Method for Making Selective Lesions of the Hippocampus in Macaque Monkeys Using NMDA and a Longitudinal Surgical Approach

Robert R. Hampton,* Cindy A. Buckmaster, Dawn Anuszkiewicz-Lundgren, and Elisabeth A. Murray

ABSTRACT: We describe a method for making selective lesions of the hippocampus in macaque monkeys, using a magnetic resonance imaging (MRI)-guided stereotaxic approach in which the excitotoxin N-methyl-D-aspartic acid (NMDA) is injected at intervals along a single needle track that extends longitudinally through the rostrocaudal extent of the hippocampus. Procedures were conducted on six rhesus monkeys (Macaca mulatta) and were assessed with either in vivo MRI (n = 3) or postmortem microscopic examination of the tissue after standard histological processing of the brains (n = 3). Based on our extensive experience with the standard stereotaxic procedure in which ibotenic acid (IBO) is injected via the dorsal approach, we report that the new method provides a viable and potentially advantageous alternative to the standard procedure. First, the longitudinal approach combined with N-methyl-D-aspartic acid (NMDA) injections increases the reliability and efficacy of hippocampal excitotoxic lesions, probably by limiting the leakage of injectant into the ventricles. Second, the present procedure led to more rapid postoperative recovery compared with that after the standard procedure. Third, because the new method requires fewer needle penetrations than the standard method, it most likely reduces the chances of infarction in extrahippocampal tissue. Finally, the new surgical approach may provide a mechanism for infusing agents into the hippocampus from a single cannula. Published 2003 Wiley-Liss, Inc.†

KEY WORDS: MRI; longitudinal; ibotenic acid; excitotoxin; medial temporal lobe

INTRODUCTION

Since the discovery that bilateral damage to the medial temporal lobe in humans is associated with selective memory deficits (e.g., Scoville and Milner, 1957), work in nonhuman animals has been aimed at identifying the particular structures important for memory. The amygdala and hippocampus were the targets of early neuropsychological investigations in nonhuman primates (e.g., Orbach et al., 1960; Drachman and Ommaya, 1964; Correll and Scoville, 1965a,b, 1967). In efforts to mimic the removal in H.M. and other patients who became severely amnesic after bilateral resection of the medial temporal lobes (Scoville and Milner, 1957; Penfield and Milner, 1958), the experimental re-

movals made from monkeys included not only the amygdala and hippocampus, but much of the subjacent perirhinal, entorhinal, and parahippocampal (TF/TH) cortex as well. Removal of at least some portions of these parahippocampal regions was necessary, in any event, to permit access to the more deeply located hippocampus and amygdala. While these early investigations proved informative, they also highlighted the need for more precise surgical approaches that would permit identification of the distinct mnemonic contributions of different temporal lobe structures. Indeed, by the late 1980s, it was evident that lesions limited to the perirhinal and parahippocampal cortical fields (Zola-Morgan et al., 1989) or to the perirhinal and entorhinal cortical fields (Murray et al., 1989) produced profound memory loss, bringing into question the cognitive functions of the hippocampus and amygdala (for review, see Murray, 1996).

In macaques, extensive interanimal variability in distance of target structures from the auditory meatus (Wagman et al., 1975), from which stereotaxic coordinates are typically derived, severely limits the value of stereotaxic atlases of the brain for guiding surgery. One approach to making selective lesions of limbic structures involves x-ray localization of bony landmarks, which, when used together with the position of the auditory meatus, provide improved reliability in predicting the locations of forebrain structures (Aggleton and Passingham, 1981; Aggleton, 1985). However, magnetic resonance imaging (MRI) provides even greater accuracy in localization of brain structures. Consequently, after the advent of MRI, surgical techniques based on MRI-guided stereotaxic approaches combined with injection of excitotoxins (Saunders et al., 1990) or radiofrequency (RF) lesions (Alvarez-Royo et al., 1991) were developed. This latter technique made possible neuropsychological investigations capable of characterizing the specific contributions of the hippocampus (and other structures) to cognition in macaques, independent of the surrounding cortex (e.g., Murray and Mishkin, 1998; Beason-Held et al., 1999; Zola-Morgan et al., 2000).

STATEMENT OF THE PROBLEM

To date, all the published studies that have examined the cognitive effects of excitotoxic lesions of the hippocampus in macaque monkeys have used a dorsal surgi-
surgical approach. In this method, a micromanipulator holding a syringe is held upright on a stereotaxic frame and the syringe needle is introduced roughly perpendicular to the dorsal surface of the brain. The needle then passes through dorsal premotor, primary motor, somatosensory, or parietal cortex (depending on the rostrocaudal level), as well as through the caudate and putamen, en route to the hippocampus. Because the body of the hippocampus is elongated and almost cylindrical in shape, and because it is oriented along a rostrocaudal axis, a series of five to seven penetrations extending along a line nearly parallel to the midline is typically used to destroy the body of the hippocampus. In addition, at least two more penetrations are needed to inject excitotoxins into the uncus portion of the hippocampus. Although this standard dorsal surgical approach has yielded selective lesions, there are difficulties associated with it. First, despite use of stereotaxic surgery, there is tremendous variation in the extent of the lesions resulting from dorsal ibotenic acid (IBO) injections (Málková et al., 2001). Sometimes lesions of the hippocampus are complete, but in many other cases there is substantial unintended sparing of tissue within the targeted area. Examination of the results of four recent studies in monkeys, using IBO injected into the hippocampus via the dorsal approach, illustrates the dramatic variation in lesion size both within and between studies. Mean percent damage across the four studies was 47.0%, with percent damage ranging from 8.8% to 98.0% in individual animals (Table 1).

Second, as already indicated, the approach requires a series of many needle penetrations. Given that each penetration passes through about 35 mm of tissue before reaching its target, there is considerable opportunity for inadvertent damage to structures lying along the track of the needle.

Third, a side effect observed commonly after dorsal IBO injections in monkeys is respiratory depression of variable duration (personal observations; L. Málková, personal communication, August 7, 2002; J. Bachevalier, personal communication, August 8, 2002). While healthy blood-gas levels can be maintained with proper intubation and ventilation, full recovery is often quite prolonged even after animals have been removed from the ventilator. Furthermore, once monkeys begin breathing on their own, they may not be able to maintain adequate blood oxygenation unless given supplemental oxygen (personal observations; J. Bachevalier, personal communication, August 8, 2002).

To address the problem of incomplete lesions, Málková et al. (2001) developed methods for in vivo assessment of the effectiveness of a particular series of injections (see also Alvarez-Royo et al., 1991; Ben-Horin et al., 1996; Nemanic et al., 2002). Assessment of in vivo lesions permits planning of additional series of injections if necessary, before beginning cognitive testing. While this solution is a reasonable and effective one for ensuring complete lesions, repeated operations increase the probability of surgical complications and unintended brain damage, they are laborious and expensive, and they are undesirable from the perspective of animal welfare. The primary aim of the present study was to determine whether modification of the standard surgical procedure would improve the reliability with which complete hippocampal lesions are achieved in a single stage.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Case</th>
<th>Left</th>
<th>Right</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murray et al. (1998)</td>
<td>H1</td>
<td>70.5</td>
<td>71.7</td>
<td>71.1</td>
</tr>
<tr>
<td></td>
<td>H2</td>
<td>68.2</td>
<td>81.2</td>
<td>74.7</td>
</tr>
<tr>
<td></td>
<td>H3</td>
<td>37.1</td>
<td>26.8</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>H4</td>
<td>30.8</td>
<td>25.0</td>
<td>27.9</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>51.7</td>
<td>51.2</td>
<td>51.4</td>
</tr>
<tr>
<td>Murray and Mishkin (1998)</td>
<td>AH1</td>
<td>90.0</td>
<td>62.0</td>
<td>76.0</td>
</tr>
<tr>
<td></td>
<td>AH2</td>
<td>46.0</td>
<td>92.0</td>
<td>69.0</td>
</tr>
<tr>
<td></td>
<td>AH3</td>
<td>98.0</td>
<td>98.0</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>AH4</td>
<td>92.0</td>
<td>89.0</td>
<td>90.5</td>
</tr>
<tr>
<td></td>
<td>AH5</td>
<td>75.0</td>
<td>59.0</td>
<td>67.0</td>
</tr>
<tr>
<td></td>
<td>AH6</td>
<td>68.0</td>
<td>48.0</td>
<td>58.0</td>
</tr>
<tr>
<td></td>
<td>AH7</td>
<td>55.0</td>
<td>55.0</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>74.9</td>
<td>71.9</td>
<td>73.4</td>
</tr>
<tr>
<td>Beason-Held et al. (1999)</td>
<td>HF1</td>
<td>8.8</td>
<td>13.1</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>HF2</td>
<td>24.9</td>
<td>18.6</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td>HF3</td>
<td>32.8</td>
<td>42.1</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>HF4</td>
<td>26.3</td>
<td>20.0</td>
<td>23.2</td>
</tr>
<tr>
<td></td>
<td>HF5</td>
<td>13.3</td>
<td>11.7</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>21.2</td>
<td>21.1</td>
<td>21.2</td>
</tr>
<tr>
<td>Zola et al. (2000)</td>
<td>IBO1-1</td>
<td>40.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBO1-2</td>
<td>52.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBO1-3</td>
<td>53.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBO1-4</td>
<td>39.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBO1-5</td>
<td>34.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBO2-1</td>
<td>60.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBO2-2</td>
<td>30.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBO2-3</td>
<td>29.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IBO2-4</td>
<td>13.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>38.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grand mean (N = 25)</td>
<td>47.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Although the cause of the variability in hippocampal lesions produced by the standard method is unknown, four categories of suspected problems can be distinguished. First, it is possible that there are variations in the potency of IBO. Our experience strongly suggests that variation in the potency of the IBO cannot account for much of the inadvertent sparing of hippocampal tissue, at least within our own studies. In some operations, both the left and the right hippocampi were injected simultaneously with IBO drawn from the same vial, yet the hippocampus in one hemisphere is damaged as intended, while the other is largely spared. This finding rules out issues regarding preparation and handling of the excitotoxin as a major source of failure to produce a lesion.

**TABLE 1.**

Percentage Damage to Hippocampus Reported in Four Recent Studies

**SOURCES OF VARIABILITY IN EXTENT OF EXCITOTOXIC HIPPOCAMPAL LESIONS**
A second possible cause of variation is that the needle placements for the injections may not be accurate. If this were the case, one would expect to see lesions outside the hippocampus, where the toxin was deposited. Although unintended damage is occasionally observed, for example in the ventrally adjacent parahippocampal cortex, we have frequently found no evidence of a lesion anywhere after making a series of injections targeting the hippocampus. Consequently, it seems unlikely that inaccuracy of needle placement accounts for the inconsistent results.

A third possibility is that the toxin simply fails to be injected into the target structure. However, between needle penetrations, we ensure that the needle is patent and that toxin is indeed exiting through the needle tip. In addition, we have used gas-tight syringes from which the needle tip is the only exit for the IBO. Thus, given the foregoing considerations, it seems unlikely that toxin is not injected.

A fourth possibility is that when the injections fail to work, either the toxin was accidentally injected directly into the ventricle overlying the hippocampus, or it leaked back through the needle track into the ventricle. This would account for ineffective injections, even when the potency of the IBO was confirmed, and for the failure to find misplaced injection sites.

Rather than systematically test these various hypotheses, we attempted to address many of the potential problems at once. In the present report, we describe in detail a longitudinal (occipital) surgical approach similar to that used in marmosets by Ridley et al. (1995), modified for use with MRI. This approach permits injections to be made throughout the body of the hippocampus with a single needle penetration, but it does not directly target the genu and uncus, which together constitute roughly 20–25% of the hippocampus. Because the injection needle passes through the ventricles only near the beginning of the operation, before the injections have taken place, and again at the end of the series of injections, at a time after much of the toxin would have diffused into its target tissue. In addition to improving the efficacy of the lesions, we also hoped to reduce the side effects observed following the traditional approach. Based on the association of IBO with respiratory depression and prolonged recovery in previous work, we chose N-methyl-D-aspartic acid (NMDA) as an alternative neurotoxin in an effort to prevent depression of vital signs. NMDA has been shown to produce lesions in the hippocampus that are indiscernible from those made by IBO (Jarrard and Meldrum, 1993).

**MATERIALS AND METHODS**

**Subjects**

Six young adult male rhesus macaque monkeys (*Macaca mulatta*) weighing 7.2–8.7 kg underwent surgery. These monkeys were either subjects of ongoing behavioral studies examining the effects of hippocampal lesions (n = 3) or received surgery for the purpose of assessing this new surgical approach after completing duty in behavioral experiments (n = 3). The lesions in three of the monkeys were evaluated after standard histological processing of the brains, and the lesions in the other three monkeys were evaluated using in vivo MRI.

**Preoperative MRI Scan**

Before surgery, each monkey underwent an MRI scan. The monkeys were anesthetized with a combination of ketamine (8.5 mg/kg) and xylazine (0.35 mg/kg) and were placed in a nonferrous stereotaxic frame (National Institutes of Health [NIH] Mechanical Design and Fabrication Branch, Bethesda, MD). Vitamin E in the earbars provided a fiducial mark visible in the scan. To ensure that each monkey’s head would be repositioned accurately in the stereotaxic frame days later at surgery, a micromanipulator (David Kopf Instruments, Tujunga, CA) with a specially made pointer (NIH Mechanical Design and Fabrication Branch, Bethesda, MD) was used to note the location of landmarks on the upper lateral incisors (Saunders et al., 1990). The monkeys were then placed on the scanner bed and the stereotaxic frame leveled, using adjustable feet and a leveling bubble mounted on the frame. T1-weighted scans were performed in a 1.5-tesla (T) magnetic resonance scanner (GE Medical Systems, Waukesha, WI), in both the coronal and sagittal planes (SPGR, TE6, TR25, flip angle 30, NEX 4, 256-square matrix, FOV 100 mm, 1-mm slices). The tips of both earbars were included in the scanned areas.

**Calculation of Needle Trajectory**

Two methods for determining the placement of the needle in the hippocampus are described: the phantom method and the trigonometric method. The two methods have all but the last step in common. The measurements necessary for planning the needle trajectories were determined from the MRI scans, using standard viewer software (GE Medical Systems) as follows. The numbered steps correspond to the numbers in Figures 1 and 2:

1. We determined the mediolateral (ML) coordinates of the sagittal sinus, either at earbar 0 or at the level of the rostral hippocampus, using the coronal scan series. Locating the sinus permits calibration of all measurements in the ML dimension, as the sinus can be visualized at surgery.
2. The dorsoventral (DV) and anteroposterior (AP) coordinates of the left and right earbar were determined from the sagittal scan series.
3. The approximate ML value of the parasagittal plane passing through the center of the hippocampus in each hemisphere was determined using the sagittal scan series. This was accomplished by sorting through the sagittal images and by selecting the section showing the largest area of the body of the hippocampus in each hemisphere.
4. Using the sagittal sections just selected, the angle of the line of best fit through the length of each hippocampus was determined visually. In practice, because the posterior hippocampus curves abruptly dorsally, we ignored the most posterior aspect of the hippocampus in determining the angle of the line. As will be evident in
the Results, despite not directly targeting this area with injections, extensive cell loss was observed.

5. We noted the location of the deepest point of penetration desired along the line of best fit, i.e., the point at which the rostral hippocampus intersected the ventricle, in DV and AP coordinates. To reduce the risk of the needle exiting the genu of the hippocampus, we routinely placed the first injection site 2 mm caudal to this point.

6. The location at which the line of best fit exited the hippocampus caudally was also noted, in DV and AP coordinates. This permitted calculation of the distance over which injections would be made.

7. Finally, we determined the point at which the line of best fit exited the skull. This measure permitted calculation of the minimum needle length required for the injections.

Once the coordinates were obtained from the MRI scans, the locations of the target sites were calculated relative to the sagittal sinus and earbar 0. Finally, the resulting values were translated from the MRI coordinate system to the stereotaxic coordinate system.

**Distance over which injections are to be made**

Using the DV and AP position of the deepest penetration into the hippocampus (5 in Fig. 1), and the posterior exit point from the hippocampus (6), relative to the vitamin E-filled earbars (2), the distance over which the injections are to be made can be calculated. This distance was determined by calculating the difference between the earbar values and these other two points, as shown in Figure 2. A right triangle can be generated using these values, in which the length of the adjacent leg (horizontal dashed line) is the difference between (5) and (6) in AP coordinates, and the height of the opposite leg (vertical dashed line) is the difference between (5) and (6) in DV coordinates (Fig. 2). The length of the hypotenuse can then be determined using the Pythagorean theorem, yielding the distance over which injections are to be made.

**Micromanipulator angle**

The angle of deviation from vertical of the desired needle path is the complement of the angle noted from the MRI scan (4 in Fig. 1).

**ML setting for the micromanipulator**

The distance of the desired needle trajectory from the sagittal sinus was determined by finding the difference between the ML value for the needle path (3) and the ML position of the sinus (1). After locating the sinus during surgery, and centering the needle above it, achieving the desired ML position was simply a matter of moving the needle the prescribed distance laterally.

**DV and AP setting for the micromanipulator**

We have used two methods for determining the DV and AP coordinates required for the micromanipulator. Unless an exact
duplicate frame is available, both methods require that the monkey be removed from the stereotaxic frame between the preoperative MRI scan and surgery. Two precision micromanipulators (David Kopf Instruments) were fitted with gas-tight microliter syringes (Hamilton, Reno, NV). The syringes were fitted with replaceable 26-gauge, double wall, 2-inch needles of point style 4. Before beginning calibration of the needles as described below, we ensured that they were straight, as judged by visual inspection using an electrode angle calibrator (David Kopf Instruments) as a straight edge.

**Trigonometric Method**

The micromanipulator holding the syringe needle was placed onto the stereotaxic frame, tilted to the appropriate angle for surgery, and then moved along the arm of the frame until the line defined by the needle passed through earbar 0. The DV position of the syringe needle was then adjusted so as to center the barrel of the needle on earbar 0. Because the body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2). The lengths of the remaining sides can be determined using trigonometric rules. Advancing the micromanipulator in the AP dimension the distance between the bars of the needle on earbar 0. The body of the hippocampus is largely superior and rostral to the earbar, the needle now projected along a line both inferior and parallel to the path passing through the hippocampus (Fig. 2, parallel black lines). To position the needle correctly in surgery, the dimensions of the triangle shown in red in Figure 2 must be determined. All three angles are known: there is a right angle between the adjacent and opposite sides of the triangle, angle (4) is determined from the MRI, and the remaining angle is the complement of angle (4). The height of the opposite side is the difference in DV coordinates between (5) and (2).

**Phantom Method**

The phantom method requires a third micromanipulator, to which a pointer, such as a stiff wire, is attached. The tip of the pointer was moved to the position of the deepest point of penetration into the hippocampus by moving it the appropriate distance rostral and superior to the earbar (5 in Fig. 2). The manipulator holding the syringe needle to be used for injections was tilted to the appropriate angle for surgery, and then moved along the arm of the stereotaxic frame until the line defined by the needle passed through the point occupied by the tip of the pointer. Note that the phantom and the syringe needle must occupy different ML planes so that they do not collide. The DV position of the needle was then adjusted to bring the bevel of the needle to the tip of the phantom, which is the desired target. These stereotaxic coordinates were recorded for use at the time of surgery.

**Surgical Preparation**

A period of 2–15 days intervened between the preoperative scan and surgery. Because ketamine hydrochloride blocks NMDA receptors, and might therefore interfere with the neurotoxic action of NMDA, it was not used. A combination of medetomidine (0.1 mg/kg; Domitor, Pfizer) and butorphanol (0.3 mg/kg), followed by 10 min, during which the animal was not disturbed, was found to immobilize the monkeys reliably. Atropine (0.1 mg/kg) was administered to prevent bradycardia and excessive secretions. After intubation, the monkeys were anesthetized with isoﬂurane gas, and the medetomidine was reversed with atipamezole (0.5 mg/kg; Antisedan, Pfizer). During surgery, isoﬂurane gas (1.0–3.5%, to effect) was used to maintain a surgical plane of anesthesia.

**Surgery**

An aseptic technique was used. A midline incision was made, and the skin and galea were retracted to expose the cranium. The sagittal sinus was visualized using one of two methods. In three cases (QQ, M, VO), a small hole was drilled in the dorsal cranium, centered on the sagittal suture, in the coronal plane of the earbar, taking care not to damage the underlying sinus. In three other cases in which dorsal injections into the genu and uncus were made, a bone flap that was ~4 cm square and centered on bregma, was turned (B, PJ, F). The bone flap was stored in sterile saline until closing. Each syringe needle was positioned directly over the sagittal sinus and ML coordinates noted. The ML coordinates for each hemisphere could then be achieved by moving the needles laterally the distance between the sinus and the center of the hippocampus, as determined from the preoperative MRI scan.

The micromanipulators were set on the predetermined AP coordinates, and the angle was set. The needles were then advanced using the DV adjustment to points near the skull. The points of entrance were marked directly on the cranium, and craniotomies ~1 cm in diameter were made. The needles were then passed through small slits in the dura, and advanced to the first (most rostral) injection site, just caudal to the genu of the hippocampus. NMDA (62.5 mg/ml; 0.42 M) was injected at 2-mm intervals throughout the length of the hippocampus; at each site a total of 2.0 μl NMDA was injected at a rate of 0.25 μl/min. After each injection was completed, the needle was left in place for 3 min before being withdrawn to the next injection site. In addition, after the last injection in the series we routinely left the needle in place for 5 min before withdrawing the syringe needle completely from the brain. There were eight or nine injection sites per hemisphere along the longitudinal trajectory of the needle. In three cases (B, PJ, F) two or three additional injections were made into the genu and uncal portions of the hippocampus, using a dorsal approach, in an effort to more completely damage the hippocampus. Methods for the dorsal approach have been described elsewhere (Saunders et al., 1990; Alvarez-Royo et al., 1991; Murray and Mishkin, 1998). In the cases with the additional injections, the bone flap was replaced. The surgical opening was then closed in anatomical layers. Before being removed from gas anesthesia, monkeys received ketoprofen (10–15 mg) for analgesia. The monkeys were monitored closely.
for seizures for several hours; diazepam (1.0 mg/kg, i.m., to effect) was administered if seizures occurred.

Dexamethasone sodium phosphate (0.5 mg/kg) and cefazolin were administered 1 day before surgery and for 1 week after surgery to reduce inflammation and to prevent infection, respectively. Ketoprofen (10–15 mg bid) was administered for 2 days, and acetaminophen (10 mg/kg, BID) for an additional 5 days.

RESULTS

Surgery and Recovery

Tachycardia was almost uniformly observed approximately 1 h into the injection series. We found that heart rate should be carefully monitored. In some operations, we interrupted the injection series when the heart rate climbed substantially. In these cases, the heart rate typically decreased within about 30 min, and injections were then continued.

Recovery from surgery was generally rapid and free of complications. In our experience, monkeys that receive dorsal injections of IBO typically recover slowly, sometimes taking many hours to regain reflexes and mobility. By contrast, monkeys that received longitudinal NMDA injections in the present study recovered quite rapidly; they were typically sitting up in the recovery cage within 1 h of the end of surgery. Seizures were observed during the first 30 min of recovery in two of six cases but were readily controlled with diazepam. In no case were seizures observed after the initial 30 min of recovery. Furthermore, whereas monkeys receiving dorsal injections of IBO often display temporary unidirectional rotation and torticollis, no such behaviors were observed in monkeys after longitudinal NMDA injections.

Evaluation of the Injection Series Using In Vivo MRI

At 5–14 days after surgery, each monkey was anesthetized with a mixture of ketamine and xylazine (or medetomidine), placed in the stereotaxic frame, and given a T2-weighted MRI scan (two-dimensional [2D] spin-echo pulse sequence, TE 17, TE2 102, TR 3000, NEX = 3, FOV 11 cm, 1.5-mm slices), to assess the effect of the injections. In these postoperative scans, edema resulting from effective injections is indicated by a “hypersignal” that appears white in the scans (Fig. 3B,C). T2-weighted scans covering the rostrocaudal extent of the hippocampus in monkey QQ are shown in Figure 4.

FIGURE 3. Preoperative and postoperative magnetic resonance images (MRIs) from monkey QQ. A: T1-weighted image of the intact brain, obtained before surgery. B,C: T2-weighted images obtained 2 days after N-methyl-D-aspartic acid (NMDA) was injected into the right hippocampus (B) and 5 days after injection of NMDA into the left hippocampus (C). White hypersignal indicates edema resulting from the injections. D: T1-weighted image obtained 250 days after the second surgery; note the substantial shrinkage of the hippocampus bilaterally.
Surgery was considered successful if hypersignal was evident throughout the body of the hippocampus. In the second (VO, right hemisphere), the small amount of hypersignal evident in the MRI scan made it clear that the needle had been placed laterally, near the ventricle, over most of the length of the hippocampus. After repeating the injection series in this hemisphere in a separate operation carried out four weeks later, hypersignal was evident in this hemisphere as well. Thus, 11 of 13 attempts were considered successful. In the three cases in which injections were not made into the genu and uncal portions of the hippocampus (via a dorsal approach), little or no signal was evident in this area, as expected.

Lesion Estimation Based on In Vivo MRI

The three monkeys currently participating in behavioral experiments (QQ, PJ, B) underwent T1-weighted scans 103–250 days after surgery. To estimate the extent of the lesions in these monkeys, we compared the preoperative and postoperative volumes of each hippocampus. For this purpose, we defined the hippocampus as including not only the dentate gyrus and CA1-CA3 fields, but also the subiculum, which is difficult to discriminate from the hippocampus proper in MRI scans. The area of the hippocampus in each 1-mm section in the preoperative MRI scan of each monkey was determined using Scion Image (Scion, Frederick, MD). At each level, the measure was taken on three separate occasions, and the average of the three measurements was used as the final estimate of the surface area of the hippocampus on each section. Because the measurements were taken at 1-mm intervals, the sum of areas for each hemisphere estimates the volume of that hippocampus, in mm³. The same procedure was applied to postoperative scans. The proportion of hippocampal volume lost was determined from the preoperative and postoperative measurements. Recently, Málková et al. (2001) determined the relationship between volume reduction evident in MRI and lesion size as assessed by microscopic examination of tissue after standard histological processing of the brain (see also Nemanic et al., 2002). Based on the regression function generated by Málková et al. (2001), our lesions were estimated to be 74.0%, 79.7%, and 84.4% of the total volume of the hippocampus (Table 2). Notably, the largest lesion of the three evaluated in this manner (that in monkey QQ) resulted from surgery using only the longitudinal approach, without additional (dorsal) injections into the genu and uncus.

Lesion Estimation Based on Postmortem Histological Examination of the Tissue

The remaining three monkeys, F, M, and VO, survived 28, 109, and 137 days, respectively. The monkeys were given ketamine hydrochloride (10 mg/kg), followed by a lethal dose of sodium pentobarbital (100 mg/kg, i.v., to effect) and perfused transcardially with physiological saline followed by 10% formalin. The brains were extracted and stored, refrigerated, in 10% formalin. Before sectioning at 50 μm on a freezing microtome, the brains were cryoprotected in buffered formalin/glycerol. Sections were

![FIGURE 4. T2-weighted magnetic resonance images (MRIs) through the hippocampus in monkey QQ. Coronal sections A–F are from anterior to posterior at 3-mm intervals. Images were obtained 2 days after injection of N-methyl-D-aspartic acid (NMDA) into the right hippocampus (right panels) and 5 days after injection of NMDA into the left hippocampus (left panels). Edema resulting from the injections is indicated by hypersignal (white region).](image-url)
mounted on gelatin-coated slides, stained with thionin, and coverslipped. Regions of cell loss and gliosis were determined with a stereoscopic microscope and plotted on standard rhesus monkey brain sections. The volume of the lesion was then determined using a digitizer tablet together with software for determining surface area. The lesions were substantial in all cases, affecting 58.5–93% of the entire hippocampus including the genu and uncus (Table 3). Note that monkey F, which received additional dorsal injections into the genu and uncus, sustained the largest lesion. Photomicrographs from monkey VO, a monkey that received only the longitudinal injections, are compared with matched images of an intact macaque hippocampus in Figure 5.

**Comparison With Ibotenic Acid Injected Via a Dorsal Approach**

Just before our experimentation with these methods, we had completed a series of hippocampal injections in seven monkeys, using IBO and the traditional dorsal approach (Hampstead et al., 2001). To assess the relative efficacy of the two surgical approaches, we have compiled the results from the 14 hemispheres receiving IBO via the dorsal approach with the 12 hemispheres receiving NMDA via the longitudinal approach. Success was assessed by inspection of the T2-weighted postoperative MRIs, and was based on examination of the body of the hippocampus only, excluding the genu and uncus. As before, surgery was considered successful if hypersignal was evident throughout the body of the hippocampus. Only the first surgical attempt on each hemisphere was considered. The lesion was successful in a significantly larger proportion of attempts, using the methods described in the present report than was the case using a dorsal approach and IBO (83% vs 36% success, respectively: $X^2_{1, n = 26} = 6.0, P < 0.01$).

## DISCUSSION

Our primary finding is that NMDA injected via a longitudinal approach into the hippocampus of macaque monkeys produces reliable lesions that cover all, or nearly all, of the targeted region. In our hands, this method leads to a substantial improvement in efficacy of the injections over that achieved using a dorsal surgical approach and IBO. A second finding is that use of the longitudinal approach and NMDA yields a more rapid recovery than does use of the dorsal surgical approach and IBO. We note that other studies using NMDA in much lower concentrations have successfully produced cell loss in rats (e.g., Jarrard and Meldrum, 1993) and marmosets (Ridley et al., 1995, 2001). Indeed, of the wide range of NMDA concentrations used and reported in the literature, 0.4 M is the high end. Thus, lower concentrations than that described in the present study may be equally effective.

Because excitotoxin (IBO and NMDA) and surgical approach (dorsal and longitudinal) were not manipulated independently of one another in this study, we cannot say definitively whether it was the change in toxin or the change in surgical approach that was responsible for the increased reliability of the lesions. However, a dorsal surgical approach almost certainly yields more leakage of excitotoxin into the temporal horn of the lateral ventricle than does

### TABLE 2.

**Hippocampal Volumes for the Three Monkeys With NMDA-Induced Hippocampal Lesions for which Histology Is Not Available**

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Type of surgery</th>
<th>Preoperative volume (mm³)</th>
<th>Postoperative volume (mm³)</th>
<th>Shrinkage (%)</th>
<th>Damage (%)</th>
<th>Mean damage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>PJ</td>
<td>Combined⁶</td>
<td>618.1</td>
<td>577.6</td>
<td>257.7</td>
<td>239.5</td>
<td>58.3</td>
</tr>
<tr>
<td>QQ</td>
<td>Longitudinal⁵</td>
<td>587.3</td>
<td>593.3</td>
<td>223.7</td>
<td>174.2</td>
<td>61.9</td>
</tr>
<tr>
<td>B</td>
<td>Combined</td>
<td>513.2</td>
<td>525.8</td>
<td>204.7</td>
<td>181.8</td>
<td>60.1</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>572.8</td>
<td>565.6</td>
<td>228.7</td>
<td>198.5</td>
<td>60.1</td>
</tr>
</tbody>
</table>

NMDA, N-methyl-D-aspartic acid.

⁶Estimated from Malková et al. (2001).

⁵The lesion was made using both the longitudinal approach (body of hippocampus) and dorsal approach (uncus only).

⁶Only the longitudinal approach was used.

### TABLE 3.

**Hippocampal Damage in Three Monkeys With NMDA-Induced Hippocampal Lesions**

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Type of surgery</th>
<th>Hemisphere</th>
<th>Mean damage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>left</td>
<td>right</td>
</tr>
<tr>
<td>F</td>
<td>Combined⁶</td>
<td>92.0</td>
<td>94.0</td>
</tr>
<tr>
<td>VO</td>
<td>Longitudinal⁵</td>
<td>58.0</td>
<td>76.0</td>
</tr>
<tr>
<td>M</td>
<td>Longitudinal⁵</td>
<td>49.0</td>
<td>68.0</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>66.3</td>
<td>79.3</td>
</tr>
</tbody>
</table>

⁶The lesion was made using both the longitudinal approach (body of hippocampus) and dorsal approach (uncus only).

⁵Only the longitudinal approach was used.
the longitudinal approach. Furthermore, a direct comparison of the effects of IBO and NMDA, injected into the hippocampus of rats under identical conditions, showed that the two toxins produced essentially identical lesions (Jarrard and Meldrum, 1993). Consequently, it seems likely that the longitudinal surgical approach, not the change in excitotoxin, was the primary factor leading to improved reliability in the present study.

The effectiveness of the lesions aside, the methods described in the present report have several other advantages. First, monkeys undergoing simultaneous bilateral injections with the present methods showed essentially no postoperative complications other than some incidence of seizures immediately after surgery, which were readily controlled with diazepam. In contrast to monkeys injected with IBO via a dorsal approach, these monkeys recovered rapidly after surgery and did not show the depressed vital signs characteristic of the standard procedure. However, as indicated earlier, because we did not systematically investigate these phenomena we cannot pinpoint the factors leading to improved recovery. It seems likely that both the type of excitotoxin and the type of surgical approach affect the rate of recovery from injection of excitotoxins into the hippocampus. A second benefit of this new procedure is that one longitudinal needle penetration replaces at least five dorsal needle penetrations, probably resulting in a lower probability of infarction. Third, using lower concentrations of NMDA, and smaller injection volumes, this technique may be useful in making selective subtotal lesions of the hippocampus, for example of CA1 only (Ridley et al., 1995).

For making lesions limited along the rostrocaudal axis of the hippocampus, the technique may be less useful. Although penetrations limited to the caudal portion of the hippocampus could yield selective damage to this area, similar selective damage to the rostral hippocampus would be more problematic given the chance of toxin leaking along the needle track into caudal areas. Finally, we suggest that the surgical approach described might be particularly well suited to infusion studies of the hippocampus, for example studies of reversible inactivation, where it is desirable for an agent to distribute throughout the hippocampus from a single cannula (e.g., Heiss et al., 2002).

Acknowledgments

The authors thank A. Chaudry, D.V.M., for optimization of sedation and anesthesia protocols and assistance during surgery. R. Saunders and the staff of the NIH In Vivo MRI Center provided invaluable help with scanning. We thank L. Jarrard and R. Ridley for helpful comments and encouragement.

FIGURE 5. Comparison of an intact hippocampus and the lesion in case VO. A–F: Photomicrographs of Nissl-stained coronal sections, from anterior to posterior, through the right hippocampus and underlying cortex from a normal (intact) macaque brain. G–L are approximately matched sections from case VO, a monkey that received N-methyl-D-aspartic acid (NMDA) injected into the hippocampus via the longitudinal approach. No dorsal injections into the genu or uncus of the hippocampus were made in this case. Scale bar = 5 mm.
REFERENCES

Murray EA. 1996. What have ablation studies told us about the neural substrates of stimulus memory? Semin Neurosci 8:13–22.