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# DEVELOPMENT OF EMBRYONIC RHOMBENCEPHALON TRANSPLANTED INTO ADULT RAT CEREBELLUM

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## ABSTRACT

*The present experiment on neural transplantation is an attempt to investigate the mechanisms of development of embryonic neural tissues which have been grafted into the brain of adult animals. The main objectives of the present study are to find out whether grafted embryonic neural cells can survive, grow and differentiate into specific cell or neuronal types, contact with afferent fibers and integrate into host brain environment, especially when the host brain has been freshly lesioned.*

*Embryonic rhombencephalic cell suspension isolated from E15 stage rat embryos were injected into the cerebellar vermis of adult rats which had been lesioned with Kainic acid (KA) one week before the transplantation. Histological observations were performed at 2, 3 and 4 weeks after transplantation. The results indicated that grafted neural cells did not only grow and differentiate into Purkinje cells (PC's) like those in the adult cerebellar cortex, but also to a certain extent integrated into damaged host brain, to replace the lost neurons. The present evidence indicates that, under freshly lesioned conditions, the host brain may be receptive to the neural grafted cells and provide a sufficiently neuronotrophic environment for further development.*

## INTRODUCTION

Damage to the central nervous system (CNS) usually results in severe deficits in cognitive and/or motor functions, 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 neural circuitry [1]. The potential of intracerebral grafts to improve functional or behavioral recovery in brain-damaged recipients depends on multiple factors, such as trophic and neurohormonal factors which enable grafted cells to survive, grow, differentiate and develop the synaptic interactions which integrate the grafts to the host brain [2,3]. Of these effects, the establishment of reciprocal graft-host synaptic connections is one of the best signs of a successful transplantation [4,5].

The present study attempts to either answer, or at least to provide further information bearing on, the following questions: 1) Whether the precursor of neuroblasts from the rhombencephalic cell suspension injected into the adult cerebellum can survive, grow and differentiate into PC's; 2) Whether the grafted cells, once developed into PC's, are capable

of migrating through the host cerebellar cortex to replace those of the hostûs PC's which have been damaged and lost by KA lesioning; and 3) Whether there is any evidence for the establishment of some synaptic relationships between grafted and host neurons.

## 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. Eight-week-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 2 groups: 1) Kainic acid lesioned group without transplantation, which served as the lesioned control (n=12), and 2) Transplantation group after kainic acid lesioning (n=12). In addition, unoperated 4-week-old rats (n=4) served as a normal control.

### Lesioning with Kainic acid

Animals were anesthetized with nembutal (IP, 50mg/kg) and placed in a David-Koff stereotaxic frame for rats. KA 1ul (10nM in 0.9% sterile saline) was injected via a Halminton syringe at a rate of 1ul/min into the vermis of lobes V-VII of the cerebellum. The coordinates are: posterior 5.0mm to interaural line in the midline, to a depth of 6.0mm, according to the sterotaxic atlas for rats[6]. 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 (7). The E15 stage embryos were removed from the pregnant females after caesarian section. A part of the rhombencephalic lip was dissected and collected in a glass petri dish containing the basic medium (0.6% D-Glucose in 0.9% sterile saline) at room temperature. Then the tissues were incubated with 300-500ul of 0.1% crude trypsin solution (Sigma type II) for 20 mins 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 10ul 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, as shown in Fig. 1, was monitored microscopically for cell viability by staining with tryphan blue. Viable cell counts typically ranged from 30,000-40,000 cells per microliter of suspension. From the known cell densities, a small volume of basic medium fluid containing 100,000 cells (approximately 3-5 µl) were then used for injection.

### Histological observations

Animals in both the lesioned control and the transplantation groups were allowed to survive 2 weeks (each n=4), 3 weeks (each n=4) and 4 weeks (each n=4) after transplantation (for the transplantation group), or 3 weeks, 4 weeks and 5 weeks, respectively, after Kainic acid lesioning for both groups. In addition, another 4 unoperated normal controls (aged 4 weeks) were included in the study, for comparison with the (same developmental stage) grafts at 4 weeks post-transplantation. Animals were perfused intracardiacally with 0.9%

saline, in order to wash away blood, and followed by a fixative containing 1% paraformaldehyde combined with 1.25% glutaraldehyde in 0.1M phosphate buffer, pH 7.4. The brains were removed from the skulls, dehydrated through a series of graded alcohol and embedded in paraplast. Sections of 15 microns each were cut in the parasagittal plane, and alternate sections were mounted on 2 series of glass slides. One series was stained by Nisslûs method for cell bodies, while the other series was stained by Bodian method for cellular processes, thereby allowing optimal identification of both cell type and possible synaptic connections. The slides were examined and photographed under the Olympus research model microscope. The diameters of the grafted PC's were measured with a calibrated ocular grid under the light microscope to compare the size of grafted PC's at different survival periods following either Kainic acid lesioning or KA lesioning with subsequent transplantation, as appropriate for the 2 groups, to the size of normal PC's. Statistical analyses were performed by the Epistat microcomputer statistical program, using both paired and unpaired T-test independent variables, in order to test for significant difference of each value.

## RESULTS

### Kainic acid lesioned group

Morphological study after injection of KA 10nM into the cerebellar vermis showed similar findings when observed 2, 3 and 4 weeks after injection (Fig. 2). There was a central necrotic area around the tip of the injection site, in which all neuronal cell types were lost. At the peripheral part of the injection site, granule cells appeared normal, but vaccuolations of lost PC's were observed along the junction between the molecular layer and the granular layer, as PC's became totally degenerated and disintegrated. Therefore, KA injection resulted in the removal of the host PCûs around the injection site.

### Transplantation group

#### Morphological studies

Observations indicated that cells from the rhomencephalic tissue suspension survived and developed in the adult host cerebellum that had 1 week previously been lesioned with KA. Neuroblasts from the grafted tissues were able to grow and gradually differentiate into mature PC-like neurons, migrating into the host molecular layer. At 2 weeks after transplantation, the morphology of the grafted PCûs was characterized by a pear-shaped cell body with more cytoplasm at its apical cone, and a big round pale nucleus, as shown in Fig. 3. These cells appear to be larger, and a thick apical dendritic shaft could be observed by 3 weeks post- transplantation. At 4 weeks post-transplantation, these large neurons showed the typical morphology of adult PC's (Fig. 5), with a pear-shaped cell body, big round nuclei, denser basal cytoplasmic staining (Nissl's stain) than at the apical process. With Bodian stain, which impregnates nerve fibers, the apical dendritic process of the grafted PC's could be clearly observed. It appeared in all respects to resemble the thick shaft of the dendritic tree of PC'sobserved in normal control animals (Fig. 6). In many cases, incoming afferent fibers which projected out of the host granular layer to make synaptic contact with

**Fig. 1.** Micrographs of the Nissl' s stained smear of sample fluid of the cell suspension from Rhombic lip tissue from the E15 stage embryos, composed of neuroepithelial cells in Fig. 1b and some identified neuroblasts (arrow) in Fig. 1a.

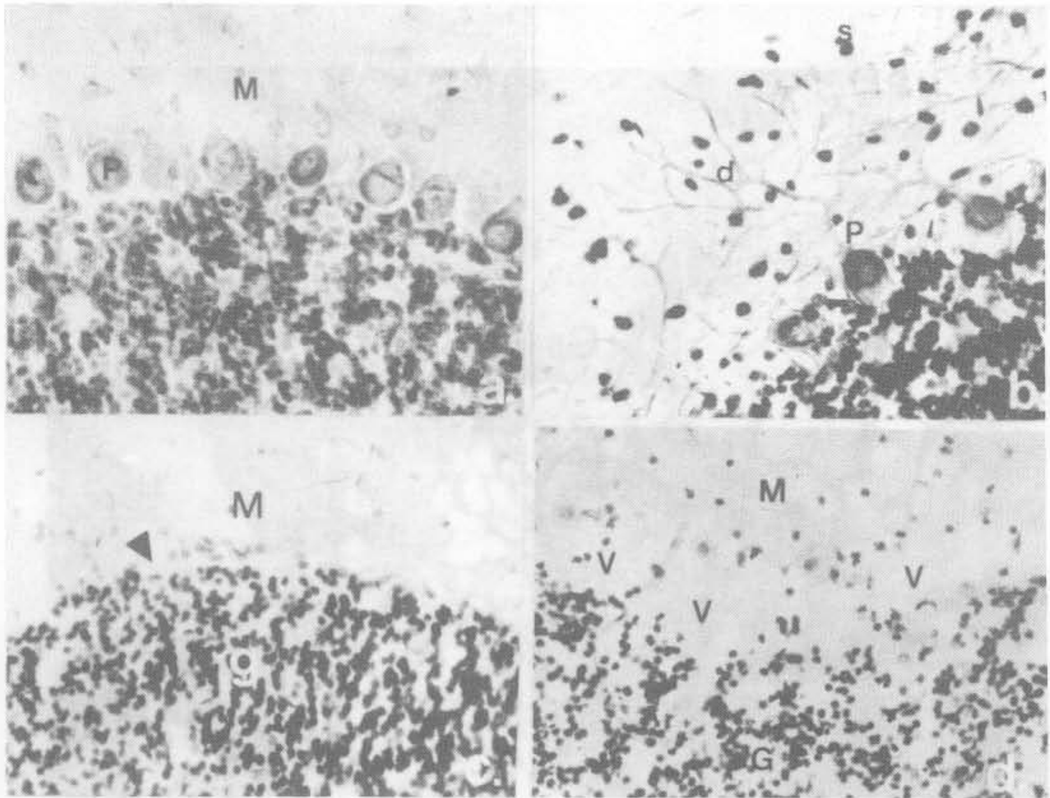



**Fig. 2.** Parasagittal sections of the cerebellar cortex of a normal 8-week-old rat (Figs. 2a and 2b), compared to the cortex of a same-age rat, 1 week after lesioning with 10nM KA (Figs. 2c and 2d). The morphological changes after lesioning were similar when observed 2, 3 and 4 weeks after injection with KA. Fig. 2d shows degeneration and loss of Purkinje cells (marked by "V"), whereas other neural elements appear to be intact. M=molecular layer, g=granular layer, P=Purkinje cell, d=dendrites of Purkinje cells.
(2a, 2c and 2d: Nissl's stain, 2b: Bodian stain)

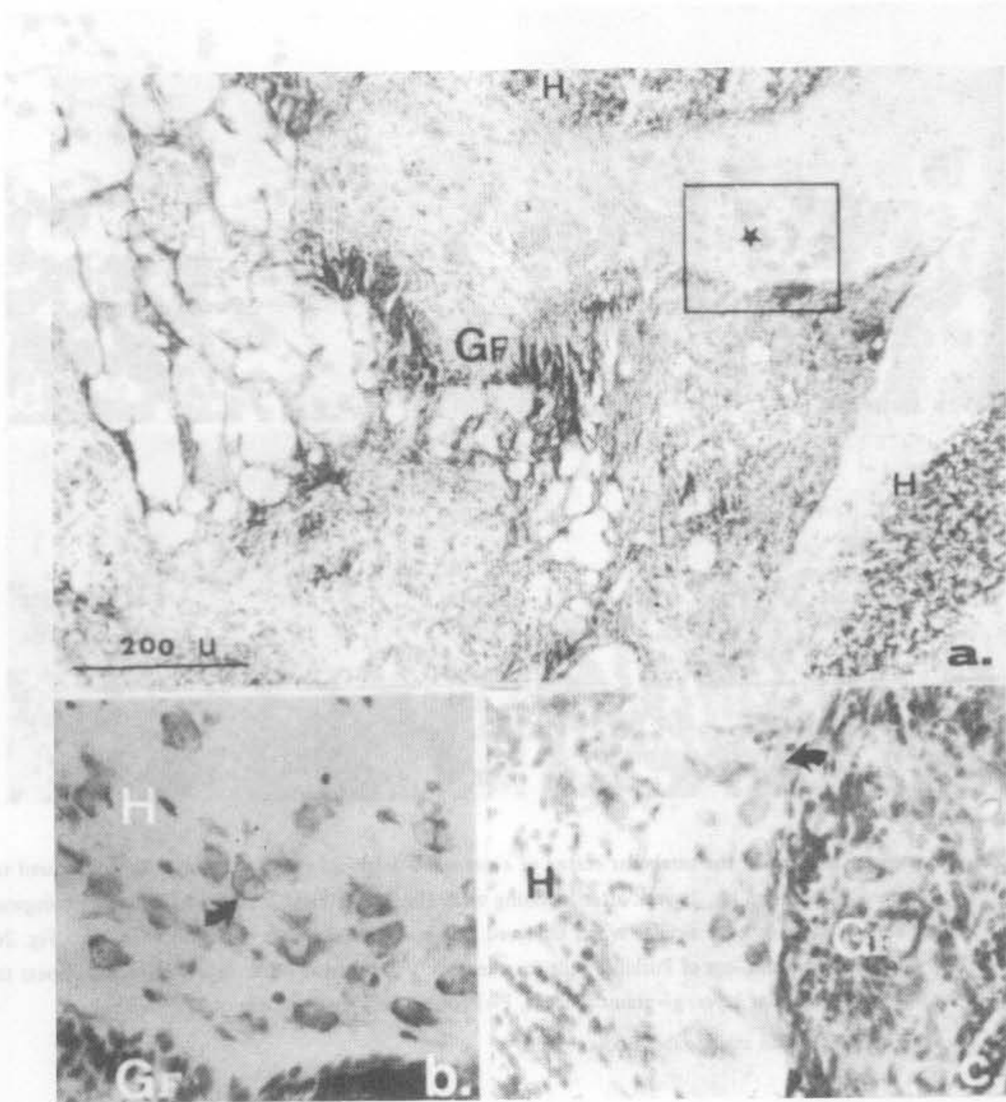



**Fig. 3.** Micrographs of the parasagittal sections through the graft at 2 weeks after transplantation. A cluster of large neurons in the graft (* in Fig. 3a) appeared to migrate into the host molecular layer (H). The area inside the window in Fig. 3a is magnified and shown in Fig. 3b., where the arrow points to the large PC-like neuron, which has a pear-shaped cell body, a big round nucleus and a little apical cytoplasm. Fig. 3c shows the migration of large neurons (arrow) into the host molecular layer in another graft (Gf). (Nissl's stain)

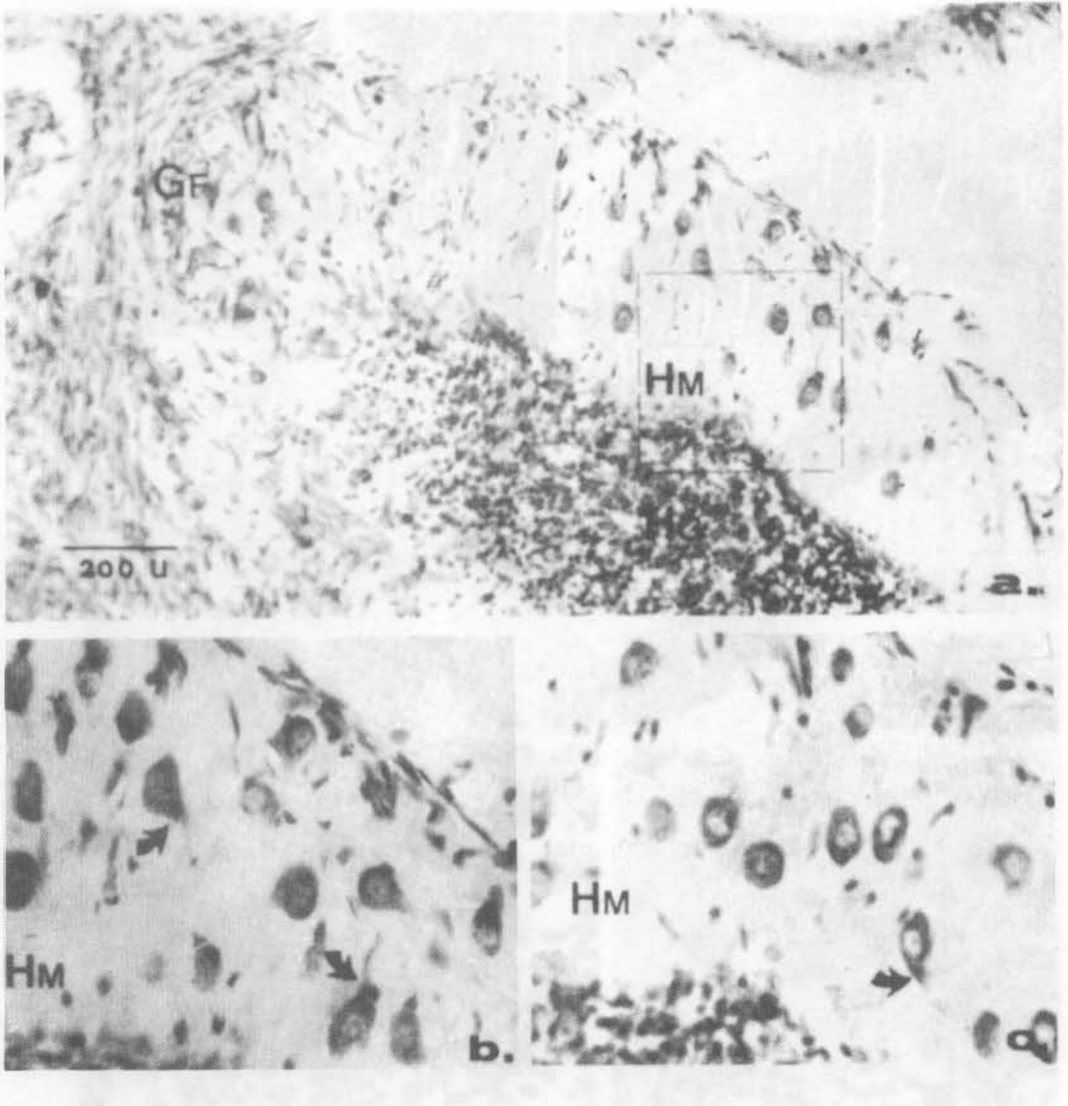



**Fig. 4.** Micrographs of the parasagittal sections through the graft (Gf) at 3 weeks after transplantation (Fig. 4a). Larger neurons which migrate into the host molecular layer (Hm) appear more mature at this stage. A higher magnification of the area inside the window in Fig. 4a is shown in Figs. 4b and 4c. The thick apical dendritic shaft can be observed (arrow).

(Nissl's stain)

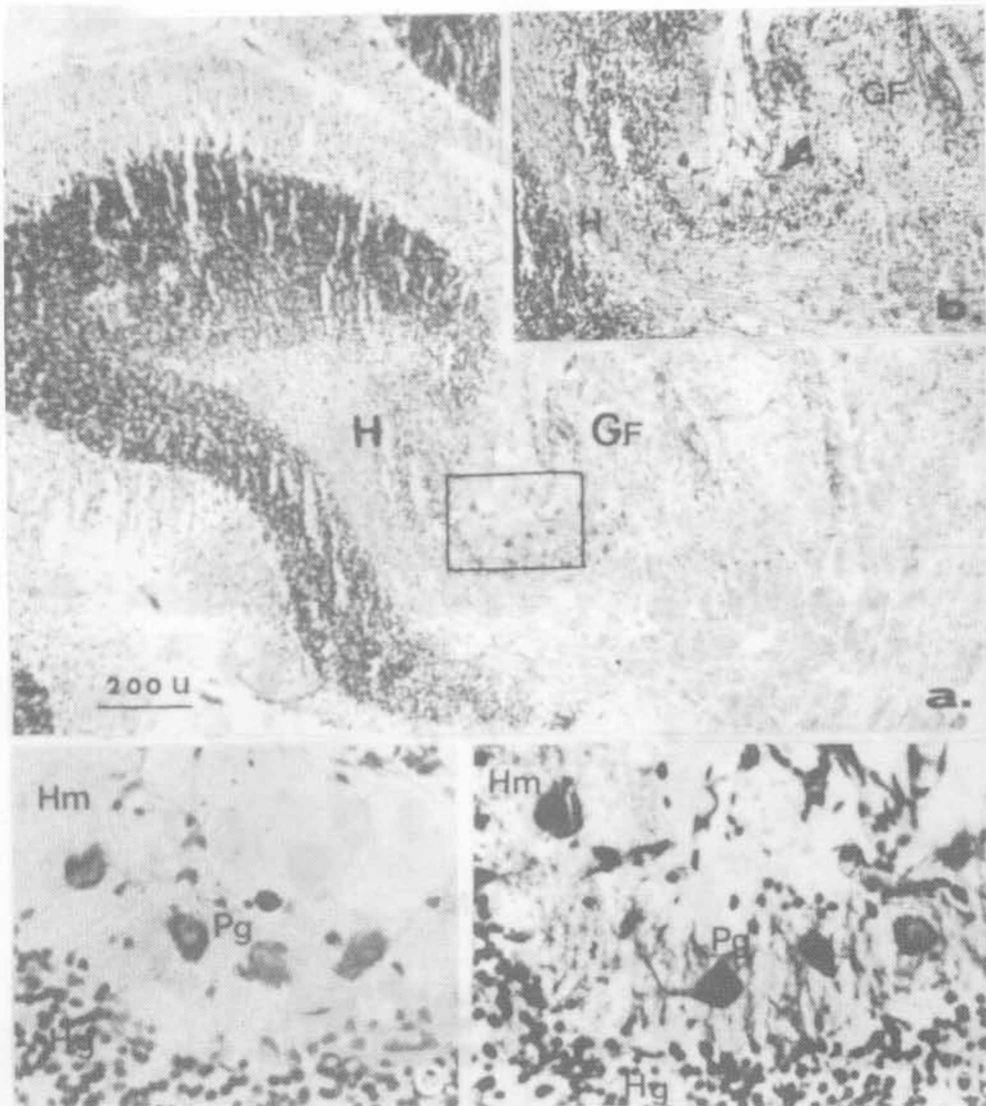



**Fig. 5**. Micrographs of the parasagittal sections through the graft at 4 weeks after transplantation. Large neurons which migrate into the host molecular layer (Hm) are organized into a single cell-thick laminar. The small area marked by a window in Fig. 5a is magnified and shown in Fig. 5b. The inset in Fig. 5b shows a stream of migration of large neurons (arrow) from the graft (Gf). Higher magnification of these grafted Purkinje cells (Pg) are shown in Fig. 5c (Nissl's stain) and Fig. 5d (Bodian stain).

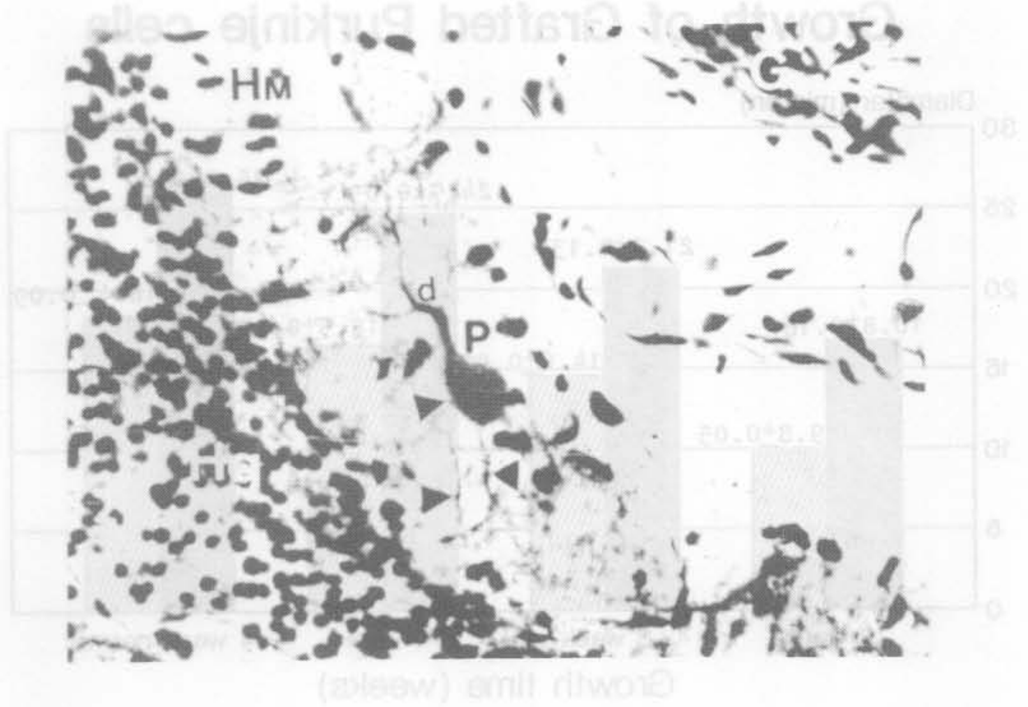
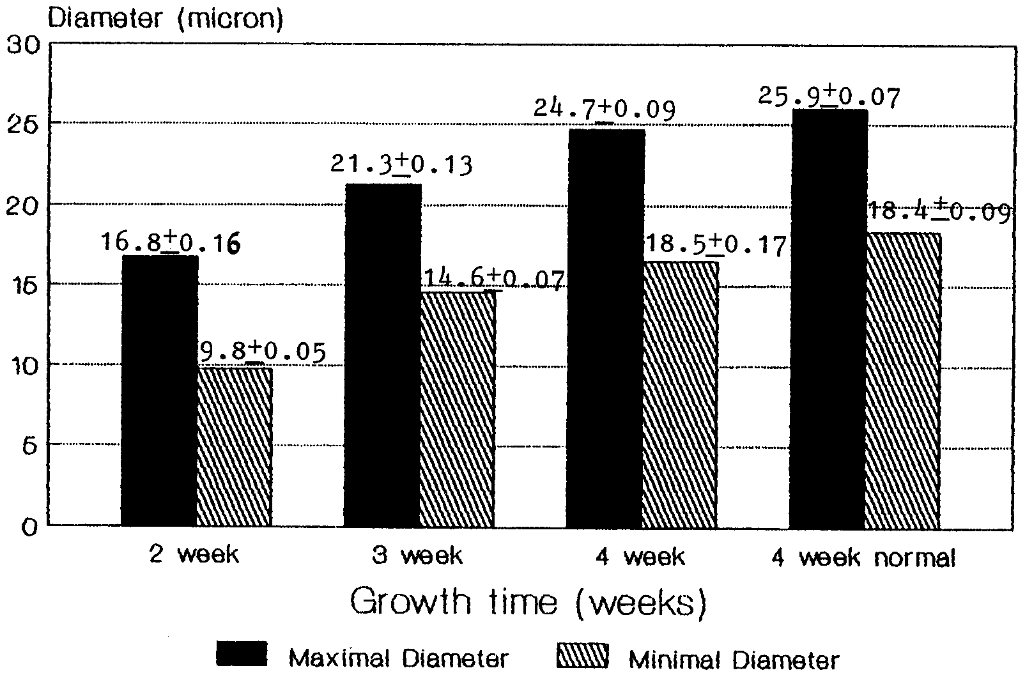



**Fig. 6.** Micrograph showing the incoming afferent fibers to innervate the grafted Purkinje cell (P). The arrow points to the afferent fibers and their terminals. (d=dendrite of Purkinje cells, Hm=host molecular layer, Hg=host granular layer) (Bodian stain)



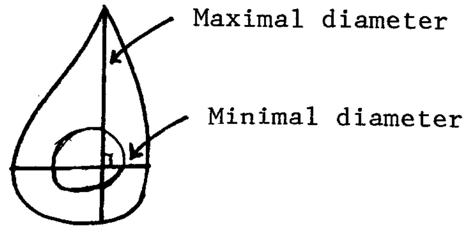




**Fig. 7.** Bar graph comparing the maximal and minimal diameters of grafted Purkinje cells at 2, 3 and 4 weeks after transplantation with normal cells. The values represent MEANq SEM, with n=100 in each group. The graph shows significant differences ($p<0.0001$) in the maximal and minimal diameters of the cells in each group, as well as showing a significant increase ($p<0.0001$) in the maximal and minimal diameters of grafted Purkinje cells from 2, 3 to 4 weeks.

the thick dendritic shaft of grafted PC's could also be observed (Fig. 6).

**Quantitative studies**

Maximal and minimal diameters of grafted PC's at 2 weeks (n=100), 3 weeks (n=100) and 4 weeks (n=100) post-transplantation were measured and displayed in graph form (Fig. 7), and also compared to normal PC's. The data show significant differences ($p<0.0001$) between maximal and minimal diameters of these groups of neurons. Both maximal and minimal diameter sizes are also seen to increase significantly ($p<0.0001$) from 2 through 4 weeks post- transplantation.

## DISCUSSION

The embryonic rhombencephalic tissue from E15 stage fetuses used in this study were composed mainly of neuroepithelial cells and neuroblasts [8,0]. The neuroblast cells, when transplanted into the adult host cerebellum (previously lesioned with KA), continued to develop, grow and differentiate into PC's. Other neuroepithelial cells could also develop into cerebellar granule cells [16,17], however this study focused mainly on the development of PC's. The findings indicate that embryonic cells from E15 stage fetal rhombencephalic tissue had already been largely predetermined as to what morphological characteristics they would display in their fully-differentiated stage [8,16]. The cell separation and suspension procedure neither disturbed nor interfered with their specific developmental programs (7,9).

It was not possible to conclude that all large neurons in the graft were PC's, since the cerebellar cortex also included other large neurons, e.g. Golgi cells (GC's). However, PC's usually outnumber GC's in the normal cerebellum [9]. The other possibility also existed that some of these large neurons were deep cerebellar neurons. From the graph in Fig. 7, a significant difference ($p<0.0001$) between the maximal and minimal neuronal diameters in all groups of grafted large neurons would seem to indicate that their cell bodies were not round, but have a pear-shaped morphology typical of normal PC's, rather than other types of neurons. The Bodian stain of these large neurons at 4 weeks post-transplantation (Fig. 6) showed mature neuronal cells, having a thick shaft process like the dendritic tree of adult PC's. For these reasons, we believe that the observed large neurons in the graft were mainly PC's, although definitive identification of the various neuronal types may have to await specific staining of neuronal type-specific antigens by immunocytochemical method in the future.

Some parts of the grafted cells did not penetrate out from the graft mass into the host brain. The grafted tissue in this area continued to develop into a minicerebellum, composed of three cell layers (Granular, Purkinje and Molecular), as in normal cerebellum, and incoming fibers from host tissue to innervate the grafted PC's was not observed. In some cases, glial cells were seen to form a barrier between grafted tissue and host in these areas. This glial response might inhibit and prevent migration and integration of the grafted tissue. An astroglial response to transplanted neural tissue has also been reported in a hippocampal transplant [13].

Some parts of the grafted PC's migrated out of the graft into the host molecular layer

where PC's had previously been lost. This type of migration occurred rapidly if cells were placed into a suitable environment, e.g. in the molecular layer where host PC's had been destroyed by KA. In contrast, migration to an area where host PC's were intact was rarely observed. Therefore, the area of host cerebellum which was damaged by the toxicity of KA probably created a growth stimulation or some trophic factors in the host brain which induced the grafted neurons to migrate and replace the lost neurons [13]. These growth factors might stimulate the grafted neuroblasts in the area to develop into mature PC's, and might stimulate incoming fibers from the host to innervate them. Several other reports have demonstrated that neuronotrophic factors could significantly enhance the survival and integration of grafted neurons to the host brain environment [10,11,12,13]. However, these effects seemed to correlate with time delay between the injury (or the lesioning) and the subsequent transplantation, which could significantly alter the production and accumulation of trophic factors in the brain as a response to injury [13]. In this study, the transplantation was made 1 week after the KA lesioning, thus, the environment of the host might have already established the proper conditions for enhancement of the survival, migration and integration of grafted neurons into the host brain.

Significant increases ($p<0.0001$) in the maximal and minimal diameters of grafted Purkinje cells were also observed from 2, 3 to 4 weeks (Fig. 7). This result suggests that the grafted large neurons in this experiment from 2, 3 to 4 weeks were the same group of neurons that continue to grow and nearly reach the size of normally developed PC's. The finding that the grafted PC's at 4 weeks post-transplantation were smaller, compared to the normal PC's of the unoperated, normal rats, could be explained by the fact that the graft tissues were taken from the embryonic stage 1 week before birth (E15), or might be due to the growth interruption by the preparation of the cell suspension, which then caused a delay in development.

Observations from the Bodian stained sections of grafted PC's at 4 weeks post-transplantation (Fig. 6) indicate incoming afferent fibers from the host to make synaptic contacts on grafted PC's. These fibers were possibly climbing fibers, because they made synaptic contacts on the PCûs dendrites [14]. These results suggest a situation in which the host climbing fibers that had lost their normal targets from the toxic effects of KA might be reorienting themselves to make new contact with the grafted PC's. However, the possibility of whether or not the efferent fibers from the grafted PC's could innervate the host deep cerebellar nuclear neurons could not be ascertained in the present experiment. This is because of the long distances from grafted PC's to the targets, thereby requiring a longer observation period. In many other studies [15,16,17,18], the experimental data also suggested that efferent connections from the graft to innervate the host brain could occur to the same extent as receiving afferent connections from the host brain.

In conclusion, the transplanted rhombencephalic tissues not only grow and differentiate into mature PC's, but also can integrate into host cerebellar circuitry. However, the question of whether or not the grafted tissues can function properly in the host environment was not addressed in this study. For assessment of the survival and functioning of the graft in relation to clinical improvement following neural transplantation, positron emission tomography (PET) has been recently used in a small number of patients with

Parkinsonism [19]. Detailed studies with longer post-transplantation survival periods, combined with behavioral studies, will be useful in furthering our understanding of the mechanisms of neural graft-host interactions.

## ACKNOWLEDGEMENT

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## บทคัดย่อ

การทดลองเกี่ยวกับการปลูกถ่ายเนื้อเยื่อประสาทในปัจจุบัน ต่างพยายามที่จะอธิบายถึงกลไกการทำงานของเนื้อเยื่อประสาทที่ปลูกถ่ายเข้าไป การทดลองนี้มีจุดมุ่งหมายเพื่อศึกษาว่าเนื้อเยื่อประสาทที่ปลูกถ่ายเข้าไป สามารถมีชีวิต, เจริญเติบโตต่อไปตามปกติ และสามารถแทรกตัวเข้าไปในวงจรประสาทของตัวรับ (host) เพื่อทดแทนวงจรประสาทที่ขาดหายไปได้

เนื้อเยื่อประสาทส่วนรอมเบนเซฟฟาลอน จากเอมบริโอหนู อายุ 15 วัน ถูกนำมาทำให้แยกเป็นเซลเดี่ยวๆ จากนั้นนำไปฉีดเข้าสู่สมอง ส่วนซีรีเบลลัมของตัวรับที่ถูกทำให้เกิดบาดแผลด้วยการฉีดกรดไคนิค (Kainic acid) หลังจากการปลูกถ่าย 2, 3 และ 4 สัปดาห์ ได้ทำการศึกษาเนื้อเยื่อที่ปลูกถ่ายเข้าไป โดยใช้กล้องจุลทรรศน์ จากการทดลอง สามารถสรุปได้ว่าเซลล์ประสาทตัวอ่อน เมื่อปลูกถ่ายเข้าสู่สมองส่วนซีรีเบลลัมของตัวรับที่เซลล์บัวดินจิถูกทำลายด้วยกรดไคนิค สามารถเจริญเติบโตพัฒนาเป็นเซลล์บัวคินจิพร้อมกับแทรกตัวเข้าไปทดแทนวงจรประสาทที่ขาดหายไปของตัวรับ ซึ่งอาจเป็นกลไกที่สำคัญกลไกหนึ่งของการปลูกถ่ายเนื้อเยื่อสมองจากตัวอ่อน เพื่อทดแทนหน้าที่ของเซลล์ประสาทที่หายไป ข้อมูลจากการทดลองเสนอว่าบาดแผลที่เกิดในสมองใหม่ๆ อาจกระตุ้นให้เกิดการสร้างสาร หรือปัจจัยที่เป็นตัวกระตุ้นการเจริญเติบโตของเซลล์ประสาทที่ปลูกถ่ายเข้าไป