

PREPARATION AND MAINTENANCE OF HIGHER MAMMALS DURING NEUROSCIENCE EXPERIMENTS

REPORT OF A NATIONAL INSTITUTES OF HEALTH WORKSHOP



U.S. Department of Health and Human Services • Public Health Service • National Institutes of Health

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FOREWORD

There are some parallels between research on humans and research on animals. The most important element in such a comparison is the need for all experimentation to be conducted utilizing the highest ethical standards. For example, there are specific ethical guidelines dealing with the inability to obtain truly informed consent from patients unable to fully communicate for various reasons. The need for a patient advocate in such a circumstance becomes essential to protect the patient from inappropriate interventions. This same line of reasoning can be applied to animals used in research. There needs to be an active advocacy to ensure that research animals are not subjected to unnecessary pain or suffering. This responsibility clearly rests with the biomedical research community in general and with individual Institutional Animal Care and Use Committees in particular. I would hope that this workshop report will be read and studied against this ethical backdrop and that the scientific community at large will incorporate this concern within its everyday activities in the laboratory.

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I. INTRODUCTION

Neuroscience, and in particular visual neuroscience, has emerged as an important scientific discipline in recent years and has made significant contributions to clinical medicine. The use of experimental animals plays an essential role in this endeavor and, as a result, neuroscientists and their institutions need accurate information to use and care for experimental animals appropriately and to carry out their research effectively.

The U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy), the *National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Guide)* and the U.S. Animal Welfare Act require that research scientists, veterinarians and Institutional Animal Care and Use Committees (IACUCs) cooperate to ensure that experiments using live vertebrate animals are designed and carried out in a humane manner that complies with all applicable laws, policies and guidelines. However, recent revisions of PHS Policy and the *NIH Guide*, and amendments to the U.S. Animal Welfare Act, have created a number of new challenges to the continued use of higher mammals in neuroscience research. In some instances, current animal care and use guidelines may appear to be incompatible with the scientific needs of contemporary experiments on brain function. As a result, dilemmas can arise when flexibility in applying these guidelines is required in order to achieve the objectives of a proposed neuroscience research project. This situation has led to the application of different standards of review when research protocols are submitted to IACUCs at different institutions. These discrepancies usually have arisen, not because of different attitudes about what is humane or ethical, but because of lack of information.

It is important to recognize that the language of the *NIH Guide* provides for flexibility in interpreting its recommendations. The problem is that while the *NIH Guide* tends to be very specific about those things that are required or prohibited, it usually makes only rather general statements about what is allowed. In an open acknowledgment of this policy, the Introduction to the *NIH Guide* states,

"Nothing in the *Guide* is intended to limit an investigator's freedom - indeed, obligation - to plan and conduct animal experiments in accord with scientific and humane principles." (*pages 1-2*)

"Finally, it should be understood by all who use the *Guide* that it is deliberately written in general terms so that the recommendations can be applied in the diverse institutions that produce or use animals for research, testing, and education. *Professional judgment is essential in the application of these guidelines.*" (*page 2*)

In an effort to provide some assistance in the preparation and review of neuroscience research proposals, a group of neuroscientists, members of IACUCs, and veterinary specialists in laboratory animal medicine gathered at a Workshop organized by the National Eye Institute (NEI), sponsored by NEI and the National Institute of Mental Health, and held at the National Institutes of Health on June 5-6, 1989. The major purpose of this Workshop was to bring together a group of knowledgeable individuals so that they could exercise their collective professional judgment in applying current animal care and use guidelines to neuroscience research. Other goals were to facilitate the continued exchange of information about ongoing developments in the procedures used to prepare and maintain animals during these experiments, and to promote the training of animal care specialists in this area. Finally, a subgroup of the participants was designated to prepare this report of the Workshop, which is intended to serve as a resource to assist researchers, veterinarians, and IACUCs in interpreting and implementing current animal care and use laws, policies and guidelines. It should be emphasized that this report is being distributed for informational purposes only. It does not establish NIH policy nor does it reflect a change in official animal care and use guidelines.

The general format of the Workshop was a group discussion of a range of animal care and use concerns, followed by presentation of a number of experimental preparations commonly used in visual neuroscience. The discussion of these example preparations led to the development of a list of general research themes designed to reflect the spectrum of contemporary approaches to neuroscience research. These general research themes, and the special problems that they pose for animal welfare, are discussed in section II. During the Workshop, a set of six pervasive animal care and use concerns emerged during discussion of the general research themes. Section III describes each of these animal care and use concerns in detail, and discusses various measures that IACUCs can take into consideration when attempting to mitigate them. Finally, section IV presents a variety of sample neuroscience research protocols, drawn from each of the six general research themes described in section II. These examples are not meant to delimit what is allowable and what is not, but rather to illustrate the various kinds of solutions that can be reached when researchers, veterinarians, and IACUCs cooperate in the application of professional judgment to the interpretation and implementation of current animal care and use guidelines.

II. NEUROSCIENCE RESEARCH THEMES

The conduct of contemporary neuroscience research on higher mammals involves the use of a broad array of methods, procedures and approaches. For the purposes of this report, a set of six general research themes was developed. These themes are intended to be broad enough in their scope to encompass most neuroscience research proposals that might be submitted to an IACUC for review and approval. Each of these general research themes is described below, along with a brief overview of the special problems that it poses from the standpoint of animal welfare.

1. Prolonged Non-survival Recording Experiments

Recordings of neural activity have provided some of the most valuable information in neuroscience over the last several decades. As they provide the most direct information about neural signals, recording experiments have fundamentally shaped our understanding of the processing of information throughout the brain. In addition, the results of brain recording experiments have probably had more influence on the fields cognate to neuroscience than any other kind of experiment; their influence can be traced into fields as disparate as behavioral psychology, image processing, and computer design.

The great majority of these experiments are carried out in anesthetized animals and must often be extended over many hours or days. An additional difficulty that often occurs in experiments on the visual system is that they usually require that images on the retina be stationary, or that image motion be controlled precisely. Most investigators achieve this by immobilizing the animal with curariform muscle relaxants, thereby paralyzing both the extraocular muscles and all other voluntary muscles. Under these conditions, assessing whether an experimental animal is experiencing pain or distress is not straightforward. The particular problems associated with this situation are discussed here, but were considered in more detail in the report of a recent workshop on Anesthesia and Paralysis in Experimental Animals (*Visual Neuroscience* 1:421-426, 1988).

The most critical issues in recording experiments arise in the context of anesthesia, maintenance of physiological state, and monitoring of the animal's condition. The choice of anesthetic must jointly satisfy the need of the experimenter to perturb the preparation as little as possible and his/her obligation to ensure that the animal remains free of pain and distress. Maintaining an anesthetized (and often immobilized) animal in sound physiological condition is a considerable technical challenge and monitoring both the anesthesia and the animal's general condition require careful attention to a number of kinds of measurement.

2. Survival Anatomical Procedures

Survival neuroanatomical experiments most commonly involve the use of tracer substances to label and subsequently visualize neural pathways. When these tracers are injected into nervous tissue, they are incorporated into neuronal cell bodies and/or processes and subsequently transported anterogradely and/or retrogradely. Transport of a tracer generally occurs over period of several days. An injected animal must therefore be allowed to survive for a short period of time before being perfused and studied. Various factors will determine the extent to which approval of a proposed protocol for a particular survival anatomical procedure will require the IACUC to exercise some degree of flexibility in interpreting and implementing the recommendations of the *NIH Guide*.

The invasiveness of the procedure required to inject a tracer will establish whether it constitutes a major survival surgery, which should be performed in a facility intended for that purpose. Thus, while an injection into the eye is a relatively minor surgical procedure similar to a biopsy, an injection into a central brain structure, which usually requires performing both a craniotomy and a durotomy, is usually considered a major surgical procedure. On the other hand, when the target is a central brain structure, the method used to place an injection will have a direct bearing on the feasibility of performing the procedure in a dedicated surgical facility. In some cases injections can be placed under direct visual control, but in others hidden brain structures can be injected only by using a stereotaxic apparatus and/or by recording the neurophysiological response properties of the neurons they contain. These more complicated procedures often require a good deal of specialized equipment and therefore can be difficult to perform successfully in the confines of a general use surgical facility.

Another important consideration is the chemical properties of the substance to be injected. For instance, many tracers are sensitive to temperature and cannot be heat sterilized prior to injection. Others may exhibit high levels of tissue toxicity and can cause a marked local inflammatory response at the injection site. Some neural tracers are radioactive and proposed procedures for their use must be reviewed for compliance with institutional policy on radiation safety.

The scientific requirements of certain experiments may require subjecting a single animal to multiple injection procedures. As a result, when the injection site is a central brain structure, it will be necessary to subject an animal to multiple major survival surgeries. Experiments of this sort include those in which two or more tracers are to be injected and it is known that they require markedly different survival periods for transport to occur. Another example is experiments in which injections will be made at different ages in the same animal in order to label the arrangement of connections at different stages in the development of a neural pathway.

3. Perinatal Procedures

A major challenge for neuroscience research is to learn how the brain develops. This is an issue of relevance to elucidating the causes of congenital neurological defects and learning disabilities, and to understanding the effects of the *in utero* (maternal) and the postnatal environment on brain development. Modern developmental neuroscience experiments involve not only the study of postnatal development, but also the examination of fetal animals even at very early stages of brain development.

Studies of the developing nervous system employ techniques drawn from the broadest range of modern neuroscience methods and also from recent methods specifically designed for the study of the fetal nervous system. Two broad experimental approaches are generally used. In the first, brain development is observed in order to establish the sequence of events associated with normal development. Observation itself is not always benign since it is often necessary to introduce neuroanatomical tracers into the developing nervous system, or to use portions of the brain for *in vitro* anatomical or physiological studies. The second experimental approach is to perturb or manipulate the nervous system in order to test hypotheses pertaining to developmental mechanisms. Such manipulations commonly involve lesions or ablations introduced surgically or via chemical neurotoxins, transplantation techniques, or chronic drug treatment.

While these experimental approaches frequently pose the same concerns as those arising from similar studies in adult animals, the study of the developing nervous system does involve several unique issues. The first is that the studies always entail the use of animals at a variety of

different ages, with each age posing different experimental and animal welfare challenges. The second is that, especially when fetal studies are involved, the welfare of both mother and fetus must be monitored. And finally, special consideration must be given to animal care and use concerns regarding anesthesia, monitoring and maintenance of physiological state, physical environment, multiple survival surgeries, and training and supervision, as detailed in sections III and IV of this report.

4. Inducing Neurological Deficits

A major class of neuroscience experiments involves the induction of neurological deficits in research animals. These experiments have provided valuable insights into the function of the central nervous system. Selected lesions or other manipulations of central nervous system structures have been used to learn how different parts of the brain function in behavior or perception. This approach has also been used to produce models for naturally occurring diseases and conditions, so that treatments leading to recovery can be developed.

A number of invasive techniques are currently employed to induce neurological deficits. These include surgical ablations, vascular occlusions (stroke models), electrical or radiation lesions, and chemical interventions (involving neurotoxins or other neuroactive drugs). A potential alternative to the use of these invasive techniques is the study of animals or humans with naturally occurring neurological diseases or damage. However, most naturally occurring conditions show great variability and they are often accompanied by undesirable side-effects. As a result, it is difficult or impossible to reach conclusions regarding how the affected parts of the brain function in behavior. In humans, there are ethical, legal, and practical limitations on invasive experimentation. In addition, it is not possible to obtain human tissue for histological verification of brain damage or analysis of the effects of treatments until the time of death, and human post-mortem tissue typically cannot be optimally fixed or otherwise treated for anatomical analysis. Thus, at present there is often no feasible alternative to the use of invasive techniques to induce neurological deficits in animals.

The care and use of animals with induced neurological deficits poses a number of special problems for researchers, veterinarians and IACUCs. For example, depending on the site and extent of their deficits, these animals may exhibit only limited abilities to care for themselves. In these cases, the scientific need to induce such debilitating deficits must be rigorously investigated by the IACUC, and researchers and veterinarians must demonstrate that they are capable of providing the special care that these animals will require. In these, as in every neuroscience experiment, mammals should be replaced by nonmammals whenever possible, the number of animals exposed to a debilitating deficit should be minimized, and the experimental protocol should be refined to reduce or eliminate pain, discomfort, and mortality and to increase the probability of obtaining a successful outcome to the study.

5. Repeated or Prolonged Exposure to Agents or Treatments

There is a category of neuroscience experiments that is characterized by the need for repeated or prolonged treatment of live animals with chemical agents or electrical stimulation to alter the pattern of activity in a population of neurons. Examples of this experimental approach include the repeated application of chemical toxins to destroy a population of neurons over time, the chronic administration of activity-blocking chemical agents or neurotransmitter analogs, and exposure to chronic electrical stimulation. Some experiments in this category seek to examine the role of particular types of experience or neuronal activity in the development of the nervous system. Others are designed to isolate the function of certain neural structures or pathways by reversibly or permanently inactivating them. Both developmental and inactivation studies have

proven valuable for understanding the human nervous system and for devising therapeutic approaches to disease and dysfunction.

The prolonged and repeated nature of the treatments employed in this category of experiments can make it difficult to conduct them in strict accord with the recommendations of the *NIH Guide*. For example, in some cases the neuroactive agents or treatments themselves have the potential to cause pain and distress. It can be problematic both to evaluate the likelihood of this adverse side-effect and, when appropriate, to design a method for avoiding it without compromising the scientific goal of the experiment. In other cases, the agents that are to be applied exhibit some form of toxicity. Once again, it can be difficult to determine whether their repeated administration is likely to compromise an animal's general health and to develop strategies for alleviating this problem. In some studies, certain aspects of the procedures for repeatedly applying an agent or a treatment might be considered a form of repeated survival surgery. In these experiments, it is necessary to evaluate the appropriateness of repeatedly exposing an animal to the stress of general anesthesia. It is also necessary to determine the level of asepsis that is both desirable and feasible to maintain during repeatedly performed procedures and whether these procedures should be performed in a laboratory setting. Finally, in some experiments the chronic alteration of neural activity or the destruction of a population of neurons can induce a permanent neurological deficit, raising the considerations discussed above (see 4. Inducing Neurological Deficits).

6. Awake Behaving Preparations

The study of many important neuroscience questions requires the use of an alert, usually behaving, animal. Awake behaving preparations make it possible to study the "higher" functions of the brain by involving the active participation of the organism. Trained animals not only can serve as subjects in experiments on motor control, but also can be sophisticated participants in psychophysical studies concerning the processes involved in perception and memory. Since the behavioral repertoires of many mammals resemble those of humans, data generated on awake, behaving preparations can be expected to have considerable relevance when extrapolated to humans.

Experiments on awake, behaving animals require a lengthy initial phase during which the animal is trained to perform a task and implanted with the various devices needed to monitor its performance. Data are then collected in daily sessions over a period that can extend for years. Most experiments on the brain require that the animal be restricted to a constrained working space and that a head-holder be implanted chronically on its skull so that the head can be immobilized during the experimental sessions. This kind of restraint can be accomplished without causing undue discomfort, if the animal is conditioned to the restraint properly. Major surgery to implant devices for head restraint and data collection can be accomplished with standard aseptic surgical technique and typically can be performed in a facility dedicated to aseptic surgery. The fact that animals are awake during the actual experimental sessions obviates many of the concerns associated with studies on anesthetized animals.

The extreme amount of preparation that goes into each animal and the duration of its participation in a typical experiment pose a number of difficulties that require the exercise of professional judgment and considerable flexibility in interpreting the recommendations of the *NIH Guide*. These difficulties include the determination of what kind of reinforcement to use for behavioral training, the criteria for evaluating the condition of animals whose food or fluid intake must be restricted to motivate them to perform a behavioral task, the conditions under which multiple survival surgeries are allowed, and the determination about whether a given surgical procedure must be performed in a dedicated surgical facility using aseptic technique. Once an animal has recovered from a major surgical procedure, overt pain and distress are

seldom a problem in awake behaving preparations. However, various factors must be taken into account in determining whether the overall training and experimental regimen is having an adverse affect on the animal's general well-being.

III. ANIMAL CARE AND USE CONCERNS IN NEUROSCIENCE RESEARCH

Consideration of the general research themes described in section II gives rise to six animal care and use concerns that are pervasive in neuroscience research. This section describes these concerns and gives the *NIH Guide* recommendations that are relevant to each of them. It also discusses factors that the members of an IACUC should take into consideration when they are reviewing a neuroscience research protocol from an investigator whose proposed use of animals appears to require that the IACUC grant an exception to the recommendations of the *NIH Guide*.

1. Physical Environment and Asepsis

Perhaps no other *NIH Guide* recommendations are more problematic for reviewers of proposed neuroscience procedures than those designed to ensure that the physical environment is appropriate and that an adequate level of asepsis is achieved. The *NIH Guide* states that:

"Aseptic surgery should be conducted only in facilities intended for that purpose. These facilities must be maintained and operated to ensure cleanliness and directed and staffed by trained personnel." (page 37)

"Aseptic technique must be used on most animals, including lagomorphs, that undergo major survival surgery. This technique includes wearing of sterile surgical gloves, gowns, caps, and face masks; use of sterile instruments; and aseptic preparation of the surgical field. Major survival surgery is defined as any surgical intervention that penetrates a body cavity or has the potential for producing a permanent handicap in an animal that is expected to recover. Survival surgery on rodents does not require a special facility but should be performed using sterile instruments, surgical gloves, and aseptic procedures to prevent clinical infections." (page 37)

"Functional areas for aseptic surgery should include a separate surgical support area, a preparation area, the operating room or rooms, and an area for intensive care and supportive treatment of animals. The interior surfaces of this facility should be constructed of materials that are impervious to moisture and easily cleaned. The surgical support area should be designed for storing instruments and supplies and for washing and sterilizing instruments. Items that are used on a regular basis, such as anesthesia machines and suture materials, can be stored in the operating room." (page 47)

"There should be a separate surgical preparation area for animals. An area equipped with surgical sinks should be close to, but apart from, the operating room. A dressing area should be provided for personnel to change into surgical attire." (page 47)

"If explosive agents are to be used, floors should be conductive and outlets should be explosion-proof and located not less than 5 ft (1.52 m) off the floor. Provision should be made for scavenging or exhausting waste gases from anesthesia machines. Explosion-proof hoods are preferable if volatile, explosive agents like ether are to be used. Consideration should be given to providing positive air pressure in the operating room to reduce contamination." (pages 47-48)

"A separate facility for rodent surgery is not necessary. A rodent surgical area can be a room or portion of a room that is easily sanitized and not used for any other purpose during the time of surgery." (page 48)

In its position statement of December, 1987, The American Association for Accreditation of Laboratory Animal Care (AAALAC) has interpreted the *NIH Guide's* recommendations regarding facilities for survival surgery. AAALAC's position is that major

survival surgery must be performed in a closed, single-purpose operating room. If aseptic operative procedures are performed frequently, this room cannot be used for any other procedure, but if procedures are performed only occasionally, the room may be used for other purposes. This room should contain only the equipment and supplies that are required to support the procedure being performed and whose presence is scientifically or professionally justified. In high-volume programs, the activities associated with surgeons' preparation, animal preparation, the surgical procedure, surgical support, and post-operative care may each require a separate room. However, it is acceptable to have as few as three rooms: one used exclusively for surgery, one for surgical support and surgeons' preparation, and one for preparation and post-surgical care of the animal.

Most neuroscience procedures can be performed in full compliance with the *NIH Guide's* recommendations regarding asepsis and the physical environment. Even in those cases where full compliance may not be possible, most aspects of these recommendations can still be met (e.g., the use of sterile surgical gloves, gowns, caps and face masks; the use of sterile instruments; aseptic preparation of the surgical field; and post-surgical care procedures). On the other hand, when a survival surgical procedure requires the use of specialized equipment, facilities, and/or substances, it can be very impractical, if not completely infeasible, to perform it in a manner that fully complies with all recommendations.

An example of this problem occurs when a survival procedure must be performed under the control of neurophysiological recordings. The apparatus needed to obtain the recordings typically will include amplifiers, filters, oscilloscopes, audio monitors, stimulators, and computers. Usually it will not be possible to place this equipment remotely because the monitors must be directly available and other equipment will require frequent adjustment during the procedure. In addition, moving extensive equipment back and forth between the laboratory and a dedicated surgical suite generally will not be possible because of the risk of damaging fragile items and the need for extensive recalibration each time they are moved. Even if this were feasible, the difficulties encountered in attempting to sterilize or isolate the necessary racks of equipment would be considerable. For example, most large equipment requires good air flow for heat exchange and cannot be wrapped or contained. Finally, the microelectrodes used to record from the brain may be difficult to sterilize, either because they are physically too fragile to withstand the rigors of sterilization, or because the materials used in their construction are easily damaged by exposure to heat or chemicals.

Even when neurophysiological control is not required and a survival surgical procedure can be performed under visual or stereotaxic control, it may be difficult to sterilize and relocate to a dedicated surgical suite all the pieces of equipment needed to accomplish a procedure (e.g., iontophoretic or pressure injection equipment, stereotaxic apparatus, micromanipulators, specialized microscopes, etc.) each time it is to be performed. Similarly, many neuroactive agents or tracer substances that are commonly injected into the brain may be impossible to sterilize and it even can be difficult to sterilize the devices used to inject them (e.g., micropipettes, combination injection/recording microelectrodes, precision microsyringes, etc.). Finally, the extra safety and monitoring precautions required when dealing with toxic, radioactive, or other hazardous substances can make it difficult to use them safely in a general purpose surgical facility.

In light of the various constraints outlined above, IACUCs frequently will be asked to allow survival neuroscience procedures to be performed in a modified laboratory setting. Prior to granting such an exception to the *NIH Guide's* recommendations, an IACUC should consider the extent to which it will subject animals to an increased risk of infection. In particular, the various steps that can be taken to minimize this risk should be carefully reviewed by the committee. These mitigating measures include aseptic preparation of a separate area of the laboratory in which the the surgery will be conducted; the use of aseptic surgical attire,

instruments, and supplies; and aseptic preparation and maintenance of the surgical field during the procedure.

Several things can be done to make a laboratory setting a more acceptable site for performing major survival surgery. In many situations, it may be possible to partition a large, general purpose laboratory, thereby physically isolating a smaller surgical area from the remainder of the space. In any case, the room in which surgery is to be performed must be capable of being sanitized immediately prior to each procedure. Thus, the walls, ceiling and floor should have smooth surfaces that are impervious to moisture and easily cleaned. The room should be free of fixed equipment and should not be used for general purpose storage. Prior to a surgical procedure, all unnecessary items should be removed from the room to allow thorough sanitization. Further specific details about conducting survival surgery in a laboratory setting are discussed below in the sample protocol on survival anatomical procedures in section IV.

An additional mitigating factor that should be considered is the proposed duration of the post-surgical survival period, since a brief period offers less risk of an infection developing. Finally, in some cases it may be advisable to consider administering a prophylactic dose/doses of a systemic antibiotic to healthy animals prior to a survival surgery, and to continue this regimen post-operatively. Usually this procedure is considered inadvisable since it can promote the development of resistant strains of bacteria. However, there are several reasons for considering this course of action in neuroscience experiments of this type. Chief among these is the fact that operated animals often will be allowed to survive for a period of only a few days before they are euthanized and perfused with fixatives. Furthermore, during the survival period they often can be caged individually and isolated from other animals.

The decision to allow a survival procedure to be performed in a modified laboratory setting also should be contingent on the development of a stringent set of post-surgical monitoring and reporting procedures. For example, the IACUC may wish to approve a temporary exception to the *NIH Guide's* recommendations, subject to receiving a status report from veterinary staff on the health and welfare of animals during the post-surgical survival period. In this way, the efficacy of the various procedures proposed to mitigate against the risk of post-surgical infection can be verified before a project is given full approval. When the proposed experiments involve introducing microelectrodes or other devices into the brain, this verification process can include culturing a sample of these devices to ensure that they are free of microbial pathogens. Another potential verification technique is histopathological examination of tissue sections taken from the brains of operated animals. Since the brains of animals used in neuroscience experiments often will be prepared for histological analysis as part of the experimental protocol, selected sections can be examined for signs of infection secondary to the surgical procedure.

IACUCs also may find it difficult to evaluate some aspects of the experimental procedures used with awake behaving animals for compliance with the *NIH Guide's* recommendations for ensuring asepsis. Awake behaving preparations frequently require the attachment of a chronic percutaneous implant to the skull. These implants require regular inspection and cleaning to keep them functional and to minimize local infection. This can involve a variety of manipulations, including tightening the bolts used for head restraint, thinning the dura inside a recording chamber, flushing, cleaning, or repositioning physiological monitors, and removing or replacing subcutaneous monitoring devices. In general, these minor procedures are no more invasive than a biopsy or a venipuncture and as such they may be conducted in a laboratory setting using aseptic technique.

Finally, neuroscience experiments that involve prolonged non-survival recordings should be mentioned in the context of the *NIH Guide's* recommendations regarding asepsis and the physical environment. Animals do not survive these procedures and, since they should be

anesthetized throughout their duration, they should not be subjected to significant pain or distress. On the other hand, the duration of many of these non-survival procedures is more than sufficient to allow infection to develop and as a result the quality of the preparation may suffer. For this reason, it is desirable to perform this type of experiment in as aseptic a manner as is compatible with the constraints of the particular procedures involved. Principal investigators should be encouraged to seek veterinary professional advice in this regard.

2. Anesthesia and Analgesia

Experimenters must employ appropriate anesthetic or analgesic drugs to ameliorate pain and discomfort in experimental animals. Because neuroscience experiments often involve surgical intervention, the use of these drugs is a regular feature of research in this field. However, because anesthetic and analgesic drugs act on the system under study - the nervous system - neuroscientists must make uniquely difficult choices in selecting their pharmacological agents. This difficulty is further confounded by the fact that certain experiments require also that the animal be immobilized with a skeletal muscle relaxant, which prevents the expression of the most reliable behavioral signs of pain and distress. The *NIH Guide* states,

"The proper use of anesthetics, analgesics, and tranquilizers in laboratory animals is necessary for humane and scientific reasons. In accordance with the U.S. Animal Welfare Act, the choice and use of the most appropriate drugs are matters for the attending veterinarian's professional judgment. The veterinarian must provide research personnel with guidelines and advice concerning choice and use of these drugs." (page 37)

"If a painful procedure must be conducted without the use of an anesthetic, analgesic, or tranquilizer -- because such use would defeat the purpose of an experiment -- the procedure must be approved by the committee and supervised directly by the responsible investigator." (page 37)

"Muscle relaxants or paralytic drugs (e.g., succinylcholine or other curariform drugs) are not anesthetics. They must not be used alone for surgical restraint, although they can be used in conjunction with drugs known to produce adequate analgesia." (page 37)

The goal of this discussion is to provide general guidance to principal investigators, veterinarians and IACUCs in the difficult task of making this selection in a way that both ensures that pain and distress are prevented, and interferes least with the neurobiological measurements being undertaken. It is important to appreciate at the outset that the *NIH Guide* provides relatively little specific guidance on matters of anesthesia, quite properly leaving the specific choices to be dictated by the needs of the experiment as jointly judged by the principal investigator and the attending veterinary staff. This is an area in which few clear categorical boundaries exist, and in which the flexibility with which the regulatory functions of IACUCs and veterinarians are performed is of the utmost importance to the conduct of sound experiments. It is often necessary to take into account the effect of (sometimes subtle) variations in technique when making recommendations for the choice of anesthetic and analgesic drugs. For this reason, this discussion for the most part eschews specific prescriptions and proscriptions and instead encourages the flexible and knowledgeable application of the professional judgment of all concerned with a particular experiment. The sample research protocols provided in section IV contain some specific examples of anesthetic regimens that have proved useful in specific conditions, but these are not intended to serve as specific or quantitative guidelines.

In considering how best to provide guidance on the choice and use of suitable analgesic and anesthetic procedures in neuroscience experiments, we have broken our treatment down along several different dimensions. The first important consideration in choosing an anesthetic is

whether or not the animal is to survive the procedure requiring anesthesia. An anesthetic agent can have an adverse side-effect that renders it unacceptable for use in animals intended for survival, but it may also possess a special property that makes it perfectly reasonable for use in animals that are euthanized at the end of the procedure. An example is urethane, an effective anesthetic that exhibits long-term toxicity, but has little or no direct effect on neurons in the primary visual pathway, making it an ideal candidate for use in an acute physiological recording experiment. A second consideration is whether the anesthetic is to be used in association with a muscle relaxant, since the inability of a paralyzed animal to react to noxious stimulation creates serious problems in monitoring anesthetic depth. Finally, procedures used for fetal and neonatal surgery may be quite different from those used in adult animals. Consideration of the various plausible combinations of these cases reveals that some conditions require only the straightforward application of modern anesthetic practice, while others pose complex challenges for investigators and veterinary staff alike.

Anesthesia vs. analgesia. An important issue that is imprecisely treated in the *NIH Guide* is the distinction between general anesthesia and analgesia. It has been common in neuroscience experiments to use techniques designed to produce total loss of awareness - general anesthesia - to relieve pain and distress, at least during acute surgical procedures. More recently clinicians (and some neuroscientists) have come increasingly to use tranquilizers and opiates that produce a state of neuroleptanalgesia, a condition in which a loss of awareness is not assured, but in which freedom from pain and distress is produced. Neuroleptanalgesia has a number of virtues for some experimental situations and it seems wise to encourage the use of this sort of technique when it is judged appropriate. However, some objections might be raised because a neuroleptanalgesic state is not "true" anesthesia. Despite this superficial difficulty, the use of these techniques should be considered seriously when it is well established that they produce freedom from pain and distress, and when surgery is not being performed.

In addition to barbiturates, volatile anesthetic agents, and neuroleptanalgesics, a number of other anesthetic regimens are available. Dissociative agents, such as ketamine or tiletamine, "dissociate" sensation and perception. They produce unconsciousness and analgesia, while retaining muscle tone and leaving many reflexes intact. They are seldom used as the sole anesthetic for major surgical procedures, but may be combined with a variety of other agents as part of a balanced anesthesia protocol.

Non-survival experiments without systemic paralysis. In experiments not involving muscle relaxants, in which the animal is rendered unconscious or insensible to pain and is euthanized before regaining consciousness, few complexities arise. Because the animal is capable of expressing its reactions to painful or distressful stimuli should it regain sensitivity to them, the basic veterinary requirement of such experiments is that suitable monitoring be undertaken to ensure that the animal remains free of pain and distress (see 5. Monitoring and Maintenance of Physiological State). Certainly the professional advice of veterinarians and anesthesiologists are essential in selecting drug regimens that both are effective and minimally affect the measurements being undertaken.

Survival procedures undertaken without systemic paralysis. This is the case closest to conventional clinical veterinary practice, so it should not pose any particular problems for either veterinarians or principal investigators. As in the previous case, the experimental situation does not prevent the animal from expressing normal reactions, so the regulation of anesthetic state is without special complications. Features of the experiment may dictate the use of anesthetic agents that are uncommon in normal clinical practice, but the suitability and effectiveness of these can be easily checked. It should, however, be noted that neuroscience researchers may be unaware of some modern advances in anesthetic practice (e.g. the use of neuroleptanalgesic agents mentioned above) that can significantly improve the quality of surgery and thus the post-operative success of the experiment. It is therefore not always advisable to continue use of

long-standing procedures, however well-tried they may seem. The paramount considerations in choosing among drugs thought to be effective in controlling pain and distress should be survivability and ease of post-operative recovery. This is an area in which the advisory functions of an experienced consulting veterinarian can be exercised very valuably.

Post-operative use of analgesics and sedatives. Good veterinary practice requires the use of suitably chosen analgesics and/or local anesthetics to relieve post-operative pain. Such medication does not usually interfere with the conduct of neuroscience experiments and does not present a problem in the context of this report. Note should be made, however, that reducing discomfort may have the undesirable side-effect that the experimental animal does not protect the region of injury (see 5. Monitoring and Maintenance of Physiological State).

Experiments involving systemic paralysis. A common experimental situation, especially in visual neuroscience experiments, is one in which the experimental animal must be immobilized with a curariform muscle relaxant, thereby paralyzing the extraocular muscles as well as all other voluntary muscles. Since most neuromuscular blockers do not interact significantly with anesthetic and analgesic drugs, the basic criteria for choosing those drugs are not altered by paralysis. However, because of its inability to respond behaviorally, the animal's freedom from pain or distress is difficult to evaluate. This specific issue was the subject of a previous Workshop held in 1984, from which a report entitled, "Anesthesia and Paralysis in Experimental Animals," has recently been published (*Visual Neuroscience* 1:421-426, 1988). The remainder of this discussion summarizes the recommendations of this previous report, with some modifications based on subsequent experience.

Observable signs of pain and distress that may be used under paralysis include: autonomic signs such as lacrimation and salivation; reactivity of heart rate and arterial blood pressure to noxious stimuli; and electroencephalographic recordings. These signs, either singly or in combination, can provide valuable information about the animal's condition. However, they are not infallibly linked to the animal's state and for that reason they should be used with caution and validated for each particular experimental situation.

The *Visual Neuroscience* report recommended that principal investigators conduct trial experiments in which muscle relaxants were not administered, but which are in other respects identical to "real" experiments. This was intended to test the utility of the particular anesthetic regimen used and also to allow direct correlation of behavioral evidence of distress with particular signs that can be assessed under paralysis. This suggestion has not been widely taken up by the neuroscience research community, largely because of the practical difficulties associated with conducting a full-length trial experiment, although as a general method it seems to be the best available.

As a more practical alternative, it seems reasonable to conduct a modified trial, usually abbreviated to a period long enough for the animal's condition to stabilize, but substantially shorter than an entire recording experiment (which may last many days). Because the early phases of a prolonged procedure - while the preparation is stabilizing - are the most critical, an abbreviated trial can often serve the purpose of a full one. This method is particularly attractive when using drug regimens that are free of potentiation or tolerance effects.

Another recommendation of the *Visual Neuroscience* report was that muscle relaxants be periodically withheld during experiments, so that the state of the unparalyzed animal can be conventionally assessed at intervals during the experiment. This recommendation still seems prudent and reasonable. The use of recently formulated relaxants whose action is of short duration (e.g., Pavulon or Norcuron) can materially ease this process. The combination of an abbreviated trial preparation with the occasional release of the animal from paralysis provides a

good approximation to the results of the full trial recommended by the *Visual Neuroscience* report.

Measuring the EEG should in principle provide a good deal of information about the animal's state, but the conventional EEG is not always reliable, can be difficult to interpret, and can be altered by certain drugs that have only minor effects on the animal's mental state. Some special EEG methods were noted as potentially more useful in the *Visual Neuroscience* report, but development of more sophisticated techniques for EEG recording and interpretation has progressed little in the succeeding period. In the absence of new evidence on the matter, it remains prudent to use EEG measurements only when it is possible to establish a clear association between EEG measure and overt behavioral evidence of pain or distress.

Flexible application of anesthetic techniques in paralyzed animals is particularly important when the experimental subject has been previously prepared for study in a way that permits atraumatic restraint and renders most pain-producing surgical procedures unnecessary. These methods are commonly used for animals that will survive an experimental procedure, have the relaxant withdrawn, and be returned to their home cage for subsequent use in further experimental sessions. The use of this sort of technique does not relieve the experimenter of the obligation to use drugs to ensure freedom from distress, because the experimental situation may include stressors other than frank surgical wounds and pressure points, such as paralysis itself. Nonetheless, it should be possible to obtain satisfactory conditions using milder drugs and/or smaller doses than would be needed in animals undergoing surgical procedures. The *Visual Neuroscience* report considers a number of factors, such as respiratory distress, postural discomfort, uncontrolled body temperature, and others that can produce distress in paralyzed animals. Clearly, when more of these factors can be eliminated, a lower level of anesthetic or analgesic potency will serve to maintain the animal in a satisfactory condition.

Experiments in fetal and neonatal animals. When fetuses or neonates are the subjects of an experiment, both the monitoring of anesthetic state and the choice of anesthetic present complex problems. These individuals often do not show the overt signs of pain and distress that are demonstrated by older animals, making conventional monitoring of anesthetic state difficult. Moreover, drug regimens that are safe and effective in older animals can be lethal in very young ones.

For fetal surgery, the welfare of the mother should be of primary concern. Neural mechanisms subserving the perception of pain are developing and immature in fetal brains and it is difficult to establish whether the perception of pain is present. However, most anesthetic drugs freely cross the placental barrier and it is therefore likely that any regimen that prevents pain and distress in the mother will also be effective for the fetus.

For neonatal surgery, anesthetic practice should conform as far as possible to that used in human neonates; veterinary practice and experience offer few useful guidelines because neonatal animals are rarely encountered clinically. Anesthetics used for adult animals often have a much narrower margin of safety in neonates and, as in human neonates, the goal of ensuring viability must often control the technique chosen. One technique that is applicable to altricial neonates that have not yet developed effective thermoregulatory mechanisms is hypothermia, which has a wide margin of safety and appears to be an effective anesthetic. The choice of methods to be used in neonates is another area in which the flexible cooperation of principal investigators and veterinarians is particularly important.

Other exceptional cases. It must be recognized that in some experiments, for example those concerned with pain mechanisms, pharmacological relief of pain and distress may be incompatible with the aims of the experiment. In such cases the principal investigator and

veterinary staff should work to ensure that any episodes of unrelieved pain are as brief and as low in intensity as is compatible with the successful conduct of the study.

3. Training and Supervision

The responsibility for training of personnel and supervision of neuroscience experiments rests jointly with the principal investigator and the institution. The *NIH Guide* states that,

"It is an institutional obligation to ensure that professional and technical personnel and students who perform animal anesthesia, surgery, or other experimental manipulations are qualified through training or experience to accomplish these tasks in a humane and scientifically acceptable manner. Special training programs should be provided for technicians and faculty, as well as undergraduate, graduate, and postdoctoral students." (page 5)

In applying for PHS funding, the principal investigator must complete the Grant Application Form (PHS 398). The General Information section on Vertebrate Animals states,

"Research investigators are entrusted with an essential role in assuring the humane care and use of animals. In activities they conduct or which are conducted under their direction, they have a direct and continuing responsibility to see that animals are adequately cared for and used. Investigators must comply with the PHS Policy . . ." (pages 5-6, 10/88 Rev.)

While the principal investigator is ultimately responsible for assuring that appropriate training has been provided, it is an *institutional obligation* to make available training in humane practices of animal maintenance and experimentation that minimize pain and distress. Such training might well be considered a normal part of graduate education for students who will use animals in experimentation. The responsibility for reviewing this training rests with the IACUC, which must consider the qualifications of personnel involved in conducting research as part of its review process.

Training of research personnel should include procedure-specific training in neuroscience research techniques, which the principal investigator is usually best suited to oversee, and more general training in areas such as regulations, aseptic technique, anesthesiology, euthanasia techniques, and animal handling, which members of the veterinary or animal care staff are generally most qualified to teach. The level of training in these areas will of course vary depending upon the duties and responsibilities of the staff involved. Where procedures have the potential for producing pain and distress, mechanisms must be in place to assure that principal investigators have demonstrated competency in their performance. In those areas of a more general nature, the level of training may be more suitably directed toward a level of general awareness in terms of the responsibilities and duties of the individuals involved.

There are no specific requirements for training principal investigators, but in general it can be assumed that the requirements of the PHS Policy will be met if the goal of training is to assure that individuals are aware of their responsibilities to the institution and the animals in their care and that they demonstrate competency in performing techniques which assure that pain and distress are kept to minimum.

Adequate training and supervision in the techniques necessary for the conduct of specific neuroscience experiments must be provided. The principal investigator is ultimately responsible for ensuring that this training is available and for ensuring that all applicable laws, guidelines, and policies are followed and understood in the context of neuroscience research.

In general, neuroscience experiments do not require any special exceptions to the *NIH Guide* and PHS Policy recommendations regarding training and supervision. On the other hand, implementation of the guidelines can be challenging because aseptic techniques are frequently required, particularly in unconventional settings (e.g., see 1. Physical Environment and Asepsis). Also, many surgical procedures in neuroscience experiments are more familiar to human neurosurgical practice than to standard veterinary practice. Thus, a close collaboration between veterinary and scientific personnel may be required to achieve the requisite level of expertise necessary for the principal investigator to meet the requirements of the *NIH Guide*. Moreover, as veterinary medicine advances, appropriate improvements in relevant techniques should be shared with investigators. These issues imply that the process of training is an ongoing one, with continual communication and collaboration on both sides. Finally, training should not be restricted to the local level, but should involve veterinarians and scientists throughout the country. A serious problem in this regard is that currently there is no way in which this kind of training at the national level can be implemented.

4. Multiple Survival Procedures

The *NIH Guide* states that,

"Multiple major survival surgical procedures on a single animal are discouraged. However, under special circumstances they might be permitted with the approval of the (IACUC) committee. One situation in which multiple survival surgical procedures might be justified is when they are related components of a research project. Cost savings alone is not an adequate reason for performing multiple survival surgical procedures." (pages 9-10)

While, in general, multiple surgical procedures should be discouraged, under several circumstances they may be allowed. This is clearly the case in many neuroscience experiments where multiple procedures are necessary for the scientific conduct of the study. Multiple survival surgeries may also be permitted when allowing them to occur reduces the number of animals that must be subjected to an initial, major, traumatic surgery. A key consideration here is that the level of pain and distress involved in the additional surgical procedure(s) should be significantly less than that involved in the initial surgical preparation. In addition, any time animals will undergo additional survival procedures during the subsequent phase(s) of an experiment, the scientific need for each animal to have been subjected to the initial major procedure must be clear. Finally, in any specific case, the balance between the welfare of the individual animal and the need to reduce the use of animals must be carefully weighed by the members of the IACUC.

Multiple surgeries may also be allowed is when an individual animal model is difficult to develop or maintain and an adequate sample for a valid scientific outcome requires multiple procedures. In all circumstances, close monitoring of the animal in consultation with the institutional veterinary staff is necessary to assure that the multiple procedures are in fact as benign as feasible. Techniques should be continually refined to hold any pain and distress to a minimum. The level of monitoring should be appropriately matched to any anticipated level of pain and distress. In no case is the cost alone an acceptable reason for performing multiple surgeries.

Factors to be considered in evaluating the justification for multiple surgeries include the following:

- (1) serial surgeries essential to the design of a given study.

- (2) entry into a body cavity such as that required for cannula or microelectrode placement and which does not produce more than momentary pain.
- (3) clinical procedures which may be necessary for the health and well-being of the animal, such as repair of an implant.
- (4) procedures which are commonly performed multiple times in a clinical setting, such as multiple hysterotomies for delivery of the contents of a pregnant uterus.
- (5) animal conservation such as commonly occurs with nonhuman primates and difficult to obtain or develop animal models. Examples of the latter include highly trained animals, breeding animals that are difficult to obtain, animals reared in special circumstances, and animals whose use is critical to the outcome of a study but whose supply is restricted, such as unique genetic models or endangered species.

Some specific examples of the need for multiple survival procedures in neuroscience research include the following. In studies of awake-behaving animals, it is common to implant a movement transducer first, train the animal, and then implant a chronic recording chamber. Often, the experiment requires weeks or even months of recording of movements as a prelude to recording from brain cells. The health of the animal and the implants are much better if devices are added only as needed, usually in surgeries that have a rather short duration and do not place a severe physiological stress on the animal. A second example is one in which studies of several different brain areas are conducted in a single, awake, behaving animal. An initial major surgery is required to prepare the animal for the first set of studies; less traumatic procedures are required for implantation of subsequent recording devices. This approach reduces the total number of animals subjected to the major traumatic surgery. Another example involves prolonged non-survival experimentation, where it can be advantageous to perform an initial survival surgery to implant devices that then allow the atraumatic restraint of an animal and access to the brain during the subsequent non-survival procedure. This approach is often used to prepare animals for a non-survival recording experiment that requires the use of paralytic agents. A fourth case can arise when the basic design of an anatomical tract-tracing experiment requires the use of two or more tracers, each of which has a different post-injection survival time, or in studies of development, when two different tracers must be injected into the brain at two separate times.

5. Monitoring and Maintenance of Physiological State

The topic of monitoring and maintenance encompasses the periodic assessments of animal health and physiological state, along with associated record keeping, that must be carried out in the course of any study using experimental animals. The *NIH Guide* states that adequate veterinary care includes,

"observing all animals daily to assess their health and welfare; ...Daily observation of animals can be accomplished by someone other than a veterinarian; however a mechanism of direct and frequent communication should be adopted so that timely and accurate information on problems in animal health, behavior, and well-being is conveyed to the attending veterinarian." (page 33)

"using appropriate methods to prevent, control, diagnose, and treat disease and injuries;" (page 33)

"monitoring surgery programs and postsurgical care. (page 33) ...Postsurgical care should include observing the animal to ensure uneventful recovery from anesthesia and surgery; administering supportive fluids, analgesics, and other drugs as required; providing adequate care for surgical incisions; and

maintaining appropriate medical records. ...Proper monitoring by trained personnel should be provided during recovery." (page 38)

Particular monitoring and maintenance issues fall into a continuum between two extremes. At one extreme are measures necessary to ensure animal health and welfare. These issues, which are referred to below as "animal welfare" issues, are appropriate subjects for regulation, as indicated by the passage quoted above. At the other extreme are issues dealing with the optimal monitoring and maintenance procedures for obtaining the best experimental data. These are referred to below as "advisory" issues. It is important for neuroscientists, veterinarians, and animal care personnel to consult with and learn from one another regarding these advisory issues. However, they usually are not appropriate subjects for regulation, since they will differ significantly from one experiment to another.

Prior to discussing specific monitoring and maintenance problems that arise in particular types of neuroscience experiments, it is important to list three overriding considerations that apply to every research project in which potential problems of monitoring and maintenance are an animal care and use concern.

Consultation. It is essential that neuroscientists and veterinarians consult with each other in the design and implementation of monitoring and maintenance procedures. Many of the problems addressed below do not at the present time have well established solutions. For this reason, as well as others, their solution requires continuous discussion and collaboration among principal investigators and veterinarians. This interaction should begin before experiments are initiated. Investigators should recognize veterinary consultation as a positive feature in the overall design of animal research programs. The interaction between the investigator and the veterinarian should be an opportunity for mutual education.

Responsibility. Monitoring and maintenance activities must be carried out and documented by neuroscientists in the ordinary course of their experiments. There is in general no necessity for a veterinarian to be present to carry out routine monitoring and maintenance activities. However, it should be borne in mind that it is the ultimate responsibility of the veterinarian to ensure that monitoring and maintenance are carried out appropriately and adequately documented.

Record-keeping. It is not enough merely to carry out appropriate monitoring and maintenance procedures. It is essential to keep adequate records of them as well. These records must be written as close as is practical to the time at which the monitoring and maintenance activities are performed. The method of documentation should not be overly cryptic; it should be clear to another person reading the record of monitoring and maintenance activities. This documentation serves at least four purposes:

- (1) It facilitates detection of gradual changes in the condition of an animal that might not be apparent in a single observation period. Since there are great differences among animals, it is often the case that a change in condition is more informative than the immediate condition itself.
- (2) The keeping of appropriate records of monitoring and maintenance activities requires the experimenter to decide in advance what should be monitored and what should be the frequency of monitoring. Filling in the blanks of a form designed for monitoring a particular experiment helps make the experimenter more diligent in carrying out monitoring and maintenance activities.
- (3) These records become an archive for use over several years in improving experiments.

- (4) Such documentation establishes beyond any challenge that appropriate monitoring and maintenance activities were carried out.

The remainder of this discussion considers monitoring and maintenance activities that are appropriate for different experimental approaches. For the purposes of this discussion, neuroscience experiments have been divided into two general categories -- non-survival and survival. A number of "animal welfare" and "advisory" issues are mentioned for each of these categories.

Non-survival experiments. In non-survival experiments, intra-procedural monitoring arises as an animal welfare issue. A primary issue for monitoring is the control of pain. If the animal is not subjected to neuromuscular blockade, monitoring of the normal signs of anesthesia, such as withdrawal reflexes, can suffice. In the case of paralyzed animals, the monitoring of the adequacy of anesthesia is more difficult. This issue has been addressed previously (*Visual Neuroscience*, 1:421-426, 1988) and is discussed elsewhere in section III of the present report (see 2. Anesthesia and Analgesia). In addition to basic pain control, the investigator should monitor those physiological systems that are vital to the continued maintenance of the experimental preparation. Core temperature and some measure of cardiovascular and respiratory function, such as color and capillary refill time, are desirable. All appropriate monitoring should be carried out and recorded routinely, so that gradual as well as acute changes may be noted. It is important to note that the recordings of neuronal activity obtained in many non-survival neuroscience experiments may themselves constitute an extremely sensitive monitor of the physiological state of the animal; such direct observation of the brain often can help monitor the adequacy of various physiological maintenance procedures.

Numerous advisory issues may also arise in non-survival experiments. In general, it is important for both the principal investigator and the veterinary consultant to become knowledgeable of the physiology of the prolonged maintenance of unconscious animals. The complex issues that arise (e.g., hyperalimentation, the maintenance and monitoring of ventilation and lung function and the prevention or reduction of infections) are typical of those that pertain to intensive care patients and there is no general or simple formula for their solution.

Survival experiments. In survival experiments, monitoring and maintenance issues arise pre- and post-surgically, intra-procedurally, and in relation to long-term survival and animal health. A medical records chart must be kept for each animal throughout the course of an experiment. Before any surgery or treatment, it is important to note and record at least the weight and general health of the animal, as well as any known peculiarities of that individual animal. During a procedure, many of the same monitoring and maintenance concerns arise as in non-survival experiments, along with one additional issue, asepsis, which is discussed further in section III (see 1. Physical Environment and Asepsis). Post-surgically, at least temperature and hydration should be monitored, maintained, and recorded until recovery from anesthesia. Immediately following recovery from anesthesia it can be desirable to monitor a number of factors, unless forcible restraint and/or sedation would be required to enable monitoring to take place. These factors include temperature, heart rate, chest sounds, and fluid adequacy. If an experimental manipulation has the possibility of producing a chronic deficiency or debilitation, the principal investigator should consult with the veterinarian to devise specific long-term monitoring procedures that are appropriate for a particular experiment. These procedures can involve monitoring indicators of cardiovascular, pulmonary, renal, metabolic, and neurological function.

The issue of pain and analgesia during the post-surgical period is seldom different in neuroscience experiments from conventional clinical veterinary practice. It should be noted that with some treatments, the time of surgical or chemical insult may not be the time of maximum

pain, requiring that monitoring and possible maintenance on analgesic treatment be continued for an appropriate time. It also should be noted that blocking the ability to perceive any pain during the postsurgical recovery period can lead to complications. Thus, in many cases local anesthetics should not be used, so that an animal can help protect a wound site from further damage. The benefits of administration of non-steroidal anti-inflammatories should be weighed carefully.

The post-surgical monitoring of pain and general health is a delicate matter of necessarily subjective clinical judgment. This judgment depends upon evaluating a variety of behavioral factors. It is important to recognize that the interpretation of these factors differs greatly among species; in general the more removed a species of animal is from domestication, the more likely it is to mask pain or distress from the observer. Among the aspects of animal behavior that should be monitored for signs of pain or failing health are general alertness, responsiveness, possible reluctance to move or difficulty in movement, grooming, and ease of feeding, drinking and elimination. The monitoring of awake, behaving animals has the special advantage that the performance of the animal in its behavioral task can be a sensitive measure of the animal's general condition (see section IV, 6. Awake Behaving Preparations). In the case of all long-term survival animals, an aspect of monitoring that sometimes receives inadequate attention is the importance of recording baseline measurements. In many cases, it is a change in some behavioral measurement, rather than the particular repertoire of behaviors that is present at any one observation, that is particularly informative. Accordingly, thorough record-keeping is an essential aspect of any ongoing behavioral monitoring program.

6. Physical Restraint

In neuroscience research, prolonged restraint of awake animals is an essential procedure in a number of instances. Restraint may be used for clinical reasons in the post-surgical recovery period or for scientific reasons to provide a restricted workspace and allow measurement of physiological variables in awake behaving preparations. The *NIH Guide* allows prolonged restraint and is quite specific about the procedures that must be followed:

"Brief restraint of animals for examination, collection of samples, and a variety of other clinical and experimental manipulations can be accomplished manually or with devices such as restraint stocks or squeeze cages. It is important that such devices be suitable in size and design for the animal being held and operated properly to minimize stress and avoid injury to the animal."

"Prolonged restraint of any animal, including the chairing of nonhuman primates, should be avoided unless essential to research objectives. Less restrictive systems, such as the tether system or the pole and collar system, should be used when compatible with research objectives (Wakeley et al., 1974; Byrd, 1979; Bryant, 1980; Anderson and Houghton, 1983; McNamee et al., 1984). The following are important guidelines for the use of restraint equipment:

Animals to be placed in restraint equipment should be conditioned to such equipment prior to initiation of the research.

The period of restraint should be the minimum required to accomplish the research objectives. Prolonged restraint for any reason must be approved by the (IACUC) committee.

Restraint chairs or similar devices are not to be considered 'normal' methods of housing, although they may be required for specific research objectives.

Restraint chairs or similar devices must not be used simply as a convenience to investigators in handling or managing animals. When such devices are used, their use must be specifically approved by the (IACUC) committee.

Attention must be paid to the possible development of lesions or illnesses associated with restraint, including contusions, decubital ulcers, dependent edema, and weight loss. If these or other problems occur, veterinary care must be provided to treat the animal, which if necessary must be temporarily or permanently removed from the restraint device." (page 9)

Methods of restraint. It is important to select a method of restraint that is appropriate for the species in question and allows the animal to rest in a natural position. For primates, specially-designed chairs are commonly used for restraint. For other species, it is common to place the animal in a loosely fitting bag and then to place the bag within a box or tube. For most neurophysiological procedures, it is necessary to prevent the animal from moving its head during physiological recordings. This is accomplished by implanting transcutaneous devices in the skull in a survival surgical procedure and then using those devices to affix the animal's head to a stable platform during experiments. The proper use of restraint includes conditioning the animal gradually until it achieves behavioral adaptation to the restraint device. The ultimate demonstration of behavioral adaptation would be voluntary movement by the animal into restraint, but other criteria indicating ready acceptance of restraint should suffice.

Restraint during experiments. Most awake behaving preparations require that the animal be restrained or confined to a certain workspace. This provides a way to bring the animal into proximity with the behavioral task, to provide sensory stimuli in a quantifiable manner, and to monitor physiological variables, including the electrical activity of brain cells. In most cases, there is no viable alternative. Thus, some form of restraint is intrinsic to almost all neuroscience research done on awake, behaving preparations.

The *NIH Guide* treats restraint explicitly and permits the elements of restraint as practiced for scientific reasons in experiments on awake behaving preparations. As long as the requirements of the *NIH Guide* are met, restraint for a normal working day should be treated as a routine experimental technique both for primates and for other mammalian species. Overnight restraint brings a number of additional problems and requires closer scrutiny and special consideration by the IACUC.

Post-surgical restraint. In the period immediately following surgery, clinical considerations may make it appropriate to maintain the animal in restraint for a period of time. Experience has demonstrated that post-operative restraint can be the most appropriate procedure for a number of reasons, including but not limited to:

- (1) keeping the animal still and preventing it from injuring itself in the acute post-surgical recovery phase;
- (2) minimizing the risk of wound contamination and protecting the wound site during healing by limiting the animal's access to the surgical site; or
- (3) protecting an implant, such as a chronic, indwelling cardiac catheter, during the acute phase of healing.

In the last case, clinical considerations may dictate prolonged restraint. Restraint should also be allowed for clinical reasons outside the normal post-surgical recovery period if the veterinarian and principal investigator decide together that a period of continuous restraint may be the most appropriate method for promoting the healing around an implant. For post-surgical restraint it is acceptable to use the devices described above, but less restrictive devices such as jackets or

tethers should be considered if they are appropriate for the goals of the restraint. The *NIH Guide* is quite explicit on the procedures for monitoring restrained animals, and these procedures clearly apply here.

IV. SAMPLE NEUROSCIENCE RESEARCH PROTOCOLS

The sample research protocols that follow were selected to illustrate each of the six neuroscience research themes presented in section II. It should be noted that the content and format of these examples are *not* meant to correspond to what might appear in an actual protocol submitted to an IACUC for review and approval. For example, these sample protocols generally avoid recommending detailed animal use procedures or specific drug regimens. Instead, they provide typical examples of the animal care and use concerns that these kinds of research protocols frequently present to neuroscientists, IACUC members, and veterinarians and they suggest possible strategies for resolving these concerns to the satisfaction of all concerned.

1. Prolonged Non-Survival Recording Procedures

This example protocol discusses some of the difficulties that arise when it is necessary to make measurements of neuronal activity in animals under systemic paralysis. This situation occurs frequently in visual neuroscience experiments and is not uncommon in some other situations. Several issues arise that require the *NIH Guide* to be interpreted as a flexible document in reviewing protocols of this sort.

The first difficulty is that monitoring and regulating anesthesia and analgesia under paralysis requires special measures, since behavioral indicators of pain and distress are suppressed by systemic paralysis. This problem is compounded by the fact that anesthesia must be carefully regulated to ensure that the effect of the anesthetic on the neurophysiological responses being measured is minimal. Another problem concerns monitoring and maintenance of the animal's physiological state, especially when the proposed experiments are of long duration (recording sessions in excess of 48 hr are common). Finally, issues of asepsis can pose problems, since many aspects of non-survival brain recording experiments are inherently non-sterile and an experimental session may last long enough for infection to develop.

A typical regimen used in experiments of this kind involves a 2-4 hr period of acute surgical preparation, followed immediately by a recording session whose duration varies from a few hours to several days. All surgical procedures are completed before systemic paralysis is induced, so control of anesthesia during the preparatory surgery can be achieved using conventional methods. A variant of this procedure involves performing a separate survival surgery several days prior to recording, during which a pedestal and chamber (used to secure the animal and the recording device, respectively) are implanted on the cranium. With this latter approach, on the day of the non-survival recording session, surgical procedures are minimal (e.g. venipuncture, endotracheal intubation) and they require relatively light anesthesia.

There are no unambiguous methods for ensuring that animals under paralysis are free from pain and distress. Therefore, it is desirable to establish dose ranges and reliable signs of pain and distress for a particular species and situation. This can be achieved by using a "trial" preparation in which the animal is prepared for recording but is not paralyzed, to establish suitable dose ranges. When there is reason to suppose that the effects of anesthetic drugs may vary with time, this trial should be long enough to establish the range of this variation. For drugs whose plasma level can be stably maintained, it should be sufficient to establish stable anesthetic levels over a period of a few hours. This trial can also be used to establish the physiological signs that will be monitored under paralysis to verify that the animal is maintained in a suitable condition. Among the physiological measures that can be useful are EEG, EKG, arterial blood pressure, urine production and pH, end-expiratory PCO₂ and/or blood gas concentration, and general autonomic signs of arousal (e.g., salivation, pupil size). Attention to

many of these measures is also useful for ensuring the maintenance of proper physiological state during recording.

The issues involved in maintaining an experimental animal in good physiological condition during a prolonged recording experiment are similar to those that arise in other situations that require the long-term maintenance of animals in clinical situations. In most cases, these methods do not involve controversial choices.

In experiments involving systemic paralysis, it is of course necessary to provide artificial ventilation. The parameters for this are selected according to the usual techniques. In some cases, gaseous anesthetics like N_2O are included in the gas mixture; in other cases, room air alone is used. It may be helpful to ventilate animals with a 50:50 mixture of N_2O and O_2 (the N_2O is an adjuvant to intravenous anesthetics). Whether or not N_2O is added to the gas mixture, it is useful to hyperinflate the animal hourly in order to prevent the partial collapse of the pulmonary alveoli (atelectasis). It is also helpful to hydrate the inspired gases in order to prevent desiccation of the lungs. The measurement of end-expiratory CO_2 is a simple method of monitoring the adequacy of the artificial respiration.

Typically, an intravenous infusion provides the animal with an osmotically-balanced source of fluid and metabolites. An isotonic lactated Ringer's solution with dextrose is suitable for this purpose and it may be desirable to supplement this with additional potassium and/or amino acids during long experiments. Adequate volume should be infused to make up for inevitable losses through the skin and lungs, as well as to maintain renal function. Proper renal function is particularly important during artificial ventilation, because of the role of the kidneys in correcting pH and osmotic imbalances that may arise after prolonged artificial ventilation. As a rough guide, infusion of 0.5 to 2 times the animal's blood volume every 24 hr seems appropriate.

It is prudent to rearrange the position of a paralyzed animal's limbs and body periodically and to massage its large muscle masses to prevent venous pooling. Heated, circulating water pads are useful for maintaining body temperature. Regular doses of antibiotics, vitamins, and anti-inflammatory agents may also be helpful in maintaining stable conditions and preventing systemic infections. Core body temperature should be monitored routinely and supplemented as necessary with circulating water heating pads.

The need for aseptic technique in these experiments is an issue that is open to interpretation under current guidelines. On the one hand, asepsis might seem to be called for because experiments often extend beyond 24-48 hr. On the other hand, because the animal doesn't survive, the usual rationale for aseptic procedure is absent. Most laboratories do not use aseptic technique for non-survival procedures. However, when a preparatory survival surgical procedure is conducted to implant a pedestal and chamber, it is performed under aseptic conditions. Most implanted devices and their carriers can be disinfected, but because a typical neurophysiological recording laboratory contains many other items that cannot be sterilized, the full application of aseptic technique during a recording session is usually impossible. A frequent approach to solving this problem is to create and maintain a sterile field that contains any openings into major body cavities that are made during the conduct of a surgery in a prolonged non-survival recording session.

2. Survival Anatomical Procedures

This example describes a protocol for injecting neural tracers into the brain of the cat. Experiments of this general type seek to reveal the neuroanatomical projections into and out of known brain areas by injecting these areas with antero- and/or retrogradely transported tracer

substances. In this example, multiple intracortical microinjections of tracers are placed in the occipital neocortex of the cat to study the arrangement of its visual cortical pathways. This approach is used to compare the mature distribution of these connections to that present in young kittens, in an effort to characterize the stages of the normal developmental process.

The general procedure involves a single survival surgery to inject neural tracers into the brain. Following a short (2-4 day) survival period, the injected animal is euthanized and tissue sections from its brain are analyzed using a variety of histological techniques. The pre-surgical monitoring and preparation of the animal and the induction of anesthesia during surgery conform with standard veterinary practice. The level of anesthesia during surgery and the general physiological state of the animal during and after surgery are monitored in routine fashion. Euthanasia is accomplished by barbiturate overdose followed by transcardiac perfusion with fixatives.

The tracer injection surgery is performed within a modified laboratory setting. The primary basis for allowing this exception to *NIH Guide* and AAALAC recommendations is that numerous items of dedicated laboratory equipment are needed during this specialized surgical procedure (e.g., amplifiers, oscilloscopes, audio monitors, micropipette puller, pressure microinjection device, micromanipulators, etc.) and it would be infeasible to move this equipment into the surgery each time the procedure is to be done. Other mitigating factors include:

- (1) the use of a radioactive tracer substance;
- (2) the need to manufacture, fill, and position into the brain several injection micropipettes during the course of a single procedure;
- (3) the relatively short duration of the post-surgical survival period (2-4 days);
- (4) the suitability of the proposed laboratory area for aseptic surgery;
- (5) the infrequency of the procedure (less than once per month, on average);
- (6) verification of the absence of post-surgical infection or other complications in a series of animals from a pilot project.

The surgical procedure is performed in a room that is closed off from outside corridors and other rooms and is thus free of all unnecessary traffic during the procedure. A telephone/intercom and emergency power and lighting are available, as well as all necessary surgical support equipment (respirator, monitors, oxygen, heating pad, etc.). The air supply for this room is filtered to remove dust. The room is free of clutter and capable of being scrubbed with disinfectants. Care is taken to avoid contaminating the room during preparation of the animal for surgery. Similarly, the area in which the surgeon scrubs and gowns is separated from both the surgical and the animal preparation areas. Equipment in the immediate vicinity of the surgery area is draped to avoid contamination of the surgical field during the procedure. Provision is made for scavenging and exhausting waste gases from the anesthesia machine and for meeting all institutional safety requirements regarding the use of a radioactive substance.

Surgery is performed using a two-step procedure. Standard aseptic technique is used to expose the surface of the occipital cerebral cortex. At this point the animal is redraped and the surgeon breaks sterility in order to fill and insert sterile injection micropipettes into the micromanipulator, position the pipettes into the brain, and adjust and activate the pressure injection device. Throughout this period, the surgeon has no direct contact with the wound site or the surgical field. When the injections have been made, the surgeon re-gloves (and re-gowns,

if necessary), the top level of drapes is removed, and the wound is closed using standard aseptic technique. The animal is allowed to regain consciousness and placed in a heated cage for post-surgical observation.

3. Perinatal Procedures

The following are two examples of neuroscience experiments that involve the use of animals during the perinatal period. Both illustrate cases in which part of the procedure poses a challenge to execution of the experiment within the guidelines of the *NIH Guide*. The solutions to these challenges are discussed.

The first example is that of performing fetal neurosurgery for observing development of connections in the brain. The aim of the experiment is to inject a tracer substance into the brain (cat/ferret/monkey) to assess the developmental state of axonal connections. The tracer itself is nontoxic and the general procedure involves Cesarean section and fetal neurosurgery.

In these experiments, two rounds of surgery are usually required. First, a Cesarean section is performed and a small amount of tracer is placed into a particular brain location. A second Cesarean section is performed 24 hours to 2 weeks later to remove the fetus(es) for final analysis, during which the fetal brain is fixed by aldehyde perfusion or rapidly removed for preparation of tissue slices for *in vitro* physiological studies.

Animals are anesthetized in conformity with standard veterinary practice. Anesthesia and the general physiological state of the mother are monitored in routine fashion. However, determining the state of the fetus during and after surgery is not straightforward. The state of anesthesia of the fetus during surgeries is assumed to be adequate if that of the mother is adequate, since the anesthetics chosen for use (inhaled gases) cross the placenta. Adequate monitoring of the physiological condition of the fetus during surgeries and during intervening periods remains a problem, but since the condition of the fetus is intimately related to that of the mother, every effort is made to ensure maternal health and comfort.

Aseptic surgical technique is used throughout all procedures. In general, this is eminently straightforward. However, a potential problem common to many neuroscience experiments is that occasionally it is not possible to sterilize the neuroanatomical tracers, which are used and available only in minute amounts. Experience has shown that in such instances, there is little or no risk of infection and neither fetal nor maternal viability is affected. In any specific case, this can be verified by a variety of approaches, including histological examination of the injection site, making bacterial cultures of samples taken from the amniotic or uterine sacs or peritoneal cavity, and culturing a sample of the tracers that are to be injected.

Due to the nature of the experimental procedures needed for the final analysis (e.g., aldehyde perfusion requiring a fume hood and/or *in vitro* electrophysiology requiring elaborate hardwired recording equipment), the surgical suite is not located within the general surgical facilities of the veterinary staff but rather immediately adjacent to the laboratory of the principal investigator. This location is necessitated because fetal brain tissue must be processed using neurophysiological or neuroanatomical equipment within 1-2 minutes after the fetus is removed at the time of the final Cesarean section.

The procedures described above require significant training in general preparative and surgical techniques and in fetal neurosurgical techniques. This is accomplished in several steps, all of which involve a close collaboration between scientific and veterinary staff.

- (1) All students are required to take a course in general surgical procedures given by the veterinary staff.
- (2) Students first observe, then participate in, the required procedures. At all times at least one member of the surgical team has sufficient experience in all of the techniques used to be able to accomplish the procedure successfully and in conformity with animal care and use concerns.
- (3) Members of the veterinary staff observe the surgeries and make further suggestions regarding updated or more desirable surgical procedures.

The general success of this experimental approach is demonstrated by a fetal mortality rate of less than 25% and a maternal survival rate of almost 100%.

The second example is that of performing neonatal recovery surgery for inducing rearrangement of connections in the brain. The aim of this and similar experiments is to induce nerve cell axons to innervate novel targets in the brain, in order to learn more about the factors that normally regulate the formation of connections in the intact brain.

In these studies, two surgical procedures are done. The first, a survival procedure, is done on the day of birth and involves the removal of a small part of the brain. The second, a non-survival, anatomical or physiological experiment, is done at a later age. The procedure for the acute experiment is similar to that used for non-survival recordings in the visual system of other higher mammals. The following description pertains to the first procedure.

As with fetal animals, a major issue is how to administer anesthesia at a safe level and how to monitor it adequately in neonates. Experience indicates that many anesthetics safe for use in adult animals have very small margins of error in immature animals. For non-primate neonates, such as rats and ferrets, a safer approach is to use hypothermia as an anesthetic. Human clinical studies indicate that an adequate plane of anesthesia can be reached at about 27°C, a temperature at which there is no observable breathing after the animals are cooled to a state of areflexia. Hypothermia has the additional advantage that it constricts blood vessels and reduces bleeding substantially during surgery. Again, due to the small size and delicacy of the animals, monitoring of anesthesia by conventional means is not possible. However, initially the withdrawal reflex, respiration, and temperature can be monitored and temperature can be monitored throughout the procedure. The very high rate of survival following this approach indicates its feasibility. Moreover, during the second phase of the experiment, in which neurophysiological studies are often performed, no adverse effects have been noted on the physiological properties of neurons in the visual pathway of animals that have undergone hypothermia.

4. Inducing Neurological Deficits

This example describes a protocol involving adult cats in which neurological deficits are induced by the researcher. The aims of this general type of experiment are to learn how different parts of the brain function in behavior, to develop animal models for naturally occurring neurological disorders, and to produce treatments designed to lead to the recovery of neurological function.

The general procedure is to produce an initial neurological deficit by removing or destroying a region of the brain and then to test the animal for a loss of behavioral function. After the behavioral symptoms of this initial lesion have stabilized (usually from one to several months following surgery), a second surgery is performed to lesion another region of the brain,

with the goal of alleviating some of the deficits induced by the initial lesion. Following this second surgery the animal's behavioral recovery is assessed until it has stabilized, again usually after several months. Finally, the animal is euthanized with an overdose of barbiturate anesthetic and the brain is anatomically evaluated for the extent of damage. It should be emphasized that the performance of multiple survival surgeries on a single animal is a necessary part of the design of this experiment.

Animals are anesthetized according to standard practice and the attending veterinarian's professional judgment. During surgery, the physiological condition of the animal is monitored and maintained within normal limits. Aseptic technique is maintained during all types of surgery, even those in which the approach to the brain is via an inherently non-sterile surgical field such as the roof of the mouth or the nasal sinuses. In these latter cases, prophylactic antibiotic therapy is initiated prior to surgery, with the choice of antibiotic agent(s) and regimen based upon the recommendation of the attending veterinarian after consideration of the types of bacteria most likely to be encountered. Another potential source of infection stems from the impossibility of sterilizing some of the chemical neurotoxins that are injected into the brain. However, as discussed in the previous example (see 3. Perinatal Procedures), these substances are injected in extremely small amounts and do not cause bacterial infection.

In this protocol, special problems arise involving the post-operative state of the animals. Destruction of some brain regions may result in severe, short-term (up to 72 hours) motor disturbances which, if the animal is not sedated, can lead to discomfort and even inadvertent injury. Thus, animals are sedated and monitored continuously until motor disturbances abate and they are able to eat, drink, void, thermoregulate, and engage in normal behavior. In the rare instances in which these functions are not regained within 72 hours, the principal investigator and the attending veterinarian intervene to ensure the animal's welfare. The most common intervention is fluid replacement to prevent dehydration. Another standard post-operative procedure is to provide the animal with soft, rather than hard, food. This is especially important in animals with intra-oral sutures, which could become dislodged with hard food.

In addition to the clinical post-surgical monitoring mentioned above, it may be necessary from a scientific standpoint for members of the research staff to monitor the post-operative behavioral recovery of lesioned animals for periods lasting up to 3 days. Carrying out this behavioral testing during the immediate post-surgical period requires close cooperation between researchers and veterinary staff. In such cases, animals must be allowed to recover in a specially designed and fully equipped recovery cage that is readily accessible at all times to both researchers and animal care staff. If the behavioral testing involves the use of dedicated items of research equipment, the recovery cage may have to be located adjacent to, or in some cases even within, a laboratory setting. In any case, the attending veterinarian must conduct regular visits to monitor the animal's overall condition and to review records of post-surgical care.

These protocols involve complex neurosurgical procedures and they require extensive training of all personnel who participate in the surgery and/or the post-surgical care of the animals. All training is done in cooperation with the veterinary staff. Personnel take a required general course in veterinary procedures and then undergo further specialized training with the principal investigator. The decision as to when a new investigator is qualified to perform surgery independently is made jointly by the principal investigator and the veterinarian. The surgeon and at least one trained assistant are in the operating room at all times. Adherence to these training procedures and policies ensures an extremely low mortality rate (<3%), and experimental animals that thrive over an extended period of time.

5. Repeated or Prolonged Exposure to Agents or Treatments

This first example describes an experiment in which repeated intracranial injections of drugs are used to discover the function of physiologically identified regions of the brain by reversibly inactivating these regions in awake, behaving cats. Although some of the injected drugs are capable of exerting powerful systemic effects, they are injected in such small amounts that they act only locally. The main problems that this type of experiment raises center around devising implanted devices that are small enough to be carried by the animal between recording sessions without discomfort, maintaining sterility when positioning injection pipettes into the brain, and dealing with the issue of multiple survival surgeries.

In an initial survival surgical procedure, an aluminum crown is attached to the skull and an eye coil is implanted under the scleral conjunctiva. Surgery is performed in a dedicated suite, and anesthesia, post-surgical recovery, and monitoring are routine. Post-surgically, cats undergo behavioral training and during this period they are examined daily and weighed regularly to monitor the effects of the food restriction that is part of the training paradigm. When the training is complete, a stainless steel guide tube is implanted on the skull to guide probes into the brain during the succeeding stages of the experiment. This tube cannot be implanted during the initial surgery because the specific experiment (and thus the nature and site of the implant) depends in part upon the animal's performance during the behavioral training, which can only be assessed with the aluminum crown and the eye coil in place. The implantation procedure is quite minor and it takes only about 30 minutes if neurophysiological recordings are required and 15 minutes if they are not. Although little or no pain is involved, the cat is tranquilized to prevent struggling during the procedure. The head is restrained by the aluminum crown and no skin incisions are needed. The acrylic cover is disinfected, aseptic technique is used to drill a 3-mm hole through the skull, and the sterile guide tube is lowered into the brain and cemented in place, thereby resealing to the skull.

The animals are alert within a few hours following this second procedure and will work at their behavioral task the next day. Some experiments require electrophysiological recordings to guide implantation of the guide tube during the second surgery. In many of these cases, it may not be possible to perform the procedure in a dedicated surgical suite, and so it must be carried out using aseptic technique in a modified laboratory setting. This is permissible because of:

- (1) the minor nature of the surgery;
- (2) the fact that procedures are specifically designed so as to minimize the risk of infection;
- (3) the use of monitoring procedures that would alert one immediately to adverse consequences for the animal's health;
- (4) the regular neurohistological verification in each animal at the end of the experiment that infections have not been caused by these procedures.

Between training or data collection sessions, a sterile stylus is screwed into the guide tube and a fibreglas cap with a silicone rubber gasket covers the aluminum crown and the acrylic-covered region of the skull. This cap keeps the area of the implant clean, making aseptic changing of probes easier. During sessions, the cat is restrained in a canvas bag and held by the aluminum crown. Sterile solutions are injected through heat-sterilized micropipette probes that are advanced into the brain through the guide tube. The daily placement of these pipettes into the brain is considered a minor surgical procedure and is done in the laboratory with the animal in the behavioral apparatus. After the first guide tube is no longer useful because the targets it

can reach have already been studied, an additional one or two guide tubes can be attached to the implant. Each of these additional minor procedures is carried out using the techniques described above. It is permissible to conduct multiple surgical procedures on each cat because of the scientific goals of the experiment.

The area of skull covered by acrylic eventually deteriorates from the outer surface inward. This deterioration usually does not lead to infections and does not appear to cause discomfort to the animal, but it can cause the implant to loosen. Consequently, this problem must be monitored carefully so that a study can be terminated and the affected animal can be euthanized before its health and well-being are jeopardized. All members of the laboratory are trained directly by the principal investigator in the experimental procedures involved in all aspects of this study. A series of lectures on the use of laboratory animals is available to all members of the institution and graduate students are required to attend the relevant lectures.

The second and third examples are experiments in which neural activity is chronically altered by repeated oral administration of toxic substances or by repeated injection of neuroactive agents into the vitreous humor of the eye. A fourth example involves chronic electrical stimulation of fiber tracts within the central nervous system in awake animals. These experiments will not be described in detail; instead, only those aspects of them that arouse special concerns are noted.

A particular concern with each of these experiments is the potential of the neuroactive agents or treatments themselves to cause pain. Acute pain can be an undesirable side-effect of electrical or chemical stimulation of the nervous system. Such stimulation may affect not only the nerve fibers or cells whose activation is desired, but also nearby somatic sensory afferents or innervated connective tissue, the activation of which produces pain. In unanesthetized animals, such phasic, acute pain is made evident by the responses of the animal, such as vocalization, startle, and escape behavior. An experimenter who monitors the behavior of the animal carefully during delivery of the treatment, particularly after each change in the effective stimulus parameters, can be assured of preventing all but the briefest episodes of such pain. Injection of chemicals to block activity in the central nervous system is unlikely to cause pain as long as the surrounding skin and connective tissues are desensitized.

A second concern in such experiments is that some neuroactive agents are toxic and may compromise the overall health of the animals treated with them. There are a number of powerful neurotoxins (e.g., tetrodotoxin) that have become important tools in neuroscience research. In some experiments, these drugs are applied in doses that would be lethal if they were delivered acutely, but appear to be innocuous in their systemic effects because they are delivered slowly to the site at which their action is desired. An overdose of these toxins can result in death by respiratory paralysis. Other substances, such as those used to block axonal transport (colchicine) or to inactivate some classes of retinal ganglion cells (acrylamide) can have widespread effects on many cells throughout the body. The appropriate response to these unintended effects is regular and careful attention to the overall demeanor, attitude, alertness, and health of the treated animals. If the treated animals are tested behaviorally each day, this can provide a very effective observation period. Especially in young, developing animals, daily observation and at least twice-weekly weighing of treated animals in comparison to untreated littermates is a sensitive indicator of the extent to which the animals are continuing to thrive under the conditions of the experiment. In older animals, monitoring of food or water intake can be a valuable additional sign.

Many of the repeated treatments require repeated application of general anesthetics. An example is the repeated injection (e.g., every 2-3 days for a total of as many as 20 injections) of substances into the vitreous humor of the eye during development of the visual system. In humans such a procedure could be performed using a local anesthetic block. In animals, the

need for stability of the eye during the injection procedure, and the impossibility of adequate mechanical restraint, necessitate the use of a general anesthetic. As a result, questions arise regarding anesthetic safety and recovery and possible long-term effects of repeated exposure to anesthetic agents. The use of an inhalation anesthetic such as halothane, with its rapid onset and more-rapid recovery, solves most of the practical difficulties. Adverse long-term effects of repeated anesthetics are avoided by carefully monitoring the health of animals throughout the course of an experiment.

Many experiments involving repeated treatments like intraocular or intravenous injections do not require the use of a dedicated surgical suite. Since they do not involve opening a major body cavity, these procedures are not considered a form of major survival surgery. Accordingly, they may be performed in a laboratory setting using aseptic techniques that have been demonstrated to be sufficient to prevent incidents of clinical infection. It is important to recognize that sterility may not be feasible for a number of reasons. In many cases, the investigator may have only microscopic quantities of a neuroactive agent to work with, much less than the 200 ul that is wasted in any attempt to sterilize by millipore filtration. In addition, some experiments may require the use of unique or immobile equipment that can not be brought into a conventional surgical suite. The issues to be considering when evaluating a request to perform a survival surgical procedure in a modified laboratory setting are discussed further in section III (see 1. Physical Environment and Asepsis).

6. Awake Behaving Preparations

This example discusses some of the difficulties that arise in preparing and maintaining animals that will participate in experiments while they are awake and, in many cases, performing a behavioral task. In many instances, this class of experiment becomes practical to perform only if the IACUC and the veterinary staff exhibit some flexibility in interpreting the recommendations of the *NIH guide*.

Experiments on awake behaving animals almost always involve training animals to perform a specific behavioral task. This allows the investigator to elicit and monitor the same movement repeatedly, to present sensory stimuli under highly controlled conditions, and to obtain psychophysical discriminations from the animal. In addition, providing animals with a challenging and rewarding behavioral task to perform ensures their cooperation in the experiment, helps eliminate boredom and struggling, and generally facilitates smooth collection of quality data.

There are a variety of methods for training animals and different tasks will be suitable for different experimental goals. In most neuroscience experiments on awake behaving animals, traditional operant conditioning paradigms are used. These paradigms require an animal to perform some act to achieve a desired consequence. The most common consequences used in neuroscience experiments are appetitive or avoidance.

Appetitive consequences often involve the delivery of a small quantity of a desirable solid or liquid food (e.g., pureed or solid pieces of fruit, beef baby food, apple juice, milk, water). In these cases, the goal of the conditioning is to use the food substance as a reward to induce animals to perform an unrelated task. There is no optimal food for a reward -- different species and different individuals within a species prefer different rewards. It is rarely possible to find a food that both provides reliable behavior and serves as a reward for an animal when the animal's access to that food is not otherwise restricted. In most cases, animals are allowed to work to satiation in daily experimental sessions. When a liquid is used as a reward, the behavioral task is often the only time the animal is allowed access to liquids. When a solid is

used, the animal usually is allowed to eat a measured amount of solid food supplement daily. In all cases, weekend supplements should be given to ensure that animals remain healthy.

Avoidance consequences generally involve exposing animals to some form of noxious stimulus (e.g., mild electric shock, bitter tasting food, unpleasant sound). In some experiments, avoidance consequences may be the most appropriate means of training animals to perform a task, because they yield highly reliable behavior with smaller differences between individuals. Accordingly, avoidance consequences generally will require an investigator to use fewer animals, and in addition will be less likely to upset basic metabolic functions, than appetitive consequences.

Investigators must provide a sound rationale for employing either an appetitive or an avoidance consequence in a proposed awake behaving preparation. If access to liquid or solid food is to be restricted, the proposed level of dietary control must be justified and workable monitoring procedures must be described. These procedures can be based on either the literature or an investigator's personal experience and must include criteria used to determine when an unhealthy animal will be removed from a particular conditioning paradigm. The goal of these monitoring procedures is to keep animals in a highly motivated state while at the same time maintaining their health and welfare. As a result, acceptable procedures often will involve monitoring both an animal's performance on the behavioral task and various physiological indices.

No single physiological measurement will always provide a reliable index of an animal's well-being throughout the course of an awake behaving preparation, but regular monitoring of several factors (e.g., food and water intake, weight, urine and feces, fur and skin) usually permits adequate evaluation. Since each animal has different needs for solid and liquid food, flexible monitoring procedures are preferable to rigid prescriptions for how much solid or liquid food animals should receive daily. Investigators and veterinary staff should be aware that animals will often lose weight during the initial phase of a conditioning paradigm. This is because during this initial phase it often will be necessary to restrict an animal's access to all solid or liquid food to those training sessions when it is first learning how to perform a specific task, in order to attract the animal's attention to that task. However, once the basic elements of a task have been learned, animals rewarded with solid food should be brought back gradually to their normal food ration and fluid-rewarded animals should be allowed to work for the levels of fluid they need.

In most experiments on awake behaving preparations, the prolonged nature of the initial training and data collection phases will require multiple survival surgeries. The use of multiple surgeries in these experiments, even surgeries to repair implants, is permitted by the *NIH Guide*, because they are "related components of a research project." A common approach is to start with each animal by conditioning it to the restraint, shaping it to the appetitive or avoidance consequence and teaching it to perform the basic behavioral task. At some point in the training, it becomes necessary to monitor the animal's performance and an initial surgery is performed under aseptic conditions to implant monitoring devices and a receptacle for head restraint. The animal then undergoes further training and additional aseptic surgeries are performed as needed to implant stimulating devices and to expose the dura for stimulation, recording, or localized brain lesions. This stepwise surgical preparation of the animal often makes sense from a veterinary point of view. It subjects the animal to a small number of short and relatively innocuous surgeries, rather than to one prolonged and debilitating surgery. It also maximizes the useful lifetime of implants by placing them in/on the animal only when they are needed. In all cases, the devices implanted on the animal's head are designed so that the skin can heal around them and the devices can be used without causing the animal any pain or distress.

It is often necessary to implant devices in the brain under physiological control. These procedures are commonly conducted in a laboratory setting, because they usually require specialized equipment that either cannot or may not be taken into a dedicated facility for aseptic surgery. If the laboratory can be cleaned and prepared to allow aseptic technique, the full surgery and implant is commonly done as a single survival procedure in the laboratory. If it is impractical to achieve such a modified aseptic procedure in the laboratory, implants can be done in a two-step procedure. The site for the implant is prepared and the calvarium opened in a dedicated surgical facility and a temporary cap is placed over the site of the future implant. The following day, the animal is brought to the laboratory, restrained via the previously implanted hardware for head restraint, and the temporary cap is removed. The device is then implanted into the brain, maintaining asepsis in the area immediately around the site. This two-step procedure makes it possible to implant devices chronically in the brain even in an awake animal.

There is no need to treat all procedures performed on awake behaving preparations as major survival surgeries that must be performed in a facility dedicated to aseptic surgery. Certainly, the long tenure of these animals in the research setting mitigates in favor of exercising maximum precautions to avoid infection. However, the practical problems involved in maintaining implants in a laboratory setting often makes the use of fully aseptic surgical technique unrealistic. As a result, minor surgical procedures such as treating surgical wounds as they heal, cleaning and maintaining implants, and removing the scar tissue that forms over the dura are commonly performed under light anesthesia in a laboratory setting, using aseptic techniques within a local sterile field.

The introduction of microelectrodes and other devices for recording and stimulating in the brain is usually done without anesthesia and does not require full aseptic procedure. The brain itself does not have sensory endings, so the use of these probes does not cause the animal any sensation. There is no consensus about whether the recording and stimulating devices themselves can be sterilized. In many cases, the materials used to manufacture these probes cannot withstand rigorous sterilization procedures. Consequently, many laboratories do not sterilize microelectrodes and apparently do not introduce infections into the brain because microbes do not survive on the materials typically used to fabricate the electrodes. However, whenever possible it makes sense to sterilize devices before they are introduced into the brain.

Perhaps the greatest challenge in the maintenance of awake behaving preparations is the determination of the overall status of the animal. It is important to realize that the animal's overall demeanor and conduct in its cage is a sensitive indicator of its psychological and physical status and the investigator and veterinarian must work together to follow each animal through the progression of the experimental regimen. Finally, while standard clinical tests will reveal serious pathology, the more insidious, gradual deterioration of an animal's status can be recognized and treated only by regular observation and the exercise of professional judgment.

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