Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5227–5230, August 1985
Neurobiology

Cholinergic ventral forebrain grafts into the neocortex improve passive avoidance memory in a rat model of Alzheimer disease

(neuronal transplantation/nucleus basalis/learning)

ALAN FINE*†, S. B. DUNNETT‡, A. BJÖRKLUND§, AND S. D. IVERSEN¶

*Neurochemical Pharmacology Unit, Medical Research Council Centre, Medical School, Hills Road, Cambridge, United Kingdom; ‡Experimental Psychology Department, University of Cambridge, Cambridge, United Kingdom; §Histology Department, University of Lund, Lund, Sweden; and ¶Neuroscience Research Centre, Merck Sharp and Dohme, Terlings Park, Harlow, Essex, United Kingdom

Communicated by Max F. Perutz, April 18, 1985

ABSTRACT **The memory dysfunction of Alzheimer disease has been associated with a cortical cholinergic deficiency and loss of cholinergic neurons of the nucleus basalis of Meynert. This cholinergic component of Alzheimer disease can be modeled in the rat by ibotenic acid lesions of the cholinergic nucleus basalis magnocellularis. The memory impairment caused by such unilateral lesions, as reflected in passive avoidance behavior, is reversed by grafts into the deafferented neocortex of embryonic neurons of the cholinergic ventral forebrain, but not by grafts of noncholinergic hippocampal cells.**

Postmortem brains of patients with senile dementia of the Alzheimer type show reduced cerebral cortical choline acetyltransferase (ChoAcTase; acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) and acetylcholine (1–3). These biochemical deficits are thought to reflect degenerative changes occurring in the cortical cholinergic projection neurons of the forebrain nucleus basalis magnocellularis (NBM) (4–6). The memory deficit characteristic of Alzheimer disease has been attributed to this cholinergic loss (7, 8), although involvement of other neurotransmitters cannot be excluded (9, 10). In animal experiments, both anticholinergic drugs (11) and lesions of the NBM (12–14) have been shown to disrupt learning or memory in a number of paradigms, the most sensitive of which has been the passive avoidance test. It has also been shown that transplanted embryonic neurons can reinnervate the rat brain to restore certain functions of dopaminergic (15, 16) or cholinergic (17) systems in rats with NBM lesions. In the present study, we examined the ability of embryonic neurons from the cholinergic ventral forebrain to reinnervate the neocortex of animals with NBM lesions. We show that such grafts survive and ameliorate a memory deficit caused by lesions in the rats as measured in a passive avoidance paradigm.

The long time course of transplantation experiments requires the use of animals with stable lesions, large enough to preclude compensatory sprouting. Large bilateral lesions of the NBM in the rat produce profound disruption of appetitive behaviors, which may be attributable to damage to adjacent hypothalamic and pallidal structures, and which often have fatal consequences. By contrast, large unilateral NBM lesions leave one side intact to maintain normal eating and drinking, have no detectable effect on spontaneous locomotor activity, and provide an internal control for biochemical and histochemical assessment. Thirty-three female Sprague–Dawley rats (180–210 g) received unilateral ibotenic acid lesions at two sites in the right NBM (see Fig. 1). Such lesions spare fibers of passage (18); indeed, identical injections of ibotenic acid directly into the more ventrally located median forebrain bundle completely spare the ascending catecholaminergic fibers (19). Manipulations were restricted to one side of the brain and to animals of one sex in order to reduce possible sources of variability. Eight of the injected animals were killed 1 week later for biochemical assay of ChoAcTase (20); the lesions reduced ChoAcTase content in dorsolateral frontal and parietal cortex to 43.2% ± 5.4% and 41.2% ± 3.2% (mean ± SEM), respectively, of intact contralateral levels. This corresponds to a decrease in measured activity from 16.24 to 6.83 nmol of acetylcholine synthesized per mg of protein per hr and from 15.74 to 6.38 nmol per mg of protein per hr in the two respective sites. Since cortical undercutting indicates that 20–50% of cortical ChoAcTase is derived from intrinsic cortical neurons (21), this suggests that these NBM lesions are nearly complete. The remaining 25 rats with lesions were subdivided into three groups 1 week after surgery. Twelve animals received transplants of cholinergic-rich tissue dissected from the developing ventral forebrain (including septum, diagonal band, nucleus basalis precursors) of 15- to 16-mm long (crown–rump) Sprague–Dawley rat embryos and dissociated to a single-cell suspension (1 ventral forebrain per 10-μl suspension) as described elsewhere (22). Each animal received two 2-μl aliquots of cell suspension stereotaxically injected into dorsolateral frontal and parietal cortex ipsilateral to the lesion (Fig. 1). Six rats received control transplants of cholinergic-poor cell suspension prepared from developing hippocampus of 17-mm long (crown–rump) embryos, injected into the same cortical sites. Seven rats with lesions (but ungrafted) remained as controls. These latter two groups did not differ significantly at any phase of the behavioral testing, and they were combined in the analysis of this experiment into a single control group, to augment statistical significance. Finally, a further 12 normal animals served as unoperated controls.

Six months after experimental surgery, "step-through" passive avoidance training was carried out. Single-trial passive avoidance, as conventionally used, does not allow separation of acquisition and retention impairments in animals with lesions. We have therefore given multiple training trials in a single session to establish a uniform acquisition level for all animals, so that differences in performance on subsequent days reflect differences in retention. The apparatus was a 50-cm square aluminum box, divided into two compartments of equal size connected by a small central open door. The first compartment had a Perspex front wall for observation and was brightly lit from above by two 60 W bulbs. The second compartment had an electrifiable grid floor, a closed roof, and no illumination. The testing room was without light but with constant background white noise. All 37 rats were habituated to the apparatus on 2 successive

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NBM, nucleus basalis magnocellularis; ChoAcTase, choline acetyltransferase; AcChoEase, acetylcholinesterase.

†To whom reprint requests should be addressed.

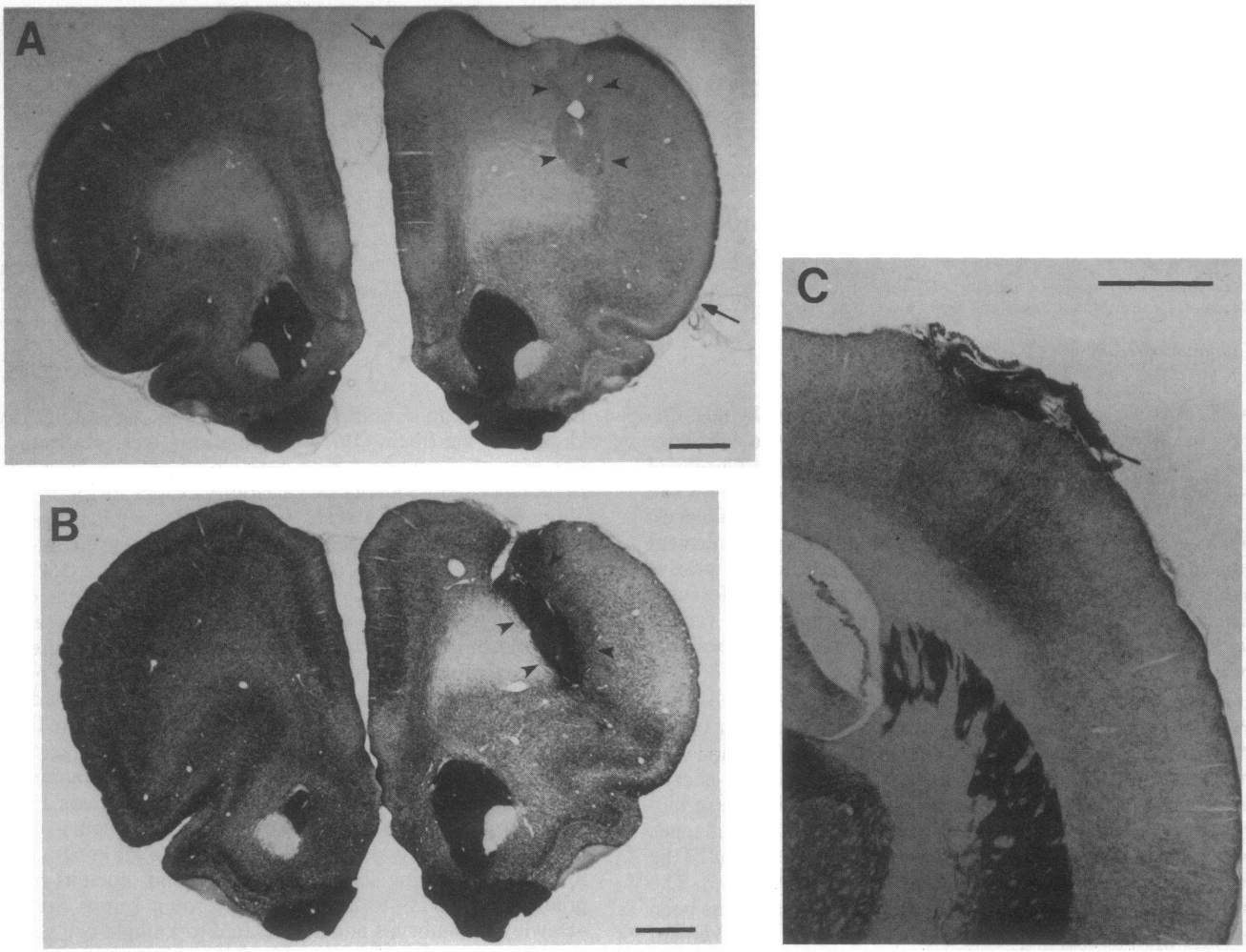

FIG. 1. AcChoEase-stained 40-μm sections through neocortex of experimental animals 6 months after unilateral lesions of the NBM. Lesions were made by injecting ibotenic acid (5 μg in 0.5 μl of 0.1 M phosphate buffer, over 2 min) to two sites in the right NBM (coordinates from bregma, midline, and dura, with incisor bar +5.0: A = 0.2, L = 3.4, V = 7.0 and A = 1.0, L = 2.6, V = 7.3). One week after lesioning, animals to be grafted received two injections of cell suspension, one in dorsolateral frontal cortex (A = 3.4, L = 3.5, V = 3.0) and one in dorsolateral parietal cortex (A = −1.0, L = 5.0, V = 2.0) ipsilateral to the lesion. AcChoEase staining was by the thiocholine method (18) with 0.1 mM ethopropazine as inhibitor of pseudocholinesterase, and 0.25% silver nitrate enhancement. (*A*) Frontal cortex. Reduced cortical AcChoEase staining (between arrows) is unaffected by control graft of hippocampal cells (arrowheads). (*B*) Frontal cortex. Graft of ventral forebrain cells (arrowheads) has brought about substantial restitution of AcChoEase staining in lesioned cortex. (*C*) Parietal cortex ipsilateral to the lesion, through a ventral forebrain graft. Dense AcChoEase-positive fiber outgrowth from the graft spreads in laminar fashion into surrounding denervated cortex. (Bars = 1.0 mm.)

days, so that by the third (training) day all rats entered the dark compartment promptly upon being placed in the light compartment (range, 2–32 sec). Immediately upon entering the dark compartment on the training day, each rat received a 2-sec, 0.5 mA, 50 Hz scrambled footshock. The rat was removed from the apparatus at once, and returned to her home cage. One minute later, the rat was reintroduced to the light compartment. Acquisition of the avoidance response was judged successful if the animal remained in the light compartment 120 sec. Animals entering the dark compartment before 120 sec immediately received an identical shock; they were removed at once and the process was repeated until each animal satisfied the acquisition criterion.

The ventral forebrain graft rats with lesions and control group with lesions did not differ in their rates of acquisition, all animals requiring at least two shock presentations (range, 2–4) to reach criterion. Both groups, however, were significantly slower ($\chi^2_4 = 20.19$; $P < 0.001$) to learn the avoidance than unoperated controls, only six of which required a second shock to reach criterion. Independent measurement of spontaneous locomotion showed no significant group differences in activity.

Retention of this passive avoidance learning was measured in two further tests 24 and 72 hr later. Latency to enter the dark compartment was measured up to a maximum of 300 sec, during which time no further shocks were given. Analysis of variance indicated the three groups differed significantly [$F(2,34) = 4.70$, $P < 0.02$] in overall passive avoidance retention (Fig. 2); ventral forebrain graft animals with lesions did not differ significantly from unoperated animals ($P > 0.1$), whereas controls with lesions reentered the dark chamber significantly more rapidly than the other two groups ($P < 0.025$). The significant differences between control group with lesions and the unoperated and ventral forebrain graft groups were primarily evident at 72 hr. The difference between control and ventral forebrain graft operated groups was not significant at 24 hr [$t(34) = 1.313$, $P < 0.1$].

To eliminate the possibility that these results reflected a graft effect on extinction rather than retention, a second, similar experiment was performed with a different set of animals, examining passive avoidance retention only once, at a longer post-training interval. Unoperated controls, lesioned only, lesioned/ventral forebrain grafted, and lesioned/hippocampal grafted animals were prepared as described above.

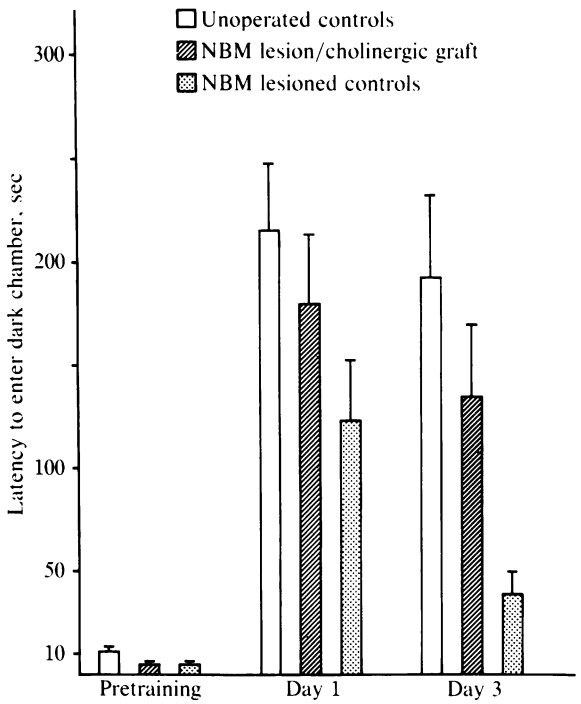

FIG. 2. Step-through passive avoidance performance of the three groups of animals. Values are the mean latencies to enter the dark chamber for the three groups, prior to and on days 1 and 3 after training. Error bars indicate SEM. One and 3 days after training, NBM lesioned rats entered the dark chamber significantly sooner than unoperated rats. By contrast, lesioned rats with ventral forebrain grafts reinnervating the deafferented neocortex showed substantial improvement in retention and did not differ significantly from unoperated controls.

Three months after the time of transplantation, the animals were trained to criterion in the passive avoidance apparatus. On the basis of additional experience gained with other animals in this paradigm, training parameters were slightly modified in this experiment: a 1-sec 0.45-mA footshock was used, and animals were kept in the dark chamber for 15 sec before being returned to their home cages between sessions. Training continued until a 300-sec avoidance criterion was achieved. Animals were tested for retention only once, 5 days later. Under these conditions, unoperated controls required 2.0 ± 0.3 trials to reach acquisition criterion, compared with 2.8 ± 0.4 trials for lesioned only, 3.8 ± 0.4 trials for hippocampal grafted, and 4.0 ± 0.6 trials for ventral forebrain grafted animals. Newman–Keuls tests indicated that the two grafted groups needed significantly more trials to reach criterion than unlesioned controls ($P < 0.01$) but did not differ from each other. By contrast, cholinergic ventral forebrain grafts, but not hippocampal grafts, dramatically restored retention (as measured by latency to enter the dark chamber) to normal (Table 1).

Upon completion of behavioral testing in the first experiment, all rats were killed. The brains of control animals with lesions were assayed regionally for ChoAcTase; brains of grafted animals were perfused with 4% paraformaldehyde, sectioned, and stained for Nissl substance, acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.6) (23), and immunohistochemically for substance P, neuropeptide Y, somatostatin, vasoactive intestinal polypeptide, and [Met]enkephalin (24). The NBM lesions produced a marked loss of ipsilateral cortical AcChoEase staining that persisted even after 6 months (Fig. 1*A*). ChoAcTase in the cortex of lesioned but ungrafted animals also remained profoundly decreased after 6 months, with levels in dorsolateral frontal and parietal cortex ipsilateral to the

Table 1. Effects of NBM lesions and cortically placed ventral forebrain or hippocampal cell grafts on passive avoidance retention 5 days after training

Group	Mean latency, sec
Unlesioned controls	155.04 ± 39.15* (n = 13)
Lesion/ventral forebrain	148.26 ± 42.56* (n = 10)
Lesion/hippocampal	38.60 ± 29.60† (n = 5)
Lesion only	40.17 ± 18.60† (n = 9)

Values (untransformed means ± SEM) are latencies to enter dark chamber. Analysis of variance and mean separation by the Student–Newman–Keuls multiple range test was performed on logarithmically transformed data. Values with superscript * are significantly different from values with superscript † ($P < 0.005$); values sharing the same superscript do not differ significantly from each other.

lesions 52.0% ± 7.8% and 55.2% ± 5.8%, respectively, of contralateral (unlesioned) levels. The stability of these depletions is in contrast to another report (25) and presumably reflects the more complete lesions in the present study. The cortically placed hippocampal control grafts survived, but had no effect on cortical cholinergic innervation (Fig. 1*A*). The grafted ventral forebrain cells, however, were not only heavily AcChoEase-positive themselves (Fig. 1*B*) but gave rise to an extensive outgrowth of AcChoEase-positive fibers often extending beyond 3 mm into adjacent deafferented host neocortex. Frequently, the distribution of graft fiber outgrowth resembled the normal laminar distribution of cortical AcChoEase staining (Fig. 1*C*). While small numbers of some peptidergic neurons and fibers were seen within the ventral forebrain grafts, only cholinergic cells extensively innervated the host neocortex (24). Although cortical ChoAcTase levels were not measured in the grafted animals of this study, in a separate experiment with identically prepared lesions and ventral forebrain grafts (24), ChoAcTase in frontal cortex adjacent to the graft was raised from 39.2% (in lesioned only animals) to 62.7% of intact contralateral levels [$t(15) = 2.25$, $P < 0.05$] 1 month after implantation.

These results confirm previous observations that NBM lesions, even unilaterally, impair passive avoidance learning (12–14). The present results indicate that this deficit involves both acquisition and retention (consolidation and/or retrieval) components of the task. Thus, even when additional training enables animals with lesions to achieve a uniform level of acquisition, they nevertheless show a significant retention impairment with respect to normal animals. This retention deficit is most likely due to a direct memory impairment rather than to changes in shock sensitivity (12, 13) or in general level of fear or motivation, as indicated by the absence of group differences in pretraining habituation. The impairment of acquisition by NBM lesion is not ameliorated by cortically placed ventral forebrain grafts. By contrast, the lesion-induced retention deficit is substantially corrected by the ventral forebrain grafts. The results suggest the possibility that diffuse cholinergic input from these grafts to the cortex is sufficient to sustain normal memory; however, we cannot yet exclude a role for other neuroactive substances released from the grafts. The grafts' inability to influence the acquisition deficit does not necessarily imply the absence of a cholinergic involvement in this component; rather, cholinergic function in acquisition may require patterned activation dependent on normal presynaptic input. Alternatively, a higher level of acetylcholine or cholinergic inputs to other cortical areas not influenced by the present grafts may be necessary for normal acquisition. The possibility must be borne in mind that the cholinergic input does not function in memory *per se*; instead, it may serve to suppress cortical activity that would, in its absence, interfere with normal memory.

Interpretation of functional effects of brain lesions is often made difficult by nonspecificity of the lesions. In particular, effects of ibotenic acid lesions of the NBM region may reflect damage to adjacent structures that do not project to the neocortex, rather than the direct result of neocortical cholinergic denervation. For example, Flicker *et al.* (13) have shown that ibotenic acid lesions of the adjacent dorsolateral globus pallidus result in a similar, though less severe, impairment of passive avoidance learning. Intracerebral transplantation provides an important tool that may help to circumvent this problem: functional recovery after innervation of the neocortex by cholinergic ventral forebrain grafts far removed from the basal forebrain lesions provides strong support for the hypothesis that observed impairments following the lesions are the specific consequence of loss of cortical NBM (presumably cholinergic) input. Furthermore, systematic transplantation to different sites in the neocortex may allow analysis of the cortical topography of the particular NBM-dependent function.

We thank Drs. G. C. Preston, J. E. Alpert, T. W. Robbins, P. C. Emson, and J. Salamone for criticism and suggestions; A. Porter-Goff, S. T. Bunch, D. Conner, and K. Fogelstrom for technical assistance; and L. Evans and M. Wynn for typing the manuscript. This work was supported by the Medical Research Council and Merck Sharp and Dohme. A.F. is the Pinsent-Darwin Student of Mental Pathology at the University of Cambridge.

1. Davies, P. & Maloney, A. J. F. (1976) *Lancet* **ii**, 1403.
2. Bowen, D. M., Smith, C. B., White, P. & Davison, A. N. (1976) *Brain* **99**, 459–496.
3. Perry, E. K., Tomlinson, B. E., Blessed, G., Bergmann, K., Gibson, P. H. & Perry, R. H. (1978) *Brit. Med. J.* **2**, 1457–1459.
4. Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T. & DeLong, M. R. (1982) *Science* **215**, 1237–1239.
5. Pearson, R. C. A., Sofroniew, M. W., Cuello, A. C., Powell, T. P. S., Eckenstein, F., Esiri, M. M. & Wilcock, G. K. (1983) *Brain Res.* **289**, 375–379.
6. Wilcock, G. K., Esiri, M. M., Bowen, D. M. & Smith, C. C. T. (1983) *Neuropathol. Appl. Neurobiol.* **9**, 175–179.
7. Bartus, R. T., Dean, R. L., Beer, B. & Lippa, A. S. (1982) *Science* **217**, 408–417.
8. Coyle, J. T., Price, D. L. & DeLong, M. R. (1983) *Science* **219**, 1184–1190.
9. Morrison, J. H., Rogers, J., Scherr, S., Benoit, R. & Bloom, F. E. (1985) *Nature (London)* **314**, 90–92.
10. Roberts, G. W., Crow, T. J. & Polak, J. M. (1985) *Nature (London)* **314**, 92–94.
11. Deutsch, J. A. (1971) *Science* **174**, 788–794.
12. LoConte, G., Bartolini, L., Casamenti, F., Marconcini-Pepeu, I. & Pepeu, G. (1982) *Pharmacol. Biochem. Behav.* **17**, 933–937.
13. Flicker, C., Dean, R. L., Watkins, D. L., Fischer, S. K. & Bartus, R. T. (1983) *Pharmacol. Biochem. Behav.* **18**, 973–981.
14. Friedman, E., Lerer, B. & Kuster, J. (1983) *Pharmacol. Biochem. Behav.* **19**, 309–312.
15. Perlow, M. J., Freed, W. J., Hoffer, B. J., Seiger, A., Olson, L. & Wyatt, R. J. (1979) *Science* **204**, 643–647.
16. Björklund, A., Dunnett, S. B., Stenevi, U., Lewis, M. E. & Iversen, S. D. (1980) *Brain Res.* **199**, 307–333.
17. Dunnett, S. B., Low, W. C., Iversen, S. D., Stenevi, U. & Björklund, A. (1982) *Brain Res.* **251**, 335–348.
18. Schwarcz, R., Hökfelt, T., Fuxe, K., Jansson, G., Goldstein, M. & Terenius, L. (1979) *Exp. Brain Res.* **37**, 199–216.
19. Winn, P., Tarbuck, A. & Dunnett, S. B. (1984) *Neuroscience* **12**, 225–240.
20. Fonnum, F. (1975) *J. Neurochem.* **24**, 407–409.
21. Emson, P. C. & Lindvall, O. (1979) *Neuroscience* **4**, 1–30.
22. Schmidt, R. A., Björklund, A. & Stenevi, U. (1981) *Brain Res.* **218**, 347–356.
23. Koelle, G. B. (1954) *J. Comp. Neurol.* **100**, 211–228.
24. Fine, A., Dunnett, S. B., Björklund, A. & Iversen, S. D. (1985) *Neuroscience*, in press.
25. Wenk, G. L. & Olton, D. S. (1984) *Brain Res.* **293**, 184–186.