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1. **Title page**

A. Type of Grant: Research Training Grant
B. Project title: The role of neurogenesis in the young and old brain in mediating cognitive outcome following anesthesia

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G. Proposed Starting Date: July 2005

2. Biographical sketch

Greg Stratmann

POSITION TITLE
Assistant Professor of Anesthesia

EDUCATION INSTITUTION AND LOCATION, DEGREE, YEAR(s)FIELD OF STUDY
MD, University of Hamburg School of Medicine 1994
PhD, University of Hamburg School of Medicine 1996 Biochemistry

A. Positions

1997-2000	Intern/Resident Anesthesia, University of California San Diego
2000-2001	Chief Resident Anesthesia, University of California San Diego
2001-2002	Fellow Pediatric Cardiac Anesthesia, University of California at San Francisco
2002-2003	Fellow Intraoperative Echocardiography, University of California at San Francisco
2003-	Assistant Professor of Anesthesia, University of California at San Francisco

B. Selected peer-reviewed publications (in chronological order).

Bickler PE, Warner DS, Stratmann G, Schuyler J: gamma-Aminobutyric acid-A receptors contribute to isoflurane neuroprotection in organotypic hippocampal cultures. *Anesthesia and Analgesia* 2003, 97:564-71

C. Research Support

None

3. Budget

FAER Application Budget **Part 2 – Justification**

1. *Justification of costs and identification of departmental and other funding sources or contributions to the project*

   Efforts not covered by the FAER award will be covered by departmental funds.
   The 80% research effort will be directed to the proposed project.

   Greg Stratmann's fringe benefits are 17% of the salary.
   GAEL insurance stands for general auto employee liability insurance and is required by UCSF.

   Animals: 306 rats, 16 months old ($ 130/rat). 306 rats 2 mths old ($ 29/rat)
   Procurement: 16.4% of purchase price
   Shipping: $ 5/rat

   Reagents: We estimate that $ 1500/mth will be spent on reagents, mostly antibodies ranging in price from 124 – 374 / ml

   The fridge is necessary for storage of brain slices until immunocytochemical staining

   Use of the confocal microscope in the core research facility at UCSF requires a training and introductory session that costs roughly $900. The hourly use fee for the confocal microscope is $37. We estimate that 190 hours of confocal microscopy time will be required, 105 in year 1 and 85 in year 2.

   Animal housing and disposal: Housing is $ 0.86/rat/day. The disposal fee is 1.4% of rat purchase price.

2. *If the mentoring funds are not required to support the mentor's research time commitment, please justify how the funds will be used, indicating which funds will be used directly in support of this project.*

   In the first year the entire mentoring funds will be made available for purchase of animals. In year 2 the mentor funds will be used to pay for the salary of a yet-to-be named postdoctoral research fellow.

3. *Describe facilities, equipment, supplies and services that are available for the completion of this project*

   The projects will mostly be performed in the laboratory of the mentor, Dr. Phil Bickler. Occasionally experiments will be performed in the laboratory of Dr. Liu. The core research facilities of UCSF will be used for confocal microscopy. Dr. Bickler's salary will be paid to 60% by NIH grant RO1 GM52212-09 and to 40% by the Department of Anesthesia, UCSF.

3.a *Are any resources (cell lines, transgenic animals, experimental drugs, etc) necessary for successful completion of the project currently not available to you? If so please explain.*

None

4. *Describe briefly other current research projects that involve the applicant and the mentor, and indicate the time commitment and source of funding for each. Indicate the extent of overlap with the present project.*

No overlap with other research projects currently exists nor is any overlap with other projects planned for the term of the grant.
No other project is currently in progress involving the applicant and the mentor.

Dr. Bickler's own project studies the role of small changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in the injury defense mechanism of neurons deprived of oxygen. It further explores changes in $[Ca^{2+}]_i$ as a basis for anesthetic action and neuroprotection, and as a variable influencing age-dependent changes in sensitivity to anoxia or ischemia. As a model for elucidating new insights into neuroprotection, the project makes use of naturally hypoxia-tolerant neurons from freshwater turtles, and from neonatal rat hippocampus.

5. *List all applied for extramural funding by the applicant. Give the budgeted time commitment for each grant and include a copy of the application's budget page(s). State the overlap with the FAER project and the plan if both grants are awarded. If additional grants are submitted prior to the awarding of the FAER grant, this information must be forwarded to the FAER office.*

No other extramural funding has been applied for.

4. Abstract

General anesthesia is associated with a high incidence of cognitive decline in elderly patients. Recent data suggest that the brain can generate new neurons from neural stem cells (neurogenesis). Neurogenesis is important in maintaining cognitive function. Augmentation of neurogenesis causes cognitive improvement and reduction of neurogenesis causes cognitive decline. Cognitive dysfunction following anesthesia has been demonstrated in aging rats. However, in young rats isoflurane improves cognitive function. Our preliminary data show that isoflurane causes increased neurogenesis in young rats. We hypothesize that anesthesia increases cognitive function in young rats by increasing neurogenesis and that anesthesia decreases neurogenesis in old rats causing cognitive decline. To test this hypothesis we will first evaluate neurogenesis following isoflurane or propofol anesthesia in young and old rats by immunocytochemical techniques including BrdU (marker of dividing cells) labeling of neural stem cells in the dentate gyrus of the hippocampus. Second we will assess electrophysiologic function of the synaptic network in hippocampal slices by measuring long term potentiation, the electrophysiologic correlate of learning and memory. Third we will assess neurobehavioral function in vivo.

Collectively, this novel and comprehensive approach, which will include cellular and molecular biology, in vitro hippocampal electrophysiologic function and neurobehavioral function, will permit the study of the role of neurogenesis in anesthesia-induced cognitive improvement in the young and cognitive decline in the elderly.

6. Research Plan

Following anesthesia 10-14 % of elderly patients develop cognitive decline (*1*), the mechanism of which is unknown. The adult human hippocampus generates roughly 10,000 new functional neurons from endogenous neural stem cells per day(*2, 3*), a process which appears critical for hippocampal function(*4*). Hippocampal dysfunction causes cognitive decline(*4*). Suppression of neurogenesis, for example by irradiation or stress, causes neurocognitive dysfunction in mice and rats (*5*). Conversely, stimulation of neurogenesis, for example by voluntary exercise, learning, antidepressants, electroconvulsive therapy and certain growth factors(*5*) improves neurocognitive function in animals. Animal studies have consistently shown long-term neurocognitive deficits in aged rats following general anesthesia(*6*), the mechanism of which is not yet known. However in young rats isoflurane anesthesia improves cognitive function(*7*), the mechanism of which is equally elusive. Our preliminary data show increased neurogenesis following a 4-hour isoflurane anesthetic in young rats. We hypothesize that anesthesia affects neurocognitive function via an effect on neurogenesis. To test this hypothesis we will assess neurogenesis and cognitive outcome in young and old rats following anesthesia with isoflurane or propofol.

a. <u>Specific Aims</u>

We hypothesize that isoflurane causes a differential effect on neurogenesis and functional outcome in young and old rats (increase in young, decrease in old rats).

Aim 1: To characterize the long-term effects of isoflurane on neurogenesis in the dentate gyrus of the hippocampus of young (2 month-old) and old (16 month-old) rats. We hypothesize that isoflurane increases neurogenesis in young rats and decreases neurogenesis in old rats.

Aim 1a: To compare the time course and dose-response curve for the effect of isoflurane on neural stem cell proliferation between young and old rats.

Aim 1b: To compare the dose-response curve for the effect of isoflurane on neuronal differentiation and survival between young and old rats.

Aim 2: To determine the functional significance of the effect of isoflurane on neurogenesis. We hypothesize that isoflurane improves functional outcome in young rats while worsening functional outcome in aged rats.

Aim 2a: To measure and compare long-term potentiation (LTP), the electrophysiologic correlate of learning and memory, at the medial perforant pathway to dentate granule cell synapse at various intervals after exposure to isoflurane in hippocampal slices made from young and old rats.

Aim 2b: To assess and compare long-term neurobehavioral outcome between young and old rats up to 4 months after exposure to isoflurane.

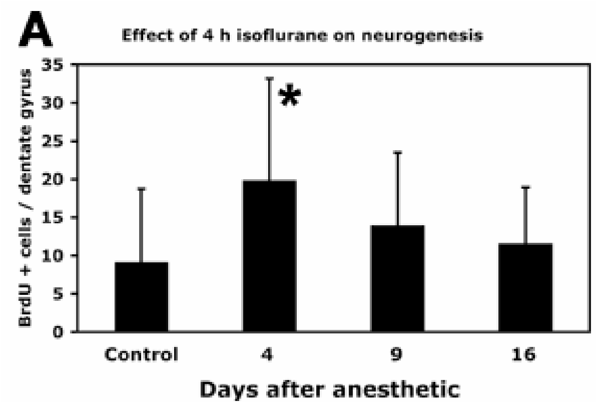
Aim 3: To determine whether the observed effects of isoflurane are unique to isoflurane or secondary to the state of general anesthesia *per se* by comparing neurogenesis, LTP and neurocognition following exposure to isoflurane and propofol. We hypothesize that propofol differs from isoflurane and does not cause a differential effect on neurogenesis and functional outcome in rats of different ages.

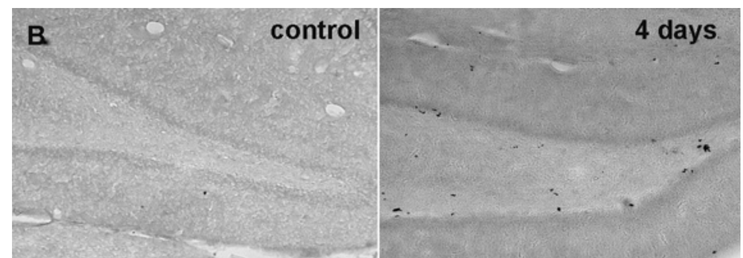
b. Background And Significance

Neural stem cells and neurogenesis in the adult CNS. Postoperative cognitive decline occurs in 10-14% of elderly patients(*1*). The adult brain contains self-renewal and multipotential neural stem cells at the subventricular zone (SVZ) of the lateral ventricles and at the subgranular zone (SGZ) of the dentate gyrus (DG). Proliferating SVZ cells form a migrating chain and differentiate into neurons at the olfactory bulb in animals (*8*). Adult hippocampal progenitor cells divide at the SGZ and form neurons in the granule cell layer. Newly born cells become functionally integrated into the DG and have passive membrane properties, action potentials and functional synaptic inputs similar to those found in mature dentate granule cells(*2, 9, 10*). Most important are findings that newly generated neurons play a significant role in synaptic plasticity(*10, 11*) and that a reduction in the number of these cells impair learning and memory(*12*). Hormones, neurotransmitters, environmental stimuli, and growth factors are among the mediators involved in the regulation of adult hippocampal neurogenesis(*9*). Glutaminergic deafferentiation and NMDA receptor antagonists induce dentate gyrus progenitor proliferation with increased newborn neurons in the granule cell layer(*13*). Adrenal steroids and stress causes the opposite effect(*14*). Enriched environments result in more total granule cell neurons by increasing the survival rate of the progeny of the dividing progenitor cells(*15*). Voluntary wheel running increases both the proliferation of dentate gyrus stem cells and the survival of their progeny(*16, 17*). Cerebral ischemia (*18, 19*), hypoglycemic brain injury(*20*), seizure(*21*), and traumatic brain injury(*22*) also increase dentate gyrus neurogenesis. Impairment of hippocampal neurogenesis may be linked to cognitive decline in aging, Alzheimer's disease and major depression (*23-25*).

The effect of anesthesia on neurogenesis is not known. Anesthesia causes cognitive improvement in young and cognitive decline in elderly rats(*6, 7*), the mechanism of which is also unknown. Decreased neurogenesis in the elderly by anesthesia might explain the postoperative cognitive decline in elderly patients. We propose to address this important issue by studying the effect of isoflurane or propofol anesthesia on neurogenesis and cognitive outcome in young and old rats. We will define the importance of neurogenesis in mediating cognitive improvement following anesthesia in young and cognitive decline following anesthesia old rats. This will provide the scientific foundation on which to base future therapies of postoperative cognitive decline in the elderly.

c. <u>Preliminary Studies</u>: Our preliminary data demonstrate that isoflurane increases neurogenesis in young rats. Data of this experiment demonstrate the feasibility of measuring stem cell proliferation and neuronal differentiation and survival by BrdU-labelling (aim 1b). Then the feasibility of aim 1a (immunohistochemistry, unbiased stereology) will be demonstrated. Although we have not used these techniques to test the effect of anesthesia we have used them in an ischemia model. The depicted data only serve to demonstrate the feasibility of aim 1a and do not express or imply involvement of ischemia as any part of this study. We will also show neurobehavioral outcome data collected in water maze experiments. We have not performed these experiments to test the effect of anesthesia but instead the effect of brain ischemia. The purpose of presenting these data is only to demonstrate the feasibility of aim 2b.





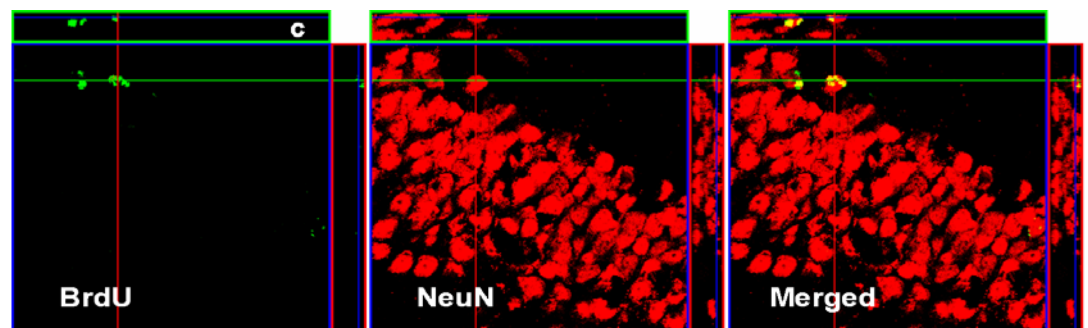


Figure 1: Isoflurane increases neurogenesis in young rats. We performed experiments similar to those proposed in aim 1 to demonstrate the feasibility of aim 1. **A,** When compared to a control group (n=4) a 4 hour general anesthetic with 1.8% (1 minimum alveolar concentration in rats) of isoflurane more than doubles the number of BrdU+ cells (newly formed cells, mostly neurons) in the subgranular zone **(B)** of the hippocampus of young rats (10-week old) 4 days (n=8) after the anesthetic. **C,** Over the following 4 weeks, these BrdU + cells differentiate mostly into mature neurons (the yellow and red cell crossed by both the horizontal and the vertical lines in the right hand panel) as demonstrated by BrdU/NeuN colocalization using confocal microscopy.

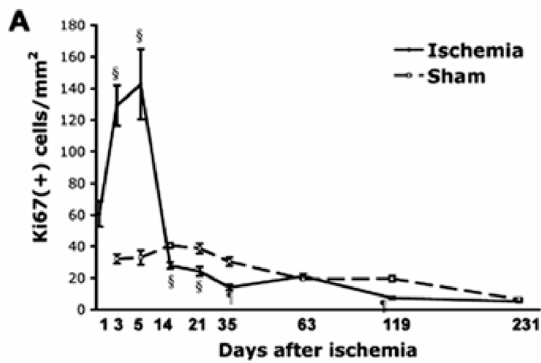
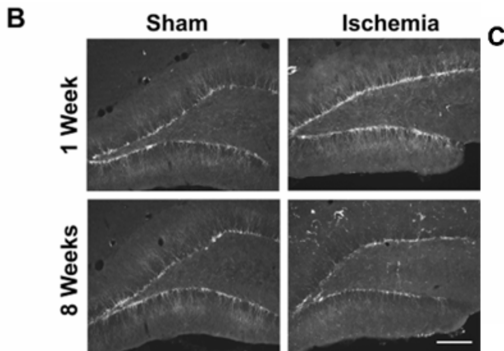
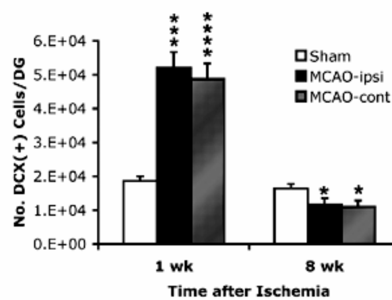


Figure 2. Feasibility of aim 1a (Ki-67, double cortin immunohistochemistry, stereology). We show a transient upregulation of progenitor cell proliferation in the hippocampus followed by a sustained decrease in neurogenesis after brain ischemia. We used an ischemic stroke model only to demonstrate the feasibility for aim 1. **A,** Ki67 immunohistochemistry indicated that cell proliferation in

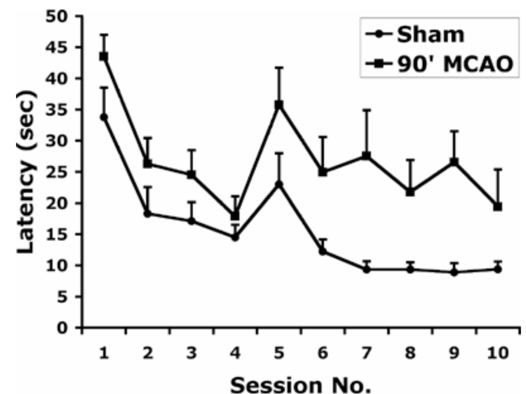




the dentate gyrus subgranular zone (SGZ) peaked around 5 days after 90 min middle cerebral artery occlusion (MCAO) and decreased to baseline 2 weeks after MCAO. Age-matched sham-operated rats showed a gentle decline in proliferative activity over 8 months of time (¶: p<0.005; §: p<0.001). N=8 in each group per time point. **B,** Immunohistochemistry staining of double cortin, representing immature neuroblasts, showing a significant increase in the dentate gyrus 1 wk after MCAO followed by a decrease below baseline level 8 wks after MCAO compared to age-matched sham rats. Scale bar, 250 μm. **C,** Unbiased stereology estimated a significant increase in the amount of double cortin expressing cells 1 wk after MCAO, and a significant decline 8 wks after MCAO both ipsilaterally and contralaterally (*p<0.05; ***p<0.005; ****p<0.001).

Figure 3: Feasibility of neurobehavioral outcome testing. To demonstrate feasibility of aim 2.b, water maze test was performed in rats subjected to 90 min middle cerebral artery occlusion (MCAO) compared to sham-operation. We have not performed these experiments to test the effect of anesthesia and present these data only to demonstrate feasibility of aim 2b.

Fig 3a. Escape latency of 90' MCA occlusion and sham-operated rats to locate the platform in the water maze test. Both groups learned to locate the visible (sessions 1–4) and hidden platform (sessions 5–10), but there was a significant difference in their ability to locate the submerged platform as reflected by the escape latency (p<0.05). (Sham: n=6; MCAO: n=8).



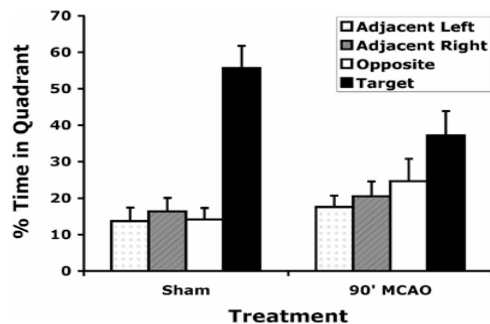


Fig 3b. Performance of 90'-MCA occluded and sham operated rats during the probe trial (hidden platform removed) of the water maze test. After 5 days of water maze training, rats were tested for a single probe trial. Percentages of time spent in the pool quadrants were recorded and calculated. Sham-operated group showed an increased preference ($p < 0.001$, compared with any of the other quadrants by ANOVA and Tukey-Kramer posthoc test) to search the target quadrant where the platform used to be. However, rats with MCA occlusion failed to spend most of their time searching the target quadrant indicating that there was a severe spatial learning retention deficit in the ischemic rats.

d. Experimental design and methods

Aim 1: **To characterize the long-term effects of isoflurane on neurogenesis in the dentate gyrus and subventricular zone (SVZ) of young and old rats**.

Aim1a: To compare the time course and dose-response curve for the effect of isoflurane on neural stem cell proliferation between young and old rats.

Time course of progenitor cell proliferation and neurogenesis in the dentate gyrus and SVZ will be determined in young and old rats from 2 days to 4 months after isoflurane anesthesia.

Experiment 1: 2 and 16 month-old Sprague Dawley rats will either undergo no anesthetic (control) or a 4-hour general anesthetic with isoflurane as described in the detailed methods. Based on our preliminary data rats will be killed at 2, 4, 8, 16, 32, 68 and 136 days after the anesthetic. Progenitor cell proliferation will be assessed by Ki67 immunohistochemistry. Total number of neuroblasts, an indication of neurogenesis, will be quantified by double cortin immunohistochemistry using unbiased stereology. A minimal group size of 6 animals is required to reach statistical power (power analysis: alpha=0.05, beta=0.8, delta= 2.9, effect size d=2.22). A total of 168 rats are required including 2 treatment groups (isoflurane plus control) at 2 age groups for 7 time points.

Experiment 2: The dose-related impact of isoflurane on progenitor cell proliferation and neurogenesis will be determined by varying the duration of isoflurane exposure (30, 60, 120 and 240 min). The rationale for varying the duration of anesthetic rather than the concentration is to provide controlled physiologic variables (hemodynamics and oxygenation) as described in the detailed methods. The effect of dose on neurogenesis will be compared between young and old animals. A total of 96 rats are required including 2 treatment groups at 2 age groups for 4 doses.

Aim1b: To compare the dose-response curve for the effect of isoflurane on neuronal differentiation and survival between young and old rats.

Experiment: The fate and phenotypes of the surviving newborn (BrdU+) cells will be determined and compared between age groups following anesthetics. BrdU, which is incorporated into the DNA of dividing cells, will be administered during the peak of cell proliferation to label a large pool of cells (ascertained from aim1a). Surviving newborn cells in the granule cell layer and the SVZ at 2 and 4 wks after labeling will be quantified by unbiased stereology. Phenotypes will be determined by double immunofluorescence with double cortin, Tuj1, NeuN, NG2 and GFAP using confocal microscopy. A total of 48 rats are required including 2 treatment groups (isoflurane plus control) at 2 age groups for 2 time points.

Possible outcomes, potential problems and alternative strategies: We expect to see a differential effect of anesthesia on progenitor cell proliferation in animals of different age. We anticipate that isoflurane might cause a sustained depression of neurogenesis in the old rats due to a possible increased susceptibility of aged neural stem cells to isoflurane. Isoflurane toxicity has been demonstrated only in neonatal but not in adult animals(*26*). Evidence of progenitor cell death will be assessed by TUNEL and BrdU/Caspase-3 double labeling. It is also possible that age might influence neuronal differentiation and survival among the newborn progenies, with possible decreased neuronal differentiation and survival in old rats.

Others have demonstrated no effect of an anesthetic regimen similar to the one proposed herein on hemodynamics and oxygenation in rats(*7*). Further, in a large human trial even severe hypotension and severe hypoxemia did not correlate with postoperative neurocognitive decline(*1*). Despite this we will measure and control hemodynamic variables and oxygenation in a subset of animals to ensure tight control of these parameters within physiologic ranges as described in the detailed methods.

Aim 2: To determine the functional significance of the effect of isoflurane on neurogenesis by measuring LTP, the electrophysiologic correlate of learning and memory, at various intervals after exposure to isoflurane in the in vitro hippocampal slices made from old and young rats, and by assessing long-term neurobehavioral outcome between young and old rats up to 4 months after exposure to isoflurane.

LTP at the medial perforant path to dentate granule cell synapse will be assessed in the *in vitro* hippocampal slices with a multi-electrode array recording system following exposure to isoflurane. We will also use a battery of established neurobehavioral tests to assess cognitive function to determine if functional outcome following anesthesia correlates with the degree of neurogenesis assessed in aim 1. Age matched controls will be included to rule out age-related cognitive decline.

Aim 2a: Functional outcome in hippocampal slices. Acutely prepared hippocampal slices will be positioned on 64- multi-electrode array recording chips so that a stimulating electrode is in or near the medial perforant pathway. Recordings of potentials will be made from a recording electrode closest

to the molecular layer in the medial dentate gyrus. During testing, slices will be perfused with standard artificial ACSF, bubbled with 95% oxygen and 5% CO_2 and recorded as described later in the detailed method. A minimal group size of 6 is required to reach statistical power (power analysis: alpha=0.05, beta=0.8, delta= 2.9, effect size d=2.22). A total of 48 rats are required including 2 treatment groups (isoflurane, control) in young and old rats at 2 time points (1 and 4 months) following anesthesia.

Aim 2b: Neurobehavioral testing. At 1 and 4 months following anesthesia, rats will be tested by the open field, novel object recognition and water maze tests for various aspects of memory. A minimal group size of 12 is required to reach statistical power (power analysis: alpha=0.05, beta=0.8, delta 2.77, effect size d=1.60**).** A total of 96 rats are required including 2 treatment groups in 2 age groups at 2 time points.

Possible outcomes, potential problems and alternative strategies:

We anticipate that the degree of neurogenesis will be reflected both in the strength of field EPSPs and strength of LTP in the dentate region, both in absolute magnitude and in comparison to the CA1 synapse in the same slices. We also expect that following isoflurane a much worse functional outcome in spatial learning and memory will be observed in aged rats compared to the young rats, reflecting the expected difference in the degree of neurogenesis. It is possible that the functional outcome will be compounded by brain injury at sites other than the neurogenic sites. Although there has been no strong evidence to suggest brain injury induced by isoflurane, we will examine this question rigorously by carefully assessing signs of cell death using updated techniques as mentioned in the aim 1.

Aim 3: To determine whether the observed effects of isoflurane are unique to this drug by comparing isoflurane to propofol. We hypothesize that propofol differs from isoflurane with respect to changes in neurogenesis, LTP and neurocognition.

We will add a propofol group to the experiments pertaining to aim 1 and 2 to allow comparison of the effects of isoflurane and propofol on neurogenesis and functional outcome. Propofol was chosen on the grounds of its wide clinical use and the pharmacologic differences between this agent and isoflurane (see below).

Experiments: Rats will be anesthetized with an intraperitoneal injection of propofol 10 mg/kg. Following insertion of an intravenous (iv) cannula into the internal jugular vein an iv infusion of propofol 0.5 mg/kg/min for young and 0.4 mg/kg/min for old animals will be administered for 30 min, 1, 2 and 4 hour. The peak of stem cell proliferation will be determined as described in aim1a. At the peak of stem cell proliferation BrdU labeling will occur as described in aim 1b to quantify surviving newborn cells in the granule cell layer and the SVZ by unbiased stereology. Phenotypes will be

determined as described in aim 1b. Functional outcome will be assessed in vitro and in vivo as described in aim 2. An additional 204 rats will be required for completion of this aim based on the sample sizes calculated above and the assumption of no difference in the tested endpoints between propofol and the control group.

Possible outcomes, potential problems and alternative strategies:

We anticipate that the effect of propofol on neurogenesis and functional outcome will differ from isoflurane because propofol and isoflurane are distinctly different anesthetic agents. Propofol belongs to the class of intravenous agents, acts via a different GABA receptor subtype than isoflurane(*27*) and, in contrast to isoflurane(*28*), is not also an antagonist at the NMDA receptor. It is possible that the two agents are similar in their effect on neurogenesis and cognitive outcome, which would be a potentially very important finding signifying that the state of general anesthesia per se might affect neurogenesis differentially in the young and old. It is possible that iv and arterial cannula insertion confounds the results. We will perform a sham incision and iv / arterial cannulation in a small number (n=4 - 8) of animals in the isoflurane group of subaim 1. If the results suggest that we have to control for this insult, we will do so by adding another group of animals (isoflurane plus sham iv cannulation) to the relevant experimental steps (dose response at peak of proliferation, functional outcome). We have considered the following limitations to the study: 1) Although it is plausible that anesthesia differentially affects neurogenesis in young and old animals, it is also possible that isoflurane induces a more severe injury in old animals for a given degree of neurogenesis. The balance of injury and neurogenesis might be tipped towards injury in this case. Despite the fact that, to our knowledge, histologic isoflurane neurotoxicity has not been shown in adult animals in vivo, we will investigate this possibility by quantifying cell death for both agents as described in the detailed methods. 2) The proposed study addresses neither the mechanism of anesthesia-related alteration of neurogenesis nor the role of the surgical procedure in post surgical neurocognitive dysfunction, nor possible treatment strategies thereof. Any of the above would be meritious future investigations.

Detailed methods

Rat-anesthesia: Animals will be anesthetized in a plexiglas cylinder by insufflation of 1.8% isoflurane. Old animals will receive 1.6% due to a decreased MAC in old age. Arterial blood pressure will be measured invasively. A 22-gauge catheter will be inserted into the tail artery of anesthetized rats. Arterial blood gas analysis will be performed every 15 minutes. pH, PaO_2 and $PaCO_2$ will be controlled within the physiologic ranges. Pericranial temperature will be measured and controlled within a tight range of 37.5 – 38.5 °C using a heat lamp or a wet rag applied onto the plexiglas tube anesthetic chamber. Propofol anesthesia will be administered via an intravenous (iv) cannula inserted into the internal jugular vein of the rat. Propofol (10mg/kg) will be intermittently administered

intraperitoneally for insertion of the iv cannula. We will determine the propofol infusion rate necessary to achieve and maintain 1 MAC-equivalent over 4 hours by tail clamping experiments as previously described(*29*). The propofol dose so determined will be administered over 4 hours followed by closure of the neck incision.

BrdU labeling and immunohistochemistry BrdU (50 mg/kg; Sigma, St. Louis, MO) will be administered intraperitoneally twice per day for 4 days during the peak of progenitor cell proliferation after anesthesia. Animals will be anesthetized with isoflurane and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer, pH 7.4 (PB). The brains will be removed, post-fixed for 6 hours in 4% PFA/PB, and placed in 40% sucrose overnight. 40-μm coronal sections will be cut on a microtome and stored in PB. For immunocytochemical detection of BrdU-labeled nuclei, DNA will be denatured to expose the antigen. For immunohistochemical detection of NeuN, Ki67, double cortin, NG-2 and GFAP, similar procedures will be applied without the denaturation steps described(*18, 24*).

Double immunofluorescence staining, detection and quantification For phenotype determination, sections will be double labeled with anti-BrdU, and anti-NeuN, or anti-GFAP or anti-NG-2 antibodies sequentially as described(*18, 24*). Fluorescence signals will be detected using a Zeiss LSM 510 confocal imaging system with a sequential scanning mode for Alexa 488 and 594. Stacks of images (1024 x 1024 pixels) from consecutive slices of 0.7 μm in thickness will be obtained by averaging four scans per slice and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA). The number of BrdU/NeuN, BrdU/NG-2, and BrdU/GFAP double-labeled cells will be estimated by multiplying the percentages of co-localization (determined by confocal microscopy) to the total number of BrdU-labeled cells (determined by Zeiss Axioskope 2 plus epifluorescent microscope, Zeiss, New York)(*18, 24*)

Quantification of BrdU+ cells: Unbiased stereology (Stereo Investigator; MicroBrightField, Williston, VT) will be used to assess the volume of the hippocampus at the various time points. Every 10th slice will be counted. Counting frames (15 x 15 x 20 μm) will be placed at the intersection of a matrix (30 x 30 μm for Ki67 and 200 x 200 μm for NeuN and BrdU estimation) randomly superimposed onto the region of interest by the program. Ki67 and BrdU immunoreactive cells will be counted in the subgranular zone and SVZ in the same fashion. The volume of the dentate granule layer will be determined using Cavalieri's principal with a 60 x 60 μm grid matrix(*20, 24*).

Preparation of hippocampal slices: After decapitation during 2% halothane anesthesia, hippocampi are rapidly removed and placed in ice-cold artificial spinal fluid (aCSF). Both hippocampi are sectioned with a tissue chopper or vibratome (Pella, Inc., Saint Louis, MO) into 400 μM transverse

slices, yielding 10-16 slices with intact CA1, CA3 and dentate cell body fields. Following sectioning, slices remain in oxygenated aCSF at room temperature for about an hour as described(*28*).

Measurements of synaptic potentials and long term potentiation. Electrophysiologic recordings will be made with a multielectrode array recording system from Multi Electrode Systems, Inc. (Whitestone, NY). Slices are positioned on 64 microelectrode array chips (ultradense arrays, electrodes spaced 100 microns, 20 micron electrode size) in an interface type recoding chamber perfused with 95% oxygen/CO_2 equilibrated standard artificial CSF at room temperature (22 1°C) at about 6 ml/min. Based on direct visualization of electrode positions in each slice, software is used to select stimulus and recording sites. Multiple stimuli and multiple recording locations can be accessed and recorded simultaneously; i.e. recordings of excitatory postsynaptic potentials and LTP can be recorded at the medial perforant path/dentate granule cell layer and the Schaffer collateral/CA1 neuron synapse without manipulating the slice position or the need to introduce different electrodes. Signals will be amplified and filtered using an active notch filter (high pass 20 Hz, low pass corner frequency 5Khz, Med64 computer and amplifier system). Evoked responses will consist of an orthodromically-evoked, negative population spike superimposed on a positive excitatory post-synaptic potential. Population spike amplitude will be defined as the mean of the amplitudes of its rising and falling phases and population spike latency is the elapsed time from stimulus to the peak of the population spike. These measures as well as population spike slope are all calculated from averaged potentials using software supplied with the Med 64 system.

Behavioral testing:

(**a). Water maze learning** In the water maze test, the ability of the mice to locate a hidden submerged platform in a circular pool (180 cm in diameter, 50 cm deep) filled with warm (24°C) opaque water will be assessed. The rats are trained first to locate a visible platform (cued training, day 1-2), and then to locate a hidden platform (day 3-5); in a subsequent trial the hidden platform is removed (probe trial, day 5). The rats receive two training sessions per day for five consecutive days. Each session consists of three trials with a 10-minute inter-trial interval. The interval between the two daily sessions is 3 hours. Once the rats locate the platform they will be allowed to remain on it for 10 seconds. Time to reach the platform (latency), path length, and swimming speed will be recorded with a Noldus Instruments EthoVision video tracking system set to analyze two samples per second. Because the time required to locate the hidden platform is determined by both path length and swim speed we will, in addition to latency, also analyze these parameters(*24*).

(**b). Open field activity** Open field activity is assessed to determine possible differences in overall activity levels. To quantify open field activity, rats are placed in brightly lit, individual automated infrared photocell activity cages (16 x 16 photocells for measuring horizontal movements, 8 photocells

for measuring rearing) interfaced with a computer (Hamilton & Kinder). The open field is divided in 9 zones, in which the center zones contain 6 x 6 photocells and the side zones 5 x 5 photocells. The data are converted into a zone map file, and the following parameters are calculated per interval: active time (defined as time (to within 1 sec) in which a new beam is broken), distance moved, rearing (raising of both forefeet off the ground and extension of the body) times and events, corner entries, center entries, and percentage of time spent in the center. The open field activity is recorded for three consecutive 10-min intervals after a 1-min adaptation period.

(c). Novel object recognition test Novel object recognition will be measured in an open field (16 x 16 inches) with a video tracking system (Noldus). Rats are individually habituated to the open field for 3 days. During the training session, two novel objects are placed in the open field and the animal is allowed to explore for 15 min. During retention tests (3 and 24 hours after the training session), the animals are placed back in the open field, in which one of the familiar objects is replaced by a novel object, and allowed to explore for 5 min. The time spent exploring each object during the training and retention sessions is recorded. The ratio of the amount of time spent exploring any of the two objects (training session) or the novel one (retention session) over the total time spent exploring both objects, is calculated and used to measure object recognition memory.

Detection of cell death TUNEL will be performed as described previously(*25*) using ApopTag kit (ApopTag, Intergen Company, Purchase, NY). Frozen sections will be fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS), pH 7.4 for 5 minutes followed by quenching endogenous peroxidase activity using 3% hydrogen peroxide in 0.1 mol/L PBS for 5 minutes. Sections will then be incubated with equilibration buffer for 30 seconds followed by TdT enzyme for 1 hour. Anti-digoxigenin peroxidase conjugate will be applied to slides and incubated for 30 minutes. 3,3'-diaminobenzidine-tetrachoride (DAB; Sigma Chemical Co, St. Louis, MO) will be applied for 6 minutes. Sections will be counterstained with 0.5% methyl green for 10 minutes.

Statistical analysis: Data will be expressed as mean±SD. Data will be analyzed by "mixed model regression" using SAS Version 9 (SAS Institute, Cary, NC) Proc MIXED. Post hoc tests will be used to compare the least squares means and Bonferroni method will be used to adjust for multiple comparisons. Within group comparisons will be made using ANOVA and between group comparisons will be made using the student T-test. A p-value of <0.05 is considered significant.

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f. Personal Development

The FAER research training grant is essential to my development into an independent investigator. It would otherwise not be possible to commit a period of 2 years almost entirely to research. Mastering the techniques outlined herein and establishing credibility in the field of neuroscience has been my desire for some time. The FAER award would make this possible. Data generated by this project will serve as preliminary data for an RO1 grant application to the NIH.

7. Mentoring Plan

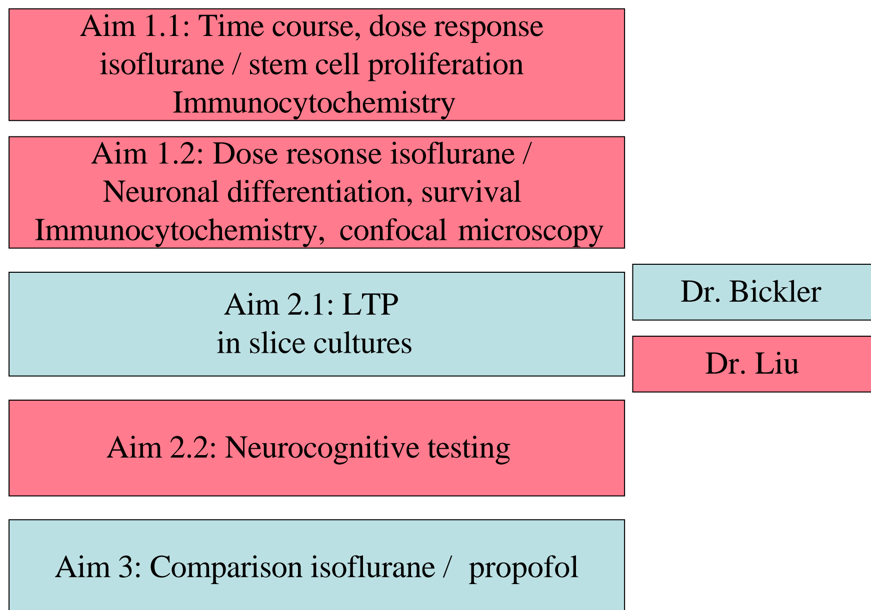
Both mentors are NIH funded, world-class scientists.

Philip Bickler, M.D. Ph.D.: Dr. Bickler has directed a NIH funded laboratory for about 15 years and has mentored over a dozen postdoctoral fellows and junior faculty with interests in neuroscience, anesthesia mechanisms, neuroprotection and comparative respiratory physiology.. Dr. Bickler's interest and expertise in neuroprotection, intracellular signaling mechanisms during anesthesia, and electrophysiologic recording sfrom hippocampal slices provides an essential framework for the proposed work. Mentoring by Dr. Bickler has already resulted in a peer-reviewed publication (see references, biosketch). Dr. Bickler has generously provided funding and space to generate some of the preliminary data presented herein and is a strong supporter of my career in general. He will dedicate 20% of his time to the support of this project.

Jialing Liu, Ph.D.: Dr. Liu is an internationally renowned expert in the field of neural stem cell research. She will dedicate 20% of her research time towards the proposed work. In addition to assisting with the design and planning of the experiments, Dr. Liu has provided and will provide hands-on instruction of animal tissue preparation and immunocytochemical techniques. Her involvement has been invaluable for the generation of the preliminary data presented in this proposal. Additionally Dr. Liu will instruct in and assist with the neurocognitive testing techniques that are well established in her laboratory.

Both mentors hold themselves and others to the highest standards of efficiency and integrity that are essential for a budding scientist like myself. Both will continue to encourage me whenever necessary both in moving forward and in taking a step back to rethink or redo experiments. As established and productive scientists, each mentor will be in a strong position to assist me with data analysis, presentation and publication of the results from these studies.

A synopsis of the involvement of both mentors in the individual specific aims is given.



8. Letters of Commitment and Recommendation

a. Chairman, Dr. Ronal D. Miller, M.D.
b. Mentors, Dr. Philip E. Bickler, M.D., Ph.D. and Dr. Jialing Liu, PhD
c. Two senior faculty members
   Isobel Russell ,M.D. Ph.D. Department of Anesthesia, UCSF
   Piyush Patel, M.D., Department of Anesthesia , UCSD

9. Animal Use Review

An application to the institutional animal care and use committee has been submitted and a revision is currently in review. Approval is expected in short order.

10. Acceptance of Conditions