1. Therapeutic Na+ channel blockers: Receptor & Drug Design: The goals of this proposal are (1) to map the topology of the local anesthetic (LA) receptor in voltage-regulated Na+ channels, (2) to design better Na+ channel blockers toward this LA receptor, and (3) to utilize these blockers in vivo as long-acting LAs. The putative receptor for LAs and their quaternary ammonium (QA) derivatives has been assigned within the α-subunit of the voltage-regulated Na+ channel. We plan to mutate various amino acid residues on this putative LA receptor by the site-directed mutagenesis method. Mutants and wildtype muscle Na+ charmers of rat µI clones will be expressed in transiently transfected mammalian cells, and their binding to conventional LAs and newly synthesized QA drugs will be assessed in patch membranes and/or in planar lipid bilayers at the single channel level with batrachotoxin present. The binding contacts to the tertiary amine, the intermediate chain, the phenyl ring, and the putative second hydrophobic group of LA/QA drugs will be charted within the LA receptor. In conjunction, we will continue to design a series of QA derivatives from various LAs; among LAs used are tetracaine, procaine, tetracaine, and etidocaine. These high-affinity Na+ channel blockers, in turn, will be employed to resolve further the topology of the LA receptor. Newly synthesized drugs will be tested first in vitro for their binding affinities in native Na+ channels and subsequently in vivo for their efficacy in rat sciatic nerve block and in rat spinal block. N-butyl tetracaine and its related QA derivatives that block sciatic sensory and motor functions for more than one week will be examined for their local anesthetic and neurolytic characteristics. Neurolytic compounds that destroy nerve fibers but retain the tissue integrity for later nerve regeneration will then be explored as potential ultralong acting local anesthetics. Together, these experiments should give us a clearer view of the LA receptor at the molecular level and likely provide us better Na+ channel blockers with longer duration of block. These high affinity Na+ channel blockers may be beneficial for patients with chronic as well as intractable cancer pain.

2. Molecular Basis of Ligand-Na+ Channel Interactions: The broad objectives of this project are (1) to understand better the molecular basis of state dependent interactions between voltage-gated Na+ channels and local anesthetics (LAs) and (2) to explore the interplay between LAs and the Na+ permeation pathway. This pathway, in part, consists of a selectivity filter, permeant ion binding sites and an inactivation gate. Among LAs included are putative inactivation enhancers, such as benzocaine and tricaine, putative open-channel blockers, such as cocaine, bupivacaine, and quaternary ammonium (QA) compounds, and putative dual blockers such as tetracaine and procaine. In this proposal we plan to examine the structural basis that distinguishes these three distinct LA types. Two separate hypotheses will be tested: first, only one single receptor is present within the Na+ permeation pathway for all three types of LAs and second, the common amino group on the phenyl ring of inactivation enhancers and dual blockers preferentially stabilizes the inactivated state of the Na+ channel. Both whole cell and single channel currents will be measured in order to obtain detailed kinetic information on the dynamic interactions between Na+ channels and LAs. Because ion-ion repulsion within the pore is a common trait for ion permeation, demonstration and further characterization of a knockout phenomenon of LA/QA ions by the inflowing cations through the Na+ selectivity filter will be obtained to provide crucial evidence that the LA binding site is indeed located within the Na+ permeation pathway. Concurrently, we will delineate LA channel interactions at the molecular level. At first, the LA binding toward cloned 41 muscle Na+ channels will be studied with and without subunit -present. Subsequently, the roles of two separate regions of µI Na+ channels, including the internal QA binding site (probably within the pore and S6 regions) and the inactivation-related loop (between domain III and IV), on LA binding affinities will be examined by the macropatch technique in Xenopus oocytes injected with wildtype and µI mutant mRNAs. Together, these studies should provide a clearer understanding of LA Na+ channel interactions as well as the whereabouts of the LA/QA binding site within the Na+ permeation pathway.

3. Activation Gating in Human Heart Na+ Channels: The long-term objective of this project is to understand better how the activation gate of voltage gated Na+ channels works during state transitions. As
a first step, we plan to delimit the whereabouts of this activation gate. We hypothesize that the Na+ channel activation gate is adjacent to the batrachotoxin (BTX) binding site and is situated at the S6 segment-crossing region. Our rationale is based on the fact that BTX affects the activation gate of Na+ channels drastically and that its receptor encompasses multiple S6 segments. Our specific aims are (1) to create, express, and characterize a series of cysteine-substituted mutants at positions 15-28 of S6 segments (~28 residues/S6) in all four homologous domains (DI-D4), (2) to determine the reactivity rate of selected S6 mutants with cysteine-modifying reagents or Cd2+, ions, and (3) to create, express, and characterize additional mutants with residues of different size, hydrophobicity, and polarity at the putative activation gate. Mutants of the human heart α-subunit Na+ channel (hH1) clone will be created and subsequently expressed in human embryonic kidney cells by transient transfection. Mutant Na+ channels and their gating properties will be characterized under whole-cell voltage-clamp conditions. The effects of BTX binding on activation gating will be also measured in these mutants. Selected mutants will be further assayed in inside-out patches before and after application of cysteine-modifying reagents or Cd2+ ions. Repetitive pulses will then be applied to obtain the cysteine reactivity rate during open state transitions. If needed, BTX will be used to measure the cysteine reactivity rate of the open state. These gating and BTX binding profiles along with the cysteine reactivity rate will allow us to infer the location of the activation gate. Subsequent characterizations of this specific region with additional mutations may unravel how the Na+ channel opens upon depolarization at the molecular level. This activation gate also governs the access of various clinical drugs such as local anesthetics, antiarrhythmics, and anticonvulsants to their overlapping receptor site within the Na+ channel inner vestibule. Detailed mapping of the Na+ channel activation gate may provide insights for the design of new therapeutic drugs that target this important region.