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Expression of Voltage-Gated Potassium Channel Genes

by Neonatal Rat Peripheral Neurons.

by

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Abstract:

Voltage-gated potassium currents make important contributions to neuronal function. Postsynaptically, potassium currents regulate excitability to depolarizing inputs and determine action potential firing frequency. Furthermore, voltage-gated potassium channels are known to affect the shape of the presynaptic action potential, so as to influence calcium influx and neurotransmitter release. I have focused on the expression of voltage-gated potassium channel genes by peripheral neurons. Among my objectives were to determine which voltage-gated potassium channel genes are expressed by sympathetic neurons of the superior cervical ganglia (SCG) and measure mRNA levels for these genes during normal neonatal development and during development in culture. I found that P7 SCG neurons expressed low levels of Kv1.4, Kv2.2 and Kv3.1 mRNAs, moderate levels of Kv2.1 and Kv3.3 mRNAs, and high levels of Kv3.4 and Kv4.2. Where the expression level of Kv1.4, Kv2.1, Kv2.2, Kv3.1 and Kv3.3 mRNAs did not change significantly, neither during development *in vivo* nor *in vitro*, both Kv3.4 and Kv4.2 mRNA levels underwent significant developmental changes. I found that Kv3.4 mRNA levels increased sixfold during neonatal development, and remained expressed at high levels or increased during culture. In contrast Kv4.2 mRNA levels increased thirty-fivefold during neonatal development, but in culture Kv4.2 mRNA levels decreased to low or very low levels. Additionally, culturing SCG neurons in the presence of the cytokine ciliary neurotrophic factor (CNTF) failed to influence the *in vitro* developmental mRNA levels of Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.3 nor Kv4.2. Only the expression level of Kv3.4 was significantly increased by CNTF. I also measured the mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 in sensory neurons of the nodose and trigeminal ganglia. I found that Kv2.1, Kv3.1, Kv3.3 were expressed at similar levels to SCG neurons, Kv3.2 mRNA was detectable, Kv1.4 and Kv2.2 mRNAs were expressed at significantly higher levels by sensory neurons, where as Kv3.4 and Kv4.2 mRNAs were expressed at significantly lower levels by nodose and trigeminal neurons.

Kv4.2 and Kv1.4 likely contribute to the SCG rapidly inactivating transient current (IAf). Furthermore, changes in Kv4.2 mRNA levels may account for the developmental changes in IAf density *in vivo* and *in vitro*. Kv2.1, Kv2.2, Kv3.1, Kv3.3 and Kv3.4 channels likely contribute to the SCG slowly inactivating transient current (IAs), perhaps as heteromeric channels. Changes in mRNA levels do not account for developmental changes in IAs current density. Moreover, the increase in Kv3.4 mRNA levels in SCG neurons cultured with CNTF does not account for the sustained expression of IAf and IAs. Similar to SCG neurons, Kv1.4 and Kv4.2 likely contribute to IAf on nodose and trigeminal neurons, whereas, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3 and Kv3.4 likely contribute to nodose and trigeminal IAs.

Résumé:

Les canaux potassiques voltage-dépendants ont un rôle de première importance dans la fonction neuronale. Par exemple, au niveau postsynaptique, ce sont des courants potassiques qui contrôlent l'excitabilité en réponse aux inputs dépolarisants et déterminent la fréquence des potentiels d'action. D'autre part, les canaux potassiques voltage-dépendants modulent la forme des potentiels d'action présynaptiques de façon à influencer l'entrée de calcium et la libération de neurotransmetteur. Je me suis concentré sur l'expression des gènes des canaux potassiques voltage-dépendants par les neurones périphériques. Un de mes objectifs était de déterminer lesquels de ces gènes sont exprimés par les neurones sympathiques des ganglions cervicaux supérieurs (GCS) et de mesurer les niveaux d'ARNm de ces gènes durant le développement néonatal normal ainsi que durant le développement en culture. J'ai ainsi découvert que des neurones P7 (septième jour post-natal) du GCS expriment de bas niveaux d'ARNm pour Kv1.4, Kv2.2 et Kv3.1, des niveaux modérés pour Kv2.1 et Kv3.3 et des niveaux élevés pour Kv3.4 et Kv4.2. Alors que les niveaux d'expression des ARNm de Kv1.4, Kv2.1, Kv2.2, Kv3.1 et Kv3.3 n'ont pas changé de manière significative durant le développement *in vivo* ou *in vitro* des neurones, ceux de Kv3.4 et Kv4.2 ont tous deux subi des variations significatives. J'ai trouvé que les niveaux d'ARNm de Kv3.4 sont multipliés par six au cours du développement néonatal et, en culture, sont maintenus élevés ou augmentent aussi. Par contre, les niveaux d'ARNm de Kv4.2 sont multipliés trente-cinq fois au cours du développement néonatal mais, en culture, chutent jusqu'à des niveaux bas ou même très bas. De plus, les cultures de neurones de GCS ne sont pas affectées par le facteur neurotrophique ciliaire (CNTF), une cytokine, en ce qui concerne les niveaux d'ARNm de Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3 et Kv4.2: seule l'expression de Kv3.4 augmente avec le CNTF. J'ai également mesuré les niveaux d'ARNm de Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.3, Kv3.4 et Kv4.2 dans les neurones sensoriels des ganglions nodoses et trijumeaux. J'ai découvert que les ARNm de Kv2.1, Kv3.1 et Kv3.3 y sont exprimés à des niveaux semblables aux GCS, que l'ARNm de Kv3.2 y est détectable et que les ARNm de Kv1.4 et Kv2.2 y sont exprimés à des niveaux significativement plus élevés dans les neurones sensoriels tandis que que ceux de Kv3.4 et Kv4.2 le sont à des niveaux significativement plus bas.

Kv4.2 et Kv1.4 contribuent probablement au courant transitoire IAf qui s'inactive rapidement dans les GCS. De plus, les variations des niveaux d'ARNm de Kv4.2 pourraient expliquer les changements observés dans les densités de IAf au cours des développements *in vivo* et *in vitro*. De leur côté, les canaux Kv2.1, Kv2.2, Kv3.1, Kv3.3 et Kv3.4 participent probablement au courant transitoire IAs qui s'inactive lentement dans les GCS, peut-être en formant des canaux hétéromères. Cependant, les variations des niveaux d'ARNm de ces gènes ne pourraient expliquer les changements observés dans les densités de IAs. Par ailleurs, la hausse des niveaux d'ARNm de Kv3.4 dans les neurones de GCS cultivés avec du CNTF n'expliquent pas que l'expression de IAf et IAs y soit maintenue. Enfin, comme dans les neurones des GCS, Kv1.4 et Kv4.2 contribuent probablement au courant IAf dans les neurones des ganglions nodoses et trijumeaux et Kv2.1, Kv 2.2, Kv3.2, Kv3.3 et Kv3.4, au courant IAs.

Contributions to original knowledge:

In chapter three, I measured the mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1 Kv3.2, Kv3.3, Kv3.4 and Kv4.2 in SCG neurons, this was the first quantification of potassium channel mRNA expression by native cells. I also demonstrated for the first time that multiple Kv genes from the same subfamily were coexpressed by the same cells, suggesting the possibility that heteromeric channels are expressed in native cells. Furthermore, I demonstrated for the first time that Kv3.4 is the most abundant mRNA transcript expressed by SCG neurons. This work was first reported in abstract form: Fraser and Cooper, Society for Neuroscience abstracts, 1994.

In chapter four, I present the first quantitative measurement of potassium channel mRNA levels in native cells during *in vivo* and *in vitro* development. Furthermore, I showed that the mRNA levels of many Kv genes were not developmentally regulated during neonatal life. This implies that the regulation of voltage-gated potassium channel expression occurs at a later stage of processing. In contrast, Kv3.4 mRNA levels increased in expression level both *in vivo* and *in vitro*, and Kv4.2 mRNA levels underwent developmental regulation *in vivo* and *in* vitro that was dependent on the presence of extrinsic factors, which were absent in our culture system. Furthermore, the mRNA expression level of Kv4.2, closely correlated to the expression of SCG IAf density, suggesting that transcriptional control of Kv4.2 leads to changes in IAf density. I also showed that CNTF selectively regulates the expression of Kv3.4 mRNA in SCG

neurons, which is the first evidence that trophic factors can influence potassium channel mRNA levels by native cells.

In chapter five, I measured the mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1 Kv3.2, Kv3.3, Kv3.4 and Kv4.2 in nodose and trigeminal neurons. This was the first quantitative measurement of potassium channel mRNA expression by sensory neurons. These experiments show that while sensory neurons expressed some potassium channel mRNAs at the same levels as did sympathetic neurons, other potassium channel mRNAs are expressed at different levels. Furthermore, I showed first that Kv3.4 is the most abundant Kv transcript expressed by sensory neurons. This work was first reported in abstract form: Fraser and Cooper, Society for Neuroscience abstracts, 1994.

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Table of Abbreviations

Rapidly Inactivating Transient Current	IAf
Slowly Inactivating Transient Current	IAs
A-Current	IA
D-Current	ID
Muscarine Blockable Current	IM
Delayed rectifier Current	IK
Superior Cervical Ganglia	SCG
Nodose	Nod
Trigeminal	Tri
Tetraethyl Ammonia	TEA
4-Amino Pyridine	4-AP
Protein Kinase C	PKC
Cell Adhesion Molecule	CAM
Cilary Neurotrophic Factor	CNTF
Calcium/Calmodulin Dependent Kinase	CAMKII
Phencyclidine	PCP
Excitatory Postsynaptic Potential	EPSP
Double Distilled Water	ddH_2O
Postnatal Day One	P1
Postnatal Day Seven	P7
Postnatal Day Fourteen	P14
Postnatal Day Twenty-one	P21
Embryonic Day Eighteen	E18
Postnatal Day One Cultured Seven Days	P1C7
Postnatal Day One Cultured Fourteen Days	P1C14
Postnatal Day One Cultured Twenty-one Days	P1C21
Postnatal Day Fourteen Cultured Seven Days	P14C7
Postnatal Day Fourteen Cultured Fourteen Days	P14C14

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Chapter 1:

Introduction

1.1 General introduction

Neurons use a variety of different ion channels and receptors to initiate and propagate electrical signals. To function properly, these proteins must be expressed in precise locations at the correct density. Neurons achieve this functionality through a combination of intracellular mechanisms and cell-cell interactions that act to regulate gene transcription, translation, and influence the assembly and targetting of proteins. Recent advances in molecular biology have made important contributions to understanding these various processes.

The generation of the action potential involves the gating of ion channels in response to changes in membrane voltage. Sodium channels, calcium channels and potassium channels are among the different classes of ion channels that form the superfamily of voltage-gated ion channels. Mutagenesis studies have been crucial in providing an understanding of the structure-function relationship of ion channels. However, it is through studying ion channel genes in the context of native systems that we will learn how neurons control ion channel gene expression as to affect their physiological properties.

For my doctoral studies, I have focused on the expression of voltage-gated potassium channel genes. I hope that through elucidating why specific potassium channel genes become expressed in a defined population of neurons over development that we will better understand the general rules governing how neurons come to express their particular sets of ion channels and receptors.

In order to provide a framework for understanding my experiments, I first present an overview of the electrophysiological characterization of outwardly conducting voltage-gated potassium currents, then I review studies that investigated extrinsic influences on the expression of these currents. In addition, I describe the recent work on the structure-function relationship of potassium channels.

1.1.1 Physiological properties of voltage-gated potassium channels

Neurons employ a number of different potassium currents to fullfill their specific functional needs (Salkoff and Jelga, 1995). These include: rapidly inactivating potassium currents, delayed-rectifier potassium currents, calcium-activated potassium currents, cyclic nuceotide gated potassium currents and inward rectifier potassium currents. Rather than list the properties of individual potassium currents described on many cell types in different species (Llinas, 1989; Rudy, 1988), I have summarized the work on two well characterized systems, hippocampal pyramidal neurons and sympathetic neurons, to illustrate the diversity of potassium currents and to show how potassium currents are used to fulfill distinctive functional roles.

1.2 Hippocampal pyramidal neurons

The hippocampus is important for the storage of declarative memory and for associative learning. Declarative memory requires conscious reflection for acquisition and recall, and involves cognitive processes such as evaluation, comparison and inference; whereas associative memory involves learning a relationship between two stimuli, as occurs during classical conditioning. The hippocampus contains a trisynaptic pathway. If one of these synapses are presynaptically stimulated then potentiation occurs at that synapse. Different hippocampal synapses are capable of either associative or nonassociative potentiation; many researchers feel that this potentiation reflects the underlying mechanisms involved in learning and memory (Kandel *et al.*, 1991; for reviews, see Miyamoto and Fukunaga, 1996; Nicoll and Malenka, 1995).

The important role of the hippocampus in memory formation and the capacity of the hippocampus to develop potentiation have led many scientists to investigate the electrophysiological properties of hippocampal neurons. Membrane currents on pyramidal neurons have been well characterized using a number of complementary preparations including: 1) acutely isolated slices from adult animals, 2) organotypic slice cultures, 3) acutely dissociated hippocampal cells and 4) hippocampal cell cultures. Hippocampal pyramidal neurons use a large number of membrane currents on their cell bodies, axons and dendrites. Of direct relevance to this thesis are the potassium currents.

Table 1.1 Summary of hippocampal pyramidal neuron voltage-gated potassium channel properties.

Channel	Voltage-Sensitivity of Activation	Rate of Activation	Voltage-Sensitivity of Inactivation	Rate of Inactivation	Block by 4-AP	Block by TEA
IA	activates above -60 mV	activates rapidly	inactivated above -60 mV	inactivates rapidly	blocked by 1 mM 4-AP	not blocked by 10 mM TEA
ID	activates above -80 mV	activates rapidly	inactivated above -80 mV	inactivates slowly	blocked by 30 μM 4-AP	not blocked by 25 mM TEA
IK	activates above -40 mV	activates slowly	not clear	inactivates slowly	not blocked by 5 mM 4-AP	blocked by 25 mM TEA
IM	activates above -60mV	activates slowly	does not inactivate	does not inactivate	not blocked by 1 mM 4-AP	blocked by 10 mM TEA

(rapid activation occurs over milliseconds; slow activation ocurrs over tens of milliseconds; rapid inactivation occurs over tens of milliseconds; slow inactivation occurs over seconds; adapted from: Storm, 1990)

There are at least four different outward voltage-gated potassium currents expressed by pyramidal neurons that can be distinguished through electophysiological and pharmacological dissection (for a review, see Storm, 1990). These include a delayed-rectifier current (IK), a rapidly-inactvating transient current (IA), a slowly activating transient current (ID) and a slowly activating muscarine-blockable current (IM). Below, I describe the physiological properties of these pyramidal currents (see table 1.1).

1.2.1 Pyramidal IK

The pyramidal delayed -rectifier current (IK) (Segal and Barker, 1984; Numann *et al.*, 1987; Madison *et al.*, 1984; Storm, 1988) closely resembles the delayed-rectifier current originally described by Hodgkin and Huxley (1952) on squid giant axon. Hippocampal IK activates slowly, at potentials above -40 mV (Segal and Barker, 1984). As pyramidal IK only activates above threshold potential for action potential firing, IK is unlikely to contribute to subthreshold phenomena. Instead, pyramidal IK activates during the action potential to influence spike repolarization as does IK on the squid giant axon. Delayed-rectifier currents were initially described as non inactivating currents, however, pyramidal IK inactivates over many seconds (Segal and Barker, 1984; Numann *et al.*, 1987; Storm, 1988), similar to most other currents also classified as delayed-rectifier currents (Rudy, 1988). This IK also is expressed on pyramidal cell dendrites at the same current-density as on the cell body (Hoffman *et al.*, 1997)

Pyramidal IK is blocked by moderate concentrations of TEA and insensitive to block by moderate concentrations of 4-AP (Doerner *et al.*, 1988). Blockage of IK with TEA leads to action potential broadening, consistent with a role in action potential repolarization (Storm, 1990).

The psychotomimetic drug phencyclidine (PCP) produces a psychotic reaction in humans that closely resembles schizophrenia (Nabeshima *et al.*, 1996). Interestingly, PCP blocks hippocampal IK (ffrench-Mullen *et al.*, 1988), suggesting that alteration of IK channel function in the hippocampus or similar channels in other brain areas plays a role in the development of schizophrenia, although the PCP effect is not specific to hippocampal neurons or block of IK on CNS neurons.

1.2.2 Pyramidal somatic IA

A fast transient outward potassium current that influences action potential encoding and contributes to spike repolarization on hippocampal pyramidal cell bodies was first described by Gustafsson *et al.*, (1982) and Zbicz and Weight (1985). This current resembles the A-current initially described by Connor and Stevens (1971) in molluscan neurons and later by others in different species (Hille, 1984; Rogawsky, 1985; Rudy, 1988). The term A-current (IA) is now commonly applied to describe any rapidly inactivating potassium current and refers to a whole family of channels composed of different molecular subunits. Currents classified as A-currents show a range of pharmacological and voltage-sensitivities (Rudy, 1988). Pyramidal IA activates in

response to depolarization above -60 mV and requires hyperpolarization to fully remove inactivation. The A-current shows both rapid activation and inactivation and removal of inactivaton is quick (Storm, 1988). Because hippocampal IA activates rapidly from subthreshold potentials, this current delays onset of firing (Gustafsson *et al.*, 1982; Segal *et al.*, 1984; Storm, 1984, 1987, 1988). Similarly, in invertebrate and vertebrate neurons IA delays response to depolarizing stimuli and permits slow repetitive firing (Connor and Stevens, 1971b; Aghajanian, 1985). As such, pyramidal IA contributes to the integration of synaptic inputs. Furthermore, IA may be involved in action potential repolarization and may influence firing frequency.

The A-currents on hippocampal neurons is sensitive to block by moderate levels of 4-AP but is not blocked by high concentrations of TEA, similar to the molluscan IA (Gustafsson *et al.*, 1982; Segal *et al.*, 1984; Segal and Barker, 1984; Nakajima *et al.*, 1986; Numann *et al.*, 1987; Storm, 1988). Dendrotoxin from mamba snake venom produces convulsions similar to those observed in epilepsy and also blocks hippocampal IA (Harvey and Karlsson, 1982; Dolly *et al.*, 1984; Halliwell *et al.*, 1986). Concieveably, certain forms of epilepsy may be related to the activity of these channels or simlar channels in other brain areas.

1.2.3 Pyramidal dendritic IA

Through combining whole-cell recording with calcium sensitive fluorescence imaging in the hippocampal slice preparation, Hoffman and colleagues (1997) recorded

an A-current on hippocampal pyramidal cell dendrites that is similar to IA on pyramidal cell bodies. This dendritic IA is expressed at high density, limits large rapid dendritic depolarizations and is modulated by local post-synaptic depolarization. Furthermore, dendritic IA is implicated in the induction of associative plasticity. Associative plasticity is a type of synaptic potentiation that occurs when two or more input fibers to a neuron are activated, and when both the contributing fibers and postsynaptic neuron are activated together (association). Moreover, this plasticity is specific to the activating pathway (Kandel *et al.*, 1991).

When dendrites receive excitatory synaptic input, the size and shape of the EPSP is limited by IA activation, thereby preventing the formation of dendritic action potentials. Similarily, IA limits the amplitude of back propagated action potentials from the cell body into the dendrites. However, following the generation of an EPSP, there is a brief period of time when the A-current recovers from inactivation and the dendrite is more excitable. During this time the amplitudes of back propagated action potentials are less attenuated. This dampening effect of dendritic IA heightens the associativity of the pairing between back propagated action potentials and EPSPs, such that synaptic activity causing postsynaptic depolarization can locally modulate the A-current, thereby permitting full amplitude back-propagated action potentials capable of evoking calcium influx. The increase in intracellular calcium signals through calcium/calmodulin kinases and protein kinase C leading to associative and nonassociative potentiation, however, the

exact mechanism by which this potentiating occurs is not yet understood. (Kandel *et al.*, 1991).

Pharmacological evidence suggests that dendritic IA may consist of distinct currents: one component that is blocked by moderate concentrations of 4-AP and moderate concentrations of TEA and another component that is blocked by 4-AP but not TEA. Yet, only a small component of dendritic IA is blocked by dendrotoxin whereas somatic IA is readily blocked by dendrotoxin. The pharmacologically different components of dendritic IA may indicate that more than one type of IA channel, which overlap in voltage-sensitive properties, are colocalized in pyramidal cell dendrites.

1.2.4 Pyramidal ID

The cell bodies of pyramidal neurons also have a slowly inactivating transient current (ID), which activates slowly at potentials near resting membrane potential (Storm, 1988). This current permits pyramidal neurons to integrate depolarizing inputs over long times and is responsible for the latency to action potential firing seen in these cells. Also, the D-current activates in the subthreshold range and takes tens of seconds to recover from inactivation making the encoding properties of the cell sensitive to the prevailing membrane potential. In this way the firing properties of the neuron are determined by the recent electrophysiological activity of that neuron.

The D-current can be dissected from the rapidly inactivating A-current on the same cells because it activates and inactivates ~20 mV more hyperpolarized and much

more slowly than IA. Yet, ID may have been mistakenly included as part of hippocampal IA in the initial characterization of currents, as ID and IA overlap in voltage-sensitive and pharmacological properties (Gustafsson *et al.*, 1982; Zbicz and Weight, 1985).

Low concentrations of 4-AP block ID on rat pyramidal neurons, but ID is not blocked by high concentrations of TEA. Yet, a 4-AP blockable current similar to ID has not been recorded from cultured embryonic rat hippocampal neurons (Segal and Barker, 1984; Segal *et al.*, 1984). The source of this discrepancy is not obvious. One possibility is that some aspect of dissociation could cause pyramidal cells to lose ID in culture. For example, enzymatic treatment or mechanical dissociation of the neurons could disrupt the channel proteins leading to loss of ID. Alternatively, the loss of extrinsic influences *in vitro* may account for the disappearance of ID. Synaptic activity, cell contact, diffusible factors or extracellular matrix proteins each play important roles in neuronal differentiation and development (for reviews, see Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996; Katz and Shatz, 1996; Tessier-Lavigne and Goodman, 1996). More specifically, many studies show that extrinsic factors influence the expression of voltage-gated potassium currents on neurons (Yu and Barish, 1992, 1994; Dourado and Dryer, 1992; Raucher and Drier, 1994; McFarlane and Cooper, 1992, 1993; for a review, see Barish, 1995). Neurons removed from their non-neuronal cells and grown *in vitro* undergo different patterns of potassium current development than *in vivo*. (Barish, 1995; Yu and Barish, 1992, 1994; Dourado and Dryer, 1992; Raucher and Drier, 1994; McFarlane and Cooper, 1992, 1993).

Despite the apparent absence of ID *in vitro* (Numann *et al.*, 1987; Segal and Barker, 1984; Segal *et al.*, 1984), ID has since been recorded from cultured pyramidal cells by Yu and Barish (1992, 1994). It is not clear why Numann and coworkers, and Segal and coworkers did not detect ID on cultured hippocampal neurons. Perhaps, Segals group failed to resolve ID because they were not looking for a second transient current, and ID overlaps in voltage-sensitive and pharmacological properties with IA. There are other studies which have failed to resolve distinct potassium currents due to similarities in functional properties (Belluzzi and Sacchi, 1985; Galvan and Sedlmeir, 1984; Nerbonne and Gurney, 1989).

1.2.5 Pyramidal IM

The pyramidal M-current (IM) is an important current that influences action potential amplitude, duration and firing patterns and is also regulated by neurotransmitter action. The neurotansmitter acetylcholine acts to supress IM through M_2 subtype of muscarinic receptors by the phosphoinositide second messenger system (Dutar and Nicoll, 1988). The M-current activates slowly over tens of msec, at potentials above -60 mV and does not inactivate even over long voltage-steps, though some deactivation does occur (Storm, 1990). On pyramidal neurons, IM underlies the early phase of action potential firing frequency adaptation (Madison and Nicoll, 1987) and may contribute to the after hyperpolarizing potential, which follows action potential firing (Storm, 1989).

The M-current does not seem to influence the resting membrane potential as this potential is ~10 mV more negative than the threshold for activation of IM.

In addition to acetylcholine, TEA blocks IM at moderate concentrations but 4-AP has no effect. A similar M-current which is also supressed by acetylcholine is present on sympathetic neurons (Brown and Adams, 1980; Adams *et al.*, 1982; Atkins *et al.*, 1990) and neurons of the olfactory cortex (Constanti and Sim, 1987), but these M-currents are less sensitive to TEA. This difference in pharmacological sensitivities suggests that IM channels may be composed of different subunits in hippocampal pyramidal cells and other types of neurons.

The activation of cholinergic medial septal inputs to the hippocampus blocks the M-current, facilitates repetitive firing, increases action potential amplitude and prolongs action potential duration in hippocampal neurons (Halliwell and Adams, 1982; Nakajima *et al.*, 1986). This facilitation allows hippocampal neurons to respond more robustly to synaptic input. As presynaptic facilitation is important for the development of synaptic potentiation (Kandel *et al.*, 1991), cholinergic modulation of IM could be a mechanism involved in memory formation.

1.2.6 Development of hippocampal potassium currents

To function properly, neurons must first arrive in the correct place and then send out axons to proper targets as they receive specific innervation. Furthermore, neurons must express appropriate receptors and ion channels in order to integrate and transmit

signals. The acquisition of excitable properties occurs through a complex interplay of extrinsic factors and genetic programs reflecting a neurons particular developmental history (for a review, see Spitzer, 1991). To learn more about how hippocampal neurons acquire their unique repertoires of ion channels, Wu and Barish (1992, 1994) and Ficker and Heinemann (1992) examined the expression of voltage-gated potassium currents by embryonic pyramidal neurons.

The A-current recorded from embryonic hippocampal pyramidal neurons had the same voltage-dependent, time-dependent and pharmacological properties as IA on adult hippocampal neurons recorded using other systems (Gustafsson *et al.*, 1982; Segal and Barker, 1984; Segal *et al.*, 1984; Zbicz and Weight, 1985; Halliwell *et al.*, 1986; Nakajima *et al.*, 1986; Numann *et al.*, 1987; Storm, 1988). As the properties of pyramidal IA channels did not change, this suggests that the composition of the IA channels remained constant during development and there were no changes in the modulation of this channel.

Embryonic pyramidal ID activates and inactivates slowly, is insensitive to TEA and sensitive to low concentrations of 4-AP as is adult ID (Storm, 1988). Yet, the voltage-sensitivities for activation and inactivation of the D-current are ~30-60 mV more positive in embryonic neurons (Storm, 1988). As the voltage-sensitive properties of ID shift over development, this change could reflect either a change in composition of the ID channel itself or alterations in ID channel function brought about through interactions with other proteins. Modification of the ID channel composition could occur through

distinct channel subunits with differing voltage-sensitive properties being incorporated into ID channels. This change in the composition of ID could produce a similar current but with different voltage-sensitivities. Alternatively, the composition of the ID channel itself may remain constant while shifts in voltage-sensitivity may be due to interactions with other proteins. For example, coexpression of a voltage-gated potassium channel gene with low molecular weight brain mRNA thought to encode a modulatory subunit, significantly shifted the voltage-sensitivity of the observed currents in the negative direction in a heterologous expression system (Serodio *et al.*, 1996).

1.2.7 Influence of non-neuronal cells on the expression of hippocampal potassium currents

Cell-cell interactions influence the expression of potassium currents in the hippocampus. Ficker and Heinemann (1992) showed that acutely isolated embyronic day 17-19 (E17-E19) neurons all expressed ID but only half expressed IA. However, by the end of the first week of postnatal development most hippocampal cells expressed a high density of IA (Ficker and Heinemann, 1992) and this increase was dependent on the presence of non-neuronal cells (Wu and Barish, 1992). Non-neuronal cells influenced neuronal development by producing growth factors and extracellular matrix components (Giulian *et al.*, 1988; Beneveniste, 1992). IA and ID expression was regulated in culture by the presence of astroglia (Wu and Barish, 1992). When E15 neurons were removed

Table 1.2 Summary of superior cervical ganglion neuron voltage-gated potassium channel properties.

Channel	Voltage-Sensitivity of Activation	Rate of Activation	Voltage-Sensitivity of Inactivation	Rate of Inactivation	Block by 4-AP	Block by TEA
SCG IAf	half activated at -2 mV	activates rapidly	half inactivated at -65 mV	inactivates rapidly	half blocked by 1 mM 4-AP	not blocked by 10 mM TEA
SCG IAs	half activated at 10 mV	activates rapidly	half inactivated at -40 mV	inactivates slowly	not known	not known
SCG IK	half activated at 22 mV	activates slowly	does not inactivate	does not inactivate	not known	not known
SCG IM	activates above -60mV	activates slowly	does not inactivate	does not inactivate	not known	not blocked by 10 mM TEA

(rapid activation occurs over milliseconds; slow activation occurs over tens of milliseconds; rapid inactivation occurs over tens of milliseconds; slow inactivation occurs over seconds; adapted from: Belluzzi and Sacchi, 1991; McFarlane and Cooper, 1992)

from the influence of non-neuronal cells, over three days in culture, they typically lost about half of their IA, and their ID increased threefold. Therefore, there is some influence present *in vivo* that is not found in culture. Neurons grown on glia maintained their IA density and relative low expression of ID, whereas, neurons grown touching glia expressed an intermediate phenotype and neurons grown not touching glia expressed the same phenotype as neurons grown in the absence of glia (Wu and Barish, 1994). Furthermore, the glia must be alive to produce this effect. Fixed glial cells do not influence the potassium current expression by pyramidal cells.

There is some aspect of contact with non-neuronal cells that influences the expression of voltage-gated potassium currents in pyramidal cells. This effect could be due to a number surface cell adhesion molecules (CAMs) produced by glial cells such as N-CAM, Ng-CAM/L1, N-cadherin, and L2/HNK-1 or through the production of extracellular matrix proteins such as laminin, fibronectin or tenascin (for reviews, see Fu and Gordon, 1997; Luckenbill-Edds, 1997; Yamaguchi, 1997).

We do not know which genes encode the different potassium channels expressed by hippocampal neurons, through what mechanisms the properties of IA and ID change nor what specific interplay of genetic and environmental influences are involved. To better understand the voltage-gated potassium currents expressed by hippocampal pyramidal cells, it is necessary to identify which specific voltage-gated potassium channel genes are expressed in pyramidal cells and to be able to associate physiological function with gene expression. However, the hippocampus contains several different neuronal cell

types in a complex organization making it a difficult system to study developmental principles. A simpler preparation would facilitate the elucidation of the developmental mechanisms which control the expression of ion channels.

1.3 Sympathetic neuronal potassium currents

Superior cervical ganglion (SCG) neurons are a useful system to investigate the changes that underlie the acquisition of electrophysiological properties. The SCG ganglia is the largest and most rostral of the sympathetic chain ganglia. Preganglionic neurons of the intermediolateral region of the grey spinal make cholinergic synapses on SCG neurons (Rubin, 1985b). SCG neurons provide norandernergic innervation on targets including the iris, glands and vasculature (Rubin, 1985c).

The SCG contains a relatively homogeneous population of neurons that are electrophysiologically well characterized (Galvan and Sedlmeir, 1984; Belluzzi *et al.*, 1985; Nerbonne *et al.*, 1986, 1989; McFarlane and Cooper, 1992, 1993). SCG neurons undergo interesting changes in expression of potassium currents over development dependent on the influence of extrinsic factors (McFarlane and Cooper, 1992, 1993). Furthermore, these neurons are easily dissected and the influence of denervation or axotomy can be investigated through cutting the pre or post ganglionic fibers. Moreover, SCG neurons grow well in dissociated culture so that they may be investigated in isolation from the intact organism, and extrinsic factors may be added back to evaluate their influence.

Sympathetic neurons express at least four electrophysiologically distinct outward voltage-gated potassium currents, a delayed rectifier current (IK), a rapidly inactivating transient current (IAf), a slowly inactivating transient current (IAs), and a slowly activating current that is blockable by acetylcholine (IM). These sympathetic currents are described below (see table 1.2).

1.3.1 Sympathetic IK

A delayed-rectifier current (IK) has been identified on sympathetic neurons (Belluzzi and Sacchi, 1991; Belluzzi *et al.*, 1985a; Nerbonne *et al.*, 1989; Cooper and McFarlane, 1992, 1993). This current activates slowly and provides sustained current much like the delayed-rectifier current described by Hodgkin and Huxley (1952). SCG IK activates at potentials above -30 mV (Belluzzi *et al.*, 1985a). Belluzzi and coworkers detected inactivation of IK during prolonged depolarizations, especially visible at more depolarized potentials, which he attributed to possible extracellular K^+ accumulation (Belluzzi *et al.*, 1985). Further studies illustrated that the inactivating component is a distinct current and that SCG IK does not inactivate even over very long voltage-steps (McFarlane and Cooper, 1991, 1992). TEA dramatically reduces the spike falling rate, markedly prolonging the action potential duration (Galvan and Sedlmeir, 1984). This suggests that IK makes a contribution to action potential repolarization.

1.3.2 Sympathetic IAf

A rapidly inactivating A-current (IAf) that is responsible for spike repolarization and may contribute to the integration of presynaptic input has been reported on sympathetic neurons by a number of researchers (Belluzzi and Sacchi, 1991; Galvan and Sedlmeir, 1984; Belluzzi *et al.*, 1985b; Nerbonne *et al.*, 1989; McFarlane and Cooper, 1992). Adult SCG IA activates rapidly from potentials above -60 mV (Belluzzi *et al.*, 1985b) and its amplitude increases markedly with greater depolarization. This IA is completely inactivated when the membrane potential is held at -50 mV (Belluzzi *et al.*, 1985b). Inactivation of IA is rapid, occuring over tens of msec and proceeds with a single exponential time course (Galvan and Sedlmeir, 1984), suggesting that a homogeneous population of A-channels is present on mature neurons. Testing Galvan and Sedlmeir predictions will require identifying which specific voltage-gated potassium channel subunits contribute to IAf on SCG neurons. Moderate concentrations of 4-AP block IAf (Galvan and Sedlmeir, 1984), but IA is only slightly sensitive to block by high levels of TEA (Belluzzi *et al.*, 1985b). The pharmacological and voltage-sensitive properties of SCG IA are similar to those of molluscan IA and pyramidal cell IA (Connors and Stevens, 1971a, b; Storm, 1990). The SCG IA plays an important role in rapid membrane repolarization following the action potential upstroke (Belluzzi *et al.*, 1985b, 1988), but the short duration of the activated current suggests that it is not involved in slow neuronal behavior such as determining spike threshold and interspike interval. Belluzzi and Sacchi (1990) argued that IA is the only potassium current sufficiently large in amplitude and

rapid in activation in adult neurons to charge the cell capacitance during normal action potential firing.

1.3.3 Sympathetic IAs

The slowly inactivating transient current was first identified on SCG neurons by McFarlane and Cooper (1991, 1992, 1993). The current previously classified as a delayed-rectifier current (Galvan and Sedlmeir, 1984; Belluzzi *et al.*, 1985a; Nerbonne *et al.*, 1986, 1989) can be separated into two currents, a slowly inactivating transient current that inactivates over hundreds of msec and is fully available for activation from –60 mV and a non inactivating delayed-rectifier current that activates at potentials above -30 mV. As IAs activates at depolarized membrane potentials, activation only occurs during the action potential and likely contributes to spike repolarization. The apparent lack of inactivation of IAs, over shorter voltage-steps, explains why this current was mistakenly included as a component of the delayed-rectifier current by previous investigators (Galvan and Sedlmeir, 1984; Belluzzi *et al.*, 1985a; Nerbonne *et al.*, 1986, 1989). The pharmacology of IAs has not been characterized.

1.3.4 Sympathetic IM

An acetylcholine-blockable current has been reported on bull-frog, mouse and rat sympathetic neurons (Hamilton, *et al.*, 1997; Constanti and Brown, 1981; Adams *et al.*,

1982; Belluzzi and Sacchi, 1990, 1991). IM activates slowly at membrane potentials between -60 and -10 mV, does not inactivate over long voltage steps, is not blocked by TEA and requires extracellular calcium for activation (Brown and Adams, 1980; Belluzzi and Sacchi, 1991; Belluzzi *et al.*, 1990). Sacchi and Belluzzi deduced that IM has little influence on action potential firing by rat sympathetic neurons because it is typically expressed at very low levels and activates slowly (Sacchi and Belluzzi, 1990).

1.3.5 Development of sympathetic neuronal potassium currents

Voltage-gated potassium currents on rat sympathetic neurons undergo marked changes in density during development, beginning from very early times when the SCG is forming. Rubin (1985a) found that postganglionic processes appeared around E12 and reached remote targets as early as E15. Rubin (1985c) also asserts that synapse formation occured early, specifically that preganglionic axons first entered the SCG between E12-E13, that postynaptic responses can be evoked at E13 and that by E14 the strength of innervation resembles the mature form. This is possible even though the number of synapses present at birth is a small fraction of the adult number (Smolen and Raisman, 1980). Dendrites appear beginning E14 and synaptogenesis begins to focus on these new processes as connections continue to be remodelled through early postnatal development (Rubin 1985b). Yet, McFarlane and Cooper (1992) showed that there does not seem to be a postnatal influence of denervation or axotomy on the developmental expression of potassium channels properties by sympathetic neurons.

Typically, voltage-gated potassium currents densities are relatively uniform on SCG neurons of the same age (McFarlane and Cooper, 1992). Early in development at E15, a voltage-gated potassium current which shows no inactivation over short voltage-steps is detectable on SCG neurons as they as they are coalescing to form a ganglion (Nerbonne *et al.*, 1989; Rubin, 1985a). This current has properties of both IAs and IK. By E19, IAf, a fast transient potassium current that only activates from hyperpolarized potentials and rapidly inactivates over tens of msec, is detectable on sympathetic neurons (Nerbonne *et al.*, 1986).

At postnatal day one, IAs is the predominant outward current expressed by most SCG neurons, while IAf and IK are expressed at comparatively lower levels (McFarlane and Cooper, 1992). Over the first two weeks of development, the expression of IK remains low, IAs decreases twofold while the expression of IAf increases threefold, to become the predominant current. This increase is consistent with the IAf making a strong contribution to action potential repolarization in adult SCG. IK remains low through normal development (McFarlane and Cooper, 1992). The M-current is expressed at low levels in adult sympathetic neurons 1-2 orders of magnitude less than the density of IK or IAf (Galvan and Sedlmeir, 1984), but IM expression has not been investigated over development.

Over normal development between P1 and P14, there are shifts in the voltage-dependent properties of IAf and IAs (McFarlane and Cooper, 1992). The greatest change observed was in IAs, where the voltage-sensitivity for activation is shifted positive by 16

mV. The voltage-sensitivity for inactivation is also shifted positively by 5 mV. The changes in the other parameters are smaller and less significant. For IAf, voltage-sensitivity for activation is shifted positively 6 mV. The rates of activation and inactivation of IAf and IAs also show slight changes. Between P14 and adulthood, there appears to be a further shift in the voltage-sensitivity of IAf to more negative potentials (Belluzzi *et al.*, 1985). The mechanisms underlying these changes in properties have not been identified.

1.3.6 P1 SCG neurons in culture

When sympathetic neurons are placed in culture, the voltage-gated potassium currents which they express undergo a very different pattern of development. At birth, IAs is the predominant conductance and IAf is present at lower levels. Over the first two weeks in culture, the expression of both IAs and IAf decreases dramatically while the expression of IK increases steadily. By the fourth week in culture, IAf is undetectable. IAs is expressed at very low levels and IK has increased fivefold to become the predominant conductance (McFarlane and Cooper, 1993). There appears to be a reciprocal relationship between the expression level of the transient currents, IAf and IAs, and the delayed-rectifier current, IK. However, the total density of potassium currents does not change over development (McFarlane and Cooper, 1992).

1.3.7 P14 SCG neurons in culture

Postnatal day fourteen sympathetic neurons typically express high levels of IAf, moderate levels of IAs and low levels of IK. If these neurons are placed in dissociated cell culture, then the delayed-rectifier current IK increases to high levels as is observed in cultured P1 neurons and the expression of the transient currents IAf and IAs decreases to moderate and low levels respectively. Yet, the expression of the transient currents does not decrease to undetectable levels as is observed in cultured P1 SCG neurons. Therefore, changes seem to occur during the first two weeks of development, involving factors absent in cell culture which regulate SCG neurons such that they become resistant to loss of A currents (McFarlane and Cooper, 1992).

1.3.8 Influence of non-neuronal cells on SCG neuronal potassium currents

Neonatal SCG neurons directly contact the heart, vasculature, and ganglionic non-neuronal cells and receive synaptic contact from preganglionic axon-terminals (Rubin 1985b, c). It is clear that extrinsic influences originating from such cells play an important role in neuronal differentiation including expression of voltage-gated potassium currents. When rat sympathetic neurons are cultured in the presence of non-neuronal cells, IAs expression decreases moderately, while IAf and IK remain expressed at close to P1 levels through time in culture (McFarlane and Cooper, 1993). This activity

is not specific to ganglionic non-neuronal cells as co-culture with heart or skin produces a similar developmental pattern. Moreover, this effect seems to be due to a soluble factor secreted by non-neuronal cells, rather than direct contact, as conditioned media from non-neuronal cells produces the same effect on cultured SCG neurons (McFarlane and Cooper, 1993). The candidate soluble factor may belong to a class of molecules called cytokines. Ciliary neurotrophic factor (CNTF) was isolated based on its ability to support the survival of chick ciliary neurons (Mehler and Kessler, 1995). CNTF is expressed at high levels in the rat sciatic nerve (Lin *et al.*, 1989) and has effects on the differentiation of SCG neurons (Ernsburger *et al.*, 1989; Saadat *et al.*, 1989). McFarlane and Cooper (1993) found that CNTF influenced the expression of voltage-gated potassium currents in the same way as does conditioned media from non-neuronal cells. For this reason it is possible that CNTF or a closely related molecule regulates the expression of potassium currents over normal development. This is not the complete story because even though CNTF and conditioned media influence the expression of currents, they do not produce the normal developmental phenotype.

1.4 Molecular Biology of Voltage-Gated Potassium Channels

Earlier in this chapter, I described the voltage-gated potassium currents and their expression patterns by hippocampal neurons and SCG neurons. For my doctoral studies, I have chosen to identify which specific voltage-gated potassium channel genes are expressed by SCG neurons and to compare their expression to the relative current

densities of IAf, IAs and IK. Numerous mammalian voltage-gated potassium channel genes exist which give rise to voltage-gated potassium currents with unique functional properties when expressed in heterologous expression systems. These channels contain well characterized functional domains that are responsible for conduction of potassium, gating and pharmacological properties. In some cases, differences in functional channel properties can be attributed to specific residues. In this section, I describe the initial characterization of *Shaker*, the first cloned voltage-gated potassium channel gene and I review the structure-function work on voltage-gated potassium channels.

1.4.1 Cloning of the *Shaker* locus

Potassium channels are widely distributed in a number of tissues, yet, they were difficult to purify because there is no known irreversible ligand for potassium channels, and no enriched sources of potassium channels exist. Previously, genes for ion channels have been cloned only after the isolation of a partial protein sequence. For example, a partial amino acid sequence from the nicotinic acetylcholine receptor (Noda *et al.*, 1983) and voltage-gated sodium channel gene (Noda *et al.*, 1984) were used to create antisense oligonucleotides which were then used to screen a cDNA library. Whereas, the first potassium channel locus cloned was identified through molecular analysis of the *Drosophila* mutant *Shaker*.

Shaker is a behavioral mutant whose legs shake when anaesthetized with ether. An electrophysiological investigation of the mutant revealed that *Shaker* lacked the

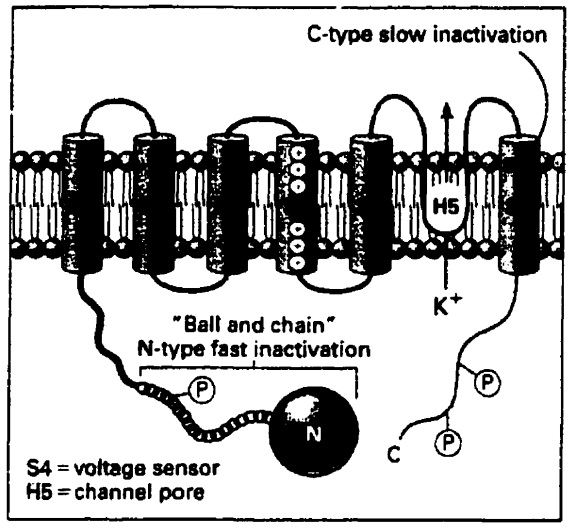

Figure 1.1 Voltage-gated potassium channel subunit.

Each individual voltage-gated potassium channel subunit is composed of cytoplasmic amino and carboxyl termini, and six transmembrane spanning α-helicies (S1-S6). The linker between S5 and S6 contains the selectivity filter (H5) of the channel pore. The S4 region, which contains a positively charged residue every third position, acts as the voltage sensor. Rapidly-inactivating voltage-gated potassium channel subunits include a "ball and chain" structure at the amino-terminal which can physically obstruct the channel pore. Residues in S6, near the outer mouth of the pore are important for slow-inactivation. (modified from Ackerman and Clapham, 1997).

rapidly inactivating A-current normally present in muscle (Salkoff and Wyman, 1981a, b). Based on this observation, it was hypothesized that the *Shaker* locus encoded a potassium channel gene. As several mutations within the *Shaker* locus were caused by chromosomal translocations, these mutations were used to localize the *Shaker* locus to a particular region of the *Drosophila* X chromosome. After the approximate location of *Shaker* was established, three different labs used chromosomal walking as an approach to clone the *Shaker* locus (Papazian *et al.*, 1987; Kamb *et al.*, 1987; Pongs *et al.*, 1988; Jan and Jan, 1997). *Shaker* is a large gene locus, consisting of more than twenty exons spanning greater than 110 kbp (Tempel *et al.*, 1987; Kamb *et al.*, 1987; Pongs *et al.*, 1988; Mottes and Iverson, 1995). Based on hydrophobicity analysis, the *Shaker* protein was predicted to contain six transmembrane domains as well as cytoplasmic amino and carboxyl-termini. Furthermore, the *Shaker* protein sequence bears strong homology to one quarter of the voltage-gated sodium channel sequence suggesting that functional channels are formed by the assembly of four *Shaker* subunits.

Timpe and colleages (1988a) and Iverson and colleagues (1988) expressed *in vitro* transcribed mRNA in *Xenopus* oocytes to confirm that the *Shaker* locus encodes a potassium channel. The rapidly inactivating potassium currents were similar to those recorded from wild-type *Drosophila* muscle (Salkoff and Wyman, 1981a, b, 1983; Timpe *et al.*, 1988; Inverson *et al.*, 1988; Zagotta *et al.*, 1989), thus confirming their hypothesis. *Shaker* is alternatively spliced at the amino and carboxyl-termini giving rise to currents with differing electrophysiological properties (Kamb *et al.*, 1988; Stocker *et al.*, 1990;

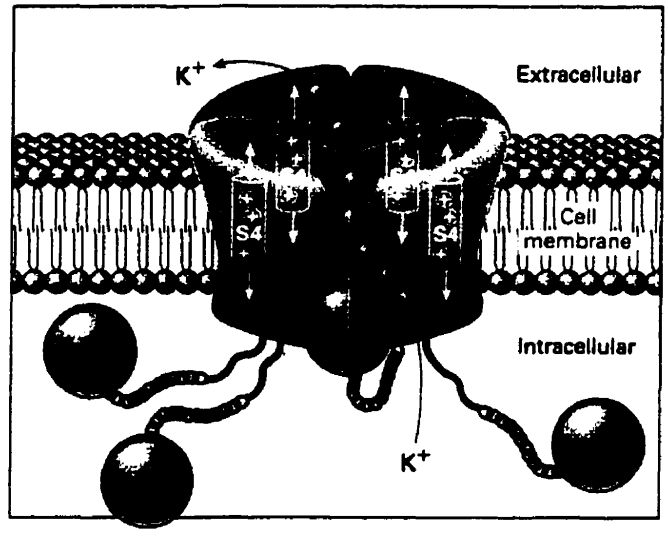

Figure 1.2 Voltage-gated potassium channel.

Four voltage-gated potassium channel subunits are required to form a functional channel. The topology of the pore region was revealed through determining the NMR-derived structures of scorpion toxins and examining their high affinity interactions with potassium channels (Aiyar *et al.*, 1995). The pore has wide intracellular and extracellular vestubles ~3 nm wide and 0.6 nm deep that leads to a restricted pore ~1 nm in diameter. The restricted pore tapers to ~0.5 nm at its narrowest point. (modified from Ackerman and Clapham, 1997).

Schwarz *et al.*, 1988, 1990; Timpe *et al.*, 1988a, b; Inverson *et al.*, 1988; Inverson and Rudy, 1990).

1.4.2 Cloning of *Shab*, *Shaw* and *Shal*

The *Shaker* mutant lacks a rapidly inactivating potassium current in skeletal muscle, but this mutant still expresses other fast transient potassium currents (Solc *et al.*, 1987). As other potassium currents were present, Butler and colleagues (1989) deduced that there must be other loci besides *Shaker* which encode transient currents. They (Butler *et al.*, 1989) used a *Shaker* cDNA probe to screen a *Drosophila* cDNA library at low stringency. This strategy yielded three additional potassium channel genes, *Shab*, *Shaw* and *Shal* that are closely related to *Shaker*, but are distinct. Each of the genes *Shaker*, *Shab*, *Shaw* and *Shal* are encoded by separate loci, and when expressed in a heterologous expression system, each gave rise to voltage-gated potassium currents with unique voltage-sensitive and pharmacological properties. *Shaker* activates and inactivates the most rapidly and is blocked by 4-AP, but not TEA. *Shab* also activates rapidly, inactivates slowly and is less blocked by 4-AP, but more blocked by TEA. *Shal* activates and inactivates rapidly, is blocked by 4-AP, but is less blocked by TEA. *Shaw* activates slowly, does not appear to inactivate, is blocked by 4-AP, but is less blocked by TEA (Wei *et al.*, 1990; Tsunoda and Salkoff, 1995).

Table 1.3 Standardized Kv gene nomenclature

STANDARDIZED NOMENCLATURE	NAMES OF PUBLISHED SEQUENCES
Shaker-related (Kv1 subfamily):	
Kv1.1	RCK1 / RBK1 / RK1
Kv1.2	RBK2 / RCK5 / NGK1 / RK2
Kv1.3	RCK3 / RGK5 / KV3
Kv1.4	RCK4 / RHK1 / RK3
Kv1.5	KV1 / RK4
Kv1.6	KV2 / RCK2
Kv1.7	RK6
Shab-related (Kv2 subfamily):	
Kv2.1	DRK1
Kv2.2	
Kv2.3	CDRK
Shaw-related (Kv3 subfamily):	
Kv3.1*	KV4 / NGK2
Kv3.2*	RKSHIIIA / RSHAW12
Kv3.3*	RKSHIIID / RAW4
Kv3.4	RAW3 / RKSHIIIC
Shal-related (Kv4 subfamily):	
Kv4.1	MSHAL
Kv4.2*	SHAL1 / RK5
Kv4.3	
Other possible subfamilies:	
Kv5, Kv6, Kv7 or Kv8?	

1.4.3 Classification of voltage-gated potassium channels

There is typically 40% homology between any two of these *Drosophila* genes (Salkoff, 1992), with the highest homology found in the core region of the protein and considerably lower homology in the amino and carboxyl-termini. *Shaker*, *Shab*, *Shaw* and *Shal* define four subfamilies of voltage-gated potassium channels which extend across phylogeny. In mammals, each subfamily contains from three to seven separate genes, with 60-70% sequence homology between family members. A standardized system of nomenclature has been adopted due to the large number of voltage-gated potassium channel genes that have been cloned by different labs (see table 1.3; Jan and Jan, 1990, 1997; Strong *et al.*, 1993; Chandy *et al.*, 1991). The pore forming subunits are called α-subunits to distinguish them from the other subunits which assemble with them. Furthermore, in this system the α-subunits are designated the prefix Kv. Members of the *Shaker*-related (Kv1) subfamily are Kv1.1-Kv1.7, the *Shab*-related (Kv2) subfamily are Kv2.1-Kv2.3, the *Shaw*-related (Kv3) subfamily are Kv3.1-Kv3.4, and *Shal*-related (Kv4) subfamily are Kv4.1-Kv4.3. Different alternative splice variants are designated with letters, for example Kv3.2A and Kv3.2B indicate alternatively spliced isoforms of Kv3.2 (alternatively spliced Kv genes are marked with an asterisk in table 1.3). Recently, four other putative Kv genes have been cloned that only share 40% homology with members of the existing subfamilies (Zhao *et al.*, 1994; Hugnot *et al.*, 1996; Drew *et al.*, 1992; Baumann *et al.*, 1988). These genes may represent new Kv subfamilies Kv5-Kv8. Yet,

Table 1.4 Summary of Kv channel properties.

Channel	Voltage-Sensitivity of Activation	Rate of Activation	Voltage-Sensitivity of Inactivation	Rate of Inactivation	Block by 4-AP	Block by TEA
Kv1.1	half activated at -30 mV	activates rapidly	half inactivated at -47 mV	inactivates slowly	half blocked by 0.3 mM	half blocked by 0.3 mM
Kv1.2	half activated at 27 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.6 mM	half blocked by 0.6 mM
Kv1.3	half activated at -10 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.4 mM	not blocked by 10 mM
Kv1.4	half activated at -22 mV	activates rapidly	half inactivated at -74 mV	inactivates rapidly	half blocked by 12.5 mM	not blocked by 10 mM
Kv1.5	half activated at 0 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.3 mM	half blocked by 0.3 mM
Kv1.6	half activated at -15 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.3 mM	half blocked by 4 mM
Kv2.1	half activated at 0 mV	activates rapidly	half inactivated at -35 mV	inactivates slowly	half blocked by 0.5 mM	half blocked by 10 mM
Kv2.2	not known	activates rapidly	not known	inactivates slowly	not known	half blocked by 7.9 mM
Kv3.1	half activated at 19 mV	activates rapidly	half inactivated at 10 mV	inactivates slowly	half blocked by 0.1 mM	half blocked by 0.2 mM
Kv3.2	half activated at 6 mV	activates rapidly	not known	inactivates slowly	not known	not known
Kv3.3	half activated at 5 mV	activates rapidly	half inactivated at 5 mV	inactivates moderately	half blocked by 1.2 mM	half blocked by 0.14 mM
Kv3.4	half activated at 10 mV	activates rapidly	half inactivated at -53 mV	inactivates variably	half blocked by 0.5 mM	half blocked by 0.5 mM
Kv4.1	half activated at 10 mV	activates rapidly	half inactivated at-65 mV	inactivates rapidly	half blocked by 5 mM	not blocked by 10 mM
Kv4.2	half activated at -5 mV	activates rapidly	half inactivated at -55 mV	inactivates rapidly	half blocked by 5 mM	not blocked by 10 mM
Kv4.3	half activated at -15 mV	activates rapidly	half inactivated at -60 mV	inactivates rapidly	half blocked by 2 mM	not blocked by 10 mM

(rapid activation occurs over milliseconds; rapid inactivation occurs over tens of milliseconds; moderate inactivation occurs over hundreds of milliseconds, slow inactivation occurs over seconds; the variable inactivation of Kv3.4 channels occurs over tens of milliseconds to seconds; adapted from: Yokayama *et al.*, 1989; Luneau *et al.*, 1991; Rettig *et al.*, 1992; Vega-Saenz de Miera *et al.*, 1992; Covarrubias *et al.*, 1994; Swanson *et al.*, 1990; Duprat *et al.*, 1995; Stuhmer *et al.*, 1989; Pak *et al.*, 1991; Baldwin *et al.*, 1991; Serodio *et al.*, 1996; Taglialatela *et al.*, 1992; Hwang *et al.*,1992; Grissmer *et al.*, 1994)

three of these genes do not produce functional channels when expressed in heterologous expression systems.

Voltage-gated potassium channels appear to be the most highly conserved protein between species, even more so than histones. For example, there is typically more than 99% amino acid homology between the corresponding voltage-gated K channels of mice and man (Salkoff, 1992). Among the four *Drosophila* voltage-gated channels and votage-gated channels of other species, there are highly conserved functional domains including a pore region (H5), six putative transmembrane spanning regions (S1-S6), a voltage sensor (S4), and and inactivation ball and receptor.

1.4.4 Other potassium channel gene families

We are now aware of eight different potassium channel gene families with shared homology (for a review, see Wei *et al.*, 1996). In addition to the Kv family, there are five other potassium channel families that contain six transmembrane spanning regions. The KQT family is related to the human KVLQT1 channel, which is implicated in one form of long QT syndrome, a hereditary cardiac disorder (Wang *et al.*, 1996). The *eag*-like family was first discovered in *Drosophila* and its human homologue *herg* is also implicated in long QT syndrome (Curran *et al.*, 1995; Smith *et al.*, 1996; Trudeau *et al.*, 1995). The calcium-activated *slo*-like potassium channel family also was first cloned in *Drosophila* (Elkins *et al.*, 1986; Atkinson *et al.*, 1991). This family contains large-conductance potassium channels that are gated by both voltage and intracellular calcium

(Adelman *et al.*, 1992; Butler *et al.*, 1993). The cyclic-nucleotide-gated cation channels are associated with primary sensory neruons of the retina and olfactory epithelium, and are gated by intracellular-cyclic nucleotides (Kaupp *et al.*, 1989). Another calcium-activated potassium channel gene family was recently identified in *C.elegans* and encodes a small conductance calcium-activated potassium channels (Kohler *et al.*, 1996). There is a family of inward-rectifying potassium channels composed of two transmembrane spanning domains and a pore region which are homologous with the fifth through sixth transmembrane spanning regions of the six transmembrane spanning potassium channel families (Doupnik *et al.*, 1995). Additionally, there is a family of four transmembrane spanning domain channels which resemble two inward-rectifying channels linked in tandem (Lesage *et al.*, 1996). However, in my thesis, I have limited my experiments and discussions to the Kv family of voltage-gated potassium channels.

Anatomy of a Kv channel

1.4.5 Pore region

A definitive feature of voltage-gated potassium channels is conduction of potassium. This conduction is accomplished through the pore region (H5) that consists of 21 amino acid residues which lie between the fifth and sixth transmembrane spanning regions and is believed to span the membrane twice (Yellen *et al.*, 1991; Yool and Schwarz, 1991; for a review, see Kerr and Sansom, 1997). As there is no three-

dimensional structure available for voltage-gated potassium channels, our current understanding of pore structure is derived from mutagenesis studies (Gross *et al.*, 1994; Heginbotham *et al.*, 1994; Yool and Schwartz, 1995) and cysteine-scanning mutagenesis technique (Lu and Miller, 1995; Kurz *et al.*, 1995; Pascual *et al.*, 1995). In the cysteine scanning mutagenesis technique, individual potassium channel residues are mutated to cyteine which contains a sulfhydryl moeity. Then other reagents for detection are coupled to this moiety under specific experimental conditions. If the mutated channel residue is accessible to react with the labelling reagent in either the intracellular or extracellular environment, then a statement about the position of the residue can be made (Akabas *et al.*, 1992, 1994; Javitch *et al.*, 1994).

The H5 region is the most highly conserved sequence among voltage-gated potassium channels and is the primary determinant of ionic selectivity (Aiyar *et al.*, 1996; Heginbotham *et al.*, 1992; 1994; DeBiasi *et al.*, 1993; Taglialatela *et al.*, 1993, 1994; Kirsch *et al.*, 1995, 1992; Yool and Schwarz, 1991; MacKinnon and Yellen, 1990; Hartman *et al.*, 1991). The pore also determines susceptibility to block by TEA (Harris and Isacoff, 1996; Taglialatela *et al.*, 1994; Choi *et al.*, 1993; MacKinnon and Yellen, 1991; Kirsh *et al.*, 1992; 1990; Busch *et al.*, 1991), charybdotoxin (Gross *et al.*, 1994; Heginbotham and MacKinnon, 1992; MacKinnon and Miller, 1989) and agitoxin (Gross amd MacKinnon, 1996).

Though we do not know the conformation of the pore region, three structurally distinct types of models are being considered. 1) In the eight stranded ß-barrel model, the

H5 region adopts a membrane spanning ß-hairpin conformation (Kerr and Samson, 1997). 2) In the eight staved α-helix bundle model, each H5 region adopts a partially membrane spanning α-helical hairpin conformation (Kerr and Samson, 1997). 3) In mixed secondary structure models, α-helix or ß-sheets are combined with randomly coiled residues to produce the pore structure (Lipkind *et al.*, 1995; Durell and Guy, 1996). However. in each of these models, the voltage-gated potassium channel conducts via a single-file aqueous pore structure composed of multiple ion binding sites with a vestibule at each end (Yool and Schwarz, 1991) and the H5 region does not fully traverse lipid bilayer (Durell and Guy, 1992, 1996; Kerr and Samson, 1997). Also, the membrane spanning regions act as barrel staves while H5 extends into the aqueous pore from the outside of the membrane to act as a selectivity filter (MacKinnon, 1995).

Gating

1.4.6 Activation and the voltage sensor

Voltage-gated potassium channels open in response to depolarization. In their descripion of currents on the squid giant axon, Hodgkin and Huxley (1952) developed the idea that charged particles moving in an electric field may be responsible for channel gating. The charged particles are described as gating charges, while their movement which results in channel opening is called gating current (Hodgkin and Huxley, 1952; Hille, 1984). The fourth transmembrane spanning domain (S4) which contains a

positively charged amino acid (basic arginine or lysine) every third position is the voltage sensor or gating charge (Lopez *et al.*, 1991; Papazian *et al.*, 1991; Larsson *et al.*, 1996; Bezanilla *et al.*, 1991). When the channel is closed, only five S4 residues lie in the plane of the membrane, but when the membrane is depolarized, this segment is displaced outward through the plane of the membrane and four basic residues are drawn into the membrane (Larsson *et al.*, 1996). In fact, during channel activation, at least seven residues of S4 move from a buried position to the extracellular environment (Mannuzzu *et al.*, 1995). The resultant movement translocates basic charges outwards across the transmembrane core of the channel, resulting in conformational changes which open the channel. This movement of the basic residues is the gating current.

1.4.7 Rapid-inactivation and the tethered plug

Voltage-gated potassium channels inactivate through two different mechanisms: N-type and C-type. Rapid inactivation or N-type inactivation occurs through the obstruction of the pore region by a tethered ball structure. The ball corresponds to the twenty amino-terminal region residues and is linked to the core region of the subunit by a chain that is sixty residues long (Hoshi *et al.*, 1990; Zagotta *et al.*, 1990a; MacKinnon 1990). Antz and coworkers (1997) recently deduced the ball structure using high resolution nuclear magnetic resonance (NMR) spectroscopy. The structure is compact with all basic residues clustered on one side of the molecule. In some channels the ball is the target of modulation by serine phoshorylation through protein kinase C (PKC)

(Covarrubias *et al.*, 1994) or through oxidation/reduction of a cysteine residue (Stevens *et al.*, 1995; Ruppersburg *et al.*, 1991).

1.4.8 Slow-inactivation

C-type inactivation occurs more slowly than N-type, though there is a relationship between the two types. The outer channel mouth is involved in C-type inactivation (Choi *et al.*, 1991; Hoshi *et al.*, 1991; Lopez-Barneo *et al.*, 1993) and the conformational change leading to C-type inactivation can only occur when the potassium binding site at outer mouth of the pore is vacant (Baukrowitz and Yellen, 1995, 1996a, b). During N-type inactivation, this potassium binding site is maintained vacant when the inner mouth of the pore is occluded by the inactivation ball (Baukrowitz and Yellen, 1995). Thus the vacant potassium binding site in N-type activation faciliates C-type inactivation.

1.4.9 Assembly of functional channels

Functional voltage-gated potassium channels are believed to be formed from four Kv subunits (Jan and Jan, 1997). The assembly of tetrameric channels is supported by coexpression studies of Kv subunits having different pharmacological and biophysical properties (MacKinnon, 1991; Ruppersburg *et al.*, 1990; Isacoff *et al.*, 1990; Christie *et al.*, 1990; Taglialatela *et al.*, 1994) and through the expression of linked Kv subunits in heterologous systems (MacKinnon, 1990; Liman *et al.*, 1992).

Regulatory mechanisms specifiy the interactions among Kv subunits involved in the formation of voltage-gated potassium channels and limit the formation of heteromeric channels to members of the same subfamily (Isacoff *et al.*, 1990; Christie *et al.*, 1990; Ruppersburg *et al.*, 1990; Covarrubias *et al.*, 1991; Jan and Jan, 1997). The cytoplasmic amino-terminal and first membrane spanning domain are important in subunit assembly and the specificity of subunit assembly. It seems the first membrane spanning domain, the S1 region, is essential for the association of subunits to occur (Babilia *et al.*, 1994), but it is the amino-terminal cytoplasmic region that provides specificity of assembly (Li *et al.*, 1992; Shen *et al.*, 1993; Jan, and Jan, 1992). Although the exact code determining specificity of assembly has not been deciphered, the hydrophilic motif appears to reside in a 20-30 residue stretch of the amino-terminal, which is highly conserved between subunits of the same subfamily and is called the A region (Shen and Pfaffinger, 1995; Xu *et al.*, 1995). So far there is one reported exception to the formation of channels between subfamilies (Shadidullah *et al.*, 1995a, b).

1.4.10 Modulation of Kv channels

The gating of certain Kv channels is coupled to the oxidative/reductive metabolism of the neuron and regulated through second messenger systems. Hydrogen peroxide is generated during normal cell metabolism by a variety of enzymes including monoamine oxidase (MAO), superoxide dismutases (SODs), and NAD(P)H oxidases. H_2O_2 oxidizes a cysteine residue in the inactivation balls of Kv1.4, Kv3.3 and Kv3.4.

(Kv3.4 cysteine 6; Kv1.4 cysteine 13; Ruppersberg *et al.*, 1991; Vega-Sanez de Miera, and Rudy, 1992; Stephens and Robertson, 1995; Duprat *et al.*, 1995; Stephens *et al.*, 1996). This oxidation of the inactivation gate stabilizes the channel open state presumably through inhibiting interactions with the acidic inactivation ball receptor near the cytoplasmic mouth of the channel (Isacoff *et al.*, 1991; Yool and Schwarz, 1995).

Numerous second messenger systems modulate Kv channels. Protein kinase C dramatically slows the inactivation of Kv3.4 through serine phosphorlyation of the inactivation ball (serine 15 and serine 27; Covarrubias *et al.*, 1994). Similar to oxidation, phosphorlyation is thought to stabilize the channel open state through inhibiting the interaction of the basic inactivation ball with its receptor. Calcium/calmodulin dependent kinase slows Kv1.4 inactivation through serine phosphorylation of the inactivation ball chain (serine 123; Roeper *et al.*, 1997), also presumably through inhibiting interactions with the ball receptor. In contrast, protein kinase A activation increases the inactivation rate of *Shaker* through phosphorylation of the carboxyl-terminal (Drain *et al.*, 1994). It seems that the effect of phosphorylation on the Kv channel gating depends on where the phospho-acceptor is located. Still other pathways modulate potassium channels, phospholipase A_2 releases arachadonic acid from the cell membrane when activated by G-proteins or elevated intracellular calcium, in response to signaling by neurotransmitters or hormones (Chang *et al.*, 1987; Bursh, 1989). Arachadonic acid selectively supresses Kv4 currents (Villarroel and Schwarz, 1996). This suppression by arachadonic acid is direct but the site of action on Kv4 channels is unknown.

1.4.11 Contribution of auxiliary subunits

Auxilary subunits influence the voltage-sensitive properties and localization of Kv channels. These auxilary subunits are called ß-subunits and are cytoplasmic proteins which interact with the amino or carboxyl-termini of the pore forming α -subunits. Three Kvß subunits have so far been cloned, Kvß1, Kvß2 and Kvß4, which restrict their co-assembly to specific α -subunit subfamilies (Fink *et al.*, 1996; Rettig *et al.*, 1994; England *et al.*, 1995; Yu *et al.*, 1996). Kvß1 associates selectively with Kv1 subunits (Sewing *et al.*, 1996) through a motif found in the amino-termini of Kv1 subunits. The Kvß1 subunit is related to the NAD(P)H-dependent oxidoreductase superfamily, though no enzymatic function of Kvß1 has been demonstrated (McCormack and McCormack, 1994). A splice variant of Kvß1, Kvß1.1, contains an N-terminal structure that functions like the "ball and chain" of rapidly inactivating channels; Kvß1.1 conveys rapid inactivation on slowly inactivating Kv1 channels and increases the rate of N-type inactivation on rapidly inactivating Kv1.4 channels (Rettig *et al.*, 1994; Yu *et al.*, 1996). Kvß1.2 and Kvß1.3 also increase the rate of N-type inactivation and, directly or indirectly, influence C-type inactivation and voltage sensitivity of activation (Castellino *et al.*, 1995; England *et al.*, 1995; Majumder *et al.*, 1995; Rasmusso *et al.*, 1995). Kvß2 interacts with two members of the Kv1 subfamily, Kv1.2 and Kv1.4, and accelerates N-type inactivation of Kv1.4 and C-type inactivation on Kv1.2 (Uebele *et al.*, 1996). Kvß4

associates specifically with Kv2.2, through interacting with the carboxyl-terminal of Kv2.2 (Fink *et al.*, 1996). A "chaperone" role is proposed for Kvß4 because it is believed to facilitate the integration of Kv2.2 in the plasma membrane (Fink *et al.*, 1996). A Kv4 specific ß-subunit is believed to influence the voltage-sensitive properties of these channels. This putative subunit has not yet been cloned, however, co-expression of Kv4 channels in *Xenopus* oocytes with small molecular weight mRNA from brain shifts the voltage-sensitive properties of Kv4 channels ~30 mV hyperpolarized.

1.4.12 PDZ domains and subcellular localization

The specific functional role of a voltage-gated potassium channel depends on its subcellular localization. For example, if a voltage-gated potassium current is to contribute to integration of synaptic input, the channel must be localized postsynaptically either on dendrites or the neuronal somata. There are proteins containing PDZ domains which determine the localization of ion channels. The PDZ domains bind selectively to a short motif at the carboxyl-temini of target proteins and function as modules for specific protein-protein interaction. These domains were first recognized as sequence repeats in PSD-95, *Dlg* and ZO-1 (Sheng and Kim, 1996; Hsueh *et al.*, 1997). PDZ domains are found in a wide variety of developmentally important proteins including: syntropin, p55 and *disheveled* (Sheng and Kim, 1996; Doyle *et al.*, 1996; Songyang *et al.*, 1997). Furthermore, molecules containing these domains act as molecular scaffolding for signal conduction complexes such as the components of the *Drosophila* signaling cascade.

Of the PDZ domain containing molecules, MAGUKs (membrane associated guanylate kinases), which includes the PSD-95 subfamily, are implicated in the synaptic clustering of several classes of ion channels. The PSD-95 subfamily, which contains four members: PSD-95/SAP90 (Cho *et al.*, 1992; Kistner *et al.*, 1993); SAP97/hdlg (Lue *et al.*, 1994; Muller *et al.*, 1996); chapsyn-110/PSD-93 (Brenman *et al.*, 1996; Kim *et al.*, 1996); and SAP102 (Muller *et al.*, 1996), is involved in clustering and localization of voltage-gated and ligand-gated ion channels at synaptic sites. PSD-95 colocalizes with Kv1 channels in the vertebrate CNS (Hunt *et al.*, 1996) and causes clustering of Kv1 channels in heterologous cells (Kim *et al.*, 1995). *Dlg* (Disc large) is the *Drosophila* homolog of PSD-95 which binds *Shaker* channels and colocalizes with *Shaker* in the larval neuromuscular junction (Cho *et al.*, 1992; Tejedor *et al.*, 1997; Francisco *et al.*, 1997). The activity of PSD-95 is specific to members of the Kv1 subfamily, but as immunocytochemistry shows specific subcellular localization for individual Kv genes, then it is probable that similar PDZ containing molecules exist and direct the targetting of other voltage-gated potassium channels.

1.5 Kv gene expression in hippocampal pyramidal cells

For my doctoral studies, I have set out to identify which genes encode the voltage-gated potassium currents expressed by rat sympathetic neurons. There is a similar body of work which contributes to the identification of the voltage-gated potassium channel genes that are expressed in hippocampal pyramidal neurons. Our understanding of gene

expression in the hippocampus is the result of *in situ* hybridization and immunohistochemistry experiments. These two techniques are limited because statements about mRNA or protein expression are at best semiquantitative, and in a complex structure like the hippocampus, localization of signal to a specific cell type is difficult, especially if the signal is perisynaptic.

1.5.1 Kv genes which contribute to pyramidal IK

Hippocampal pyramidal cells express a number of different genes that could contribute to the delayed-rectifier current. Kv1.1, Kv1.2, Kv1.3, Kv1.5; Kv1.6, Kv2.1, Kv2.2, Kv3.1, Kv3.2 and Kv3.3 have been localized to hippocampal pyramidal cells through *in situ* hybridization (Veh *et al.*, 1995; Sheng *et al.*, 1992, 1994; Hwang *et al.*, 1992, 1993; Rettig *et al.*, 1992; Beckh and Pongs, 1990). Furthermore, immunocytochemistry shows that Kv1.3 and Kv1.6 protein appears to be expressed in pyramidal cell dendrites, but the signal instead may be due to the mossy fiber-terminals (Veh *et al.*, 1995). Kv1.2 mRNA is highly expressed in pyramidal cells but the signal for Kv1.2 protein is very low or absent in dendrites (Veh *et al.*, 1995). Kv1.5 staining is detectable in all pyramidal cell bodies and proximal dendrites at light to moderate staining (Maletic-Savatic *et al.*, 1995), whereas Kv1.1 protein is only localized to the dendrites (Sheng *et al.*, 1992, 1993; Veh *et al.*, 1995).

Each of these Kv1 genes, when expressed in heterologous expression systems, encode slowly inactivating potassium channels with voltage-sensitive properties which

resemble those of hippocampal IK. Kv1 channels typically activate slowly above the threshold for action potential generation and are partially inactivated at resting membrane potential. Kv1 channels show a range of sensitivities to block by TEA and sensitivity to block by 4-AP. The pharmacolgical properties of Kv1 channels are different from those of pyramidal IK, which is sensitive to block by moderate concentrations of TEA and not blocked by 4-AP. Even though differences exist in the subcellular localization of individual Kv1 proteins, the physiological properties are similar and we would not expect strong differences in physiological currents due to differential expression of Kv1 subunits.

The properties of pyramidal IK are the same on dendrites and axons (Hoffman *et al.*, 1997). Unless Kv1 channels are in some way modified, thereby altering their pharmacological properties, then it is unlikely that they are the principle channels contributing to hippocampal IK.

Kv2.1 mRNA is more highly expressed in hippocampal pyramidal cells than is Kv2.2, as observed through *in situ* hybridization (Hwang *et al.*, 1992). Both Kv2.1 and Kv2.2 have been immunohistologically localized to the dendrites and somata of hippocampal pyramidal cells (Maletic-Savatic *et al.*, 1995). The Kv2.1 staining is a discrete punctuate pattern on pyramidal body extending into proximal dendrites (Hwang *et al.*, 1993), whereas, Maletic-Savatic and colleagues (1995) report that Kv2.2 immunoreactivity is present faintly and homogenously on the soma and proximal dendrites of pyramidal cells.

Each of these Kv2 genes encode a slowly inactivating potassium channels. Kv2.1 activates at depolarized potentials and is half steady-state inactivated at -20 mV (Shi *et al.*, 1994). Moderate concentrations of TEA block Kv2.1 similar to hippocampal IK. However, unlike hippocampal IK, Kv2.1 is also by blocked by moderate concentrations of 4-AP (Ikeda *et al.*, 1992). Kv2.2 seems to encode a similar delayed-rectifier current as Kv2.1, but it has not been well characterized. Like Kv1 channels, Kv2 channels show similar voltage-sensitive properties to hippocampal IK, yet differ in pharmacology, therefore it is difficult to explain how Kv1 channels contribute to IK.

Kv3.1, Kv3.2 and Kv3.3 are detectable at low to moderate levels in hippocampal pyramidal cells using *in situ* hybridization, whereas, Kv3.4 is undetectable (Weiser *et al.*, 1994). These three Kv3 channels encode slowly inactivating currents which inactivate at depolarized potentials (Rettig *et al.*, 1992; Vega-Saenz de Miera *et al.*, 1992). Unlike hippocampal IK, Kv3 channels are sensitive to block by both low concentrations of 4-AP and TEA (Rettig *et al.*, 1992). As for Kv1 and Kv2 channels it is difficult to account for differences in pharmacology between Kv3 channels and pyramidal IK.

1.5.2 Kv genes contributing to pyramidal IA

In situ hybridization shows that four genes (Kv1.4, Kv4.1, Kv4.2 and Kv4.3) expressed by hippocampal pyramidal neurons encode rapidly inactivating transient currents when expressed in *Xenopus* oocytes. (Sheng *et al.*, 1992, 1994; Hwang *et al.*, 1992, 1993; Serodio *et al.*, 1996). Immunocytochemistry shows that Kv1.4 protein is

lacking from pyramidal cell bodies but may be expressed on axons (Sheng *et al.*, 1992; Maletic-Savatic *et al.*, 1995). Kv4.2 has a somatodendritic location and is especially prevalent in the distal dendritic processes (Maletic-Savatic *et al.*, 1995).

Similar to pyramidal IA, Kv1.4 encodes a rapidly inactivating transient current that activates rapidly from depolarized potentials, requires hyperpolarization to remove inactivation and is blocked by high 4-AP but not TEA (Stuhmer *et al.*, 1992). It is possible that Kv1.4 contributes to an A-current present distally on axons.

The Kv4 genes also give rise to rapidly inactivating channels *in vitro* that have similar voltage-sensitivity to pyramidal IA when expressed *in vitro* (Baldwin *et al.*, 1991; Serodio *et al.*, 1996). Pyramidal IA has similar pharmacological sensitivity as Kv4 channels, both are insensitive to block by TEA and sensitive to block by 4-AP, similar to hippocampal IK (Baldwin *et al.*, 1991; Serodio *et al.*, 1996). Therefore, it is quite likely that Kv4.2 channels contribute to the A-currents recorded from pyramidal cell dendrites. Yet, it is not clear what genes contribute to the TEA sensitive component of dendritic IA.

1.5.3 Kv genes contributing to pyramidal ID

None of the Kv genes described above encode a slowly inactivating current that activates from subthreshold potentials, when expressed in heterologous expression systems. The above genes give rise to channels that typically fall into two categories: 1) Kv1.1, Kv1.2, Kv1.3, Kv1.6, Kv2.1 and Kv2.2 encode slowly inactivating channels which activate at depolarized potentials and 2) Kv1.4, Kv4.1, Kv4.2 and Kv4.3 encode

rapidly inactivating currents which activate above the threshold for action potential generation (-55 mV; Storm, 1990; Pak *et al.*, 1991; Baldwin *et al.*, 1991; Serodio *et al.*, 1996; Grissmer *et al.*, 1994). Additionally, ID is ten times more sensitive to block by 4-AP than is any Kv channel. Therefore, it is not clear how Kv channels could contribute to ID.

1.5.4 Kv genes contributing to pyramidal IM

The M-current on hippocampal pyramidal cells is typical of a class of potassium currents recorded *in vivo* which seem to be distinct from the other voltage-gated potassium currents. None of the Kv genes that have been cloned give rise to a low threshold, slowly activating, noninactivating current with odd pharmacological properties. It has been suggested that another class of potassium channels *eag* (*ether-a-go-go*) encode the M-current (Stanfeld *et al.*, 1997; Mathie and Watkins, 1997).

1.6 Summary

Neurons are able to respond to a wide range of physiological stimuli and intensities, integrating input spatially and temporally to initiate and propagate action potentials at the proper frequency and correct shape. For a neuron to function properly, it is important that it expresses the correct repertoire of ion channels in the proper location. While other classes of channels such as sodium channels, calcium channels, calcium activated potassium channels and inward rectifying channels are also important to the

electrical behavior of neurons, we elected to focus on outward voltage-gated potassium channel genes as a model for the expression of different classes of ion channels and receptors.

How do the sixteen or more voltage-gated potassium channel genes contribute to physiological currents in native cells? How does voltage-gated potassium channel gene expression change over development and what factors regulate this gene expression? What role, if any do changes in mRNA levels have on the expression of voltage-gated potassium channels.

It is difficult to address these questions of gene expression in the CNS as neurons are not typically found in discrete homogeneous populations. Moreover, CNS neurons are difficult to isolate and grow in culture as a pure population, where their external environment can be manipulated. In contrast, SCG neurons offer many advantages for the study of developmental gene expression. The SCG ganglia contain relatively homogenous population of neurons that are easily dissected and grow well in culture. It should therefore be possible to identify the voltage-gated potassium channel genes that are expressed in these neurons and examine changes of gene expression over normal neonatal development. Also, it should be possible to grow these neurons alone in dissociated culture, to investigate the influence of extrinsic factors on Kv gene expression.

1.7 Overview

For my doctoral thesis, I investigated the expression of voltage-gated potassium channel genes in neonatal SCG neurons. The objectives of my studies presented in this thesis were to identify candidate voltage-gated potassium channel genes that may be expressed in P7 SCG neurons using a PCR based approach, and then quantify the expression of these genes in P7 SCG neurons. I also wished to measure the change in expression of these genes by SCG neurons over neonatal development and development in culture, and to investigate the effect of the cytokine CNTF on the expression of voltage-gated potassium channels genes in culture. Finally, I wanted to compare the expression of voltage-gated potassium channel genes between SCG neurons and sensory neurons from the nodose and trigeminal ganglia because these are also peripheral neurons but have a different functional role.

(A) Chapter 2: This chapter is a general methods section describing molecular biology and cell culture techniques used for experiments discussed in Chapters 3, 4 and 5.

(B) Chapter 3: This chapter discusses the use of molecular biology techniques to identify candidate genes that may encode the voltage-gated potassium currents expressed by P7 SCG neurons, which were described by McFarlane and Cooper (1992). Then, I went on to use RNase protection assay to directly measure the expression level of these Kv genes in P7 SCG neurons. I found that of the eight Kv genes investigated, seven were expressed by SCG neurons, these seven genes were expressed exclusively in the neuronal fraction

of the ganglia and these Kv genes were expressed at levels comparable to the nicotinic acetylcholine receptor genes also expressed by these neurons.

(C) Chapter 4: This chapter discusses studies investigating the expression of voltage-gated potassium channel genes by SCG neurons. I used a combination of molecular biology and cell culture techniques in these studies to measure gene expression over normal neonatal development and in dissociated cell culture. Voltage-gated potassium currents are known to undergo interesting developmental changes that are dependent on extrinsic influences (McFarlane and Cooper, 1993). The results show that most voltage-gated potassium channel genes examined were expressed at relatively constant levels throughout development and in culture with two exceptions. Both Kv3.4 and Kv4.2 transiently increased from low levels over neonatal development and Kv4.2 expression virtually disappeared in culture. Moreover, CNTF treatment increased the gene expression of Kv3.4 and Kv4.2 in culture.

(D) Chapter 5: This chapter discusses studies investigating the expression of voltage-gated potassium channel genes by nodose and trigeminal neurons. Similar to sympathetic neurons, sensory neurons express three voltage-gated potassium currents, IAf, IAs and IK (McFarlane and Cooper, 1991, 1992). However, in sensory neurons these currents are not regulated over development (McFarlane and Cooper, 1992). I show that all eight Kv genes investigated were expressed in nodose and trigeminal neurons, but with notable differences in expression level between the three different ganglia.

(E) Chapter 6: This chapter is a general discussion in which I briefly summarize my finding from earlier chapters. Furthermore, I discuss the unresolved questions arising from my thesis and approaches that could be used to address these issues.

Chapter 2:

General Methods

2.1 Identification of candidate Kv genes

2.1.1 Total cellular RNA isolation

Total cellular RNA was extracted from SC, nodose or trigeminal ganglia by a method based on Chomoczynski and Sacchi (1987). The ganglia were dissected and kept in a petri dish containing ice-cold L15-Air until the end of the dissection. They were then transferred to a 15 ml polypropylene tube (Falcon 2059), and the L15 was removed and replaced with 3 ml of ice-cold solution D (see section **2.3.9**). The ganglia were then homogenized using a polytron. For many experiments, the ganglia were dissociated and neurons were separated from non-neuronal cells (see section **2.2.6**) prior to RNA extraction. In that case, the dissociated neurons were rinsed once with ice-cold modified HBSS (plus 1 mM $CaCl_2$). Then 2-4 dishes from a given condition were typically pooled together and 300-400 µl of solution D was used to collect the RNA. To maximize yield of RNA extraction, 100 µl of solution D was added to the center well of one petri dish, pipetted up and down 5 times and the solution was transferred to a second well. A second 100 µl of solution D was used to rinse the same center well.

The following solutions were added sequentially to the solution D containing neuronal extracts while vortexing between each one: sodium acetate 2 M (pH 4.1) at a 1:10 ratio with solution D; phenol (see section **2.3.9**) at a 1:1 ratio with solution D; chloroform:isoamyl (24:1) at a 2:10 ratio with solution D. This mixture was kept on ice for 15 min, centrifuged for 20 min at 4°C (10 000 g), and the aqueous phase was recovered and mixed with equal volume of isopropanol and stored at -20°C for 1 hour or overnight. The precipitated RNA was then centrifuged, resuspended in 0.1 to 0.5 ml of solution D (depending on the amount of RNA) and reprecipitated with an equal volume of isopropanol at -20°C for 1 hour or overnight. The precipitated RNA was rinsed with 70% EtOH, air dried and resuspended in DEPC-H_20 (100-400 µl) and the optical densities (OD260 and OD280) were measured on a spectrophotometer to quantify the amount of RNA (OD260 of 1 = 40 µg/ml RNA) as well as the ratio of RNA:DNA. A ratio of OD260/OD280 = 1.8-2.1 was considered satisfactory for RNase protection assays. The RNA from cultured neurons was resuspended in only 100 µl and the OD was measured using an RNase free microcuvette (100 µl; Beckman) that was precleaned with DEPC-H_2O containing 100 mM NaOH and 1 mM EDTA. In this manner, the ODs could be measured for the whole RNA samples, without extra dilutions. The RNA was then reprecipitated and kept at -20°C until use for RNase protection assays. Our average yield of total cellular RNA from 4 petri dishes with ~6000 SCG neurons cultured for 7 days was 2.0 ± 0.2 µg.

2.1.2 First strand cDNA synthesis

The cDNA was reverse transcribed from postnatal day seven SCG total cellular RNA by methods developed by Saiki et al. (1985) using the cDNA cycle kit (from Invitrogen). For a 20 µl reaction, 1 µl of random hexamer primers (1 µg/ul) were annealed to RNA (2-10 µg in 10 µl DEPC-H_20), in the presence of 2.5 µl ß-mercaptoethanol (0.7 M), by denaturing the mixture for 2 min at 65°C and then allowing the mixture to stand for 10 min at 21°C. Then the following reagents were sequentially added: 4 µl 5x AMV buffer, 4 nucleotide bases (1 µl of 25 mM) dATP, dCTP, dGTP and dTTP, each at a final concentration of 1.25 mM, 1 µl placental RNAse inhibitor (5 units) and 1 µl avian murine reverse (AMV) transcriptase (5 units), gently mixed and incubated at 42°C for 1 hour. Some reactions were "spiked" with ^{32}P-dCTP (10 µCi/tube) to permit visualization of the amount and size distribution of cDNA produced on an agarose gel (see section **2.1.4**). Reverse transcription was repeated by denaturing the new cDNA-RNA hybrids at 95°C for 3 min, adding 1 µl new reverse transcriptase (5 units) and incubating for another hour at 42°C. To help precipitate the cDNA, 1 µl mussel glycogen (1ug/ul) was added, before 100 µl ddH_2O and 100 µl Tris-saturated phenol:chloroform (50:50) were added and vortexed well in order to remove all proteins. Then, the cDNA was precipitated with a 0.1 volume of 3 M NH_4Ac (pH 5.2) and 2 volumes of 100% EtOH, washed with 70 % EtOH, allowed to air dry for 15 min and resuspended in 15 µl of TE-buffer.

To verify that the cDNA synthesis reactions were successful, 1 µl of each "spiked" reaction was counted using a liquid scintillation analyzer (1600 TR, Packard). Six thousand cpm of each reaction were run on an agarose gel (1%). The gel was dried at 65°C for 2 hour and exposed to X-ray film (Kodak) for at least 3 hour. We confirmed that the majority of cDNAs were longer than 1 kbp through comparing the distribution of the cDNA synthesis products to size markers run alongside on the same gel.

2.1.3 First round PCR amplification with degenerate primers

The cDNA was PCR amplified according to methods described by Saiki et al. (1985). For a 25 µl PCR reaction, ~1ug of cDNA in 1 µl, reverse transcribed (Invitrogen, cDNA cycle kit) from total cellular RNA isolated from P7 SCG ganglia, was combined with 14.5 µl of ddH_2O, 2.5 µl 10x Taq buffer (see section **2.3.9**), and 1 µl of each sense and antisense degenerate primers 25 µM (see appendix I) for a final concentration of 1 µM. A control reaction with no cDNA was amplified in parallel. The reaction mixture was denatured by heating at 98°C for five min and allowed to cool. Then a cocktail was added to each reaction tube containing 3 µl of distilled H_2O, 0.5 µl of 10 mM dATP, dCTP, dGTP, and dTTP and 1 unit (1 µl) of Taq DNA polymerase (USB). During the first five cycles of amplification, annealing was conducted at low stringency (35°C for one min), before ramping the temperature at a rate of 1°C/sec to 72°C for elongation (1 min). Elongation was followed by denaturation at 94°C for one min, before beginning the

next cycle. These five rounds of low stringency amplification were followed by 25 cycles of higher stringency amplification annealing at 55°C instead of 35°C.

2.1.4 Size selection

The PCR products (see section **2.1.3**) were precipitated with 2.5 µl (0.1 volume) 3 M NH_4Ac, 50 µl (2 volumes) of 100% EtOH and 1 µl (1 µg/ul) mussel glycogen (Boehringer Mannhiem) and pelleted at 10 000 g for 20 min. The cDNA pellet was washed with 70% EtOH, vacuum dried and resuspended in 8 µl of TE. The entire cDNA sample from each reaction was run on an agarose gel (3%) see below, containing 0.1 µg/ml ethidium bromide (see section **2.3.9**) at 65 mV for 2 hour along with a 123 bp ladder (Gibco). A band corresponding to the correct size 121 base pair fragment was visualized with UV light and cut from the gel with a razor blade. This excised gel slice was soaked in 1 ml TE for 15 min in order to dilute the ethidum bromide. The cDNA was extracted from the slice by spin filtering through a 0.5 uM PZ523 spin column (5prime-3prime Inc.) at 4000 g for 5 min, leaving the size selected product in ~100 µl TE.

2.1.5 Second round amplification

A 1 µl aliquot of size-selected and spin-purified product (see section **2.1.4**) was further amplified using nondegenerate nested primers. The product was amplified for 30 cycles, annealing for 1 min at 55°C, then ramping the temperature at 1°C/sec up to 72°C

for a one min elongation followed immediately by a one min denaturation at 94°C. These reaction products were again size selected on an agarose gel (3%), spinfilter purified (see section **2.1.4**), precipitated with 2.5 µl (0.1 volume) 3 M NH_4Ac, 50 µl (2 volumes) of 100% EtOH and 1 µl (1 µg/µl) mussel glycogen (Boehringer Manhiem), pelleted at 10 000 g for 20 min, washed in 70% EtOH and resuspended in 10 µl ddH_2O.

2.1.6 Restriction digest of PCR product and cloning vector

Both the purified PCR product and cloning vector (M13 mp18 RF bacteriophage or pBS KS+) were digested simultaneously with appropriate restriction enzymes (XbaI and EcoRI; Boehringer Mannhiem) to produce compatible restriction sites for subcloning. 10x digestion buffer (2 µl; Boehringer Mannhiem), 1 µl of each restriction enzyme and M13 cloning vector (5 µg) or PCR product (500 ng) in 16 µl of ddH_2O were combined and incubated overnight (12-18 hour) at 37°C. To help precipitate the cDNA, 1 µg mussel glycogen was added, before 100 µl ddH_2O and 100 µl water-saturated phenol:chloroform (50:50) were added and vortexed well in order to remove all proteins. The digested products were resuspended in 10 µl TE, purified on an agarose gel (3%) to remove unwanted plasmid polylinker and uncut material, and spinfilter purified (see section **2.1.4**). The products were precipitated with 2 µl (0.1 volume) 3 M NH_4Ac, 40 µl (2 volumes) of 100% EtOH and 1 µl (1 µg/µl) mussel glycogen (Boehringer Mannhiem) and pelleted at 10 000 g for 20 min, washed in 70% EtOH and resuspended in 10 µl ddH_2O.

2.1.7 Ligation of PCR product and cloning vector

The restriction digested cloning vector and PCR product were ligated together using T4 DNA ligase as described by Sambrook *et al.* (1989). The PCR product (~200 ng) and M13 mp18 RF bacteriophage (~400 ng) (each in 4 µl ddH_2O; 10:1 molar ratio), were mixed with 1 µl T4 DNA Ligase 10X buffer (NEB), and 1 µl T4 Ligase (20 units; NEB) and then incubated at 21°C overnight (12-18 hour).

2.1.8 Creating competent bacterial cells

DH5αF' bacteria were made competent using the $Ca(PO_4)_2$ precipitation technique described by Sambrook *et al.* (1989). A single DH5αF' colony was grown for 16-20 hour at 37°C and then transferred to 100 ml LB (see below) and grown for ~3 hour at 37°C. The culture was cooled (10 min at 0°C) and then pelleted at 4000 g for 10 min at 4°C. The fluid was decanted and the cells resuspended in 10 ml ice-cold 0.1 M $CaCl_2$. Then the cells were repelleted at 4000 g for 10 min at 4°C, resuspended in 2 ml ice-cold 0.1 M $CaCl_2$, aliquoted into sterile 1.5 ml microcentrifuge tubes and stored at -70°C until needed.

2.1.9 Bacterial cell transformation

The competent cells were transformed using methods described by Sambrook et al. (1989). Freshly thawed competent DH5αF' bacteria (100 µl; see below) were added

to half of the ligation mixture (5 µl; see section **2.1.7**) in a 1.5 ml microcentrifuge tube that had been cooled on ice for 10 min. The DNA was allowed to settle onto the cell membranes for 15 min prior to heat shocking the cells to increase cell membrane permeability (42°C for 2 min). The bacteria were left to recover for 5 min on ice, before restoring normal calcium levels by adding 500 µl of 2xYT (see section **2.3.9**) and then incubating at 37°C for 30 min.

2.1.10 Plating

The transformed bacteria were plated according to the methods described by Sambrook *et al.* (1989). DH5αF' bacteria were streaked on an agar plate and left overnight at 37°C. An overnight culture was made by infecting 50 ml of 2xYT medium with a single bacterial colony. For each 15 mm agar plate, 200 µl of overnight culture was added to a mixture of 1-100 µl of the transformed bacteria, 50 µl of Isopropylthio-ß-D-Galactosidase (100 mM, Gibco BRL) and 10 µl of X-Gal (2% in DMSO, Gibco BRL). To this mixture was added 3 ml of melted top loading agarose (7 g agarose/l of 2xYT media). After gentle vortexing, the plating solution was poured onto the agar plate. The plates were left for 4-6 hour at 37°C for bacterial colonies to develop. The infective cycle of M13 bacteriophage does not involve cell lysis; however, infected bacteria grow more slowly. As such, colonies of infected bacteria appear as plaques on a turbid background lawn of non-transformed bacteria.

2.1.11 Isolation of single stranded plasmid DNA

Selected colonies were touched with a sterile micropipet tip which was ejected into 3 ml of 2xYT media (see below) along with 100 µl of an overnight bacterial culture of untransformed DH5αF' (see section **2.1.10**). The culture was shaken at 37°C for 6 hour, before 500 µl of the culture was removed to be stored frozen at -70°C in 65% glycerol, 0.025 M Tris-Cl (pH 8.0) and 0.1 M $MgSO_4$. The remaining 2.5 ml of culture was centrifuged for 15 min at 11 000 g to remove cells and the supernatent containing the M13 bacteriophage was recovered. The M13 bacteriophage was precipitated with 0.25 volume 3.5 M ammonium acetate/20% polyethylene glycol and pelleted by centrifugation for 30 min at 11 000 g. The pellet was resuspended in 500 µl ddH_2O and 500 µl Tris-saturated phenol:chloroform (50:50) were added and vortexed well in order to remove all proteins. Then, DNA was precipitated with a 0.1 volume of 3 M NH_4Ac (pH 5.2) and 2 volumes of 100% EtOH, washed with 70 % EtOH, allowed to air dry for 15 min, resuspended in 10 µl ddH_2O and then quantified (see section **2.1.1**).

2.1.12 DNA sequencing

The DNA inserts were sequenced using a modified version of the dideoxy sequencing method developed by Sanger and co-workers (1977) (Pharmacia T7 sequencing kit). About 2 µg of DNA in 10 µl of ddH_2O (see section **2.1.11**) was primed with 2 µl of T7 Universal primer (0.8 µM) and 2 µl of Pharmacia annealing buffer. In the

labeling reactions (5 min, 21°C), T7 polymerase elongates from the annealed primer along the template strand incorporating dCTP, dGTP, dTTP and dATPα^{35}S. Termination reactions (5 min, 37°C) followed, where the labeling mixture was transferred into four separate microcentifuge tubes containing ~0.1 mM dATP, dCTP, dGTP, and dTTP and ~0.005 mM of one of the dideoxynucleotide triphosphates ddATP, ddCTP, ddGTP or ddTTP. The dideoxynucleotide triphosphates lack the 3-hydroxyl group that is essential for elongation, so that when a ddNTP is incorporated, further elongation is halted. When these reactions were fractionated by electrophoresis on a denaturing acrylamide gel, the pattern of bands showed the distribution of terminations for a particular ddNTP. Reaction products were run on a 200 μm thick, 6% polyacrylamide 8 M urea gel at 65 watts for 3 hour. The gel was then placed on Whatman paper, covered with plastic wrap and vacuum dried for 45-60 min. The dried gel was exposed to X-ray film at 21°C for 1-4 days.

2.2 RNase protection assay

2.2.1 Generating probe templates

The probe templates for use in RNase protection assay were created in one of two ways. The sequences for Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4, and Kv4.2 templates were PCR cloned, while the probe template for Kv1.4 was created through the restriction digest and ligation of a full length Kv1.4 (courtesy of Ehud Isacoff). PCR amplification was conducted as described by Saiki *et al.* (1985) using cDNA reverse

transcribed from total cellular RNA isolated from P7 SCG (see section **2.1.2**) as the starting material. For a 25 µl PCR reaction ~1 ng of cDNA in 1 µl was combined with 14.5 µl of ddH_2O, 2.5 µl 10x Taq buffer (see section **2.3.9**), and 1 µl (25 µM) each of sense and antisense primers specific for the target sequence (see appendix I; final concentration of 1 µM). The mixture was denatured by heating at 98°C for five min and then allowed to cool. Then, a cocktail containing 3 µl of ddH_2O, 0.5 µl of 10 mM dATP, dCTP, dGTP, and dTTP and 1 µl of Taq DNA polymerase (1 unit/µl; USB) was added to each reaction tube. During the cycles of amplification, annealing took place at 57°C for one min, before ramping 1°C/sec to 72°C for elongation (1 min). Elongation was immediately followed by denaturation at 94°C for one min, before beginning the next cycle. These PCR products were then size-selected on an agarose gel (1%; see section **2.1.4**). As not enough cDNA was generated from one single amplification to subclone, a portion of the first round amplification product was reamplified, a 1 µl 1/100 dilution of gel purified product was used as starting material for a second round of PCR amplification conducted under the same conditions described above (see section **2.1.4**). These products were again size selected from an agarose gel (1%; see above). The PCR products and cloning vector (pBS KS+, Stratagene) were digested with the appropriate restriction enzymes (see appendix I), ligated (see section **2.1.7**), and used to transform competent bacteria (see section **2.1.9**). Transformed bacteria (1-100 µl) were spread plated onto agar plates just previously spread with 50 µl of Isopropylthio-ß-D-

Galactosidase (100 mM, Gibco BRL), and 10 µl of X-Gal (2% in DMSO, Gibco BRL) (Sambrook *et al.* 1989).

2.2.2 Double stranded plasmid isolation

Individual colonies were selected and grown up in 3 ml 2xYT over night (12-16 hour), 500 µl of the culture was removed to be stored frozen at -70°C in 65% glycerol, 0.025 M Tris-Cl (pH 8.0) and 0.1 M $MgSO_4$, but as pBS does not exist as a bacteriophage the double stranded DNA was isolated through alkali lysis (Sambrook *et al.*, 1989). The bacteria (1.5 ml) were transferred to a microcentifuge tube and pelleted at 21°C for 1 min. The supernatent was aspirated and the bacteria resuspended in 100 µl glucose solution (see section **2.3.9**) by gentle vortexing. The cells were lysed by adding 200 µl of 0.2 N NaOH/1% SDS, gently mixed by inverting the tube and cooled on ice for 10 min. The cellular debris was precipitated by adding 150 µl 5 M KOAc, incubating on ice for 10 min and then pelleted at 21°C for 5 min. The supernatent was removed to a clean tube and RNA was degraded by adding 1 µl of RNase A and incubating at 37°C for 30 min. The remaining protein and debris were removed through 2 rounds of 50% tris-phenol/chloroform extraction (500 µl). The DNA was precipitated by adding 1 ml EtOH and pelleted at 21°C for 10 min, washed with 70% EtOH and resuspended in ddH_2O. The amount of plasmid DNA was quantified (see section **2.1.1**) and sequencing using the Sanger didioxy method (see section **2.1.12**).

2.2.3 Large scale preparation of double stranded plasmid DNA

The bacterial colonies containing the inserts of interest were subcultured from frozen stocks. 3 ml of 2xYT was inoculated and grown ~8 hour at 37°C, 1 ml of the miniculture was added to 100 ml of 2xYT and grown overnight (12-16 hour; 37°C). Double stranded DNA was isolated using the Qiagen Maxi kit. Similar to above (**2.2.2**), the bacteria were pelleted, resuspended, lysed under alkali, and cellular debris precipitated and pelleted. The supernatent was applied to a column containing an anion exchange resin (Qiagen). In the Qiagen kit, the plasmid DNA is bound by the resin under low salt, low pH conditions, washed at medium salt concentrations, and eluted under concentrated salt conditions. The eluted plasmid DNA was precipitated with isopropanol, washed with 70% EtOH, resuspended in ddH_2O, and quantified as above. A portion of the total plasmid preparstion was linearized with the appropriate restriction enzyme (appendix I; see section **2.1.7**).

2.2.4 Riboprobe synthesis

Riboprobe synthesis for RNase protection assays were performed according to that described by Krieg and Melton (1987). [^{32}P] radiolabeled antisense RNA probes were transcribed *in vitro* from linearized plasmids containing subcloned portions of the cDNAs of interest. For the transcription reaction, the following reagents were added sequentially to a microcentrifuge tube: 1 µl of ATP, GTP and CTP (10 mM stock; final concentration:

500 µM; Promega), 8.5 µl ^{32}UTP (800 Ci/mmol; Dupont), 1 µl RNase inhibitor (30 units Pharmacia), ~0.5 µg linearized template cDNA (~1 µl) and 0.2 to 1.0 µl of RNA polymerase (either SP6, T3 or T7, 5-10 units). The final volume was ~20 µl. After mixing well, the solution was incubated at 37°C for 50-60 min. The template DNA was then digested with 10 units of DNase I (Pharmacia) for 20 min at 37°C. To help precipitate the probes, 10 µg tRNA (Sigma) was added. before 100 µl DEPC-H_2O and 100 µl water-saturated phenol:chloroform (50:50) was added and vortexed well in order to remove all proteins. After a 5 min centrifugation (10 000 g), the aqueous phase was collected and mixed with 9 µl of 10 M NH_4Ac and 322 µl EtOH. The probes were precipitated for 30 min and centrifuged for 20 min at 10 000 g.

In order to eliminate any smaller radiolabeled RNA resulting from incomplete transcription, which could interfere with the full length riboprobes in the hybridization reactions, the probes were gel purified. The pelleted probes were resuspended in loading buffer (see section **2.3.9**) and separated by electrophoresis on a 5% polyacrylamide 8 M urea gel (see section **2.3.9**) for 1 hour at constant voltage (240 V) with 1x TBE (see section **2.3.9**) as the running buffer. Following electrophoresis, the gel was absorbed onto Whatman paper and covered with plastic wrap, then exposed to Kodak X-ray film (in a dark room) for approximately 1 min to reveal the probe signals. The outline of the film was drawn with a marker onto the Whatman paper during the exposure period; the developed X-ray film could then be placed back on top of the gels to excise the probes (through the film) with a razor blade. The highly radioactive piece of gel was then put in

a microcentrifuge tube containing elution buffer (see section **2.3.9**). The probes were eluted (vigorously shaken at 37°C) for 2-18 hour. The eluate was mixed with equal volume of 100% EtOH to precipitate the probes which were kept at -20°C until use (up to 3 days).

2.2.5 RNase protection assay reactions

RNase protection assays were performed according to protocols described in Krieg and Melton (1989), Sambrook *et al.* (1989) and Ausubel *et al.* (1989), with minor modifications. Typically, 2 μg of total cellular RNA was combined with the radiolabeled probes (50 000 - 200 000 cpm each), both as EtOH precipitates. The mixture was centrifuged at 10 000 g for 20 min, the EtOH was removed and the radioactive pellet resuspended in 30 μl hybridization buffer (see section **2.3.9**), by 30 triturations. We found that this method gave us much more reproducible results than resuspending pellets by vortexing. The RNA mixture was denatured at 85-90°C for 10 min and incubated overnight at 60°C. The following day, the non-hybridized riboprobes (or all single stranded RNAs) were digested by 30 min incubation at 37°C with RNase T1 (1000 units, Sigma), in 350 ml of digestion buffer (see section **2.3.9**). To stop the digestion reaction, 50 μg of proteinase K (Sigma) and 10% sodium dodecyl sulfate (SDS) were added for a 15 min incubation at 37°C. Then, 10 μg of tRNA was added to help precipitate the RNA/riboprobe duplexes which were first extracted with phenol:chloroform (50:50). After vortexing and centrifuging (5 min, 10 000 g), the aqueous phase was recovered and

combined with 1 ml of 100% EtOH to precipitate the RNA/riboprobe duplexes, which took 30 min at room temperature. After centrifugation (20 min, 10 000 g) the EtOH was removed and the pelleted RNA/riboprobe duplexes were resuspended in 30 µl of loading buffer by 30 "triturations", separated by incubating at 85-90°C for 5 min and electrophoresed on a 5% polyacrylamide 8 M urea gel for 2-3 hour at 230 volts. The gel was then placed on Whatman paper, covered with plastic wrap and vacuum dried for 45-60 min. The dried gel was first exposed to a phosphor imaging plate for 1-2 hour at room temp to quantify the hybridization signals (Fujix BAS 2000, Bio image analyzer). Then the gel was exposed to X-ray film at -70°C with an intensifying screen for 1-4 days.

2.2.6 Quantification of RNase Protection Assay

After, the dried gel was exposed to a phosphor imaging plate, the region of the hybridization signal was selected and the signal in that area was quantified. A region of the same size was quantified directly above each hybridization signal region and then that signal was subtracted from the corresponding hybridization signal to control for the background signal. Some hybridization signals were present at very low levels. In certain experiments, these signals were not readily detectable on an autoradiogram (1-4 day exposure). However, these low level signals could be visualized through contrast adjustment of phosphor imager or if the X-ray film were exposed to the gel for a very long time (>1 week). In each RNase protection assay figure, the name of the gene indicates the level of the protected fragment. Each quantification was repeated in at least

three separate experiments and consistently gave reproducible results. The specific activity of each riboprobe was calculated from the number of adenine bases (see appendix II) and to quantify the levels of mRNA among different transcripts, the relative intensities of the hybridization signals were divided by the specific activity of the corresponding riboprobe.

2.2.7 Neuronal Dissection

Nodose, trigeminal (Tri) and superior cervical (SCG) ganglia were dissected under sterile conditions from newborn rats (C.D. strain Charles River, Canada) killed by cervical dislocation. The dissected ganglia were placed in a petri dish containing plating medium. In some experiments, the ganglia were removed from rat pups E18, P1, P7, P14 or P21.

2.2.8 Dissociation

SC ganglia were dissociated and the neurons cultured as originally described by Mains and Patterson (1973), Hawrot and Patterson (1979) and Hawrot (1980). The ganglia were dissociated at 37°C in enzyme containing media: HBSS (see below), 8% (v/v) FVM (see below), 4 mM HEPES (pH 7.4), collagenase (1 mg/ml, type 1; Sigma) and neutral protease, dispase (grade 2, 2.4 mg/ml; Boehringer Mannheim). After 15 min in this solution, the ganglia were gently triturated using a fire polished glass pipette. This pipette was first coated with plating media, consisting of a L-15 solution containing 10%

horse serum (Gibco) to prevent ganglia from sticking to the sides. When the chunks of ganglia had settled, the solution was removed and replaced with media similar to that described above, except the collagenase was omitted. Dispase was used because it is a mild proteolytic enzyme that causes minimal damage to neurons. The dissociation was continued at 37°C and every 15 min the solution was triturated 100 times; after 3-4 hour there were no chunks visible to the eye and the dissociation was stopped. When the cells were fully dissociated, 5 ml of plating medium were added to the cell suspension to inactivate the enzymes and the cells were centrifuged (International Clinical Centrifuge, International Equipment Co.) at 1335 RPM. Cells were then resuspended in 0.5 ml of plating media and added on the top of 5.7 ml of a 35% percoll solution (see below). The percoll solution was centrifuged for 15-20 min to establish a gradient of cells. The neurons, which are more dense than the non-neuronal cells, form a the pellet at the bottom of the tube, while the non-neuronal cells remain in suspension. More than 95% of the neurons pelleted to the bottom of the tube, while ~80% of the non-neuronal cells remained in suspension. The top 3.5 ml of solution was either discarded or kept for non-neuronal cells. The pelleted neurons were washed twice by centrifugation in plating medium, resuspended in plating medium (0.1 ml/culture dish) and added to the center well of a modified petri dish (see section **2.3.8**). The cell suspension was restricted to the center well of the culture dish by a sterile glass ring. The glass ring was removed 24 hour later, at a time when neurons had attached to the substrate. In spite of the percoll separation a few non-neuronal cells remained in the neuronal suspension of SCG. To

eliminate these in neuronal culture, cytosine arabino-furanoside (Ara-C; Sigma), which kills dividing cells, was added to the growth media for the first 3-4 days of culture.

2.2.9 Isolation of ganglionic non-neuronal cells

The non-neuronal cells of dissociated SCG were obtained from the percoll separation step (see section **2.2.7**). After the centrifugation, the non-neuronal cell fraction remains at the top of the percoll solution, while the neurons pellet to the bottom. Therefore, we collected the top layer (3.5 ml) of solution and transferred it to another 15 ml centrifuge tube and washed the cells twice by centrifugation in plating medium. Non-neuronal cells from SCG were used in RNA isolation (see section **2.1.1**).

2.2.10 Incubation

The cultures were incubated in a humid atmosphere of 95% Air and 5% CO_2 at 37°C and were fed growth medium every 3-4 days.

2.2.11 Culture solutions and media

L15-Air:

Leitzbovitz 15 (L15; Gibco) was used as a base for all media (plating and growth) L15 medium powder (14.9 g, Gibco Inc.) was dissolved in 1080 ml of double distilled water. To the mixture was added: 60 mg imidazole, 15 mg glutamic acid, 15 mg proline, 10 mg inositol, 15 mg aspartic acid, 15 mg cystine, 5 mg B-alanine, 2 mg vitamin B12, 10 mg choline chloride, 0.5 mg lipoic acid, 0.02 mg biotin, 5 mg ß-aminobenzoic acid, 25 mg fumaric acid (all from Sigma), and 0.4 mg coenzyme-A (Pharmacia Inc.). The pH of the solution was adjusted to 7.35 with 1 N HCl and filtered through a 0.2 μM membrane (nucleopore). L15 was stored at 4 °C. This modified L15 medium is termed L15-Air.

L15-CO2:

Before filtering, 170 ml of 150 mM $NaHCO_3$ was added to 850 ml of base L15 Air medium. When used in growth media, exposure of the solution to an atmosphere containing 95% air and 5% CO_2 keeps the pH of the solution at 7.4. L15-CO_2 was stored at 4°C.

Plating media:

Plating media consisted of 50 ml of modified L15 Air medium to which was added: 5.0 ml horse serum (Gibco) and 2 ml FVM (see below).

Fresh Vitamin Mix :

FVM consisted of 1.25 mg/ml ascorbic acid, 65 μg/ml glutathione and 12 μg/ml 6,7-dimethyl-5,6,7,8-tetrahydropterine (Sigma), supplemented with 100 mg/ml glucose (Fisher), L-glutamine (50 mM, Gibco), penicillin (12000 units/ml, Gibco) and streptomycin (12 000 μg/ml, Gibco).

Growth media:

Growth media consisted of L15-CO_2 medium supplemented with 2 ml FVM and 5% rat serum and 7S-Nerve Growth Factor (10 nM; see below).

Nerve Growth Factor:

7S-Nerve Growth Factor was purchased from Almone labs.

Ciliary Neurotrophic Factor:

In some cultures of SCG neurons ciliary neurotrophic factor (CNTF) was added to the growth media. Recombinant rat CNTF (kindly provided by Dr. P. Richardson) was used at a concentration of 25 ng/ml.

Percoll solution (35%):

2 ml percoll (Pharmacia), 3.7 ml L15-Air and 0.1 ml of 1 M HEPES (pH 7.4).

Rat serum:

The rat serum was prepared by bleeding male retired breeders (Sprague Dawley, C.D. strain, Charles River Canada Inc.) that were asphyxiated in a CO_2 saturated bell jar. The blood was allowed to clot on ice and centrifuged at 16 000 rpm at 4°C for 30 min to pellet the blood cells. The supernatent (serum) was kept at 4°C overnight, recentrifuged, filtered through a 0.22 μm membrane and stored in 2.5 ml aliquots at -20°C.

2.3 *In situ* hybridization

For *in situ* hybridization, neurons were grown on small (5 mm^2) coverslips to minimize the amount of riboprobe required. The riboprobes were synthesizedusing digoxigenin (DIG)-labeled UTP and detected by immunocytochemistry, using an anti-DIG antibody conjugated to alkaline phosphotase (DIG-Ab-AP).

2.3.1 Preparation of fixative solution

The fixative solution consisted of 4% paraformaldehyde in 0.1 M phosphotase buffer (pH 7.4). 25 ml of DEPC-H_2O was warmed up to 50-60°C in a clean beaker containing a stirring bar, then 4 g of paraformaldehyde (16% solution: w/v) was added to the warmed DEPC-H_2O. While stirring, ~10 pasteur drops of 10 M NaOH were added until almost all the paraformaldehyde was dissolved. A drop of phenol red was added to approximate the pH (ie pink solution). Drops of 10 M HCl were then added until the color changed to red-orange (the accuracy of the pH is not critical at this step). The

solution was filtered (0.22 μM) to remove traces of undissolved paraformaldehyde and kept cold (4°C) afterward until needed (within 24 hour.). At the time of fixation, the paraformaldehyde stock (16%) was mixed with phosphate buffer (pH 7.4; 0.5 M stock). The fixative solution was warmed to 37°C before use.

2.3.2 Fixation of cells

Freshly prepared fixative solution (see section **2.3.9**) was added (~1 ml) to culture dishes for 15 min. The fixed neurons were rinsed three times with Tris-buffered saline (TBS; see below), permeablized with 100% ethanol at -20°C for 15 min, rehydrated with three rinses of TBS, and incubated in blocking buffer (see section **2.3.9**) for 30 min.

2.3.3 Prehybridization

Salmon sperm DNA (final: 250 μg/ml; see below) and tRNA (final: 500 μg/ml) were boiled for 5 min, chilled on ice, and then mixed with pre-hybridization buffer (see below), containing RNase inhibitor, vanadyl sulfate ribonucleoside complex (VSRC; 20 mM; Boehringer Manheim). The mixture was rapidly added to the neurons in the center well of each dish (e.g. 20-30 μl) (see section **2.3.2**) and the cells were incubated for 1-3 hour at 43°C in a humid incubator.

2.3.4 Riboprobe hybridization

Precipitated DIG-labeled cRNA probes were pelleted by 20 min centrifugation, rinsed with 70% ethanol and air dried for 10 min. Salmon sperm DNA and tRNA (same final concentrations as above) were added to the pellet and thoroughly mixed by 30 triturations. The mixture was boiled 5 min after which the hybridization buffer, VSRC (20 mM final), and DTT (60 mM final) were added. After removing the pre-hybridization buffer from the center well of each culture dish, the cRNA probe-containing hybridization buffer was added (30 µl). The cells were incubated with the probes (20 ng of DIG-labeled riboprobes were mixed with 30 µl of hybridization buffer) for 16 hour at 43°C in a humid incubator.

2.3.5 Rinsing procedure and digestion of non-hybridized RNA

After hybridization, the cells were rinsed twice with ~1 ml of warm (~43°C) 4x SSC (see section **2.3.9**) while gently shaking on a rotary plate in a 43°C incubator for 10-15 min. Two more rinses were repeated with 2x SSC and then the neurons were rinsed once with RNase buffer (see section **2.3.9**), followed by a 30 min incubation at 37°C with RNase T1 (2 µg/ml; Sigma) in RNase buffer, in order to digest any non-hybridized cRNA probes. This was followed by 3 rinses with 1x SSC for 10-15 min and 1 rinse with 0.1x SSC for 15 min at 43°C.

2.3.6 DIG-riboprobes: DIG-antibody incubation and alkaline phosphotase reaction

Cells were rinsed once with DIG-Ab-AP buffer (see section **2.3.9**) and incubated with DIG-Ab-AP blocking solution (see below) for 30 min at room temperature. Then, the cells were incubated for 1 hour with DIG-Ab-AP (sheep antibody; Boehringer Mannheim; 1/200 dilution in DIG-Ab-AP blocking solution). The incubation was followed by three rinses with DIG-Ab-AP buffer for 10-15 min on a shaking table at room temperature. Cells were rinsed once more with AP reaction buffer (see section **2.3.9**) and then AP reaction buffer plus NBT (180 µg/ml; see below) and BCIP (360 µg/ml; see section **2.3.9**) to allow the enzymatic reaction. When a strong brown staining appeared (~30 min), the reaction was terminated with stopping buffer (see section **2.3.9**).

2.3.7 DIG-riboprobe synthesis

DIG-labeled cRNA antisense probes were synthesized according to the method described in section **2.2.4** with some modifications. The following reagents were added sequentially in a microcentrifuge tube: 6 µl of ATP, GTP, and CTP (10 mM stock; final = 1.5 mM), 6 µl of UTP:DIG-UTP mix (75:25; both 10 mM stocks; UTP-DIG from Boehringer Mannheim), 4 µl 10x transcription buffer, 10 µg linearized template DNA, 2 µl (60 units) RNase inhibitor (Pharmacia), 0.5-2 µl RNA polymerase (T3 or T7), completed to 40 µl with nuclease free ddH_2O. Probes were synthesized for 1 hour and

fresh polymerase was added for a second round of synthesis. As described in section **2.2.4**, the template DNA was digested, the probes were extracted with phenol-chloroform and then precipitated with ethanol. Precipitated probes were resuspended in DEPC-H_2O, reprecipitated with EtOH and ammonium acetate and kept at -20°C until use.

2.3.8 Culture dishes

Culture dishes were prepared by cutting a hole (10-15 mm) in the center of a plastic petri dish (Falcon #1008, 35 mm) and attaching an aclar (Allied Plastics) coverslip to the outside of the dish with a silicone elastomer (Sylguard, Dow Corning). The day before a culture, the petri dishes were sterilized with ultraviolet light (20 min) and laminin (kindly provided by Dr. S. Carbonetto) at a concentration of 30 µg/ml (diluted in L15-Air) was added to the center wells and left overnight. Prior to plating, the dishes were washed three times with L15-Air to remove excess laminin.

2.3.9 Solutions and reagents for molecular biology

2xYT Medium:

For 1 liter of ddH_2O, 16 g bactotryptone, 10 g bacto-yeast extract (Fischer), and 5 g NaCl; pH adjusted to 7.0 with NaOH 5 N; sterilized by autoclaving 20 min.

Agar plates:

2xYT plus medium plus 15 mg/ml bacto-agar; autoclaved 20 min, cooled down to 50°C and 20 μg/ml of ampicillin was added before liquid (30-35 ml) was poured in 10 cm plates and were stored at 4°C protected from light for a month.

Denhardt's solution 100x:

2% (w/v) Ficoll (Sigma mol. biol. grade), 2% (w/v) poly-vinylpyrollidine (mol. biol. grade; Sigma), 2% (w/v) BSA (fraction V; Sigma), in ddH_2O; filtered through a 0.45 μm membrane, aliquoted (~1 ml) and stored at -20°C.

DEPC- H_2O (Diethyl pyrocarbonate-treated ddH_2O):

ddH_2O was stirred with 0.1% of DEPC for several hours and autoclaved for 1 hour to hydrolyze the DEPC (from Fisher).

DIG-Ab-AP buffer (Digoxigenin-antibody-alkaline phosphatase buffer):

150 mM NaCl, 100 mM Tris-HCl (pH 7.5).

DIG-Ab-AP Blocking solution:

DIG-Ab-AP buffer + 3% Fetal Calf Serum (v/v) and 1% blocking reagent (Boehringer Mannheim).

Digestion buffer:

300 mM NaCl, 10 mM Tris-Cl (pH 7.5) and 5 mM NaEDTA in ddH_2O.

DTT (dithiothoureitol):

5 M DTT (mol. biol. grade; Sigma), dissolved in 0.01 M sodium acetate (pH 5.2); filtered through 0.45 μM. aliquoted (~1 ml) and stored at -20°C.

Elution buffer:

2 M ammonium acetate, 1% SDS, 33 μg/ml tRNA, and DEPC-H_2O.

Hybridization buffer for in situ hybridization:

Same as pre-hybridization buffer plus 10% dextran sulfate.

Hybridization buffer for RNase protection assays:

80% deionized formamide (Fisher), 10% 10x hybridization buffer (400 mM PIPES [piperazine-N,N'-bis [2-ethanesulfonic acid]] buffer, pH 6.4, 4 M NaCl 10 mM EDTA) and 10% DEPC-H_2O.

Loading buffer:

80% formamide, 1 mM EDTA, 0.02% (w/v) xylene cylanol, 0.02% (w/v) bromophenol blue and DEPC-H_2O.

Phenol for DNA preparation:

The phenol (Fisher) was saturated with 0.1 M Tris pH 8.0 according to the method of Sambrooke et al. (1989). Hydroxyquinoline (0.1% w/v) was added to reduce oxidation, RNase activity and to facilitate the distinction between the aqueous and organic phases.

Phenol for RNA prepartation: The phenol was saturated with ddH_2O (+ 0.1% hydroxyquinoline).

Phenol:chloroform:

1 volume of phenol (DNA or RNA) type; see above) was mixed with 1 volume of chloroform:amyl (24:1).

Polyacrylamide-8 M Urea gel (5%):

A stock solution (50 ml) containing 24 g of urea (BRL), 5 ml of 10x TBE, 6.25 ml of 24:1 acrylamide:bis-acrylamide (38:2% stock, Fisher) and DEPC-H_2O was prepared. Once the ingredients were dissolved, the solution was filtered through Whatman paper. 500 µl of 10% ammonium persulfate (BRL) and 10 µl of TEMED (N,N,N',N'-tetramethylethylenediamine, Fisher) were added to catalyze gel polymerization, which took approximately 1 hour.

Pre-hybridization buffer for in situ hybridization:

50% formamide (super pure grade, Fisher), 4x SSC, 1x "Denhardt's", 20 mM phosphate buffer (pH 7.0), 20% (w/v) sarcosyl; filtered through 0.45 μM filter, aliquoted (~1 ml) and stored at -20°C.

RNase buffer:

300 mM NaCl, 100 mM Tris-HCl, 5 mM NaEDTA, ddH_2O.

Salmon Sperm DNA:

Salmon sperm was prepared according to Sambrook *et al.* (1989).

SSC:

20x solution: NaCl (175.3 g) and sodium citrate (88.2 g) in 1 liter of ddH_2O, pH adjusted to 7.0 with 10 N NaOH; sterilized in autoclave (20 min).

Solution D:

4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl (w/v) sarcosy; filtered through 0.45 μM filter.

10x Taq buffer:

100 mM Tris-HCl, pH 8.3, 500 mM KCl.

T4-DNA ligase buffer 10x:

500 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM.

Top loading agarose:

7 g agarose/l of 2xYT medium.

TBE:

892 mM Tris-HCl, 890 mM boric acid and 25 mM EDTA, pH adjusted to 8.3 with HCl; sterilized by autoclaving.

TE-buffer:

10 mM Tris-HCl [pH 8.0], 1 mM EDTA.

Transcription buffer:

400 mM Tris-HCl [pH 7.9], 60 mM $MgCl_2$, 20 mM spermidine, 10 mM DDT; from NEB.

Chapter 3:

The Expression of Kv genes by P7 SCG Neurons

3.1 Introduction

Voltage-gated potassium channels serve distinctive physiological roles in neurons. Postsynaptically, potassium currents regulate excitability to depolarizing inputs (see section **1.2.2-1.2.5**), and determine action potential firing frequency (see section **1.2.2**). Furthermore, voltage-gated potassium channels are known to affect the shape of the presynaptic action potential, so as to influence calcium influx and neurotransmitter release (Augustine, 1990). Potassium currents can serve different functional roles due to the unique physiological properties of underlying voltage-gated potassium channels (see section **1.1.1**). For example, voltage-gated potassium channels differ in their voltage-sensitivity, rates of activation and inactivation, and susceptibility to modulation (see sections **1.2.2-1.2.5** and **1.3.2-1.3.4**). This diversity of function is largely achieved through the existence of a large number of Kv genes that encode voltage-gated potassium channel subunits (see section **1.4.3**) and the possible formation of heteromeric channels (see section **1.4.9**).

Molecular biology studies have clarified much about the structure-function relationship of voltage-gated potassium channels (see sections **1.4.1-1.4.12**), yet, we

know little about how these different Kv genes contribute to the physiological currents observed on native cells (see section **1.1**). A first step towards this understanding is to determine which Kv genes are expressed by identified neurons. For my studies on Kv channel gene expression, I have used neonatal rat sympathetic neurons because these neurons a) are well characterized in terms of electrophysiological properties, b) are relatively homogeneous in their electrical properties and c) exist as a discrete readily dissectable population (see section **1.3**).

Voltage-gated potassium currents expressed by rat sympathetic neurons are well characterized (Galvan & Sedlmeir, 1984; Belluzzi *et al.*, 1985a, b, 1988; 1990; Nerbonne *et al.*, 1986, 1989; McFarlane and Cooper, 1992, 1993). SCG neurons express four electrophysiologically distinct outward voltage-gated potassium currents: a rapidly inactivating transient current (IAf) that inactivates over tens of msecs and requires hyperpolarization to -90 mV to fully remove inactivation, (see section **1.3.2**); a slowly inactivating transient current (IAs) that inactivates over seconds and is fully available for activation from resting membrane potential (see section **1.3.3**); a non inactivating delayed-rectifier current (IK) that activates at positive potentials (see section **1.3.1**); and a slowly activating current that is blockable by acetylcholine (IM; see section **1.3.4**).

In this chapter, my aim was to identify the Kv genes that are expressed by SCG neurons. I used a PCR strategy which targets highly conserved structural features typical of that protein family. Specifically, this type of PCR strategy is appropriate for identifying candidate genes because many different genes exist in the Kv family and it is

possible that yet uncharacterized Kv genes are expressed by SCG neurons. Then I use RNase protection assay to quantify the expression of the genes which I identified using PCR. These results were previously reported in abstract form: Fraser and Cooper, Society for Neuroscience abstracts, 1994.

3.2 Methods

3.2.1 PCR screening for candidate Kv genes

The RNA was extracted from P7 SCG (see section **2.1.1**). The cDNA was reverse transcribed from P7 SCG total cellular RNA (see section **2.1.2**). The cDNA was amplified with degenerate primers (see section **2.1.3**). The PCR product was size selected (see section **2.1.4**). The size selected product was reamplified with nested primers (see section **2.1.5**) and size selected again (see section **2.1.4**). The PCR product and cloning vector were digested with restriction enzymes (see section **2.1.6**) and then gel purified (see section **2.1.4**). The PCR product and cloning vector were ligated (see section **2.1.7**). The bacteria were made competent (see section **2.1.8**), transformed (see section **2.1.9**) and were plated (see section **2.1.10**). The single strand cDNA was isolated from individual bacterial colonies (see section **2.1.11**). The cDNA was sequenced (see section **2.1.12**) and the DNA sequences were read directly from the X-ray film (see section **2.1.12**).

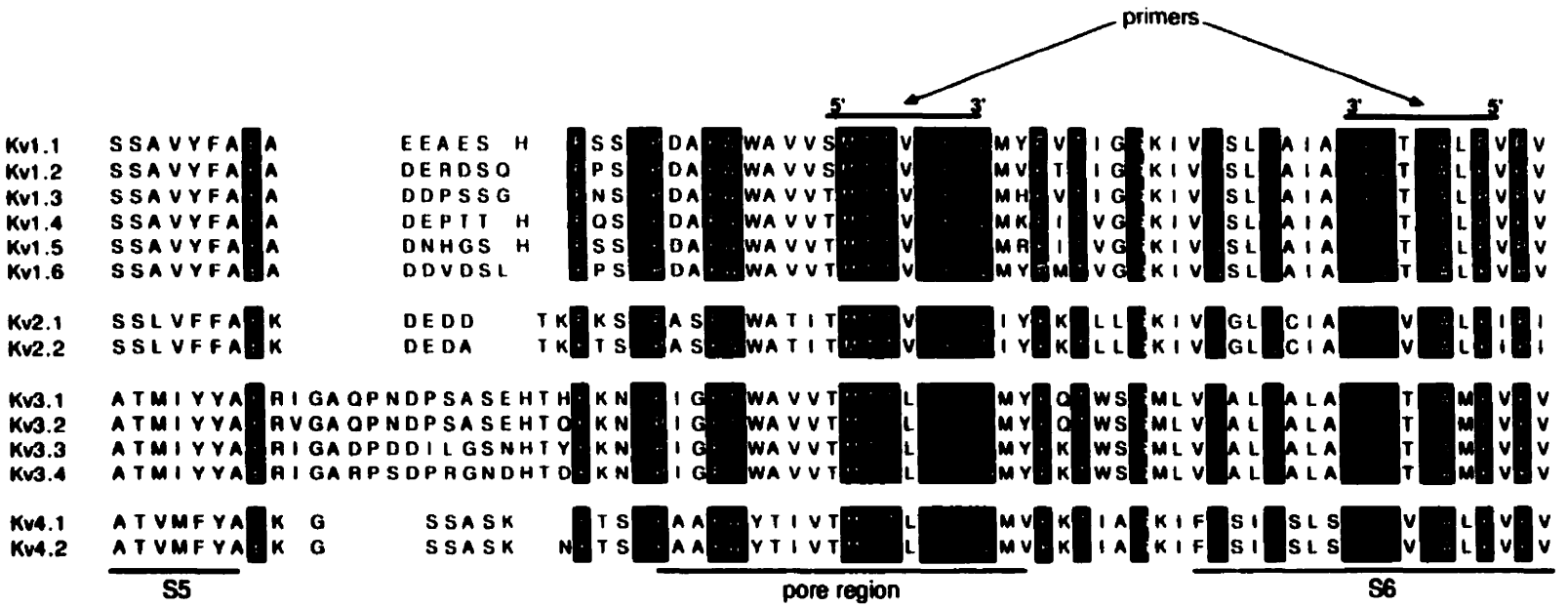

Figure 3.1 PCR primers are targeted to highly conserved regions found in all Kv genes.

The degenerate primers used to identify candidate voltage-gated potassium channel genes that may be expressed in SCG neurons were designed to be able to amplify Kv genes from any species. These primers target the highly conserved pore region and sixth transmembrane spanning domain.

3.2.2 RNase protection assay

The RNA was extracted from P7 SCG (see section **2.1.1**). For some experiments SCG neurons were separated from their non-neuronal cells prior to RNA extraction (see section **2.2.9**). The radiolabelled cRNA probes were transcribed, purified and quantified (see section **2.2.5**). The RNA and cRNA probes were hybridized (see section **2.2.5**). The single stranded RNA and cRNA probe were digested (see section **2.2.5**). The cRNA probe signal was detected (see section **2.2.6**).

3.2.3 *In situ hybridization*

P7 SCG neurons were separated from their non-neuronal cells, plated (see section **2.2.7**) and fixed (see section **2.3.2**). The DIG-labeled cRNA probe was transcribed, purified and quantified (see section **2.3.7**). The fixed cells and cRNA probes were hybridized (see section **2.3.3**). The unhybridized cRNA probe and cellular RNA were digested (see section **2.3.5**). The cRNA probe was detected (see section **2.3.6**).

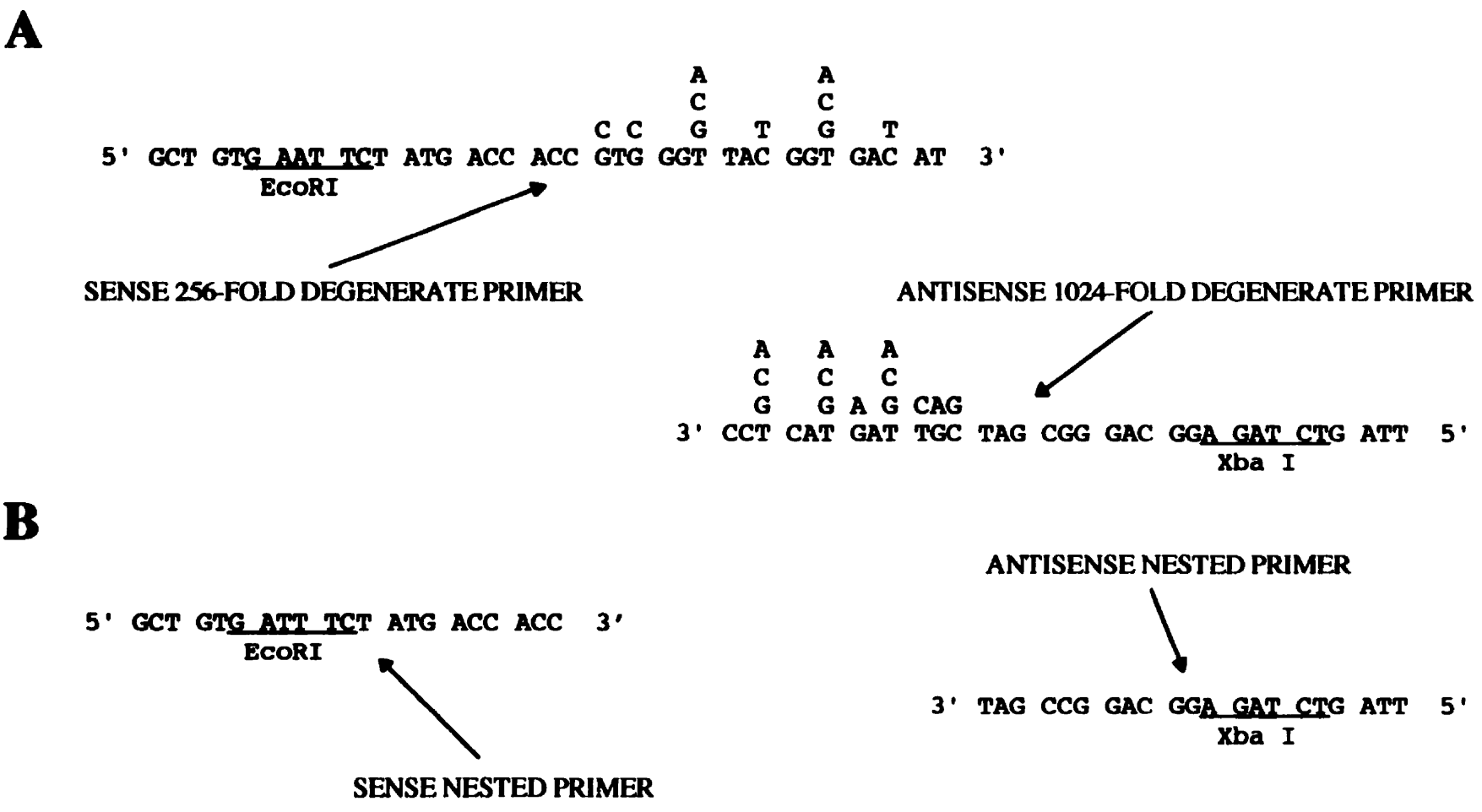

Figure 3.2 Primers contain fixed and variable regions.

(A) The degenerate primers used in first round PCR amplification contained both fixed and variable regions as well as restriction sites to facilitate subcloning. The variable regions of the sense and antisense primers were designed with 256-fold and 1024-fold degeneracy respectively. **(B)** The nested primers were designed to perfectly correspond to the fixed region of the degenerate primers and thereby provide uniform amplification of cDNAs which have incorporated the degenerate primers.

3.3 Results

3.3.1 PCR identification of candidate genes

To identify Kv genes expressed by neonatal rat sympathetic neurons, we took advantage of a PCR strategy developed by Larry Salkoff and coworkers (Jelga and Salkoff. 1997). This protocol uses degenerate primers that target the conserved H5 and S6 region of Kv channels (Figure 3.1). These degenerate primers contained fixed and variable regions (Figure 3.2A). In this strategy, I reverse-transcribed cDNA from rat P7 SCG total cellular RNA and then PCR amplified the cDNA using degenerate primers. When electrophoresed on a 3% agarose gel, the PCR product yielded a faint 121 bp band and a smaller nonspecific amplification product (Figure 3.3A., 1st round PCR amplification). The 121 bp band was size selected and further amplified using nested primers identical to the 5' region of the degenerate primers (Figure 3.2B). This second round of PCR amplification gave a bright 121 bp band (Figure 3.3B). The nested primers uniformly amplified any cDNA that had incorporated the degenerate primers and included restriction sites to facilitate subcloning. The second round amplification product was subcloned into an M13 vector and this construct was then used to transform competent DH5αF' bacteria. The transformed bacteria were plated and then plasmid cDNA from individual bacterial colonies was sequenced to determine the identities of the inserts.

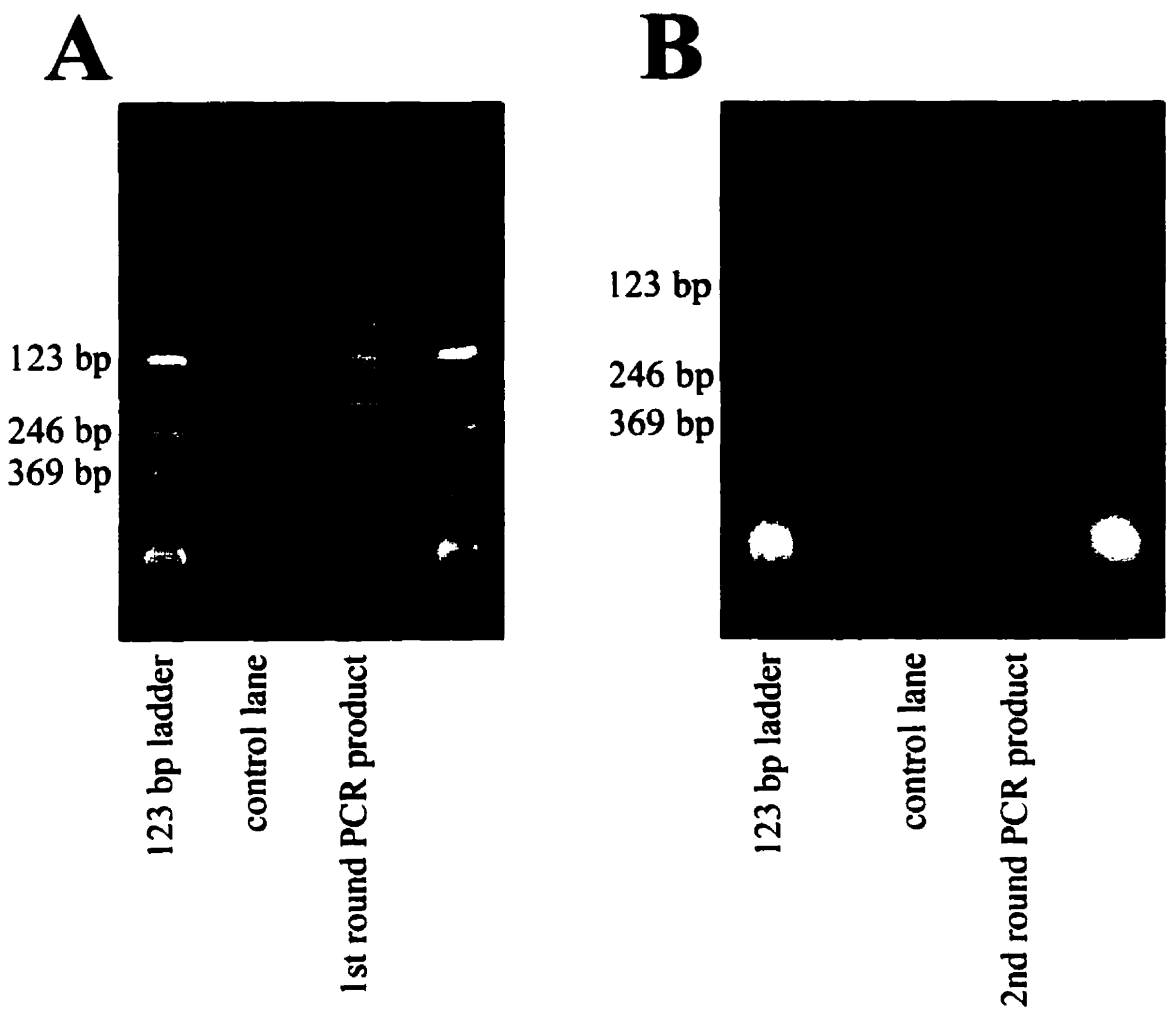

Figure 3.3 PCR amplification product.

(**A**) The PCR products of first round amplification, using degenerate primers at low stringency, were electrophoresed on a 3% agarose gel alongside a 123 bp ladder. The control lane contains the products of a PCR reaction that was conducted without cDNA starting material. The band present in the control lane was the nonspecific amplification product of the degenerate primers amplifying themselves. The nonspecific band is also found in the experimental lane. Additionally, the experimental lane contains a 121 bp band corresponding to the target sequence. The target sequence was amplified from cDNA reverse-transcribed from P7 SCG total cellular RNA. This product was cut from the gel, purified and reamplified. (**B**) Further amplification with nested primers at high stringency of the purified first round amplification product gave a single specific band corresponding to the amplified target sequence in the experimental lane. There is no nonspecific amplification product in the control lane.

Table 3.1 Frequency of Kv gene clones from PCR screening

Sequence	Frequency
Kv3.4	21/50
Kv3.3	12/50
Kv3.1	2/50
Kv1.4	4/50
Kv2.2	2/50
Kv4.2	1/50
db39	3/50

To determine which voltage-gated potassium channel genes were amplified, I picked at random and sequenced plasmid cDNA from fifty bacterial colonies. Six different Kv sequences appeared repeatedly in these fifty clones (sequences in appendix III). Using the BLAST (Basic Local Alignment Search Tool) program on the NIH server (www.ncbi.nih.nlm.gov/blast), I compared these sequences to those in the GenBank database and identified six of them as previously characterized voltage-gated potassium channel gene sequences, Kv1.4, Kv2.2, Kv3.1, Kv3.3, K3.4 and Kv4.2. Therefore, three of the four Kv3 subfamily genes were observed, along with one representative gene from each of the other subfamilies, Kv1, Kv2 and Kv4. When expressed in *Xenopus* oocytes, three of the genes Kv1.4, Kv3.4 and Kv4.2 give rise to rapidly inactivating currents, Kv3.3 gives rise to a slowly inactivating transient current while Kv2.2 and Kv3.1 are believed to encode "delayed-rectifier" type currents (Vega-Saenz de Miera *et al.*, 1992; Rettig *et al.*, 1992; Luneau, *et al.*, 1991; Covarrubias, *et al.*, 1994; Yokoyama, *et al.*, 1989; Hwang, *et al.*, 1992; Baldwin, *et al.*, 1991; Stuhmer, *et al.*, 1989; Blair, *et al.*, 1991). One additional sequence was detected which was not reported to be a voltage-activated potassium channel: we called this sequence db39.

The frequency of voltage-gated potassium channel clones are shown in table 3.1. Kv3.4 occurred at the highest frequency, accounting for more than half of the total colonies that were sequenced. Two other members of the Kv3 subfamily were also present. Kv3.3, was present in more than a quarter of the colonies that were sequenced, while Kv3.1 was present in only two colonies. The high frequency of Kv3.4 and Kv3.3 in

randomly selected colonies suggested that both may be expressed at high levels in SCG neurons. However, this may not be an accurate representation of genes expression, because this PCR approach is semiquantitive and some sequences may have been preferentially amplified due to primer bias. Members from the Kv1, Kv2 and Kv4 subfamily were also detected through the PCR screening. Kv1.4 was present in four colonies. while Kv4.2 occurred in only one colony, and one member of the Kv2 subfamily, Kv2.2 was also present in two colonies.

3.3.2 Kv mRNA quantification

Our PCR screening for candidate genes suggested that at least 6 voltage-gated potassium genes were expressed by SCG neurons. Furthermore, it implied that Kv3.4 and Kv3.3 were expressed at high levels relative to the other Kv genes that were detected. As this PCR strategy was semiquantitive, I decided to directly measure mRNA levels for specific transcripts using RNase protection assay. The cDNA sequences generated to identify candidate genes were too short and nonspecific for use as probe templates in RNase protection assays, because these cDNA sequences were 114 bp in length, almost half of which (56/114 bp) was primer region. Furthermore, the primer regions corresponded to highly conserved structural regions found in all potassium channel genes, which could have lead to nonspecific hybridization and also these regions contain nucleotide mismatches which could have interfered with probe-mRNA hybridization leading to loss of signal.

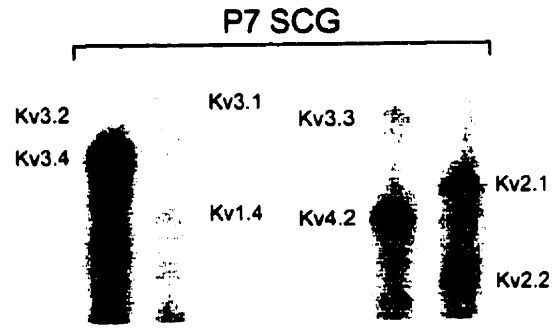

Figure 3.4 RNase protection assay: Kv mRNA expression by P7 SCG.

RNase protection assay for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 mRNAs. A 2 μg sample of P7 SCG total cellular RNA was protected in each lane. Signals were detectable for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.3, Kv3.4, and Kv4.2. The level of each gene on this figure indicates the location of the protected fragment. Similarly, the level of each gene on figures **3.6**, **3.8**, **4.1**, **4.3**, **4.5**, **4.7** and **5.1** also indicate the location of the protected fragment.

In order to produce probes that were sufficiently distinct to discriminate between different mRNA species, I PCR cloned longer cDNA sequences using specific primers corresponding to the genes: Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2. These PCR amplification products were subcloned into a cloning vector (pBS+). In the case of Kv1.4, I transferred a region of Kv1.4 (courtesy of Dr. E. Isacoff) from the expression vector (PVC/en) into a cloning vector (pBS+). These constructs were used to transform competent DH5αF' bacteria. The transformed bacteria were plated and individual colonies grown and sequenced to isolate the appropriate clones for use as probe templates in RNase protection assay. Kv2.1 was not present in my initial PCR screening, however, Kv2.1 is expressed by PC12 cells which are a cell line derived from the same lineage as are SCG neurons (Sharma *et al.*, 1993). Furthermore, if Kv2.1 is expressed by SCG neurons it may combine with Kv2.2 to produce heteromeric channels. For these reasons, I used PCR to produce a sequence specific probe for Kv2.1. Similarly, as three Kv3 genes were present in my initial screening at high frequency, then it was possible that Kv3.2 could combine with other Kv3 subunits to produce heteromeric channels if it is expressed by SCG neurons. For this reason, I also used PCR to generate a probe for Kv3.2. Each probe was designed to target the mRNA sequence corresponding to the S1-S5 membrane region. Alternative splicing is known to occur at the amino or carboxyl termini of some channels (Ghanshani *et al.*, 1992; Rettig *et al.*, 1992), however, the core regions of these genes (S1-S6) consist of continuous exons. Therefore, our probes could detect which ever

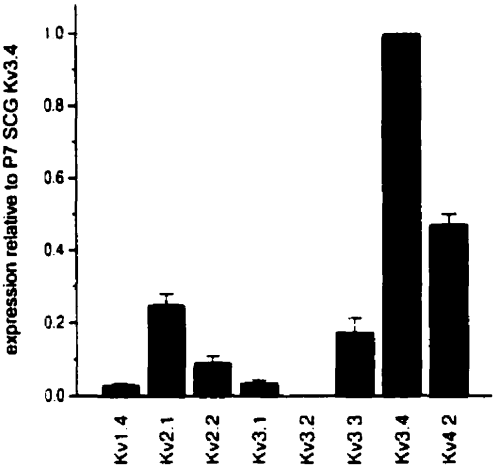

Figure 3.5 Quantification of Kv mRNA expression by P7 SCG.

The means (± SEM) of mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 in P7 SCG are plotted as a histogram. Each mean reflects at least three separate experiments. Individual signals were measured using a phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of P7 Kv3.4 mRNA.

splice variants were present, but were unable to distinguish between the expression of different splice variants of a channel.

Figure 3.4 is a representative RNase protection assay showing mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 in P7 SCG. I quantified the hybridization signals with a phosphorus imaging system and plotted the mRNA levels of each transcript relative to Kv3.4 mRNA level in Figure 3.5. Three of the four Kv3 transcripts were detectable. Kv3.4 was expressed at high levels while Kv3.3 and Kv3.1 were detectable at low levels (17.4% Kv3.4) and very low levels (3.5% Kv3.4) respectively, and Kv3.2 was not detectable. Kv2.1 and Kv2.2 were expressed at moderate levels (24.8 % Kv3.4) and low levels (10% Kv3.4) respectively, Kv4.2 was expressed at moderate levels (47.2% Kv3.4) and Kv1.4 was detectable at low levels (2.9% Kv3.4).

3.3.3 Kv genes localized to SCG neurons

Since the SC ganglia contains Schwann cells and fibroblasts in addition to neurons, the total cellular RNA isolated from this ganglia contained mRNA from neurons and non-neuronal cells. As such, the signals observed in my PCR screening (Table 3.1) or the RNase protection assay (Figure 3.4) may have been due to non-neuronal cell mRNA. Therefore, I performed an RNase protection assay on neurons separated from non-neuronal cells to localize the source of Kv mRNA signal. Figure 3.6 shows that each of Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.3, Kv3.4 and Kv4.2 were expressed exclusively in the neuronal fraction. Figure 3.7 is an *in situ* hybridization showing Kv3.4 mRNA

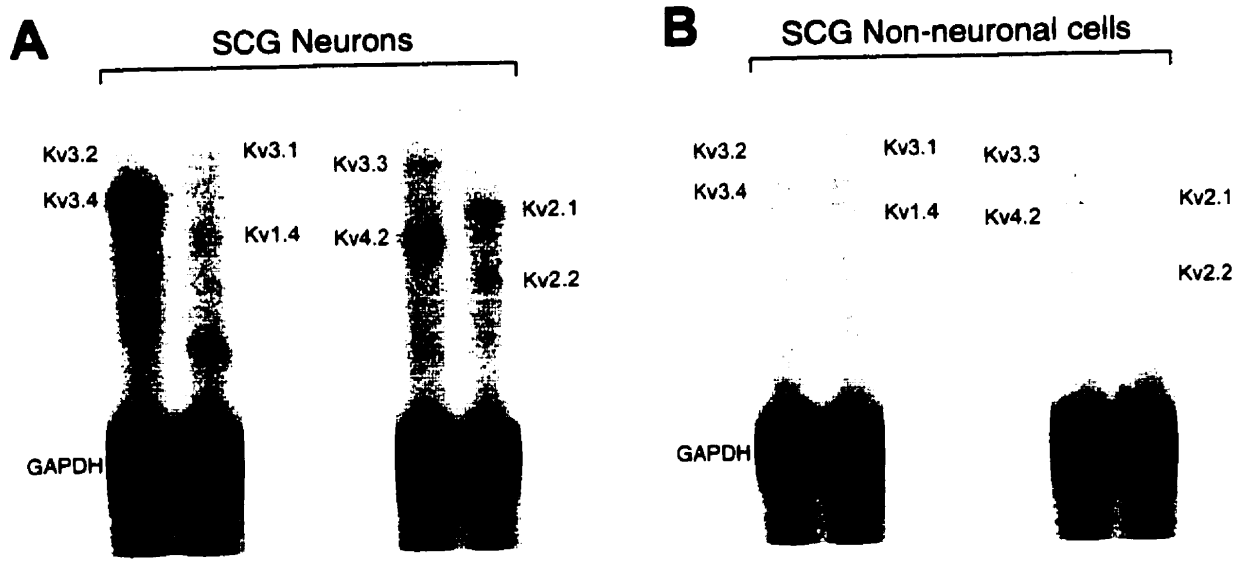

Figure 3.6 RNase protection assay: neuron and non-neuronal separation.

RNase protection assay for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 mRNAs. For this experiment, the P7 SCG neurons were separated from their non-neuronal cells using enzymatic and mechanical dissociation and total cellular RNA was isolated from each fraction. A 2 μg sample of total cellular RNA from P7 SCG neurons (**A**) or P7 SCG non-neuronal cells (**B**) was protected in each lane. Signals corresponding to the Kv mRNAs were detectable in the neuronal fraction, but not in the non-neuronal fraction. GAPDH was detected throughout. The level of each gene on this figure indicates the location of the protected fragment.

localization in dissociated cells from P7 SCG neurons that were grown in culture overnight. Kv3.4 mRNA was localized to neurons and is expressed in every neuron. There was no signal in control neurons hybridized with the sense DIG-labeled Kv3.4 cRNA probe.

3.3.4 Absolute expression level of Kv3.4

Kv3.4 mRNA signal was readily detectable in SCG neurons, however, this level was very low compared to GAPDH mRNA expression (Figure 3.6). Furthermore, it was not clear how Kv3.4 mRNA expression compared to the expression of other receptors or ion channels in SCG neurons. To determine if Kv3.4 mRNA was expressed at levels similar to those of other ion channel mRNA, I compared the expression of Kv3.4 to that of the α 3 nAChR (neuronal nicotinic acetylcholine receptor). The acetylcholine current is present at similar densities to the voltage-gated potassium currents and α 3 is the most highly expressed subunit (Mandylzes *et al.*, 1992). Figure 3.8 is an RNase protection assay showing that Kv3.4 is expressed at comparable levels to α 3. In a separate set of experiments in our lab, De Koninck and Cooper (1995) determined that there were ~5000-7000 α 3 transcripts per SCG neuron. We therefore would expect a similar number of Kv3.4 transcripts per cell.

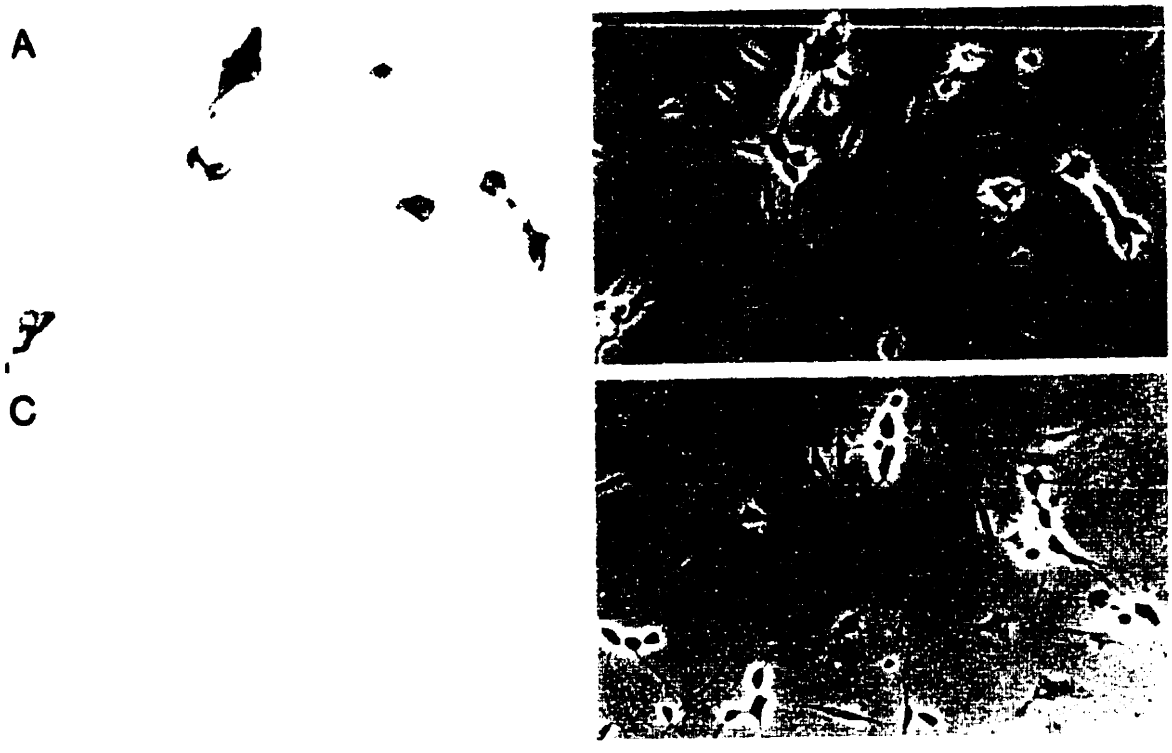

Figure 3.7 *In situ* hybridization: Kv3.4 mRNA in SCG neurons with DIG-labeled antisense Kv3.4 riboprobe.

(**A**) Bright field photomicrograph of P1SCG neurons grown overnight in culture, fixed, incubated with DIG-labeled Kv3.4 antisense riboprobes and detected with an immuno-alkalinephosphotase reaction. Staining was restricted to neurons and appeared uniform in all neurons. (**C**) Similar bright field photomicrograph to (**A**) except that fixed cells were incubated with "control" DIG-labeled Kv3.4 sense riboprobe. There was no signal detectable in these neurons. (**B**) and (**D**) are phase contrast micrographs of the same fields of view shown above in (**A**) and (**C**) respectively. In (**B**) and (**D**) both neurons and non-neuronal cells were clearly visible.

Figure 3.8 RNase protection assay: absolute expression of Kv3.4.

RNase protection assay for Kv3.4 and $\alpha 3$ nAChR mRNAs in a 2 μg sample of P7 SCG total cellular RNA. Signals for Kv3.4 and $\alpha 3$ were present at approximately equal levels.

3.3.5 Identification of the db39 PCR sequence

In section **3.3.1**, I reported the identification of a cDNA sequence that was not listed in the GenBank database, but even though the sequence which I PCR cloned was short, db39 does bear strong homology to voltage-gated potassium channel genes. I used 5' RACE (5' Rapid amplification of cDNA ends; 5'-Amplifinder race kit, Clontech) in order to get a larger nucleotide sequence to better understand the structure and identity of the protein. Briefly, I reverse transcribed cDNA from SCG poly-A RNA using a sequence specific primer and AMV reverse transcriptase, and then degraded the poly-A RNA. Next, I ligated an anchor primer cDNA onto the reverse transcribed cDNA using single stranded RNA ligase. Finally, I PCR amplified the ligated construct with sequence specific primers to amplify the sequence of interest. The RACE approach was unsuccessful. Additionally, repeated tries using RNase protection assay, with an antisense cRNA probe derived from the db39 sequence of interest, detected no signal in SCG RNA. These failed attempts may be attributed to the db39 sequence being of *E. coli* origin rather than rat.

A recent screening of the GenBank database revealed the db39 sequence to have homology to the GenBank sequence *E. coli* nucleoside permease NupG. Figure 3.9 shows the homology of the db39 protein sequence to the NupG protein sequence. As the nucleotide sequence lying between the primers corresponds perfectly to this GenBank sequence, it is likely that the db39/NupG clone represents an *E. coli* sequence and not the rat equivalent of this gene.

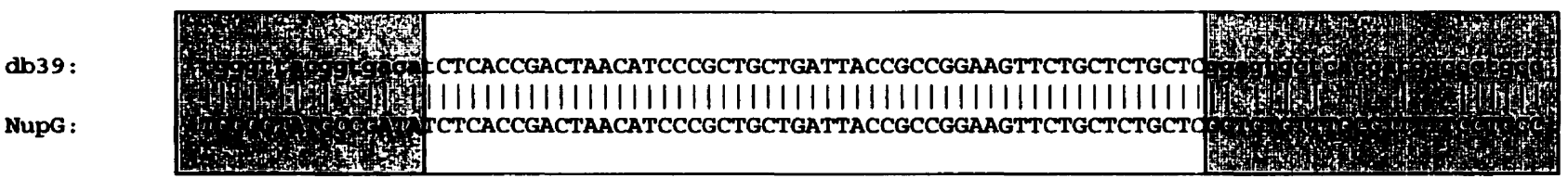

Figure 3.9 Alignment of db39 nucleotide sequence and nucleoside permease (NupG) nucleotide sequence.

The PCR clone (db39) which I identified in section **3.3.1** (top sequence) is shown aligned with the Genbank sequence for *E. coli* nucleoside permease (NupG) (bottom sequence). The outer sequences (darkly shaded) are the PCR primer regions and the internal region (lightly shaded) is the PCR amplified sequence. The conserved residues are marked with vertical bars. There is perfect conservation between the interprimer region of db39 and NupG implying that they are the same gene.

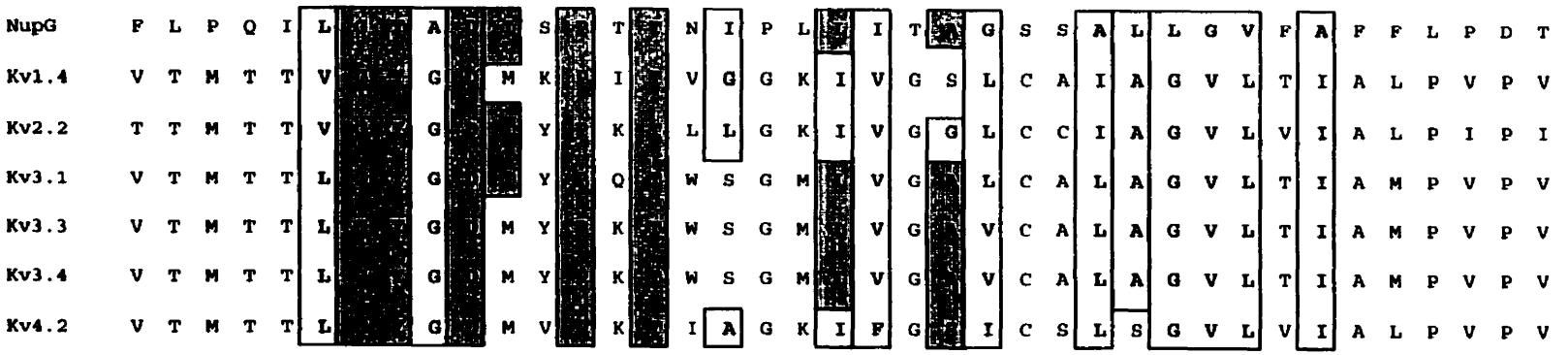

Figure 3.10 Alignment of homologous regions of NupG to Kv1.4, Kv2.2, Kv3.1, Kv3.3, Kv3.4 and Kv4.2.

The Genbank amino acid sequence of *E. coli* nucleoside permease (NupG) is compared to the homologous regions of Kv1.4, Kv2.2, Kv3.1, Kv3.3, Kv3.3, Kv3.4 and Kv4.2. The amino acids that are conserved between NupG and Kv sequences are darkly shaded. The percentage of conserved amino acids, between the different sequences in the homologous region, are shown in table 3.2. Amino acids that are considered to be conservative substitutions are lightly shaded.

Table 3.2 NupG protein homology to Kv channels

	NupG	Kv1.4	Kv2.2	Kv3.1	Kv3.3	Kv3.4
Kv1.4	5/28 (18%)					
Kv2.2	6/28 (21%)	20/28 (71%)				
Kv3.1	8/28 (29%)	17/28 (61%)	18/28 (64%)			
Kv3.3	7/28 (25%)	18/28 (64%)	16/28 (57%)	26/28 (93%)		
Kv3.4	7/28 (25%)	18/28 (64%)	16/28 (57%)	26/28 (93%)	28/28 (100%)	
Kv4.2	5/28 (18%)	17/28 (61%)	17/28 (61%)	15/28 (54%)	17/28 (61%)	17/28 (61%)

Table 3.3 Summary of SCG neuron voltage-gated potassium channel properties and Kv channel properties.

Channel	Voltage-Sensitivity of Activation	Rate of Activation	Voltage-Sensitivity of Inactivation	Rate of Inactivation	Block by 4-AP	Block by TEA
SCG IAf	half activated at –2 mV	activates rapidly	half inactivated at -65 mV	inactivates rapidly	half blocked by 1 mM	not blocked by 10 mM
SCG IAs	half activated at 10 mV	activates rapidly	half inactivated at -40 mV	inactivates slowly	not known	not known
SCG IK	half activated at 22 mV	activates slowly	does not inactivate	does not inactivate	not known	not known
SCG IM	activates above -60mV	activates slowly	does not inactivate	does not inactivate	not known	not blocked by 10 mM
Kv1.1	half activated at -30 mV	activates rapidly	half inactivated at -47 mV	inactivates slowly	half blocked by 0.3 mM	half blocked by 0.3 mM
Kv1.2	half activated at 27 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.6 mM	half blocked by 0.6 mM
Kv1.3	half activated at -10 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.4 mM	not blocked by 10 mM
Kv1.4	half activated at -22 mV	activates rapidly	half inactivated at -74 mV	inactivates rapidly	half blocked by 12.5 mM	not blocked by 10 mM
Kv1.5	half activated at 0 mV	activates rapidly	not known	inactivates slowly	not known	half blocked by 0.3 mM
Kv1.6	half activated at -15 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.3 mM	half blocked by 4 mM
Kv2.1	half activated at 0 mV	activates rapidly	half inactivated at -35 mV	inactivates slowly	half blocked by 0.5 mM	half blocked by 10 mM
Kv2.2	not known	activates rapidly	not known	inactivates slowly	not known	half blocked by 7.9 mM
Kv3.1	half activated at 19 mV	activates rapidly	half inactivated at 10 mV	inactivates slowly	half blocked by 0.1 mM	half blocked by 0.2 mM
Kv3.2	half activated at 6 mV	activates rapidly	not known	inactivates slowly	not known	not known
Kv3.3	half activated at 5 mV	activates rapidly	half inactivated at 5 mV	inactivates moderately	half blocked by 1.2 mM	half blocked by 0.14 mM
Kv3.4	half activated at 10 mV	activates rapidly	half inactivated at -53 mV	inactivates variably	half blocked by 0.5 mM	half blocked by 0.5 mM
Kv4.1	half activated at 10 mV	activates rapidly	half inactivated at-65 mV	inactivates rapidly	half blocked by 5 mM	not blocked by 10 mM
Kv4.2	half activated at -5 mV	activates rapidly	half inactivated at -55 mV	inactivates rapidly	half blocked by 5 mM	not blocked by 10 mM
Kv4.3	half activated at -15 mV	activates rapidly	half inactivated at -60 mV	inactivates rapidly	half blocked by 2 mM	not blocked by 10 mM

(rapid activation occurs over milliseconds; slow activation occurs over tens of milliseconds; rapid inactivation occurs over tens of milliseconds; moderate inactivation occurs over hundreds of milliseconds; slow inactivation occurs over seconds; the variable inactivation of Kv3.4 channels occurs over tens of milliseconds to seconds; adapted from: Yokoyama *et al.*, 1989; Luneau *et al.*, 1991; Rettig *et al.*, 1992; Vega-Saenz de Miera *et al.*, 1992; Covarrubias *et al.*, 1994; Swanson *et al.*, 1990, Duprat *et al.*, 1995; Stuhmer *et al.*, 1989; Pak *et al.*, 1991; Baldwin *et al.*, 1991; Serodio *et al.*, 1996; Taglialatela *et al.*, 1992; Hwang *et al.*, 1992; Belluzzi and Sacchi, 1991; McFarlane and Cooper, 1992; Grissmer *et al.*, 1994)

3.4 Discussion

In this chapter, I set out to identify the voltage-gated potassium channel genes expressed by rat sympathetic neurons. I used a PCR strategy to identify candidate genes that may be expressed by SCG neurons. Our PCR strategy successfully predicted the high expression levels of Kv3.4, the moderately low to low expression of Kv2.2, Kv3.2 and Kv1.4, and the undetectable expression of Kv3.1. However, our strategy did not predict the high expression level of Kv4.2, the moderate expression of Kv2.1, nor the low expression of Kv3.3. Overall, this PCR approach was useful in providing a starting point for our studies, but failed to identify all of the different Kv genes expressed by SCG neurons. As such, we cannot be sure that there are not still other Kv genes expressed in SCG neurons that we failed to detect. A recently published study (Dixon and McKinnon, 1996), which was independent of our work, supports our findings by confirming the expression of Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.3, Kv3.4 and Kv4.2 in P21 SC ganglia. Yet, these researchers (Dixon and McKinnon) failed to determine which of these genes were expressed in SCG neurons versus non-neuronal cells and also failed to quantitate the relative expression of these Kv mRNAs.

Our studies show that at least seven separate Kv genes are expressed in SCG neurons. As heteromeric channel formation occurs between different subunits of the same subfamily and modifications in channel behavior due to interactions with other proteins are possible, potentially several different potassium channels may be found on SCG

neurons. Below, I discuss the physiological voltage-gated potassium currents in relation to the voltage-gated potassium channel genes that are expressed by these neurons.

3.4.1 Molecular components of SCG IAf channels

The rapidly inactivating transient current, IAf, is present at high levels on P7 SCG neurons. IAf activates in msecs at potentials more positive than -70 mV and inactivates over tens of msecs (McFarlane and Cooper, 1992, 1993). IAf is completely steady-state inactivated at -40 mV, so that at normal resting membrane potential, about half of IAf is available for activation. Three of the genes I detected using RNase protection assay encode rapidly inactivating transient currents when expressed *in vitro*: Kv1.4, Kv4.2 and Kv3.4, while the other four: Kv2.1, Kv2.2, Kv3.1 and Kv3.3 are thought to encode slowly inactivating transient currents or delayed-rectifier channels.

3.4.1.1 Possible contribution of Kv4 to SCG IAf

Kv4 channels each possess similar voltage-gated and pharmacological properties when expressed *in vitro*. When expressed in *Xenopus* oocyte, each Kv4 gene encodes a rapidly inactivating transient current, which activates and inactivates at similar voltages and over the same time scale as does IAf (Pak *et al.*, 1991b; Baldwin *et al.*, 1991; Serodio *et al.*, 1996; McFarlane and Cooper. 1992,1993). The Kv4.2 channel has the same voltage sensitivity for activation as IAf, however, the voltage sensitivity for inactivation of Kv4.2

occurs ~10 mV more depolarized than IAf. Both IAf and Kv4 channels have similar pharmacology, as IAf and Kv4 channels are blockable by moderate concentrations of 4-AP and are unaffected by high concentrations of TEA (Belluzzi and Sacchi, 1988; Schofield and Ikeda, 1989; Pak *et al.*, 1991b; Baldwin *et al.*, 1991; Blair *et al.*, 1991; Serodio *et al.*, 1996). Arachadonic acid (AA) also modulates sympathetic neuron IAf and Kv4 channels in *Xenopus* oocytes. In both cases, arachadonic acid suppresses the fast transient current, however, the concentrations necessary to block sympathetic neuronal IAf are much higher than those that block Kv4 channels in a heterologous expression system (Villarroel, 1993; Villarroel and Schwarz, 1996).

Of the Kv4 subfamily members, Kv4.2 and Kv4.3 mRNAs are abundantly expressed in the brain in distinct and sometimes overlapping patterns, while Kv4.1 is only weakly expressed (Serodio *et al.*, 1996). Kv4.2 is believed to contribute to most rapidly inactivating transient currents expressed by the brain, as Rudy and colleagues showed that antisense arrest of Kv4.2 but not Kv4.1 expression was sufficient to block >90% of the fast transient potassium current from the brain mRNA that was expressed in *Xenopus* oocyte (Serodio *et al.*, 1994). Thus, Kv4.2 seems to be an important gene in the central nervous system. Similarly, Kv4.2 and Kv4.3 are also expressed at high levels in the heart where they are believed to be responsible for the transient outward current (I_{TO}) (Dixon and McKinnon, 1996). I found that Kv4.2 is expressed at high levels in SCG neurons. Both SCG IAf and Kv4 channels expressed *in vitro* have similar pharmacological sensitivities. We do not know if Kv4.3 is expressed in SCG neurons, but Kv4.1 is

expressed in P21 SCG neurons at levels comparable to Kv4.2 (Dixon and McKinnon, 1996). Based on this co-expression, Kv4 channels may contribute to IAf as heteromeric channels, but as Kv4 channels are so similar in behavior, there may not be a detectable difference between heteromeric and homomeric channels.

The carboxy-termini of Kv4.2 channels seem to influence the time course of inactivation. Kv4.2 has two alternative splice variants: one variant "Shal1" has an additional 139 amino acids on the carboxy-terminal and differs in the last 14 amino acids from the other variant "RK5" (Baldwin *et al.*, 1991; Blair *et al.*, 1991). Blair reports that the "RK5" variant with the shorter carboxy-termini has one rapid component to inactivation while the other longer "Shal1" splice variant inactivates with both a rapid and slow component to inactivation. SCG IAf inactivates with a single rapid component to inactivation (Belluzzi *et al.*, 1985b), implying that the shorter splice variant is the predominant channel expressed in SCG neurons.

In an attempt to determine the subcellular localization of Kv4.2 and Kv1.4 on SCG neurons, I used subunit specific antibodies obtained from Morgan Sheng for immunohistochemistry (Sheng *et al.*, 1992). These studies were performed on acutely isolated P7 SCG neurons grown in culture overnight. I failed to detect Kv4.2 or Kv1.4 protein on rat SCG neurons through my immunocytochemistry studies. The lack of Kv4.2 and Kv1.4 signal may reflect the low levels of protein expression for these channels, as I was consistently able to detect Tau-1 (a cytoskeletal protein) in my controls. Perhaps

confocal microscopy is necessary to visual signal for these ion channels. Alternatively, the antibodies that I used could have been degraded at the time I received them.

Sheng and colleagues (1992) showed that Kv4.2 has somatodendritic localization in the hippocampus. If Kv4.2 contributes to SCG IAf, then the somatic localization of Kv4.2 protein is consistent with recordings from acutely isolated SCG neurons that express high levels of IAf but lack processes. A somatodendritic localization of Kv4.2 is appropriate to regulate postsynaptic excitability, which is a proposed role for IAf (Sacchi and Belluzzi, 1990).

3.4.1.2 Possible contribution of Kv1 to SCG IAf

SCG neurons also express other genes that are known to encode inactivating channels. Kv1.4 activates rapidly and inactivates at a range of different rates dependent on the phosphorylation and oxidation state of the channels, and Kv1.4 activates ~20 mV more hyperpolarized and inactivates ~10 mV more depolarized than IAf. Both SCG IAf and Kv1.4 have similar pharmacological sensitivity: IAf and Kv1.4 are both sensitive to 4-AP and insensitive to TEA (Belluzzi and Sacchi, 1988b, 1991; Schofield and Ikeda, 1989; Stuhmer *et al.*, 1989). I was unable to determine the subcellular localization of the Kv1.4 protein using immunocytochemistry, however, the Kv1.4 protein is reported to be targeted to axons and nerve terminals (Sheng *et al.*, 1992). The axonal location of an A-current is not typical. Usually, delayed-rectifier currents are considered to be important repolarizing current in classic axon preparations, such as the squid giant axon and frog

nodes of Ranvier (Hille, 1984). Since there are no potassium current recordings from rat sympathetic neuron axons, we do not know which type of currents repolarize these axons. As Kv1.4 mRNA is expressed at low levels on P7 SCG neurons, I predict that the total amount of current attributable to Kv1.4 channels is small and could easily be masked by more highly expressed rapidly inactivating Kv channels, for example, the current due to Kv4 channels. As the pharmacology and voltage-sensitivities of Kv1.4 and Kv4.2 channels are similar, dissecting the individual contribution of these different channels on the same neurons would be difficult using voltage-clamp protocols or pharmacological block. Yet, even a channel expressed at low levels may have an important physiological consequence. A small number of Kv1.4 channels localized presynaptically could dramatically affect synaptic transmission, through acting to modulate the amount of calcium influx during an action potential so as to affect neurotransmitter release which has been observed at the squid giant axon nerve terminal (Augustine, 1990).

Calcium/calmodulin dependent kinase (CaMKII) modulates the Kv1.4 channel, such that an increase in the intracellular calcium concentration results in the phosphorylation of a serine residue on the Kv1.4 inactivation ball (serine 123; Roeper *et al.*, 1997). The serine phosphorylation causes a 5-10x slowing in inactivation and accelerated recovery from N-type inactivation. Furthermore, oxidation removes fast inactivation from Kv1.4 (Duprat *et al.*, 1995; Stephens *et al.*, 1996). The oxidative/reductive metabolism of the cell includes the reactions of monoamine oxidase (MAO), which catalyses the oxidative deamination of catacholamines in the nerve

terminals, and NAD(P)H oxidases which are membrane associated enzymes with ubiquitous distribution (McCormack and McCormack, 1994). If Kv1.4 is located presynaptically in sympathetic neurons, then the shape of the presynaptic action potential could be coupled to the oxidative metabolism of the cell and regulated by synaptic activity through calcium influx acting through the CaMKII pathway. As calcium influx is generally considered to be a consequence of the action potential, modulation of the presynaptic action potential by calcium could represent a negative feedback mechanism which plays a role in neurotransmitter release.

Other Kv1 channels may contribute to IAf as the Kvß1-subunit conveys rapid inactivation on Kv1 channels. According to Dixon and McKinnon (1996), Kv1.1, Kv1.2, Kv1.5 and Kvß1 mRNAs are expressed at moderate levels in the SCG while Kv1.3 and Kv1.6 mRNA are expressed in high levels in the SCG. However, it is not clear if any of these messages are localized to neurons. Both Kv1.1 and Kv1.5 mRNAs are expressed in Schwann cells (Mi *et al.*, 1995) and similarly, Kv1.2, Kv1.3, Kv1.6 and Kvß1 may be exclusively expressed in non-neuronal cells. The rapidly inactivating channels formed by the Kvß1-subunit with Kv1 channels activate ~20 mV more positive and inactivate ~40 mV more positive than IAf (Sewing *et al.*, 1996). Therefore, judging by their voltage-sensitive properties, it is unlikely that these channels contribute to IAf.

3.4.1.3 Possible contribution of Kv3 and Kv2 to SCG IAf

Kv3.4 encodes a potassium current which activates rapidly (Schroter *et al.*, 1991) and inactivates with a range of inactivation rates depending on phosphorylation and oxidation/reduction status of the channel. Typically, activation and inactivation of Kv3.4 occurs at ~15-35 mV more depolarized potentials than IAf. The pharmacology of Kv3.4 is different from that of IAf; Kv3.4 is more sensitive to 4-AP than is SCG IAf and is more sensitive to TEA, whereas IAf is insensitive to block by 10-50 mM TEA (Schroter *et al.*, 1991; Belluzzi and Sacchi, 1988; Schofield and Ikeda, 1989). Due to the differences in pharmacology and voltage-sensitive properties between Kv3.4 and IAf, I feel that it is unlikely that Kv3.4 contributes to IAf, even though Kv3.4 mRNA is expressed at very high levels in SCG neurons.

Both Kv3.3 and Kv3.1 channels are very different in their voltage-sensitive and pharmacological properties compared to IAf, as Kv3.3 and Kv3.1 activate and inactivate at much more depolarized potentials than IAf (Vega-Saenz de Miera and Rudy, 1992; Luneau *et al.*, 1991). Furthermore, Kv3.1 and Kv3.3 inactivate much more slowly and are much more sensitive to block by 4-AP and TEA than IAf (Vega-Saenz de Miera and Rudy, 1992). It is unlikely that Kv3.1 or Kv3.3 contribute to IAf as homomers. Like most Kv3 channels, Kv2 channels also manifest physiological properties far different from IAf. Kv2.1 and Kv2.2 channels activate slowly at depolarized potentials and Kv2.1 channels inactivate over very long voltage-steps (Ikeda *et al.*, 1992). Kv2.1 channels are more

sensitive to block by 4-AP than is IAf and Kv2 channels are also sensitive to block by TEA where IAf is not (Ikeda *et al.*, 1992; Shi *et al.*, 1994).

3.4.2 Molecular components of SCG IAs

The slowly inactivating transient current, IAs is also expressed at high levels in P7 SCG neurons (McFarlane and Cooper, 1991, 1992). IAs activates over tens of msec and inactivates over hundreds of msec, and activates and inactivates at potentials more positive than IAf.

3.4.2.1 Possible contribution of Kv1 and Kv4 to SCG IAs

When expressed in *Xenopus* oocyte, Kv4.2 encodes channels which activate and inactivate more rapidly than IAs and at potentials ~20-30 mV more negative than IAs. Therefore the Kv4.2 channel likely does not contribute to IAs. Kv1.4 activates more rapidly than IAs and inactivates with a range of rates dependent on phosphorylation and oxidation. Additionally, the voltage-sensitivity of Kv1.4 is ~20-30 mV more hyperpolarized than IAs. As Kv1.4 is expressed at low levels and the voltage-sensitive properties of Kv1.4 are quite different from IAs, then it is unlikely that it makes a substantial contribution to IAs as a homomeric channel. Nevertheless, as other Kv1 channels may be expressed by SCG neurons, it is possible that Kv1.4 could contribute to

IAs through forming heteromeric channels with other Kv1 subunits that have more depolarized voltage-sensitivities and activate and inactivate more slowly.

3.4.2.2 Possible contribution of Kv3 to SCG IAs

When expressed in *Xenopus* oocyte, the Kv3.4 current is similar in rates and voltage-sensitivity of activation and inactivation to IAs. Kv3.4 channels are highly susceptible to modulation through modification of the inactivation-ball structure. For example, protein kinase C eliminates rapid inactivation on Kv3.4 through phosphorylation of serine residues (serine 15 and serine 21) in the inactivation-ball (Covarrubias *et al.*, 1994). In addition, oxidation of a cysteine residue (cysteine 30) in the inactivation ball also removes rapid inactivation from the Kv3.4 channel (Vega-Saenz de Miera and Rudy, 1992; Duprat *et al.*, 1995). Both protein kinase C and oxidation abolish N-type inactivation of Kv3.4 channels, but neither the effects of phosphorylation nor oxidation have been examined on the potassium currents of sympathetic neurons. Therefore, it is not clear if IAs is susceptible to the same modulation.

The physiological contribution of the Kv3.4 to SCG IAs depends on the subcellular location of these channels. Kv3.4 seems to have an axonal localization (Veh *et al.*, 1995). Presynaptically, IAs could influence the shape of the action potential so as to influence calcium influx and neurotransmitter release. However, Kv3.4 channels open during recovery from inactivation and current flow at hyperpolarized membrane potentials could control firing frequency (Ruppersburg *et al.*, 1991). Kv3.4 is expressed at

very high levels in SCG neurons, so we would anticipate that it makes a significant contribution to currents on these neurons. As Kv3.4 closely resembles the electrophysiological properties of IAs, then Kv3.4 likely contributes to this current. Both Kv3.3 and Kv3.1 mRNAs are expressed by SCG neurons, and Kv3 channels activate at similar depolarized potentials as does IAs. The Kv3.3 channel inactivates over hundreds of msecs similar to IAs, but ~30 mV more positive than IAs. while Kv3.1 channels inactivate very slowly over seconds (Luneau *et al.*, 1991; Vega-Saenz deMiera *et al.*, 1992; Weiser *et al.*, 1994). Close examination of nodose IAs revealed that this current has two time constants for inactivation. One component of IAs inactivates over hundreds of msec, while another component activates over seconds (McFarlane and Cooper, 1991, 1992). These two time constants of IAs likely represent distinct populations of channels with different kinetics. Similarly, SCG IAs may also contain channels which inactivate over seconds. As the expression level of Kv3.1 and Kv3.3 mRNA is low, then if these genes contribute to SCG IAs as homomeric channels, we would expect their overall contribution to be small and the current due to these channels may be masked by the more highly expressed currents. Unlike alternatively spliced *Drosophila Shaker*, alternatively spliced Kv3 channels are not reported to have distinctive functional properties (Rettig *et al.*, 1992).

In the central nervous system, each Kv3 mRNA has a unique expression pattern, but Kv3 signal appears to be only in neurons and not non-neuronal cells. Kv3.4 mRNA is expressed at low levels in brain compared to the other Kv3 transcripts (sixfold less), and

is expressed at much higher levels in skeletal muscle but not heart (Weiser *et al.*, 1994; Rettig *et al.*, 1992). Kv3.4 channels seem to be more important peripherally than centrally. Other Kv3 transcripts are detected at very high levels in brain but are only faintly detected in the periphery. Kv3.4 mRNA is found mainly expressed in brain areas that express Kv3.1 or Kv3.3 mRNA (Weiser *et al.*, 1994). This is the same pattern we observe in SCG neurons. Kv3.2 signal appears to be localized distinctly from other Kv3 mRNAs (Weiser *et al.*, 1994) and Kv3.2 is absent in SCG neurons where the other Kv3 mRNAs are expressed. Kv3.4 mRNA is expressed in all SCG neurons and as other Kv3 members are expressed by SCG neurons, this necessitates that multiple Kv3 subunits are co-expressed at least in some cells. We do not know if the channels formed by different Kv3 subunits in the same cells are homomeric or heteromeric. If the proteins are targeted to different regions of the neuron then heteromeric channels will not form. Co-expression studies that examine the properties of heteromeric channels show that a single inactivation ball is all that is required to convey rapid inactivation of a channel, though the rate of inactivation increases with increasing number of inactivating subunits (Isacoff *et al.*, 1990; McCormack *et al.*, 1990; Ruppersberg *et al.*, 1990; MacKinnon *et al.*, 1991; Weiser *et al.*, 1994; Antz *et al.*, 1997). In sympathetic neurons, the expression pattern is reversed from the CNS in that Kv3.4 is expressed at high levels relative to Kv3.1 and Kv3.3. Therefore, we may expect homomeric Kv3.4 channels or possibly heteromeric channels containing a single Kv3.1 or Kv3.3 subunit to be formed in these neurons.

3.4.2.3 Possible contribution of Kv2 to SCG IAs

Both Kv2.1 and Kv2.2 channels activate at depolarized potentials as does IAs (Shi *et al.*, 1994). Kv2.1 has a similar voltage-sensitivity to inactivation as IAs but inactivates over seconds (Shi *et al.*, 1994). The voltage-sensitive properties of Kv2.2 have not been well characterized, but Kv2.2 channels do not show inactivation over short voltage-steps. Kv2 channels could contribute to IAs, perhaps a more slowly inactivating component of Ias, than is contributed by Kv3 channels.

Kv2 channels are predominantly localized to neurons in brain (Hwang *et al.*, 1993). However, Kv2.1 is expressed at high levels peripherally in excitable tissues such as the atria, ventricle and skeletal muscle, whereas Kv2.2 is undetectable or only expressed at low levels in these tissues. Kv2.1 and Kv2.2 proteins have been localized to the dendrites and soma of some populations of CNS neurons (Maletic-Savatic *et al.*, 1995). Kv2.1 staining is discrete and punctuate on cell bodies and extends into proximal dendrites (Hwang *et al.*, 1993), whereas Kv2.2 immunoreactivity is present faintly and homogeneously on soma and dendrites (Maletic-Savatic *et al.*, 1995). It is possible that Kv2 channels make a substantial contribution to IAs but are masked by a more rapidly inactivating current.

3.4.3 Molecular components of SCG IK

The delayed-rectifier current that is expressed by SCG neurons activates slowly at depolarized potentials and shows no inactivation even over long steps. This slow activation of IK at depolarized potentials occurs much more slowly than the activation of Kv channels. Moreover, a relatively unique property of SCG IK is the complete lack of inactivation even over very long voltage-steps. So far, every Kv channel that has been examined over long voltage-steps inactivates slowly, which is attributed to C-type inactivation (see section **1.2.8**). Oxidation/reduction and phosphorylation modulation has been shown to slow or abolish N-type inactivation, yet no reported modification of Kv channels removes C-type inactivation. Therefore, it seems unlikely that any Kv gene encodes SCG IK. The SCG delayed-rectifier current is likely encoded by genes from one of the other potassium channel gene families (see section **1.4.4**).

3.4.4 Molecular components of SCG IM

As described in section **1.3.5**, M-currents are a class of currents recorded *in vivo* which appear to be distinct from other voltage-gated potassium currents. No known Kv gene give rise to a low threshold, slowly activating current that is blockable by acetylcholine. It has been suggested that another class of potassium channels (*eag ether-a-go-go*) encodes the M-current (Stanfeld *et al.*, 1997; Mathie and Watkins, 1997).

3.4.5 Ratio of Kv mRNA/channel

The typical total outward voltage-gated potassium current on a P7 SCG neuron is ~2 nA (McFarlane and Cooper, 1992, 1993), and the single channel current of an IAf or IAs channels is ~2pA. Therefore, the total number of Kv channels per neuron is ~1000. In section **3.3.4**, I showed that there were ~5000 Kv3.4 mRNA molecules per P7 SCG neuron. As Kv3.4 mRNA composes about half of the Kv mRNAs that I detected in P7 SCG neurons then there are ~10 000 Kv mRNA molecules per cell (more if additional Kv mRNAs are expressed). This means that there is at least a 10:1 ratio of Kv mRNAs to Kv channels. The 10:1 ratio implies that tenfold more Kv mRNA is transcribed than is translated into protein. Yet, alternative explanations may explain this ratio. For example, whole-cell recording is limited to the region of the neuron that can be voltage-clamped and *in vivo* and *in vitro* SCG neurons extend axons which extend millimeters. This distance is considerable, as the cell body of such a neuron is only micrometers in diameter. It is probable that the ionic currents on distal axons are not detectable from cell body recordings. Therefore, these channels are not represented in the total outwardly rectifying current recorded from these neurons leading to an inflation in the ratio of mRNA/channels. Additionally, intracellular pools of channels may exist. Similar to channels in distal locations, these channels in intracellular pools would not be represented in total outward current on SCG neurons and would therefore also skew the ratio of Kv mRNA to Kv channels.

3.4.6 Resemblance of NupG to Kv channels

As described in section **3.3.5**, the clone db39 was determined to be NupG (nucleoside permease) an *E. coli* transmembrane protein. Voltage-gate potassium channels and NupG represent distinct classes of proteins, yet there is strong homology between proteins in the regions amplified by the PCR primers (see section **3.3.5**). This region of homology in the db39 sequence corresponds to the coding region of NupG gene. At some stage during the preparation of cDNA or during the PCR reaction, contamination with *E. coli* must have occurred. NupG is a membrane protein with strong structural similarities in this region to the cloned Kv channels, however, NupG is not an ion channel. NupG is an *E. coli* nucleoside transport system. When I compared the entire amino acid sequence of NupG to that of Kv channels from each of the four different families, I found that only a small region of NupG contains shared homology with the Kv genes. Figure 3.10 compares this homologous NupG amino acid sequence to those of the voltage-gated potassium channels, which I PCR cloned. The conserved amino acids are darkly shaded while conservative substitutions are lightly shaded. Sequence homology extends from the region between the primers into the primer regions. There is curious homology between NupG and the Kv channels in the region targeted by the sense primer, which in Kv channels is referred to as the GYGD signature sequence. The GYGD motif is believed to contribute to the selectivity filter of the Kv channels (Aiyar *et al.* 1995, 1996; Heginbotham *et al.*, 1992, 1994; Kirsh *et al.*, 1995; Ranganathan *et al.*, 1992) and is therefore considered a definitive feature of potassium channels.

To further investigate the homology of NupG to Kv channels, I compared the 28 amino acids of the conserved region in Table 3.2. There is 18-29% homology observed between NupG and the Kv genes. The highest homology is between the NupG and Kv3.1 (29%) while the lowest homology is between the unknown gene and Kv1.4 (18%). There is higher homology (57-71%), in this region, between the different subfamilies, and extraordinarily high homology (93-100%), within members of the Kv3 subfamily. Furthermore, the region in NupG amplified by the PCR primers is the exact length as the regions of Kv channels. It is not clear whether these observed similarities between NupG and the Kv channels are due to a common ancestral gene or if the conserved motif arose independently.

It is difficult to account for the three voltage-gated potassium currents we record from SCG neurons, but various possibilities may explain how these gene products give rise to the currents that we see. 1) Subunits within the same subfamily may form heteromeric channels. 2) Currents may overlap in physiological properties, such that they are indistinguishable from each other and appear as a single current. 3) Channels may be expressed at remote locations, such as the nerve terminal and thus be physiologically undetectable when recording from the cell body. 4) Channel function may be modulated or altered through interaction with other proteins.

In this chapter, I showed that rat SCG neurons, which are known to have three distinct voltage-gated potassium currents, also express at least seven different voltage-gated potassium channel genes. We are uncertain how these individual genes give rise to

the currents we record, however, through investigating changes in Kv gene expression over normal neonatal development and development in culture, we hope to better understand their contribution.

Chapter 4:

The Expression of Voltage-Gated Potassium Channels Genes by SCG Neurons During Neonatal Development and in Culture

4.1 Introduction

Individual populations of neurons acquire their unique electrophysiological properties through a complex interplay of genetic and extrinsic factors (see section **1.1**). These electrophysiological properties undergo changes in a fixed temporal pattern and there is diversity in observed developmental patterns (for a review, see Spitzer, 1991). In particular, the developmental expression of voltage-gated potassium currents are well characterized in certain types of neurons (see sections **1.2.6** and **1.3.5**) and non-neuronal cells provide important developmental influences that affect the expression of voltage-gated potassium currents (see sections **1.3.6-1.1.3.8** and **1.2.7**; for a review, see Barish, 1995).

Superior cervical ganglion neurons express four distinct outward voltage-gated potassium currents, a rapidly inactivating transient current (IAf) that inactivates over tens of msecs and requires hyperpolarization to -90 mV to fully remove inactivation (see section **1.3.2**), a slowly inactivating transient current (IAs) which inactivates over hundreds of msecs and is fully available for activation from -60 mV (see section **1.3.3**), a

non inactivating delayed rectifier current (IK) that activates at positive potentials (see section **1.3.1**) and a slowly activating current that is blockable by acetylcholine (IM) (see section **1.3.4**). SCG neurons undergo marked changes in expression of voltage-gated potassium currents over neonatal development. Briefly, postnatal day one (P1) SCG neurons express high levels of IAs (~70 pA/pF), moderate levels of IAf (~25 pA/pF), and very low levels of IK (~10 pA/pF). Over the first two weeks of normal postnatal development, dramatic changes occur in the relative expression of the different conductances. IAs decreases by half (~35 pA/pF), while IAf expression increases greater than twofold (~70 pA/pF), becoming the predominant potassium conductance. Throughout postnatal development, IK remains at low levels (~10 pA/pF; McFarlane and Cooper, 1992). However, if P1 SCG neurons are grown in culture, a different developmental pattern occurs. During three weeks in culture, both IAs and IAf decrease to low levels (~10 pA/pF and ~2 pA/pF respectively) and IK increases fivefold (~60 pA/pF) to become the predominant conductance. Yet, if postnatal day fourteen (P14) neurons are grown in culture IAf and IAs decrease to moderate (~40 pA/pF) and low levels (~10 pA/pF) respectivly, whereas IK increases fivefold to become the predominant current (~60 pA/pF). These results suggest that a factor is present *in vivo* which is absent in our culture system. If P1 SCG neurons are grown in culture with conditioned media from non-neuronal cells, IAs and IAf are maintained at their P1 levels. This effect can be mimicked if neurons are grown in ciliary neurotrophic factor (CNTF), a neuropoietic

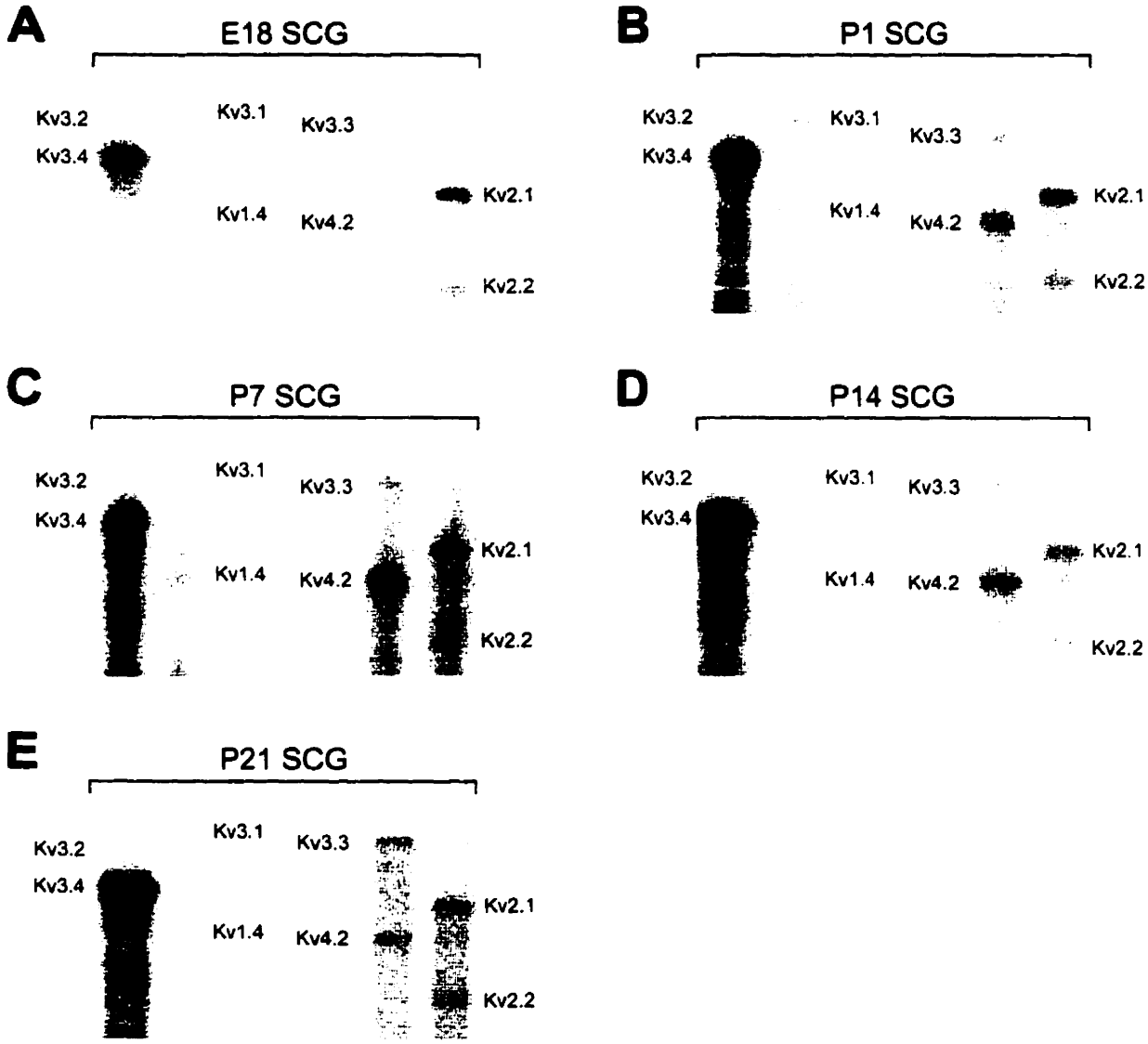

Figure 4.1 RNase protection assays: Kv mRNA expression through neonatal development.

RNase protection assay for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 mRNAs. A 2 μg sample of (**A**) E18, (**B**) P1, (**C**) P7, (**D**) P14 or (**E**) P21SCG total cellular RNA was protected in each lane. Signals for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.3, Kv3.4 and Kv4.2 were detectable.

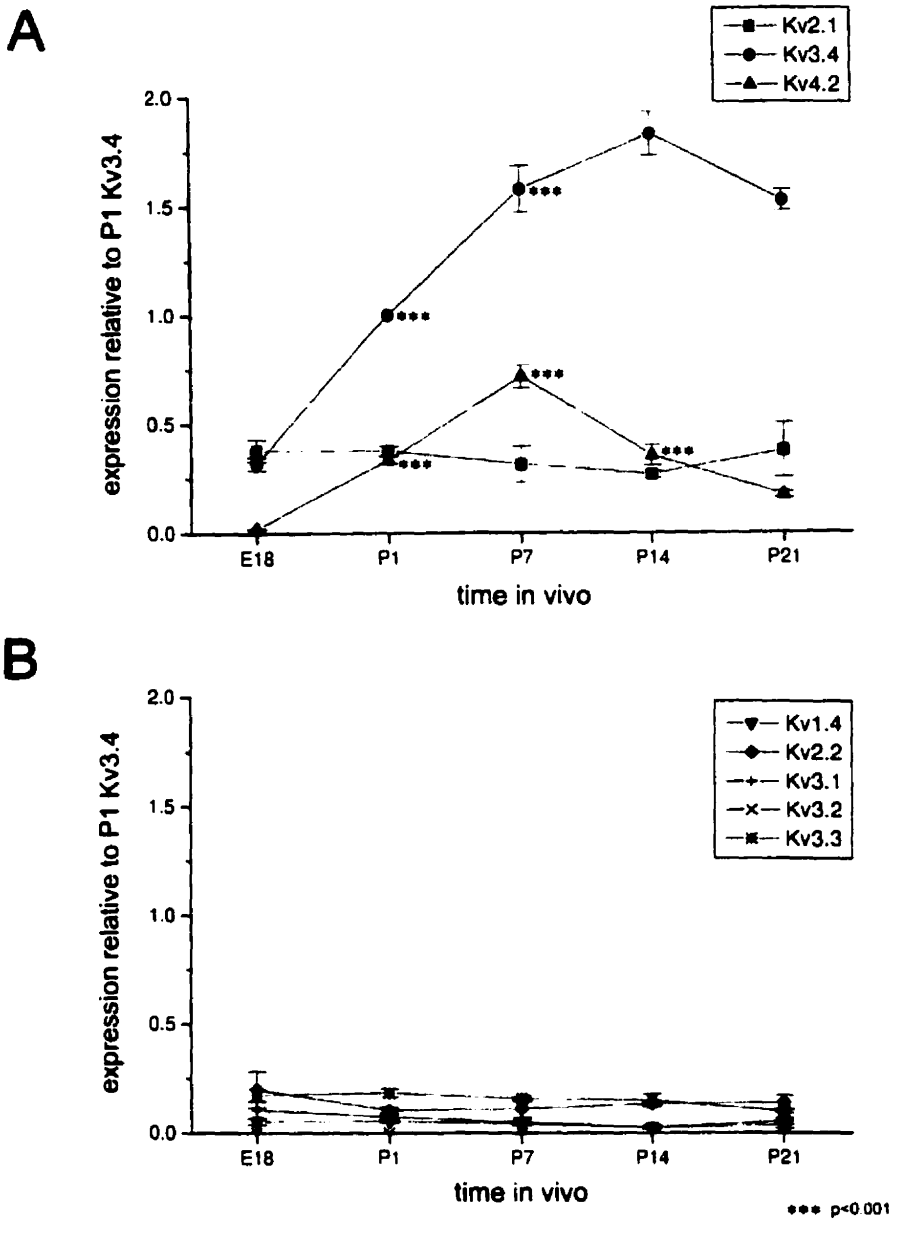

Figure 4.2 Summary of Kv mRNA expression in SCG during neonatal development.

The means (± SEM) of mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 in SCG at different developmental times are plotted as a line-symbol graph. Each mean reflects at least three separate experiments. (**A**) shows the *in vivo* mRNA expression of Kv2.1, Kv3.4 and Kv4.2. Both Kv3.4 and Kv4.2 mRNA levels increase significantly between E18 to P1, and between P1 and P7, and Kv4.2 mRNA levels decrease significantly between P7 and P14 ($p<0.001$; ANOVA). Individual signals were measured using a phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of P1 Kv3.4 mRNA.

cytokine expressed by non-neuronal cells, which is capable of supporting neuronal survival and influencing neuronal phenotype (McFarlane and Cooper, 1993).

Various possibilities may account for the *in vivo* and *in vitro* developmental changes in voltage-gated potassium current density by SCG neurons. For example, as these developmental changes occur over days, it is possible that they reflect changes in mRNA levels. Through studying Kv mRNA levels under different developmental conditions and comparing this to changes in current density, we may better understand how individual Kv genes contribute to the expression of voltage-gated potassium currents. Sympathetic neurons are appropriate for these studies as they change their voltage-gated potassium current expression over a short period of time (days). SCG neurons are easily dissected, they dissociate well from non-neuronal cells and grow well in culture, making it easy to manipulate their external environment (Mains and Patterson, 1973; Hawrot, 1980).

In this chapter, my aim was to measure changes in Kv mRNA levels over neonatal development and as these neurons develop in culture. I also investigated whether CNTF influences Kv mRNA levels. Furthermore, I correlated expression level of Kv mRNAs with current densities to determine if changes in mRNA levels can account for the changes in current density.

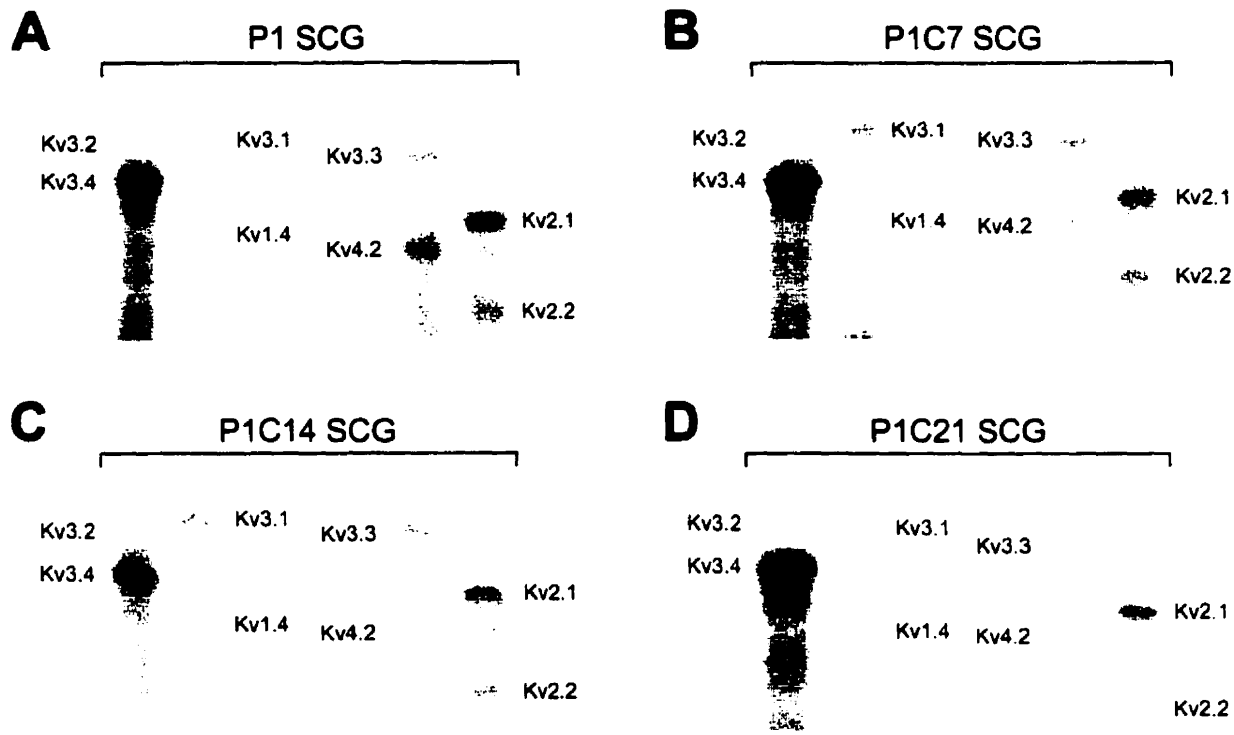

Figure 4.3 RNase protection assays: Kv mRNA expression by P1 SCG neurons during *in vitro* development.

RNase protection assay for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 mRNAs. A 2 µg sample of (**A**) P1, (**B**) P1C7, (**C**) P1C14 or (**D**) P1C21 SCG total cellular RNA was protected in each lane.

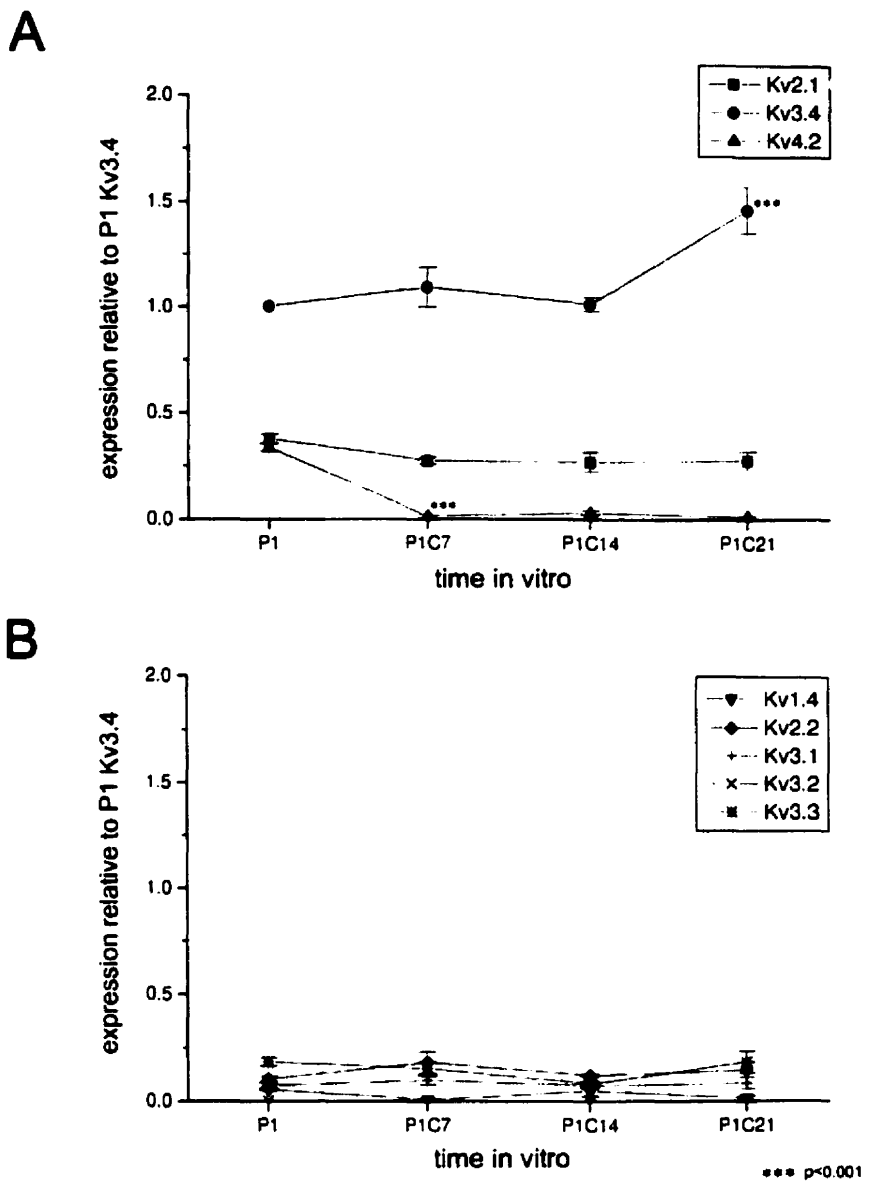

Figure 4.4 Summary of Kv mRNA expression by P1 SCG neurons during time in culture.

The means (± SEM) of mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 for P1 SCG neurons at different developmental times in culture are plotted as a line-symbol graph. Each figure reflects at least three separate experiments. (**A**) shows the *in vitro* mRNA expression of Kv2.1, Kv3.4 and Kv4.2. Kv4.2 mRNA levels undergo a significant decrease to very low levels during the first week *in vitro* and Kv3.4 mRNA levels undergo a significant increase during the third week *in vitro* ($p<0.001$; ANOVA). (**B**) shows the *in vitro* mRNA expression of Kv1.4, Kv2.2, Kv3.1, Kv3.2 and Kv3.3. Individual signals were measured using a phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of P1 Kv3.4 mRNA.

4.2 Methods

4.2.1 RNase protection assay

Total cellular RNA was extracted from E18-P21 SCG (see section **2.1.1**). For some experiments SCG neurons, were separated from their non-neuronal cells and grown in dissociated cell culture (see sections **2.2.8** and **2.2.10**) prior to RNA extraction (see section **2.2.9**). The radiolabeled cRNA probes were transcribed, purified and quantified (see section **2.2.5**). The total cellular RNA and cRNA probes were hybridized (see section **2.2.5**). The single stranded total cellular RNA and cRNA probe were digested (see section **2.2.5**). The cRNA probe signal was detected (see section **2.2.5**).

4.3 Results

4.3.1 Kv mRNA expression by SCG neurons *in vivo*

In chapter 3, I showed SCG neurons expressed at least seven different Kv genes. As the current densities of voltage-gated potassium currents on SCG neurons change over neonatal development (see section **1.3.5**), I chose to investigate the expression of voltage-gated potassium channel genes during this time period to determine if changes in Kv mRNA levels reflect changes in current density. Figure 4.1 is a representative RNase protection assay showing the developmental mRNA expression of the voltage-gated potassium channel genes, Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2,

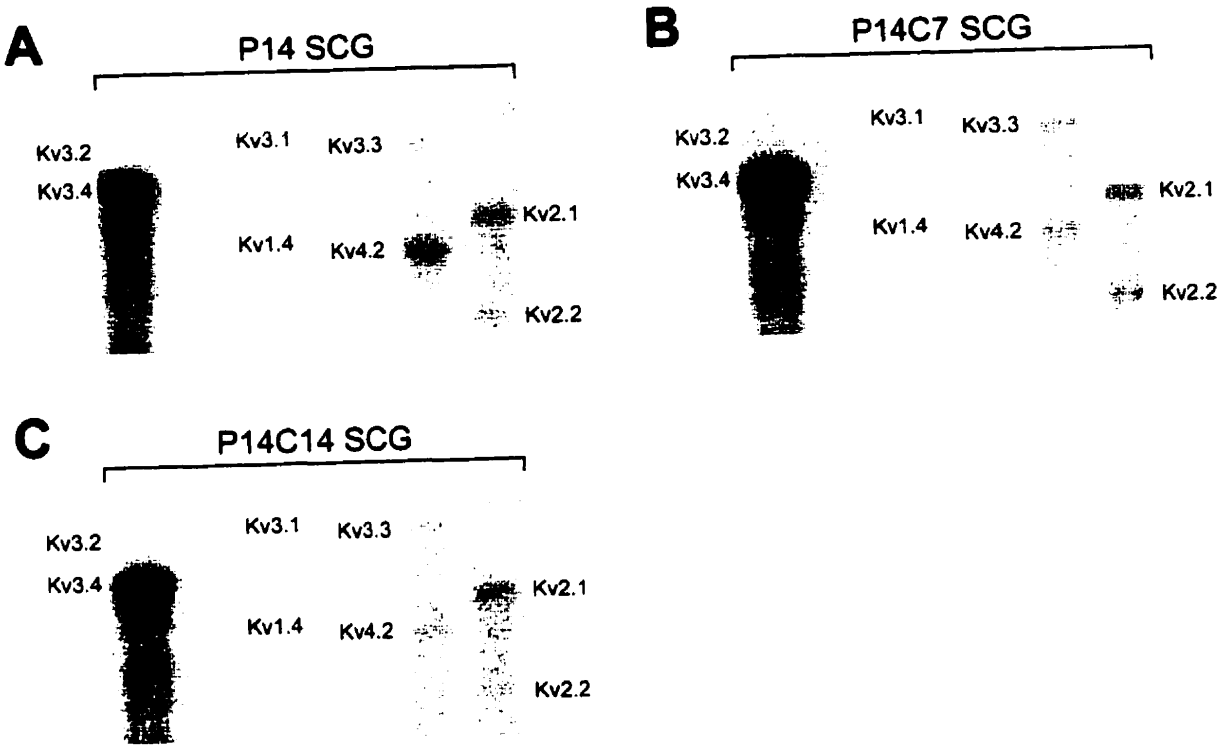

Figure 4.5 RNase protection assays: Kv mRNA expression over *in vitro* development in P14 SCG neurons.

RNase protection assay for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 mRNAs. A 2 μg sample of (**A**) P1, (**B**) P1C7, (**C**) P1C14 or (**D**) P1C21 SCG total cellular RNA was protected in each lane.

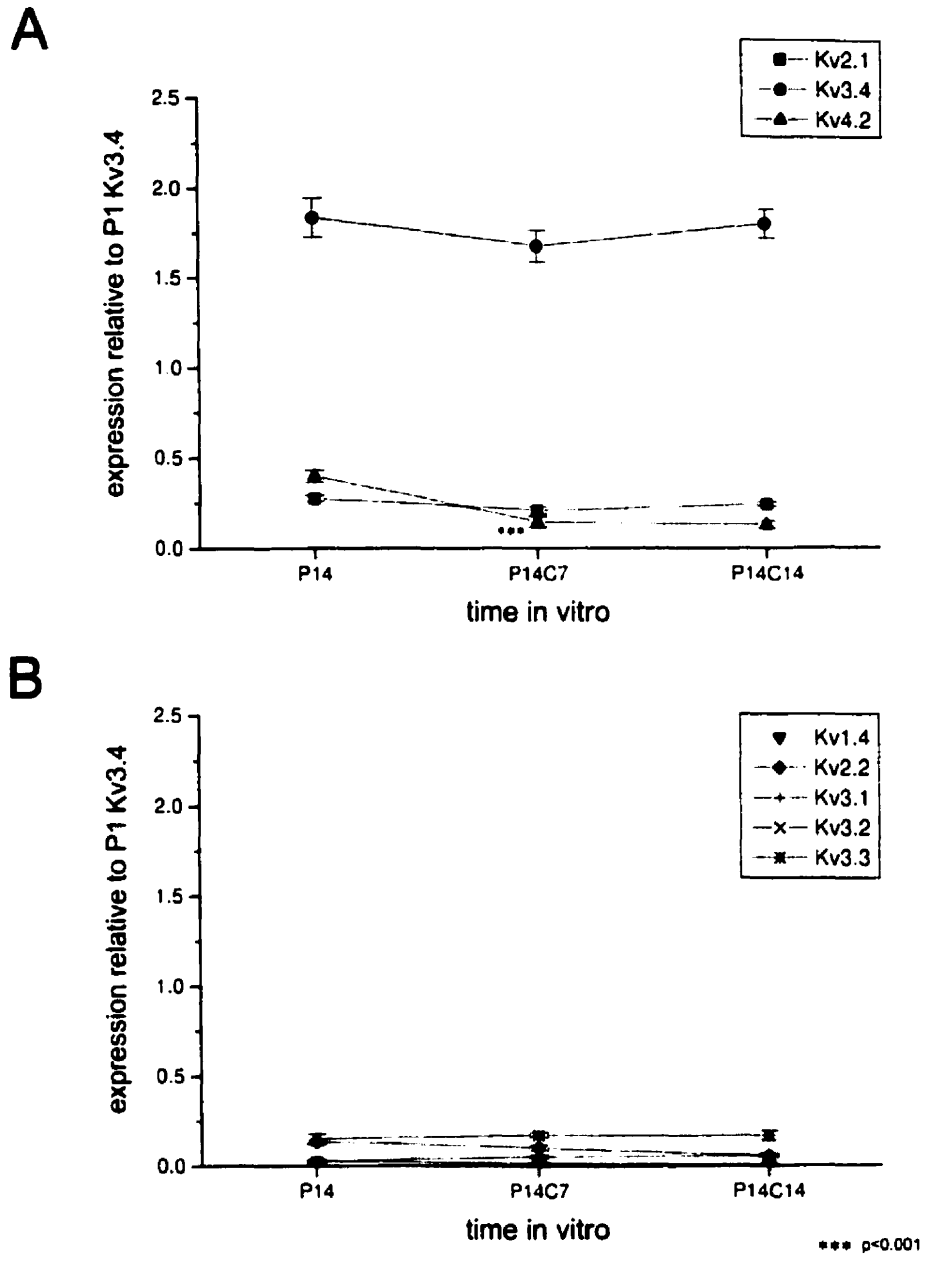

Figure 4.6 Quantification of Kv mRNA expression by P1 SCG neurons during time in culture.

The means (± SEM) of mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 for P14 SCG neurons at different developmental times in culture are plotted as a line-symbol graph. Each mean reflects at least three separate experiments. (**A**) shows the *in vitro* mRNA expression of Kv2.1, Kv3.4 and Kv4.2. Kv4.2 mRNA levels undergo a significant decrease during the first week in culture. (**B**) shows the *in vitro* mRNA expression of Kv1.4, Kv2.2, Kv3.1, Kv3.2 and Kv3.3. Individual signals were measured using a phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of P1 Kv3.4 mRNA.

by neonatal SCG neurons. Figure 4.2 is the corresponding phosphorus imager quantification showing that seven of eight Kv genes investigated were detectable through neonatal development. Kv mRNAs were expressed at a range of levels. While some Kv genes were expressed at constant levels through development, other Kv genes underwent clear developmental changes in expression.

Prenatally, mRNA for three of the four Kv3 genes, Kv3.1, Kv3.3 and Kv3.4 were expressed at low to moderate levels. At E18, Kv3.1 was expressed at low levels (10.5% P1 Kv3.4) and Kv3.3 was expressed at moderate levels (17.3% P1 Kv3.4) and their expression did not change significantly through neonatal development. Figure 4.2 shows that Kv3.4 was expressed at the highest levels of all Kv transcripts. Furthermore, the increase in expression of Kv3.4 fold was sixfold from E18 level. Kv3.2 was not detectable at any time during neonatal development.

At E18, Kv4.2 was expressed at very low levels (1.9% P1 Kv3.4). The change in levels of Kv4.2 from E18 to P7 was a greater than thirty-fivefold increase in expression. Kv1.4 was expressed at consistently low levels, Kv1.4 was expressed at 5.3% P1 Kv3.4 at E18 and did not change significantly through neonatal development. Figure 4.2 shows that, at E18, Kv2.1 was expressed at moderate levels (37.7% P1 Kv3.4), Kv2.2 was expressed at low levels (19.9% P1 Kv3.4) and Kv2 mRNA expression did not change significantly over neonatal development.

4.3.2 Kv mRNA expression by P1 SCG neurons *in vitro*

The normal *in vivo* developmental pattern of potassium current expression depends on influences that are present *in vivo*. When SCG neurons are dissected and placed in culture, a very different developmental pattern occurs. In culture, both IAf and IAs, which are initially present at high levels on P1 neurons, drop to very low levels, whereas the delayed rectifier current, which is initially present at low levels, increases fivefold to become the predominant conductance. To investigate if changes in Kv mRNA expression contributes to the changes in current density observed *in vitro*, I used RNase protection assay to measure the Kv mRNA levels on SCG neurons grown in culture.

Figure 4.3 is a representative RNase protection assay showing the developmental mRNA expression of the voltage-gated potassium channel genes, Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2, by P1 rat SCG neurons grown in culture. Figure 4.4 is the corresponding phosphorus imager quantification showing that seven of eight Kv genes investigated were detectable through *in vitro* development. Some Kv mRNAs were expressed at relatively constant levels, while others underwent significant developmental changes.

Figure 4.4 shows that at birth Kv3.4 mRNA was expressed at high levels (100% P1 Kv3.4) and remained unchanged through the first two weeks in culture, but by P1C21, Kv3.4 expression had increased significantly (146% P1 Kv3.4). Kv3.1 and Kv3.3 mRNA were expressed at low levels (7.4% P1 Kv3.4) and moderate levels (18.5% P1 Kv3.4) respectively and did not significantly change their expression levels during time in

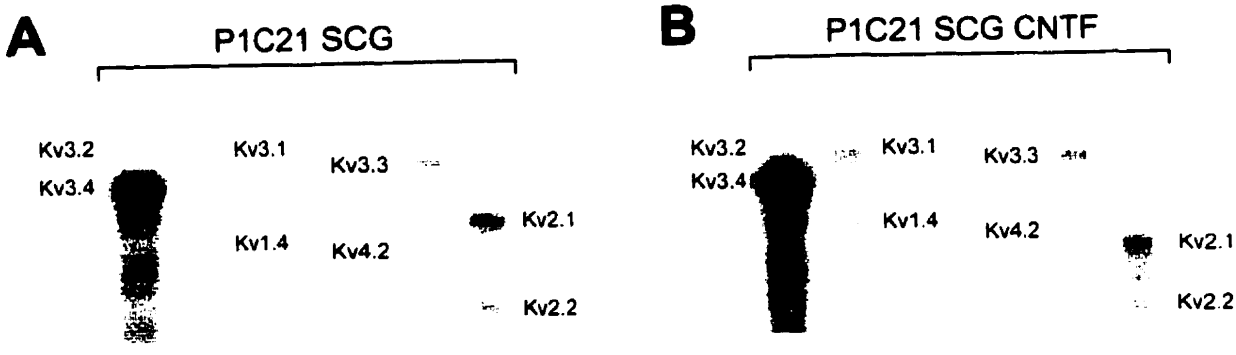

Figure 4.7 RNase protection assays: Kv mRNA expression by P1 SCG neurons grown in culture for three weeks with or without CNTF.

(**A**) RNase protection assay for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 mRNAs. A 2 μg sample of total cellular mRNA isolated from P1 SCG neurons grown in culture for three weeks (**A**) in the absence of CNTF or (**B**) in the presence of CNTF was protected in each lane.

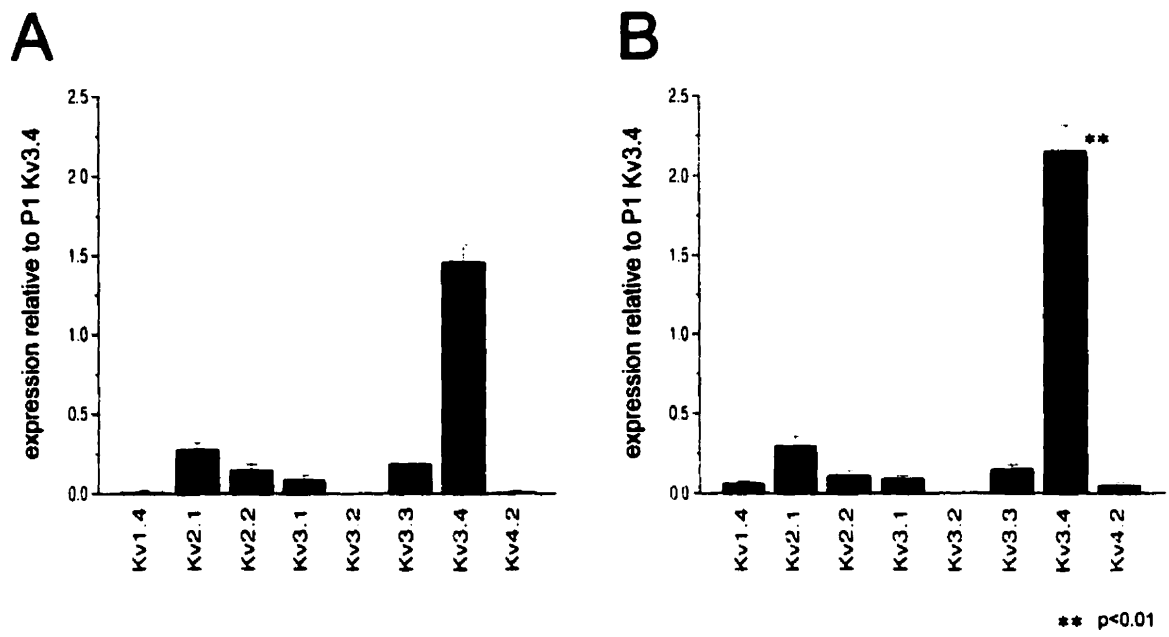

Figure 4.8 Quantification of mRNA expression in P1 SCG neurons cultured for three weeks with CNTF.

(**A**) The means (± SEM) of mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 are plotted for SCG neurons grown in culture for three weeks. Each mean reflects at least three separate experiments. (**B**) Similar to (**A**) except the values plotted are for P1 SCG neurons grown in culture for three weeks with CNTF. Individual signals were measured using phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of P1 Kv3.4 mRNA. There is no significant difference in the means between the experimental and control conditions except for Kv3.4 mRNA that is expressed at higher levels in neurons grown with CNTF.

culture. Kv3.2 mRNA was not detectable in culture. Kv4.2 mRNA expression decreased during time in culture. At P1, Kv4.2 mRNA was expressed at moderate levels (33.4% P1 Kv3.4), but by the end of the first week in culture, Kv4.2 expression had decreased significantly to virtually undetectable levels. Kv1.4 was expressed at low levels (5.4% P1 Kv3.4) and did not change during time in culture. At P1, Kv2.1 was expressed at moderate levels (37.6% P1 Kv3.4) and Kv2.2 was expressed at low levels (10.4% P1 Kv3.4). Neither Kv2.1 nor Kv2.2 levels significantly changed during time in culture. Since *in vitro* changes in Kv mRNA levels do not correspond to changes in current density, these results imply that changes in Kv mRNA expression do not account for all the developmental changes in current density in culture.

4.3.3 Kv mRNA expression by P14 SCG neurons *in vitro*

When P14 SCG neurons are grown in culture, there is an increase in the expression of IK, as occurs for P1 neurons grown in culture. Also, cultured P14 SCG neurons reduce their A-current density. However, this A-current density does not decrease to the same low level observed in cultured P1 neurons (see section **1.1.18**). I examined Kv gene expression in cultured P14 neurons using RNase protection assay to determine if there were changes in Kv mRNA levels that could account for changes in current density.

Figure 4.5 is a representative RNase protection assay showing the developmental mRNA expression of the voltage-gated potassium channel genes, Kv1.4, Kv2.1, Kv2.2,

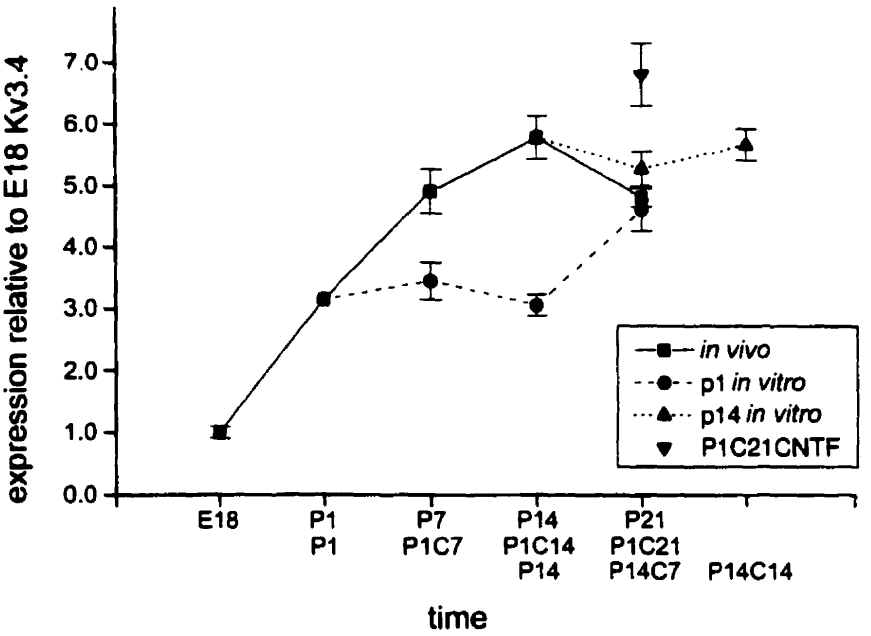

Figure 4.9 Summary of Kv3.4 expression under different conditions.

The means (± SEM) of SCG neuronal Kv3.4 mRNA levels during development *in vivo* and *in vitro* are plotted as a line-symbol graph. Each mean reflects at least three separate experiments. Individual signals were measured using phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of E18 Kv3.4 mRNA. The *in vivo* levels of Kv3.4 mRNA are plotted with squares. The *in vitro* levels of Kv3.4 mRNA are plotted with circles (cultured P1 SCG neurons) or triangles (cultured P14 SCG neurons). The level of Kv3.4 mRNA from SCG neurons cultured for three weeks with CNTF is indicated by the inverted triangle.

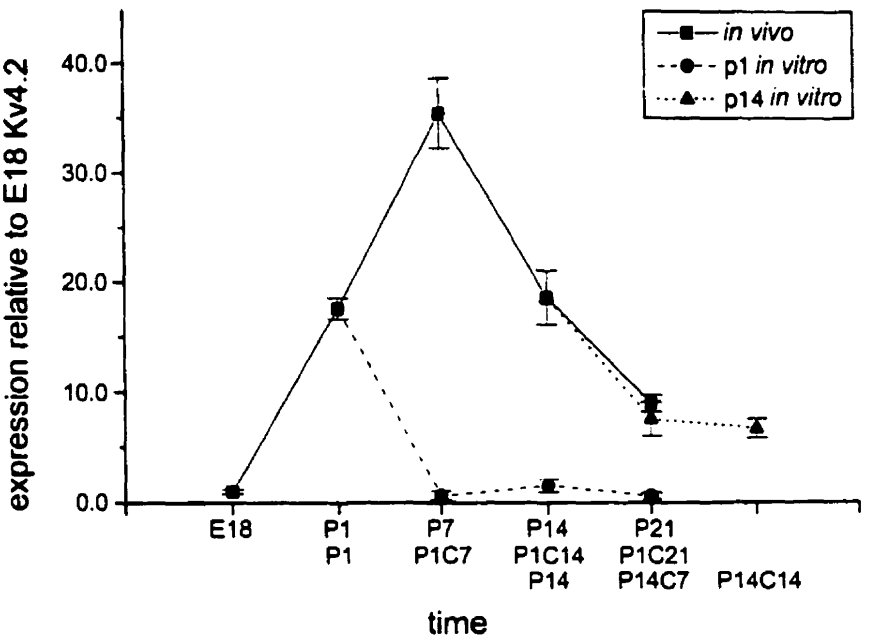

Figure 4.10 Summary of Kv4.2 expression under different conditions.

The means (± SEM) of SCG neuronal Kv4.2 mRNA levels during development *in vivo* and *in vitro* are plotted as a line-symbol graph. Each mean reflects at least three separate experiments. Individual signals were measured using a phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of E18 Kv4.2 mRNA. The *in vivo* levels of Kv4.2 mRNA are plotted with squares. The *in vitro* levels of Kv4.2 mRNA are plotted with circles (cultured P1 SCG neurons) or triangles (cultured P14 SCG neurons).

Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2, by P14 SCG neurons grown in culture. Figure 4.6 is the corresponding phosphorus image quantification. Many Kv mRNAs did not undergo significant changes over development. Figure 4.6 shows Kv3.4 mRNA was expressed at high levels by P14 SCG neurons (183.3% P1 Kv3.4) that did not change significantly during two weeks in culture. Kv3.1 and Kv3.3 mRNA were expressed at low levels (2.6% P1 Kv3.4) and moderate levels (15.1% P1 Kv3.4) respectively and did not change significantly during two weeks in culture. Kv3.2 mRNA was not detectable. Kv4.2 mRNA was initially present at moderate levels (39.6% P1 Kv3.4), but Kv4.2 expression had decreased significantly to low levels (14.4% P1 Kv3.4) by P14C7, where it remained through P14C14. Kv1.4 mRNA was initially expressed at very low levels (2.6% P1 Kv3.4) and did not change significantly during time in culture. Kv2.1 and Kv2.2 mRNA were initially expressed at moderate levels (27.4% P1 Kv3.4) and low levels (13.5% P1 Kv3.4) and did not change significantly during time in culture.

4.3.4 Kv mRNA expression by SCG neurons cultured with CNTF

CNTF has been shown to sustain the expression of A-currents and depress the expression of IK on cultured P1 SCG neurons (see section **1.1.19**). To determine whether this effect is due to changes in mRNA levels, I grew SCG neurons in culture for three weeks with CNTF.

Figure 4.7 is a representative RNase protection assay comparing the expression of voltage-gated potassium channel mRNAs, Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3,

Kv3.4 and Kv4.2, by SCG neurons grown in culture for three weeks in either the presence or absence of CNTF. Figure 4.8 is the corresponding phosphorus imager quantification of Kv mRNA expression. There was no significant difference between the mRNA expression levels of Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3 and Kv4.2 in experimental and control conditions. Furthermore, there was no significant change in mRNA levels of Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2 and Kv3.3 from P1 levels when SCG neurons were grown in culture with or without CNTF. Moreover, Kv4.2 mRNA levels decreased from moderate (33% P1 Kv3.4) to very low levels when cultured with CNTF, as was observed in the absence of CNTF. Curiously, CNTF significantly increased Kv3.4 mRNA expression. Kv3.4 mRNA, which was highly expressed by P1 SCG neurons, increased over three weeks in culture by ~50% (145.5% P1 Kv3.4), but when P1 SCG neurons were cultured for three weeks with CNTF, then Kv3.4 expression increased by >100% (212.6% P1 Kv3.4).

In this chapter, the majority of voltage-gated potassium channel mRNAs that I examined, Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2 and Kv3.3, were expressed at constant levels under different developmental conditions (see sections **4.3.1-4.3.4**). Yet, there were two Kv mRNAs that underwent significant developmental changes Kv3.4 and Kv4.2. To more clearly illustrate the changes that occur in mRNA expression, I plotted the changes in mRNA levels of Kv3.4 and Kv4.2 under different conditions together.

4.3.5 Kv3.4 mRNA expression by developing SCG neurons

Figure 4.9 shows the developmental changes in expression of Kv3.4 mRNA, during *in vivo* and *in vitro* development. Over neonatal development, Kv3.4 mRNA expression increased sixfold from moderate levels to high levels. When P1 SCG neurons were grown in culture, Kv3.4 mRNA expression remained constant for two weeks before increasing to similar levels as *in vivo*. When P14 neurons were grown in culture they sustained Kv3.4 mRNA expression at high levels, however, when P1 neurons were grown in culture with CNTF the expression of Kv3.4 was increased significantly above *in vivo* and *in vitro* levels.

4.3.6 Kv4.2 mRNA expression by developing SCG neurons

Figure 4.10 shows the developmental changes in expression of Kv4.2 mRNA both over *in vivo* and *in vitro* development. Over normal development Kv4.2 increased more than thirty-fivefold, from very low levels to high levels before decreasing to low levels, but this developmental pattern did not occur when P1 SCG neurons were grown in culture. Instead Kv4.2 mRNA levels immediately decreased to very low levels. If P14 neurons were grown in culture, Kv4.2 mRNA expression closely resembled *in vivo* expression. CNTF did not significantly increase the expression of Kv4.2 in mRNA culture.

4.4 Discussion

As voltage-gated potassium currents on SCG neurons undergo marked changes in relative current densities during different developmental conditions, the objective of this chapter was to determine if changes in Kv mRNA levels could account for changes in current density. However, the changes in voltage-gated potassium current densities of SCG neurons that occur over normal development and in culture can not be totally explained by changes in mRNA levels of the seven Kv genes expressed by SCG neurons.

The developmental expression of Kv mRNA in neonatal SCG neurons typically followed two different patterns. Surprisingly, the majority of voltage-gated potassium channel mRNAs, Kv1.4, Kv2.1, Kv2.2, Kv3.1 and Kv3.3, were expressed at relatively constant levels during development, whereas Kv3.4 and Kv4.2 underwent developmental changes in mRNA expression levels. Possibly, the transcriptional regulation of additional Kv genes could be involved in the development regulation of SCG voltage-gated potassium currents. Alternatively, developmental changes in potassium currents may reflect a post-transcriptional mechanism such as translational control. Such an occurrence was reported for Kv2.1 in PC12 cells. NGF treatment of PC12 cells leads to an increase in the expression of the Kv2.1 peptide without an increase in the expression of mRNA (Sharma *et al.*, 1993). Moreover, this threefold increase is detectable 12 hours after NGF treatment.

Another possibility is that the composition of channels in the SCG cell membrane remains constant through development and the activity of these channels is modified to

produce the changes in current density. For example, Kv3.4 channels change from rapidly inactivating channels to slowly inactivating channels through oxidation or phosphorylation (see section **1.2.10**). As Kv3.4 mRNA is expressed at high levels through development, this type of modulation of Kv3.4 channels could explain the decrease in expression of IAs and the increase in expression of IAf. Though this does not explain the observed difference between Kv3.4 channels expressed *in vitro* and SCG IAf (see section **3.4.1**).

In the remaining sections of this chapter, I discuss how Kv mRNA levels may contribute to changes in the current densities of IAf, IAs and IK during neonatal SCG neuronal development (**4.4.1**), development of P1 SCG neurons in culture (**4.4.2**), development of P14 SCG neurons in culture (**4.4.3**), and P1 SCG neurons during development in culture with CNTF (**4.4.4**).

4.4.1 Normal SCG neuronal development

4.4.1.1 Developmental changes in IAf *in vivo*

IAf is first detectable prenatally on E18 SCG neurons. By P1, IAf is present at moderate levels (~25 pA/pF) and increases in expression level threefold over the first two postnatal weeks and remains high into adulthood, as described by McFarlane and Cooper (1992). I described how the voltage-sensitive properties and pharmacological properties of SCG IAf closely resemble Kv4.2 expressed *in vitro* (see section **3.4.1**). I found that

Kv4.2 mRNA was expressed at very low levels at E18 and increased to greater than thirty-fivefold by P7. This increase in Kv4.2 mRNA expression parallels the developmental increase in IAf density, but in contrast to the sustained expression of IAf, Kv4.2 mRNA expression decreased after P7. There are different explanations that may account for the Kv4.2 mRNA expression pattern. It is possible that during growth, SCG neurons express high levels of Kv4.2 mRNA to increase IAf current density as their surface area is expanding. Yet, when IAf reaches high density, only a lower level of Kv4.2 mRNA expression is necessary to maintain it.

Alternatively, Kv4.1 and perhaps Kv4.3 are expressed by SCG neurons, though the expression level of these transcripts is unknown (see section **3.4.1**). Possibly, the Kv4.2 subunit is the predominant contributor to IAf early in development, but Kv4.1 or Kv4.3 subunits make a greater contribution at later times either as homomeric or heteromeric channels. If changes in the subunit composition of IAf channels occur over development, then this may account for the 6 mV positive shift in the voltage-sensitivity of activation of IAf channels (McFarlane and Cooper, 1992). In addition, low molecular weight mRNA, which is believed to encode ß-subunits, has been shown to shift the voltage-sensitive properties of Kv4 channels (Serodio et al., 1997). Perhaps the action of ß-subunits shift IAf voltage-sensitivity over development. IAf has similar voltage-sensitive and pharmacological properties as Kv1.4 (see section **3.4.1**). However, Kv1.4 is expressed at very low level through development so that it is unlikely that Kv1.4 makes a large contribution to IAf (see section **3.4.1**).

The voltage-sensitive and pharmacological properties of IAf are different from Kv3.4 (see section **3.4.1**). However, the *in vivo* expression pattern of Kv3.4 mRNA does closely resemble the changes in current density of IAf. Both IAf and Kv3.4 mRNA are present at E18, increase through early neonatal life and continue to be expressed at high and moderate levels respectively. Therefore, it is not clear if Kv3.4 contributes to IAf.

4.4.1.2 Developmental changes in IAs *in vivo*

Prenatally, we are uncertain of the expression level of IAs, however, Nerbonne and coworkers (1989) have reported the presence of a delayed rectifier current at E15 that shares similar properties with IAs and IK (Cooper and McFarlane, 1992). More recent studies show that the current originally described as the delayed rectifier consists of two distinct currents, a delayed rectifier current and a slowly inactivating transient current, so that it is not clear what the proportion of the prenatal current is IAs versus IK.

As IAs is present at high current density (~70 pA/pF) at P1 and decreases by 50% to moderate levels over the first two weeks of postnatal life, we would presume that the mRNA encoding IAs is expressed before birth. When Kv3.4 is expressed *in vitro*, Kv3.4 channels have similar voltage-sensitive properties as IAs and may contribute to IAs as a homomeric channel or through combining with Kv3.1 or Kv3.3 subunits to form heteromeric channels (see section **3.4.2**). I found that at E18, Kv3.4 mRNA was expressed at moderate levels, and that it increases fourfold by P1. This pattern is consistent with Kv3.4 contributing to IAs, however, Kv3.4 mRNA remained expressed at

high levels where as the density of IAs decreases to moderate levels. Two other members of the Kv3 family, Kv3.1 and Kv3.3 were also expressed at relatively constant levels through development, at low and moderate levels respectively.

Over normal development between P1 and P14, there are shifts in the voltage-dependent properties of SCG IAs. The voltage-sensitivities for steady-state inactivation and activation become 16 mV and 5 mV more positive respectively (McFarlane and Cooper, 1992). These changes could reflect changes in the subunit stoichiometry or modulation of existing channels. For example, three out of four Kv3 subfamily mRNAs were expressed, Kv3.4 at high levels and Kv3.1 and Kv3.3 at much lower levels. As the expression level of Kv3.4 mRNA increases greater than sixfold during development, and the expression of Kv3.1 and Kv3.3 remains constant, the ratio of Kv3.4 expression to the expression of other Kv3 subunits changes from ~1:1 to >10:1. There may not be a directly linear relationship between mRNA expression and protein expression in the membrane, however, the stoichiometry of Kv3 channels could shift from heteromeric three Kv3.4 and one Kv3.1 or Kv3.3 to predominantly homomeric Kv3.4 channels.

Both Kv2.1 and Kv2.2 when expressed *in vitro* give rise to currents with similar properties as IAs (see section **3.4.2**). However, unlike IAs that decreases in current density through postnatal development, both Kv2.1 and Kv2.2 mRNA were expressed at relatively constant levels through neonatal life. At E18 Kv2.1 mRNA was expressed at moderate levels and remains so through development, while at E18, Kv2.2 mRNA was expressed at lower levels and remained so over development. Either of these genes could

contribute to IAs either as homomeric channels or through the formation of heteromeric channels.

4.4.1.3 Developmental changes in IK and IM *in vivo*

Every Kv gene expressed *in vitro* yields a current with rapid or slow activation, unlike SCG IK that shows no inactivation even over very long voltage-steps (see section **3.4.3**). However, like the SCG IK, the Kv mRNAs which encode "delayed rectifier" type channels, Kv2.1, Kv2.2, Kv3.1, and Kv3.3, were expressed at low to moderate levels and did not significantly change in expression level over development. There is no research on the developmental expression of the M-current, only that it is expressed at very low levels in adult SCG neurons (Sacchi and Belluzzi, 1990). Furthermore, it does not seem that IM is encoded by Kv channels (see section **1.5.3**).

4.4.2 P1 SCG *in vitro*

4.4.2.1 Developmental changes in IAf in cultured P1 neurons

When P1 SCG neurons are grown in culture the transient currents IAf and IAs decrease to very low levels while IK increases fivefold to become the predominant potassium current (McFarlane and Cooper, 1992). Similar to what was observed *in vivo*, the mRNA expression levels of the majority of potassium channel genes, Kv1.4, Kv2.1,

Kv2.2, Kv3.1 and Kv3.3, remained constant over time in culture, whereas Kv3.4 and Kv4.2 underwent developmental changes.

The IAf current density rapidly decreases to very low levels on cultured P1 SCG neurons. If a decrease in mRNA expression is responsible for this decrease in current density, then we would expect a decrease in expression of the mRNAs which contribute to IAf. Of the Kv genes expressed in SCG neurons, only Kv4.2 mRNA showed a significant drop in expression over time in culture. Kv4.2 mRNA was initially expressed at moderate levels but Kv4.2 expression dropped to virtually undetectable levels over the first week *in vitro* and remained low though out time in culture. This is consistent with Kv4.2 channels contributing to IAf. Kv1.4 was expressed at very low levels through time in culture, and as such likely does not make a major contribution to IAf. The Kv3.4 channel shares some similar properties with IAf (see section **3.4.1**), however, Kv3.4 mRNA expression undergoes a different developmental pattern *in vitro* than does IAf. Where IAf decreases to low levels *in vitro*, Kv3.4 mRNA remains constant for two weeks before increasing. The change in expression level of Kv3.4 mRNA was not consistent with changes in the expression of IAf.

4.4.2.2 Developmental changes in IAs in cultured P1 neurons

The IAs current density decreases to low levels in culture as does IAf. Kv3.4, which has similar properties to IAs, continued to be expressed at high levels in culture before increasing to similar levels as *in vivo*. The developmental pattern of Kv3.4 is

curious as Kv3.4 encodes a transient current, while the only current that increases in density *in vitro* is the delayed rectifier current. The expression levels of the other members of the Kv3 subfamily remained constant. If the Kv3 subfamily encodes IAs, then it does not appear that the decrease in IAs current density is due to changes in mRNA levels. Kv2.1 and Kv2.2, which also share similar properties with IAs, remain expressed at moderate and low levels respectively, through *in vitro* development. If Kv2 genes contribute to IAs (see section **3.4.2**), then the developmental increase in IAs density is not through an increase in the levels of these mRNAs.

4.4.2.3 Developmental changes in IK in cultured P1 neurons

The IK current density increases fivefold in culture. If an increase in mRNA levels contribute to this increase in current density, then we would expect the genes responsible for this change to be developmentally upregulated *in vitro*. However, the expression level of Kv1.4, Kv2.1, Kv2.2, Kv3.1 and Kv3.3 mRNA remained constant, Kv4.2 mRNA expression decreased and only Kv3.4 mRNA levels increased after two weeks in culture. Furthermore, none of the Kv genes give rise to a current similar to IK when expressed *in vitro* (see section **3.4.3**).

4.4.3 P14 SCG *in vitro*

4.4.3.1 Developmental changes in IAf in cultured P14 neurons

If P14 neurons are grown in culture, then IK increases and both IAf and IAs decrease to moderate current densities over time. I observed *in vivo* and in cultured P1 neurons that the majority of Kv mRNAs were expressed at relatively constant levels, while Kv4.2 mRNAs expression decreased to low levels. Since IAf decreases in current density on P14 SCG neurons grown *in vitro*, then we would predict a decrease in the mRNA expression of the Kv genes which encode this current. Kv4.2 mRNA expression does decrease in cultured P14 SCG neurons, though not to the low levels of cultured P1 neurons. As observed in other conditions, Kv1.4 mRNA expression remains very low in P14 neurons in culture. Even though Kv1.4 shares common properties with IAf (see section **3.4.1**), it is unlikely that Kv1.4 makes a substantial contribution to IAf on SCG neurons. Kv3.4 mRNA remains expressed at high levels whereas IAf decreases to moderate levels. So it is unlikely that changes in Kv3.4 mRNA expression cause the developmental decrease of IAf observed in P14 neurons grown in culture.

4.4.3.2 Developmental changes in IAs in cultured P14 neurons

As IAs density decreases to low levels on P14 SCG neurons grown in culture, if expression of IAs channels are due to changes in mRNA levels, then we predict that the Kv mRNAs which encode IAs decrease in expression level over development in culture.

Kv3.4 mRNA expression remained high on cultured P14 SCG. Kv3.1 and Kv3.3 mRNA expression remained at low levels in culture. The developmental expression of Kv3 mRNA does not explain the changes in IAs current density. Kv2.1 and Kv2.2 channels share similar properties with IAs (see section **3.4.2**), but Kv2.1 and Kv2.2 mRNAs are expressed at moderate and low levels respectively through time in culture. Though the Kv2 channels may contribute to IAs, they do not contribute to changes in IAs through alterations in their mRNA expression level.

4.4.3.3 Developmental changes in IK in cultured P14 neurons

The IK current density increases markedly in culture. If changes in mRNA levels contribute to this increase in current density, then we would expect an increase in the expression level of the corresponding mRNA. However, the expression level of Kv1.4, Kv2.1, Kv2.2, Kv3.1 and Kv3.3 mRNA remained constant, Kv4.2 mRNA expression decreased and only Kv3.4 mRNA levels increased after two weeks in culture. Furthermore, none of the Kv genes give rise to a current similar to IK when expressed *in vitro* (see section **3.4.3**).

4.4.4 Influence of non-neuronal cells on the expression of voltage-gated potassium currents

Non-neuronal cells influence the expression of voltage-gated potassium currents on different types of neurons. I described the influence of non-neuronal cells on the expression of potassium currents by SCG neurons and hippocampal neurons in previous sections (see sections **1.3.8** and **1.2.7**). Non-neuronal cells also influence the expression of potassium currents in chick parasympathetic neurons of the ciliary ganglia (Dourado and Dryer, 1992) and chick lumbar sympathetic neurons (Raucher and Drier, 1994).

By E13, ciliary neurons develop a large transient current, with fast and slow components of inactivation, and a delayed-rectifier current. If these neurons are instead grown in culture for the last four days, then the delayed-rectifier current is present but the transient current is absent, suggesting that the development of the transient current depends upon some extrinsic influence. The missing factor seems to be soluble in nature (Dourado and Dryer, 1994) as coculturing with other cells such as striated muscle cells, extracts of chick brain or acidic fibroblast growth factor (FGF) increased expression of the transient current, though the current observed demonstrated only rapid inactivation. However, co-culturing ciliary neurons on either lysed muscle cells or lysed fibroblasts was not sufficient to enhance transient current amplitude.

Cell contact regulates the expression of transient currents in chick lumbar sympathetic neurons. E9 neurons that have been grown in culture for 4 days (C4) and do not contact other cells have low to undetectable expression of transient currents, while

E9+C4 in contact with either satellite cells or other neurons have current densities similar to E13 neurons. The type of cell contacted seemed to make little difference, as neurons in contact with either ventral spinal cord explants, cardiac myocytes or aortic smooth muscle have similar current densities, all lacking the rapidly inactivating current. However, unlike E13 neurons, E9+C4 neurons express entirely slowly inactivating current (Raucher and Drier, 1994). This effect appears to be specific to cell contact, as conditioned media from either ventral spinal cord explants, cardiac myocytes or aortic smooth muscle had no effect on current-density. Contacted cells did not have to be alive, as cells grown on remnants of sympathetic neurons also expressed high levels of rapidly inactivating current (Raucher and Drier, 1994).

In SCG neurons, hippocampal neurons, ciliary neurons and chick lumbar sympathetic neurons, removing non-neuronal cells has the effect of slowing the inactivation rate of the voltage-gated potassium currents expressed. In each case, non-neuronal cells promote the expression of rapidly inactivating transient currents.

4.4.4.1 P1 SCG neurons cultured in CNTF

The neuropoetic cytokine, ciliary neurotrophic factor (CNTF), maintains the *in vitro* expression of potassium currents by P1 sympathetic neurons, just as does conditioned media from non-neuronal cells (McFarlane and Cooper, 1993). Ciliary neurotrophic factor belongs to a large family of diffusible proteins called cytokines which

are known to influence neuronal growth and differentiation. CNTF was initially isolated from chick eye extracts and characterized as being capable of supporting the survival of parasympathetic neurons of the chick ciliary ganglion (Barbin *et al.*, 1984). When P1 SCG neurons were grown in culture with CNTF, there was a reduction in the decrease in expression of A-currents and a reduced increase in IK compared to normal culture conditions. Furthermore, CNTF selectively increases the delayed rectifier current in SK-S-SH cells, which are neuroblastoma cells of sympathetic origin (Lesser and Lo, 1995).

The expression of Kv genes in P1 SCG neurons grown in culture with CNTF for three weeks are similar to control cultures in that the expression levels of most Kv genes do not change. The only exception is Kv3.4, which was expressed at higher levels by SCG neurons grown in CNTF. It is possible that elevated Kv3.4 mRNA expression contributes to the maintenance of IAs density.

CNTF is expressed by Schwann cells of the peripheral nervous system and astrocytes of the CNS (Sendtner *et al.*, 1991). An odd characteristic of CNTF is that it has no signal sequence for secretion, thus it is likely that neurons would encounter this factor in the event of rupture of the supporting cells, as may occur in injury. However, there are other closely related neuropoeitins which may be secreted, bear close functional and structural homology and signal through common receptors. It is not clear how exactly CNTF exerts its influence on the expression of A-currents or how it selectively increases gene expression. CNTF binds to a receptor complex containing LIF receptor beta, gp130 and CNTF receptor alpha (Ip *et al.*, 1992, 1993), which signals through Jak/Stat kinases.

NGF, another growth factor, has been shown to induce transcriptional changes in PC12 cells through phosphorylation of transcription elongation factor eIF-4E (Green and Tischler, 1976; Garnels and Shubert, 1979). It is possible that CNTF induces transcription of Kv3.4 through a similar mechanism.

Chapter 5:

The Expression of Kv Genes by Rat Sensory Neurons

5.1 Introduction

Individual populations of neurons express ion channels and receptors that are appropriate for their specific physiological roles. Sympathetic neurons of the SCG ganglia transmit motor commands from the CNS to target tissues including the iris, glands and vasculature (see section **1.3**). These neurons express four well characterized voltage-gated potassium currents: a rapidly inactivating transient current (IAf), a slowly inactivating transient current (IAs), a delayed-rectifier current (IK) and a slowly activating current blockable by acetylcholine (IM) (see sections **1.3.1-1.3.4**). Furthermore, these voltage-gated potassium currents are uniformly expressed on SCG neurons and undergo marked developmental changes in density, which are dependent on the presence of extrinsic factors (see sections **1.3.5-1.3.8**).

To better understand how voltage-gated potassium channel genes contribute to physiological currents on native cells, I identified the voltage-gated potassium channel genes which sympathetic neurons express. In chapter three, I showed that SCG neurons express at least seven different voltage-gated potassium channel mRNAs. These seven mRNAs represent all four voltage-gated potassium channel gene subfamilies and include

three distinct Kv genes (Kv1.4, Kv3.4 and Kv4.2) that encode rapidly inactivating voltage-gated potassium channels when expressed in heterologous expression systems.

Sympathetic neurons of the SCG and sensory neurons of the nodose and trigeminal ganglia are among the neurons of the peripheral nervous system, yet, these different types of neurons serve distinct physiological roles. While SCG neurons receive extensive innervation on their dendrites and make noradernergic synapses onto sympathetic targets, sensory neurons lack dendrites and extend a single axonal process with two branches. Nodose neurons project peripherally in the vagus nerve to targets including the heart, lungs, gastrointestinal tract and vasculature, and centrally to the tractus solitarus in the medulla (Zhuo *et al.*, 1997). Trigeminal neurons project in the fifth cranial nerve, providing the sensory innervation to the face including pain innervation to the teeth, and trigeminal neurons project centrally to the trigeminal sensory nuclei of the brain stem (Barlow and Mollon, 1987).

Sensory neurons like sympathetic neurons express a rapidly inactivating transient current (IAf), a slowly inactivating transient current (IAs) and a delayed-rectifier current (IK) (McFarlane and Cooper, 1991). These currents are similar in voltage-sensitive properties to those of SCG neurons, yet, voltage-gated potassium currents are not uniformly expressed on nodose neurons nor developmentally regulated (McFarlane and Cooper, 1991; Stansfeld *et al.*, 1986) and trigeminal voltage-gated potassium currents are poorly characterized (Spigelman and Puil, 1989; McFarlane PhD. thesis, 1992). In this chapter, my aim was to determine if sensory neurons of the nodose and trigeminal ganglia

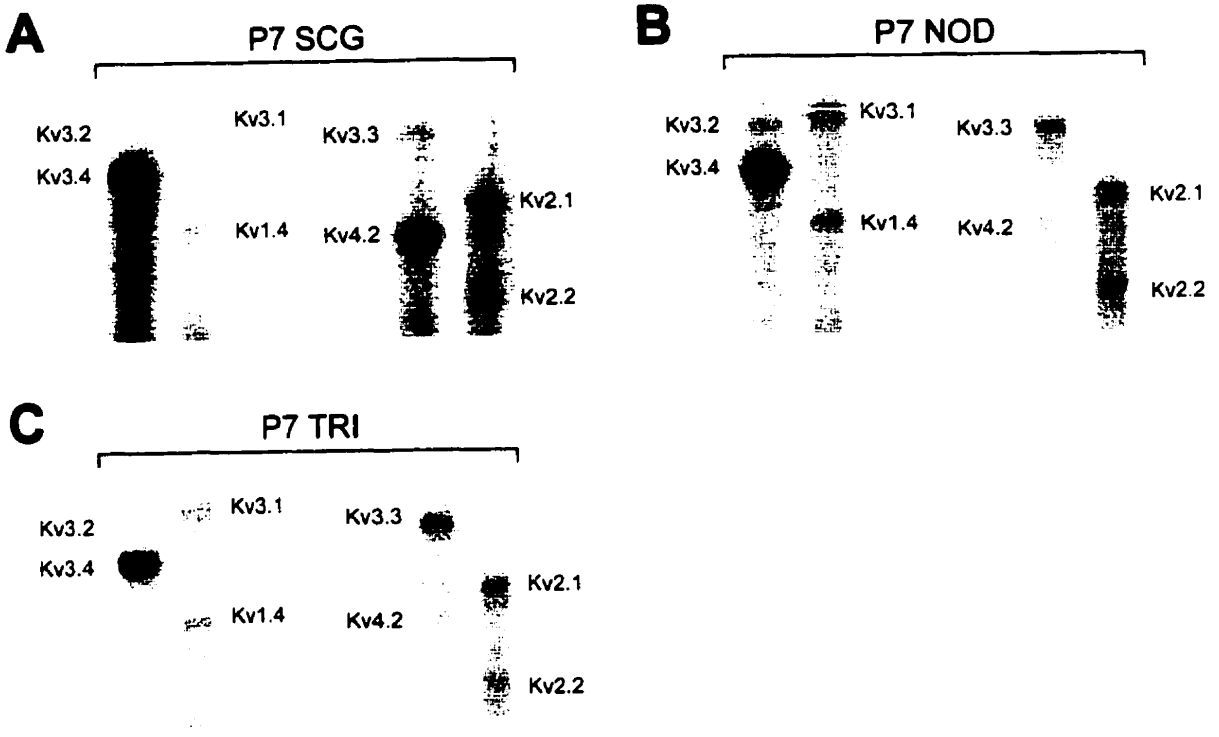

Figure 5.1 RNase protection assays showing Kv mRNA expression in P7 superior cervical, nodose and trigeminal ganglia.

RNase protection assay for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 mRNAs. A 2 μg sample of P7 (**A**) SCG, (**B**) nodose ganglia or (**C**) trigeminal ganglia total cellular RNA was protected in each lane.

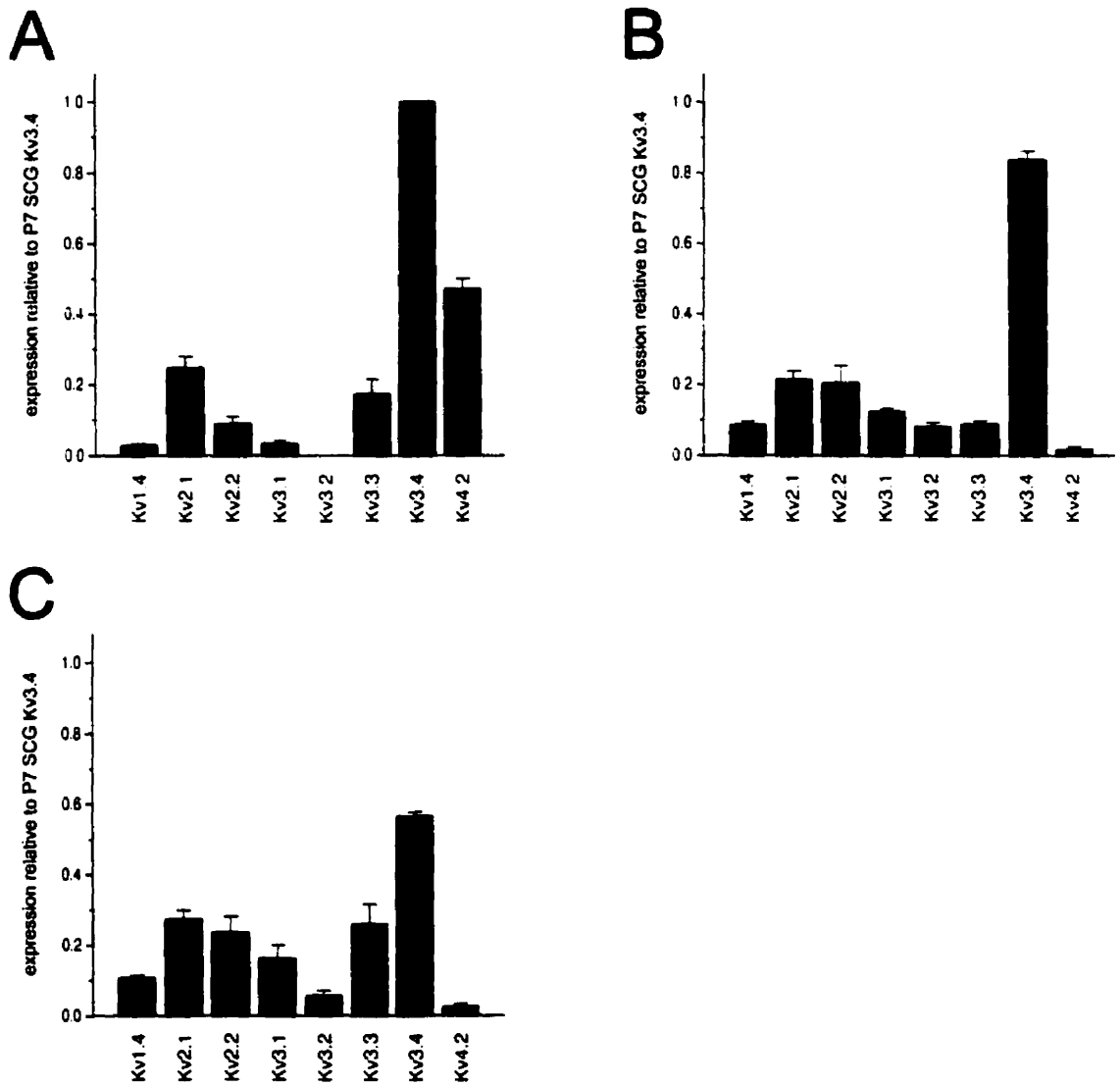

Figure 5.2 Quantification of Kv mRNA expression in P7 superior cervical, nodose and trigeminal ganglia.

The means (± SEM) of mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 are plotted relative to P7 SCG Kv3.4 as a histogram. Each mean reflects at least three separate experiments. The values reflect signals from (**A**) P7 SCG RNA, (**B**) P7 nodose ganglia RNA and (**C**) P7 trigeminal ganglia RNA. Individual signals were measured using a phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of P7 SCG Kv3.4 mRNA.

express the same Kv genes as do neurons of the superior cervical ganglia. For these studies, I used RNase protection assay to quantify the expression of voltage-gated potassium channel RNA in nodose and trigeminal total cellular RNA. These results were previously reported in abstract form: Fraser and Cooper, Society for Neuroscience abstracts, 1994.

5.2 Methods

5.2.1 RNase protection assay

The RNA was extracted from P7 nodose, trigeminal and SC ganglia (see section **2.1.1**). The radiolabelled cRNA probes were transcribed, purified and quantified (see section **2.2.5**). The RNA and cRNA probes were hybridized (see section **2.2.5**). The single stranded RNA and cRNA probe were digested (see section **2.2.5**). The cRNA probe signal was detected (see section **2.2.6**).

5.3 Results

I used RNase protection assay to measure Kv mRNA expression in nodose and trigeminal neurons, all eight Kv transcripts were detectable in both neuronal populations. Most transcripts were expressed at similar levels in sensory and sympathetic neurons, however, significant differences in mRNA expression existed. Figure 5.1 shows RNAse

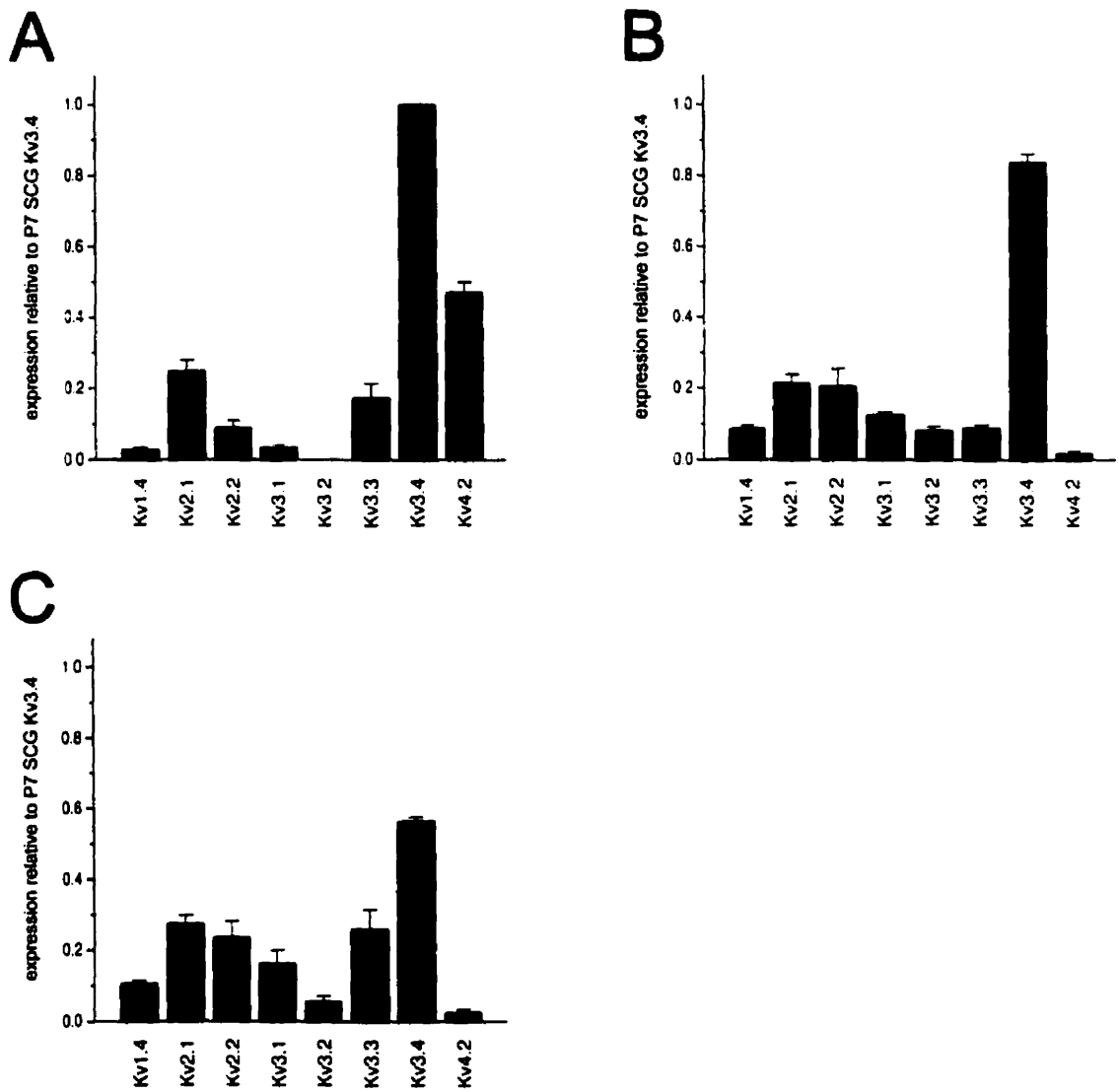

Figure 5.2 Quantification of Kv mRNA expression in P7 superior cervical, nodose and trigeminal ganglia.

The means (± SEM) of mRNA levels for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 are plotted relative to P7 SCG Kv3.4 as a histogram. Each mean reflects at least three separate experiments. The values reflect signals from (**A**) P7 SCG RNA, (**B**) P7 nodose ganglia RNA and (**C**) P7 trigeminal ganglia RNA. Individual signals were measured using a phosphorus imaging system, normalized by dividing by the GAPDH signal and plotted relative to the level of P7 SCG Kv3.4 mRNA.

protection assays for Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3, Kv3.4 and Kv4.2 mRNA levels in P7 superior cervical, nodose and trigeminal ganglia. Figure 5.2 is the phosphorus imager quantification showing the means and SEM of the relative expression levels for the individual Kv transcripts.

5.3.1 Expression of Kv3 mRNA by P7 SCG, nodose and trigeminal neurons

Kv3.4 mRNA was expressed at high, but significantly lower levels in nodose (86.9% P7 SCG Kv3.4) and trigeminal neurons (56.5% P7 SCG Kv3.4). The three other Kv3 mRNAs were detectable in both nodose and trigeminal ganglia. Kv3.1 mRNA was expressed at very low levels in SCG (3.5% P7 SCG Kv3.4), and low levels in nodose (12.5% P7 SCG Kv3.4) and trigeminal ganglia (16.3% P7 SCG Kv3.4). Kv3.2 mRNA, which was not detectable in SCG, was detectable at low levels in nodose (8.3% P7 SCG Kv3.4) and in trigeminal (5.8% P7 SCG Kv3.4). Kv3.3 mRNA was expressed at low to moderate levels in SCG (17.4% P7 SCG Kv3.4), nodose (8.9% P7 SCG Kv3.4) and in trigeminal (26.0 % P7 SCG Kv3.4).

Table 5.1 Summary table of SCG, nodose and trigeminal neuron voltage-gated potassium channel properties and Kv channel properties.

Channel	Voltage-Sensitivity of Activation	Rate of Activation	Voltage-Sensitivity of Inactivation	Rate of Inactivation	Block by 4-AP	Block by TEA	Single Channel Conductance
SCG IAf	half activated at -2 mV	activates rapidly	half inact. at -65 mV	inactivates rapidly	half blocked by 1 mM	not blocked by 10 mM	not known
SCG IAs	half activated at 10 mV	activates rapidly	half inact. at -40 mV	inactivates slowly	not known	not known	not known
SCG IK	half activated at 22 mV	activates slowly	does not inactivate	does not inactivate	not known	not known	not known
SCG IM	activates above -60mV	activates slowly	does not inactivate	does not inactivate	not known	not blocked by 10 mM	not known
Nod IAf	half activated at -21 mV	activates rapidly	half inact. at -73 mV	inactivates rapidly	half blocked by 1 mM	not blocked by 10 mM	22 pS
Nod IAs	half activated at -2 mV	activates rapidly	half inact. at -51 mV	inactivates slowly	half blocked by 2 mM	half blocked by 10 mM	22 pS
Nod IK	half activated at 16 mV	activates slowly	does not inactivate	does not inactivate	not blocked by 5 mM	half blocked by 10 mM	not known
Tri IAf	not known	activates rapidly	not known	inactivates rapidly	not known	not known	not known
Tri IAs	not known	activates rapidly	not known	inactivates slowly	not known	not known	not known
Tri IK	not known	activates slowly	not known	does not inactivate	not known	not known	not known
Kv1.1	half activated at -30 mV	activates rapidly	half inact. at -47 mV	inactivates slowly	half blocked by 0.3 mM	half blocked by 0.3 mM	10 pS
Kv1.2	half activated at 27 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.6 mM	half blocked by 0.6 mM	18 pS
Kv1.3	half activated at -10 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.4 mM	not blocked by 10 mM	14 pS
Kv1.4	half activated at -22 mV	activates rapidly	half inact. at -74 mV	inactivates rapidly	half blocked by 12.5 mM	not blocked by 10 mM	8 pS
Kv1.5	half activated at 0 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.3 mM	half blocked by 0.3 mM	10 pS
Kv1.6	half activated at -15 mV	activates rapidly	not known	inactivates slowly	half blocked by 0.3 mM	half blocked by 4 mM	not known
Kv2.1	half activated at 0 mV	activates rapidly	half inact. at -35 mV	inactivates slowly	half blocked by 0.5 mM	half blocked by 10 mM	15 pS
Kv2.2	not known	activates rapidly	not known	inactivates slowly	not known	half blocked by 7.9 mM	13 pS
Kv3.1	half activated at 19 mV	activates rapidly	half inact. at 10 mV	inactivates slowly	half blocked by 0.1 mM	half blocked by 0.2 mM	11 pS
Kv3.2	half activated at 6 mV	activates rapidly	not known	inactivates slowly	not known	not known	14 pS
Kv3.3	half activated at 5 mV	activates rapidly	half inact. at 5 mV	inactivates moderately	half blocked by 1.2 mM	half blocked by 0.14 mM	not known
Kv3.4	half activated at 10 mV	activates rapidly	half inact. at -53 mV	inactivates variably	half blocked by 0.5 mM	half blocked by 0.5 mM	14 pS
Kv4.1	half activated at 10 mV	activates rapidly	half inact. at-65 mV	inactivates rapidly	half blocked by 5 mM	not blocked by 10 mM	not known
Kv4.2	half activated at -5 mV	activates rapidly	half inact. at -55 mV	inactivates rapidly	half blocked by 5 mM	not blocked by 10 mM	~4 pS
Kv4.3	half activated at -15 mV	activates rapidly	half inact. at -60 mV	inactivates rapidly	half blocked by 2 mM	not blocked by 10 mM	not known

(rapid activation occurs over milliseconds; slow activation occurs over tens of milliseconds; rapid inactivation occurs over tens of milliseconds; moderate inactivation occurs over hundreds of milliseconds; slow inactivation occurs over seconds; the variable inactivation of Kv3.4 channels occurs over tens of milliseconds to seconds; adapted from: Yokayama *et al.*, 1989; Luneau *et al.*, 1991; Rettig *et al.*, 1992; Vega-Saenz de Miera *et al.*, 1992; Covarrubias *et al.*, 1994; Swanson *et al.*, 1990; Duprat *et al.*, 1995; Stuhmer *et al.*, 1989; Pak *et al.*, 1991; Baldwin *et al.*, 1991; Serodio *et al.*, 1996; Taglialatela *et al.*, 1992; Hwang *et al.*,1992; Belluzzi and Sacchi, 1991; McFarlane and Cooper, 1991, 1992; McFarlane PhD thesis, 1992; Grissmer *et al.*, 1994)

5.3.2 Expression of Kv2.1 and Kv2.2 mRNA by P7 SCG, nodose and trigeminal neurons

Kv2.1 mRNA was expressed at moderate levels in SCG (24.8 % P7 SCG Kv3.4), nodose (27.5% P7 SCG Kv3.4) and trigeminal (21.4% P7 SCG Kv3.4). Kv2.2 mRNA was expressed at low levels in SCG (10% P7 SCG Kv3.4), and moderate levels in nodose (24.8% P7 SCG Kv3.4) and trigeminal (27.5% P7 SCG Kv3.4).

5.3.3 Expression of Kv1.4 and Kv4.2 mRNA by P7 SCG, nodose and trigeminal neurons

Kv1.4 mRNA was expressed at very low levels in SCG (2.9 % P7 SCG Kv3.4), and at low but significantly higher levels in nodose neurons (8.6% P7 SCG Kv3.4) and trigeminal neurons (10.7% P7 SCG Kv3.4). In contrast to SCG neurons, which expressed high levels of Kv4.2 (47.2% P7 SCG Kv3.4) nodose, (1.8% P7 SCG Kv3.4), and trigeminal (2.6% P7 SCG Kv3.4) expressed significantly lower levels of Kv4.2 mRNA.

The differences in expression level of each Kv mRNAs between SCG, nodose and trigeminal neurons are more clear when they are plotted relative to the corresponding SCG transcript as in figure 5.3. Significant differences in Kv gene expression between SCG, nodose and trigeminal existed for Kv1.4, Kv2.2, Kv3.1, Kv3.2 and Kv4.2. I found that Kv1.4 mRNA was expressed threefold higher in nodose and trigeminal. Kv2.2 mRNA was expressed twofold higher in nodose and trigeminal. Kv3.1 mRNA was

expressed greater than threefold higher in nodose and trigeminal. Kv3.2 was not detectable in SCG but was present at low levels in nodose. Kv4.2 mRNA, which is present at moderate levels in SCG, was faintly detectable in nodose or trigeminal.

5.4 Discussion

In this chapter, I set out to determine if sensory neurons from the nodose and trigeminal ganglia express the same voltage-gated potassium channel mRNAs at similar levels as do SCG neurons. Sensory neurons express the seven voltage-gated potassium channel mRNAs, Kv1.4, Kv2.1, Kv2.2, Kv3.1, Kv3.3, Kv3.4 and Kv4.2, expressed by SCG neurons and additionally, Kv3.2 mRNA. I found that neurons of the nodose and trigeminal ganglia demonstrate similar expression levels of Kv mRNA as do SCG neurons. However, nodose and trigeminal neurons are more similar in Kv mRNA expression to each other than to sympathetic neurons.

5.4.1 Sensory neurons respond to diverse physiological stimuli

Sensory neurons are much less uniform in their expression of voltage-gated potassium currents than are sympathetic neurons (Cooper and Shrier, 1989; McFarlane and Cooper, 1991, 1992, 1993; Spigelman and Puil, 1989). This heterogeneity likely reflects the diversity of the individual types of "sensory units". A "sensory unit" is a term that is used to describe a sensory neuron together with the associated morphologically

distinct structure that combined with the peripheral nerve terminals, form the sensory transducing receptor. Sensory axons may be either myelinated or unmyelinated, there are three subdivisions of myelinated fibers based on size and conduction velocity, and different fiber types are associated with different sensory transducing structures. Furthermore, there are at least fifteen distinct types of sensory units that are able to detect information in different sensory modalities.

Cutaneous mechanoreceptors have high sensitivity to indentation or pressure on the skin, whereas visceral mechanoreceptors provide information about distention or stretch. Moreover, mechanoreceptors can be classified into two subpopulations depending on whether they adapt rapidly or slowly to stimuli. This rate of adaptation is likely to be determined by the repertoire of potassium channels expressed by specific sensory neurons. Thermoreceptors respond to either warm or cold stimuli, while nociceptors detect either painful mechanical or thermal stimuli. In addition, the trigeminal ganglia provides unique innervation to the tooth pulp which gives rise to the sensation of pure pain. Furthermore, visceral chemoreceptors include slowly adapting pH receptors and non-adapting glucoreceptors (Kandel *et al.*, 1991; Barlow and Mollon, 1987).

5.4.2 A rapidly inactivating transient current on SCG, nodose and trigeminal neurons

A rapidly inactivating transient current is present on a subset of both nodose (Bosso *et al.*, 1985; Stansfield *et al.*, 1986; Cooper and Shrier, 1985, 1989; McFarlane and Cooper, 1991) and trigeminal neurons (Puil and Spigelman, 1989). Yet, the trigeminal IAf is not characterized in terms of voltage-sensitive nor pharmacological properties. The nodose IAf activates rapidly, inactivates over tens of msec and requires hyperpolarization to remove inactivation (Cooper and Shrier, 1985, 1989; McFarlane and Cooper, 1991). Furthermore, nodose IAf has different voltage-sensitivity compared to SCG IAf: nodose IAf activates at potentials 20 mV more hyperpolarized than SCG IAf and inactivates 8 mV more hyperpolarized (McFarlane and Cooper, 1991, 1992). As a result, nodose IAf is almost completely steady-state inactivated near the resting membrane potential and requires hyperpolarization to remove this inactivation. This difference in voltage-sensitivity could reflect differences in the Kv genes that are expressed by sensory and sympathetic neurons or alternatively, differences in the modulation of the same types of channels (see sections **1.4.10** and **1.4.11**). The ensemble average of nodose IAf single channels activate 10-15 mV more positively than the macroscopic current, suggesting that there are different populations of ion channels that contribute to IAf (McFarlane and Cooper, 1992).

Three of the Kv mRNAs that I detected (Kv1.4, Kv3.4 and Kv4.2) encode rapidly inactivating transient currents when expressed *in vitro*. Nodose and trigeminal neurons

express low levels of Kv1.4 and very low levels of Kv4.2, unlike sympathetic neurons which express very low levels of Kv1.4 and high levels of Kv4.2. Kv3.4 is expressed at high levels in sensory neurons as is observed in SCG. In chapter three, I suggested that Kv4.2 contributes to IAf on SCG neurons, because the voltage-sensitive and pharmacological properties of Kv4.2 expressed in *Xenopus* oocyte closely resemble SCG IAf and because Kv4.2 mRNA is expressed at high levels in SCG. Similarites also exist between nodose and trigeminal IAf. Nodose IAf activates and inactivates on the same time scale as do Kv4.2 channels expressed in *Xenopus* oocyte and similarly is blocked by low concentrations of 4-AP but not by TEA. However, Kv4.2 channels show voltage-sensitivity for activation ~20 mV more positive and inactivation ~30 mV more positive than does nodose IAf and Kv4.2 single channel conductance is much smaller (~4 pS) than that of nodose IAf channels (22pS) (Baldwin *et al.*, 1991, McFarlane and Cooper, 1991). Other Kv4 channels, Kv4.1 and Kv4.3, which may be expressed in nodose neurons, also have similar voltage-sensitive and pharmacological properties as IAf (Serodio *et al.*, 1996). Low molecular weight mRNA which is thought to encode a ß-subunit can shift the voltage-sensitivity of Kv4 channels towards more hyperpolarized potentials (see section **1.4.11**). Through this mechanism Kv4 genes may give rise to the channels with the properties of the rapidly inactivating current on nodose neurons. IAf is found only on 60% of nodose neurons at low to moderate expression levels (McFarlane and Cooper, 1991). As sensory neurons are electrophysiologically heterogeneous, Kv4.2 mRNA may

only be expressed by a subpopulation of sensory neurons. Therefore, Kv4.2 may only contribute to nodose IAf on a subpopulation of cells.

Kv4.2 typically has a somatodendritic localization on mulipolar neurons such as hippocampal pyramidal neurons (see section **3.4.1**). I found that Kv4.2 is expressed at high levels by SCG neurons which are also multipolar in morphology (see section **3.4.1**). Both of these neuronal types receive extensive innervation on their dendrites, so that Kv4.2 likely plays a postsynaptic role in the integration of synaptic input. Nodose and trigeminal neurons, in comparison, are pseudo-unipolar in morphology, lack dendrites, and do not typically receive synaptic input on their cell bodies (Barlow and Mollon, 1987). Therefore, the role of Kv4.2 channels on nodose and trigeminal neurons is unclear.

In chapter three, I suggested that Kv1.4 may make a small contribution to SCG IAf, because the voltage-sensitive and pharmacological properties of Kv1.4 closely resemble those of SCG IAf, but Kv1.4 mRNA is expressed at very low levels in SCG neurons. Yet, Kv1.4 is expressed at significantly higher levels in nodose and trigeminal neurons. Kv1.4 encodes a rapidly inactivating transient current which activates and inactivates on the same scale as nodose and trigeminal IAf. Furthermore, Kv1.4 channels are similarly blockable by 4-AP and insensitive to block by TEA as is nodose IAf. Additionally, Kv1.4 channels have a similar voltage-sensitivity for activation as does nodose IAf, but Kv1.4 channels inactivate ~30 mV more positive and the single channel conductance of Kv1.4 (8 pS) is smaller than that of nodose IAf channels (22 pS). Perhaps, the voltage-sensitivity for inactivation of Kv1.4 is shifted negatively *in vivo* due to the

interaction with other proteins, as is believed to be the case for the Kv4 channels (see section **1.4.11**). The single channel conductance of channels formed by the Kv1.4 subunit on sensory neurons may be larger than that predicted from Kv1.4 single channel conductance due to interactions with other Kv1 subunits.

For rapid inactivation of a heteromeric channels to occur, only one inactivation ball for each channel is required (Ruppersburg, *et al.*, 1990). *In vivo* Kv1.4 and Kv1.2 channels are known to assemble as heteromers (Sheng, *et al.*, 1993). If other Kv1 channels are expressed by nodose or trigeminal neurons then possibly they contribute to nodose IAf through combining with ß-subunits. However, when other Kv1 channels associate with a ß1.1-subunit, they form rapidly inactivating channels with voltage-sensitivities ~50 mV more positive than nodose IAf (see section **1.4.11**).

In the brain, Kv1.4 channels have an axonal location. As nodose and trigeminal neurons extend an axon both peripherally and centrally, then Kv1.4 may be involved in the sensory coding of information. The terminals of some neurons act as rapidly adapting receptors, while others act as slowly adapting receptors (Paintal, 1973). Alternatively, I suggested that Kv1.4 channels could be involved in neurotransmitter release.

Kv3.4 also encodes a transient current that activates rapidly and inactivates over a range of rates (see section **3.4.1**). However, Kv3.4 channels activate ~35 mV more positive and inactivate ~45 mV more positive than IAf and are blockable by low concentrations of 4-AP and low concentrations of TEA, whereas nodose IAf is blockable by low concentrations of 4-AP but not by TEA. Given these differences in voltage-

sensitive and pharmacological properties, it is difficult to explain how Kv3.4 could contribute to IAf. Kv3.3 encodes a slowly inactivating transient current which activates and inactivates more slowly than does IAf; Kv3.3 activates ~25 mV more positive than IAf and inactivates ~75 mV more positive than IAf. Kv2.1, Kv2.2, Kv3.1 and Kv3.2 encode delayed rectifier currents which activate at more depolarized potentials than does IAf (Frech *et al.*, 1989; Ikeda *et al.*, 1992; Hwang *et al.*, 1992).

5.4.3 A slowly inactivating transient current on SCG, nodose and trigeminal neurons

Nodose and trigeminal neurons express a slowly inactivating transient current. Nodose IAs is similar to SCG IAs but the voltage-sensitivities are shifted ~10 mV more negative than IAs on SCG. IAs on trigeminal is not well characterized (McFarlane PhD. thesis, 1992). As sensory neurons are known to be more electrophysiologically heterogeneous than are sympathetic neurons, it is more likely that different subpopulations of neurons within the same ganglia express unique subsets of genes. Nodose IAs seems to be composed of at least two different populations of channels, as there are two components to IAs inactivation, a slowly inactivating component which inactivates over hundreds of msec and a very slowly inactivating component which inactivates over seconds (Cooper and Shrier, 1989). Additionally, there is a component of IAs that is not pharmalogically blockable by 4-AP. The slowly inactivating transient

current expressed by nodose neurons was originally believed to be a component of the rapidly inactivating current found on the same cells (Cooper and Shrier, 1985; Cooper and Shrier 1989). Nodose IAs activates over tens of msec and inactivates over hundreds of msec at potentials more positive than IAf.

When expressed in *Xenopus* oocytes, both Kv4.2 and Kv1.4 channels activate and inactivate more rapidly than IAs. Moreover, these channels activate and inactivate at voltages >20 mV more positive than IAs. In chapter three, I proposed that Kv3.4 contributes to IAs, perhaps through the formation of heteromeric channels. When Kv3.4 channels are expressed *in vitro*, they encode a current that activates and inactivates at similar rates and has similar voltage-sensitivities for activation and inactivation as does nodose IAs. Also, Kv3.4 channels have a similar single channel conductance as do nodose IAs channels. When expressed *in vitro*, Kv3.3 encodes a transient current that activates and inactivates more slowly than IAs, and Kv3.3 activates ~10 mV more positive and inactivates ~60 mV more positive than IAs. In addition, Kv3 channels are blockable by low concentrations of TEA and 4-AP and a large component of nodose IAs is blockable by low concentrations of TEA and 4-AP. This is good evidence that Kv3 channels contribute to nodose IAs. In the brain, Kv3.4 has an axonal localization (see section **3.4.1**). Therefore, the role of Kv3.4 in sensory neurons may be sensory transduction, peripherally, or control of presynaptic excitability, centrally.

In chapter three, I suggested that Kv2.1 and Kv2.2 contribute to IAs. Both Kv2.1 and Kv2.2 channels activate at depolarized potentials as does nodose IAs (Shi *et al.*,

1994). Kv2.1 has similar voltage-sensitivity to inactivation as nodose IAs and inactivates over seconds, similar to the slow time constant of IAs. Kv2.1 is blockable by low concentrations of 4-AP and high TEA, similar to nodose IAs. The voltage-sensitive and pharmacological properties of Kv2.2 are not well characterized, but Kv2.2 channels are blockable by high concentrations of TEA and do not show inactivation over short voltage-steps and have similar single channel conductance (15 pS) as does nodose IAs (22 pS). Given these similarities, Kv2 channels likely contribute to the slowly inactivating component of nodose IAs. In some neurons, Kv2 channels are localized to the cell bodies and somata. Kv2.1 staining is discrete and punctate on the cell bodies and extends into proximal dendrites, whereas Kv2.2 staining is faint and homogeneous on neuronal cell bodies (see section **3.4.2**). It is not clear what the functional role of these channels could be on nodose or trigeminal neurons if their expression is restricted to neuronal cell bodies.

5.4.4 A delayed-rectifier current on SCG, nodose and trigeminal neurons

The nodose delayed rectifier current activates slowly and does not inactivate even over very long voltage-steps. The voltage-sensitive properties of the nodose delayed rectifier current closely resemble those of the SCG delayed-rectifier. The slow activation of nodose IK at depolarized potentials occurs more slowly than that of Kv channels

expressed in heterologous expression systems. Furthermore, nodose and SCG IK do not inactivate even over very long voltage-steps. Trigeminal neurons also express a delayed-rectifier current similar to nodose IK. Yet, neither the voltage-sensitive nor pharmacological properties of the trigeminal current have been well characterized (McFarlane PhD. thesis, 1992). Every Kv gene that has been characterized *in vitro* undergoes slow inactivation (see section **1.2.8**). It is not likely that any of the Kv genes encode nodose IK.

Chapter 6:

General Discussion and Conclusions

6.1 Potassium currents expressed by peripheral neurons

Superior cervical ganglia neurons express three distinct voltage-gated potassium currents: a rapidly inactivating transient current (IAf), a slowly inactivating transient current (IAs) and a delayed-rectifier current (IK). *Xenopus* oocyte expression studies show that Kv1.4 and Kv4.2 channels have voltage-sensitive and pharmacological properties which resemble IAf, whereas Kv2.1, Kv2.2, Kv3.1, Kv3.3 and Kv3.4 channels more closely resemble IAs. *In vivo*, IAf density increases from moderate to high levels during the first two weeks of postnatal life. A thirty-fivefold neonatal increase in Kv4.2 mRNA levels may account for this regulation. Furthermore, IAf decreases to low levels in culture and Kv4.2 mRNA levels also undergo a similar decrease in expression level. Yet Kv 1.4 mRNA remained expressed at low levels *in vitro* and *in vivo*. Thus, it seems likely that regulation of Kv4.2 mRNA levels lead to changes in IAf current density. There is less direct correspondence between the regulation of IAs and the expression of Kv2.1, Kv2.2, Kv3.1, Kv3.3 or Kv3.4 mRNA levels. Where IAs decreases over *in vivo* and *in vitro* development, the expression of levels of Kv2.1, Kv2.2, Kv3.1, Kv3.3 and Kv3.4 mRNA either remain constant, or in the case of Kv3.4 mRNA undergo a developmental

increase. Similarly, the influence of CNTF on voltage-gated potassium channel mRNAs does not account for the persistence of A-currents on SCG neurons in culture. CNTF sustains the expression of IAf, but does not significantly influence the expression of Kv4.2 or Kv1.4. Furthermore, CNTF sustains the expression of IAs but does not influence the expression of Kv2.1, Kv2.2, Kv3.1 or Kv3.3 mRNA. Only Kv3.4 mRNA expression was significantly elevated relative to the corresponding *in vitro* level. It is not precisely clear how these Kv genes contribute to SCG IAf and IAs, but regulatory processes must be in place at the translational or post-translational levels because the expression of Kv mRNAs does not perfectly correspond to the changes in expression of SCG IAf and IAs. Additionally, as multiple subunits of the same family are coexpressed by SCG neurons it is likely that heteromeric channels are formed.

Similar to SCG neurons, nodose and trigeminal neurons also express three distinct voltage-gated potassium currents, IAf, IAs and IK. Furthermore, as in SCG neurons, Kv1.4 and Kv4.2 channels are similar in properties to nodose and trigeminal IAf, whereas the Kv2.1, Kv2.2, Kv3.1, Kv3.2, Kv3.3 and Kv3.4 channels more closely resemble nodose and trigeminal IAs. Unlike the SCG currents, the nodose and trigeminal currents are not uniformly expressed by individual neurons and it is not clear to what extent heterogeneity exists in the expression of Kv genes by sensory neurons. Moreover, nodose voltage-gated potassium currents are not developmentally regulated as are SCG currents, so that it is not clear how these different types of neurons express a similar repertoire of Kv genes, yet manifest different patterns of current expression.

6.2 Specific contribution of individual Kv genes

Even though I identified a number of Kv genes expressed by SCG neurons, it is not clear what specific contributions particular Kv subunits make to the individual currents. We do not know if IAf and IAs consist of a single population of homogenous channels or if distinct subsets of channels are involved. To address the above questions, we have chosen to use an adenovirus system as a way to manipulate voltage-gated potassium channel mRNA and protein expression in SCG neurons. Through using a virus containing either ß-galactosidase or green fluorescence protein, we can efficiently infect 80-90% of neurons in dissociated cell culture. Moreover, this system has been used successfully in our lab to over-express the $\alpha 3$ nAChR mRNA in SCG neurons. Alternatively, we could create transgenic mice to investigate the role of individual Kv genes.

6.2.1 Over-expression of Kv mRNAs

The first stage of our adenovirus studies is to over-express individual Kv genes in SCG neurons. This will enable us to examine the physiological properties of specific Kv genes expressed by real neurons, as it is not clear that the properties of Kv channels expressed in heterologous expression systems, such as *Xenopus* oocytes or cell lines, are identical to those observed in native cells, due to interactions with other proteins or

differences in post-translational processing. For example, to investigate whether Kv4.2 contributes to IAf, we plan to express Kv4.2 mRNA in cultured P1 SCG neurons. These neurons typically express very low levels of both IAf and Kv4.2 mRNA. Our prediction is that over-expression of Kv4.2 will increase IAf density on these neurons. Similarly, we plan to over-express Kv3.4, Kv2.1 and other Kv mRNAs to see if this leads to an increase in IAs expression. A drawback of this approach is that post-transcriptional regulation could prevent the expression of functional channels in neuronal membranes. Therefore, an effect on the membrane currents may not be observable.

6.2.2 Expression of antisense Kv mRNA

The next stage of the adenovirus project is to infect SCG neurons, with an antisense RNA producing virus, in an attempt to "knock-out" specific transcripts. The technique of antisense hybrid arrest involves destroying or otherwise preventing the translation of a specific mRNA sequence, by hybridizing it with the complementary sequence (Serodio *et al.*, 1994; Lotan, 1992; Dangle *et al.*, 1991; Prives and Foukal, 1991; Cazenave *et al.*, 1987; Rebagliati and Melton, 1987). In this way, we will be able to address the contribution of individual Kv genes to voltage-gated potassium currents on SCG neurons. For example, by using an adenovirus construct containing a Kv3.4 cDNA sequence in the reverse orientation, we will be able to selectively eliminate Kv3.4 mRNA and determine if there is a reduction in IAs. In addition to applying the adenovirus construct to "knock-out" gene expression *in vitro*, we will also be able to infect neurons

in the intact rat with virus and assess the phenotype of acutely isolated neurons. Furthermore, this approach will allow us to address the roles of auxiliary subunits and other regulatory proteins which are known to modulate Kv channel function or localization. Yet, the adenovirus approach is not perfect. The infection level may not be sufficient to produce enough antisense transcripts to eliminate all of the target mRNA. So, in the example of an antisense Kv3.4 containing virus, the number of Kv3.4 subunits produced by infected neurons may be unaffected or only partially reduced, therefore resulting in little or no reduction in the corresponding current. Furthermore, there is the danger that hybridization of the antisense transcript may be nonspecific due to the stretches of highly conserved residues in different Kv mRNAs. This could result in the nonspecific reduction of transcripts, and therefore a nonspecific decrease in currents. Additionally, the antisense approach may be limited by the rate of turnover of Kv channels in the cell membrane. If the duration of adenovirus infection is too short relative to the turnover of Kv channels, then an effect on currents may not be observable. Additionally, if targeting of the viral antisense RNA is to a different subcellular region than the native mRNA, then this would fail to inhibit the expression of current.

6.2.3 Dominant/negative expression of Kv subunits

An interesting property of Kv subunits is their tetrameric assembly in a subfamily specific manner (see section **1.4.9**). In SCG neurons, which express multiple Kv mRNAs of individual subfamilies, it is probable that some heteromeric voltage-gated potassium

channels exist, as has been reported in other systems (Shamtienko *et al.*, 1997; Wang *et al.*, 1993). Through infecting SCG neurons with a virus containing the truncated form of a Kv subunit, we should be able to "knock-out" at the protein level all Kv channels of a particular subfamily. This dominant/negative approach has been used successfully to investigate the contribution of individual Kv subunits in cerebellar granule cells (Johns *et al.*, 1997) and *Xenopus* spinal neurons (Ribera *et al.*, 1996). However, as with the antisense approach, an incomplete "knock-out" of a particular subunit may be difficult to interpret. Furthermore, as for the antisense approach, the success of the dominant/negative approach depends on the turnover rate of Kv channels in the cell membrane. If the duration of time that a Kv channel remains inserted in the cell membrane is very long relative to the duration of infection, then an effect may not be observable. Moreover, if the viral Kv subunits are targeted to a different region of the cell than are other members of the same Kv subfamily, then they would be ineffective in interacting with them and producing the dominant/negative effect.

6.2.4 Transgenic approaches

An alternative approach to adenovirus infection is the generation of transgenic animals. This transgenic approaches offers both benefits and disadvantages for investigating the contribution of individual genes to voltage-gated potassium current. Though traditional targeted gene disruption can completely abolish the expression of functional protein, there may be compensatory changes that occur during development

that affect the phenotype of the organism in unforeseen ways. A more elegant approach to address our questions would be to create a transgenic animal with an inducible "knock-out" using the Cre/loxP system (Akagi *et al.*, 1997; Khun *et al.*, 1997). Still, the transgenic approach is expensive. Additionally, as the work we have conducted was done in rat, then we would have to confirm our findings in the mouse before proceeding.

6.3 Specific Targeting of Kv channels

As discussed in chapters one, three and five, the subcellular localization of voltage-gated potassium channels is an important determinant of channel function. Yet, voltage-gated potassium channels were hard to localize until recently, due to the lack of subunit specific antibodies. The adenovirus system provides further utility, because expressed cDNAs can be designed to include an epitope-tag. This epitope can then be detected with an antibody to reveal the subcellular localization of proteins. In this way, we will be able to determine the subcellular localization of Kv4.2, Kv3.4, Kv2.1 and other Kv subunits. Furthermore, we will be able to assess if the subcellular localization of channels is conserved between sensory and sympathetic neurons or if intracellular pools of Kv channels exist.

Determining the subcellular localization of Kv channels is important to understanding the role of particular Kv subunits, but we will not fully understand the physiology of the Kv channels until we determine the subunit composition of particular channels. This determination can be accomplished through work with Kv specific

antibodies, which are becoming increasingly available. Co-localization of Kv subunits with antibodies can be accomplished through confocal microscopy and the exact subunit composition of Kv channels could be determined through immunoprecipitation. Yet, this remains to be a challenge for potassium channels that are expressed at low numbers, in regions such as presynaptic nerve terminals.

6.4 Regulation of voltage-gated potassium currents

6.4.1 Regulation of Kv mRNA levels

After we have determined how particular Kv subunits contribute to the individual potassium current on SCG neurons, we will be able to investigate better the question of developmental regulation of voltage-gated potassium currents: "Why does a neuron come to express its particular repertoire of currents?" For example, in the case of Kv4.2, mRNA levels could be responsible for the developmental changes in SCG IAf expression over normal development and in culture. Yet, the question remains, why do changes in Kv4.2 mRNA levels occur? The mRNA levels I detected through RNase protection assay reflect the regulation of different control points: transcriptional control, RNA processing control, RNA transport control and mRNA degradation control (Alberts *et al.*, 1994).

Control of the initiation of transcription is the predominant form of regulation for most genes. Gene transcription depends on the primary nucleotide regulatory sequences preceding and flanking the gene as well as the presence of regulatory proteins in the cell.

The initiation of transcription in eukaryotes requires the ordered assembly of transcription factors at the promoter site, which is located just upstream from the transcriptional start site. The assembly of transcription factors provide many steps which can provide positive or negative regulation.

The promoter region of the Kv3.1 gene has been investigated using the CAT reporter system (Gan *et al.*, 1996). In these studies, the 5' region upstream from Kv3.1 is attached to a CAT gene and CAT activity is measured. The Kv3.1 promoter region conveys neuron restrictive promoter activity, thereby preventing the expression of a Kv3.1 construct in NIH 3T3 cells. This activity may be due to a silencing element, which is located -743 to -717 bp 5' of the gene. A similar silencing element has been observed in the RNAII channel (Mau *et al.*, 1990). Furthermore, this promoter is regulated by cAMP levels as 1μM 8-bromo cAMP increases the expression threefold in 24 hours. This regulation occurs through CREB (cAMP response element binding protein) binding to CRE (cAMP response element). There also appears to be regulation due to intracellular calcium as the calcium ionophore, ionomycin can increase expression 50%.

6.4.2 Post-transcriptional regulation of Kv proteins

Following transcription, there are a number post-transcriptional mechanisms of regulation. Alternative splicing can produce different forms of the same protein. Voltage-gated potassium genes are each composed of a central exon containing the core region and other exons containing the amino and carboxyl-termini. Among the Kv channels, a

number of genes are alternatively spliced at the amino or carboxyl-terminals. In *Drosophila*, *Shaker* is extensively alternatively spliced, producing channels with distinct gating properties. In mammals, Kv3.1, Kv3.2, Kv3.3 and Kv4.2 are alternatively spliced though the observed differences in channel properties are subtle or not detectable (see section **1.4.3**).

RNA transport from the nucleus can be regulated. RNA is actively transported from the nucleus and requires a specific 5' nucleotide cap and a 3' poly-A tail, but before this process can occur, the mRNA must free itself from the splicosome components to which it is tethered. Therefore, any process that prevents the completion of splicing, could prevent RNA from exiting the nucleus (Izaurralde and Mattaj, 1992; Maquat, 1991). Moreover, the stability of mRNA transcripts can be changed. For example, RNA molecules are initially poly-adenylated in the nucleus, but begin to lose adenylation once they reach the cytosol. However, some mRNAs undergo selective poly-A addition or removal in the cytosol. The addition of poly-A greatly increases stability and translation (Wickens, 1990). Furthermore, many unstable mRNAs contain specific sequences that stimulate their degradation such as AU-rich sequences that stimulate the removal of the poly-A tail or repeat sequences that promote cleavage by specific endonucleases (Sach, 1993; Theil, 1990). Five such AU-rich sequences that are found in the 3' untranslated region of the longer of two forms of Kv1.4 mRNA (4.5 kbp and 3.5 kbp) are believed to facilitate degredation of this transcript. Moreover, these AU-rich regions reduce

translational efficiency, leading to a four to fivefold decrease in recorded current when the 4.5 kbp transcript is expressed in *Xenopus* oocyte (Wymore *et al.*, 1996).

6.4.3 Translational regulation

Surprisingly, five out of seven Kv mRNAs that I detected did not undergo significant changes in expression level. As sympathetic voltage-gated potassium currents are strongly developmentally regulated, then some type of control likely occurs at the translational level, during targeting or in the control of protein activity.

A number of different stimuli decrease protein synthesis through phosphorylation of the initiation factor eIF-2. This elongation factor is involved in the formation of the translation complex. Typically, eIF-2 forms a complex with GTP and mediates the binding of the methionyl initiator tRNA to the 40S ribosomal subunit which binds the 5' mRNA cap (Hinnebusch, 1990; Rhoads, 1993; Sarre, 1989). Additionally, the translation of some mRNA molecules is blocked by specific repressor proteins that bind near the 5' end of mRNA molecules (Melefors and Hentze, 1991).

Any attempt to understand the physiological contribution of Kv genes and gene products should consider the complexities involved in their regulation and their protein-protein interactions. Many distinct Kv genes are expressed by individual neurons and their regulation is controlled at different points. Furthermore, even though similar sets of genes are expressed by different populations of neurons, this does not necessarily mean that the different neurons have similar electrophysiological properties. Moreover,

voltage-gated potassium channel subunits interact with each other and heterologous proteins in a manner that affects the functional properties and localization of the channel that is formed. To understand the contribution of a single Kv gene, we will most likely be required to combine a variety of different cell and molecular biology approaches to address this complex question.

6.5 Relevence to disease

Elucidating the physiological roles of Kv genes is important to understanding the pathology of certain genetic diseases and developing therapeutic treatments, as mutations in ion channels may have dire consequences. This is observed in the genetic diseases involving mutations in voltage-gated potassium channel genes. Epilepsy (Biervert *et al.*, 1998), heart arrhythmias (Wang *et al.*, 1995, 1996; Curran *et al.*, 1995; Sanguinetti *et al.*, 1995) and motor disorders (Brown *et al.*, 1994) have each been linked to point mutations in voltage-gated potassium channel genes (for a review of genetic ion channel diseases, see Sanguinetti and Spector,1997; Akerman and Clapham, 1997). Understanding the physiological consequences of such mutations at the cellular and molecular levels will facilitate the development of treatments for these disorders.

Appendix I PCR primers used for cDNA amplification

Primer	5'->3' Sequence	Restriction Site	Target Sequence GB Accession #	Target Sequence
Kv2.1 sense	GCTCTGAATTCTTC GTGGAG	EcoRI GAATTC	X16476	1952-1970
Kv2.1 antisense	GCACGCTCTAGAG CAGCTGAC	Xba I TCTAGA	X16476	2295-2317
Kv2.2 sense	CCAGGATCCTTTG CAACCTGAC	BamHI GGATCC	M77482	2374-2396
Kv2.2 antisense	CTGACAGAATTCT GAAACATCAG	EcoRI GAATTC	M77482	2625-2647
Kv3.1 sense	ACCGAATTCGAGG GTGTCTGCGT	EcoRI GAATTC	X62840	1022-1044
Kv3.1 antisense	CGCGTCGACCAAC ATCCCAGACC	Sal I GTCGAC	X62840	1518-1540
Kv3.2 sense	GTGGAATTCTTTT GAATTTTTAGTC	EcoRI GAATTC	X62839	1062-1088
Kv3.2 antisense	CGCGTCGACCATC CCCTGACC	Sal I GTCGAC	X62839	1533-1552
Kv3.3 sense	TTTGAATTCCTCAT GCGCGTCACC	EcoRI GAATTC	M84210	1306-1329
Kv3.3 antisense	CGCGTCGACCAGC ATCCCAGACC	Sal I GTCGAC	M84210	1766-1778
Kv3.4 sense	ACCGAATTCGAGG GCGTGTGCGT	EcoRI GAATTC	X62841	1246-1268
Kv3.4 antisense	CGCGTCGACCAGC ATTCCTGACC	Sal I GTCGAC	X62841	1742-1764
Kv4.2 sense	CATAAGCTTTACG GTTGAGTAC	Hind III AAGCTT	S64320	1262-1283
Kv4.2 antisense	TTCTCTGTCTAGA ACATAACCG	Xba I TCTAGA	S64320	1570-1591

Appendix II RNase protection assay probe information.

Sequence	RNA polymerase	Length of Antisense Probe	Length Protected	#A in Probe Template
Kv1.4	T3	451	393	72
Kv2.1	T7	382	345	80
Kv2.2	T3	353	273	70
Kv3.1	T3	538	507	90
Kv3.2	T3	511	480	107
Kv3.3	T3	493	462	84
Kv3.4	T3	424	393	85
Kv4.2	T7	356	321	58

```
Kv1.4 g aat tct atg acc acc gtc ggt tat ggc gac atG AAG CCC ATC ACA GTG GGA GGA AAG ATT GTG GGG TCC CTG TGT GCC ATT GCG ggc gtc ctt atc atc gcc ctg cct cta ga
                          V   G   Y   A   D   M   Y   P   I   T   V   G   G   Y   I   V   G   S   L   C   A   I   A   G   V   L   I

Kv2.2 g aat tct atg acc acc gtg gga tat ggg gat atT TAC CCT AAA TCA CTG TTA GGG AAA ATT GTG GGT GGC CTT TGC TGC ATT GCA ggc gtc ctt acc atc gcc ctg cct cta ga
                          V   G   Y   G   D   I   Y   P   Y   S   L   L   G   Y   I   V   G   G   L   C   C   I   A   G   V   L   T

Kv3.1 g aat tct atg acc acc ctc ggc tac ggg gat atG TAT CCT CAG ACG TGG TCT GGG ATG TTG GTG GGA GCA CTG TGC GCC CTG GCT ggt gtt ctg acg atc gcc ctg cct cta ga
                          L   G   Y   G   D   M   Y   P   Q   T   W   S   G   M   L   V   G   A   L   C   A   L   A   G   V   L   T

Kv3.3 g aat tct act acc acc gtg gga tat gga gac atG TAC CCC AAG ACA TGG TCT GGG ATG CTG GTC GGG GCA GTG TGT GCC CTG GCT ggt gtg tct gtc atc gcc ctg cct cta ga
                          V   G   Y   G   B   M   Y   P   K   T   W   S   G   M   L   V   G   A   V   C   A   L   A   G   V   L   V

Kv3.4 g aat tct atg acc acc ctc ggg tat ggg gac atG TAT CCT AAG ACA TGG TCA GGA ATG CTG GTG GGT GCG CTG TGT GCA CTG GCT ggt gtg ctc gcc atc gcc ctg cct cta ga
                          L   G   Y   G   D   M   Y   P   K   T   W   S   G   M   L   V   G   A   L   C   A   L   A   G   V   L   A

Kv4.2 g aat tct atg acc acc gta ggc tac ggg gac atG GTA CGA AAA ACC ATA GCA GGG AAG ATT TTC GGG TCT ATC TGC TCA CTG AGC ggt gtg ctt acc atc gcc ctg cct cta ga
                          V   G   Y   G   D   M   V   R   K   T   I   A   G   K   I   F   G   S   I   C   S   L   S   G   V   L   T
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Appendix III Sequences of candidate Kv genes identified through PCR screening.

The cDNA sequences of PCR cloned Kv channel fragments are shown above. The primer region of the cDNA sequence is shown in lower case letters and the interprimer region is shown in upper case letters. Below each cDNA sequence is the predicted protein sequence.

Bibliography:

Adams, P.R., Brown, D.A., and Constanti, A. (1982). M-current and other potassium currents in bullfrog sympathetic neurons. J. Physiol. 330, 537-572.

Adelman, J.P., Shen, K.Z., Kavanaugh, M.P., Warren, R.A., Wu, Y.N., Lagrutta, A., Bond, C.T. and North, R.A. (1992). Calcium-activated potassium channels expressed from cloned complementary DNAs. Neuron 9, 209-216.

Aghajanian, G.K. (1985). Modulation of a transient outward current in serotonergic neurones by a_1-adrenoceptors. Nature 315, 501-503.

Aiyar, J., Withka, J.M., Rizzi, J.P., Singleton, D.H., Andrews, G.C., Lin, W., Boyd, J., Hanson, D.C., Simon, M., Dethlefs, B. and Chandy, K.G. (1995). Topology of the pore-region of a K^+ channel revealed by the NMR-derived structures of scorpion toxin. Neuron 15, 1169-1181.

Aiyar, J., Rizzi, J.P., Gutman, G.A., and Chandy, K.G. (1996). The signature sequence of voltage-gated potassium channels projects into the external vestibule. J. Biol. Chem. 271, 31013-31016.

Akabus, M.H., Stauffer, D.A., Xu, M. and Karlin, A. (1992). Acetylcholine receptor channel structure probed in cysteine-substitution mutants. Science 258, 307-310.

Akabus, M.H., Kaufmann, C., Archdeacon, P., and Karlin, A. (1994). Identification of acetylcholine receptor channel-lining residue in the entire M2 segment of the alpha subunit. Neuron 13, 919-927.

Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M. Berns, A. (1997). Cre-mediated somatic site-specific recombination in mice. Nucleic Acids Research 25, 1766-1773.

Akerman, M.J. and Clapham, D.E. (1997). Ion channels-basic science and clinical disease. New Engl. J. Med. 336, 1575-1586.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1994). Molecular biology of the cell. Garland Publishing, Inc. New York and London.

Alkon, D.L. (1984). Calcium-mediated reduction of ionic currents: A biophysical memory trace. Science 226, 1037-1045.

Antz, C., Geyer, M., Fakler, B. Schott, M.K., Guy, H.R., Frank, R., Ruppersburg and Kalbitzer, H.R. (1997). NMR structure of inactivation gates from mammalian voltage-dependent potassium channels. Nature 385, 272-275.

Atkins, P.T., Surmeier, J., and Kitai, S.T. (1990). Muscarinic modulation of a transient K^+ conductance in rat neostriatal neurons. Nature 344, 240-242.

Atkinson, N.S., and Robertson, G.A. and Ganetzky, B. (1991). A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. Science 253, 551-555.

Augustine, G. (1990). Regulation of transmitter release at the squid giant axon synapse by presynaptic delayed rectifier potassium current. J. Physiol. 431, 343-364.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1989). Current protocals in molecular biology. Greene Publishing Associates with Wiley-Interscience, New York.

Babilia, T., Moscucci, H.W., Weaver, F.E. and Koren, G. (1994). Assembly of voltage-gated potassium channels: evidence for an important role of the first transmembrane segment. Neuron 12, 615-626.

Baldwin, T.J., Tsaur, M.L., Lopez, G., Jan, Y.N. and Jan, L.Y. (1991). Characterization of a mammalian cDNA for an inactivating voltage-sensitive K channel. Neuron 7, 471-483.

Barbin, G., Manthorpe, M., Varon, S.J., (1984). Purification of chick eye cilary neurotrophic factor. J Neurochem. 43, 1468-1478.

Barish, M.E. (1995). Modulation of the electrical differentiation of neurons by interactions with glia and other non-neuronal cells. Perspect. on Dev. Biol. 2, 357-370.

Barish, M.E. (1986). Differentiation of voltage-gated potassium current and modulation of excitability in cultured amphibian spinal neurones. J. Physiol. 375, 229-250.

Barlow, H.B. and Mollon, J.D. (1987). The senses. Cambridge University Press, Great Britain.

Baukrowitz, T. and Yellen, G (1995). Modulation of K^+ current by frequency and external [K+]: a tale of two inactivation mechanisms. Neuron 15, 951-960.

Baukrowitz, T. and Yellen, G (1996a). Two functionally distinct subsites for the binding of internal blockers to the pore of voltage-activated K^+ channels. PNAS 12, 13357-13361.

Baukrowitz, T. and Yellen, G (1996b). Use-dependent blockers and exit rate of the last ion from the multi-ion pore of K^+ channels. Science 2, 653-656.

Baumann, A., Grupe, A., Ackermann, A. and Pongs, O. (1988). Structure of the voltage-dependent potassium channel is highly conserved from *Drosophila* to vertebrate central nervous systems. EMBO J. 7, 2457-2463.

Beckh, S. and Pongs, O. (1990). Members of the RCK potassium channel family are differentially expressed in the rat nervous system. EMBO J. 9, 777-782.

Belluzzi, O., Sacchi, O. and Wanke, E. (1985a). Identification of delayed potassium and calcium current in the rat sympathetic neurone studied under voltage-clamp conditions. J. Physiol. 358, 109-129.

Belluzzi, O., Sacchi, O. and Wanke, E. (1985b). A fast transient outward current in the rat sympathetic neurone studied under voltage-clamp conditions. J. Physiol. 358, 91-108.

Belluzzi, O. and Sacchi, O. (1988). The interaction between the potassium and sodium currents in generating action potentials in the rat sympathetic neurone studied under voltage-clamp conditions. J. Physiol. 412, 493-512.

Belluzzi, O. and Sacchi, O. (1990). The calcium dependent conductance in rat sympathetic neurones. J. Physiol. 422, 561-583.

Belluzzi, O. and Sacchi, O. (1991). A five-conductance model of the action potential in the rat sympathetic neurone. Prog. Biophys. Mol. Biol. 55, 1-30.

Belmonte, C. and Gallego, R. (1983). Membrane properties of cat sensory neurones with chemo-receptor and baroreceptor endings. J. Physiol. 304, 603-614.

Benveniste, E.N. (1992). Inflammatory cytokines within the central nervous system: sources, function and mechanisms of action. Am. J. Physiol. 263, c1-c16.

Bezanilla, F., Perozo, E., Papazian, D.M. and Stefani, E. (1991). Molecular basis of gating charge immobilization in *Shaker* potassium channels. Science 254, 679-683.

Biervet, C., Schroeder, B.C., Kubisch, C., Berkovic, S.C., Propping, P., Jentsch, T.J.and Steinlein, O.K. (1998). A potassium channel mutation in neonatal human epilepsy. Science 279, 403-406.

Blair, T.A., Roberds, S.L., Tamkun, M.M. and Hartshorne, R.P. (1991). Functional characterization of RK5, a voltage-gated K^+ channel cloned from the rat cardiovascular system. FEBS 295, 211-213.

Bossu, J.L., Dupont, J.L. and Feltz, A. (1985). I_A current compared to low threshold calcium currents in cranial sensory neurones. Neurosci. Lett. 62, 249-254.

Breitwieser, G.E. (1996). Mechanisms of K^+ channel regulation. J. Membrane Biol. 152, 1-11.

Brenman, J.E., Christopherson, K.S., Craven, S.E., McGee, A.W., and Bredt, D.S. (1996). Cloning and charaterization of postsynaptic density 93, a nitric oxide synthase interacting protein. J. Neurosci. 16, 7407-7415.

Brown, A.M. (1993). Functional basis for interpreting amino acid sequences of voltage-dependent K^+ channels. Annu. Rev. Biomol. Struct. 22, 173-198.

Brown, D.A., and Adams, P.R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+ current in a vertebrate neurone. Nature 283,673-676.

Brown, D.A., Constanti, A. and Adams, P.R. (1982) Calcium dependence of a component of a component of transient outward current in bullfrog ganglion cells. Soc. Neurosci. Abstr. 8:252.

Brown, D.L., Gancher, S.T., Nutt, J.G., Brunt, E.R.P., Smith, E.A., Kramer, P. and Litt, M. (1994). Episodic ataxia/myokymia syndrome is associated with point mutations in the human potassium channel gene, KCNA1. Nature Genetics 8, 136-140.

Bunge, R., Johnson, M. and Ross, C.D. (1978). Nature and nurture in development of the autonomic neuron. Science 199, 1409-1416.

Burch, R.M., Luini, A. and Axelrod, J. (1989). Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α 1-andernergic stimulation in FRTL5 thyroid cells. PNAS 83, 7201-7205.

Busch, A.E., Hurst, R.S., North, R.A. Adelman, J.P. and Kavanaugh, M.P. (1991). Current inactivation involves a histidine residue in the pore of the rat lymphocyte potassium channel RGK5. Biochem. Biophys. Res. Commun. 179, 1384-1390.

Butler, A., Wei, A., Baker, K. and Salkoff, L. (1989). A family of putative potassium channel genes in *Drosophila*. Science 243, 943-947.

Butler, A., Tsunoda, S., McCobb, D.P., Wei, A. and Salkoff, L. (1993). Mslo, a complex mouse gene encoding 'maxi' calcium-activated potassium channels. Science 261, 221-224.

Cassell, J.F. and McLachlan, E.M. (1986). The effect of a transient outward current (IA) on synaptic potentials in sympathetic ganglion cells of the guinea pig. J.Physiol 374, 273-288.

Cassell, J.F., Clark, A.L. and McLachlan, E. (1986). Characterization of phasic and tonic sympathetic ganglion cells of guinea pig. J. Physiol. 372, 457-483.

Castellano, A., Chiara, M.D., Mellstom, B., Molina, A., Monje, F., Naranjo, J.R. and Lopez-Barneo, J. (1997). Identification and functional characterization of a K^+ channel α -subunit with regulatory properties specific to brain. J. Neurosci. 17, 4652-4661.

Chandy, K.G., Douglas, J., Gutman, G.A., Jan, L., Joho. R., Kazmerek, L., McKinnon, D., North, R.A., Numa, S.A., Philipson, L., Rivera, A.B., Rudy, B., Salkoff, L., Swanson, R., Steiner, D., Tanouye, M., and Tempel, B.L. (1991). Simplified gene nomenclature. Nature 352, 26.

Chang, J., Musser, J.H., and McGregor, H. (1987). Phospholipase A_2: function and pharmacological regulation. Biochem. Pharmacol. 36, 2429-2436.

Chomoczynski, P. and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Ann. Biochem. 162, 156-159.

Cho, K-O., Hunt, C.A., and Kennedy, M.B. (1992). The rat brain postsynaptic density fraction contains a homolog of the *Drosophila* discs-large tumour suppressor protein. Neuron 9, 929-942.

Choi, K.L., Aldrich, R.W., and Yellen, G. (1991). Tetraethylammonium blockade distinguishes two inactivation mechanisms in voltage-activated K^+ channels. PNAS USA 88, 5092-5095.

Choi, K.L., Mossman, c., Aube, J. and Yellen, G. (1993). The internal quanternary ammonium receptor site of *Shaker* potassium channels. Neuron, 10, 533-541.

Christie, M.J., North, R.A., Osborne, P.B., Douglass, J. and Adelman, J.P. (1990). Heteropolymeric potassium channels expressed in *Xenopus* oocytes from cloned subunits. Neuron 4, 405-411.

Connor, J.A. and Stevens, C.F. (1971a). Voltage clamp studies of a transient outward membrane current in gastropod neural somata. J. Physiol. 213, 21-30.

Connor, J.A. and Stevens, C.F. (1971b). Prediction of repetitive firing behaviour from voltage clamp data on isolated neural somata. J.Physiol. 213, 31-53.

Constanti, A. and Brown, D.A. (1981). M-currents in voltage-clamped mammalian sympathetic neurones. Neurosci. Lett. 24, 289-294.

Constanti, A. and Sim, J.A. (1987). Calcium-dependent potassium conductance in guinea-pig olofactory cortex neurones *in vitro*. J. Physiol. 387, 173-194.

Cooper, E. and Shrier, A. (1985). Single-channel analysis of fast transient potassium currents from rat nodose neurons. J. Physiol. 369, 199-208.

Cooper, E. and Shrier, A. (1989). Inactivation of A currents and A channels on rat nodose neurons in culture. J. Gen. Physiol. 94, 881-910.

Covarrubias, M., Wei, A. and Salkoff, L. (1991). *Shaker*, *Shal*, *Shab*, and *Shaw* express independent K^+ current systems. Neuron 7, 763-773.

Covarrubias, M., Wei, A., Salkoff, L. and Vyas, T.B. (1994). Elimination of rapid potassium channel inactivation by phosphorylation of the inactivation gate. Neuron 13, 1403-1412.

Curran, M., Splawski, I., Timothy, K.W., Vincent, G.M., Green, E.D. and Keating, M.T. (1995). A molecular basis for cardiac arrhythmia: *Herg* mutations cause long QT syndrome. Cell 80, 299-307.

Dangle, J.M., Weeks, D.L., and Walder, J.A. (1991). Pathways of degradation and mechanisms of action of antisense oligonucleotides in *Xenopus* laevis embryos. Antisense Res. Dev. 1, 11-20.

DeBiasi, M., Hartmann, H.A., Drewe, J.A., Taglialatela, M., Brown, A.M. and Kirsch, G.E. (1993). Inactivation determined by a single site in K^+ pores. Pflugers Arch. 422, 354-363.

Dekin, M.S., Getting, P.A. and Johnson, S.M. (1987). In vitro characterization of neurons in the ventral part of the nucleus tractus solitarius. II. Ionic basis for repetitive firing patterns. J.Neurophys. 58, 215-229.

De Koninck, P. and Cooper, E. (1995). Differential regulation of neuronal nicotinic ACh receptor subunit genes in cultured neonatal rat sympathetic neurons: Specific induction of α7 by membrane depolarization through a Ca^{2+}/calmodulin-dependent pathway. J. Neurosci. 15, 7966-7978.

Dixon, J.E. and McKinnon, D. (1996). Potassium channel mRNA expression in prevertebral and paravertebral. European J. Neurosci. 8, 183-191.

Dobrea, G.M., Unnerstall, J.R., and Rao, M.S. (1992). The expression of CNTF message and immunoreactivity in the central and peripheral nervous system. Dev. Br. Res.. 66, 209-219.

Doerner, D., Pitler, T.A. and Alger, B.E. (1988). Protein kinase C activators block specific calcium and potassium current component in isolated hippocampal neurons. J. Neurosci. 8, 4069-4078.

Dolly, J.O., Halliwell, J.V., Black, J.D., Williams, R.S., Pelchen-Matthews, A. Breeze, A.L., Mehraban, F., Othman, I.B. and Black, A.R. (1984). Botulinium neurotoxin and dendrotoxin as probes for studies of transmitter release. J. Physiol. 79, 280-303.

Doupnik, C.A., Davidson, N. and Lester, H.A. (1995). The inward-rectifier potassium channel family. Curr. Opin. Neurobiol. 5, 268-277.

Dourado, M.M. and Dryer, S.E. (1992). Changes in the electrical properties of the chick ciliary ganglion neurones during embryonic developmemt. J. Physiol. 449, 411-428.

Dourado, M.M. and Dryer, S.E. (1994). Regulation of A-currents by cell-cell interactions and neurotrophic factors in the developing chick parasympathetic neurones. J. Physiol. 474, 367-377.

Doyle, D.A., Lee, A., Lewis, J., Kim, E., Sheng, M. and MacKinnon, R. (1996). Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. Cell 85, 1067-1076.

Drain, P., Dubin, A.E. and Aldrich, R.W. (1994). Regulation of *Shaker* K^+ inactivation gating by cAMP-dependent protein kinase. Neuron 12, 1097-1109.

Drewe, J.A., Verma, S., Frech, G. and Joho, R.H. (1992). Distinct spatial and temporal expression patterns of K^+ channel mRNAs from different subfamilies. J. Neurosci. 12, 538-548.

Duprat, F., Guillemare, E., Romey, G., Fink, M., Lesage, F., Lazdunski, M. and Honore, E. (1995). Susceptibility of cloned K^+ channels to reactive oxegen species. PNAS 92, 11796-11800.

Durell, S.R. and Guy, H.R. (1992). Atomic scale and structure and functional models of voltage-gated potassium channels. Biophys. J. 62, 238-250.

Durell, S.R. and Guy, H.R. (1996). Structural model of the outer vestibule and selectivity filter of the *Shaker* voltage-gated K^+ channel. Neuropharmacology 35, 761-773.

Dutar, P. and Nicoll, R.A. (1988). Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second messenger systems: electrophysiological studies *in vitro*. J. Neurosci. 8, 4214-4224.

Elkins, T., Ganetzky, B. and Wu, C.F. (1986). A *Drosophila* mutation that eliminates a calcium-activated potassium current. PNAS 83, 8415-8419.

England, S.K., Uebele, V.N., Kodali, J., Bennet, P.B. and Tamkun, M.M. (1995). A novel K+ channel ß-subunit hKv ß1.3 is produced via alternative mRNA splicing. J. Biol. Chem. 270, 28531-28534.

Ernsberger, U., Sendtner, M., and Rohrer, H. (1989). Proliferation and differentiation of embryonic chick sympathetic neurons: effects of cilary neurotrophic factor. Neuron 2, 1275-1284.

ffrench-Mullen, J.M.H., Rogawski, M.A. and Barker, J.L. (1988). Phenyclidine at low concentrations selectively blocks the sustained but not the transient voltage-dependent potassium current in cultured hippocampal neurons. Neurosci. Lett. 88:325-330.

Ficker, E. and Heinemann, U. (1992). Slow and fast transient potassium currents in cultured rat hippocampal cells. J. Physiol 445, 431-455.

Fink, M. Dupratt, F. Lesage, F., Heurteaux, C., Romey, G., Barhanin, J and Lazdunski, M. (1996). A new K^+ channel subunit to specifically enhance Kv2.2 expression. J. Biol. Chem. 271, 26341-26348.

Francisco, J.T., Bokhari, A., Rogero, O., Gorczyca, M., Zhang, J., Kim, E., Sheng, M, and Budnik, V. (1997). Essential Role for *Dlg* in synaptic clustering of *Shaker* K^+ channels *in vivo*. J. Neurosci. 17, 152-159.

Fraser, A.B. and Cooper, E.C. (1994). *Shaw*-related subfamily of voltage-gated potassium channel genes expressed by rat peripheral neurons. Soc. Neurosci. Abst. 36.12.

Frech, G.C., Van Dongen, A.M.J., Schuster, G., Brown, A.M. and Joho, R.H. (1989). A novel K channel with delayed rectifier properties isolated from rat brain by expression cloning. Nature 340, 642-645.

Freschi, J. E. (1983) Membrane currents of cultured rat sympathetic neurons under voltage clamp. J. Neurophys. 50, 1460-1478.

Fu, S.Y. and Gordon, T. (1997). The cellular and molecular basis of peripheral nerve regeneration. Molecular Neurobiology 14, 67-116.

Furshpan, E.J., Potter, D.D. and Landis, S.C. (1982). On the transmitter repertoire of sympathetic neurons in culture. Harvey Lect. 76, 149-191.

Galvan, M. and Sedlmeir, C. (1984). Outward currents in voltage-clamped rat sympathetic neurones. J. Physiol. 356, 115-133.

Gan, L., Perney, T.M. and Kaczmarek, L.K. (1996). Cloning and characterization of the promoter for a potassium channel expressed in high frequency firing neurons. J. Biol. Chem. 271, 5859-5865.

Gautam, M. and Tanouye, M. (1990). Alteration of potassium channel gating: molecular analysis of the *Drosophila* Sh^5 mutation. Neuron 5, 67-73.

Ghanshani, S., Pak, M., McPherson, J.D., Strong, M., Dethlefs, B., Wasmuth, J.J., Salkoff, L., Gutman, G.A. and Chandy, K.G. (1992). Genomic organization, nucleotide sequence, cellular distribution of *Shaw*-related potassium channel gene, Kv3.3, and mapping of Kv3.3 and Kv3.4 to human chromosomes 19 and 1. Genomics 12, 190-196.

Greene, L.A. and Tischler, A.S. (1976). Establihment of a norandernergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. PNAS 73, 2424-2428.

Gross, A., Abramson, T. and MacKinnon, R. (1994). Transfer of scorpion toxin receptor to an insensitive potassium channel. Neuron 13, 961-966.

Gross, A and MacKinnon, R. (1996). Agitoxin footprinting the *Shaker* potassium channel pore. Neuron 16, 399-406.

Gustaffson, B., Galvan, M., Grafe, P. and Wigstrom, H. (1982). A transient current in a mammalian central neurone blocked by 4-aminopyridine. Nature 299, 252-254.

Halliwell, J.V. and Adams, P.R. (1982).Voltage-clamp analysis of muscarinic excitation in hippocampal neurons. Brain Res. 250, 71-92.

Halliwell, J.V., Othman, I.B., Pelchen-Matthews, A. and Dolly, J.O. (1986). Central actions of dendrotoxin: selective reduction of a transient K conductance in hippocampus and binding to localized acceptors. PNAS 83, 493-497.

Hamilton, S.E., Loose, M.D., Qi, M., Allan, I.L., Hille, B., McKnight, G.S., Idzerda, R.L. and Nathanson, N.M. (1997). Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current and seizure activity in mice. PNAS 94, 13311-13316.

Harris, G.L., Henderson, L.P and Spitzer, N.C. (1988). Changes in densities and kinetics of delayed rectifier potassium channels during neuronal differentiation. Neuron 1, 739-750.

Harris, R.E. and Isacoff, E.Y. (1996). Hydrophobic mutations alter the movement of Mg^{2+} in the pore of voltage-gated potassium channels. Biophysical J. 71, 209-219.

Hartmann, H.A., Kirsch, G.E., Drewe, J.A., Taglialatela, M., Joho, R.F. and Brown, A.M. (1991). Exchange of conduction pathways between two related K^+ channels. Science 251, 942-944.

Harvey, A.L. and Karlsson, E. (1982). Protease inhibitor homologues from mamba venoms: facilitation of acetylcholine release and interactions with prejunctional blocking toxins. Br. J. Pharmacol. 77, 153-161.

Hawrot, E. (1980). Cultured sympathetic neurons: Effects of cell-derived and synthetic substrata on development. Dev. Biol. 74, 136-151.

Heginbotham, L., Ambramson, T., and MacKinnon, R. (1992). A functional relation between the pore of distantly related ion channels as revealed by mutant K^+ channels. Science 258, 1152-1155.

Heginbotham, L. and MacKinnon, R. (1992). The aromatic binding site for tetraethylammonium ion on potassium channels. Neuron 8, 483-491.

Heginbotham, L., Lu, Z., Ambramson, T. and MacKinnon, R. (1994). Mutations in the K^+ channel signature sequence. Biophys. J. 66, 1061-1067.

Heinemann, S.H., Rettig, J., Wunder, F and Pongs, O. (1995). Molecular and functional characterization of a rat Kvß3 potassium channel subunit. FEBS 383-389.

Hille, B. (1984). Ionic Channels in Excitable Membranes. (Sunderland, MA: Sinauer Associates, Inc.)

Hinnebusch, A.G. (1990). Involvement of an initiation factor and protein phosphorylation in translational control of GCN4 mRNA. Trends Biochem. Sci. 15, 148-152,

Hodgkin, A.L. and Huxley, A.F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol. 117, 500-544.

Hoffman, D.A., Magee, J.C., Colbert, C.M. and Johnston, D. (1997). K+ channel regulation of signal propagation in dendrites of hippocampal pyramidal neurons. Nature 387, 869-875.

Hoshi, T., Zagotta, W.N. and Aldrich, R.W. (1990). Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. Science 250, 533-538.

Hoshi, T., Zagotta, W.N. and Aldrich, R.W. (1991). Two types of inactivation in Shaker K^+ channels: Effects of alterations in the carboxy-terminal region. Neuron 7, 547-556.

Hsueh, Y-P., Kim, E. and Sheng, M. (1997). Disulphide head-to-head multimerization in the mechanism of channel clustering by PSD-95. Neuron 18, 803-814.

Hugnot, J.P., Salinas, M., Lesage, F., Guillemare, E., de Weille, J.,Heurteaux, C., Mattei, M.G., and Ladunski, M. (1996). Kv8.1, a new neuronal potassium channel subunit with specific inhibitory properties towards *Shab* and *Shaw* channels. EMBO 15, 3322-3331.

Hunt, C.A., Schenker, L.J. and Kennedy, M.B. (1996). PSD-95 is associated with the postsynaptic density and not with the presynaptic membrane at forebrain synapses. J. Neurosci. 16, 1380-1388.

Hwang, P.M, Glatt, C.E., Bredt, D.S., Yellen, G. and Snyder, S.H. (1992). A novel K^+ channel with unique localizations in mammalian brain: Molecular cloning and characterization. Neuron 8, 473-481.

Hwang, P.M, Fotuhi, M., Bredt, D.S., Cunningham, A.M., and Synder, S.H. (1993). Contrasting immunohistochemical localizations in the rat brain of novel K^+ channels of the *Shab* subfamily. J. Neurosci. 13, 1569-1578.

Ikeda, S.R., Soler, F., Zuhlke, R.D. Joho, R.H. and Lewis, D.L. (1992). Heterologous expression of the human potassium channel Kv2.1 in clonal mammalian cells by direct cytoplasmic microinjection of cRNA. Pfugers Arch 422, 201-203.

Ip, N.Y., Nye, S. H., Boulton, T.G. Davies, S., Taga, T., Li, Y., Birrem, S.J., Yasakawa, K., Kishimoto, T., Anderson, D.J., Stahl, N. and Yancopoulos, G.D. (1992). CNTF and LIF act on neuronal cells via a shared signalling pathway that involve the IL-6 signal transducing receptor gp130, Cell 69, 1121-1132.

Ip, N.Y., McClain, J., Barrezueta, N.X., Aldrich, T.H., Pan, L., Li, Y., Wiegand, S.J., friedman, B., Davis, S. and Yancopoulos, G.D., (1993). The alpha component of the

CNTF receptor is required for signalling and defines potential CNTF targets in the adult and during development. Neuron 10, 89-102.

Isacoff, E.Y, Jan, Y.N. and Jan, L.Y. (1990). Evidence for the formation of heteromultimeric potassium channels in *Xenopus* oocytes. Nature 345, 530-534.

Isacoff, E.Y, Jan, Y.N. and Jan, L.Y. (1991). Putative receptor for the cytoplasmic gate in the *Shaker* K^+ channel. Nature 353, 86-90.

Iverson, L.E., Tanouye, M.A., Lester, H.A., Davidson, N. and Rudy, B. (1988). A-type potassium channels expressed from *Shaker* locus cDNA. PNAS 85, 5723-5727.

Iverson, L.E. and Rudy, B. (1990). The role of the divergent amino and carboxyl domains on the inactivation properties of potassium channels derived from the *Shaker* gene of *Drosophila*. J. Neurosci. 10, 2903-2916.

Izaurralde, E. and Mattaj, I.W. (1992). Transport of RNA between nucleus and cytoplasm. Semin. Cell. Biol. 3, 279-288.

Jan, L.Y. and Jan, Y.N. (1997). Cloned potassium channels from eukaryotes and prokaryotes. Annu. Rev. Neurosci. 20, 91-123.

Jan, L.Y. and Jan, Y.N. (1992). Specification of subunit assembly by the hydrophobic amino terminal domain of the *Shaker* potassium channel. Science 257,1225-1230.

Jan, L.Y. and Jan, Y.N. (1990). How might the diversity of potassium channels be generated? TINS 13, 415-419.

Javitch, J.A. Li, X., Kaback, J., and Karlin, A. (1994). A cysteine residue in the third membrane spanning segment of the human D2 dopamine receptor is exposed in the binding site crevice. PNAS 91, 10355-10359.

Jelga, T. and Salkoff, L. (1997). A novel subunit for *Shal* K^+ channels radically alters activation and inactivation. J. Neurosci. 17, 32-44.

Johns, D.C., Nuss, H.B., and Marban, E. (1997). Suppression of neruonal and cardiac transient outward currents by viral gene transfer of dominant-negative Kv4.2. J. Biol. Chem. 272, 31598-31603.

Kamb, A., Iverson. L.E. and Tanouye, M.A. (1987). Molecular characterization of *Shaker*, a *Drosophila* gene that encodes a potassium channel. Cell 50, 405-413.

Kandel, E.R., Schwartz, J.H. and Jessel, T.M. (1991). Principles of neural science. Elsevier Science Publishing Co. New York, NY.

Kaupp, B.U., Niidome, T., Tanabe, T., Terada, S., Bonigk, W., Stuhmer W., Cook N., Kangawa, K., Matsuo, H., Hirose, T. and Numa, S. (1989). Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. Nature 342, 762-766.

Kamb, A., Tseng-Crank, J. and Tanouye, M.A. (1988). Multiple products of the *Drosophila Shaker* gene may contribute to potassium channel diversity. Neuron 1, 421-430.

Katz, L.C. and Shatz, C.J. (1996). Synaptic activity and the construction of cortical circuits. Science 274, 1134-1138.

Kerr, I.D. and Sansom, M.S.P. (1997). The pore-lining region of the *Shaker* voltage-gated potassium channels: comparison of ß-barrel and α-helix bundle models. Biophys. J. 73, 581-602.

Khun, R., Schwenk, F., Aguet, M. and Rajewsky, K. (1995). Inducible gene trapping in mice. Science 269, 1427-1429.

Kim, E, Niethammer, M. Rothschild, A. Jan, Y.N. and Sheng, M. (1995). Clustering of *Shaker*-type channels by interaction with a family of membrane-associated guanylate kinases. Nature 378, 85-88.

Kim, E. and Sheng, M. (1996). Differential K+ channel clustering activity of PSD-95 and SAP97, two related membrane-associated putative guanylate kinases. Neuropharmacol. 35, 993-1000.

Kirsch, G.E., Drewe, J.A., Taglialatela, M., Joho, R.H., DeBiasi, M., Hartmann, H.A. and Brown, A,M. (1992). A single nonpolar residue in the deep pore of related K^+channels acts as a K^+:Rb^+ conductance switch. Biophys. J. 62, 136-144.

Kirsch, G.E., Pascual, J.M. and Shieh, C.C. (1995). Functional role of a conserved asparagine in the external mouth of voltage-gated potassium channels. Biophys. J. 68, 1804-1813.

Kistner, U., Wenzel, B.M., Veh, R.W., Cases-Langhoff, C., Gardner, A.M., Appeltauer, U., Voss, B., Gundelfinger, E.D. and Garner, C.C. SAP90, a rat presynaptic protein related to the product of the *Drosophila* tumour suppressor gene *Dlg*. J. Biol. Chem. 268, 4580-4583.

Kohler, M., Hirschberg, B., Bond, C.T., Kinzie, J.M., Marrion, N.V., Maylie, J., and Adelman, J.P. (1996). Small conductance calcium-activated potassium channels from mammalian brain. Science 273, 1709-1714.

Krieg, P.A. and Melton, D.A. (1987). *In vitro* RNA synthesis with SP6 polymerase. Methods in Enzymology. 155, 397-415.

Kurz, L.L., Zuhlke, R.D., Zhang, H.J. and Joho, R.H. (1995). Side-chain accessibilities in the pore of a K+ channel probed by sulfhydryl-specific reagents after cysteine-scanning mutagenesis. Biophys. J. 68, 900-905.

Larsson, H.P., Baker, O.S., Dhillon, D.S. and Isacoff, E.Y. (1996). Transmembrane movement of the *Shaker* K^+ channel S4. Neuron 16, 387-397.

Lesage, F., Guillemare, E., Fink, M., Duprat, F., Lazdunski, M., Romey, G. and Barhanin, J. (1996). *Twick*-1, a ubiquitous human weakly inward rectifying channel with a novel structure. Eur. Molec. Biol. Org. J. 15, 1004-1011.

Lesser, S.S., and Lo, D.C., (1995). Regulation of voltage-gated ion channels by NGF and cilary neurotrophic factor in SK-N-SH neuroblastomal cells. J. Neurosci. 15, 253-261.

Li, M., Jan, Y.N. and Jan, L.Y. (1992). Specificity of subunit assembly by the hydrophillic amino-terminal domain of the *Shaker* potassium channel. Science 257, 1225-1230.

Liman, E.R., Tytgat, J. and Hess, P. (1992). Subunit stoiciometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. Neuron 9, 861-871.

Lin, L.F.H., Drzislav, M., Lile, J.D., Lyman, G.A., Butler, E.T. Vannice, J.L. and Collins, F. (1989). Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science 246, 1023-1025.

Lipkind, G.M., Hanck, D.A. and Fozzard, H.A. (1995). A structural motif for the voltage-gated potassium channel pore. PNAS 92, 9215-9219.

Llinas, R.R. (1988). The intrinsic electrophysiological properties of mammalian neurons: Insights into central nervous system function. Science 242, 1654-1664.

Lopez, G.A., Jan, Y.N. and Jan, L.Y. (1991). Hydrophobic substitution mutations in the S4 sequence alter voltage-dependent gating in Shaker K^+ channels. Neuron 7, 327-336.

Lopez-Barneo, J., Hoshi, T., Heinemann, S.H. and Aldrich, R.W. (1993). Effects of external cations and mutations in the pore region on C-type inactivation of *Shaker* potassium channels. Receptor Channels 1, 61-71.

Lotan, I. (1992). Hybrid arrest technique to test for functions roles of cloned cDNAs and identify homologies among ion channel genes. Methods Enzymol. 207, 605-612.

Lu, R.A., Marfatia, S.M., Branton, D. and Chishti, A.H. (1994). Cloning and characterization of *hdlg*: the human homologue of the *Drosophila* discs large tumour suppressor binds to protein 4.1. PNAS 91, 9818-9822.

Lu, Q. and Miller, C. (1995). Silver as a pore-forming residues in a potassium channel. Science 268, 304-307.

Luckenbills-Edds, L. (1997). Laminin and the mechanism of neuronal outgrowth. Brain Res.23,1-27.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. Science 274, 1109-1114.

Luneau, C.J., Williams, J.B., MarShall, J., Levitan, E.S., Oliva, C., Smith, J.S., Antanavage, J., Folander, K., Stein, R.B., Swanson, R., Kaczmarek, L.K. and Sukrow, S.A. (1991). Alternative splicing contributes to K channel diversity in the mammalian central nervous system. PNAS 88, 3932-3936.

MacKinnon, R. (1990). Determination of subunit stoichiometry of a voltage-activated potassium channel. Nature 350, 232-235.

MacKinnon, R. and Miller, C. (1989). Mutant potassium channels with altered binding of Charybdotoxin, a pore-blocking peptide inhibitor. Science 245, 1382-1385.

MacKinnon, R., Heginbotham, L. and Abramson, T. (1990). Mapping the receptor site for charybdotoxin, a pore-blocking potassium channel inhibitor. Neuron 5, 767-771.

MacKinnon, R. and Yellen, G. (1990). Mutations affecting TEA blockade and ion permeation in voltage-activated K^+ channels. Science 250, 276-278.

MacKinnon, R. (1991). Determination of the subunit stoichiometry of a voltage-activated potassium channel. Nature 350, 232-235.

MacKinnon, R., Aldrich, R.W. and Lee, A.W. (1993). Functional stoichiometry of Shaker potassium channel inactivation Science 262, 757-759.

MacKinnon, R. (1995). Pore loops: an emerging theme in ion channel structure. Neuron 14, 889-892.

Madison, D.V., Lancaster, B. and Nicoll, R.A. (1987). Voltage clamp analysis of cholinergic action in the hippocampus. J.Neurosci. 7, 733-741.

Majumder, K., DeBiasi, M., Wang, Z. and Wible, B.A. (1995). Molecular cloning and functional expression of a novel potassium channel ß-subunit from human atrium. FEBS 13, 13-16.

Maletic-Salvatic, M., Lenn, N.J., and Trimmer, J.S. (1995). Differential spatiotemporal expression of K^+ channel polypeptides in rat hippocampal neurrons developing *in situ* and *in vitro*. J. Neurosci. 15,3840-3851.

Mandelzys, A. and Cooper, E. (1992). Effects of ganglionic satellite cells and NGF on the expression of nicotinic acetylcholine currents by rat sensory neurons. J. Neurophys. 67, 1213-1221.

Mannuzzu, L.M., Moronne, M.M. and Isacoff, E.Y. (1995). Direct physical measure of conformational rearrangement underlying potassium channel gating. Science 271:117-260.

Mathie, A. and Watkins, C.S. (1997). Is *Eag* the answer to the M-current? Trends Neurosci. 20, 14.

Maquat, L.E. (1991). Nuclear mRNA export. Curr. Opin. Cell Biol. 3, 279-288.

Mau, R.A., Kramer, S.D., Goodman, R.H. and Mandel, G. (1990). Neuron 4, 223-231.

McCormack, J.W., Lin, L.E., Iverson, L.E. and Rudy, B. (1990). Molecular cloning of a member of a third class of *Shaker* family K^+ channel genes in mammals. PNAS 87, 5227-5231.

McCormack, T. and McCormack (1994). *Shaker* K+ channel ß subunits belong to an NAD(P)H- dependent oxidoreductase superfamily. Cell 79, 1133-1135.

McFarlane, S. and Cooper, E. (1991). Kinetics and voltage-dependence of A-type currents on rat sensory neurons in culture. J. Neurophys. 66, 1380-1391.

McFarlane, S. and Cooper, E. (1992). Postnatal development of voltage-gated K currents on rat sympathetic neurons. J. Neurophys. 67, 1291-1300.

McFarlane, S. (1992). Developmental control of voltage-gated potassium currents on postnatal rat peripheral neurons. PhD. thesis McGill University.

McFarlane, S. and Cooper, E. (1993). Extrinsic factors influence the expression of voltage-gated K currents on neonatal rat sympathetic neurons. J. Neurosci. 13, 2591-2600.

Mehler, M.F., and Kessler, J.A. (1995). Cytokines and neuronal differentiation. Critical reviews in neurobiology. 9:419-446.

Melefors, O. and Hentze, M.W. (1993). Translational regulation by mRNA/protein interactions in eukaryotic cells: ferritin and beyond. Bioassays 15, 85-90.

Mi, H., Deerinck, T.J., Ellisman, M.H. and Schwarz, T.L. (1995). Differential distribution of closely related potassium channels in rat Schwann cells. J. Neurosci. 15, 3761-3774.

Miller, C.(1991). Annus mirabilis of potassium channels. Science 252, 1092-1096.

Miyamoto, E. and Fukunaga, K. (1996). A role of Ca^{2+}/ calmodulin-dependent protein kinase II in the induction of long-term potentiation in hippocampal CA1 area. Neuroscience Research 24, 117-122.

Mottes, J.R. and Iverson, L.E. (1995). Tissue-specific alternative splicing of hybrid *Shaker*/lacZ genes correlate with kinetic differences in *Shaker* K^+ currents *in vivo*. Neuron 14, 613-623.

Muller, B.M.,Kistner, U., Kindler, S., Chung, W.J., Kuhlendahl, S., Lau, L.F., Veh, R.W., Hunganir, R.L., Gundelfinger, E.D., and Garner, C.C. (1996). SAP102, a novel postsynaptic protein that interacts with the cytoplasmic tail of the NMDA receptor subunit NR2B. Neuron 17, 255-265.

Nabeshima T. Kitaichi, K. and Noda Y. (1996) Functional changes in neuronal systems induced by phencyclidine ANYAS 801,29-38.

Nakajima, J., Nakajima, S., Leonard, R.J., and Yamaguchi, K., (1986). Acetylcholine raises excitability by inhibititing fast transient potassium current in cultured hippocanpal neurons. PNAS 83, 3022-3026.

Nawa, H. and Sah, D.W. (1990). Different biological activities in conditioned media control the expression of a variety of neuropeptides in cultured sympathetic neurons. Neuron 4, 279-287.

Nerbonne, J.M, Gurney, A.M. and Rayburn, H.B. (1986). Development of the fast, transient outward K current in embryonic sympathetic neurones. Brain Res.378, 197-202.

Nerbonne, J.M. and Gurney, A.M. (1989). Development of excitable membrane properties in mammalian sympathetic neurons. J. Neurosci. 9, 3272-3286.

Nicoll, R.A. and Malenka, R.C. (1995). Contrasting properties of two forms of long-term potentiation in the hippocampus. Nature 14, 115-118.

Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M.A., Hirose, T., Inayama, S., Hagashida, H., Miyata, T. and Numa, S. (1984). Primary structure of *Electrophorus electricus* sodium channel deduced from cDNA sequence. Nature 312, 121-127.

Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S. and Numa, S. (1983). Cloning and sequence analysis of calf cDNA and human genomic DNA encoding α -subunit precursor of muscle acetylcholine receptor. Nature 305, 818-823.

Numann, R.E., Wadman, W.J. and Wong, R.K.S. (1987). Outward currents of single hippocampal cells obtained from the adult guinea pig. J. Physiol 393, 331-353.

Paintal, A.S. (1973). Vagal sensory receptors and their reflex effects. Physiol. Revs. 53, 159-227.

Pak, M., Covarrubias, M., Ratcliffe, A. and Salkoff, L. (1991a). A mouse brain homolog of the *Drosophila Shab* K^+ channel with conserved delayed-rectifier properties. J. Neurosci. 11, 869-880.

Pak, M., Baker, K., Covarrubias, M., Butler, A., Ratcliffe, A. and Salkoff, L. (1991b). *mShal*, a subfamily of A-type K channel cloned from mammalian brain. PNAS 88, 4386-4390.

Papazian, D.M., Schwarz, T.L., Tempel, B.L. Jan, Y.N. and Jan, L.Y. (1987). Cloning of genomic and complementary DNA from *Shaker*, a putative potassium channel gene from *Drosophila*. Science 237, 749-753.

Papazian, D.M., Timpe, L.C., Jan, Y.N. and Jan, L.Y. (1991). Alterations of voltage-dependence of *Shaker* potassium channels by mutation in the S4 sequence. Nature 349, 305-310.

Pascual, J.M., Shieh, C.C., Kirsch, G.E. and Brown, A.M. (1995). K^+ pore structure revealled by reporter cysteines at inner and outer surfaces. Neuron 14, 1055-1063.

Papazian, D.M., Timpe, L.C., Jan, Y.N. and Jan, L.Y. (1991). Alteration of voltage-dependence of *Shaker* potassium channel by mutations in the S4 sequence. Nature 349, 305-307.

Pongs, O., Kecskemethy, N., Muller, R., Krah-Jentgens, I., Baumann, A., Kiltz, H.H, Canal, I., Llamazares, S. and Ferrus, A. (1988). *Shaker* encodes a family of putative potassium channel proteins in the nervous system of *Drosophila*. EMBO J. 7, 1087-1096.

Ranganathan, R., Lewis, J.H. and MacKinnon, R. (1996). Spacial localization of the K^+ selectivity filter by mutant cycle-based structure analysis. Neuron 16, 131-139.

Rao, M.S. and Landis, S.C. (1990). Characterization of a target-derived neuronal cholinergic differentiation factor. Neuron 5, 899-910.

Rasmusso, R.L., Morales, M.J., Castellino, R.C., Zhang, Y., Cambell, D.L. and Strauss, H.C. (1995). C-type inactivation controls recovery in a fast inactivating K^+ channel (Kv1.4) expressed in *Xenopus* oocyte. J. Physiol. 489, 709-721.

Raucher, S., and Dryer, S.E. (1994). Functonal expression of A-current in embryonic chick sympathetic neurones during development in situ and in vitro. J. Physiol. 2666, 77-93.

Rebagliati, M.R., and Melton, D.A. (1987). Antisense RNA injections in fertilized frog eggs reveal an RNA duplex unwinding activity. Cell 48, 599-605.

Rettig, J., Wunder, F., Stocker, M., Lichtinghagen, R., Mastiaux, F., Beckh, S., Kues, W., Pedarzani, P., Schroter, K.H., Ruppersberg, J.P., Veh, R. and Pongs, O. (1992). Charactorization of a *Shaw*-related potassium channel family in rat brain. EMBO 11, 2473-2486.

Rettig, J., Heinemann, S.H., Wunder, F., Lorra, C, Parcej, D. N., Dolly, J.O., and Pongs, O. (1994) Inactivation properties of voltage-gated potassium channels are altered by the presence of ß-subunit. Nature 369, 289-294.

Roeper, J., Lorra, C. and Pongs, O. (1997). Frequency-dependent inactivation of mammalian A-type K^+ channel Kv1.4 regulated by Ca^{2+}/calmodulin-dependent protein kinase. J. Neurosci. 17, 3379-3391.

Rogawski, M.A. (1985). The A-current: how ubiquitous a feature of excitable cells is it? TINS 8, 214-219.

Rhoads, R.E. (1993). Regulation of eukaryote protein synthesis by initiation factors. J. Biol. Chem. 268, 3017-3020.

Ribera, A.B. (1996). Homogenous development of electrical excitabilty via heterogenerous ion channel expression. J. Neurosci. 16, 1123-1130.

Roufa, D., Johnson, M. and Bunge, M. (1983). Influence of ganglion age, non-neuronal cells and substratum on neurite outgrowth in culture. Dev. Biol. 99, 225-239.

Ruben, E. (1985a). Development of the rat superior cervical ganglion: ganglion cell maturation. J.Neurosci 5, 673-684.

Ruben, E. (1985b). Development of the rat superior cervical ganglion: Ingrowth of preganglionic axons. J.Neurosci 5, 685-696.

Ruben, E. (1985c). Development of the rat superior cervical ganglion: Initial stages of synapse formation. J. Neurosci 5, 697-704.

Rudy, B. (1988). Diversity and ubiquity of K channels. Neurosci. 25, 729-749.

Rudy, B., Hoger, J.H., Lester, H.A. and Davidson, N. (1988). At least two mRNA species contribute to the properties of rat brain A-type potassium channels expressed in *Xenopus* oocytes. Neuron 1, 649-658.

Ruppersberg, J.P., Schroter, K., Sakmann, B., Stocker, M., Sewing, S. and Pongs, O. (1990). Heteromultimeric channels formed by rat brain potassium channel proteins. Nature 345, 535-537.

Ruppersburg, J.P., Frank, R., Pongs, O. and Stocker, M. (1991). Cloned neuronal IK(A) channels reopen during recovery from inactivation. Nature 353, 657-660.

Saadat, S., Sendtner, M. and Rohrer, H., (1989). Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. J. Cell Biol. 108, 1807-1816.

Sach, A.B. (1993). Messenger RNA degradation in eurkaryotes. Cell 74, 413-421.

Sah, P., Gibb, A.J. and Gage, P.W. (1988). Potassium current activated by depolarization of dissociated neurons from adult guinea pig hippocampus. J. Gen. Physiol. 92, 263-278.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985). Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230, 1350-1354.

Salkoff, L.B. and Wyman, R. (1981a). Outward currents in developing *Drosophila* flight muscle. Science 212, 461-463.

Salkoff, L.B. and Wyman, R. (1981b). Genetic modification of potassium channels in *Drosophila* Shaker mutants. Nature 293, 228-230.

Salkoff, L. and Wyman, R. (1983). Ion channels in *Drosophila* muscle. TINS 6, 128-133.

Salkoff, L., Baker, K., Butler, A., Covarrubias, M., Pak, M.D. and Wei, A. (1992). An essential 'set' of K^+ channels conserved in flies, mice and humans. TINS 15, 161-166.

Salkoff, L. and Jelga, T. (1995). Surfing the DNA databases for K^+ channels nets yet more diversity. Neuron 15, 489-492.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning, a laboratory manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. PNAS 74, 5463-5467.

Sanguinetti, M.C. and Spector, PS. (1997). Potassium channelopathies. Neuropharmacol. 36, 755-762.

Sarre, T.F. (1989). The phosphorylation of eukaryote initiation factor 2: a principle of translational control in mammalian cells. Biosystems 22, 311-325.

Schofield, G.G. and Ikeda, S.R. (1989). Potassium currents of acutely isolated adult rat superior cervical ganglion neruons. Brain Res. 485, 205-214.

Schroter, K.H., Ruppersberg, J.P., Wunder, F., Rettig, J., Stocker, M. and Pongs, O. (1991). Cloning and functional expression of a TEA-sensitive A-type potassium channel from rat brain. FEBS 278, 211-216.

Schwarz, T.L., Tempel, B.L. Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988). Multiple potassium-channel components are produced by alternative splicing at the *Shaker* locus in *Drosophila*. Nature 331, 137-142.

Schwarz, T.L., Papazian, D.M., Caretto, R.C., Jan, Y.N. and Jan, L.Y. (1990). Immunological characterization of K channel components from the *Shaker* locus and differential distribution of splicing variants in *Drosophila*. Neuron 2, 119-127.

Scott, V.E.S., Muinz, Z.M., Sewing, S., Lichtinghagan, R., Parcej, D.N., Pongs, O. and Dolly, J.O. (1994). Antibodies specific for distinct Kv subunits unviel a heterooligomeric basis for subtypes of α-dendrotoxin-sensitive K^+ channels in bovine brain. Biochemistry 33, 1617-1623.

Sendtner, M. Arakawa, Y., Stockli, K.A. Kreutzberg, G.W. and Thoenen, H. (1991). Effect of ciliary neurotrophic factor (CNTF) on motorneuron survival J. Cell Science 15, 103-109.

Segal, M. and Barker, J.L. (1984). Rat hippocampal neurons in culture: potassium conductance. J. Neurophys. 51, 1409-1433.

Segal, M., Rogawski, M.A. and Barker, J.L. (1984). A transient potassium channel regulates the excitability of cultured hippocampal and spinal neurons. Nature 336, 379-381.

Serodio, P., Vega-Saenz de Miera, E. and Rudy, B. (1996). Cloning of a novel component of A-type K+ channels operating at subthreshold potentials with unique expression in theheart and brain. J. Neurophysiol. 75, 2174-2179.

Serodio, P., Kentros, C.. and Rudy, B. (1994). Identification of molecular components of A-type channels operating at subthreshold potentials. J. Neurophysiol. 72, 1516-1529.

Sewing, S. Roeper, J. and Pongs, O. (1996). Kvß1 subunit binding specific for *Shaker*-related potassium channel α subunits. Neuron 16, 455-463.

Shen, N.V. and Pfaffinger, P.J. (1995). Molecular recognition and assembly sequences involved in subfamily-specific assembly of voltage-gated K^+ channel subunit proteins. Neuron, 14 625-633.

Shen, N.V., Chen, X., Boyer, M.B. and Pfaffinger, P.J. (1993). Deletion analysis of K^+ channel assembly. Neuron 11, 67-76.

Sheng, M., Tsaur, M., Jan, Y.N., and Jan L.Y. (1992). Subcellular segragation of two A-type K^+ channel proteins in rat central neurons. Neuron 9, 271-281.

Sheng, M., Liao, Y.J., Jan, Y.N. and Jan, L.Y. (1993). Presynaptic A-current based on heteromultimeric K^+ channels detected in vivo. Nature 365, 72-75.

Sheng, M. and Kim, E. (1996). Ion channel associated proteins, Curr. Opin. Neurobiol. 6, 602-608.

Shahidullah, M., Hoshi, N., Yokoyama, S., Kawamura, T. and Higashida, Haruhiro, H. (1995a). Slow inactivation conserved in hetertomeric voltage-dependent K^+ channels between *Shaker* (Kv1) and *Shaw* (Kv3) subfamilies. FEBS 371, 307-310.

Shahidullah, M., Hoshi, N., Yokoyama, S., Kawamura, T. and Higashida, Haruhiro, H. (1995b).Slow inactivation conserved in heteromeric voltage-dependent K^+ channels between *Shaker* (Kv1) and *Shaw* (Kv3) subfamilies. Proceedings of Royal Society of London 261, 309-317.

Shi, G., Kleinklaus, A.K., Marrion, N.V., and Trimmer, J.S. (1994). Properties of Kv2.1 K channel expressed in transfected mammalian cells. J. Biol. Chem. 269, 23204-23211.

Shi,G., Nakahira, K., Hammond, S., Rhodes, K.J., Schechter, L.E., Trimmer, J.S. (1996) ß-subunits promote K^+ channel surface expression through effects early in biosynthesis. Neuron 16, 843-852.

Shimahara, T. (1983). Presynaptic modulation of transmitter release by the early outward current in Aplysia. Brain Res.263, 51-56.

Smolen, A. and Raisman, G. (1980). Synapse formation in the rat superior cervical ganglion during normal development and after neonatal deafferentation. Brain Res.181, 315-323.

Smith, P.L., Baukrowitz, T. and Yellen, G. (1996). The inward rectifying mechanism of the *Herg* cardiac potassium channel. Nature 379, 833-836.

Solc, C.K., Zagotta, W.N. and Aldrich, R.W. (1987). Single-channel and genetic analyses reveal two distinct A-type potassium channels in *Drosophila*. Science 236, 1094-1098.

Songyang, Z., Fanning, A.S., Fu, C., Xu, J., Marfatia, S.M., Chishti, A.H., Crompton, A., Chan, A.C., Anderson, J.M., and Cantley, L.C. (1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. Science 275, 73-77.

Spigelman, I. and Puil, E. (1989). K-channel blockade in trigeminal root ganglion neurons: Effects on membrane outward currents. J. Neurophys. 62, 802-809.

Spitzer, N.C. (1979). Ion channels in development. Ann. Rev. Neurosci. 2, 263-297.

Spitzer, N.C. (1985). The control of development of neuronal excitability. In: Molecular Bases of Neural Development, G.M. Edelman, W.E. Gall, and W.M. Cowan, Eds., (New York, NY: Rockefeller University Press, New York).

Spitzer, N.C. (1991). A developmental handshake: Neuronal control of ionic currents and their control of neuronal differentiation. J. Neurobiol. 22, 659-673.

Stansfeld, C.E. and Wallis, D.I. (1985). Properties of visceral primary afferent neurons in the nodose ganglion of the rabbit. J. Neurophys. 54, 245-260.

Stansfeld, C.E., Marsh, S.J., Halliwell, J.V. and Brown, D.A. (1986). 4-Aminopyridine and dendrotoxin induce repetitive firing in rat visceral sensory neurones by blocking a slowly inactivating outward current. Neurosci. Lett. 64, 299-304.

Stansfeld, C. and Feltz, A. (1988). Dendrotoxin-sensitive K channels in dorsal root ganglion cells. Neurosci. Letts 93, 48-55.

Stansfeld, C.E., Roper, J., Ludwig, J., Wesseloh, R.M., Marsh, S.J., Brown, D.A. and Pongs, O. (1997). Elevation of intracellular calcium by muscarinic receptor activation induces a block of voltage-activated rat *ether-a-go-go* channels in a stably transfected cell line. PNAS 3, 9910-9914.

Stephens, G.J., Owen, D.G. and Robertson, B. (1996). Cysteine-modifying reagents alter the gating of cloned potassium channel Kv1.4 Pflugers Arch Eur J Physiol 431,435-442.

Stocker, M., Stuhmer, W., Wittka, R., Wang, X., Muller, R., Ferrus, A. and Pongs, O. (1990). Alternative *Shaker* transcripts express either rapidly inactivating or inactivating K^+ channels. PNAS 87, 8903-8907.

Storm, J.F. (1984). Two components of slow prepotentials in CA1 hippocampal pyramidal cells. Acta Physiol. Scand. 120:15A.

Storm J.F. (1987). Action potential repolarization and a fast after-hyperpolarization in rat hippocampal pyramidal cells. J. Physiol. 385, 733-759.

Storm, J.F. (1988). Temporal integration by a slowly inactivating K current in hippocampal neurons. Nature 336, 379-381.

Storm, J.F. (1989). An after-hyperpolarization of medium duration in rat hippocampal pyramidal cells. J. Physiol. 409, 171-190.

Storm, J.F. (1990). Potassium currents in hippocampal pyramidal cells. Progress in Brain Res.83, 161-187.

Strong, M., Chandy., K.G. and Gutman, G.A. (1993). Molecular evolution of voltage sensitive ion channel genes: on the origins of electrical excitability. Mol. Biol. Evol. 10, 1-20.

Stuhmer, W., Stocker, M., Sakmann, B., Seeburg P., Baumann, A., Grupe A. and Pongs, O. (1988). Potassium channels expressed from rat brain cDNA have delayed rectifier properties. FEBS 242, 199-206.

Stuhmer, W., Ruppersberg, J.P., Schroter, K.H., Sakmann, B., Stocker, M.,Giese, K.P., Perschke, A., Baumann, A. and Pongs, O. (1989). Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. EMBO J. 8, 3235-3244.

Swanson, R., MarShall, J., Smith, J.S., Williams, J.B., Boyle, M.B., Folander, K., Luneau, C.J., Antanavage, J., Oliva, C., Buhrow, S.A., Bennett, C., Stein, R.B. and Kaczmarek, L.K. (1990). Cloning and expression of cDNA and genomic clones encoding three delayed rectifier potassium channels in rat brain. Neuron 4, 929-939.

Taglialatela, M., Kirsch, G.E., VanDongen, A.M., Drewe, J.A., Hartmann, H.A., Joho, R.H., Stefani, E. and Brown, A.M. (1992). Gating currents from a delayed rectifier K^+ Biophysical J. 62, 34-36.

Taglialatela, M., Drewe, J.A. and Brown, A.M. (1993). Barium blockade of a clonal potassium channel and its regulation by a critical pore residue. Mol. Pharmacol. 44, 180-190.

Taglialatela, M., Champagne, M.S., Drewe, J.A. and Brown, A.M. (1994). Comparison of H5, S6, and H5-S6 exchanges on the pore properties of voltage-dependent K+ channels. J. Biol. Chem. 269, 13867-13873.

Taglialatela, M. and Brown, A.M. (1995). Structural motifs underlying voltage-dependent K^+ channel function. Kidney Int. 48, 918-922.

Takimoto, K., Gealy, R., Fomina, A.F., Trimmer, J.S., and Levitin, E.S. (1995). Inhibition of voltage-gated K^+ channel gene expression by the neuropeptide thyrotropin-releasing hormone. J. Neurosci. 15,449-457.

Tejedor, F.J., Bokhari, A., Rogero, O., Gorczyca, M. Zhang, J., Kim, E., Sheng, M. and Budnik, V. (1997). Essential role for *Dlg* in synaptic clustering of *Shaker* K^+ channels *in vivo*. J. Neurosci. 17, 152-159.

Tempel, B.L., Papazian, D.M., Schwarz, T.L., Jan, Y.N. and Jan, L.Y. (1987). Sequence of a probable potassium channel component encoded at *Shaker* locus of *Drosophila*. Science 237, 770-774.

Tempel, B.L., Jan, Y.N. and Jan, L.Y. (1988). Cloning of a probable potassium channel gene from mouse brain. Nature 332, 837-839.

Theil, E.C. (1990). Regulation of ferritin and transferrin receptor mRNAs. J. Biol. Chem. 265, 4771-4774.

Tessier-Lavigne, M. and Goodman, C.S. (1996). The molecular biology of axon guidance. Science 274, 1123-1133.

Timpe, L.C., Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. and Jan, L.Y. (1988a). Expression of functional potassium channels from *Shaker* cDNA in *Xenopus* oocytes. Nature 331, 143-145.

Timpe, L.C., Jan, Y.N . and Jan, L.Y. (1988b). Four cDNA clones from the *Shaker* locus of *Drosophila* induce kinetically distinct A-type potassium currents in *Xenopus* oocytes. Neuron 1, 659-667.

Tomida, M., Yamamoto-Yamaguchi, Y. and Hozumi, M. (1984). Three different cDNAs encoding mouse D-factor/LIF receptor. J. Biol Chem. 259, 10978-10982.

Trimmer, J.S. (1991).Immunological identification and characterization of a delayed rectifier K^+ channel polypeptide in rat brain. PNAS 88,10764-10768.

Trudeau, M.C., Warmke, J.W., Ganetzky, B. and Robertson, G.A. (1995). *Herg*, a human inward rectifier in the voltage-gated potassium channel family. Science 269, 92-95.

Tsunoda, S., and Salkoff, L. (1995). Genetic analysis of drosophila neurons: *Shal*, *Shaw*, and *Shab* encode most embryonic potassium currents. J. Neurosci. 15, 1741-1754.

Varon, S., Manthorpe, M. and Adler, R. (1979). Cholinergic neurotrophic factors. I. Survival, neurite outgrowth and choline acetyltransferase activity in monolayer cultures from chick embryo ciliary ganglia. Brain Res.173, 29-45.

Vega-Saenz de Miera, E., and Rudy, B. (1992). Modulation of K+ channels by hydrogen peroxide. Biochem. and Biophys. Res. Comm. 186,1681-1687.

Veh, W., Lichtinghagen, R., Sewing, S., Wunder, F., Grumbach, I.M., Pongs, O. (1995). Immunohistochemical localization of five members of the Kv1 channel subunits: contrasting subcellular locations and neuron-specific colocalizations in rat brain. J. Neurosci. 7, 2189-2205.

Villarroel, A. (1993). Suppression of neuronal potassium A-current by arachadonic acid. FEBS 2, 184-188.

Villarroel, A. and Schwarz, T. (1996). Inhibition of the Kv4 (*Shal*) family of transient K^+ currents by arachadonic acid. J. Neurosci. 16, 1016-1025.

Uebele, V.N., England, S.K., Chaudhary, A. Tamkun, M.M. and Snyders, D.J. (1996). Functional differences in Kv1.5 currents expressed mammalian cell lines are due to the presence of endogenous Kv ß 2.1 subunits. J. Biol. Chem. 271, 2406-2412.

Wang, Q., Curran, M.E., Spawski, I., Burn, T.C., Millholland, J.M., VanRaay, T.J., Shen, J., Timothy, K.W., Vincent, G.M., de Jager, T., Schwartz, P.J., Towbin, J.A., Moss, A.J., Atkinson, D.L., Landes, G.M, Conners, T.D. and Keating, M.T. (1996). Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias. Nature Genetics 12, 17-23.

Wang, H., Kunkel, D.D., Martin, T.M., Schwartz, P.A. and Tempel, B.L. (1993). Heteromultimeric K^+ channels in terminal and juxtaparanodal regions of neurons. Nature 365, 75-79.

Wang, H.S. and MacKinnon (1995). Potassium currents in rat prevertebral and paravertebral sympathetic neurones: control of firing properties. J. Physiol. 485, 319-335.

Weiser, M., Vega-Saenz de Miera, Kentros, C., Moreno, H., Franzen, L., Hillman, D., Baker, H. and Rudy, B. (1994). Differential expression of *Shaw*-related K^+ channels in the rat central nervous system. J. Neurosci. 14, 949-972.

Wei, A., Covarrubias, M., Butler, A., Baker, K., Pak, M. and Salkoff, L. (1990). K current diversity produced by an extended gene family conserved in *Drosophila* and mouse. Science 248, 599-603.

Wei, A., Jelga, T. and Salkoff, L. (1996). Eight potassium channel families revealed by the *C. elegans* genome project. Neuropharmacol. 35, 805-829.

Wickens, M. (1990). In the beginning is the end: regulation of poly(A) addition and removal during early development. Trends Biochem. Sci. 15, 320-324.

Wu, R.L., and Barish, M.E., (1992). Two pharmacologically and kinetically distinct transient potassium currents in cultured embryonic mouse hippocampal neurons. J. Neurosci.. 12, 2235-2246.

Wu, R.L., and Barish, M.E., (1994). Astroglial modulation of transient potassium current development in cultured mouse hippocampal neurons. J. Neurosci. 14, 1677-1687.

Wymore, R.S., Negulescu, D., Kinoshita, K., Kalman, K., Aiyar, J. Gutamn, G.A. and Chandy, K.G. (1996). Charaterization of the transcription unit of mouse Kv1.4, a voltage-gated potassium channel gene. J. Biol.Chem. 271, 15629-15634.

Xu, J., Yu, W., Jan, Y.N., Jan, L.Y., Li, M. (1995). Assembly of voltage-gated potassium channels: conserved hydrophillic motifs determine subfamily-specific interactions between the alpha subunits J. Biol. Chem. 270, 24761-24768.

Yamaguchi, Y. (1997). Brevican: a major proteoglycan in adult brain. Perspectives on Developmental Neurobiology 3:307-17.

Yellen, G., Jurman, M.E., Abramson, T. and Mackinnon, R. (1991). Mutations affecting internal TEA blockage identify probable pore forming region of a K^+ channel. Science 251, 939-941.

Yokoyama, A., Imoto, K., Kawamura, T., Higashida, H., Iwabe, N., Miyata, T. and Numa, S. (1989). Potassium channels from NG108-15 neuroblastoma-glioma hybrid cells. Primary structure and functional expression from cDNAs. FEBS 259, 37-42.

Yool, A.J. and Schwarz, T.L.(1991). Alteration of ionic selectivity of a K^+ channel by mutation of the H5 region. Nature 349, 700-703.

Yu, W., Xu, J. and Li, M. (1996). NAB Domain is essential for the subunit assemble of α-α and α-ß complexes of *Shaker*-like potassium channels. Neuron 16, 441-453.

Yuste R. (1997). Dendritic shock absorbers. Nature 387, 851-853.

Zagotta, W.N. and Aldrich, R.W. (1989). Gating of single *Shaker* potassium channels in *Drosophila* muscle and in *Xenopus* oocytes injected with *Shaker* mRNA. PNAS 86, 7243-7247.

Zbicz, K. and Weight, F. (1985). Transient voltage and calcium-dependent outward currents in hippocampal CA3 pyramidal neurons. J. Neurophys. 53, 1038-1058.

Zhao, B., Rassendren, F., Kaang, B.K., Furakawa, Y., Kubo, T. and Kandel, E.R. (1994). A new class of noninactivating K^+ channels from aplasia capable of contributing to the resting potential and firing pattern of neurons. Neuron 13, 1205-1213.

Zhuo, H., Ichikawa, H. and Helke, C.J. (1997). Neurochemistry of the nodose ganglion. Progress in Neurobiology. 52, 79-107.