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A rat model of neurolathyrism: repeated injection of L-β-ODAP induces the paraparesis of the hind legs

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Summary. Neurolathyrisim is a motor neuron disease characterized by spastic paraparesis in the hind legs, and is caused by grass pea, *Lathyrus sativus*, which contains the excitotoxic amino acid, 3-*N*-oxalyl-L-2,3-diaminopropanoic acid (L-β-ODAP), an α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-type glutamatergic receptor agonist. In an attempt to make a useful model of this disease, the CNS distribution and toxicity of L-β-ODAP was studied in rat neonates after parenteral administration. L-β-ODAP was detected in the spinal cord as well as in the pons/medulla oblongata, though only small amounts in the latter. Repeated injection of L-β-ODAP resulted in rats with paraparesis of the legs, though at a low incidence rate of 0.032. These paralyzed rats displayed the severe atrophy of the ventral root of the lumbar cord as well as degenerations of motor neuron. The rats were useful models for the study of motor neuron degeneration in the spinal cord.

Keywords: Neurolathyrism – L-β-ODAP – Spinal cord – Excitatory amino acids – Neurotoxin – *Lathyrus sativus*

Abbreviations: CNS, central nervous system; PBS, phosphate buffered saline; L-β-ODAP, 3-*N*-oxalyl-L-2,3-diaminopropanoic acid

Introduction

Neurolathyrism is an upper motor neuron disease caused by the overconsumption of grass pea seeds (*Lathyrus sativus L.*) (Spencer et al., 1993; Hugon et al., 2000). Symptoms of this disease include severe and irreversible paraparesis of the hind legs (Ludorph et al., 1996). The cause of this disease is 3-*N*-oxalyl-L-2,3-diaminopropanoic acid (L-β-ODAP, or another abbreviation of L-BOAA), a non-protein amino acid which is biosynthesized in this species through a specific pathway (Ikegami et al., 1991; Ikegami et al., 1999). It is well known that this compound is an AMPA-type glutamatergic receptor agonist, and exerts its toxicity through the action on this receptor (Brigs et al., 1988; Kusama-Eguchi et al., 1996).

In vivo studies using experimental animals to analyze the toxic action of L-β-ODAP or grass peas have been performed using guinea pigs (Jahan et al., 1993; Amba et al., 2002), monkeys (Lakshmanan et al., 1977; Rao 1978), and chicks (Shamim et al., 2002). In these studies, exogenous L-β-ODAP showed various toxicities to the CNS including convulsions, retarded motor ability, and strange behavior such as rotation. In monkeys, when given intrathecally it caused paraparesis (Rao et al., 1967).

In an attempt to develop a more useful rodent model to study the pathogenesis of neurolathyrism, we studied the distribution of L-β-ODAP in five CNS areas of rat neonates after subcutaneous injection, because the toxin would be attainable to the CNS around these periods to a detectable level. We then treated them with repeated injections of L-β-ODAP to make crippled rats. In fact, as shown below, this resulted in rats with spastic paralysis in the hind legs. The cases that showed obvious defects in the hind legs were limited in number because of death due to the toxicity of L-β-ODAP, and some due to unknown reasons. However, their behavior resembled that of humans showing paraparesis in the hind legs. Anatomical analysis of the rats revealed that they had severe damage to the spinal cord, especially the anterior horn. This is the first case showing that only 6 continuous subcutaneous injections produced animals defective in motor function that resembles human neurolathyrism. If the number of cases was larger, and the analysis was done while the

symptoms were developing, it would be quite useful for the study of neurolathyrism. It would also be useful to analyze drugs that prevent or block the development of motor diseases like neurolathyrism.

Materials and methods

Chemicals

3-*N*-oxalyl-L-2,3-diaminopropanoic acid (L-β-ODAP) was obtained from Tocris Cookson Ltd., and later obtained from Lathyrus Technologies, Hyderabad, India. All other reagents were of reagent grade and purchased from Wako Pure Chemicals Inc., Tokyo, Japan.

Animal treatment

Wistar/ST clean rats were purchased from Japan SLC Inc., and kept in a standardized animal care facility (room temperature 23 ± 1°C, humidity 50%). The handling of the rats and all procedures performed were approved in accordance with the Principles of Laboratory Animal Care (NIH publication no. 86–23, revised 1985). For the distribution experiment, rat neonates within 20 hr of birth were injected subcutaneously with 400 mg/kg of L-β-ODAP in the skin at the back or at the side of the body using a 29G needle, and their behavior was observed. Rats were sacrificed by sudden decapitation at 5, 15, and 60 min after the treatment. Blood samples were directly taken from the injured neck surface. For repeated treatment, male rat neonates within 20 hr of birth were injected with 200 mg/kg of 25 mg/ml L-β-ODAP subcutaneously as above. They were fed with their mother rats a month, followed by a separate cage with littermates (control rats). Their body weights were measured daily, and behaviors were observed as well. They were sacrificed 13 weeks after the treatment.

Amino acid analysis

Brain tissue was removed, and was divided into five areas; cerebral cortex, thalamus/hypothalamus, pons/medulla oblongata, rostral half of the spinal cord, and caudal half of the spinal cord. Tissues were weighed, rinsed with PBS, blotted and homogenized with 3 volumes of 5% perchloric acid at room temperature using a Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at $2{,}100 \times g$ for 10 min, and the supernatant was collected. Samples were lyophilized, dissolved in 0.02 *N* HCl, and analyzed using an automatic amino acid analyzer (Hitachi: model 835) after filtration (0.45 μm) as previously described (Murakoshi et al., 1984).

Preparation of organ and tissue samples

Rats were raised with normal diets and water ad libitum until use. On the day of sacrifice (13 weeks after the last treatment day), they were anesthetized with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital. From their cardiac ventricule, 50 ml of 50 U/ml heparin, 100 ml of PBS, followed by 250 ml of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) were perfused. Tissue samples were additionally fixed by soaking in 4% paraformaldehyde for 24 hr at 4°C.

Statistical analysis

The body weight data were analyzed by one-way analysis of variance (ANOVA) followed by paired *t*-test. *P* values smaller than 0.05 were considered to be statistically significant.

Results

Distribution of L-β-ODAP in the CNS of rat neonates

Rat neonates within 20 hr of birth were injected subcutaneously with 400 mg/kg of L-β-ODAP in 25 mg/ml solution. After a few minutes, they showed severe convulsions of the limbs. In some cases, they showed hyperactivity with vocalization. The distribution of L-β-ODAP was examined from the CNS samples of the rats by perchloric acid extraction of the tissue. Figure 1A shows the change in the L-β-ODAP concentration in the serum ($n = 6$). The concentration of L-β-ODAP increased abruptly after injection, and reached a level as high as *ca*. 1 mM in 60 min. Figure 1B shows the distribution of L-β-ODAP in five portions of the neonate CNS 15 min after subcutaneous injection ($n = 4$). The concentration of L-β-ODAP in the CNS was quite low compared to the serum. By amino acid

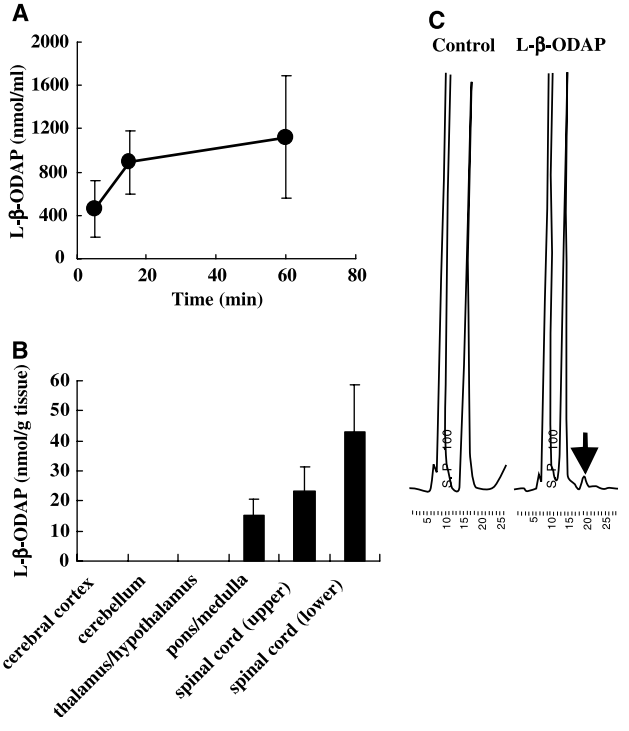

Fig. 1. Distribution of parenteral L-β-ODAP in rat neonates. Wistar rats within 20 hr of birth were injected subcutaneously with 400 mg/kg of L-β-ODAP. Rats were sacrificed at 15, 30 and 60 min., and the CNS and blood samples were subjected to amino acid analysis as described in Materials and methods. **A** Time course of L-β-ODAP concentration in the serum. **B** Content of L-β-ODAP in cerebral cortex, diencephalon (thalamus and hypothalamus), pons/medulla, spinal cord (rostral half), and spinal cord (caudal half) 15 min after subcutaneous injection. **C** Profile of the amino acid analysis of the caudal half samples of the spinal cord. The arrow on the right (L-β-ODAP treatment sample) shows the position of L-β-ODAP elution that was confirmed by the authentic sample (not shown)

analysis, no L-β-ODAP was found in the cerebral cortex, diencephalon, or cerebellum. In the lower part of the CNS, however, trace levels of L-β-ODAP were found in extracts of the pons/medulla oblongata, where the two regions were taken together. In spinal cord samples, L-β-ODAP was separated as a distinctive small peak of the HPLC profile indicated by the arrow (Fig. 1C). The spinal cord was divided into two portions; the cervical and most of the thoracic cord were included in the 'rostral half', and the rest of thoracic cord, lumbar cord, and sacral cord were included in the 'caudal half'. Much more was found in the spinal cord, especially in the caudal half.

Occurrence of rats with hind-leg paralysis after a repeated L-β-ODAP treatment

To make a rodent model of human neurolathyrism, rat neonate males within 20 hr of birth were treated with L-β-ODAP subcutaneously. Commercial L-β-ODAP at 200 mg/kg resulted in potent acute toxicity. As shown in Table 1, as many as 34.8% of the neonates died within the treatment period, or were killed because of cannibalism by their mothers. Interestingly, a limited numbers of rats grew up with their hind-legs completely paralyzed. Figure 2 shows one of these cases. The incidence rate was three out of 92 rats (3.26%). Interestingly, one of the three handicapped rats showed defects mainly on one side of the hind-legs (monoplegia). In addition, at least 4 additional rats showed similar, bilateral paralysis during and just after the treatments. However, some of them died afterwards, and others recovered from their handicaps. The incidence of the latter 'reversible' or 'incomplete defect' case was 4.34% (Table 1). The rats of

Table 1. The incidence of hind-leg paralysis in Wistar/ST rats treated with L-β-ODAP. Wister/ST newborn male rats within 20 hr of birth were injected subcutaneously with 200 mg/kg of L-β-ODAP once daily for 6 days. Rats were raised normally until sampling. They were classified into irreversible paralysis, reversible paralysis, no sign of paralysis of hind legs, and dead

Treatments	Control		L-β-ODAP	
Condition	Number	Incidence (%)	Number	Incidence (%)
Irreversible paralysis	0	0	3*	3.26
Reversible paralysis	0	0	4	4.34
No sign of paralysis	42	93.3	53	57.6
Dead**	3	6.66	32	34.8

* One case was monoplegia. The other two were paraplegia
** Including rats killed by cannibalism

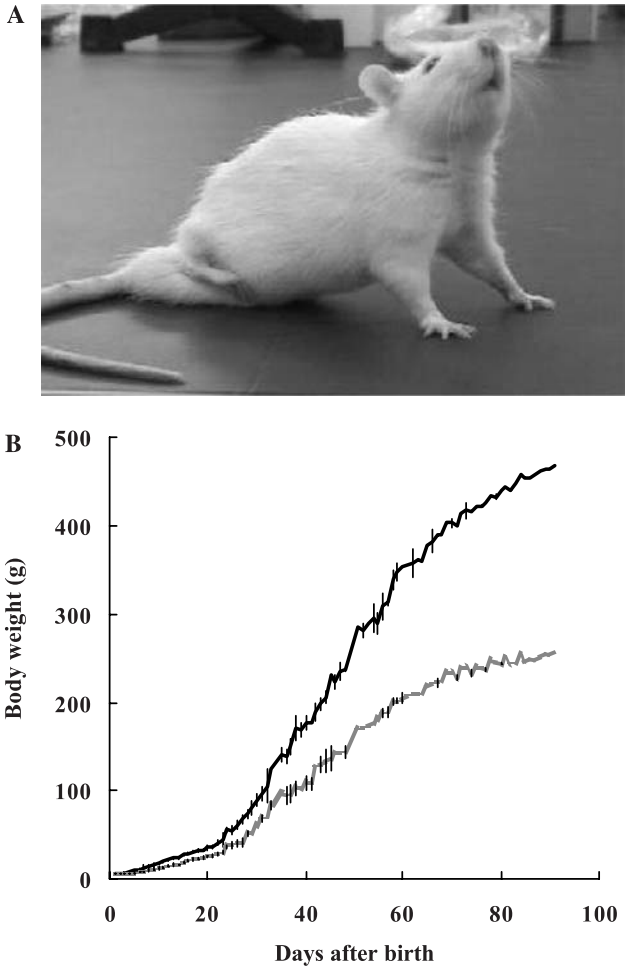

Fig. 2. An example of a neurolathyrism model rat. **A** A Wistar rat was treated with 200 mg/kg of L-β-ODAP for 6 continuous days. It was fed for a month with its mother rat followed by the separate lodging with its littermate (control rat). At this time, it was 5 weeks old after the treatment. Note that both hind legs were completely paralyzed, and it walked with forelimbs. This handicap persisted until it was sacrificed. **B** Change in body weight of the control ($n = 5$) and hind-leg paralyzed rats ($n = 3$). Data are shown as the average ± S.E.M. The value of the treated group was significantly different from that of control ($p < 0.05$)

irreversible defect in hind-legs showed no sign of incapabilities in forelimbs that enabled them to crawl with forelimbs quickly. The paraparesis rats were brought up until 13 weeks after the last day of injections. Their growth curve is shown in Fig. 2B. They all showed growth curves with lower body weights being almost half that of control rats. The difference was significant from the day 5 of the treatments to the last period ($p < 0.05$).

Gross anatomy of paralyzed rat

A hind leg-paralyzed rat was sacrificed by bleeding and fixed by paraformaldehyde perfusion from the left

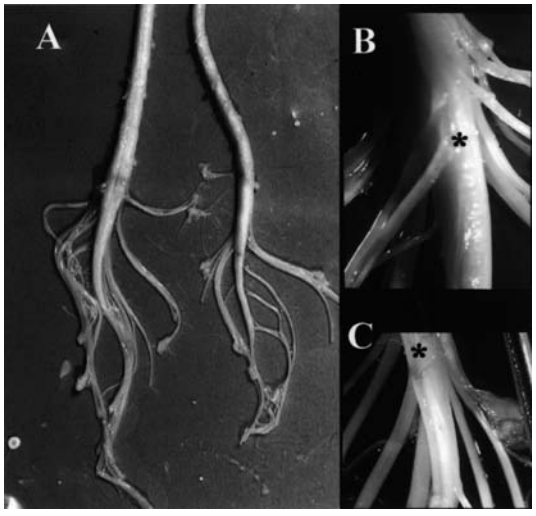

Fig. 3. Spinal cord sample of control and the paraparesis rats. Thirteen week-old rats with typical paraparesis in hind legs and a control were sacrificed as described in Materials and methods. **A** Ventral view of the spinal cord of a control (left) and L-β-ODAP-treated rat (right). **B** A higher magnification side view of lower (lumbar to sciatic) part of the control sample. On the left there are anterior roots (asterisk), and on the right are posterior roots. **C** Similar magnification view to B of L-β-ODAP-treated rat. Note that almost no anterior roots (asterisk) are seen at the left side compared to the right side, i.e. the posterior roots

ventricle under anesthesia. The spinal cord of this rat revealed a marked difference from the control in the following points: The spinal cord was smaller in case of L-β-ODAP treatment (Fig. 3A, right); the anterior roots were so thin and fragile that they were almost invisible macroscopically as shown in Fig. 3C (asterisk) compared to the anterior root of control (Fig. 3B, asterisk), and the number of fibers of caudal equina was obviously scant (Fig. 3A).

Discussion

Neurolathyrism is caused by the overconsumption of grass pea seeds. In these peas, the toxic amino acid L-β-ODAP is sometimes contained at concentrations high enough to cause the motor neuron disease neurolathyrism, with spastic paraparesis in the legs. The grass pea is a drought-resistant plant, and is the only crop of high nutritive value that grows after the disastrous floods or drought. Epidemic occurrence of this disease has been reported recently in Ethiopia in 1997 (Getahun et al., 1999). Patients typically cannot walk without two sticks, and sometimes have to crawl. It is a serious problem because it prevents younger people from working in the fields or engaging other occupations.

Our goal was to develop an animal model of this disease to study the pathophysiological mechanisms of the disease. For this purpose, we first applied a single high dose to rat neonates to see whether the toxic amino acid went to the rat CNS, and to quantify it. As neonate rats have immature blood-brain barriers, analysis of the distribution of this toxin in the CNS was possible. As shown in Fig. 1, L-β-ODAP was distributed in the CNS in quite low levels. Among six divided areas, the highest concentration of L-β-ODAP was found in the caudal (lower) part of the spinal cord. The second highest was found in the rostral (upper) part, followed by a trace amount in the medulla oblongata/pons. Previous results using radioactive L-β-ODAP administered intrathecally to rhesus monkeys, or intraperitoneally to 10 day-old rats also showed that the highest radioactivity was found in the spinal cord (Lakshanan et al., 1977; Rao, 1978). These reports did not show identification of the compound by any method than radioactivity. Our present results clearly show that L-β-ODAP itself distributed to the spinal cord, especially in the caudal region. The distribution pattern should be the background of the specific toxicity of this compound to the spinal cord.

Interestingly, repeated treatment with synthetic L-β-ODAP at 200 mg/kg subcutaneously produced hind leg-paralyzed rats (Fig. 2). Only male rats were chosen for this experiment because in the case of humans, male patients develop neurolathyrism at a rate three times higher than females (Getahun et al., 2002). Therefore we speculated that more cases would occur in male rats. Two rats were completely unable to move hind legs or showed spastic paraparesis in which their legs were flexed beside the bodies. In another case, only one of the legs was paralytic similarly (monoplegia). There forelimbs, however, were moving normally that enabled them to crawl quickly. Previous results showed that guinea pigs that were treated with grass pea seeds being made deficient in vitamin C turned out to be lathyric (Jahan et al., 1993). In our case, no such nutritive condition was necessary, although the incidence was still quite low at 3.26% (one out of *ca.* 30).

The rats with hind leg paralysis did not show incontinence, indicating that the function of the Onuf's nucleus in the posterior horn of sciatic spinal cord was not impaired as is the case in amyotrophic lateral sclerosis (Mannen et al., 1977; Schroder et al., 1984). They showed similar seeking behavior as control or normal rats when put in a strange place, suggesting they did not have problems with the brain functioning. Though their body weights were lower, organ weights were not significantly different from controls when values were normalized by the body weight. However, some organs including adrenal, brain, and spinal cord tended to be bigger (adrenal) or smaller (the latter two, data not shown).

The most important changes were observed in the spinal cord. The anterior roots of the lumber cord were most prominent. The roots were quite thin and hardly visible macroscopically as shown in Fig. 3. Along with the lower weight and shorter size, the spinal cord tissue was atrophic, especially in the anterior horn motor conducting system. These observations were not seen in control rats. Therefore, it was clear that L-β-ODAP injected exogenously caused these defects. The tissue samples made from the spinal cords showed that in the anterior horn there was severe damage, with fewer motor neurons 13 weeks after the last injection (Kusama-Eguchi et al., unpublished results). The damage was similar, to some extent, to the previous reports that Nissl body was decreased, or innumerable vacuolizations seen in motor neurons (Ludorf et al., 1996). More precise analysis of tissue samples is currently under way.

In conclusion, the present report clearly showed penetration of L-β-ODAP in the lower part of the spinal cord of rat neonates. We also developed hind-leg paralyzed rats with repeated, subcutaneous injections. These results show that L-β-ODAP exerts toxicity in the spinal cord to produce irreversible, and sometimes reversible, degeneration in the anterior horn as well as in the anterior root of the spinal cord.

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