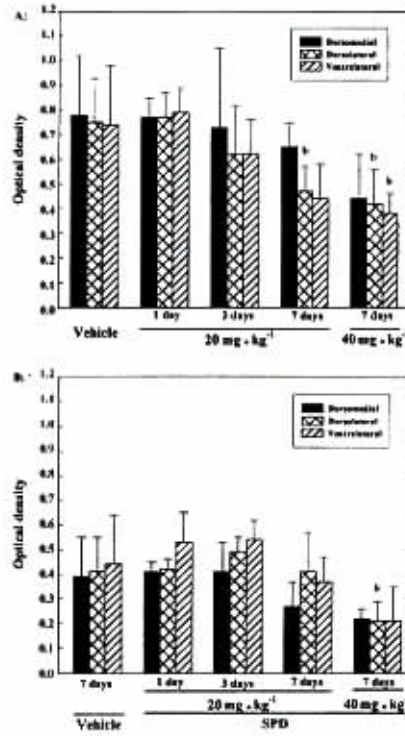

Fig 3. Relative contents of PENK mRNA in the subregions of denervated (A) and intact (B) striatum after the administration of SPD. $n=4$. $\bar{x}\pm s$. $^bP<0.05$ vs vehicle.

of SPD 40 mg/kg for 7 d also lowered significantly the level of PENK mRNA in the intact side of striatum, although its control value was lower than that in the denervated (Fig 3B). It showed that SPD 20 mg/kg for 7 d could not remarkablely reduce the level of PENK mRNA in the intact striatum.

PDYN mRNA Following SPD treatment (20 – 40 mg/kg) for 7 d, there was no difference of PDYN mRNA levels between intact and denervated striatu of 6-OHDA-lesioned rats or between vehicle and SPD treatment (Fig 4).

Fos expression Twenty out of 30 lesioned rats were simultaneously used for Fos expression besides *in situ* hybridization experiments of PENK and PDYN mRNA.

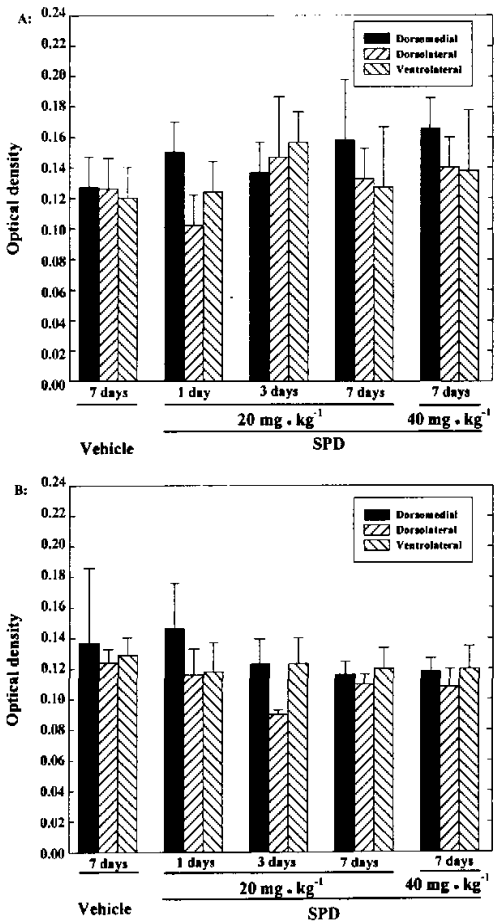

Fig 4. Relative contents of PDYN mRNA in the subregions of denervated (A) and intact (B) striatum after the administration of SPD. $n = 4$. $\bar{x} \pm s$.

In the vehicle group, there was a few positive Fos nuclei in both sides of the striatum of lesioned rats. After administration of SPD 20 mg/kg for 1 d, Fos positive nuclei appeared remarkably in both denervated ($P < 0.01$) and intact sides of the striatum ($P < 0.05$) (Fig 5C, D, Tab 2). Moreover, there were many more positive Fos nuclei in the denervated striatum than that in the intact striatum. The distributive area of induced Fos immunoreactive nuclei covered all of the striatal subregions, particularly dense in the dorsomedial part of the striatum.

However, Fos expression was reduced gradually by consecutive administration of SPD 20 mg/kg for 3 – 7 d or with SPD 40 mg/kg for 7 d. the Fos expression was easily reducible in the intact striatum (Fig 5D) than that in the denervated ($P < 0.05$ *vs* group 2, Tab 2, Fig 2C), and yet the Fos expression remained at a high level ($P < 0.05$ *vs* vehicle).

DISCUSSION

In the previous work[13], the reliability of preparation of 6-OHDA unilateral lesioned rats have been established with the measurement of loss of DA content and tyrosine hydroxylase reactivity in immunocytochemistry. This Parkinson's disease model was used widely in the experiments of the present study.

At first, SPD was demonstrated as a D_1 agonist once again, which was drawn under the following programm designed 3 sets of serial rotational tests during the period of SPD treatment for 1, 3, and 7 d, corresponding with the previous reports[11,12].

After the last rotational test was finished, the rats were used for Fos immunocytochemistry, PENK mRNA and PDYN mRNA experiments. The results showed that the D_1 agonistic rotation induced by SPD was in pace with the Fos-expression and the reduction of elevated PENK expression in the denervated side of striatum, but not with

Tab 2. Fos immunoreactive nuclei in the striatum. $\bar{x} \pm s^*$. $^bP < 0.05$, $^cP < 0.01$ *vs* Group 1; $^eP < 0.05$, $^fP < 0.01$ *vs* Group 2.

Group	SPD mg/kg	Day	Intact striatum			Denervated striatum		
			DM	DL	VL	DM	DL	VL
1	Vehicle	7	23 ± 12	16 ± 186	15 ± 22	32 ± 18	21 ± 14	27 ± 22
2	20	1	366 ± 112^c	130 ± 94	160.6 ± 93^b	526 ± 126^c	462 ± 14^c	291 ± 152^b
3	20	3	134 ± 117	62 ± 46	93.9 ± 47^b	474 ± 256^b	267 ± 136^b	180 ± 146
4	20	7	123 ± 43^{c,f}	43 ± 44	45.3 ± 66	256 ± 111^{c,e}	217 ± 162^e	104 ± 85
5	40	7	99 ± 70^e	38 ± 50	31.9 ± 54	319 ± 156^b	248 ± 90^{c,f}	140 ± 133

Mean total numbers of nuclei displaying FLI in three areas of the denervated and intact striatum of rats treated with either vehicle or SPD. DM, dorsomedial; DL, dorsolateral; VM, ventrolateral.

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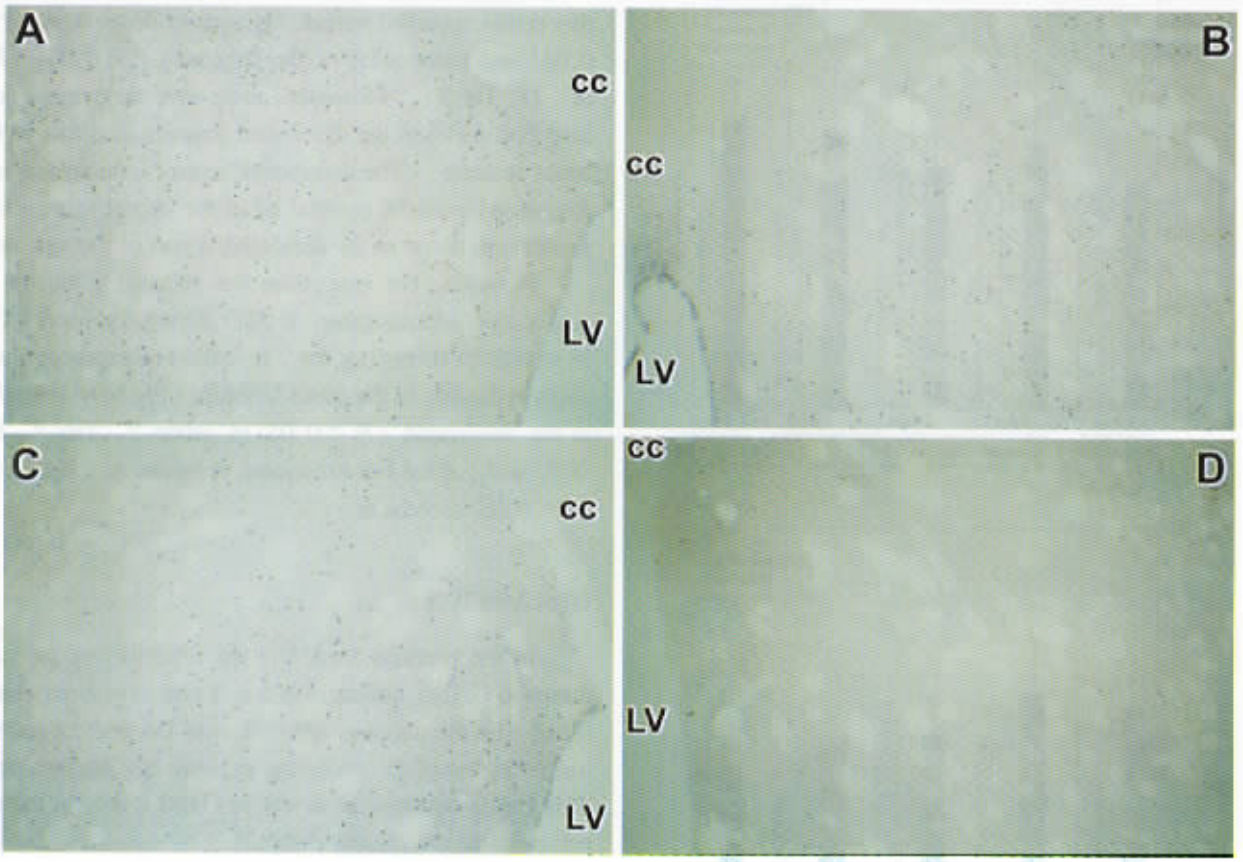

Fig 5. Fos in the denervarted (A, C) and intact (B, D) striata of 6-OHDA-lesioned rats treated with SPD for 7 days (C, D) or 1 day (A, B).

the PDYN expression.

DA receptor drugs hold the ability to induce Fos expression in the striatum under some circumstance. The present and previous studies[21] observed Fos expression by SPD in both sides of denervated and intact striata of 6-OHDA-lesioned rats with different mechanisms. In the denervated striatum the high Fos expression was due to the activation of D_1 receptors by SPD, which was similar to that by SKF 38393[22], a selectiwe D_1 agonist, while in the intact striatum Fos expression depends on the D_2 antagonistic action of SPD in the present study and previous studies[21] as well as D_2 antagonist[23]. However, the intensity of Fos expression in the denervated striatum was quite more abundant than that in the intact striatum. It is implied that the agonistic action of SPD on supersensitive D_1 receptor was more potent in the expression of Fos. Nevertheless, the Fos expression is easily dissipated owing to the characteristics of action of immediate-early gene c-*fos*. Thus the duration between Fos expression and D_1 agonistic action of SPD had an incoordinate tendency. The former became downward, and the latter upward in period of administration of SPD.

In the present study, the expression of PENK mRNA significantly increased in the denervated striatum of 6-OHDA-lesioned rats, which was correspondent to the other reports[5-7]. The elevated expression of PENK mRNA in the denervated striatum implied the increment of inhibitory effect of the indirect striatal projection neurons on its efferent target cells[13]. Such increment of PENK expression could be reversed by D_2 receptor agonist, while D_2 receptor antagonists elevated the PENK expression only in the normal as well as intact striata[8,16]. Furthermore, the D_2 receptor knockout could result in the elevation of PENK expression[17]. Therefore, PENK expression in the striatum was well negatively correlated with the activation of D_2 receptors. D_2 antagonist could

modify the interaction between endogenous DA and D_1 receptors by a release of $G_{\beta\gamma}$ subunits of G_i protein from D_2 receptors. The $G_{\beta\gamma}$ subunits would interact with α-subunits of G_s protein, which was cooperated with D_1 receptors. The 6-OHDA lesion might change the state of D_1/D_2 interaction operating at the receptor level[24].

SPD decreased the expression level of PENK in the denervated striatum in the present study as well as in the previous report[13]. This effect was considered to be D_1 agonistic action. Moreover, the down-regulation of SPD on PENK expression could be explained with the interaction between D_1 agonistic/D_2 antagonistic effects[24]. It might result in disinhibition on the target neurons in the indirect circuit pathway by the dual action of SPD.

As to the striatal PDYN expression following 6-OHDA-lesion, it is uncertain whether it is decreased[18] or unchanged[19,20]. The PDYN expression in the denervated striatum can be elevated by the D_1 selective agonist[19] and D_1 agonistic action of DA receptor agonist[20]. SPD elevated the PDYN mRNA expression to be relevant to weak D_1 agonistic action at the high dose for 21 d[13], but this effect was not observed in the present work due to short treatment.

Based on the present and previous work, it could be suggested that the D_1 agonistic action of SPD challenged the rotation behavior in the 6-OHDA-lesioned rats in pace with the expression of Fos, and redaction of elevated PENK mRNA, but not closely with PDYN mRNA or DYN.

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左旋千金藤立定诱导 6-羟基多巴胺损毁大鼠的纹状体 Fos、前脑啡肽 mRNA、前强啡肽 mRNA 表达与旋转行为的关系[1]

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关键词 千金藤立定; 纹状体; 6-羟基多巴胺; 原位杂交; 基因表达; 脑腓肽类; 强啡肽类; *fos* 基因

目的: 研究左旋千金藤立定(SPD)调节 6-羟基多巴胺(6-OHDA)损毁大鼠纹状体的 Fos、前脑腓肽(PENK)和前强啡肽(PDYN)mRNA 表达水平与旋转行为的关系. **方法**: 用免疫细胞化学法观察 Fos 表达, 用地高辛非同位素法标记的寡核苷酸探针检测纹状体 PENK 和 PDYN mRNA, 进行图像处理作半定量分析. **结果**: (1) SPD 给予 1, 3, 7 d 后, 损毁大鼠旋转行为仍维持在高水平; (2) SPD 诱导双侧纹状体 Fos 显著表达, 尤以损侧为甚. SPD 重复应用使双侧纹状体的 Fos 诱导表达下降, 尤以健侧为显著; (3) 与健侧相比, 损毁侧纹状体的 PENK mRNA 表达水平增加非常显著. SPD 重复应用 7 d, 使这种增加的 PENK mRNA 水平明显下降. 同时, SPD 也使健侧 PENK *m*RNA 水平降低. 然而, 6-OHDA 损毁和 SPD 处理对双侧纹状体的 PDYN mRNA 水平无明显影响. **结论**: SPD 激发 6-OHDA 损毁大鼠旋转行为维持在高水平, 与损侧纹状体的 Fos 表达和 PENK mRNA 水平下降是同步的. 但是, 6-OHDA 损毁和 SPD 均未显示出对 PDYN mRNA 表达的影响.

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