Intracerebral drug delivery in rats with lesion-induced memory deficits

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Pharmacological treatments directed at increasing cortical acetylcholine activity in patients with Alzheimer's disease have largely been disappointing, perhaps because denervated areas of brain may not be exposed to adequate amounts of drug. A new method has been developed to enable localized intracerebral delivery of neurotransmitter substances using a polymeric drug delivery system. Microspheres of a polyanhydride sebacic acid copolymer were impregnated with bethanechol, an acetylcholinesterase-resistant cholinomimetic. Twenty rats received bilateral fimbria-fornix lesions, producing cholinergic denervation of the hippocampus and marked impairment in spatial memory. The animals were trained for 2 weeks to run an eight-arm radial maze, after which they received bilateral intrahippocampal implants of saline (five rats), blank polymer (five rats), or bethanechol-impregnated polymer (10 rats). Following implantation, spatial memory was assessed by radial-maze performance testing for 40 days. Untreated lesioned rats showed persistently poor spatial memory, entering maze arms with near random frequency. Similarly, animals treated with saline and blank polymer did not improve after implantation. Rats treated with bethanechol-impregnated microspheres, however, displayed significant improvement within 10 days after implantation; this improvement persisted for the duration of the experiment (p < 0.05, Student's t-test). Histological analysis of regional acetylcholinesterase staining showed widespread loss of activity throughout the hippocampus bilaterally in all animals. The microsphere implants were visible within the hippocampus, with minimal reactive changes in surrounding brain. It is concluded that intracerebral polymeric drug delivery successfully reversed lesion-induced memory deficits, and has potential as a neurosurgical treatment method for Alzheimer's disease and other neurodegenerative disorders.

KEY WORDS • Alzheimer's disease • dementia • bethanechol • drug delivery • rat
Radial-Maze Testing

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This new method of regionally specific neurotrans-
mitter delivery may be useful in the study and treatment
of Alzheimer's disease. Hippocampal cholinergic de-
ervation reliably produced profound spatial memory

deficits in rats. The cholinomimetic agent bethanechol
was impregnated into polyanhydride microspheres and
implanted in denervated hippocampus in rats to pro-
vide selective local administration of the drug.

Materials and Methods

Fimbria-Fornix Lesions

Twenty-two adult Sprague-Dawley rats, each weigh-
ing 250 to 300 gm, were anesthetized with 1.6 ml/kg of
intra-peritoneal Chloropen (chloral hydrate, magne-
sium sulfate, pentobarbital, ethyl alcohol, and propyl-
ene glycol) and placed in a small-animal stereotactic
head frame. Through a midline scalp incision and small
bilateral frontoparietal craniectomies, bilateral stereo-
tactic fimbria-fornix transections were performed using
a Beaver ophthalmic knife blade mounted on the mi-
cromanipulator. For each transection, the tip of the
knife blade was placed initially in the midline, 1.5 mm
posterior to the bregma and 4.5 mm deep to the cortical
surface, then advanced laterally and anteriorly at an
angle 30° anterior to the coronal plane for a distance of
5.0 mm. After scalp closure the rats were allowed to
recover for 1 week, during which time food and water
were provided ad libitum. Two animals were subse-
sequently excluded from analysis when postmortem
AChE histochemical studies revealed inadequate fornix
lesions.

Radial-Maze Testing

The eight-arm radial maze, as described by Olton
and Samuelson, was used to assess spatial memory in
the lesioned animals. Prior to initiation of the maze
trials, the rats underwent food restriction over a 2-week
period until they reached 85% of their baseline weight.
During this period of weight reduction, the rats were
gradually acclimatized to the radial maze by allowing
them to randomly roam the maze surface for short
periods of time.

The radial maze consists of an elevated octagonal
central platform with eight “choice arms” radiating
from the center (Fig. 1). The maze is placed in a well-
lighted room with visually prominent objects on all
walls. Olton and Samuelson have shown that rats
utilize visual features from the surrounding room rather
than olfactory cues to spatially orient themselves within
the maze. Sliding doors control access to each choice
arm, and at the end of each arm is a food well. During
trial runs, a rat is placed on the central platform and
food rewards are placed in the food wells. All doors are
opened and the rat enters a choice arm and eats the
food reward at the end of the arm. When the rat returns
to the central platform, all doors are closed for a 5-
second period, then simultaneously opened. Rats with
intact spatial memory will learn to avoid entering choice
arms that no longer have food rewards, and instead
select arms which they have yet to enter.

During individual trial runs, the rats in this study
were placed on the maze until either all food wells were
empty, 10 minutes had elapsed, or a total of 15 arm
choices had been made. Between test periods rats were
given water ad libitum and supplemental food to main-
tain body weight at 85% of baseline plus 5 gm/wk for
growth. Following 1 week of recovery from fimbria-
fornix surgery and 1 week of weight reduction, rats were
tested on the maze once daily for 2 weeks to determine
baseline performance (Fig. 2).

Bethanechol-Polymer Microspheres

The PCPP-SA was prepared by solvent removal as
previously described. Bethanechol was placed in
PCPP-SA solution in an organic solvent which was then
dispersed as microspheres into silicon oil. The polymer
solidified in the silicon oil and was extracted as drug-
loaded microspheres. Microspheres of the bethanechol-
(PCPP-SA) compound ranged in size from 1 to 5 μ (Fig.
3). Blank polymer was prepared as described above
without impregnated bethanechol. The specimens were
stored at -4°C until the time of implantation.

Hippocampal Implants

Rats were anesthetized with inhaled halothane and
placed in a small-animal stereotactic head frame. The
previous midline scalp incision was reopened and a
total of four burr holes were placed, two over each
hippocampal formation. Two implants were placed into
each hippocampus, either saline (in five rats), blank
polymer (in five rats), or bethanechol-impregnated
microspheres (in 10 rats). Implant coordinates refer-
cenced to the bregma were: 1) 3.3 mm posterior, 1.5
mm lateral, and 3.8 mm ventral; and 2) 5.3 mm pos-
terior, 3 mm lateral, and 3.6 mm ventral.

Microsphere implants were created by placing the
powder into a small cone, allowing the powder to settle,
then passing a No. 22 blunt-tipped needle into the cone.
The microsphere cylinder within the needle was comp-
acted into a length of 1.8 mm by means of a stainless
steel stylette. The loaded blunt-tipped needle-stylette
assembly was attached to a micromanipulator and di-
rected to the target coordinates described above. Once
in the proper position, the stylette was kept stationary
to maintain implant position, while the blunt-tipped
needle was slowly withdrawn (Fig. 4). Saline was im-
planted by drawing up fluid into the distal 1.8 mm of
the injection assembly and injecting it into the hippo-
Intracerebral polymer microsphere drug delivery in rats

Fig. 1. Diagram of the eight-arm radial maze. Choice arms containing food rewards extend out from a central platform. As they learn the task, rats with intact spatial memory recall which arms have already been explored and do not reenter the empty choice arms.

Following implantation surgery, all rats resumed daily radial-maze testing. After 40 days of postimplantation testing, rats were deeply anesthetized with intraperitoneal pentobarbital and perfused transcardially with a 10% neutral buffered formalin. The brains were removed and postfixed in 30% sucrose-formalin for 3 days.

Histological Studies

Frozen coronal sections, 40 μm thick, were cut from the perfused brains. Alternating sections were processed for cresyl violet staining or AChE histochemistry using Naik’s method.22 To confirm hippocampal cholinergic denervation, AChE-staining densities in hippocampal regions CA1 through CA4 were analyzed using a Drexel DUMAS microcomputer image analysis system. To control for variability in staining technique between animals, mean AChE optical density in the hippocampus was compared to optical density within the thalamus for each rat. Normal values for AChE optical density were determined by examining hippocampal sections from four unlesioned rats. Two rats with hippocampal AChE-staining density more than 2 standard deviations (SD’s) greater than the mean staining density of all lesioned rats were considered to have inadequate fimbria-fornix lesions and were excluded from analysis. Cresyl violet-stained sections were examined to determine the location of the polymer implants and to assess parenchymal changes in the vicinity of the implants.

Statistical Analysis

Radial-maze performance was assessed according to a weighted daily performance scale (Table 1). The probability of entering a baited maze arm (correct choice) by chance decreases logarithmically as successive arms are entered, and the weighted score reflects that probability difference. Mean performance scores over 10-day epochs were determined for each animal, beginning with the 10 days preceding polymer implantation. Radial-maze performance was compared between groups as the mean ± SD for each 10-day epoch using Student’s t-test.

Fig. 2. Diagram of experimental design. The rats underwent bilateral fimbria-fornix lesions followed by radial-maze training. After 2 weeks of maze shaping and weight reduction, the rats received hippocampal implants of saline (five rats), blank polymer (five rats), or bethanechol-impregnated polymer (10 rats). Following implantation, the rats were tested for 40 additional days, then sacrificed and processed for histological study.

Fig. 3. Scanning electron micrograph of bethanechol-impregnated polyanhydride microspheres. The average sphere diameter is 3 μm. Microspheres were in powder form at room temperature. × 1500.

FIG. 4. Microsphere implantation technique. Left: Microspheres are impacted into a No. 22 stainless steel cylinder to a depth of 1.8 mm. Center: The implantation assembly is stereotactically placed into the hippocampal formation. Right: The implantation stylette is held stationary as the stainless steel cylinder is slowly raised, leaving a polymer implant column within the hippocampus.

**Results**

*Histological Findings*

The polymer implants were visible by light microscopy in all rats treated with blank or bethanechol-impregnated microspheres. The microspheres maintained their cylindrical configuration within the brain parenchyma, and a thin rim of reactive glial tissue was noted surrounding the needle track and polymer column (Fig. 5). A similar pattern of mild gliosis was noted along the implant tracks of rats in the saline-treated group. There was no evidence of hemorrhage in any of the specimens examined. The implant location varied slightly between animals. The majority of implants were contained entirely within the hippocampus, usually extending from the CA1 region into the more ventral CA4 region. However, several implants extended further ventrally into the thalamus.

The AChE histochemical findings revealed widespread loss of hippocampal staining optical density in the fimbria-fornix lesioned rats compared to the unoperated control rats. Figure 6 shows the regional optical density determinations for AChE staining in an unlesioned control versus a lesioned rat. Following fimbria-

**TABLE 1**

Performance scores for radial maze

<table>
<thead>
<tr>
<th>No. of Arms Entered</th>
<th>No. of Correct Choices</th>
<th>Performance Score</th>
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<tr>
<td>8</td>
<td>8</td>
<td>6.0309</td>
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<td>7</td>
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<td>4</td>
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<td>6</td>
<td>6</td>
<td>1.5091</td>
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<td>5</td>
<td>0.77343</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>0.13012</td>
</tr>
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</table>

FIG. 5. Photomicrograph of a hippocampal section from a rat with bethanechol-impregnated microspheres implanted 40 days before. The polymer column has retained its configuration. A thin rim of gliosis is evident around the implant. Cresyl violet, × 40.
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Fornix lesioning, there was a consistent 50% to 70% reduction in optical density throughout all regions of the hippocampus except the medial portion of CA1, which showed only slight reductions in AChE staining.

Maze Performance

All rats tolerated the hippocampal implants well and awoke rapidly after halothane anesthesia was discontinued. No difference in general activity level was noted among the various treatment groups. The animals implanted with bethanechol-(PCPP-SA) entered fewer than eight arms in 7.3% of all trials, compared with 8.4% in the blank polymer-treated groups, and 7.0% in the saline-treated group. In the succeeding weeks, the rats treated with saline and blank polymer continued to perform poorly, showing no improvement in performance scores above baseline at any of the time intervals tested (Fig. 7). As a group, the rats treated with bethanechol-impregnated microspheres improved significantly following implantation (p < 0.05). Individual performances, however, varied within this improved treatment group; some rats ran the maze without mistakes, while others showed only modest improvement (Fig. 8). The time course of improvement was rapid, reaching statistical significance during the first 10-day epoch. Beneficial treatment effects persisted for the entire 40 days of the experiment.

Discussion

In this study a new method of intracerebral regional neurotransmitter delivery was utilized to reverse lesion-induced spatial memory deficits in rats with bilateral fimbria-fornix lesions. Implantation of polymer microspheres containing bethanechol into cholinergically denervated hippocampus resulted in improved maze performance in lesioned animals.

Brain histochemical abnormalities involving a variety of neurotransmitters have been reported in patients with Alzheimer's disease. The neurotransmitter system most consistently and profoundly affected, however, is the basal forebrain cholinergic system. This collection of cholinergic neurons consists of the medial septal nucleus (MSN), the nucleus of the diagonal band of Broca, and the nucleus basalis of Meynert (NBM). In humans as well as in rodents, axons from these basal forebrain neurons project to all regions of the cerebral cortex. The MSN neurons project to the hippocampus by way of the fimbria-fornix, and the NBM neurons project in a

![Fig. 6. Photomicrographs of hippocampal sections stained for acetylcholinesterase (AChE), x 1.25. Specimens are from a rat with a fimbria-fornix lesion (left) and from a control rat (right). The numbers indicate the average regional optical densities for lesioned and control rat groups on the respective photomicrographs. The AChE optical staining densities are markedly decreased in the group with fimbria-fornix lesions compared to the control group, confirming hippocampal cholinergic denervation.](image)

![Fig. 7. Group maze performance scores in the three treatment groups: sham (five saline-treated rats), blank polymer (five rats), and bethanechol-impregnated polymer (10 rats). The vertical axis denotes the mean corrected group performance score over each 10-day epoch ± standard deviation; the horizontal axis denotes the number of days before and after hippocampal implantation. The performance of the bethanechol-impregnated polymer group shows significant improvement compared to baseline at all postimplant time intervals. Asterisks indicate statistically significant changes (p < 0.05, Student's t-test).](image)
The "cholinergic hypothesis" of Alzheimer's disease is based on the assumption that loss of basal forebrain cholinergic neurons is the primary pathological process contributing to the cognitive deficits characteristic of this disease. This hypothesis is supported by animal experimental studies showing that selective lesions of the basal forebrain cholinergic system cause cortical cholinergic denervation and impaired memory function. A variety of treatments have been developed based on this cholinergic hypothesis. Several investigators have reported cortical reinnervation and functional improvement in rodent models of Alzheimer's disease following implantation of cholinergic fetal brain grafts. Others report no improvement and temporal lobes.

In the current experiment, microspheres of PCPP-SA polymer were impregnated with the AChE-resistant cholinergic agonist bethanechol. The efficacy of this method of regional cholinergic replacement was tested using an animal model of cholinergic denervation. Axons of the medial septal nucleus portion of the basal forebrain cholinergic system travel as a compact fiber bundle in the fimbria-fornix and terminate in the hippocampus. The rodent septohippocampal projection system is easily disrupted surgically, resulting in selective denervation of the hippocampus and reproducible deficits in spatial memory function. The hippocampal polymer implantation sites used in the present experiment are comparable to the cholinergic brain grafting sites used by Dunnett, et al., to successfully reverse behavior deficits in rats with fimbria-fornix lesions.

Histological analysis substantiated the stereotactic

Drugs administered via intraventricular infusion lack regional selectivity for denervated areas of the brain, and this may contribute to poor clinical results. Cholinergic receptors are located throughout the brain and many are not involved with the NBM projection system. Just as systemic treatment causes detrimental stimulation of peripheral cholinergic receptors, whole-brain administration of bethanechol may excessively stimulate cholinergic receptors in nondenervated regions of the brain.

Regional intracerebral delivery of neurotransmitters using sustained-release polymers may be a useful alternative to these conventional pharmacological methods. A variety of polymers have been synthesized to release biologically active molecules. Polymeric drug delivery systems have also been used to deliver neuronal tracers and immunizing antigen, and delivery of chemotherapeutic agents into brain tumors. Polymeric drug delivery systems have also been used to deliver neuronal tracers and heraldin to perivascular sites. In preliminary experiments, we have characterized the release of radio-labeled acetylcholine from PCPP-SA in rat hippocampus. Approximately 40% of radiolabeled transmitter was released from the polymer in a linear fashion during the 6 weeks following implantation of PCPP-SA microspheres impregnated with 3H-acetylcholine. Diffusion of the tracer was limited to 1 to 2 mm of the adjacent brain parenchyma.

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Histological analysis substantiated the stereotactic
administration of polymer to the hippocampal sites and showed that the implants retained their cylindrical configuration over time. Host brain reaction was minimal, consisting of a thin concentric ring of gliosis around each implant. This lack of inflammatory response is consistent with previous reports describing a noninflammatory interaction between polymers and corneal tissue. The exact location of the polymer implants was somewhat variable: most implants were contained within the hippocampus but several extended more ventrally into the thalamus. Because this technical imprecision may contribute to variability in maze performance, an improved technique of intracerebral polymer implantation is currently being developed.

Behavioral effects of polymer implants were objectively assessed using an eight-arm radial maze, a paradigm that has been well-characterized and is thought to accurately reflect spatial memory function in the rat. In the current experiment, rats with bilateral fimbria-fornix lesions performed poorly prior to polymer implantation, entering baited and empty choice arms at near-chance frequencies. Following implantation surgery, rats with hippocampal implants of saline or blank polymer showed no improvement during the 6-week testing period. As a group, the rats implanted with bethanechol-impregnated microspheres showed significant improvement in maze performance after implant surgery. This improvement was evident within the first 10 days and persisted for the entire 40-day postimplantation test period. The magnitude of improvement for individual rats within this treatment group varied: some rats executed the maze task perfectly following implantation, while others showed only moderate improvement. This heterogeneous performance pattern may be a consequence of variability in implant locations, differences in the degree of injury caused by the fimbria-fornix lesion surgery, or differences in the quantity of microspheres implanted, among other possible explanations.

An additional caveat must be considered when interpreting the results of the current experiments. Patients with Alzheimer's disease lose cholinergic projections to all regions of the cerebral cortex, while rats with fimbria-fornix lesions lose cholinergic projections predominantly to the hippocampus. Although it seems likely that cholinergic intraparenchymal drug delivery will enhance function in other regions of cholinergically denervated brain, this has yet to be proven. This issue will soon be addressed experimentally using rats with lesions of the nucleus basalis. This lesion results in cholinergic denervation throughout the cerebral cortex. The behavioral effects of cholinergic polymeric implants in several brain regions can be tested using this model.

References


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