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Transplantation of neural tissues in the brains of laboratory mammals: Technical details and comments

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Summary. Various technical details on the transplantation of the embryonic neural tissues in the brains of the neonatal and adult rats are presented. Conditions determining successful or leading to unsuccessful survival, growth and differentiation of these transplants are critically examined.

Transplantation of neural tissues in the brains of the laboratory mammals has been attempted by some investigators in the past[2–6]. Greene[7,8], using a trocar, was successful in transplanting embryonic brain tissue and other tissues, including fragments of tumors, in the brains of adult guinea-pigs, mice and hamsters. Das and Altman[9] used forceps to insert the transplants in the brains of neonatal animals; and Stenevi et al.[10] employed a metal cannula for transplantation. When all these investigations are viewed comprehensively it becomes evident that these researchers were successful in variable degrees, and that to a large extent this was due to differences in the techniques employed by them. Over the years, in this laboratory the technique of transplantation has been improved to such an extent that one can perform a large number of transplantations in one session, and achieve successful survival and growth of the transplants practically in every case. Some aspects of this technique were described earlier[11]. In this paper we submit the technique of transplantation in some detail and its modifications in relation to the donors and hosts of different ages. Although the technique described in this paper is applicable to rats specifically, with some modifications it can be successfully employed for other laboratory mammals such as rabbits and guinea-pigs.

The procedure described below offers an efficient method of performing surgeries on a large number of host animals in 1 session, and at the same time permitting the use of as many donor embryos from 1 pregnant female as possible without an undue waste of time. This is achieved by a team of at least 3 individuals - the first to prepare the pregnant animal, remove the embryos, dissect out the embryonic neural tissues, prepare the transplants, and later inject the transplants; the second to anesthetize and prepare the host animals and maintain the proper level of anesthetic state in them; and the third to suture the epidermal incision in the host animals after they have received the transplants. For these experiments in addition to appropriate surgical instruments for microsurgery a surgical microscope or a good dissecting microscope with long working distance is indispensable. Some basic surgical skills, manual dexterity and knowledge of location of neural structures in an embryo, needless to say, are important requirements for successful transplantation.

Syringe for transplantation

Of various surgical instruments required in these experiments, a glass tuberculin syringe fitted with a glass needle is the most important (figure 1, a, b).

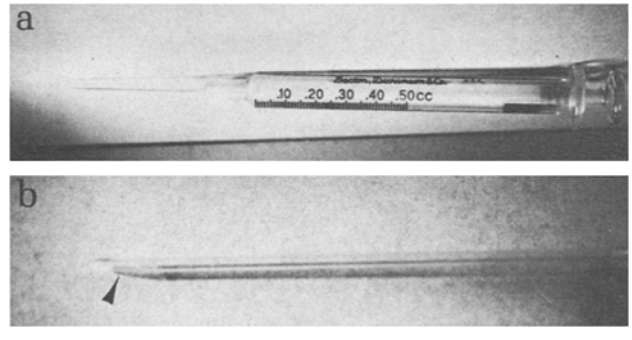

Fig. 1. *a* Glass tuberculin syringe with glass needle. *b* High power view of the glass needle. Arrowhead points to the oblique opening of the glass needle.

Such a syringe, the tool for transplantation, can be easily prepared by the investigator himself. For this a glass tuberculin syringe with glass neck and 0.5 cm^3 capacity, and a 3 cm long thin-walled capillary glass tube, 0.8 mm in outer diameter and 0.6 mm in inner diameter are required. The latter fits well into the bore of the neck of the syringe. The capillary glass tube is gently polished at one end on a fine grade sharpening stone in such a fashion that the opening is oblique and sharp. This is the free end of the glass needle. The other end of the capillary glass tube is inserted into the neck of the syringe and sealed airtight with epoxy cement. After 1 day or 2 when the epoxy cement is completely dried the needle and the syringe should be examined under a dissecting microscope for smoothness of the open end of the glass needle, and for any small glass pieces or dirt present inside. The syringe should be thoroughly cleaned and chemically sterilized. It is valuable to have a few such syringes available at hand on the day of the experiment. The length of the glass needle as suggested is optimum. A needle longer than 3 cm, being fragile, is liable to break right during the surgery; and a needle shorter than 3 cm will not contain adequate amount of neural tissue for transplantation. Repeated measurements have indicated that with the use of a 3 cm long glass needle 6–8 mm^3 of neural tissue can be transplanted. Further, using a glass syringe provides a better control on the plunger of the syringe, and with this the transplant can be gently and smoothly injected. Disposable plastic syringes do not offer such a fine control on the plunger. Using glass needles has many advantages over the use of metallic needles, and the most important one being the facility of checking the nature and purity of the neural transplants prior to injecting them. Of course, the major shortcoming of such fine glass needles is their fragile character. If the investigator does not have full control on his hands or if the animal wiggles about during the act of transplantation, the glass needle is certain to break. Only the experience of handling syringes with glass needles and trying out a number of surgeries as a pilot study will aid the investigator in performing successful surgeries. If a glass needle is chipped at its open end or has accumulated dirt that is uncleanable, it is better not to use it at all. The syringe with its damaged or dirty glass needle may be placed in acetone to dissolve the epoxy cement, and the needle removed. After cleaning the syringe it can be reused for mounting a new glass needle.

In the following the technique is described in 4 sections for convenience. Although these 4 sections represent distinct operations one following the other in its appropriate temporal sequence, they should be viewed comprehensively as the procedure for transplantation.

1. Preparation of pregnant animals to provide embryos

At the start it is important to plan the entire experiment some weeks, better still 1 month or 2, in advance so that the donor embryos and the neonatal or adult host animals of appropriate ages are available on the day of the experiment. In order to obtain rat embryos of a specific age to provide neural tissues for transplantation adult female rats are bred overnight. Only those female animals are taken for the experiment that show sperm-positive smears on the following morning. The day when such vaginal smears are obtained is considered as the day 1 of gestation. Other animals that do not show sperm-positive smears are kept aside, and are bred, if necessary, after 3–4 weeks if they are not already pregnant. Using female animals for breeding over 2–3 nights successively inevitably results in having donor embryos of unspecifiable gestational stages. On the day of the experiment only 1 pregnant animal at a time is used to provide the donor embryos. For inducing anesthesia sodium pentobarbital is used at a dose of 35 mg/kg b.wt, and it is administered i.p. When the animal is deeply anesthetized, it is placed on its back in an instruments tray and its limbs held down firmly with the aid of an adhesive tape. Following laparotomy the uteri are gently exposed towards the surface, and the number of embryos available and their nature determined. Note that the embryos should not be exposed at this stage. Their condition can be established from a superficial examination of the uterus. Generally, the healthy and viable embryos can be identified by the presence of a firm and reddish muscular wall of the uterus surrounding them. The nonviable or partially resorbed embryos have smaller swellings of the uterus, and the muscular wall of the uterus surrounding them is somewhat bluish in color. Only viable embryos are used to obtain the neural tissue for transplantation. The number of viable embryos available will help determine the number of host animals to be prepared for transplantation. At this stage the incision is closed temporarily with a hemostat and covered with a gauze pad soaked with lactated Ringer's solution. The animal is kept warm till it is required at a later stage to provide embryos.

2. Preparation of host animals

Having determined the number of viable embryos available, one can plan for the number of hosts to be used for transplantation. As a rule the number of host animals to be prepared should be less than the number of viable embryos available, and only 4 host animals at a time should be prepared. Since there are some differences in preparing the neonatal and the adult host animals, they are treated separately. For neonatal animals, i.e. up to 20 days after birth, ethyl

ether for anesthesia is the suitable anesthetic. It is relatively easy to administer with the aid of a small beaker containing a moistened piece of gauze; is fast acting; and the level of anesthetic state can be easily manipulated. Once an animal is deeply anesthetized, depending upon the location of the brain whether forebrain or cerebellum where the transplant is to be injected, an appropriate incision is made in the skin. If the transplant is to be injected in the forebrain a V-shaped incision is made in the cranium with the aid of a sharp-pointed scalpel blade (figure 2, a). Generally, an incision anterior or posterior to the coronal suture 2–3 mm lateral to the sagittal suture provides a good access for transplantation. Obviously an anterior incision helps place transplants in the anterior regions of the telencephalon, and a posterior incision helps inject neural transplants in the posterior regions of telencephalon and midbrain. It is very important to see that the incision in the cranium is small and of right size to allow the needle snugly. If the incision is too large the neural tissue oozes back on the surface of the cranium as it is being transplanted. Of course, if the incision is too small it is not possible to penetrate the needle at all without damaging it as well as the host brain. Further, any other type of incision in the cranium, except a V-shaped one, tends to damage the bone and leave the host brain in that region exposed permanently. A V-shaped incision in the cranium, in contrast to any other type of incision, has the advantage of closing itself and keeping the transplants intact inside the host brain after the needle is withdrawn following transplantation. If the transplant is to be injected into the cerebellum, it is best to approach from the cisterna magna (figure 2, b). After parting the muscular tissues overlying the cisterna magna a small incision is made in the meningeal membranes with the sharp point of a scalpel blade, and some cerebrospinal fluid allowed to flow out naturally. In this instance also the incision has to be just right in size to allow the glass needle penetrate snugly. With an incision larger than the thickness of the glass needle the transplants tend to ooze out; and the needle with a greater freedom to move about tends to damage the host cerebellum. A small incision fitting snugly the glass needle not only helps to hold the transplant inside the brain but also repairs itself fast. After having prepared the neonatal host animals the epidermal incision is temporarily closed with a serrefine or bulldog clamp and covered with a pad of gauze soaked in the lactated Ringer's solution. At a time 4 host animals are prepared in this fashion for receiving the transplant and are kept deeply anesthetized and warm.

In the case of adult animals sodium pentobarbital at a dose of 35 mg/kg b.wt is used as the anesthetic. After they are deeply anesthetized, the head and the neck regions are shaved and thoroughly cleaned. The rest of the procedure is similar to that followed for the neonatal animals, except for drilling holes instead of making incisions in the cranium. The holes are drilled with the aid of a fine bone finishing bur (1.5 mm diameter) attached to a variable speed drilling motor (⅛ HP, 1200–14,000 rpm). This is done gently and slowly at a low speed. The holes should be drilled round, and the drilling should be stopped just at the point when only a thin translucent film of the bone is left. In other words, a hole should not be drilled all the way through the bone as the dura mater and the host brain will be damaged by the drilling bur. At this stage the thin film of the bone is gently broken by a Dumont No. 5 micro-dissecting forceps and the underlying dura mater punctured. This causes some cerebrospinal fluid to flow out, but it does not affect the transplantability of the neural tissues in any way. The hole made in this manner should be small and of right size to let the glass needle penetrate snugly. For transplanting neural tissues in the forebrain region a hole is made in the frontal bone, and for transplanting in the posterior regions of telencephalon or midbrain

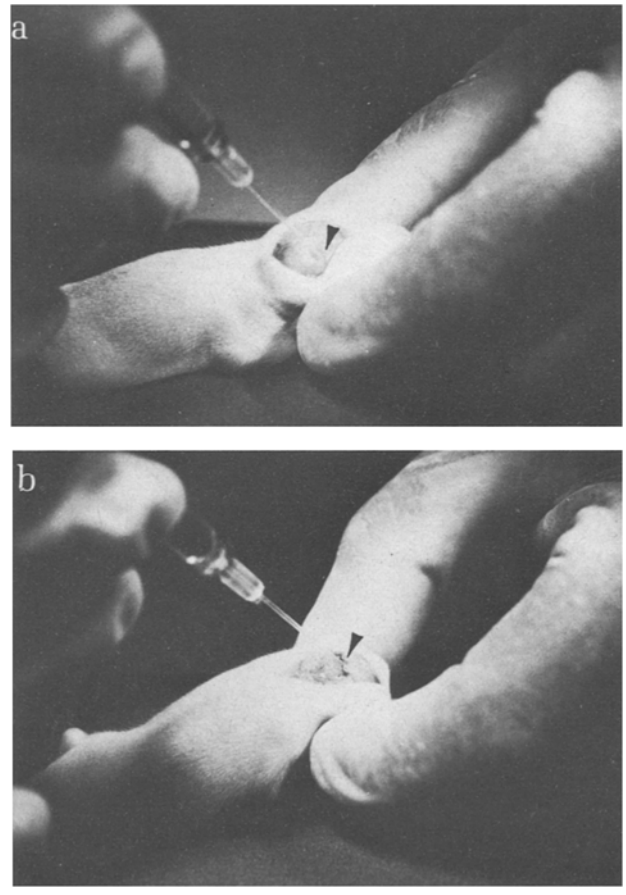

Fig. 2. Preparation of the neonate host animals. *a* Opening in the cranium anterior to the coronal suture for transplanting in the forebrain is indicated by arrowhead. *b* Arrowhead points to the opening in the meningeal membranes of the cisterna magna for transplantation in the cerebellum. These illustrations also show the manner of holding the host animal and the orientation of the syringe for inserting the transplant in the host brain.

a hole is made in the parietal bone at the appropriate spot (figure 3, a). In order to transplant neural tissue in the cerebellum from dorsal aspect an appropriate hole is made in the interparietal bone (figure 3, b). It is important that holes should not be drilled in the middle of any suture or in the lateral aspects of the cranium. However, for transplantation in the cerebellum an approach from the cisterna magna is also possible (figure 3, c). This is identical to that described for the neonatal animals. This approach suffers from 2 shortcomings. a) In order to approach the cisterna magna a large amount of muscular tissue in the neck region has to be parted along the midline. There are many points in this region where an inadvertent cut can cause severe bleeding. b) At the time of transplantation through a hole in the meningeal membranes of the cisterna magna the animal has to be bent far excessively for the glass needle to reach the cerebellum in a straight path. Even a slight movement on the part of the animal or a slight relaxation by the investigator will cause the needle to break into pieces or penetrate into the critical regions of the brain stem. After having prepared an animal for transplantation the incision is temporarily closed with the aid of a serrefine, and covered with a pad of gauze soaked in the lactated Ringer's solution. In the case of the adult host animals also 4 animals are prepared at a time for receiving the transplant.

During this procedure for preparing the host animals, as well as while injecting the neural transplant later on, the animal is held firmly in the left hand, and all operations are performed by the right hand. Once the host animals are prepared to receive the transplant they are made to wait till the transplant is ready for injection. This waiting period may vary from 15 to 45 min, and during this interval the animals are kept warm and properly anesthetized. The waiting period does not in any manner affect the acceptance of the neural transplants by the host brain. This procedure actually helps avoid unnecessary waiting interval for the neural transplants, since injecting the transplants as quickly as possible, after they are prepared, is of crucial importance.

3. Preparation of embryos and neural transplants

At this stage embryos can be removed from the pregnant female rat that has been prepared at the start. Before doing so a beaker containing 300–400 ml of the lactated Ringer's solution at room temperature, watch glasses (100 mm diameter, made of transparent glass, having smooth rounded edge) containing about 1.0–1.5 ml of the lactated Ringer's solution at room temperature, Dumont No. 5 and No. 7 micro-dissecting forceps, other dissecting and micro-dissecting forceps, and sterile gauze pads should be close at hand. The beaker and the watch glasses containing

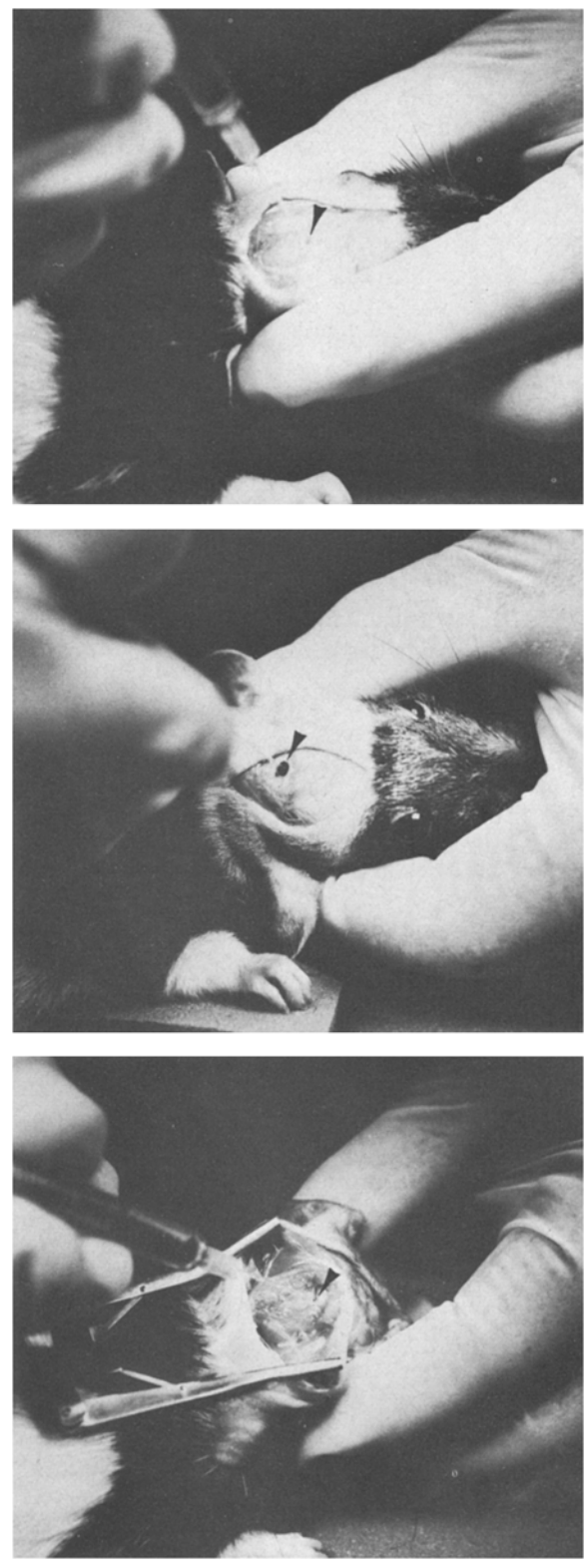

Fig. 3. Preparation of the adult host animals. *a* Arrowhead indicates the opening in the cranium anterior to the coronal suture for transplantation in the forebrain. *b* The opening indicated by the arrowhead in the interparietal bone provides an approach for transplantation in the cerebellum from dorsal aspect. *c* Arrowhead points to an opening in the meningeal membranes of the cisterna magna for transplantation in the cerebellum from posterior aspect. Note the different ways of holding the animal's head and different orientations of the needle for transplanting neural tissues in different regions of the host brain.

the lactated Ringer's solution should be kept covered with other watch glasses of appropriate size. With the aid of dissecting forceps one uterus at a time is exposed to the surface, and laid on the gauze pad. Using No. 5 and No. 7 Dumont micro-dissecting forceps the muscular wall of the uterus, away from the placenta of an embryo, is gently torn apart to expose the embryo. The slit in the uterus wall should be as small and as clean as possible so that minimal bleeding is caused and only one embryo with its membranous structures is popped out. Other embryos usually remain intact inside the uterus. It is important to avoid pulling the uterus forcefully outside and damaging the placenta. The embryo should be removed with all its surrounding membranes intact, and this is achieved by simply pinching the embryo with its membranes from the placenta with a forceps (figure 4). Only 1 embryo should be removed at a time. After removing an embryo the peritoneal incision of the female rat is covered, and it is kept aside for providing more embryos as the experiment progresses. The embryo is freed from the membranous structures and examined under the surgical microscope for the presence of heart beat and reflexive response of limbs to a tactile stimulus. These signs help establish the viability of an embryo.

The embryos of 17, 18, 19, 20, 21 and 22 days gestational stages can be dissected, and neural tissues removed with considerable ease. It may be pointed out that in our experience, where the animals are bred by the investigator himself and not by the animal house caretaker, the pregnant female animals from the laboratory-bred stocks of Wistar-albino or Long Evans hooded rats give birth to the litters on day 23 of gestation (the day of sperm-positive smears being day 1 of gestation). These embryos have soft osteoid cranium and overlying integument which can be removed with 2 Dumont micro-dissecting forceps and, thus, the embryonic central nervous system exposed (figure 5, a, b). This procedure is carried out under a surgical microscope and the embryo placed on a pad of gauze moistened in the lactated Ringer's solution. It is preferable, and easy too, to start dissection from the region of brain stem and then proceed rostrally or caudally depending upon the neural tissue desired for transplantation. It is undesirable to waste time and keep the embryonic brain exposed by dissecting the entire central nervous system just to take out a small fragment of neural tissue, say cerebral cortex, for transplantation. The embryo and its exposed organs should be kept moist with this solution all the time. Use of scissors or scalpel blade for exposing the

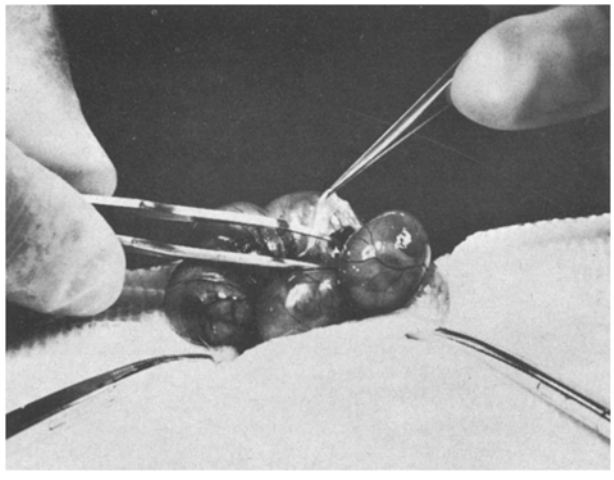

Fig. 4. Removing an 18-day-old embryo from uterus. The illustration shows the embryo along with its membranes being pinched off from the placenta.

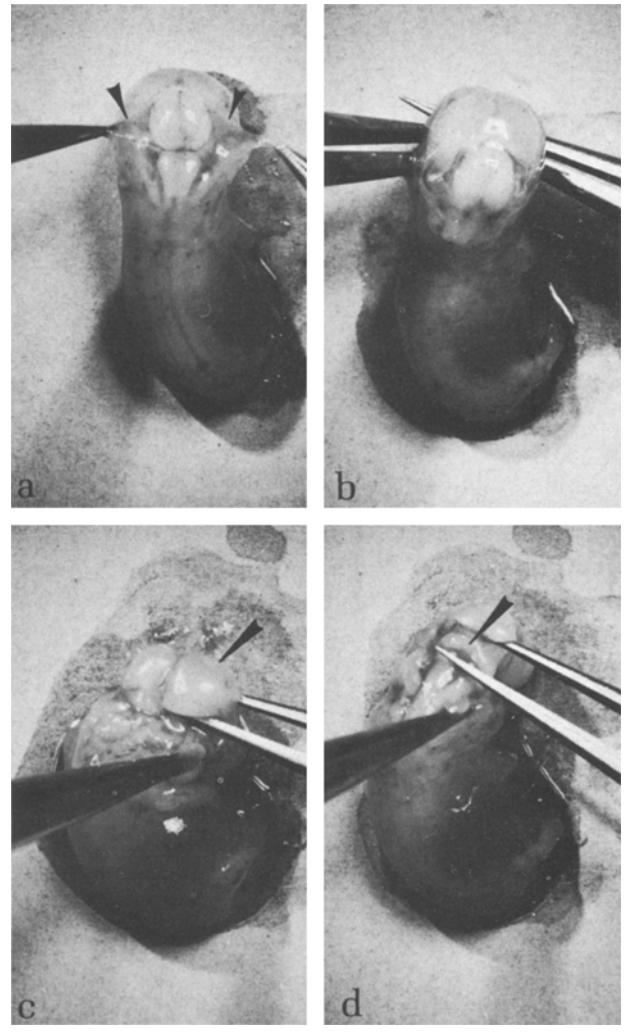

Fig. 5. Dissection of an 18-day-old embryo and removing neural tissues for transplantation. *a* The brain of the embryo is exposed by dissecting away the osteoid cranium and integument (arrowheads). *b* After the brain is exposed it is lifted out from the base of the cranium. *c* Left cerebral hemisphere (long arrowhead) is pinched out from rest of the brain. *d* Cerebellar mass (long arrowhead) is removed with the aid of forceps. These illustrations show that either the whole brain or well-defined regions of it can be removed directly from the embryonic brain, which after further dissection can be used for transplantation. During this phase of dissection care is taken to keep the embryo moist with the lactated Ringer's solution.

central nervous system is not recommended as the embryonic brain tissue may be inadvertently damaged. Depending upon the type of neural tissue to be transplanted, say neocortex or cerebellum, the desired region of the embryonic brain is pinched out with the forceps (figure 5, c, d), placed in a watch glass containing the lactated Ringer's solution, and the watch glass covered with another watch glass. The neural tissue should be kept completely submerged in the lactated Ringer's solution. Any part of it exposed to air will dry up, and will contribute to the nonviability of the transplant. This part of the procedure, i.e. removing the embryo, dissecting it and removing a part of the embryonic brain, is crucially important and should be carried out as accurately and as fast as possible. Any delay in this part of the procedure will contribute to the drying up of the embryo and its neural tissues. Such neural tissues should not be transplanted, for they are nonviable. While pinching out a part of the embryonic brain every care should be taken to see that no extraneous mesenchymal tissue of the embryo is taken out along with the transplant. Transplanting mesenchymal tissue, however small, along with neural tissue causes extensive degeneration in the growing transplant as well as in the host brain. Although with experience it is possible to expose the entire central nervous system of an embryo and different regions from it dissected out fast for transplantation, there is always the possibility that some dissected portions of the brain may become dry and, therefore, nonviable and nontransplantable. It is advisable to remove only one part of the brain from an embryo for transplantation and discard rest of the tissue.

The brain tissue in the watch glass is now prepared for transplantation, and this procedure too is carried out under the surgical microscope. Using 2 Dumont micro-dissecting forceps the meningeal membranes from the brain tissue are removed, and the brain tissue in the watch glass is further dissected into smaller fragments of well-delineated neural structures (figure 6, a, b). For instance when telencephalic tissue is removed from an embryo it contains cortical tissue, hippocampus, basal ganglia, thalamus, hypothalamus and olfactory bulbs. It is important to identify different neural structures accurately in fresh state and to gently dissect them out. Only the desired neural structure is saved for transplantation, and the rest is discarded. Finally, the tissue is freed from any meningeal membranes and choroid plexus attached to it.

This entire procedure is carried out with the neural tissue submerged in the lactated Ringer's solution in the watch glass, taking precaution not to expose it to air. As an indication it may be pointed out that viable neural tissue has a relatively firmer consistancy than the nonviable tissue. The latter tends to be very soft, mushy and difficult to manipulate with forceps. It may be stressed that a thorough knowledge of topographic anatomy of the embryonic nervous system at various stages of its development is indispensable for accurate dissection of the brain tissue and preparing it as a transplant.

In the case of embryos of 15 and 16 days of gestation the osteoid cranium and the overlying integument are undifferentiated, soft and closely attached to the embryonic brain. These conditions make it extremely difficult, if not impossible, to dissect out the embryonic brain intact without contaminating it with mesenchymal or other nonneural tissue. Even if one were to painstakingly dissect out the embryonic brain in its fresh state the time spent on it is so unduly long that in the process of doing so the brain tissue dries up and, thus, becomes nonviable and nontransplantable. The best way of collecting neural tissues from such young embryos is to take it in the glass needle directly. For this the embryo is positioned appropriately on a moistened gauze pad, and under the surgical microscope the glass needle is so penetrated into the embryo that its tip is close to the neural structure desired for transplantation. In other words the neural structure is approached from inside instead of outside, and it is directly sucked in the glass needle and transplanted (figure 7, a, b). This method circumvents the steps of dissecting the brain in the embryo

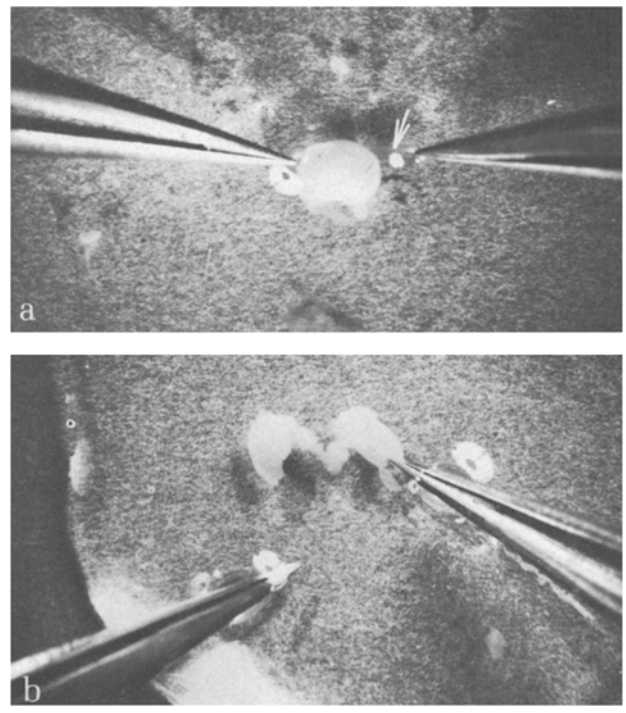

Fig. 6. Embryonic neural tissue in watch glass and its dissection. *a* Right cerebral hemisphere obtained from the embryo is submerged in the lactated Ringer's solution in a watch glass, and the meningeal membranes (white arrow) are removed. *b* The neural tissue is further dissected into smaller pieces appropriate for transplantation into cortical tissue, diencephalic tissue and the tissue containing basal ganglia. During this phase care is taken not to allow the neural tissue be exposed to air.

and brain tissue in the watch glass. In this case a great care is taken to avoid taking in the mesenchymal tissue in the needle. The neural tissue is obtained most satisfactorily when no damage to the embryo other than what is inevitable due to the penetration of the glass needle is caused. If by accident in the process of handling an embryo extensive damage is caused to it, it is safe to discard it altogether. No amount of salvaging it will help get neural tissues without contamination from the mesenchymal and other non-neural tissues. Here also only viable embryos, one at a time should be used. The viability of an embryo is easily established by the presence of heart beat. Since the neural tissue is collected directly from an embryo it is imperative that one should collect it as purely as possible. Even a slight error in identifying the neural structures grossly could result in transplanting impure neural tissues, which in turn may induce extensive pathological changes in the host brain. Finally, this method demands not only a high degree of precision in collecting the transplants from the embryonic brain but also a high speed in executing the operations of collecting the neural tissue in the glass needle and transplanting it in the brain of the host animal.

The rat embryos of day 14 gestation or younger are found to be rather difficult donors. Small size and undifferentiated state of their brains make it practically impossible to obtain pure neural transplants in sufficient quantity even by direct means. No matter how careful one tries to be the mesenchymal tissue invariably gets in the needle along with the neural tissue, thus making transplantation of neural tissues unfruitful. Further, as described below transplants of small volume are extremely difficult to inject in the host brain. They usually remain adhered to the glass needle. Although it is feasible to take out some neural tissue directly in a very small-caliber glass needle from an embryo of 14 days gestational stage, other steps of microsurgery dealing with live and wiggling host animals contribute to a higher incidence of breaking of such fragile glass needles right in the midst of surgery. A broken piece of glass needle left inside the brain of the host animals is definitely not conducive to successful transplantation.

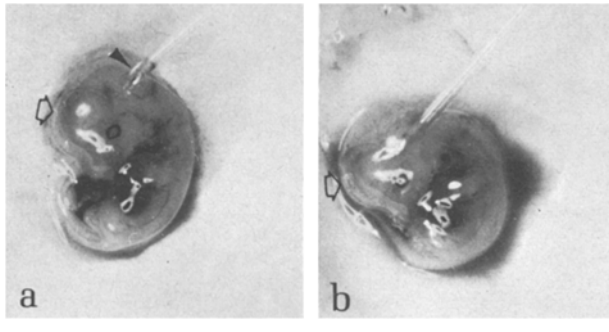

Fig. 7. Taking neural tissue directly into the glass needle from a 16-day-old embryo. *a* The glass needle with its opening oriented outward (arrowhead) penetrates postero-anteriorly to reach the cerebral cortex (open arrow) from inside. *b* Once the opening of the glass needle has reached the region of cerebral cortex (open arrow) the neural tissue is directly sucked in the needle. By appropriately modifying the approach of penetration of the needle, different neural masses can be taken in the glass needle directly for transplantation.

4. Transplantation of the neural tissue

The neural transplant submerged in the lactated Ringer's solution in a watch glass is now taken in the glass needle of the syringe for transplantation. Prior to taking in the transplant the syringe and the needle should be well moistened with the lactated Ringer's solution. If the syringe and the needle are dry they do not provide optimum air-tightness in the barrel of the syringe. Due to this it is extremely difficult to take in the transplant in the needle, and even after this is achieved the tissue will stick to the walls of the needle, thus, making transplantation practically impossible. At the same time the syringe should not be over-moistened for this could lead to an inadequate control on the plunger and a premature slippery ejection of the transplant much before it is taken close to the host animal. The optimum moistening of the syringe is determined by holding it vertically and checking that the plunger does not slide down by itself but moves down slowly only when a gentle pressure by the forefinger is offered. The ideal method of taking transplant in the needle is to move the fragment of neural tissue out of the lactated Ringer's solution in the well and slide it gently towards the dry edge of the watch glass with the help of the glass needle (figure 8, a, b, c). This treatment aids in draining away the excessive lactated Ringer's solution from the transplant and holding it firmly in a dry terrain without letting it slide back into the lactated Ringer's solution in the well of the watch glass. In other words, one should not attempt to take the transplant in the needle directly from the well of the watch glass where it is submerged in the lactated Ringer's solution. With this approach the transplant eludes from getting in the needle and one ends up having the solution in the syringe. While taking in the transplant the oblique opening of the needle should face down and be covered completely by the tissue, and the tissue slowly sucked in. The pressure applied to suck in the transplant in the needle should be extremely gentle so that the transplant remains in the needle alone and there are not air bubbles in it. An excessive or jerky application of pressure at this step tends to suck in the transplant along with air bubbles all the way inside the barrel. It is extremely difficult to transplant the tissue that is in the barrel of the syringe. And, it is undesirable to inject air bubbles along with the transplant as they cause extensive damage to the host brain. It is always desirable to take in the sufficient amount of neural tissue so as to fill the needle and about 0.01–0.02 ml in the barrel of the syringe. In

actuality only the tissue in the glass needle gets injected out while transplanting, and rest of the tissue in the barrel remains behind in the needle. If one were to take in small amount of neural tissue to fill the needle partially it is very difficult to inject it out satisfactorily. Since in this research one is attempting to transplant embryonic neural tissues and not the individual neuroblasts it is possible to obtain sufficient quantity of neural tissue of any type from an embryo to satisfy the above presented technical requirement.

In the case of younger embryos of 15 or 16 days gestational stage, as described earlier, the neural tissue desired for transplantation is directly taken in from the embryonic nervous system into the glass needle. As a matter of fact it is here that a great care is required to see that the mesenchymal tissue, air bubbles and cerebrospinal fluid or other body fluids of the embryo are not taken in along with the neural tissue in the glass needle. With experience it will be evident that the use of glass needle, instead of a metallic needle, has the advantage of having a clear and full view of the transplant as it is taken in from the embryonic brain. If inadvertently mesenchymal tissue or fluids are taken in the needle the investigator can see it on the spot and immediately withdraw the needle, discard the contents and make another attempt to take in the neural tissue only either from the same embryo, if it is still viable and intact, or from another fresh embryo. In these small embryos different regions of brain are not yet delineated and the brain as a whole is very small. Therefore, it is futile to attempt to take in a neural tissue from a narrowly defined region of the brain, e.g. anterior thalamic nuclei, for it is practically and technically not possible. Many neural structures in such young embryos are either not yet formed or have just started to come into existence. One must be content to collect as much of neural tissue uncontaminated with mesenchymal tissue of the embryo as possible even if it comes from more than one neural structure. Finally, from such small embryos neural tissue for transplantation should be obtained only once. If a second attempt is made to take more neural tissue from an embryo, which is probably dry and mutilated by now, only mesenchymal and other nonneural tissues will be taken in the syringe.

After having collected pure neural tissue in the glass needle and in the syringe barrel one should proceed to transplanting it as quickly as possible. The host animals that are appropriately prepared to receive the transplant and are deeply anesthetized should be taken for transplantation one at a time. That is, a given neural transplant in the needle should be injected completely only once in the brain of a host animal. It is undesirable to use one neural transplant collected in the needle to inject in aliquots in multiple brain regions of one host animal or singly in the brains of several host animals. The host animal is held firmly in the left hand in such a fashion that it cannot wiggle its head freely and at the same time its breathing is not interfered with. If the animal's head is held very tightly the internal pressure increases, and the transplant as it is injected in instead of staying inside the brain oozes out. And, if the head is held rather loosely the animal with the glass needle containing the transplant in its brain may wiggle its body and head, thus, causing the glass needle to break or an extensive damage to the host brain or both. The most successful approach for transplantation involves holding the head of the animal firmly, inserting the glass needle containing the neural transplant in the desired region of the host brain, holding the syringe firmly in a vertical or at best in a slanted position to the head of

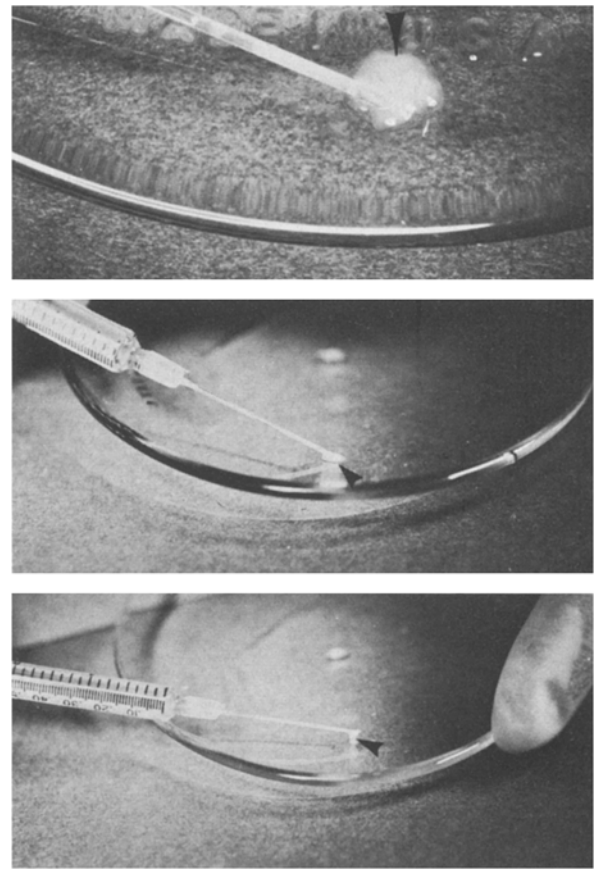

Fig. 8. Taking neural tissue from watch glass into the glass needle. *a* Surgical microscopic view showing the neural tissue close to the edge of the watch glass being taken in the glass needle (arrowhead). The neural tissue after dissection and remaining in the lactated Ringer's solution for some time loses its morphogenetic characteristics. *b* With the aid of the glass needle the neural tissue is removed from the lactated Ringer's solution by sliding it to the periphery of the watch glass. This helps drain away excessive solution from the neural tissue. With the obliquely cut openings of the glass needle facing downward and covering the neural tissue completely, the transplant is gently taken in (arrowhead). *c* Close to the end of this operation the needle and the transplant are slightly lifted up from the watch glass (arrowhead) and the tissue is taken in completely. This helps avoid the excessive lactated Ringer's solution as well as air bubbles from entering the needle.

the animal, slowly injecting in the transplant, holding the animal and the syringe in the same position without even a slight movement for about 20 sec, slowly releasing the pressure slightly on the animal's head but still holding it firmly, twisting the needle around its axis and slowly withdrawing it along the same path that it penetrated in, and quickly covering the hole in the cranium with the skin as soon as the needle is out (figures 2, a, b and 3, a, b, c). After this the syringe is kept aside for cleaning with the lactated Ringer's solution, but the animal's head is still held firmly in the left hand. The pressure on the animal's head is slowly released over an interval of 20–30 sec, and then the animal is let free. Finally, the incision is sutured and coated with 6% celloidin.

During the above described step of transplantation of neural tissue in the host brain 3 important precautions should be observed. a) If the transplant has to be placed in the host cerebellum via cisterna magna, the head of the host animal has to be bent so that the needle can penetrate into the cerebellum in a straight path. If the animal's head is not bent adequately, it will not be possible to penetrate the needle in the cerebellum. The needle may hit the vital structures of the brain stem and damage them. And, at the same time if the animal's head is bent too much it may interfere with its breathing and circulation, and, thus, prove to be fatal to the animal. b) While penetrating the needle in the host brain, injecting in the neural tissue, or withdrawing it the movements of the hand and the needle should be under complete control. If there is any wiggling of the needle, it could damage the brain tissue and cause extensive bleeding inside the host brain, and the transplant in such a damaged brain region will neither survive nor grow. This implies that as far as possible the host brain should not be damaged and that there should be no blood surrounding the transplant when it is left in the host brain. c) If by accident the transplant, after injecting it in the host brain does not stay in but oozes out it is safe to discard the animal from the experiment rather than attempt to reinject the transplant in. If an attempt is made to reinject the oozed out neural tissue or new transplant in the same region of the host brain, it invariably fails, and in addition to it the host brain is left extensively damaged.

Concluding comments

Although the description presented above gives detailed information on various technical aspects of transplantation of neural tissue, there are some other observations made over the years in our laboratory which may also be of some value. They are:

1. For the surgeries for transplantation described, it is important to use sterilized lactated Ringer's solution, instruments and dishes. The investigators actively participating in the surgeries should wear surgical gloves. Other than these precautions, a clean and isolated surgical room, and an overall cleanliness on the part of the investigators themselves, no other measures are taken to have absolutely sterile conditions. Except for the failures attributable to some flaws in the technique or the use of nonviable neural tissues as the transplants, we have had close to 100% success in the survival and growth of the transplants. No amount of improvement in the sterile procedure for surgery over that described above, will compensate for poor technique or use of nonviable neural tissue.

2. Neural tissues obtained from any region of the embryonic central nervous system can be successfully transplanted homo- or heterotopically. In the case of homotopic transplantations, e.g. embryonic neocortex transplanted in the forebrain of the host animal, it is important to employ some labelling technique to distinguish transplanted neural elements from those of the host brain. In our experience the best technique for this purpose is thymidine-H^3 autoradiography, and for this the investigator should have sound knowledge of neurohistogenesis in the rat. Heterotopic transplantations, e.g. embryonic neocortex transplanted in the cerebellum of the host brain, in contrast can be easily identified with suitable basophilic stains. This is aided by the fact that the cellular and cytorachitectural characteristics of a fully differentiated neural transplant are not at all altered by the region of the host brain where it is deposited. A neural transplant during its differentiation achieves cellular and cytoarchitectural characteristics identical to those of its counterpart in a normal brain.

3. The neural transplants should be deposited in the brain tissue and not in the ventricles of the host brain. If they are injected in the ventricles they fail to grow and differentiate. If they grow they invariably tend to block the ventricles. A transplant injected in the anterior region of the 4th ventricle will block the cerebral aqueduct, and that deposited near the anterior region of the lateral ventricles will block the foramen of Monroe. Both these conditions induce hydrocephaly and progressive degeneration in the telencephalic structures of the host brain. This observation holds true for neonate as well as adult host animals. The danger of depositing the transplants in the ventricles of the host brain is greater in the neonatal animals than in the adult animals.

4. Importance of using pure transplants free from meningeal membranes and mesenchymal tissue must be once again stressed. If a transplant has meningeal membranes surrounding it, they will grow very fast around it, will not permit the neural tissue establish any physical continuity with the host brain, and eventually by isolating the transplant will not allow it to survive for long. Such isolated neural transplants

may be treated as similar to in vitro or tissue culture preparations with brief life span. If mesenchymal tissue is transplanted along with neural tissue it tends to grow faster than the neural transplants, blocks the ventricular system and eventually causes hydrocephaly of the brain and concomitant degenerative changes in the telencephalic structures of the host brain. Similarly transplantation of spinal ganglia or segments of peripheral nerves, which are ensheathed with connective tissue, does not yield successful results. The connective tissue in such cases forms a barrier between the neural elements of the transplants and the host brain. The transplants as a consequence fail to survive and grow. An uncontaminated neural transplant not only survives, grows and differentiates but also becomes an anatomically integrated part of the host brain and survives for the life of the animal.

5. The neural transplants seem to grow better in the brains of the neonatal animals than in those of the adult animals. This is not due to differences in their acceptability of the transplants, but due primarily to differences in the overall room available for their growth. The brain and the cranium of the neonatal animals at the time of transplantation has a potential to grow large, whereas those of an adult animal lack this potential. The former condition provides more room for the transplants to grow as the host animals grow than the latter. Similarly age of the embryo which provides neural tissue for transplantation has a bearing on the growth of the transplant. Transplants obtained from embryos of earlier gestational stages, e.g. day 15 or 16 of gestation, grow far more extensively than those obtained from embryos of advanced developmental state, e.g. day 20 or 21 of embryogenesis. The former grow extensively because the neural tissue used for transplantation contains largely neuroepithelial cells that have the potential to proliferate even after transplantation and give rise to neuroblasts that will differentiate into neurons according to their fate. The latter transplants contain mostly post-mitotic neuroblasts that, after transplantation, do not undergo any further mitoses but simply differentiate into neurons. Thus, the ages of both the donor embryos and the host animals should be taken into consideration when evaluating the ultimate growth of a neural transplant. Of course, in normal host animals the magnitude to which a neural transplant grows is directly related to the amount of the host brain displaced and, probably, lost. The transplant during its growth does take a toll of the host brain.

6. The technique for transplantation described above, after having tried many variations over it, has been found to be by far the most satisfactory. As long as, at the time of actual transplantation, the hands are under control and the needle does not wiggle there is minimal bleeding in the host brain due to the penetration of the needle. This is a very important condition for the survival of the transplant as it settles as an alien tissue in the host brain. With minimal bleeding the transplant is able to have physical continuity with and establish its initial footing in the host brain following the physical trauma that it undergoes. This is the most critical requirement for the initial survival of the transplant. If there is extensive bleeding, which is very common with other methods of transplantation, the transplant fails to establish its initial footing in the host brain, floats in the blood along with other fragments of the damaged host brain and soon degenerates. Even with satisfactory transplantation, following the technique presented here, there is some degeneration in the transplant. This is localized in the center, but the periphery of the transplant remains viable and attaches itself to the host brain. This initial physical continuity between the transplant and the host brain actually prevents the penetration of pia mater between the two, and these conditions foster the survival and growth of the transplants. As the transplant grows it occupies the region left by the degenerated portion at its center, and depending upon the age of the host brain pushes the host neural structures aside. Particularly in the host animals of neonatal stages of development the host brain does not seem to offer any resistance to the growing transplant. As a consequence of this some portions of the host brain where the transplant is growing are slowly damaged. During the development of the transplant both its degenerated fragments and the atrophied portions of the host brain are cleared away. Eventually the transplant stops growing as it acquires the destined number of neuroblasts and as they differentiate into neurons. No transplant grows indefinitely.

7. During the entire period of surgery the pregnant female rat providing donor embryos is kept under deep anesthesia. If it has 10–12 embryos it may remain under anesthetic state for 60 min or longer till all the viable embryos are used for obtaining the neural transplants. Use of sodium pentobarbital and ether as the anesthetics does not seem to affect the viability of the embryos. The neural tissues obtained from the embryos that were removed from the female rat that was anesthetized for more than 90 min were seen to survive, grow and differentiate normally well when transplanted in the host brain. This holds true so long as bleeding in the uterus is under control. If, while removing the embryos, placenta or other major blood vessels are severely damaged the ensuing bleeding invariably leads to the nonviability of the embryos in the uterus irrespective of the anesthetic state or the type of anesthesia used.

8. In our estimate it takes about 5–8 min from removing and dissecting an embryo to finally injecting the transplant in the host brain. In some pilot studies this time interval was extended to 25–30 min,

and still the transplants survived and grew well in the host brain. It was observed that in all these cases keeping the neural tissues submerged in the lactated Ringer's solution was beneficial for the anaerobic metabolism of the embryonic tissue. The lactated Ringer's solution left at room temperature in a watch glass for a prolonged period of time did not seem to get contaminated. In contrast to this such prolonged immersion of neural tissues in the tissue-culture media at room temperature did not yield good results with survival and growth of the transplants. It is possible that either the tissue-culture media exposed for long time at room temperature or injecting some tissue-culture medium along with the transplant in the host brain or both may have contributed to the negative results.

9. Technically it is possible to deposit more than one transplant in more than one region of the host brain, but such multiple transplants do not seem to grow to their full extent and they seem to cause more damage to the host brain than the single transplants. The real danger in multiple transplantation lies in that one of them may grow in the ventricles, block the flow of cerebrospinal fluid in the ventricular system of the host brain, and induce hydrocephaly. This is true for both neonatal and adult host animals.

10. Neural tissues can be successfully transplanted in any given strain of rats, whether it is Wistar-albino or Long-Evans hooded. Such transplantations can also be performed between the 2 strains of animals, e.g. Wistar-albino rats as donors and Long-Evans hooded rats as hosts or vice versa. Transplantations performed between the 2 strains of rats do not appear to grow as well as those within a single strain of animals but they do survive, grow, differentiate and live permanently. Further, in our experience transplantations between 2 different species of laboratory mammals, e.g. rat embryos providing donor tissue and guinea-pigs or rabbits serving as hosts or vice versa, have not been successful. In such preparations the transplants at best survive in isolated condition in the ventricles. They are never integrated with the host brain.

11. Finally, in no instance of transplantation, whether single or multiple transplants, the host animals have shown any observable functional or behavioral abnormality or deficiency. This holds true for all conditions of transplants obtained from embryos of different ages and of neonatal or adult host animals. If an animal shows any functional abnormality or deficiency this is invariably related to pathological changes in the host brain ensuing poor transplantation. In all the animals where transplantation of neural tissue is judged successful the transplants are seen to survive the initial trauma of the surgery, grow and differentiate into normal neural masses, share neuropil and coursing fibres with the host brain, and survive for the life of the host animals (figure 9, a, b). These anatomical characteristics, in actuality, help define anatomical integration between the transplant and host brain. Anatomically the neural transplants become a part and parcel of the host brain.

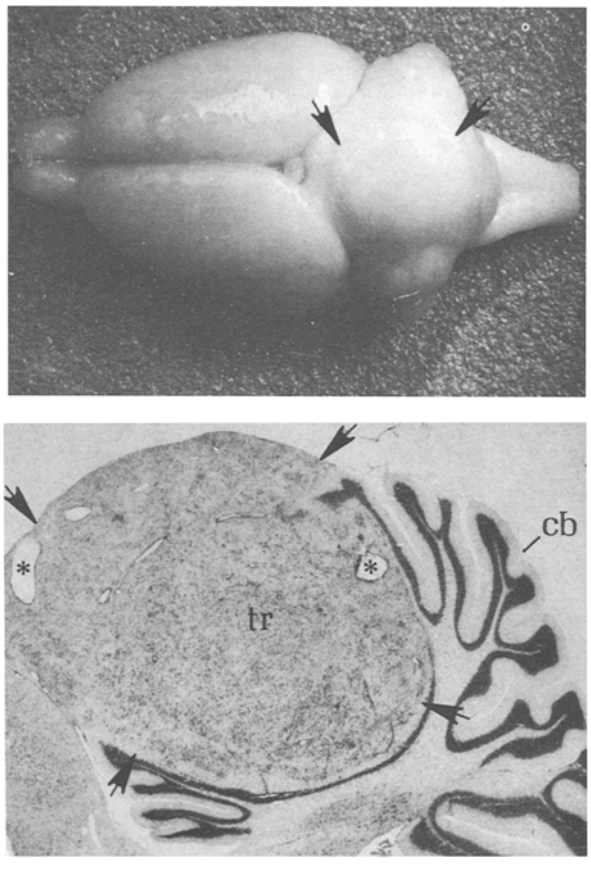

Fig. 9. Fully grown cortical transplant in the cerebellum of the host animal 90 days after transplantation. *a* Macroscopic view showing transplant (arrows) in the cerebellum. The transplant appears like a large swelling and lacks the characteristic fissures of the cerebellum. ×3. *b* Microscopic view of the transplant. The transplant (tr) bounded by arrows is distinct and yet anatomically continuous with the host cerebellum (cb). Open areas (*) are the ventricular structures derived from the ependymal cells in the transplant. CV stain, × 10.

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