Experiment Proposal:
Using siRNA to knockdown receptors utilized by Inhaled Anesthetics
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The mechanisms by which inhaled anesthetics produce immobility remain enigmatic. The current dogma is that effects exerted upon various receptor types (e.g. NMDA, Glycine) in the spinal cord sum to produce anesthesia by inhibiting excitatory receptors and exciting inhibitory receptors.

RNA interference (RNAi - explained below) allows one to selectively silence genes, alone or in combination, at the messenger RNA level. We intend to infuse small interfering RNA (siRNA), the functional component of RNAi, into the fluid surrounding the spinal cord. This methodology will allow us to efficiently test the involvement of various receptors in producing immobility during anesthesia.

Spinal cord receptors involved in mediating inhaled anesthetic action turn over with regular frequency (on a roughly twenty-four hour cycle*). RNAi produces detectable activity within twenty four hours that lasts five to seven days. Therefore we can expect our intrathecal siRNA infusions to produce an animal devoid of selected receptor(s) for 4-6 days. Our aim is to test the role of various receptors on inhaled anesthetic action. Specifically, we will examine the minimum alveolar concentration (MAC), the standard measure of inhaled anesthetic potency, in response to the knock-down of selected receptors. The behavioral tests will be complemented with molecular validation of our targets (explained below).

Currently two general methods predominate in testing theories regarding the production of anesthetic immobility: pharmacological perturbation of various receptors; and genetic engineering methods - generally the creation of mouse strains (i.e. knock-in, knock-out of a specific receptor). RNAi can complement these methods by offering several key advantages. RNAi allows access to receptor types that both are and are not pharmacologically accessible. siRNA will be introduced into developmentally competent adult rats, allowing minimization of untoward (e.g. developmental) effects. Moreover, the time involved in generating knock-downs and conducting MAC tests (1-2 weeks) makes it a relatively "high-throughput" method for in vivo genetic testing.

RNAi is a highly specific method of knocking down genes. If the knock-down of a specific receptor directly influences the MAC value, this will constitute direct evidence of that receptor's involvement. Conversely, knockdown of a receptor believed to mediate immobility, along with an unchanged MAC value will: 1) rule out that receptor as indispensable to producing immobility; or 2) strongly suggest redundancy in the physiological program mediating immobility. Furthermore, simultaneous testing of various receptors in combination will test the theory that effects by multiple receptor types sum to produce anesthetic immobility (and if true, is it true for all involved receptors?).

Background:
RNAi is an efficient tool for selectively inhibiting gene expression in a wide range of species. This process of gene silencing occurs through naturally occurring pathways. RNAi is characterized by sequence-specific degradation of mRNA transcripts.

The mechanism by which siRNA works is as follows. dsRNAs are introduced into the cytoplasm and cleaved by the Endonuclease complex, called Dicer, into short 21 base pair duplexes. The siRNA is the actual agent responsible for the mRNA degradation. This occurs when the siRNA enters a protein complex called RISC (RNA induced silencing complex). RISC separates the duplex: one strand, the "passenger strand", is discarded; the other strand, the "guide strand", guides RISC to the target mRNA. The various components of RISC then "silence" the mRNA.

There are two general approaches to utilize RNAi in vivo. The first is by introduction of synthetic dsRNA directly into an animal. Synthetic siRNA does not integrate into the genome, and maintains a transient effect detectable in 24 hours post-transfection and lasting 5-7 days. The second approach is to deliver a specific DNA template into the genome by use of a plasmid or viral vector (and therefore is longer lasting). This results in expression of dsRNA by the animal (specifically in the form of short hairpin or shRNA), and similarly results in mRNA degradation by activating the same RISC pathway. We will use the former method.

Experimental Design:
Our procedure for surgically inserting a catheter into the intrathecal space will follow an earlier protocol (AN077436). Briefly, a 32 gauge polyurethane catheter (Micor Inc., Allison Park, PA) will be placed through the atlantooccipital membrane of adult Spague Dawley rats, and into the intrathecal space. Under sterile conditions, the atlantooccipital membrane is exposed through a 1-2 cm vertical incision posteriorly. A small incision is made in the membrane, sufficient to reveal cerebrospinal fluid. The 32 gauge polyurethane catheter then is advanced acaudally for 4-6 cm. The catheter then is tunnelled through tto the external auditory meatus and sutured in place. The skin over the posterior would is sutured closed. Bupivicaine, up to 0.25mL of 0.25%, is injected into the would site. The catheter is brought through the external auditory meatus to protect it from dislodgment from the rat. the rats will be monitored until recovered from anesthesia and will be returned to their home cages.

siRNA does not cross the blood brain barrier. We will use the cationic lipid carrier, iFECT transfection reagent (Neuromics, Minneapolis Minn., USA), as the transfection vehicle. iFect has been successfully employed in delivering siRNA via intrathecal catheter to the spinal cord. 2ul of siRNA will be complexed with iFECT at a ratio of 1:4 (w:v) in a total volume of 10ul. Multiple siRNA sequences can be mixed.

siRNA sequences will be purchased from an siRNA manufacturing company that has libraries of "off the shelf" siRNA targets (such as Applied Biosystems or Integrated

DNA Technology, IDT). In the case where a target is needed without a readily available sequence, sequences can be designed by these companies. We will use three similar sequences against any gene of interest to determine the optimal sequence specificity (a standard practice).

We will perform several control experiments. A vehicle control will consist of the transfection reagent conjugated to a fluorophore. This will allow us to confirm visually that our vehicle has indeed penetrated the spinal cord (e.g. the ventral horn). A positive control will consist of the vehicle with siRNA targeting a ubiquitously expressed gene (such as HPRT or GAPDH). A negative control (mismatch control) will consist of the vehicle with a random scrambled sequence that targets no known mRNA. Additionally, we will corroborate our MAC results with quantitative RT-PCR to confirm the RNAi has succeeded in knocking down the intended target.

The actual experiment will consist of cohorts of rats (eight to a cohort) divided into control and experimental groups (i.e. infused and non-infused animals, respectively). siRNAs against our gene of interest will be administered on days 1, 2 and 3, to the experimental group by intrathecal infusion at a rate of 2 ul/min over 5 minutes for a total of 10ul. The control group will receive an aCSF infusion on the same schedule. On day 4 a MAC study will be conducted on all 8 rats.