

A Squirrel Monkey Model of Poststroke Motor Recovery

Randolph J. Nudo, Diane Larson, Erik J. Plautz, Kathleen M. Friel, Scott Barbay, and Shawn B. Frost

Abstract

Nonhuman primate models of poststroke recovery have become increasingly rare primarily due to high purchase and maintenance costs and limited availability of nonhuman primate species. Despite this obstacle, nonhuman primate models may offer important advantages over rodent models for understanding many of the brain's mechanisms for self-repair due to greater similarity in cortical organization to humans. Since the mid-1990s, surgical, neurophysiological, and neuroanatomical methods have been developed to understand structural and functional remodeling of the cerebral cortex after an ischemic event, such as occurs in stroke. These methods require long surgical procedures and entail constant physiological monitoring. With careful attention to intraoperative and postsurgical monitoring, these procedures can be repeated multiple times in individual monkeys without untoward events. This model provides a statistically powerful approach for tracking brain plasticity in the ensuing weeks and months after a stroke-like injury, reducing the number of animals required for individual experiments. This methodology is described in detail, and many of the resulting findings that are relevant for understanding stroke recovery and the effects of rehabilitative and pharmacotherapeutic interventions are summarized.

Key Words: motor cortex; neurophysiology; physiological monitoring; squirrel monkey; stroke

Introduction

After a stroke has affected the motor cortex, motor deficits are common in the upper extremity contralateral to the injury. Deficits include paralysis or weakness and abnormal muscle tone, posture, movement synergies, and interjoint coordination (Cirstea and Levin 2000; Nakayama et al. 1994; Twitchell 1951; Wade et al. 1985). Although dexterity with the impaired hand may be

permanently affected, significant recovery occurs during the first several weeks after the injury (Duncan et al. 2000).

At least part of the recovery process involves resolution of pathophysiological events associated with cortical injury. In addition, recent results from human and animal studies suggest that cerebral cortex is capable of significant functional and structural remodeling after injury and that motor experience is a major modulator of neurophysiological and neuroanatomical changes that take place in the undamaged tissue. The notion that the brain can be remodeled after injury is an exciting prospect because it may lead to the development of new pharmacotherapeutic and physiotherapeutic interventions for restitution of function after stroke.

To understand the underlying mechanisms of stroke recovery at molecular, synaptic, cellular, and systems levels of organization, animal models, including nonhuman primate models, are essential. This article summarizes one such model that has been developed in our laboratory over the past several years. The purposes of this model have been to determine (1) the detailed neurophysiological and neuroanatomical changes that occur in undamaged cortical tissue after a focal ischemic lesion, and (2) the effects of behavioral interventions on this cortical tissue. In this review, we describe the unique surgical, neurophysiological, and behavioral procedures associated with the model, and a few of the resulting findings regarding neuroplasticity after stroke.

Need for Primate Models of Stroke

Although rodent species have been very valuable in furthering our understanding of the cascade of events that follow acute stroke, and have begun to be utilized for studies of recovery mechanisms (Jones et al. 1999), the need for nonhuman primate models of stroke has received increasing attention. The advantages and disadvantages of nonhuman primate models for stroke are reviewed in detail by Fukuda and del Zoppo in this issue (Fukuda and del Zoppo 2003). Briefly, from a heuristic standpoint, nonhuman primate species share a more recent common ancestry with humans, increasing the probability that neural processes related to stroke-induced deficits and recovery are similar to those of human stroke. It is clear that certain features of the primate motor system, especially in cortical motor structures, are more highly differentiated and elaborated compared with those of other mammalian species (Heffner and Masterton 1983; Nudo et al. 1995).

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Recently, the interest in nonhuman primate models has grown, due largely to the failure of clinical trials using putative neuroprotective drugs that were successful in rodent models. The possible reasons for failure have recently been reviewed by Gladstone et al. (2002) and include questionable study design in some clinical trials, premature clinical trials due to insufficient preclinical results, differences in therapeutic windows between preclinical and clinical studies, emphasis on neuroprotection of peri-infarct tissue and on gray matter protection in preclinical models, and inappropriate outcome measures. Several reasons for clinical trial failures have also focused on the appropriateness of the animal model, such as the small size of the rodent brain (Neff 1997), differences in recovery rates between rodents and humans, and other differences in the genetic and proteomic makeup between rodents and humans (Gladstone et al. 2002). As a result, recent recommendations for priorities in preclinical stroke research have emphasized the need for better models of stroke disease, especially nonhuman primate models (Report of the Stroke Progress Review Group, April 2002 [www.ninds.nih.gov]).

Characteristics of the Squirrel Monkey Model

The most common approach to mimicking clinical stroke in primate species is similar, in principle, to approaches applied in rodents. Usually the approaches involve occlusion of the middle cerebral artery (MCA¹) or the internal carotid artery (Fukuda and del Zoppo 2003). Other investigators have used direct surgical dissection (craniectomy) and permanent occlusion of the MCA over the lateral portions of cerebral cortex (Marshall et al. 2000). The intent of experiments in our laboratory is not to mimic clinical stroke per se, but instead to provide a model for examining mechanisms of neuronal plasticity that may underlie recovery after stroke. Thus, we have developed an injury model that allows the generation of focal ischemia over a functionally identified cortical area of predictable extent.

Our primate model of choice is the adult squirrel monkey (*Saimiri* spp., males and females, ages 2.5–15 yr) chosen primarily because the motor hand area is contained within a relatively flat, unfissured sector of frontal cortex, allowing direct access for neurophysiological examination and infarct induction. Because the availability of squirrel monkeys has been unreliable over the past few years, we use a broad range of ages. To avoid major confounds with developing brains, we use squirrel monkeys at least 2.5 yr of

age (i.e., young adults) or older. Because squirrel monkeys from commercial suppliers usually include retired breeders, we often utilize much older animals. Our upper age limit is 15 yr, considered by most to correspond to older adult squirrel monkeys. However, beyond the age of about 12, we have found that squirrel monkeys are not as physiologically stable during our long surgical/neurophysiological procedures (15–20 hr). Thus, we limit the use of older monkeys to procedures that are of shorter duration (<10 hr). Although we have not systematically examined the effects of age on neuroplasticity or the behavioral response to cortical injury, we have not observed major differences to date. Thus, for our particular experimental design, an age range of 4 to 8 yr is optimal. Because clinical stroke predominantly occurs in the elderly, the need to produce generalizable results in older nonhuman primates will likely become increasingly important.

Likewise, no major differences have yet been found between males and females with regard to neuroplasticity or the behavioral response to cortical injury. However, because females are typically smaller than males in this species, they often present additional surgical challenges (e.g., tracheal intubation, venous cannulation). We have found that a weight of at least 700 g is optimal for these experiments but have been successful utilizing squirrel monkeys as small as 600 g. Body weights in squirrel monkeys also change seasonally and vary as a function of species and subspecies.

Using electrical stimulation of the deep layers of cerebral cortex (output layers) via microelectrodes, we can define the functional boundaries of the motor cortex with great precision (<250 μ m resolution). The hand area has been the focus of these experiments because the hand is often affected in human patients with cortical motor infarcts. Also, we have been successful in training refined movements of the hand in squirrel monkeys. We have used squirrel monkeys in our studies of cortical plasticity for nearly 20 yr and have compiled a large database of behavioral, anatomical, and physiological information regarding the cortical control of movement in this species.

Methods for Defining Functional Boundaries in Motor Cortex

Motor cortex is classically defined as the part of the cerebral cortex that requires the least amount of electrical stimulation to evoke movement of skeletal musculature. In primates, including humans, motor cortex is subdivided into several regions: primary motor cortex (M1¹), premotor cortex, the supplementary motor area, and the cingulate motor area(s). Each is thought to play a separate role in the cortical control of movement (Picard and Strick 1996; Preuss et al. 1996; Tanji and Kurata 1989; Wise 1996; Wu et al. 2000).

The global topography of motor representations in M1 follows an orderly progression, from the hindlimb at the most medial locations, through the trunk, forelimb, and finally the face in the most lateral locations (Figure 1) (Pen-

¹Abbreviations used in this article: AM, morning; BUN, blood/urea/nitrogen; GLU, glucose; HCT, hematocrit; ICMS, intracortical microstimulation; M1, primary motor cortex; MCA, middle cerebral artery; NMDA, *N*-methyl-D-aspartate; PCV, packed cell volume; PM afternoon; SMBRR, Squirrel Monkey Breeding and Research Resource; TP, total protein.

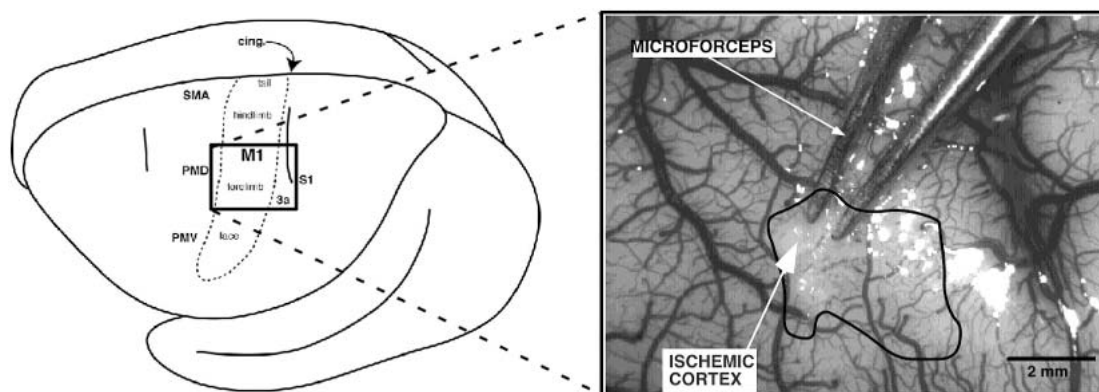


Figure 1 Left: Location of motor areas in the frontal cortex of a squirrel monkey. At least five separate motor areas can be identified in this and other primate species. These areas include the primary motor cortex (M1), supplementary motor area (SMA), dorsal premotor area (PMD), ventral premotor area (PMV), and cingulate motor area (cing.). This arrangement is similar in other primates studied to date, although the cortical sulci are much deeper in the majority of primate species (including humans). Right: Method for creating ischemic infarct in the M1 hand area. Infarct procedure is in progress. Microforceps connected to a bipolar electrocoagulator are used to permanently occlude the entire vascular bed of the target tissue. Black line outlines the M1 hand area defined by microelectrode stimulation techniques.

field and Boldrey 1937; Penfield and Rasmussen 1950). Microelectrode stimulation mapping studies in nonhuman primates (Gould et al. 1986; Wu et al. 2000), as well as recent neuroimaging studies in humans (Grafton et al. 1991; Rao et al. 1995; Sanes et al. 1995), have replicated this basic homuncular somatotopy in M1. However, the representations of individual movements and muscles are widely distributed and overlapping (Asanuma and Rosén 1972; Donoghue et al. 1992; Gould et al. 1986; Kwan et al. 1978; Nudo et al. 1992; Poliakov and Schieber 1999; Sanes et al. 1995; Schieber 1999; Sessle and Wiesendanger 1982; Strick and Preston 1982).

Surgical Techniques

The surgical techniques for defining functional boundaries in motor cortex are standardized (Nudo et al. 1992). Under sterile conditions and after induction of halothane/nitrous oxide anesthesia, a craniectomy is made over the lateral portions of the frontal cortex contralateral to the monkey's preferred hand. The dura is removed over an area approximately 1 cm², directly exposing the hand area of M1. For neurophysiological procedures, after the dura is removed, a small plastic cylinder is fitted over the craniotomy and filled with warm sterile silicone oil to prevent desiccation.

To define functional boundaries within M1, we use a common technique known as intracortical microstimulation (ICMS¹). Ketamine is the most common agent used for these studies and has been successfully utilized in our laboratory for several years. The halothane/nitrous oxide used for the surgical procedure is withdrawn gradually, and ketamine is administered intravenously until the animal is stabilized. Supplemental doses of ketamine are administered throughout the remainder of the experiment as needed to

maintain a stable level of anesthesia. Diazepam or acepromazine are delivered to reduce excessive muscle tone. Heart rate, respiration rate, expired CO₂, oxygen saturation, and temperature are monitored and maintained within normal physiological limits throughout the experiment. Core temperature is maintained with a homeothermic blanket system. Lactated Ringer's solution with 3% dextrose is infused at the rate of 10 mL/kg/hr. The entire surgical/neurophysiological procedure lasts approximately 15 to 20 hr, during which blood chemistry (hematocrit; sodium; potassium; chloride; blood, urea, nitrogen [BUN¹]; total protein; and glucose) is monitored approximately every 6 hr using a portable blood analysis system.

Using a video frame-grabber, a magnified photograph of the cortical surface vasculature is derived. A glass micropipette filled with 3.5 M NaCl is introduced on a fine grid pattern, sited with reference to the surface vasculature and then advanced perpendicularly to the cortical surface to a depth of 1700 to 1800 μm. Thresholds for evoking movements are minimal at this depth. Motor fields are defined by determining movements elicited by ICMS using near-threshold and suprathreshold electrical stimulation (<30 μA). These procedures are now widely used for mapping the functional topography of motor cortex and have been utilized in our laboratory for more than 15 yr (Donoghue et al. 1992; Friel et al. 2000; Gould et al. 1986; Neafsey et al. 1986; Nudo et al. 1990, 1992, 1996a; Plautz et al. 2000b; Waters et al. 1990). At each site, current is gradually increased from zero until a response is just visible in at least 50% of the train bursts. After two observers agree on the movement activated at threshold, the movement is recorded. In some experiments, electromyographic activity is recorded from forearm muscles.

The various sites defined by the grid pattern are probed sequentially until the entire hand area (digit, wrist, and fore-

arm) is explored. After neurophysiological procedures are complete, the plastic cylinder is removed, the dura is replaced with gelfilm, the bone flap is cemented in place with dental acrylic, the skin is sutured, and the wound is dressed with an antibacterial agent. This procedure is repeated before and several weeks to months after the infarct procedure.

This so-called map-remap procedure has several advantages for tracking changes in motor representations within individual animals at the resolution required for these experiments (250 μm between penetrations). Foremost, because of the high degree of individual variability in cortical motor map organization (Nudo et al. 1992), these studies would be much less feasible if between-group designs were used (requiring ~10–12 monkeys per group). Using a repeated measures statistical design, it is possible to achieve sufficient statistical power with four to six monkeys per group.

It is essential for the mapping procedure to have minimal effect on the integrity of motor cortex organization. Therefore, for ICMS, we use thin glass micropipettes that have a relatively small diameter compared with insulated metal electrodes. The diameters of the electrodes are approximately 15 to 20 μm at the tip and approximately 100 μm at 2 mm from the tip. Tips are beveled to a sharp point, allowing them to pass through the pia with minimal resistance. These electrodes have excellent electrical properties (~750k to 1M impedance) allowing currents of up to 100 μA with standard constant current isolators and reasonable recording of multiunit and some single-unit responses. With the dura removed, highly magnified digital photographs of the cerebral vasculature are used as a guide to position microelectrodes with great precision and accuracy. Thus, it is possible to relate maps to each other that are derived at different time points in the same animal.

Cortical Infarct Procedure

Following the neurophysiological mapping procedure, or in a separate surgical procedure, M1 is exposed using the techniques described above. If present, the plastic cylinder is removed, and the silicone oil is flushed with warm saline. Warm saline is applied periodically to the cortical surface to prevent desiccation. The infarct procedure incorporates vascular coagulation using a bipolar electrocoagulator primarily applied to venous drainage and, to a lesser extent, small end-arteries supplying the targeted tissue (Figure 1). The infarcted vessels include very fine capillaries as well as larger vessels. We specifically avoid any bypassing arterial supply to other areas.

We chose this technique because it enables us to make reliable infarcts within a restricted functionally defined zone under visual guidance. Histological examination suggests that the tissue adjacent to the injury is structurally indistinguishable from normal tissue, at least based on Nissl (cresyl violet) and myelin (Gallyas) staining. Qualitative analysis of neuronal type, somatic size, cortical lamination, neuronal

packing density, nuclear profile, axonal size, and axonal myelination reveals no pathologies in the peri-infarct tissue. The normal appearance of this tissue is likely due to (1) the infarct procedure that restricts blood flow permanently and completely over a focal region, and (2) long survival times that extend several months. Although we continually examine the feasibility of other techniques for producing cortical infarcts (excitotoxic lesions, photochemical lesions, MCA occlusions) (Jaskiw et al. 1990; Marshall et al. 2000; Watson et al. 1985), our current vascular infarct technique is very reliable in our hands and reproduces many of the events that occur naturally after an embolic stroke. The procedure allows us to track the neurophysiological properties of the spared tissue before and after injury.

Direct measurement of the precise size of infarcts using any lesion technique is problematic in chronic recovery models. Techniques that compare the remaining volume of tissue in the infarcted hemisphere with the intact hemisphere as used in many rodent experiments are not reliable for our model in squirrel monkeys because the lesions account for only a small percentage of the total cortical tissue. We can induce lesions of predictable size with greater accuracy than if we were using more traditional lesion techniques (e.g., aspiration or electrolytic). After vascular electrocoagulation, the ischemic cortex becomes blanched. The boundaries between the ischemic and normal tissue are clear-cut. Thus, the extent of the damaged cortex can be measured directly by comparing pre- and postinfarct photographs. In postinfarct maps, a digital photograph of the intact vasculature can be superimposed on the preinfarct photograph, and the cortical territory spared by the infarct can be evaluated. In addition, laser-Doppler images of reduced blood flow taken 1 hr after infarct are used to verify the extent of the ischemic damage.

Monitoring Physiological State During Long-term Surgical/Neurophysiological Procedures

One of the unique challenges of this model is that the motor mapping procedures require an extended surgical/neurophysiological procedure that can last from 10 to 20 hr. The length of the procedure is the result of our need to resolve functional boundaries within the motor map with high resolution. The procedure must be repeated at least twice (pre- and poststroke) in each monkey.

Vital Signs

Several physiological variables are monitored continuously and recorded every 15 min during the procedure. Because of the long duration of the neurophysiological procedures, it is extremely important to maintain these values within normal physiological ranges for the following reasons: (1) Heart rate, respiration rate, and expired CO_2 are rough indicators of the animal's anesthetic state, which can affect the ability

to evoke movements via cortical stimulation or can change the required current. (2) Core temperature can affect the degree of cortical injury via an ischemic infarct. Low cortical temperatures can have a neuroprotective effect in some stroke models. (3) Maintenance of vital signs within normal physiological ranges contributes to a more rapid and uneventful postsurgical recovery.

Heart rate and oxygen saturation are monitored using a Nonin 8600 pulse oximeter (Nonin Medical, Inc., Plymouth, MN). Because of the small size of squirrel monkeys, the sensor cannot be placed on an individual finger, therefore the sensor is usually placed on the plantar surface of the

foot, where readings are most reliable. Other locations have also been used, such as the proximal femoral area and the palmar surface of the hand. Temperature is monitored and controlled by a homeothermic blanket system using a rectal probe (Harvard Apparatus, Inc., Holliston, MA). Expired CO_2 and respiration rate are monitored by an Ohmeda 5200 CO_2 monitor. In addition, we periodically monitor electrocardiographic activity using a neonatal electrocardiographic monitor (Hewlett-Packard Company, Palo Alto, CA).

Physiological state is relatively stable throughout the entire procedure (Figure 2). In a sample of seven squirrel monkey procedures examined for this report, only one

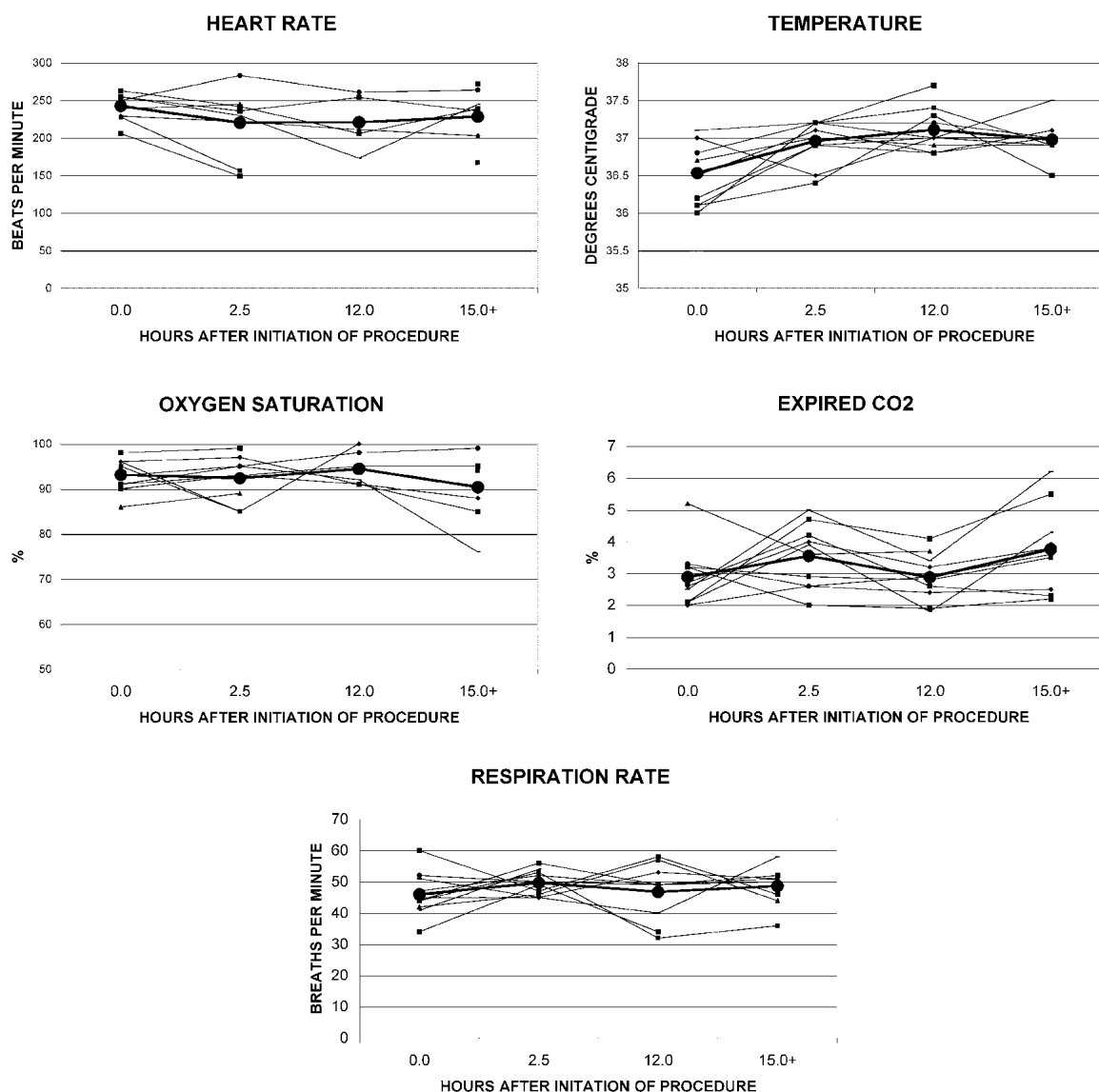


Figure 2 Physiological values over the course of long-term surgical/neurophysiological procedures. Thin lines and small symbols depict individual animals; thick lines and large symbols depict mean values. Values are relatively constant throughout the entire procedure. 0.0 hr: 30 min after initiation of anesthesia marking beginning of surgical/neurophysiological procedure; 2.5 hr: early in postsurgery neurophysiological procedure, or 1 hr after transfer to ketamine; 12 hr: approximately 12 hr after initiation of surgical/neurophysiological procedures; 15.0+ hr: 30 min after halothane/nitrous oxide is withdrawn during anesthetic recovery period, minimum 15 hr after initiation of surgical/neurophysiological procedures. Some data are omitted due to unreliable readings (see text).

physiological variable, rectal temperature, changed over time ($F = 6.24$, $p = 0.0017$); however, even this difference was quite small. Although the monkeys are placed on a heating pad controlled by a homeothermic feedback circuit, temperatures at time zero, corresponding to a period before surgery when the monkey is on halothane/nitrous oxide, were slightly lower than those at 2.5, 12, and 15 hr, but only by about 0.5°C ($p = 0.0047$, 0.0003 , and 0.0042 , respectively; Fisher's least significant difference). The slight reduction in temperature at time zero is a typical response to halothane/nitrous oxide anesthesia and is within the expected range. When these temperatures occasionally fall below 36°C , we supplement the heating pad with an infrared heat lamp. Care should be taken to prevent injury to the skin by maintaining the heat lamp at a safe distance from the animal (at least 3 ft) and by covering any exposed skin of the monkey with surgical drapes. None of the other physiological measures, including heart rate, expired CO_2 , respiration rate, and oxygen saturation, showed any statistically significant changes over the course of the procedures.

Although physiological variables rarely deviate far from normal values, we use this information (1) to make adjustments to anesthetic levels, and (2) occasionally to ventilate the animal using an ambulatory bag. The latter is performed if both oxygen saturation and expired CO_2 levels are verified to be low.

It should be noted, however, that the optical sensors of oxygen saturation monitors are not entirely reliable. As the extremities cool and peripheral circulation decreases, it is difficult to obtain a reliable reading. When heart rate (derived from the oxygen saturation monitor) decreases to 150 or less, the heart rate and percentage of oxygen saturation are suspect. Thus, some of the values in Figure 2 may be slightly biased. When we have verified suspiciously low heart rates using electrocardiographic signals or a stethoscope, we have typically found the optical readings to be incorrect in these instances. Likewise, when respiration occasionally becomes shallow, respiration rates are unreliable. Suspect values have been eliminated from the data presented in Figure 2. In practice, when readings are suspect, we use less automated means to determine heart and respiration rate (usually a stethoscope for heart rate and visual observation of thoracic movements for respiration rate). Despite periods of unreliability in automated monitoring systems, we can conclude that physiological values are maintained within normal ranges throughout these extended procedures.

Serum Chemistry and Hematological Values

During surgical/neurophysiological procedures, we also monitor glucose (GLU^1), total protein (TP^1), BUN, packed cell volume (PCV^1) and hematocrit (HCT^1), typically two times per day. GLU and BUN are measured using an IRMA SL Blood Analysis System (Diametrics Medical, Inc., St. Paul, MN); TP is measured using a clinical refractometer

(Schuco 5711-2020; Schuco International, Ltd., London, UK); and PCV is measured using standard optical reading of a capillary tube after centrifugation. More recently, we have begun recording electrolyte levels, but these data are not presented herein.

We give the monkeys free access to food up to the time of surgical preparation in an attempt to provide as much nourishment as possible before the long procedures. Although some monkeys experience emesis, this problem has not been significant because a tracheal cannula is inserted very soon after preanesthesia is administered. After preanesthesia with ketamine, the monkeys are initially placed on halothane/nitrous oxide for the surgical phase lasting approximately 60 to 90 min and then are transferred to ketamine/diazepam for the neurophysiological phase, lasting 15+ hr. The first, morning (AM^1) reading is obtained as soon as the intravenous line is in place before surgery. If hematological values are far from the normal range after the AM reading, the experiment is canceled and the veterinary staff is notified. These events are rare. Normally, values correspond to in-house normal ranges.

Once normal hematological values are verified, the monkey is infused continuously with lactated Ringer's (3 or 5% dextrose; see below) at the rate of 8 to 10 mL/kg/hr for the duration of the procedure. The second reading is obtained approximately 6 hr after the first, typically in the late afternoon (PM^1).

For this article, we have compiled values from 33 surgical/neurophysiological procedures in squirrel monkeys. These values differ somewhat from reference values reported by the Squirrel Monkey Breeding and Research Resource (SMBRR¹) at the University of South Alabama Primate Research Laboratory (<http://www.saimiri.usouthal.edu/prl/>). However, it is not uncommon for hematological readings to vary from laboratory to laboratory based on different species or subspecies, diet, time of day the sample is collected, type of assay, and the equipment used for the assay. GLU values are especially variable in squirrel monkeys. For example, our AM GLU values (77.64 ± 23.87 mg/dL; mean \pm standard deviation; Figure 3) were lower than reference values reported by SMBRR. In addition, AM TP (7.42 ± 0.95 g/dL) and AM HCT ($51.8 \pm 5.68\%$) were slightly higher than SMBRR reference values, but within the normal range of variability. AM BUN values (37.35 ± 1.61 mg/dL) were very similar to those reported by SMBRR.

Our initial protocol called for the continuous infusion of lactated Ringer's with 5% dextrose. When we compared AM and PM values in the same animals ($N = 9$), we found that PM glucose values increased beyond our in-house normal range (Figure 3). For this reason, we reduced the amount of dextrose to 3% and found that although PM glucose values were lower than PM values using 5% dextrose, this reduction was not statistically significant. Analysis of variance indicated a main effect of time (AM vs. PM; $F = 42.582$, $p < 0.0001$), but not percentage of dextrose ($F = 1.693$, $p = 0.20$) or time by percentage of dextrose

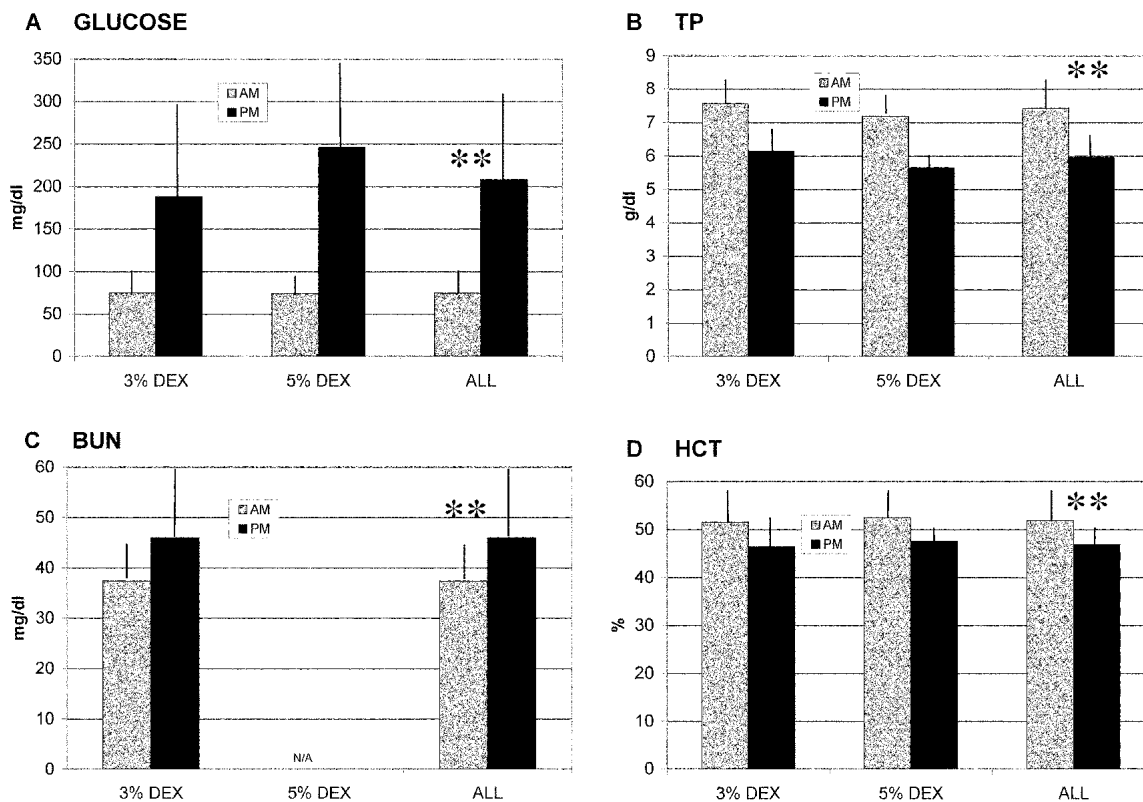


Figure 3 Serum chemistry/hematological values at the beginning of the surgical/neurophysiological procedure (AM), and after approximately 6 hr of anesthesia (PM). We observed a significant increase in glucose and blood-urea-nitrogen (BUN) and a significant decrease in total protein (TP) and hematocrit (HCT). These changes are not thought to be clinically significant, given the relatively high infusion rate of lactated Ringer's solution with dextrose (DEX).

interaction ($F = 1.804$, $p = 0.19$). Although the PM glucose values may seem high (mean = 207.8 mg/dL for all cases), we have found that squirrel monkey glucose values vary widely within individuals over time, even in the awake condition. Because surgical recovery from these extended procedures has been generally excellent, we suspect that it may be advantageous to maintain slightly elevated glucose levels during the initial postsurgical period. We do not plan to reduce the amount of dextrose further.

Other measurements were also altered over time. We observed a statistically significant decrease in TP and HCT ($F = 24.45$, $p < 0.0001$; $F = 5.63$, $p = 0.024$, respectively; Figure 3, B and D), but these changes are relatively small and are not thought to be of physiological importance. The high rate of fluid infusion (8-10 mL/kg/hr) may account for some of these changes, but we have chosen to err on the side of slight overhydration. In addition, BUN increased significantly in the afternoon ($F = 12.5$, $p = 0.0009$; Figure 3C). Although the mechanism responsible for the slight increase in BUN values is not known, this change is not likely to be physiologically significant.

Postsurgical Recovery

Monkeys recover from these procedures remarkably well. After the completion of the surgical and neurophysiological

procedures, monkeys are placed in a temperature-controlled incubator (28°C ambient air) for monitoring. Each animal is monitored continuously until it is somewhat alert and has reliable reflex responses. This state often occurs within 1 hr but more typically occurs in a range of 2 to 3 hr. Then the animal is monitored periodically until it appears to have normal balance, is moving about the incubator voluntarily, and is reasonably responsive to auditory, visual, and tactile stimuli. Notations are made in the monkey's postsurgical recovery record regarding fluid intake and excretion of urine and feces. The animal is then transported back to its home cage. Often, monkeys will be alert within the incubator but will not eat monkey chow or fresh fruit until placed back into their home cage. For this reason, we try to return them as soon as it is feasible. Typically the return occurs approximately 12 hr after the completion of the procedure, although these times have been as short as 8 hr and as long as a few days (rare).

Most monkeys display no untoward effects other than that intended by the ischemic cortical infarct procedure. Occasionally, monkeys that undergo the surgical/neurophysiological procedure but are not subjected to the infarct display mild motor impairments contralateral to the cortex that was examined. These deficits typically subside in a few days and are thought to be related to edema associated with the surgical procedure. For this reason, we now

administer dexamethasone before and 1 or 2 days after the surgery. We are now evaluating the effectiveness of this approach. Despite our efforts, a few deaths have occurred either late in the neurophysiological procedure or during postsurgical recovery. Although a single pathology has not been identified, we suspect that age is a key factor. As noted above, we now limit the duration of surgical/neurophysiological procedures in older squirrel monkeys (>12 yr) to 10 hr.

Anesthetic Protocols for Motor Mapping Experiments

The vast majority of the experiments we have performed in squirrel monkeys have utilized bolus injections of ketamine/diazepam during the extensive neurophysiological period. Physiological, hematological, and recovery data presented above were collected under this anesthetic protocol. Although constant infusion is convenient and would appear to offer the optimal means of maintaining a constant anesthetic state, we have found that the anesthetic state varies considerably during constant infusion. For microelectrode stimulation of motor activity to be effective, a narrow anesthetic window is required. However, anesthetic state under ketamine tends to wax and wane over the course of several hours. Our method of monitoring anesthetic state (i.e., degree of muscle tone and ability to evoke movements of peripheral musculature with low currents of stimulation), in addition to vital signs, is extremely sensitive to these fluctuations. We have experienced greater control with bolus injections of diluted ketamine. We have been less successful in maintaining the anesthetic state within the narrow window required for these experiments using continuous infusion of diluted ketamine. Because of individual differences in the effects of ketamine, monkeys are occasionally rendered too deep anesthetically to evoke movements with cortical stimulation at safe current levels ($\leq 30 \mu\text{A}$ in our studies). Return to optimal anesthetic state is usually rapid using bolus injections (<30 min) but can be quite prolonged (1–2 hr) when using continuous infusion.

Although the normal ketamine concentration is 100 mg/mL, we dilute the ketamine with normal saline (0.9%), to 20 mg/mL. Bolus injections are given approximately every 15 min, at the rate of 20 mg/kg/hr. In addition, to reduce the total amount of ketamine delivered during a given procedure, we periodically supplement the ketamine with either diazepam or acepromazine at times when muscle tone is excessive. Both are diluted to 0.1 mg/mL. Care must be taken with diazepam or acepromazine because small amounts can greatly reduce muscle tone and block the stimulation-evoked movements. A typical bolus injection is 0.01 to 0.02 mg per animal. Because of individual differences in body weights and individual differences in reaction to these drugs, bolus injections correspond to a range of 0.006 to 0.02 mg/kg of body weight.

Because ketamine is a noncompetitive *N*-methyl-D-

aspartate (NMDA¹) receptor antagonist, there is some concern for its use in plasticity experiments, especially those that involve ischemic infarcts. First, because NMDA receptors have been implicated in synaptic mechanisms of neuroplasticity, the use of an antagonist during the mapping procedures may be questionable. Second, the use of NMDA receptor antagonists (especially MK-801) has been found to be neuroprotective during ischemic stroke in rat models (Park et al. 1988). However, ketamine is a relatively weak NMDA receptor antagonist compared with typical neuroprotective agents. In addition, our method of inducing ischemic damage is quite focal, so that the size of the penumbra (the adjacent area containing vulnerable neurons where neuroprotective agents have their greatest effect) is minimized. Although the residual influence of the intraoperative use of ketamine on neural plasticity is not yet known, there are few alternatives for conducting the neurophysiological procedures.

We have evaluated the use of other anesthetics for use in the neurophysiological phase. Propofol, an injectable emulsion that is classified as an alkylphenol, is typically used as a sedative-hypnotic agent for general, pediatric, neuro-, and cardiac anesthesia. For the purposes of our model, it has the advantages of rapid effect and short half-life. Most importantly, it is one of the few agents other than ketamine that does not abolish stimulation-evoked movements in the periphery. The neurophysiological results and surgical recovery times have been comparable to ketamine (Plautz et al. 2000a). Aside from its higher cost, we have concluded that propofol is a feasible and safe alternative to ketamine for motor mapping procedures. Because propofol has been recommended for frail, elderly, and neurological patients, we will evaluate its use in our older squirrel monkeys.

We have also evaluated the use of tiletamine-zolazepam (TelazolTM; Fort Dodge Animal Health, Fort Dodge, IA) for the motor mapping procedures. TelazolTM is a dissociative anesthetic similar to ketamine combined with a minor tranquilizer. We have found the neurophysiological results to be comparable to ketamine. However, for long-term motor mapping experiments, TelazolTM has the disadvantage of slow postsurgical recovery. Animals were not alert for many hours, and some animals were not alert for 2 to 3 days. Although their eventual recovery was uneventful, we strive to move animals to their home quarters as quickly as possible. Thus, the use of TelazolTM for these experiments has been abandoned.

We have limited experience with the use of alphaxalone-alphadolone (SaffanTM; Glaxovet, Harefield, Uxbridge, UK) for these experiments. Preliminary results suggest that Saffan also can be used safely for long-term procedures, such as motor mapping procedures.

Methods for Assessing Motor Skill in Squirrel Monkeys

M1 is thought to mediate skilled voluntary movements, especially of the distal musculature (Phillips and Porter 1977).

M1 lesions result in weakness or paralysis in the contralateral musculature and disruption of skilled limb use (Bucy 1944; Fulton and Kennard 1934; Passingham et al. 1983; Whishaw et al. 1991). However, during the ensuing weeks and months, gradual recovery of motor abilities is commonly observed (Denny-Brown 1960; Lashley 1924; Travis and Woolsey 1956; Twitchell 1951). At least in humans, complete recovery of function in distal musculature, including independent control of digits, is uncommon (Gowland 1987).

Ischemic infarcts in our model are more limited than those that occur in human stroke, encompassing the entire distal forelimb, or hand representation, but sparing proximal upper extremity representations. Nevertheless, a similar pattern of weakness or paralysis of distal musculature followed by gradual recovery is seen in squirrel monkeys after such infarcts. We use a standardized behavioral technique to monitor manual skill before and after cortical infarct. We also provide motor training after the infarct procedure to determine the effects of physiotherapy on recovery. Details of each of the behavioral procedures are described below.

Manual Skill: Automation and Assessment

The primary task used for assessment and training of manual skill is based on a so-called “Klüver board” task used in many experiments from this laboratory and others (Glees 1961; Lawrence and Kuypers 1968; Nudo et al. 1992; Xerri et al. 1998). This task is used for several reasons: (1) The task is quickly learned, (2) task difficulty can be varied (well size), (3) squirrel monkeys perform it in a highly stereotyped way with practice, (4) the motor performance measures can be easily quantified, (5) the kinematics of the movements used in the task are highly correlated with specific changes in functional motor map topography during motor learning, and (6) the time course of deficits and recovery of manual skill on this task have been described in detail and are reproducible.

We have recently automated this task, allowing for efficient and objective motor assessment and training. The automated apparatus consists of a plastic disc containing five food wells of different diameters (9.5–25 mm) evenly spaced around the perimeter. Flavored 45-mg food pellets (banana, cherry, chocolate, plain, depending on each monkey’s preference; BioServe, Inc., Frenchtown, NJ) are automatically dispensed at a fill position at the rear of the apparatus. Once the target well is filled with a single pellet, the disc rotates to the front of the apparatus. Then, one or both access doors are automatically opened to allow access to the well by the right hand, left hand, or both hands. The doors and target well are located so that only the desired hand can gain access to the well. The apparatus records the opening of the door, the target well size, the hand used to retrieve the pellet (if both doors are open; i.e., hand preference), successful retrievals, time to initiate trials, number of total reaches, and time that fingers are in the well.

During preinfarct manual skill training, monkeys are food restricted for 18 hr before each daily training session. In some experiments, a jacket is placed on the monkey with a sleeve extending the length of the nonpreferred forelimb, covering the hand and forcing the monkey to use the impaired hand for tasks requiring manual dexterity. The monkey wears the jacket for the remainder of the experiment except during surgical procedures. The target well is gradually titrated to produce progressively more retrievals from the smaller wells. Preinfarct training continues until 600 pellets are retrieved from the smallest well on each of 2 consecutive days. In addition, probe trials are conducted during each daily session. On probe trials, a food pellet is placed randomly into one of the five wells, and the well is rotated in front of the animal for retrieval. Probe trials allow daily assessment of hand preference and motor performance with each hand. If the criterion number of pellets is not retrieved on a given day, a measured amount of monkey chow is used as a supplement to bring the daily food intake to at least 3% of ad libitum weight.

Because behavioral training involving food restriction takes place 5 days per week for several weeks to months, body weights are closely monitored. In most monkeys, body weights increase slightly over the course of training compared with initial ad libitum weights. The flavored food pellets used as rewards are eaten readily by most squirrel monkeys, although some have specific flavor preferences.

For the studies described in this article, we examined body weights from a random sample of eight squirrel monkeys (four females, four males) that underwent behavioral training for an average of 5.3 mo (Figure 4). The average

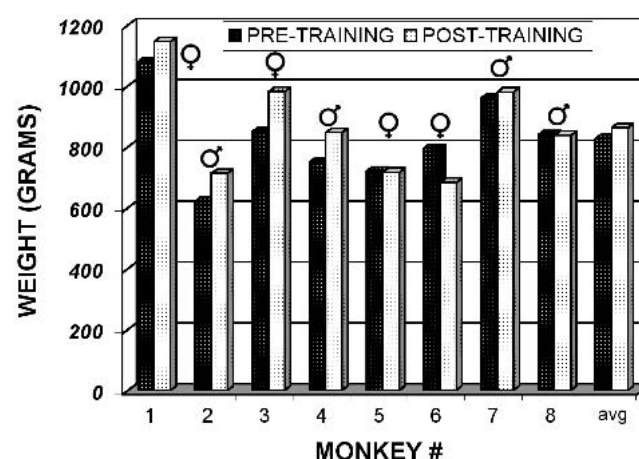


Figure 4 Representative body weights of squirrel monkeys before and after behavioral training requiring food restriction. Behavioral training in these monkeys lasted several weeks to months. Most monkeys either maintained their ad libitum body weight or increased their weight (e.g., #3). Only one monkey in the group (#6) experienced a decrease in body weight, but this value was still within the reference range for squirrel monkeys (see text and <http://www.saimiri.usouthal.edu/prl/>).

weight change was a gain of 4.6% (not statistically significant; $t = 1.3$, $p = 0.23$). Seven of the eight monkeys either maintained the same weight (<1% change) or gained weight. Only one monkey (#6 in Figure 4) experienced any substantial weight loss (14.1%). This loss occurred at the beginning of behavioral training after a jacket was put on the monkey. Weight was then stable for the duration of the behavioral training. Even this weight was within the normal range of reference values for squirrel monkeys. Thus, in our hands, monkeys do not lose weight during the course of behavioral training involving food rationing. Although rare, when weight loss does occur, it is typically not of clinical significance.

Motor Performance: Initial Deficits and Course of Recovery

In our squirrel monkey model, the degree of the motor deficit produced by the ischemic infarcts and the extent and

time course of recovery depend largely on the extent of the injury. We have produced both “small” and “large” lesions, all within the physiologically identified hand area of the M1 within the hemisphere that the monkey’s preferred hand is represented (i.e., contralateral to the preferred hand). Small lesions destroy approximately 35% of the M1 hand area. Large lesions destroy more than 90% of the M1 hand area. As expected, large lesions generally induce more severe and long-lasting deficits. However, the response of individual monkeys to the infarct is variable, even when the location and size of the infarct are constant. Examples of recovery profiles after large lesions are shown in Figure 5.

Acute Phase (0-2 Wk After Stroke)

After small infarcts (~35% of M1 hand area), flaccid paralysis of the hand is often seen during the first day after the infarct. Monkeys typically hold the elbow in extension and move the hand only reflexively. Movement about the shoulder appears normal. Hand preference usually changes to the

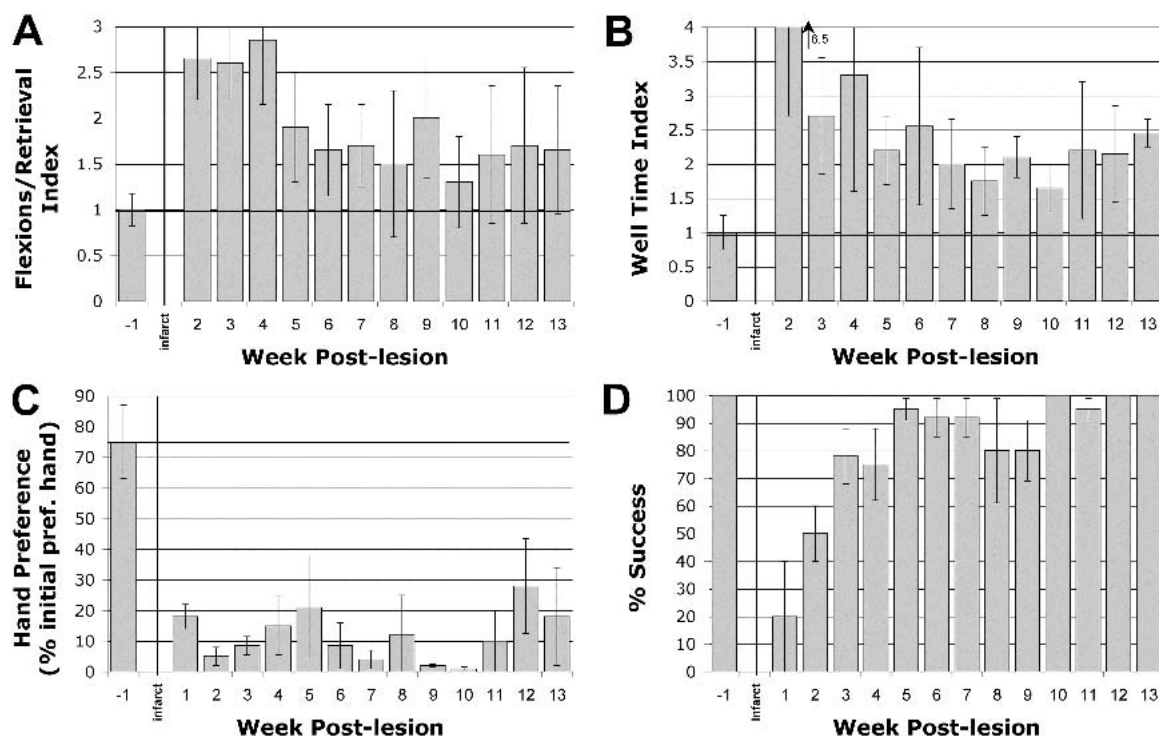


Figure 5 Recovery of motor skill following an ischemic infarct in the primary motor cortex hand area (N = 3). In each case, the infarct destroyed at least 80% of the hand representation but spared proximal upper extremity representations. The performance scores were based on motor performance on a task requiring the extraction of food pellets from small wells. All data were calculated by the automated trainer, except for flexions/retrieval, which were derived from frame-by-frame analysis of videotapes. These graphs represent spontaneous recovery (i.e., in the absence of intensive rehabilitative training). The individual scores are (A) numbers of finger flexions per retrieval, relative to baseline (preinfarct) performance; (B) the time fingers were inside the food well, relative to baseline (preinfarct) times; (C) hand preference, with reference to the hand preferred before infarct; and (D) percentage of successful pellet retrievals. Although monkeys are typically able to retrieve pellets successfully within a few weeks, residual deficits are seen in motor skill, as defined by flexions per retrieval and well time. Except for the initial week, the automated well time measure parallels the number of flexions per retrieval. With lesions of this size or larger, monkeys typically prefer to use the less impaired hand. This change in hand preference appears to be long-lasting, persisting for at least several months.

less-impaired hand. Although few monkeys will engage in a reach and retrieval task within the first 1 to 2 days, most will make attempts with the impaired hand by the fourth or fifth day. Clear deficits in motor skill can be observed during the first week after infarct, as measured by the total number of finger flexions required to retrieve a small pellet from a food well or the total time the fingers are in the well before retrieval (Friel et al. 2000). Interestingly, presumed cutaneous and proprioceptive deficits are also seen during the first week, which may partially account for the deficits in motor skill (Nudo et al. 2000). Although it is quite difficult to dissociate sensory from motor deficits, we have inferred that motor performance decrements are at least partially due to problems in utilizing sensory information in a goal-directed task (i.e., a sensory-motor disconnection). Monkeys display behaviors similar to a sensory agnosia, which has been noted after pure somatosensory cortex lesions (Xerri et al. 1998). For example, monkeys reach into the well, extract pellets, and then look into the hand as if they are not aware that the pellet is present. Often, no pellet is present and repeated reaches are necessary, partially accounting for the deterioration in motor performance.

During the second week after infarct, sensory errors subside and motor skill begins to improve. Occasionally, a behavioral relapse occurs near the end of the second week or beginning of the third week. The neural basis for this phenomenon is not yet known (Nudo et al. 1996b).

After large infarcts (>80% of M1 hand area), the period of flaccid paralysis and extension of the elbow is extended to several days (Frost et al. 2000). The elbow appears to be impaired in addition to the hand. Again, control of the shoulder appears normal. When monkeys engage in the pellet retrieval task, they use the less-impaired hand almost exclusively. By the end of the first week, some monkeys will use the impaired limb on a reach and retrieval task, but only when physical barriers prevent the use of the other hand. Motor skill is markedly impaired (Figure 5). Pellets are successfully retrieved only on a small number of trials. Most monkeys now hold the impaired limb close to the body, with the elbow flexed. Like the monkeys with small infarcts, some improvement in skill is seen during the second week, but deficits are still clearly seen. Pellets are retrieved on only about half of the trials.

Subacute Phase (2 Wk to 3 Mo After Stroke)

After small infarcts, motor skill continues to improve during the third and fourth weeks. By the end of the first month, motor skill typically returns to near baseline levels, although, again, the time course of this spontaneous recovery is quite variable. When movement kinematics are closely examined, it is clear that many monkeys adopt a different movement strategy during recovery (Friel and Nudo 1998). For example, before the infarct, a monkey may stereotypically retrieve pellets from a small food well by using a finger flexion/forearm supination movement. During the first week after the infarct, the movement patterns appear to

be somewhat random. By the end of 1 mo, a different stereotypic strategy (e.g., a finger flexion/ulnar deviation of the wrist movement) may develop. The development of compensatory behaviors, even with these relatively small lesions, is an important mechanism in the spontaneous resolution of motor impairments after stroke. During the subsequent 2 mo, motor skill is relatively stable.

After large infarcts, motor deficits improve during the first month (Figure 5). More pellets are successfully retrieved, but skill level is still markedly affected. Typically, when the monkey is not engaged in a behavioral task, the impaired limb is still held next to the body with the elbow flexed. This posture begins to resolve by 3 mo. Behavioral improvements are difficult to detect after 2 mo, and monkeys typically have a mild to moderate deficit in motor skill that persists.

Chronic Phase (3 Mo to 2+ Yr After Stroke)

Few changes in behavior are seen with either small or large infarcts during the chronic period. Animals with large infarcts continue to use their less-impaired limb for pellet retrievals on the majority of trials when offered the choice, even 2+ yr after infarct. In summary, although motor deficits following small infarcts are resolved by about 1 mo, large infarcts result in a chronic impairment in motor skill.

Neuroplasticity as a Basis for Functional Recovery After Stroke

Studies over the past 2 decades have demonstrated that the structural and functional organization of the cerebral cortex of adults can be modified in several ways (Donoghue and Sanes 1988; Donoghue et al. 1990; Nudo 1997; Nudo et al. 1990, 1997; Sanes et al. 1988). For example, motor cortex can be altered by motor training (Karni et al. 1998; Nudo et al. 1992, 1996a,b; Pascual-Leone et al. 1994; Plautz et al. 2000b; Schlaug et al. 1994). The areas of the cortex devoted to use of the hand are enlarged following the development of manual motor skills. Alterations in synaptic and dendritic structure also occur in adults as a consequence of experience (Green et al. 1983; Greenough et al. 1985; Johansson 2000; Jones et al. 1997, 1999; Kleim et al. 1996, 2002; Kolb 1995; Rosenzweig et al. 1964; Withers and Greenough 1989). Thus, motor skill learning results in widespread structural and functional alterations in M1.

After an ischemic infarct in the M1 hand area, the intact cortical motor representations adjacent to the infarct reorganize. If monkeys recover spontaneously (i.e., without postlesion behavioral training or encouragement to use the affected limb), the remaining, undamaged hand representation decreases in size (Nudo and Milliken 1996). Because it has long been suggested that physical therapeutic interventions might improve recovery after injury to motor cortex (Johansson 1996; Ogden and Franz 1917), we have examined the effects of postlesion motor training on recovery of

motor maps (Nudo et al. 1996b). In contrast to spontaneously recovering monkeys, after postinjury behavioral training, monkeys retain undamaged hand representations. Similar results have been found in rats (Castro-Alamancos and Borrel 1995).

Recent studies in human stroke patients suggest that both the intact, peri-infarct zone and more remote cortical motor areas may play a role in neurological recovery (Cramer et al. 1997; Furlan et al. 1996; Nelles et al. 1999; Rapisarda et al. 1996). Using transcranial magnetic stimulation after stroke, it has been shown that the excitability of motor cortex is reduced, and the cortical representation of the affected muscles is decreased (Cicinelli et al. 1997; Traversa et al. 1997). It is likely that this effect occurs from a combination of disruption of activity in spared cortical tissue (diaschisis) (von Giesen et al. 1994) and disuse of the affected limb (Liepert et al. 2000). Perilesional changes in cortical activity have been shown using a variety of neuroimaging techniques (Classen et al. 1997; Cramer et al. 1997; Kamada et al. 1997; Seitz et al. 1995). Furthermore, after several weeks of rehabilitation, motor representations in the injured hemisphere are enlarged relative to the initial postinjury map (Traversa et al. 1997). Also, constraint-induced movement therapy, in which the unimpaired hand is constrained to induce goal-directed movement with the impaired hand, produces a significant enlargement of the representation of the paretic limb (Liepert et al. 1998, 2000; Weiller and Rijntjes 1999), closely paralleling results observed in nonhuman primates (Nudo et al. 1996b). Although the location of the injury is frequently unknown and/or uncontrolled, these human studies have consistently shown that functional changes occur in several cortical areas after stroke or other cortical damage, paralleling results from animal experiments (Aizawa et al. 1991; Cramer and Bastings 2000).

These studies suggest that functional and structural remodeling of undamaged parts of the cerebral cortex underlie motor recovery after stroke. Primate models provide a means to assess various therapeutic interventions and their effects on motor recovery and cortical physiology and anatomy. To date, most of these interventions have utilized behavioral or physiotherapeutic approaches. However, pharmacotherapeutic methods may play a much larger role in future studies (Goldstein 2000).

Summary

The primary motor cortex of humans is often damaged in clinical stroke, resulting in significant impairment of motor function, especially of the upper extremity. Nonhuman primate models of motor recovery after stroke are extremely important in the development of pharmacological and physiotherapeutic interventions to improve recovery. The squirrel monkey possesses unique qualities that make it an attractive model for understanding the neurophysiological and neuroanatomical mechanisms that may underlie stroke

recovery. To determine the functional boundaries of motor areas in sufficient detail, very long surgical/neurophysiological procedures are required. However, with proper care and monitoring, the motor behavior and neurophysiological properties of individual squirrel monkeys can be tracked over many months or years after a stroke-like injury.

One major disadvantage of working with any primate species is their lack of availability. The availability of squirrel monkeys has become a more critical issue in the past few years. There are now few federally funded domestic breeding colonies (Abee 2000). South American breeding resources and feral sources have become unpredictable. Commercial suppliers have squirrel monkeys available only sporadically. This supply shortage has resulted in escalating prices. It remains to be seen whether support from the National Institutes of Health will be sufficient to maintain the viability of this and other nonhuman primate models in the future.

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