related to the variety of transport, enzymatic, and receptor activities which are likely to be properties of this membrane[7,8].

The presence of the dye on the inner plasma membrane of some coated vesicles enclosed in the syncytial cytoplasm, may be due to the section plane, or can be interpreted as the final step of an endocytotic process started at the time of fixation and not suddenly blocked by the fixative. The latter is a problem of general experimental cell biology which reaches far beyond the scope of the present investigation, and deserves further discussion.

Finally the RRS pattern of the trophoblastic basement membrane may be related to the presence of acidic glycoproteins since they were demonstrated by a previous biochemical study in the basement membrane of the human trophoblastic epithelium[9].

1 J.H. Luft, Anat. Rec. *171*, 347 (1971).
2 B.A. Fowler, Histochemie *22*, 155 (1970).
3 K. Yamada, Histochemie *23*, 13 (1970).
4 D. Reissig, Verh. anat. Ges. *71*, 499 (1977).
5 A.G. Everson Pearse in: Histochemistry, vol. 2, p. 1275, 3rd edn. Churchill and Livingstone, Edinburgh and London 1972.
6 J. Martin, S.S. Spicer and N.M. Smythe, Anat. Rec. *178*, 796 (1974).
7 L.K. Kelley, B.F. King, L.W. Johnson and C.H. Smith, Exp. Cell Res. *123*, 167 (1979).
8 M. Sideri, G. de Virgiliis, G. Fumagalli and G. Remotti in: Electron microscopy, vol. 2, p. 30. Eds P. Brederoo and W. De Priester. 7th Eur. Congr. Electron Microsc. Found., Leiden 1980.
9 M. Ohno, R.B. Nagle, E. Meezan and K. Brendel, J. Supramolec Struct. *12*, 457 (1979).

Stereotaxic technique for transplantation of neural tissues in the brain of adult rats[1]

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Summary. A technique of neural transplantation in the brains of adult animals, using stereotaxic apparatus, is described. It facilitates transplantation of neural tissues of small volumes in precisely defined structures of the host brain, and yields a high percentage of successful transplantations.

The successful transplantation of neural tissues in the brains of laboratory mammals depends heavily upon the technique of transplantation. A sound technique aids in keeping the neural transplants viable for long durations, inducing minimal or no pathological reaction in the host brain, causing minimal bleeding at the site of transplantation, and achieving a very high percentage of success in the survival, growth and integration of the transplants. The technique described in our earlier publications[2,3] meets these requirements satisfactorily. That technique requires the use of a glass tuberculin syringe with a glass needle sealed into it for the injection of neural transplants into the host brain. The injection is done with one hand, while the host animal is held firmly in the other hand. That technique has the advantage of freedom of holding the host animal in any position and of injecting the neural transplants from any angle. But, it does not aid in injecting the transplants in precisely defined structures of the host brain.

Neuroendocrinologists have conventionally used stereotaxic apparatus for implanting non-neural tissues[4] or chemical substances[5] in the hypothalamus of the experimental animals. In recent years the same technique has been used for implantation of neural tissues in the brains of the host animals[6]. In all these studies the investigators have used trocar, or metalic cannulae or needles of 1 mm or more in diameter for carrying the implant inside the host brain and expelling it at the desired site. With this instrumentation one has the advantage of using the stereotaxic apparatus, but disadvantages of metalic cannulae or needles. During the past few years, in some studies, we have employed a modified procedure by adapting the glass syringe with a glass needle to a stereotaxic apparatus for transplantation. In the following some details on the assembly of instrumentation and technical advantages of it are presented.

Instrumentation and its assembly. The setup for transplantation includes 3 glass syringes and a stereotaxic unit. The 3 syringes include a transplantation syringe (Luer-Slip, 0.5 ml), a carrier syringe (Luer-Lok, 5.0 ml), and a hydraulic syringe (Luer-Lok, 5.0 ml). The relationship between these syringes is shown in figures 1 and 2. The carrier syringe, to start with, is used for holding the transplantation needle in position and for lowering or raising it with the aid of the hydraulic syringe. In order to achieve these functions the syringe is held upside down, and attached firmly to an electrode holder with appropriate clamping device. Into its Luer-Lok socket, which is at its top, an 18-gauge metal needle is attached. This needle is connected to the hydraulic syringe, which also has an 18-gauge metal needle in its Luer-Lok socket, with the aid of a 60–70 cm long clear polyethylene tubing (PE-60). These 2 syringes and the polyethylene tubing are filled with deionized water. By moving the plunger in the hydraulic syringe to-and-fro the plunger in the carrier syringe can be moved synchronously up-and-down.

The outer end of the plunger of the carrier syringe, which faces downward, has a Luer-Lok socket attached at its head with the aid of epoxy cement. Luer-Lok sockets which are used in this setup can be readily extracted from discarded syringes. Before gluing this Luer-Lok socket a large hole, 3-4 mm in diameter, is made on its side. It is important to note that the Lue-Lok socket is attached to the head of the plunger in such a fashion that the central vertical axes of both are coincidental.

The transplantation needle (fig. 2 inset), that is affixed to the Luer-Lok socket, consists of a bevelled glass capillary needle, a 17-gauge metalic needle hub, and 60–70 cm long polyethylene tubing (PE-60). The glass needle is made from a capillary tubing (0.8 mm outer diameter, 0.6 mm inner diameter, 3.0 cm long), and it is beveled to a smooth tip at one end as described in earlier publications[2,3]. The other end of the glass needle is inserted into the polyethylene tubing and their junction sealed with epoxy cement. The glass needle is then inserted into the metalic needle hub, sealed with epoxy cement, and allowed to dry overnight. It

is important that the central vertical axes of the hub and the glass needle should be coincident.

The assembly of this setup involves first coating the plungers of all 3 syringes with glycerin, and testing them for smooth movement in the barrels of their respective syringes. Following this the carrier syringe is attached upside down to an electrode holder in the stereotaxic unit. The hydraulic syringe and polyethylene tubing are filled with deionized water, and then attached to the carrier syringe. The system should be free from air bubbles. This arrangement will permit equal and synchronous movement of plungers in the 2 syringes, in opposite directions.

Following this the transplantation needle is connected to the transplantation syringe as follows. The free end of the polyethylene tubing of the transplantation needle is inserted through the central hole of the Luer-Lok socket attached to the head of the carrier syringe plunger and slowly pulled out through the side hole (fig. 2, inset). The metal hub with the glass needle is carefully screwed into the Luer-Lok socket. The free end of the polyethylene tubing is then attached to a 21-gauge metal needle of the transplantation syringe. Through the glass needle sterile lactated Ringer's solution is taken in to fill the needle, the polyethylene tubing, and about half of the transplantation syringe. It is of utmost importance that the plunger in this syringe should move smoothly as it will ultimately determine how well the neural tissue is transplanted.

Transplantation of neural tissues. For transplantation the host animal is anesthetized, and prepared according to the surgical protocol and placed in the stereotaxic unit. The carrier syringe and the electrode holder are then attached to the unit. The glass needle is used to determine the landmarks on the cranium to drill the hole. Stereotaxic atlases on rat brain should be consulted for determining the stereotaxic coordinates and the surface landmarks before making holes in the cranium[7,8].

During this period the pregnant animal providing the donor embryos is anesthetized. The details on removing the embryos, dissecting them, and preparing neural tissues for transplantation are given in our earlier publications[2,3]. The glass needle along with the metal hub is unscrewed from the Luer-Lok socket attached to the head of the plunger of the carrier syringe, and pulled out gently along with the polyethylene tubing without detaching the polyethylene tubing from the transplantation syringe. Under a surgical microscope the desired neural tissue is taken in the glass needle by manipulating the transplantation syringe. It is then taken back by pulling out the polyethylene tubing and locked into the Luer-Lok socket. The glass needle on the carrier syringe is brought to the desired antero-posterior and medio-lateral co-ordinates. It is then lowered with the aid of the screw on the stereotaxic unit for vertical movement till the tip of the glass needle touches the opening in the dura mater, the zero point along dorsoventral coordinates. The needle is lowered through the hole to the desired coordinates inside the brain of the animal, and the transplant injected in by slowly manipulating the transplantation syringe. After the neural tissue has been injected in, the individual operating the transplantation syringe retains his control on the plunger to hold the transplant in place, and another individual slowly raises the glass needle out by operating the hydraulic syringe. With experience the 2 individuals operating the 2 syringes can achieve a highly coordinated and smooth mode of operation such that the transplant is retained inside the brain instead of oozing back along the track of the glass needle as it is raised. At the end the animal is removed from the stereotaxic unit, the incision sutured, and the animal placed back in its cage.

Some precautions. 1. During transplantation once the glass needle is lowered to a given region of the brain of the experimental animal, the direction of the bevel of the needle will determine the direction in which the transplant will tend to settle. By reducing the angle of the bevel of the glass needle it is possible to place the transplant exactly in the spot around the tip of the needle.

2. As mentioned earlier the central vertical axes of the glass needle, the metal hub attached to it, the Luer-Lok socket on the head of the plunger of the carrier syringe, and the plunger of the carrier syringe should be all coincident. Failure to achieve this will result in failure in transplanting the neural tissues in precise locations.

3. The hydraulic and transplantation syringes should be always at a lower level than the glass needle attached to the carrier syringe. If the hydraulic syringe is at a higher level, the plunger in the carrier syringe will not be held in a stationary position at any time. It will come sliding down. If the transplantation syringe is at a higher level, the neural tissue will not stay in the glass needle. It will be ejected out immediately.

4. Lowering of the glass needle containing the transplant should be done by operating the screw to move the electrode holder in the vertical plane. But raising it, after the transplant has been injected in should be done by pulling out the hydraulic syringe. This permits a slow and smooth mode of raising the glass needle. If it is raised by operating the screw, the jerky motion will cause unnecessary damage to the host brain.

5. The neural tissue should be injected very slowly. If it is done rapidly with force the transplant gets scattered away inside the brain, often being located in the ventricular or sub-arachnoid cavities, causing extensive damage to the host brain.

6. Care should be taken to inject only the neural tissue and not the lactated Ringer's solution in the brain of the host animal. Even inadvertent injection of Ringer's solution could lead to undesirable pathological reaction in the host brain.

7. After the transplant has been injected, it is helpful to let the needle stay in that position for about 10 sec. This seems to facilitate the transplant in settling inside the parenchyma of the host brain. If the needle is withdrawn immediately after the injection of the transplant, the neural tissue invariably oozes out along the needle track. As the needle is withdrawn slowly it is important to maintain slight pressure on the plunger of the transplantation syringe. This prevents upward displacement of the neural transplant along the needle track.

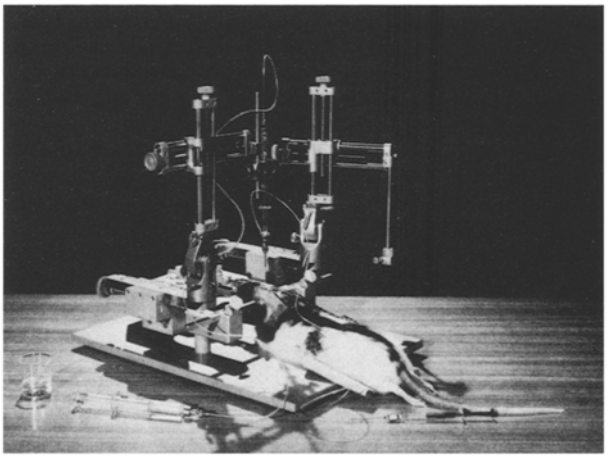

Figure 1. An adult rat in a stereotaxic unit with carrier syringe, and transplantation and hydraulic syringes in position.

8. Following each transplantation the needle should be cleaned with sterile lactated Ringer's solution, and its bevel checked for the presence of cracks, dirt, dried blood, etc. If a needle is very dirty or damaged it should be replaced with a new clean glass needle.

Advantages. The setup described in this report has many advantages which help achieve a very high percentage of successful transplantations. Without going into details of all of them the following few may be emphasized.

1. The most important advantage of using this setup is that neural tissues, as small as 0.5 mm^3 in volume, can be successfully transplanted precisely in any region of the host brain. If the injection of the transplant is done carefully and slowly it is possible to achieve very little damage to the host brain, minimal amount of bleeding at the site of transplantation, and a good parenchymal apposition between the transplant and the host brain parenchyma. These conditions promote better survival and growth of the transplants, and maximum possible anatomical integration between the neural transplants and the host brain (fig. 3).

2. The use of the glass needle for carrying neural transplants inside the host brain has many advantages over using trocar, or metal cannulae or needles, and they can be summed up as the advantages of being able to see directly the neural transplants and their movement while injecting them. Firstly, the glass needle can be calibrated for transplanting exact volumes of neural tissue. This is achieved by obtaining a droplet of Ringer's solution of known volume from a µl-syringe, taking it in the glass needle, and marking its level with an indelible and nontoxic ink. A droplet of 1.0 µl is equal to 1.0 mm^3. Calibration of the glass needle helps maintain consistency in transplanting exact volumes of neural tissues in different animals. Secondly, while taking neural tissue inside the needle it is possible to check if the transplant is purely neural or contains air bubbles and Ringer's solution. Injecting air bubbles and excessive Ringer's solution in the host brain reduces the chances of survival of the transplants and contributes to pathological reactions in the host brain. Thirdly, as the neural tissue is injected in, the investigator can observe the actual move-

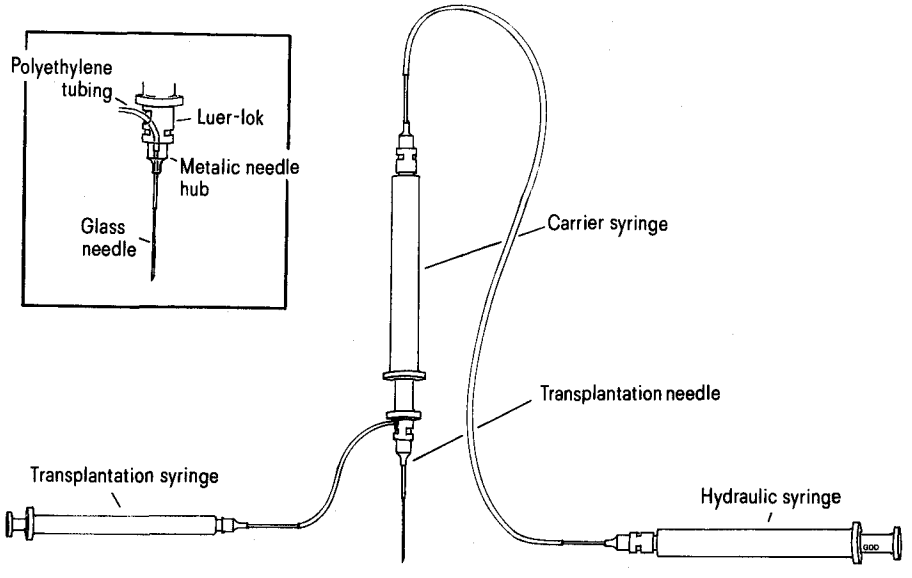

Figure 2. A schematic drawing to show the arrangement of the carrier, transplantation and hydraulic syringes. Inset shows the arrangement of the glass needle and polyethylene tubing through the metalic needle hub. Note the hole on the side of the Luer-Lok socket through which the polyethylene tubing is drawn out.

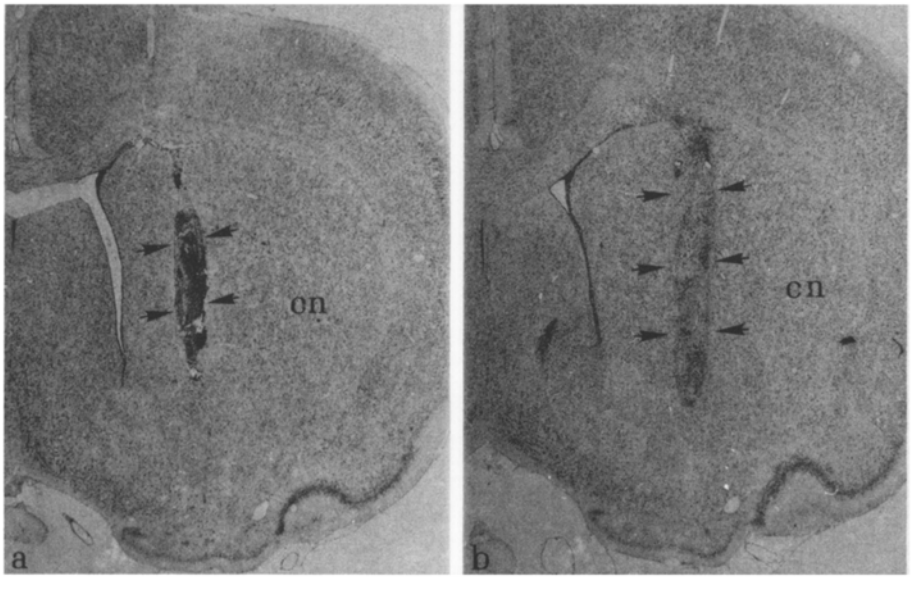

Figure 3. Neural transplant in the caudate nucleus (cn) of an adult rat. *a* Neural transplant (arrows) 30 min after injection. The transplant was 0.5 mm^3 in volume, and it was obtained from the tegmentum of a 17-day-old rat embryo. Note that the transplant is closely apposed to the host brain tissue, and is not surrounded by blood or excessive Ringer's solution. *b* Neural transplant (arrows) 30 days after injection. The transplant has grown large, contains well-differentiated normal-looking neurons, and has established parenchymal integration with the host brain along its entire interface. This is a case of complete intraparenchymal transplantation. Note absence of pathological reaction in the transplant and the host brain. The tubular shape of the transplant was determined by the track of the glass needle along which it had grown. Cresyl-violet stain, × 10.

ment of the transplant, and thereby regulate the rate of injection. Fourthly, the glass capillary used for making glass needle is of very fine caliber (0.8 mm outer and 0.6 mm inner diameters), and it causes least amount of physical damage to the host brain. And, it being of glass there is no danger of its inner wall rusting over time.

3. The use of the stereotaxic apparatus aids in transplantation of neural tissues in exactly defined structures of the host brain according to the stereotaxic coordinates. This in turn facilitates subsequent manipulations of the fully grown transplants, such as making electrolytic lesions in the transplants or injecting chemicals into them for the study of their connectivity, with a high degree of precision.

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2 G.D. Das, T.I.T.J. Life Sci. *4*, 93 (1974).
3 G.D. Das, B.H. Hallas and K.G. Das, Experientia *35*, 143 (1979).
4 B. Flerko' and J. Szentagothai, Acta endocr. *26*, 121 (1957).
5 P.G. Smelik, in: Neurohormones and neurohumors, p.277. Ed. J. Ariens Kappers. Springer, Wien-New York 1969.
6 U. Stenevi, A. Bjorklund and N.-A. Svendgaard, Brain Res. *114*, 1 (1976).
7 J.F.R. Konig and R.A. Klippel, The rat brain, a stereotaxic atlas. Williams and Wilkins, Baltimore 1963.
8 L.J. Pellegrino, A.S. Pellegrino and A.J. Cushman, A stereotaxic atlas of the rat brain. Plenum Press, New York 1979.

Lens regeneration from the dorsal iris in *Eurycea bislineata*, the two-lined salamander[1]

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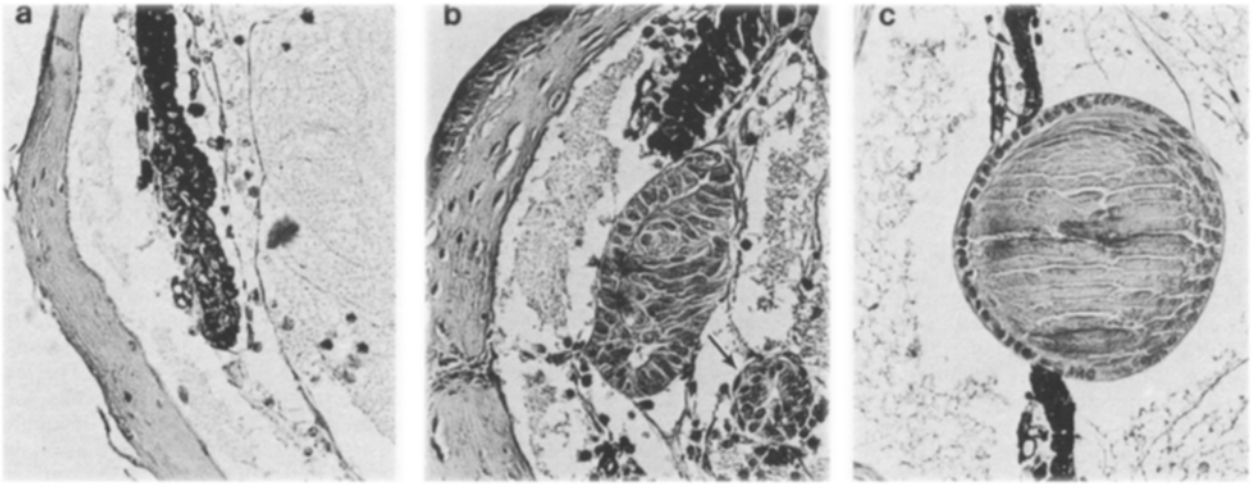
Summary. Following lens removal from the eye of adult *Eurycea bislineata*, the northern (USA) 2-lined salamander, it was found that this salamander has the capacity for lens regeneration. Its widespread distribution and high percentage of regenerative success suggests it as a suitable organism for the study of this differentiative phenomenon.

Eurycea bislineata, the northern 2-lined salamander, is a member of the family *Plethodontidae* (lungless salamanders) and native to the northeastern United States. Stone[3] reported that one species of the genus *Eurycea (E. lucifuga)* can regenerate the lens of the eye after lentectomy, while another in the same genus *(E. longicauda melanopleura)* cannot; both inhabit the twilight zone of caves. In order to determine the ability of *E. bislineata*, a much more common organism, to regenerate a lens (from the dorsal iris), histological examination, of eyes fixed at varying periods after lentectomy, was undertaken.

Adult salamanders were collected in Montgomery County, Pennsylvania, anesthetized using 1:2000 ethyl-m-aminobenzoate methane sulfonate (Tricaine, Sigma), and the lenses removed with the aid of a binocular dissecting microscope. A naso-temporal incision was first made in the cornea with a scapel. After the cut was completed, the open tips of a pair of watchmaker's forceps were pressed gently against the eye perpendicular to the incision. This pressure forced the lens out through the slit cornea. Only those animals whose lens was removed intact were utilized. The salamanders were then kept in a moist environment at 21 ± 1 °C until they were sacrificed at 2 weeks, 3 weeks, an 1 month post-lentectomy. The eyes were isolated and fixed in Bouin's fixative, then prepared for histological observation.

Morphological staging of lens regeneration as described by Yamada[4] was used to characterize the regenerated lenses seen in 10 µm serial sections. Briefly, these stages recognize initial depigmentation of cells along the margin of the



Representative lens regeneration stages of *E. bislineata* (hematoxylin and eosin; ×278): *a* stage III/IV - depigmentation of the dorsal iris epithelium and initiation of vesicle formation; *b* stage VIII, with 2° fibers beginning to form - the connection of the lens regenerate to the dorsal iris is not evident in this section (arrow indicates portion of tortuous neural retina); *c* stage XI - the fully-regenerated lens has detached from the dorsal iris and fills the pupillary space.