

PROTOCOL FOR ANIMAL USE AND CARE

Email to: campusvet@ucdavis.edu

CNPRC

EH&S USE ONLY

PROTOCOL: 10506

EXPIRES: 3/27/04

Investigator		Contact	
Last Name:		Last Name:	
First:		First:	
Middle:		Middle:	
email:		email:	
Department:		Department:	
Phone / Fax:		Phone:	
After hrs. #:		After hrs. #:	

Species (common names):	Number:	Source:
Rhesus macaques (<i>M. mulatta</i>)	74	CNPRC

Project Title: **Functional Organization of the Hippocampal Formation: Development**

Overnight housing location:: CNPRC Day use: CNPRC

Animals will be maintained by: Vivarium Investigator (*If investigator maintained, attach husbandry SOP*)

Procedures: Provide a one or two sentence layman's description of the procedures employed on the animals in this project. This information will help the animal care staff understand any conditions they may encounter while caring for your animals.

Rhesus macaques will be anesthetized and undergo various experimental procedures, including stereotaxically guided lesions and/or neuroanatomical tracer injections into the brain. All animals will also be subject to non-invasive, in vivo, Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) analysis of their brain at various time points prior to euthanasia. The brain will then be processed to allow mapping of the brain connectivity and stained for various neurochemical markers to study the postnatal development of the functional organization of the hippocampal formation.

Special Husbandry Requirements: Describe any special requirements your animals have with respect to food, water, temperature, humidity, light cycles, caging type, bedding, or any other conditions of husbandry.

none

Other instructions for animal care staff: (check applicable entries)

Sick Animals	Dead Animals	Pest Control
<input checked="" type="checkbox"/> Call Investigator	<input checked="" type="checkbox"/> Call Investigator	<input type="checkbox"/> Call Investigator
<input checked="" type="checkbox"/> Clinician to treat	<input type="checkbox"/> Save for Investigator	<input checked="" type="checkbox"/> OK to use pesticides
<input type="checkbox"/> Terminate	<input type="checkbox"/> Bag for disposal	<input type="checkbox"/> No Pesticides in animal area
<input type="checkbox"/> Necropsy	<input checked="" type="checkbox"/> Necropsy	

Hazardous Materials (*only if in the animal room*):

Infectious Agents?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Agent(s):	
Radioisotopes?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Agent(s):	
Chemical Carcinogens?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Agent(s):	
Toxic Chemicals?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Agent(s):	

Funding source:	NIH	Previously approved?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No
Is the project already funded?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	Previous protocol number (if any):	

What Veterinarian or veterinary clinic will provide care for your animals? (check one)

<input type="checkbox"/>	Lab Animal Health Clinic (2-0514)	<input checked="" type="checkbox"/>	California Primate Research Center (2-0447)
<input type="checkbox"/>	VMTH Large Animal Field Service (2-0292)	<input type="checkbox"/>	Another Veterinarian

If you checked "Another Veterinarian", please provide:

Veterinarian:		Address:	
Day phone:			
Emergency phone:		Email:	

If your veterinarian is not affiliated with one of the three service units listed above, please contact the campus veterinarian, 2-2357 (email ptillman@ucdavis.edu) for current information about training and record keeping requirements.

Summary of Procedures:

a) Briefly describe the **overall intent** of the study. Include in your description a statement of your hypothesis, the objectives and significance of the study. Your target audience is a faculty member from a discipline unrelated to yours. Do not use jargon.

The goal of our research program is to characterize the functional organization of the mammalian hippocampal formation, with the aim of understanding the mechanisms by which the human hippocampal formation contributes to normal memory. In this series of experiments, we focus on the postnatal structural development and functional maturation of the hippocampal formation. The development of the hippocampus is of particular interest because of its importance in higher cognitive processes and its vulnerability to pathology in various neurodevelopmental, genetic and neurodegenerative conditions. The proposed studies will provide essential information regarding the postnatal maturation of the functional organization of the primate hippocampal formation. These data will provide a foundation for interpreting studies of the structural and functional maturation of the human hippocampal formation, with implications for studies of normal memory processes, in particular infantile amnesia, as well as neurodevelopmental and genetic disorders, such as autism and Down syndrome. We will investigate the postnatal maturation of the functional organization of the primate hippocampal formation through five complementary experimental approaches.

Specific Aim 1: To characterize the degree of elaboration of the connectivity of the primate hippocampal formation at birth.

We will place discrete injections of anterograde and retrograde neuroanatomical tracers in the entorhinal cortex (the main interface of the hippocampal formation with the neocortex) of newborn monkeys in order to study the organization of the connections constituting the major afferent and efferent pathways of the primate hippocampal formation at birth. These projections have already been studied in the adult primate, and explicit principles of organization have been described. These studies will determine whether these connections are already mature at birth, enabling neocortical-hippocampal interaction in much the same manner as in the adult, or if the connections are immature, resulting in a fundamentally different interaction between the neocortex and the hippocampus.

Specific Aim 2: To conduct stereological analyses of the primate hippocampal formation throughout postnatal development.

We will implement modern design-based stereological techniques to measure the volume, count the number of neurons and characterize neuron morphology in the hippocampal formation throughout postnatal development (i.e., at 1 day, 3 months, 6 months, 9 months, 1 year and 5-10 years of age). In addition, we will employ immunohistochemical techniques to detect intrinsic and extrinsic cell proliferation markers in order to characterize the time and origin of the cells generated postnatally and their possible integration in the hippocampal circuitry. These studies will provide fundamental information about the volumetric and neuronal population changes underlying the structural maturation of the monkey hippocampal formation throughout postnatal development.

Specific Aim 3: To conduct neurochemical analyses of the primate hippocampal formation

throughout postnatal development.

We will implement chemical and immunohistochemical techniques to characterize the degree of maturation of four major neurotransmitter systems (i.e., glutamatergic, GABAergic, cholinergic and serotonergic) in the hippocampal formation throughout postnatal development. In addition, we will investigate the developmental patterns of myelination and of the expression of non-phosphorylated neurofilaments (SMI-32 antibody) and synaptic markers (synaptophysin). These studies will provide fundamental neuroanatomical information about the neurochemical changes underlying the functional maturation of the monkey hippocampal formation throughout postnatal development.

Specific Aim 4: To conduct non-invasive PET/MRI studies of the postnatal functional maturation of the medial temporal lobe structures in normal monkeys and monkeys with early hippocampal damage.

We will implement non-invasive Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) techniques to investigate the normal functional maturation of the primate hippocampal formation in vivo. In addition, we will produce selective ibotenic acid lesions of the CA1 field of the hippocampus in 2-week-old monkeys and investigate, in vivo, the development of compensatory functional mechanisms in other brain areas that might enable the partial recovery of declarative memory function observed following early hippocampal lesions in humans. These studies will also establish a much-needed link between detailed post-mortem neuroanatomical studies in monkeys and non-invasive PET/MRI studies of normal and pathological memory processes in humans.

Specific Aim 5: To investigate the reorganization of the connectivity of the medial temporal lobe structures following early hippocampal damage in primates.

We will place discrete injections of anterograde and retrograde neuroanatomical tracers in the entorhinal cortex of normal monkeys and monkeys that received early selective hippocampal lesions (see Specific Aim 4), when they reach one year of age. This study will evaluate empirically the hypothesis that reorganization of the connectivity of the medial temporal lobe structures might subserve the partial recovery of declarative memory function observed in humans with early hippocampal damage. This experiment will also yield additional information on the organization of normal monkey hippocampal connectivity at one year of age.

b) Procedures employed in this project:

Please check the appropriate boxes if any of these procedures will be employed in your project:

- | | | |
|---|--|--|
| <input type="checkbox"/> Monoclonal Antibody Production ** | <input type="checkbox"/> Food or water restriction | <input type="checkbox"/> Special diets; food or water treatment. |
| <input type="checkbox"/> Polyclonal Antibody Production ** | <input type="checkbox"/> Non-recovery surgical procedures | <input type="checkbox"/> Induced illness, intoxication, or disease |
| <input type="checkbox"/> LD 50 or ID50 studies. | <input checked="" type="checkbox"/> Survival surgical procedures | <input type="checkbox"/> Death as an endpoint (see i below) |
| <input checked="" type="checkbox"/> catheters, blood collection, intubation | <input checked="" type="checkbox"/> Multiple survival surgery | <input type="checkbox"/> Trapping, banding or marking wild animals |
| <input type="checkbox"/> Prolonged restraint. (8 hrs+) | <input type="checkbox"/> Behavioral modification. | <input type="checkbox"/> |
| <input checked="" type="checkbox"/> Fasting prior to a procedure. | <input type="checkbox"/> Aversive conditioning. | <input type="checkbox"/> |

** If this protocol only describes antibody production, you may use the attached antibody production page in lieu of completing section c below.

c) Describe the use of animals in your project in detail, with special reference to any of procedures checked above. Include any physical, chemical or biological agents that may be administered. List each study group, and describe all the specific procedures that will be performed on each animal in each study group. Use terminology that will be understood by individuals outside your field of expertise. (Note: This cell will expand to whatever length you require. You may make this section as long as you wish, but try to be concise. Some projects may require one or two pages.)

Specific Aim 1: Connectivity of the primate hippocampal formation at birth.

Eighteen, 1-7-day-old rhesus monkeys, *Macaca mulatta* (9 males, 9 females), are requested to conduct this project. On the day of the surgery, MRI scans will be performed to define the surgical coordinates for tracer injections into the entorhinal cortex. Monkeys will be anesthetized with ketamine hydrochloride (15 mg/kg i.m.) and medetomidine (30 µg/kg), intubated with a tracheal cannula and placed in an MRI-compatible stereotaxic apparatus (Crist Instruments Co., Damascus, MD, USA). Brain images will be acquired on a General Electric 1.5 Tesla Gyroscan magnet: 1.00 mm thick sections will be taken using a T1-weighted Inversion Recovery Pulse sequence (TR=21, TE=7.9, NEX 3, FOV=8cm, Matrix 256 X 256). The MRI images will be analyzed and a stereotaxic atlas prepared for each individual to determine the coordinates for injection of the neuroanatomical tracers. The animal, remaining in the stereotaxic apparatus, will be placed on a mechanical ventilator where a surgical level of anesthesia will be maintained with a combination of isoflurane (1%) and i.v. infusion of fentanyl (7-10 mg/kg/hr). Using sterile procedures, the skull will be exposed and a small hole will be made at a site appropriate for the injection. Electrophysiological recordings will be performed to confirm the appropriate dorsoventral coordinate for placement of the injection: A tungsten microelectrode will be lowered through the intended injection site and extracellular single- and multi-unit responses recorded along its trajectory. Each monkey will receive a discrete injection of anterograde or retrograde neuroanatomical tracer in four distinct locations within the entorhinal cortex. Two different anterograde tracers (*Phaseolus vulgaris-leucoagglutinin* [Vector; PHAL, 2.5% solution in 0.1M PO₄ buffer, pH 7.4] and biotinylated dextran amine [Molecular Probes; BDA, 10% solution in 0.1M PO₄ buffer, pH 7.4]; and two different retrograde tracers (cholera toxin beta subunit, [Molecular Probes; CTB594 and CTB488, 10% solution in dH₂O, pH 7.4]) will be used. All tracer substances will be iontophoretically dispensed (30-minute injections with 5 µAmp DC pulses; 7 seconds ON, 7 seconds OFF) through glass micropipettes (20 and 30 µm tips). After the last injection, the wound will be sutured and the animal will recover from anesthesia. Analgesics (0.15 mg/kg of oxymorphone given three times daily) will be administered immediately postsurgically and a prophylactic regime of antibiotics (20 mg/kg of Cefazolin, three times daily) will be administered during the first 5 days of the survival period. After a 7-day survival period, animals will be deeply anesthetized with pentobarbital (Nembutal, 50 mg/kg i.v.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains will be postfixed for 6 hours in the same fixative, cryoprotected in 10% and 20% glycerol solutions in 0.1 M phosphate buffer (pH 7.4; for 24 and 72 hours respectively), rapidly frozen in isopentane and stored at -70°C until sectioning. Sections will be cut at 30 µm on a freezing, sliding microtome and processed for the visualization of the tracer substances.

Specific Aims 2 and 3: Stereological and neurochemical analyses.

Twenty-four rhesus monkeys, *Macaca mulatta*, (four neonates [2M, 2F], four 3-month-old [2M, 2F], four 6-month-old [2M, 2F], four 9-month-old [2M, 2F], four 1-year-old [2M, 2F] and four adults [5-10 years old; 2M, 2F]) are requested to conduct this study. The small degree of variability in the stereological measures observed in a preliminary study indicates that four subjects per age group will bring sufficient power to identify the developmental changes that occur between birth and adulthood. All animals will be acquired from the time-mating program established at the CNPRC, so that we will know the ancestry and the exact gestational age of every animal used in our study. Each monkey will be injected with the cell-proliferation marker, 5'-bromo-2-deoxyuridine (BrdU; Boehringer Mannheim, 150 mg/kg i.p. or i.v.) four weeks prior to euthanasia. For the four animals collected at birth (natural birth will provide sufficient age control, no c-section will be performed to control for the exact gestational age at birth), we will inject the mothers with the same concentration of BrdU four weeks prior to the expected delivery date. The injection will take place in the pre-surgical suite at the CNPRC and the animals returned to their cage following the procedure. The interval of four weeks between BrdU injection and euthanasia has been chosen to allow sufficient time for cell differentiation, migration and possible integration into the hippocampal circuitry (et al. 1999). Animals will be deeply anesthetized with pentobarbital (Nembutal, 50 mg/kg i.v.) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains will be postfixed for 6 hours in the same fixative, cryoprotected in 10% and 20% glycerol solutions in 0.1 M phosphate buffer (pH 7.4; for 24 and 72 hours respectively), rapidly frozen in isopentane and stored at -70°C until sectioning.

Sections will be cut at 30 μm on a freezing, sliding microtome. One in 8 series will be processed for Nissl and the other series will be stored in cryoprotectant solution at -70°C until processing for different immunohistochemical markers.

Specific Aim 4: Non-invasive PET/MRI studies of the postnatal development of the medial temporal lobe structures in normal monkeys and monkeys with early hippocampal damage.

We propose to carry out non-invasive PET/MRI imaging studies of the postnatal development of the medial temporal lobe structures in normal monkeys and monkeys that received neonatal hippocampus lesions. In a first cross-sectional study, we will perform in vivo PET/MRI imaging of the brain of the monkeys that will be killed for the stereological and neurochemical studies (Specific Aims 2 and 3). These animals of different, but exactly known, ages (1 day, 3 months, 6 months, 9 months, 1 year and 5-10 years) will be imaged immediately prior to euthanasia. The structural and functional information derived from these in vivo PET/MRI studies will be compared to the detailed post-mortem neuroanatomical information obtained for the same animals. These data will provide a comprehensive and unprecedented normative database of the developing and adult primate hippocampal formation. All imaging procedures will be the same as the ones described below for the longitudinal study.

In a second experiment, we propose to conduct a longitudinal, in vivo, PET/MRI study of the postnatal development of the medial temporal lobe structures in normal monkeys and monkeys that received neonatal hippocampus lesions. Although we will not have detailed stereological and neurochemical data for these animals, the longitudinal aspect of this study will allow precise comparison of the maturation of the medial temporal lobe structures between normal and early hippocampus-lesioned animals.

Twelve newborn rhesus monkeys, *Macaca mulatta*, (6 males, 6 females), are requested to conduct this study. The lesion procedures will follow the experimental procedures previously developed in our laboratory and described in et al. (et al. 2001). Six infant rhesus monkeys (3 males, 3 females) will receive selective hippocampus lesions at 2 weeks of age, and six additional infants (3 males, 3 females) will be subject to a sham surgery. All infants will be returned to their mothers and reared in two social groups in outdoor enclosures to ensure normal development in a semi-naturalistic setting. Each social group will include 3 hippocampus-lesioned infants and their mothers, 3 sham-operated controls and their mothers and an adult male. We have established a rigorous protocol to prepare the infant and the mother prior to surgery in order to guarantee a successful reunification following the surgical procedure. On postnatal day four, eight and eleven, each infant will be temporarily removed to accustom the mother to the separation procedure necessary for surgery. During these separations, the infant's head will be shaved and scrubbed with Betadine and 70% ethanol to mimic the appearance and odor of pre-surgical preparations and habituate the mother to these manipulations. This procedure has resulted in a 100% successful reunion rate for the 30 neonatal surgeries we have performed over the last three years. This protocol was developed in collaboration with the CNPRC veterinary and animal care staff. There was no need for milk expression of the dams during these separations or surgeries. However, if judged necessary by the veterinary staff, trained animal care technician from the CNPRC will perform the procedure.

On the day of the surgery (at 14 days of age), the infant will be anesthetized with ketamine hydrochloride (15 mg/kg i.m.) and medetomidine (30 $\mu\text{g}/\text{kg}$), and placed in an MRI-compatible stereotaxic apparatus (Crist Instruments Co., Inc., Damascus, MD). Its brain will be imaged using a General Electric 1.5 T Gyroscan magnet; 1.0 mm thick sections will be taken using a T1-weighted Inversion Recovery Pulse sequence (TR = 21, TE = 7.9, NEX 3, FOV = 8cm, Matrix, 256 x 256). The MRI images will be evaluated for the location of the hippocampus and a stereotaxic atlas for the subject animal prepared to calculate the coordinates for injection of ibotenic acid. Electrophysiological recordings will be performed to confirm the dorso-ventral location of the CA1 field of the hippocampus: A tungsten microelectrode will be lowered through the hippocampus and extracellular single- and multi-unit responses will be recorded along its trajectory. Bilateral ibotenic acid injections will be made at multiple rostrocaudal locations within the CA1 field of the hippocampus using two 10 μl Hamilton syringes (26-gauge beveled needles). 0.2 μl of ibotenic acid (Biosearch Technologies Inc., 10 mg/ml in 0.1 M phosphate buffered saline) will be injected at each site at a rate of 100nl/min. The total amount of ibotenic acid injected will range from 2.4 to 3.2 μl per animal bilaterally. During surgery a stable level of anesthesia will be maintained with a combination of isoflurane (1%) and intravenous infusion of fentanyl (7-10 $\mu\text{g}/\text{kg}/\text{hr}$). The use of ibotenic acid lesions to create selective CA1 hippocampal field damage has major advantages over whole-brain ischemic procedures. First, ibotenic acid lesion procedures are easier to control than global ischemic events, thus producing more reliable and reproducible lesions of the targeted area (et al. 1999). Second, ibotenic acid selectively kills

neurons at the site of injection, leaving other brain areas intact. In contrast, although subjects who have suffered an ischemic episode have hippocampal damage limited to CA1, they may also exhibit pathology outside the hippocampal formation (et al. 1986). Consequently, the use of selective ibotenic acid lesions guarantees that compensatory mechanisms observed in the medial temporal lobe structures or other more distant brain areas are directly associated with the early selective damage of the CA1 field of the hippocampus.

At 1 month, 3 months, 6 months, 9 months and 1 year of age post-lesion, each infant will undergo MRI and PET imaging procedures. The MRI procedure will be the same as described for the lesion surgery. For the PET images, we will use a primate microPET 4-ring system scanner with a 22 cm animal port (et al. 2001). The microPET P4 provides a resolution in the reconstructed image of approximately 2 mm fixed width half maximum (FWHM) in all directions for an 8.0 cm field of view. Prior to any imaging of subjects, the scanner will be calibrated using a rotating rod source (^{68}Ge) which orbits at the edge of the field of view. Heart rate, blood oxygenation level, respiration rate and blood pressure will be monitored. The subject will be anesthetized with ketamine and a line will be placed into the saphenous vein for injection of a bolus of the radioactive glucose analogue 2-deoxy-2- ^{18}F fluoro-D-glucose (FDG; P.E.T.NET Pharmaceuticals, Sacramento, CA), using up to 1.0 millicurie per kilogram (mCi/kg). After 45 minutes to allow the deposition of FDG in the brain (Mori, Schmidt et al. 1990), the subject will be placed in the PET scanner. A stable level of anesthesia will be maintained with a combination of isoflurane (1%) and intravenous infusion of fentanyl (7-10 $\mu\text{g}/\text{kg}/\text{hr}$). We will use blood sampling to produce an arterial input function, following the standard operating procedures of the CNPRC. 2-deoxy-2- ^{18}F fluoro-D-glucose is commonly used in humans to conduct similar studies, it has a half-life of 110 minutes and is eliminated from the organism in less than 24 hours. The animals will be returned to their mother and maintained in the metabolism room in the main animal wing of the CNPRC for radioactivity monitoring for 24 hours (sufficient time to reach background levels) prior to their return to their home cage.

Specific Aim 5: To investigate the reorganization of the connectivity of the medial temporal lobe structures following early hippocampal damage in primates.

The rearrangement of synaptic connections is potentially the most important biological mechanism underlying recovery of function after brain injury. Mammals, including humans, exhibit remarkable recovery of behavioral function after circumscribed brain injuries, particularly those occurring early in life. Recently, Vargha-Khadem and colleagues reported that early lesions of the hippocampus in humans produce a selective disruption of declarative memory, affecting episodic memory but leaving semantic memory seemingly intact. Although massive neonatal medial temporal lobe lesions have been shown to produce long-term disruptions in distant brain circuitry, restricted hippocampal lesion are likely to induce neuronal reorganization within the medial temporal lobe structures that might enable partial functional recovery capable of subserving semantic (declarative) memory processing.

The animals used for this study will be the same animals requested for Specific Aim 4. Therefore, no additional animal are requested specifically for this project. All procedures for the injection of neuroanatomical tracers, brain acquisition and tissue processing will be identical to those described in Specific Aim 1. We will place discrete injections of neuroanatomical tracers (two anterograde tracers, PHA-L, BDA, and two retrograde tracers, CTB594, CTB488) in the entorhinal cortex of normal monkeys and monkeys that received neonatal selective hippocampal lesions (Specific Aim 4), when they reach one year of age. This study will investigate the degree of reorganization of the connectivity of the medial temporal lobe structures following early selective lesions of the hippocampus. Most importantly, this study will provide direct experimental evaluation of the hypothesis that early selective hippocampal damage induces a reorganization of the connectivity of the adjacent medial temporal cortical structures that might lead to the partial recovery of declarative memory function following early hippocampal lesion in humans.

All experimental procedures have been developed in close collaboration with the veterinary staff at the CNPRC. All procedures, including, anesthesia, blood sampling, post-operative care follow the standard operating procedures established at the CNPRC.

Fasting is only preliminary to surgery to prevent vomiting and aspiration.

d) **Study Groups and Numbers:** Define, in the form of a table, the numbers of animals to be used in each experimental group described above. The table may be presented on a separate page as an attachment to this protocol if you prefer. The Normal format should be three columns: Study Group, Procedure, Number of animals. The number of rows should follow from the number of study groups; **you may add as many rows as you require.** The chart must fully account for the number of animals you intend to use under this protocol. Assign each group to an invasiveness category according to the chart below.

Group	Procedures / Drugs	Number of Animals	Category
I Specific aim 1	MRI, tracer injection surgery * Euthanasia one week after surgery	18 infant rhesus	3
II Specific aims 2&3	BrdU injection four weeks prior to MRI/PET imaging * Euthanasia: immediately following imaging	24 rhesus of various ages 4 neonates ** 4 3-month-old 4 6-month-old 4 9-month-old 4 1-year-old 4 adult (5-10-year-old)	2
III Specific aims 2&3	BrdU injection 4 weeks prior to delivery of experimental neonates ** Returned to the colony after giving birth	4 adult female rhesus	1
IV Specific aims 4&5	Lesion surgery * MRI/PET imaging * At 1 month, 3 months, 6 months, 9 months and 1 year post-lesion Tracer injection surgery * at 1 year of age Euthanasia: one week after tracer surgery	12 neonates rhesus 6 hippocampal lesions 6 sham lesions	3
V Specific aims 4&5	Animals used to provide normal social environment and rearing conditions for the experimental infants Returned to the colony at the end of the experiment (see below)	16 adult rhesus (14F, 2 M)	1
	*Choice of anesthetics at the discretion of the veterinarian from those listed below.		

Categories of invasiveness

Category	Description
1	Little or no discomfort or stress Examples: domestic flocks or herds being maintained in simulated or actual commercial production management systems; the short-term and skillful restraint of animals for purposes of observation or physical examination; blood sampling; injection of material in amounts that will not cause adverse reactions by the following routes: intravenous, subcutaneous, intramuscular, intraperitoneal, or oral.
2	Minor stress or pain of short duration Examples: cannulation or catheterization of blood vessels or body cavities under anesthesia; minor surgical procedures under anesthesia, such as biopsies or laparoscopy; short periods of restraint beyond that required for simple observation or examination, but consistent with minimal distress
3	Moderate to severe distress Examples: major surgical procedures conducted under general anesthesia, with subsequent recovery; prolonged (several hours or more) periods of physical restraint; induction of behavioral stresses such as maternal deprivation
4	Severe pain near, at or above the pain tolerance threshold Examples: exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs, chemicals, or infectious agents at levels that markedly impair physiological systems and which cause death, severe pain, or extreme distress; Surgical experiments which have a high degree of invasiveness.

Further descriptions of these categories are included in the instructions following this document.

e) **Rationale for species and numbers:** How did you determine that 1) the species choice was appropriate and 2) the number of animals in each study groups was the minimum number necessary to achieve sound scientific results?

The ultimate goal of these studies is to understand the structure and function of the human brain. The macaque monkey provides the best available animal model for experimental analysis of primate neuroanatomy. The number of animals used is the minimal number needed to provide convincing and reliable descriptions of neuroanatomical patterns. By using the same animals to pursue several specific aims, we maximize the amount of information generated by the proposed studies, while increasing the reliability and power of analysis by reducing inter-animal variability, which results in a reduction in the number of animals. The use of imaging techniques prior to any neurosurgical procedure has reduced the risk of missed injections to nearly zero, reducing the total number of animals necessary for these studies.

For specific aim 1 (animal group I), 18 animals will provide an adequate number of cases (four injections per animal) to characterize the connectivity of the monkey hippocampal formation at birth. This number is based on our experience in conducting such studies in the adult monkey.

For specific aims 2 and 3 (animal group II), pilot studies have demonstrated that 4 monkeys per age group will bring sufficient power to identify the developmental changes that occur between birth and adulthood. The specific ages (birth, 3 months 6 months, 9 months, 1 year and 5-10 years) have been selected based on preliminary research suggesting developmental changes within the first year of life. The systematic sampling will allow to characterize the age at which the structures of the hippocampal formation become structurally mature.

For specific aims 4 and 5 (animal group IV), 6 animals per group is the minimal number of animal necessary to demonstrate the functional maturation and the structural reorganization of the medial temporal lobe following lesions. This number is based on our experience in conducting lesion studies in adult and infant monkeys.

f) **Surgery:** If the project involves survival surgery, where will the surgery be conducted?

Building:

CNPRC

Room:

CNPRC Surgical Suite

Who will be the surgeon?

and various trained students.

g) **Anesthetics, Analgesics, Tranquilizers, Neuromuscular blocking agents:**

Post procedural analgesics should be given whenever there is possibility of pain or discomfort that is more than slight or momentary. If postoperative analgesics are not to be given, justify the practice under part (i) below.

Provide the following information about any of these drugs that you intend to use in this project.

Species	Drug	Dose (mg/kg)	Route	When and how often will it be given?
Rhesus	Ketamine	10-20 mg/kg	i.m.	MRI/PET, transport, presurgery
	Atropine	0.4mg/kg	s.q.	MRI/PET, transport, surgery
	Medetomidine	30µg/kg	i.m.	MRI/PET, transport, presurgery
	Xylazine	0.5-2 mg/kg	IM	MRI/PET, transport, presurgery
	Isoflurane	1-2.5%	inhalation	Surgery
	Fentanyl	7-10 µg/kg/hr	i.v.	Surgery
	Oxymorphone	0.15 mg/kg	i.m.	Post-operative care (3 times daily)
	Buprenorphine	0.1 mg/kg	i.m.	Post-operative care (3 times daily)

h) **Neuromuscular blocking agents** can conceal inadequate anesthesia and therefore require special justification. If you are using a neuromuscular blocking agent, please complete the following:

Why do you need to use a neuromuscular blocking agent?

N/A

What physiologic parameters are monitored during the procedure to assess adequacy of anesthesia?

N/A

Under what circumstances will incremental doses of anesthetics-analgesics be administered?

N/A

i) **Adverse effects:**

Describe any potential adverse effects of the experiment on the animals (such as pain, discomfort; reduced growth, fever, anemia, neurological deficits; behavioral abnormalities or other clinical symptoms of acute or chronic distress or nutritional deficiency).

There will be postoperative pain associated with the craniotomy. Analgesics (oxymorphone or buprenorphine) will be provided and animals will be monitored daily for complications by the veterinary staff at the CNPRC.

Ibotenic acid: we do not expect any adverse effect with the use of ibotenic acid. We have performed 11 amygdala and 8 hippocampal lesions in neonate infants within the last three years without having any adverse effect.

Long-term anesthesia: we do not expect to see any adverse effect of long-term anesthesia, both infant and adult monkeys recover readily from anesthesia and are typically fully awake within 1 hour following surgery.

This level of radiation is similar to that approved for human PET studies. No adverse effect is expected from the use of 2-deoxy-2-[¹⁸F]fluoro-D-glucose.

How will the signs listed above be ameliorated or alleviated? If signs are not to be alleviated or ameliorated by means of post-operative analgesics or other means, explain why this is necessary.

Anesthetics and analgesics are appropriate, at the discretion of the CNPRC veterinary staff

Note: if any unanticipated adverse effects not described above do occur during the course of the study, a complete description of those effects and the steps taken to mitigate them must be submitted to the committee as an amendment to this protocol.

Is death an endpoint in your experimental procedure? [] Yes [x] No

(Note: "Death as an endpoint" refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation). If death is an endpoint, explain why it is not possible to euthanize the animals at an earlier point in the study. If you can euthanize the animals at an earlier point, describe the clinical signs which will dictate that an animal will be euthanized.

j) Literature search for alternatives and unnecessary duplication:

Federal law specifically requires this section. You are required to conduct a literature search to determine that either 1) there are no alternative methodologies by which to conduct this class/lab, or 2) there are alternative methodologies, but these are not appropriate for your particular class/lab. "Alternative methodologies" refers to reduction, replacement, and refinement (the three R's) of animal use, not just animal replacement. You must also show that this use of animals is not **unnecessarily** duplicative of other studies.

UC Davis provides on-line access to a number of databases that can be used to search for alternatives. Visit

http://trc.ucdavis.edu/jawelsh/Databases_Med_Vet_Researchers.htm (email: jawelsh@ucdavis.edu)

or http://www.vetmed.ucdavis.edu/Animal_Alternatives/main.htm (email: mwood@ucdavis.edu)

What was the date on which you conducted this search?

2/13/03

List the databases searched or other sources consulted (there should be more than one). Include the years covered by the search.

Database Name	Years Covered	Keywords / Search Strategy
PubMed (NLM)	1966 - present	Hippocampus, development
Current Contents	1993 - present	Hippocampus, development
BIOSIS Previews	1985 - present	Hippocampus, development

What were your findings with respect to alternative methodologies?

There are no alternative strategies to determine the connectivity and maturation of the primate brain.

We have reduced the number of animals to the minimum and maximized the amount of information obtained from every single experimental animal by using non-invasive, in vivo, imaging techniques followed by detailed post-mortem analysis of their brains.

Has this study been previously conducted?

Yes No

If the study has been conducted previously, explain why it is scientifically necessary to replicate the experiment.

k) Disposition of animals: At what point in the study, if any, will the animals be euthanized?

Death is not an endpoint. Experimental animals will be killed immediately after PET/MRI imaging or one to two weeks after the neural tracers have been injected in order to allow histological analysis of the brain.

l) Methods of euthanasia: Even if your study does not involve killing the animals, you should show a method that you would use in the event of unanticipated injury or illness. If anesthetic overdose is the method, show the agent, dose, and route.

Species	Method	Drug	Dose (mg/kg)	Route
Rhesus (<i>M. mulatta</i>)	Perfusion	Deep pentobarbital anesthesia	50-100 mg/kg	i.v.

m) Surplus animals: What will you do with any animals not euthanized at the conclusion of the project?

All experimental animals will be euthanized.

The adult animals used to provide normal social rearing conditions for the experimental infant animals will be returned to the colony at the end of the experiment

