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Acetylcholine release from intrahippocampal septal grafts is under control of the host brain

(neuronal transplantation/microdialysis/hippocampus)

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ABSTRACT **The activity of intrahippocampal transplants of cholinergic neurons was monitored by microdialysis in awake, freely moving rats. Fetal septal–diagonal band tissue was implanted into rats with a complete transection of the fimbria–fornix cholinergic pathway either as a cell suspension injected into the hippocampus or as a solid graft implanted in the lesion cavity. The grafts restored baseline acetylcholine release in the graft-reinnervated hippocampus to normal or supranormal levels. The graft-derived acetylcholine release was dependent on intact axonal impulse flow, and it was markedly increased during behavioral activation by sensory stimulation or by electrical stimulation of the lateral habenula. The results demonstrate that the septal grafts, despite their ectopic location, can become functionally integrated with the host brain and that the activity of the transplanted cholinergic neurons can be modulated from the host brain during ongoing behavior. Anatomical observations, using immunohistochemistry and retrograde tracing, indicate that direct or indirect brainstem afferents to the graft could mediate this functional integration. Host afferent control of the graft may thus play a role in the recovery of lesion-induced functional deficits seen with these types of transplants.**

Previous behavioral and electrophysiological studies have shown that grafts of fetal cholinergic-rich tissue can restore at least some aspects of normal function in rats with lesions of the septohippocampal cholinergic pathway (1, 2). These results raise the question as to the mechanism(s) of action underlying graft-induced functional recovery in the subcortically deafferented hippocampus. While available data indicate that such grafts can restore synaptic cholinergic neurotransmission in the previously denervated target, it remains unclear to what extent they can become functionally integrated with the host neuronal circuitry.

In the intact animal, the activity of the septohippocampal cholinergic neurons is known to change with alterations in ongoing behavior and in response to arousing or behaviorally activating stimuli (3–5). Hippocampal activation, as reflected in the induction of theta rhythm in the hippocampal electroencephalogram, depends on the integrity of the septohippocampal connections, and lesions of the septohippocampal cholinergic pathway by fimbria–fornix (FF) transection or medial septal lesions are known to remove a principal route for subcortical activation of the hippocampal formation (1, 5).

In the present study, we have used the intracerebral microdialysis technique to monitor acetylcholine (AcCho) release in the subcortically denervated hippocampus reinnervated by fetal septal solid or suspension grafts. The experiment was performed in awake, freely moving rats both under baseline conditions and during behavioral activation of types that are known to induce AcCho release from intact septohippocampal cholinergic neurons (3, 4).

METHODS

Young adult female Sprague–Dawley rats (ALAB, Stockholm) were given a unilateral aspirative FF lesion (including the supracallosal striae) in order to produce a complete and permanent cholinergic denervation of the hippocampus (6). Five days later, the septal–diagonal band area was dissected out from 15- to 16-day rat fetuses of the same outbred strain and grafted to the previously lesioned rats either into the cavity produced by the FF lesion (one bilateral septal–diagonal band area per recipient; $n = 16$) or as a cell suspension (7) stereotaxically injected into the ipsilateral hippocampal formation ($n = 17$) at the following coordinates: (*i*) A = −4.5 (behind bregma), L = 3.5 (from the midline), V = 3.0 (ventral to dura); (*ii*) A = −6.0, L = 5.0, V = 5.0. The incisor bar was set at zero level and 1.5 μl of suspension was injected at each site. Nine to 12 months later, normal age-matched control rats ($n = 13$), lesioned-only rats ($n = 5$), and lesioned and grafted rats (solid grafts, $n = 6$; suspension grafts, $n = 12$) had microdialysis probes of the loop type (4 mm of exposed membrane) implanted in the ipsilateral hippocampal formation (normal, lesioned controls and suspension-grafted rats at A = −6.0, L = 4.0, V = 4.0; rats with solid grafts at A = −4.5, L = 2.5, V = 3.5; with incisor bar at zero). In the same surgical session a stimulation electrode was implanted in the caudal part of the ipsilateral nucleus of the lateral habenula (LHb) (A = −4.8 or 4.3, L = 0.7 or 0.9, V = 5.0, with incisor bar at −3.3). Probe and electrode were kept in place by skull screws and dental acrylic. A unilateral transection of the fasciculus retroflexus was performed with an L-shaped knife lowered 5.3 mm ventral to dura immediately lateral (about 0.1 mm) to the LHb stimulation electrode and then rotated 100° medially, thereby undercutting the LHb. A transection between a solid graft and the host hippocampus was performed stereotaxically with a sharp scalpel blade at A = −3.0, L = from midline to −3.5, V = from dura to 4.5 (incisor bar at −3.3).

Dialysis was performed in unrestrained awake rats 1 and 2 days after probe implantation. The probes were perfused at a rate of 2 μl/min with Ringer solution (pH 7) containing 5 μM neostigmine bromide (Sigma); samples were collected every 15 min and immediately frozen in liquid nitrogen. On the first day the rats were subjected to sensory stimulation by gentle handling (stroking the fur and tail) and electrical stimulation of the LHb (15 Hz, 0.3 mA). The LHb stimulation produced a pronounced hyperactivity, which made it necessary to hold the rats during stimulation. KCl (100 mM) and tetrodotoxin

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Abbreviations: AcCho, acetylcholine; FF, fimbria–fornix; LHb, lateral habenula; TTX, tetrodotoxin.

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(TTX; Sigma, 1 μM) were added to the perfusion medium on the second day. In the transection experiment, performed in a separate set of normal (n = 7) and suspension-grafted (n = 6) rats, the LHb was stimulated twice and the fasciculus retroflexus was cut (under halothane anesthesia) between the stimulations. AcCho was assayed by HPLC with post-column enzyme reaction and electrochemical detection (4). The detection limit was ≈0.2 pmol of AcCho. The *in vitro* recovery of AcCho across the probe membrane was 10.8 ± 1.0% (mean ± SEM). The reported AcCho values have not been corrected for the recovery.

After completion of the dialysis experiments, most rats were perfused and immersion-fixed with an ice-cold buffered solution of 4% paraformaldehyde. The brains were sectioned (20 μm) on a cryostat and processed for acetylcholinesterase histochemistry (8). The rats used for tyrosine hydroxylase and serotonin immunohistochemistry (n = 15) were perfused using a pH-shift protocol (9), the brains were sectioned on a vibrating microtome (40 μm), and the sections were processed free-floating with antibodies against serotonin (1:1500 dilution; courtesy of H. W. M. Steinbusch, Free University, Amsterdam) and against tyrosine hydroxylase (1:1000; Pel-Freez Biologicals) by the peroxidase-antiperoxidase technique (10). In another group of five rats that received solid grafts, the retrograde tracer Fluoro-Gold (2%) (11) was iontophoretically injected into the transplant under visual guidance and perfused for fluorescence microscopy after 2–4 weeks survival.

RESULTS

Baseline AcCho levels, which averaged about 1.7 pmol/15 min in the perfusates from the intact hippocampi, were reduced by about 70% in the FF-lesioned hippocampi (Kruskal–Wallis test followed by pairwise Mann–Whitney U tests, $P < 0.05$; Figs. 1*A* and 2*A*). In rats with both lesions and grafts, both types of transplants restored baseline AcCho release to normal or supranormal levels ($P < 0.05$, compared to the FF-lesioned group; Fig. 1). The highest levels were observed in the suspension-grafted hippocampi ($P < 0.05$, compared to the normal group).

Depolarizing concentration of KCl (100 mM) increased AcCho overflow by 340% (range, 120–540%) in the intact hippocampi, whereas no response was detected in the FF-lesioned rats (Fig. 1*A*). All grafted rats responded significantly to the KCl stimulation (for suspension grafts, mean increase was 140%, range was 75–250%; for solid grafts, mean increase was 210%, range was 117–356%). In three of the rats in the suspension group (Fig. 1*B*), AcCho overflow remained high also after the stimulation, unlike the normal rats and the other grafted rats, where the levels were reduced to prestimulation baseline immediately after KCl infusion. Interestingly, in those three rats the probe was found to be located very close to or partly within the transplant, whereas in rats with a normal response pattern the probe was located in the graft-reinnervated areas of the hippocampus at some distance from the grafts (Fig. 1*B*).

Addition of TTX to the perfusion fluid reduced AcCho release in both the intact and the grafted hippocampi to levels seen in the FF-lesioned hippocampi (Fig. 1). This reduction, which amounted to about 75% in the intact hippocampi and to about 80% in the grafted ones, was evident in the second TTX sample and persisted for at least 45 min after the TTX application. No effect of TTX was seen in the FF-lesioned rats.

In three of the rats with solid grafts the connections between the graft and the dialyzed area of the hippocampus were transected during ongoing dialysis (≈2 hr after TTX application, when baseline AcCho levels were restored back to normal) by a knife cut passing through the graft–host border zone (see Fig. 4). This cut caused an immediate drop

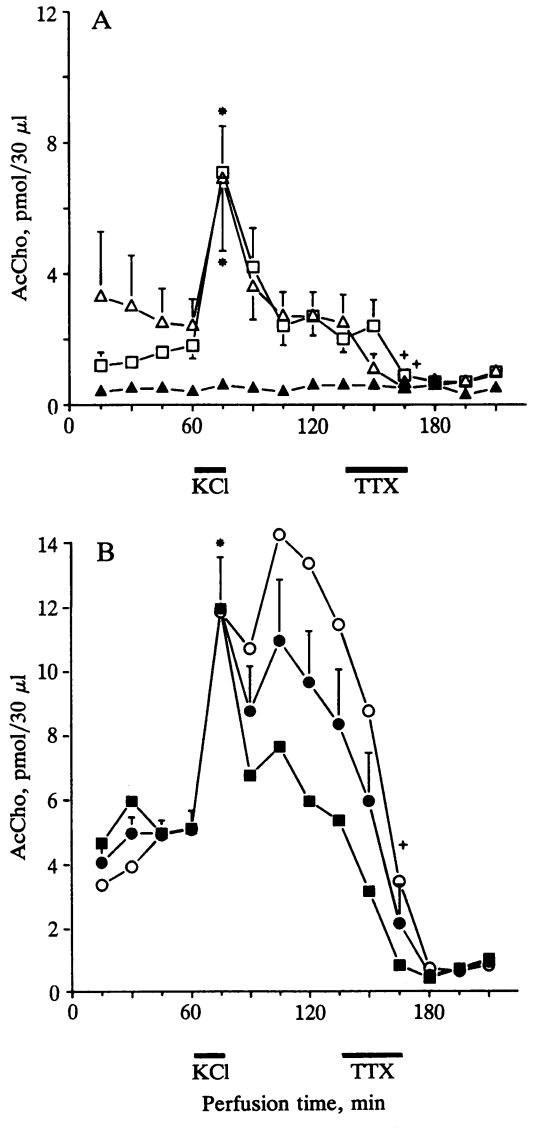

FIG. 1. Effects on AcCho release (in pmol/30 μl, i.e., 15-min sample) of adding KCl (100 mM) or TTX (1 μM) to the neostigmine-containing (5 μM) perfusion fluid. (*A*) Normal (□; n = 5), FF-lesioned (▲; n = 5), and septal solid-grafted (△; n = 6) rats. (*B*) Septal suspension-grafted rats (●; n = 6), including those having the dialysis probe distant from the graft (■; n = 3) or adjoining the graft (○; n = 3). Asterisks indicate significant effects of KCl compared to prestimulus baseline level, and plus signs indicate significant effects of TTX compared to previous sample (at 135 min) in the normal, solid-grafted, and suspension-grafted rats. No effects were seen in the rats with FF lesions only. Statistical analyses in this and subsequent figures were made within each group by two-sided paired t tests at $P < 0.05$.

in baseline AcCho release, by on average 60%, down to a level similar to that seen after TTX application (*cf.* ref. 4).

Sensory stimulation by gentle handling (Fig. 2*A*) or electrical stimulation of the LHb (Fig. 2*B*) produced a marked, 2- or 3-fold increase in hippocampal AcCho release in the normal rats (4). These stimulus-induced changes were entirely absent in the FF-lesioned rats. In both types of grafted rats AcCho release responded to both handling and LHb stimulation, although the magnitude of the response was generally lower than in the normal rats (Fig. 2 *C* and *D*). The peak increase in AcCho release in response to handling was on average 57% (range, 22–100%; $P < 0.05$) in the suspension-grafted rats and 64% (range, 34–112%; $P < 0.05$) in the rats with solid septal grafts. The mean increase in response to LHb stimulation was 65% (range, 20–119%; $P < 0.05$) in

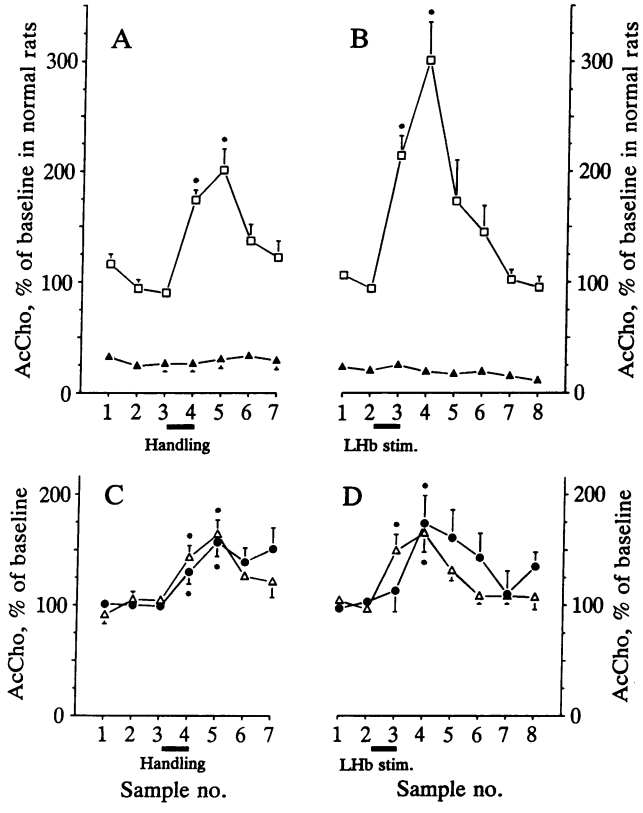

FIG. 2. Effects on AcCho release of sensory stimulation by handling (*A* and *C*) and LHb stimulation (15 Hz, 0.3 mA) (*B* and *D*) in the different groups, expressed as a percentage of the prestimulation baseline level; 15-min samples were collected. (*A* and *B*) Normal (□) and FF-lesioned (▲) rats. The values in the FF-lesioned rats are expressed as a percentage of baseline in normal rats. (*C* and *D*) Suspension-grafted (●) and solid-grafted (△) rats. Asterisks indicate significant effects of the stimulations compared to the mean of the prestimulus baseline ($P < 0.05$).

the solid-grafted rats and 68% (range, 15–160%; $P < 0.05$) in the suspension-grafted rats. As in the normal rats the AcCho increase was evident in the sample collected during the 15-min stimulation period and persisted or was further increased in one or two samples following the stimulation. The behavioral activation induced by handling or LHb stimulation followed a similar pattern: the rats were markedly activated by both types of stimuli and they remained more active in the cage during the sampling period following the stimulation period. Transection of the fasciculus retroflexus between two subsequent LHb stimulations almost completely abolished the response in hippocampal AcCho release in both the normal and the grafted animals (Fig. 3). Previous experiments in normal rats have shown that repeated LHb stimulations evoke the same increase in AcCho release before and after sham surgery (4).

In the microscopic analysis, the probes were found to be located in the dentate gyrus and CA1 area of the dorsocaudal hippocampus (Figs. 4 and 5) or (in two rats with suspension grafts) in the CA2–CA3 region. In the FF-lesioned rats the dorsal two-thirds of the hippocampal formation (including the area around the probe) was devoid of acetylcholinesterase-positive innervation (Fig. 5*B*). In the grafted animals, a dense graft-derived acetylcholinesterase-positive innervation had been reestablished in the area around the probe (Fig. 5*C*). This graft-derived terminal network was generally denser in the rats with suspension grafts (Fig. 5*C*), and in some of these specimens the density was clearly higher than normal (compare with Fig. 5*A*).

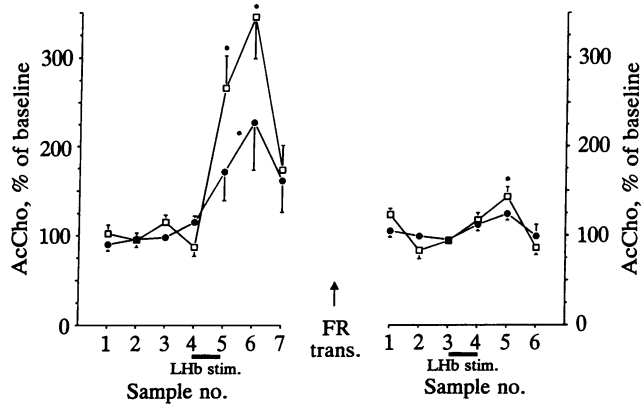

FIG. 3. Effects on AcCho release of LHb stimulation (15 Hz, 0.3 mA) before and after transection of the fasciculus retroflexus (arrow). Values are expressed as percentages of prestimulation baseline before each LHb stimulation in normal (□; $n = 7$) and septal suspension-grafted (●; $n = 6$) rats. Asterisks indicate significant effects compared to the mean of baseline samples before each LHb stimulation ($P < 0.05$). Normal-group data are also part of a parallel study (4).

The cell suspension grafts appeared as one or several tissue masses, either embedded within the hippocampus (Fig. 5*C*) or the hippocampal fissure or attached to the dorsal or ventral hippocampal surfaces. The solid septal grafts filled part of the FF lesion cavity (Fig. 4), where they were attached to the surface of the dorsal thalamus. They had fused with the septum rostrally (Fig. 5*D*) and the hippocampus caudally, thus forming a tissue bridge across the cavity. In many cases the grafts had fused also with the lesioned surface of the caudate-putamen laterally.

Immunohistochemistry revealed areas of serotonin- and tyrosine hydroxylase-positive fibers in both types of grafts

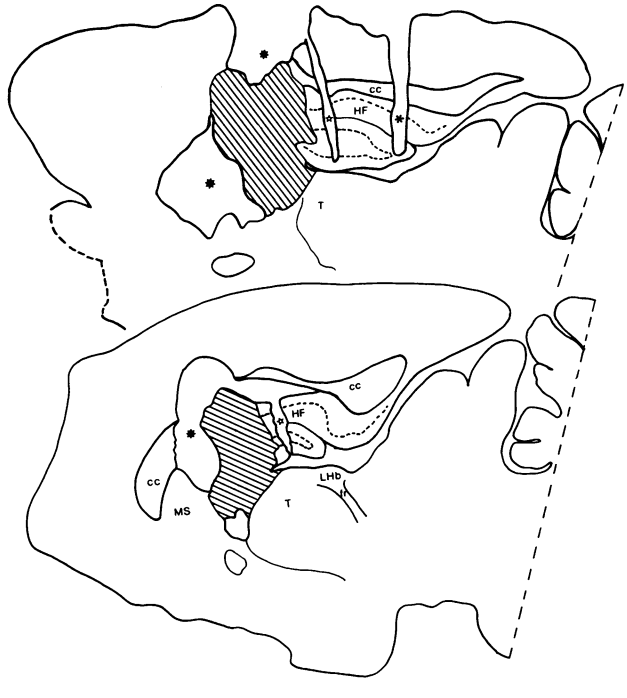

FIG. 4. Camera lucida drawings of sagittal sections from a rat with a solid septal graft (hatched area) placed in the lesion cavity (filled stars) that was subjected to a knife cut (open stars) between the graft and dialysis probe (asterisk) in order to transect all graft-derived fibers. The solid grafts had fused with the septum (MS) rostrally, the thalamus (T) ventrally, and the hippocampal formation (HF) caudally. cc, Corpus callosum; fr, fasciculus retroflexus.

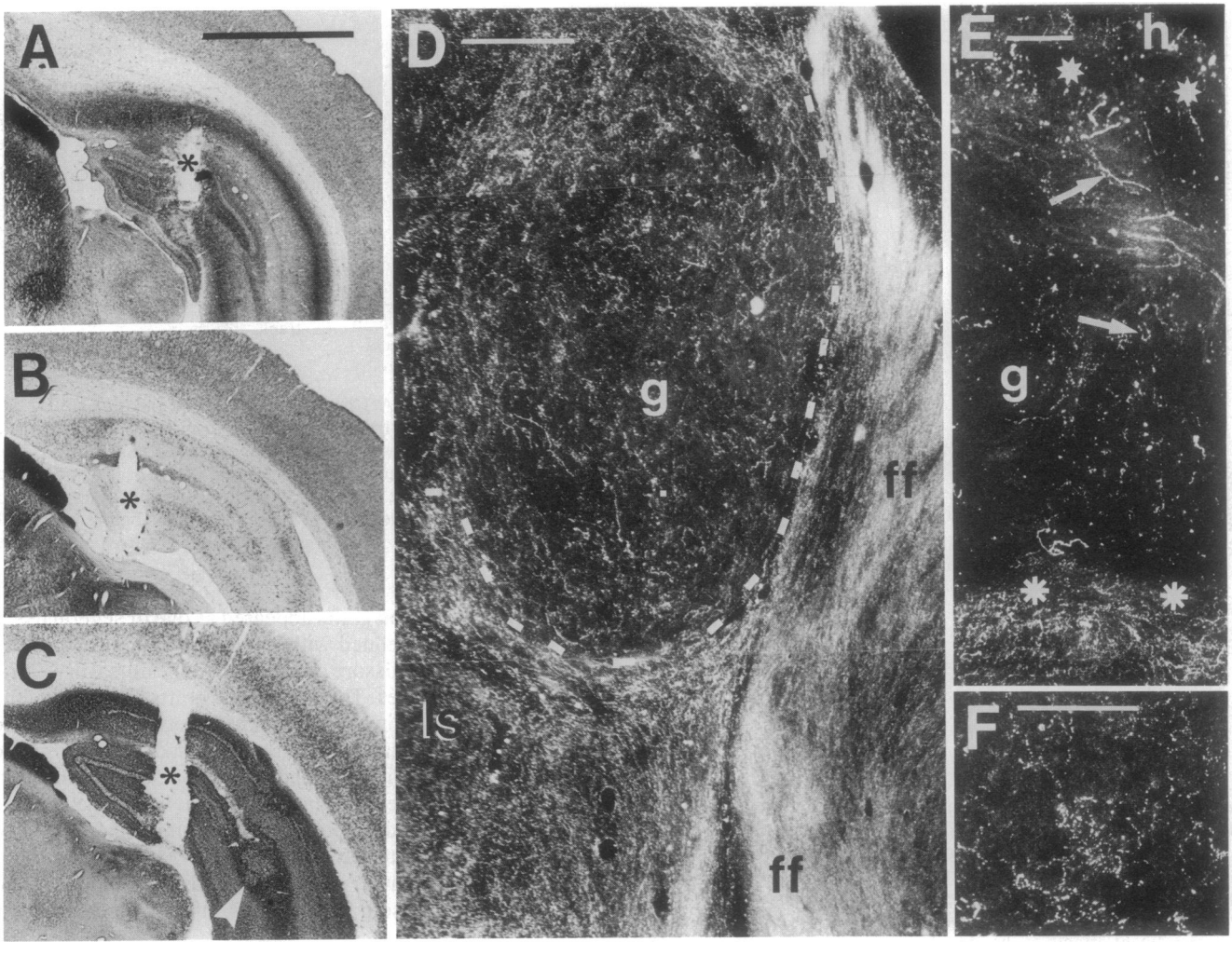

FIG. 5. (*A–C*) Coronal sections of the dorsal hippocampus from a normal rat (*A*), FF-lesioned rat (*B*), and septal suspension-grafted rat (*C*) processed for acetylcholinesterase histochemistry. Arrowhead points at graft tissue. Asterisks indicate probe tract. [Bar (in *A*) = 2 mm.] (*D*) Overview from a coronal section of a solid septal graft (g) in the caudal part of the septal area, showing abundant host-derived serotonin-immunoreactive fibers passing from the lateral septum (ls) to form a terminal network in large parts of the graft. Dashed line indicates graft–host border. ff, Fimbria–fornix. (Bar = 0.25 mm.) (*E*) Serotonin-positive fibers (arrows) in a suspension graft (g) that had fused with both the diencephalon below (border indicated by asterisks) and the hippocampus (h) above. Stars indicate border between graft and hippocampus. (Bar = 0.1 mm.) (*F*) Tyrosine hydroxylase-positive terminal network in the depth of a solid septal graft. (Bar = 0.1 mm.)

(Fig. 5 *D–F*), although the density was clearly higher in the solid septal grafts. In several cases, it was possible to trace immunostained fibers from the host into the transplant tissue, and it was particularly evident in the solid grafts (Fig. 5*D*), where serotonin- and tyrosine hydroxylase-positive fibers were seen to pass in large numbers from the septum. Serotonin-positive cell bodies were not found in the grafts, whereas tyrosine hydroxylase-positive perikarya of the small fusiform type normally found in the preoptic region (12) occurred in widely varying numbers. However, a clear tyrosine hydroxylase-positive innervation was present also in grafts with a very small number of intrinsic tyrosine hydroxylase-positive cell bodies (Fig. 5*F*).

Fluoro-Gold injected into the depth of solid septal grafts labeled cells in the medial septum–diagonal band area, in the ipsilateral host hippocampus (especially the hilus of the dentate gyrus), and in one case also in the ipsilateral lateral hypothalamus. Areas caudal to the hypothalamus were not analyzed. In the five rats included in the analysis, the Fluoro-Gold injection sites were confined to the graft, although there was some leakage into the lesion cavity and the adjacent lateral ventricle (seen as Fluoro-Gold staining of the cavity walls). Previous experiments indicate, however, that such tracer leakage into the cerebrospinal fluid is probably unable to produce neuronal labeling of the type seen here (11).

DISCUSSION

The results show that intrahippocampal septal grafts can restore baseline and K^+-evoked AcCho release in the graft-reinnervated hippocampus to levels that are close to or even above those seen in intact animals. The TTX and knife-transection experiments indicate that the graft-derived AcCho release (as in the intact septohippocampal system) is dependent on axonal impulse flow. This suggests that the grafted cholinergic neurons are spontaneously active at a level approximating that of the intact septal cholinergic neurons, despite their ectopic location.

The principal finding was the observation that the graft-derived hippocampal AcCho overflow increased in response to behavioral activation in a way similar to (although smaller in magnitude than) that seen in the intact hippocampus. Gentle handling can be characterized as an arousing general sensory stimulus, probably with a stressful component, of a type that induces hippocampal theta rhythm as well as activation of the septohippocampal neurons in the intact animal (3, 13). The activation of the septum by sensory stimuli is likely to be relayed via the mesencephalic reticular formation (5).

The effect of the LHb stimulation might, in part, be mediated by a small direct projection to the nucleus of the

diagonal band, but the principal action is more likely to be relayed through the mesencephalic reticular formation, which is extensively innervated from the LHb (14, 15). The LHb has been shown to be a prominent activator of the ascending serotonergic and noradrenergic systems (16); in addition, this nucleus is known to project to the mesencephalic dopamine and AcCho cell groups, which, in turn, provide afferents to the septal area. Transection of the fasciculus retroflexus, which carries the efferent output of the LHb, blocked the effect of LHb stimulation on hippocampal AcCho release, both in the intact and in the grafted animals, indicating that the effect was not due to a nonspecific volume-conducted spread of current.

The present data provide direct evidence that the activity of intrahippocampal septal grafts is under functional control of the host brain and that the activity of the grafted cholinergic neurons can be modified in a seemingly normal way during ongoing behavior. In the intact brain, the most important regulation of the septum appears to come from the brainstem, above all the mesencephalic reticular formation. Studies on the brainstem regulation of the septohippocampal neurons have proposed a role for serotonergic, dopaminergic, noradrenergic, and cholinergic ascending projections (5, 17–20). While AcCho and norepinephrine have been reported to activate the septohippocampal neurons (18, 19), the effects of the serotonergic and dopaminergic afferents have been ascribed to disinhibitory and inhibitory mechanisms, respectively (17, 18, 20). The present immunohistochemical analysis demonstrated host serotonergic and catecholaminergic afferents, probably of brainstem origin, to both types of septal grafts, and the preliminary observations after retrograde tracer injections into the solid septal grafts indicated the presence of afferents also from the septal–diagonal band area, hippocampus, and lateral hypothalamus of the host.

The two different transplantation procedures used here should provide quite different possibilities for circuitry reconstruction. In the case of the solid septal grafts, the tissue was implanted as a bridge across the lesion cavity, which should optimize the possibilities for host axons, lesioned by the FF lesion, to grow into the graft or even across the lesion cavity (21). In the suspension-grafted rats, on the other hand, the chances for host axons to regenerate across the lesion are minimal. Consistent with this, Buzsaki *et al.* (22) observed recovery of behavior-dependent theta rhythm in the hippocampus only in rats with solid septal grafts, whereas rats with septal suspension grafts displayed only short-duration bursts of theta activity, mainly during immobility. However, in the present study, hippocampal AcCho release in response to LHb stimulation and handling was similar for both types of grafts. This suggests that brainstem influences can reach the grafts not only through the host septum, but also via other routes, such as the so-called ventral pathway through the amygdaloid–piriform lobe, the entorhinal cortex, and the host hippocampus, as demonstrated in previous studies (2, 23–25). Indeed, this may explain why septal suspension grafts can ameliorate lesion-induced functional deficits in the absence of any anatomical reconnection across the FF lesion cavity (26, 27).

In conclusion, the present results provide evidence that grafted septal neurons can reinstate cholinergic neurotransmission in the subcortically denervated hippocampal formation and that the grafted neurons, despite their ectopic position, can become functionally integrated with the host brain. Direct or indirect brainstem afferents, allowing the activity of the grafted neurons to be modulated from the host brain during ongoing behavior, may play a role in the recovery of lesion-induced behavioral deficits seen with these types of transplants.

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