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Involvement of Shaker-Like Potassium Channels in Control of Nervous System Hyperexcitability

by

Sharon Louise Smart

A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy
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Approved by _____
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Program Authorized
to Offer Degree Pharmacology

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University of Washington

Abstract

Involvement of Shaker-Like Potassium Channels in Control of Nervous System Hyperexcitability

by Sharon Louise Smart

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Department of Pharmacology

Mutations at the Drosophila *Shaker* locus cause motor hyperexcitability in flies, and allowed the cloning of the first voltage-gated potassium channel gene. Subsequently, at least sixteen different related potassium channel genes have been identified in each of rat, mouse, and human species. The family of voltage-gated potassium channels has been implicated in a variety of functions in the CNS, serving to limit excitability at both cellular and whole nervous system levels. Here, we present an investigation of two specific aspects of potassium channel modulation of nervous system hyperexcitability. First, we addressed the role of the widely-distributed Shaker-like channel Kv1.1 in maintenance of normal neuronal function. We have disrupted the *Kcna1* gene locus in the mouse, thereby deleting the entire open reading frame of the Kv1.1 gene. Homozygous Kv1.1-null animals display frequent spontaneous seizures, alterations in nerve conduction, and changes in excitability of the hippocampal CA3 pyramidal cell population. These observations confirm the importance of this delayed rectifier potassium channel in stabilization of neuronal activity, and the surprisingly limited ability of other potassium channels to compensate for its absence. Second, we addressed the role of voltage-gated potassium channels in astrocytes, a cell type thought to be involved in buffering of extracellular potassium in the CNS to prevent neuronal hyperexcitability. As an initial step, we identified expression of a specific channel, Kv1.6, in mouse cortical astrocytes. Kv1.6 appears to underly a portion of the sustained outward potassium current in these cells. We then used a dominant negative channel subunit under control of the GFAP promoter in an attempt to ablate the function of Kv1.6 and any related Shaker-like channels in astrocytes of transgenic mice. Astrocytes in these transgenic

animals appear to express the dominant negative construct, yet no abnormalities at whole animal or cellular levels have been identified.

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LIST OF ABBREVIATIONS

4-AP	4-aminopyridine
BSA	bovine serum albumin
bp	base pair(s)
°C	temperature in degrees centigrade
cDNA	complementary DNA
cRNA	complementary RNA
CAP	compound action potential
cm	centimeter
CNS	central nervous system
cpm	counts per minute
CTP	cytidine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTX	dendrotoxin-I
EA	episodic ataxia/myokymia
EDTA	ethylenediamine-N,N,N',N-tetraacetic acid
EEG	electroencephalogram
EGTA	ethylene glycol-bis(β-amino-ethyl ether)
ES cells	embryonic stem cells
F1	first filial generation which results from cross of parental individuals
FITC	fluorescein isothiocyanate
GFAP	glial fibrillary acidic protein
HBK	Hepes buffered Kreb's Ringer
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hr	hour(s)
I	current
kb	kilobase(s)
mg	milligram
min	minute(s)
ml	milliliter(s)
mM	millimolar

mmole	millimole(s)
msec	millisecond(s)
mV	millivolt(s)
neo	neomycin
ng	nanogram
nM	nanomolar
ORF	open-reading frame
pA	picoampere(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	negative log of hydrogen ion concentration
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
sec	second(s)
SEM	standard error of the mean
TEA	tetraethylammonium
TK	thymidine kinase
Tris	tris(hydroxymethyl) aminomethane
Tris-HCL	tris(hydroxymethyl)aminomethane hydrochloride
tRNA	transfer ribonucleic acid
µA	microampere(s)
µg	microgram(s)
µl	microliter(s)
µm	micrometer(s)
µM	micromolar
UTP	uridine triphosphate
UV	ultraviolet
V	voltage

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Dr. Philip Schwartzkroin and members of his laboratory, especially Carol Robbins, have been instrumental in the study of epilepsy in the Kv1.1-null mice, and I thank them. In particular, I am grateful to Dr. Schwartzkroin for helping to provide me with a basis in physiological research. I would like to also thank Dr. Albee Messing for his involvement in the original production of the Kv1.1-null animals, and Dr. Chris Clegg for his assistance in generation of transgenic animals. I am indebted to Linda Robinson for her extensive work with and impeccable care of all mice involved in this research.

DEDICATION

This work is dedicated to my parents, Lynne and Wilson Smart, for their constant encouragement and faith in my abilities.

CHAPTER 1: INTRODUCTION

Potassium currents have been observed in a variety of both excitable and non-excitable cell types (Rudy, 1988). Much emphasis has been placed on their role in the nervous system, and a description of an outward potassium current was included in Hodgkin and Huxley's original discussion of the mechanism of action potential conduction in the squid giant axon (Hodgkin and Huxley, 1952; Hille, 1992). Potassium conductances have been observed to display dependence on specific ranges of membrane potential, concentration of intracellular calcium, or binding of ligands to receptors for activation (Hille, 1992). Among those conductances gated by membrane potential, differences exist in voltage dependence of activation and inactivation and kinetics. This diversity of biophysical properties allows modulation of specific neuronal functions by different voltage-gated potassium currents. For example, a rapidly activating, slowly inactivating potassium current with an activation threshold more negative than spike threshold may enable hippocampal pyramidal neurons to integrate separate depolarizing inputs over long time periods (Storm, 1988). Transient outward potassium currents may modulate action potential spacing during repetitive neuronal firing (Connor and Stevens, 1971). Delayed rectifier currents contribute to action potential repolarization (Marsh and Brown, 1991), and are involved in regulation of synaptic terminal neurotransmitter release (Augustine, 1990).

By limiting electrical excitability at the level of the individual neuron, potassium currents may prevent hyperexcitability of the nervous system as a whole, as evidenced by the ability of selective potassium channel blockers to produce seizures in rodents (Rutecki et al., 1990; Bagetta et al., 1992). That behavioral hyperexcitability can be caused by mutation of a potassium channel gene, resulting in abnormal potassium current activity, was first demonstrated by the positional cloning of the *Shaker* locus in Drosophila. Motor hyperexcitability in the *Shaker* fly was associated with prolonged action potential duration (Tanouye et al., 1981) and transmitter release at the neuromuscular junction (Jan et al., 1977). Pharmacological and genetic analyses suggested production of an abnormal potassium channel protein as the underlying cause. The *Shaker* locus was indeed found to encode a family of A-type or transient potassium channel α subunits, defective in Shaker mutants (Papazian et al., 1987; Tempel et al., 1987; Timpe et al., 1988; Iverson et al., 1988; Pongs et al., 1988).

Using Shaker sequences for reduced stringency screening, three additional potassium channel genes subsequently were cloned in Drosophila--*Shab*, *Shal* and *Shaw* (Wei et al., 1990). A large number of homologous mammalian genes have been isolated and characterized into four subfamilies on the basis of sequence homology to the fly genes (Gutman and Chandy, 1993). A nomenclature has been adopted for the mammalian potassium channels genes, such that the Shaker-like genes are designated as Kv1.X, the Shab-like genes as Kv2.X, the Shaw-like genes as Kv3.X, and the Shal-like genes as Kv4.X (Gutman and Chandy, 1993). For the gene loci, the symbol *Kcna*X has been assigned to Shaker-like channel gene loci, and the Shab-like, Shaw-like and Shal-like loci have been assigned *Kcnb*X, *Kcnc*X, and *Kcnd*X respectively.

These genes appear to each encode a single polypeptide, referred to as an alpha (α) subunit, comprised of a hydrophobic core region of six transmembrane alpha-helices (S1-S6) flanked by amino and carboxyl terminal cytoplasmic domains. The region between S5 and S6 transmembrane domains has been implicated in channel pore formation (Yellen et al., 1991), and positively charged residues in the S4 domain are thought to function as a voltage sensor for voltage-dependent activation (Papazian et al., 1991). Four α subunits come together to form the functional channel (Mackinnon, 1991). A second category of potassium channel subunits, the beta (β) subunits, are thought to potentially modify the properties of this functional potassium channel (Rettig et al., 1994). It should be noted that more recent cloning efforts have identified a separate and distinct voltage-gated potassium channel gene family of inwardly rectifying channels (Kubo et al., 1993). The inward rectifier genes encode subunits containing only two transmembrane regions. These channels have optimal conductance at hyperpolarized membrane potentials, where the voltage across the membrane drives potassium into rather than out of the cell, against the potassium concentration gradient.

When expressed in Xenopus oocytes or transformed mammalian cell lines, each voltage-gated potassium channel or combination of coexpressed channels gives rise to currents with distinct biophysical properties. However, despite the availability of detailed electrophysiological characterization of potassium currents in specific cellular locations, and the extensive cloning and *in vitro* study of potassium channel genes and gene products, the assignment of identity between *in vivo* currents and specific molecularly identified channel proteins has not been forthcoming. Several factors contribute to this difficulty in assigning channel to current. The cellular and subcellular distribution of the various channel proteins are unique yet may overlap extensively, and

are unknown as yet for some potassium channels. The tetrameric structure of the functional channel provides additional complexity, allowing formation of either homomeric channels or heteromultimeric channels among α subunits within each of the *Shaker*, *Shab*, *Shaw* and *Shal* subfamilies (Covarrubias et al., 1991). Finally, the potential influence of various posttranslational modifications and other subunit types on channel properties further hinders definitive assignments of identity.

Exemplary are studies on Kv1.1, the first potassium channel clone isolated from mammals (Baumann et al., 1988; Tempel et al., 1988). When expressed alone in either Xenopus oocytes or cultured mammalian cells, Kv1.1 gives rise to a rapidly activating, sustained outward potassium current (Stuhmer et al., 1988; Bosma et al., 1993; Grissmer et al., 1994; Hopkins et al., 1994). When Kv1.1 is coexpressed with α subunits encoded by other members of the Shaker gene subfamily, unique potassium currents arise reflecting contributions from different subunits assembled into heteromultimeric channels (Isacoff et al., 1990; Ruppersberg et al., 1990). When coexpressed with β1 subunits, the Kv1.1 current inactivates rapidly (Rettig et al., 1994). *In vivo*, Kv1.1 has been shown to form heteromultimers with other Shaker-like α subunits (Sheng et al., 1993; Wang et al., 1993) and is likely to associate with β subunits (Scott et al., 1994; Rhodes et al., 1995). Kv1.1 is broadly distributed in the mouse nervous system at sites including the juxtaparanodal regions of myelinated axons and synaptic terminals (Wang et al., 1993; Wang et al., 1994; Mi et al., 1995; Veh et al., 1995). This localization suggests that Kv1.1 may strongly influence action potential repolarization and conduction and neurotransmitter release. However, because of the overlapping distribution of relevant α and β subunits and the lack of pharmacological tools with specificity for individual channels, the actual physiological role of Kv1.1 is unknown. We sought to analyze the role of Kv1.1 in modulation of cellular function, and thereby in the nervous system as a whole, by using homologous recombination technology to generate mice lacking Kv1.1.

In addition to neuronal functions, potassium channels may also play an important role in the other abundant cell type of the central nervous system (CNS), the glial cells (Barres et al., 1990b). The astrocyte, with its location near to cell somata, dendrites and synapses (Kuffler and Nicholls, 1976) is of particular interest. Astrocytes display transient outward, sustained outward, calcium activated, and inwardly rectifying potassium conductances both in culture and upon acute isolation (Bevan et al., 1987; Nowak et al., 1987; Tse et al., 1992). Astrocytes appear to regulate extracellular potassium concentration, with possible involvement of astrocytic voltage-gated

potassium channels (Barres, 1990b; Sontheimer, 1994). Neuronal activity can cause extracellular potassium concentration to rise (Dietzel and Heinemann, 1986), and this elevated extracellular potassium may alter neuronal function, increasing excitability (Rutecki et al., 1985). Therefore, a mechanism for removal of neuronally released potassium would seem important for maintenance of normal brain function.

Original evidence implicating glial cells in potassium buffering came from studies on Necturus optic nerve glia (Orkand et al., 1966). Stimulation of the optic nerve in this preparation caused a slow glial depolarization, the magnitude of which was altered by changes in extracellular potassium concentration as predicted by the Nernst equation for a specifically potassium permeable membrane. These experiments supported the idea that potassium released from axons during action potential propagation accumulates in intercellular clefts and depolarizes the glial membrane. Orkand proposed that potassium current enters glial cells in areas of high extracellular potassium, moves between cells coupled by gap junctions, and exits in areas of lower extracellular potassium concentration. This "spatial buffer" hypothesis has been supported by calculations of the passive electrical properties of glial cells of cat sensorimotor cortex (Trachtenberg and Pollen, 1970), and by demonstration of potassium movement into glial cells in response to applied cortical current (Gardner-Medwin and Nicholson, 1983). Gardner-Medwin related data to a mathematical model of potassium movement to suggest spatial buffering of potassium by glia exceeds extracellular diffusion for large or long-lasting potassium elevation (Gardner-Medwin, 1983). Intracellular use of potassium-sensitive microelectrodes has shown directly the elevation of potassium levels inside cortical glial cells when neuronal activity evokes a rise in extracellular potassium concentration (Ballanyi et al., 1987). Only a portion of this elevation was blocked by ouabain, indicating involvement of mechanisms of potassium flux other than via the sodium-potassium ATPase.

Whether potassium entering glial cells of the CNS is accumulated, or spatially distributed, and what the role of voltage-gated potassium channels might be in this process, remain unclear. However, direct evidence for spatial buffering of potassium has been provided for Mueller cells, a retinal glial cell related to astrocytes (Newman, 1985). Inwardly rectifying potassium channels provide a high potassium conductance localized particularly to the Mueller cell endfeet (Newman, 1993). These channels appear to directionally shunt potassium into and out of the cell preferentially through the endfoot processes (Newman, 1985). Astrocytes express both inward and outward conducting

potassium channels, raising the possibility that a spatial separation of these channels to distinct locations on the astrocyte processes could mediate a similar shunting of potassium. The inward rectifier channels would then allow entrance of potassium into the cell at sites of extracellular elevation, and the outward conducting channels would mediate potassium exit at sites of lower extracellular potassium concentrations. Quite recently, a novel ATP-dependent inward rectifier potassium channel was cloned and appears to have an expression pattern limited to astrocytes and other glial cells (Takumi et al., 1995). This channel might be involved in astrocyte potassium buffering. The role of specific outward potassium channels remains less clear (Sontheimer, 1994). To investigate the role of voltage-gated outward potassium channels in astrocytes, particularly regarding extracellular potassium regulation, we first sought to identify specific outwardly conducting channel(s) in astrocytes from mouse cerebral cortex. Next, we used a dominant negative channel subunit under control of the glial fibrillary acidic protein (GFAP) promoter in an attempt to ablate the function of the identified channel(s) in astrocytes of transgenic mice.

CHAPTER 2: POTASSIUM CHANNEL TARGETED DELETION CAUSES EPILEPSY IN MICE

Introduction

Kv1.1, the first potassium channel clone isolated from mammals, is broadly distributed in the nervous system at sites including the juxtaparanodal regions of myelinated axons and synaptic terminals (Wang et al., 1993; Wang et al., 1994). This localization suggests that Kv1.1 may strongly influence action potential repolarization and conduction, and neurotransmitter release (Black et al., 1990; Augustine, 1990). Pharmacological studies have implicated potassium channels in control of neuronal hyperexcitability, both *in vitro* and *in vivo* , although these studies lack molecular specificity (Rutecki et al., 1990; Bagetta et al., 1992). In order to analyze the role of a single potassium channel in maintenance of normal neuronal functions, we used homologous recombination technology to generate mice lacking Kv1.1.

Experimental Procedures

Generation of Kv1.1-null Mice

For construction of the *Kcna*1 targeting vector, the complementary DNA sequence of Kv1.1 (Tempel et al., 1988) was used to isolate genomic clones from a 129/Sv mouse liver genomic library (Stratagene). A 4.2kb PvuII/HindIII fragment was subcloned into SmaI/HindIII cut pBSSK$^+$ (Stratagene). The neomycin resistance cassette PGKneobpA (Hasty et al., 1991) (provided by Dr. R. Behringer) was inserted into HindIII and XhoI sites downstream of the 5' flanking sequence. To obtain the 3' flanking sequence, a 5.1kb NotI fragment was subcloned into the NotI site of pBSSK$^+$. A portion of the NotI subclone extending from the DraI site at the end of the Kv1.1 ORF to an XbaI site in the 3' polylinker was cloned into the polylinker 5' to the thymidine kinase cassette MC1tkpA (Hasty et al., 1991) (provided by Dr. R. Behringer), and the combined 3' flanking/TK fragment was inserted into the XhoI site 3' to PGKneobpA. The entire Kv1.1 coding region should be deleted by homologous recombination with this construct. The targeting vector (5μg) was linearized at a unique ClaI site and electroporated into AB-1 embryonic stem (ES) cells (gift of Dr. A. Bradley). The ES cells were then subjected to positive-negative selection for eight days in 300μM G418 (Syntex) and

200nM FIAU (Bristol-Myers). Doubly-resistant clones were expanded and analysed by Southern blot hybridization as previously described (Ramirez-Solis et al., 1993), and the predicted mutant EcoRV band was found in six of 109 clones examined. Homologous recombination was confirmed with hybridization of the 3' XbaI/BglII probe to both an 8kb and a 3.8kb XbaI band. Three of these clones were expanded and injected into C57BL/6J blastocysts (U. of Cincinnati Core Facility), and the chimaeric mice generated from these blastocysts were bred to test for germline transmission of the mutant allele. The initial chimaeric mice were mated in three types of crosses (129/SvJ for inbred offspring, and C57BL/6J or Swiss Black for hybrid offspring) to permit evaluation of the Kv1.1-null allele in different genetic backgrounds. Two of the clones (K23 and K25) yielded heterozygous offspring, and these F1 generation mice were intercrossed to produce homozygous Kv1.1-null animals. DNA preparations (10μg) from the tails of all offspring were analyzed by Southern blotting to determine genotype. Zetabind (Cuono) nylon membrane was used for all blots. Procedures were as described (Sambrook et al., 1989).

Ribonuclease Protection

Total RNA was isolated from 7-12 week old mouse brains in the presence of 4M guanidinium isothiocyanate according to the method described by Chomczynski and Sacchi (1987). RNA integrity and purity were checked by formaldehyde gel electrophoresis and ethidium bromide staining. RNA concentration was quantitated by spectrophotometric absorbance measurement. Ribonuclease protection assay of 10μg of total brain RNA was performed as described (Ausubel et al., 1991). Riboprobes were synthesized in the presence of ^{32}P-UTP, and 5 x 10^5 cpm was added to each sample for overnight hybridization at 55°C. As a control for sample loading and RNA quality, each sample was hybridized with a riboprobe specific for mouse cyclophilin (Ambion), in addition to the Kv1.1 or Kv1.2 specific riboprobe. The samples were then digested with RNase A and RNase T1, concentrated, and separated on a denaturing 5% acrylamide sequencing gel. The fixed and dried gel was exposed to film overnight. PhosphorImager analysis of each gel was performed, and density measurements were made of protected fragments to quantitate transcript levels (Fred Hutchinson Cancer Research Center). Three littermate groups were used to obtain the Kv1.1 data, and two littermate groups were used to obtain the Kv1.2 data.

Immunocytochemistry

Cryostat sections of brains from seven week old male mice were used. Procedures for antibody purification, fixation and immunocytochemistry were as described (Wang et al., 1993), except for the use of 0.2% Triton X-100 in the antibody dilution buffer.

Histology

Brains from eight week old male mice were dissected into buffered formalin for use in histological analysis. The brains were embedded in paraffin and 5μm sagittal sections were stained with haematoxylin and eosin (Histology Laboratory, Dept. of Pathology, U. Washington).

Flurothyl-Induced Seizures

Seven to nine week old homozygote Kv1.1-null mice and their heterozygous and wildtype littermates were exposed to the convulsant agent flurothyl (2,2,2-trifluoroethyl ether, Aldrich) as a vapor (Prichard et al., 1969). Mice were placed individually into an air tight plexiglass chamber. Liquid flurothyl was administered by an infusion pump at a constant rate of 40μl/minute through the chamber lid onto filter paper from which it vaporized. The time from start of flurothyl infusion to the onset of a tonic-clonic seizure was taken as seizure latency. Animals were removed into fresh air immediately following seizure onset. Littermates were tested within the same experimental session. The testing chamber was aerated and cleaned between each animal. The order of testing was random relative to genotype. Each animal was exposed to flurothyl only once.

Electroencephalography (EEG)

EEGs were recorded from mice at nine weeks of age, using littermates of each genotype. Under a dissecting microscope, two burr holes were drilled in the cranium of animals under methoxyflurane anesthesia. One hole was located over each cerebral hemisphere halfway between bregma and lambda, and 1.0mm lateral to the sagittal suture. An eletrode pair was then implanted epidurally and fixed to the cranium with dental acrylic (Jensen et al., 1991). A reference electrode was placed subdermally 8-10mm anterior to the recording electrodes, and a ground electrode was positioned subdermally 8-10mm posterior to the recording electrodes. The Telefactor DEEG/Video

Monitoring System was used to record referential EEGs and simultaneous video images of the mouse's behavior.

Compound Action Potential (CAP) Measurement

Sciatic nerves were obtained from two to four month old homozygous Kv1.1-null and age-matched control heterozygous and wildtype mice. Monophasic compound action potentials were recorded from sciatic nerve trunks using standard methods (Kocsis et al., 1983). Briefly, desheathed sciatic nerves were placed across two pairs of electrodes with an intervening pool of perfusing oxygenated saline solution that allowed for a short conduction distance of about 0.5 cm. One pair of electrodes was used for stimulation, while the other pair was used for recording. The nerve segments overlying the electrodes were covered with Vaseline. In addition, the nerve was crushed between the recording electrodes to allow monophasic recording of compound action potentials. Supramaximal and brief (0.01msec) stimuli were used. Sciatic nerves in the recording chamber were continuously perfused with a Kreb's solution that contained (mM): NaCl 130.0; $NaHCO_3$ 20.0; KCl 3.0; KH_2PO_4 1.2; $CaCl_2$ 2.4; $MgSO_4$ 1.3; HEPES 3.0; and glucos, 10.0; and was continuously oxygenated with 95% O_2, 5% CO_2 (pH 7.4). Temperature was maintained at either 22°C or 37°C. Different sets of nerves were tested at different temperatures.

CA3 Field Potential Measurement

Adult (8-12 weeks old) homozgous Kv1.1-null mice and wildtype littermate controls were sacrificed by decapitation, and their brains quickly removed. Horizontal hippocampal slices 400μm thick were cut, and maintained in oxygenated (95% O_2-5% CO_2) artificial cerebrospinal fluid (aCSF) with the following composition (in mM): NaCl 124.0, KCl 3.0, NaH_2PO_4 1.25, $CaCl_2$ 2.0, $MgSO_4$ 2.0, $NaHCO_3$ 26.0. Individual slices were transferred to an interface chamber and perfused with oxygenated aCSF, at a temperature of 33-35°C. Each slice was perfused with either standard aCSF, or with aCSF to which KCl had been added to give a final concentration of 6mM KCl, without correction for changes in osmolality.

Extracellular recordings were made using 2-10MΩ microelectrodes filled with 2M NaCl. The recording electrode was placed in the CA3b-c subfield. Spontaneous activity was recorded on videotape. A bipolar stimulating electrode was used to deliver

stimuli to the fimbria. The resultant population spike was measured by its amplitude from baseline. Single pulse stimulations were delivered at various intensities at 0.1Hz. Paired-pulse stimulations (at 0.1Hz) were given at interpulse intervals (IPIs) of 3, 4, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 msec using twice the threshold stimulus intensity. The ratio of the amplitude of the population spike evoked by the second pulse (P2) to the amplitude of the population spike evoked by the first pulse (P1) was calculated. Data collection was done using pClamp software (Axon Instruments).

Results

Production of Kv1.1-Null Mice

Embryonic stem (ES) cells were electroporated with a targeting vector containing genomic DNA sequences from regions flanking the Kv1.1 open reading frame (Figure 1A). On homologous recombination, this construct disrupts the *Kcna*1 gene locus, deleting the entire Kv1.1 coding region. Targeted ES cell clones were identified by Southern blot analysis using appropriate probes (Figure 1A, bottom). Independent mouse lines were produced from two ES cell clones and heterozygote intercrosses were used to provide homozygous Kv1.1-null offspring (Figure 1B). Chi squared analysis of the genotypes of 182 offspring of heterozygote matings indicates a good fit between the observed genotype assortment--45 wildtype, 96 heterozygous and 41 homozygous null pups-- and that expected from Mendelian theory, suggesting that disruption of the *Kcna*1 gene is not embryonic lethal (Hassard, 1991). Using a ribonuclease protection assay specific for Kv1.1 RNA, no Kv1.1 transcript was detected in the brains of homozygous Kv1.1-null mice and an approximately 50% reduction of Kv1.1 transcript was seen in brains of heterozygous littermates (Figure 2A and C). A ribonuclease protection assay specific for the closely related Kv1.2 channel showed apparently normal levels of Kv1.2 transcript in brains from both homozygous and heterozygous Kv1.1-null mice, suggesting a lack of compensation by Kv1.2 for the loss of Kv1.1, at the level of transcription (Figure 2B and C). Brains from null mutant animals appear grossly normal in form and structure. Histological analysis shows normal cortical, cerebellar and hippocampal architecture (Figure 3A). Immunocytochemistry with channel-specific antibodies confirmed the absence of the Kv1.1 channel protein, but the apparently normal expression and distribution of the closely related Shaker-like potassium channel Kv1.2 (Figure 3B and C).

Kv1.1-Null Mice Display Seizure Propensity and Increased Mortality

At two to three weeks of age, homozygous Kv1.1-null mice begin to display episodic eye blinking, twitching of vibrissiae, forelimb paddling, arrested motion and a hyperstartle response. Incoordination or overt ataxia are not apparent. Approximately 50% of the Kv1.1-null mice die suddenly between two and four weeks of age. Several animals have been observed having apparent generalized seizures of variable duration preceding death. Surviving homozygotes continue to display spontaneous seizure behavior. In seven to nine week old Kv1.1-null animals, a significantly reduced threshold to generalized seizure is found with exposure to the volatile convulsant flurothyl (Figure 4). Homozygotes seize upon flurothyl exposure with a mean latency of 1.48 minutes (n=7), a 60% reduction from the 3.72 minute mean seizure latency (n=18) of wildtype littermates. Heterozygous Kv1.1-null mice seize upon flurothyl exposure with a mean latency of 3.39 minutes, a significant reduction from wildtype.

Electroencephalographic Characterization of Spontaneous Seizures

Spontaneous seizures, a defining feature of epilepsy, occur in young adult homozygous Kv1.1-null mice but were never observed in their wildtype or heterozygous littermates. We implanted epidural electrodes to obtain continuous electroencephalographic (EEG) and simultaneous video monitoring [experiments done by Carol Robbins]. A variety of ictal EEG patterns and behaviors were observed. Obvious seizure behavior was always accompanied by an ictal EEG pattern. However, in some instances an ictal EEG pattern was observed in the absence of obvious behavioral changes, perhaps reflecting either cortical discharge not generalizing to motor output regions or the occurrence of only very subtle seizure behaviours. Ictal episodes often began with a sudden voltage depression followed by either well organized high amplitude spiking or irregular rhythmic spiking. Paroxysmal discharges of polyspikes, burst suppression patterns and runs of rapid spikes were also observed. Ictal behaviors included circling, facial clonus, rearing and falling, bilateral and alternating fore- and/or hindlimb clonus, and generalized tonic and/or clonic episodes. Seizures typically lasted from 20 seconds to 2 minutes. However, one animal was documented to remain in status epilepticus for over 3 hours. Selected EEG traces recorded during a motor seizure from one homozygous animal are shown in Figure 5D. Interictal spiking recorded from the

same mouse is displayed in Figure 5C. Representative wildtype and heterozygote traces are shown for comparison (Figure 5A and 5B, respectively). All flurothyl and EEG data reported here were obtained from Swiss Black/129 hybrid animals. Similar behavioral phenotypes, including motor seizures and increased mortality, have been seen in homozygous Kv1.1-null mice with other genetic backgrounds (129/Sv inbred and C57BL/6/129 hybrid).

Altered Compound Action Potentials in Sciatic Nerve

In humans, missense mutations in the *Kcna*1 gene are associated with episodic ataxia/myokymia, a disorder in which affected individuals display symptoms in peripheral musculature (myokymia) thought to arise from impulse generation within peripheral nerve (Browne et al., 1994; Brunt and van Weerden, 1990). In the peripheral nervous system of rodents, Kv1.1 is localized to juxtaparanodal regions of sciatic nerve (Mi et al., 1995; Wang et al., 1995). Therefore, we searched for possible effects of the absence of Kv1.1 on the excitability of sciatic nerve [experiments done in collaboration with Dr. S.Y. Chiu]. Compared to heterozygous and wildtype littermates, compound action potentials from Kv1.1-null sciatic nerves exhibited a subtle, prolonged after-depolarization during the final 10% of the repolarization phase (Figure 6A and 6B). This after-depolarization may represent repetitive firing at the individual fiber level following a single stimulation, as seen with application of 4-AP to young sciatic nerve (Chiu and Ritchie, 1981; Kocsis et al., 1983). The small size of the after-depolarization suggests that only a small fraction of fibers may be affected by the elimination of Kv1.1, perhaps due to the presence of the colocalizing channel Kv1.2 (Wang et al., 1993).

A paired-pulse protocol was used to assess any difference in the refractory period of the homozygous Kv1.1-null sciatic nerve. Two identical supramaximal stimuli were applied at various interstimulus intervals, and the peak amplitude of the second response relative to the first response was plotted as a function of the interstimulus interval (Figure 6C). The homozygote curve is shifted to the right of the control curve indicating an increase in the refractory period of the homozygote nerve. The refractory period, defined as the time at which the second response recovered to 50% of the initial response, was increased in the homozygous Kv1.1-null sciatic nerves by approximately 0.1 millisecond (msec) over control at 37°C and by approximately 1.0 msec at 22°C. This increase in refractory period, though small, is consistent with changes found in various human

peripheral neuropathies (Kimura, 1981) and may cause abnormal conduction at sites of Kv1.1 expression in both central and peripheral fiber tracts.

Hippocampal Physiology

Because of the similarity between seizure behaviors observed in the Kv1.1-null mice with those known to be of limbic origin in other seizure models (Lothman and Collins, 1981), and the wildtype expression of the Kv1.1 channel protein in recurrent axon collaterals of the CA3 pyramidal cell layer (Wang et al., 1994), we assessed CA3 population activity using extracellular recording techniques in acute hippocampal slices from adult homozygous Kv1.1-null mice and their wildtype littermates. Six mice of each genotype were studied. Spontaneous activity but no synchronous bursting was observed in slices bathed in normal aCSF (3mM KCl) from wildtype (n=10) and homozygous (n=11) animals. Stimulation of the fimbria resulted in a single population spike in the CA3 pyramidal cell layer, in response to either a single stimulus or in response to each of a pair of stimuli. A respresentative voltage trace is shown (Figure 7A) and such traces did not appear obviously different between homozygous Kv1.1-null and wildtype slices. Use of a paired-pulse protocol revealed a slight increase in facilitation in homozygous slices (Figure 7B). In slices bathed in high potassium aCSF (6mM KCl), the paired-pulse ratio was depressed in homozygous compared with wildtype slices (Figure 7C). The meaning of this depression is unclear. It might indicate increased refractoriness of CA3 axons, perhaps due to a problem in repolarization caused by the combination of elevated extracellular potassium and the absence of the Kv1.1 channel. Spontaneous bursting was observed in 6mM KCl in a subset of homozygous Kv1.1-null hippocampal slices; five slices from two mice exhibited bursting, out of 13 slices from six mice studied (38.5%). Spontaneous bursting was never observed in hippocampal slices from wildtype littermate controls; eight slices from six mice were studied.

Discussion

Our studies of Kv1.1-null mice reveal the essential role of this voltage-gated potassium channel in counterbalancing neuronal excitability and thereby preventing seizure activity.

Kv1.1-null animals have frequent spontaneous seizures which are evident both behaviorally and electrographically. The subcellular distribution of Kv1.1 to cell somata,

apical dendrites, axons and synaptic terminals in many regions of the mouse central nervous system (CNS) (Wang et al., 1994) positions this channel to impact both intrinsic neuronal properties such as membrane potential and neuronal network properties such as synaptic neurotransmission. Alteration of either category in the direction of net excitability could potentially generate epileptiform activity (Dichter and Ayala, 1987), as clearly occurs in the Kv1.1-null mice.

At present we do not understand how seizures originate and spread in the Kv1.1-null mice. However, some of the observed behaviors such as facial twitching and blinking, and rearing and bilateral clonus are similar to behaviors in kainic acid treated rats experiencing limbic seizures (Lothman and Collins, 1981). Our observation of altered excitability of the CA3 region of the hippocampus of Kv1.1-null mice supports possible hippocampal involvement. The recurrent excitatory collateral pathways of the CA3 pyramidal cell region have been suggested to contribute greatly to generation of synchronous bursting in this region of the hippocampus (Miles and Wong, 1987). The absence of the Kv1.1 channel from these collaterals may increase this burst propensity sufficiently to produce spontaneous synchronous activity. Depth electrodes in kainate treated rats revealed high frequency spiking in the hippocampus during periods in which the animals displayed motionless staring spells, raising the possibility that the periods of arrested motion which may occur in the Kv1.1-null mice without abnormal cortical EEG activity actually represent focal hippocampal seizure activity. Obvious motor seizures may then reflect secondary generalization from a hippocampal or other initial focus.

Epilepsies with genetic determinants represent an important category of inherited human neuropathologic disorders (Noebels, 1988) though few of the human epilepsies have yielded to linkage studies or positional cloning efforts to identify a genetic error (Shoffner et al., 1990; Steinlein et al., 1995). The advent of targeted homologous recombination in embryonic stem cells has very recently allowed the unexpected identification of several genes the inactivation of which results in an epileptic phenotype in the mouse (Noebels, 1996). Derangement of inhibitory neurotransmission (Waymire et al., 1995; Li et al., 1995), absence of a neurotransmitter receptor (Tecott et al., 1995) or alterations in second messenger systems (Butler et al., 1995; Matsumoto et al., 1996) have been linked to seizures of variable severity. Our finding that Kv1.1-null mutants have epilepsy reveals that deletion of a direct effector of ionic conduction can profoundly influence the balance of electrical activity in the CNS.

Recently, several different point mutations in the human Kv1.1 gene have been identified in patients with the autosomal dominant neurological disorder episodic ataxia/myokymia (EA) (Browne et al., 1994). The episodic attacks of imbalance and incoordination (ataxia) and persistent myokymia of the human disorder (Brunt and van Weerden, 1990) are distinct from the seizures which are the dominant feature of the Kv1.1-null mouse phenotype. This distinction may result from species differences in localization, expression, or function of Kv1.1 in humans and mice, or may reflect differential consequences of the various point mutations in EA in contrast to a null mutation in mice.

The mutations identified in EA individuals have recently been introduced into a cloned human Kv1.1 sequence and the physiological properties of these mutant channels studied in Xenopus oocytes (Adelman et al., 1995). Two of the six EA subunits studied produce functional homomeric channels with altered voltage dependence or kinetics, and similarly alter the gating properties when assembled into heteromultimers with wildtype Kv1.1 subunits. The other four EA subunits are nonfunctional alone but reduce the potassium current when coexpressed with wildtype Kv1.1 subunits. Based on previous coexpression studies, the ability of EA subunits to dominantly diminish the function of the Kv1.1 channel suggests they could have a similar effect upon other Shaker-like α subunits. The net result of expression of one EA mutant allele may then be diminution of all coexpressed Shaker-like channels but ablation of none. Assuming that similar rules for assembly and expression exist *in vivo* in human tissue, the physiological impact would depend upon the EA allele in a given individual, as well as upon the complement of potassium channels and their level of expression in a particular cell. In contrast, in the Kv1.1-null mouse, Kv1.1 is exclusively and uniformly ablated. Given the uncertainty involved in generalizing results from one system or organism to another, it would be interesting to analyze the consequences of EA mutant Kv1.1 subunits expressed in mice.

The family of voltage-gated potassium channels is significantly larger than that of either sodium or calcium channels (Hille, 1992). Its members differ sometimes only subtly in biophysical characteristics, suggesting that cellular functions of each channel may overlap. Studies identifying potassium channel functions have frequently lacked molecular specificity. Both motor hyperactivity in the Drosophila *Shaker* mutant (Tanouye et al., 1981) and seizures in animals given potassium channel toxins (Bagetta et al., 1992) indicated a role for voltage-gated potassium channels in modulation of neuronal excitability. Yet the defect in *Shaker* resulted from functional ablation of the

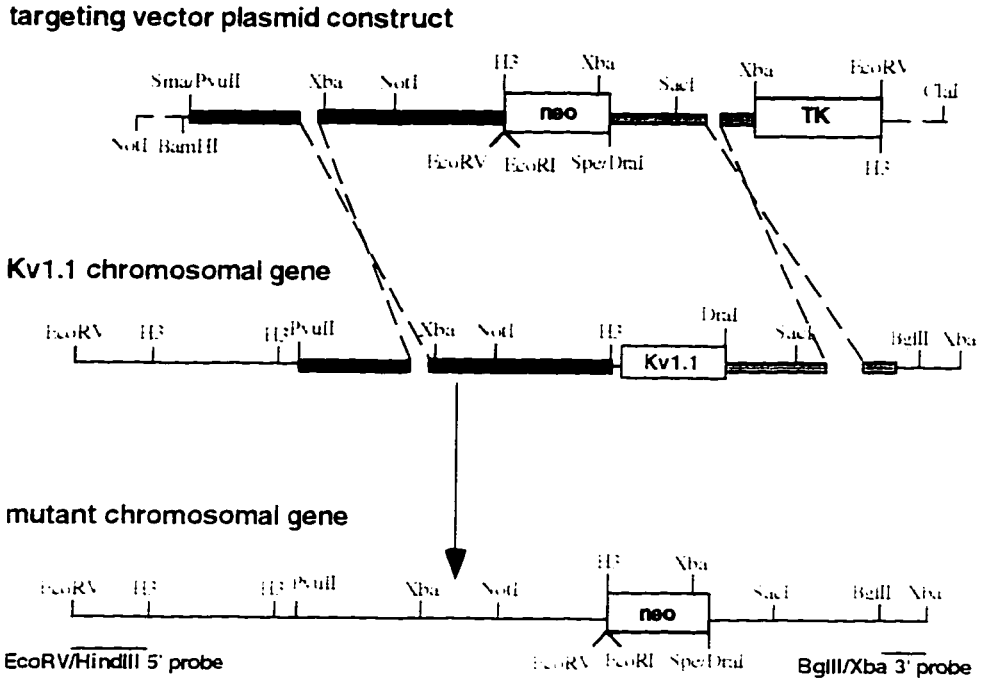
entire family of *Shaker* A-type potassium channels (Gisselman et al., 1989), and the blockers 4-aminopyridine and dendrotoxin used to produce seizures each block multiple potassium channel simultaneously (Rehm and Tempel, 1991; Pongs, 1992). The epileptic phenotype of the Kv1.1-null mice has now defined an important role for a single channel, Kv1.1, in limiting CNS hyperexcitability. The severity of this phenotype suggests an absence of compensation by related channels. Our studies have revealed no compensatory elevation in the expression of the channel Kv1.2, the most likely candidate for a compensatory role given its extensive colocalization with Kv1.1 in wildtype mouse brain. Given the number of potassium channels, their overlapping expression patterns, and the existence of multiple potassium currents in many cells, this inability of other channels to compensate for the lack of Kv1.1 suggests a high level of functional specificity within the family of voltage-dependent potassium channels. This specificity is supported by the dissimilarity between the Kv1.1-null phenotype and that reported for mice lacking the Kv3.1 voltage-gated potassium channel (Ho and Joho [1995] Soc. for Neurosci., abstract); Kv3.1-null mice display specific motor deficits with no evidence for seizure propensity.

Figure 1. Generation and Characterization of Mice Deficient in the Delayed Rectifier Potassium Channel Kv1.1

(A) Diagram of the targeting strategy used to remove the Kv1.1 open reading frame (ORF). The targeting construct is shown at the top. Homologous recombination of 5' and 3' flanking regions in the targeting vector is predicted with the *Kcna1* chromosomal locus (middle). The targeted locus is shown at the bottom, where the neomycin resistance cassette has replaced the deleted Kv1.1 ORF. The EcoRV/HindIII probe external to the 5' region of the targeting construct recognizes a very large (>23 kilobase [kb]) fragment in EcoRV-digested wildtype genomic DNA but an 8kb fragment in successfully targeted genomic DNA. Similarly, the 3' BglII/XbaI probe recognizes an 8kb and a 3.8kb fragment in XbaI-digested wildtype and targeted genomic DNA respectively.

(B) Southern blot of genomic DNA from offspring of a heterozygote intercross. EcoRV digestion followed by hybridization with the 5' EcoRV/HindIII probe generates a >23kb band alone for wildtype (+/+) mice, both a >23kb and an 8kb band for heterozygous (+/-) mice, and an 8kb band alone for homozygous (-/-) Kv1.1-null animals.

A


targeting vector plasmid construct
neo
TK
Kv1.1 chromosomal gene
Kv1.1
mutant chromosomal gene
neo
EcoRV/HindIII 5' probe
BglII/Xba 3' probe

B

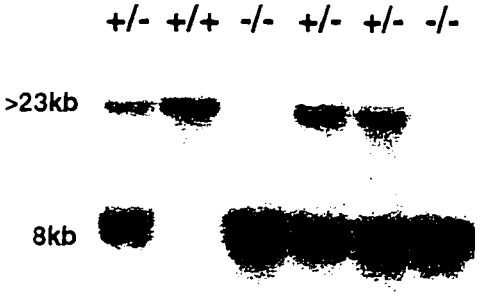

+/- +/+ -/- +/- +/- -/-
>23kb
8kb

Figure 2. Ribonuclease Protection Assay of Total Brain RNA from Wildtype, Heterozygous and Homozygous Kv1.1-Null Mice

(A) Detection of Kv1.1 transcript. Protection of a 252bp band with a Kv1.1 specific antisense riboprobe is seen in lanes containing wildtype or heterozygous, but not homozygous Kv1.1-null mouse brain RNA. As a control for sample loading and quality, RNA samples were simultaneously hybridized with a riboprobe specific for mouse cyclophilin transcript, which protects a 103bp band in each lane.

(B) Detection of Kv1.2 transcript. Protection of a 154bp band with a Kv1.2 specific antisense riboprobe is seen in lanes containing wildtype, heterozygous and homozygous Kv1.1-null mouse brain RNA. RNA samples were simultaneously hybridized with a riboprobe specific for mouse cyclophilin transcript, which protects a 103bp band in each lane.

(C) Comparison of Kv1.1 and Kv1.2 transcript quantity between homozygous, heterozygous and wildtype mice. Kv1.1 and Kv1.2 transcript levels were measured by PhosphorImager analysis of ribonuclease protection assay gels and density measurements of protected fragments, each normalized to cyclophilin fragment density. Homozygous and heterozygous levels were then normalized to wildtype littermate protected fragment density within each assay, to give a ratio of transcript expression. Such ratios were combined from several assays to provide the averaged ratio data shown above. For Kv1.1 transcript, the homozygote null to wildtype ratio was 0.011 and the heterozygote to wildtype ratio was 0.54 (SEM=0.027). For Kv1.2 transcript, the homozygote to wildtype ratio was 0.80 (SEM=0.056), and the heterozygote to wildtype ratio was 1.01 (SEM=0.09). This difference in Kv1.2 ratios did not appear to be significant ($p>0.1$). The homozygous Kv1.1-null mice then appear to produce no Kv1.1 transcript yet a normal level of Kv1.2 transcript. Their heterozygous littermates produce only approximately 50% of the wildtype Kv1.1 transcript level, yet normal levels of Kv1.2 transcript.

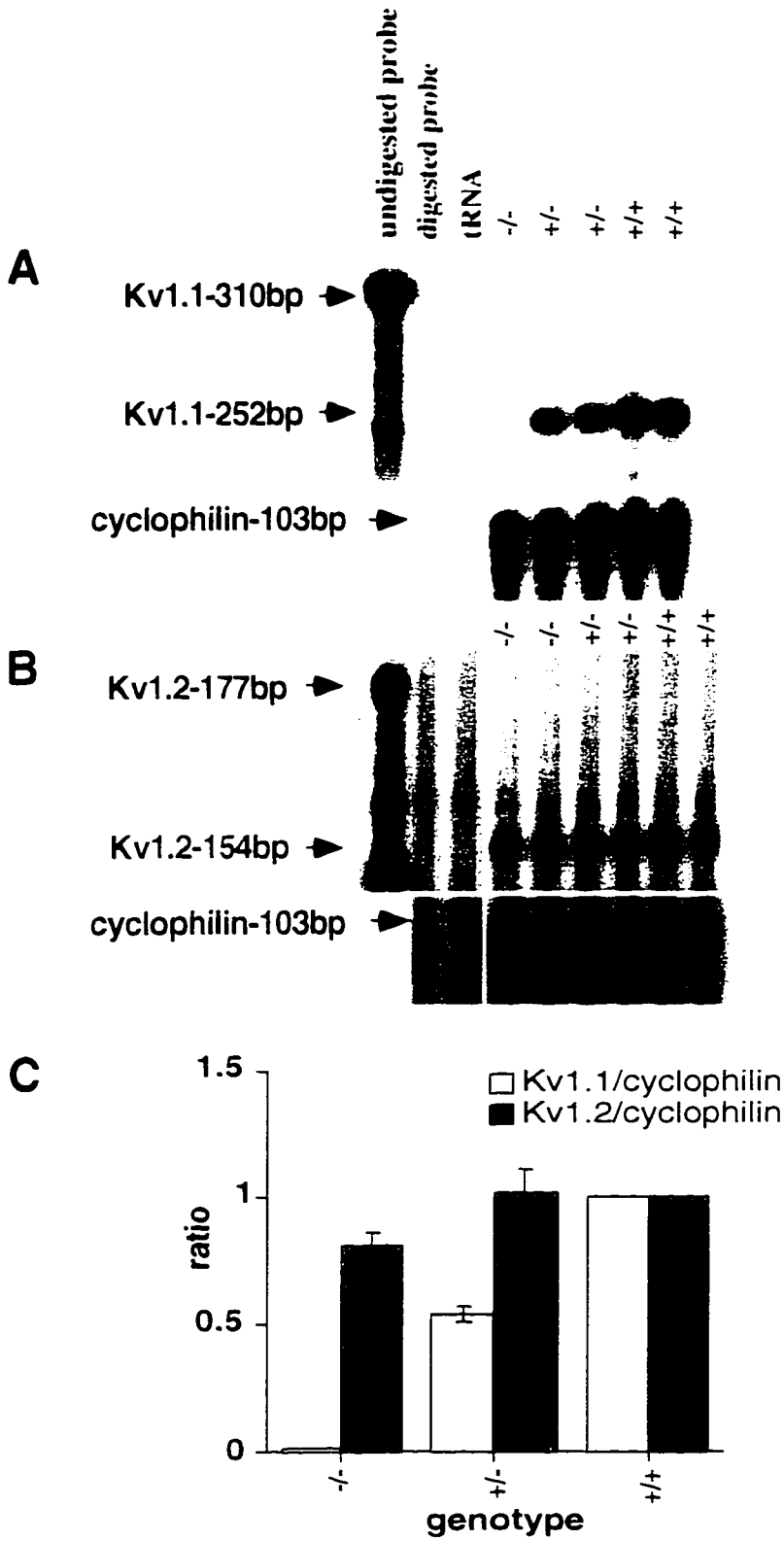

A
undigested probe
digested probe
tRNA
-/-
+/-
+/-
+/+
+/+
Kv1.1-310bp
Kv1.1-252bp
cyclophilin-103bp
B
-/-
-/-
+/-
+/-
+/+
+/+
Kv1.2-177bp
Kv1.2-154bp
cyclophilin-103bp
C
1.5
1
0.5
0
ratio
Kv1.1/cyclophilin
Kv1.2/cyclophilin
-/-
+/-
+/+
genotype

Figure 3. Analysis of Kv1.1-Null Mouse Brain Morphology and Patterns of Kv1.1 and Kv1.2 Protein Expression.

(A) Histological analysis of homozygous Kv1.1-null mouse brain sagittal sections. Haematoxylin-eosin stained sections show normal structure of cerebral cortical cell layers I-VI (upper left), cerebellar folium (upper right), and hippocampus (lower left). The CA3 pyramidal cell layer, a site of Kv1.1 expression in wildtype mice, is shown at higher magnification (lower right). The scale bar represents approximately 1000μm in upper left panel, 2000μm in upper right and lower left panels, and 400μm in lower right panel.
(B) Immunocytochemical staining of mouse cerebellar sagittal sections. No specific labeling is seen with Kv1.1 antibody (α-Kv1.1) in the cerebellum of a homozygous Kv1.1-null mouse (upper left). Staining of the cerebellum of a wildtype littermate with α-Kv1.1 shows the expected strong labeling of basket cell terminal fields (upper right). Immunocytochemical staining of homozygous (lower left) and wildtype (lower right) cerebellar sections with Kv1.2 antibody (α-Kv1.2) shows a similar labeling pattern in each.
(C) Immunocytochemical staining of mouse cochlea. No specific labeling is seen with α-Kv1.1 in thecochlea of a homozygous Kv1.1-null mouse (upper left). A strong α-Kv1.1 signal is seen in spiral ganglion cell somata and processes in wildtype cochlea (upper right). Staining of homozygous (lower left) and wildtype (lower right) sections with α-Kv1.2 shows staining in axons and peripheral processes yet relatively little signal in spiral ganglion cell somata, indicating maintenance of Kv1.2 specific subcellular localization patterns in the absence of Kv1.1. The scale bar represents 95μm.

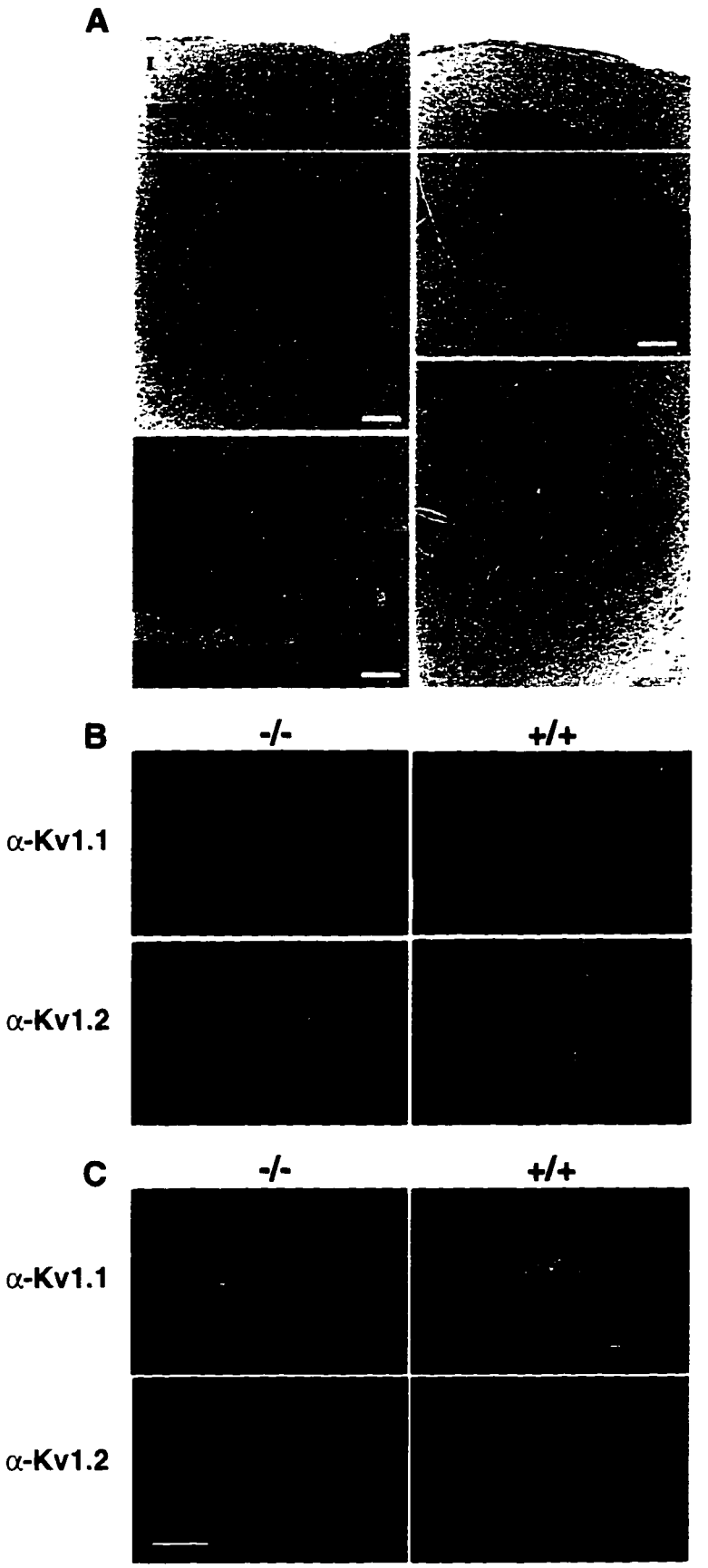

A
B
-/-
+/+
α-Kv1.1
α-Kv1.2
C
-/-
+/+
α-Kv1.1
α-Kv1.2

Figure 4. Decreased Latency to Flurothyl-Induced Seizures in Kv1.1-Null Mice

Genotype and sex are plotted on the abscissa, and mean latency to seizure onset in minutes is plotted on the ordinate. Error bars indicate standard error of the mean (SEM). The numbers of animal studied in each category are as follows: male homozygotes (-/-), n=3; male heterozygotes (+/-), n=11; male wildtype (+/+), n=9; female homozygotes (-/-), n=4; female heterozygotes (+/-), n=15; female wildtype (+/+), n=9. The mean seizure latency of male homozygotes (1.73 min.) differs significantly from the latency of male wildtype littermates (3.75 min.) at the $p<0.01$ level (t-test). Similarly, the mean seizure latency of female homozygotes (1.30 min.) differs significantly from the latency of female wildtype littermates (3.68 min.). Combining male and female data gives a mean homozygote seizure latency that is 60% shorter than that of wildtype littermates ($p<0.01$), and a mean heterozygote seizure latency that is 9% shorter than wildtype ($p<0.01$).

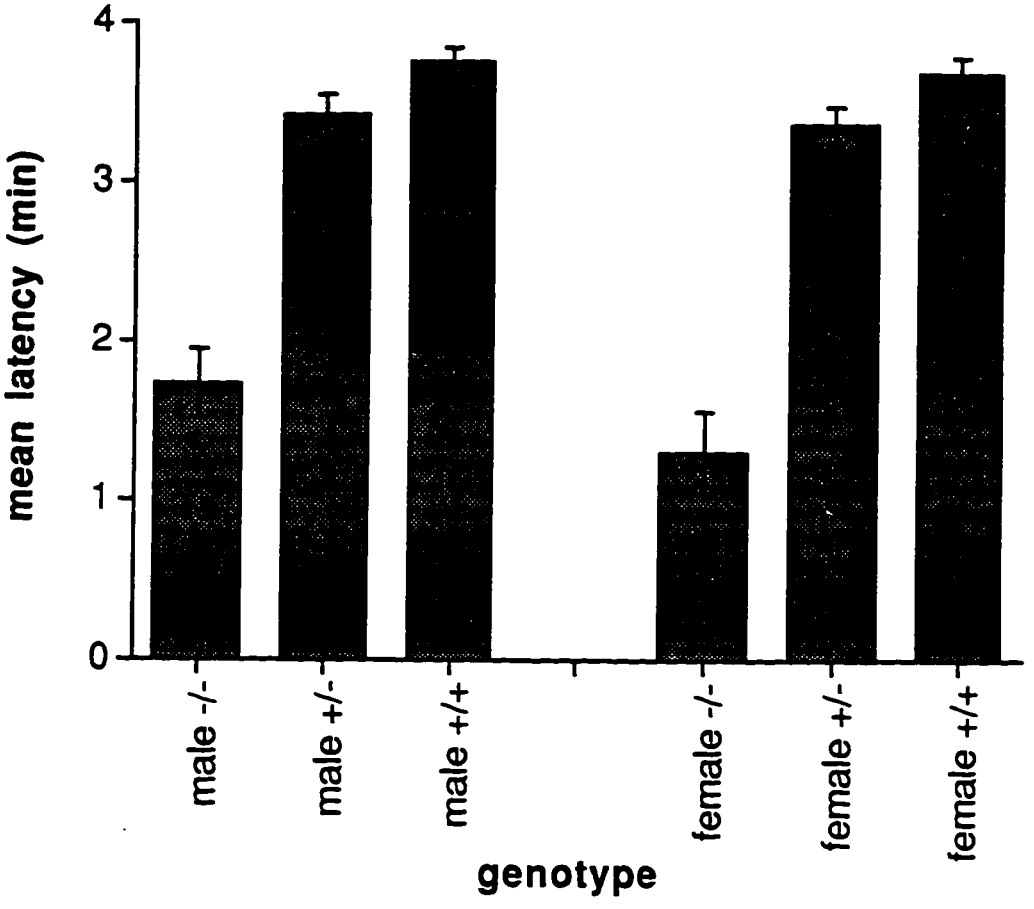

mean latency (min)
4
3
2
1
0
male -/-
male +/-
male +/+
female -/-
female +/-
female +/+
genotype

Figure 5. Electroencephalographic Analysis of Spontaneous Seizures

Representative EEG recordings from a wildtype (A) and a heterozygote (B) showing lower voltage and desynchronized patterns, compared with a homozygous Kv1.1-null mouse demonstrating interictal patterns with higher voltage and intermittant spiking and polyspikes (C), and typical ictal EEG patterns (D). Tracings are continuous with the exception of a 38 second discontinuity indicated in D (asterisk). Tracings from one channel (left) only are shown as no difference was apparent between left and right hemispheric EEG activity in the sections displayed. EEGs were recorded from 12 mice, four of each genotype. EEGs were recorded an average of 3 times for a total of 6 to 12 hours for each animal, between 4 and 14 days following electrode implantation. A total of 28 seizures were recorded, excluding a single episode of status epilepticus.

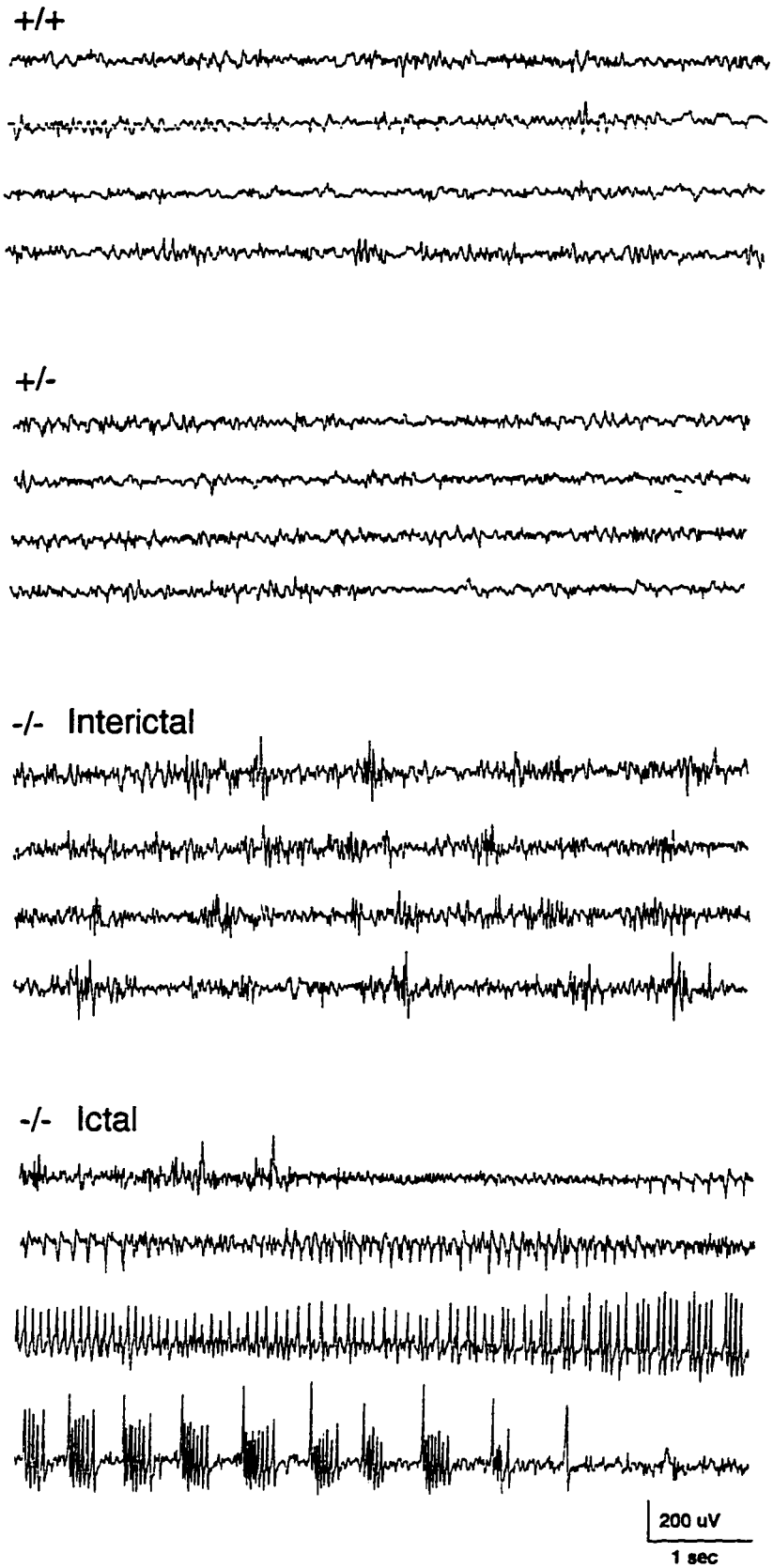

+/+
+/-
-/- Interictal
-/- Ictal
200 uV
1 sec

Figure 6. Compound Action Potential (CAP) Waveform and Refractory Period in Scintic Nerves of Homozygous Kv1.1-Null Mice and Controls

(A) Average CAPs for mutant (-/-) and control (combined +/+ and +/-) nerves at 37°C. Before the average trace was computed, we normalized the peak voltage amplitude and shifted the time axis in individual action potentials so that the peak amplitude and peak time of all action potentials from different nerves coincided. The amplitude of the average Kv1.1-null mutant CAP differs significantly from control over the time range of 0.81-1.4 milliseconds (msec), where the slow after-depolarization of the Kv1.1-null mutant CAP is most pronounced ($p<0.01$,t-test; mutant, n=10 [5 animals]; control, n=13 [8 animals]; each mouse contributed either n=2 data points representing traces from each of its two sciatic nerves , or in later experiments, n=4 data points when each of the two main bundles of a single desheathed sciatic nerve were separated and recorded from individually). The inset shows the data replotted logarithmically.

(B) CAPs for Kv1.1-null mutant (-/-) and control (combined +/+ and +/-) nerves at 22°C. Averages were computed as in (A). The mutant CAP amplitude differs significantly from control over the time range 1.0-5.0 msec ($p<0.01$, t-test; mutant, n=10 [3 animals]; control, n=12 [4 animals]). The inset shows the data replotted logarithmically . Here, the mutant data differs significantly at 1.75 msec from both the wildtype and heterozygote data ($p<0.01$, t-test; mutant, n=10 [3 animals]; heterozygote, n=4 [2 animals]; wildtype, n=8 [2 animals]). Heterozygote data does not differ significantly from control.

(C) Recovery of the amplitude of the second action potential in paired stimulus experiments. Paired stimuli were applied at various interstimulation intervals, and the amplitude of the second response relative to the first response was plotted as a function of the interstimulation intervals, at 37°C and 22°C. The mutant recovery curve is shifted significantly to the right of the control curve near the 50% recovery level. At 37°C, the mutant (-/-) data differ from control (combined +/+ and +/-) over an interstimulation interval of 0.48-0.78 msec ($p<0.05$, t-test; mutant, n=8; control, n=13), and at 22°C, they differ over an interstimulation interval of 1.8-4.5 msec ($p<0.01$, t-test; mutant, n=10; control, n=12). The inset shows 37°C recovery amplitude data during the first one msec interstimulation interval at an expanded time scale, as well as a raw data response, normalized as in (A), from a paired stimulation (0.48 msec interstimulation interval).

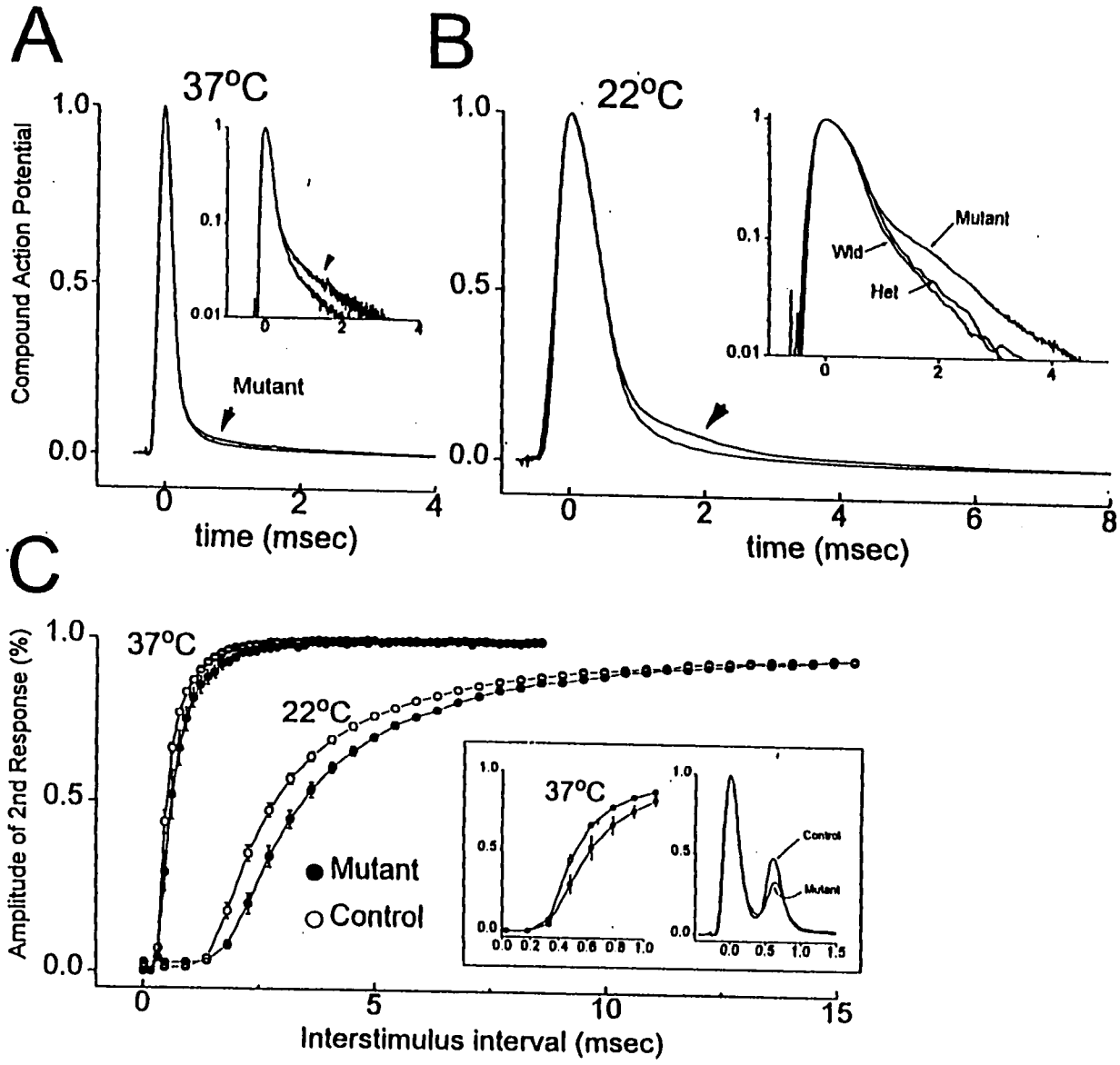

A
37°C
1.0
0.5
0.0
Compound Action Potential
Mutant
0
2
4
time (msec)
B
22°C
1.0
0.5
0.0
Mutant
Wld
Het
0.1
0.01
0
2
4
6
8
time (msec)
C
37°C
22°C
1.0
0.5
0.0
Amplitude of 2nd Response (%)
Mutant
Control
0
5
10
15
Interstimulus interval (msec)
Control
Mutant

Figure 7. Extracellular Recordings in the Hippocampal CA3 Pyramidal Cell Layer

Paired-pulse ratios as a function of interpulse interval (IPI), at approximately twice the threshold intensity, recorded in CA3.

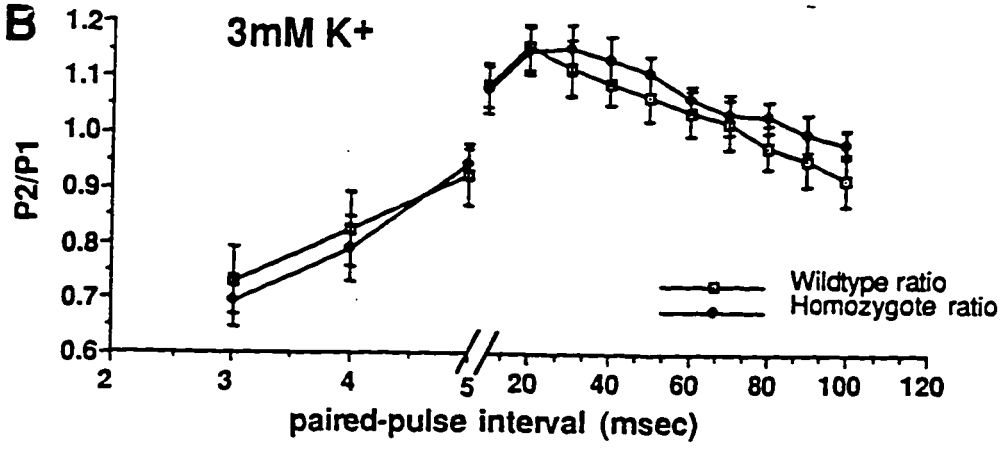
(A) A representative voltage trace.

(B) Population spike ratios (P2/P1) from slices bathed in 3mM KCL aCSF (for wildtype, n=6; for homozygote, n=6).

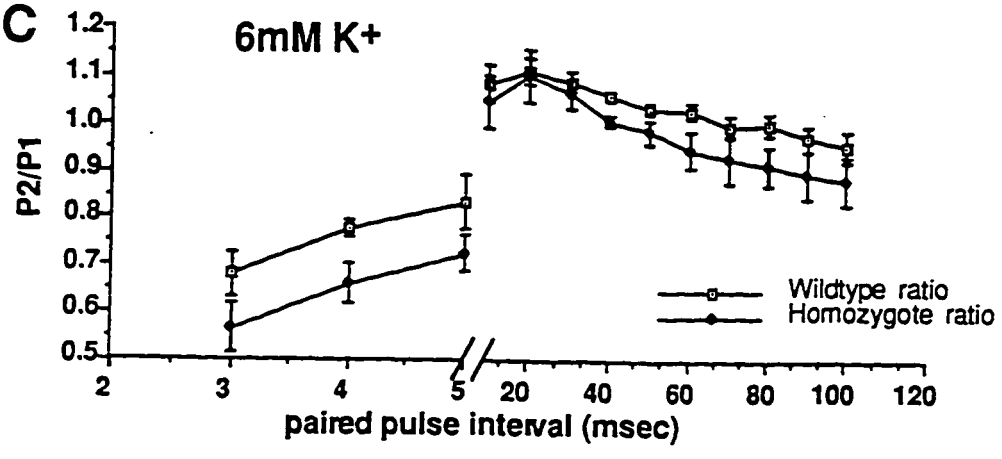
(C) Population spike ratios (P2/P1) from slices bathed in 6mM KCL aCSF (for wildtype, n=6; for homozygote, n=10).

A

B


3mM K+
P2/P1
1.2
1.1
1.0
0.9
0.8
0.7
0.6
2
3
4
5
20
40
60
80
100
120
Wildtype ratio
Homozygote ratio
paired-pulse interval (msec)

C


6mM K+
P2/P1
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
2
3
4
5
20
40
60
80
100
120
Wildtype ratio
Homozygote ratio
paired pulse interval (msec)

CHAPTER 3: IDENTIFICATION OF A DELAYED RECTIFIER POTASSIUM CHANNEL, KV1.6, EXPRESSED IN CULTURED ASTROCYTES

Introduction

Patch- and whole-cell voltage-clamp studies of cultured astrocytes have revealed the presence of voltage-dependent potassium conductances in these cells (Sontheimer, 1994). Transient or A-type (K_A) and noninactivating or delayed rectifier (K_D) outward currents have been observed, as well as inwardly-rectifying (K_{ir}) potassium currents. Recordings from acutely dissociated astrocytes (Tse et al., 1992), astrocytes in the acute hippocampal slice (Sontheimer and Waxman, 1993) and tissue prints from optic nerve (Barres et al., 1990a) demonstrate the presence of similar currents, supporting a correlation between the *in vitro* astrocyte culture system and the *in vivo* current phenotype.

The molecular identities of the specific channels mediating recorded currents in glial cells have been unknown until recently. A novel ATP-dependent inward rectifier potassium channel has been cloned and appears to have an expression pattern limited to astrocytes and other glial cells (Takumi et al., 1995). This channel, K_{AB}-2, may underlie at least some portion of the astrocytic K_{ir}. Kv1.1, a member of the Shaker-like potassium channel gene family, has been identified in C6 glioma cells, a tumor cell line thought to be of astrocytic origin (Wang et al., 1992). Kv1.1 appears to underlie the delayed rectifier current in these cells. Members of the Shaker-like channel family have also been identified in glia of the peripheral nervous system, the Schwann cells (Mi et al., 1995). The identity of a K_D channel or channels in astrocytes has remained unknown.

In the study presented here, we sought to identify channels mediating outward potassium currents in astrocytes. Using a mouse cerebral cortical astrocyte culture system as a source of a relatively pure astrocyte population, we screened for expression of several members of the Shaker-like potassium gene family (Kv1.1-Kv1.6). Our results identify Kv1.6 as expressed at both RNA and protein levels in astrocyte cultures.

Experimental Procedures

Cell Culture

Primary astrocyte cultures were prepared from cerebral cortices of four day postnatal C3HFeB mouse pups by a method modified from that of McCarthy and DeVellis (1980). Mouse pups were sacrificed by rapid decapitation and the brains removed onto ice. The meninges were carefully removed, and the cortex from each hemisphere peeled off. Cortical tissue was cut with forceps into smaller pieces and combined into a small petri dish containing HEPES buffered Kreb's Ringer (HBK). Tissue was washed twice by passage through fresh HBK. An incubation in 0.25% trypsin for 20 minutes at 37°C was followed by trituration in complete medium (DMEM:Ham's F12, 10% heat-inactivated calf serum, 1x penicillin-streptomycin) (Gibco). The cell suspension was then plated at approximately 10^5cells/cm^2 in plastic tissue culture flasks (Falcon). Cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. Media was replaced completely every 2-3 days.

Cultures were grown to confluence (10-14 days) and then shaken overnight at 260rpm to remove overlying process-bearing cells. A mild (0.02%) trypsinization (30sec.) and Ca^{2+}-free incubation (5min.) were additionally used. At this stage, cultures were either used for RNA isolation or replated onto poly-D-lysine (Sigma) coated tissue culture flasks. A 48-hour exposure to 2μM cytosine arabinoside (Sigma) was used to limit fibroblast contamination of some secondary cultures. For immunocytochemistry, cells were replated onto 35x10mm poly-D-lysine coated petri dishes (Falcon). Secondary cultures were used for RNA isolation 1-2 weeks after replating.

Anti-glial fibrillary acidic protein monoclonal antibody (α-GFAP, Boehringer) and anti-fibronectin polyclonal antibody (α-fibronectin, Gibco) were used to assess the purity of the astrocyte culture system. Cells labeled with α-fibronectin were present in many primary and some secondary clutures, indicating contamination presumably of meningeal origin. Therefore, meninges removed during preparation of astrocyte cultures were cultured in parallel and used for RNA isolation to provide a control for meningeal contamination. Both primary and secondary cultures contained predominantly α-GFAP-positive cells, with secondary cultures comprised almost entirely of a polygonal, flat monolayer and primary cultures containing more process-bearing astrocytes and α-fibronectin-positive cells.

Isolation of Total RNA

Cells from primary and secondary astrocyte cultures and meningeal cultures were lysed in Ultraspec RNA isolation solution (Biotecx). Brains from adult C3HeB mice

were dissected and homogenized in Ultraspec solution. Total cellular RNA was extracted with chloroform and precipitated with one volume of isopropanol. RNA was then extracted with phenol/chloroform, chloroform alone, and precipitated a second time with isopropanol. The pellet was washed with 75% ethanol, dried, and resuspended in water. RNA concentration was determined by spectrophotometric absorbance measurement. RNA condition and purity were checked by formaldehyde gel electrophoresis and ethidium bromide staining. RNA from multiple cultures was pooled into several groups.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For use in RT-PCR, 50μg of total RNA from primary astrocyte cultures, secondary astrocyte cultures, meningeal cultures, and adult mouse brain were further purified by digestion with RNase-free DNaseI (Boehringer) for 1 hour at 37°C, followed by phenol/chlorofom extraction and ethanol precipitation. In each reaction, reverse transcription of 100ng RNA to cDNA was carried out with M-MLV Reverse Transcriptase (Gibco) and random hexamer (Pharmacia) priming for 30 minutes at 42°C. As a control for genomic DNA contamination, a 100ng aliquot of each RNA sample was digested with DNase-free RNase (Boehringer) and included in the subsequent amplification. cDNA reverse transcription product was amplified with channel-specific primers by the polymerase chain reaction: denature 94°C, 1 min.; anneal 65°C, 1 min.; extend 72°C, 1 min.; 25 cycles. The following primer pairs were used:

mKv1.1: 5'-GCCTCTGACAGTGACCTCAGC-3'
5'-GGGACAGGAGTCGCCAAGGG-3'
mKv1.2: 5'-CGTCCTCCCCTGACCTAAA-3'
3'-GATGTCGTAGACCAAGACGTACC-5'
mKv1.3: 5'-CGAGAAGCTGGAGGCTCC-3'
3'-CCGTCACCGAGCATCGCC-5'
mKv1.4: 5'-CTCCTCCCATGATCCTCAAGG-3'
3'-CCACAAGCATGTGACTGGACG-5'
mKv1.5: 5'-CTGGCAGAGCAACAACCAGGG-3'
3'-GACAATCAGGGGTCGCACTTC-5'
mKv1.6: 5'-GCTTGGCAAACCTGACTTTGC-3'
3'-CCGGACGTCCTTTTGTCC-5'

A 10µl aliquot of each 100µl reaction product was purified by electrophoresis on a 1% agarose gel, followed by Southern transfer (Sambrook et al., 1989) to Nytran (Schleicher and Schuell) nylon membrane. Blots were incubated with α^{32}P-dCTP radioactive probes, labeled with random hexomer priming, in 4x SSPE, 4x Denhardt's, 1% SDS, 0.1 mg/ml salmon sperm DNA at 65°C. Blots were then washed for 3 hours at 65°C in 0.5x SSC, 0.1% SDS, and exposed to film.

Ribonuclease Protection Assay

Sequences specific to Kv1.6 and 18S ribosomal RNA were protected from RNase digestion as described (Ausubel et al., 1991). 10µg of RNA isolated from primary and secondary astrocyte cultures, meningeal cultures, and adult mouse brain were used in each assay. Riboprobes were synthesized in the presence of ^{32}P-UTP, and $5x10^5$ cpm were added to each RNA sample for overnight hybridization at 50°C. The samples were then digested wiht RNase A and RNase T1, concentrated, and separated on a denaturing 5% acrylamide sequencing gel. The fixed and dried gel was exposed to film overnight.

Immunocytochemistry

Purity of astrocyte cultures was assessed with α-GFAP (5µg/ml) and α-fibronectin (1:200). Cells grown on 35x10mm dishes (Falcon) were fixed in 4% paraformaldehyde for 10 minutes and washed 4 times, 10 minutes each, in 1x PBS. Primary antibodies were diluted in 3% bovine serum albumin (BSA), 3% normal goat serum, 0.3% Triton X-100 in 1x PBS, and added to dishes for overnight incubation at 4°C. Cells were washed four times, 10 minutes each, in 1x PBS. Secondary antibodies were diluted 1:200 in 3% BSA, 3% normal goat serum, 0.1% Triton X-100 in 1x PBS, and added to dishes for a one hour incubation at room temperature. Goat anti-mouse IgG-FITC (Molecular Probes) and goat anti-rabbit IgG-FITC (Molecular Probes) were used. Cells were again washed 4 times, 10 minutes each, in 1x PBS, followed by a final rinse in distilled water. Cells were air-dried and coverslipped with Vectashield fluorescent mounting medium H-1000 (Vector Laboratories). Cells were viewed under a flurorescence microscope.

Expression of Kv1.6 protein was assessed with anti-Kv1.6 polyclonal antibody (α-Kv1.6, Alomone Labs), used at a concentration of 20 µg/ml. The procedure for α-Kv1.6 immunocytochemistry was similar to the above description with the following

exceptions: use of 2% paraformaldehyde for fixation; inclusion of 0.2% Triton X-100 in the dilution buffer for primary and secondary antibodies. For α-Kv1.6/α-GFAP double-labeling experiments, staining was done by simultaneous incubation with both primary antibodies. Images of α-Kv1.6 labeled cells were collected with a BioRad MRC 600 Laser Scanning Confocal Imaging System (Fred Hutchinson Cancer Research Center).

Electrophysiology

Process-bearing presumptive astrocytes were used for electrophysiology 7-9 days after initial plating. Membrane currents were measured using the perforated patch voltage clamp technique at room temperature. Nystatin (Sigma) was diluted 1:100 in pipette solution from a 30 mg/ml stock solution. To facilitate seal formation, the pipette tip was filled with solution free of nystatin by dipping the tip in pipette solution, and the pipette was then backfilled with the nystatin-containing solution. Patch pipettes were fabricated from VWR 75μl hematocrit glass using a two-pull method and fire-polished to 1-2μm tip diameter. The pipette solution contained: 140mM KCL, 1mM $MgCl_2$, 10mM EGTA, 5mM HEPES, pH 7.4. This solution was diluted 15% prior to use (Barres et al., 1990a). The bath solution contained: 140mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM KCl, 5mM HEPES, pH 7.4. Experiments were conducted with continuous bath perfusion, and solution changes were made by switching between normal bath solution and drug-containing solution. Fraction I of dendrotoxin was purified by HPLC as in Newitt et al (1991). Dishes for electrophysiology were viewed through a Nikon Diaphot microscope, and pipettes were manipulated using a Newport XYZ translator with one motorized dimension mounted on an Orbital stage (Meridian Instruments). Currents were recorded in the whole-cell configuration using an Axopatch 200 amplifier (Axon Instruments). Currents were sampled on-line and voltages delivered using an ITC16 interface board, Pulse software (Instrutech), and Centris 650 microcomputer (Apple Computer).

Results

Screening for Shaker-Like Potassium Channel Expression

RNA isolated from primary astrocyte cultures and secondary cultures which had been replated to enrich for type-I-like astrocytes (flat, non-process bearing) was used in RT-PCR amplifications with primer pairs specific for individual Shaker-like potassium

channel cDNA sequences (Kv1.1-Kv1.6). RNA from adult mouse brain was used as a positive control for the reaction, and RNA from cultured meningeal cells was used as a control for channel expression observed in astrocyte cultures potentially arising from meningeal contaminants. Ribonuclease-treated aliquots of each RNA sample were used as negative controls. RT-PCR reaction products were electrophoresed on agarose gels and transferred to nylon membrane. The Southern blots were then hybridized with radiolabeled probes specific to the sequence of the channel amplified by a given primer pair. Blots were exposed to film to determine the presence or absence of specific PCR product from each RNA sample. The results of the RT-PCR screen are shown in Figure 8. Primer pairs for each channel produced PCR product of appropriate size from brain RNA. No specific product was produced from meningeal RNA. Primers for Kv1.1 and Kv1.2 amplified low levels of specific product from primary and secondary astrocyte culture RNA, whereas the primer pair specific for Kv1.6 produced a relatively high level of product from both samples of astrocyte RNA. Due to potential variability in amplification efficiency between primer pairs, this RT-PCR screen was non-quantitative. However, the strength of the Kv1.6 amplification product was suggestive of astrocytic expression of Kv1.6 transcript, warranting further investigation.

Kv1.6-Specific Ribonuclease Protection

To confirm and quantitate Kv1.6 expression in our astrocyte cultures, a ribonuclease protection assay was used. A probe specific for Kv1.6 RNA protected a fragment of appropriate size in brain, primary and secondary astrocyte, but not meningeal RNA (Figure 9). This result confirms the presence of Kv1.6 transcript in astrocyte cultures, and indicates that its presence does not arise from meningeal contamination. Quantitation of the results of two RNase protection assays indicates that primary astrocyte cultures express approximately 65% and secondary cultures 16% of the Kv1.6 transcript level of adult mouse brain. This decrease in Kv1.6 RNA in secondary cultures could be due to a decrease in expression with replating or longer times in culture, or from loss during purification/replating of a distinct cell type, such as stellate astrocytes, which might express a higher level of the channel.

Localization of Kv1.6 Protein in Astrocyte Cultures

A polyclonal antibody specific for the Kv1.6 channel protein was used to characterize the distribution of Kv1.6 in primary astrocyte cultures 14 days after initial plating. The Kv1.6 antibody specifically labels astrocyte cultures, as indicated by the contrast between the signal from α-Kv1.6 stained astrocyte cultures, the background signal from α-Kv1.6 stained meningeal culture controls, and the absence of signal with secondary antibody alone (Figure 9A, left to right). Double staining with both α-Kv1.6 and α-GFAP shows α-Kv1.6 labeling of GFAP-positive cells (Figure 9B). Expression of the Kv1.6 channel protein appears variable, with certain cells showing much stronger labeling than others. Several process-bearing astrocytes showed strong α-Kv1.6 staining, with clear labeling of cell body and processes (Figure 9B). Labeling of many other cells did not appear to extend to all surfaces. The α-Kv1.6 labeling of secondary astrocyte cultures at 28 days after initial plating revealed a significant decrease in Kv1.6 protein (Figure 9C), in agreement with the decrement of RNA expression indicated by ribonuclease protection.

Blockade of a Delayed Rectifier Current in Process-Bearing Cultured Astrocytes

Because of the relatively strong labeling of some process-bearing astrocytes (identified by morphology) in primary culture with α-Kv1.6, we recorded from several such cells at 7-9 days in culture using the nystatin perforated patch voltage clamp technique (Stephens et al., 1993). After a seal is made between the recording pipette and the cell membrane, nystatin gradually partitions into the membrane forming pores, until electrical continuity is achieved between pipette and cell interiors. Large molecules such as ATP remain within the cell cytoplasm, unable to pass through the pores, and current rundown is minimized. Step depolarizations from a holding potential of -70mV resulted in the appearance of an outward current in each of the 16 cells examined (Figure 11A). Sensitivity of this current to the potassium channel blocker dendrotoxin (DTX) was tested. DTX is relatively selective for blockade of the Shaker-like channels Kv1.1, Kv1.2 and Kv1.6 (Swanson et al., 1990; Pongs, 1992). In two of the 16 cells tested, no DTX block was seen. In each of the other 14 cells tested, 100nM DTX blocked a portion of the outward current (Figure 11B). The proportion of current blocked varied among cells, ranging from approximately 15-45%. This DTX block was largely reversible (Figure 11C). Subraction of the DTX-insensitive current from current prior to DTX application reveals the DTX-sensitive component (Figure 12). The DTX-sensitive current activates just positive to -40mV, and displays much less inactivation than does the DTX-resistant

current. These properties are consistent with Kv1.6 underlying the DTX-sensitive current in process-bearing astrocytes. An additional contribution to the DTX-sensitive current from Kv1.1 or Kv1.2 cannot be ruled out. We were unable to assess DTX effect on type 1-like (flat, non-process bearing) astrocytes with the perforated patch technique; degeneration of the seal occurred as nystatin diffused into the pipette tip.

Discussion

Previous electrophysiological studies have clearly demonstrated the presence of voltage dependent, delayed rectifier potassium currents in astrocytes (Sontheimer, 1994). Here, we have provided evidence that cultured mouse cortical astrocytes express a specific potassium channel, Kv1.6.

Kv1.6 transcript was found in astrocyte cultures at a level approximately 60% in primary cultures and 16% in secondary cultures of the level found in adult mouse brain. Other Shaker-like potassium channel transcripts, Kv1.1 and Kv1.2, were also identified in astrocyte cultures, yet at only apparently low levels; expression of these channels was not analyzed further. Labeling with antibody specific for Kv1.6 protein was associated with GFAP-positive cells. The antibody labeling was stronger in primary than in replated cultures, in agreement with the transcript levels. The α-Kv1.6 staining was also non-uniform thoughout the cell population. Atrocytes appear, then, to be heterogeneous with respect to Kv1.6 expression, and this expression may be affected by the culture system.

The Kv1.6 potassium channel is specific to the nervous system (Grupe et al., 1990; Swanson et al., 1990). Kv1.6 has been shown to have a specific neuronal distribution (Veh et al., 1995) but glial expression has not been reported previously. *In vitro* expression of this channel produces an outward current with an activation threshold around -40mV and little inactivation during a 200msec pulse (Swanson et al., 1990). The current is sensitive to low concentrations of TEA, 4-AP and DTX (Swanson et al., 1990). This localization to the nervous system, voltage dependence and kinetics are in agreement with Kv1.6 providing at least a portion of the astrocytic K_D. We have used the sensitivity of Kv1.6 to the quite selective potassium channel toxin, DTX-I, to obtain suggestive pharmacological evidence for involvement of Kv1.6 in providing a delayed rectifier component of the potassium currents in process-bearing astrocytes. Variability of DTX blockade in process-bearing astrocytes supports the heterogeneity of channel expression seen with Kv1.6 antibody labeling.

The function of Kv1.6 in astrocytes is unknown. Outward potassium currents have been proposed to assist in spatial buffering of the extracellular potassium released during neuronal activity (Sontheimer, 1994), a function for which inward rectifier channels have already been strongly implicated (Newman, 1993). A role in potassium buffering would position these channels to modulate global CNS excitability. A delayed rectifier channel such as Kv1.6 could also be involved in controlling astrocyte proliferation. Blockade of voltage-gated potassium channels with TEA or 4-AP has been shown to inhibit Schwann cell proliferation (Chiu and Wilson, 1989). Similar results have also been obtained with cultured spinal cord astrocytes (Sontheimer, 1994).

The identification of Kv1.6 expression in cultured astrocytes provides a molecular tool for specific genetic, pharmacological and immunohistochemical analysis of localization and function of astrocyte voltage-gated potassium channels. These results require confirmation *in vivo*, as the functional role of Kv1.6 might depend on a specific subcellular distribution which may be lost in culture.

Figure 8. Screening for Expression of Shaker-Like Potassium Channels in Cultured Astrocytes

RNA isolated from primary and secondary astrocyte cultures was used in the reverse transcriptase-polymerase chain reaction (RT-PCR) with primers specific for Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5 or Kv1.6 cDNA sequences. Brain RNA and cultured meningeal cell RNA were used as positive and negative controls, respectively. An aliquot of each RNA sample was treated with RNase to control for genomic DNA contamination, and such samples are designated with (-). RT-PCR products were loaded onto each gel in the following order: 1kb ladder, brain, brain(-), meninges, meninges(-), primary astrocyte, primary astrocyte(-), secondary astrocyte, secondary astrocyte(-). Autoradiographs of resultant Southern blots are shown here. For Kv1.4, an overnight exposure is shown while all others are 3 hour exposures. The strongest product band in astrocyte lanes was produced by the Kv1.6-specific primer pair. Weaker bands were amplified by Kv1.1, Kv1.2 and Kv1.5 primers.

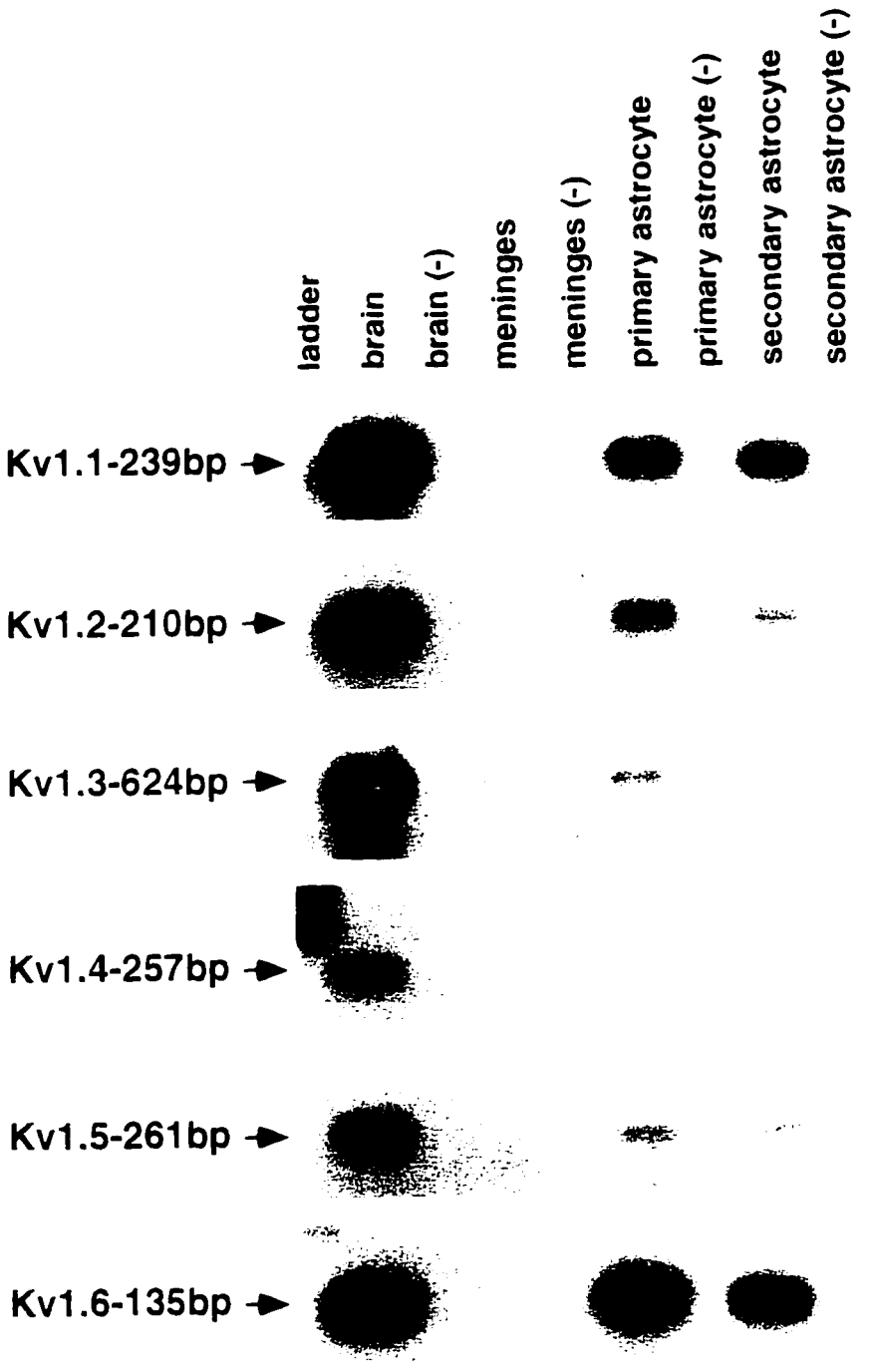

ladder
brain
brain (-)
meninges
meninges (-)
primary astrocyte
primary astrocyte (-)
secondary astrocyte
secondary astrocyte (-)
Kv1.1-239bp
Kv1.2-210bp
Kv1.3-624bp
Kv1.4-257bp
Kv1.5-261bp
Kv1.6-135bp

Figure 9. Ribonuclease Protection Assay of Cultured Astrocyte RNA

(A) Protection of a 197bp band with a Kv1.6-specific antisense riboprobe is seen in lanes containing brain or astrocyte, but not meningeal RNA. As a control for sample loading and quality, RNA samples were diluted $5x10^{-5}$ and hybridized with a riboprobe derived from the 18S rRNA gene, which protects a 340bp band in all sample lanes.
(B) The averaged results of two assays are represented graphically. Kv1.6 transcript levels were measured by PhosphorImager analysis of RPA gels and density measurements of protected fragments, each normalized to 18S fragment density. Results were then normalized to brain, and indicate that primary astrocyte cultures express 65% the Kv1.6 transcript level in brain, secondary astrocytes express 16% the brain level, and cultured meningeal cells have negligible Kv1.6 transcript (1% of brain level).

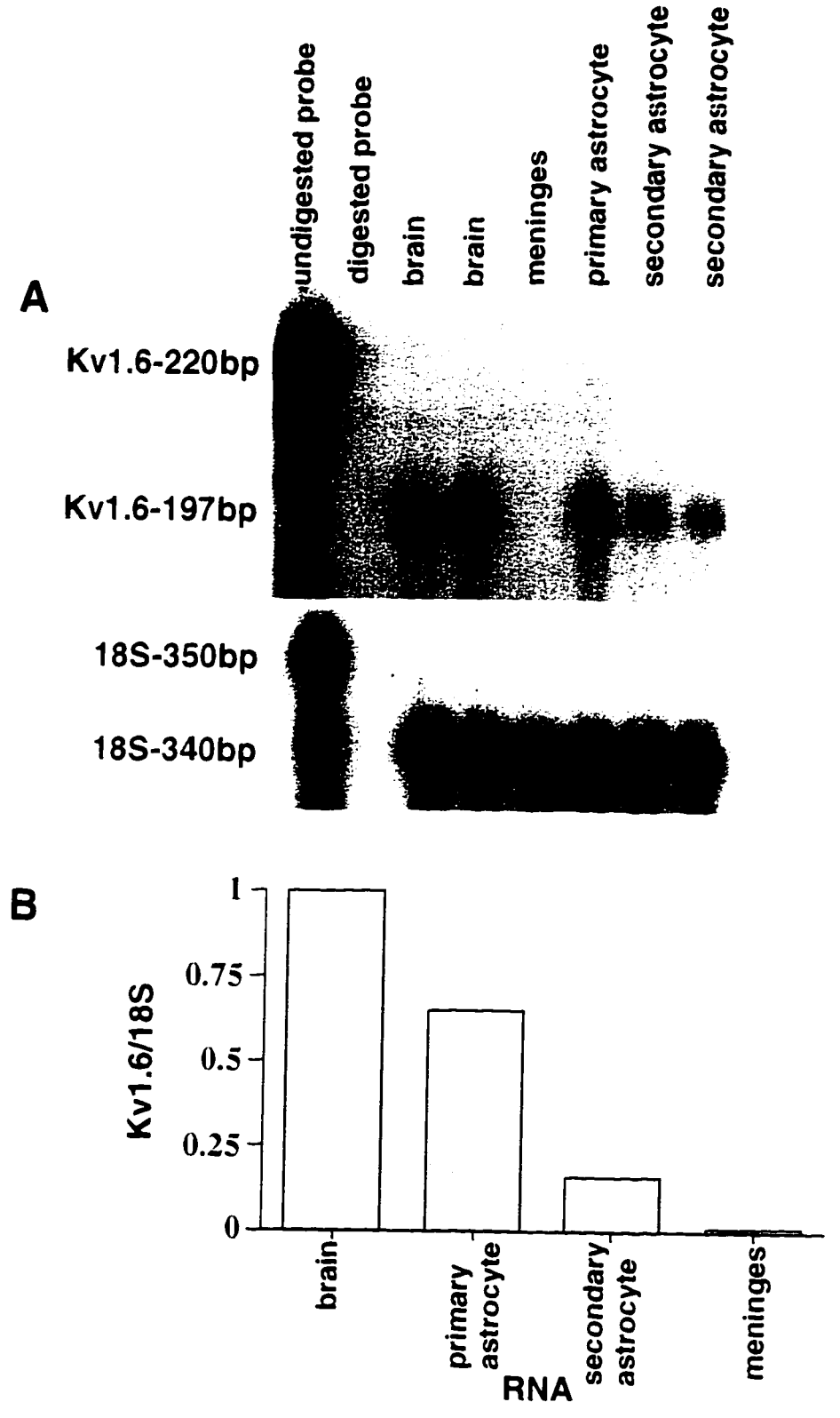

A
undigested probe
digested probe
brain
brain
meninges
primary astrocyte
secondary astrocyte
secondary astrocyte
Kv1.6-220bp
Kv1.6-197bp
18S-350bp
18S-340bp
B
Kv1.6/18S
1
0.75
0.5
0.25
0
brain
primary astrocyte
secondary astrocyte
meninges
RNA

Figure 10. Immunocytochemical Staining of Astrocyte Cultures

(A) Staining of primary astrocyte cultures (day 14) with α-Kv1.6. The α-Kv1.6 antibody specifically labels astrocyte cultures (left panel). A background signal is seen with staining of meningeal cultures (middle panel) and no signal is seen with secondary antibody alone (right panel). Magnification is 40x.

(B) Double labeling of primary astrocyte cultures (day 14) with α-Kv1.6 and α-GFAP. For each pair of images, α-GFAP labeling is shown on the left and α-Kv1.6 is shown on the right, in the same field. The α-Kv1.6 labeling of GFAP-positive cells is evident, although Kv1.6 protein expression appears quite variable between cells. Striking labeling of the cell body and processes of process-bearing astrocytes is evident in the left and right pairs of panels. Magnification is 40x.

(C) Double labeling of secondary astrocyte cultures (day 28) with α-Kv1.6 and α-GFAP. For each pair of images, α-GFAP labeling is shown on the left and α-Kv1.6 shown on the right, in the same field. Much weaker Kv1.6 signal is seen in this secondary culture than was evident in the primary culture shown in (B). Magnification is 40x.

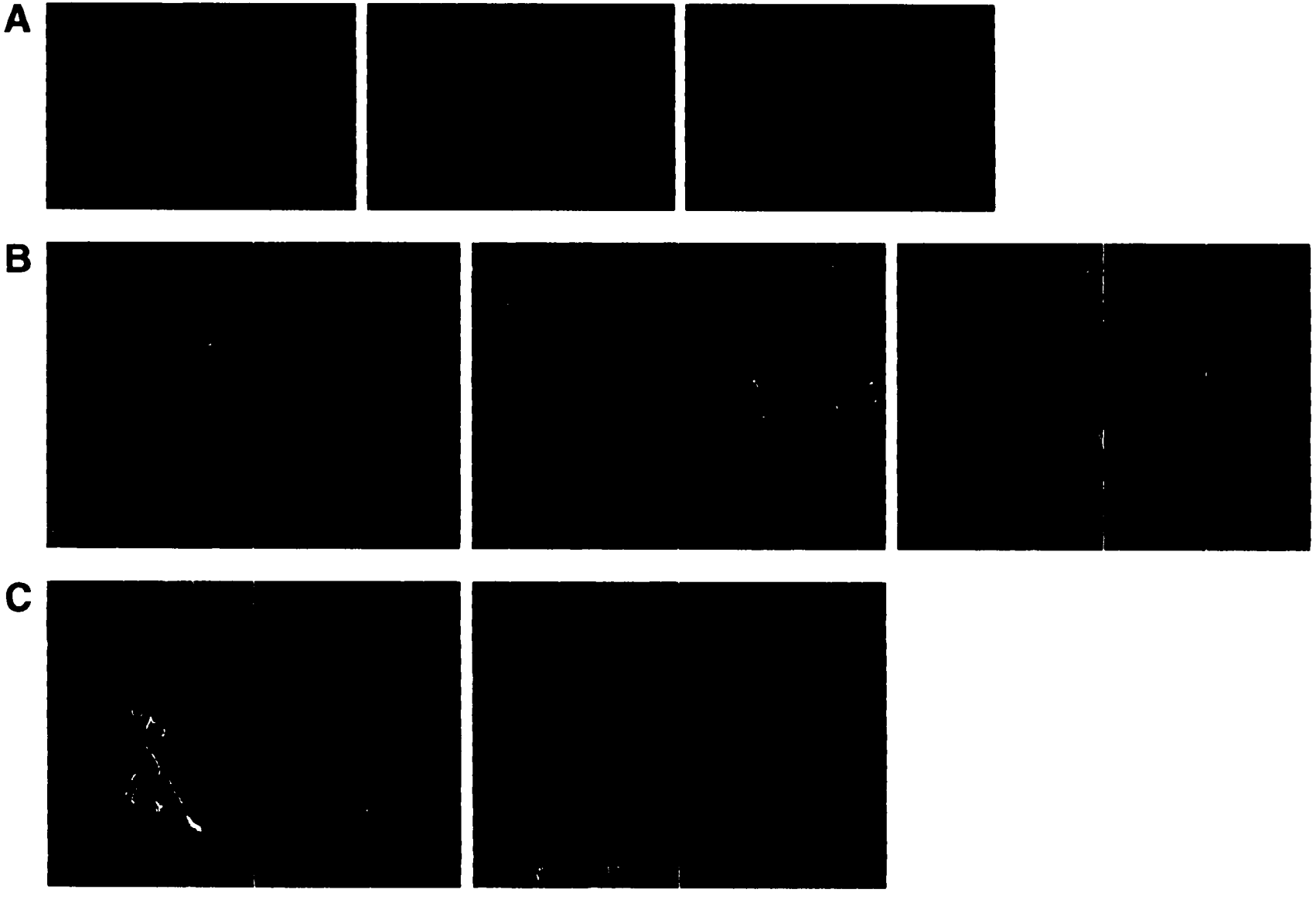

A
B
C

Figure 11. Outward Currents in Response to Depolarization of a Process-Bearing Astrocyte

Perforated patch voltage clamp recording was done on a presumptive stellate astrocyte at day 8 in primary culture.

(A) Outward current traces obtained in response to a family of depolarizing pulses from a holding potential of -70mV. Voltage was stepped from -40mV to +95mV in 15mV increments.

(B) Outward current traces obtained in the presence of 100nM DTX. Voltage pulses were given from a holding potential of -70mV to steps between -40mV and +95mV in 15mV increments. Traces shown were obtained 5 minutes after the start of perfusion with 100nM DTX.

(C) Outward currents generated by depolarization to +20mV in the presence and absence of 100nM DTX. The DTX inhibition was largely reversible after a 10 minute wash.

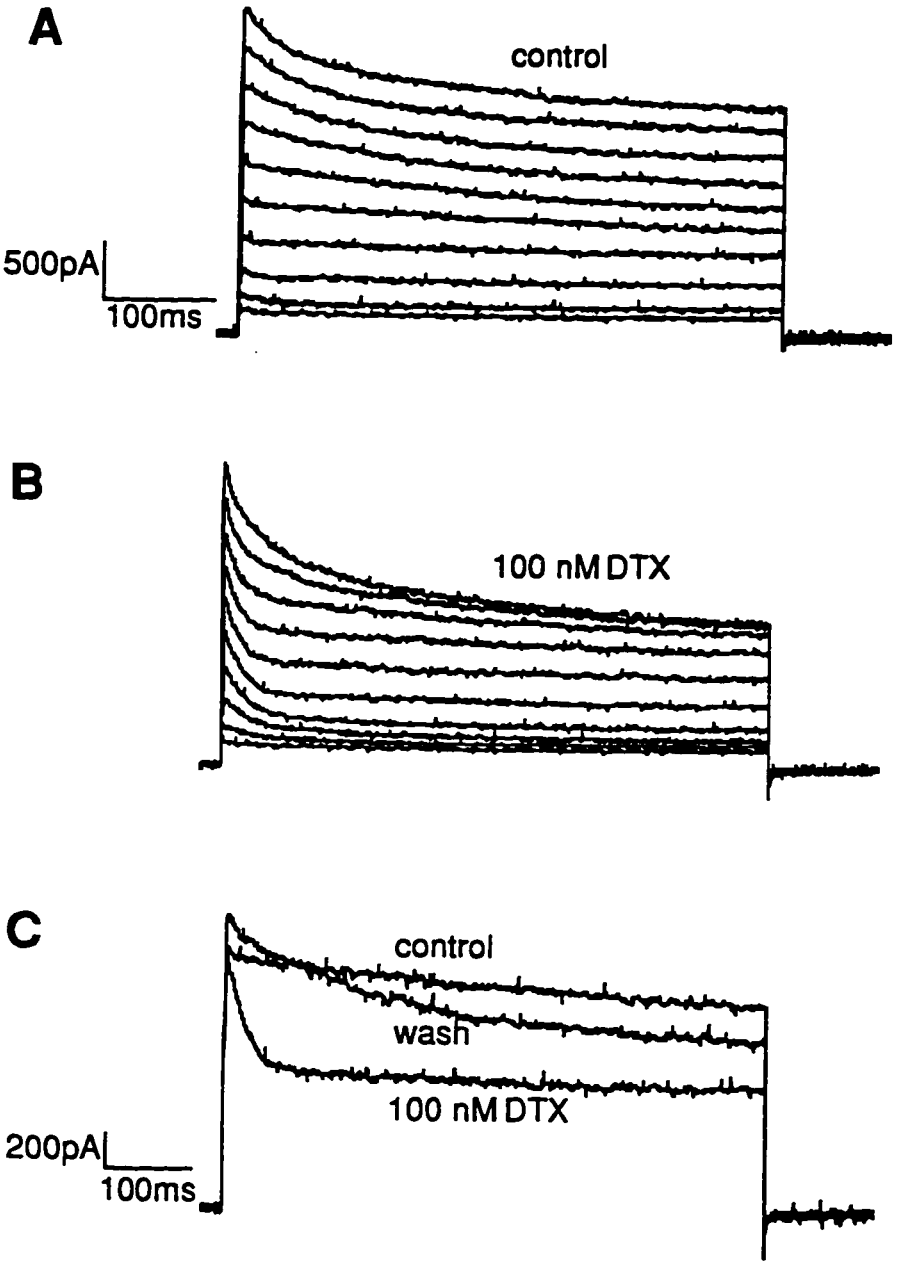
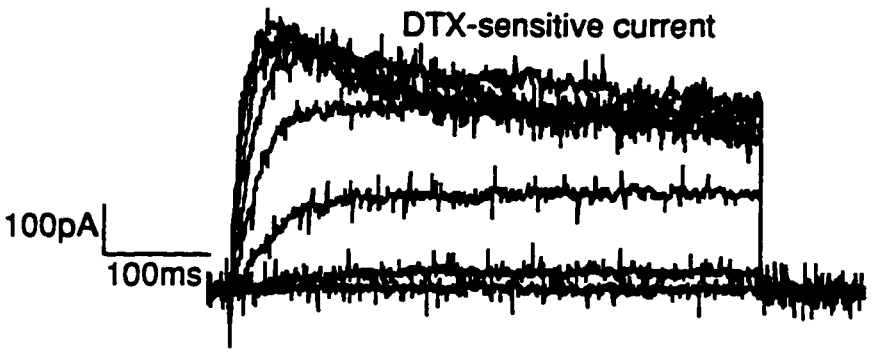
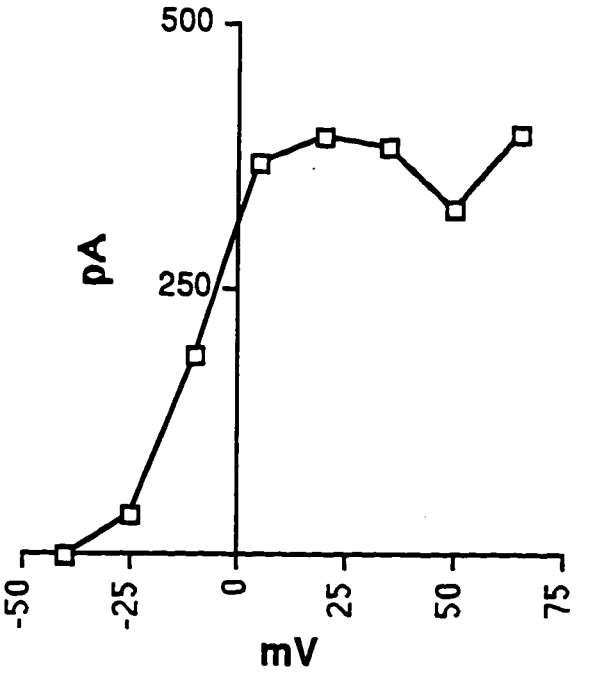

A
control
500pA
100ms
B
100 nM DTX
C
control
wash
100 nM DTX
200pA
100ms

Figure 12. Dendrotoxin-Sensitive Current

Current traces were obtained by subtraction of traces in Figure 11B from traces in A to reveal the DTX-sensitive current in a process-bearing astrocyte. Currents resulting from steps between -40mV and +65mV are shown. The I-V relationship is displayed graphically below.


DTX-sensitive current
100pA
100ms


500
250
pA
-50
-25
0
25
50
75
mV

CHAPTER 4: USE OF A GFAP PROMOTER CONSTRUCT TO DISRUPT SHAKER-LIKE POTASSIUM CHANNEL EXPRESSION IN ASTROCYTES OF TRANSGENIC MICE

Introduction

We have identified a Shaker-like delayed rectifier potassium channel, Kv1.6, in cultured astrocytes from mouse cortex, as described in Chapter 3. Kv1.1 and Kv1.2, two related Shaker-like channels, were also found at low levels. The physiological function of these channels is unknown. Astrocytes are thought to play an important role in removal of extracellular potassium released by neurons during periods of activity (Coles and Tsacopoulos, 1979; Ballanyi et al., 1987). Extracellular potassium removal appears to involve both the Na+/K+-ATPase (Ballanyi et al., 1987) and an anion transporter that co-transports K+ and Na+ with Cl- (Walz et al., 1985). Potassium may also be transported by astrocytes from areas of high extracellular potassium to areas of low extracellular potassium in a process called spatial buffering thought to involve voltage-gated ion channels. Evidence already strongly supports involvement of an inward rectifier potassium channel (Newman, 1993) in this process. Delayed rectifier channels such as Kv1.6, Kv1.1 and Kv1.2 might act together with the inward rectifier channels to mediate potassium buffering (Sontheimer, 1994).

To investigate the physiological function of Shaker-like potassium channels in astrocytes, we placed a channel subunit shown to interact in a dominant negative manner with members of the Shaker-like potassium channel subfamily under control of the human GFAP promoter. This promoter has previously been shown to direct expression specifically to astrocytes (Brenner et al., 1994). We used this construct to generate transgenic mice. Astrocytes in these transgenic animals appear to express the dominant negative construct, yet no abnormalities at whole animal or cellular levels have been observed.

Experimental Procedures

Generation of Transgenic Mice

PCR-based mutagenesis was used to make the following changes to a Kv1.2 cDNA clone: 18 amino acids were deleted from the H5 (pore-forming) region (pro361--

asp376); the 10 amino acid minimal human c-myc epitope (glu-gln-lys-leu-ile-ser-glu-glu-asp-leu) was inserted near the amino terminus (between gly17 and his18), upstream of the channel subunit association domain. This cDNA (Kv1.2ΔH5) was transcribed *in vitro* using the Megascript In Vitro Transcription kit (Ambion) for use with an SP6 promoter, and the cRNA was first mixed with an equivalent concentration of wildtype Kv1.2 cRNA, Kv1.6 cRNA, or was injected alone into Xenopus oocytes. Two-electrode voltage clamp was used for measurement of whole-cell currents. The current expression ratio was measured for Kv1.2 alone and Kv1.2+Kv1.2ΔH5 injected cells of the same batch of oocytes, and similarly, Kv1.6 current alone was compared with Kv1.6+Kv1.2ΔH5 current in the same batch of oocytes. Oocyte preparation and electrophysiology were done as described (Hopkins et al., 1994).

The Kv1.2ΔH5 cDNA was subcloned downstream of a 2.2kb portion of 5' flanking DNA of the human GFAP gene extending from -2163 to +47bp, with the ATG at +15 changed to a TTG (Besnard et al., 1991). A complete copy of the human growth hormone gene (hGX) which had been modified to prevent production of active growth hormone (Idzerda et al., 1989) was inserted downstream of Kv1.2ΔH5, producing the final construct (Kv1.2ΔH5gfa+hGX).

Dr. C. Clegg (Bristol Meyers Squibb) injected the Kv1.2ΔH5gfa+hGX DNA into fertilized mouse eggs for production of transgenic animals (C3HeB/C57BL/6 F1). Sixteen founder animals were selected by dot blot analysis, and mated to C3HeB animals. Each transgenic line was maintained by hemizygote intercross. Specific physiological experiments required transgenic homozygotes to be established and bred in addition.

Dot Blotting

Mice were screened for the presene of the transgene by dot blot analysis of genomic DNA prepared from tail clips. 1-10 μg of genomic tail DNA was boiled in 2M NaCl, 100mM NaOH buffer and applied to Nytran (Schleicher and Schuell) nylon membrane using the BioDot apparatus (BioRad). The membrane was rinsed in 2x SSC and baked under vacuum at 80°C. Blots, in duplicate, of potential founder mouse DNA were probed with both hGX and human GFAP promoter sequences. Subsequent generations of mice were screened only with hGX (the entire BamHI/HindIII 2.2kb fragment). Blots were incubated in 4x SSC, 5x Denhardt's, 100μg/ml salmon sperm DNA, and 1% SDS at 65°C. Washing was done for two hours at 65°C in 1x SSC, 0.1% SDS, followed by a higher stringency wash for one hour at 65°C in 0.1x SSC, 0.1% SDS

at 65°C, and exposure to film. Mice screening positively on both blots were selected as founders.

RT-PCR

To determine expression of transgene RNA in offspring of each of the 16 founder animals, total brain RNA isolated from a transgenic mouse of each line was screened by RT-PCR. RNA from two non-transgenic littermates was used as a control. RNA isolation and RT-PCR procedures were as described in Chapter 3. The following primers were used:

5' Kv1.2 forward (Kv1.2f):	5'-GTAAAGCACACTTCTCAAGCC-3'
5' Kv1.2 reverse (Kv1.2r):	3'-GCCTACTCCATGAAGCTAGG-5'
myc reverse (mycr):	3'-CTCGTCTTCGACTAGTCGCT-5'
hGFAP forward (hgfaf):	5'-AGGAGACGCATCACCTCCGC-3'

The 5' Kv1.2 forward and reverse primers were used to amplify a fragment including the 30bp myc epitope from the transgene and to amplify endogenous Kv1.2 sequence as a positive control. The myc reverse/hGFAP forward and hGFAP forward/5' Kv1.2 reverse primer pairs were used to amplify product uniquely from transgenic RNA. A 751bp SacI fragment spanning the hGAP promoter/Kv1.2 junction of Kv1.2ΔH5gfa+hGX was radiolabeled and used as a probe. Seven transgenic lines with expression levels ranging from high to low were selected for further breeding.

Cell Culture

Homozygous transgenic animals were intercrossed with homozygotes of the same line (when homozygotes of both sex were available) or with wildtype littermates, to produce offspring of known transgenic genotype. Litters from these breeding pairs were used to produce astrocyte cultures, by the procedure described in Chapter 3.

Electrophysiology

Type-1-like astrocytes (flat, non-process bearing) in 35x10mm dishes (Falcon) coated with poly-D-lysine (Sigma) were used for electrophysiology 2-7 days after replating. Membrane currents were measured using the whole-cell patch clamp technique at room temperature. Patch pipettes were fabricated from VWR 75μl hematocrit glass

using a two-pull method and fire-polished to 1-2μm tip diameter. Recording pipettes were filled with 140mM KCL, 1mM $MgCl_2$, 10mM EGTA, 5mM HEPES, pH 7.4. This solution was diluted 15% prior to use (Barres et al., 1990a). The bath solution contained 140mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM KCl, 5mM HEPES, pH 7.4. Equipment and software were as described in Chapter 3.

Immunocytochemistry

Expression of transgenic protein in cultured astrocytes was assessed with anti-c-myc monoclonal antibody 9E10 (α-myc, Santa Cruz Biotechnology). Replated cells in 35x10mm tissue culture dishes (Falcon) were labeled with either α-GFAP or α-myc, by the procedure described in Chapter 3. The α-myc antibody was used at a concentration of 0.5μg/ml. Goat anti-mouse IgG-FITC (Molecular Probes) at a dilution of 1:200 was used as the secondary antibody. Labeled cells were examined using a fluorescence microscope.

Expression of transgenic protein in sections of adult mouse brain was also assessed using α-myc. Mice were perfused with approximately 40ml 2% paraformaldehyde in 1x PBS. Brains were removed, incubated in 2% paraformaldehyde in 1x PBS for 4 hours at 4°C, and rinsed twice with cold 1x PBS. Each brain was then taken through a series of overnight sucrose incubations consisting of 20% sucrose in 1x PBS, 30% sucrose in 1x PBS, 30% sucrose in 1x PBS: OCT (1:1), and frozen in OCT at -80°C. 20μm parasaggital sections were cut on a Riechert-Jung 2800 Frigocut N and mounted on silanized slides. For α-myc immunocytochemistry, brain sections were washed twice for 5 minutes each in 0.1M Tris, pH 8.0, rinsed twice in dH_2O, and air-dried. Sections were then incubated in blocking buffer (3% BSA, 3% normal goat serum, 0.2% Triton X-100, 0.1M Tris, pH 7.4, 150mM NaCl) for 4 hours at room temperature. An incubation in α-myc diluted 1:300 in blocking buffer was carried out for 60 hours at 4°C in a moist chamber, followed by 5 rinses for 10 minutes each in TBS (0.1M Tris, pH 7.4, 150mM NaCl) at room temperature. Sections were then incubated in goat anti-mouse IgG-Texas Red (Molecular Probes) at 1:300 in blocking buffer for 1 hour at room temperature, rinsed as before in TBS with the addition of a final dH_2O rinse, and air-dried. The brain sections were coverslipped with Vectashield fluorescent mounting medium H-1000 (Vector Laboratories). Images were collected using an MRC-1000 Laser Scanning Confocal Imaging System (BioRad).

Flurothyl-Induced Seizures

Adult transgenic mice and their wildtype littermates were exposed to the convulsant agent flurothyl (2,2,2-trifluroethyl ether, Sigma) at ages 2-7 months. Procedures were as described in Chapter 2, with two changes. First, testing was done completely blind to genotype, as the transgenic animals display no overt phenotype. Also, this group of animals displayed a clear temporal separation between initial seizure and tonic extension and both time points were recorded.

Results

Generation and Characterization of Transgenic Mice

Kv1.2 cDNA was altered by insertion of the minimal human c-myc epitope to allow specific detection and by removal of the H5 or pore-forming region to create a channel subunit which would inhibit the function of normal subunits of the Shaker-like subfamily when co-expressed in the same cell. Studies of this altered subunit (Kv1.2ΔH5) in the Xenopus oocyte expression system showed that co-injection of wildtype Kv1.2 or Kv1.6 cRNA and Kv1.2ΔH5 cRNA in a 1:1 ratio resulted in only 11% and 6% respectively of the current produced by injecting either of the wildtype cRNAs alone (Figure 13). Injection of Kv1.2ΔH5 cRNA produced negligible current alone. Kv1.2ΔH5 appears then to act as a dominant negative inhibitor of Shaker-like potassium channel function in Xenopus oocytes. Research which has demonstrated a lack of interaction between subunits from different channel subfamilies (Covarrubias et al., 1991; Rettig et al., 1992) suggests that Kv1.2ΔH5 would not interfere with Shal-, Shab- or Shaw-like potassium channel function.

The dominant negative construct was placed downstream of a 2.2kb 5' sequence derived from the human GFAP gene which has been shown to contain the basal promoter and additional sequences both necessary and sufficient for cell-type specific expression (Besnard et al., 1991). This promoter region has been shown to direct expression of a lacZ reporter gene specifically to astrocytes in transgenic mice (Brenner et al., 1994). A complete human growth hormone gene (hGX), altered to prevent production of active growth hormone, was attached 3' of mKv1.2ΔH5 to enhance expression (Figure 14).

The final construct (Kv1.2ΔH5gfa+hGX) was injected into fertilized mouse eggs for productin of transgenic mice. Animals were screened by dot blot analysis of tail genomic DNA (Figure 15). Twenty-one of 144 animals proved positive for integration of

the transgene. Of these mice, 16 were chosen as fouders and crossed to C3HeB partners. Expression of transgenic transcript was assessed in transgenic offspring of each of the founders. RT-PCR analysis of brain RNA from the transgenic mice and wildtype littermate controls with primer pairs specific for transgenic sequence revealed a range of levels of expression of transgene message (Figure 16). Two lines showed low expression (10502, 10545), three lines showed quite high expression (10568, 10572, 10604), and the remainder showed a middle range of message levels. A primer pair recognizing sequences in the amino terminus of both the Kv1.2ΔH5 construct and the endogenous Kv1.2 channel were included to provide a positve control for RNA quantity and condition (Kv1.2f/Kv1.2r). On the basis of RT-PCR results, fertility and consistent passage of transgene to offspring, seven transgenic lines were selected for continued maintenance and study (10502, 10544, 10602, 10628, 10529, 10568, 10604). One low, two high and four medium expressing lines were maintained.

α-Myc Labeling of Cultured Astrocytes and Brain Sections from Transgenic Mice

Astrocyte cultures from several transgenic lines (10602, 10502, 10529, 10544) were stained with α-myc antibody to assess expression of Kv1.2ΔH5gfa+hGX protein. Labeling was apparent, yet non-uniform, indicating intercellular variability in production or stability of the transgenic protein (data not shown). This variability was confirmed by α-myc staining of brain sections from a homozygous transgenic mouse of the 10529 line (Figure 18) [experiment done by Janice Hallows]. The α-myc clearly labeled stellate cells with the morphological appearance of astrocytes (Figure 18A). However, staining of adjacent sections with α-GFAP demonstrated the presence of many more GFAP-positive than myc-positive cells in the same brain region (Figure 18B). These results indicate that transgenic protein is produced in cells which appear to be astrocytes, yet represent only a subset of the total astrocyte population. The reasons for this variability and the expression pattern in brains of mice from other transgenic lines remain unknown.

Potassium Currents in Cultured Astrocytes from Transgenic Mice

Homozygous transgenic animals were intercrossed when possible, or mated with non-transgenic littermates, to produce litters of known genotype (homozygous or hemizygous transgenic, respectively). The mouse pups were used for production of cortical astrocyte cultures, and whole-cell voltage clamp recording was used to assess

expressed currents at two to seven days following replating. Astrocytes from four transgenic lines were analyzed (10502, 10602, 10628, 10529) and all displayed outward delayed rectifier potassium currents not obviously different in amplitude or waveform from currents in non-transgenic astrocytes (Figure 17).

Measurement of Flurothyl-Induced Seizure Threshold in Transgenic Mice

To address the question of an effect of the transgene on CNS physiology, potentially through alteration of extracellular potassium buffering by astrocytes, transgenic mice and their non-transgenic littermates from several lines were exposed to the volatile convulsant flurothyl. Latencies to seizure and to terminal tonic extension were measured. No differences in latency to tonic extension were found. Only in animals of the 10529 line was a difference in seizure threshold from wildtype noted (Figure 19). The 10529 hemizygous transgenic mice had a latency to seizure of 2.13 minutes compared with a wildtype latency of 2.85 minutes. The 10529 homozygous transgenic mice had a futher decreased latency of 1.26 minutes. The presence of a difference in seizure threshold in only one of the four lines tested, coupled with the observation that homozygous transgenic animals of only the 10529 line appear smaller than their littermates, raises the possibility that the integration site rather than expression of the Kv1.2ΔH5gfa+hGX transgene in this line might underly the decreased latency to flurothyl-induced seizure.

Discussion

We have produced transgenic mice expressing a dominant negative potassium channel subunit shown to interfere with wildtype Shaker-like potassium channel currents in Xenopus oocytes. We had previously identified transcripts for the Shaker-like channels Kv1.1, Kv1.2 and Kv1.6 in cultured astrocytes, as discussed in Chapter 3, and attempted to ablate the function of such channels in astrocytes *in vivo* through expression of the Kv1.2ΔH5gfa+hGX transgene.

The transgene is apparently expressed at RNA and protein levels, and preliminary histological analysis suggests that this expression may be limited to astrocytes. However, transgenic protein is apparent in only a subset of the astrocyte population. *In vivo* dominant negative action of this protein is also unclear, as no obvious diminution of voltage-gated outward potassium currents in cultured astrocytes was observed. It is

possible that the small number of cells from which currents were recorded did not include any of a subset producing transgenic protein. Also, there may exist a sufficient number of potassium channels from other subfamilies in astrocytes to mask results of any ablation of Shaker-like current. Whatever is occurring at the cellular level, no impact of the transgene on whole animal physiology was apparent. Transgenic mice appear overtly normal. Flurothyl seizure latency measurement demonstrated a reduced seizure threshold in only one transgenic line, and an influence of the integration site remains a possibility. We may conclude then that the production of transgenic mice was technically successful, but may not have achieved the initial goal to diminish outward potassium currents *in vivo* in astrocytes and allow elucidation of their role in whole animal physiology.

Further studies should be done to clarify both the level of transgene expression and its functional impact in these mice. Immunocytochemical colocalization of myc and GFAP would indicate how large a subset of astrocytes is expressing the transgenic protein in each of the transgenic lines. Assessment of any decrease in DTX block of current in transgenic compared with wildtype astrocytes would allow quantification of transgenic effect on endogenous potassium channels. Finally, assessment of any alteration in potassium dynamics or in the response to elevation of extracellular potassium levels in transgenic brain slices might reveal subtle physiological alterations in these animals.

Figure 13. Inhibition of Kv1.2 and Kv1.6 Current Expression in Xenopus Oocytes by Kv1.2ΔH5

Xenopus oocytes were injected with either Kv1.2 or Kv1.6 cRNA alone, or a 1:1 ratio of Kv1.2 or Kv1.6 cRNA with Kv1.2ΔH5 cRNA. Two-electrode voltage clamp was used for measurement of whole cell currents. Average current responses to a 50mV voltage pulse are shown. The average currents were: for Kv1.2 alone, 14.89μA (n=8, SEM=2.63); for Kv1.2:Kv1.2ΔH5, 1.70μA (n=8, SEM=0.35); for Kv1.6 alone, 4.04μA (n=6, SEM=0.49); for Kv1.6:Kv1.2ΔH5, 0.24μA (n=6, SEM=0.15). Injection of Kv1.2ΔH5 alone produced no current and is not shown.

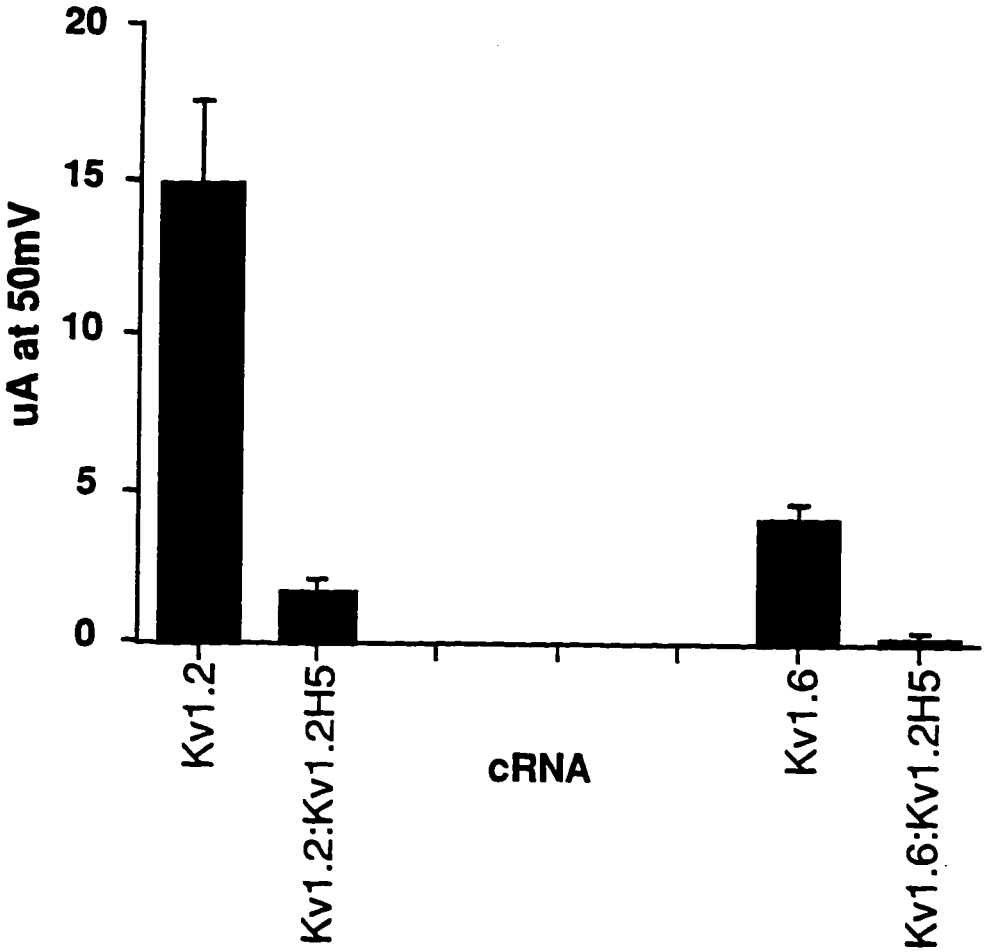

20
15
10
5
0
uA at 50mV
Kv1.2
Kv1.2:Kv1.2H5
Kv1.6
Kv1.6:Kv1.2H5
cRNA

Figure 14. Transgene Construct

A 2.2kb promoter region of the human glial fibrillary acidic protein gene (hgfa) was placed upstream of the dominant negative channel subunit (Kv1.2ΔH5), which was then followed by a copy of the human growth hormone gene altered to prevent production of functional hormone (hGX).

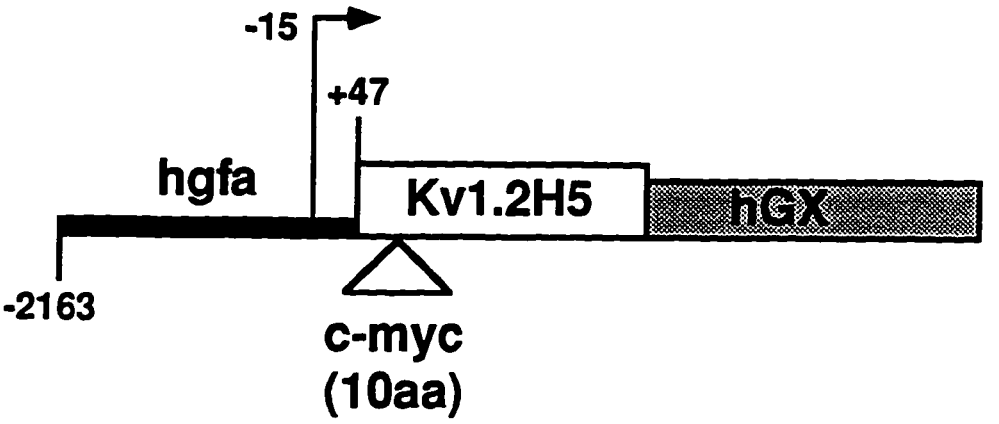
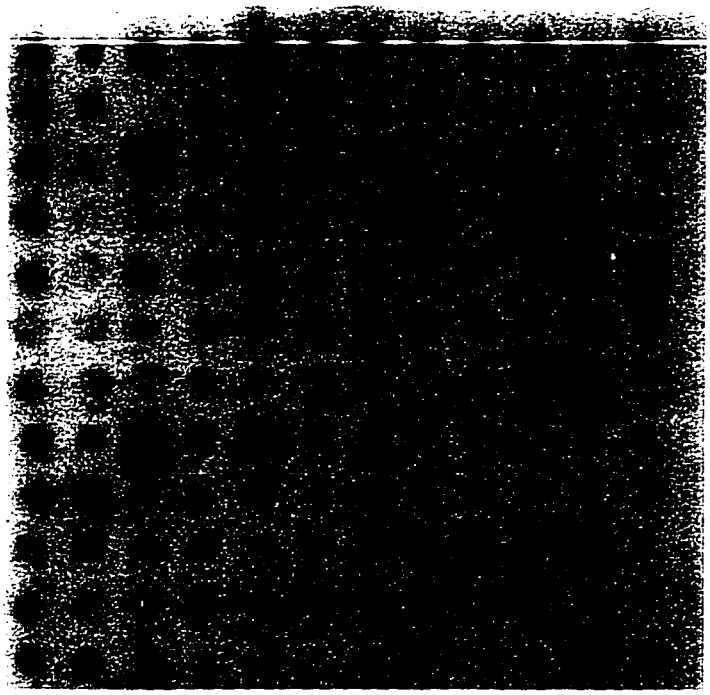

-15
+47
hgfa
Kv1.2H5
hGX
-2163
c-myc
(10aa)

Figure 15. Dot Blot Analysis of Potential Transgenic Founder Mice

Tail genomic DNA from offspring of fertilized eggs injected with transgene construct DNA was analyzed by dot blot. Duplicate blots were probed with hGX or hgfa probes, and 16 mice found positive for transgene presence on both blots were selected for breeding as founders.

hgfa



hGX

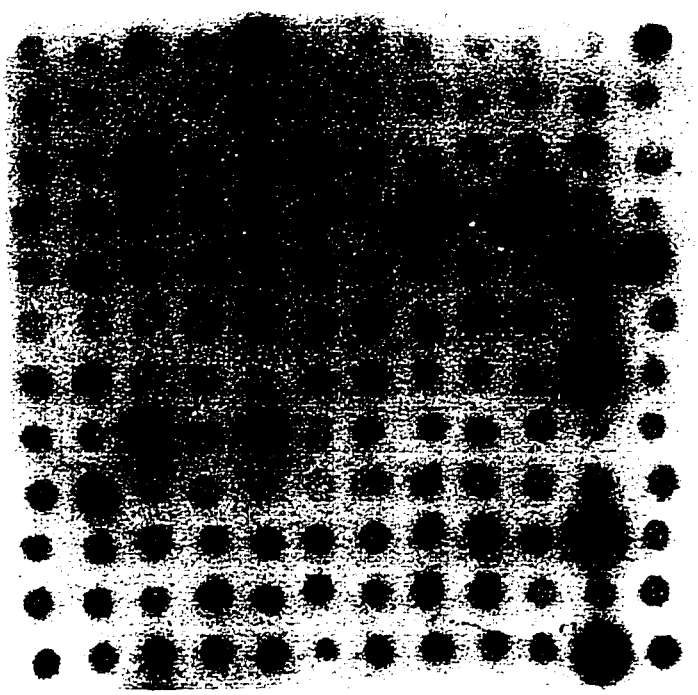


Figure 16. Screening for Transgene Expression by Founder Offspring

RNA isolated from brains of transgenic mice of each founder line, and two wildtype littermate controls, was used in RT-PCR amplification with two primer pairs specific for transgenic sequence (hgfaf/Kv1.2r, hgfaf/mycr) and one primer pair (Kv1.2f/Kv1.2r) which recognized transgenic as well as wildtype Kv1.2 sequence. An aliquot of each RNA sample was treated with RNase to control for genomic DNA contamination, and such samples are designated with (-). Autoradiographs of RT-PCR Southern blots are shown here, and indicate a range of transgene expression among founders.

hgfaf/Kv1.2r-313bp
Kv1.2f/Kv1.2r-240bp,270bp
hgfaf/mycr-144bp
tg10502
tg10502(-)
wildtype
wildtype(-)
tg10538
tg10538(-)
tg10544
tg10544(-)
tg10545
tg10545(-)
tg10602
tg10602(-)
hgfaf/Kv1.2r-313bp
Kv1.2f/Kv1.2r-240bp,270bp
hgfaf/mycr-144bp
tg10514
tg10514(-)
tg10515
tg10515(-)
tg10529
tg10529(-)
tg10568
tg10568(-)
tg10572
tg10572(-)
tg10604
tg10604(-)
hgfaf/Kv1.2r-313bp
Kv1.2f/Kv1.2r-240bp,270bp
hgfaf/mycr-144bp
tg10489
tg10489(-)
tg10512
tg10512(-)
tg10574
tg10574(-)
wildtype
wildtype(-)
tg10625
tg10625(-)
tg10628
tg10628(-)

Figure 17. Outward Currents in Cultured Transgenic Astrocytes

Current traces were obtained in response to a family of depolarizing steps between -40mV and +95mV (15mV increments) from a holding potential of -60mV, using the whole-cell voltage clamp technique. Type-1-like (flat, non-process bearing) astrocytes prepared from transgenic mouse pups of several lines were used for recording 2-7 days after replating. Outward delayed rectifier currents were observed in astrocytes of all transgenic lines tested which appeared similar to wildtype.

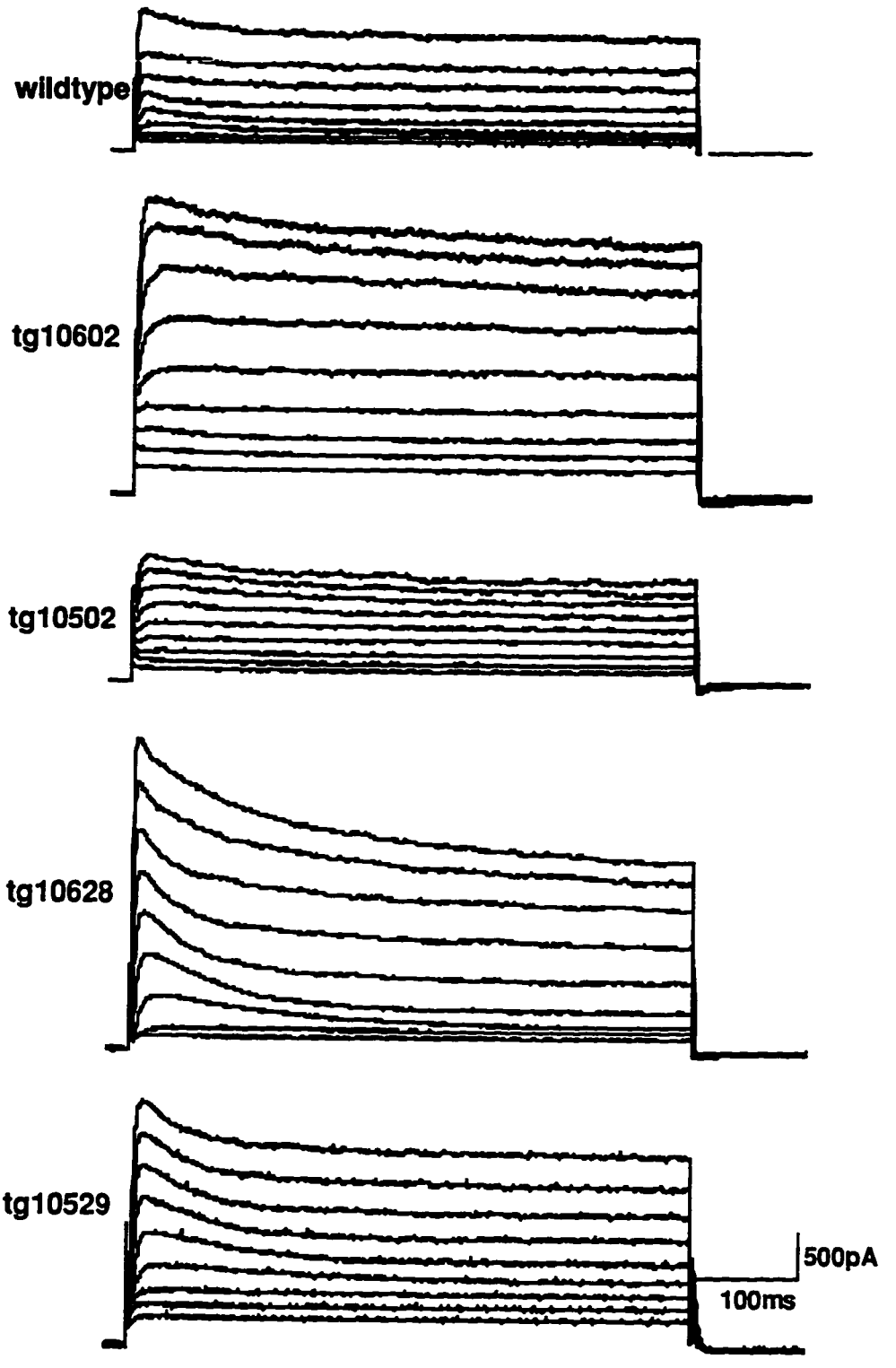

wildtype
tg10602
tg10502
tg10628
tg10529
500pA
100ms

Figure 18. Immunocytochemical Staining of Astrocytes in Transgenic Mouse Brain Sections

Brain sections from a homozygous transgenic adult mouse of the 10529 line were labeled with either α–myc or α-GFAP.

(A) A stellate cells with the morphological appearance of an astrocyte was labeled with α-myc.

(B) A comparison of α-GFAP and (C) α-myc labeling in adjacent brain sections suggests that only a subset of GFAP-positive astrocytes express the transgenic protein.

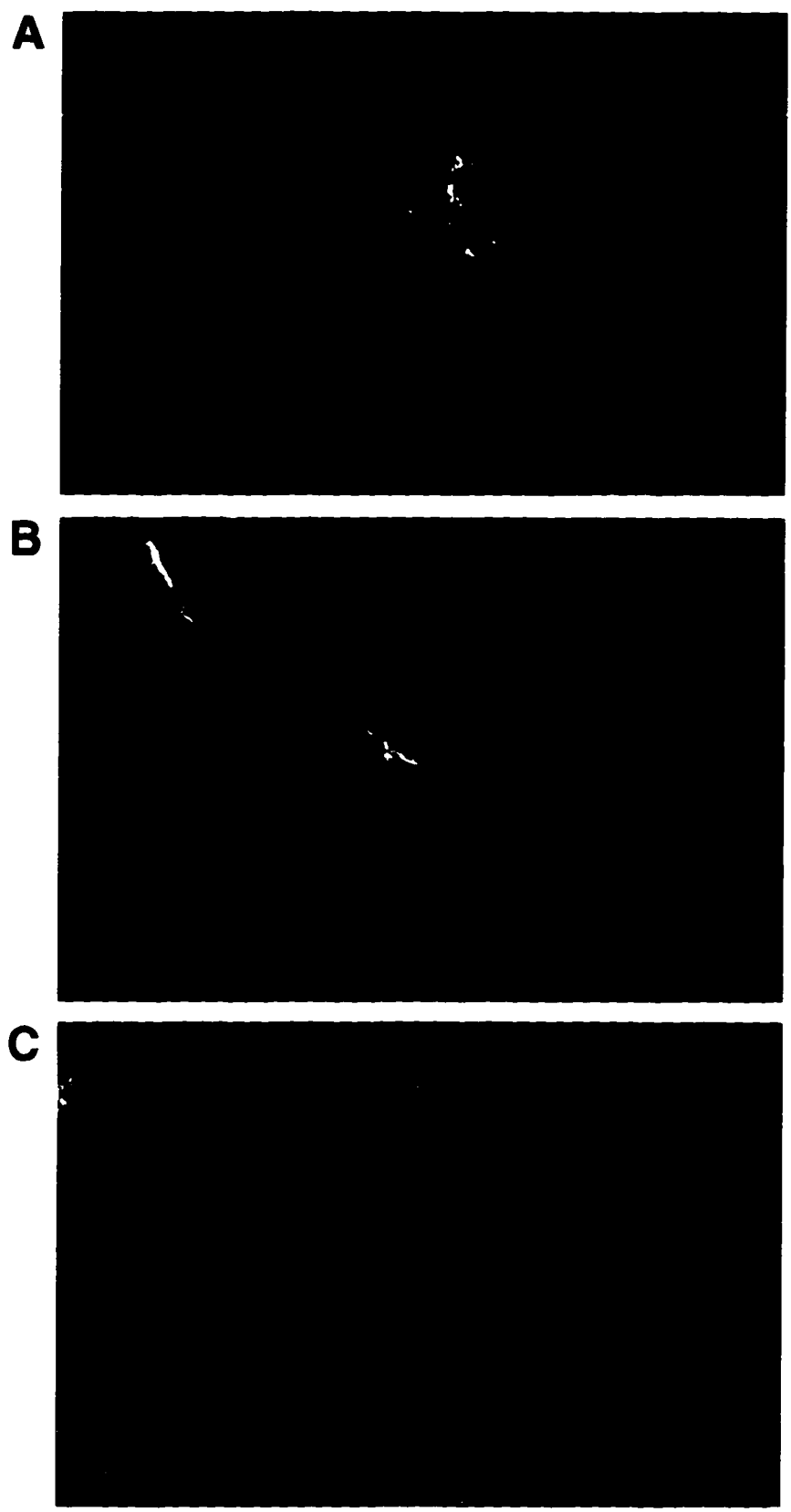
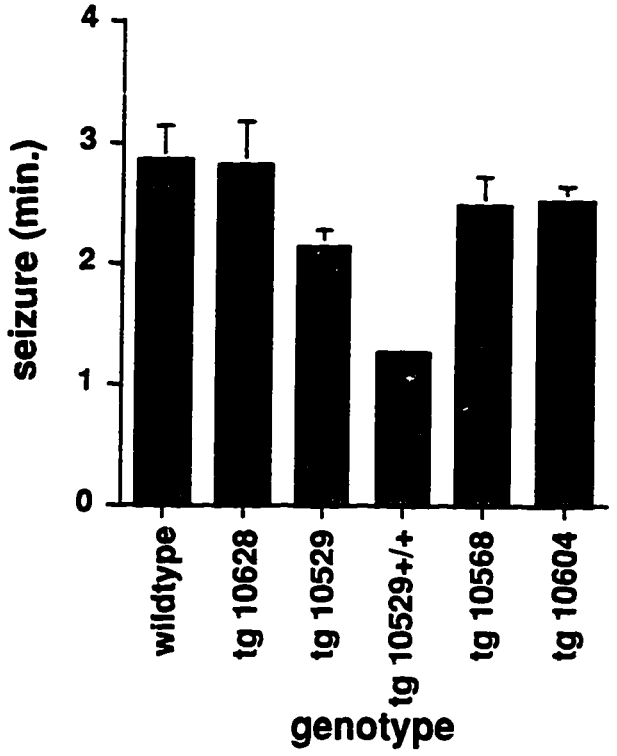
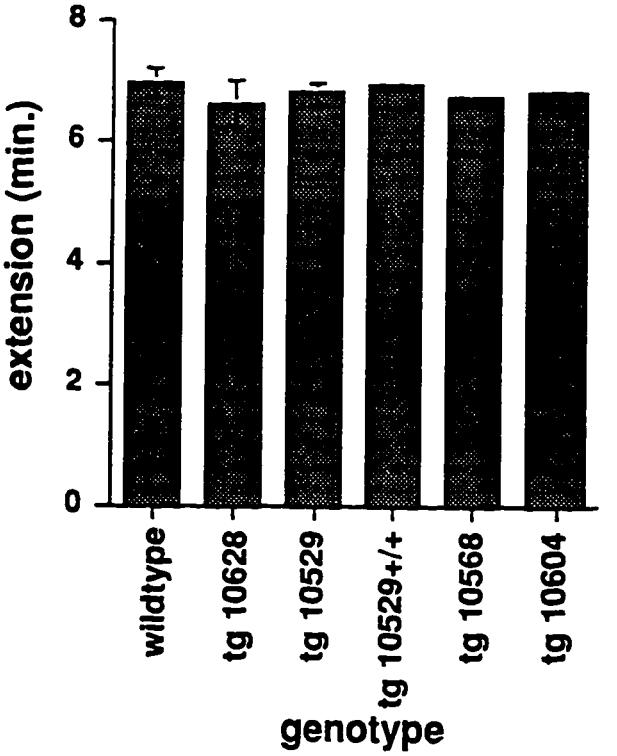

A
B
C

Figure 19. Latency to Flurothyl-Induced Seizure and Tonic Extension in Male Transgenic Mice

Genotype is plotted on the abscissa, and mean latency to seizure (top graph) or tonic extension (lower graph) in minutes is plotted on the ordinate. The wildtype mean was obtained from all male wildtype littermates of lines tested. Error bars indicate standard error of the mean (SEM). Data shown is from hemizygous transgenic animals, with the exception of data from two homozygous transgenic mice of the 10529 line (tg 10529 +/+). Only the 10529 mice seized with an apparently shortened latency, of 2.13 minutes (SEM=0.15) compared with a wildtype latency of 2.85 minutes (SEM=0.29), a difference significant at the level of $p<0.1$. The 10529 homozygous mice showed a further decrease in latency of 1.26 minutes ($p<0.05$). No other differences were noted in seizure or tonic extension latencies.


4
3
2
1
0
seizure (min.)
wildtype
tg 10628
tg 10529
tg 10529+/+
tg 10568
tg 10604
genotype


8
6
4
2
0
extension (min.)
wildtype
tg 10628
tg 10529
tg 10529+/+
tg 10568
tg 10604
genotype

CONCLUSIONS

Modern molecular genetic techniques may serve as highly specific tools for dissection of the complex functions of the mammalian central nervous system. These methods facilitate identification of single gene products and their roles in nervous system physiology, and provide insight into human genetic diseases. In the mouse, historically the best-studied model system of mammalian genetics and physiology (Dietrich et al., 1995), two complementary approaches are available, forward (phenotype to gene) and reverse (gene to phenotype) genetics (Takahashi, 1994). Forward genetics represents the more established method, originally applied to study of simpler organisms such as Drosophila, and involves the initial observation of an abnormal phenotype of interest followed by genomic mapping and cloning of the responsible gene. The forward genetic method depends upon the occurrence of spontaneous mutants, or the use of chemical mutagenesis to induce random mutations and the identification of resultant phenotypic abnormalities. Disadvantages include the labor intensity and time required for isolation of the mutant gene, as well as the possibility that a mutation will not arise which effects the nervous system function of paricular interest to the researcher. The forward genetic method does provide the clear advantage of potential identification of previously unknown genes of critical physiological importance.

Within the past decade, technological advancements have made possible the alternate reverse genetic approach, in which transgenic techniques are used to alter or inactivate a normal gene. Identification and cloning of the gene of interest is the first step. Targeted inactivation or alteration of this gene by replacement in cultured ES cells follows and the targeted ES cells are used to produce mice carrying the mutant allele. The initial step in a forward genetic approach is the final step in the reverse genetic approach--careful assessment of any abnormal phenotype. This method allows evaluation of the physiological impact of inactivation of a gene of particular interest, and can produce phenotypic abnormalities which have not occurred previously by spontaneous mutation.

The study of mice having specific mutations in identified genes, resulting from either of the discussed approaches, allows inferences to be drawn about the normal functions of the gene product through assessment of functional deficits occurring in its absence. Such inferences must be qualified by the recognition that both developmental effects of the mutation and compensatory responses may contribute to the observed

physiology. Conclusions about the normal role of the mutant gene product gained from genetic studies should therefore be validated through comparison with results obtained from histological and pharmacological studies in wildtype mice. Because the mutant animal represents the systemic response to absence of the particular gene product throughout its lifespan, it serves as a more appropriate model of genetic disease than would pharmacological intervention in the normal adult mouse (Davies et al., 1994). Genetic mouse models provide both clues to genetic causes of similar disease states in humans, and an accessible system for invasive study of the cellular physiological derangements underlying the overt phenotype.

A typical reverse genetic strategy has been successfully used in the production of the Kv1.1-null mice. These mice display an overtly epileptic phenotype, confirming the suspected role of Kv1.1 in controlling overall neuronal hyperexcitability. They provide a system for study of specific cellular functions of Kv1.1 suggested by previous pharmacological and immunocytochemical analysis, and a model system of epilepsy. As discussed above, inferences drawn about specific functions of Kv1.1 from study of mutant mice must be qualified by the possible influence of developmental and compensatory changes. We have seen no indication of gross developmental anomalies or embryonic lethality, yet Kv1.1 is expressed at early stages in the developing embryo (Janice Hallows, personal communication). Research into Kv1.1-null mouse early development and detailed histological analysis of the Kv1.1-null mouse adult brain would help to address the issue of a developmental impact. The most likely candidate for compensation for absence of Kv1.1 is the related Kv1.2 channel, which is extensively colocalized with Kv1.1 in the normal mouse (Wang et al., 1994). No compensatory upregulation of Kv1.2 transcript has been found, and the Kv1.2 protein distribution appears unchanged in the Kv1.1-null mouse. However, many other delayed rectifier channels are also present in the mouse nervous system, and some compensation from any of these channels cannot be entirely ruled out. More remote compensation, such as postsynaptic receptor downregulation in response to increased transmitter release from terminals normally expressing Kv1.1, is also possible.

Although seizures in the Kv1.1-null mice raises the possibility of an as yet unidentified identical genetic deficit in heritable forms of human epilepsy, these animals provide a useful model for the study of epilepsy even in the absence of a specifically analogous human disease. They define a key cellular site of vulnerability to seizure induction, and will allow delineation of the specific pathways leading from a persistent

cellular deficit in repolarization to episodic synchronized population activity. Mechanisms of spread of such activity from one brain region to another can also be analyzed. Depth electrodes may be useful for identification of the initial focus or foci. Interaction of seizure disorders and development may also be studied in these animals. Many homozygous Kv1.1-null pups die at an early age, raising the question of modulation of seizure severity and outcome by specific developmental factors. Some mice do survive, and it is unknown how they differ from their littermates who die. Is there a precipitating environmental event prior to the fatal seizure? Is there an influence of genetic background or maternal care, as the inbred 129Sv/J strain appears to have a higher mortality rate than do the hybrid Swiss Black/ 129Sv/J mice? Early pharmacological intervention to prevent seizures will help to determine whether seizures themselves, as is suspected, or another primary cause is responsible for the deaths. Anticonvulsant treatment, and comparison of treated with untreated animals, will also be informative about occurrence of seizure-induced damage, its influence on the epileptic phenotype, and whether there are critical periods during which pharmacological intervention can have the greatest beneficial impact.

The Kv1.1-null heterozygotes may also be useful, as a model of potential genetic susceptibility to induction of epilepsy by a secondary insult. They appear overtly normal yet show a decreased latency to flurothyl-induced seizures, and several very old (one year) heterozygotes have displayed seizure behaviors. Can a second factor, from the environment or the aging process, induce epilepsy in these animals more readily than in wildtype mice? Are such factors similar to events precipitating seizures in humans?

A less common reverse genetic approach was taken to produce mice deficient in astrocytic delayed rectifier potassium channel activity. A dominant negative channel subunit was engineered and used in the production of transgenic mice. The transgene was expected to be expressed only in astrocytes because of the specificity of the GFAP promoter, and to produce a subunit able to combine with and inactivate endogenous astrocytic Shaker-like potassium channels, particularly Kv1.6. This approach provides the potential advantage over direct gene inactivation of cell type specificity of effect. Any physiological deficit or observed phenotype should then be ultimately traceable to altered astrocyte function. A direct knockout of the Kv1.6 gene would be expected to impact neuronal activity directly and possibly mask effects of altered astrocyte physiology. The ability of the dominant negative subunit to assemble with any member of the Shaker-like potassium channel subfamily additionally increases the probability of

effect, in the event that not Kv1.6 but rather a related channel is predominant in astrocytes *in vivo*.

The transgenic mice display no overt phenotype, and voltage clamp recordings from cultured astrocytes from transgenic mouse cortex have normal appearing delayed rectifier potassium currents. Technical problems with the dominant negative strategy are one possible explanation for these findings. Immunocytochemical analysis of brain tissue from one transgenic mouse revealed expression of the transgene apparently in only a subset of astrocytes. The predominant Shaker-like potassium channel identified in astrocytes, Kv1.6, also shows variable expression, raising the possibility of only a small population of astrocytes with overlapping expression of both the transgene and the targeted Kv1.6 channel. Production of too little transgenic protein or failure of assembly of transgenic subunits with endogenous channel subunits could also prevent dominant negative activity. Alternatively, the transgene may effectively ablate astrocytic Shaker-like channel function in the transgenic mice but this ablation may be without strong physiological effect on the animal under normal conditions. The dendrotoxin studies of process-bearing astrocytes show sensitivity of only approximately 15-45% of the outward current, a difference which would not have been obvious in our recordings from the transgenic astrocytes. Assessment of DTX block of current in transgenic compared with wildtype cultured astrocytes would allow quantitation of transgenic effect. Immunocytochemical colocalization of myc and GFAP in brain slices and cultured astrocytes from transgenic animals from multiple lines would also be informative about how large a population of astrocytes really expresses the transgene, and at what level. Positive findings of transgene effect and reasonable expression in such studies would suggest the importance of evaluation of these mice for subtle physiological changes, or changes which only become apparent under abnormal conditions.

Particular attention should be paid to the 10529 line. The observation of a decrease in seizure threshold only in this transgenic line, of four lines tested, raises two possibilities. Either 10529 represents the only line in which the dominant negative transgene is successfully achieving strong effect, or it represents the result of an insertional mutation. The small size of homozygotes of this line is potentially suggestive of the latter possibility. In either case, the 10529 mice should be given priority for further electrophysiological and immunocytochemical study, and possibly more thorough seizure theshold testing.

The Kv1.1-null mice and the Kv1.2ΔH5gfa+hGX transgenic mice reveal the possibilities for success as well as difficulties in using a reverse genetic approach to investigate the specific physiological impact of a gene product. Clearly, any reverse genetic experiment is very expensive given the large number of animals involved, requires years before results are obtained, and may be ultimately uninformative about a gene's function if no phenotypic abnormality results. Because of these considerations, this approach should be applied only after formation of a specific hypothesis which cannot be tested in a simpler *in vitro* system, and reasonable expectation, from prior thorough experimentation, of a useful mammalian model system resulting. This experimental approach, when used appropriately, can however yield uniquely valuable results, as is particularly well illustrated by the Kv1.1-null mice.

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1. Smart, S.L., Robbins, C.A., Chiu, S.Y., Wang, H., Schwartzkroin, P.A., Messing, A., and B.L Tempel. Potassium channel targeted deletion causes epilepsy in mice. (Submitted).

2. Smart, S.L. and B.L Tempel. Identification of a delayed rectifier potassium channel, Kv1.6, expressed in astrocytes. (In Preparation).

3. Smart, S.L., Hopkins, W.F. and B.L Tempel. Mutagenesis of the S3 region of mKv1.2 alters voltage-dependence of activation. (In Preparation).

Abstracts

1. Smart, S.L., Hopkins, W.F. and B.L Tempel. (1994) Mutagenesis of the S3 region of mKv1.2 alters voltage-dependence of activation. *Biophysical Society Meeting*. New Orleans, LA.

2. Smart, S.L., Messing, A., Chiu, S.Y., Schwartzkroin, P.A. and B.L Tempel. (1995) Potassium channel targeted deletion causes epilepsy in mice. *Society for Neuroscience Meeting*. San Diego, CA.