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Studies About the Effect of Excitatory Amino Acid Receptor Antagonist on Traumatic Spinal Cord Injury

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ABSTRACT

The slow development of histopathological changes and long period required for stabilization of lesions have suggested that secondary injury processes exacerbate the effect of initial mechanical insult after traumatic spinal cord injury (SCI). The importance of glutamate receptors in the normal functions of spinal cord, in concert with the large body of evidence that points to their involvement in neurotoxicity due to both ischemic and traumatic insults to the CNS, suggested a probable role of glutamate receptors in secondary injury process after traumatic SCI.

In order to investigate the involvement of excitatory amino acid in the secondary injury process after SCI, this study examined the effect of dextrorphan, a noncompetitive NMDA receptor antagonist, on the recovery of hindlimb function and the residual tissue at injury site following SCI. Locomotor function was assessed using open field test (21 point scale). At 8 weeks spinal cord tissue was examined using quantitative histopathologic technique. Prior to surgery female Long-Evans rats were adapted to the test environment. Rats received laminectomies (T9/T10), and spinal cord contusions (NYU impactor) were produced by a 10 gm weight dropped 25 mm. DXT (15 or 30 mg/kg, i.p.) or saline was injected 15 min before contusion. Behavioral testing resumed 2 days post-injury and continued twice a week for 8 weeks. No differences between DXT and saline groups were found for hindlimb function and sparing tissue at the lesion site. These results suggest that NMDA receptor might not be involved in secondary injury processes after traumatic SCI.

Key Words: Spinal cord injury, Secondary injury process, Dextrorphan, NMDA receptor
Abbreviation: SCI; spinal cord injury, EAA; excitatory amino acid, DXT; dextrorphan

INTRODUCTION

It was well documented that post-traumatic tissue damage following spinal cord injury (SCI) may result from more delayed events that develop in response to initial mechanical injury (Allen 1914). Studies during past decade have provided strong support for this concept of secondary, autodestructive tissue damage (Siesjö and Wieloch, 1985, Faden and Vink, 1989; Noble and Wrathall, 1989; Panter and Faden, 1992). Many physiological and biochemical changes after SCI have been described that may be involved in the secondary injury processes (Blight, 1991; Hall, 1993; Tator and Fehlings, 1991; Young, 1993). The importance of excitatory aminoacid (EAA) receptors in the normal functions of the spinal cord

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(Headley and Grillner, 1990; Salt and Herrling, 1991), in concert with the large body of evidence that points to their involvement in neurotoxicity due to both ischemic and traumatic insults to the CNS (McIntosh *et al.*, 1992; Meldrum and Garthwaite, 1990; Olney, 1978), suggest a probable role of EAA receptors in traumatic SCI (Faden and Simon, 1988; Faden *et al.*, 1990). Selective antagonists of NMDA subclass of EAA receptors were shown to reduce the functional deficits produced by experimental traumatic SCI (Faden and Simon, 1988; Faden *et al.*, 1988; Gomez-Pinella *et al.*, 1989). However, treatment of NMDA receptor antagonists had no tissue sparing effect at lesion site in traumatic SCI models (Faden *et al.*, 1988; Gomez-Pinella *et al.*, 1989). The mismatch of functional and morphological results raise questions on the involvement of NMDA receptors in secondary injury processes after traumatic SCI.

Dextrorphan is dextrorotatory morphinan and a non-competitive NMDA receptor antagonist (Kemp *et al.*, 1987; Steinberg *et al.*, 1991), and it has high possibility for clinical uses of CNS injury (Albers *et al.*, 1991).

In order to investigate the involvement of EAA in secondary injury process after SCI, this study examined the effects of an NMDA receptor antagonist, dextrorphan, on the functional deficits of hindlimbs and morphological changes at lesion site after standardized traumatic SCI.

MATERIALS AND METHODS

Animals

Female Long-Evans rats weighing 250~300 gm were used. 2 rats were kept in a cage under a light-dark cycle with light on from 7:00 to 19:00. Prior to surgery, animals were adapted to the open-field environment.

Injury procedure

Animals were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and were given prophylactic antibiotics (gentamycin 1 mg/kg, i.m.). A catheter was placed through tail artery for recording blood pressure and arterial blood sampling. Laminectomies were performed at T9 and T10, after which the adjacent spinal processes were rigidly clamped in New York University (NYU) impactor. The dura remained intact and observed to be taut. The NYU impactor is a standardized weight-drop device which release a 10 gm rod from various heights onto the exposed cord. Movement of the impact rod and vertebral column are recorded using optical potentiometers (Gruner, 1992). For present study, the rod was dropped from height of 25 mm. The surgical site was irrigated with warm aseptic saline after contusion and immediately closed. Rectal temperatures were maintained at 37±0.5℃ with a heating pad during surgery and recovery period from anesthesia. Arterial blood pressure was monitored on polygraph (Grass 7D) during 15 min before and after contusion. Blood samplings (0.1 ml) for arterial blood gas analysis were performed at 5 min before and after contusion.

Experimental protocol

All experiments were performed according to a randomized block design. Three groups of rats (n =10~15 per group) were subjected to contusive SCI. One group was control which was received vehicle (saline) and the other groups received intraperitoneal dextrorphan 15 mg/kg or 30 mg/kg. Drug administration was performed 15 min before contusion. Dextrorphan HCl was gift of Hoffman La-Roche.

Post operative maintenance and behavioral test

After recovery from anesthesia, 2 rats were kept in a cage maintaining ambient temperature at 22~24℃ and received food and water ad libitum. Manual expression of bladders was performed twice a day until a reflex bladder was established. 2 rats were sacrificed during the first postoperative week due to autotomy.

The recovery of hindlimb motor function was assessed using open field test developed by Basso *et al.* (1994). For this test, a molded plastic wading pool with dimpled floor (100 cm diameter, 21 cm wall height) was used. Animals were tested individually for 4 minutes and scored the hidlimb function according to Basso *et al.* (1994) locomotor rating scale. This locomotor rating scale is a 21 point scale in which categories 0~7 measure isolated hindlimb joint movement during the early stage of recovery; categories 8~13 measure frequency of stepping and coordination during the intermediate stage; and, categories 14~21 mea-

Table 1. Basso, beattie & bresnahan locomotor rating scale

Score	Description
0	No observable hindlimb (HL) movement
1	Slight movement of one or two joints, usually the hip &/or knee
2	Extensive movement of one joint Extensive movement of one joint and slight movement of one other joint
3	Extensive movement of two joints
4	Slight movement of all three joints of the HL
5	Slight movement of two joints and extensive movement of the third
6	Extensive movement of two joints and slight movement of the third
7	Extensive movement of all three joints of the HL
8	Sweeping with no weight support Plantar placement of the paw with no weight support
9	Plantar placement of the paw with weight support in stance only (i.e. when stationary) Frequent to Consistent weight supported dorsal stepping and no plantar stepping
10	Occasional weight supported plantar steps, no FL-HL coordination
11	Frequent to consistent weight supported plantar steps and occasional FL-HL coordination
12	Frequent to consistent weight supported plantar steps and occasional FL-HL coordination
13	Frequent to consistent weight supported plantar steps and frequent FL-HL coordination
14	Consistent weight supported plantar steps, consistent FL-HL coordination; and, Predominant paw position during locomotion as rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of stance Consistent FL-HL coordination and occasional dorsal stepping
15	Consistent FL-HL coordination; and The toes are frequently to consistently dragged across the walking surface Predominant paw position is parallel to the body at initial contact
16	Consistent FL-HL coordination during gait: and Toes are occasionally dragged Predominant paw position is parallel at initial contact and rotated at lift off
17	Consistent FL-HL coordination during gait; and Toes are occasionally dragged Predominant paw position is parallel at initial contact and lift off
18	Consistent FL-HL coordination during gait; and Toes are no longer dragged Predominant paw position is parallel at initial contact and rotated at lift off
19	Consistent FL-HL coordination during gait; and Toes are no longer dragged Predominant paw position is parallel at initial contact and lift off; and, Tail is down part or all of the time
20	Consistent coordinated gait; no toe drages; Predominant paw position is parallel at initial contact and lift off; and Trunk instability Tail consistently up
21	Coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance, consistent trunk stability; tail consistently up

sure paw rotation and dragging of toes during the late stage (Table 1).

Morphologic analysis of injured spinal cord

After 8 weeks behavioral testing, the rats were anethetized with pentobarbital sodium (100 mg/kg, i.p.) and perfused intracardially with saline followed by 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). Spinal cord tissue was removed from the vertebral canal. The cord was labelled to identify rostral-caudal orientation. A 4 cm segment centered on the injury site left in the fixative for an additional hour, and passed through graded alcohol to paraffin wax. The spinal cord specimen was then transected at right angles to its long axis through the site of maximal injury. Segments of spinal cord rostral and caudal to this point were imbedded in individual paraffin blocks. Sections 20 μm thick were made through the entire region of injury until normal appearing spinal cord was reached. All sections were numbered according to their distance from the point of cross-section through the epicenter. Histologic staining was carried out with luxol fast blue/hematoxylin and eosin. The lesion size of spinal cord was calculated using volumetric assessment developed by Finkelstein *et al.* (1990). Briefly, the epicenter (the site of greatest histologic evidence of injury) was determined for each spinal cord. And then cross-sections 750 μm and 1,500 μm above and below were identified. Line drawings of each section were made using projection microscope; areas (mm^2) of residual tissue were measured on a digitizing pad (Summa Sketch II) and computer program (BQ Intro, R & B Biometrics, Inc.). Attention was directed only to residual tissue.

Morphometric analysis was performed on tissue sections from the epicenter, 750 μm rostral and caudal, 1,500 μm rostral and caudal, and normal spinal cord rostral and caudal, respectively. To normalize for variations in spinal cord diameter, the amount of residual tissue was expressed as a percentage of the theoretical normal cross-sectional area. Based the geometry of right circular cone, the following formula was used to calculate

$$A(x)=[\frac{x+x_2}{x_1+x_2}(\frac{A_1}{A_2}-1)+1]^2A_2$$

theoretical normal cord areas at the epicenter and at points 750 μm and 1,500 μm rostral to the epicenter where x_1 and x_2 are rostral and caudal distances from the epicenter to the cross-sections of normal tissue with circular areas A_1 and A_2. The variable x represents the distance from the epicenter that will equal 0 μm, 750 μm, or 1,500 μm. Areas caudal to the epicenter may be found using the above formula with A_1 and A_2 interchanged and x_1 and x_2 interchanged. The amount of residual tissue of each group was expressed mean $\pm$ SEM of the percentage of normal area at the epicenter or the percentage of normal areas at the five levels.

Statistical analysis

Locomotor scores of hindlimb function were compared with the Wilcoxon test. For comparison of percent of normal areas of residual tissue, ANOVA was used.

RESULTS

Spinal cord contusion significantly increased mean arterial pressure (MAP). The hypertension was produced immediately after contusion and gradually recovered. Neither basal MAP nor spinal contusion-induced MAP change was affected by intraperitoneal DXT treatment (15 mg/kg or 30 mg/kg)(Table 2). Arterial blood gas values (pH, Po_2 and Pco_2) were not significantly affected by spinal contusion and DXT treatment (Table 3).

Table 2. Mean arterial pressure changes after dextrorphan (DXT) treatment and SCI

Treatment	n	Pretreatment	Pretrauma (10 min after treatment)	Peak after trauma
Saline	15	100±2.4	99±2.9	130±4.7*
DXT 15 mg/kg	10	103±3.8	101±4.5	132±5.8*
DXT 30 mg/kg	10	102±2.3	97±5.3	126±4.6*

MAP: mmHg

*Asterisk indicates significant difference compared to corresponding pretrauma value *$p<0.01$.

Table 3. Arterial blood gas values on 5 min before and after trauma from saline- and dextrorphan (15 mg/kg or 30 mg/kg)− treated rats

Saline-Treated (n=15)						Dextrorphan-Treated (n=20)					
before			after			before			after		
pH	Po_2	Pco_2	pH	Po_2	Pco_2	pH	Po_2	Pco_2	pH	Po_2	Pco_2
7.36±	80±	42±	7.35±	82±	41±	7.36±	81±	43±	7.35±	79±	44±
0.009	1.5	1.2	0.011	2.1	2.4	0.013	2.3	1.8	0.006	2.3	2.1

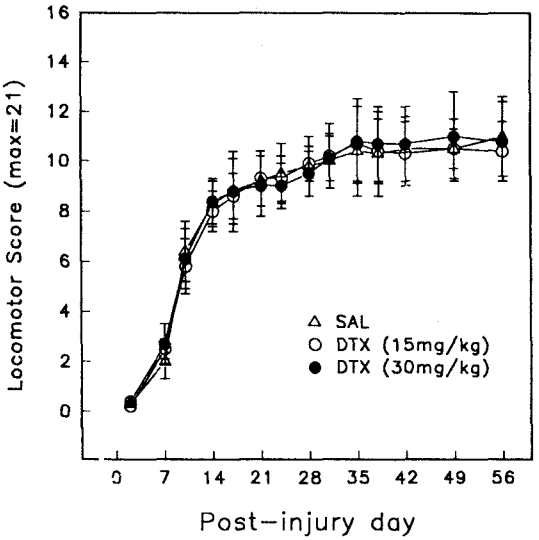

Fig. 1. Effect of intraperitoneal dextrorphan (15 mg/kg and 30 mg/kg) treatment compared to vehicle (saline) treatment, on open field test score over time after injury. Data points represent the average of 10~15 rats per group. Vertical bars are SEM.

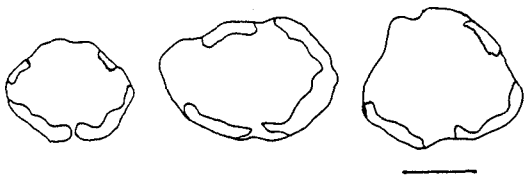

Fig. 2. Typical tracing of sections through the lesion epicenters. Sections show an incomplete rim of peripheral white matter. Scale bar; 1 mm.

Recovery hindlimb function

The rats injured spinal cord in the present study showed no hindlimb movement during first 2 or 3 days. The flaccid paralysis of hindlimbs was characteristic finding during this period. The animals then began to display slight isolated movements of one or two of the hindlimb joints. By the 7~10 days after injury, the animals were producing extensive simultaneous movements of the hip, knee and ankle. And then animals began to position the paw so that the plantar surface was in contact with the ground (plantar placement), to show weight support and abnormal stepping. Some animal showed abnormal but consistent stepping by the end of 4th week. After this period there were no more recovery of hindlimb function. Dextrorphan treatment either 15 mg/kg or 30 mg/kg did not improve the recovery of hindlimb function (Fig. 1).

Histologic results

The contusion epicenters were similar to those previously reported (Behrmann *et al.*, 1992; Bresnahan *et al.*, 1987). Briefly, an outer rim of white matter was spared surrounding a central core lesion consisting of cystic cavities and gliotic area (Fig. 2). The lesioned area of cord exhibited an elongated ovoid form with maximal tissue loss at the site of initial impact and rostral and caudal to the epicenter the lesion tapered. There were little remnants of the grey matter at the epicenters of lesioned spinal cord in the present study. The percentage of normal area at the epicenter and at the five levels (epicenter and 750, 1,500 μm caudal and rostral to the epicenter) showed no differences among the saline-and the dextrorphan-treated groups (Fig. 3).

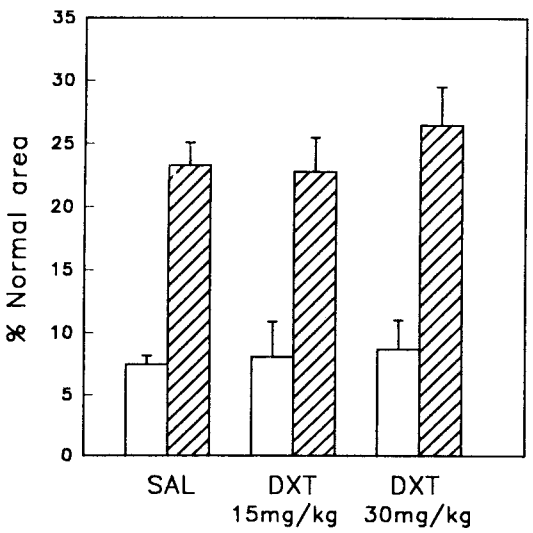

Fig. 3. Effect of dextrorphan (15 mg/kg and 30 mg/kg, i.p.)-pretreatment on morphometric measures of the spinal cord lesion at 8 weeks after injury. Each column shows mean of 10 animals, vertical bars are SEM. Open columns show % residual area at epicenter section. Hatched columns show mean % residual area of 5 cross sections. See the text for details.

DISCUSSION

The present study utilized a standardized experimental spinal cord injury model in the rat which is a modified weight-drop method. This model has been well characterized in terms of biomechanics (Gruner 1992; Huang and Young, 1994) as well as behavioral and histopathological (Basso *et al.*, 1994) outcome measures. Preliminary experiments using this device showed good correlationship between injury intensity (height) and behavioral and histopathologic outcome (unpublished observation). In the present study behavioral and histological results showed low variability between individual animals, which indicated this model's stability (Fig. 1, 3). The arterial blood gas values (pH, Po_2, Pco_2) were not significantly changed by the spinal cord contusion and the administration of dextrorphan in the present study (Table 3). These results indicated that change of blood chemistry did not affect the behavioral and histologic results after injury.

The open field test utilized in this study have been reported as a sensitive and reliable assessment tool especially to precisely evaluate pharmacological treatments after spinal cord injury (Basso *et al.*, 1994).

Histopathological analysis in present study were performed at the epicenter as well as multiple sections above and below the epicenter. The morphometry at the multiple cross-sections showed greater statistical corelation with functional outcome than that at the epicenter section (Finkelstein *et al.*, 1990).

Dextrorphan is a dextrorotatory morphinan and a noncompetitive NMDA receptor antagonist (kemp *et al.*, 1987; Steinberg *et al.*, 1991). This drug has high possibility for clical use in ischemic CNS injury (Albers *et al.*, 1991).

The slow development of histopathologic changes and lengthy period required for stabilization of the lesion (Allen 1914; Faden and Vink, 1989; Noble and Wrathall 1989; Panter and Faden, 1992) have suggested that secondary autodestructive injury processes exacerbate the effects of initial mechanical insults after traumatic SCI. Several lines of evidence suggest that EAA may be involved in traumatic SCI. EAA play an important role in the normal functioning of spinal cord (Headly and Grillner 1990). Traumatic SCI significantly increased local level of the EAA, glutamate and aspartate (Painter *et al.*, 1990; Liu *et al.*, 1991). The selective antagonists of the NMDA-receptor significantly reduce some of the consequences of experimental SCI. Faden and Simon (1988) initially found that MK-801, a noncompetitive NMDA antagonist, significantly reduced hindlimb functional impairments at 4 weeks after injury. However the behavioral effects were not associated with tissue sparing at the epicenter (Faden *et al.*, 1988). Gomez-Pinella *et al.* (1989) demonstrated that MK-801 improved function in some behavioral test (locomotion) but not others (inclined test, withdrawal reflexs) and there were no significant effect on lesion length or volume. The discrepancy of the functional and the histological results from above studies raised questions about involvement of NMDA receptor in secondary injury processes after traumatic SCI.

In the present study intraperitonal administration of dextrorphan in the doses of 15 mg/kg or 30 mg/kg affected neither the recovery of hindlimb motor function nor lesion volumes at 8 weeks after SCI (Fig. 1, 3). The doses of dextrorphan have shown maximal protective effect on focal ischemic insults in the rat (Xu *et al.*, 1994).

Recently, Wrathall *et al.* (1994) reported that a non-NMDA receptor antagonist, NBQX, dose-dependently reduced tissue loss and functional impariment after spinal cord trauma and suggested that non-NMDA receptor may be major participant in secondary injury process after SCI.

These result suggested that NMDA receptor might not be involved in secondary injury processes after SCI.

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=국문초록=

척수신경손상에 대한 흥분성 아미노산 수용체 길항제의 효과에 대한 연구

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외상성 척추신경손상후에 병리조직학적 변화가 서서히 일어나며, 손상부위가 안정화되는데 걸리는 시간이 길어서 2차 손상과정이 최초의 기계적 손상을 악화시킴이 오래전부터 잘 알려져 있다. 이러한 2차 손상과정의 개입은 척추신경손상을 약리학적으로 조절하는 시도에 대한 논리적 배경을 제공하고 있다.

흥분성 아미노산은 척수의 정상기능뿐아니라 허혈성 및 외상성 손상에 의한 세포사망에 관여함이 알려져 있어 외상성 척수손상의 2차 손상과정에 이 흥분성 아미노산 수용체가 관여할 것임이 시사되고 있다. Dextrorphan은 화학적으로 우선성 morphinan계통의 약물로 흥분성 아미노산 수용체의 하나인 NMDA 수용체의 선택적 길항제이고 임상적으로 사용가능성이 조사되고 있는 약물이다. 본 연구는 dextrorphan의 처리가 쥐의 척수신경손상후에 나타나는 후지운동기능의 회복과정 및 조직학적 변화에 어떻게 영향을 미치는지를 관찰하여 척수손상 후 일어나는 2차 손상과정에 NMDA 수용체의 개입여부를 확인하고자 하였다.

후지운동기능 회복은 open field test (21 point scale)를 사용하였으며 조직학적 손상의 크기는 손상 8주후에 정량적 조직병리학 방법을 사용하였다. 성숙 자성 Long-Evans 쥐 (무게 250~300 gm)를 pentobarbital sodium 마취 (복강내, 40 mg/kg)하에서 9번째 및 10번째 흉추부위의 추궁절재술을 시행하여 10 g 무게의 막대를 25 mm의 높이에서 노출된 경막에 떨어뜨려 척추손상을 유발하였다. 대조군 (n=15)은 생리식염수를 약물투여군 (각 n=10)은 dextrorphan 15 mg/kg, 30 mg/kg을 척추손상유발 15분전에 복강내로 투여하였다.

Dextrorphan 처리는 척수손상에 의한 동맥압 변동 및 동맥혈액 가스치 (pH, Pco_2, Po_2)에 영향을 미치지 않았다. 척수손상 2일부터 open field test를 시작하여 일주일에 두번씩 술후 8주간 시행하였다. 첫 2~3일 후에는 후지운동을 전혀 관찰할 수 없었으며 그후 2주일까지 약간 빠른 회복을 보여 후지관절의 운동을 보이고 몸무게를 지탱하였으며 4~5주에는 일부 동물에서는 비정상적인 걸음을 보였다. 그 이후에는 더 이상의 후지운동기능의 회복은 볼 수 없었다. Dextrorphan 투여는 이러한 후지운동회복기능 뿐 아니라 조직손상의 크기에도 전혀 영향이 없었다.

이상의 결과는 NMDA 수용체는 외상성 척추손상 후 일어나는 2차 손상과정에 관여하지 않음을 시사하고 있다.