J.Sci.Soc.Thailand, 24(1998) 81-90

BEHAVIOURAL EFFECTS OF RHOMBENCEPHALIC CELL SUSPENSION TRANSPLANS INTO THE RAT AFTER CEREBELLAR LESIONING WITH KAINIC ACID

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(Received March 3, 1998)

ABSTRACT

The embryonic rhombencephalic tissue suspensions isolated from E15 stage rat embryos were injected into the cerebellar vermis of adult rats which had been lesioned with kainic acid 10 nM one week before transplantation. The result from the histological studies indicated that the grafted neural cells grow and differentiate into adult Purkinje cells, migrate into the host molecular layer and organize into a single cell layer to replace the Purkinje cells lost after kainic acid lesioning. The results from the behavioural studies indicated that the transplantation of the rhombencephalic cell suspension into the lesioned area in the cerebellar cortex can improve the motor deficits as shown by a decrease in the frequency of dysmetria and an increase in the frequency of normal rearing behaviour. At 3 months after transplantation, there was no significant difference in the frequencies of dysmetria and normal rearing behaviour between the transplantation and the control groups. However, when observed with neurological tests, the percent of correct response of the transplantation group was larger than the lesioned group, but still had a significance difference ($p<0.05$) from the control group. In summary, the embryonic rhombencephalic cell suspension can be functionally integrated into a host brain and restore the motor deficits caused by the lesioning in the cerebellum, and although this restoration does not reach normal levels, it is significantly different from the lesioned animals. The results from this experiment suggest that the open-field testing and the neurological examination are useful for evaluation of functional recovery as effects of the grafted tissues, with the neurological examination being more sensitive for detecting the functional effects of transplanted tissue than open-field testing.

INTRODUCTION

Damage to the central nervous system (CNS) usually result in severe deficits in cognitive and/or motor function and recovery from which is often incomplete. Transplantation of embryonic neurons into an injured area may provide a means of replacing damaged cells and restoring the original circuitry.[1] Several experiments have been reported, showing that the transplantated neuronal tissue can survive, differentiate and integrate into host brain circuitry.[2,3]

Cerebellum is a part of the brain which is suitable for studying the effects of transplantation because of it is well known in circuitry and function. Moreover, the successful transplantation of embryonic rhombencephalic tissue into host cerebellum previously lesioned with Kainic acid has been reported.[4] Morphological studied has been shown that transplantation of rhombencephalic tissue from E15 stage of rat embryos can grew, differentiated and become integrated into the host cerebellum.[4,16] However, it is difficult to conceive that true transplants that are anatomically integrated may also be functionally integrated with the brain of host animals. Lesions of the cerebellum can cause ataxia, dysmetria and intention tremor, thus it is interesting to observe whether the neuronal transplantation can restore the disabilities or not. The present study, a series of behavioural tasks composed of: 1) the open-field testing (which assesses rearing behaviour and dysmetria) and 2) the neurological examination (which provide the information of many levels of CNS dysfunction) were tested in order to provide further information on behavioural effects of rhombencephalic tissues transplantation in the rats.

MATERIALS AND METHODS

The experimental animals used in the present study were obtained from the breeding colony of the National Experimental Animal Center, Mahidol University, Salaya Campus. Eigth weeks old Wistar Albino strain rats were used as hosts for the transplantation. Embryonic brain tissues were obtained from E15 stage fetuses removed from pregnant females. The animals were divided into three groups; 1) Unoperated rats (n=12) which represents a normal control group. 2) Kainic acid lesioning without transplantation (n=12), which represents a lesioned control group and 3) Kainic acid lesioning with transplantation (n=14) which represent a transplantation group.

Lesioning with Kainic acid

Animals in the lesioned and transplantation groups were anesthesized with nembutal (ip, 50mg/kg) and placed in a David-Koff stereotaxic frame for rats. Kainic acid (KA) 10 nM in 0.9% sterile saline was injected via a Halminton syringe at a rate of 1 μl/min into the vermis of the cerebellum between lobe V - VII. The coordinates are: posterior 5.0 mm to interaural line in the midline, to a depth of 6.0 mm, according to the stereotaxic atlas for rat.[5] After an initial 1 week survival time, animals in the transplantation group were transplanted with an injection of embryonic rhombencephalic tissues.

Transplantation

The preparation of the cell suspension was done as described by Bjorklund *et al.*, 1983.[6] The E15 stage embryoes were removed from pregnant females after caesarian section. The rhombencephalic lip, a part of the brain which will develop into the cerebellum, was dissected and collected in a glass petridish containing the basic medium (0.6% D-Glucose in 0.9% sterile saline) at room temperature. Tissues were then incubated with 300-500 μl of 0.1% crude trypsin solution (Sigma type II) for 20 minutes at 37°C. Following incubation, the trypsin was removed by washing 4 times with 400-500 μl of fresh basic medium and finally brought to a volume equivalent to 10 μl per tissue pieces with basic medium. The tissue pieces were then dissociated into a cell suspension by repeated pipetting through a pasteur pipette. The resulting milky fluid, was monitored microscopically for cell viability by staining with tryphan blue. Viable cell counts typically ranged from 30,000 to 40,000 cells per μl of suspension. From the known cell densities, a small volume of basic medium fluid containing 100,000 cells (approximately 3 to 5 μl) was used for each transplantation.

Behavioural Testing

Animals in each group were tested on a series of behavioural tasks and compared with the other groups. The lesioned group was tested at 1, 2 and 3 months after lesioning while the animals in the transplantation group were tested at 1, 2 and 3 months after transplantation. The following behavioural measures were employed; 1) an open-field tests which assesses rearing behaviour and dysmetria, and 2) a neurological examination to provide the information on the function and integrity of a number of levels of the CNS.

Open-field. This test is employed to observed general activity as well as central nervous system dysfunction. The apparatus consists of an open box with plexiglas sides and a floor marked out into 10x10 cm squares. Frequency of dysmetria and normal rearing behaviour are determined. Observations are recorded every minute for 4 five minutes sessions in each experiment.

Neurological examination.[7] This test consists of 10 separate categories which provide information on the function and integrity of many levels of the CNS. The general procedure for each of the test is to present a test stimulus and observe the reaction. In scoring for a correct response, a 1 is put on the score sheet; if no response or an inappropriate response is emitted, a 0 is used. The individual tests, manner of administration and appropriate responses on the neurological examination are as follows:

Flexion reflex : the rat is picked up and the toes are pinched with forceps. The response is to move the foot away; ***Grasping reflex*** : the rat is picked up and the palm is touched with a wire. The response is to grip the wire; ***Righting reflex*** : when the rat is put on its back, it turn over immediately ***or*** the animal is held in the lower back and when the body is tilted, its head moves opposite; ***Placing reaction*** : put the rat on the table with a leg hanging over the edge of the table, it will placed back on the table ***or*** the rat is held by the tail over the table until its whiskers get near, the paws are then put on the table; ***Equilibrium test*** : the rat is placed on a narrow bar, it should be able to stay there for approximately 3 minutes; ***Corneal reflex*** : the eye of the rat is examined in the dim light, when a bright light is turned on, the pupil should constrict; ***Auditory startle*** : when the rat is quite and calm, give a lound hand clap; it should extend all limbs and arch the body and look startled; ***Toes spreading*** : the rat is put on a piece of plexiglas over a mirror and the plexiglas is tilted, the rats spreads the toes; ***Head shaking*** : when a puff of air is blown through a rubber tube to the ear, the rat shakes its head; ***Elevated walkway*** : the apparatus consists of the walkway 1.3 cm in width, 1 m in length and elevated 80 cm off the ground. Food pellets are used at the end of the runway for a reward. Behaviourals observed are general locomotor capacity and the number of falls. If the rat walks with general locomotor capacity and does not fall, the score 1 is used. If the rat walks with abnormal locomotor capacity such as ataxia and fall down during walking, then the score 0 is used.

All datas from the behavioural testing were statistically analysed by the INSTAT microcomputor program, by using the Tukey-Kramer multiple comparisons test in order to check for variation among the columns.

Morphological studies

Animals in lesioned and transplantation groups were perfused intracardiacally with 0.9% normal saline in order to wash away blood, followed by a fixative containing 1% paraformaldehyde combined with 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH7.4. The brains were removed from the skull, dehydrated through a series of graded alcohol and embedded in paraplast. Sections of 15 μm each were cut in the parasaggital plane, and were stained by Nissl's and Bodian's method. The slides were mounted and examined under an Olympus research model microscope.

RESULTS

Morphological observation

Kainic acid lesioning

Kainic acid (10 nM) was injected into the cerebellar vermis of the rat between lobes V and VII as shown in Figure 1. In the majority of cases, the lesions were confined within the cerebellar vermis region and did not extend into the fourth ventricle and the brainstem. One week after lesioning, there was a necrotic area around the center of the injection site, in which all neuronal cell types were lost. In contrast, at the peripheral of the injection site, total degeneration and disintegration of the Purkinje were observed as represents by the vacuolations at the junction between the granular cell layer and the molecular cell layer (Figure 2A), while the granule cells remain intact. In all cases, the deep cerebellar nuclei were still present, although reduced in size and ectopic. The nodulus and paraflocculi were also remain intact in all instances.

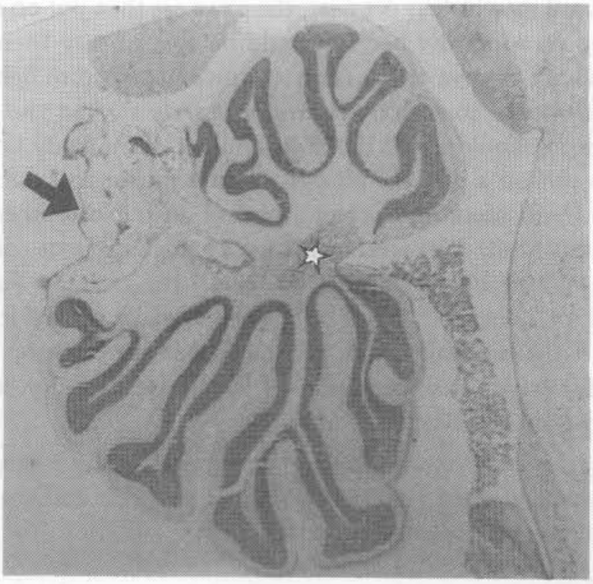

Fig.1 Photograph of midsaggital section of the cerebellum at one week after exposure to Kainic acid 10 nM. The arrow point to the cavities around the lesioned area between lobes V-VII in the vermis region. Note that the deep cerebellar nuclei (☆) are still persent.

Transplantation

Observation indicated that cells from the rhombencephalic tissue suspension survived and developed in host cerebellum that had been one week previously lesioned with kainic acid. Neuroblasts from the grafted tissues were able to grow and gradually differentiate into mature Purkinje cell-like neurons, migrating into the host molecular layer (Fig.2B). At 4 weeks after transplantation, the transplanted neurons had pear-shaped cell bodies, big round nuclei, dense basal cytoplasmic staining than at the apical process which is the typical morphology of the adult Purkinje cell.

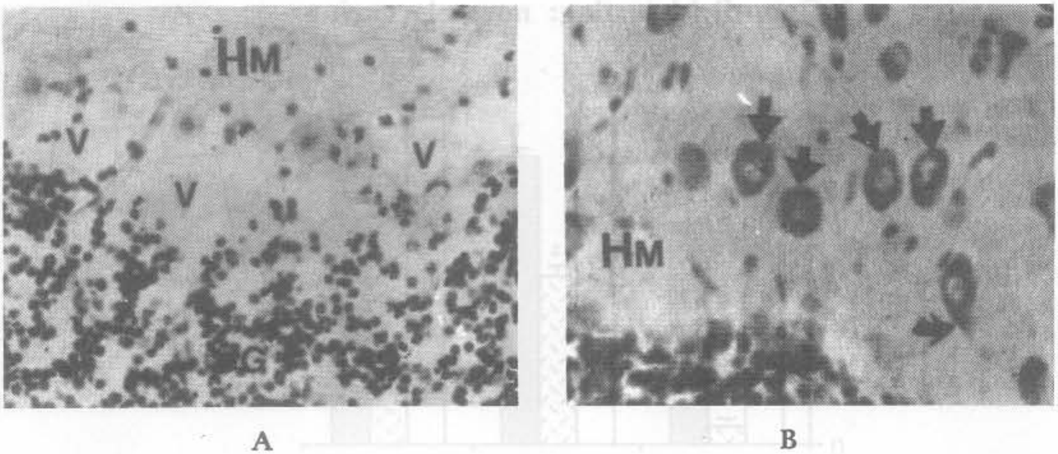

Fig.2 A) Parasaggital section of the cerebellar cortex of the rat (Nissl's stain) after exposure to 10 nM Kainic acid. The area marked by "v" are vacuolations which represent the loss of Purkinje cells at the peripheral of the lesion area. B) Photomicrograph of grafted Purkinje cells (arrow) at 1 month after transplantation (Bodian stain) which migrated into the host molecular layer and organized into a single cell thick laminar. HM=host molecular layer.

Behavioural observations

Kainic acid lesioned group

One month after lesioning with KA 10 nM, animals showed a decrease ($P<0.001$) in the frequency of normal rearing and increase ($P<0.001$) in the frequency of dysmetria, when compared to the normal control group (Fig.3 and 4). However, when observed at 2 and 3 months after lesioning, we found an improvement in the lesioned animals as represented by a decrease in the frequency of dysmetria and an increase in the frequency of normal rearing behaviour. At the end of the third month, the lesioned group still had a motor deficit as shown by a significant difference in the frequencies of dysmetria and normal rearing behaviour, when compared to the control group. The results from the neurological examination also supported the results from the open-field observations. One month after lesioning with KA, the animals performed worse on the neurological examination than the control group (Fig.5). The percent correct response of the lesioned group was less than the control group ($P<0.001$). When observed at 2 and 3 months after lesioning, the animals performed the neurological tests better than at 1 month after lesioning. Although the percent of the correct responses in the lesioned animals observed at 2 and 3 months post lesioning was more than when observed at 1 month post lesioning as shown in Fig.5, it still did not reach normal levels when compared to the control group.

Transplantation Group

Transplantation of rhombencephalic cell suspensions can improve the motor deficits in Kainic acid lesioned animals, when observed with both open-fields testing and on neurological examinations.

One month after transplantation, these experimental animals showed a decrease ($p<0.001$) in the frequency of dysmetria and an increase ($p<0.001$) in the frequency of normal rearing behaviour when compared to the lesioned group (Fig.3 and 4). The transplantation group also performed better neurological tests than the lesioned group (Fig.5), Although this improvement still did not reach normal levels at this period.

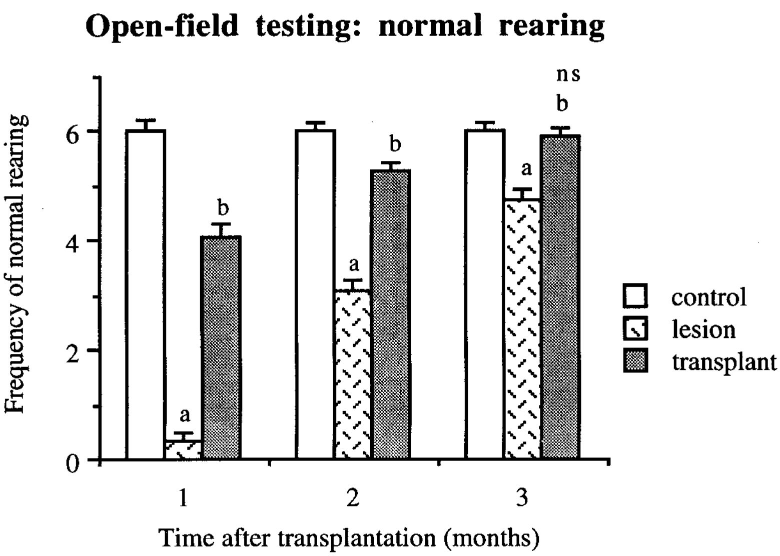

Fig.3 The bar graph show the frequency of normal rearing, compared between the control (n=12), lesioned (n=12) and transplantation groups (n=14) at 1,2 and 3 months after the experimental procedure. The values represent mean±SEM.

a = $p<0.001$ compared to the control group.

b = $p<0.001$ compared to the lesioned group.

ns = not significantly different from the control group.

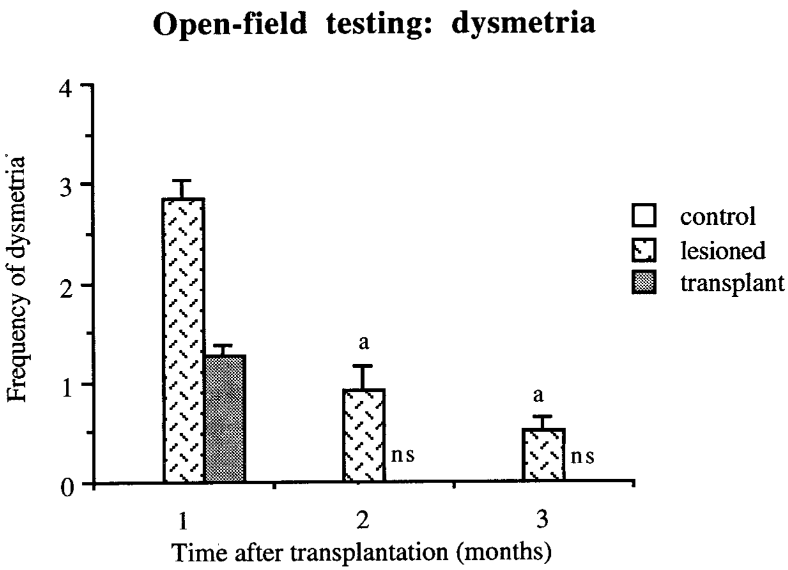

Fig.4 The bar graph shows the frequency of dysmetria compared between the control (n=12), lesioned (n=12) and transplantation groups (n=14) at 1,2 and 3 months after the experimental procedure. Rats in control group did not show dysmetria throughout the study. Transplantation rats show a reduction in frequency of dysmetria to the normal level at 2 and 3 months after transplantation. The values represent the means ±SEM.

a = $p<0.001$ compare to control and transplantation group.

ns = not significant difference from control group.

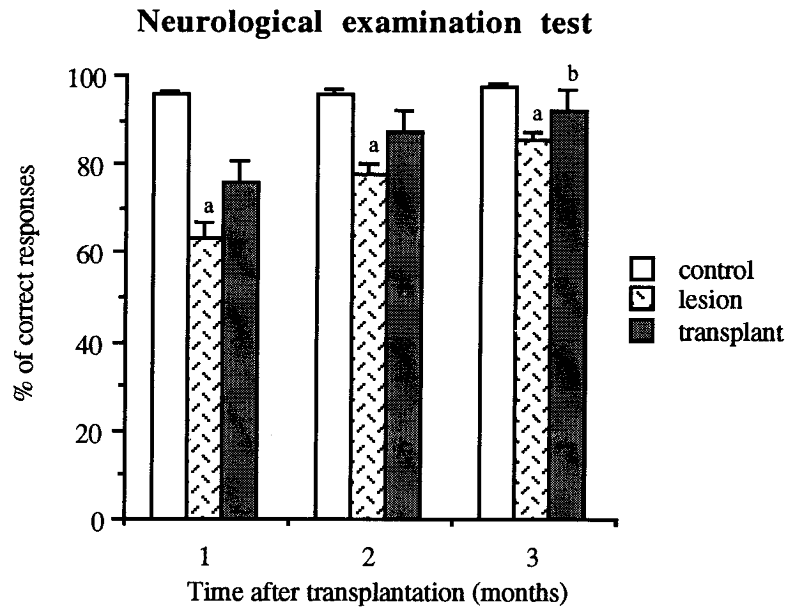

Fig.5 The bar graph shows the percent of correct responses on the neurological examination compared between the control (n=12), lesioned (n=12) and transplantation groups (n=14) at 1,2 and 3 months after the experimental procedure. The values represent the means±SEM.
a = $p<0.001$ compared to the control and transplantation group.
b = $p<0.05$ compared to the control group.

Two and three months after transplantation, the animals in these groups showed a significant improvement in motor functions when observed, both with the open-fields testing and on the neurological examination. The improvement nearly reached the normal levels by the end of the third month after transplantation. During this period, there was no significant difference in the frequency of dysmetria and in the frequency of normal rearing behaviour between the transplantation group and the normal control group. The transplantation group also showed a decrease ($p<0.001$) in the frequency of dysmetria and an increase ($p<0.001$) in the frequency of normal rearing behaviour when compared to the lesioned group, as shown in fig.3 and fig.4. The transplantation group also showed an increase in the percent of correct responses on the neurological examination, comparable to the normal control group, although this still did not reach normal levels on this period. Fig.5 shows that the percent of correct responses on the neurological examination from the transplantation group is still different ($p<0.05$) from the control group, however, it is still significantly higher ($p<0.001$) than in the lesioned group.

DISCUSSION

Several experiments have beeen reported that transplanted neuronal tissues can survive, differentiate and integrate into host brain circuitry.[8,9,10,11] These experimental data suggest that the efferent connections from the grafted to innervate the host brain could occur to the same extent as receiving afferent connections from the host brain. Previous studied in cerebellum, reported that, rhombencephalic tissues suspension can survive, differentiate and integrate into host cerebellar cortex which had been lesioned with either kainic acid or acrylamide.[4,16] Many attempts try to evaluate the long term effects of cerebellar transplantation by using open-field testing and balance rod testing as an indicator for the improvement of motor function.[17-18] In order to observe functional integration of transplanted tissues in to host cerebellum, the open-field testing together with the neurological examination were used in the present studies. There are still not have the evidence for evaluation of motor function after cerebellar transplantation by using neurological examination. Neurological examination consisted of 10 separate categeries which provide more details and informations of the functional integrity of many levels of the CNS. Several categories of the neurological examination provide information on the motor function which suitable for evaluate cerebellar function such as; elevate walkway, equilibrium test (the same as balance rod testing), righting reaction and placing reaction. Other categories of this test also benefit and allow us to make sure that the lesion was confined with in the cerebellum and not extend to the other brain areas, such as; corneal reflex, grasping reflex, flexion reflex, toe spreading and head shaking. Abnormal of these reflexs indicate that the lesioned may extend in to the brainstem structure which locate near the cerebellum. Thus, neurological examinations are suitable not only for localization of the lesion, but also for evaluation of the disturbance and recovery of motor function after cerebellar transplantation.

Kainic acid 10 nM injected into the vermis of the cerebellum results in the total loss of Purkinje cells while granule cell remain intact at the peripheral of the injection site. One week later, in order to replacing the damaged cells, rhombencephalic cell suspensions were injected into the same area. It has been reported that damaged brain area was able to create growth stimulation factors in the host brain which enhanced the survival, growth, differentiation and integration of the grafted tissues.[12,13,14,15] From the present study, one month after transplantation, the transplanted cells differentiated into adult Purkinje cells and integrated into the host circuitry. Thus, comparison between the lesioned and the transplanted animals is only the difference between them in terms of the number of Purkinje cell in the cerebellar cortex. In the lesioned animals, the number of Purkinje cells in the cerebellar cortex was less than in the transplanted animals, where the losts of Purkinje cells, was replaced by transplantation of rhombencephalic tissue suspensions.

Kainic acid lesioning results in motor deficits as shown by an increase in frequency of dysmetria and a decrease in the frequency of normal rearing behaviour when compared to the control group at one month after lesioning. When examined a neurological testing, the percent of correct responses of the lesioned animals was also less than the control animals. When observed again at 2 and 3 months after lesioning, the motor deficits were improved, as shown by a decrease in the frequency of dysmetria, an increase in the frequency of normal rearing behaviour and an increase in the percent of correct responses on neurological testing. However, this improvement did not reach normal levels at 3 months post lesioning. There was still a difference ($p<0.001$) in the frequency of dysmetria, in the frequency of normal rearing behaviour and in the percent of correct responses on neurological examination between the lesioned and control groups. In summary, the data indicated the motor deficits in the lesioned animals at 3 months after lesioned when compared to the control animals.

Transplantation of rhombencephalic cell suspensions into the lesioned area in cerebellar cortex can improve the motor deficits, as shown by a decrease in the frequency of dysmetria, an increase in the frequency of normal rearing behaviour and an increase in the percent of correct responses on neurological examinations compared to lesioned animals. At one month post transplantation, the animals show a decrease ($p<0.001$) in the frequency of dysmetria and an increase ($p<0.001$) in the frequency of normal rearing behaviour, compared to lesioned animals. At three months post transplantation there was no significant difference in either the frequency of dysmetria or of normal rearing behaviour between the transplanted and control groups. Transplanted animals also performed better on the neurological examination than lesioned animals. At one month post transplantation, their percent of correct responses was significantly increased ($p<0.001$) compared to lesioned animals. The data at 2 and 3 months post transplantation indicated that there was an improvement in the percent of correct responses on neurological examinations compared to the lesioned group. However, in contrast to the dysmetria and normal rearing behaviour, the responses on neurological examination at 3 months post transplantation had still not quite reached normal levels. Fig.5 shows a significant difference ($p<0.05$) in the percent of correct responses on neurological examinations between the transplantation and control groups. The results from the present experiment indicate that the neurological examination which detects the neurological deficits is more sensitive for detecting the functional effects of the transplanted tissue than open-field testing. The result suggests that transplantation of rhombencephalic cell suspensions can be functionally integrated into the host brain and restore functional deficits in the lesioned animals. Although this functional restoration does not reach the normal levels, it is significantly different from lesioned animals.

The present study shows that transplantation can restore motor deficits to nearly normal condition, Although there are still some neurological deficit, as shown by the significant difference ($P<0.05$) in the percent of correct responses on neurological examination. It has been reported that astroglial cells are increased around grafted tissues in hippocampal transplant[15] and form a barrier between the graft and the host brain. This glial response might inhibit and prevent the migration and integration of the grafted tissues and this may be the reason for the incomplete functional integration in the present experiment.

In conclusion; the transplantation of rhombencephalic tissue suspensions can restore motor deficits caused by Kainic acid 10 nM injected into the cerebellum. The transplantation results in functional recovery which reachs the normal levels when observed with open-field testing. However, the recovery is still incomplete when assesses on neurological tests. The result indicates that the neurological examination is more sensitive than open-field testing for the assessment of motor deficits caused by lesions in the cerebellum. The question of whether the functional recovery is temporary or permanant is sitll controversial. However, base on this experiment, we can conclude that the recovery remains successful up to 3 months after transplantation. Studies with longer survival periods after transplantation will be useful in furthering our understanding of the mechanisms of neural graft-host interaction.

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บทคัดย่อ

การทดลองนี้มีจุดมุ่งหมายเพื่อศึกษาว่า เนื้อเยื่อสมองส่วน Rhombencephalon จาก เอมบริโอหนูอายุ 15วัน ที่ปลูกถ่ายเข้าไปในสมองส่วน cerebellum ที่ถูกทำให้เกิดบาดแผลก่อนหน้านี้ด้วยกรด kainic นั้น สามารถเจริญเติบโตและสามารถแก้ไขหรือลดพฤติกรรมผิดปกติที่มีสาเหตุมาจากบาดแผลในสมองส่วนนั้นได้หรือไม่

ผลการทดลองเมื่อศึกษาภายใต้กล้องจุลทรรศ์พบว่า เนื้อเยื่อสมองส่วนที่ปลูกถ่ายเข้าไปสามารถมีชีวิตอยู่, เจริญเติบโต และพัฒนาไปเป็นเซลล์ Purkinje ที่โตเต็มวัย พร้อมกับแทรกตัวเข้าไปทดแทนเซลล์ Purkinje ที่ขาดหายไปในสมองส่วนที่มีพยาธิสภาพได้ นอกจากนั้นเมื่อศึกษาการเปลี่ยนแปลงทางพฤติกรรม พบว่าหนูในกลุ่มที่ได้รับการปลูกถ่ายเนื้อเยื่อสมองมาเป็นเวลา 1 เดือน มีการเคลื่อนไหวแบบกะระยะไม่ถูก (dysmetria) ลดลง และมีการเคลื่อนไหวแบบ normal rearing เพิ่มขึ้นเมื่อเปรียบเทียบกับหนูในกลุ่มที่มีพยาธิสภาพในสมองส่วน cerebellum แต่ความแตกต่างจะค่อยๆลดลงหลังปลูกถ่ายเนื้อเยื่อสมองได้ 2 เดือน และหลังปลูกถ่าย 3 เดือนจะไม่พบความแตกต่างดังกล่าวระหว่างหนูทั้งสองกลุ่ม เมื่อทำการทดสอบทางระบบประสาท (Neurological examination) พบว่าหนูกลุ่มที่ได้รับการปลูกถ่ายเนื้อเยื่อสมองมาแล้วเป็นเวลา 1,2 และ 3 เดือน มีจำนวนครั้งของการตอบสนองที่ถูกต้อง (% of correct response) สูงกว่าหนูในกลุ่มที่มีพยาธิสภาพในสมองส่วน cerebellum

โดยสรุป การทดลองนี้ได้ค้นพบว่าเนื้อเยื่อสมองส่วน Rhombencephalon ที่ปลูกถ่ายเข้าไปนั้น สามารถแก้ไขหรือลดพฤติกรรมผิดปกติที่มีสาเหตุจากบาดแผลในสมองส่วน cerebellum ได้ นอกจากนั้นการตรวจแบบ open-field ซึ่งประกอบด้วยการตรวจการเคลื่อนไหวแบบกะระยะไม่ถูก และการตรวจการเคลื่อนไหวแบบ normal rearing ร่วมกับการตรวจทางระบบประสาท มีประโยชน์ในการแยกแยะความผิดปกติทางพฤติกรรมของสัตว์ทดลองได้ โดยการตรวจทางระบบประสาทจะมีความไวกว่าแบบ open-field